Thesis Ref. №. _____

PATHOGENICITY OF *TRYPANOSOMA VIVAX* ISOLATES FROM TSETSE AND NON-TSETSE INFESTED AREAS OF NORTHWEST ETHIOPIA IN EXPERIMENTALLY INFECTED CALVES: BIOCHEMICAL, CYTOKINE AND CLINICO-PATHOLOGICAL EXAMINATION

MSc THESIS

BY

ADDISU AWUKEW

DEPARTMENT OF VETERINARY PATHOLOGY AND PARASITOLOGY

SEPTEMBER, 2014

BISHOFTU, ETHIOPIA

PATHOGENICITY OF *TRYPANOSOMA VIVAX* ISOLATES FROM TSETSE AND NON-TSETSE INFESTED AREAS OF NORTHWEST ETHIOPIA IN EXPERIMENTALLY INFECTED CALVES: BIOCHEMICAL, CYTOKINE AND CLINICO-PATHOLOGICAL EXAMINATION

A thesis submitted to the College of Veterinary Medicine and Agriculture of Addis Ababa University in partial fulfillment of the requirements for the degree of Master of Science in Veterinary Pathology

By

Addisu Awukew

September, 2014

Bishoftu, Ethiopia

Addis Ababa University

College of Veterinary Medicine and Agriculture

Department of Veterinary Pathology and Parasitology

As members of the Examining Board of the final MSc open defense, we certify that we have read and evaluated the Thesis prepared by **Addisu Awukew** entitled: **Pathogenicity of *Trypanosoma vivax* isolates from tsetse and non-tsetse infested areas of Northwest Ethiopia in experimentally infected calves: biochemical, cytokine and clinico-pathological examination** and recommend that it be accepted as fulfilling the thesis requirement for the degree of Masters of Science in **Veterinary Pathology.**

Dr. Fufa Abuna (DVM, MSc, Assoc. Prof)	_____	_____
Chairman	Signature	Date
Dr. Thomas Cherenet (DVM, MSc, PhD)	_____	_____
External Examiner	Signature	Date
Dr. Bulto Giro (DVM, MSc, PhD fellow)	_____	_____
Internal Examiner	Signature	Date
Dr. Shimelis Dangachew (DVM, MSc, PhD fellow)	_____	_____
Major Advisor	Signature	Date
Dr. Getachew Terefe (DVM, MSc, PhD)	_____	_____
Co- Advisor	Signature	Date
Dr. Tilaye Demissie (DVM, MSc, Asst. Prof)	_____	_____
Co- Advisor	Signature	Date
Prof. Gooddeeris B. M.: Leuven University, Belgiu	_____	_____
Co- Advisor	Signature	Date
Dr. Hagos Ashenafi (DVM, MSc, PhD)	_____	_____
Department chairperson	Signature	Date

TABLE OF CONTENTS

THIS RESEARCH PAPER WAS DEDICATED FOR

MY DAD AND MOM

STATEMENT OF AUTHOR

First, I declare that this thesis is my *bonafide* work and that all sources of material used for this thesis have been duly acknowledged. This thesis has been submitted in partial fulfillment of the requirements for an advanced (MSc) degree at Addis Ababa University, College of Veterinary Medicine and Agriculture and is deposited at the University/College library to be made available to borrowers under rules of the Library. I solemnly declare that this thesis is not submitted to any other institution anywhere for the award of any academic degree, diploma, or certificate.

Brief quotations from this thesis are allowable without special permission provided that accurate acknowledgement of source is made. Requests for permission for extended quotation from or reproduction of this manuscript in whole or in part may be granted by the head of the major department or the Dean of the College when in his or her judgment the proposed use of the material is in the interests of scholarship. In all other instances, however permission must be obtained from the author.

Name: Addisu Awukew Signature:_____

College of Veterinary Medicine and Agriculture, Bishoftu

Date of Submission: Semptember, 2014

ACKNOWLEDGEMENTS

I would like to express my deep sense of indebtedness to Dr. Shimelis Dagnachew, Dr.Getachew Terefe and Dr. Tilaye Demissie, my academic advisors for their scholarly and intellectual guidance, unreserved help and reference material provision and encouragement during the research work. Their skillful, meticulous, correction of the manuscript also deserves appreciation.

Wolaita Sodo University School of Veterinary Medicine and their respective official deserve special thanks for allowing me to join the graduate school.

I am also great full to GALV med, Thematic and Ethio-Belgium for their overall support of my research work.

I would like to thank the department of Pathology and parasitology and graduate studies office, collage of veterinary medicine and agriculture for their continual support to complete this work.

I cannot get a word to express my feeling and gratitude to Ato Alemu Tola and Yosef Cherinet for provision of all the necessary materials during my laboratory activities and for their moral and technical support.

A special word of thanks goes to NAHDIC and the staff members in pathology department particularly Dr. Asegadech Sirak, Dr. Getnet Abie, Ato Tewodros, w/o Aynalem and Ato Solomon for provision of all the necessary materials during my laboratory activities and for their incalculable help and unreserved advice and intellectual guidance in the laboratory at NAHDIC.

I wish to express my sincere thanks to members of the research project Dr. Biniyam Tsegaye, Dr, Frehiwot Tesfu and Dr. Melkamu for their help and cooperation during sample collection and laboratory work.

Staff of the Ethio-Belgium laboratory at CVMA, Sisay deserves special appreciation for her support, understanding and tolerance during the height of my work in the laboratory.

Lastly, but not least, blessing goes to my family and relatives whose prayer and thoughts have been always with me.

LIST OF TABLE **PAGES**

LIST OF FIGURES PAGES

LIST OF ANNEXES **PAGES**

LIST OF ABBREVIATION

AAT	African Animal Trypanosomosis
ALP	Alkaline Phosphatase
ALT	Alanine Amino Transferase
AST	Aspartate Aminotransferase
CD	Cluster of Differentiation
DAG	Diacylglycerol
DNA	Deoxyribonucleic Acid
EGF	Epidermal Growth Factor
ELISA	Enzyme-Linked Immunosorbent Assay
FAO	Food and Agriculture Organization
GGT	Gama-Glutamyl Transferase
GPI	Glycosyl Phosphatidyl Inositol
Hgb	Hemoglobin
HE	Haematoxylin and Eosin
IFN-γ	Interferon Gamma
Ig	Immunoglobulin
IL	Interleukin
IL1A	Interleukin-1, Alpha
IL1RN	Interleukin-1 Receptor Antagonist
LPS	Lipopolysaccharide
MHC	Major Histocompatibility Complex
NIC	Non-infected control
nm	nano meter
NO	Nitric Oxide
NT1	Non tsetse one
NT2	Non tsetse two
OD	Optic Density
PBMC	Peripheral Blood Mononuclear Cells

PCR	Polymerase Chain Reaction
PCV	Packed Cell Volume
PI	Post Infection
PIG	Phospho Inositol Glycan
RBC	Red Blood Cell
SIRS	Systemic Inflammatory Response Syndrome
sVSG	Soluble Variable Surface Glycoprotein
Th	T- helper
TLTE	Trypanosome Released Triggering Factor
TNF-α	Tumor Necrosis Factor-α
TT	Tsetse
VAT	Variable Antigen Type
VSG	Variable Surface Glycoprotein
WAD	West African Dwarf

ABSTRACT

Trypanosomosis is a protozoan disease of animals and humans in sub-Saharan Africa. In Ethiopia, particularly the northwest region is affected by both tsetse and non-tsetse transmitted trypanosomosis. The objectives of the present study were to determine biochemical changes, cytokine responses and clinicopathological findings and compare differences in virulence of *Trypanosoma vivax* infection between tsetse and non-tsetse infested areas of northwest Ethiopia. Sixteen calves were experimentally infected with *T. vivax* isolates from tsetse and tsetse free area of selected sites which was originally isolated from naturally infected cattle to study the hematological, biochemical, histopathological and major cytokine alterations during the evolution of the disease. The animals were divided in four groups, TT, NT1, NT2 and NIC, each consisting of four infected (TT, NT1 and NT2) and four (NIC) control animals, respectively. The animals were kept in strict hygienic conditions and on a zero grazing schedule. Animals of group TT, NT1 and NT2 were exposed to 1 x 10^6trypanosome/ml (2ml of blood) intravenously. The course of the experimental infection was followed up to 11 weeks. Non-infected control animals demonstrated a progressive and significantly higher weight gain (16.3% or 12kg at the end of the study period) compared to infected groups (P<0.001). Hematological analyses of the infected calves revealed a marked decline in hemoglobin (P<0.001) and packed-cell volume (P<0.001). In addition there was a significant difference in Hgb values between TT and NT2 (P<0.013); in PCV values between TT and NT1 (P<0.024); TT and NT2 (P<0.001). Biochemical analyses showed decreased serum albumin (P<0.001), serum cholesterol (P<0.001), glucose (P<0.001) values and increased AST (P<0.05), ALT (P<0.001) and ALP (P>0.05) values in all challenged groups following infection as compared to the control. On the other hand, serum total protein (P>0.05) showed decrease in the mean values at early stage of infection and start to increase throughout the experiment in all groups. There was no significant decrease in mean glucose values between TT and NT1 (P>0.05), but there was significant decrease among TT and NT2 (P<0.001). However, there was no significant decrease in mean

values of albumin, cholesterol and total protein between TT and NT1; TT and NT2. There was significant increase in values of AST between TT and NT1 (P<0.05); no significant increase between TT and NT2 (P>0.05). In addition, there was no significant increase in mean values of ALT and ALP between TT and NT1; TT and NT2. In this study cytokine assays also showed an increase in the production of TNF- α, IFN- γ, IL-10 and IL-12 except for group NT1 for all cytokine assayed. There was significant increase in IFN- γ between TT and NT2 (P<0.001), TT and non-infected control (P<0.001). Moreover there was significant increase in the values of IL-10, IFN γ and IL-12 between TT and NIC (P<0.001); TT and NT1 (P<0.001); TT and NT2 (P<0.001). At necropsy, infected calves showed enlarged and hemorrhagic spleen; swollen, edematous and enlargement of prescapular lymph nodes; pneumonic and emphysematous lung; liver was enlarged, edematous with rounded edge. However, the post mortem examination of the animal in the control group did not reveal any significant gross lesions. The histopathological examination also conform the presence of significant abnormalities on major organs characterized by lymphoid hyperplasia in the white and red pulp of the spleen, interstitial pneumonia, zonal sever hepatic necrosis, tubulo-interstitial nephritis, multifocal myocarditis, meningoencephalitis, lymphoid hyperplasia in lymph node and mononuclear cell infiltration. In conclusion *T. vivax* isolates from both areas showed a variety of virulence factors leading to the development of acute clinical signs, reduction of PCV, decrease in biochemical values as well as increase in serum enzymes. In addition excessive secretion of cytokines, gross pathological lesion and histopathological abnormalities on major organs in the infected groups are manifestations of pathogenicity. However, the occurrence of parasitaemia and clinical signs were earlier in the NT groups compared to TT group. Therefore, equal attention is required for the control of trypanosomosis in both tsetse and non-tsetse infested areas of northwest Ethiopia.

Keywords: Calves, Experimental infection, Pathogenicity, *T. vivax*, North West Ethiopia

1. INTRODUCTION

Trypanosomosis is among the well-known constraints to livestock production in Africa as it causes a serious and often fatal disease of livestock mainly in the rural poor community and rightfully considered as a root cause of poverty in the continent (Vreysen, 2006). In sub-Saharan Africa, the main pathogenic trypanosomes in domestic animals are *Trypanosoma vivax, T. congolense* and *T. b. brucei* which are generally transmitted cyclically by tsetse flies. However, *T. vivax* is exceptionally important in Africa and in South America because the parasite can also be mechanically transmitted by other biting flies. The disease causes an acute or chronic infection, characterized by anemia and immunosuppression, resulting in decreased productivity and economic losses that are estimated at U$4 billion annually (Kristajonson *et al*., 1999).

In Ethiopia, particularly, the northwest region is affected by both tsetse and non-tsetse transmitted trypanosomosis (Sinshaw *et al*., 2006; Cherenet *et al*., 2006; Shimelis *et al*., 2007). In tsetse infested areas of Dembecha and Jabitehenan districts of northwest Ethiopia, the prevalence of trypanosomosis was 17.07% (Shimelis *et al*., 2005) of which 26% of the infection was *T. vivax*. Epidemiological investigation by Cherenet *et al*., (2006) on bovine trypanosomosis in tsetse-infested and tsetse-free areas of northwest Ethiopia indicated 15.6% and the dominant species was *T. vivax*. Similar study by Sinshaw *et al*., (2006) indicated 9.1% *T. vivax* infection prevalence in areas bordering Lake Tana in northwest Ethiopia.

Trypanosomosis is known to cause anemia, hypoproteinemia, leukocytosis, immunosuppression, hypoglycemia and changes in serum enzyme and cholesterol levels. But little is known of the impact of *T. vivax* than of the better studied *T. brucei* and *T. congolense* in East Africa. Isolates of *T. vivax* from different parts of the world have been shown to differ in their pathogenicity, infectivity for laboratory rodents, requirements for

cultivation in vitro and reactivity with repetitive DNA sequence probes (Osório *et al*., 2008). It is generally believed that the East African strain of *T. vivax* is less pathogenic and causes a chronic form of infection; however, occasional outbreaks of *T. vivax* infection have been reported to cause severe mortality in Kenya and Uganda (Mwongela *et al*., 1981).

Recently, Morrison *et al*., (2010) suggested that even though the parasite genetic component is responsible for causing disease in the host, an integrated host-parasite approach is required for future studies on trypanosome pathogenesis. For instance, the existence of immunosuppression has been known for long time but the unresolved question is whether the immunosuppression was mediated by the macrophages or the T cells or simply by parasite products. There are suggestions that both cells might be involved (Tabel *et al*., 2008). One of the common pathological features observed in trypanosomosis is anemia which might be due to either loss of RBC; for example, the cytokine activated macrophages cells are suggested to be responsible for the enhanced phagocytosis of parasites as well as the RBCs or due to inability to mount a vigorous compensatory erythropoietic response (Stijlemans *et al*., 2008). In the major cattle pathogens, *T. vivax* and *T. congolense*, a wide range of virulence phenotype variation between isolates in clinical pathology are identified (Bengaly *et al*., 2002).

Therefore, the contribution of the host to the control of disease and the parasite to the pathogenesis must both be examined in order to produce a holistic picture of host-parasite interactions and the survival of the host. Recent studies on the temporal transcriptional response of bovine peripheral blood mononuclear cells (PBMC) in vivo to a controlled trypanosome infection and identified time points indicated that transcript levels for the IL2, IL8 and IL1RN genes were significantly down regulated across the time course. In addition, there were increases in transcripts for genes encoding pro-inflammatory mediators (IFN-γ, IL1A, TNF, and IL12) in N'Dama by 14 day post infection compared with pre-infection levels and at the peak of parasitaemia a type 2 helper T cell (TH2) like cytokines were prevalent in the trypanosusceptible Boran with increases in transcripts for the IL6 and IL10 genes in *T. congolense* infection in Ireland (O'Gorman *et al*., 2006).

Similarly, blood cellular damage and histopathological changes in liver, kidney, spleen and other related organs were found in cattle experimentally infected with *T. vivax* in Nigeria (Kadima *et al*., 2000).

However, whether *T. vivax* from tsetse and non-tsetse areas cause similar damage to host tissues and elicit similar immunological responses is not clear. Therefore, it is hypothesized that method of transmission and host adaptation affects pathogenicity of parasites and immunological responses of hosts which could be detected through measurement of biochemical and immunological parameters. Information on both the capacity of the parasite to elicit pathology and host responses against different strains of the parasite is believed to help in the design of future vaccines and trypanocides and selection of hosts for tolerance or resistance to the parasites.

OBJECTIVES

General aim of the research

The general aim of the research was to evaluate the pathogenicity of three isolates of *T. vivax* (from tsetse infested and non-tsetse infested areas) as measured by serum biochemical changes cytokine profile and Clinico-pathological examination in experimentally infected calves.

Specific objectives of the research

The specific objectives of the research were to:

1. Measure hematological and serum biochemical changes, cytokine profile and clinico-pathological features caused by infection with different isolates of *T.vivax* in calves.
2. Compare hematological, biochemical, cytokine and clinico-pathological changes between calves infected with *T. vivax* isolates from tsetse-infested areas and those infected with *T.vivax* isolates from non-tsetse infested area

2. LITERATURE REVIEW

2.1. **Trypanosomosis**

Tsetse transmitted trypanosomosis is the main constraint to livestock production in the continent of Africa, preventing full use of land to feed the rapidly increasing human population (Murray *et al*., 1991). It affects 37 sub- Saharan countries, extending over 10 million km^2 of land (Erkelens *et al*., 2000). African animal trypanosomosis (AAT) is considered by many to be the single greatest health constraint to increased livestock production in sub-Saharan Africa with direct annual production losses in cattle estimated at US$ 600–1200 million. Estimates of the overall annual lost potential in livestock and crop production have been as high as US$ 4750 million. An estimated 45 to 60 million cattle and tens of millions of small ruminants are at risk from trypanosomosis (Gilbert *et al*., 2001). Food and Agricultural Organization (FAO) estimates that about three million cattle die each year due to AAT (FAO, 2000). Other valuable livestock, such as camels, also suffer from trypanosomosis (ICIPE, 2006).

Direct costs due to AAT involve decreased livestock productivity (mortality, fertility, milk yield, ability to work as traction animals) to which can be added expenditure on controlling the disease (Shaw, 2004). The impact of trypanosomes in African agriculture is most obviously seen in the birth and mortality rate of young animals (Erkelens *et al*., 2000). In susceptible cattle breeds, the disease reduces calving by up to 20% and causes the death of another 20% of young stock. Such direct losses are estimated to amount between US$ 1-1.2 billion each year. The indirect impact of AAT on agriculture in sub-Saharan Africa is believed to exceed the amount estimated for direct losses. The overall impact extends to the restricted access to fertile and cultivable areas, imbalances in land use and exploitation of natural resources and compromised growth and diversification of crop-livestock production systems (Mattioli *et al*., 2004). A pondered evaluation extrapolated for the total tsetse-infested lands values the total losses, in terms of agricultural Gross Domestic Product, at US$ 4.75 billion per year (FAO, 2004).

Furthermore, about 50 million people in Africa are exposed to the risk of contracting sleeping sickness (Anne *et al*., 2001).

Infections of African trypanosomes are characterized by successive waves of parasitaemia. Trypanosome infections take a variable course depending on factors associated with both the host and the parasites, but are characterized in most instances by the intermittent presence of parasites in the blood and intermittent fever. Infection of African trypanosomes may run an acute, sub-acute or chronic course (Anne *et al*., 2001). In the acute case, anemia and general loss of body condition are the first clinical signs when trypanosomes invade and multiply in the blood stream of the infected animals. About one or two weeks later, the sick animals usually have recurrent fevers for up to 3 months. After the first bout of fever, this occurs normally within 12 days, the number of parasites in the circulation declines. The animals continue to be anemic and lose body condition resulting in reduced productivity and frequently high mortality rates. Infections with *T. b. brucei* and *T. b. evansi* can result in the invasion of the brain, eyes and skin, leading to nervous signs, discharges from the eyes and edematous swellings under the skin. In the sub-acute or chronic course, the disease lasting many month or even years is more common (Kettle, 1995).

2.2. **Development of immunity against trypanosomes**

2.2.1. *Host-Parasite interaction and pathogenesis*

The outcome of disease occurrence depends on the interplay of the host, the agent (the parasite) and environmental factors. By adding or modifying the factors the frequency of the occurrence of the disease can be changed (Thrusfield, 1995).

Upon the bite of the mammalian host by a trypanosome infected tsetse fly, the parasites multiply locally in the skin and elicit a local host response that manifests itself as an indurated skin lesion called the chancre. Eventually, the parasites enter the blood circulation via lymph vessels and can survive in the blood circulation throughout the

infection of the host. Thus, they remain continually exposed to the host's immune system (Tabel *et al*., 2008). Trypanosomes have evolved very sophisticated evasion mechanisms to survive in the chronically infected host. Well documented evasion mechanisms include antigenic variation of the VSG and the induction of alterations in the host's defense system, such as excessive activation of the complement system leading to persistent hypocomplementemia, down regulation of nitric oxide production, polyclonal B-lymphocyte activation and marked immunosuppression (Pan *et al*., 2006; Guilliams *et al*., 2007). Most likely African trypanosomes induce other, yet undiscovered, changes in the physiology of the infected host, which might interfere with effective control of the parasite (Wei and Tabel, 2008).

2.2.2. *Trypanosome antigens and exposure to host's immunity*

Trypanosomes causing morbidity and mortality in both man and livestock are unusual among the protozoan parasites in that they never enter the host's cells and yet persist for extended periods of time in mammalian blood and tissues. In the mammalian host, these parasites are completely surrounded by a dense immunogenic surface coat (12–15 nm thick) of a single polypeptide species referred to as the variant surface glycoprotein (VSG) that shields invariant surface antigens from immune recognition. Moreover, trypanosomes constantly modify their VSG by the process of antigenic variation, resulting in the fluctuating waves of parasitaemia that characterizes African trypanosomosis (Borst, 2002; Pays, 2006).

During the course of African trypanosomosis, a complex interaction between the host immune responses and parasite survival strategies occurs. As such, natural selection has enabled African trypanosomes to develop very sophisticated mechanisms to evade immune killing to survive in the chronically infected host and hence allow transmission to the next host, via the tsetse vector. Well documented evasion mechanisms include antigenic variation of the VSG (Morrison *et al*., 2009) and the induction of alterations in the host's defense system (Shi *et al*., 2006; Namangala *et al*., 2007; Stijlemans *et al*., 2007; Namangala *et al*., 2008; Magez *et al*., 2008). Antigenic variation remains one of

the most spectacular adaptive mechanisms exhibited by African trypanosomes and is the central most important immune escape mechanism by these parasites (Barry and McCulloch, 2001; Borst, 2002). Trypanosomes contain up to 1000 different genes in their genome which afford them extensive opportunities to escape host adaptive immune responses by displaying new coat antigens. The parasite has intrinsic mechanisms that ensure only one VSG gene is transcribed at any one given time. By switching VSG genes and expressing a new variant antigenic type, trypanosomes evade B and T-cell mediated immune responses. Furthermore, expression of VSG is central to the process of antigenic variation that eventually leads to exhaustion of the host immune system for the benefit of the trypanosome (Borst, 2002; Pays, 2006; Morrison *et al*., 2009).

During infection of a mammalian host, African trypanosomes are in constant contact with the host's immune system. Because of the strong selection pressure from continuous contact with the host's immune system, the trypanosomes seem to have developed several ways of evading immune killing through alteration of the host's natural and adaptive immune responses. For instance, *T. congolense* and *Trypanosoma brucei brucei* have been documented to induce a generalized state of immunosuppression following infection of cattle or mice (Namangala *et al*., 2000). The mechanisms of such immune depression seem to be mediated by both macrophages (Namangala *et al*., 2000; Gomez-Rodriquez *et al*., 2009) and T cells (Namangala *et al*., 2000) with suppressive phenotypes. In the case of macrophages, the suppressive phenotype may be exhibited by both classically (caMFs) and alternatively activated cells (aaMFs) (Namangala *et al*., 2001; Noel *et al*., 2002; Stijlemans *et al*., 2010). These macrophages subsets are antagonistically regulated, and their development is influenced by the cytokine environment. Whereas classically activated macrophages are induced by type I cytokines (TNF-α, IL-12, IFN-c) and inhibited by type II cytokines (IL-4, IL-10, IL-13), the reverse is true for alternatively activated macrophages (Kodelja *et al*., 1998). Furthermore, whereas classically activated macrophages mediate pro-inflammatory responses and are microbicidal, alternatively activated macrophages are anti-inflammatory and actively participate in tissue healing and contribute to peripheral tolerance (Munder *et al*., 1999).

2.2.3. *Host responses to trypanosomes*

The inoculation of trypanosomes into their mammalian hosts triggers a series of events involving, at first, innate immunity and, secondarily, specific immunity. The latter requires an efficient presentation of parasitic antigens, activation of T and B cells implying specific antigen receptor recognition, and the development of effector cells and molecules. These mechanisms are highly regulated by multiple signals delivered through a large number of receptors transduced across the plasma membrane and processed. During co-evolution with their hosts, trypanosomes have learnt to cope with host immune systems, by penetrating, diverting, and altering the numerous steps leading to the generation of an effective immune response. Major modifications of immune systems have been observed in trypanosomiasis: lymphadenopathy, splenomegaly (up to thirty times the normal size) with destruction of lymphatic tissue architecture and hypergammaglobulinemia. However, their effectiveness is limited as, most of the time, parasites cannot be eliminated and immuno-pathological phenomena, which induce tissue alterations (Philippe and Bernard, 2006).

Variable surface glycoproteins constitute an important molecular interface between trypanosomes and the host immune system. Variable surface glycoproteins prevents trypanosome lysis by complement alternative pathway, and, above all, enables them to avoid the specific immune response via the phenomenon of antigenic variation (trypanosomes sequentially express antigenically distinct VSG). VSG also has several effects on immune elements such as induction of autoantibodies and cytokines, in particular tumor necrosis factor (TNF)-α (Magez *et al*. 2008). According to Olsson *et al*., (1991) other trypanosome components and soluble factors, such as a trypanosome released triggering factor (TLTF) which triggers interferon (IFN)-γ production by T cells, are also involved in modulation of the immune system by acting on the synthesis of immune elements. Elaboration of escape mechanisms to host immune defenses and induction of parasite growth factor production are well developed by trypanosomes. In a recently discovered escape mechanism, host arginase induction, trypanosomes decrease

immune response efficiency and increase the production of L-ornithine, an essential growth factor (Vincendeau *et al*., 2003).

2.2.4. *Antibodies to VSG*

The production of antibodies to the VSG of *T. brucei* or *T. congolense* is the major early immune response. The first antibody to the VSG is of immunoglobulin M (IgM) class and is produced independently of T cells (Reinitz and Mansfield, 1990). Antibodies to the VSG are able to mediate control of the parasitaemia. There are three known mechanisms of anti-VSG mediated control of parasitaemia (Tabel *et al*., 2008). These are:

(i) Antibody/complement-mediated immune lysis,

(ii) Phagocytosis by macrophages, and

(iii) Cytotoxic effect of induced nitric oxide

Although anti-VSG antibodies can mediate some degree of complement mediated immune lysis, the parasites have developed mechanisms to evade an effective lysis (Frevert and Reinwald, 1990). Considering that anti-VSG antibody can induce shedding of soluble VSG (sVSG) into the circulation and mediate the formation of soluble complexes of sVSG covalently bound to degradation products of complement component C3, immune lysis might be more harmful than beneficial. In fact, *T. congolense* infected complement C5 deficient mice have about the same survival time as infected wild type mice, indicating that immune lysis of trypanosomes is not an efficient control of infection (Table *et al*., 2008).

2.2.5. *Role of cytokines*

Cytokines such as IL-1, IL-3, IL-4, IL-6, IL-10, and TNF-α and IFN-γ were found to have no direct effect on the growth of *T. congolense* (Kaushik *et al*., 1999). Susceptible Boran cattle and trypanotolerant N'Dama cattle, when infected with *T. congolense*, express elevated levels of IL-10 mRNA. Interferon-γ induced NO production by macrophages from infected cattle was decreased and in vitro IFN-γ induced synthesis of

NO by peripheral blood mononuclear cells from uninfected cattle was suppressed by IL-10 (Tabel *et al*., 2000).

In recent studies, it was found that, whereas IFN-γ down regulated the synthesis of IL-10 in macrophages stimulated with bacterial lipopolysaccharide (LPS), IFN-γ up regulated the production of IL-10 in cultures of BALB/c macrophages pulsed with *T. congolense* (Kaushik *et al*., 2000). The findings of Tabel *et al*., (2000) indicate that the regulation of IL-10 synthesis by macrophages simultaneously stimulated by IFN-γ and *T. congolense* is different from the regulation of IL-10 synthesis upon combined stimulation with IFN-γ and LPS. The amount of IL-10 produced by macrophages was positively correlated with the amount of *T. congolense* homogenate used for stimulation (Tabel *et al*., 2000).

The effects of lipopolysaccharide (LPS) in inducing endotoxic shock in experimental animals have been studied extensively. Many cytokines are involved in endotoxic shock, such as IL-1, IL-12, TNF-α, IL-10, and IFN-γ (Billiau *et al*., 2001; Esche *et al*., 2001). The result of sepsis leading to a multiple organ dysfunction has been termed systemic inflammatory response syndrome (SIRS), associated with high serum levels of IL-6 and high IL-6/IL-10 ratio (Robertson and Coopersmith, 2006). Tabel *et al*., (2008) showed that susceptible BALB/c mice infected with *T. congolense* succumb to the infection due to the development of SIRS within 10 days of infection. This inflammatory syndrome is associated with hyper activated macrophages, an outburst of cytokine release, enlarged capillary bed, decreased blood pressure, drop of body temperature, hypomotility, and piloerection towards the terminal stage (Shi *et al*., 2003). SIRS also occurs in infected resistant C57BL/6 mice, when treated with antibodies blocking the IL-10 receptor (IL-10R). This early mortality is mediated by IFN-γ produced by MHC class II restricted CD4+ T cells. IL-10 appears to be crucial in controlling the lethal SIRS. It appears that IL-10 plays a dual role in African trypanosomiasis: it exerts a detrimental role in mediating immunosuppression, but it is beneficial by controlling excessive lethal cytokine release by macrophages (Shi *et al*., 2003).

2.2.6. *Trypanosome induced professional antigen presenting cell defects*

Macrophages and dendritic cells play a central role in the immune response as professional antigen presenting cells. This function includes internalization of trypanosomes and/or their VSG through phagocytosis, processing of parasite antigens in the acidic compartment of the endocytic pathway, costimulation and presentation of the immunogenic trypanosome peptides to antigen specific T-helper cells (Th cells) in the context of MHC Class II molecules (Shi *et al*., 2007; Dagenais *et al*., 2009). Following activation, VSG specific Th cells, through their secretory products, deliver a specific signal to both the innate and adaptive immune systems, aimed at destroying the infecting trypanosomes. Thus, through antigen presentation, macrophages and dendritic cells modulate downstream events that impact on the development of adaptive anti-trypanosomal immunity (Dagenais *et al*., 2009). Modulation of antigen presentation function of macrophages and dendritic cells is an obvious way of the parasite to escape killing by immune cells and may contribute to global immunosuppression occurring during trypanosomosis. As such, selective pressure from the host immune system could have possibly induced the trypanosome to evolve several ways of altering the antigen presentation for its own survival (Pan *et al*., 2006; Shi *et al*., 2007; Dagenais *et al*., 2009). Several studies have reported alterations of antigen presenting cell membrane phenotype at different time point post infection resulting in impaired antigen presentation (Dagenais *et al*., 2009).

2.2.7. *Immunopathology*

Infection by trypanosomes results in a rising parasitaemia. Subsequently, a T-cell-independent antibody response to each VSG (Reinitz and Mansfield, 1990) leads to massive phagocytosis of trypanosomes by macrophages, especially in liver and spleen. Most of the early produced anti-VSG is of IgM class. The phagocytosis of trypanosomes then leads to rapid activation of the Kupffer cells in the liver with release of monokines and enlargement of the Kupffer cells (Shi *et al*., 2004). Tabel *et al*., (2008) indicated that highly susceptible BALB/c mice infected intra peritoneal with 10^3 *T. congolense* clone TC13 die within 8 to 9 days after infection. Up to 5 days post-infection, there is little

trypanosomal antigen detectable in the liver, and the Kupffer cells are not visibly enlarged. At day 6, however, there is a dramatic accumulation of parasite antigen in Kupffer cells, and the Kupffer cells are greatly enlarged. An impressive fivefold increase in size of the Kupffer cells develops towards the terminal stage of infection, and about 10% of the Kupffer cells undergo apoptosis, indicating that a profound derangement of the macrophage system has occurred. Infection of mice with *T. congolense* is associated with an outburst of production of monokines [IL-1, TNF-α, IL-6, IL-12, monocyte chemotactic protein-1 (MCP-1), IL-10] (Shi *et al*., 2004) and T-cell cytokines [interferon-γ (IFN-γ), IL-10, IL-4] (Shi *et al*., 2003). Tabel *et al*., (2008); in his experimental study of mice indicate that, some of the cytokine production precedes the massive phagocytosis by Kupffer cells that is detectable at day 6.

2.3. **Biochemical response during trypanosomosis**

The infection of cattle with *T. vivax* cause cellular and tissue damage, as indicated by the changes in the biochemical parameters observed, the changes being marked as the second parasitaemia build up. Infections of zebu cattle (Bos indicus) with the Samaru strain of *T. vivax* cause an acute infection and a variety of changes in the host biochemical parameters. These changes seem to vary with the level of parasites in blood early in the disease, but as the disease progressed the changes displayed no particular pattern, thus suggesting possible liver/kidney damage and the ineffective regulation of these biochemical parameters by the affected organs (kadima *et al*., 2007).

2.3.1. Aspartate amino transferase, Alanine amino transferase and Alkaline phosphatase

Enzymes are protein catalysts synthesized by all living things. As catalysts their only biologic activity is to alter the rate at which equilibrium is established between reactants and their products. Serum (plasma) enzymes can be placed in to two distinct classes. The first consists of the plasma specific enzymes which are those that have a definite and

specific function in plasma. Their normal site of action is in the plasma, and they are present in higher level in plasma than in most tissue cells. The second class consists of the non-plasma specific enzymes which are present in concentration much lower than their concentration in certain tissues. This group is divided into two subclasses: enzymes associated with cellular metabolism and enzymes of secretion. Enzymes of cellular metabolism are located within tissue cells and are present there in high concentrations as long as the cell remains healthy and the membrane is intact. The level of these enzymes in extracellular fluids and plasma is low. Secretory enzymes are rapidly disposed of through excretory channels such as the intestinal tract, urine, and bile and by inactivation and degradation their normal plasma levels are relatively low and are constant (Coles, 1986).

Alterations in serum enzymes activity due to malfunctioning of the liver occur as a result of three processes: (1) an elevation of enzymes due to disruption of hepatic cells as a result of necrosis or as a consequence of altered membrane permeability. Included in this group are the enzymes alanine amino transferase (ALT) (formerly known as glutamic pyruvic transaminase [GPT]), aspartate amino transferase (AST) (formerly called glutamic oxaloacetic transaminase [GOT]), and lactic dehydrogenase (LDH). (2) A decrease in concentration in the serum resulting from impaired synthesis by the liver (choline esterase). (3) An elevation in enzyme levels due to cholestasis. The enzymes affected include alkaline phosphatase (ALP), Gama-glutamyl transferase (GGT) and leucine amino peptidase (LAP) (Coles, 1986).

The increase in AST values during trypanosomosis (*T. vivax*) on days 3 to 5 post infection could not have been due to any tissue damage, but rather due to parasites secreting it as part of their metabolites into blood circulation, since alanine amino transferase and AST have been observed in homogenates and suspensions of trypanosome (Kadima *et al*., 2000). However, the decreases following the second parasitaemic build-up suggest a possible progressive liver fibrosis occurring as the disease progresses. Similarly, the increase in values of BUN early in the disease could be due to body catabolic breakdown of proteins as a result of the fever of the parasitaemia,

as has been reported in acute Trypanosoma infections while the significantly low levels observed as the disease progressed suggest possible kidney damage and accelerated kidney excretion of urea (Coles, 1986). In addition Abeer and Shaymaa (2011) indicate that; there is a significant increases in the activities of hepatic enzymes (ALT, AST ALP and GGT) which were observed as a result of hepatic damage which was supported cytologically by the present fatty degeneration which followed by hepatic necrosis.

2.3.2. *Glucose*

The only sugar found in blood is glucose, which is stored in the form of polymer glycogen. Normally, glycogen is found only in the intracellular form, whereas glucose is found almost exclusively in extracellular fluids. The level of glucose is maintained within a relatively narrow range and is controlled by several factors such as hepatic and renal uptake and release of glucose; glucose removal by the peripheral tissue; effects of hormonal influences on these processes; and intestinal absorption of glucose, which has only temporary effect on blood glucose levels (Coles, 1986).

However during a disease situation there is a decrease in glucose level in the blood. For instance, according to Kadima *et al*., (2000) Serum glucose levels were significantly low ($P<0.05$) on days 3, 4 and 5 post infection of cattle with *T. vivax*, corresponding with the first parasitaemia build up, followed by a significant increase ($P<0.05$) on days 7 and 8 post infection. This also coincided with disappearance of parasites from blood. Subsequently, a gradual decrease from day 10 post infection to below normal values was observed on days 11 to 13 post infection. Opperdoes *et al*., (1986) indicated that; the high parasitaemia observed coincided significantly with low levels of glucose. This situation could be explained by the parasites' need for glucose for their cellular metabolism through their glycolytic pathway.

2.3.3. Total cholesterol

Assay of triglycerides is one of the best method for diagnosing hyperlipemia, which is syndrome characterized by negative energy balance and rapid mobilization of fatty acids derived from adipose tissue. The mobilized fatty acids ultimately result in fatty liver and subsequent hypertriglyceridemia (Forhead, 1994). Decrease in plasma levels of cholesterol and phospholipids have been reported in sheep infected with *Trypanosome congolense* (Katunga- Rawakishaya *et al*., 1995) and in man infected with *T. brucei*. These have been attributed to the fact that trypanosomes take up lipids for growth in the infected host (Katunga- Rawakishaya *et al*., 1995).

2.3.4. *Total proteins*

The total plasma protein measurement which includes fibrinogen values may be affected by altered hepatic synthesis, rapid albumin breakdown or excretion during disease. According to Witola and Lovelace, (1997) serum total proteins are reported to increase in trypanosome infected animals. The increase in serum total protein could be attributed to gammaglobulinemia which is a predominant feature of trypanosomiasis, primarily due to increases in IgM levels (Ogwu *et al*., 1986). In contrast, a decrease in serum total protein concentration was reported in *T. congolense* infected sheep (Katunguka-Rwakishaya *et al*., 1995).

2.3.5. *Albumin*

Bisalla *et al*., (2007) indicated that, serum albumin concentration decreased significantly from pre-infection values of 41.2 +2.86 g/dl and 40.2 +1.9 g/dl in the infected group and infected immunomodulated group respectively to 27.6 +5.1 g/dl and 27.5 +1.8 g/dl in the infected group and infected immunomodulated group respectively to 27.6 +5.1 g/dl and 27.5 +1.8 g/dl in the infected group and infected immunomodulated group respectively in *T. congolense* infected sheep. Decreases in serum albumin are in agreement with the observations in *T. congolense* infected sheep (Katunguka-Rwakishaya *et al*., 1995), and

T. vivax infected goats (van Dam *et al*., 1998). However, Witola and Lovelace (1997) reported that there was no significant variation in mean albumin values of *T. congolense* infected goats. The decrease in serum total protein could be attributed to a decrease in serum albumin probably from decreased hepatic biosynthesis.

2.4. Pathology

There are no pathognomonic gross or histopathological lesions found in the animals that die of trypanosomiasis. Most of the observed lesions are due to the circulatory disturbances caused by anemia. Mice infected with *T. congolense* developed a severe anemia one week after infection which was accompanied by a marked increase in the plasma levels of acute phase proteins (Ngure *et al*., 2008). Animals that die of acute infections have variable atrophy of muscle and adipose tissues and pale carcasses. Often the lymph nodes, spleen and the liver are enlarged. Also, there may be edema of the lungs, ascites, hydrothorax and hydro-pericardium. The skeletal muscles of the carcass may be wasted and pale in chronic cases. Marked splenomegaly with foci of necrosis and prominent white pulp is also observed in chronic cases. Inflammatory foci are found in spleen, liver, lung, heart, skeletal muscles, brain, skin and kidney (Ngure *et al*., 2008).

3. MATERIALS AND METHODS

3.1. Experimental study site

The experimental study was carried out in the premises of College of Veterinary Medicine and Agriculture at Bishoftu. The experimental house is a state of the art fly proof compartment constructed by the GALV- med (Global alliance for livestock veterinary medicine) and is equipped with all basic facilities (feeding, watering, weighing balance, and postmortem room, storage, etc).

3.1.1. Origin of the parasites

T. vivax isolates were collected from Jabitehnan and Bahir Dar zuria districts (tsetse and non-tsetse infested areas respectively of northwest Ethiopia), propagated on donor calves and stablets cryopreserved in liquid nitrogen. The isolates were identified as ETSH2, ETPD2 and 3. This isolates were obtained from Dr. Shimelis Dagnachew (PhD fellow).

3.2. Experimental animals

Sixteen indigenous zebu calves aged approximately one year were used for the study. The calves were purchased from known trypanosome free areas (Debre Berehan) about 130 km north of Addis Ababa. All the animals were treated with long acting Oxytetracycline (Alamycin LA, Norbrook, Ireland) at the purchasing site and transported to Bishoftu. Immediately after their entry in Bishoftu they were placed under quarantine where they were sprayed for ecto-parasites with Diazinol 60% and Deltamethrin 1% (Tagros India) and dewormed with Albendazole and treated with Ivermectine (Chengdu Qiankun, China). Additional treatment was also made with trichlabendazole (Fasionox, East African pharmaceuticals P.L.C., Ethiopia) after a week for internal parasites. The animals were acclimatized for one month before they are placed in standard fly proof experimental house and divided into groups. During this period the animals were ear notched and tagged for identification and screened for endo, ecto and hemoparasitic infections. Baseline hematological data were obtained for PCV, hemoglobin, total and differential WBC count and temperature.

3.3. Feeding and animal management

Animals of both groups were housed in four different pens under a zero grazing system. The animals were fed with grass straw ad libitum. Restricted amount of concentrate in the form of wheat bran with sufficient quantity of mineral mixture was also provided. Drinking water was available ad libitum in water troughs. The handling of animals during the experiment was based on international guiding principles for biomedical research

involving animals proposed by the Council for International Organizations of Medical Sciences (1985).The research was authorized by the Animal Research Ethics Review Committee of the College of Veterinary Medicine and Agriculture of the Addis Ababa University.

3.4. Experimental Design

The experimental animals were assigned randomly into four groups of four animals each. Group I (TT) was inoculated with *T. vivax* isolates from tsetse infested areas, Group II (NT1) and III (NT2) were inoculated with *T. vivax* isolates from non-tsetse infested areas (obtained from two different animals) and Group IV (NIC) remained non-infected control and received blood from non-infected donor.

3.5. Animal Inoculation

Trypanosoma vivax isolated from a pure natural infection of cattle herd in tsetse infested and non-tsetse infested area of North West Ethiopia was used for the study. Stabilets of the parasites are prepared and stored by Dr. Shimelis Dagnachew (PhD fellow). *Trypanosome vivax* estabilates from both tsetse and non-tsetse infested area were inoculated to the donor calves to check the viability and propagation of the parasite. Following establishment of infection, the parasite burden per milliliter of blood was quantified using rapid matching method (Herbert and Lumsden, 1976; Annex 1). Then Group TT, Group NT1 and Group NT2 animals were intravenously inoculated with 2ml of fresh blood via jugular venipuncture using a 5ml syringe and 22G needles with blood directly from the donor calves and infective dose of 1×10^6 motile trypanosomes/ml. The non-infected control group (NIC) received 2ml of blood from non-infected donor.

3.6. Data collection and analysis

3.6.1. Clinical examination

During the experimental period (March to May 2014), all animals were clinically examined daily for parameters such as rectal temperature, color of mucous membranes, size of peripheral lymph nodes, coat condition, fecal consistency, appetite, edema, the presence of lacrimation etc until the end of the experiment. All animals were weighed weekly using a digital weighing scale (TAL TEC, Model LS4, and South Africa). All measurements and examinations were made in the morning from 9:00 AM to 10:00 AM.

3.6.2. Parasitological examination

A small quantity of blood sample was taken from heparinized vacutainer tube and examined for the presence of trypanosomes using the following parasitological method: wet blood smears and buffy coat technique. Wet blood smear was used to examine the movement or motility of the parasite to determine the score of parasitaemia. A drop of blood was expelled on a microscopic slide and covered with a cover slip. The slide was examined under 40X objective and 10X eyepieces. Buffy coat technique was also used to concentrate the parasite. After centrifugation the capillary tube was cut below the buffy coat to include the upper most layers of the RBCs and 3mm above to include the plasma. The content was dropped on to slide, homogenized and covered with cover slip. Similarly, the slide was examined under 40X objective and 10X eyepieces.

3.6.3. Hematological examination

Two milliliters of the blood (once per week) was obtained from all the infected calves and non-infected calves into test tubes from the jugular vein containing anticoagulant for hematological analysis. Hematological parameters (packed cell volume and hemoglobin concentration) were determined using conventional procedures (Schalm *et al*., 1975). Packed red cell volume (Annex 2) was measured with microhaematocrit reader after centrifuged for five minutes (Annex 2). Hgb was determined by the cyanomethaemoglobin technique using a hemoglobin meter. The Hgb values were measured in g/dl (Annex 3).

3.6.4. Serum biochemical analysis and cytokine assays

3.6.4.1. Serum biochemical tests and enzyme assays

Five milliliters of the blood sample was collected with plane vacutainer tube and allowed to clot. The blood sample was then centrifuged at 3200 rpm for ten minutes to obtain serum which was aspirated into eppendorf tubes and stored at -20 ^{O}C. The blood samples were collected once per week until the end of the experiment. Biochemical tests for glucose, total protein, albumin and cholesterol were performed using spectrophotometric method by using humastar 80 clinical chemistry analyzer (Reitman and Frankel, 1957). Enzymatic colorimetric assay using humastar 80 clinical chemistry analyzer (Reitman and Frankel, 1957) was done for determination of the concentrations of alanine aminotransferase (ALT), aspartate aminotransferase (AST), alkaline phosphatase (ALP), (the details of the procedures are indicted on annex 4).

3.6.4.2. Serum cytokine assays

Five milliliters of the blood sample was collected with non-heparinized vacutainer tube. The blood sample was then centrifuged at 3200 rpm for ten minutes to obtain serum which was aspirated into eppendorf tubes and stored frozen at -80^{o}c for cytokine assays. The blood samples were collected once per week until the end of the experiment. The concentration of serum cytokines (IFN-γ, TNF-α, IL-10 and IL-12) secreted during the infection was determined by antibody ELISA kits for bovine cytokines according to the manufacturer's instructions (Bethyl Laboratories, Inc. www.bethyl.com). The levels of cytokines (IL-12 and IL-10) were tested using bovine IL-12 and bovine IL-10 ELISA (Cloud-Clone Corp, USA) kits respectively, while TNF-α and IFN-γ was assayed using bovine TNF-α vetset and bovine IFN-γ vetset ELISA (BIOTECH, INC., USA) kits respectively (Annex 5, 6, and 7).

3.6.5. Gross and histopathological examination

Gross pathological changes such as change in size, weight, color, consistency and texture were noted on each examined organ. Animals were euthanized humanely using overdose of pentobarbitone sodium 20% intravenously during the course of the infection as a result of showing severe clinical signs (animal welfare) or at the end of the experiment (70 days post infection). Two animals showing PCV below 15% were euthanized before the end of the intended experimental period because of welfare concerns. Representative tissue samples were taken from lymph nodes, spleen, liver, heart, kidney, lung and brain, and fixed in 10% neutral buffered formalin for histopathological studies. Formalin fixed tissues were dehydrated in ascending grades of alcohol, cleared by two changes of xylene, impregnated with molten paraffin wax, embedded, sectioned at 3-5µm thickness and finally stained with haematoxylin and eosin for microscopic examinations (Bush, 1975). Under oil emersion magnification, cellular changes such as macrophage and leucocyte infiltrations, cell and tissue damages etc were recorded (Annex 8).

3.7. Data management and statistical analysis

The entire data generated from the study was recorded in Excel spread sheets. Data management and analysis was done using SPSS soft wares of recent version. The statistical tests used were ANOVA and T- test.

4. RESULTS

4.1. Clinical findings

4.1.1. Clinical signs

All the inoculated calves developed clinical trypanosomosis, which was characterized by fever, pale mucous membranes, dullness, reduced weight gain, weight loss, emaciation, recumbency, mucopurulent ocular discharges, reduction in feed intake, and forced euthanasia of two infected calves due to ethical considerations (Table 1). The mean rectal temperatures of infected calves first peaked on day 7 PI with fluctuating values thereafter throughout the study period. The highest mean temperature noticed during the study was 40.15^{o}C, on day 7 post infection (TT and NT1groups). Rectal temperatures of all infected groups were significantly higher than those of the control group (P<0.001). On the other hand, there was no significant variation in mean rectal temperature between infected groups (Figure 1).

Table 1: Number of animals showing clinical signs with or without infection by *T. vivax*. Symptoms listed are those frequently observed and days indicate days of post infection. TT= tsetse infected, NT1= non-tsetse infected, NIC = non-infected control

Days after infection	Day 0				7				14				21				28				35				42				49				56				63				70			
Group	TT	NT1	NT2	NIC	TT	NT1	NT2	NIC	TT	NT1	NT2	NIC	TT	NT1	NT2	NIC	TT	NT1	NT2	NIC	TT	NT1	NT2	NIC	TT	NT1	NT2	NIC	TT	NT1	NT2	NIC	TT	NT1	NT2	NIC	TT	NT1	NT2	NIC	TT	NT1	NT2	NIC
Swollen lymph node					3	4	1		4	1	2		3	4	4		4	1	3		3	1	2		1	1	2		1	1	2		1	1	3		2	1	2		2	1	3	
Pale mucus membrane					3	3	4		4	3	4		4	4	4		4	4	4		3	4	3		2	2	3		3	3	4		2	3	4		3	3	4		3	3	4	
Rough hair coat					2	3	1		3	3	2		3	4	3		4	4	4		4	4	4		2	3	4		3	3	4		2	3	3		3	3	4		3	3	4	
Lacrimation					1	0	0		0	0	1		1	0	2		1	2	0		3	2	4		2	3	4		2	2	2		1	2	4		1	0	4		2	2	3	
Forced Euthanasia																	1		1																						3	3	4	4

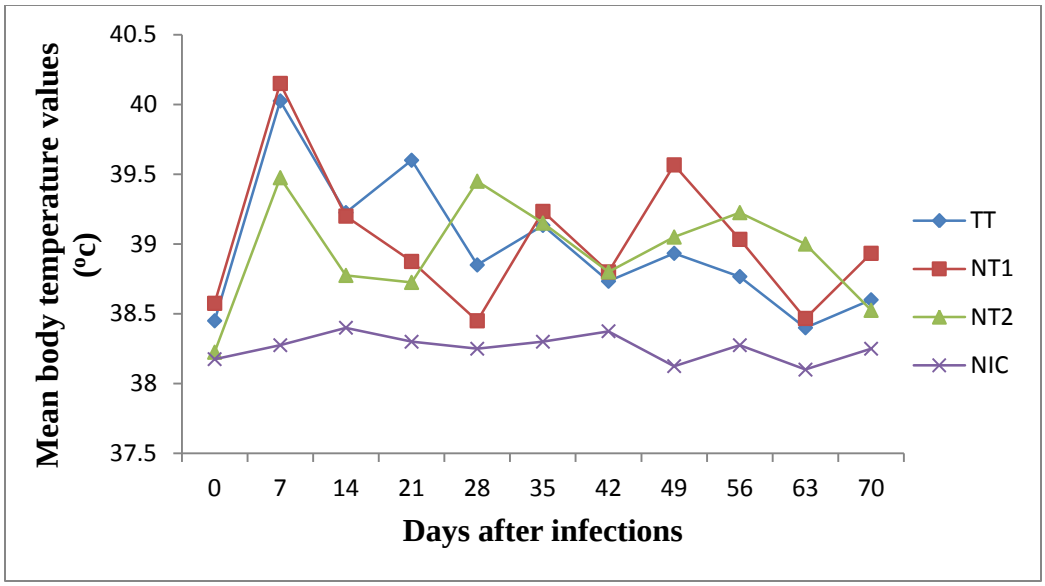

Figure 1. Mean body temperature of calves experimentally infected with tsetse and non-tsetse isolates of *T. vivax.*

4.1.2. Body weight

Pre-infection mean body weight values of all groups significantly varied (Mean for groups TT = 88.25Kg, NT1= 69Kg, NT2= 72Kg and NIC= 77Kg) at day 0. Hence the data after infection was normalized by dividing each PI mean value by the day 0 mean value and multiplying it by 100. Accordingly, non-infected control animals demonstrated a progressive and significantly higher weight gain (16.3% or 12kg at the end of the study period) compared to infected groups (P<0.001). On the other hand, there was an initial weight loss for the first three to four weeks in all infected groups followed by an attempt to restoration to pre-infection level. However, at the end of the experiment, only group NT2 has regained its initial weight (with 2% gains) whereas group TT was the biggest loser. Hence, a significant difference in mean body weight was observed between tsetse infected group and non-tsetse infected groups (P <0.001). No significant difference in body weight between NT1 and NT2 (Figure 2).

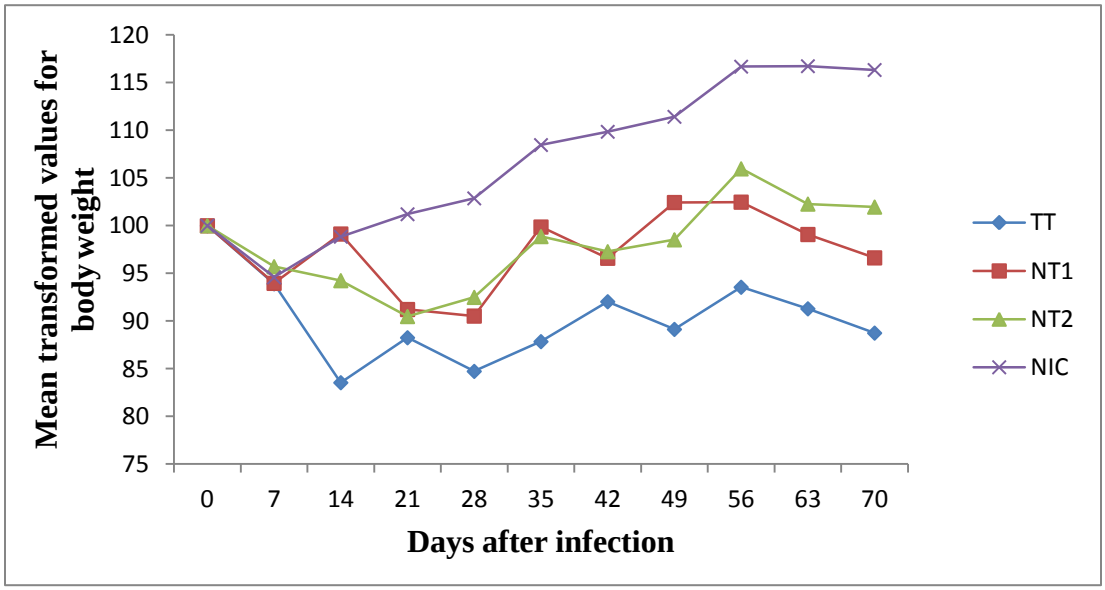

Figure 2. Mean body weight of calves experimentally infected with tsetse and non-tsetse isolates of *T. vivax.*

4.2. Development of parasitaemia

Trypanosomes were not detected in non-infected control animals throughout the study period. Parasitaemia was detected at the fourth day and seventh day post-infection (PI) in animals infected with *T. vivax* isolated from non-tsetse (NT1 and NT2) and tsetse infested areas (TT) respectively. Peak parasitaemia was seen on day 56 PI for group NT2 and day 14 PI for groups TT and NT1. Infected animals remained trypanosome positive with fluctuating parasitaemia throughout the experimental period (Figure 3). Two calves were euthanized on day 28 because of welfare issue, one from group TT and one from group NT1 post infections, during which time their levels of parasitaemia were (4+ and 5+) per microscopic field respectively.

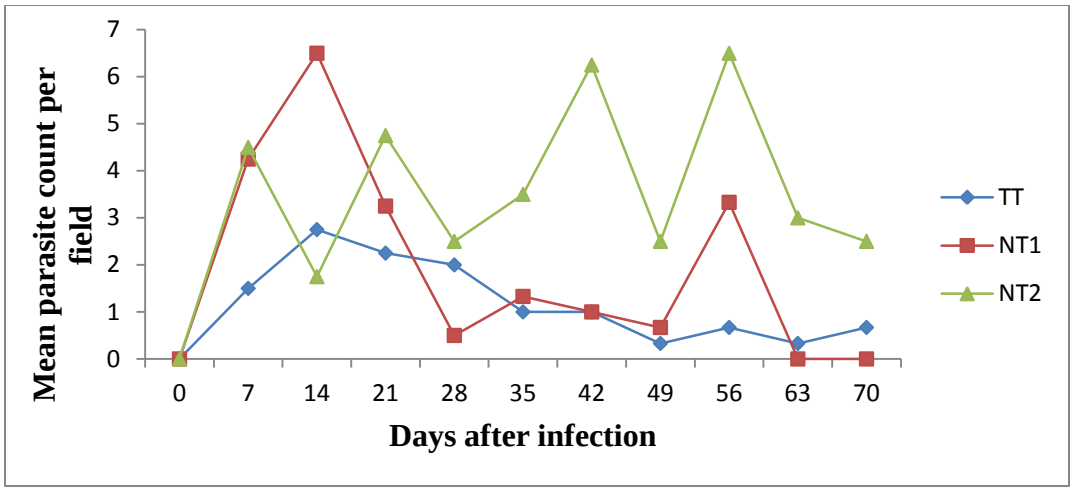

Figure 3: Changes in mean parasite counts per field of calves experimentally infected with *T. vivax* isolates from non-tsetse (NT1, NT2) and tsetse infested areas (TT).

4.3. Hematological findings

All along the course of the infection, a gradual but significant decrease in PCV values were detected in all infected groups compared to the negative control group ($P < 0.001$) where the values remained fairly constant (figure 4). The lowest mean PCV value recorded was 19±1.0 % in group TT, 15.67±2.082 % in group NT1 and 16.25±2.630 % in group NT2, which was noticed on day 42, 70 and 35 post infections respectively. The overall mean PCV value (for the 11 weeks) of animals infected with the isolates from non-tsetse area (NT1= 19.97±3.635%; NT2= 19.20±4.517%) was significantly lower than that of calves infected with the isolate from tsetse area (22.32 ±3.465%). Similarly, a significant decline in Hgb values was detected in all infected groups (Figure 5) compared to the pre-infection levels and the negative control group ($P < 0.001$). In addition, Hgb values for the group infected NT2 isolate (1.028±0.335g/dl) were remarkably lower than that of TT group ($P < 0.013$). However, differences between the other combinations were not significant.

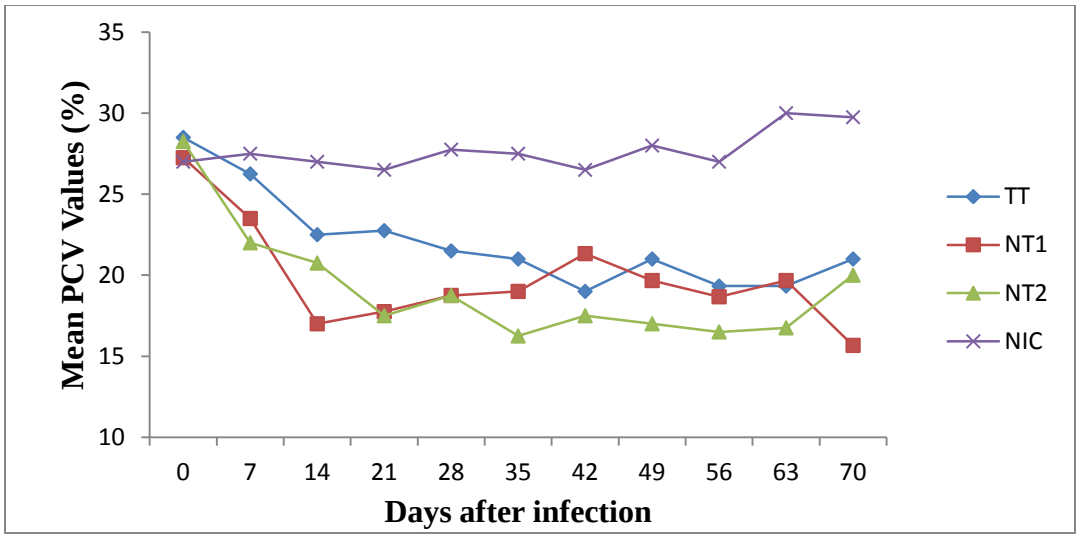

Figure 4: Changes in mean PCV values of calves experimentally infected with *T. vivax* isolates from non-tsetse area (NT1, NT2) and tsetse infested area (TT).

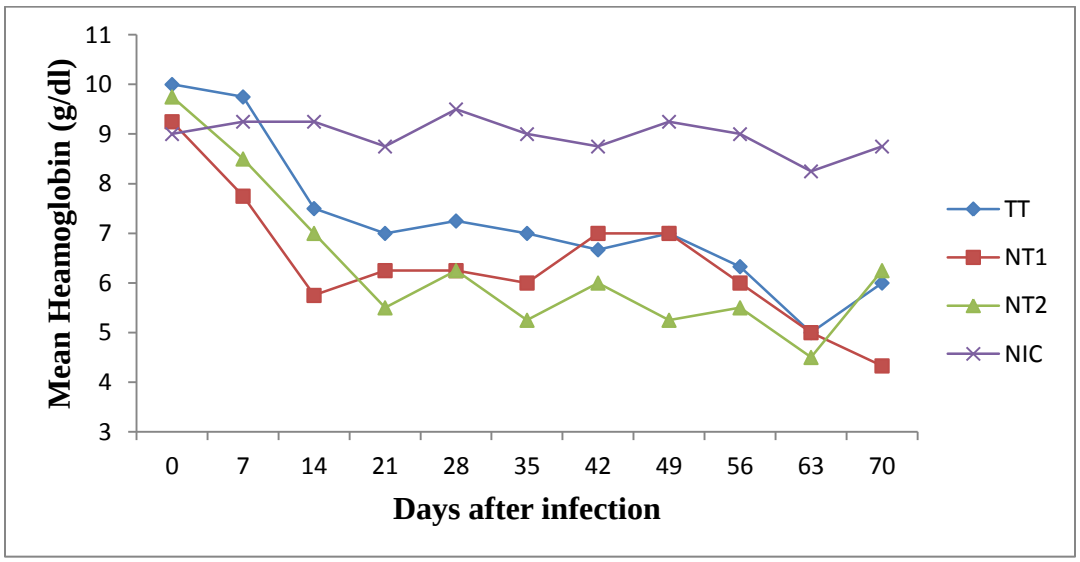

Figure 5: Changes in mean Hgb values of calves experimentally infected with *T. vivax* isolates from non-tsetse area (NT1, NT2) and tsetse infested area (TT).

4.4. Serum Biochemical Analysis

4.4.1. Glucose concentration

Pre-infection mean serum glucose values of all groups significantly varied (Mean for groups TT = 39.75mg/dl, NT1= 41.5mg/dl, NT2= 33.75mg/dl and NIC= 26.25mg/dl) at day 0. Hence the data after infection was normalized by dividing each PI mean value by the day 0 mean value and multiplying it by 100 (Figure 6). Accordingly, groups TT and NT1 have lost glucose until day 21 to 28 PI whereas groups NT2 and NIC have maintained their serum glucose levels above pre-infection values throughout the experimental period. The mean serum glucose concentration for all infected groups was lower than that of the non-infected control values (P<0.001). Moreover, glucose concentrations were lower for group TT compared to that of group NT2 (P<0.001). However, it has no significant variation when compared to group NT1.

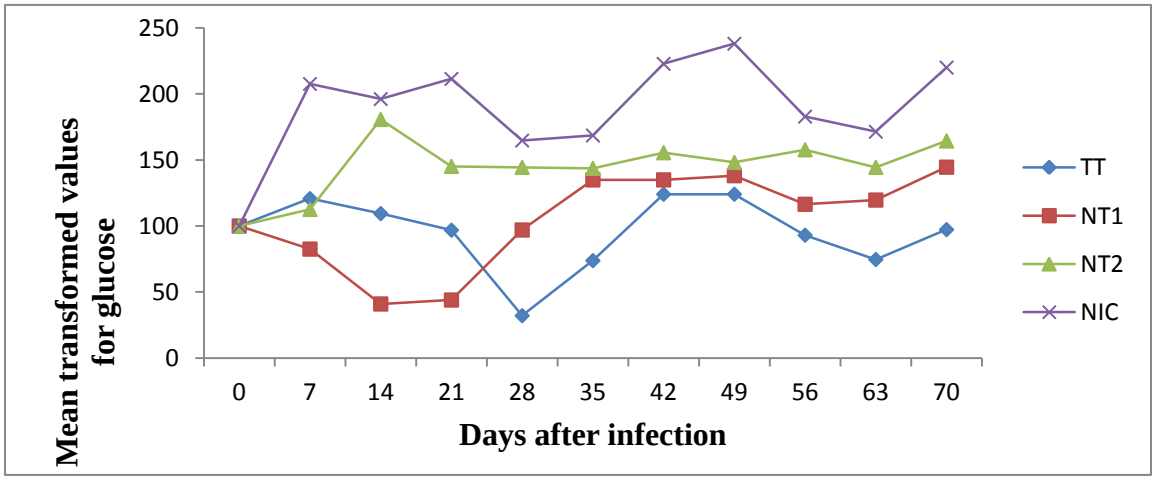

Figure 6: Changes in mean Glucose values of calves experimentally infected with *T. vivax* isolates from non-tsetse area (NT1, NT2) and tsetse infested area (TT).

4.4.2. Total cholesterol concentration

The mean cholesterol concentration declined until day 14 (NT2), 21 (NT1) and 42 (TT) post infection (Figure 7). Then, there was a gradual increase until the end of the experiment but values still remaining significantly lower than that of control animals (P<0.001). However, the mean cholesterol concentration between infected groups was not significant (P> 0.05).

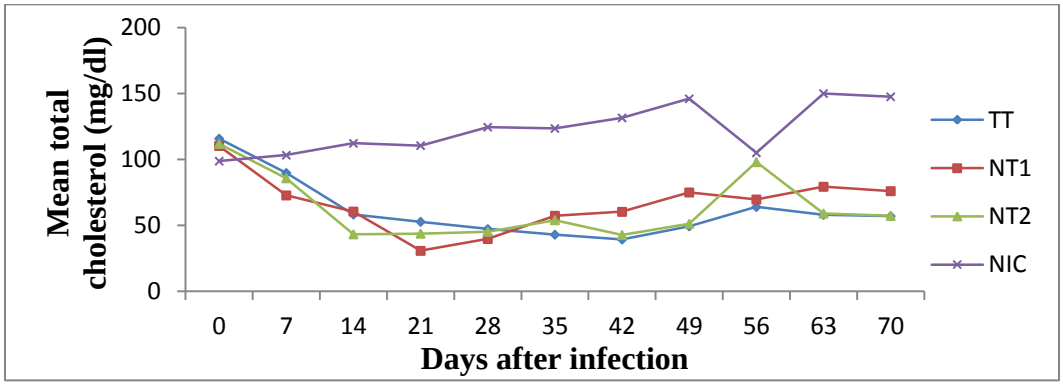

Figure 7: Changes in mean Cholesterol values of calves experimentally infected with *T. vivax* isolates from non-tsetse area (NT1, NT2) and tsetse infested area (TT).

4.4.3. Albumin concentration

The mean albumin concentration declined from day 0 to day 21 PI for all groups. While control animals which have received non-infected blood have regained their albumin concentration to normality, the values for the infected group remained significantly lower (P<0.001) until day 63 PI. There was no notable variation in albumin concentration between infected groups all along the experimental period (Figure 8).

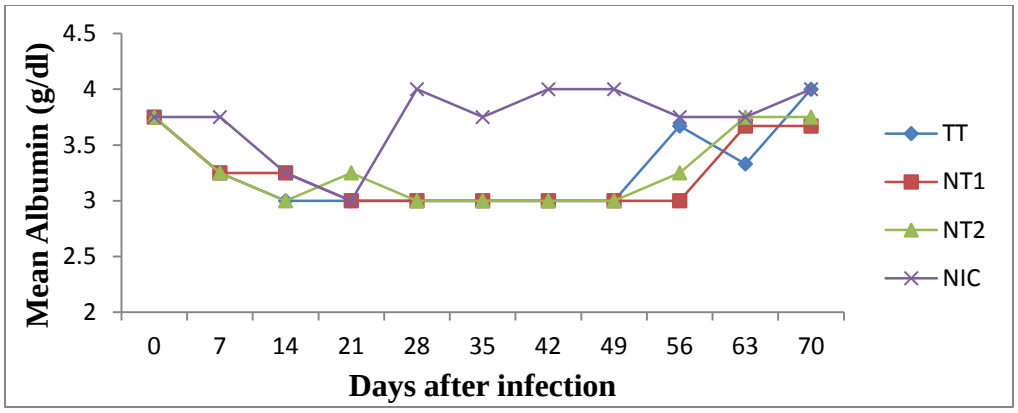

Figure 8: Changes in mean Albumin values of calves experimentally infected with T. Vivax isolates from non-tsetse area (NT1, NT2) and tsetse infested area (TT).

4.4.4. Total protein concentration

The mean total protein concentration between infected and non-infected groups was not significantly different ($P>0.05$). In addition, the mean protein concentration between infected groups was not significant ($P> 0.05$). However, a gradual decrease in the mean values of total proteins was observed until day 21-28 PI in the infected animals which later steadily increased to values higher than the pre-infection levels including for the control group (Figure 9).

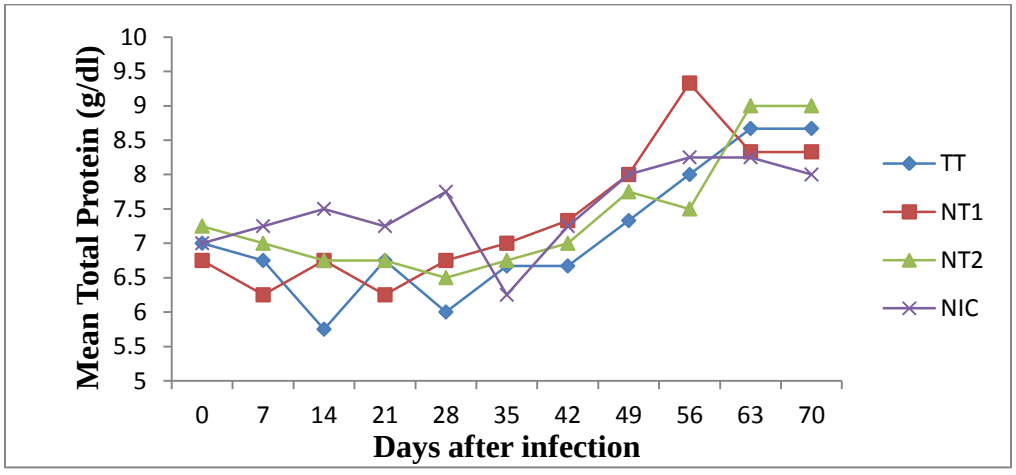

Figure 9: Changes in mean Total protein values of calves experimentally infected with *T. vivax* isolates from non-tsetse area (NT1, NT2) and tsetse infested area (TT).

4.4.5. Aspartate aminotransferase (AST)

An acute increase in serum aspartate aminotransferase (AST) level was observed in NT1 and NT2 groups 7 days PI. There was significant difference between values of TT and NT1 groups and NT1 and NIC ($P < 0.05$). Values for NT1 remained higher than those in other groups for the most part of the infection period (Figure 10) whereas the other groups including the NIC demonstrated a continuous gradual increase in serum AST level until the end of the experiment and difference was significant only between TT and NIC from 28 to 56 day PI ($P< 0.01$). The highest mean level of AST was recorded on day 7 (for NT1= 141.75±24.309 IU/l), day 42 (for TT= 106.67±21.197 IU/l) and day 63 (NT2= 95.75±20.597 IU/l) post infection.

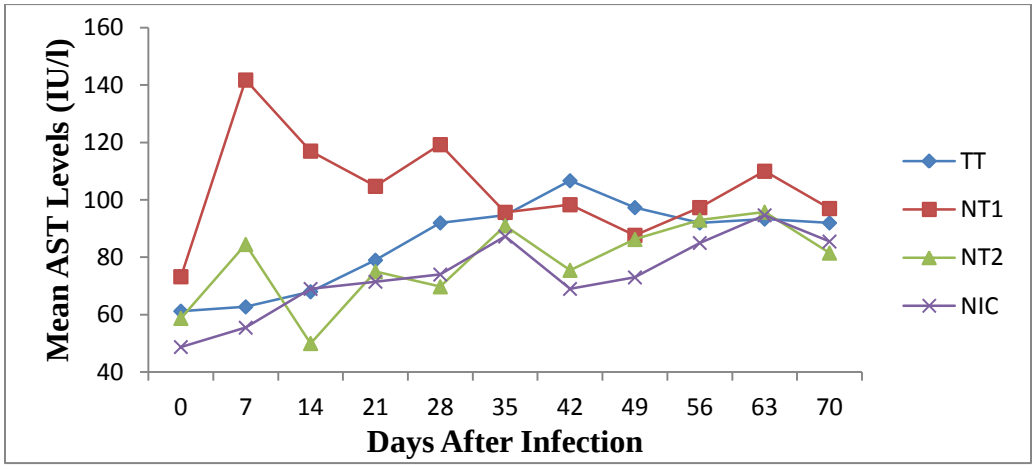

Figure 10: Changes in mean AST values of calves experimentally infected with *T. vivax* isolates from non-tsetse area (NT1, NT2) and tsetse infested area (TT).

4.4.6. Alanine aminotransferase (ALT)

The mean serum alanine aminotransferase (ALT) concentration peaked early on day 7 PI for NT1 (65.5 IU/l) followed by TT (65.0IU/l) and NT2 (69.25IU/l) values (Figure 11). For the most part of the infection period, values for infected groups remained significantly higher than control ($P<0.001$). However, the mean ALT concentration between the infected groups was not significant ($P > 0.05$).

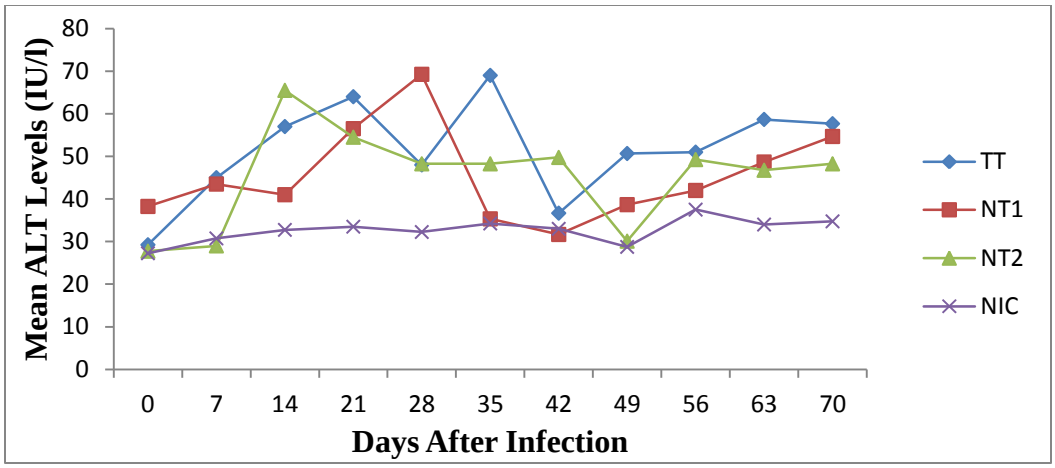

Figure 11: Changes in mean ALT values of calves experimentally infected with *T. vivax* isolates from non-tsetse area (NT1, NT2) and tsetse infested area (TT).

4.4.7. Alkaline phosphatase (ALP)

The mean serum alkaline phosphatase (ALP) concentration first peaked on day 14 PI for NT1 (549.75 IU/l) followed by TT (561.67IU/l) and NT2 (502.5IU/l) on day 35 PI. However, for the most part of the infection period, values for infected groups remained comparable to that of control animals ($P>0.05$). Moreover, there was no significant difference between the infected groups for levels of ALP (Figure 12).

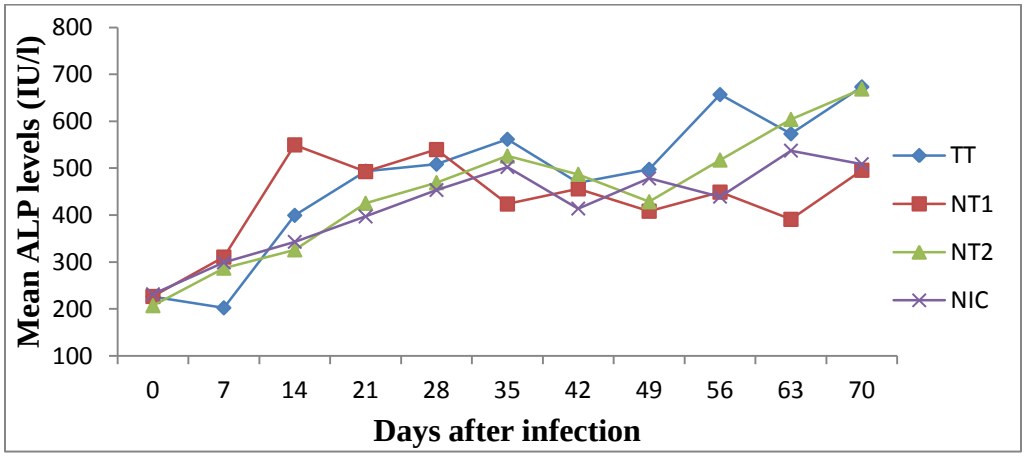

Figure 12: Changes in mean ALP values of calves experimentally infected with *T. vivax* isolates from non-tsetse area (NT1, NT2) and tsetse infested area (TT).

4.5. Cytokine analysis

4.5.1. Tumor necrosis factor- alpha (TNF-α)

Pre-infection OD values of all groups significantly varied (Mean for groups TT and NT2= 0.4425nm, NT1= 1.2748nm and NIC= 0.9098nm) at day 0. Hence the data after infection was normalized by dividing each PI OD value by the day 0 OD value and multiplying it by 100. Accordingly, a bimodal peak was observed in TNF-α OD value for TT and NT2 groups whereas values remained close to control levels for NT1 group except at day 14. The rapid up surge of TNF-α in this two groups shows a significantly increased cytokine production compared to control animals and group NT1 ($P<0.001$). On the other hand, values do not significantly differ between NT1 and NIC and between TT and NT2 (Figure 13).

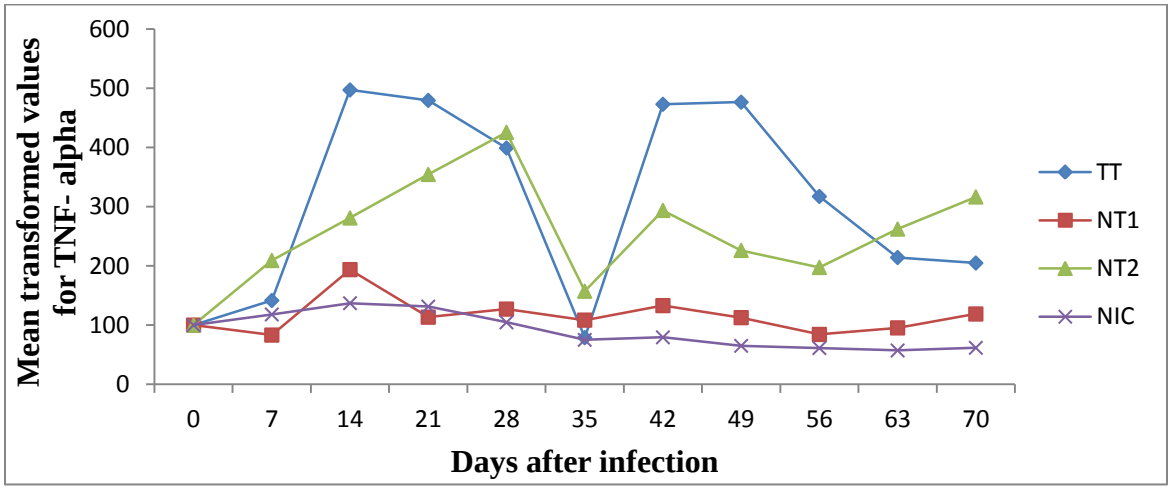

Figure 13: Changes in mean TNF-α value of calves experimentally infected with *T. vivax* isolates from non-tsetse area (NT1, NT2) and tsetse infested area (TT).

4.5.2. Interferon gamma (IFN-γ)

Pre-infection OD values of all groups significantly varied (Mean for groups TT= 0.2178nm, NT1= 0.9612nm, NT2= 0.2907nm, and NIC= 0.4352nm) at day 0. Hence, the data after infection was normalized by dividing each PI OD value by the day 0 OD value and multiplying it by 100. A very significant increase in the mean OD values of IFN-γ in

group TT until day 49 PI followed by a sharp decline was observed and the difference from values registered for other groups was remarkable (P<0.001). On the other hand, IFN-γ OD readings for NT1 and NT2 are comparable to that of NIC group although few animals in each group have demonstrated higher values (Figure 14).

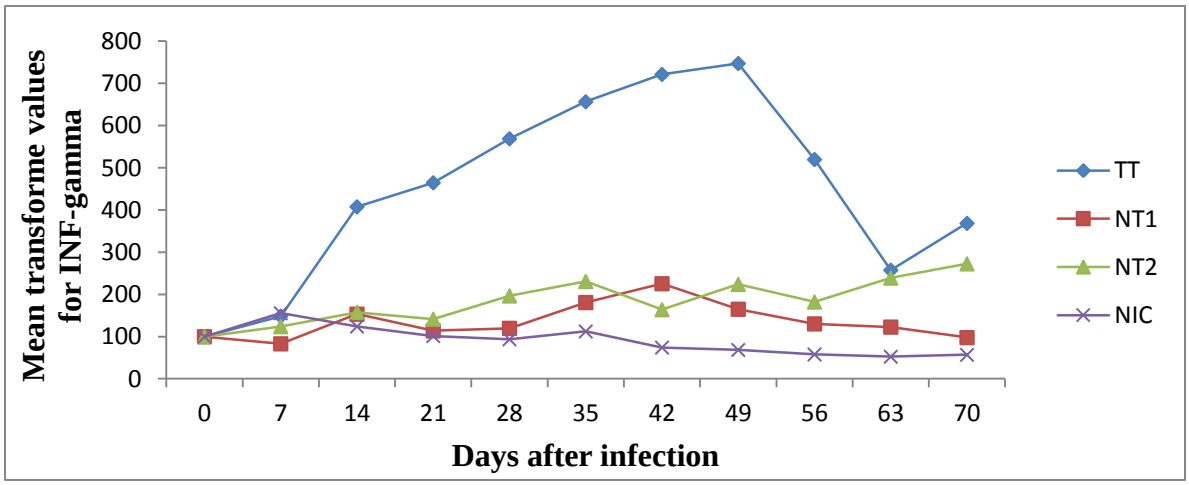

Figure 14: Changes in mean IFN-γ value of calves experimentally infected with *T. vivax* isolates from non-tsetse area (NT1, NT2) and tsetse infested area (TT).

4.5.3. Interleukin- 10 (IL-10)

Pre-infection OD values of all groups significantly varied (Mean for groups TT= 0.3042nm, NT1= 1.5700nm, NT2= 0.843nm, and NIC= 0.9687nm) at day 0. Hence, the data after infection was normalized by dividing each PI OD value by the day 0 OD value and multiplying it by 100. Moreover, although serum samples were collected until day 70 PI, due to loss of part of IL-10 reagent during shipment process, samples were processed only until day 35 PI. Accordingly, similar to IFN-γ, a very high increase in the mean OD values of IL-10 in group TT was observed until day 28 PI followed by a decline and the difference from values registered for other groups was significant (P<0.001). On the other hand, IL-10 OD readings for NT1 and NT2 are comparable to that of NIC group (Figure 15).

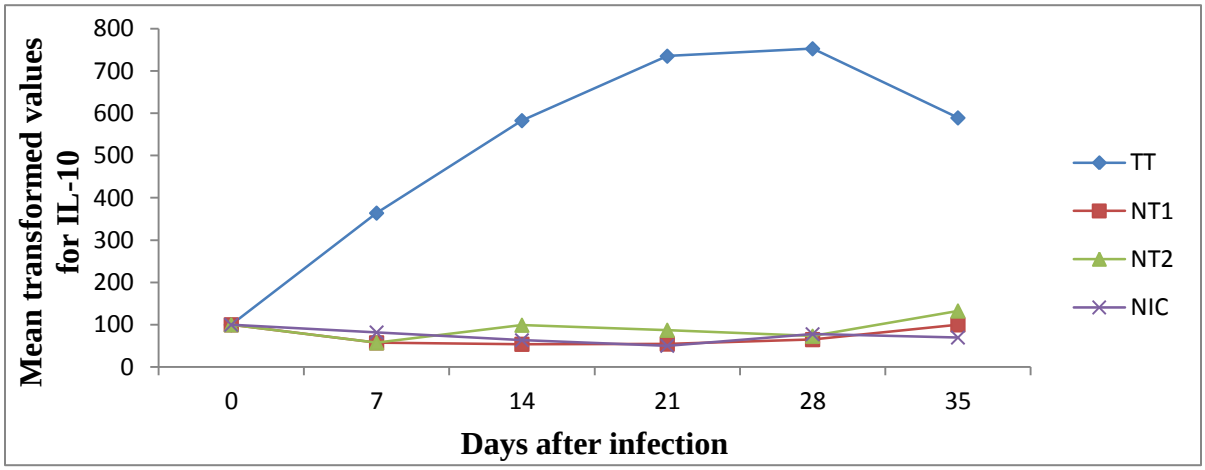

Figure 15: Changes in mean IL-10 value of calves experimentally infected with *T. vivax* isolates from non-tsetse area (NT1, NT2) and tsetse infested area (TT).

4.5.4. Interleukin 12 (IL-12)

Pre-infection OD values of all groups significantly varied (Mean for groups TT= 0.5467nm, NT1= 2.1562nm, NT2= 1.5097nm, and NIC= 1.5097nm) at day 0. Hence, the data after infection was normalized by dividing each PI OD value by the day 0 OD value and multiplying it by 100. Moreover, although serum samples were collected until day 70 PI, due to loss of part of IL-10 reagent during shipment process, samples were processed only until day 35 PI. Accordingly, similar to IFN-γ and IL-10, a very high increase in the mean OD values of IL-12 in group TT was observed until day 35 PI and the difference from values registered for other groups was significant ($P<0.001$). On the other hand, IL-12 OD readings for NT1 and NT2 are comparable to that of NIC group although few animas in each group have demonstrated higher values (Figure 16).

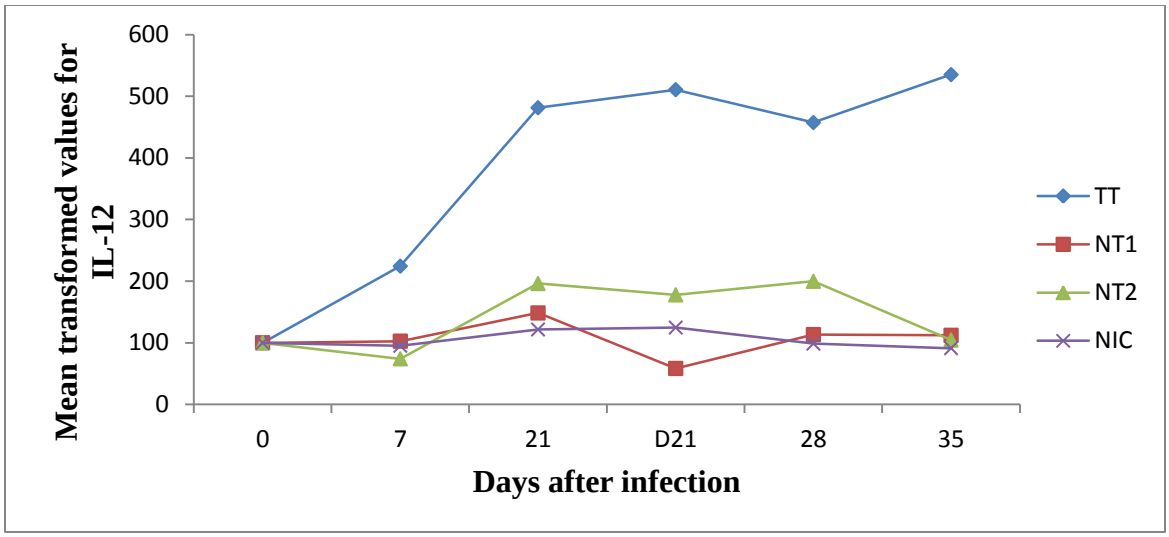

Figure 16: Changes in mean IL-12 value of calves experimentally infected with *T. vivax* isolates from non-tsetse area (NT1, NT2) and tsetse infested area (TT).

4.6. Gross pathological analysis

The most common gross lesions include splenomegaly with hemorrhagic spleen (Figure 17A); lymphadenopathy with hemorrhagic and edematous lymph nodes (Figure 17B); pneumonic (dorso caudal part) especially of interstitial type and lungs were rubbery inconsistency and meaty in appearance (Figure 18A). Liver of most infected calves were covered with fibrineous exudates (gray mass) and were adhered with peritoneum (Figure 18B). In some animals the livers were congested (bronze), enlarged, and edematous. In two of infected calves (D66 and D67) the livers were adhered to the kidney and intestine. Other significant and frequent lesions in infected calves were widespread ecchymotic hemorrhages on internal organs especially on kidney (Figure 20B) and the heart. Subepicardial ecchymotic and/or petechial hemorrhages were the common gross lesion on the heart (Figure 19) and this lesion repeat itself than any other lesion among the infected calves. In some infected calves the hearts were adhere with chest wall, flappy and with serous atrophy of fat. The brain of most infected calves was edematous and in some there were severe edema and focal hemorrhages and meningitis (Figure 20A). However, the post mortem examination of the animal in the control group did not reveal any significant gross lesions.

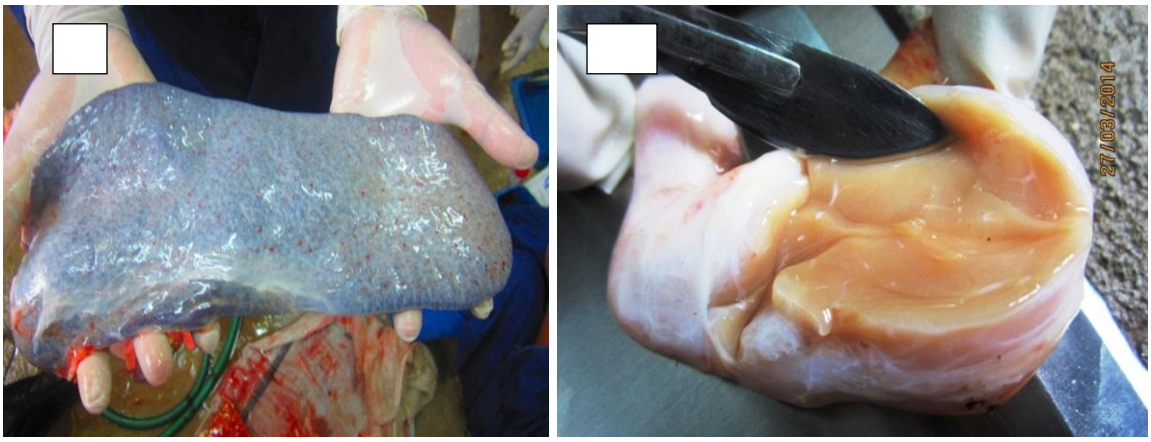

Figure 17: Hemorrhagic and enlarged spleen with rounded edge (A). Enlarged and edematous lymphnode (B).

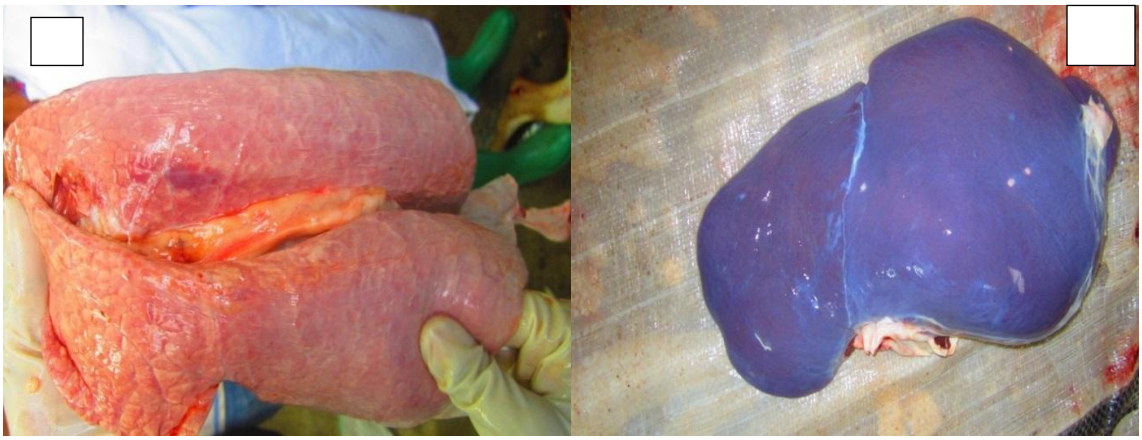

Figure 18: Lung showing rubbery, meaty in appearance, and distended interstitium; (A). Enlarged, and edematous liver with rounded edge and focal necrotic lesion (B).

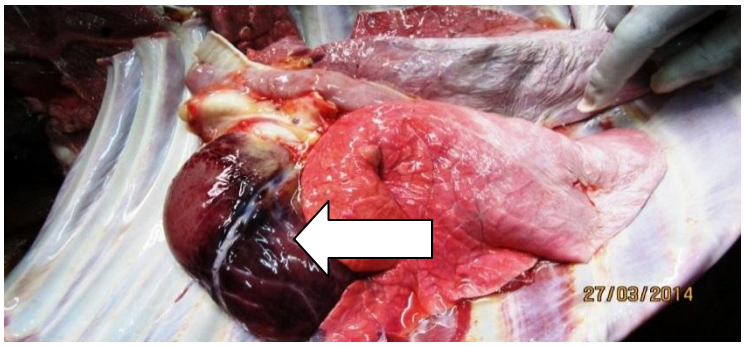

Figure 19: Heart with widespread ecchymotic hemorrhages

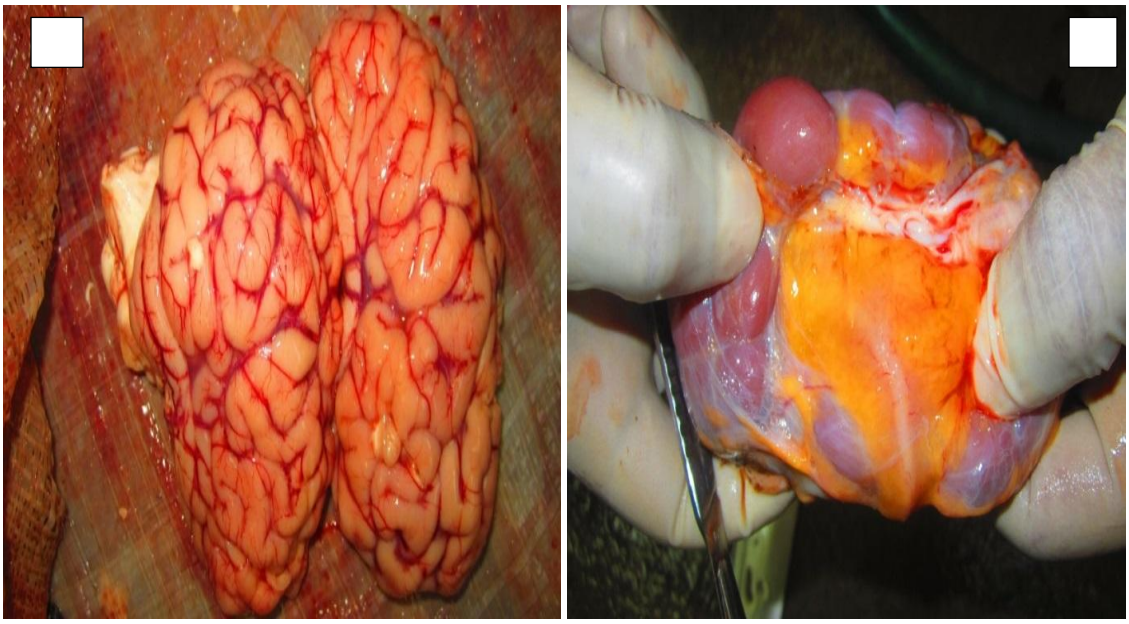

Figure 20: Edematous brain (A). Swollen hemorrhagic kidney with yellowish discoloration (B).

4.7. Histopathological analysis

Spleen: Lymphoid hyperplasia in the white and red pulp of spleen was the common microscopic lesion in spleen of infected calves. In some calves there was lymphoid depletion with formation of cavity at the center of lymphoid follicles in the white pulp. At the red pulp the spleen showed lymphoid hyperplasia with formation of follicles dominated by lymphoblast (Figure 21A).

Lymph node: The most frequent and common lesion of lymph nodes of infected calves were marked lymphoid hyperplasia with prominent germinal centers. There was a marked increase of large lymphocytes and plasma cells (Figure 21B).

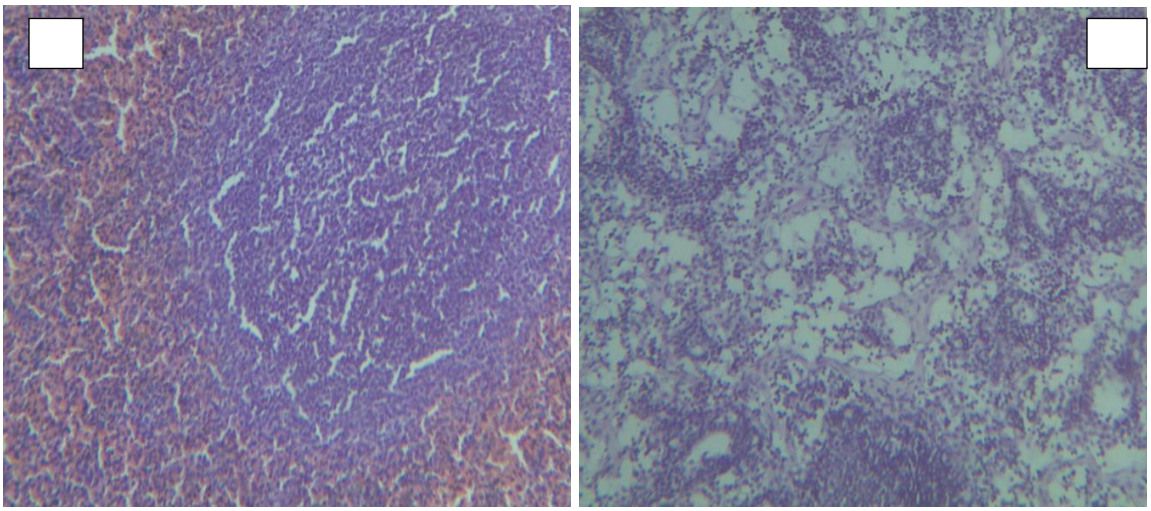

Figure 21: Spleen with severe lymphoid proliferation forming lymphoid follicles in the red zone (A). Lymph node showing severe lymphoid hyperplasia which forms lymphoid cords in the medullary region of the lymph node (B). (H & E).

Lung: In almost all infected calves microscopic examination of the lung showed interstitial pneumonia with severe thickening of interstitial wall due to proliferation of cells in the area (smooth muscle and fibroblasts) which resulted in severe compression of alveolar lumen. The alveoli located at the counter position of those compressed were ruptured resulting in emphysema like lesions (Figure 22).

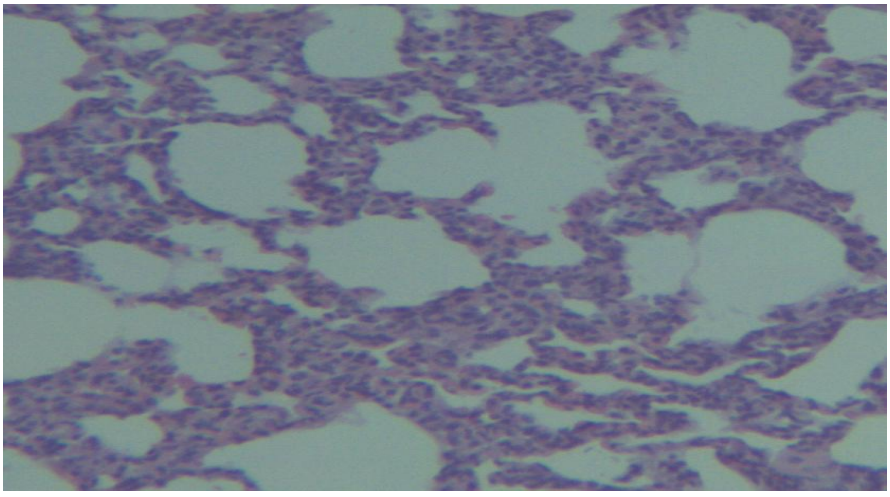

Figure 22: Lung with severe proliferative interstitial pneumonia with severe compression of the alveoli lumens in affected part. The nearby alveoli are ruptured (H & E).

Liver: The major microscopic changes in the liver of the infected calves were zonal sever hepatic necrosis especially of the centri-lobular and peri-portal region of the liver. Hepatocytes at the center of necrotized regions were either totally lysed with karyorrhectic nuclei or some are with condensed pykinotic nuclei. There were heavy infiltration of mononuclear leukocytes especially lymphocytes in the portal areas of the liver. In addition, histopathological examination of the liver revealed the blood vessels of the liver are dilated and filled with proteinacious hyaline membranes and fibrin deposits (Figure 23A and B).

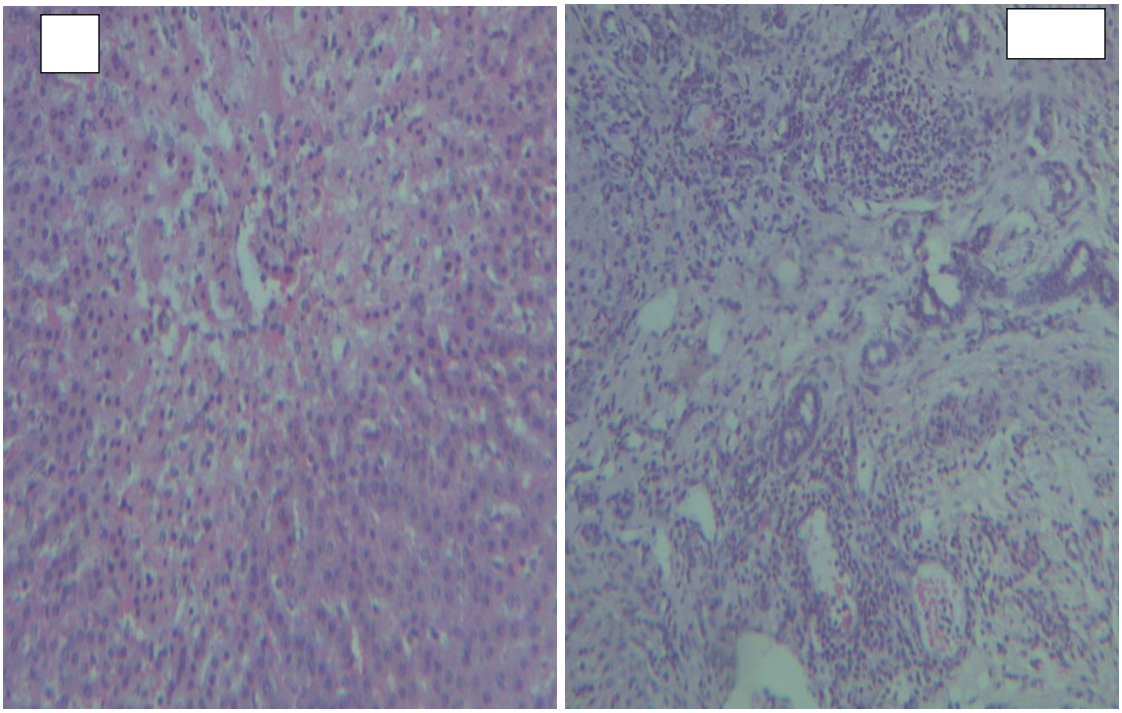

Figure 23: Liver showing centrilobular hepatocyte necrosis. Most hepatocytes in affected area have condensed (pyknotic) nuclei, some with rrhectic nuclei, some with nuclei lysis and they stains eosinophilic (A), cholangio-hepatitis with dense accumulation of mononuclear leukocytes especially lymphocytes, plasma cells, macrophages, proliferation of the biliary ducts and dilation of the vasculatures (B) (Haematoxylin and Eosin stain).

Kidney: The kidneys showed tubulo-interstitial nephritis with thickening of the interstitium due to multiplication of fibroblasts and tubular rrhexis with deposition of hyaline membranes in affected tubules (Figure 24).

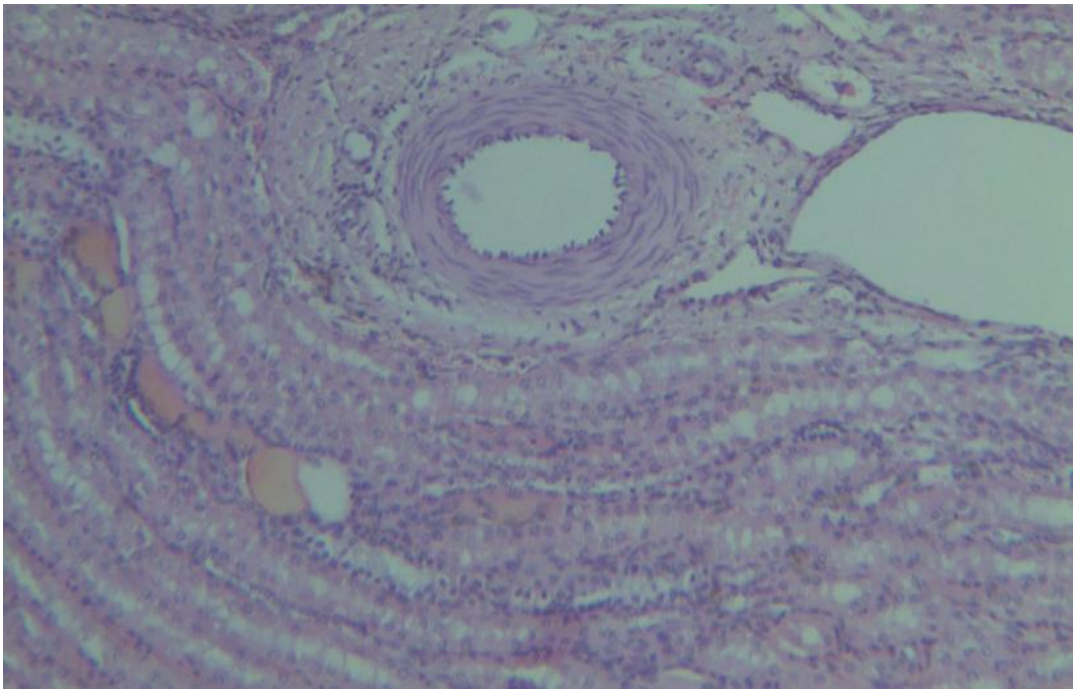

Figure 24: Kidney showing tubulo-interstitial nephritis characterized by sloughing of the tubular epithelium, deposition of uniformly staining hyaline substance and infiltration of inflammatory cells into interstitial tissues (Haematoxylin &Eosin stain).

Heart: The histopathological investigation of the heart showed multifocal myocarditis with infiltration of mononuclear cells especially of lymphocytes, few plasma cells and macrophages. The infection also induced heavy mononuclear cells infiltration to perimycial connective tissue with perivasculatis with domination of lymphocytes. The heart showed myocarditis and myocardial necrosis, sever myocardial degeneration with vacuolization; sever myocardial necrosis with mononuclear cells infiltration (especially lymphocytes). In addition, histopathological examinations of the hearts showed sever inflammation or perivasculatis of blood vessels of perineurium with massive infiltration of inflammatory cells (lymphocytes, plasma cells and macrophages). The blood vessels also showed fibrin deposits (Figure 25).

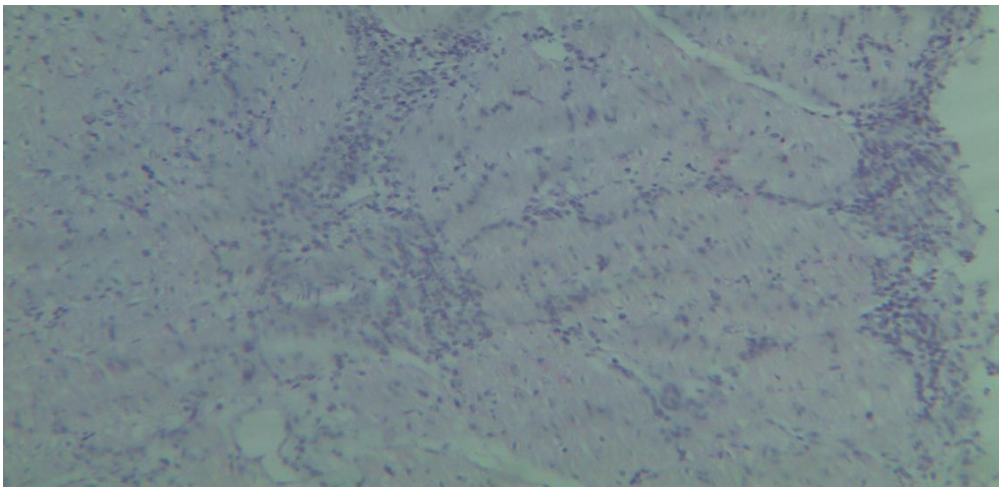

Figure 25: Myocarditis with heavy infiltration of mononuclear cells especially of lymphocytes

Brain: In almost all infected calves there is a meningoencephalitis of varying severity in brain. The brain showed neuronal necrosis with shrunken angular neuron and pyknotic somas. In some brains with neuronal necrosis there were gitter cells, small glial nodules (neurophagia), ischemic type neuronal necrosis. There were astrogliosis with hypertrophy of astrocytes in infected brains. Lesions in vasculature of the brain were perivasculatis with mononuclear cuffs and focal gliosis (Figure 26).

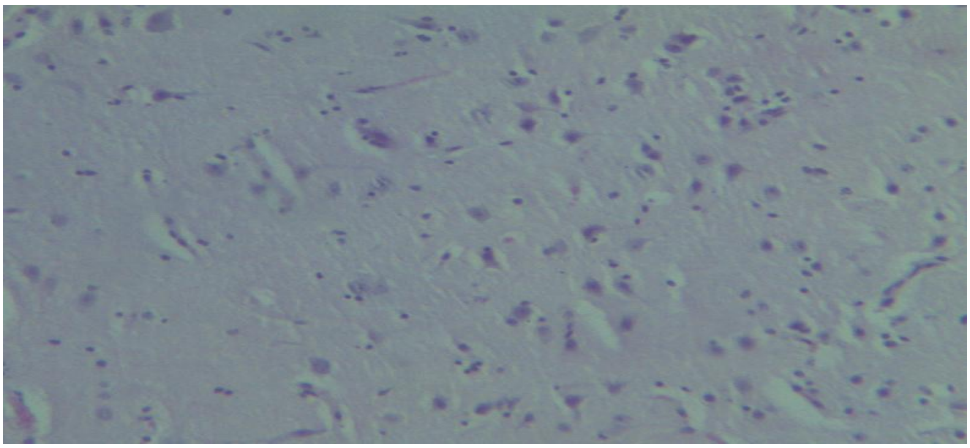

Figure 26: Brain with neuronal necrosis which is characterized by angular and shrunken cell bodies, increased gliosis and small glial nodules (upper right corner) (Haematoxylin & Eosin stain).

5. DISSCUSSION

The *Trypanosoma vivax* isolates used in this experiment were collected from naturally infected cattle from tsetse and non-tsetse infested areas. The results of this experimental study describes that *T. vivax* isolates from both tsetse and non-tsetse infested areas were infective for young zebu cattle which were brought from the central highland region of Ethiopia. It was observed that parasitaemia was detected four and seven days post infection and persisted until the end of the experiment.

5.1. Impacts of trypanosome infection on various parameters of infected calves

Fever, reduced weight gain, reduced feed intake, swollen lymph nodes and pale mucus member were common clinical signs observed in almost all infected animals but the number of animals showing such signs has reduced towards the end of the experimental study. This finding agrees with previous works done on experimentally or naturally infected animals with different species of trypanosomes (Allam *et al*., 2011; Mersha *et al*., 2012). For the most part of the experimental period, infected animals lost significant weight as compared to the continuous increment in non-infected animals. This finding agrees with that of Faye *et al*., (2005) who reported decrease in body weight gain in west African dwarf goats experimentally infected with *T. congolense* but it disagree with the finding of Osman *et al*., (2008) who reported no reduction in body weight gain in Nubian goats, Nilotic dwarf goats and Garag ewes experimentally infected with a mechanically transmitted *T. vivax* stock. Such variation might be attributed to variations in animal hosts and strain of parasites.

Development of anaemia as measured by PCV and blood Hgb values often gives a reliable indication of the disease status and productive performance of trypanosome infected animals (Ekanem *et al*., 2005, 2006). Trypanosome infection may cause anaemia as a result of massive erythrophagocytosis by an expanded and active mononuclear phagocytic system (MPS) of the host (Suleiman and Adeyemi, 2010). In agreement with the above fact, this study revealed that both tsetse and non-tsetse isolates of *T. vivax* caused significant reductions in PCV and Hgb concentration in the experimentally

infected young zebu cattle. This result also agrees with the findings of Silva *et al*., (1998) and Osman *et al*., (2008) in *T. vivax* and *T. congolense* infections in goats and cattle respectively and Sharma *et al*., (2000) in *T. evansi* infection in Barbari goats. According to Osman *et al*., (2008), anaemia and leukopenia are cardinal signs of trypanosomosis and reported that anaemia in trypanosomosis might be due to the haemolysins induced by the trypanosomes, different immunological factors, increased erythrophagocytosis, haemodilution and dyshaemopoiesis.

Following trypanosome infection, hypoglycemia was considered only important in hyper acute infections characterized by enormous numbers of trypanosomes in the circulation (Katunguka-Rwakishaya *et al*., 1999). Similarly, this study revealed that early reduction in glucose level was pronounced on two of the infected groups whereas the third group showed rather an increase in the level of serum glucose suggesting that changes in glucose concentration following trypanosome infections are not consistent. Faye *et al*., (2005) and Van Dam (1996) reported an increment of glucose concentration following infection of West African Dwarf goats probably showing a positive energy balance.

Abnormalities of lipid metabolism have been demostrated in several laboratory and domestic animals infected with various species of trypanosomes. When rabbits were infected with various species of trypanosomes (Allam *et al*., 2011), an increase in plasma cholesterol and total lipids occurred. In contrast, *T. rhodesiense* infection in cattle (Welde *et al*., 1989) and *T. congolense* infection in sheep (Katunguka- Rwakishaya *et al*., 1999) were associated with a decrease in serum cholesterol concentration. Our experimental findings are in agreement with the latter observation whereby all infected groups exhibited reduced serum total cholesterol levels compared to non-infected control animals. The present finding also in agreement with the finding of Biryomumaisho *et al*., (2003) in which he observed a rapid drop in cholesterol concentration in small east African goats infected with *T. congolense* and *T. brucei* and suggested that trypanosomes may require cholesterol for membrane synthesis.

In this study, there was a significant decrease in serum albumin concentration until day 49 PI in all infected groups as compared to the non-infected control whereas total protein concentration showed a gradual increment until the end of the experiment. This is in accord with the finding of Aquino *et al*., (2002) in his study of hematological, biochemical and anatomo-pathological aspects of the experimental infection with *T. evansi* in dogs. He reported that infected dogs showed hyperglobulinemia with decreased albumin globulin ratio. It is suggestive that fall in albumin levels was secondary to hyperglobulinemia as a compensatory mechanism for the maintenance of normal blood viscosity increased by high globulin levels.

A gradual decrease in the mean values of serum total proteins in the first four weeks and a compensatory rise above pre-infection levels until the end of the experiment was observed for all infected groups. Up to 35 days of post infection in group NT1 and NT2; 42 days post infection in group TT, observed in the infected animals during this study was in agreement with previous findings of Biryomumaisho *et al*., (2003) in small east African goats experimentally infected with *T. congolense* and *T. brucei* and Allam *et al*., (2012) in gilts experimentally infected with *T. brucei* and partly in accord with Taiwo *et al*., (2003) who observed no change in levels of total serum proteins from pre-infected values at the initial stage of the infection, but in the later stage the levels increased significantly above pre-infection levels in sheep infected experimentally with *T. brucei.* The increases in total serum protein in the later stage in all infected groups were in line with the earlier observations in Gambian trypanosomosis of vervet monkeys (Abenga and Anosa, 2005). Protein levels usually drop in trypanosome infections as a result of depressed albumin levels. The increase in protein levels during the chronic phase of the infection is usually due to the increase in globulin levels. This is as a result of the immune response by the animals to the infection as (Allam *et al*., 2011).

The general increase in AST levels pointed out in this study agrees with the results obtained during an infection in gilts by *T. brucei* (Allam *et al*., 2011), sheep by *T. brucei* (Taiwo *et al*., 2003), *T. vivax* infection of cattle and sheep, *T. congolense* infection of goats (Adah *et al*., 1992) and also dogs infected with *T. brucei* (Omotainse *et al*., 1994).

However, it contradicts with the observations that were made by Taiwo *et al*., (2003) during an infection of sheep with *T. congolense*. The causes of the elevation of AST levels in the serum of animals could be necrosis of the liver, skeletal muscles and kidneys (Lording and Friend, 1991).

The increase in ALT levels recorded in this study was in line with the results obtained during an infection in dogs by *T. evansi* (Aquino *et al*., 2002). Increases in ALT and AST activity have been also observed in a *T. evansi* naturally infected dogs (Sandoval *et al*., 1994). Elevated levels of serum ALT enzyme may suggest liver damage. It may also indicate damage to other organs such as kidney, muscle and heart where this enzyme could also be produced in small amount. Raised activities of ALP can be seen in inflammatory conditions of the gut and liver, while active hepatocellular damage is reflected by increases in plasma levels of AST and ALT (Wurochekke *et al*., 2008; Oyewole and Malomo, 2009).

The present study shows that generally secretion of TNF-α, IL-10, IL-12 and IFN-γ increased after experimental infection of calves with *T. vivax.* The patterns of appearance of IL-10, IL-12, TNF-α and IFN-γ production were essentially similar. The increase in TNF- α recorded in this study largely agrees with the results obtained during infection of mice *by T. congolense* (O'Gorman *et al*., 2006; Kitani *et al*., 2004). The more severe influences on lymphoid organs seen during infection such as the greater parasite burden in the spleen and the stimulation of splenic lymphoid cells appear to be required for IFN induction by this organism (Kitani *et al*., 2004). Similarly, the increased production of IL-10 revealed for group TT in this study largely agrees with the result obtained during an infection in mice by *T. Congolense* (Kitani *et al*., 2004). Both IFN-γ and IL-10 serum levels are increased after trypanosome infection and can influence survival. This is supported by the finding of Hertz *et al*., (1998) in which he investigated that, *Trypanosoma brucei rhodesiense* infected C57BL/6 mice that were deficient for the IFN-γ gene developed higher parasitemias and shorter survival rates than wild-type counterparts. Similarly the increase in the production of IL-12 in group TT in this study agrees with Uzonna *et al*., (1999) in his experimental study of mice infected with *T.*

congolense and with O'Gorman *et al*., (2006) in his experimental study of cattle infected with *T. congolense*.

The gross pathological examination the different organs from positive control animals showed no visible areas of gross lesion. In contrast organs from infected groups (both tsetse and non-tsetse isolates) were revealed different lesions. The splenomegaly and hepatomegaly observed in this study agrees with the findings of Taiwo *et al*., (2003). The various forms of congestion observed were also in consonance with findings of Taiwo *et al*., (2003). The histopathological examination of tissue sections from positive control animals showed no visible area of lesions. In contrast sections of tissues from infected groups (both tsetse and non-tsetse isolates) revealed gross distortion of tissue architecture with complete loss of cellular morphology marked by pronounced inflammatory changes. The pathological lesions observed in the tissues of the infected calves are in agreement with Opara and Fagbemi, (2010), who also reported organ degenerative changes in animal trypanosomiasis.

The lesions in the lymph nodes of trypanosome infected animals included increased presence of enlarged lymphoid nodules as well as increased plasma cell populations. The perivascular mononuclear cell infiltration, mainly of lymphocytes, and plasma cells in the liver was also a common feature in calves' trypanosomosis. These changes conform to the finding described by Omotainse and Anosa (2009) in ovine infected with *T.vivax, T. congolense* and *T. brucei*. Perivascular mononuclear cell infiltration, disorganization of the hepatic cords, hepatocyte degeneration and hyperplasia were common lesions observed in the liver in this study. The lesions associated with *T.vivax* in this study were in good agreement with Omotainse and Anosa (2009) in ovine trypanosomosis (infected with *T. vivax, T. congolense,* and *T. brucei*). This might have played a significant role in the inability of the animals to successfully recover from the anaemia experienced in trypanosomosis (Omotainse and Anosa, 2009).

In this study, hyperplasia of the red pulp, enlargement of the lymphoid nodules and proliferation of plasma cells were the lesions seen in the spleen in all infected groups

(both isolates). This histopathological findings pointed out in our experimental study was in line with the finding of Omotainse and Anosa (2009) in ovine infected with *T.vivax*, *T. congolense* and *T. brucei*. In histopathological investigation of this study, neuronal necrosis with shrunken angular neuron, pyknosis, perivasculatis, mononuclear cell infiltration like lymphocytes, plasma cells and macrophages were the lesions seen in the brain. In addition, in histopathological studies of the brain increased astrogliosis with hypertrophy and increased microglial cells were the lesions seen in all infected groups. The findings observed in this study were in agreement with Moulton (1986) in goats infected by *T. congolense;* Aquino *et al*., (2002) in dogs infected by *T. evansi.*

Multifocal myocarditis with infiltration of mononuclear cells especially of lymphocytes and few plasma cells, mononuclear cells infiltration to perimycial connective tissue with perivasculatis, myocardial necrosis, sever myocardial degeneration with vacuolization was the lesions seen in the heart of all infected groups. These findings observed in this study agree with the finding of Aquino *et al*., (2002) in his experimental study of dogs infected with *T. evansi*.

Interstitial pneumonia with severe thickening of interstitial wall (proliferation), decreasing of alveolar lumen, and rupture of alveoli in relatively thinner alveolar walls was the lesions seen in the lung of all infected groups. The finding investigated in this study is in line with the finding of Opara and Fagbemi (2010) in his experimental study of Grasscutter infected with *T. congolense* and *T. vivax*. Similarly, the histopathological findings of kidney in this study agree with the finding of Opara and Fagbemi (2010) in his experimental study of Grasscutter infected with *T. congolense* and *T. vivax*. In conclusion, the gross and microscopic lesions investigated in this study were similar in all infected group.

5.2. Comparison between tsetse and non-tsetse isolates of *T. vivax* in the induction of pathophysiological changes in calves

Parasitaemia was more pronounced in non-tsetse trypanosome infected groups than in the group infected by *T. vivax* drawn from tsetse infested area. Such variations in parasite load could be attributed to variations in the genetic makeup or strain of the parasites. The disappearance and resurgence of parasitaemia at different intervals may be as a result of dynamic of antigenic variation activity of the parasites (Ogbaje *et al*., 2011).

However, there was no significant variation between infected groups in the demonstration of common clinical signs such as raised rectal temperature, reduction in feed intake, emaciation, pale mucous membrane and enlargement of peripheral lymph nodes. On the contrary, body weight gain varied between isolates in such a way that animals infected with isolates from tsetse area lost significant body mass compared to those infected with isolates from non-tsetse area. Such differences could be explained by the relative pathological changes exhibited by the animals or individual variations in feed intake arising from infection related changes such as fever etc. This was in accord with Martins *et al*., (2008) in which there was no significant difference in clinical signs in cattle of high land and lowland infected with *T. vivax*.

Development of anaemia as measured by PCV and Hgb concentration was more sever in non-tsetse isolate infected groups than in the group infected with isolate from tsetse area. This is in line with degree of parasitaemia recorded for the two varieties and hence could be related to difference in pathology caused by the two isolates. This finding was inagreement with Martins *et al*., (2008) in which he investigated difference in PCV and Hgb concentration between cattle of Pantanal lowlands and highlands infected with *Trypanosoma vivax*.

Serum glucose level for TT infected group was significantly lower than the level for non-tsetse trypanosome infected group suggesting the existence of variations in the utilization of glucose by the two isolates or differences on the impact of compensatory mechanisms of the host animal. On the contrary, no significant difference was observed in the levels of serum total cholesterol, albumin and total proteins. The finding of this study for serum cholesterol and albumin agrees with the finding of Biryomumaisho *et al*., (2003) in which he reported a decrease in *T. brucei* and *T. congolense* infected goats but it concur with the

finding of total protein in which he investigated an increase in *T. congolense* and decrease in *T .brucei*.

As a measure to the extent of vital organ damages such as in the liver, levels of AST, ALT and ALP were compared between tsetse and non-tsetse *T. vivax*-infected groups. The findings demonstrated that in all cases enzyme levels were elevated and only level of serum AST was significantly higher in group TT when compared to group NT1 and NT2 suggesting that organ damages are similarly important in both isolates. This was in accord with Taiwo and Anosa (2000) in which all the animals infected with either *T. congolense* or *T. vivax* developed varying degrees of AST, ALT and ALP.

Contrary to the findings on pathological changes where we demonstrated almost similar situations between the two isolates and sometimes severe cases for non-tsetse T. vivax-infected groups (the case of PCV and Hgb), cytokine expression profiles unequivocally showed that animals infected with *T. vivax* isolates collected from tsetse infested area were immunologically more active than those infected with *T. vivax* from non-tsetse area. This was shown by the fact that group TT mounted remarkably higher levels of TNF-α, INF-γ, IL-12 and Il-10 than the NT1 and NT2 groups. This may indicate that although the latter isolate is as potent as the former in causing pathology, it is unable to elicit strong immunological response. The lower parasite level registered for the group TT might be the result of such strong immunological response demonstrated by the animals in the group. For example Hertz *et al*., (1998) showed that resistance to the animal trypanosomosis is INF-γ dependent.

6. CONCLUSION AND RECOMMENDATIONS

The present study was set out to investigate the impact of *T. vivax* infection on various clinical and pathophysiological parameters of experimentally infected calves and compare the extent of these changes in relation to two different isolates of the parasite. Blood parasite load was more important in groups infected with non-tsetse *T. vivax* where early onset was also detected. Irrespective of the isolates however, *Trypanosoma vivax* infection caused comparable significant changes in clinical, hematological, biochemical

and cytokine parameters in young zebu cattle. Anemia was more sever in non-tsetse *T. vivax* infected cattle while body weight loss was more pronounced in animals infected with tsetse adapted *T. vivax* isolate. Despite the similar clinic-pathological findings, only animals infected with the tsetse adapted *T. vivax* have demonstrated pronounced cytokine profile as an indicator of strong immunological response mounted against the infection. Altogether, this finding suggests the following further studies to be done: 1) the mechanisms of development of immunity as it may vary based on the strain of the parasite and 2) molecular characterization of the different isolates as it relates to both pathogenicity and immunogenicity.

7. REFERENCES

Abeer, A. A. E. and Shaymaa, I. S., 2011. Clinico-pathological and Cytological Studies on Naturally Infected Camels and Experimentally Infected Rats with *Trypanosoma evansi*. *World Appl. Sci. J.*, **14** (1): 42-50.

Abenga, J. N., and Anosa, V. O., 2005. Serum total proteins and creatinine levels in experimental gambian trypanosomosis of vervet monkeys. *Afri. J. Biotechnol*. **4** (2): 187-190.

Adah, M. J., Otesile, E. B., Joshua, R. A., 1992. Changes in level of transaminases in goats: experimentally infected with *T. congolense*. *Revd Elev. Med Vet. Pays. Trop*., **45**: 284-286.

Allam, L., Oguu, D., Agbede, R. I. S., Sackey, A. K. B., 2012. Posterior paresis in pregnant gilts experimentally infected with *T. brucei*. *J. Vet. Med. Anim. Health*. **4**(3): 27-35.

Allam, L., Ogwu D., Agbede R. I. S., Sackey A. K. B., 2011. Hematological and Serum Biochemical changes in gilts experimentally infected with *Trypanosoma brucei*. *Vet. arhiv*., **81**: 597-609.

Anne, B. M., Onah, D. N. and Nawa, Y., 2001. Drug resistance in pathogenic African trypanosomes: what hopes for the future? *Veterinary Parasitol*, **96**: 83-100.

Aquino, L. P. C. T., Machado, R. Z., Alessi, A. C., Santana, A. E., Castro, M. B., Marques, L. C., Malheiros, E. B., 2002. Hematological, biochemical and anatomopathological aspects of the experimental infection with *Trypanosoma evansi* in dogs. *Arq. Bras. Med. Vet. Zootec*., **54**(1).

Barry, J. D., and McCulloch, R., 2001. Antigenic variation in trypanosomes: enhanced phenotypic variation in a eukaryotic parasite. *Adv Parasitol*, **49**: 1–70.

Bengaly, Z.., Sidibe, I., Ganaba, R., Desquesnes, M., Boly, H., Sawadogo, L., 2002. Pathogenicity of three genetically distinct types of *Trypanosoma congolense* in cattle: clinical observations and haematological changes. *Vet. Parasitol*. **108**(1):1-19.

Billiau, A, Vandenbroeck, K., 2001. IFN-g. In: Oppenheim, J. J, Feldman M, eds. Cytokine Reference. A Compendium of Cytokeines and Other Mediators of Host Defense, San Diego: Academic Press, **1**: 641–688.

Biryomumaisho, S., Katunguka-Rwakishaya, E., Rubaire-Akiiki, C. M., 2003. Serum biochemical changes in experimental *Trypanosoma congolencse* and *Tryapanosoma brucei* infection in small east African goats. *Vet. arhiv*., **73**: 167-180.

Bisalla, M., Ibrahim, N. D. G., Lawal, I. A. and Esievo, K. A. N., 2007. Serum total proteins, albumin and albumin globulin ratio in Yankasa sheep experimentally

infected with *Trypanosoma congolense* and immunomodulated with levamisole. *J. Protozool. Res*., **17**: 39-43.

Borst, P., 2002. Antigenic variation and allelic exclusion. Cell, **109**: 5–8.

Bush, B. M., 1975. Veterinary Laboratory Manual, Heinmann Medical Books. London.

Cherenet, T., Sani, R. A., Speybroeck, N., Panandam, J. M., Nadzr, S., Van den Bossche, P., 2006. A comparative longitudinal study of bovine trypanosomosis in tsetse-free and tsetse-infested zones of the Amhara region, northwest Ethiopia. *Vet. Parasitol*., **140**:251–258.

CIOMS, 1985. Council for international organizations of medical sciences.

Coles, E. H., 1986. Veterinary Clinical Pathology. 4th ed. W. B. S. Saunder Co. Philadelphia.

Dagenais, T. R., Freeman, B. E., Demick, K. P., 2009. Processing and presentation of variant surface glycoprotein molecules to T cells in African trypanosomiasis. *J Immunol.*, **183**: 3344–3355.

Ekanem, J. T., Majolagbe, O. R., Sulaiman, F. A., Muhammad, N. O., 2006. Effects of honey supplemented diet on the parasitaemia and some enzymes of *Trypanosoma brucei*-infected rats. *Afr J Biotechnol*., **5**: 1557-1561.

Ekanem, J. T., Sulyman, F. A., Adeyemi, O. S., 2005. Therapeutic properties and serum iron in *T. brucei* infected rats treated with amodiaquine and mefloquine. *Bio*., **17**: 115-21.

Erkelens, A. M., Dwinger, R. H., Bedane, B., Slingenbergh, J. H. W. and Wint, W., 2000. Selection of priority areas for tsetse control in Africa; a decision tool using GIS in Didessa Valley, Ethiopia, as a Pilot Study. In: Animal Trypanosomosis: Diagnosis and Epidemiology. FAO/IAEA Coordinated Research Programme on the Use of Immunoassay Methods for Improved Diagnosis of Trypanosomosis and Monitoring Tsetse and Trypanosomosis Control Programmes, International Atomic Energy Agency, Vienna, Austria.

Esche, C, Shurin, M. R., Lotze, M. T., 2001. IL-12. In: Oppenheim JJ, Feldman M, eds. Cytokine Reference. A Compendium of Cytokines and Other Mediators of Host Defense, San Diego: Academic Press, **1**: 187–201.

FAO, 2000. Impacts of trypanosomosis on African agriculture, by B. M. Swallow. PAAT Technical and Scientific Series No. 2. Rome. Pp. 52.

FAO, 2004. The state of food and agriculture 2003-2004. Food and Agriculture Organization of the United Nations (FAO), Rome, Italy.

Faye, D., Fall, A., Leak, S., Losson, B. and Geerts, S., 2005. Influence of an experimental *Trypanosoma Congolense* infection and plane nutrition on milk production and some biochemical parameters in West African dwarf goats. *Acta trop*., **93**: 247 – 257.

Forhead, A. J., 1994. Relationship between plasma insulin and triglyceride concentrations in hyper trigluceridemic donkeys. *Rev. Vet. Sci*., **56**: 389 – 392.

Frevert, U., Reinwald, E., 1990. *Trypanosoma congolense* bloodstream forms evade complement lysis in vitro by shedding of immune complexes. *Eur J Cell Biol*.; **52**: 264–269.

Gilbert, M., Jennifer, C., Pender, J., Rogers, D., Slingenbergh, J., and Wint, W., 2001. The development and use of the Programme against African Trypanosomosis information system (PAATIS). Newsletter on Integrated Control of Pathogenic Trypanosomes and their Vectors (ICPTV), **3**: 10-12.

Gomez-Rodriquez, J., Stijlemans, B., De Muylder, G., 2009. Identification of a parasitic modulatory protein triggering the development of suppressive M1 macrophages during African trypanosomiasis. *J Infect Dis*., **200**: 1849–1860.

Guilliams, M., 2007. African trypanosomiasis: naturally occurring regulatory T cells favor trypanotolerance by limiting pathology associated with sustained type 1 inflammation. *J Immunol*., **179**:2748–2757.

Herbert, W. J. and Lumsden, W. H. R., 1976. *Trypanosoma brucei*: A rapid 'matching' method for estimating the host's parasitaemia. *Experimental parasitol*., **40**: 427-431.

Hertz, C. J., Filutowic, H. z. and Mansfield, J. M., 1998. Resistance to the African Trypanosomes is IFN-γ dependent. *J. Immunol*., **161**: 6775-6783.

ICIPE, 2006. African Insect Science for Food and Health. Nairobi, Kenya. http://www.icipe.org/research_areas/animal_health/tsetse/index.html

Kadima, K. B., Gyang, E. O., Saror, D. I., Esievo, K. A. N., 2000. Serum biochemical values of *Trypanosoma vivax*-infected cattle and the effects of lactose in saline infusion. *Vet. Arhiv.*, **70**: 67- 74.

Katugunka-Rwakishaya, E., Parkins, J. J., Fishwick, G., Murray, M. and Holmes, P. H., 1995. The influence of energy intake on the pathophysiology of *Trypanosoma congolense* infection in Scottish Blackface sheep. *Vet. Parasitol*., **59**: 207-218.

Katunguka-Rwakishaya, E., Murray, M., Holmes, P. H., 1999. The influence of energy intake on some blood biochemical parameters in Scottish Blackface sheep infected with *Trypanosoma congolense*. *Vet*. *Parasitol*., 84: 1–11.

Kaushik, R. S., Uzonna, J. E., Gordon, J. R., Tabel, H., 1999. Innate resistance to *Trypanosoma congolense* infections: differential production of nitric oxide by macrophages from susceptible BALB/c and resistant C57Bl/6 mice. *Exp. Parasitol.*, **92**: 131–143.

Kaushik, R. S., Uzonna, J. E., Zhang, Y., Gordon, J. R., Tabel, H., 2000. Innate resistance to experimental African trypanosomiasis: Differences in cytokine (TNF-alpha, IL-6, IL-10 and IL-12) production by bone marrow-derived macrophages from resistant and susceptible mice. Cytokine, **12**: 1024–1034.

Kettle, D. S., 1995. Medical and Veterinary entomology, 2nd ed. CADI, Walling ford, **P**p. 225-227.

Kitani, H., Yagi, Y., Naessens, J., Sekikawa, K., Iraqi, F., 2004. The secretion of acute phase proteins and inflammatory cytokines during *Trypanosoma Congolense* infection is not affected by the absence of the TNF-gene. *Acta Tropica*., **92**: 35–42.

Kodelja, V., Mller, C., Politz, O., 1998. Alternative macrophage activation associated CC-chemokine-1, a novel structural homologue of macrophage inflammatory protein-a with a Th2-associated expression pattern .*J Immunol*., **160**: 1411–1418.

Kristjanson, P. M., Swallow, B. M., Rowlands, G. J., Kruska, R. L., de Leeuw, P. N., 1999. Measuring the costs of African animal trypanosomosis, the potential benefits of control and returns to research. *Agric. Systems*, **59**:79-98.

Lording, P. M., Friend, S. C. E., 1991. Data analyasis guide. Interpretation of laboratory results. Australian Vet. Pract. **21**, 186-195.

Magez, S., Schwegmann, A., Atkinson, R., 2008. The role of B-cells and IgM antibodies in parasitemia and VSG switching in *Trypanosoma brucei*-infected mice. PLoS Pathog, 4: e1000122.

Martins, C. F., Madruga, C. R., Koller, W. W., Araújo, F. R., Soares, C. O., Kessler, R. H., Melo, E. S. P., Rios, L. R., Almeida, R. C. F., Lima, M. S. C., Barros, A. T. M. and Marques, L. C., 2008. *Trypanosoma vivax* infection dynamics in a cattle herd maintained in a transition area between Pantanal lowlands and highlands of Mato Grosso do Sul, Brazil. Pesquisa Veterinária Brasileira **28**(1):51-56

Mattioli, R. C., Feldman, U., Hendrickx, G., Wint, W., Jannin, J. and Slingenbergh, J., 2004. Tsetse and Trypanosomosis intervention policies supporting sustainable animal agricultural development. *Food. Agr. Env.*, **2**: 310-314.

Mersha, C., Chemirew, A. and Basaznew, B., 2012. Hematopathology and Hematological Parametric Alterations in Indigenous Cattle Due to Trypanosomosis. Department Veterinary Paraclinical Studies, Faculty of Veterinary Medicine, University of Gondar, P.O.Box 196, Gondar, Ethiopia. Global Veterinaria **9** (5): 546-551.

Morrison, L. J., Marcello, L., and McCulloch, R., 2009. Antigenic variation in the African trypanosome: molecular mechanisms and phenotypic complex. *Cell Microbiol*, **11**: 1724–1734.

Morrison, L. J., McLellan, S., Sweeney, L., Chan, C. N., Annette MacLeod, A., Tait, A., Turner, C. M. R., 2010. Role for Parasite Genetic Diversity in Differential Host Responses to *Trypanosoma brucei* Infection. *Infect and Immun.*, **78**:1096-1108.

Moulton, J. E., 1986. Relapse Infection After chemotherapy in goats experimentally infected with *Trypanosoma brucei*: Pathological Changes in Central Nervous System: *Vet Pathol*., **23**: **P**p. 21.

Munder, M., Eichmann, K., Morn, J. M., 1999. Th1/Th2-regulated expression of arginase isoforms in murine macrophages and dendritic cells. *J Immunol*, **163**: 3771–3777.

Murray, M., Stear, M. J., Trail, J. C. M., d'Ieteren, G. D. M., Agyemang, A., Dwinger, R. H., 1991. In: Owen, J. B., Axford, R. F. E. (Eds.), Trypanosomosis in cattle: Prospects for control, breeding for disease resistance in farm animals. CAB International, Wallingford.

Mwongela, G. N., Kovatch, R. M., Frazil, M. A., 1981. Acute *Trypanosoma vivax* infection in dairy cattle in Coast Province, Kenya. *Trop Anim Health Prod*. **13**: 63-69.

Namangala, B., De Baetselier, P., Brys, L., 2000. Attenuation of *Trypanosoma brucei* is associated with reduced immunosuppression and concomitant production of Th2 lymphokines. *J Infect Dis.*, **181**: 1110–1120.

Namangala, B., De Baetselier, P., W., 2001. Alternative versus classical macrophage activation during experimental African trypanosomosis. *J Leukoc Biol.*, **69**: 387–396.

Namangala, B., Sugimoto, C. and Inoue, N., 2007. Effects of exogenous transforming factor b on *Trypanosoma congolense* infection in mice. *Infect Immun.*, **75**: 1878–1885.

Namangala, B., Yokoyama, N., Ikehara, Y., 2008. Effect of CD4+ CD25+ T cell-Depletion on Acute Lethal Infection of Mice with *Trypanosoma congolense*. *J Vet Med.*, **70**: 751–759

Ngure, R. M., Eckersall, P. D., Jennings, F. W., Mburu, J., Burke, J., Mungatana, N. and Murray, M., 2008. Acute phase response in mice experimentally infected with *Trypanosoma congolense*: a molecular gauge of parasite-host interaction. *Vet Parasitol*., **151**: 14-20.

Noel, W., Hassanzadeh, G., Raes, G., 2002. Infection stage-dependent modulation of macrophage activation in *Trypanosoma congolense*-resistant and -susceptible mice. *Infect Immun.*, **70**: 6180–6187.

O'Gorman, G. M., Park, S. D., Hill, E. W., Meade, K. G., Mitchell, L. C., Agaba, M., Gibson, J. P., Hanotte, O., Naessens, J., Kemp, S. J., MacHugh, D. E., 2006. Cytokine mRNA profiling of peripheral blood mononuclear cells from trypanotolerant and trypanosusceptible cattle infected with *Trypanosoma congolense*. *Physiol Genom.*, **28**: 1– 61.

Ogbaje, C. I., Lawal , I. A. and Ajanusi , O. J., 2011. Infectivity and Pathogenicity of Sokoto (Northern Nigeria) Isolate of Trypanosoma evansi in West African Dwarf Goats. *Int. J. Anim. Vet. Adv*., **3**(3): 117-124

Ogwu, D., Osori, D. I. K., Njoku, C. O., Ezeokoli, C. D. and Kumi-Diaka, J., 1986. Effects of the reproductive status in Zebu heifers on the immunoglobulin M. and

G. levels in Bovine *Trypanosoma vivax* infection. *Animal Reprod Sci.*, **12**: 179-187.

Olsson, T., Bakhiet, M., Edlund, C., Höjeberg, B., Van der Meide, P. H. and Kristensson, K., 1991. Bidirectional activity signals between *Trypanosoma brucei* and CD81T cells: a trypanosome-released factor triggers interferon-g production that stimulates parasite growth. *Eur. J. Immunol.*, **21**: 2447–2454.

Omotainse, S. O. and Anosa, V. O., 2009. Comparative histopathology of the lymph nodes, spleen, liver and kidney in experimental ovine trypanosomosis. *Onderstepoort J Vet Vet Res*., **76**: 377–383

Omotainse, S. O., Anosa, V. O., Falaye, C., 1994. Clinical and biochemical changes in experimental Trypanosoma brucei infection of dogs. *Israel J. Vet. Med*., **49**, 36-39.

Opara, M. N., and Fagbemi, B. O., 2010. Patho-physiological Effects of Experimental *Trypanosoma congolense* and *Trypanosoma vivax* Infections in the Grasscutter (Thryonomys swinderianus, Temminck). Department of Veterinary Microbiology and Parasitology, University of Ibadan, Ibadan Nigeria.

Opperdoes, F. R., Hart, D. T., Baudhuin, P., 1986. Biogenesis of glycosomes (microbodies) in the trypanosomatidae, *T. brucei*. *Europ. J. Cell Biol*., **41** (Suppl. 14), 30.

Osman, M., Nadia, J., Kaila, G., Eltahir, H. A., and Abdel Rahman, A. H., 2008. Susceptibility of Sudanese Nubian Goats, Nilotic Dwarf Goats and Garag Ewes to Experimental Infection with a Mechanically Transmitted *Trypanosoma vivax* Stock. Central Veterinary Research Laboratories, Khartoum, Sudan. *J Biol Sci*., **11** (3): 472 – 475.

Osório, A. L. A. R., Madruga, C. R., Desquesnes, M., Soares, C. O., Ribeiro L. R. R., Costa, S. C. G. D., 2008. *Trypanosoma* (Duttonella) *vivax*: its biology, epidemiology, pathogenesis, and introduction in the New World. *Mem. Inst. Oswaldo Cruz*., **103**:1–13.

Oyewole, O. I., Malomo, S. O., 2009. Toxicological assessment of oral administration of some anti-sickling agents in rats. *Afri J Biochem Res*., **3** (2): 024-028

Pan, W., Ogunremi, O., Wei, G., Shi, M., Tabel, H., 2006. CR3 (CD11b/CD18) is the major macrophage receptor for IgM antibody-mediated phagocytosis of African trypanosomes: diverse effect on subsequent synthesis of tumor necrosis factor alpha and nitric oxide. *Microbes Infect*, **8**: 1209–1218.

Pays, E., 2006. The variant surface glycoprotein as a tool for adaptation in African trypanosomes. *Microbes Infect*, **8**: 930–937.

Philippe, V., and Bernard, B., 2006. Immunology and immunopathology of African trypanosomiasis. *An Acad Bras Cienc*, **78**(4): 645-665.

Reinitz, D. M., Mansfield, J. M., 1990. T-cell-independent and T-cell-dependent B-cell responses to exposed variant surface glycoprotein epitopes in trypanosome-infected mice. *Infect Immun*., **58**: 2337–2342.

Reitman, S. and Frankel, S., 1957. A calorimetric method for the determination of serum glutamic oxaloacetic and glutamic pyruvic transaminase. *Amer J. of Clin. Pathol.*, **28**: 56-62.

Robertson, C. M., and Coopersmith, C. M., 2006. The systemic inflammatory response syndrome. *Microbes Infect*, **8**:1382–1389.

Sandoval, G. L.; Coppo, N. B.; Negrette, M. S., 1994. Biochemical and histopathological alteration in experimentally infected rat with *Trypanosoma evansi*. *Hora Vet*., **81**: 53-55.

Sharma, D. K., Chauhanb, P. P. S., Saxenac, V. K., Agrawal, R. D., 2000. Hematological changes in experimental trypanosomiasis in Barbari goats. *Small. Rum. Res*., **38**: 145- 149.

Shaw, A. P. M., 2004. Economics of African trypanosomosis. In: Maudlin, I., Holmes, P. H and Miles, M. A., (eds). Trypanosomosis, CAB International, Wallingford, UK, **P**p. 369- 402.

Shi, M. Q., Wei, G. J. and Tabel, H., 2007. *Trypanosoma congolense* infections: MHC class II restricted immune responses mediate either protection or disease, depending on IL-10 function. *Parasite Immunol*, **29**: 107–111.

Shi, M., Pan, W., Tabel, H., 2003. Experimental African trypanosomiasis: IFN-gamma mediates early mortality. *Eur J Immunol*., **33**: 108–118.

Shi, M., Wei, G., Pan, W., 2006. Experimental African trypanosomiasis: a subset of pathogenic, IFN-c-producing, MHC Class II-restricted CD4+ T cells mediate early mortality in highly susceptible mice. *J Immunol.*, **176**: 1724–1732.

Shi, M., Wei, G., Pan, W., Tabel, H., 2004. *Trypanosoma congolense* infections: antibody-mediated phagocytosis by Kupffer cells. *J Leukoc Biol*.; **76**:399–405.

Shimelis, D. and Getachew, A., 2007. Current status of tsetse transmitted trypanosomosis in new settlement areas of Amhara region northwest Ethiopia. In: Proceedings of the 29th meeting of the International Scientific Council for Trypanosomiasis Research and Control (ISCTRC), held in Futungu Conference Centre, Luanda, Angola, October 1 -5.

Shimelis, D., Arun, K. S. and Abebe, G., 2005. Epidemiology of Bovine Trypanosomosis in the Abay (Blue Nile) Basin Areas of Northwest Ethiopia. *Revue D"Elevage Et De Medicine Veterinaire Des Pays Tropicaux*, **58**:151-157.

Silva, R. A. M. S., Ramirez, L., Souza, S. S., Ortiz, A. G., Pereira, S. R., and Duvila, A. M. R., 1999. Hematology of natural bovine trypanosomosis in the Brazilian Pantanal and Bolivian wetlands.

Sinshaw, A., Abebe, G., Desquesnes, M., Yon, W., 2006. Biting flies and *Trypanosoma vivax* infection in three highland districts bordering Lake Tana, Ethiopia. *Vet. Parasitol*., **142**:35–46.

Stijlemans, B. Vankrunkelsven, A., Brys, L., Magez, S., De Baetselier P., 2008. "Role of iron homeostasis in trypanosomiasis-associated anemia," *Immuno boil*., **213**: 823–835.

Stijlemans, B., Guilliams, M., Raes, G., 2007. African trypanosomosis: from immune escape and immunopathology to immune intervention. *Vet Parasitol.*, **148**: 3–13.

Stijlemans, B., Vankrunkelsven, A., Brys, L., 2010. Scrutinizing the mechanisms underlying the induction of anemia of inflammation through GPI-mediated modulation of macrophage activation in a model of African trypanosomiasis. *Microbes Infect*, **12**: 389–399.

Sulaiman, F. A., and Adeyemi, O. S., 2010. Changes in hematological indices and protein concentrations in *Trypanosoma brucei* infected rats treated with homidium chloride and diminazene aceturate. *excli J*., **9**: 39-45.

Tabel, H., Kaushik, R. S., and Uzonna, J. E., 2000. Susceptibility and resistance to *Trypanosoma congolense* infections. *Microbes Infect*., **2**: 1–12.

Tabel, H., Wei, G., Shi, M., 2008. T cells and Immunopathogenesis of experimental African trypanosomiasis. *Immunol. Rev*., **225**:128–139.

Taiwo, V. O., Olaniyi M. O., Ogunsanmi, A. O., 2003. Comparative plasma biochemical changes and susceptibility of erythrocytes to in vitro peroxidation during experimental *T. congolense* and *T. brucei* infections in sheep. *Israel J.Vet. Med*., **58**: 1-10.

Taiwo, V.O. and Anosa, V.O. 2000. In vitro erythrophagocytosis by cultured macrophages stimulated with extraneous substances and those isolated from the blood, spleen and bone marrow of Boran and N'Dama cattle infected with *Trypanosoma congolense* and *Trypanosoma vivax*. *J. Vet. Res*., **67**: 273-287

Thrusfield, M., 1995. Veterinary Epidemiology. 2nd ed. Blackwell Scientific Publishers, Oxyford.

Uzonna, J. E., Kaushik, R. S., Gordon, J. R. and Tabel, H., 1999. Cytokines and antibody responses during *Trypanosoma congolense* infections in two inbred mouse strains that differ in resistance. *Parasite Immunol.*, **21**: 57–71.

Van Dam, J. T. P., 1996. The interactions between nutrition and metabolism in West African Dwarf goats infected with Trypanosomes. Ph.D. thesis, Wageningen Agricultural University,The Netherlands, **P**p. 166.

Vincendeau, P., Gobert, A. P., Daulouède, S., Moynet, D., Andmossalayi, M. D., 2003. Arginases in parasitic diseases. *Trends Parasitol*, **19**: 9– 12.

Vreysen, M. J. B., 2006. Prospects for area-wide integrated control of tsetse flies (Diptera: Glossinidae) and trypanosomosis in sub-Saharan Africa. *Revista de la Sociedad Entomológica Argentina*, **65**: 1-21.

Wei, G., Tabel, H., 2008. Regulatory T cells prevent control of experimental African trypanosomiasis. *J Immunol*, **180**:2514–2521.

Wellde, B. T., Rearson, M. J., Kovatch R. M., Chumo, W. T., Wykoff, D. E., 1989. Experimental infection of cattle with *T. brucei rhodesiense*. *Annl. Trop. Med. Parasitol*., **83**: 133 – 134.

Witola, W. H. and Lovelace, C. E. A., 1997. Serum protein changes in indigenous Zambian goats with trypanosomosis (Meeting abstract No. 2344) FASEB J. **11**(9): A1257.

Wurochekke, A. U., Anthony, A. E., Obidah, W., 2008. Biochemical effects on the liver and kidney of rats administered aqueous stem bark extract of Xemenia Americana. *Afri J Biotechnol*., **7** (16): 2777-2780

8. ANNEXES

Annex.1. Principles of the Rapid matching method

Matching chart and tables: The chart and tables required for rapid matching methods are shown below.

Organisms: *Trypanosoma vivax* isolated of tsetse infested and non-tsetse infested areas of northwest Ethiopia will be used for calibration.

Blood film: A wet film of the blood of the infected animals will be made under a 7 x 22-mm cover glass. The quantity of blood should be just insufficient to fill the whole space under the cover glass when this is pressed down gently. The film is examined under x400 magnification, and a field is chosen in which the cells are evenly distributed. Rouleaux may be present, but otherwise the cells should not form more than one layer.

Methods:-

More than one organism per microscope field: The best match should be chosen quickly without attempting to count the organisms, most attention being given to their spacing.

When large numbers are present it is best to compare a section of the field, say a quarter segment, with a similar portion of the chart. It has been noticed that with a parasitaemia of antilog 8.7 organisms/ml the trypanosomes often form a reticulated pattern with clumps of red cells between them; at antilog 9.0 organisms/ml the distribution is more even, the trypanosomes swarming round and over every erythrocyte. Parasitemias exceeding that in the circle marked 9.0 are recorded as >9.0.

One organism per field or fewer: If, when the selected field is examined, no trypanosomes, or only a single one, are seen, a count is made in 5, 10, or 20 fields. The count is first made of 5 fields. If 2 or more organisms are seen, then equivalences are read off from the "5 field" section of Figure. If fewer, recourse is had to counting 10 or 20 fields, referring in each case to the appropriate section of Figure. When no organisms are seen in 20 fields, parasitaemia is recorded as <antilog 5.4 organisms/ml.

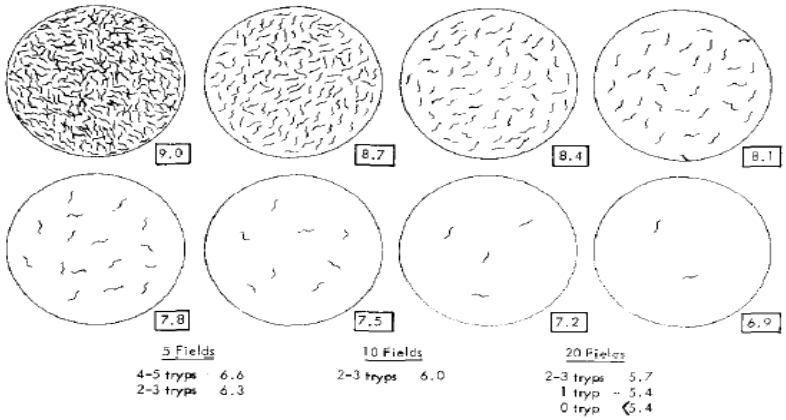

FIG. 1. Chart and table for estimating trypanosome parasitemias. The circles are used for matching when more than one organism per microscope field is present, the tables for lower concentrations. The values in the boxes in the charts and in the tables indicate the logarithm of the number of trypanosomes per milliliter as computed for *Trypanosoma brucei* infections in mouse blood inspected under ×400 magnification. For viewing at 25 cm, the circles are drawn with a diameter of 6.5 cm. They contain representations of trypanosomes (6 mm) that decrease in number by twofold steps.

Chart Showing Rationale of Computation of Values for the Circles and Tables in Fig. 1 for Wet Films of Blood from Mice Infected with *Trypanosoma brucei* Viewed under ×400 Magnification[a]

Organisms in 20 fields	Organisms in 10 fields	Organisms in 5 fields		Organisms per field	Equivalent log number of organisms per milliliter of blood
				>256	>9.0
			M	256	9.0
			A	128	8.7
			T	64	8.4
			C	32	8.1 Reference point
			H	16	7.8
			I	8	7.5
			N	4	7.2
			G	2	6.9
			C		
		4–5	O	1	6.6
		2–3	U	0.5	6.3
	2–3		N	0.25	6.0
2–3			T	0.125	5.7
1			I	0.0625	5.4
0			N	<0.0625	<5.4
			G		

[a] The base 10 logarithm value for the concentration of organisms per milliliter of blood for the reference point (the circle containing 32 "organisms") was obtained from the regression line in Fig. 2.

Annex 2. The Packed cell volume determination

Materials:

- ✓ Un-coagulated whole blood in EDTA coated vacutainer tubes
- ✓ Microhematocrit capillary tubes
- ✓ Sealing clay
- ✓ Gauze
- ✓ Microhematocrit centrifuge
- ✓ Microhematocrit reader

Procedure:

1. Capillary tubes are filled approximately ¾ of the tube with the well-mixed blood sample.
2. The top of the capillary tube is occluded with index finger to prevent spillage of the blood and excess blood is wiped carefully outside the tube by using cotton.
3. The vacant end of the tube is sealed with sealing clay.
4. The capillary tubes are placed in the microhematocrit centrifuge with the sealed end toward the periphery. Duplicate tubes are placed opposite each other for balance.
5. It is centrifuged for five minutes at full speed of 12,000rpm.
6. Using Hawksley microhematocrit reader the reading is made in percent.

Note: The bottom sealed end of the capillary tube is put at 0% and the top plasma end is adjusted at 100 % mark. Then the PCV value is read at the junction space between the buffy layer and the packed RBC's. Results are recorded to the nearest whole number.

Annex 3. Hemoglobin concentration determination

Materials and reagents:

- ✓ Un-coagulated whole blood in EDTA coated vacutainer tubes
- ✓ Micropipette with disposable tips
- ✓ Graduated hemoglobinometer tube
- ✓ Hemoglobin meter (color matching chamber).
- ✓ Glass rod
- ✓ Dropper
- ✓ 1% HCl
- ✓ Distilled water
- ✓ Piece of gauze
- ✓ Hemoglobin meter

Procedure:

1. The graduated tube of the hemoglobin meter is filled to 20 mark with 1% HCl.

2. The micropipette tip was attached to the eppendorf micropipette and 20 µl of blood was measured and the blood was wiped from outside of the tip with a piece of Gauze.
3. The blood was added into the graduated tube.
4. One drop of distilled water is added to the graduated tube and mixed with glass stirring rod.
5. The above process of adding one drop of distilled water and stirring is continued until the color of the solution in the graduated tube matches with the color of the glass standard on the hemoglobin meter.
6. The hemoglobin is read in g/dl.

Annex 4. Principles and Procedures of Biochemical Tests

Specimen Collection:

3ml of blood was collected from the individual's using a sterile vacutainer plain tube. Shaking was avoided and the blood was standing for 15-20 minutes at room temperature to allow for clot formation. Serum separated from the clot by centrifugation at 3000rpm for 5-10 minutes and transfers to tubes packed and transported on cold chain and stored at -20^{o}c prior to analysis.

Procedure for instrument set up:

Set the program on the instrument, type the individual's code no. glucose, total cholesterol total protein, albumin, AST, ALT, and ALP tests are selected from test menu. After calibration adequate controls and serum was placed in sample cup by appropriate order and enough working reagents in reagent bottles were added. The instrument by itself pipettes programmed sample volume and working reagent and after incubation, the formed color absorbance read at appropriate wavelength and the results were displayed on screen.

Glucose determination

Principle: Glucose is oxidized by glucose oxidase (GOD) to produce gluconate and hydrogen peroxide (H_2O_2). H_2O_2 is then oxidatively coupled with 4 amino- antipyrene (4-

AA) and phenol in the presence of peroxidase (POD) to yield a red quinoeimine dye that is measured at wavelength of 546 nm proportional to concentration of glucose in the sample.

Total cholesterol determination

Principle: The method for the measurement of total cholesterol in serum involves the use of three enzymes: cholesterol esterase (CE), cholesterol oxidase (CO) and POD. Cholesterol esters are first hydrolyzed by cholesterol esterase to cholesterol. The cholesterol produced by hydrolysis is oxidized by cholesterol oxidase to cholesten-3-one and H_2O_2. The hydrogen peroxide produced is then coupled with 4-AA and phenol in the presence of POD yields a quinoneimine dye that is measured at wavelength of 546 nm which is directly proportional to the total cholesterol in the sample.

Alanine Amino Transferase

Principle:

Alanine amino transferase catalyzes (ALT/GPT) catalyzes the transfer of the amino group from Alanine to oxoglutarate with the formation of glutamate and pyruvate .Pyruvate is then reduced to lactate by lactate dehydrogenase (LDH) in the presence of reduced nicotinamide adenine dinucleotide (NADH). The reaction is monitored kinetically at 340 nm by he rate of the decrease in absorbance resulting from the oxidation of NADH to NAD, proportional to the activity of ALT present in the sample.

Aspartate Amino Transferase

Principle:

Aspartate aminotransferase (AST/GOT) catalyzes the transfer of the amino group from aspartate to oxoglutarate with the formation of glutamate and oxalacetate. The latter is reduced to malate by malate dehydrogenase (MDH) in the presence of reduced nicotinamide adenine dinucleotide (NADH). The reaction is monitored kinetically at 340 nm by the rate of decrease in absorbance resulting from the oxidation of NADH to NAD+, proportional to the activity of AST present in the sample.

Alkaline Phosphatase

Principle:

Alkaline phosphatase catalyzes the hydrolysis of 4-nitopheniylphosphate (4-NPP) with the formation of free 4-nitrophenol and in organic phosphate, acting as the alanine buffer as a phosphate group acceptor. The reaction is monitored kinetically at 405nm by the rate of the formation of 4-nitrophenol, proportional to the activity of ALP present in the sample.

Total Protein

Principle:

Peptide bonds of protein react with Cu ions in alkaline medium to form colored complex (acheate) whose color intensity is directly proportional to the total protein concentration and measured at 540 nm.

Albumin

Principle

The method1 is based on the specific binding of bromocresol green (BCG), an anionic dye, and the protein at acid pH with the resulting shift in the absorption wavelength of the complex. The intensity of the color formed is proportional to the concentration of albumin in the sample.

Annex 5. Procedure for TNF- alpha

Materials/Reagent used

- ✓ Bovine TNF- alpha coated plate
- ✓ Bovine TNF- alpha standard
- ✓ Bovine TNF- alpha detection antibody

- ✓ Streptavidin-HRP
- ✓ DPBS
- ✓ Reagent diluent (4% BSA in DPBS, 0.2µm filtered)
- ✓ Standard and sample diluent
- ✓ Wash buffer
- ✓ Substrate
- ✓ Stop solution
- ✓ Plate sealer

Procedure:

1. Bring all kit components and samples to room temperature (18- 25 0 c) before use.
2. Prepare standard and sample dilutions in standard and sample diluent
3. Add 100 µl of standard or sample to appropriate wells

   Note: run each standard or sample in duplicate
4. Cover plate with plate sealer and incubate at room temperature (20-25^{o}c) for 1 hour
5. Wash plate four times with wash buffer

   Note: gently squeeze the long sides of plate frame before washing to ensure all strips remain securely in the frame. Empty plate contents. Use a squirt wash bottle to vigorously fill each well completely with 1X wash buffer, then empty plate contents. Repeat procedure three additional times for a total of four washes. Blot plate onto paper towels or other absorbent material.
6. Add 100 µl of detection antibody working solution to each well.
7. Cover plate with plate sealer and incubate at room temperature for 1 hour.
8. Wash plate four times with wash buffer as described in step 4.
9. Add 100 µl of streptavidin-HRP working solution to each well.
10. Cover plate with plate sealer and incubate at room temperature for 30 minutes.
11. Wash plate four times with wash buffer as described in step 4.
12. Add 100 µl of TMB substrate solution to each well.
13. Develop the plate in the dark at room temperature for 20-30 minutes.

    Note: Do NOT cover plate with plate sealer

14. Stop reaction by adding 100 µl of stop solution to each well.
15. Measure absorbance on a plate reader at 450 nm.

Annex 6. Procedure for IFN-gamma

Materials/Reagent used

- ✓ Bovine IFN-gamma coated plate
- ✓ Bovine IFN-gamma standard
- ✓ Bovine IFN-gamma detection antibody
- ✓ Streptavidin-HRP
- ✓ DPBS
- ✓ Reagent diluent (4% BSA in DPBS, 0.2µm filtered)
- ✓ Standard and sample diluent
- ✓ Wash buffer
- ✓ Substrate
- ✓ Stop solution
- ✓ Plate sealer

Procedure:

1. Bring all kit components and samples to room temperature (18- 25 0 c) before use.
2. Prepare standard and sample dilutions in standard and sample diluent
3. Add 100 µl of standard or sample to appropriate wells
4. Note: run each standard or sample in duplicate
5. Cover plate with plate sealer and incubate at room temperature (20-25oc) for 1 hour
6. Wash plate four times with wash buffer
7. Note: gently squeeze the long sides of plate frame before washing to ensure all strips remain securely in the frame. Empty plate contents. Use a squirt wash bottle to vigorously fill each well completely with 1X wash buffer, then empty plate contents. Repeat procedure three additional times for a total of four washes. Blot plate onto paper towels or other absorbent material.

8. Add 100 µl of detection antibody working solution to each well.
9. Cover plate with plate sealer and incubate at room temperature for 1 hour.
10. Wash plate four times with wash buffer as described in step 4.
11. Add 100 µl of streptavidin-HRP working solution to each well.
12. Cover plate with plate sealer and incubate at room temperature for 30 minutes.
13. Wash plate four times with wash buffer as described in step 4.
14. Add 100 µl of TMB substrate solution to each well.
15. Develop the plate in the dark at room temperature for 20-30 minutes.
16. Note: Do NOT cover plate with plate sealer
17. Stop reaction by adding 100 µl of stop solution to each well.
18. Measure absorbance on a plate reader at 450 nm.

Annex 7. Procedure for IL-10 and IL-12 assay.

Materials/Reagent used:

- ✓ Pre-coated 96 well strip plate
- ✓ Standard
- ✓ Detection reagent A
- ✓ Detection reagent B
- ✓ TMB substrate
- ✓ Wash buffer (30 x concentrate)
- ✓ Plate sealer for 96 wells
- ✓ Standard diluent
- ✓ Assay diluent A
- ✓ Assay diluent B
- ✓ Stop solution
- ✓ ELISA reader 450± 10 nm filter, multi-channel pipettes and disposable tips
- ✓ Eppendorf tubes for diluting samples
- ✓ Deionized or distilled water

- ✓ Absorbent paper for plotting the microtiter plate
- ✓ Container for wash solution

Reagent preparation

1. Bring all kit components and samples to room temperature (18- 25 ^{0} c) before use.
2. Standard- Reconstitute the standard with 1.0ml of standard diluent, kept for 10 minutes at room temperature, shake gently (not to foam). The concentration of the standard in the stock solution is 10,000pg/ml. First the stock solution is diluted to 1000pg/ml and the diluted standard serves as the highest standard (1000pg/ml). Then prepare 7 tubes containing 0.5ml standard diluent and use the diluted standard to produce a double dilution series. Mix each tube thoroughly before the next transfer. Set up 7 points of diluted standard such as 1000pg/ml, 500pg/ml, 250pg/ml, 125pg/ml, 62.5pg/ml, 31.2pg/ml, 15.6pg/ml, and the last EP tubes with standard diluent is the blank as 0pg/ml.
3. Detection reagent A and detection reagent B- Briefly spin or centrifuge the stock detection A and detection B before use. Dilute to the working concentration with assay diluent A and B, respectively (1: 100).
4. Wash solution- Dilute 20ml of wash solution concentrate (30X) with 580ml of deionized or distilled water to prepare 600ml of wash solution (1X).
5. TMB substrate- Aspirates the needed dosage of the solution with sterilized tips and do not dump the residual solution into the vial again.

Assay procedure

1. Determine wells for diluted standard, blank and sample. Prepare 7 wells for standard, 1 well for blank. Add 100µl each of dilution of standard, blank and samples in to the appropriate wells. Cover with the plate sealer. Incubate for 2 hours at 37^{o}c.
2. Remove the liquid of each well, don't wash.
3. Add 100µl of detection reagent A working solution to each well. Incubate for 1 hour at 37^{o}c after covering it with the plate sealer.
4. Aspirate the solution and wash with 350µl of 1x wash solution to each well using a squirt bottle, multi-channel pipette, manifold dispenser or auto washer, and let it sit for 1-2 minutes. Remove the remaining liquid from all wells completely by

snapping the plate onto absorbent paper. Totally wash three times. After the last wash, remove any remaining wash buffer by aspirating or decanting. Invert the plate and blot it against absorbent paper.

5. Add 100µl of detection reagent B working solution to each well. Incubate for 30 minutes at 37^{o}c after covering it with the plate sealer.
6. Repeat the aspiration/wash process for total 5 times as conducted in step 4.
7. Add 90µl of substrate solution to each well. Cover with a new plate sealer. Incubate for 15 – 25 minutes at 37^{o}c. Protect from light. The liquid will turn blue by the addition of substrate solution.
8. Add 50µl of stop solution to each well. The liquid will turn yellow by the addition of stop solution. Mix the liquid by tapping the side of the plate. If color change does not appear uniform, gently tap the plate to ensure thorough mixing.
9. Remove any drop of water and fingerprint on the bottom of the plate and confirm there is no bubble on the surface of the liquid. Then, run the micro plate reader and conduct measurement at 450nm immediately.

Annex: 8. Histopathological Technique:

1. ***Fixation of tissue*** by 10% formaldehyde

2. *Trimming tissue* to fit in to standard histological processing tissue cassettes (5mm thickness)

3. *Tissue processing*: dehydration, clearing and impregnating

- Dehydrating tissue by using increasing strength of alcohol; e.g. 50%, 70%, 90% and 100%. Dehydration 70% alcohol 1 hour
  90% alcohol I 1 hour
  90% alcohol II 2 hour
  100% alcohol I 1 hour
  100% alcohol II 2 hour
  100% alcohol III 2 hours
- Clearing of tissue by Xylene
  Xylene I 2 hours

Xylene II 2 hours

- Impregnation tissue with Paraffin wax

  Paraffin wax I 1 hour

  Paraffin wax II 1 hour

  Paraffin wax III 1 hour

4. *Embedding or Blocking*: Impregnated tissues are placed in a mold with their labels and then fresh melted wax (54 – 60^{o}c) is poured in it and allowed to settle and solidify.

5. *Section*: sectioning of tissue in to 4- 5 micron thickness and adhere on the surface of clear slide.

6. *Staining*: Manual staining with Haematoxylin and Eosin to give colour for sectioned tissue.

***Staining procedure*:**

- Make sure if the slides are well dried in the oven at 65^{o}c for ten minutes
- Dewax the sections with xylene 2x (cleansing)
- Hydrate the section in graded alcohols (100%2x, 95% 2x, 70% 2x) for 3 minutes each
- Rinse with tap water for 5 minutes
- Immerse the slide in myers haematoxylin for 10- 15 minutes
- Wash in running tape water for 30 seconds.
- Counter stain with eosin from 15 sec to 2 min depending on the age of the eosin and the depth of the counter stain desired
- Wash in running tape water for 30 seconds.
- Dehydrate by keeping in increasing concentration of alcohol (2-3 minutes in 50%, 70%, 95% and absolute alcohol).
- Clear it in xylene and mount with DPX or Canada balsam.

9. *Microscopic examination*: stained slide is examined under microscope.