

**SERUM BIOCHEMICAL, HAEMATOLOGICAL AND PATHOLOGICAL
CHANGES IN CALVES EXPERIMENTALLY INFECTED WITH *T. VIVAX*
ISOLATED FROM TSETSE AND NON TSETSE INFECTED AREAS OF
NORTH WEST ETHIOPIA AFTER TREATMENT WITH DIMINAZEN
ACETURATE AND ISOMETAMIDIUM CHLORIDE**

Msc Thesis



BY

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**SERUM BIOCHEMICAL, HAEMATOLOGICAL AND PATHOLOGICAL
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ISOLATED FROM TSETSE AND NON TSETSE INFESTED AREAS OF NORTH
WEST ETHIOPIA AFTER TREATMENT WITH DIMINAZEN ACETURATE
AND ISOMETAMIDIUM CHLORIDE**



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 Tropical veterinary pathology

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As members of the Examining Board of the final MSc open defense, we certify that we have read and evaluated the Thesis prepared by: Frehiwet Tesfu Entitled: Serum Biochemical, Haematological and Pathological Changes In Calves Experimentally Infected With *T. vivax* Isolated From Tsetse And Non Tsetse Infected Areas of north west ethiopia After Treatment With Diminazen Aceturate And Isometsmidium Chloride and recommend that it be accepted as fulfilling the thesis requirement for the degree of: Masters of Tropical Veterinary Pathology.

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DEDICATION

This thesis manuscript is dedicated to my family for nursing me with affection and love and for their dedicated partnership in the success of my life.

STATEMENT OF AUTHOR

First, I declare that this thesis is my original 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.

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LIST OF ABBREVIATIONS

AAT	African Animal Trypanosomosis
ALP	Alkaline phosphatase
ALT	Alanine aminotransferase
AST	Aspartate aminotransferase
DA	Diminazene aceturate
DNA	Deoxyribonucleic acid
Hb	Haemoglobin
IGM	Immunoglobulin M
IM	Intra Muscular
ISM	Isometamidium chloride
MCH	Mean Haemoglobin Concentration
MCV	Mean Corpuscular Volume
MPS	Mononuclear Phagocytic System
NT	<i>T. vivax</i> isolated from non tsetse area
P.I	post infection
PCR	Polymerase Chain Reaction
PCV	Packed Cell Volume
RBCc	Red Blood Cell Count
TT	<i>T. vivax</i> isolate from tsetse area
VSGs	Variable surface glycoproteins

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ABSTRACT

Longitudinal research on biochemical, haematological and histopathological changes in calves experimentally infected with T.vivax isolate from tsetse and non tsetse infested areas of North West Ethiopia after treatment with diminazene aceturate and Isometamidium chloride were conducted. Calves were grouped in to7 groups of 6 animals per group and then challenged by T. vivax at a dose of 1×10^6 parasites/ml intravenously (group 1&2 challenged with isolate from tsetse area, group 3,4,5,6 with isolate from non tsetse area) and group 7(control animals) were received saline water. 14 days post infection calves in group1, group3 and group5 were treated with diminazene aceturate at a dose rate of 7mg/kg body weight and group2, group4 and group6 were treated with Isometamidium chloride at a dose rate of 1mg/kg body weight IM. All trypanosome-inoculated cattle developed acute form of trypanosomosis following the detection of parasitemia in the blood of infected calves. There was variable degree of reduction in mean PCV, Hb concentration and RBC after infection. After treatment all infected groups has resulted in significant improvements in which PCV, Hb concentration and total RBC counts became similar to the pre-infection values for group1 and group2. There was significant reduction in serum glucose, total protein and cholesterol and significant increase in serum enzymes in all challenged groups. After treatment all groups has resulted in significant improvements in the values which were become equivalent to or better than the pre-infection measurements. Gross and histopathological changes were observed in different tissues of calves after treatment with diminazene aceturate and Isometamidium chloride in all groups of calves which were highly

significant in calves infected with isolate from non tsetse area. In conclusion the result of the current experiment revealed that there were improvement after treatment but differences in haematological, biochemical and pathological parameters remained significant in all groups of calves treated with both drugs in comparison to the non-infected groups. So trypanocidal drugs which can not only result in clinical cure but also cause a better improvement in pathophysiological parameters should be developed.

Key words: *Biochemical changes, haematological changes, Pathological changes, Trypanocidal drugs, TT, NT*

1. INTRODUCTION

Trypanosomosis is an important parasitic protozoal disease of humans and animals which is prevalent throughout the tropical and sub-tropical parts of the world (Fatihu *et al.*, 2008). It is transmitted by arthropod tsetse flies (*Glossina spp*) and also mechanically transmitted by tsetse and other biting flies through the transfer of blood from one animal to another (Songa *et al.*, 1990). The major pathogenic tsetse transmitted trypanosome species are *T. congolense*, *T. vivax* and *T. brucei* in cattle, sheep and goats and *T. simiae* in pigs (Sackey, 1998).

African animal trypanosomosis represents one of the most serious veterinary problems in the world (Sackey, 1998). *Trypanosoma vivax* is one of the protozoan parasites that retard economic growth and agricultural production in Africa. The parasite affects wide range of domestic animals in the region (Davila and Silva, 2000; Silva *et al.*, 2002). *T. vivax* is transmitted from herd to herd by infected cattle or sheep, and is transmitted within the herd by biting insects (Desquesnes *et al.*, 1996) and can readily be transmitted mechanically by biting flies other than tsetse (Nyindo, 1992). In Ethiopia, *T. vivax* is one of the commonest trypanosome species and has been found in almost all provinces (Langridge, 1976). This trypanosome species is most prevalent that infect cattle in tsetse free areas (Abebe and Jobre, 1996,). When sample were taken from livestock that were not in contact with tsetse, *T. vivax* was the dominant trypanosome species encountered (Langridge, 1976).

Haematological, pathological and serum biochemical aberration are characteristic of trypanosomosis in domestic animals and man, the severity of which are often determined by the strain of the infecting trypanosomes and host (Anosa, 1988). Anaemia and leucopaenia which are the consistent haematological features in trypanosomiasis (Biryomumaisho *et al.*, 2003) are normocytic normochromic in nature in *T. congolense* infected cattle (Sadique *et al.*, 2001) with the leucopaenia characterised by neutropaenia, eosinopaenia and lymphopaenia in cats experimentally infected with *T. brucei* (Nfon,

2000), as well as cattle infected with *T. congolense* (Biryomumaisho and Katunguka-Rwakishaya, 2007).

Varying observations of biochemical changes have been reported in studies of trypanosome infections in animals (Awobode, 2006). Tissue damages as evidenced by the alteration in the serum enzyme have also been reported in animal trypanosomosis. Marked elevation in the serum levels of AST, ALP and ALT have been observed in both rabbits and rats experimentally infected with *T. brucei* (Oruhe *et al.*, 2005) and *T. congolense* (Egbe-Nwiyi *et al.*, 2005).

Generally, trypanosomosis is known to cause anaemia, hypoproteinemia, leukocytosis, immunosuppression, hypoglycemia and changes in serum enzyme and cholesterol levels. But little is known about *T. vivax* than of the better studied *T. brucei* and *T. congolense* in East Africa. It has long been 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 in Kenya and Uganda (Mwongela *et al.*, 1981). Moreover, it is not yet clear whether *T. vivax* that is transmitted by purely mechanical means (in the non-tsetse area) is equally pathogenic to those in the tsetse area that are adapted to both cyclical and mechanical means of transmission. The present study focuses on *T. vivax* isolated from both tsetse-infested area (Jabitehenan) and non-tsetse area (Bahir Dar Zuria). It is hypothesized that *T. vivax* isolates from the two areas differ in their response to trypanocidal treatments and consequently infected animals respond differently to the impact of treatment which can be demonstrated by biochemical, haematological and pathological changes. Therefore the objectives of this research work are:

- To investigate haematological and serum biochemical changes in local calves experimentally infected with *T. vivax* isolates from tsetse and non tsetse infested area after treatment with DA and ISM.

- To determine pathological changes in local calves experimentally infected with *T. vivax* isolates from tsetse and non tsetse infested area after treatment with DA and ISM.

2. LITERATURE REVIEW

2.1. The parasite (Trypanosome)

Trypanosomosis was first discovered by Sir David Bruce in 1894, who found a correlation between nagana, a disease affecting cattle, the tsetse fly and trypanosomes, while investigating the deaths of many cattle in the Zululand region of South Africa (Duggan, 1977). *T. congolense*, *T. vivax* and *T. b. brucei* are the major causative agents of bovine trypanosomosis in sub-Saharan Africa (Lalmanach *et al.*, 2002; Stevens and Brisse, 2004). *T. vivax* and *T. evansi* are also transmitted mechanically by biting insects, such as tabanids and stomoxes, in areas outside the tsetse belt as well as in South and Central America and Asia (Nantulya, 1990; Osório *et al.*, 2008), while *T. equiperdium* is transmitted sexually and have a wider geographic distribution (Nantulya, 1990; Brun *et al.*, 1998). Infections by these trypanosome species are not confined to cattle since they infect a wide range of domestic animals such as horses, camels, donkeys, mules, water buffalo, pigs, goats and dogs (Nantulya, 1990; Brun *et al.*, 1998; Uilenberg *et al.*, 1998; Stevens and Brisse, 2004). African trypanosomes also affect humans, causing sleeping sickness or human African trypanosomosis. These parasites are *T. b. gambiense* found mainly in West Africa and *T. b. rhodesiense* located mainly in East Africa (Baltz *et al.*, 1985; Barrett *et al.*, 2003; Stevens and Brisse, 2004). The direct effect of trypanosomosis on humans and cattle, is the death of more than 100 people and 10 000 cattle daily (Hursey, 2001). The South American trypanosome known as *T. cruzi* is transmitted by triatomas, and causes Chagas disease in humans (Cazzulo *et al.*, 1997). Dogs and cats have recently been shown to be associated in the transmission of *T. cruzi* (Gürtler *et al.*, 2006).

2.2. Classification of trypanosomes

The classification of trypanosomes has been based solely on medical and veterinary features. The unicellular trypanosome parasites belong to the order Kinetoplastida due to the presence of a kinetoplast at the base of the flagellum (Uilenberg *et al.*, 1998; Stevens

and Brisse, 2004). This kinetoplast contains the mitochondrial DNA of the parasite (Vickerman, 1985). The family Trypanosomatidae is subdivided into Trypanosoma and Leishmania genera, which are classified according to their morphology and range of hosts the parasites, infect (Momen, 2001). The Trypanosoma genus is further subdivided into two sections, namely the Stercoraria and Salivaria, based on how the parasites are transmitted from the insect vector to the mammalian host once the parasite has completed its cyclic development (Uilenberg *et al.*, 1998). In the Stercoraria section, the metacyclic trypanosomes develop in the hindgut and are transmitted via the faeces of the insect vector. The Salivarian parasites develop into the metacyclic stage in the anterior part of the digestive tract of the tsetse fly and they are inoculated via the saliva into the mammalian host (Stevens and Brisse, 2004). A striking feature of the salivarian species is that they contain and express variable surface glycoproteins (VSGs); therefore, they are able to change their surface coats by a process known as antigenic variation (Donelson, 2003; Stevens and Brisse, 2004). Salivaria are further divided into four subgenera namely, Duttonella, Nannomonas, Trypanozoon and Pycnomonas. The Duttonella genus has a principle species known as *T. vivax* and a morphologically similar, but smaller species known as *T. uniforme*. *T. vivax* parasites mainly infect mammalian hosts in Africa and Latin America. The presence of a terminally situated kinetoplast in *T. vivax* is the distinguishing feature that separates the Duttonella genus from the Salivarian trypanosomes (Stevens and Brisse, 2004).

2.3. Clinical manifestations

It is very common for one animal to be infected with not only more than one species of Trypanosomes but also infected simultaneously with other blood parasites (*Babesia* spp., *Theilria* spp., *Anaplasma* spp. and *Ehrlrichia* spp). This makes it difficult to conclude which clinical signs are attributed to a given parasite. Few adequately controlled studies have been made and thus a typical clinical response to any trypanosome is difficult to construct. The cardinal sign observed in the African animal trypanosomosis is anaemia. Within a week of infection with the haematic trypanosome (*T. congolense* and *T. vivax*) there is usually pronounced decrease in packed cell volume, hemoglobin, red blood cells,

and white blood cells levels and within two months these may drop to below 50 percent of their preinfection value. *T. vivax* has a variable incubation period, and, although it is considered to be less virulent for cattle than *T. congolense*, mortality rate of over 50% can occur. There seems to be a marked variation in the virulence of different strains of *T. vivax* but it remains the most important causes of the AAT of cattle, sheep and goat in West Africa. It causes mild disease in horse and chronic disease in dogs. *T. vivax* is often difficult to find in blood smears and can also be demonstrated in lymph node smears (Elnasri, 2005). Certain African isolates of *T. vivax* can cause acute disease accompanied by hemorrhagic syndrome (Mwongela *et al.*, 1981; Roeder *et al.*, 1984). Typical features of these infections include high, persistent parasitemia, fever, very pronounced anemia and generalized visceral and mucosal hemorrhage particularly in the gastrointestinal tract. In the field the disease affecting adult cattle can be severe enough to lead to death or miscarriage even before diagnosis is reached and treatment can be started (Mwongela *et al.*, 1981).

2.4. Transmission

Trypanosomes are transmitted by both male and female tsetse flies of the *Glossina* genus (Uilenberg *et al.*, 1998; Aksoy, 2003). The transmission of the trypanosomes, such as *T. b. brucei*, *T. congolense* and *T. vivax*, by tsetse flies are cyclical. However, mechanical transmission of *T. vivax* and *T. evansi* also occurs outside the tsetse belt of Africa by biting flies, such as tabanids (Vickerman *et al.*, 1993; Uilenberg *et al.*, 1998). Experimentally, *T. vivax* may also be transmitted by syringe passage of infective blood (Van den Bossche *et al.*, 2000)

2.5. Diagnosis

The diagnosis of *Trypanosoma* infection is based on clinical signs and on the demonstration of the parasites by direct or indirect methods. The clinical signs of the AAT are indicative but are not sufficiently pathognomonic and diagnosis must be confirmed by laboratory methods (Elnasri, 2005). The classical direct parasitological

methods for the diagnosis of trypanosomosis, namely microscopic examination of blood or lymph node material, are not highly sensitive, but a number of techniques, including enrichment of the sample, rodent inoculation and DNA methods may increase the sensitivity (Tran *et al.*, 2009).

2.6. Pathogenesis

Pathogenicity may differ within species of trypanosomes based on their geographical region. Some *T. vivax* trypanosomes from East Africa may cause an acute haemorrhagic disease in cattle, whereas *T. vivax* trypanosomes from West Africa may result in a milder non-haemorrhagic disease (Taylor and Authie, 2004). There has also been evidence of a correlation between the biological vector species and the level of virulence of *T. vivax* isolates. The *G. pallipes* from central Kenya transmits a *T. vivax* isolate that causes acute disease and eventually leads to death in approximately one month in 70% of infected cattle, whereas the *G. fuscipes* transmits an isolate in Nyanza (a province located in the south west of Kenya), that causes a chronic infection and eventually leads to death in 100 to 160 days post infection (Osório *et al.* 2008). In bovine trypanosomosis, *T. vivax* seems to be pathogenic followed by *T. congolense* and to a lesser extent *T. b. brucei* (Taylor and Authié 2004). Even though *T. vivax* is less virulent than *T. congolense* it is responsible for over 50% of mortalities in cattle (Prowse 2005). *T. congolense* and *T. b. brucei* have a relatively low parasitaemia when compared to *T. vivax*, which is usually 108 trypanosomes/mL of blood) (Taylor and Authié, 2004).

During bovine trypanosomosis three successive stages in infection may occur, namely acute, stabilisation and chronic; however, death can occur at any stage. The initial stage is the acute phase of the disease, which may contain clinical signs such as enlarged lymph nodes and spleen, weakness, lethargy, abortion and reduced milk production. The acute phase is characterised by a continuous drop in the haematocrit value (packed red blood cell volume, PCV), haemoglobin concentration and red blood cell numbers. Death of the animal may occur in the first weeks or months of the infection due to the acute phase. If cattle survive the acute phase, infection tends to stabilise after six to eight weeks,

characterised by stable PCV values, typical of the stabilisation phase. The animal then enters the third or chronic phase of infection during which the animal may develop cachexia, intermittent parasitaemia and may become stunted, wasted and infertile. This chronic infection may lead to the death of the animal by congestive heart failure due to prolonged anaemia, damage to the heart muscles and increased vascular permeability. The haemorrhagic *T. vivax* isolates from East Africa cause a hyperacute disease that shows symptoms of high parasitaemia, severe anaemia and haemorrhages. These cattle either die in two weeks or self-cure under favourable conditions after two months (Taylor and Authie 2004). Trypanosomosis, like other infectious diseases, starts with an increase of the body temperature, a hyperthermia. This is the result of the contact between the trypanosomes multiplying in the host and the defense system of the host (Uilenberg, 1998). Following are some haematological, serum biochemical and histopathological changes known to be altered during trypanosomosis in animals.

2.6.1. Haematological changes

The trypanosome species affecting man and domestic animals have been subdivided into two groups, the haematinic group (*Trypanosoma congolense* and *T. vivax*) which remains in the plasma and the tissue invading group (*T. brucei*, *T. evansi*, *T. gambiense*, *T. rhodesiense* and *T. equiperdum*) found in extra and intra vascular spaces (Ngure *et al.*, 2008). Because of their presence in the blood, these invading parasites produce numerous changes in the cellular and biochemical constituents of blood (Taiwo *et al.*, 2003).

Trypanosome infection may cause anaemia associated with decrease in PCV, haemoglobin and RBCs counts as reported by many authors in different animal species (Silva, 1999 and Lukins, 1999) as a result of massive erythrophagocytosis by an expanded and active mononuclear phagocytic system (MPS) of the host (Igbokwe and Nwosu, 1997). The mechanism or pathophysiology of anaemia in trypanosomosis is complex and multifactorial in origin (Naessens *et al.*, 2005). Widespread phagocytosis of blood cells, such as erythrocytes and platelets, by macrophages invading bone marrow sinusoids is seen (Murray & Dexter, 1988). Anemia is the chief sign of bovine

trypanosomiasis, and its persistence is responsible for congestive heart failure. This anaemia might be due to the haemolysins such as proteases, phospholipases and neuraminidases induced by the trypanosomes (Soulsby, 1982).

Anaemia which is regarded as the most consistent finding in trypanosomosis of man and domesticated animal has been reported in *T. vivax* infected cattle and goats and *T. congolense* infected sheep (Bisalla, 2007), *T. congolense* infected dogs (Gow *et al.*, 2007), and *T. brucei* infected goats, sheep and rabbits (Taiwo *et al.*, 2003 and Seed, 1969). Study on experimentally infected Nubian goats with *T. vivax* was resulted in significant decrease in packed cell volume (PCV), total red blood cells counts (RBCs) and haemoglobin concentration (Hb) values. Significant increases were encountered in mean corpuscular volume (MCV) and mean corpuscular haemoglobin (MCH) values. No significant change was observed in mean corpuscular haemoglobin concentration (MCHC). Total white blood cells (WBCs) counts were at normal range (Osman, 2012).

2.6.2. Biochemical changes

Biochemical changes have been observed to be associated with trypanosome infection in animals and several factors have been found to influence the nature and severity of these changes. These include the strain of the infecting agent and host variability in susceptibility to infection. Biochemical evaluation of the body fluids gives an indication of the functional state of the various body organs and biochemical changes in body fluids that result from infections depend on the species of the parasite and its virulence (Anosa, 1988). Varying observations of biochemical changes have been reported in studies of trypanosomosis in animals (Awobode, 2006).

2.6.2.1. Serum glucose changes

Carbohydrate metabolism in Trypanosomatidae is compartmentalized in a unique way, and the notion that this compartmentalization must be important for the energy supply of these parasitic protozoa is widely accepted (Michel *et al.*, 2000). Serum glucose

concentration had a significant decrease in subclinical infection of camels with *T. evansi*. This is a common finding in trypanosomiasis, and is reported due to excessive utilization of blood glucose by the parasites for their metabolism (Anosa, 1988). It has also been shown that parasite count is inversely proportional to glucose concentration. Increased metabolic rate caused by fever and hepatocytes degeneration could also be a reason for hypoglycemia in trypanosomiasis (Cadioli *et al.*, 2006).

An acute infection and a variety of changes in the host biochemical parameters were reported from experiment on infection of zebu cattle (*Bos indicus*) with the Samaru strain of *T. vivax*. 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.*, 2000). Infection with the Samaru strain of *T. vivax* caused an acute disease with a very short prepatent period. This was probably due to the high dose of parasites given (Murray and Dexter, 1988). Similarly, the high parasitemia observed coincided with significantly low levels of glucose. Serum glucose levels were significantly low on days 3, 4 and 5 p.i. corresponding with the first parasitemia build up followed by a significant increase on days 7 and 8 p.i. This also coincided with disappearance of parasites from blood. Subsequently, a gradual decrease from day 10 p.i. to below normal values was observed on days 11 to 13 p.i. This situation could be explained by the parasites need for glucose for their cellular metabolism through their glycolytic pathway (Opperdoes *et al.*, 1986). Subsequent significant increases in glucose when parasites have disappeared from blood seem to further agree with the above suggestions of a parasitic/glucose relationship (Kadima *et al.*, 2000).

Similarly, significant decrease in blood glucose on haemato-biochemical studies of camels infested with trypanosomiasis were observed and one animal died showing signs of circling head pressing and convulsions with decreased blood glucose level. The nervous signs in camel can be attributed to hypoglycemic encephalopathy (Padmaja, 2012). Hypoglycaemia in *T. congolense* and *T. brucei* infected animals from 14 days p.i.

onwards were also reported on West African dwarf sheep infected with *T.congolense* and *T.brucei*. It was however, more pronounced in *T.brucei* infected animals on 14, 21 and 35 days p.i (Taiwo *et al.*, 2003).

2.6.2.2. Protein changes

There are no consistent reports on the situation of total protein during animal trypanosomosis; it could remain normal, increased or decreased (Anosa, 1988). Total proteins and gamma globulins increase while serum albumin decreases in several trypanosomes infection (Herrera *et al.*, 2002). The increase in serum total protein may have been due to increased release of tissue specific enzymes and other intracellular proteins secondary to parasite induced cell membrane disruption (Orhue *et al.*, 2005). It was also shown that an increase in total protein and globulins could be due to elevation in the gammaglobulin, as immunological response against the parasite (Orhue *et al.*, 2005; Hilali *et al.*, 2006). Hyper gammaglobulinaemias in African trypanosomosis on the other hand is usually associated with the increase in immunoglobulin M (IgM) which is a consistent finding in trypanosomosis of man and animals (Anosa, 1988). The edema reported in the different parts of the body during the chronic stage could be due to a significant decrease in the albumin levels that possibly indicates liver damage (Orhue *et al.* 2005).

On the other hand, infection caused a significant decrease in serum albumin concentration in infected Nubian goats compared to control goats (Osman *et al.*, 2011). And also, a significant difference in total protein levels was found between control and infected groups in experimentally *T. evansi* infected dogs. In which concentrations of total protein in control animals maintained at a nearly constant value during the infection, but in the infected dogs, total protein values rose significantly throughout the experiment and was found above normal ranges from week 10 to 12. Infected animals also showed hyperglobulinemia at weeks 4, 10, 11 and 12 after infection (Aquino *et al.*, 2002).

Other reports revealed that the levels of total plasma protein remained unchanged from the pre-infected levels for the first three week p.i. on West African dwarf sheep infected with *T.congolense* and *T.brucei*. On the 28th day p.i., the plasma protein levels showed significant increases in *T.congolense* and *T.brucei* infected animals. The levels of plasma globulin showed significant increases above pre-infection levels in both *T.congolense* and *T.brucei* infected animals from 28 days pi and 21 days pi, respectively till the experiment was terminated. No significant changes were observed in the levels of plasma albumin in both groups throughout the experiment (Taiwo *et al.*, 2003). In contrast, decrease in total plasma protein in experimentally *T. brucei* and *T. vivax* infected Savannah brown goats was reported (Adeiza *et al.*, 2008). Similarly, serum total protein, albumin and albumin-globulin ratio were determined in Yankasa sheep experimentally infected with *T. congolense*. In which serum protein concentration was increased while Serum albumin concentration and albumin globulin ratio decreased significantly from a pre-infection in the infected group. The significant drop in albumin globulin ratio could be due to a significant decrease in albumin concentration and an increase in globulin concentration in the infected animals (Bisalla, 2007).

2.6.2.3. Lipid changes

Abnormalities of lipid metabolism have been identified in several laboratory and domestic animals infected with various species of trypanosomes (Anosa, 1988). Determination of total serum proteins, albumin, total cholesterol, triglycerides and high and low density lipoproteins in serum showed that hypoproteinemia, hypocholesteremia, hypoalbuminaemia, low density and high density hypolipidaemia on goats experimentally infected with *T.congolense* or *T.brucei*. However, serum free fatty acid concentrations were significantly higher than those of the control. These changes suggest that the growing number of trypanosomes post infection in goats require some lipids and proteins to support their growth (Biryomumaisho *et al.*, 2003). subclinical infection of camels with *T. evansi* showed hypertriglyceridemia which could be due to defective plasma triglyceride degradation that probably cause free fatty acid unavailable for importation into hepatocytes despite of serum triglyceride elevation and no change was observed in

the serum cholesterol concentration (Sazmand *et al.*, 2011). Experimental study on sheep infected with *T.congolense* and *T.brucei* were resulted in hypocholesterolaemia from the 7th day p.i., in animals with *T.congolense*, which became more severe with progression of infection till the experiment was terminated. However, animals infected with *T.brucei* suffered more severe hypocholesterolaemia than those infected with *T.congolense* (Taiwo *et al.*, 2003). Serum lipids and cholesterol value increased progressively with time, attaining the three or four fold increases over control value nearing terminal stage of infection in rabbits infected with *Trypanosoma gambiense* (Diehl and Risby, 1974). Increased cholesterol value has been associated with hepatic malfunction resulting from impairment of liver lipid metabolism in Africa trypanosomosis (Adejinmi and Akinboade, 2000).

2.6.2.4. Serum/plasma enzymatic changes

The rise in AST activity can be attributed partly to cellular damage caused by the trypanosomes lysis, while the increase in ALT activity probably results from host destruction of trypanosomes (Enwezor and Sacky, 2005). The causes of the elevation of AST levels in the serum of animals are necrosis of the liver, skeletal muscles and kidneys (Lording and Friend, 1991). Raised levels of alkaline phosphatase (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 (Lording and Friend, 1991).

Significant increase in the serum activities of ALP, ALT, and AST on single or mixed infection of mongrel dogs with *T. congolense* and *T. brucei brucei* was reported in twenty dogs experimentally. Serum activities of ALP and AST became noticeable from day seven P.i in all the infected groups whereas that of ALT became noticeable from day 14 P.i and increased continuously until the experiment was terminated. These increases however did not differ significantly between the infected groups in most cases (Ezeokonkwo *et al.*, 2012). This result was substantially consistent with the experiment on albino rats and mongrel dogs reported significant increases in the serum activities of ALP, ALT, and AST respectively following trypanosome infections (Obidike *et al.*,

2005 and Akpa *et al.*, 2008). These significant elevations indicated that the integrity of the vital organs like the liver in the case of elevations in ALP, ALT, and AST was compromised following infection of the dogs with the trypanosomes. This might have led to the increased destruction of hepatocytes and other cells of the body like osteocytes, and skeletal muscle cells, giving rise to increased release of these substances in circulation hence the elevation in the serum. Specifically in dogs, it has been reported that the ALP and ALT levels are relatively narrow in range under normal circumstances and hence a raise in the level in the serum is an indicator of hepatic malfunction (Ezeokonkwo *et al.*, 2012).

A significant difference was found in AST and ALT activity between control and infected groups on experimental *T. evansi* infection in dogs. AST values were above normal levels on weeks 4, 6 and 8 after infection. Despite remaining within normal levels, ALT mean values fluctuated in infected animals and were higher than the values found in the control animals for most of the experimental period. There was no significant difference in ALP activity in control and infected dogs (Aquino *et al.*, 2002).

The plasma levels of ALP and AST showed no significant changes from the pre-infection levels in *T. brucei* infected animals Experimentally on West African dwarf sheep infected with *T. congolense* and *T. brucei*. Those *T. congolense* infected animals had significant increases in both ALP and AST levels in plasma from 14th day p.i. and thereafter remained above the pre-infection level till the termination of the experiment. There were no significant changes in the levels of ALT in all the infected animals as these remained consistently within the pre-infection ranges throughout the course of the experiment (Taiwo *et al.*, 2003).

2.6.3. Pathological changes

2.6.3.1. Gross pathological change

The gross post-mortem lesions observed in sacrificed *T. congolense* infected rabbits include varying degrees of emaciation, dehydration, mucopurulent oculonasal discharges and pasted perineum. The lungs were congested and there was serous atrophy of the perirenal, pericardiac and abdominal fats. There was splenomegaly and hepatomegaly. The liver had greyish depressed focal areas of necrosis. The skeletal muscles were pale (Takeet and Fagbemi, 2009). Similarly, study on comparative histopathology of the lymph nodes, spleen, liver and kidney in experimental ovine infected with *T. brucei*, *T. congolense* and *T. vivax* revealed cachexia and serous atrophy of fat, which were more pronounced in chronic infections. There was enlargement of the prescapular and prefemoral lymph nodes, spleen, liver and kidneys (Omotainse and Anosa, 2009). Another study on experimentally *T. vivax* infected zebu bulls the thyroid glands of both the infected and uninfected animals showed no gross lesions. The mean mass of the thyroid glands of the infected bulls was showed no gross lesions (Fatihu *et al.*, 2009).

2.6.3.2. Histopathological changes

Histopathological changes observed in sacrificed *T. congolense* infected rabbits include mild congestion and disruption of the splenic pulp which were filled with macrophages mild venous congestion of the liver, pulmonary congestion, oedema, acute bronchopneumonia with moderate lymphocytic infiltration and severe emphysema of the lung. There was focal centrilobular necrosis and periportal mononuclear cell aggregation in the kidney, shrunken and congested glomerulus (Takeet and Fagbemi, 2009). Another study on comparative histopathology of the lymph nodes, spleen, liver and kidney in experimental ovine infected with *T. brucei*, *T. congolense* and *T. vivax* showed that the lymphnodes and spleen demonstrated various degrees of severity of hyperplasia of the lymphoid follicles. The cortical and medullary regions of the lymph nodes showed congestion of the sinuses, proliferation of mononuclear cells, erythrophagocytosis and

haemosiderosis. Similarly, the spleen showed depopulated red pulp, proliferation of macrophages characterized by erythro phagocytosis and haemosiderosis as well as proliferation of plasma cells. There are differences in the extent of these reactions depending on the parasite species and stages of infection. The kidneys similarly demonstrated various degrees of congestion, perivascular and interstitial mononuclear cells infiltration, thickening of glomerular capsules, desquamation of tubular cells and protein casts in the tubules. The livers showed various degrees of vascular congestion, perivascular cuffing of mononuclear cells, hepatocellular degeneration and erythrophagocytosis (Omotainse and Anosa, 2009).

Histopathological observations In the experimentally *T. vivax*-infected bulls, the thyroid glands showed focal occurrence of lesions which include distended follicles, containing pale-staining colloid, with numerous peripheral vacuolations of the colloid. There was squamous metaplasia of the follicular epithelium and fibroplasia in the widened stroma (Fatihu *et al.*, 2009). Experimental study on histopathological effect of *T. b. brucei* infection on the master circadian rhythm pacemaker in rats revealed normal neurons with clearly visible round nuclei and fewer glial cells on the suprachiasmatic nucleus of uninfected control rats. On the other hand, the suprachiasmatic nucleus of infected experimental rats showed neurons with smaller and shrunken (pyknotic) nuclei, and degenerative patches were evident. There was also a marked increase in cellularity of glial and other cells. These changes may be correlated to the dysregulation of the circadian firing pattern in the suprachiasmatic nucleus (Maina *et al.*, 2013).

3. MATERIALS AND METHODS

3.1. Study Area

The experimental study was conducted at Debre Zeit in the premises of College of Veterinary Medicine and Agriculture of Addis Ababa University. The experimental house was a state of the art fly proof compartment constructed by the GALVmed (Global alliance for livestock veterinary medicine) and was equipped with all basic facilities (feeding, watering, weighing, postmortem, and storage rooms).

Trypanosoma vivax isolates used in the current experimental study were originally collected from naturally infected cattle in Birsheleko areas of Jabitehenan district (ETBS2) and Bahir Dar Zuria districts (ETBD2 and ETBD3) (tsetse and non-tsetse infested areas respectively of northwest Ethiopia) and stablates were prepared and cryopreserved in liquid nitrogen. In the present experimental study cryopreserved *T. vivax* stablates were initially propagated on donor calves. These isolates were obtained from the undergoing *T. vivax* PhD research project of the VLIR-UOS Ethio- Belgium project by Dr. Shimels Dagnachew. The *T. vivax* used in this study was confirmed by molecular (PCR) techniques (Duffy *et al.*, 2009).

3.2. Study animals

The experimental animals were local zebu cattle of 9 to 12 months of age. The total numbers of animals used in the experiment were 42 in which 27 of the animals were female and the rest 15 male. They were purchased from trypanosome free area Debre Berehan (north central Ethiopia) about 130 km north of Addis Ababa located at latitude and longitude of 9°41'N and 39°32'E coordinates and an elevation of 2,840 meters. All the animals were treated with long acting oxytetracycline in the purchasing site and transported to Debre Zeit. Immediately after their arrival they were dewormed for internal and external parasites with (Triclabendazole and ivermectin). The animals were acclimatized for one month before the beginning of the experiment and handled based on

the guidelines of animal welfare (CIOMS, 1985). All animals were housed in standard fly proof compartments, provided with adequate feed and clean water ad libitum and supplied with concentrated feeds.

3.3. Study design

Longitudinal study design was employed on randomly grouped animals as depicted in (Table 1.) below. Animals were grouped randomly into seven groups of six animals each. Three isolates were used to infect the experimental animals one isolate from tsetse infested area and the other two isolates were taken from nontsetse infested area from the same place from different animals in order to see if there is any difference in their virulence.

TABLE 1: Experimental groups

Group (Six animals /group)	Origin of trypanosome	Challenging dose	Treatment dose
Group one (TT1+DA)	Tsetse area	2 ml of blood at 10^6 trypanosomes/ml	7mg/kg bw- DA
Group two TT1+ISM	Tsetse area	//	1mg/kg bw-(ISM)
Group three (NT1+DA)	Non-tsetse area	//	7mg/kg bw-DA
Group four (NT1+ISM)	Non-tsetse area	//	1mg/kg bw-ISM
Group five (NT2+DA)	Non- tsetse area	//	7mg/kg bw-DA
Group six NT2+ISM	Non- tsetse area	//	1mg/kg bw-ISM
Group seven(NIC)		Saline water	Saline water

- Group1= (TT1+DA) Animals infected with *T. vivax* isolated from tsetse infested area and treated with Diminazene aceturate.
- Group2= (TT1+ISM) Animals infected with *T.vivax* isolated from tsetse infested area and treated with ISM.
- Group3= (NT1+DA) Animals infected with *T.vivax* isolated from non tsetse infested area and treated with DA.
- Group4= (NT1+ISM) Animals infected with *T.vivax* isolated from non tsetse infested area and treated with ISM.
- Group 5= (NT2+DA) Animals infected with *T.vivax* isolated from non tsetse infested area and treated with DA.
- Group6= (NT2+ISM) Animals infected with *T.vivax* isolated from non tsetse infested area and treated with ISM.
- Group7= (NIC) non infected control.

3.4. Trypanosome challenges

Cryopreserved trypanosome parasites were inoculated into propagator (donor) calves for each isolate. At peak parasitemia following establishment of infection, the parasite burden per milliliter of blood was quantified using the rapid matching method (Herbert, 1976) listed in (**Annex 2**). Then, two milliliters of blood containing 1×10^6 parasites/ml was prepared for each infection group of animal reference. Then the blood was given intravenously using sterile syringe.

3.5. Treating animals by trypanocidal drugs

A total of 36 animals grouped in to six groups of six animals per group (table 1) were treated with trypanocidal drugs (Diminazene aceturate and Isometamidium chloride). Calves in group one, group three and group five were treated with Diminazene aceturate (Batchno.A189A01, exp.03/2015 South Africa) at a dose rate of 7mg/kg body weight IM. Calves in group two, group four and group six were treated with Isometamidium chloride (Batchno.199A1, fab. 07/2010,exp.07/2015 France) at a dose rate of 1mg/kg

body weight IM. Calves in group seven (control group) received equal amount of normal saline.

3.6. Sample collection and processing

3.6.1. Clinical and parasitological examination

During the experimental period (from March to June 2014), all animals were clinically examined two times per week for the first two weeks before treatment and once per week for the rest of the experimental period. Parameters such as rectal temperature, color of mucous membranes, size of peripheral lymph nodes, coat condition, fecal consistency, appetite, edema, the presence of lacrimation were examined and recorded. All animals were weighed weekly using digital weighing scale (Taltec, South Africa). Wet smear and Buffy coat technique were done to determine the degree of parasitemia and relapses. Packed cell volume (PCV) determination was also done using the microhaematocrit technique on blood samples collected in capillary tubes (**Annex 3**) (Murray *et al.*, 1977).

3.6.2. Blood collection and Haematological analysis

Blood was collected in EDTA coated vacutainer tube once per week from the jugular vein of the infected calves. The total red blood cell (RBC) counts (**Annex 5**) were determined per week using a haemocytometer after the blood is diluted in Hayem's solution. Haemoglobin concentration (g/dl) was measured using haemoglobinometer (Sahlis method) (**Annex 4**). Anemia was determined based on PCV (%), RBC count MCV, MCH, (**Annex 6**) and hemoglobin concentration.

3.6.3. Serum Biochemical tests

Biochemical tests were performed on serum samples prepared from blood obtained in plain vacutainer tubes, centrifuged at 3200rpm for 10-15 minutes and stored at -20°C until the performance of the test. It was collected once per week until the end of the experimental period. Enzymatic colorimetric assay using humastar 80 clinical chemistry

analyzer (Reitman and Frankel, 1957) was done for determination of the concentrations of alanine aminotransferase (ALT/GPT), aspartate aminotransferase (AST/GOT), alkaline phosphatase (ALP), glucose, total protein, albumin and total cholesterol (**Annex 1**). Human GmbH (Wiesbaden, Germany) standard commercial test kits were used according to manufacturer's instruction.

3.6.4. Postmortem examination and histopathological analysis

Postmortem examinations were done at the end of the experiment after the animals were killed by euthanasia with high dose of phenobarbital intravenously. The skin was reflected for assessing the carcass condition. The regional lymph nodes, prescapular, mandibular, prefemoral, bronchial, intestinal and portal lymph nodes were examined for any abnormality. The pleural and peritoneal cavities as well as all internal organs were examined systemically for gross lesions or abnormalities. The head was opened and the brain removed and examined for lesions. Representative tissue samples were taken from various organs with special emphasis on lymph nodes, spleen, liver, heart, kidney, lung and brain, and fixed in 10% neutral buffered formalin (Bush, 1975) listed in (**Annex 7**). The well preserved tissue was dehydrated in ethanol and embedded in paraffin wax and sectioned at 5µm using a microtome (Leica Co., USA). The thin sections were stained using the haematoxylin and eosin method (Lowe, 1998), examined under a microscope and photomicrographs taken. Histological differences in these tissues between negative control and infected and treated animals were determined.

3.7. Statistical analysis

The entire data source was recorded in Excel spread sheets. Data management and analysis were done using SPSS soft ware of version 20. The statistical tests used were, ANOVA, T-test, Wilcoxon signed rank test. Significance differences are considered at $P < 0.05$.

4. RESULTS

4.1. Clinical findings and development of parasitemia

The detection of parasitaemia in the blood of infected animals begin on day 4 post infection (pi) for the groups infected with non-tsetse (NT) isolates and day 6 pi for animals infected with tsetse (TT) isolates. The parasitemia reached at peak load on day 8 and 12 for NT and TT isolate-infected groups respectively. The uninfected control cattle remained negative throughout the experimental period. All trypanosome-inoculated cattle developed acute form of trypanosomosis, which was characterized by fever, pale mucous membranes, dullness, swollen lymph node, lacrimation, weight loss, reduction in feed intake and dehydration until the day of treatment. The clinical manifestation like fever, swelling of lymph nodes, pale mucous membrane, and reduction in feed in take started on day 4 and day 6 post infection in NT and TT respectively corresponding to the detection of parasites in blood. Diarrhea and dehydration were observed on day 11 p.i. only on animals infected with *T.vivax* isolated from non-tsetse area. Treatment was given when PCV has fallen below 20% but above 15% for majority of the animals (day 14 p.i.) so that response to treatment could easily be visualized. Following treatment, parasitemia becomes non-detectable in the blood of all infected calves. Relapse was observed in group five (NT2+DA) and group three (NT1+DA) on day 21 and 35, respectively. Observable clinical signs disappeared starting from a week following treatment with Diminazene aceturate and Isometamidium chloride in all groups of animals except in the case of relapse where some mild signs have been seen.

4.2. Haematological findings (red blood cell indices)

Following infection with trypanosomes, there was variable degree of reduction in mean PCV, Hb concentration and RBC counts until treatment day. In all cases there was significant reduction in red blood cell indices following infection (Day -14/day of infection to Day 0/day of treatment) ($p < 0.05$). Treatment on day 0 of all infected groups has resulted in significant improvements in the values whereby PCV, Hb concentration

and total RBC counts became similar to the pre-infection values for group one (TT1+DA) and group two (TT1+ISM) in general. However, the improvement did not equal to the control values for the three indices in all groups infected by non-tsetse transmitted trypanosomes and treated by either DA or ISM ($P < 0.05$). Similarly, the PCV measurements improved to values comparable to that of the control group only for the group infected with TT trypanosomes and treated with DA whereas this was not yet restored in all other groups ($P < 0.05$) compared to the non-infected controls. Moreover, following relapse of parasitemia on day 21 p.i. in group five (NT2+DA), there was further fall in PCV value which was again significant on day 49 p.i. as compared to that of day 21 (Figure 1).

Similarly hemoglobin concentrations remained lower than that of control group in group five (NT2+DA), group four (NT1+ISM) and group six (NT2+ISM) ($P < 0.05$) whereas it regained normality in other groups. RBC counts returned to values comparable to that of control group except for group four (NT1+ISM) where it remained significantly lower ($p < 0.05$). Moreover, in one of the groups (NT2+DA) that has shown relapsing parasitemia, mean Hb and RBC count values further dropped to lower level on day 49 as compared to day -14 pi ($P < 0.05$) (Figures 2 and 3). Mean MCV and MCH value of all groups of animals after treatment with DA and ISM showed returning to normality except for group three (NT1+DA) and group four (NT1+ISM) where it was significantly lower than control values ($p < 0.05$).

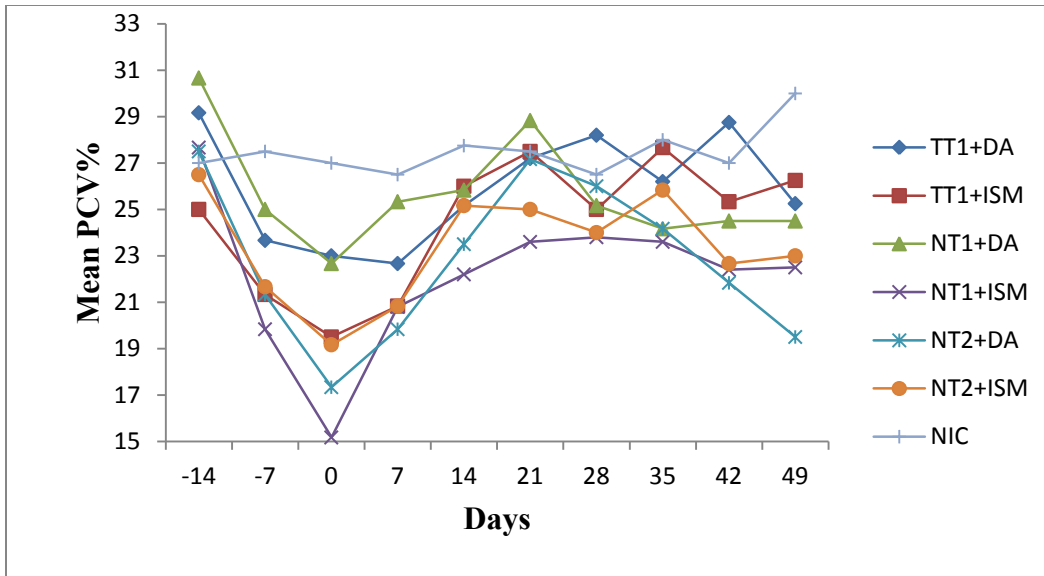


Figure 1: changes in mean PCV values in calves experimentally infected with *T. vivax* isolate from TT and NT areas and treated with DA and ISM. Animals were infected on day -14, treated on day 0 with DA and ISM and followed until day 49.

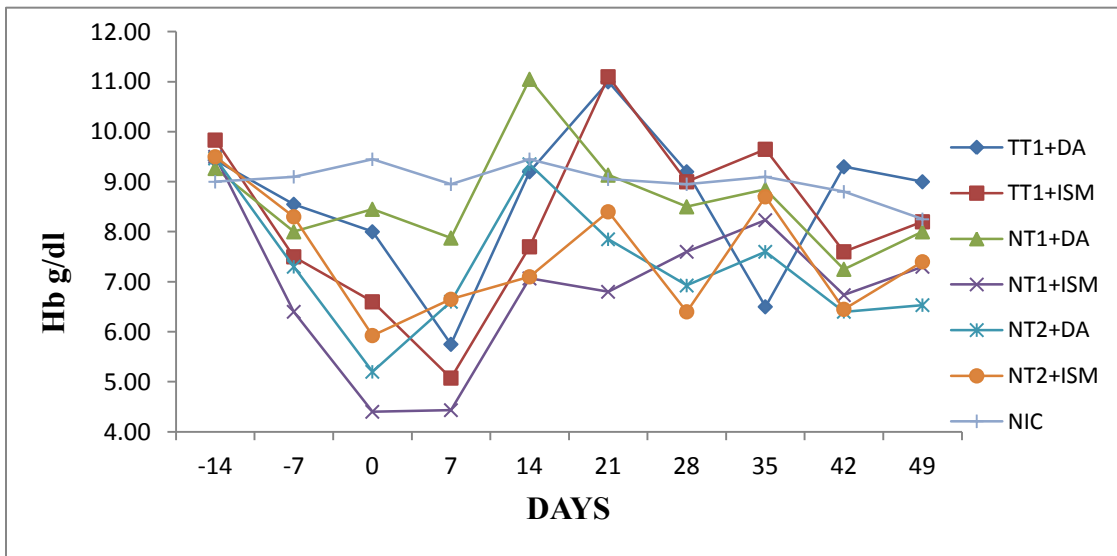


FIGURE 2: changes in mean Hb values in calves experimentally infected with *T. vivax* isolate from TT and NT areas and treated with DA and ISM. Animals were infected on day -14, treated on day 0 with DA and ISM and followed until day 49.

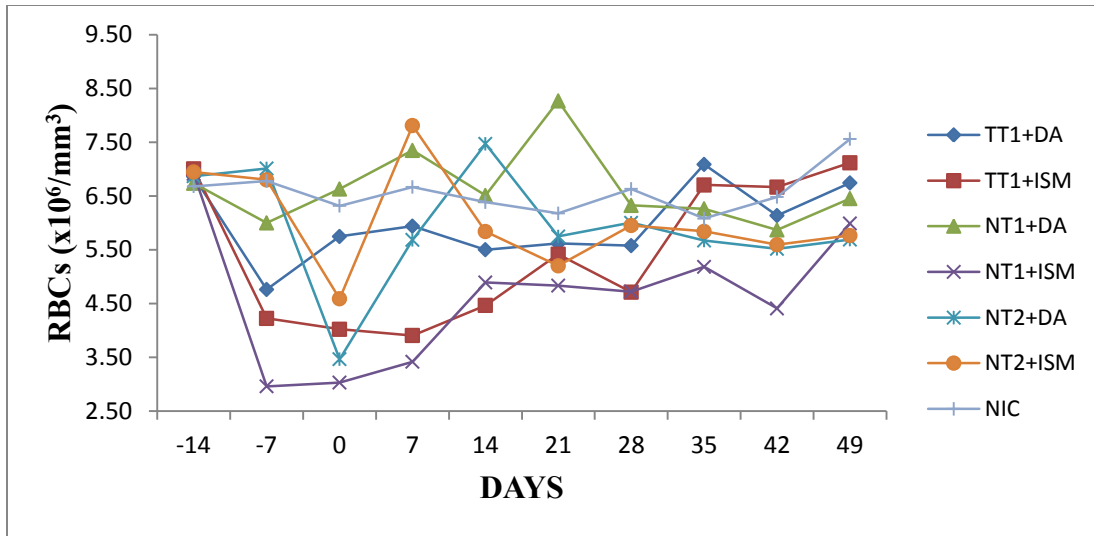


Figure 3: changes in mean RBC count values in calves experimentally infected with *T. vivax* isolate from TT and NT areas and treated with DA and ISM. Animals were infected on day -14, treated on day 0 with DA and ISM and followed until day 49.

4.3. Biochemical changes

In most cases there was significant reduction in serum biochemical parameters following infection (Day -14 to Day 0). Treatment on day 0 of all infected groups has resulted in significant improvements in the values which were either become equivalent to or larger than the pre-infection measurements. Values for control animals remained relatively constant. After treatment, there was a trend of increase back to pre-infection levels except for day 14 pi where values of glucose in group one and two (TT1-DA and TT1-ISM), albumin (all infected groups) and total protein (all groups) showed significant decline ($p < 0.05$). Similarly, in diminazene treated infected groups serum glucose level returned to control level except for group five (NT2+DA) where it was significantly below control value ($p < 0.05$). On the other hand, in Isometamidium treated groups, significant reductions ($p < 0.05$) in group two (TT1-ISM) for glucose and in group four (NT1-ISM) and group six (NT2-ISM) for total cholesterol were maintained until the end of the experiment whereas significant increase in albumin concentration was seen in

group six (NT2-ISM) ($P < 0.05$). However, complete return to normality was observed for total protein in both DA and ISM treated groups (Figures 4-7).

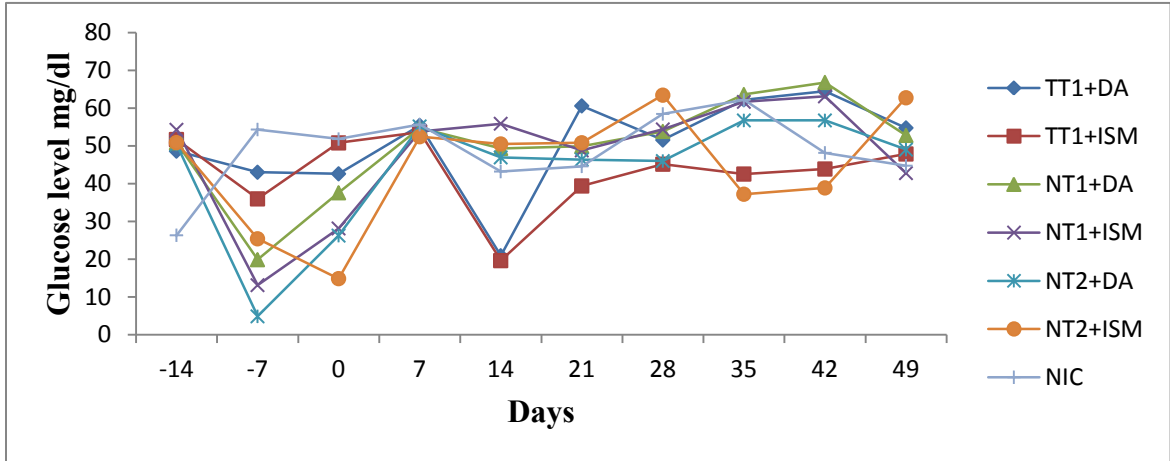


Figure 4: changes in mean serum glucose level in calves experimentally infected with *T. vivax* isolate from TT and NT areas and treated with DA and ISM animals were infected on day -14, treated on day 0 with DA and ISM and followed until day 49.

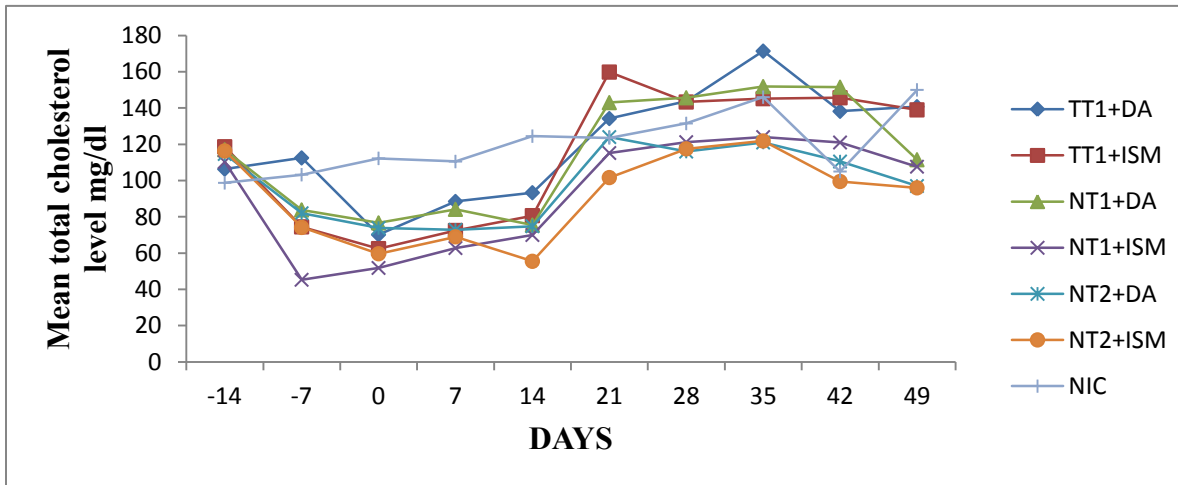


Figure 5: changes in mean total cholesterol values in calves experimentally infected with *T. vivax* isolate from TT and NT areas and treated with DA and ISM. Animals were infected on day -14, treated on day 0 with DA and ISM and followed until day 49.

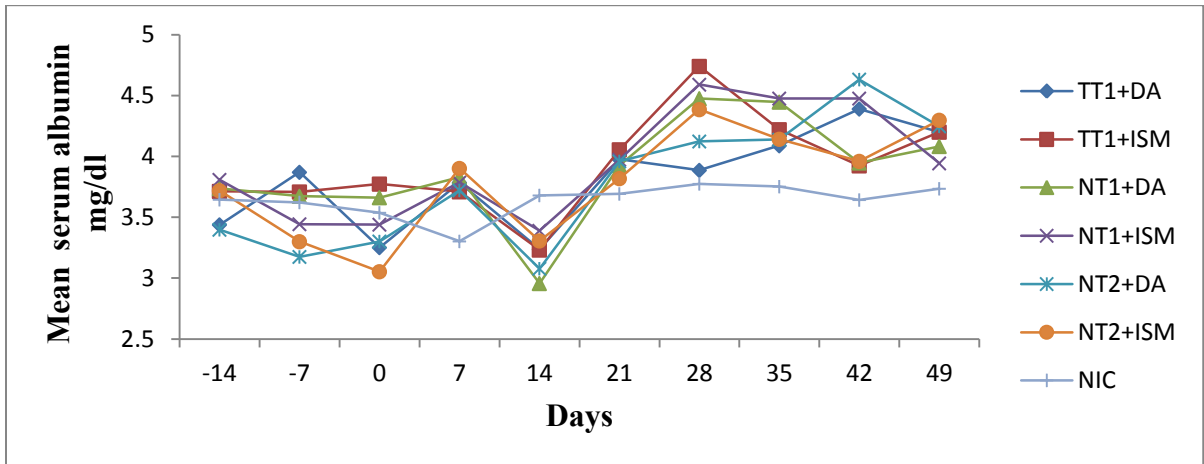


Figure 6: changes in mean serum albumin level in calves experimentally infected with *T. vivax* isolate from TT and NT areas and treated with DA and ISM. Animals were infected on day -14, treated on day 0 with DA and ISM and followed until day 49.

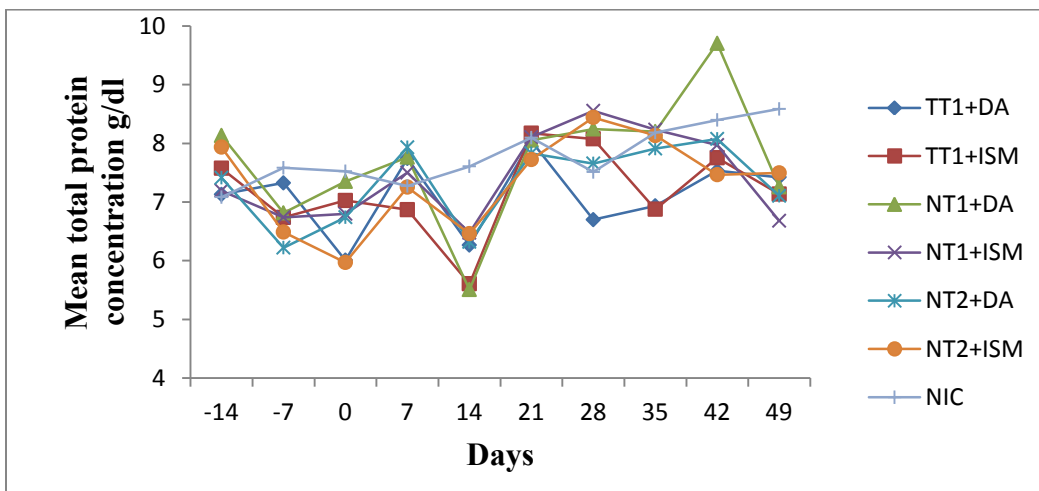


Figure 7: changes in mean total protein concentration values in calves experimentally infected with *T. vivax* isolate from TT and NT areas and treated with DA and ISM. Animals were infected on day -14, treated on day 0 with DA and ISM and followed until day 49.

In most cases there was significant increase in serum enzyme levels following infection (Day -14 to Day 0). Treatment on day 0 of all infected groups has resulted in reduction in group three (NT1+DA) and group four (NT1+ISM) or an increase which was significantly ($p < 0.05$) above the pre-infection values in other groups for serum AST. ALT values returned to pre-infection levels except for group two (TT1+ISM) and group six (NT2+ISM) where it was still lower on day 49 p.i. ALP values all returned to pre-infection levels although individual variations were high. Comparison of values with control groups also shows similar trend. A decline for AST and ALT and a rise for ALP back to values comparable to control values within seven days post treatment except that significantly higher values were still maintained for AST in group one (TT1+DA) and group two (TT1+ISM) (Figures 8-10) as compared to control group. Moreover, in the group showing early relapse group five (NT2+DA), a re-rise in serum ALT was noticed after parasites were detected in their blood.

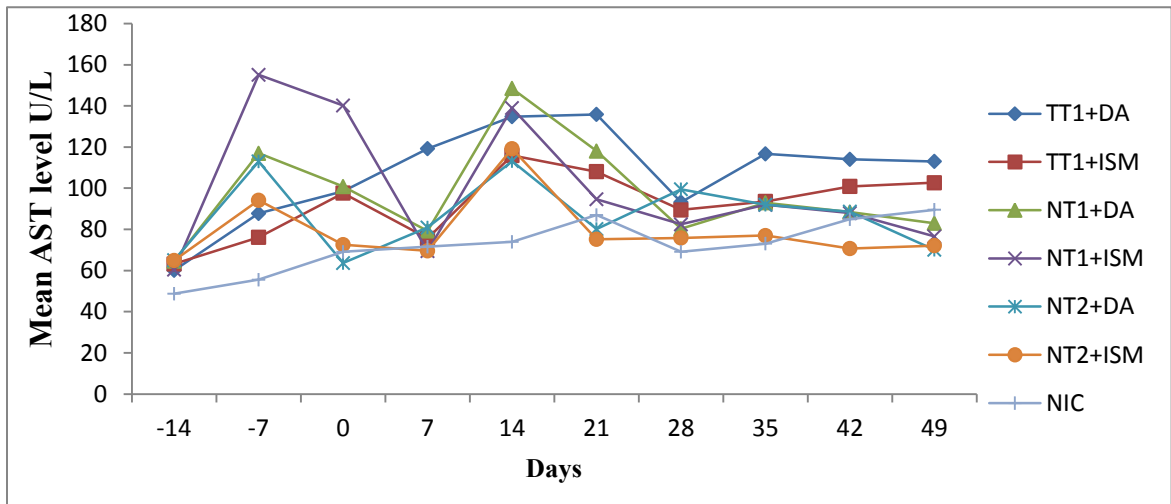


Figure 8: changes in mean AST level in calves experimentally infected with *T. vivax* isolate from TT and NT areas and treated with DA and ISM. Animals were infected on day -14, treated on day 0 with DA and ISM and followed until day 49.

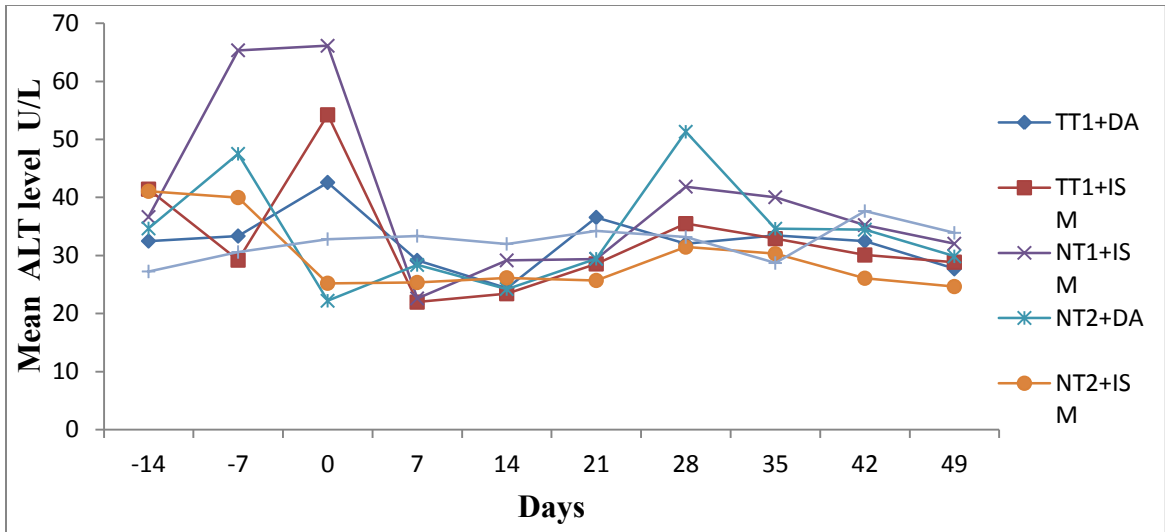


Figure 9: changes in mean ALT level in calves experimentally infected with *T. vivax* isolate from TT and NT areas and treated with DA and ISM. Animals were infected on day -14, treated on day 0 with DA and ISM and followed until day 49.

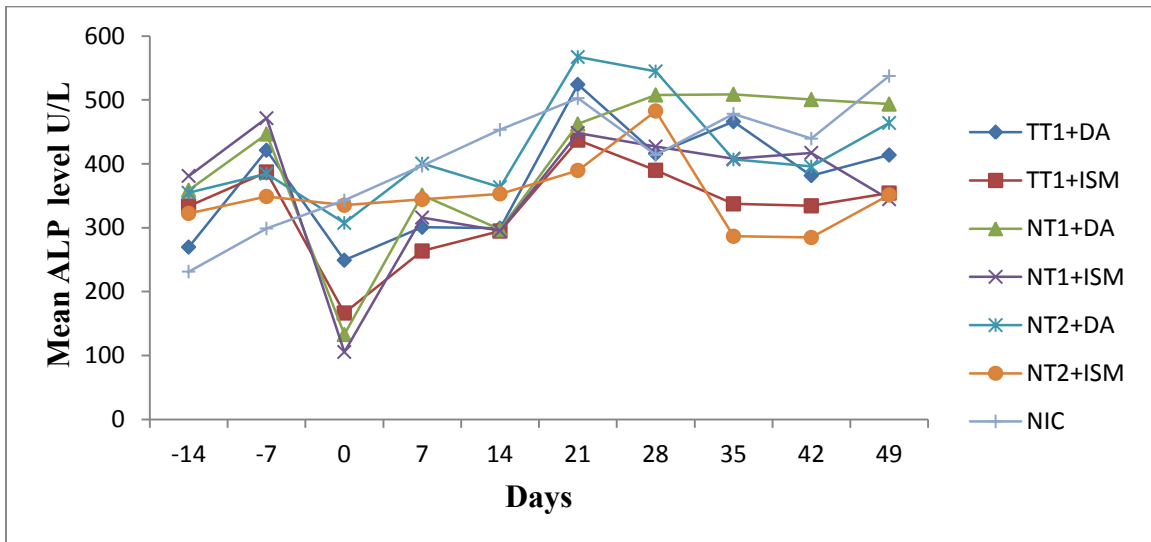


Figure 10: changes in mean ALP level in calves experimentally infected with *T. vivax* isolate from TT and NT areas and treated with DA and ISM. Animals were infected on day -14, treated on day 0 with DA and ISM and followed until day 49.

4.4. Pathological lesion

The spleen, liver, kidney, lung, heart and brain were observed grossly and histopathologically for any abnormality in young zebu cattle treated with DA and ISM following infection with *T. vivax* isolated from tsetse area and non-tsetse areas. Postmortem examinations were done mainly at the end of the experiment. The non infected control group of calves showed no any pathological changes.

4.4.1. Gross pathological changes

Spleen

Grossly, spleen was enlarged, there by characterizing marked splenomegaly and congestion in animals infected with *T. vivax* isolated from non-tsetse area and treated with DA (NT2+DA) in comparison to the non-infected control group (Figure 11). Other infected groups showed slight enlargement and congestion after treatment in comparison to the spleen of non infected control group.

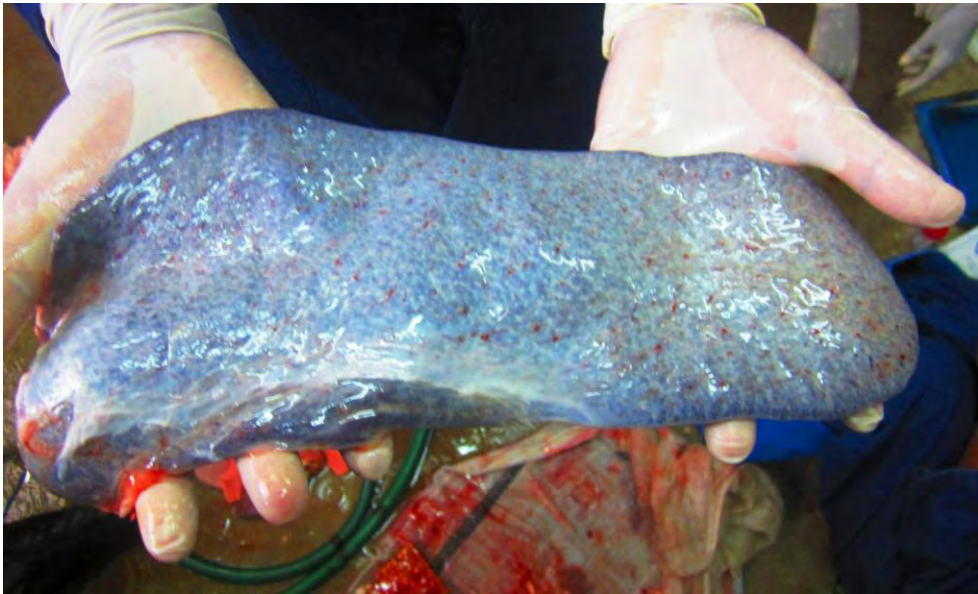


Figure 11: Enlarged and congested spleen from calves infected *T. vivax* isolated from non tsetse area and treated with DA (NT2+DA).

Kidney

Grossly kidneys were decreased in size in all groups of animals. In addition to this there was yellowish pigment of the fats present in the kidney in group three (NT1+ISM) and group six (NT2+ISM) in comparison to the non infected control group.

A)

B)

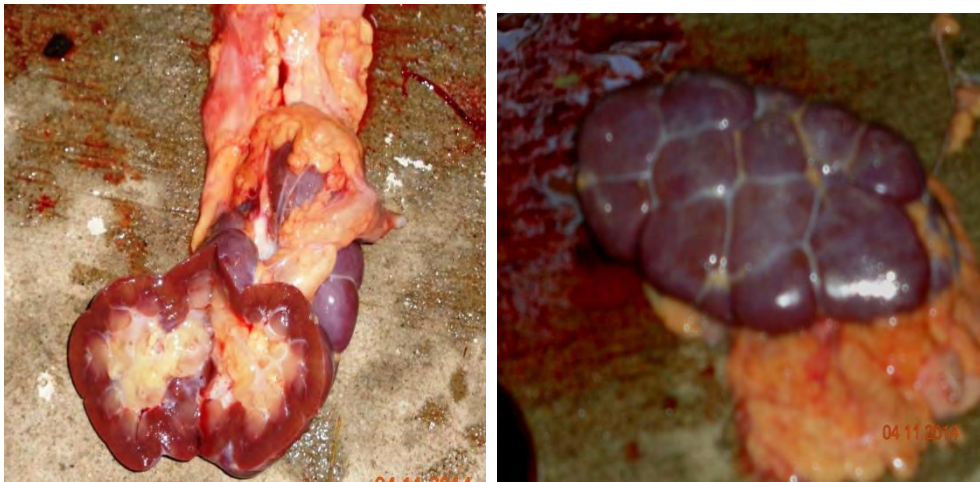


Figure 12: atrophied kidneys of calves infected with *T.vivax* isolated from tsetse (TT) (a) and non tsetse area (NT) (b) treated with DA.

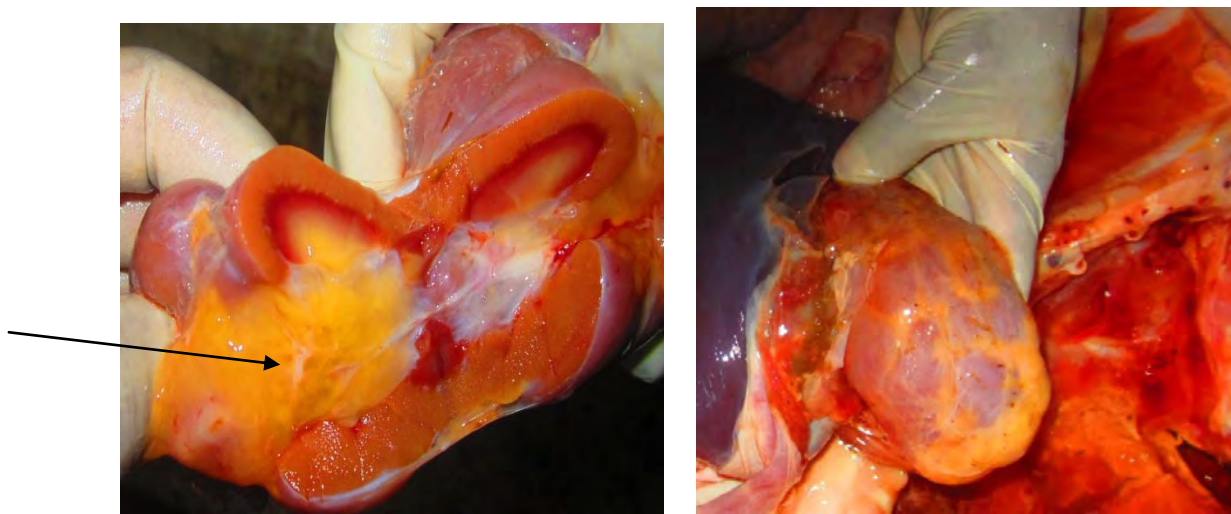
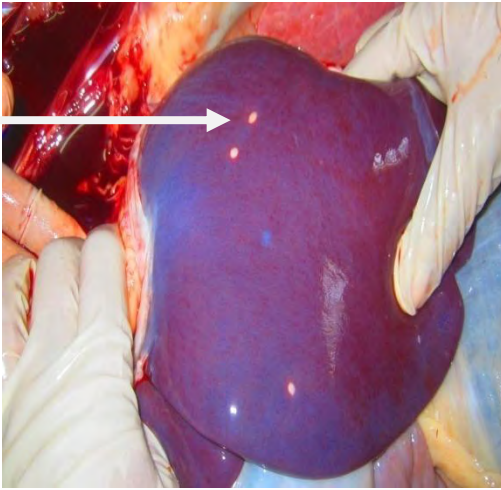


Figure 13: atrophied with yellowish pigment in the pelvis region and in the external fat of the kidney of calves infected with NT and treated with ISM.

Liver

Grossly livers were enlarged and the edges become round in all groups of the experimental animals. In addition to that group one (TT1+DA) and group three (NT2+DA) animals showed white nodule that were sharply delineated from the adjacent parenchyma (Fig 14).

A)



B)

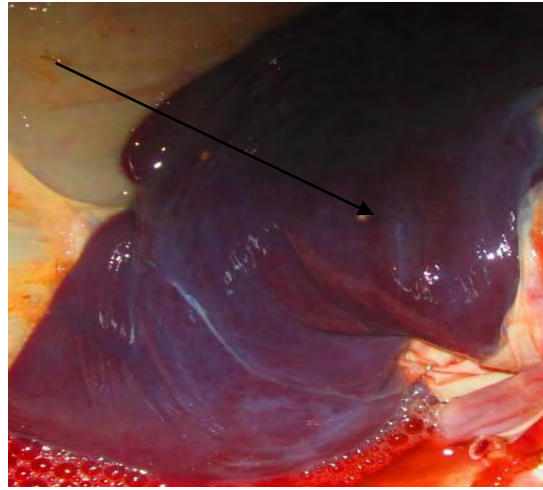


Figure 14: enlarged, edematous livers with white nodules (arrows) and rounded edges from calves infected with TT (a) and NT (b) treated with DA.

Lung

Grossly lesions observed in the lung of all groups includes emphysematous, pneumonia with consolidation but most of the lungs showed meaty consistency and were rubbery, condition characteristic for interstitial pneumonia. This gross lesions were pronounced in group three (NT1+DA) and group five (NT2+DA) (Fig 15).

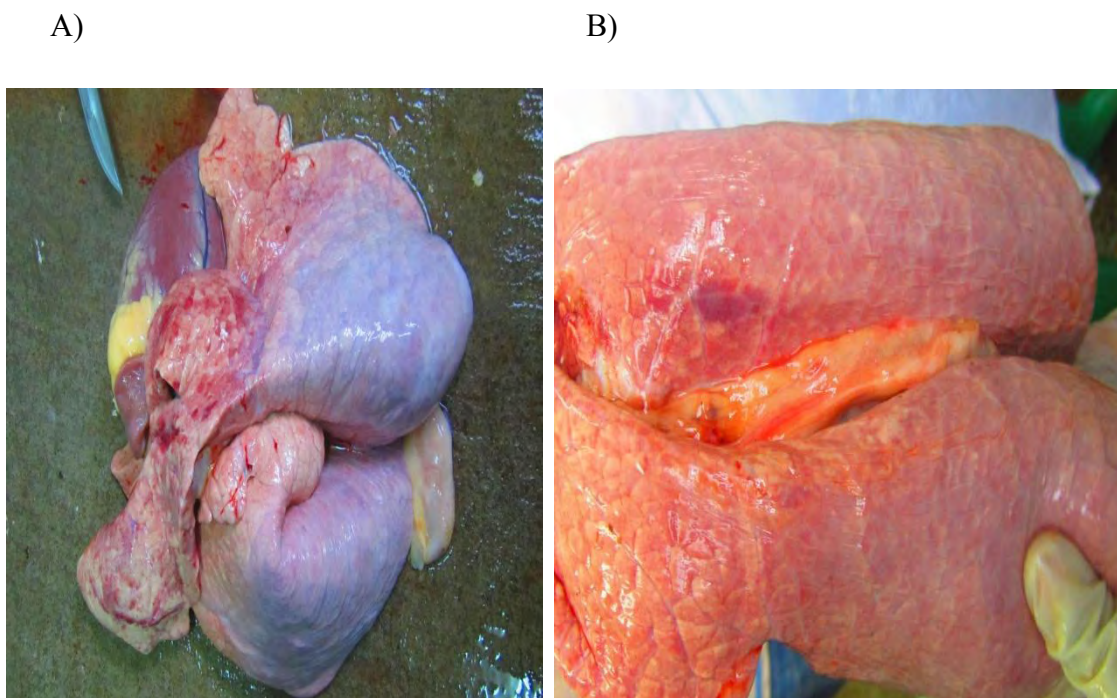


Figure 15: pneumonia with consolidation on cranial, middle and some part of caudal lobes in the right lung and cranial lobe in left lung (a) and rubbery lung with meaty appearance and edema in interstitial space (b) in calves infected with (NT) and treated with DA.

Brain

Grossly brain was edematous with severe meningitis and congestion in animals infected with *T.vivax* isolated from non tsetse area treated with DA and ISM. Animals infected with *T. vivax* isolated from tsetse area treated with DA and the other group treated with ISM grossly showed no any gross abnormality in brain.

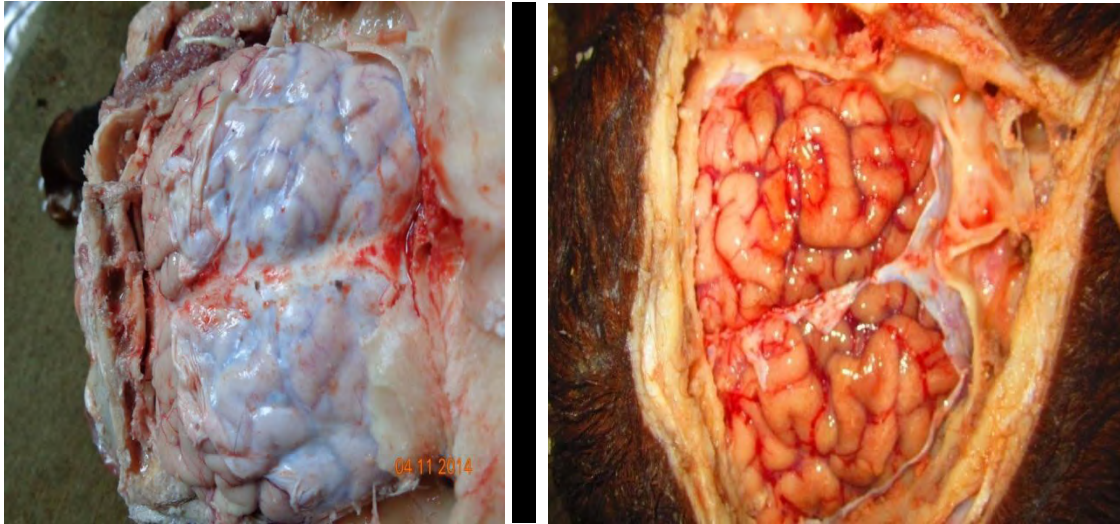


Figure 16: brain with severe edema which resulted in flattening of gyri and widening of sulci in calves infected with NT and treated with DA (a) and brain showing congestion in calve infected with NT and treated with ISM (b).

4.4.2. *Histopathological changes*

Spleen

Histopathologically spleen of experimental calves infected with *T.vivax* isolated from non tsetse area and tsetse area treated with DA showed focal necrosis with cystic lymphoid follicles (both in the red pulp and white pulp parts of spleen (Fig 17 and 18). The histopathology of spleen in non infected control group was normal in all animals.

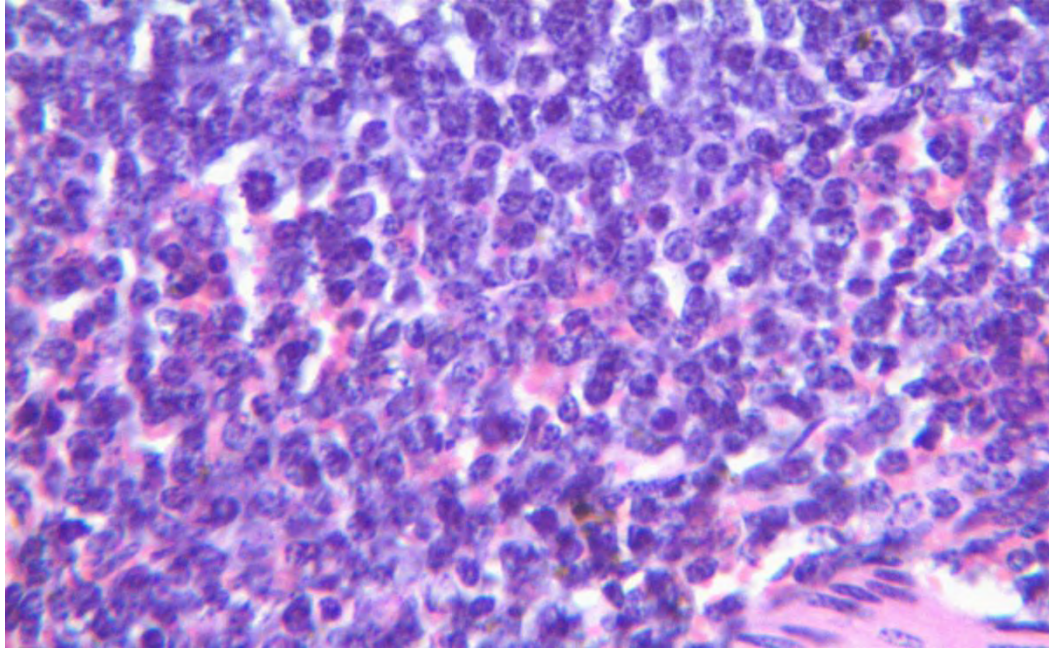


Figure 17: Photomicrograph of spleen showing necrosis of lymphoid cells in calves infected with *T. vivax* isolated from non tsetse area and treated with DA.x40

A)

B)

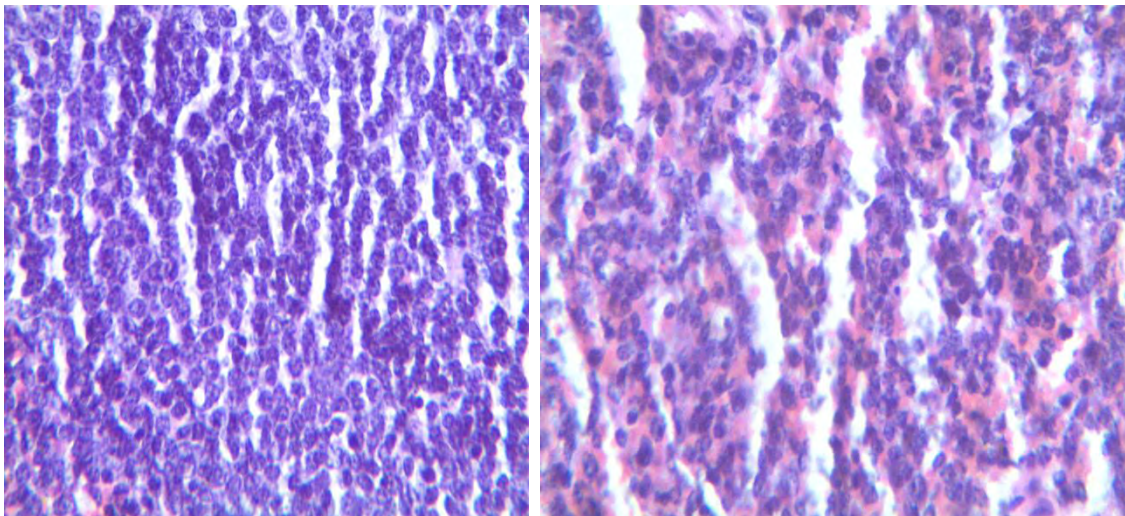


Figure 18: photomicrography of spleen showing necrosis of lymphoid cells in the white (a) and (b) red pulp of spleen in calves infected with *T. vivax* isolated from tsetse area and treated with DA.x40

Kidney

Histopathologically changes were also revealed almost similar findings in all groups of animals. There was glomerulonephritis with mononuclear cell infiltration (Lymphocytes), acute tubulointerstitial nephritis and acute tubulo necrosis with hyaline membrane in the tubules. The histopathology of kidney in non infected control group was normal in all animals.

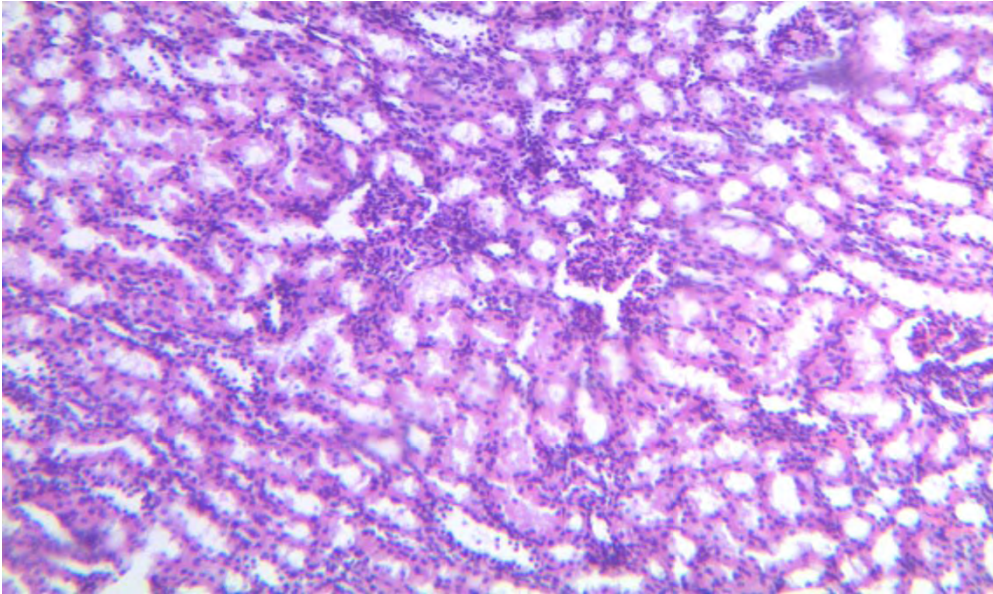


Figure 19: Photomicrograph of kidney from non tsetse infected calves treated with DA showing glomerulonephritis with mononuclear cell infiltration (lymphocytic follicles), acute tubulointerstitial nephritis and acute tubulo necrosis with hyaline membrane.x10

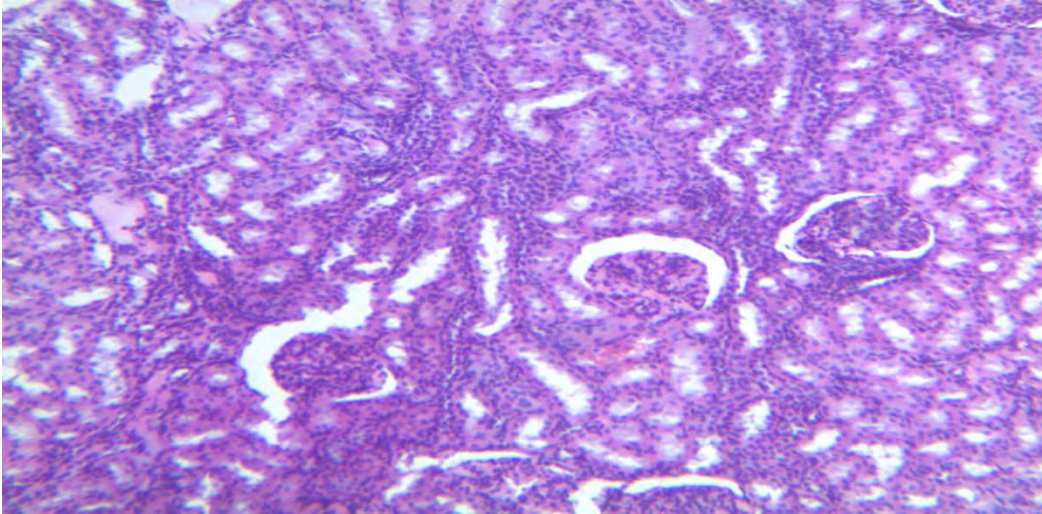


Figure 20: Photomicrograph of kidney tissue from calves infected with *T. vivax* isolated from tsetse area treated with DA showing glomerulonephritis with mononuclear cell infiltration (lymphocytes) and acute tubulointerstitial nephritis.x10

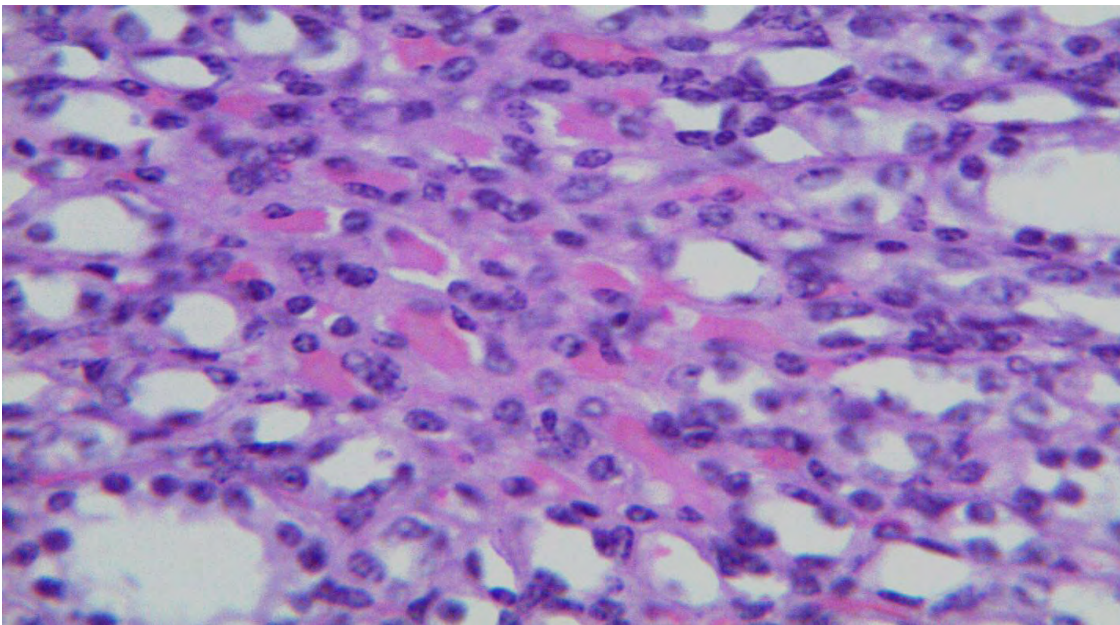


Figure 21: Photomicrograph of kidney tissue from calf infected with *T.vivax* isolated from non tsetse area and treated with ISM showing hyaline membrane in the interstitium and tubules.x40

Liver

Histopathologically liver showed hepatitis with lymphocytic infiltration at the portal region in all groups of animals, pyogranuloma in some cases (more neutrophils with few lymphocytes) in group one (TT1 + DA) and group five (NT2 + DA). The gross and histopathology of liver in non infected control group were normal in all animals.

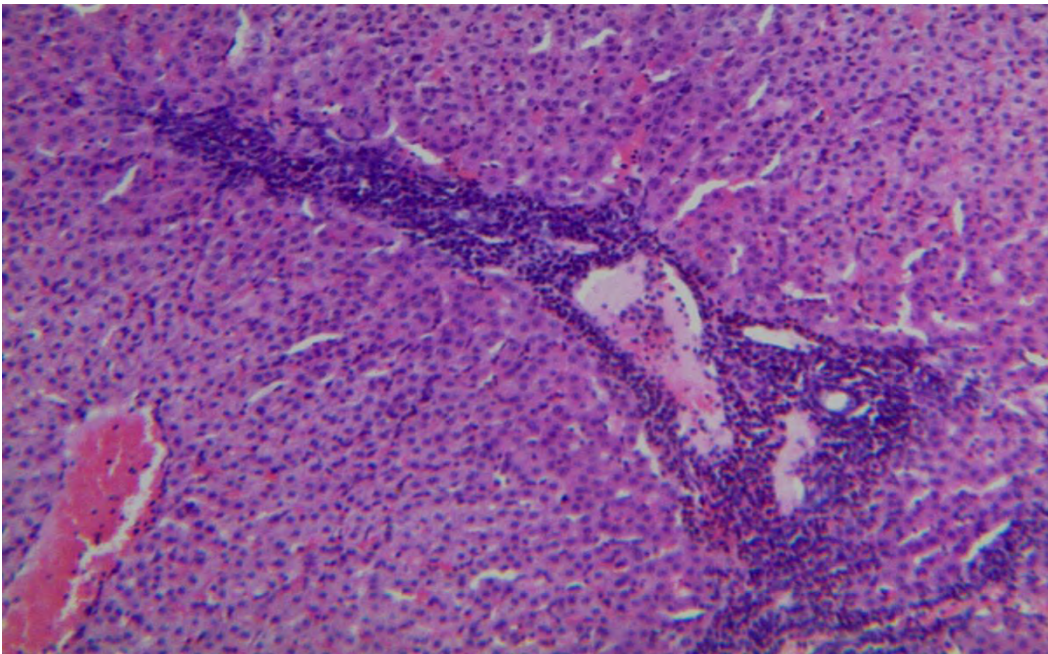


Figure 22: Photomicrograph of liver from calves infected with *T. vivax* isolated from tsetse area treated with ism showing massive lymphocytic infiltration at the portal region. x10

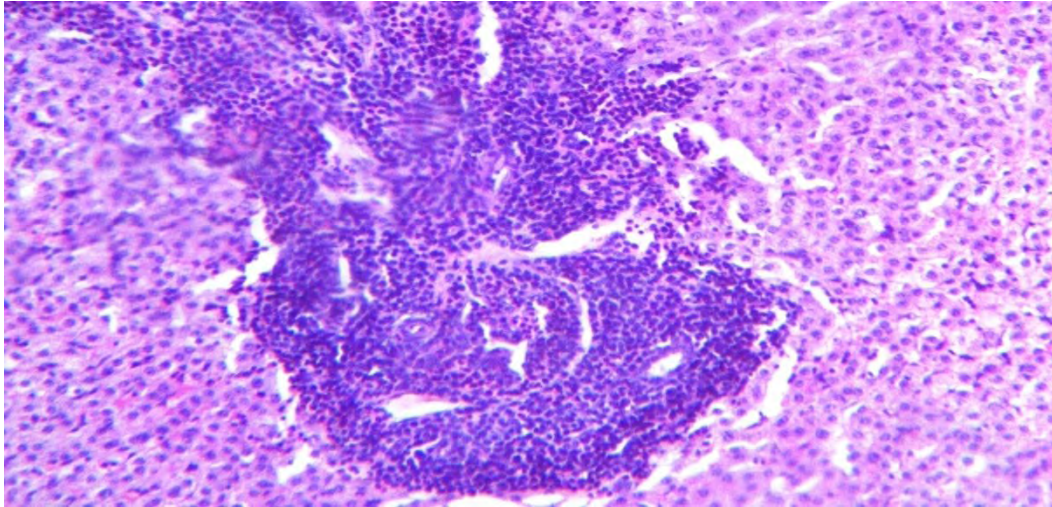


Figure 23: Photomicrograph of liver from calve infected with *T. vivax* isolated from tsetse area and treated with DA showing pyogranoloma (massive neutrophil with few mononuclear cells). x10

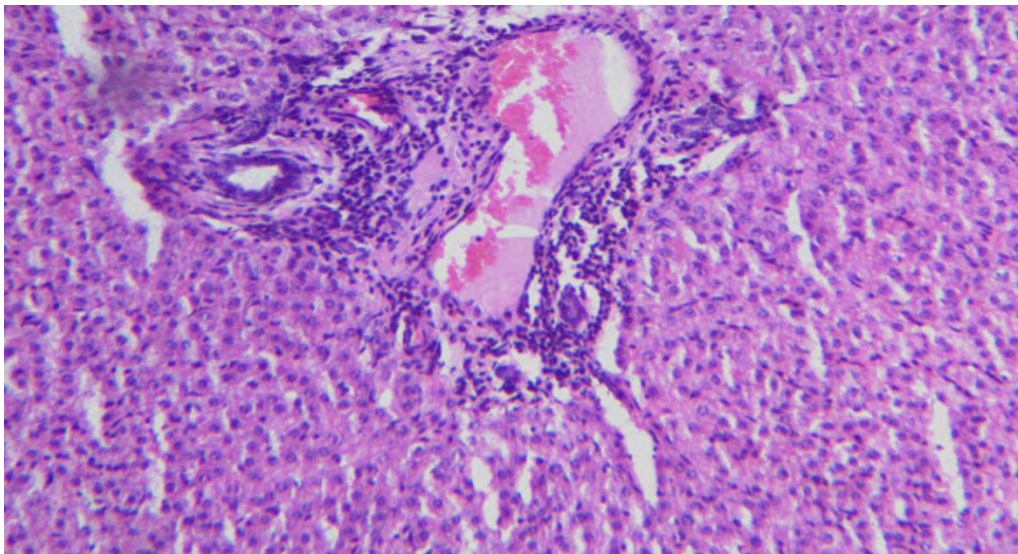


Figure 24: Photomicrograph of liver from calve infected with *T. vivax* isolated from non tsetse area treated with ISM showing lymphocytic infiltration at the portal region and perivascular infiltrations. x10

Heart

Histopathologically heart revealed myocardial necrosis and severe myocarditis that is infiltrated by mononuclear cells specially lymphocytes, plasma cells. In some cases eosiniphils infiltration were observed (few in number). The histopathology of the NT2+ISM, NT1+ISM, TT1+DA and TT1+ISM was revealed almost similar histopathological changes with the non infected control group of animals. Lesion in the heart that is myocarditis with lymphocytic infiltration was the most repeatable lesions than any lesion in any organ. The histopathology of heart in non infected control group was normal in all animals.

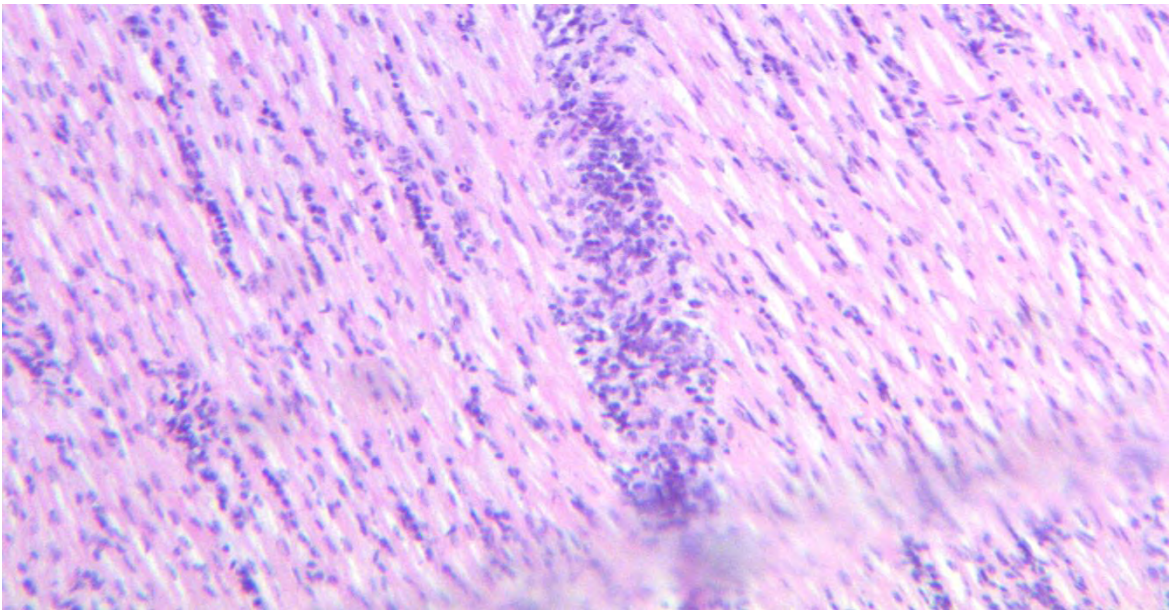


Figure 25: heart microscopy, severe myocarditis infiltrated by mononuclear cells especially lymphocytes, plasma cells in calves infected with *T. vivax* isolated from tsetse area treated with DA.

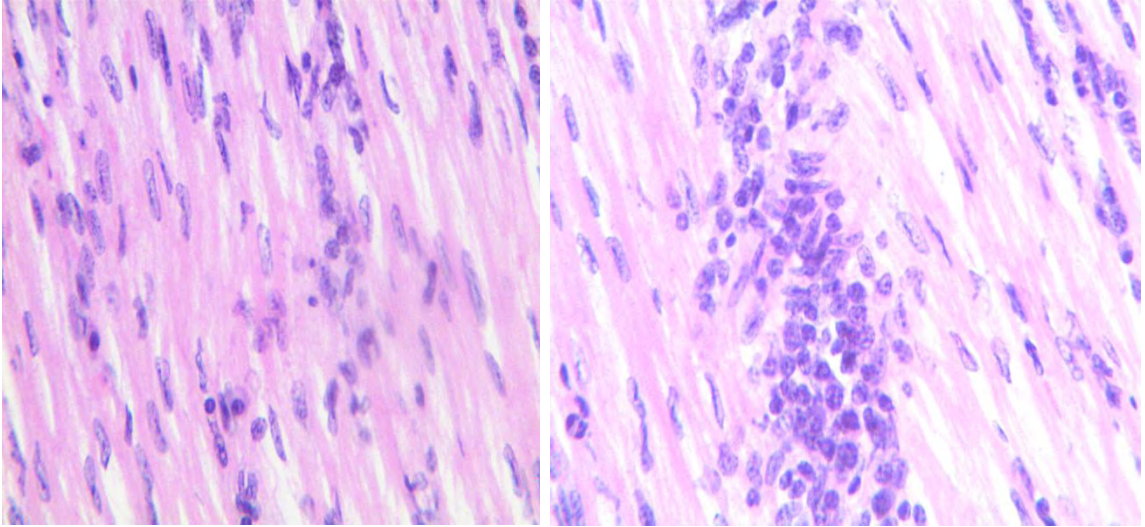


Figure 26: Photomicrograph of heart showing myocardial necrosis in calves infected with *T. vivax* isolated from non tsetse area treated with DA.x40

Lung

Microscopically most consistent lesion were interstitial pneumonia with aggressive proliferation of fibroblasts and inflammatory cells which results in sever compression of alveoli in affected region which leads to sever emphysema in the nearby parts in the lung

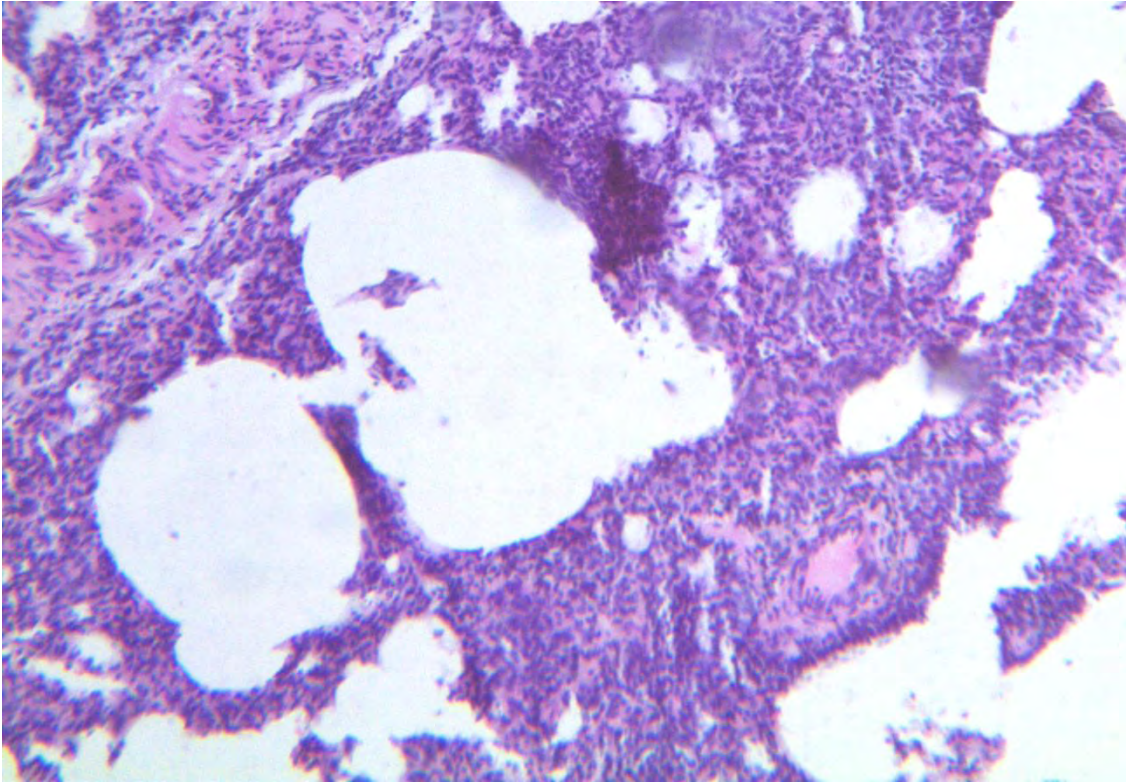


Figure 27: Photomicrograph of lung from calves infected with NT and treated with DA interstitial pneumonia and aggressive proliferation of fibroblastes and inflammatory cells which results in sever compression of alveoli. Some alveoli at the center were distended because of severe compression of nearby alveoli. x40

Brain

Microscopically brain of (NT1+DA, NT1+ISM, NT2+DA and NT2+ISM) showed neuronal necrosis in which the neurons were angular and shrunken with hypertrophied astrocytes. (Fig 28).The other groups infected with *T. vivax* isolate from tsetse area showed slight histopathological changes in comparison to the non infected control group. The histopathology of brain in non infected control group was normal in all animals.

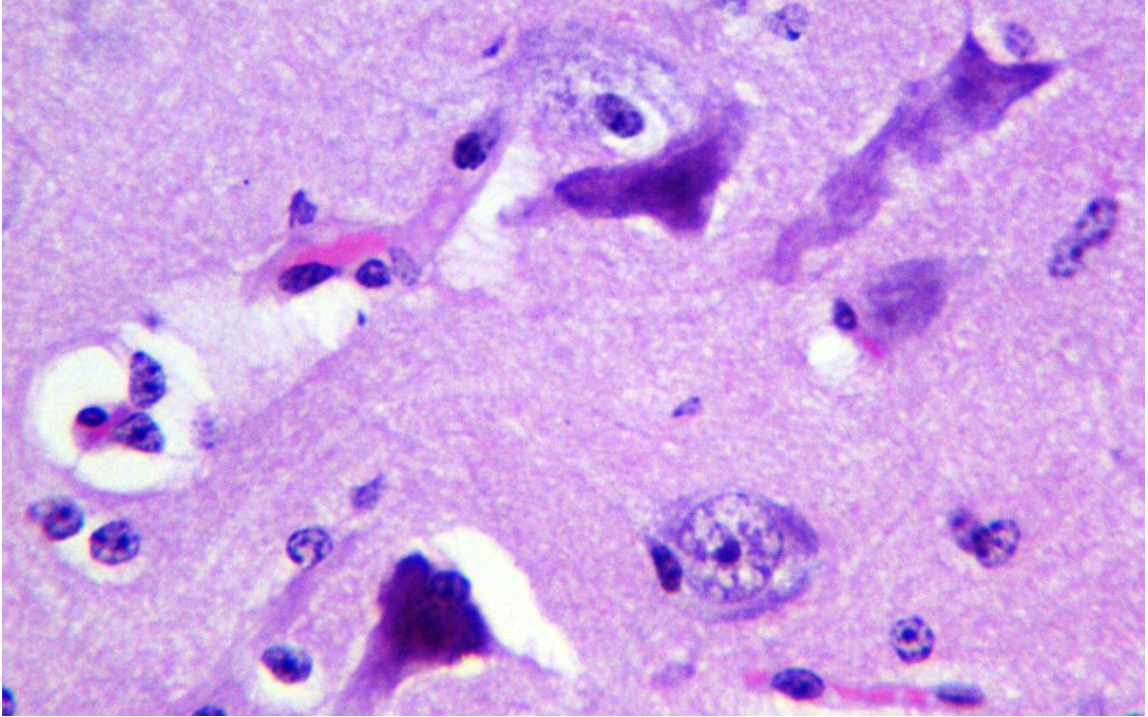


Figure 28: Photomicrograph of brain from calves infected with NT and treated with DA showed neuronal necrosis in which the neurons were shrunken with hypertrophied astrocyte.

5. DISCUSSION

Treatment with either DA or ISM has significantly improved hematological values that have been changed due to infection with *T. vivax* isolates. This was comparable to the pre-infection level or control values in animals infected with the tsetse transmitted trypanosomes whereas complete normality could not be restored at least in the 49 days period after treatment for those infected with mechanically transmitted *T. vivax*. This might indicate the severe damage the latter isolate has caused to the different organ systems with consequent delay in the healing process. This finding was in contrast to the findings of Adenike and Stephen (2010) who reported complete restoration of PCV, Hgb and RBC count after treatment with DA and ISM in trypanosoma brucei infected rats. Such differences in findings could arise from the difference in experimental animal models or inefficacy of the drugs. In the latter case, we have reported in this study that animals infected with non-tsetse trypanosomes and treated with DA have relapsed after 21 days post treatment.

Moreover, it is believed that mechanical transmission of parasites might increase their virulence. Recent works by (Spence *et al.*, 2013) supports this speculation. *Plasmodium c. chabaudi* transmitted through multiple blood transfers between mice in the laboratory loses the reset mechanism and the malaria parasite multiplies much more quickly in mice after blood transfers, and causes an increase in disease severity. It should be noted that detectable parasitaemias were observed in NT trypanosomes two days earlier than for the TT trypanosomes.

Parallel to the hematological changes, several biochemical changes indicative of pathological and functional disturbances were observed in infected groups. Significant reductions were noticed in serum glucose, total cholesterol and albumin/total protein in infected cattle compared with the control group or pre-infection level. The reduction in the serum glucose levels until 14 dpi could be explained by the parasite's need for glucose as an energy source (Opperdoes *et al.*, 1986). After treatment, these values were increased or maintained normality strengthening the above fact. Abnormalities of lipid

metabolism have been identified in several laboratory and domestic animals infected with various species of trypanosome (Anosa, 1988). Impaired synthesis which could in turn be the result of insufficient hepatocellular respiration due to hypoxia caused by anemia and reduced subsequent release of cholesterol from the liver could also be a contributory factor to the decrease in serum levels of total cholesterol observed in the trypanosome infected animals (Adamu *et al.*, 2008). Reduction after treatment in this parameter could hence mean coming to normality of the liver which is in agreement with findings in liver enzyme levels.

Changes in enzyme levels are a good marker of soft tissue damage and that damage to body cells results in the alteration of membrane permeability and consequent release of enzymes into the extracellular fluid (Obaleye *et al.*, 2007). Although a general increase in AST and ALT values were observed after infection and a corresponding reduction to normal value after treatment was seen, there is variation in the response to treatment according to treatment groups or parasite isolate. The rise in ALP level may be associated with damage to the liver or hemopoietic system, the two major sites of ALP production. Again, any positive change after treatment may show that healing process has resumed. This finding was similar to Stephen and Adenike (2012) who found significant decrease in AST level after treatment with ISM in *T. brucei* infected rats. This finding was consistent to Stephen and Adenike (2012) who found significant increase in ALT and ALP level of rats treated with ISM after infection with *T. brucei*. This significant difference of serum enzymes from the control group in ISM treated animals of both isolates indicates that absence of restoration of ALT and ALP in to their normal state after treatment.

In the current, study significant gross lesions and histopathological changes were observed in the spleen, liver, lung, kidney, heart and brain in *T.vivax* infected animals isolated from both tsetse and non-tsetse infested areas after treatment with DA and ISM in comparison to the non-infected control group although these changes were significant in animals infected with *T.vivax* isolated from non-tsetse area. This finding was in contradiction with the findings of Stephen and Adenike (2012) who reported less

distortion of tissue architecture and reduced inflammatory changes compared to the non-infected control animals in *T.brucei* infected rats after treatment with DA and ISM. The pronounced gross and histopathological changes observed in NT1+DA and NT2+DA might be due to the relapse detected after treatment or to the higher virulence of the isolate. This was in agreement with the finding of Moulton (1986) who reported pronounced histopathological changes in the brain of goats experimentally infected with *T.brucei* after treatment with DA.

6. CONCLUSION AND RECOMMENDATIONS

The response of animals infected with *T. vivax* isolated from tsetse and non tsetse area to ISM and DA was tested in young zebu cattles. This response was studied by observing haematological, biochemical and histopathological changes occurred after treatment. Thus, the result of the current experiment revealed that there were significant differences haematological, biochemical and histopathological parameters in all groups of animals treated with DA and ISM in comparison to the non-infected control groups. Moreover, treatments of non-tsetse trypanosome infected groups have resulted in weaker restoration of hematological, histopathological and biochemical values as compared to the tsetse transmitted trypanosome infected groups. Such differences could be linked to the drug resistant strain detected in this parasite population and or the inherent nature of mechanical transmission exercised by this population. Generally, *T. vivax* from North West Ethiopia can cause significant pathology in infected animals and the degree and type of pathological changes might vary between the tsetse-transmitted and non- tsetse transmitted trypanosome types. Based on the above conclusion, the following recommendations are forwarded:

- Trypanocidal drugs which can not only result in clinical cure but also cause a better improvement in pathophysiological parameters should be developed if complete cure is needed in production animals.
- The belief that east African *T.vivax* is less pathogenic does not apply at least to the studied isolates and the type of animals used in this experiment. Hence, at least equal attention to the cyclically transmitted varieties must be given to the mechanically transmitted trypanosomosis prevalent in tsetse free areas
- Further study is required to investigate the cause of failure or delay to return to normality in animals infected with non-tsetse *T. vivax*. Is it because of the virulence or the relapse after failure to clear the parasite by diminazene aceturate.

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8. APENDEX

Annex 1. 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°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 (H₂O₂). H₂O₂ is then oxidatively coupled with 4 amino- antipyrene (4-AA) and phenol in the presence of peroxidase (POD) to yield a red quinoxaline 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 the 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-nitrophenylphosphate (4-NPP) with the formation of free 4-nitrophenol and inorganic 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 (achete) whose color intensity is directly proportional to the total protein concentration and measured at 540 nm.

Albumin

Principle

The method 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 2. Rapid matching method

Principles:

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. Parasitaemias 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, parasitemia is recorded as <antilog 5.4 organisms/ml.

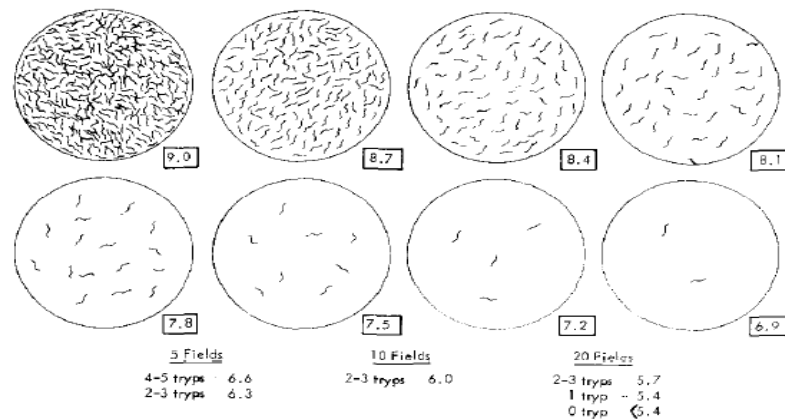


FIG. 1. Chart and table for estimating trypanosome parasitemias. The circles are used for matching when more than one organism per microscope field is present, the tables for lower concentrations. The values in the boxes in the charts and in the tables indicate the logarithm of the number of trypanosomes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

			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	<u>8.1 Reference point</u>
			H 16	7.8
			I 8	7.5
			N 4	7.2
			G 2	6.9
Organisms in				
			20 fields	
			10 fields	
			5 fields	
				4-5
				2-3
			2-3	
			1	
			0	
			C	
			O 1	6.6
			U 0.5	6.3
			N 0.25	6.0
			T 0.125	5.7
			I 0.0625	5.4
			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 3. 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:

- Capillary tubes are filled approximately $\frac{3}{4}$ of the tube with the well-mixed blood sample.

- 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.
- The vacant end of the tube is sealed with sealing clay.
- 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.
- It is centrifuged for five minutes at full speed of 12,000rpm.
- Using Hawksley microhematocrit reader the reading is made in percent.
 - 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 4. 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:

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

- The micropipette tip was attached to the eppendorf micropipette and 20 μ l of blood was measured and the blood was wiped from outside of the tip with a piece of Gauze.
- The blood was added into the graduated tube.
- One drop of distilled water is added to the graduated tube and mixed with glass stirring rod.
- 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.
- The hemoglobin is read in g/dl.

Annex 5.Total red blood cell (RBC) count

Materials and reagents:

- Un-coagulated whole blood in EDTA coated vacutainer tubes
- Micropipette calibrated to dispense 1 to 1000 μ l with disposable tips
- Vial capable of holding 2ml fluid
- hemocytometer (improved Neubauer chamber)
- A piece of gauze
- RBC diluting fluids (Hayem's solution)
- light microscope
- Cover slip.

Procedure:

- About 995 μ l of diluting fluid is measured with a micro pipette and added into a vial.
- 5 μ l of blood is measured; excess blood outside the micro pipette tip is wiped carefully by using gauze and then the blood is added in to a diluting fluid that gives a 1:200 dilution. The solution is then mixed slowly by shaking for 2-3 minute.

- Some of the solution is taken by using a micropipette. Holding the pipette slightly inclined, small volume of the fluid was introduced under the cover slip which is placed on the counting chamber. The fluid filled the space by capillarity.
- The cells are allowed to settle for 2 to 3 minutes.
- The counting chamber is placed on the stage of the microscope.
- Low power (10 x) objective is switched. Light is adjusted and the large scales in the center with 25 small squares are located.
- High power (40 x) objective is switched.
- The red blood cells in the four corner squares and in the center square are counted.
- The total number of red blood cells per cubic mm (μl) is calculated by multiplying the number of red cells counted by 10,000 (correction factor).
- The following formula is used for the calculation of red blood cells:

$$\begin{aligned} \text{Total red blood cells / cubic mm} \\ = [\text{number of red cells counted} \times \text{dilution}] / [\text{area counted} \times \text{depth} \\ \text{of fluid}] \end{aligned}$$

Where

$$\text{Dilution} = 1:200 \text{ (i.e., 200)}$$

$$\text{Area counted} = 80/400 = 1/5 \text{ sq.mm}$$

Since cells will be counted in 5 bigger squares and such square is further divided into 16 small squares.

$$\text{Each small square} = 1/400 \text{ sq.mm}$$

$$\text{Hence area of } (5 \times 16) = 80 \text{ such areas} = 80/400 \text{ sq.mm} = 1/5 \text{ sq.mm}$$

$$\text{Depth of fluid} = 1/10 \text{ mm}$$

$$\text{Number of red cells counted} = N$$

$$\begin{aligned} \text{Hence total red blood cells/cubic mm} &= [(N \times 200)] / [(1/5) \times (1/10)] = N \times 200 \times 50 \\ &= N \times 10,000 \end{aligned}$$

Annex 6. Determination of erythrocyte indices

Erythrocyte indices are calculated from the above hematological profiles. Each of the indices is calculated as follows.

Mean Corpuscular Volume (MCV) is determined indirectly by dividing the PCV by the total erythrocyte count (in millions per micro liter) and multiplying by 10.

$$\text{MCV} = \frac{\text{PCV (\%)} \times 10}{\text{RBC count}}$$

Mean Cell Hemoglobin is calculated by dividing the hemoglobin value (in gram per deciliter of blood) by the erythrocyte count (in millions per micro liter) and multiplying by 10.

$$\text{MCH} = \frac{\text{Hg con.} \times 10}{\text{RBC count in millions per micro liter}}$$

Annex 7. 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 mould with their labels and then fresh melted wax (54 - 60C^o) 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*: Automatic or manual staining with Haematoxylin and Eosin to give colour for sectioned tissue.

Staining procedure:

Put the sections fixed on slides in xylene for 3 minutes.

Then transfer to absolute alcohol for 3 minutes.

Transfer to 80% alcohol for 2 minutes.

Place in 50% alcohol for 2 minutes.

Wash the slide in running tap water for 1 minute and put in Harris's *Haematoxylin* for 5-7 minutes.

Wash in running tap water for 30 seconds

Wash excess dye in 1% acid alcohol by continuous agitation for 15 second.

Wash in running tap water for 30 seconds.

2-3 dips in ammonia water solution until tissues attain a blue colour.

Wash in running tap water for 30 seconds.

Counter stain with *eosin* for 3-5 minutes.

Wash in running tap 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.

Microscopic examination: stained slide is examined under microscope.

