The effect of Trypanosoma evansi in Determination of Blood grouping

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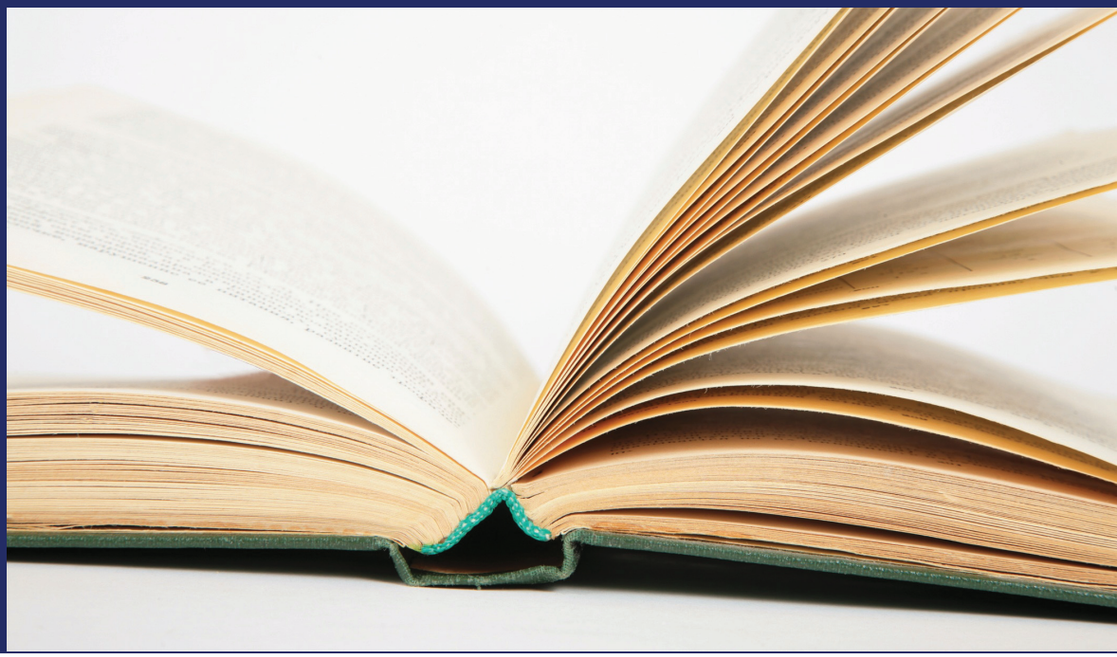

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Evaluation of the effect of Trypanosoma evansi infection on the Determination of the direct blood grouping

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Dedication

To the soul of my father

Abdelgader Elhaj

Acknowledgement

To all of our teachers, whom guide, advise and help us to promote ourselves.

Chapter One

Introduction and Literature Review

1. Introduction

1.1 Trypanosomes:

Trypanosomes found in mammals (including humans) are blood and sometimes tissue parasites of the order Kinetoplastida, family of the Trypanosomatidae, genus *Trypanosoma*.Theyare principally transmitted by biting insects, in which most of them undergo a biological cycle. They are grouped into 2 sections: *Stercoraria*, which develops in the posterior part of the insect digestive tract, including *Trypanosomacruzi,*which is both an extra- and intracellular parasite that is responsible for Chagas disease, a major human disease affecting 15 million people and threatening 100 million people in Latin America (Coura and Borges, 2010).The other is *Salivaria* which develops in the anteriorpart of the insect digestive tract, such as the main African pathogenic livestock trypanosomes, including the agents of sleeping sickness. This is a major human disease affecting around half a million people and threatening 60 million people in Africa (Rodgers, 2009).

1.1.2*Trypanosomaevansi*:

Itisa widely distributed flagellate protozoa that causes a disease called surra in domestic animals and is transmitted mechanically by biting flies such as Tabanus and Stomoxys spp. (Lun and Desser 1995; Reid, 2002; Sumba *et al.,* 1998; Otte and Abuabara 1991). The main clinical signs of *T. evansi* infection include fever, anemia, weight loss, lethargy, swelling of the hind limbs, and hemostatic abnormalities. Furthermore, *T. evansi* may exacerbate secondary infections and interfere with vaccinations because it suppresses the immunity of

infected animals (Holland *et al*., 2003; Holland *et al.,* 2001). In recent years, the damage caused by the parasite has been increasing due to the expansion of the biting fly's range as well as transportation of infected livestock. Surra occurs in all 13 regions of the Philippines and has become a serious problem for the livestock industry. The Philippines government now regards surra as the second most important disease of livestock after fasciolosis and is planning to implement a national control program for surra (Reid, 2002).

1.1.2.1 Human Trypansomiasis due to *T.evansi*:-

Although *T.evansi* can infect most domestic animals, it is a primarilya parasite of camels and horses. However *T.evansi* infection in man in India., was the first report of its kind. In attempt to explain this unusual event(Joshi *et al*.,2005) suggested that as animal trypanosomes are sensitive to human plasma components and that they will die when introduced into blood stream of human .In this case of *T.evansi* infection in man, therefore trypanosome had evidently developed the ability to resist this lytic activity which may have occurred eitherbetween the parasite mutated to aform that can resist the lytic factor in the human plasma or that the human host had a deficiency in the lytic factor in the plasma.However,surveys in India showed that this was not the only case. As it stimulated to launch an epidemiological survey by screening 1,806 people in Seoni village in India using card agglutination(CATT) test.they showed that (22.7%) were positive i.e. having anti.*T.evansi*antibodies (Joshi *et al*., 2005)

1.1.3Distribution:-

The tsetse-fly-infested area of Africa extends from the southern edge of the Sahara desert (lat. 15° N.) to Angola, Zimbabwe, and Mozambique (lat. 20° S.). Of the three African animal trypanosomes, only *T. vivax* occurs in the Western Hemisphere in at least 10 countries in the Caribbean and South and Central America (Logan ,*et. al*; 1992).).*Trypanosomaevansi* is a widely distributed

hemoflagellate parasite that affects domestic and wild mammals (Hoare, 1972).It is endemic in Asia, Africa, Central and South America, Europe, and recently a case of human infection has been reported in India making it a potential human pathogen (Joshi *et al*., 2005)Fig1. In India, the disease caused by *T. evansi* is commonly known as 'surra' (Bhatia and Shah, 2001).InSudan , it is common in camels in northernKordofan and in the eastern region (Kassala , Red Sea hill butanaandGedarif).(WHO, 2001).

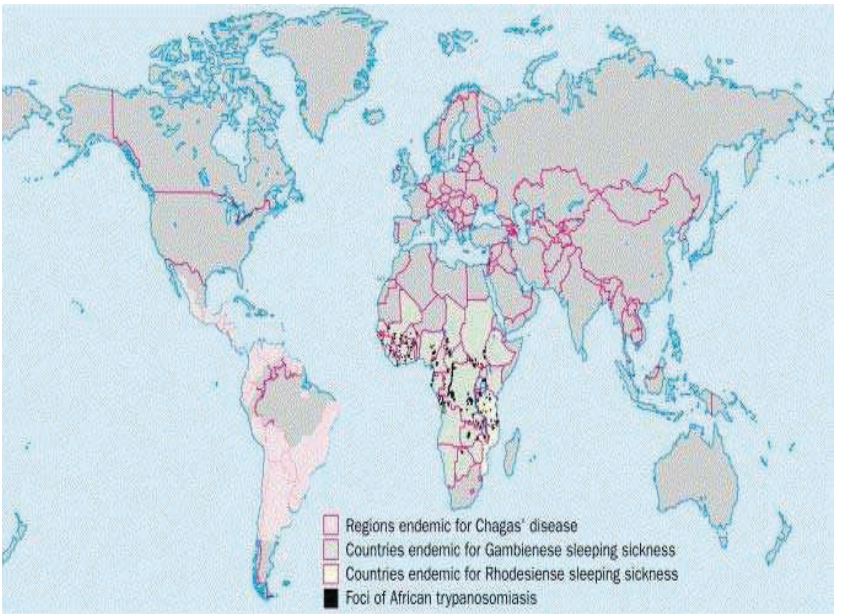


Fig.1.1 The distribution of endemic in Asia,Africa, Central and South America and Europe(WHO, 2001).

1.1.4 Morphology:-

T.evansi is a member of subgenus Trypanozoon and is described as monomorphic but may be pleomorphic in some strains with length of 15 to 34 µm. Leaf-like slender forms are characterized by a long free flagellum with

narrow and drawn out posterior end. Kinetoplast is terminal or subterminal in position. Dyskinetoplastic forms also appear after treatment of *T. evansi* with a variety of trypanocidal drugs including berenilandprothidium(Juya, 2002).

1.1.5Transmission:

African trypanosomes are transmitted by tsetse fly (Glossina). Mechanical transmission occurs by blood-sucking insect species such as Tabanus, Stomoxys, Lypersoia and Haematopota. Infection can quickly spread amongst close-living herds of cattle, water buffalo, horses and camels (Foil, 1989).*Trypanosomacruzi* is transmited by Triatomine bugs mainly in America.The main African pathogenic trypanosomes belong to three subgenera of the salivarian section, namely,NannoMonas (*Trypanosomacongolense*), Duttonella (*Trypanosomavivax*), and Trypanozoon (*Trypanosomabrucei* group). These parasites are mostly transmitted cyclically by the tsetse fly in which the procyclic forms undergo a cycle of transformations and multiplications leading to infective metacyclic forms, which may be inoculated by the tsetse flies with its saliva into a new host(Hoare, 1972).

1.1.6 Life cycle:

The tsetse fly(genusGlossina) is a large, brown, biting fly that serves as both a host and vector for the trypanosomeparasites. While taking blood from a mammalian host, an infected tsetse fly injects metacyclictrypomastigotes into skin tissue. From the bite, parasites first enter the lymphatic system and then pass into the bloodstream. Inside the mammalian host, they transform into bloodstream trypomastigotes, and are carried to other sites throughout the body, reach other body fluids (e.g., lymph, spinal fluid), and continue to replicate by binary fission.The entire life cycle of African trypanosomes is represented by extracellular stages. Atsetse fly becomes infected with bloodstream

trypomastigotes when taking a blood meal on an infected mammalian host. In the fly's midgut, the parasites transform into procyclictrypomastigotes, multiply by binary fission, leave the midgut, and transform into epimastigotes. The epimastigotes reach the fly's salivary glands and continue multiplication by binary fission.The entire life cycle of the fly takes about three weeks.(Fig.1.2)

In addition to the bite of the tsetse fly, the disease can be transmitted by:

- Mother-to-child infection: the trypanosome can sometimes cross the placenta and infect the fetus (Olowe, 1975).
- Laboratories: accidental infections, for example, through the handling of blood of an infected person and organ transplantation, although this is uncommon.
- Blood transfusion
- Sexual contact (This may be possible).(Rocha*et al.*,; 2004).

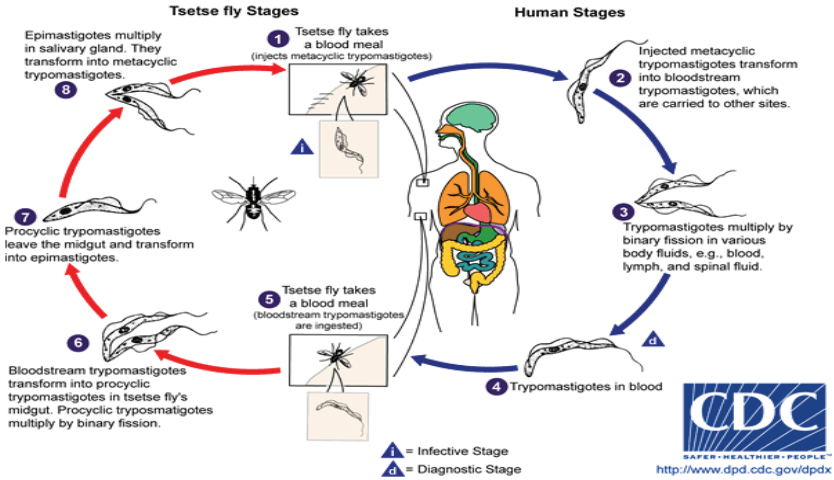


Fig.1.2 .The life cycle of trypansoma(CDC).

1.1.7 Pathology and Pathogenicity:-

T.rhodesiansi exists in eastern Africa only while *T. gambiensi* exists in western and central Africa. Human African trypanosomiasis (The gambien)is a wasting disease which is usually fatal unless treated. A painful swelling (chancre)is usually seen at site of inoculation of the trypomastigote. The early stage is characterized by high fever, sweating, headache and neck pain. While the Rhodesiandiseaseis the acute form a characterized by clinical signs similar to infection with malaria parasites.Anaemia is a major component of the pathology of surra and of African trypanosomosis generally. Anaemia in *Trypanosomaevansi* infections of camels is reportedly macrocytic and hypochromic (Jatkar and Purohit, 1971). In the early phases of infection the anaemia is haemolytic and haemophagocytic. The mechanism(s) responsible for this increased erythrophagocytic activity are not fully understood. Several have been proposed, viz, immune complexes, expanded mononuclear phagocytic system per se, haemolytic factor produced by the trypanosome, fever and disseminated intravascular coagulation (FAO, 1979). In the late stages, anemia continues to be a major factor, with probably additional causes. However, irrespective of the cause of anemia the primary abnormality of function are the anoxic conditions created by the persistent anaemia. Following this are signs of dysfunction which appear in the various organs. An increase in cardiac output due to increase in stroke volume and heart rate and a decrease in circulation time are obvious manifestations. The central nervous system is reported to be most susceptible to anoxia with consequent development of cerebral anoxia. The marked depression observed in camel trypanosomasis is a mental state and is a manifestation of depression of cerebral cortical function in various degrees. Other nervous signs reported, such as circling movement, incoordination and dullness, appear to be the results of brain tissue disturbance or damage by the

parasites. Evidence of *Trypanosomaevansi* being found in the cerebrospinal fluid has been presented (Rottcher*et al*., 1987).

1.1.7.1 Tissue damage:

The atypical lesions of multiple necrotic foci found in the liver and spleen, as well as generalized lymphoid tissue hyperplasia in camels suffering from surra on post mortem, could be attributed to pathological events that occur in the tissues of animals infected with *Trypanosomaevansi*. The degenerative changes thus observed could be due to tissue anoxia, possibly caused by anaemia, which results in a fall in tissue pH and vascular damage. Other mechanisms may also be involved. It is known that *Trypanosomaevansi* is a member of the Brucei group of trypanosomes, which have a known preference for connective tissues of a host, where they disrupt the collagen bundles and destroy the fibroblasts which produce and maintain the collagen (Boid, 1980).This disruption of host connective tissues, along with the vascular damage attributable tobrucei group trypanosomes ,would be expected to release large quantities of cytoplasmic and mitochondrial enzymes into the serum, thereby causing further tissue damage (Boid, 1980). Indeed, a two-step process in the pathology of infection with *Trypanosomaevansi* in camels based on studies of changes in serum enzymes has been proposed (Boid, 1980). The first step coincides with the appearance of trypanosomes in the host bloodstream and is characterized by a sharp and as yet unexplained rise in sorbitol dehydrogenase (SDH) activity. The second step occurs later in the infection and is characterized by a large increase in serum levels of glutamic oxaloacetic transaminase (GOT) now known as aspartate alanine transferase (AST) (Delarue*et al*., 1997) and a smaller rise in glutamic pyruvic transaminase (GPT), now known as alanine amine transferase (ALT) (Delarue*et al*., 1997). The rise in AST level can be attributed partly to cellular damage caused by the trypanosomes lysis, while the increase in ALT probably results from host destruction of trypanosomes. AST is found mostly in cell

organelles and rises when there is a great damage to the heart, kidney, skeletal muscles and liver. ALT is a specific liver enzyme found in the cell cytoplasm and its rise is associated with cell membrane damage. The reported increases in these enzymes, especially AST, is not surprising as it is indicative of organ damage and supports the post mortem reports of necrotic foci in the liver and spleen of camels suffering from surra. The fever characterized by high temperature might be due to the effects of toxic metabolites produced by dying trypanosomes (Tizard*et al*., 1978). In addition, the oedema reported in the dependant parts of the body during the chronic stage could be due to a significant decrease in the albumin levels, resulting in alterations in osmotic pressure of the blood. This leads to excessive accumulation of fluid in tissue spaces caused by a disturbance in the mechanism of fluid interchange between capillaries, the tissue spaces and the lymphatic vessels. All this possibly indicates great liver damage (Delarue*et al*., 1997). The haemorrhage and serous exudates that occurred could be caused by haemolysis involving the expanded mononuclear phagocytic system. This has also been observed in *Trypanosomabrucei*-infected donkeys, while the frequent abortions reported may be attributed to endocrine dysfunction (Losos, 1980).

1.1.8 Immune response:

Pronounced immune changes occur in African trypanosomosis. An increase in gamma-globulin (IgM) during both acute and chronic *Trypanosomaevansi*infections in camels has been reported but this is not protective, as the majority of the antibodies are auto antibodies (Boid*et al*., 1981). Leucocytosis, neutrophilia and eosnophilia have been reported in *Trypanosomaevansi* infections of camels (Anosa, 1988). These changes occur as a result of an increase in the activity of the mononuclear phagocytic system. The eosinophilia observed is a feature of parasitic infections and is associated

with immediate- type hypersensitivity reactions. The cells are expected to accumulate in tissue in response to tissue injury. In the acute phase of the disease, lymph nodes and spleen are remarkably reactive, with plasma cells predominating. This may account for the generalized lymphoid tissue hyperplasia characteristic of *Trypanosomaevansi* infections, while in the late stages the immune system becomes depleted of lymphoid cells (Losos, 1980). Circulating and tissue-mediated immune complexes have been demonstrated in laboratory animals infected with*Trypanosomabrucei*species, and much of the antibody found in them is directed against the trypanosome (FAO, 1979). Complement has also been found in association with them. These immune complexes are likely to have wide varying pathological effects including anaemia, complement activation, tissue damage and interference with both induction and effector mechanisms of the immune response (FAO, 1979). Hypocomplementaemia (decreased alteration of the complement system) has been reported, including elevated levels of immunocoaggulation and deposition of complement in the tissues. Further possible evidence of complement reactivity is found in the demonstration that the kinin system becomes activated with the release of pharmacologically active substances, thus causing microvascular dilatation and increased permeability (FAO, 1979). A state of immunosuppression later develops associated with these changes. The host immune response to a variety of antigens has been found depressed in animals under experimental conditions (Baltz*et al*., 1981; Anene*et al*., 1989; Enwezor and Ekejindu, 1998). Several hypotheses have been put forward to explain trypanosome- induced immunosuppression, and the most favoured appeared to be the action of trypanosome enzymes. Trypanosome enzymes, such as phospholipases (Tizard*et al*., 1978), neuraminidases (Esievo, 1983) and proteases (Lonsdale-Eccles and Grab, 1986) have all been implicated in membrane fluidity and cellular damage. Moreover, the phospholipases generate free fatty acids (FFAs) and these have been reported to not only have a

haemolytic effect, thus contributing to anaemia, but also to control lymphocyte reactivity through their role as prostagladin precursors. The net effect of immunosuppression is lack of pre- immunity to other diseases. Little wonder then that in most cases secondary infections, e.g. bacteria bronchopneumonia, often sets in and death may ensue if untreated.The main immune response intrypansomiasis is humoral one with stimulated B lymphocytes producing large amount of IgM followed in the later stages of infection by IgG .The antibodies are effective in destroying the trypanosomes which caused their production until the organism varies their surface antigenic structure [variant surface glycoprotein's(VSG's)].Their antigens can be grouped into somatic antigens, surface antigens and exo antigen.

1.1.9LaboratoryDiagnosis:

Two areas from a blood smear from a patient with African trypanosomiasis, thin blood smear stained with Giemsa: Typical trypomastigote stages (the only stages found in patients), with a posterior kinetoplast, a centrally located nucleus, an undulating membrane, and an anterior flagellum. The two *Trypanosomabrucei* subspecies that cause human trypanosomiasis, *T. b. gambiense* and *T. b. rhodesiense*, are indistinguishable morphologically. Patient samples that can be used for diagnosis include chancrefluid, lymph node aspirates, blood, bone marrow, and, during the neurological stage, cerebrospinal fluid. Detection of trypanosome-specific antibodies can be used for diagnosis, but the sensitivity and specificity of these methods are too variable to be used alone for clinical diagnosis. Further, seroconversion occurs after the onset of clinical symptomsduring a *T. b. rhodesiense* infection, soitis of limited diagnostic use.Trypanosomes can be detected from patient samples using two different preparations. A wet preparation can be used to look for the motile trypanosomes. Alternatively, a fixed (dried) smear can be stained using

Giemsa's or Field's technique and examined under a microscope. Often, the parasite is in relatively low abundance in the sample, so techniques toconcentrate the parasites can be used prior to microscopic examination. For blood samples, these include centrifugation followed by examination of the buffy coat; mini anion-exchange/centrifugation; and the quantitative buffy coat (QBC) technique. For other samples, such as spinal fluid, concentration techniques include centrifugation followed by examination of the sediment

Three serological tests are also available for detection of the parasite: the micro-CATT, WB-CATT, and WB-Latex. The first uses dried blood, while the other two use whole blood samples. A 2002 study found the wb-Catt to be the most efficient for diagnosis, while the wb-Latex is a better exam for situations where greater sensitivity is required.(Truc *et al,*. 2002).

1.1.9.1 Laboratory Technique:

1.1.9.2 Methods of parasite identification :

• Dried thick and thin blood smears during the febrile phase stained with Giemsa.

• Dried thick and thin smears from needle biopsies of prescapular and precrural lymph node aspirates.

• Smears from any skin exudates.

• Anticoagulated blood in EDTA and/or heparin (10 ml).

• Cerebrospinal fluid.

• Impression smears of lungs, liver, and kidney at post mortem.

• Serum samples (10–20 ml of serum), (OIE, 2009).

1.1.9.2.1Procedures :

1.1.9.2.1.1Identification of the agent:

• Direct identification of the parasite in stained thick or thin blood films or wet mounts. Diagnostic sensitivity is increased significantly by concentrating the parasites in the buffy coat layer of a heparinisedmicrohaematocrit tube The buffy coat is then examined directly at low power (Woo's method) or in a wet preparation with phase-contrast or dark-ground microscopy (Murray's method) Sensitivity is also increased when used at the herd versus individual animal level parasitaemias are highly variable during the course of infection: high during early infection, low during chronic infection, and almost nil in healthy carriers. Mini-anion exchange centrifugation technique: simplified method for detecting low parasitaemia by separating salivarian trypanosomes from host red blood cells

• Direct identification of the parasite in lymph node biopsy smears from fine needle aspirates

• Animal inoculation to reveal subclinical infections: rats or mice

• Recombinant DNA probes: detect trypanosomes in infected blood or tissue; experimental

• Polymerase chain reaction (PCR) More sensitive test than direct identification or mouse inoculation False negatives canoccur when parasitaemias are very low, which occurs frequently with chronic infections(OIE, 2009).

1.1.9.3 Serological tests :

a) Antibody detection ELISA: very useful for large-scale surveys ELISA using variable surface glycoproteins from a *T. evansi*successfully

differentiated from *T. brucei.* Protocols are available for equines, camelidae and water buffaloes

b) Card agglutination test: also makes use of *T. evansi*.

c) Latex agglutination test: also makes use of *T. evansi*.

d) The serum globulin concentration is increased and form auto-agglutination (known as rouleaux of RBC).(Oie 2009).

1.2 Rouleaux:

1.2.1 Agglutination:

True agglutination is irregular clumping and agglutination of red blood cells into grape-like clusters. True agglutination must be differentiated from the rouleaux formation (pseudoagglutination) seen in patients with paraproteins or marked hypergammaglobulinemia or fibrinogenemia, which produces more regularly spaced clusters of red blood cells adhering side-to-side ("coin stacks," see below). True red cell agglutination usually indicates the presence of a cold reactive anti-red blood cell antibody ("cold agglutinin") found in cold agglutinin syndrome or paroxysmal cold hemoglobinuria, although some warm-reactive autoantibodies with wide temperature specificity may produce similar agglutination. True agglutination and pseudoagglutination cannot always be differentiated by light microscopy, but the Coomb's test, cold agglutinin titer, and serum/urine protein analysis can provide additional information.(Riley *et. al,.*1999).

1.2.2Rouleaux formation:

Rouleaux formation ("pseudoagglutination") is a linear arrangement of RBCs ("coinstack") caused by an increased blood concentration of fibrinogen,

globulin, or paraproteins. Associated clinical disorders include acute and chronic inflammatory disorders, Waldenstrom'smacroglobulinemia, and multiple myeloma. Serum and urine protein analysis should be performed in the absence of an acute or chronic inflammatory disease to determine if a paraprotein is present.(Riley *et. al,.*1999).

1.2.2.1 Rouleaux (singular is rouleau):

Are stacks of red blood cells(RBCs) which form because of the unique discoidshape of the cells in vertebrates.

Figure.1. 3. a- red blood cell free. b-Rouleaux formation.

Rouleaux refers to a medical condition where red blood cells, also called erythrocytes, stick together, yielding an appearance similar to a stack of coins. This is an unhealthy condition with many possible causes including infection, cancer, and diabetes. The primary job of a red blood cell (RBC) is to transport oxygen throughout the body. When RBCs can't float freely, they can't perform this job properly, which can result in illness and disease. Symptoms of roleaux can include fatigue, poor circulation to the hands and feet, and dizziness.Identifying rouleaux formations is made by investgate a blood sample under a microscope. Generally, if the blood cells are largely separate, it is

probably not present. If, however, the cells are clumped together forming a chain, rouleaux is likely the cause. Red blood cells clump together like this

when the blood has a lot of protein in it. Usually, the high concentrationof protein is of one of two types : fibrinogen or globulins. Both of these proteins are produced in the liver.When red blood cells stack together, it is often an indication of inflammationin the body. Conditions that cause rouleaux include acute and chronic infections, inflammatory and connective tissue disorders, chronic liver disease, and cancers such as myeloma. Rouleauxalso sometimes occurs in people who have diabetes and can cause small blood vessels in the eyes to become damaged, causing diabetic retinopathy. Patients receiving intravenous therapy with high molecular weight fluids, such as fibrinogen, may also experience rouleaux. Others that may exhibit the condition include people suffering from allergies and those who have undergone severe trauma.Treatment for rouleaux varies. Natural remedies, such as nutritional supplements, may work for some people. Some studies show that ozonetherapy preventsrouleaux formation. In this type of therapy, a mixture of ozone and oxygen is administered by intramuscular injection, through the rectum, or intravenously. The intravenous route requires some of the patient's blood to be removed, treated with the oxygen and ozone mixture, and then reintroduced into the patient(Riley *et. al,.*1999).

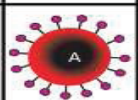
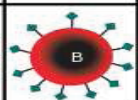
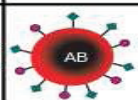
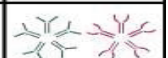
1.3 Blood:

When a peripheral blood sample is smeared on a slide and stained, it is known as a peripheral blood film. It allows for examination of the physical characteristics of the red cells, white cells and platelets under the microscope. Additionally, it helps detect parasites or abnormal cells in the blood. Thus the peripheral blood film is an important indicator of haematological and other disease. It is, however, relatively difficult to interpret, uses terminology which can be opaque to those who do not practicehematology and can be of limited specificity depending on the abnormality that is found. (Riley*et. al,.*1999).

1.3.1 Blood Grouping:-

- People can have different blood types, known as blood groups. There are four main blood groups: A, B, AB and O. Each group can then be either RhD positive or negative.(table 1)
- The genes thatare inherited from mother and father determine the type of blood group.
- blood group are identified by the antigens and antibodies present in the blood. Antigens are usually protein molecules found on the surface of red blood cells. Antibodies are found in the plasma. They are your blood's natural defence mechanism against any foreign antigens.
- Blood type (blood group) is determined,in part,by the ABO blood group a ntigens present on red blood cells.(Table.1.1)

Table.1.1.Types of ABO blood group(Maton, 1993).

	Group A	Group B	Group AB	Group O
Red blood cell type				
Antibodies in Plasma	 Anti-B	 Anti-A	None	 Anti-A and Anti-B
Antigens in Red Blood Cell	 A antigen	 B antigen	 A and B antigens	None

A blood type (also called a blood group) is a classification of bloodbased on the presence or absence of inheritedantigenic substances on the surface of red blood cells (RBCs). These antigens may be proteins, carbohydrates, glycoproteins, or glycolipids, depending on the blood group system. Some of these antigens are also present on the surface of other types of cells of various tissues. Several of these red blood cell surface antigens can stem from one allele(or very closely

linked genes) and collectively form a blood group system.(Maton,1993).Blood types are inherited and represent contributions from both parents. A total of 32 human blood group systems are now recognized by the International Society of Blood Transfusion (ISBT).(jumptable, 2008).The two most important ones are ABO and the RhD antigen; they determine someone's blood type (A, B, AB and O, with + and - denoting RhD status).

1.3.1.2Schematic showing thedirect and indirect Coombs tests:

The two Coombs tests are based on the fact that anti-human antibodies, which are produced by immunizing non-human species with human serum, will bind to human antibodies, commonly IgG or IgM. Animal anti-human antibodies will also bind to human antibodies that may be fixed onto antigens on the surface of red blood cells (also referred to as RBCs), and in the appropriate test tube conditions this can lead to agglutination of RBCs. The phenomenon of agglutination of RBCs is important here, because the resulting clumping of RBCs can be visualised; when clumping is seen the test is positive and when clumping is not seen the test is negative. Common clinical uses of the Coombs test include the preparation of blood for transfusion in cross-matching, screening for atypical antibodies in the blood plasma of pregnant women as part of antenatal care, and detection of antibodies for the diagnosis of immune-mediated haemolyticanemias. Coombs tests are done on serum from venous blood samples which are taken from patients by venipuncture. The venous blood is taken to a laboratory (or blood bank), where trained scientific technical staff do the Coombs tests. The clinical significance of the result is assessed by the physician who requested the Coombs test, perhaps with assistance from a laboratory-based hematologist(Terry andKotrla, 2013).

1.3.1.2.1Direct Coombs test:

The direct Coombs test (also known as the direct antiglobulin test (DAT) is used to detect if antibodies or complement system factors have bound to RBC surface antigens *in vivo*. The DAT is not currently required for pre-transfusion testing but may be included by some laboratories.

1.3.1.2.1.1 Examples of diseases that give a positive direct Coombs test:

The direct Coombs test is used clinically when immune-mediated hemolytic anemia (antibody-mediated destruction of RBCs) is suspected. A positive Coombs test indicates that an immune mechanism is attacking the patient's own RBC's. This mechanism could be autoimmunity, alloimmunity or a drug-induced immune-mediated mechanism.False positive means the reaction should have been negative but agglutination occurred. false negative means the reactions should have been positive but no agglutination occurred. The key is to recognize which type of problem is occurring so that appropriate testing can be performed. When evaluating an ABO discrepancy it needs to be determined whether there is a problem with the forward type (antigens on cells), the reverse type (antibodies in serum/plasma) or both.(Terry andKotrla, 2013).

1.3.1.3Problems With the Forward Type:

The forward type detects antigens present on the red bloodcells.

False negativereactions in the forward type may occur due to:

- Weak subgroups of A or B.
- A, B or AB individual transfused with massive quantities of group O blood.

- ABO non-identical bone marrow or stem cell transplant.
- Inhibitor substances which neutralize anti-A or anti-B.

False positive reactions may occur due to:

- heavy protein coating of the red blood cells.
- coating of cord blood cells with Wharton's jelly.
- antibodies to dyes used to color anti-A or anti-B

1.3.1.4Problems with the Reverse Type:

The reverse type detects antibodies present in the serum or plasma.

False negativereactions in the reverse type may be due to weak or missing anftibodies due to a variety of factors which influence the production of antibodies.

- Patient is an infant.
- Patient is elderly.
- Seriously immunocompromised due to disease, therapy or depressed immunoglobulin levels.
- Large amounts of IV fluids present due to treatment or drawing blood above an IV.
- Antibodies passively transfused in non-ABO identical products containing excessive plasma, usually platelets.

False positivereactions may be due to "unexpected antibodies", those antibodies other than anti-A or anti-B.

- False positive reactions occur in situations such as a groupA individual reacting with both the A_1 and B reagent cells. In this situation one must

think of the most frequent cause of the problem. Anti-A_1 is not uncommon in A_2 or A_2B individuals.

- Cold agglutinins are another cause of an unexpected positive in the reverse type. It is important that red blood cell reagents be allowed to warm to room temperature prior to use to prevent false positives due to cold agglutinins.(Terry andKotrla, 2013).

1.3.2Direct blood group typing of forensic samples using a simple monoclonal antibody assay.

A simple direct test for blood group antigens in samples of blood, dried blood, dried blood associated with fabric, semen, vaginal secretions, saliva and fingerprints is described.(Cecka,*et al*;1987). This test takes advantage of monoclonal antibodies which have been produced in the laboratory, but which are also becoming available commercially in ever increasing numbers. The test is sensitive and reliable as evidenced by its performance in blind studies of more than 700 blood samples. The test requires no special equipment and can be completed in 4 hours. The test is sufficiently versatile that new antibodies can be added to the same test format as they become available.(Cecka,*et al*;1987).

- Blood typing is a common tool used to solve crimes. It may allow the examiner to match or exclude a suspect from a crime scene. To detect the presence of blood proteins, you will add specific antibodies to individual drops of blood and determine whether clumping (agglutination) occurs.
- Blood left at a crime scene can be analyzed in several ways by a criminal investigator. Blood typing may provide class evidence because more than one person has the same blood type. Because white blood cells contain DNA, it is possible to determine with a high degree of certainty using DNA

profiling whether evidence blood left at a crime scene matches the blood of a suspect (or victim).

- Blood collected from a crime scene is tested using specific antibodies. The person's blood type is determined by examining antigen–antibody reactions. Remember, the resulting match is considered class evidence. However, if the blood does not match, then a particular person may be excluded as a suspect. Depending on the circumstances, blood typing may not be done at all, just DNA analysis (Anthony and Bertino , 2009)
  - The current study was conducted to assess the effect of *T.evansi* infection in determination of direct blood group.

1.4 Literature Review:

Excessive sequestration of Plasmodium falciparum-infected (pRBC) and uninfected erythrocytes (RBC) in the microvasculature, cytoadherence, and rosetting, have been suggested to be correlated with the development of cerebral malaria(Treutiger*et al*,1999). *P. falciparum* erythrocyte membrane protein-1 (PfEMP1) is the parasite-derived adhesin which mediates rosetting. It was shown that serum proteins are crucial for the rosette formation of four strains of parasites (FCR3S1, TM284, TM180, and R29), whereas the rosettes of the fifth strain (DD2) are serum independent(Treutiger*et al*,1999). Some parasites, e.g., FCR3S1, can be depleted of all rosettes by washes in heparin and Na citrate and none of the rosettes remain when the parasite is grown in foetal calf serum or ALBUMAX. Rosettes of other parasites are less sensitive; e.g., 20% of TM180 and R29 and 70% of TM284 rosettes still prevail after cultivation. A serum fraction generated by ion-exchange chromatography and poly-ethylene-glycol precipitation restored 50% of FCR3S1 and approx 40 to 100% of TM180 rosettes. In FCR3S1, antibodies to fibrinogen reverted the effect of the serum fraction and stained fibrinogen bound to the pRBC surface in transmission electron microscopy. Normal, nonimmuneIgM and/or IgG was also found

attached to the pRBC of the four serum-dependent strains as seen by surface immunofluorescens. The results suggest that serum proteins, known to participate in rouleaux formation of normal erythrocytes, produce stable rosettes in conjunction with the recently identified parasite-derived rosetting ligand PfEMP1(Treutiger*et al*,1999).

Mammalian erythrocyte aggregation increases when the levels of plasma proteins favoring aggregation rise. Red cell aggregate formation has been attributed to noncovalent bonding of adjacent erythrocyte plasma membranes by these proteins and similar macromolecules. (McMillan,*et al* 1989). The proposed membrane to membrane noncovalent bonds would keep each membrane from sliding during aggregate formation because the bonds responsible for maintaining cell-cell contact would need to be disrupted.(McMillan,*et al* 1989).Because past studies of doublet formation suggested that the membranes might slide during contact expansion, latex particles were embeded in the membranes of individual human red cells and recorded doublet formation on video tape. The cells were suspended in a buffer that contained polyvinylpyrrolidone at a concentration sufficient to cause a moderately elevated sedimentation rate. The latex particles remained stable in position relative to each host cell during doublet formation, indicating that sliding was involved.(McMillan,*et al* 1989).Individualrouleaux was stretched using a glass rod and observed that latex particles attached to red cells whose contact area was reduced by the motion maintained their position during their return to a normal shape.(McMillan,*et al* 1989). These studies showed that erythrocyte aggregate formation is accomplished by membrane sliding and that aggregate shape change and disruption during blood flow commonly involve sliding.(McMillan,*et al* 1989). The sliding motion argues that the attraction between red cell membranes generated by an array of elongated macromolecules involves a delocalized rather than a noncovalently coordinated adhesion.(McMillan,*et al* 1989).

The mechanics by which normal human erythrocytes join on a plastic cover slip into two cell doublets and larger aggregates of rouleaux were studied microscopically.(Sewchand and Canham 1979).Polyvinylpyrrolidone (PVP-360) or dextran (DX-70 or DX-110) were used as the rouleau agents. The minimum concentration of the rouleau-inducing agents required to form doublets was 1 g/L for PVP-360 and 5 g/L for the DXs. Three modes of interaction were observed in Ringer's solution with PVP or DX, cresting and flipping (involving no intercellular sliding) and a sliding mode of doublet formation (involving less gravitational work and less cellular deformation). The sliding mechanism occurred in suspensions with the lower concentrations of the rouleau agent but was also observed when geometric constraints prevented the nonsliding interaction of larger groups of cells in the higher concentrations of the rouleau agent. (Sewchand and Canham 1979). The technique provides a sensitive index for studying the combined effect of cellular flexibility and intercellular adhesion. Significant changes were observed for reduced membrane surface charge or reduced ionic calcium.(Sewchand and Canham 1979).

Erythrocyte deformation involves both viscous dissipation in the cell interior and viscoelastic motion of the cell membrane. Reports that describe reduced filterability of diabetic erythrocytes, altered response to oscillatory motion in a capillary-sized pipet, and impaired packing during centrifugation indicate a disturbance of red cell rheology in diabetes. They selected conditions that minimize the macromolecule-mediated energy of attraction between erythrocytes and studied erythrocyte motion during doublet formation. Under such conditions, doublet formation frequency is strikingly reduced in diabetes. For nondiabetic erythrocytes the formation rate is 0.73 doublets per minute, whereas for diabetic erythrocytes the rate is 0.23 doublets per minute. In addition, mean velocity of doublet formation was found to be decreased to half

of normal in diabetes. Completeness of doublet formation and regularly diminished when cell size of the two component cells were similar.This is similar for diabetic and nondiabetic erythrocytes(Mcmeillan,*et al*1983). Observation of several features of doublet formation gave a picture of the mechanical process. The initial cell making contact with the glass microscope slide was observed to remain fixed in position. The late arriving cell's ability to form a doublet was seen to decrease rapidly, apparently because it came to adhere to the glass surface. The attractive force between the cells overcomes the force of gravity, but cell deformation resistance slows doublet formation by balancing the tendency for cell-cell contact area to increase. An integral equation combining strain energy and viscous dissipation was applied to the doublet formation process. Slowing of doublet formation in diabetes appears to be produced by a doubling of resistance to rate of change of curvature of diabetic erythrocytes.(Mcmeillan,*et al* 1983).

A purpose of the present study is to make an artificial rouleau of bovine red blood cells which is not capable of rouleau formation under physiological condition. Rheological behaviors of bovine blood forming artificial rouleaux were examined. The modification of cell surface by enzyme trypsin induced rouleau formation, whereas the modification of cell surface by neuraminidase did not cause any aggregate formation. The drastic elevation of the fibrinogen content in bovine red blood cells suspension also brought about the formation of rouleau. The value of dynamic rigidity modulus G' of bovine red blood cells in saline solution containing high concentration of fibrinogen is somewhat smaller than that of trypsin treated bovine red blood cells in plasma. The value of G' of trypsin treated bovine red blood cells in plasma first increased to a maximum value and then decreased with the time. It is supposed that the removal of macro-molecules from the cell surface facilitates the mutual approach of cells

and causes the formation of rouleau which seems to be the same as that of human and horse .(Kaibara, 1983).

1.5 Rationale:-

- The principle of direct blood grouping (slide or tube method) depends on agglutination of RBCs after addition of the specific antisera.This agglutination is checked either by naked eyes or microscopically.Patients infected with trypanosome usually have non-specific auto antibodis that may lead to auto agglutination of RBCs (Rouleaux formation) which may cause difficulties in the determination of direct blood group.

- Infections by trypanosome is known to cause auto-agglutination of red blood cells.This may cause difficulty in determination of blood group.

- In Sudan there are no published data on the effect of the infections with Trypanosomes on human or animal blood groups.

- Blood type categorically denied if evidence is found not matching the blood found at the crime scene, so you must make sure that the accused is not infected with trypanosomiasis disease which affects his blood group and give false positive result.

1.6 Objectives:-

1.6.1 General Objective:-

To evaluate the effect of trypanosome parasite in blood grouping which relate with forensic work.

1.6.2 Specific Objective:

- To evaluate the auto-agglutination (Rouleaux formation) caused by trypanosome as compared to non-infected control.
- To evaluate the auto-agglutination (Rouleaux formation) caused by trypanosome infected animals after treatment (Quinapyraminsulphate).
- To correlate the degree of parasiteamia to the degree of rouleaux formation.

Chapter Two

Materials and Method

2.1 The expermental design:

A total of 18 rats were used. They were divided into three groups as in (table2.2.) (Fig2.3.).

Table. 2.2. The study groups of rats.

Group A	**Group B**	**Group C**
Infected un treated	Infected treated	Un infected untreated
6 rats inoculated with *T.evansi*Showakstabilate	6 rats inoculated with *T.evansi*Showaktabilate	6rats un infected (clean).

2.2 The Study area:

This study was conducted at the parasitology laboratory of College of VeterinaryMedicine, Sudan University of Science and Technology (SUST)App(6)(Fig.2.4).

2.3 TheStudy duration:

The studywas conducted during the period from March to November 2013.

2.4The Study subjects:

The study was conducted on White albino rats(*Rattusnorvigicus)*(Fig.2.5).The White albino Rats used wereout bred obtained from College of Veterinary

Medicine, Sudan University of the Science and Technology (SUST). Both sexes were used in the present study with weight range 95 – 160gramseach.About 18-rats used in the present study. The rats werekept in group of 6rats per cage with ranging 25-30^{0}c and relatione humidity about 70%.Rats were fed on food for mulatedlocally(Ismail,1988).Food and water were given ad lib.

Fig.2.5.White albino Rat (*Rattusnorvigicus*)

2.4.1 Accommodation and Feeding:

They were kept in a good ventilation and accommodation with a mean temperature 25- 30c˚. They were kept in wide metal cages (Fig.2.6). Saw dust was used for bedding, and was changed every other day. The food supply was prepared as formulated by Ismail,(1988).Water was provided in clean crystal bottles with rust free nozzle and ball so that it can easily be imbibed. Each rat was identified by marks on their tails using the system of Ramesh,(2004).

Fig.2.6.Cages of rats to group of study.

2.5The Study Procedure:

2.5.1 The parasite Inoculations (*T.evansi*) Showak :

*Trypanosomaevansi*was isolated naturally from infected camel and was preserved in liquid nitrogen as a stock at-197C˚.

Trypanosomaevansi, was isolated from a camel belonging to a rural sedentary family (agro-pastoralist) in a village near Showak, Gadarif State, Eastern Sudan. About 0.2ml of infected EDTA blood(stock parasite) was injected interaperitoneally (I/P) into a donor rat.The inoculated donor rat was then followed up by preforming blood parasite count until a high degree of parasitemia(5×10^6parasites/ml of blood)was obtained. The rat was then scarified. Heart blood was collected into EDTA container. Serial dilutions of the donor rat blood was done using phosphate buffered saline with glucose (PSG) withpH8.0.The parasite count was then done for each dilution until the

parasite count reaches $5x10^4$ parasites/mlthen eachof the study ratswas inoculated intraperitoeallywith 0.2 ml of the diluted blood equivalent to $1x10^4$ parasites per each rat(App.7.Fig.2.7.). Prior to inoculation, wet blood film was examined for each rat and examined for rouleaux formation and other possible parasitic infection. A total of 12 ratsweighting about 95-160g were inoculated as above, of these 6 were left without any drug treatment and the remaining 6 were given antitrypansoma drug. The drug used was QuinapyraminesulphateB.Vet.C. in adose of20mg/kg B.Wt. to each of the six rats. The drug was given after establishing infection in the 6 rats indicated by presence of the parasite in a wet blood preparation. The other 6 rats were not inoculated(control group).The rats were marked in their tail from 1 – 6 for each group during the inoculation for the purpose of individual post- infection follow up.

2.5.2 Follow-up of the inoculated rats:

2.5.2.1 A-Parasite count:

For each rat under study tail blood(Fig.2.8) was taken daily as described by Eisler , *et .al*, (2001).The collected blood was examined for presence of parasites in awet blood film until the number of parasites reaches one parasite per high power field and thereafter blood parasite count was done daily for each inoculated rat until the rat died . Parasite count was doneas follows:

Fig.2.8.Taking sample of blood from tail

2.5.2.1.1 a- Wet blood film:

A drop of fresh blood from the tip of the tail of the infected rat was placed on a clean slide, then covered with 22x22mm cover slip and examined microscopically(using ×40 0bjective and ×20 eye piece) for the presence of the parasite (>30fields). The results were reported as number of parasite perfield then converted to number of parasites per cubic millimeter (mm^3) according to Ismail,(1988), then expressed as $\log_{10}$. Parasitaemia curves were drawn in graphs.The same blood filmswere examined microscopically for the presence of the roulex and compared it with number of the parasites(Plate.2.1).

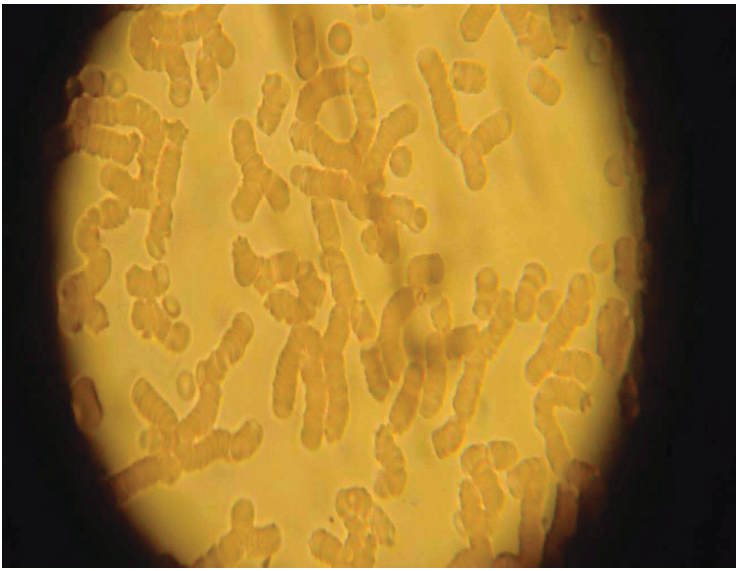


Plate.2.1. Wet blood film.

2.5.2.1.2b- Dry smear:

Thin and thick blood smears were prepared from freshly collected whole blood , stained withGiemsa stain as described by Jain, 1986.

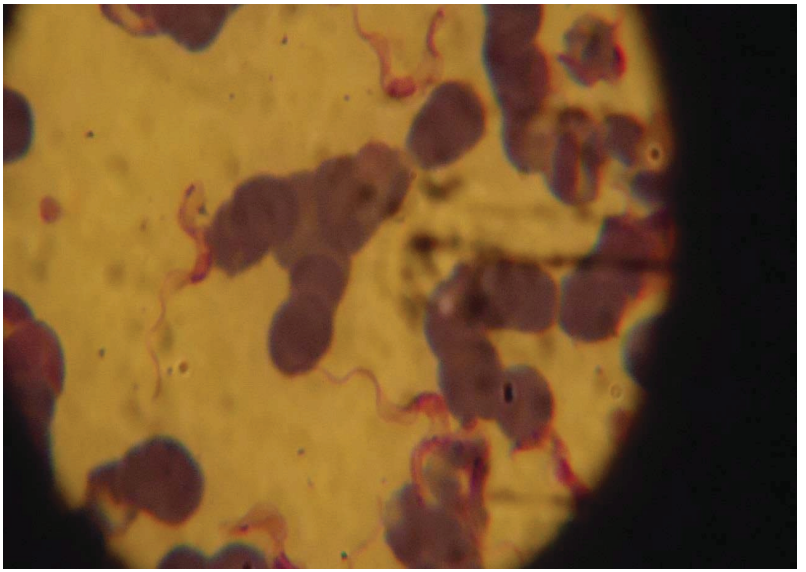


Plate.2.2. Giemsa stained blood film.

2.5.2.1.3 c-Haemocytometer :

When parasite counts reached 1/field or more by the above method,the obtained number was confirmed byhaemocytometerparasite count as reported byParis, *et al*.(1982). In this method, 0.5µl of blood were withdrow from the rat tail,fixed and stained in 45 µltryps diluting buffer.. Thentrypanosome count was determinedas for (WBC count).The number of parasiteswere then designated as number of parasites per ml of blood(Fig.2.9).

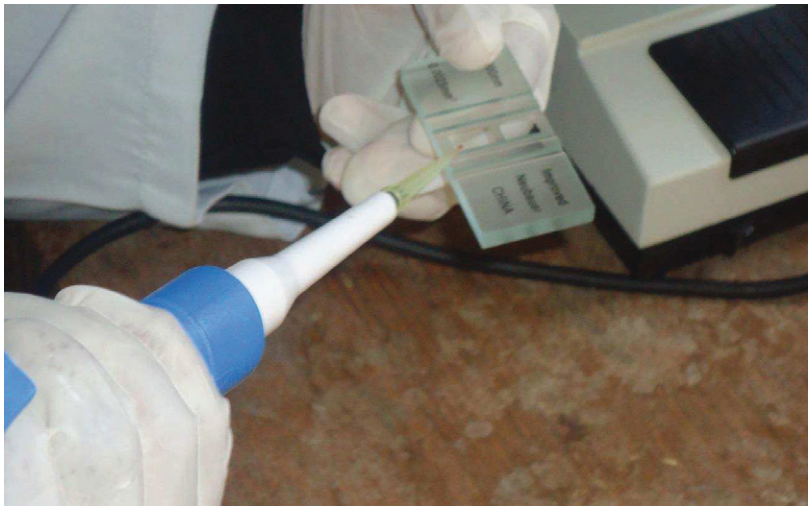


Fig 2.9.HaemocytometerTechnique.

2.5.2.2 B-grading the degree of rouleaux:

Formation of rouleaux was graded as follows:App.9.(Table.3.4)&App.11.(Table.3.6)

- **Grade 0**: when there were no formation of rouleaux ,App.1.(Plate 2.3).
- **Grade 1**: when the predominant RBCs aresingle (not involved in rouleaux formation),App.2. (Plate 2.4).
- **Grade 2**:when the number of single RBCs is almost equal to those involved inrouleaux formation,App.3.(Plate 2.5).
- **Grade 3**: when the predominant cells are involved in rouleaux,App.4.(Plate 2.6).
- **Grade 4**: when there were excessive rouleaux formation resulting in clumping of cells,App.5.(Plate 2.7).

2.6Data analysis:

Data were analyzed by computerized programSPSS (using correlation and Independent sample test) and computer(Excell).

2.7Ethicalconsidration:

The permission was taken from the Ethical committee of the national Ribat University.

Chapter three

Results

3.1 Group A (Infected and untreated):

The parasite started to appear in wet blood film on day 5 in 3 rats (1,3and 4) and by day 7 the parasite wasdetected in the wet blood film of all the 6 rats (Fig3.10). Parasite count started to increase gradually for all rats till died. The rats started to die on day 24 until day 40 at which the last rat died. The sequence of rat's death was as follows: Rat2 on day 24, rat5 on day 26, rat 6 on day 32, rat1 on day 33, rat3 on day 36 and rat4 on day40($\underline{X=31.8\pm5.5}$).

Rouleaux started to appear in wet blood preparation on day 2 for three rats (1,3and 6) and by day 5 the rouleaux was detected in the wet blood film of all the 6 rats (Fig3.10). The degree of rouleaux gradually increased for most rats reaching the maximum (+4) on day 10 and maintained in this level until day 15 thereafter started to fluctuate (Fig 3.10)(Table 3.3&3.4).

The results of this study showed directly proportional relation between the degree of parasiteamia and rouleaux formation (Fig 3.11)(table 3.7).

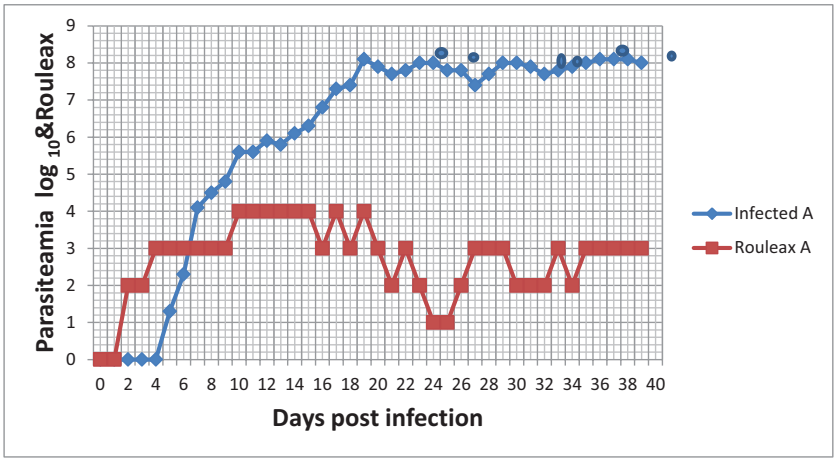


Fig.3.10 : Shows the relation between the mean log parasite count and degree of rouleaux formation for the inoculated untreated group of rats(groupA) .

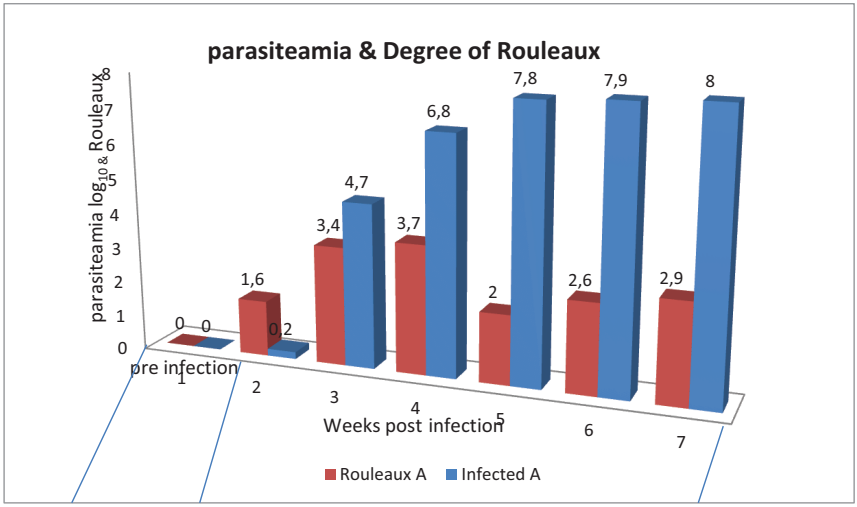


Fig.3.11: Bar.chart showing The relation between the degree of parasitaemia and rouleaux formation for group (A) rats by weeks.

3.2 Group B (Infected and treated):

The parasite started to appear in wet blood film on day 2 for 2 rats(3and 5) and by day 5 the parasite was detected in the wet blood film of all the 6 rats (Fig3.12).Treatment commenced at day 5 with parasitaemia level 7/ prepration (Fig3. 12). By day 6 Parasites were cleared from all rats in the group,indicated by absence of parasites in wet preparation and this continued to day 8.Parasites thereafter appeared in the wet blood preparation and parasitemia increased gradually reaching high level on day 36 . The increase in parasitemia was found to be statistically lower in this group compared to the group of the untreated rats (Pvalue 0.00).In this group all rats except one survived to the end of the study (40 days). That rat died on day 39 and it is observed that the degree of parasitemia was higher in it compared to the other members of the group.

Rouleaux started to appear in wet blood preparation on day 2 for one rat (5) and by day 5rouleauxwere detected in the wet blood film of all the 6 rats (Fig3.12). The degree of rouleaux fluctuated for most rats. It reached maximum on day 25 and maintained in this level until the end of the study period (Fig3. 12)(Table 3.5&3.6).

The result of this study showed a directly proportional relation between the degree of parasiteamia and rouleaux formation (Fig3.13)(table 3.8).

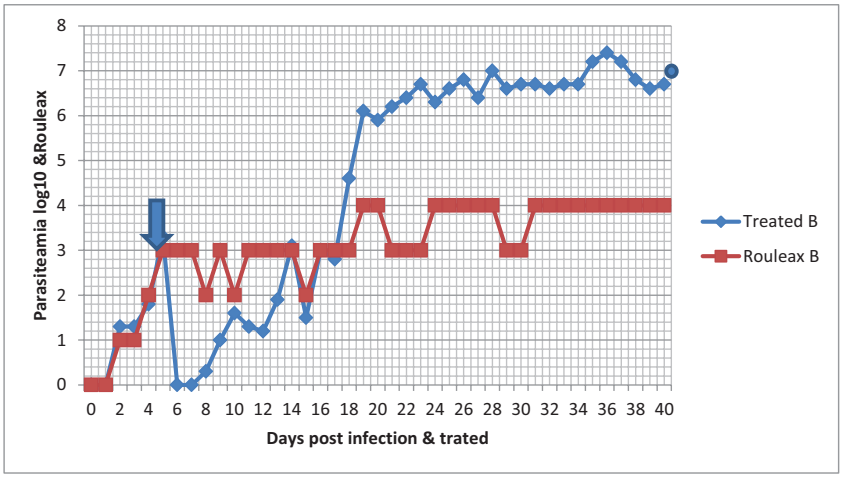


Fig.3.12: Shows thethe relation between the mean log parasite countand degree of rouleaux formation for the inoculated trated group of rats(group B) .

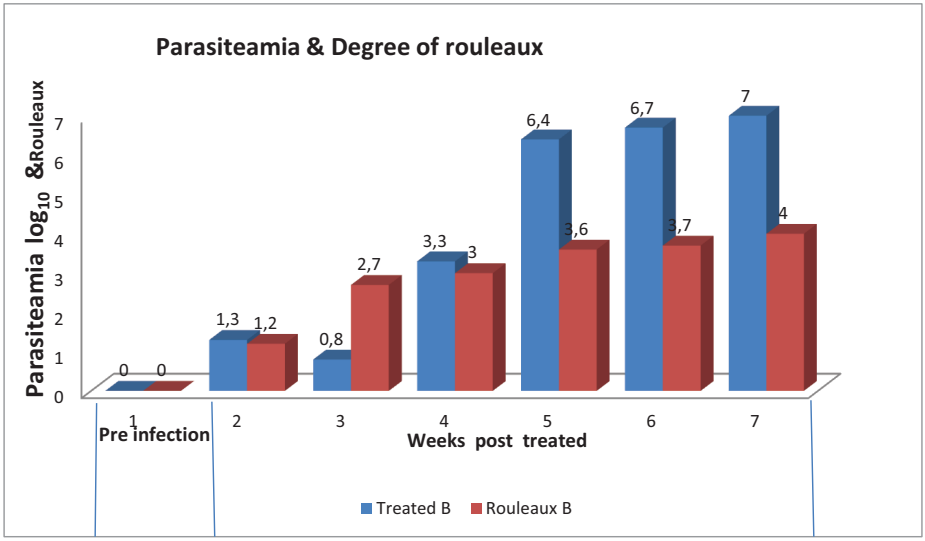
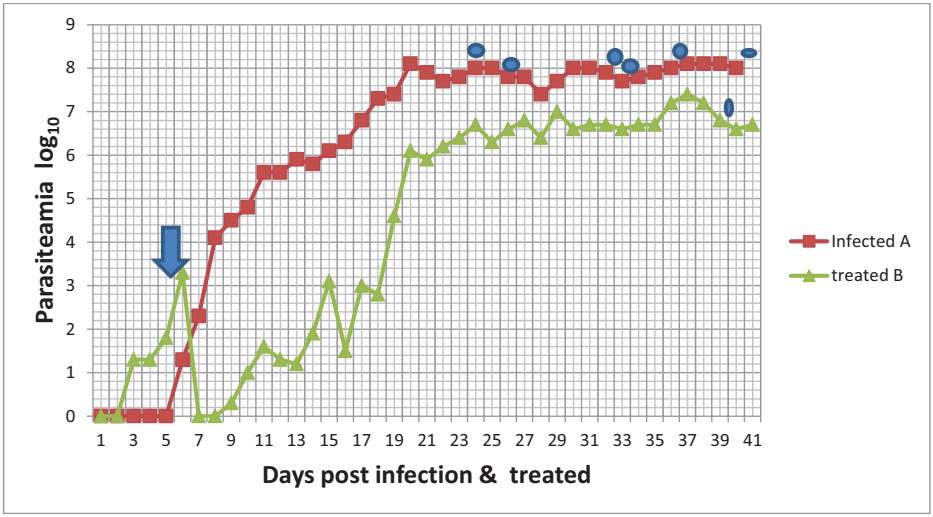


Fig.3.13:Bar chart showing the relation between the degree of parasitaemia and rouleaux formation for Group (B) rats, by weeks.

3.3 The group A and group B:

The degree of parasitaemia was significantly higher (PV 0.00) in group A than group B until day 25(mean parasitaemia of $\log_{10}$ 6.3 and$\log_{10}$8 for group A and B respectively),(Fig.3.14) . By day 29 the difference becomeinsignificant (PV 0.065) The study showed insignificant difference in the rouleaux formation between group A and group B (PV, .088) (Fig.3.16) . The study showed statistically the correlation between parasite count and rouleaux formation significant (PV 0.00) in two groups. This may point to the fact that, infection with *T.evansi* results in excessive formation of rouleaux that may cause difficulty in determination of direct blood group especially for the weak blood group like A_1(Fig.3. 15).



Time to death	Control 6 rats	Time to death	Infected and treated 6 rats
Day 24 Day 26 Day 32 Day 33 Day 36 Day 40 X=31.8±5.5	1 rat n=5 1 rat n=4 1 rat n=3 1 rat n=2 1 rat n=1 1 rat n=0	Day 40	1 rat n=5 5 rats survived.

fig.3.14: Comparison of the means of parasiteamia levels (log_{10}), between rats infected with *T.evansi* (Showakstabilate) treated by Interquin, (Quinapyramine sulphate) at adose of 20mg/kgbwt and rats infected not treated .

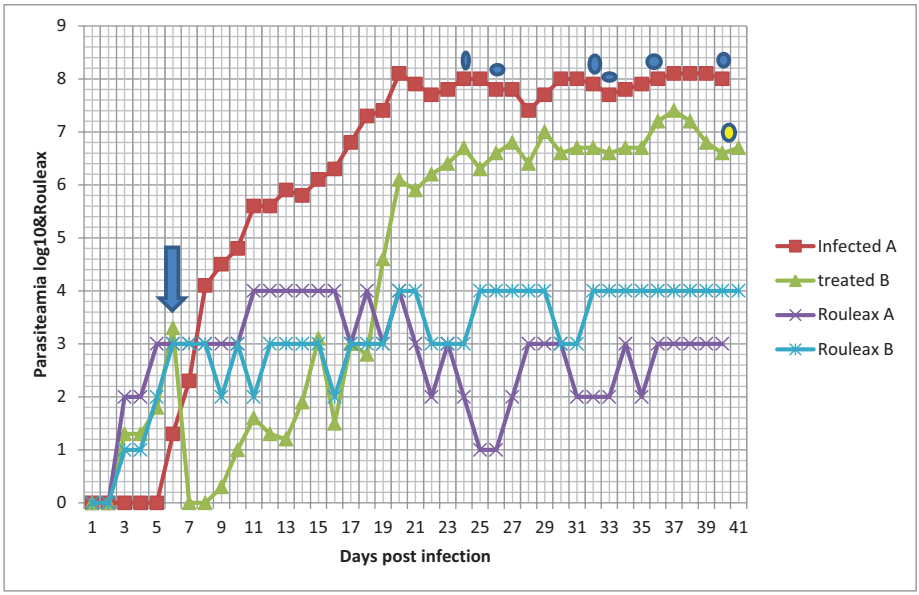


fig.3.15: Comparison of the parasiteamia level (log_{10}), between rats infected with *T.evansi* (Showakstabilate) treated and rats infected untreated. Also comparison of the degree of rouleaux formation between them.(blue dot indicated to death in group A and yellow dot to death in group B).

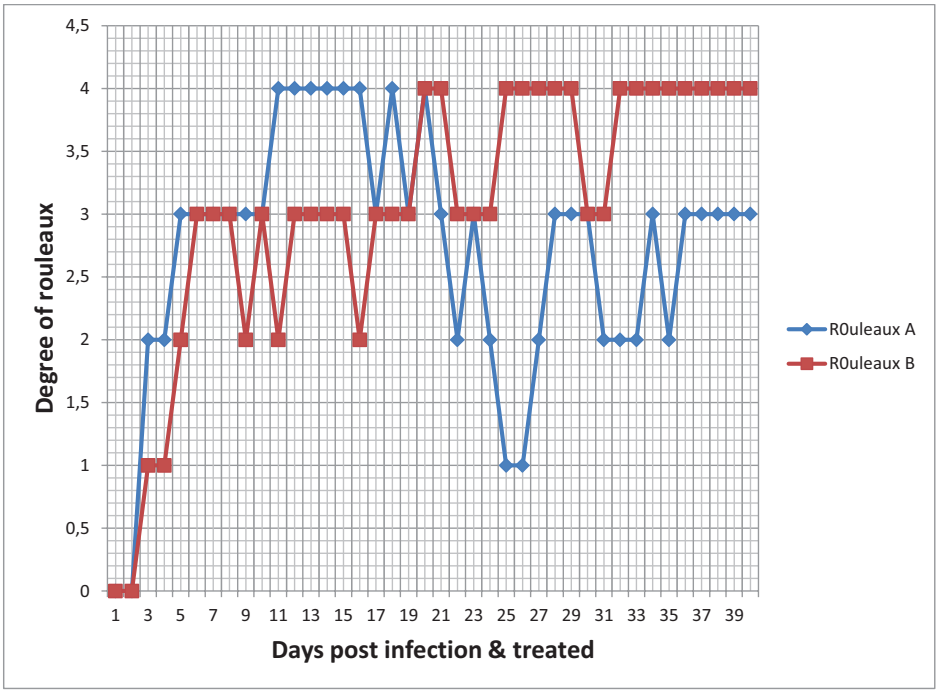


Fig.3.16. Shows the relation between degree of rouleaux for the inoculated untreated &treated group of rats (group A and B).

3.4Group C (uninfected treated):

This group were not inoculated (control group) and remained negative until the end of the study period.

Chapter four

Discussion

In this study rouleaux started to appear on day 2 for three rats of group A ,and in all rats by day 5. While in group B it started to appear on day 2 in one rat and in all rats by day 5 .On the other hand the parasite started to appear on day 5 for three rats and day 7 for all rats of group A. While the parasite appeared on the wet blood film on day2 for two rats ,and day 7 for all rats .These result revealed thatrouleaux formation occurs before appearance of the parasite in the peripheral blood, by approximately 2 days. Since all rats were prechecked and found to be negative forrouleauxbeforeinoculation, it can be concluded that rouleaux was due to *T.evansi* infection.

In the inoculated untreated rats (Group A), the degree of rouleaux formation was directly proportional to the degree of parasitaemia .statistically the correlation association between parasite count and rouleaux formation significant (pv 0.00).

In the inoculated untreatedrats, rouleaux formation started on day2 and increased gradually until day 10, mainted at the high level until day 15 and thereafter started to fluctuate but never returned to the high level. Un like group B rats, the rouleauxformation started to appear on day 2and increased gradually reaching (grade3) on day 6 then fluctuated until day19 .Then reached the highest level (grade4) on day 20,where it was maintained. The fluctuation in rouleaux formation can be due to the fact that some rats developed infection of the tailswith inflammation that is expected to affect roulaeux formation. As the degree of fluctuation was more in the untreated group of rats in which more rats were infected .The fluctuation might be due to technical errors regarding the preparation of wet blood film and peripheral blood film.

Chapter Five

Conclusion

- In this study infection with*Trypanosomeevansi*was found to cause excessive rouleaux formation.
- The degree of rouleaux was significantly found to be directly proportional to the degree of parasiteamia.
- The strain of the parasite used in this study was found to be resistant to the drug (quinopyrmin sulphate) evidenced by remission after initial disappearance of the parasite from the peripheral blood.
- The study showed insignificant difference in the degree of rouleauxformation between group A and group B of the rats.

Recommendations

- It is recommended to conduct studies to see if infection with trypanosome can change the blood group.
- Further in depth studies including large sample size are recommended to assess the relation between infection by trypanosome and rouleaux formation.
- New antitrypansoma drugs to which the parasite is sensitive are recommended to be used in the proposed studies .
- Further researchesto evaluate other factors (parasites,virus, etc) as possible causes ofrouleaux formation are suggested.

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