

## SERUM IMMUNOGLOBULIN PROFILE OF *Trypanosoma congolense* - INFECTED SHEEP IN RELATION TO PARASITAEMIA AND CHANGES IN CIRCULATING LEUCOCYTE AND ERYTHROCYTE MASS

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### ABSTRACT

Trypanosomiasis is a major problem in developing countries most especially Africa and the incriminating agents are *T. congolense*, *T. vivax*, *T. brucei* in small ruminants. It has high morbidity and mortality Rates of 70% and 20% respectively . The experimental animals were four Rams which were used as control and infected group, Parameters for this experiment were recorded before infection. Following inoculation with *T. congolense*, 2 mls of blood was collected from the sheep in vacutainers for serum biochemical assay. Enzyme linked immunosorbent assay was used to assay IGG and IGM concentrations and 1ml of blood was collected in vacutainers with anti coagulant to check leucocytic and erythrocytic index. This was done every day until day 6 and then every other day. Clinical signs observed post infection were , Anemia, Hematuria , Rhinitis, dyspnea , emaciation and paralysis of the fore and hind limbs and death . Statistical tables and charts were used for analyses. The role of Hypoproteinemia , IGG and IGM in experimental infection with *Trypanosoma congolense* were looked at. The anemia was Normocytic normochromic and later became Microcytic hypochromic, PCV Recorded on the first day prior to infection was 27% and on the last day of infection became 6% . Leucocytosis was Degenerative and Leucopenia was also observed Perhaps due to the antigenic nature of the parasite. On first day WBC count was  $8.5 \times 10^9/L$  and Prior to death Leucocytosis of  $6.1 \times 10^9 /L$ . Hypoproteinaemia was also seen with Blood urea Nitrogen and Alanine aminotransferase falling to values below normal 55U/L and 155mg/dl respectively. There were also fluctuations in the values of IGG and IGM with initial values of 1.4 and 0.71  $\mu g/ML$  and terminal values of 0.34 and 0.21  $\mu g/L$ . The organs looked at Kidneys, Lungs, Heart and Liver were all in their initial stages of destruction , they were inflamed, edematous , congested and necrotic . Hypoproteinemia, , immune suppression, dyspnea , Anemia and Leucopenia may be the cause of death in Trypanosomiasis.

**Keywords:** Leucopenia, Anemia, *Trypanosoma*, immunoglobulins, Hypoproteinemia

**DOI:** 10.7176/JBAH/11-6-06

**Publication date:** March 31<sup>st</sup> 2021

### 1.0 INTRODUCTION

#### 1.1 Statement of research problem

African trypanosomiasis continues to decimate human and livestock populations unabated and constitute an impediment to human and livestock production in the disease endemic countries of Tropical Africa (Dubois et al., 2005). Anaemia which is evidenced by low values of erythrocyte count, packed cell volume (PCV) and haemoglobin concentration, had for long been identified as one of the principal features and major causes of death in acute phase of trypanosomiasis in livestock, as well as been an aggravating factor in the development of most of the reported disorders in affected animals (Esiebo and Saror, 1991)

The recognition of the importance of this clinical feature of trypanosomiasis stimulated diverse forms of research interest aimed at elucidating the mechanism of its development in affected animals. These efforts resulted in the emergence of numerous reports that vary in concept (Igbokwe, 1989). The varying nature of the reports notwithstanding, the general consensus is that the anemia in Animal trypanosomiasis is predominantly the result of hemolytic crisis in which the red blood cells are being destroyed by mononuclear phagocytic system (Igbokwe and Mohammed, 1991).

Trypanosomosis (trypanosomiasis), a globally occurring parasitic disease, poses as a major obstacle to livestock production in tropical and subtropical regions consequent to huge economic losses it causes in affected livestock (Boada-Sucre *et al.*, 2016). The principal trypanosomes which cause disease in ruminants in sub-Saharan Africa are *Trypanosoma congolense* (*T. congolense*), *T. vivax*, *T. brucei* and *T. evansi* and a resultant decrease in serum sialic acid and erythropoietin (Losos *et al.*, 1973; Adamu *et al.*, 2009; Boada-Sucre *et al.*, 2016; Ode *et al.*, 2017). Animal trypanosomosis is characterized by periodic fevers, parasitemia, and anemia, changes in blood chemistry, body mass loss, and often death. The anemia developed by animals is considered the most characteristic symptom (Boada-Sucre *et al.*, 2016). It seems that the anemia is hemolytic in nature, occurring intravascularly in the early stages of the disease, and extravascularly in the chronic phase. There are many reported possible mechanisms of erythrocyte destruction that lead to the anaemia in affected animals (Boada-Sucre *et al.*, 2016).

Animals infected with trypanosomes develop a number of immunological disorders of varying severity. First, circulating immune complexes may be generated as a result of the persistent antigenemia and resulting immune stimulation. The deposition of these biologically active complexes on erythrocytes may contribute to the anemia often associated with *T. congolense* (Fadiga *et al.*, 2013). Serum immunoglobulin levels in infected animals show significant differences from normal. For example, serum immunoglobulin M (IgM) levels are greatly elevated (Iizard *et al.*, 1978; Kobayashi *et al.*, 1976) rising on occasion to 20 times normal values. In contrast to the rise in IgM levels, serum IgG tends to remain relatively constant, at values close to normal (Nagele *et al.*, 2013; Iizard *et al.*, 1978). Catabolic studies indicate that the half-life of all serum immunoglobulin classes is shortened (Iizard *et al.*, 1978).

Parasite antigenic variation and the induction of alterations in the hosts defense system, such as excessive activation of the complement system leading to persistent hypocomplementemia, down regulation of nitric oxide production, polyclonal B-lymphocyte activation and marked immunosuppression are the main mechanisms to survive in the chronically infected host. The production of antibodies to VSG of *T. congolense* is the major early immune response. The first antibody to the VSG is of immunoglobulin M (IgM) class and is produced independently of T cells. It was reported that at peak of parasitaemia a type 2 helper T cell (TH2) like cytokines were prevalent in the trypanosusceptible Boran cattle (Ayele *et al.*, 2017).

Hemolytic material from *T. congolense* has been shown to consist of a mixture of free fatty acids and phospholipase A. Hemolytic factors have been found in the sera of trypanosome-infected cattle (Mc crorie *et al.*, 1980). A number of immunological mechanisms have been proposed to be involved in the hemolysis. Trypanosome antigen has been demonstrated on the surface of erythrocytes. Antiglobulin tests showed that the erythrocytes of calves infected with *T. congolense* had absorbed immunoglobulins. Preformed antigen-antibody complexes may be absorbed onto erythrocytes, a phenomenon found in immune complex diseases, this would account for the detection of both antigen and immunoglobulin. Such immune complexes may fix complement on the erythrocyte surface, resulting in intravascular hemolysis or erythrophagocytosis or both. Complement has been detected on the surfaces of erythrocytes of patients infected with African trypanosomes (Ayele *et al.*, 2017; Baral *et al.*, 2007; Vali *et al.*, 1978). These observations would be consistent with antibody or antibody complement coating of erythrocytes and host parasite interactions (Nicholas *et al.*, 2009; Babatunde *et al.*, 1982).

African trypanosomiasis is one of the major disease constraints to livestock production in sub-Saharan Africa. While the pathology of the disease has been well documented, surprisingly little is known about the mechanisms that cause disease. An unusual feature associated with the immune response during trypanosome infections in man and animals is that a large fraction of the antibodies produced are IgM. (Noyes *et al.*, 2009). Although increases in serum IgM have been consistently observed in many trypanosome infections of cattle, the appearance of trypanosome non-specific antibodies was not a consistent finding. Recently, following the observation that numbers of CD5+B cells increased in blood and spleen concomitantly with an increase in serum IgM during *T. congolense*-infection of cattle (Naessens *et al.*, 1992) CD5+ B cells form a distinct B-cell lineage (B-1), and spontaneously produce IgM with the features of natural antibodies in normal individuals/animals. (Buzza *et al.*, 1997)

## 1.2 Significance of the study

In spite of the rapid development of medicine in recent years trypanosomosis continues to be a major constraint to Human health and increased livestock production, and broadly, to agricultural development in most countries of Tropical Africa (Kristjanson *et al.*, 1999; FAO, 2002). The disease still remains one of the most important diseases of livestock and Human especially in Sub-Saharan Africa (Odoya *et al.*, 2003). Verily, the prevalence of trypanosome infections in Nigeria and perhaps most African countries appears to be on the increase (Odoya *et al.*, 2003). More so, it is estimated that 46 million cattle are exposed to the hazard of contracting the disease just as are millions of other species of domestic animals in Africa (Kristjanson *et al.*, 1999). Like-wise curative and preventive treatments which have remained the only means of curbing the ravaging effects of the disease cost livestock owners and Government colossal amounts. Efforts to use vaccines as alternatives to prevent the disease have been rendered futile due to the antigenic variation of the organisms in question (Teale, 1991). For example trypanosomes have

multiple genes that code for different surface coat glycoproteins that are not vulnerable to immune response and this causes the persistence of the organism in blood.

The major source of concern is the fact that Human and Animal trypanosomes are re-emerging (Waiswa et al., 2003) while development of resistance to trypanocidal drugs by the organism is on the increase. Also, with the enormous losses incurred and the disturbing epidemiologic trends of this disease further studies need to be done so as to ascertain ways of prevention and cure.

It has recently been demonstrated that the Boran breed produces anti-trypanosome antibodies mainly of the IgM isotype, while the IgG isotype antibodies are detectable in N'Dama cattle. Since immunoglobulin class switching is dependent upon the release of cytokines from activated helper T cells, it is possible that differences in T-cell function between trypanotolerant and trypanosusceptible cattle may contribute to the differences observed in their antibody production (Gachohi et al., 2012). This may be obtainable in different breeds of sheep where the trypanotolerant ones may produce mostly IgG and the trypanosusceptible ones will produce mostly the IgM isotype (Flynn et al., 1992; Buza et al., 1997).

Another reported mechanism of erythrocyte destruction is through the action of phospholipases, possibly released in trypanosome infections, which can destroy cell membrane phospholipids leading to the erythrocyte destruction. Since trypanosomiasis continues to ravage Nigerian indigenous livestock, knowledge of immunologic responses and activities of phospholipases may give an insight and a better understanding of these mechanisms of trypanosome infection-induced erythrocyte destruction in these species of animals.

### 1.3 Objectives

Objectives of the study are to determine changes in-

1. serum concentration of immunoglobulins (IgM and IgG) in relation to the level of parasitaemia in *Trypanosoma congolense*-infected sheep
2. haematologic and eucyctic parameters of *Trypanosoma congolense*-infected sheep in relation to parasitaemia and free fatty acid and amino acid concentrations .
3. some vital organ functions (kidney and liver) in relation to parasitemia in *Trypanosoma congolense*-infection.

### 1.4 Research Questions

1. Does serum concentration of immunoglobulins (IgM and IgG) change in relation to parasitaemia in *T. congolense* - infected sheep
2. Do haematologic and eucyctic parameters of *Trypanosoma congolense*-infected sheep change in relation to parasitaemia and free fatty acid concentrations?
3. Do some of the vital organ functions (kidney, liver, heart and lungs) change in relation to parasitemia in *Trypanosoma congolense*-infected sheep?

## 2.0 LITERATURE REVIEW

### 2.1 Trypanosomiasis

#### 2.1.1 Classification

Trypanosomes are protozoa belonging to the order Kinetoplastida, family Trypanosomatidae and the genus *Trypanosoma* (Kirti Dua, 2012). The family Trypanosomatidae consist of eight genera. Four of these are monoxenous (single host) parasites of invertebrates, mainly insects. The genera include *Leptomonas*, *Herpetomonas*, *Crithidia* and *Blastocrithidia*. (Kirti Dua, 2012). The other four genera are dixenous (digenetic) parasites that require two successive hosts in their life cycles. These hosts constitute the intermediate host or vectors, which are usually

invertebrates, and the other, the definitive host, which are vertebrates or plants. Members of these genera include *Phytomonas*, *Leishmania*, *Trypanosoma* and *Endotrypanum*.

### 2.1.2 Trypanosomatidae

Members of these group are characteristically leaf-like in shape, have a single flagellum that is attached to the body by an undulating membrane. The blood stream forms of mammalian trypanosomes (Trypomastigote) are basically lanceolate in shape (Hoare, 1972). The flagellum of trypanosomes represents the locomotor apparatus and is a single structure under light microscope. The kinetoplast represents the mitochondrion.

### 2.1.3 General characteristics of Trypanosomes

Flagellated protozoan parasites that live in blood and other body fluids of vertebrate hosts, the flagellum is a contractile fibre and when it beats the cell membrane is pulled to form an undulating membrane. It is also with the help of the flagellum that the protozoa swims and penetrates the host cell. It possesses a Kinetoplast which it uses to undergo cyclic development in arthropod host. (Kirti Dua, 2012).

### 2.1.4 General life cycle of the family Trypanosomatidae

Most Trypanosomes of this group undergo a cyclical development (Kirti Dua, 2012). The final host is a vertebrate animal, while a variety of hemetogenous invertebrates serve as intermediate hosts or vectors. The vectors take the blood stream form of Trypanosomes during the feeding process. A cycle of development in the intermediate host result in the formation of special infective forms referred to as metacyclic trypanosomes, which are transmissible to a new mammalian host. Thus the intermediate stages in the vector are not infective to the vertebrate hosts, giving rise to an indirect or cyclical mode of transmission. The course of development of trypanosomes in the mammalian host begins when the metacyclic trypanosomes are introduced directly by inoculating through bite, as seen in tsetse borne salivarian species, or passively by contamination of the mucous membrane or skin which occurs in stercorarian species.

### 2.1.5 Developmental stages

The different stages involved are;

- (A) Trypomastigote stage; formerly referred to as trypanosome stage, mainly seen in the vertebrate host but can occur in arthropod vectors as infective stage for the vertebrate.
- (B) Epimastigote stage; formerly called crithidial stage, found principally in arthropods but can be found in vertebrate host as part of the vertebrate developmental life cycle.
- (C) Promastigote stage; formerly referred to as leptomonial stage and found in arthropods or plants.
- (D) Amastigote stage; also known as *Leishmania* stage and these occurs in vertebrates and arthropods.

### 2.1.6 Clinical signs and pathogenesis

The incubation period for *T. congolense* infection is usually 4 to 24 days while that for *T. vivax* is 4 to 40 days and *T. brucei* 5 to 10 days. Infected biting flies inoculate metacyclic trypanosomes into the skin of animal during feeding, trypanosomes grow at the site and cause localized swelling called chancres. They then migrate to the lymph node and then into the blood stream where they divide rapidly and initiate parasitemias (Kirti Dua, 2012). The anemia has complex pathogenesis that involves erythrophagocytosis, some hemolysis and dyshemopoiesis. The clinical signs are usually stress associated for example, transportation, inclement weather, malnutrition or concurrent infection which may cause acute or per acute form of the disease where nervous symptoms are present. In the acute form there is high fever, emaciation, lacrimation, reduced milk yield, circling movements, anemia and death within 24 hours. In chronic cases there is dullness, anorexia, intermittent fever and cachexia though the cardinal sign of this disease is anaemia (Kirti Dua, 2012). Within a week of infection there is usually a decrease in packed cell volume (PCV), hemoglobin, red blood cell and white blood cell levels which may within two months drop up to 50% their preinfection values. Mucous membrane become pale and some animals may exhibit edema of the throat and underline. The disease often always result in immunosuppression and mortality and morbidity depends on the virulence of the trypanosome, age and breed of the animal. (Kirti

Dua,2012).Necropsy findings vary and are not specific but major anomalies include, anemia, serous atrophy of fat, edema(subcutaneous edema)and excessive fluid in the body cavities, emaciation, petechial hemorrhages and an enlarged liver,the spleen may be enlarged,normal or atrophied.(Huan,1975)

### 2.1.7 Trypanosoma congolense

This organism is mostly seen in Tropical Africa and they have a sub-terminal kinetoplast and short undulating membrane,may or may not possess flagellum,and may be pleomorphic or monomorphic in shape. and causes the disease known as African trypanosomiasis,also referred to as Nagana,Tsetse disease,Trypanosomiasis,the vector is tsetse flies or Glossina and species of animals infected include cattle,pig ,sheep and goats and horses.Several reports or observations were made on the clinical disease caused by *T.congolense* in experimentally infected livestock(Sekoni et al.,1990).*T.congolense* infections are often highly pathogenic with fatal infections.The acute phase of *T.congolense* infection in sheep was reported to last for 4-6 weeks(Logan-Henfrey et al.,1992)but their susceptibility to this infection varies,a breed of sheep known as Djallonke are more trypanotolerant than other species(Leak et al .,2002).Host infected with *T.congolense* may die within 6-10weeks post infection.In chronic cases *T.congolense* may last for months or years in blood during which anemia or extreme emaciation(cachexia) may develop(Tizard and Holmes,1976).

### 2.1.8 Immunoglobulin

Animals infected with trypanosomes develop a number of immunological disorders of varying severity. First, circulating immune complexes may be generated as a result of the persistent antigenemia and resulting immune stimulation. The deposition of these biologically active complexes on erythrocytes may contribute to the anemia often associated with *T. congolense* (Boada-Sucre *et al.* , Itard *et al.*,1978). Serum immunoglobulin levels in infected animals show significant differences from normal. For example, serum immunoglobulin M (IgM) levels are greatly elevated (Iizard *et al.*,1978; Kobayashi *et al.* ,1976) rising on occasion to 20 times normal values. In contrast to the rise in IgM levels, serum IgG tends to remain relatively constant, at values close to normal(Nagele *et al.* , 2013; Iizard *et al.*,1978). Catabolic studies indicate that the half-life of all serum immunoglobulin classes is shortened(Iizard *et al.*,1978).

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Hemolytic material from *T. congolense* has been shown to consist of a mixture of free fatty acids and phospholipase A(Herbert and Miller, 1980). Hemolytic factors have been found in the sera of trypanosome-infected cattle (Mc corrie *et al.*,1980). A number of immunological mechanisms have been proposed to be involved in the hemolysis. Trypanosome antigen has been demonstrated on the surface of erythrocytes. Antiglobulin tests showed that the erythrocytes of calves infected with *T. congolense* had absorbed immunoglobulins. Preformed antigen-antibody complexes may be absorbed onto erythrocytes, a phenomenon found in immune complex diseases , this would account for the detection of both antigen and immunoglobulin. Such immune complexes may fix complement on the erythrocyte surface, resulting in intravascular hemolysis or erythrophagocytosis or both.

Complement has been detected on the surfaces of erythrocytes of patients infected with African trypanosomes (Ayele *et al.* , 2017;Baral *et al.*, 2007 ; Vali *et al.*,1978). These observations would be consistent with antibody or antibody complement coating of erythrocytes and host parasite interactions (Nicholas *et al.* , 2009; Babatunde *et al.* , 1982).

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Although increases in serum IgM have been consistently observed in many trypanosome infections of cattle,the appearance of trypanosome non-specific antibodies was not a consistent finding. Recently, following the observation that numbers of CD5+B cells increased in blood and spleen concomitantly with an increase in serum IgM during *T. congolense*- infection of cattle (Naessens *et al.* , 1992) CD5+ B cells form a distinct B-cell lineage

(B-1), and spontaneously produce IgM with the features of natural antibodies in normal individuals/animals.(Buza *et al.*, 1997)

### 2.1.9 Immunoglobulin M (IgM)

IgM is one of several isotypes of antibody (also known as immunoglobulin) that are produced by vertebrates. IgM is the largest antibody and it has a molecular weight of 500-990 Daltons, it

is the first antibody to appear in the response to initial exposure to an antigen.[1][2] In the case of humans and other mammals that have been studied, the spleen, where plasmablasts responsible

for antibody production reside, is the major site of specific IgM production.(Baron 2019)

#### Functions

- Opsonization and cytotoxic destruction of antigens
- Found on mucosal surfaces and serve as precursor for light chain Immunoglobulins
- Able to bind with light chain Immunoglobulin to effect desired response.
- Aids in diagnosis of infections in early stage.

### 2.2.0 Immunoglobulin G (IgG)

IgG is a type of antibody. Representing approximately 75% of serum antibodies in humans, IgG is the most common type of antibody found in blood circulation.[1] IgG molecules are created and released by plasma B cells. Each IgG has two antigen binding sites.(Baron 2019)

#### Functions

-IgG mediated binding of pathogens causes their immobilization and binding together via agglutination; IgG coating of pathogen surfaces (known as opsonization) allows their recognition and ingestion by phagocytic immune cells leading to the elimination of the pathogen itself;

-IgG activates all the classical pathway of the complement system, a cascade of immune protein production that results in pathogen elimination;

- IgG also binds and neutralizes toxins(Baron 2019)

### 2.2.1 Blood urea nitrogen (BUN)

BUN is a medical test that measures the amount of urea nitrogen found in blood. The liver produces urea in the urea cycle as a waste product of the digestion of protein. Normal human adult blood should contain 6 to 20 mg/dL (2.1 to 7.1 mmol/L) of urea nitrogen..The test is used to detect renal problems. It is not considered as reliable as Creatinine or Bun/Creatinine ratio blood studies.(Myoclinic.org 2019)

BUN is an indication of renal (kidney) health. The normal range is 2.1–7.1 mmol/L or 6–20 mg/dL.

The main causes of an increase in BUN are: high protein diet, decrease in glomerular filtration rate (GFR) (suggestive of kidney failure), decrease in blood volume (hypovolemia), congestive heart failure, gastrointestinal hemorrhage,[5] fever, rapid cell destruction from infections, athletic activity, excessive muscle breakdown, and increased catabolism.[1]

Hypothyroidism can cause both decreased GFR and hypovolemia, but BUN-to-creatinine ratio has been found to be lowered in hypothyroidism and raised in hyperthyroidism.(Myoclinic.org 2013)

The main causes of a decrease in BUN are severe liver disease, anabolic state, and syndrome of inappropriate antidiuretic hormone.[5]

### 2.2.2 Alanine transaminase (ALT)



.It is also called alanine aminotransferase (ALAT) and was formerly called serum glutamate-pyruvate transaminase (SGPT) or serum glutamic-pyruvic transaminase (SGPT) and was first characterized in the mid-1950s by Arthur Karmen and colleagues.[1] ALT is found in plasma and in various body tissues but is most common in the liver. It catalyzes the two parts of the alanine cycle. Serum ALT level, serum AST (aspartate transaminase) level, and their ratio (AST/ALT ratio) are commonly measured clinically as biomarkers for liver health. The tests are part of blood panels (Analyte monograph 2013)

### Function

ALT catalyses transfer of an amino group from L-alanine to Alpha ketoglutarate to produce pyruvate and L-glutamate (Transamination)

Significant levels of ALT often suggest medical problems such as Hepatitis, Congestive heart failure, Liver damage or Myopathy.

ALT is commonly used as a tool for screening for Liver problems.

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### 2.2.3 Free Fatty Acid

Free fatty acid is carboxylic acid with a long aliphatic chain, which is either saturated or unsaturated. Most naturally occurring fatty acids have an unbranched chain of an even number of carbon atoms, from 4 to 28.[1] Fatty acids usually exist as three main classes of esters: triglycerides, phospholipids,

and cholesteryl esters. In any of these forms, fatty acids are both important dietary sources of fuel for animals and they are important structural components for cells. When circulating in the plasma (plasma fatty acids), not in their ester, fatty acids are known as non-esterified fatty acids (NEFAs) or free fatty acids (FFAs). FFAs are always bound to a transport protein, such as albumin

Carbohydrates are converted into pyruvate by glycolysis as the first important step in the conversion of carbohydrates into fatty acids.[23] Pyruvate is then decarboxylated to form acetyl-CoA in the mitochondrion. However, this acetyl CoA needs to be transported into cytosol where the synthesis of fatty acids occurs. This cannot occur directly. To obtain cytosolic acetyl-CoA, citrate (produced by the condensation of acetyl-CoA with oxaloacetate) is removed from the citric acid cycle and carried across the inner mitochondrial membrane into the cytosol.[23] There it is cleaved by ATP citrate lyase into acetyl-CoA and oxaloacetate. The oxaloacetate is returned to the mitochondrion as malate.[24] The cytosolic acetyl-CoA is carboxylated by acetyl CoA carboxylase into malonyl-CoA, the first committed step in the synthesis of fatty acids. The "uncombined fatty acids" or "free fatty acids" found in the circulation of animals come from the breakdown (or lipolysis) of stored triglycerides.[23]

## 3.0 Materials and methods

### 3.1 Experimental animals

Three apparently healthy sheep of Yankasa breed aged between 1.5 and 2 years will be purchased from sheep markets in an apparently tsetse fly-free area. The ages of these animals will be confirmed using the dental eruption pattern described by Wilson and Durkin (1984). The animals will be kept in a fly-proof experimental house of the Department of Veterinary Medicine and Surgery, Faculty of Veterinary Medicine, Ahmadu Bello University, Zaria, and screened for haemoparasites. They will be fed adequately (with concentrates, cotton seed cake, groundnut and cowpea hays, corn bran and salt licks). They will also be provided with water *ad libitum*.

The animals will be dewormed using a broad spectrum dewormer ( albendazole at a dose of 5 mg/kg and with a broad spectrum antibiotic , oxytetracycline LA at a dose rate of 20 mg/kg). The animals will also be sprayed against external parasites using diazintol at a concentration of 2 ml/litre of water. Each of the animals will be ear tagged for proper identification. The sheep will be allowed to acclimatize for a period of three weeks and they will be

routinely examined and subjected to usual handlings to determine temperature, pulse and respiratory rates. Blood collection twice weekly for parasite screening and establishment of baseline haematological and biochemical analyses.

Just before commencement of the experiment, the Rams blood will be examined for the preinfection blood parameters. 1 ml of blood would be taken to parasitology to check for parasitemia, 1 ml to clinical pathology laboratory for hematologic blood analysis and 2 mls to public health laboratory to check the immunoglobulin level.

### 3.2 Trypanosome stock

The parasite, *T. congolense*, will be sourced from Nigerian Institute for trypanosomiasis research in Kaduna. The stabilate of the parasite will be inoculated into two Wistar rats intraperitoneally and subcutaneously. The infected rats will be immediately transported to the department of Veterinary Parasitology and Entomology, Faculty of Veterinary Medicine, Ahmadu Bello University, Zaria. The rats will be kept separately and fed with commercial pelleted feeds. Blood samples will be collected from each of the rats everyday for parasitaemia detection using the method of Woo (1969).

### 3.3 Infection of sheep

When parasitaemia reaches an estimated level of  $10^6$  trypanosomes/ml, the rat will be put on chloroform anaesthesia, and its jugular vein severed to obtain sufficient blood for infecting the Rams intravenously.

### 3.4 Infection and Detection of Parasitaemia in the experimental sheep

Following the infection of the Rams through the jugular vein, blood sample (3 ml) will be collected, everyday until parasitaemia is detected and then every other day, from each of the sheep, beginning from day 1 post-infection. One millilitre of the blood will be dispensed into a vacutainer containing 2 mg ethylenediamine tetraacetic acid (EDTA) to determine parasitaemia and its level, and also used in for haematologic and eucocytic analyses. The other 2 ml will be used to obtain serum for use in biochemical analyses (Woo, 1969). Parasitaemia will be detected in the *T. congolense*-infected sheep as described earlier. Estimation of the level of parasitaemia will be carried out according to the modified method of Mutayoba *et al.* (1994).

### 3.5 Haematologic and eucocytic evaluations

Packed cell volume (PCV), red blood cell count, and Leucocyte (WBC) count and differentia eucocyte count will be estimated as described by Coles (1986).

#### 3.5.1 PCV And Red blood ce count

Boob is coected in capriary tubes and then seaed at one end and centrifuged at 2000rpm for 5mins and haematocrit reader is used to determine packed red bood ce in percentage. The Red bood ce wi be counted after diuting 0.5ms of norma saine with 0.5ms of bood and then haemocytometer is used to determine the number of Red ces.

#### 3.5.2 WBC and differentia count

WBC count woud be determined by diuting 0.5ms of bood in 0.5ms of 2% acetic acid and haemocytometer woud be used to determine the number of ces. Thin bood smear woud be made on a gass side air dried and then fixed in methano for 5mins thereafter paced in giemsa for 25mins. The side is viewed under oi immersion for Differentia WBC count.

### 3.6 Determination of serum immunoglobulin levels( IgM and IgG quantitation)

Abcam's Immunoglobulin Sheep ELISA Kit (ab190546) is a two-site enzyme linked immunoassay (ELISA) for the measurement of IgG and IgM in sheep biological samples.

In this assay the IgG and IgM present in samples reacts with the anti-IgG or anti IgM antibodies which have been adsorbed to the surface of polystyrene microtitre wells. After the removal of unbound proteins by washing, anti-IgG antibodies or anti IgM antibodies conjugated with horseradish peroxidase (HRP), are added. These enzyme-labeled antibodies form complexes with the previously bound IgG or IgM. Following another washing step, the enzyme bound to the immunosorbent is assayed by the addition of a chromogenic substrate, 3,3',5,5'-tetramethylbenzidine (TMB). The quantity of bound enzyme varies directly with the concentration of Immunoglobulins in the sample tested; thus, the absorbance, at 450 nm, is a measure of the concentration of IgG



or IgM in the test sample. The quantity of IgG or IgM in the test sample can be interpolated from the standard curve constructed from the standards, and corrected for sample dilution.

### 3.7 Enzyme-linked immunosorbent assay (ELISA) for Sheep antibodies

The method of Buza *et al.*, 1997 will be adapted. Serum will be prepared from blood samples collected sequentially over a 5-week period during trypanosome infections and immediately stored at - 70° until use. Each well of a 96-well Dynatech ELISA plate (Dynatech, Plochingen, Germany) will be coated with 100 µg of a 40 µg/ml solution of trypanosome lysate in coating buffer (0.05 M bicarbonate buffer, pH 9.6) and the plates will be incubated overnight at 4°C. The plates will then be washed three times with washing buffer (phosphate-buffered saline pH 7.4, containing 0.1% Tween 20).

Thereafter, 100 µl of serum diluted 1/100 in washing buffer will be added in duplicate wells and incubated for 2 hr at 37°. The plates will then be washed and 100 µl of horseradish peroxidase-conjugated monoclonal antibodies (mAb) IL-A2, specific for sheep IgG, or mAb IL-A30, specific for IgM, were added for 1 hr at room temperature. After washing, 100 µl of substrate K-blue ELISA (Technologies, Lexington, KY) will be added to each well and the optical density determined at 450 nm on a Titertek Multiskan MCC/340 ELISA plate reader (Flow, Oy, Finland) following a 30-min incubation.

### 3.8 Measurement of Serum Alanine Aminotransferase (ALT)

1. Measurement against reagent blank

Pipette into test tubes

	Reagent blank	Sample
Sample	-	0.1ml
Solution reagent 1	0.5ml	0.5ml
Distilled water	0.1ml	-
Mix, incubate for 30mins at 37°C		
Solution reagent 2	0.5ml	0.5ml
Mix, stand for 20mins at 20 to 25°C		
Sodium hydroxide	5.0ml	5.0ml

Mix, absorbance of sample will be read against the reagent blank after 5 minutes. Activity of ALT and AST will then be calculated.

Reagent 1 = (100 mmol/l, pH 7.4 of phosphate buffer, 200 mmol/l of L-alanine, 2.0 mmol/l of α-oxoglutarate).  
 Reagent 2 = 2.0 mmol of 2,4-dinitrophenylhydrazine (Reitman, 1957; Schmidt 1963; Gometi *et al.*, 2014)

#### 3.8.1 Measurement of Blood urea Nitrogen

Creatinine gives a red colour with alkaline solution of picric acid. The red colour is measured in the presence of a high background yellow colour, the solutions will appear orange at low creatinine concentration.

	Test (ml)	Standard (ml)	Blank (ml)
Distilled water	3	-	4
Serum	1	-	-
10% sodium tungstate	1	-	-
Sulphuric acid	1	-	1
This will be mixed and centrifuged for 10mins			
Supernatant	3	-	3
Standard	-	3	-
0.75N NaOH	1	1	1
Picric acid	1	1	1

This will be mixed and allowed to stand for 15 minutes and then read at 450 nm. Activity of creatinine will then be calculated. Normal range is 72-126 µmol/l (Syal *et al.*, 2013).

## 8 Statistical analysis

Values of individual parameters in both the infected and control group will be subjected to simple descriptive statistics using tables and charts. Analysis of variance will be used to check significant changes in mean and standard deviation in the infected and control groups through comparison of data obtained.

## 9. Results and Discussion

### 9.1 Parasitaemia, Leucocytic index and variable surface glycoprotein (VSG)

The parasitaemia in *T.congolense* infection in the infected Rams were as presented in Table I .The organisms were detected in the blood of the infected Rams simultaneously on day 6 post infection.Hence the incubation period for this experiment was six days.Mean parasitemia rose from  $8 \times 10^6$  trypanosomes per milliliter(tryps/ml) of blood(2tryps/field of view) from the 6day and reached peak values of  $16 \times 10^6$  Tryps/ml of blood on day 22 post infection (5tryps/field). On day 15 post infection two of the Rams died and parasitaemia at that stage was  $8 \times 10^6$  (2tryps/field)prior to death they showed clinical signs which were Anorexia, Rhinitis, dyspnea ,hemoglobinuria,Hematuria,paper white ocular mucous membranes,paralysis of the fore and hind limbs,diarrhoea and the Rams died after this signs were observed. .Blood was taken from the Rams prior to infection so as to compare the parameters required pre and post infection. White blood cell count was normal pre infection with a mean value of  $8.5 \times 10^9/L$ . Post infection on day 7, leukopenia was observed and the value was  $3.4 \times 10^9/L$  and on day 14 leukocytosis was seen again and the value was  $7.9 \times 10^9/L$  but on day 22 there was a relapse back to leucopenia and the value was  $4.8 \times 10^9/L$  but on day 28 leukocytosis was seen again and the value was  $6.1 \times 10^9/L$ . On the 29 day the animal died.The fluctuation in Leukocytosis and Leukopenia might be as a result of the parasite varying it's surface glycoprotein at several intervals( Antigenic variation).Table 1 shows the relationship between WBC and VSG . The control Mean Leukocytic index was  $8.5 \times 10^9/L$  at the beginning of the experiment but this gradually started to decrease until day 7 probably due to the response of the body to the parasite but on day 14 there was a build up of Leukocytes to a value of  $7.9 \times 10^9/L$ . This maybe due to the parasite varying its surface glycoprotein and the immune system recognizes the parasite as new. This pattern was sustained with increasing parasitaemia until the animals died.

Table I showing the relationship between WBC, Leucocytosis and Antigenic variation

Response to infection per day	Number of months	Mean WBC count ( $10^9/L$ )	
1	24/6/2019	8.5	Leucopenia
2	25/6/2019	7.9	
3	26/6/2019	7.5	
4	27/6/2019	7.7	
5	28/6/2019	7.8	
6	29/6/2019	3.8	
7	30/6/2019	3.4	
10	3/7/2019	6.8	Leucocytosis
12	5/7/2019	7.0	
14	7/7/2019	7.9	
			Antigenic variation
16	9/7/2019	6.8	Leucopenia
18	11/7/2019	6.8	
22	15/7/2019	4.0	
24	17/7/2019	5.8	
26	19/7/2019	4.8	
28	21/7/2019	6.1	Leucocytosis
			Antigenic Variation

### 9.2 Packed cell volume(PCV)and parasitaemia

The Mean PCVs of the control Rams pre infection was 27% which was within the Normal Range. Following infection with *T.congolense* there was a gradual and progressive drop in mean PCV value. On day 6 when parasitaemia was noticed the packed cell volume had dropped considerably to 21% and parasitaemia at this stage was  $8 \times 10^6$ (2tryps/field).This parasitaemia was maintained for fourteen days post infection while the PCV at this stage was 13% and at this stage, two of the infected Rams had died(day 15).PCV continued to drop as parasitaemia increased and when parasitaemia was  $16 \times 10^6$ (5tryps/field) on day 28 ,PCV was 6% and on day 29 the last Ram was found dead.

It is therefore evident that increasing parasitaemia causes intravascular and Extravascular hemolysis because tissues observed had petechial hemorrhages,and a severe decline in PCV was observed. More so there was Hemorrhage by diapedesis and rhexis as whole blood was lost in urine(Hematuria) probably due the fact that the kidney was gradually failing to filter blood as a result of the infection with the organism in question. There was a mean PCV decline rate of  $\pm 2$ ..Table II showing relationship between PCV and parasitaemia.

Table II showing the relationship between PCV and Parasitaemia.

No of days of infection	No. of tryps/field.	Parasitaemia /ml	Mean PCV %
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24/6	Nil	nil	27
28/6	2	8 x 10 <sup>6</sup>	21
29/6	1	4 x 10 <sup>6</sup>	20
30/6	2	8 x 10 <sup>6</sup>	17
1/7	5	16 x 10 <sup>6</sup>	14
3/7	1	4x10 <sup>6</sup>	13
7/7	2	8 x 10 <sup>6</sup>	13
15/7	5	16 x 10 <sup>6</sup>	10
17/7	5	16 x 10 <sup>6</sup>	8
19/7	5	16 x 10 <sup>6</sup>	7
21/7	5	16 x 10 <sup>6</sup>	6

### 9.3 Changes seen in Vital Organs associated with *Trypanosoma congolense* infection

The Liver, Kidney, Heart and the Lungs were observed for Gross Pathologic lesions associated with this infection, but prior to that the Trachea was seen to contain froathy exudates and petechial hemorrhages and the thoracic muscles were severely emaciated. Thoracic cavity contained copious amount of Sero-fibrinous exudates which were blood tinged. But the area of interest were the changes seen in the vital organs.

#### Heart

The Muscles of the heart were inflamed especially the Pericardium and Myocardium and there was serous atrophy of fat (yellowish-red discoloration) which is an indication of starvation and inflammation. There was Edema, slight enlargement and congestion of the heart on cut surface.

#### Liver

The liver was also inflamed and the parietal Surface had areas of Focal Necrosis which were grey, round and unraised. It was slightly enlarged and edematous because the sharp edges had been lost and congestion was seen on cut surface.

#### Lungs

The Lungs were congested and inflamed slightly edematous and the left lung had Rib impression. It showed no sign of consolidation because it did not sink when a small portion was immersed in water, there were areas of petechial hemorrhages.

#### Kidney

The Kidney was inflamed because the Kidney capsule had croupous exudates and it detached easily from the Cortex. It was congested and slightly enlarged.

Table III showing Gross pathologic lesions associated with *T. congolense* infection (All the Rams had similar lesions)

Liver	Kidney	Lungs	Heart
Inflamed & congested	Inflamed & congested	Inflamed & congested with areas of petechial hemorrhages	Inflamed & congested
Enlarged & edematous	Enlarged & edematous	Enlarged & edematous	Enlarged & edematous (Compensatory hypertrophy)
Local necrotic areas		Local necrotic areas	
		Rib impression	Serous atrophy of fat

### 9.4 Blood Urea Nitrogen and Alanine amino transferase

The Mean serum concentration of Alanine amino transferase and Blood urea Nitrogen were as presented below in Table iv. The Mean pre-infection value of the control groups were, 161 U/L for Alanine amino Transferase and 70 mg/dl for Blood Urea Nitrogen. Thereafter on day 4 both values started to decline progressively giving an indication of Hypoproteinemia. The decrease in protein below the Normal Range may be as a result of Anorexia, hypovolemia and of course the infection more so cellular uptake. Significant decrease in Blood proteins was

observed on day 10 with values of 20mg/dl and 128U for BUN and ALT respectively. Intermittent surges and decline became prominent from day 12 to 26 but the values observed were less than the normal values observed on day 1.( initial protein values ALT and BUN 161U and 70mg/dl respectively).

Table IV Showing the Relationship between ALT , BUN and Hypoproteinemia in T. congolense infection.

Response to infection in days	Mean concentration of Alanine amino transferase. U/L	Mean concentration of BUN. Mg/dl	
1	161	70	Normal
2	158	70	
3	159	70	
4	119	62	
5	146	63	
6	145	60	
7	126	69	
8	135	67	Hypoproteinemia
10	128	20	
12	134	56	
14	132	43	
16	130	60	
24	140	57	
26	155	55	

### 9.5 Changes in serum IGG and IGM Concentration

The Mean serum immunoglobulin IGG and IGM were as presented in Table V .The mean values for the pre-infected groups were 1.40µg/ML and 0.71µg/ML for IGG and IGM respectively .These values are comparable and the concentration of IGG was higher than that of IGM in both groups(infected and control).On Day 7 post infection a gradual decrease of 0.30µg/ML and 0.50µg/ML was observed for both IGG and IGM respectively and on Day 14 peak values of 0.75µg/ML and 1.3µg/ML was observed for IGG and IGM respectively until day 16 when both values started to decrease to 0.35µg/ML and 0.25µg/ML respectively. Prior to the death of the Rams on Day 22 the values of IGG and IGM had decreased to 0.21µg/ML and 0.34µg/ML respectively. These fluctuations may be associated with the massive invasion of blood by the parasites and the inability of the host immune system to produce enough humoral immunity. More so the WBC,s had been compromised with a remarkable decrease in their number. Memory cells produced could not be sustained for long due to parasite varying its surface glycoprotein at irregular intervals.

Table V Differences in concentration of IGG and IGM

Duration of experiment in months and days	IGG µg/ML	IGM µg/ML
24/6 1	1.4	0.71
25/6 2	1.0	0.55
26/6 3	0.30	0.21
27/6 4	0.25	0.19
28/6 5	0.23	0.15
29/6 6	0.38	0.25
30/6 7	0.50	0.30
3/7 10	0.80	0.45
5/7 12	1.20	0.65
7/7 14	1.30	0.75
9/7 16	0.25	0.35
11/7 18	0.32	0.25
15/7 22	0.30	0.19
17/7 24	0.30	0.19
21/7 28	0.34	0.21

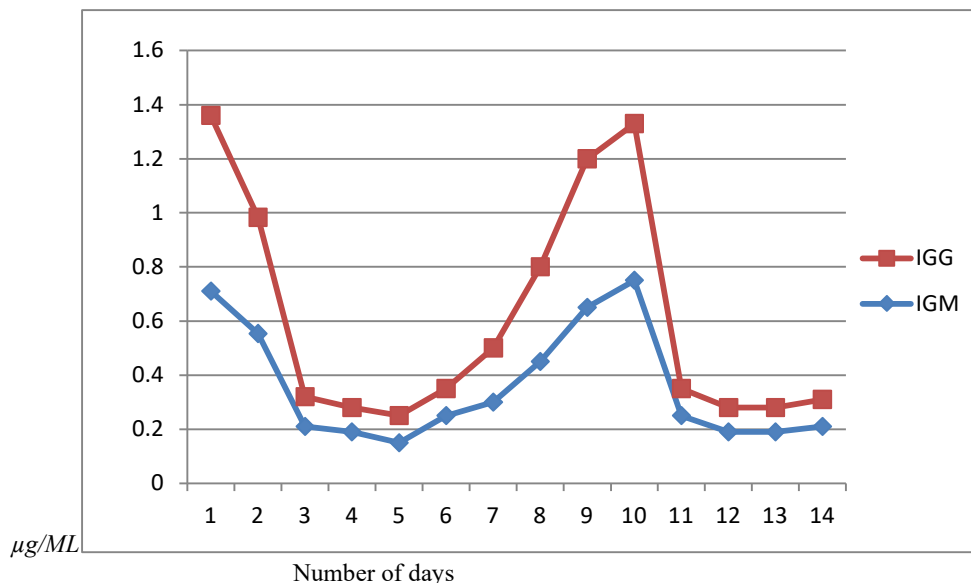


Fig I Showing differences in concentration of IGG and IGM

### 10. Conclusion

Anemia found in this experiment was hemorrhagic and hemolytic and the red blood cell morphological appearance was at first Normocytic normochromic but towards the terminal stages of the experiment became Microcytic hypochromic. The Leucocytes were degenerative with the appearance of many band cells( immature cells with fragmented and spherical nucleus with weak cell membrane) in their formative stages and these band cells were more numerous than the mature normal neutrophils. Leucocytosis was relative because the leucocytes observed in the infected group were not up to the value seen in the control. Cellular and humoral immunity were not conferred due to these reasons, more so macrophages in tissues were also overwhelmed by the parasites because most of the organs observed such as the liver, lungs, kidney and heart were in their early stages of destruction. In this experiment it is therefore evident that Anemia, leucopenia, immune suppression, hypoproteinemia and hypoglycemia may be the cause of death in *Trypanosoma congolense* infection in sheep more so the anemia was both hemorrhagic and hemolytic. This experiment has also shown that erythrocyte and leucocyte(Plasmids) proteins, lipids and carbohydrates covering their cell membranes might be a source of nourishment for the parasite in concern and despite the VSG of *Trypanosoma*, infected animals still respond to treatment so in my opinion suppressed immunity might be the major problem. I wish to acknowledge the following professors namely S. Adamu, K.A.N.Esievo, S.O Akpavie, C. N Njoku and Dr I.D Jatau all of Department of Veterinary pathology, Faculty of veterinary medicine Ahmadu Bello University, Zaria, Nigeria. Also worthy of thanks are Dr F.I Mohammed and Prof.M.N Salka of the college of Agriculture and Animal science, A.B.U Mando road Kaduna.

### 11. Recommendations

- i. Funds should be granted to researchers carrying out experiments on Trypanosomiasis because much work still needs to be carried out as it is still endemic in Africa.
- ii. Laboratories should be well equipped with standard laboratory equipments so as to be able to compete favorably with developed countries especially in areas of emerging diseases.
- iii. Training and re-training of staff to help facilitate research experiments and also in areas of emerging diseases.

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