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**EXPERIMENTAL STUDIES ON CLINICO-HEMATOLOGY OF TRYPANOSOMA
EQUIPERDUM INFECTED HORSES AND VENEREAL TRANSMISSION IN MICE**

MSc Thesis



BY

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EXPERIMENTAL STUDIES ON CLINICO- HEMATOLOGY OF TRYPANOSOMA
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A Thesis submitted to the College of Veterinary Medicine and Agriculture of Addis Ababa University in partial fulfillment of the requirements for the degree of Master of Veterinary Science in Veterinary Parasitology

BY

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June, 2017

Bishoftu, Ethiopia

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DEDICATION

This thesis is dedicated to my mother, Kene Terefe and my father, Gemeda Ayana for their uninterrupted nursing and supporting me with deep affection and love, as well as for their dedicated partnership becomes mainstay in the success of my life.

STATEMENT OF AUTHOR

First, I declare that this thesis is my original work and that all sources of material used for this thesis have been duly acknowledged. This thesis has been submitted in partial fulfillment of the requirements for an advanced MVSc degree at Addis Ababa University, College of Veterinary Medicine and Agriculture and is deposited at the University/College library to be made available to borrowers under rules of the Library. I solemnly declare that this thesis is not submitted to any other institution anywhere for the award of any academic degree, diploma, or certificate. Brief quotations from this thesis are allowable without special permission provided that accurate acknowledgement of source is made. Requests for permission for extended quotation from or reproduction of this manuscript in whole or in part may be granted by the head of the major department or the Dean of the College when in his or her judgment the proposed use of the material is in the interests of scholarship. In all other instances, however permission must be obtained from the author.

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LIST OF ABBREVIATIONS

BoTat	Bordeaux Trypanosoma antigen type strain
CATT	Card Agglutination for Trypanosomiasis Test
CFT	Complement Fixation Test
CSF	Cerebro-Spinal Fluid
DNA	Deoxyribonucleic acid
EARO	Ethiopia Agricultural Research Organization
EDTA	Ethylene Diamine Tetra Acetic Acid
ELISA	Enzyme Linked Immunosorbent Assay
FAO STAT	Food and Agriculture Statistical Database
HCT	Haematocrit Centrifugation Technique
IP	Inter-peritoneal
IV	Intravenous
MAECT	mini Anion Exchange Centrifugation Technique
MCH	Mean Corpuscular Hemoglobin
MCHC	Mean Corpuscular Hemoglobin Concentration
MCV	Mean Corpuscular Volume
NMSA	National Meteorological Service Agency
OIE	Office international des Epizooties
OVI	Onderstepoort Veterinary Institute strain
PCR	Polymerase Chain Reaction
PCV	Pack Cell Volume
RBC	Red Blood Cell
RoTat 1.2	Rhode Trypanosome antigenic type 1.2
ROTAT	Rhode Trypanosoma Antigenic Type
STIB-818	Swiss Tropical Institute Basel strain 818
VAT	Variable Antigen Type
VSG	Variable Surface Glycoproteins

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ABSTRACT

Dourine is an endemic venereal transmitted trypanosomosis causing a major health problem threatening the life and productivity of the equine population in Ethiopia. The present experimental study was conducted from November 2016 to June 2017 in Bishoftu, central Oromia, Ethiopia. The objective of the study was to evaluating clinico-hematological profile of horses experimentally infected with *Trypanosoma equiperdum*, assess the effect of cymelarsan treatment on hematological values of horses infected with *T. equiperdum* and determine the possibility of venereal transmission of *T. equiperdum* in mice. The study design was conducted on (n=4) mares and (n=4) stallions. Mares were challenged by insemination of semen originated from non-infected stallion after mixed with *T. equiperdum* at a dose of 100,000 parasite/ml by artificial insemination whereas stallion group were infected with 2ml of blood that drawn from infected mare at dose of 1×10^6 parasites /ml via intravenous route. Data on parasitaemia, rectal temperature, serology (CATT/*T. evansi* test) and hematological were determined before and after infection. Furthermore infected horses were divided into two groups designated as treated and non-treated groups. At weeks 6 of post infection treatment group was injected with cymelarsan® at dose of 0.25mg/kg body weight. Changes in clinical sign, parasitemia and hematological values were monitored. Thirty (n=30) female mice and male (n=12) were involved in experiment to check coital transmission. One infected mouse was co-housed with two healthy female mice at low level of parasitemia (1×10^1 - 1×10^3) in six replications and other one infected male mouse co-housed with two healthy female mice at high level of parasitemia (1×10^4 - 1×10^6) in six replications. Other two infected female co-housed with four healthy female as control. Thereafter the appearance of Parasitemia was monitored daily from female mice. The study revealed that, Dodola strain caused a severe change in blood parameter and a clinical sign in both stallion and mare, infection was predominantly characterized by involvement of genital, nervous and corneal signs. There was significant decrease in mean PCV, Hg, RBC, eosinophil, basophil and monocyte count while mean total WBC and neutrophil count had no significant difference ($p > 0.05$) in animals post-infection when compared to pre-infection values. Lymphocyte and MCHC were significantly ($p < 0.05$) higher at post infection as compared to pre-infection. Cymelarsan treatment at dose of 0.25 mg/kg body weight failed to

clear the parasite and improve in clinico-hematological values, rather being relapsed after 21days. Treated group had no significant improvement in RBC, Hgb, and differential leukocyte count whereas PCV and WBC which were significantly ($p < 0.05$) improved as compared to non-treated group. In healthy female mice co-housed with infected male mice using wet smear no trypanosomes were detected in all female mice and their offspring in wet smear throughout study period. In conclusion the current study revealed that dourine resulted in hematological changes that lead to normocytic hypochromic anemia, thus further elucidative investigation recommended on pathogenesis of the disease. In addition, venereal transmission of *T. equiperdum* in mice was unsuccessful; hence further molecular based test is essential to ascertain this result.

Key words: *Cymelarsan, Experimental infection, Hematological, Horse, Mice, Trypanosoma equiperdum.*

1. INTRODUCTION

The world equine population is estimated at 44 million donkeys, 11 million mules and 59 million horses (FAOSTAT, 2012). Ethiopia has one of the largest livestock population in Africa with the estimated equids, 2.1 million horses, 0.4 million mules and 7.88 million donkeys (CSA, 2016). Equine are extremely important in Ethiopian agriculture and for the national economy. Nearly 90% of agricultural operations depend on manual labor and because of the rugged mountainous terrain of the country these animals are still the main method used to transport both people and agricultural products (EARO, 1999; FAO, 2003). In addition, Equines are used for various works such as carting goods and people, carrying packs and bricks, and other construction materials, riding, tillage, weeding and water carrying (Maarten, 2009).

Despite their huge numbers and significant contribution to the communities and national economy the attention given to study the health aspects of working equids in Ethiopia is quite minimal. Among the multiple health and welfare problems affecting working equids, infectious disease are one of the major constraints to their productivity and work performance, this often leads to high morbidity and mortality (Behnke and Metaferia, 2011). The major infectious diseases that affect horses in Ethiopia are African horse sickness, anthrax, epizootic lymphangitis, dourine, equine piroplasmosis, horse mange, rabies, glanders and ulcerative lymphangitis (FAO, 1996).

Among these diseases Dourine is an insidious venereal infection of equine species caused by the protozoon parasite, *Trypanosoma equiperdum* (Radostits *et al.*, 1994). It is the only trypanosomiasis that is not transmitted by an invertebrate vector. The causative agent of dourine, *T. equiperdum*, differs from other trypanosomes in that it is primarily a tissue parasite that rarely invades the blood (OIE, 2013).

Dourine is a unique disease in the sense that it has no known vectors existing in the natural world, other than members of the equine family, including donkeys, mules and horses (Gillingwater *et al.*, 2007). Zebra has been tested and showed positive serology

but there is no conclusive evidence of infection. Male donkey can be asymptomatic carrier (Luckins, 1994). In laboratory setting, *Trypanosoma equiperdum* has been manipulated to adapt and proliferate in other species, such as dogs, rabbits, mice and rats (Hagos *et al.*, 2010). Currently few cases of Dourine have been reported owing to the wide use of artificial fertilization technology (OIE, 2013). Some concern has been raised about the possibility of dourine transmission through imported semen (Metcalf, 2001).

For the treatment of *T. equiperdum* infection the same drug which is used for *T. evansi* are available. Evidence from *in vitro* drug sensitivity determination of *T. equiperdum* indicates that Suramin, Diminazen, Quinapyramine and Cymelarsan are effective against this trypanosome species (Zhang and Baltz, 1992; Barn and Lun, 1994). Nowadays, the OIE terrestrial code considers dourine as a non-treatable disease and imposes to practice a stamping-out policy for affected animals to recover a country free aimed at isolation, castration or slaughtering of complement fixation test (CFT) positive horses (Zabotskij *et al.*, 2003). A recent study suggests that melarsamine hydrochloride has the capacity to cure infected horses (Hagos *et al.*, 2010b). To date the Cymelarsan® treatment failed to cure the two ponies at a single dosage but efficacy of a repeated treatment can be supposed depending on the stage of the disease (Cauchard *et al.*, 2016).

Blood cells are important indicators of health status and they are invaluable in diagnosis, treatment and prognosis for many diseases (Fatihu *et al.*, 2000). Blood invading parasites produce numerous changes in the cellular and biochemical constituents of blood (Taiwo *et al.*, 2003). Anemia is a cardinal sign of trypanosomosis in many domestic animals that attributed to weight loss and poor body condition, due to dyshaemopoiesis (Lukins, 1999).

There were different studies conducted on bovine trypanosomosis based on clinical signs and hematological analysis (Maxie *et al.*, 1979); (Hilali, 2006); (Bisalla, 2007); (Padmaja, 2012); (Dagnachew *et al.*, 2015). However, there is paucity of information related to the impact of *T. equiperdum* infections on alteration of clinico-hematological values for the purpose of better understanding of pathogenicity of *T. equiperdum* in

infected horses. Proper understanding and good knowledge of the venereal transmission capability and pathogenicity of *T. equiperdum* among horses as well as mice may favor the improvement of the existing possible diagnostic techniques and disease control and prevention methods. The objectives of the present study were, therefore, to:

- ✍ Evaluate clinico-hematological profile of horses experimentally infected with *T. equiperdum*.
- ✍ Assess the effect of cymelarsan treatment on hematological values of horses infected with *T. equiperdum*.
- ✍ Determine the possibility of venereal transmission of *T. equiperdum* in mice.

2. LITERATURE REVIEW

2.1. The Disease Description and Synonyms

The first description of the nature of the disease was established only in 1896 following demonstration of the trypanosomes in the blood of infected Algerian horses (Rouget, 1986). Dourine is a venereal disease of equidae caused by the protozoan, *Trypanosoma equiperdum* which can result in an acute or chronic form. Clinical signs include paralysis, swelling of the genitalia, cutaneous plaques, emaciation and neurological signs and there is a high mortality rate. It is transmitted almost exclusively during coitus, rather than requiring a vector. During acute periods, parasites can be recovered from genital secretions, but in the chronic phase parasites disappear and the animals are non-infectious (Centre for Food Security and Public Health, 2009).

The venereal disease of equines or dourine has been known under other names (Arabic "el Dourin", English "Covering disease", German "Beschalseuche", French "Mal de coit", Russian "Slucnaja Boleznj" or "Podsedal") (Hoare, 1972). In Ethiopia Dourine is locally known as "Lappessa" or "Dirressa" (Zelege *et al.*, 1980).

2.1.1. Historical back ground of *T. equiperdum*

First recognized description of dourine in Europe was by Ammon and Dirkhausen who, in 1796, observed cases in a Prussian stud. However, it was only in 1894 that Rouget demonstrated the presence of *T. equiperdum* in the blood of an infected Algerian horse. However, this parasite was lost before Rouget could reproduce the disease in horses (Zwick and Knuth, 1928). The name *T. equiperdum* was postulated by Doflein in 1901. Buffard and Schneider suggested in 1902 that the parasite might cause surra or nagana, but not dourine. However, trypanosomes had been isolated from other cases of dourine in France, Hungary, Germany and Canada (Laveran and Mesnil, 1912). Since the 19th century, dourine has occurred sporadically in Europe. Around 1918, the disease was reported only in Russia, Turkey, Hungary and Spain. During World War II, *T.*

equiperdum was reintroduced into Western Europe by Russian and Algerian horses, which were used in the German army and in France, respectively (Saurat, 1946).

In Italy, it was first eradicated in the late-40s and again in the 70s of last century following an epidemic due to the import of infected animals from former USSR. A stallion undergoing routine serological testing for stud purposes was found positive for dourine in May 2011, in Sicilia and the following tracing back detected four further outbreaks (Lelli *et al.*, 2012). Dourine was first clinically diagnosed in Namibia in 1914 and is believed to have been introduced into the country in horses imported by German settlers. Documentation exists about the wide distribution of the disease in southern Africa, including Namibia (Barrowman and Van Vuuren, 1976; Williamson and Herr, 1986).

Zelege *et al.* (1980) first reported the presence of dourine in Ethiopia. As his reports *T. equiperdum* was widely spread in the districts of Etaya, Sagure, Bokoji and Koffele in the former Arsi-Bale region. In Ethiopia, although it was not possible to trace the origin of the spread of the dourine or to associate the first occurrence of the disease with any particular event in the past, the peasant in bale administrative region consider that the disease spread from Arsi to their own region in the past two decades and horse manifested clinical sign of dourine was reported in Ethiopia (Alemu *et al.*, 1997).

2.2. Causative Agent

Dourine is a protozoa parasitic disease of equids caused by *Trypanosoma equiperdum* of the subgenus Trypanozoon (Brun *et al.*, 1998). Cross reactions can occur with other Trypanosomatids using serology, therefore diagnosis is a combination of clinical signs and CFT (Centre for Food Security and Public Health, 2009). They are haemoflagellated parasites characterized by one nucleus and one flagellum, either free or attached to the parasites body by means of an undulating membrane. They also usually contain a small compact kinetoplast, a disc-shaped DNA containing organelle, situated within a large mitochondrion (Brun *et al.*, 1998).

2.2.1. Taxonomy

Trypanosomes are unicellular organisms (Phylum Protozoa) belonging to the phylum *Sarcomastigophora*, order *Kinetoplastida*, family *Trypanosomatidae* and genus *Trypanosoma* (Levine *et al.*, 1980) (Figure 1). Each species is furthermore given two names, the first, always spelled with a capital letter, places it in a relatively small group known as the genus (plural genera) and the last name spelled with a small letter, indicates the species (Hoare, 1972). *T. equiperdum* is classified under the subgenus Trypanozoon along with *T. brucei* spp. and *T. evansi* however, the species classification of Trypanozoon remains a controversial topic because it has been hypothesized that a very close evolutionary relationship exists among the trypanosome species of Trypanozoon (Suganuma *et al.*, 2016). *T. equiperdum* strain classified as subgenus of trypanozoon based on their genome sequence (Hébert *et al.*, 2017).

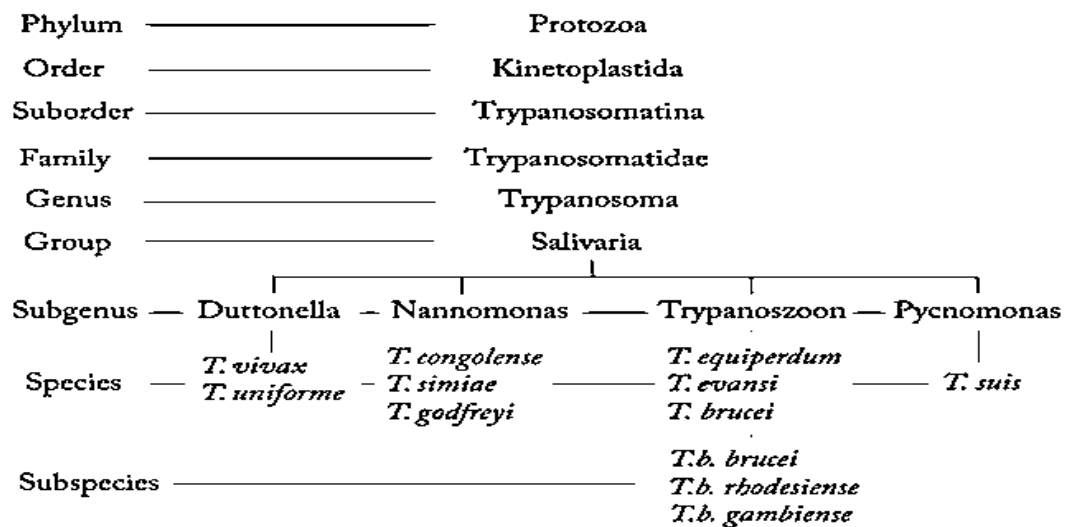


Figure 1: Classification of trypanosome (Munnender, 2013)

2.1.2. Morphological identification of *Trypanosoma. equiperdum*

Morphologically, *T. evansi* and *T. equiperdum* cannot be distinguished from the slender forms of *T. brucei brucei* or the human pathogenic subspecies *T. brucei rhodesiense* and *T. brucei gambiense*. Although pleomorphism may appear to some extent, generating stumpy and intermediate forms in some strains, *T. evansi* is generally monomorphic,

assuming a long slender form. Strains from different geographical areas and various host sources are morphologically indistinguishable (Hoare, 1972; Stephen, 1986).

Trypanosoma equiperdum is closely related to *T. evansi* and more distantly to *T. brucei*, because *T. evansi* was originally a parasite of camels and only later spread to horses. In *T. equiperdum* the nucleus lies in the center of the body, the kinetoplast is more distinct and terminal or sub terminal in position, with well-developed undulating membrane and free flagellum as in *T. evansi*. The size of *T. equiperdum* is likewise between the range of *T. evansi*, with the length of different strains varying from 15.6 μm - 31.3 μm and 1.5 μm - 2.2 μm width (Hoare, 1972). To date, phylogenetic analyses show that *T. equiperdum* and *T. evansi* are not monophyletic and should therefore be considered as subspecies of *T. brucei*, a parasite causing sleeping sickness in humans and nagana in animals (Hébert *et al.*, 2017).

T. equiperdum also infects equines under natural conditions, and causes a venerally transmitted disease called dourine. Biochemical and molecular biological data indicated that *T. evansi* and *T. equiperdum* are much more closely related to each other than to *T. brucei brucei*. The two species cannot be distinguished morphologically at the light or electron microscope level. However, based on the differences in the mode of transmission, the host range, the pathogenicity and the location of the parasite in its host, these two trypanosomes were classified as different species (Hoare, 1972).

T. equiperdum possess a free flagellum and a small sub-terminal kinetoplast. Dyskinetoplastic forms, in which the circular kinetoplast DNA (kDNA) is absent, are found in wild strains as a result of mutation, or after treatment with trypanocides such as diminazene, prothidium (Hajduk, 1978). Some dyes such as ethidium bromide can also cause the appearance of a high percentage of dyskinetoplastic forms. Dyskinetoplastic forms of *T. evansi* were also reported to appear after long term in vitro cultivation (Zweygarth *et al.*, 1990). *T. equiperdum* is also monomorphic, but occasionally exhibits pleomorphism during sub-passage in rodents (Blacklock and Yorke, 1913). At a molecular level, *T. evansi* is characterized by the complete loss of the maxicircles of the

kinetoplastic DNA, while *T. equiperdum* has retained maxicircle fragments similar to those present in *T. brucei*. *T. evansi* causes the disease known as Surra (Sánchez *et al.*, 2015) described in (Table 1).

Table 1: Comparison of the characteristics of *T. evansi* and *T. equiperdum*

Character	<i>T. evansi</i>	<i>T. equiperdum</i>
Size (length)	15-36 μm without free flagellum	
Morphology	Typically monomorphic, pleomorphic forms occur in some strains; identical ultrastructure	
Natural host	Equines, camels, cattle, buffalo, deer, Asian elephant Tigers, vampire bats, etc	Equine only
Vectors and transmission	Tabanus spp, stomoxys spp, vampire bats, mechanical transmission	Direct transmission during coitus
Drugs used for treatment	Diminazene, suramine, quinapyramine and cymelarsan	Identical to <i>T. evansi</i> usually not recommended
In vitro cultivation	Only at 37 $^{\circ}\text{C}$, no insect stages occur for either species	
Iso-enzymes	Differences were found only in two (MDH and ALAT) of 16 enzymes between strain of <i>T. evansi</i> and <i>T. equiperdum</i>	
KDNA and hybridization with PTK 420	Minicircle only: hybridized with PTK 420 probe	Minicircle hybridized with PTK 420 probe: maxicircle also present
RFLPs in rDNAs and VSG	No difference was found between <i>T. evansi</i> and <i>T. equiperdum</i> strains	

Source: Brun *et al.* (1998).

2.3.1. Trypanosome cell architecture

The trypanosome consists of a single cell varying in size from 8 to 50 μm . The trypanosome cell is elongated and has a highly polarized microtubule cytoskeleton which defines the cell shape (Matthews, 2005). The organelles in the trypanosome cell (the flagellar pocket, flagellum, kinetoplast, mitochondrion and nucleus) are precisely positioned within the cytoskeletal corset and are concentrated between the posterior end and the center of the cell (Gull, 2002) (Figure 2).

The most posterior structure is the mouth of the flagellar pocket. This is the exit point for the flagellum, which is tethered along the exterior length of the parasite. The flagellar pocket is the site of endo and exo-cytosis; this is important in bloodstream forms, in which the surface membrane is densely packed with variable surface glycoprotein (VSG) to protect against the alternative pathway of complement activation and to shield common antigenic determinants from immune recognition (Overath and Engstler, 2004).

The motility of the trypanosome is dependent upon its single flagellum, which has a conventional axonemal structure plus an associated paraflagellar rod. This is a semi-rigid structure found in the kinetoplastids and euglenoids that contributes to parasite motility, perhaps assisting trypanosome flagellar beat efficiency in the viscous mammalian blood stream (Vaughan and Gull, 2003). The mitochondrion is a single elongated structure that runs from the posterior to the anterior of the cell. In blood stream forms, the mitochondrion is a simple tubular structure devoid of cristae. This reflects the absence of mitochondrial respiration during this stage, energy generation being dependent on glycolytic reactions compartmentalized within specialized organelles termed glycosomes (Parsons, 2004). Trypanosomes are defined by the presence of a highly organized mitochondrial DNA structure, the kinetoplast (Gull, 2002) (Figure 2).

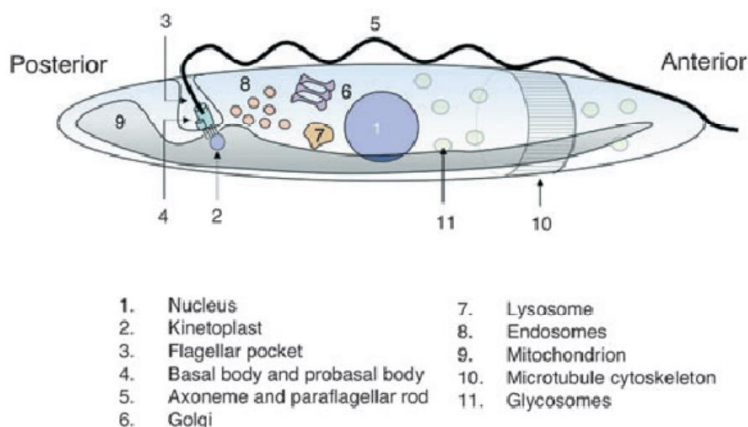


Figure 2: Schematic representation of the principal structures of trypanosomes (Mathew, 2005).

2.4. Epidemiology

2.4.1. Host range and geographical distribution

Dourine is a unique disease in the sense that it has no known vectors or fomites existing in the natural world, other than members of the equine family, including donkeys, mules and horses (Gillingwater *et al.*, 2007). The disease is more severe in improved breed of horse and milder in native ponies, mule and donkey. Zebra have tested positive serology but here is no conclusive evidence of infection. Male donkey can be asymptomatic carrier (Luckins, 1994). In laboratory setting, *Trypanosoma equiperdum* has been manipulated to adapt to and proliferate in other species, such as dogs, rabbits, mice and rats, but this has never been observed to occur naturally and without scientific manipulation (Hagos *et al.*, 2010a). Although this limits spread of the disease because it is restricted to the equine population alone, the organism has developed complex mechanisms over time to better equip itself for prolonged survival in the equine species.

Trypanosoma equiperdum infection, as a venereal disease, is even less restricted by climate and in the past has spread as far as Canada and Russia in the northern hemisphere, and as far to the south as Chile and South Africa. Its present distribution is not very well known; *T. equiperdum* is sometimes difficult to distinguish from *T. evansi*.

It has been eradicated from North America and most of Europe. Nowadays, the United States, Western Europe and Australia are considered to be free from dourine (Claes, 2003).

The recent reports of dourine (i.e. CFT positive cases) were in China, Kyrgyzstan, Kazakhstan, Botswana, Pakistan, Ethiopia, Namibia, Italy, South Africa, Brazil and Germany (Figure 3). However, due to possible cross-reactions in the complement fixation test (CFT) it is difficult to conclude that seropositive animals are real *T. equiperdum* cases (Zablotskij *et al.*, 2003).

Dourine has been found to be prevalent throughout the highlands of Ethiopia, particularly in the Arsi and Bale zones (Alemu *et al.*, 1997; Fikru *et al.*, 2010). Multiple cases have been found to be positive on the serological complement fixation test (CFT) and enzyme-linked immunosorbent assay (ELISA), or to be positive on a trypanozoon polymerase chain reaction, yet a parasitaemic horses have also been reported in the Arsi and Bale zones (Clausen *et al.*, 2003).

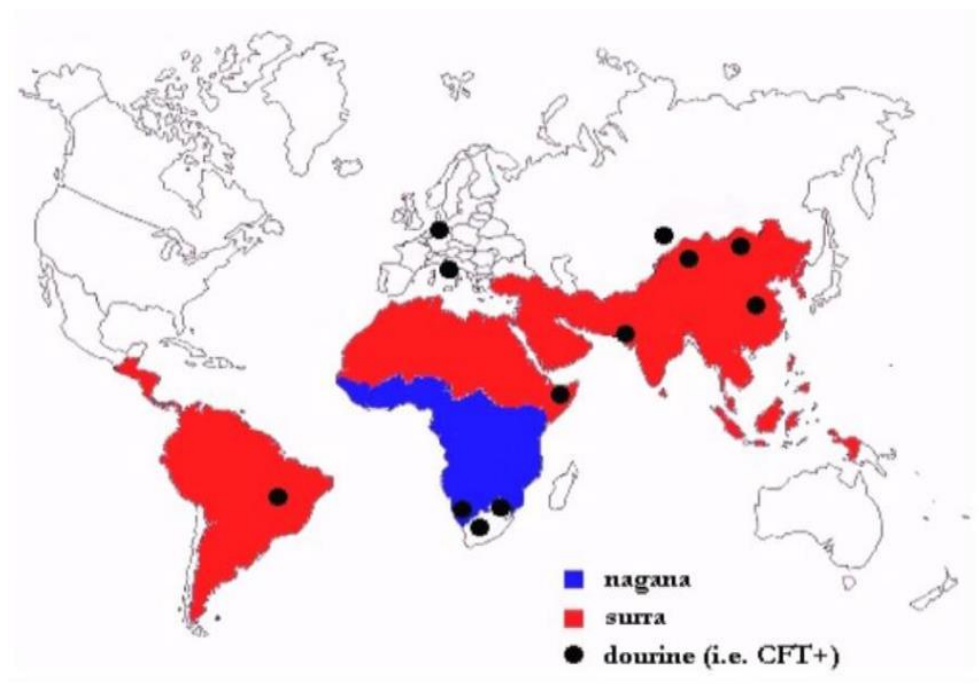


Figure 3: Map showing the global distribution of dourine and other trypanosomal disease
Source: (Adopted from Claes, 2003) as cited by Nesradin *et al.* (2017).

2.4.2. Distribution of *T. equiperdum* strain

Some authors agree on that *T. equiperdum* strains might be derived from an African *T. brucei* mutant (Hoare, 1972). However, other author also consider that some of these *T. equiperdum* strains might have evolved more rapidly, possibly due to the pressure of natural selection when they were transmitted out of Africa (Li *et al.*, 2005) (Table 2).

Table 2: The availability of laboratory strains of *Trypanosoma equiperdum*

Code	Country	Host	Year of Isolation	Reference
STIB 818	China	Horse	1979	(Lun <i>et al.</i> , 1992)
OVI	South Africa	Horse	1977	(Barrowman, 1976)
ATCC 30019	France	Horse	1903?	(Hajduk, 1976)
ATCC 30023	France	Horse	1903?	(Hajduk, 1976)
Am. Stabilate	America?	Horse	Unknown	(Hagebock <i>et al.</i> , 1993)
Can. Stabilate	Canada?	Horse	Unknown	(Hagebock <i>et al.</i> , 1993)
AnTat 4.1	Unknown	Unknown	Unknown	(Claes <i>et al.</i> , 2003)
Alfort	Unknown	Horse	1949?	(Claes <i>et al.</i> , 2003)
Hamburg	Unknown	Unknown	Unknown	(Claes <i>et al.</i> , 2003)
SVP	Unknown	Unknown	Unknown	(Claes <i>et al.</i> , 2003)
BoTat 1.1	Morocco	Horse	1924	(Claes <i>et al.</i> , 2003)
TREU 2259	Unknown	Unknown	Unknown	(Claes <i>et al.</i> , 2003)
Dodola strain	Ethiopia	Horse	2010	(Hagos <i>et al.</i> , 2010b)
IVM-t1	Mongolia	Horse	2016	(Suganuma <i>et al.</i> , 2016)

2.4.2. Status of dourine in Ethiopia

The first official report of the dourine in Ethiopia was made in 1980 when the Arsi Rural Development Unit asked the Tsetse and Trypanosomiasis Survey and Control Department to investigate a persistent disease problem in horses in the administrative regions of Arsi and Bale. According to this report, the disease was widely spread in Itaya, Sagure, Bekoji and Kofele districts of Arsi-Bale highland. In these areas the disease is known as

“Lappessa Hida kuta”, “Lappessa Dugda kuta” which means back bone breaker vernacularly or simply “Kuta” which means back bone breaker, whilst in Bale region known as “Derissa” (Zelege *et al.*, 1980; Hagos, 2005). Since then, dourine has been found to be prevalent throughout the highlands of Ethiopia, particularly in the Arsi and Bale zones (Alemu *et al.*, 1997).

Multiple cases have been found to be positive on the serological complement fixation test (CFT) and enzyme-linked immunosorbent assay (ELISA), or to be positive on a trypanozoon polymerase chain reaction, yet a parasitemic horses have also been reported in the Arsi and Bale zones (Clausen *et al.*, 2003). In Arsi-Bale highlands, dourine was major problem of equids. It is not difficult to identify the *T. equiperdum* from Surra which is caused by *T. evansi*. *T. evansi* is restricted to arid and semi-arid areas of Ethiopia where it is endemic in camel population (Kassa *et al.*, 2011). Now a days camels are forced to move to mid low lands areas in search for feed during the dry season. These might predispose equids in the Arsi-Bale high land to Surra complicating the diagnosis because *T. equiperdum* and *T. evansi* are genetically and antigenitically are similar, serological identification is not valuable (Claes *et al.*, 2003).

The high sero-prevalence of anti-trypanozoon antibodies observed in all horse-breeding districts such as Agarfa, Dinsho, Goba and Sinana among Bale highlands provides strong circumstantial evidence that trypanozoon infection is a highly prevalent endemic disease and a potential threat to the equine population. This is the first report of such sero-prevalence in these districts. The uniform and widespread distribution of the disease in this area could be attributed to the unrestricted animal movement from neighboring districts for trade and transport purposes, uncontrolled animal breeding and the absence of effective trypanocidal drugs (Hagos *et al.*, 2010c). The status of dourine in donkeys and mules in Arsi Bale highlands is demonstrated that both mule and donkeys are found to be sero-positive to CATT, LATEX and ELISA/*T. evansi* test. It was observed that active clinical cases of dourine in male and female donkeys at Dodola veterinary clinic of Bale high lands (Hagos, 2005).

2.4.2. Mode of transmission

This venereal disease is spread almost exclusively by coitus. Organisms are present in the urethra of infected stallions and in vaginal discharges of infected mares. The organism may pass through intact mucous membranes to infect the new host. Infected animals do not transmit the infection with every sexual encounter. However, as the disease progresses, trypanosomes periodically disappear from the urethra or vagina; during these periods, the animals are non-infective. Non-infective periods may last for weeks or months and are more likely to occur in the later stages of the disease. Thus, transmission is most likely early in the disease process (Henning, 1956; Barrowman, 1976). An interesting finding in the literature was a positive PCR test result from a prepuce swab taken from a dourine free stallion immediately after mounting an infected mare. The horse remained negative at all subsequent tests, supporting the theory that the parasite is present in the genital tissues but that sexual transmission is not constant (Vulpiani *et al.*, 2013).

It is possible for mares to become infected and pregnant after mating with an infected stallion. Foals born to infected mares may be infected. However it is unclear if this occurs in uterus or during birth. Because trypanosomes may occur in the milk of infected mares, these foals may be infected per os during birth or by ingestion of infected milk. Foals infected in this way may transmit the disease when mature and develop a lifelong positive CF titer. This method of disease transmission is rare, however some foals may acquire passive immunity from colostrum of infected mares without becoming actively infected; in such foals, the CF titer declines, and the animal becomes sero-negative by 4 to 7 months of age. Although the possibility of non-coital transmission remains uncertain, it is supported by dourine sporadic infections in sexually immature equids (Barrowman, 1976; Henning, 1956).

Human carelessness may also be responsible for conveying the infection, for instance when contaminated utensils are used for grooming the horses or contaminated instruments are used for artificial insemination. Other means of transmission may also be

possible, but there is no evidence that arthropod vectors play any role in transmission. Animal other than equines can be infected experimentally (OIE, 2001).

2.4.3. Course of infection

The incubation period, severity and duration of the disease vary considerably. In South Africa, the disease is typically chronic, usually mild and may persist from 6 months to 2 years (Henning, 1955). In other areas, such as Northern Africa and South America, the disease tends to be more acute, often lasting only 1-2 months or, exceptionally, 1 week. Although dourine is a fatal disease with an average mortality of 50% (especially in stallions), spontaneous recovery can occur and sub-clinical infections are recognized. The course of the disease may last several years after infection with a mild strain. Experimentally, horses have survived for up to 10 years after infection. The course is apparently more acute in the European and Asian forms of the disease in which the mortality rate is higher (OIE, 2013; Brown and Torres, 2008).

2.4.4. Antigenic variation and evasion mechanism

In the mammalian host, these parasites are completely surrounded by a dense immunogenic surface coat (12-15 nm thick) of a single polypeptide protein referred to as the variant surface glycoprotein (VSG) that shields invariant surface antigens from immune recognition. Moreover, trypanosomes constantly modify their VSG by the process of antigenic variation, resulting in the fluctuating waves of parasitemia that characterizes trypanosomosis (Pays, 2006). Trypanosomes contain up to 1000 different genes in their genome which afford them extensive opportunities to escape host adaptive immune responses by displaying new coat antigens. By switching VSG genes and expressing a new variant antigenic type, trypanosome evade B and T-cell mediated immune responses. Furthermore, expression of VSG is central to the process of antigenic variation that eventually leads to exhaustion of the host immune system for the benefit of the trypanosome (Morrison *et al.*, 2009) (Figure 4).

In this sense, *T. equiperdum* is a very efficient organism; it may infect less species than other diseases, but it infects and survives very efficiently within its specified hosts (Raibaud *et al.*, 1982).

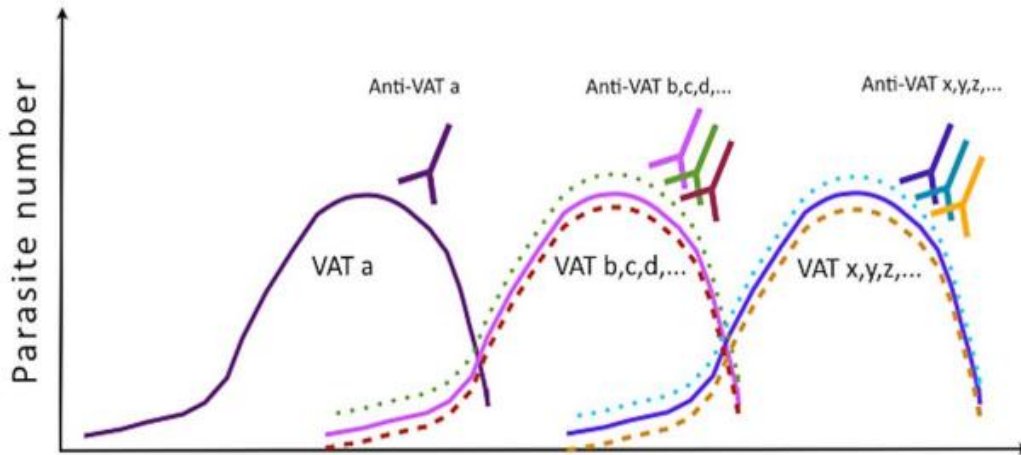


Figure 4: Representation of the concept of antigenic variation during mammalian *T. brucei* infection (Cnops *et al.*, 2015).

2.4.5. Host Immunity

The inoculation of trypanosomes into their mammalian hosts triggers a series of events involving, at first, innate immunity and, secondly, specific immunity. Cells of the macrophage lineage provide the first line of host defense against infectious diseases and, with dendritic cells, modulate downstream events that impact on the development of acquired immunity. The latter requires an efficient presentation of parasite antigens, activation of T and B cells implying specific antigen receptor recognition, and the development of effector cells and molecules. The first antibody to the VSG is immunoglobulin M (IgM) class and is produced independently of T cells (Mansfield and Paulnock, 2005).

Antibodies to the VSG are able to mediate control of the parasitemia. During co-evolution with their hosts, trypanosomes have learned to cope with these host immune systems, by penetrating, diverting and altering the numerous steps leading to the

generation of an effective immune response. Major modifications of immune systems have been observed in trypanosomiasis: lymphadenopathy, splenomegaly with destruction of lymphatic tissue architecture and hyper-gammaglobulinemia. However, their effectiveness is limited since most of the time; parasites cannot be eliminated and prevailing immuno-pathological phenomena which induce tissue alterations (Philippe and Bernard, 2006).

In case of *T. Equiperdum*, improved breeds of horses seem to be more susceptible. The disease in these animals often progresses rapidly and involves the nervous system. In contrast, native ponies and donkeys often exhibit only mild signs of the disease. Infected male donkeys, which may be asymptomatic, are particularly dangerous in the epidemiology of the disease, for they may escape detection as carriers. Some foals may acquire passive immunity from colostrum of infected mares without becoming actively infected; in such foals, the CF titer declines, and the animal becomes seronegative by 4-7 months of age (Brown and Torres, 2008).

2.5. Clinical Manifestations

2.5.1. Primary phase/genital swelling/oedema

Dourine is marked by stages of exacerbation, tolerance or relapse, which varies in duration and which may occur once or several times before death or recovery. The clinical signs of this disease most frequently noted are: pyrexia, tumefaction and local oedema of the genitalia and mammary glands, the vaginal mucosa may show raised and thickened semi-transparent patches. Folds of swollen membrane may protrude through the vulva. It is common to find oedema of the mammary glands and adjacent tissues. Depigmentation of the genital area, perineum and udder may occur. There may be vulvitis and vaginitis with polyuria and other signs of discomfort such as an elevated tail. Abortion is not a feature of infection with mild strains, but significant abortion losses may accompany infection with a more virulent strain. Conjunctivitis and keratitis are often observed in outbreaks of dourine and may be the first signs noted in some infected herds. In the stallion, the first clinical sign is a variable swelling involving the glans penis

and prepuce. The oedema extends posteriorly to the scrotum, inguinal lymph nodes and perineum, with an anterior extension along the inferior abdomen. In stallions of heavy breeds, the oedema may extend over the whole floor of the abdomen (OIE, 2013; Brown and Torres, 2008).

2.5.2. Secondary phase/cutaneous

The clearly pathognomonic for dourine is typical cutaneous plaques or skin thicknesses can be observed, which can range from very small to hand sized up to 5-8 cm in diameter and 1 cm thick the name of the disease originates from the shape of this skin lesion similar to the coin named “duro” (ancient Spanish money). The plaques usually appear over the ribs, although they may occur anywhere on the body, and usually persist for between 3 and 7 days (Claes *et al.*, 2005).

2.5.3. Tertiary phase/nervous sign

Pyrexia is intermittent; nervous signs include incoordination, mainly of the hind limbs, lips, nostrils, ears and throat. Facial paralysis is usually unilateral. In fatal cases, the disease is usually slow and progressive, with increasing anemia and emaciation, although the appetite remains good almost throughout (OIE, 2013). Conjunctivitis and keratitis are common in some reports, and ocular disease may sometimes be the first sign of dourine (OIE, 2015).

2.6. Pathology and Pathogenesis

Anemia and cachexia are consistent findings in animals that have succumbed to dourine. Edema of the genitalia and ventral abdomen become indurated later in the course of the disease. Chronic lymphadenitis of most lymph nodes may be evident. Perineural connective tissue becomes infiltrated with edematous fluid in animals with nervous signs, and a serous infiltrate may surround the spinal cord, especially in the lumbar or sacral regions (Barrowman, 1976).

At post-mortem examination, gelatinous exudates are present under the skin. In the stallion, the scrotum, sheath, and testicular tunica are thickened and infiltrated. In some cases the testes are embedded in a tough mass of sclerotic tissue and may be unrecognizable. In the mare, the vulva, vaginal mucosa, uterus, bladder and mammary glands may be thickened with gelatinous infiltration. The lymph nodes, particularly in the abdominal cavity, are hypertrophied, softened and, in some cases, hemorrhagic. The spinal cord of animals with paraplegia is often soft, pulpy and discolored, particularly in the lumbar and sacral regions (OIE, 2013).

Blood invading parasites produce numerous changes in the cellular and biochemical constituents of blood (Taiwo *et al.*, 2003). Anemia is a cardinal sign of trypanosomosis in many domestic animals that attributed to weight loss and poor body condition, due to dyshaemopoiesis (Lukins, 1999). Despite the apparent absence of parasites in the circulation, red blood cell destruction continues and insufficient erythropoietic compensation results in persistent anaemia. The anemia caused by animal trypanosomosis could be associated with decrease in PCV, haemoglobin and RBCs counts as reported by many authors in different animal species (Lukins, 1999) which may result from massive erythrophagocytosis by an expanded and active mononuclear phagocytic system (MPS) of the host.

Reports also showed that anemia with a significant reduction in PCV, total RBC count and hemoglobin concentration (Hgb) is a consistent finding in trypanosome infected cattle, goats, sheep, dogs and rabbits (Bisalla, 2007). On the other hand, significant increases were reported in mean corpuscular volume (MCV) and mean corpuscular haemoglobin (MCH) values, whereas no significant change was observed in mean corpuscular haemoglobin concentration (MCHC) (Nadia *et al.*, 2012).

Total white blood cell (WBC) counts were also varied from normal range to significant reduction (Osman, 2012) in animal trypanosomosis. The mechanism or pathophysiology of anemia in trypanosomosis is complex and multifactorial in origin (Naessens *et al.*, 2005). Widespread phagocytosis of blood cells, such as erythrocytes and platelets, by

macrophages invading bone marrow sinusoids is seen (Murray and Dexter, 1988). This anemia could also be due to the haemolysins such as proteases, phospholipases and neuraminidases induced by the trypanosomes (Soulsby, 1982).

The *T. equiperdum*, which is present in the seminal fluid and mucous membranes of the genitalia of the infected donor animal, are transferred to the recipient during sexual intercourse. It is considered that transferred trypanosomes penetrate the intact mucous membranes and initiate an infection in the recipient animal. After the infection of the genital mucosa via coitus, *T. equiperdum* invade the tissue and parasitize in the blood, lymph, CSF and sub-cutaneous lesions (Pascucci *et al.*, 2013).

2.7. Diagnosis

T. equiperdum is the only trypanosome to affect horses in temperate climates, the confirmation of the parasite or the detection of trypanosomal antibodies is sufficient for a positive diagnosis. However, in countries of Africa where overlap of *T. equiperdum* and *T. brucei* spp. exists, it is difficult to distinguish *T. equiperdum* microscopically or serologically from other members of the subgenus Trypanozoon (OIE, 1996).

T. equiperdum is morphologically indistinguishable from the two other pathogenic species, *T. brucei* and *T. evansi* (Brun, 1998). *T. equiperdum* is the causative agent of dourine; a distinctive disease that only affects equidae and is transmitted mainly via coitus (Claes, 2005). Because *T. evansi*, *T. equiperdum* and *T. brucei* species cannot be distinguished by sequences of their ribosomal RNA (rRNA) genes (Hamilton, 2008), other probes, including isoenzymes and peptidase profiles (Queiroz *et al.*, 2000) restriction fragment length polymorphisms, kinetoplast sequences and microsatellites markers (Biteau, 2000) have been used to characterize and distinguish these trypanosome species.

2.7.1. Clinical diagnosis

The diagnosis of *T. equiperdum* in equines has generally been dependent on clinical observation and the CF test. However, the clinical signs are not pathognomonic and although clinical signs for dourine are useful in making a diagnosis, some horses may not show clinical signs. In areas where dourine is endemic and susceptible animals are exposed to the disease, the clinical signs of dourine such as the edematous swellings, developing of plaques and the nervous signs may enable one to suspect the disease. Meanwhile, dourine should be differentiated in the early stages and latent cases (Hoare, 1972) from other diseases such as coital exanthema, which is manifested by the appearance of vesicles, ulcers and depigmented spots on the vaginal mucous membrane. These signs are not important in dourine. The differential diagnosis includes Surra, Anthrax, Equine infectious anemia, Equine viral arthritis and purulent endometritis such as contagious metritis should also be considered, as their signs resemble those of dourine. In some countries (e.g. in South America) *T. evansi* infection can give rise to similar signs (OIE, 2001). Direct laboratory diagnosis is also problematic, given the low number of parasites normally present in infected tissues and the mild, short-lasting parasitaemia (Pascucci *et al.*, 2013).

2.7.2. Parasitology

Direct parasitological demonstration of *T. equiperdum* is unusual as it is always very difficult to isolate the organism directly from either the blood or pathological secretions (oedemas, plaques) of infected horses, even at the earliest stages of the infection (Rouget, 1896). Some of the parasitological methods currently in use are: thin stained blood films (Shute and Maryon, 1966), the haematocrit centrifugation technique (HCT) (Woo, 1970) and the miniature-anion exchange centrifugation technique (mAECT) (Lumsden *et al.*, 1977). These methods are confirmatory since they depend on demonstration of trypanosomes. However, these parasitological detection methods have a limited analytical sensitivity (i.e. lower detection limit) and may lead to under-reporting of the prevalence of disease (Paris *et al.*, 1982).

2.7.2.1. Blood films

The wet smear method is useful, simple and cheap; it is limited by low sensitivity and cannot identify the species of trypanosomes. It is also useful in drug trials. The blood films are useful in identification of trypanosomes species but less efficient. Thick blood method is more sensitive than both wet and thin smear in detection of trypanosomes; it is limited by difficulty in recognizing the parasite species (Kendrick, 1968).

2.7.2.2. Hematocrit centrifugation technique (HCT)

This method is very sensitive in detecting parasites during the first period of infection (Wernery, 2001). It is able to detect trypanosomes infection earlier than other parasitological methods. It has more efficiency in surveys of trypanosomes that are non-infective to laboratory animals like *T. vivax* and some strains of *T. congolense*. The method detects *microfilaria* in the blood. The technique is proved to be sensitive in *T. evansi* infections; however it is less sensitive than mice inoculation technique (Monzon, 1990). The technique is used for determining the assessment of anemia and parasitemia estimation (Kendrick, 1968).

2.7.2.3. Mini Anion-Exchange Centrifugation Technique (mAECT)

The technique is based on the ability of the negatively charged RBCs to be held back in the anion column, and the less negatively charged trypanosomes to pass through with the solution. The trypanosomes are concentrated in the solution by low-speed centrifugation (François *et al.*, 2005). The concentrate is then examined in a special holder under the microscope for the presence of trypanosomes. This technique is highly sensitive than most of the other described techniques because of large blood volume (300 µl) used, which enables the detection of less than 100 trypanosomes/ml (OIE, 2008).

2.7.3. Serology

Sero-diagnosis of diseases depends mainly on antigen-antibody reaction in the blood serum. The essential element in immune-diagnosis is the availability of species-specific test antigens and/or highly specific antibodies. Because parasites contain thousands of potentially antigenic polypeptides, glycoproteins and glycolipids, many of which are shared with unrelated species and even with bacteria, there has been great difficulty in developing sufficiently specific immune-diagnostic tests (Nantulya, 1991).

Indirect methods rely on serological tests by detecting specific antibodies developed by the host against the infection or, inversely, to demonstrate the occurrence of circulating parasitic antigens in the blood by the use of characterized specific antibodies. The detection of antibodies indicates that there has been infection, but as antibodies persist for some time (weeks, sometimes months) after all trypanosomes have disappeared from the organism (either by drug treatment or self-cure) a positive result is no proof of active infection. On the other hand, circulating trypanosomal antigens are eliminated quickly after the disappearance of the trypanosomes, and their presence therefore shows almost always that live trypanosomes are present in the animal (FAO, 2004).

2.7.3.1. Complement Fixation Test (CFT)

The complement fixation (CF) test is the prescribed test for international trade, and has been used successfully in eradication programs. However, no serological test is specific for dourine, as cross-reactions occur with old world trypanosomes, especially *T. brucei* and *T. evansi*. In addition, uninfected animals, particularly donkeys and mules, often have inconsistent or nonspecific reactions (false positives) in the CFT, due to anti complementary effects in equid serum (OIE, 2015). The complement fixation test (CFT) was one of the first techniques used to diagnosis of *T. evansi* in camels. The test was used successfully for detection of *T. equiperdum* presence in horses. Cross reactions with sera of horses infected with other trypanosomes may be occurred. However, CFT is less

sensitive than ELISA in the diagnosis of the dourine disease in equines (Gillbert, 1998; Wassal *et al.*, 1991).

2.7.3.2. Enzyme Linked Immuno Sorbent Assay (ELISA)

ELISA is more reliable than CFT with normal sera and can detect antibody in anti-complementary sera. It lends itself to a considerable degree of automation which makes it suitable for the routine testing of large numbers of samples. The technique also has the advantage of being relatively economical with reagents, particularly with regard to the antigens; an important consideration since the antigen is expensive and laborious to produce (Wassal, 1991). The indirect ELISA has been shown to be as sensitive as the IFAT in detecting antibodies to *T. evansi* infection in rabbits, cattle and camels (Alemu *et al.*, 1997). Test for the detection of trypanosomal antigens followed the development of species-specific mono-clonal antibodies for use in ELISA (Luckins, 1992).

2.7.3.3. Card Agglutination Test for *Trypanosoma evansi* (CATT/*T. evansi*)

The CATT/*T. evansi* is a rapid direct agglutination test, which uses formaldehyde fixed, Coomassie stained, freeze-dried trypanosomes of *T. evansi* VAT RoTat 1.2 (Bajyana Songa and Hamers, 1988). The demonstration of specific antibodies for the diagnosis of *T. evansi* infections in camels has been used by employing a modified card agglutination test (CATT) initially developed for *T. brucei gambiense* (Diall *et al.*, 1994). The CATT test being based on the variable antigen type of trypanosomes is very specific and does not suffer from interference by other infection. However, its usefulness is limited by a relative lack of sensitivity attributable to the presence of other repertoires lacking the early VAT of the CATT test. Some infection will remain undetectable also if the VAT type used in the test is only expressed late in infection or is not very immunogenic in the species considered (Bajyana Songa and Hamers, 1988).

2.7.3.4. Latex agglutinate/ *T. evansi*

LATEX/*T. evansi* (Verloo *et al.*, 1997) is a rapid indirect agglutination test, in which the antigen consists of purified variable surface glycoprotein (VSG) of *T. evansi* VAT RoTat 1.2 covalently coupled to latex particles. The CATT/*T. evansi* and LATEX/*T. evansi* tests appear to be valuable for the detection of anti-trypanozoon antibodies, regardless of whether the causative agent is *T. evansi* (surra) or *T. equiperdum* (dourine) (Claes *et al.*, 2005).

2.7.4. Animal inoculation

Laboratory animals may be used to reveal sub clinical infections in domesticated animals. *Trypanosoma* spp. has a broad spectrum of infectivity for small rodents. 2 ml of infected blood inoculated into rabbit or mouse intraperitoneal (I/P), will demonstrate the trypanosomes in the blood after a few days (Mohiuddin, 2007). Several authors succeeded in isolating strains of *T. equiperdum* from laboratory animals injected intraperitoneally rabbits (Pautrizel *et al.*, 1962), rats (Kujumgiev, 1948) and mice (Lun, 1995) or sub-cutaneously dogs (Laveran and Mesnil, 1912). The ease with which a strain was established in rats and the absence of localization in the genitalia of rabbits were considered evidence of *T. evansi* (Killick-Kendrick, 1964).

In dogs inoculation of *T. equiperdum* produces the typical picture of dourine with trypanosomes present in the lesions, but not in the blood, the infection may last from one to several months (Stephen, 1986; Claes, 2003). The susceptibility of dogs to *T. equiperdum* is generally high (Pautrizel *et al.*, 1962) and this means that strains can be sent from remote countries after the animals have been experimentally infected. However, some breeds, like the pariah dogs in India, are less susceptible or sometimes even completely resistant. Before World War I, there was no record of *T. equiperdum* having been isolated in Russia. However, it was thought that rabbits might be useful in differentiating between *T. evansi* and *T. equiperdum* because the lack of localization in the genitalia of this animal species clearly shows horse trypanosome to be *T. evansi*.

After World War II, strains of *T. equiperdum* were successfully isolated by the intratesticular injection of rabbits with blood or material from infected horses (Zabotskij, 2003).

2.7.5. *In vitro* culture

The isolated *T. equiperdum* were well adapted and proliferated using soft agarose media but did not adapt in liquid media. This culture system will be useful for the future isolation of *T. equiperdum* from dourine-infected horses in the field (Suganuma *et al.*, 2016). One Chinese *T. equiperdum* isolate could be grown axenically in the same medium used for *T. evansi*, but an adaptation period of about 3 weeks was required until consistent growth was attained (Brun and Lun, 1994). In contrast to *T. b. brucei*, *T. evansi* and *T. equiperdum* do not go through a procyclic (insect) stage. Bloodstream forms of the latter two species do not transform into a procyclic stage when transferred into a procyclic stage-specific culture medium at 27°C (Zweygarth and Kaminsky, 1989). However, the limitation of culture may lead to many of the akinetoplastic Trypanozoon trypanosome strains have been established from a parental kinetoplastic strain by the supplementation of DNA binding drugs or RNA interference during *in vitro* culture (Schnauffer *et al.*, 2002). The predominance of the akinetoplastic *T. evansi* and *T. equiperdum* population was also induced from akinetoplastic parental trypanosome by long-term aseptic cultivation (Kaminsky *et al.*, 1997).

2.7.6. *Molecular tests*

Detection of minute amounts of trypanosomal DNA is a possible mean of identifying animals with active infections as the parasitic DNA does not remain for more than 24-48 hours in the blood of the host after the trypanosomes are killed (Desquesnes, 1997). DNA preparation is an important step that determines the success and the sensitivity of the PCR. It can be done on plain blood (generally collected with anticoagulant), or, preferably, on the buffy coat to increase the sensitivity of the test (Desquesnes and Davila, 2002). Polymerase chain reaction (PCR) based on DNA sequences of various taxonomic levels is used. The gold standard, to date, for detection of the *Trypanozoon*

subgenus are the NRP or TBR primers PCR offers the sensitivity and specificity required for detection of trypanosome infection, but it may give false-negative results (Masiga *et al.*, 1992).

2.8. Prevention and Control

There is not any available drug for dourine. The disease is considered to be incurable and, for that, seropositive horses should be removed or euthanized (OIE, 2012). However, in vitro sensitivity of different *T. equiperdum* strains to suramin, diminazene, quinapyramine and melarsomine has been reported (Zhang *et al.*, 1991). Treatment is more successful when the disease is caused by the more virulent (European) strains of the parasite. In general, treatment is not recommended for fear of continued dissemination of the disease by treated animals. Treatment may result in apparent disease carriers and is not recommended in a dourine-free territory (Brown and Torres, 2008).

According to Cauchard *et al.* (2016) two ponies were treated one day after observation of the parasites in the cerebrospinal fluid (early treatment) and two were treated after apparition of nervous clinical signs (late treatment). Following one administration of Cymelarsan® (0.5 mg/kg), *T. equiperdum* was cleared from the blood of the two lately treated ponies but a massive infection was observed in cerebrospinal fluid. Thereafter, a daily repeated Cymelarsan® (0.5 mg/kg) treatment was administrated to one of the lately treated ponies (n=5 injections) and to the two early treated ponies (n=6 injections). Following this treatment, parasites were cleared from the blood circulation of all the ponies but a massive *T. equiperdum* infection was observed in the cerebrospinal fluid one of the lately treated pony and not of the two early treated animals. In conclusion, the Cymelarsan® treatment failed to cure the two ponies at a single dosage but efficacy of a repeated treatment can be supposed depending on the stage of the disease.

Various attempts made to control the spread of trypanosome were hampered by the problem of geographical scale and in most cases the biology of the organism itself. Since it has been established that organism undergoes antigenic variation in order to evade the

action of the host immune response. In addition, resistance development to most trypanocidal drugs is on the increase and there seems to be dim hope of producing conventional vaccines against trypanosomes (Brun *et al.*, 1998; Keating *et al.*, 2015).

Hence, Immunity to trypanosomiasis is complicated. *T. equiperdum* has the ability periodically to replace major surface glycoprotein antigens, which is a strategy supporting chronic infections. No method of immunization against dourine exists at present. The most successful prevention and eradication programs have focused on serologic identification of infected animals. Infected animals should be humanely destroyed or castrated to prevent further transmission of the disease. Some geldings may still show service behavior and constitute a risk. All equids in an area where dourine is found should be quarantined and breeding should be stopped for 1-2 months while testing continues (Brown and Torres, 2008). Some authors suggest, avoiding euthanizing the infected horses in endemic areas, where the disease is extended and horses play an important role, propose a revised strategy including treatment with Cymelarsan® (Hagos *et al.*, 2010b) but results are inconclusive and additional studies are needed.

3. MATERIAL AND METHODS

3.1. Study Areas

The study was conducted in the fly proof stable located near Lake Babugaya and laboratory work conducted in molecular parasitology laboratory of Ethio-Belgium VLIR UOS funded dourine project which is found inside the compound of Addis Ababa University College of Veterinary Medicine and Agriculture, Bishoftu, Ethiopia. Bishoftu is found 47 km south east of Addis Ababa at (8° 44 N and 38° 58° E) at an altitude of 1850 meters above sea level. The mean annual rain fall is 885.4 mm with a bimodal distribution. There are alternating dry and rainy season in the area. The long rainy season extends from June to September and contributes about 84% of the total annual rainfall. While the dry season last from October to February. The short rainy season lasts from March to May. The mean annual minimum and maximum temperatures are 14°C and 26.3°C respectively with an overall average of 18.7°C. The mean relative humidity is 61.3 % (NMSA, 2015).

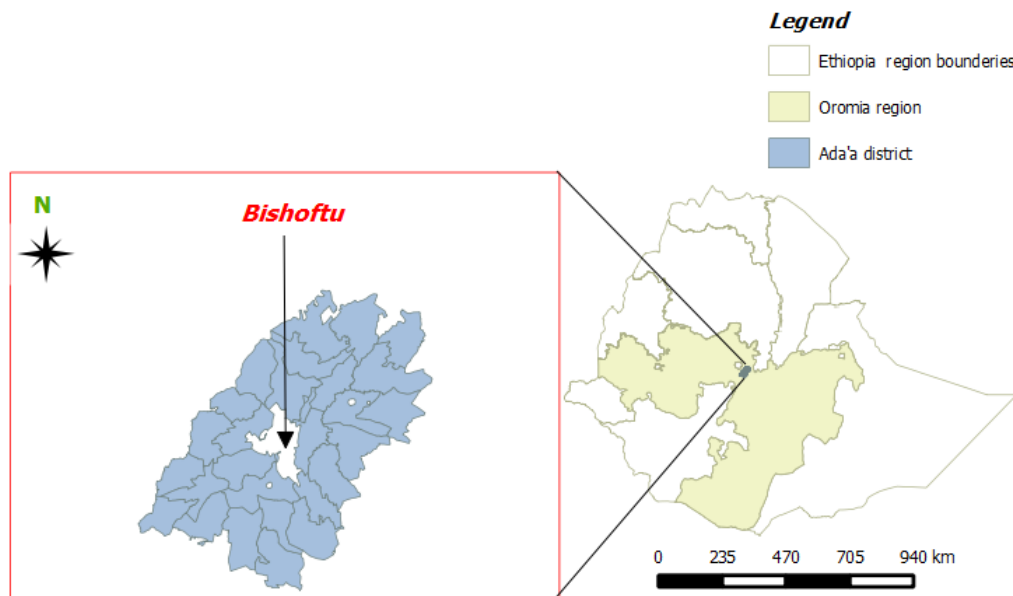


Figure 5: Map showing experimental study area created by using QGIS version 2.0.1

3.2. Study Methodology

3.2.1. Experimental animals

3.2.1.1. Horses

Eight healthy horses aged 6-12 years were purchased from Salale, north Shewa, Oromia regional state, Ethiopia, which is found about 116 km north of Addis Ababa. Serologically, all animals were found negative for CATT/*T. evansi* test and also all animals were found apparently healthy. Upon their arrival at the experimental study site, they were all treated with Ivermectin against endoparasites and ectoparasites. Triclabendazole was also given as additional treatments with feed against Fasciolosis, twice in two weeks interval. Animals were housed in fly proof stables prior to commencement of experiment. To acclimatize with new environment they were kept for a month before commencement of experiment. Horses were fed during the whole course of the study period with grass hay, concentrates and water was available *ad libitum*.

3.2.1.2. Mice

Swiss white mice, 10 weeks old, were obtained from the breeding colony of the National Veterinary Institute (NVI), in Bishoftu. Then they were kept in a conducive environment in molecular parasitology laboratory; they were allowed to acclimatize to their new environment for 21 days before the commencement of experiment. Wood shavings were provided as beddings on the floor of the metal cages. Pelleted feed and water was given *ad libitum*.

3.2.2. Source of stocks and cryostabilate preparation

Dodola isolate 943 of *T. equiperdum* stabilates originally isolated by (Hagos *et al.*, 2010b), cryopreserved and stored in liquid nitrogen at -196°C in Ethio-Belgium laboratory of AAU, CVMA were used. This *T. equiperdum* stabilate was inoculated into naive mice at a dose of 0.2 ml and once the parasite is established the level of Parasitemia was monitored daily according to (Paris *et al.*, 1982). When first parasitemia reach at peak (10^6 /ml), mice with swarming level of parasitemia were anaesthetized by Ether and then

blood was collected with EDTA coated vacutainer tube from cardiac puncture. Then 180 µl of blood sample was thoroughly mixed by vortex mixture with an equal amount of cryomedium and cryostabilates were prepared and kept at -196 °C in vapor phase for more than one hour and viability of the parasite was checked before it was immersed in liquid nitrogen for long term storage according to (Maina *et al.*, 2007).

3.3.3. Experimental design and infection procedures

Horse

Longitudinal study design was conducted on eight horses which were divided into two groups designated as mares and stallions group. Both groups of animals were kept in separated stable. To investigate transmission of *T. equiperdum* through artificial insemination, 360µl of the stabilate containing 100,000 trypanosomes/ml was added with raw semen free of *T. equiperdum*, which was harvested from non-infected stallion. Thereafter, it was inoculated to uterine body of four (4) mares via artificial insemination by using insemination n catheter. The mares were monitored daily until parasite become available in the blood and then regular monitoring of level of parasitemia within two days interval up to the end of study day by using wet blood smears, PCV and Woo test. Other clinical and laboratory parameters such as temperature, hematological analysis and serology were investigated.

About 2ml of blood was drawn from infected mare during at optimum infective dose of 1×10^6 trypanosomes/ml using ethylene diamine tetra acetic acid (EDTA) coated vacutainer tubes and transfused to each stallion directly into the jugular vein. All stallions were monitored daily with the same parameter described above for mares by using wet blood smears, PCV and Woo test. Other parameters such as temperature, hematological analysis and serology were also investigated.

Mice

For analysis of transmission by coitus in mice, 12 female and 6 male mice, which had never been exposed to mating, were randomly divided into six experimental groups. Male mice were inoculated with 0.2ml of trypanosomes stabilates through IP route with 100 unit syringe. Female mice were remained non-infected. When parasitemia reached on low level (10^1 - 10^3 trypanosomes/ml) in the infected male mice during daily checkup of parasitemia and then after one infected male mouse was co-housed with two healthy females in one cage.

Moreover, 12 female and 6 male mice were randomly divided into six experimental groups. Infected male mice were co-housed with two healthy female after high level of parasites was appeared in the blood greater than (10^4 trypanosomes/ml). In both cases, blood was taken from the female mice frequently to check presence of parasitemia approximately on day 2 of post crossing.

In other group, in order to rule out the possibility of transmission occurring via any routes (other than sexually) as control, two infected female were co-housed with four non-infected females. Then, female mice in group were checked daily for the presence of trypanosomes on wet blood smears. All non-infected female mice in all group were challenged with dexamethasone sodium phosphate 4 mg/kg on basis of body weight of mice (Batch Number; 14/08/6, MFG; 12/2014, Exp; 12/2017) intramuscularly for five consecutive days at three different interval of time to induce immune-suppression thereby reveal the obscured parasitemia. Mice in all groups were kept and followed up for 90 days.

Cymelarsan® treatment

The effect of drug studies was conducted on horses (n=7) using cymelarsan® (Lot: cym00101B, Mfd 19/01/015, Exd 19/01/018). Infected horses with *T. equiperdum* Dodola strain isolate 943 were grouped into two designated as treated and non-treated group based on their level of parasitemia. Cymelarsan® was administered intramuscularly (i.m) at a dose of 0.25 mg/kg body weight (standard dose in camels) to treated group at weeks 6 of post infection when parasitemia reach (+6) score. Thereafter, changes in level of parasitemia, clinical sign and hematological values were recorded.

3.3. Blood Collection, Parasitemia Estimation and Hematological Analysis

About 5ml of Blood samples were drawn from all experimental horses at rest, undisturbed and less excitement conditions through jugular vein in vacutainer tube with EDTA for hematology. Immediately after blood collection, the capped tubes containing the anticoagulant and the blood samples were inverted gently about ten times to avoid clotting. Samples were transported to the laboratory and the following hematological analysis was performed on weekly basis up to end of the experiment based on the methods previously described (Ghaffar *et al.*, 2014). Hemoglobin concentration (Hb), total erythrocyte counts (TEC), Total leukocyte count (TLC), Differential leukocyte count (DLC), such as monocyte, lymphocyte, neutrophils, basophil and eosinophils were estimated. In PCV determination, the capillary tubes were centrifuged at 12000 rpm for 5 minutes by micro-haematocrit centrifuge and then read on the scaling instrument (Murray *et al.*, 1983). PCV level and level of parasitemia were checked within one day of intervals. Hemoglobin concentration was determined using acid hematin method where the values were expressed in g/dl (Annex 1). Total erythrocyte counts were done using an improved Neubauer hemocytometer and the values were expressed in million cell/ μ l blood (Annex 2). Differential leukocyte counts were performed by preparing thin blood smear with differential quick stain (Diff Quick stain) , based on 100 cells per slide according to their staining reactions, shape of the nucleus, and presence or absence of granules in their cytoplasm (Coles, 1986) (Annex 4). Mean Corpuscular Haemoglobin (MCH), Mean Corpuscular Haemoglobin concentration (MCHC) and Mean Corpuscular Volume (MCV) were calculated according to (Schalm, 1975).

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

$$\text{MCH} = \text{Hgb con.} \times 10 / \text{RBC count}$$

$$\text{MCHC} = \text{Hgb con.} \times 100 / \text{PCV}$$

The rectal temperature was recorded within two days interval at the morning from all experimental horses with digital thermometer. All infected horses were being inspected and all data were recorded in any visit during the experimental period for any changes and clinical signs. Parasitemia was estimated daily by the count of trypanosomes in a hundred microscopical fields of wet blood film under a 22 x 22 coverslip using a X10

eyepiece and X40 objective. The results were expressed as parasites/ml according to methodology described by Paris *et al.* (1982).

3.4. Parasitological and Serological Examination

Blood samples were collected from jugular vein and tail bleeding of horses (n=8) and mice (n=42), respectively, following the guide line of International Animal Ethic and Welfare Committee to detect the flagellated protozoan *T. equiperdum*. Fresh wet and thin smear were made using glass slides immediately after collection of blood. Wet smear was examined under the microscope at 40x magnifications to determine level of parasitemia. The micro-hematocrit (mHCT) was performed as described by (Woo, 1971). Briefly, micro-hematocrit capillary tubes were filled with approximately 50 µl of blood from the vacutainer tube and stoppered with sealant. After centrifugation at 12,000 rpm for 5 minutes, the PCV was measured by the hematocrit centrifugation technique using a Hawksley micro-hematocrit reader and the tubes were mounted in a specially designed viewing chamber and examined under the microscope at 10x magnifications for the presence of motile trypanosomes at the level of the buffy coat.

Blood samples were collected in vacutainer tube without anti-coagulant for serology. Blood was kept overnight in laboratory at room temperature and serum was obtained by centrifugation (2500 rpm, 15 minutes) thereafter, serum stored at -20°C until CATT/*T.evansi* test was performed. The test was carried out on serum that was prediluted in CATT diluent, according to the instructions of the manufacturer (Institute of Tropical Medicine, Antwerp, Belgium) (Annex 5).

3.5. Ethical Statement

Samples from horses and mice were collected in ethical manner. At the end of the experiment, animals were humanely euthanized by using ether and pentobarbital for mice and horses, respectively. All efforts were made to minimize animal suffering. Ethical approval was obtained from the Animal Research Ethical Review Committee of Addis Ababa University College of Veterinary Medicine and Agriculture (CVMA) (Certificate

Ref. No: VM/ERC/04/01/09/2017) and VM/ERC/004/07/015, 17/04/015 for mice and horse, respectively.

3.6. Data Management and Analysis

All data were entered into an Excel spread sheet and imported into SPSS version 20 statistical software (IBM Corp, 2011). Descriptive statistics were used to describe the data. Differences in mean haematological values and body temperature between pre-infection and post-infection and treated and non-treated groups were assessed by independent sample T-test. The groups mean \pm S.E.M. was calculated for each group with its respective variables and significant difference between means evaluated by T-test. Post-test analysis was done using ANOVA, Tukey multiple comparison tests (Post Hoc/Tukey's test) to compare parameter within and between groups. Values of $P < 0.05$ were considered as statistically significant.

4. RESULTS

4.1. Demonstration of Antibody and Parasite in the Blood.

The mean time in days of both stallions and mares infected with *T. equiperdum* became seropositive at 15 days of post infection using CATT/*T. evansi* test. The route of infection did not vary the mean days of seroconversion in CATT/*T. evansi* test. Onset of Parasitaemia was detected in all mares infected with *T. equiperdum* through artificially insemination on days 6, 15, 14 and 8 for mares 1, 2, 3 and 4, respectively. Since the route of infection in stallions was directly to venous blood, the parasitemia was established earlier than mares. The level of parasitemia was fluctuating up and down throughout the study period and sometimes became aparasitaemic.

4.2. Clinical Findings

Mean rectal temperature of horses post infection (37.40 ± 0.26) was statistically higher ($p = 0.007$) than horses pre infection (35.94 ± 0.18). Body temperature of all infected animals increased after infection and thereafter oscillated usually in line with the waves of parasitaemia. The highest mean of rectal temperature (37.61°C) was recorded on day 42 post infection.

Both stallions and mares that were challenged with *T. equiperdum* revealed variable number of clinical signs of dourine (Table 3). For each type of clinical sign percentage of specific clinical sign was calculated by total number of specific signs observed in each horse by total number of infected horse and multiplied by 100.

Table 3: Summary of clinical signs observed in experimentally infected horses with *T. equiperdum*.

Horse	Emaciation	Scrotal and Prepuccail edema	Vulval Edema	Edema of limb	genital Depigmentation	Edema of mammary gland	Incoordination and paralysis	Corneal Opacity
M01	+	-	-	+	+	-	+	-
M02	+	-	+	+	+	-	+	+
M03	+	-	+	-	-	+	+	-
MO4	+	-	-	-	+	-	-	-
S01	+	+	-	+	-	-	+	-
S02	+	+	-	-	-	-	+	+
S03	+	+	-	-	-	-	+	+
SO4	+	+	-	-	-	-	+	-
Total	8	4	2	3	3	1	7	3
Percent	100	100	50	37.5	37.5	25	87.5	37.5

(+), indicates clinical sign present; (-), indicates clinical sign absent.

4.2.1. Progressive weight loss, weakness and emaciation

Gradually after infection body weight of all infected animals declined, leading to progressive emaciation and weakness (Figure 6). Eventually, reduction in body condition score of infected animal was clearly seen from a distant. The appetites of animals remain unchanged. Dull hair coat, muscular hypotrophy and loss of skin elasticity were observed throughout study period.



Figure 6: Reduction in body condition score and weight loss in stallions. (A) Body condition of S02 (pre infection), (B) Body condition of S02 (post infection), (C) Body condition of S04 (pre infection), and (D &E) Body condition of S04 (post infection).

4.1.2. Genital signs

Prepuccial swelling was manifested in all infected stallion but lately it developed to scrotal areas which produce yellowish fluid up on puncture (Figure 7 A-C).



Figure 7: Clinical sign of dourine on genital organ of stallion. (A) Prepucial edema, (B) prepucial and scrotal swelling, (C) scrotal swelling containing yellowish watery fluid.

Depigmentation was observed on skin around anal region and under the tail of stallions (n=3) and vulval areas of mares (n=3). Swelling and muco-purulent vaginal discharge which lately adhered and dried on vulva lips was also observed (Figure 7).



Figure 8: Depigmentation of perineal region in mare and stallion and genital sign in mares. Perineal depigmentation (A-E), Swollen vulva (F), Muco-purulent vaginal discharge (G-I).

4.1.3. Edema of limbs

Edema of lower hind limb below stifle joint was observed in two of the four mares. The nature of edema was painless upon palpation and disappeared within a short period of time. Ventral edema and cutaneous form ‘urticarial plaques’ were not found in current findings.

4.1.4. Nervous signs

Lameness and paralysis in hind quarter dominantly observed in all infected horses that led to partial dragging or stiffness of the hind legs, staggering gait, posterior ataxia, inability to stand upright after prolonged sternal or lateral recumbence and need support to get up (Figure 9). On standing position, asymmetrical posture and tendency to shift weight from one leg to another was also observed. Stallions had desire for copulation with the mare but they were not able to mount on the mares and breeding phantom. As the disease advanced they were unable to stand up and remain on recumbent position. They were humanely euthanized using an overdose of phenobarbital sodium intravenous administration at this stage of the disease after 90 to 120 days post-infection.



Figure 9: Difficulty in standing up due to hind quarter paralysis on stallions.

4.1.5. Corneal opacity

Inflammation and lacrimal discharge of eye was noticed in two stallions of the four and one mare of the four infected with *T. equiperdum* that eventually, resulted in corneal opacity. Permanent blindness was manifested due to inflamed and cloudy covering eye (Figure 10).



Figure 10: Lacrimation and corneal opacity recorded in mare

4.2. Hematological Findings

4.2.1. Effect of dourine infection on haematological values

The comparisons of overall mean haematological values at pre-infection and post-infection period are summarized in (Table 4). The Mean total number of RBC of pre-infection period ($7.13 \pm 0.25 \times 10^6 / \mu\text{l}$) was significantly higher ($p < 0.01$) than post-infection ($5.32 \pm 0.21 \times 10^6 / \mu\text{l}$). Similarly, the mean Hgb concentration of post-infection value was significantly lower ($P < 0.01$) than pre-infection. The significant drop ($p < 0.01$) in PCV was observed in post-infection period as compared with that of pre-infection. No significant difference was detected in the mean MCV and MCH values ($P = 0.962$ and

0.133, respectively) between pre-infection and post-infection values. However, Significant difference was observed in mean MCHC values (P = 0.03) (Table 4).

Table 4: Mean haematological values of horses during pre and post-infection with *T. equiperdum* (for 8 consecutive weeks).

Hematological values	Group	Mean ±SE	95 % CI for the mean	Significance value
TRBC ($\times 10^6$ cell/ μ l)	Pre-infection	7.13±0.25	6.59-7.68	0.000
	Post-infection	5.32±0.21	4.91-5.74	
Hemoglobin (gm/dl)	Pre-infection	10.27±0.31	9.61-10.93	0.000
	Post-infection	8.38±0.23	7.91-8.85	
PCV (%)	Pre-infection	34.43±0.56	33.21-35.64	0.000
	Post-infection	24.88±0.67	23.52-26.24	
MCV(fl)	Pre-infection	49.21±2.24	44.37-54.05	0.962
	Post-infection	49.03±1.99	45.00-53.06	
MCH (pg)	Pre-infection	14.65±0.75	13.03-16.26	0.133
	Post-infection	16.67±0.72	15.21-18.13	
MCHC (gm/dl)	Pre-infection	29.94±1.01	27.75-32.12	0.03
	Post-infection	34.28±1.04	32.17-36.39	

There was a gradual decrease in the mean total RBC count starting from day 7 post-infections, which attained significant reduction by day 35 post-infection. The lowest mean total RBC count of 4.75×10^6 cell/ μ l was noticed on day 42 post-infection, which were significantly lower (P=0.031) than the value of pre-infection (Figure 10A). Reductions in Hgb concentration at post-infection period became significant at day 21 of post-infection and persisted until the end of follow up. The lowest mean Hgb value was noticed at day 42 of post-infection, which was 7.29 gm/dl (Figure 11A). The mean PCV values of infected animals started to reach significant variation at day 14 post infection. Moreover, it became significantly and consistently decreases until the end of study period and the lowest mean PCV was recorded on day 42 of post-infection which was 21 % (Figure 11A).

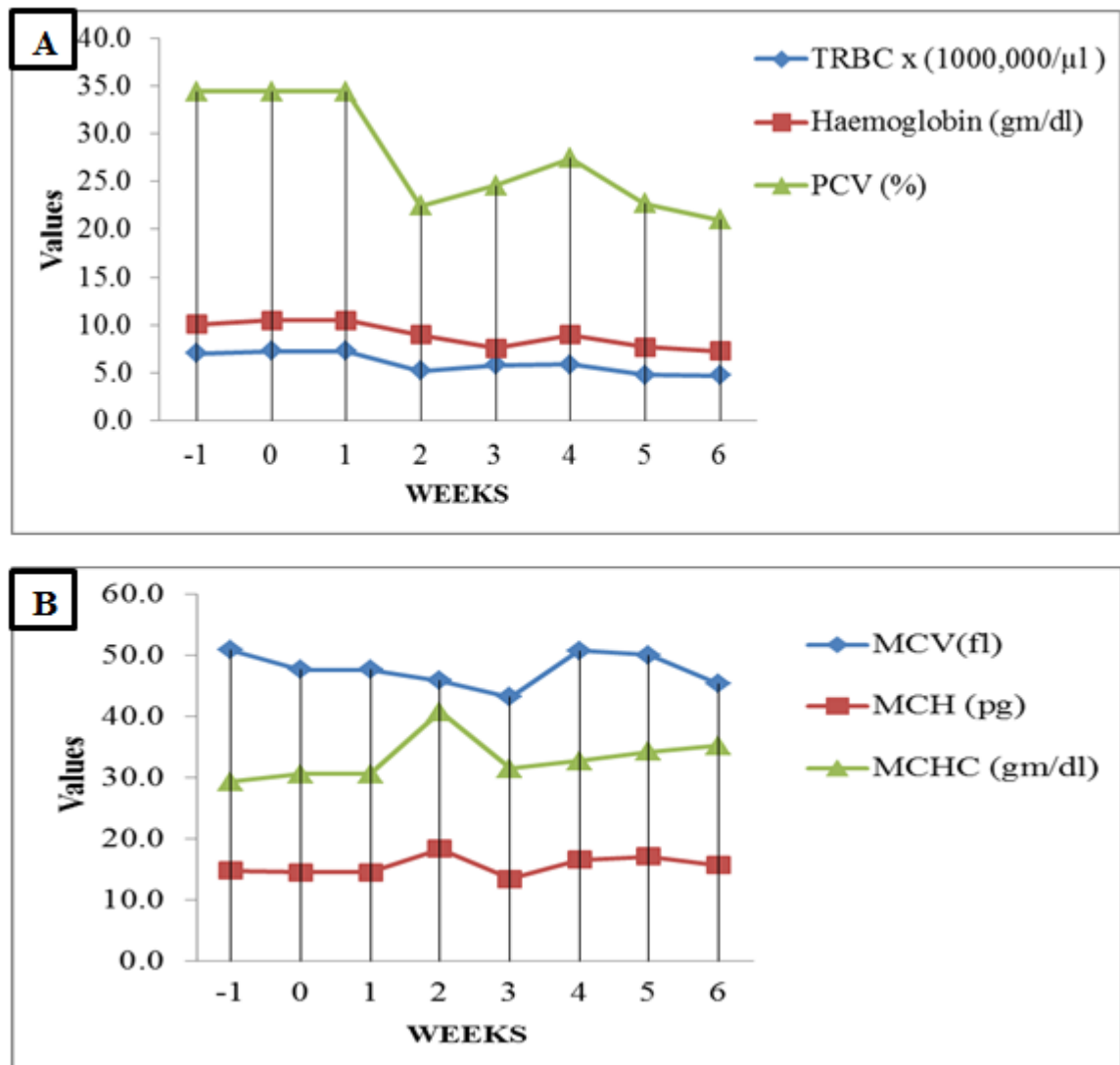


Figure 11: Mean hematological value in horses experimentally infected with *T. equiperdum* during the study period. (A) Mean PCV values in percent, mean haemoglobin concentration in (g/dl) and Mean total RBC count in (10^6 cells/ μ l), (B) change in mean level of MCV (fl), MCH (pg) and MCHC (gm/dl). Animals were infected on week 0 and followed up to 6 weeks.

The overall mean of total WBC during post-infection values had no significant difference when compared with that of pre-infection (Table 5). The mean total WBC count in post-infection period gradually dropped on day 14 to the 3.3×10^3 cell/ μ l which had significant difference ($p < 0.01$) as compared to pre-infection values (9×10^3 cell/ μ l). The mean total WBC in post-infection period significantly higher ($p = 0.001$) than that of pre-infection

period specifically on day 28 (Figure 11). The mean differential WBC in eosinophil, basophil and monocyte during post-infection period were significantly lower ($p=0.01$, 0.044 and 0.002 , respectively), than pre-infection. The mean lymphocyte count of post-infection value was significantly elevated ($p=0.004$) and remained at higher level throughout the study period as compared to pre-infection. The mean neutrophil values in post-infection period was not constant change rather, it was fluctuating up and down throughout the study period (Figure 12).

Table 5: Mean total WBC and differential WBC during pre and post-infection with *T. equiperdum* for 8 consecutive weeks.

Heamatological values	Group	Mean \pm SE	95 % CI for the mean	level of significance
TWBC ($\times 10^3$ cell/ μ l)	Pre-infection	8.96 \pm 0.29	8.34-9.58	0.403
	Post-infection	8.05 \pm 0.61	6.82-9.28	
Neutrophil ($\times 10^3$ / μ l)	Pre-infection	5.34 \pm 0.14	5.03-5.64	0.981
	Post-infection	5.33 \pm 0.22	4.88-5.77	
Eosinophil ($\times 10^3$ / μ l)	Pre-infection	0.89 \pm 0.12	0.63-1.15	0.000
	Post-infection	0.27 \pm 0.04	0.19-0.35	
Basophill ($\times 10^3$ / μ l)	Pre-infection	0.29 \pm 0.03	0.22-0.36	0.044
	Post-infection	0.20 \pm 0.02	0.15-0.25	
Monocytes ($\times 10^3$ / μ l)	Pre infection	0.89 \pm 0.08	0.72-1.05	0.002
	Post-infection	0.58 \pm 0.05	0.48-0.68	
Lymphocytes ($\times 10^3$ / μ l)	Pre-infection	2.59 \pm 0.15	2.26-2.92	0.004
	Post-infection	3.64 \pm 0.19	2.26-2.92	

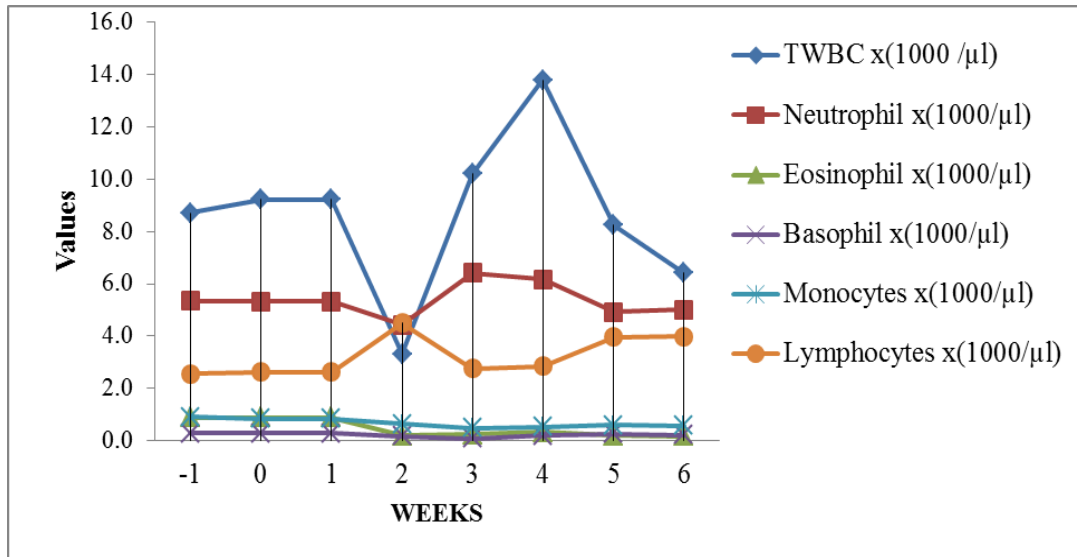


Figure 12: Change in mean total white blood cell count and differential WBC value in horses experimentally infected with *T. equiperdum* during the study period. Animals were infected on week 0 and followed up to 6 weeks.

4.2.1. Effect of cymelarsan[®] treatment on haematological values

The mean changes in haematological values of horses infected with *T. equiperdum* are shown (Table 6). Parasites were cleared from blood of all infected horses' within 48 hours of post-treatment. The relapse of parasite was noticed in blood of treated animals after 21 days of post treatment by using parasitological test (Woo test). Cymelarsan[®] treatment was completely failed to cure and no evidence of improvement in clinical signs of dourine infected horses.

The mean total RBC count in treated groups had not significant elevation ($p=0.143$) as compared with that of non-treated groups. No significant differences were observed in mean RBC count between treated and non-treated groups throughout study period (Figure 11). The treated group has shown significant improvement in mean PCV value as speculated with non-treated group. The first significant improvement in PCV was observed in treated group on 28 days of post treatment. There was a slight improvement in mean Hgb concentration of treated group but not significant ($p=0.109$) as compared

with animals that were not treated. The mean WBC count in treated group was significantly higher ($p=0.01$) than non-treated group. This significant difference was started at day 21 post treatment and persisted until the end of experiment. No significant difference was observed in mean MCV, MCH and MCHC values ($P = 0.953, 0.610$ and 0.365 , respectively) between treated and non-treated groups. The mean differential WBC count in monocyte, lymphocyte, basophil, neutrophil and eosinophil had no significant difference ($p > 0.05$) between treated and non-treated group (Table 6).

Table 6: Mean haematological values comparison of treated and non-treated groups with Cymelarsan® drug during eight (8) weeks in experimental infected horses with *T. equiperdum*.

Heamatological values	Group	Mean ±SE	95 % CI for the mean	Significance level
TRBC (x10 ⁶ cell/μl)	Treated	5.08±0.23	4.60-5.5	0.143
	Non-treated	4.60±0.21	4.16-5.04	
TWBC (x10 ³ cell/μl)	Treated	9.39±0.61	8.13-10.66	0.000
	Non-treated	6.74±0.31	6.09-7.39	
Haemoglobin (gm/dl)	Treated	7.41±0.21	7.48-8.35	0.109
	Non-treated	7.42±0.22	6.95-7.88	
PCV (%)	Treated	21.91±0.51	20.85-22.98	0.030
	Non-treated	20.00±0.69	18.58-21.42	
MCV (fl)	Treated	45.27±2.22	40.67-49.87	0.953
	Non-treated	45.47±2.46	42.07-48.67	
MCH (pg)	Treated	16.25±0.74	14.71-17.79	0.610
	Non-treated	16.83±0.86	15.06-18.61	
MCHC (gm/dl)	Treated	36.26±0.83	34.54-37.99	0.365
	Non-treated	37.78±1.43	34.81-40.75	
Neutrophil (x10 ³ /μl)	Treated	6.84±0.27	6.29-7.39	0.155
	Non-treated	6.33±0.23	5.85-6.81	
Eosinophil (x10 ³ /μl)	Treated	0.18±0.05	0.07-0.28	0.893
	Non-treated	0.18±0.03	0.11-0.25	
Basophil (x10 ³ /μl)	Treated	0.24±0.04	0.16-0.32	0.190
	Non-treated	0.3292±0.05	0.22-0.44	
Monocytes (x10 ³ /μl)	Treated	0.6417±0.07	0.49-0.79	0.388
	Non-treated	0.55±0.08	0.39-0.71	
Lymphocytes (x10 ³ /μl)	Treated	2.10±0.28	1.53-2.67	0.097
	Non-treated	2.6750±0.19	2.27-3.07	

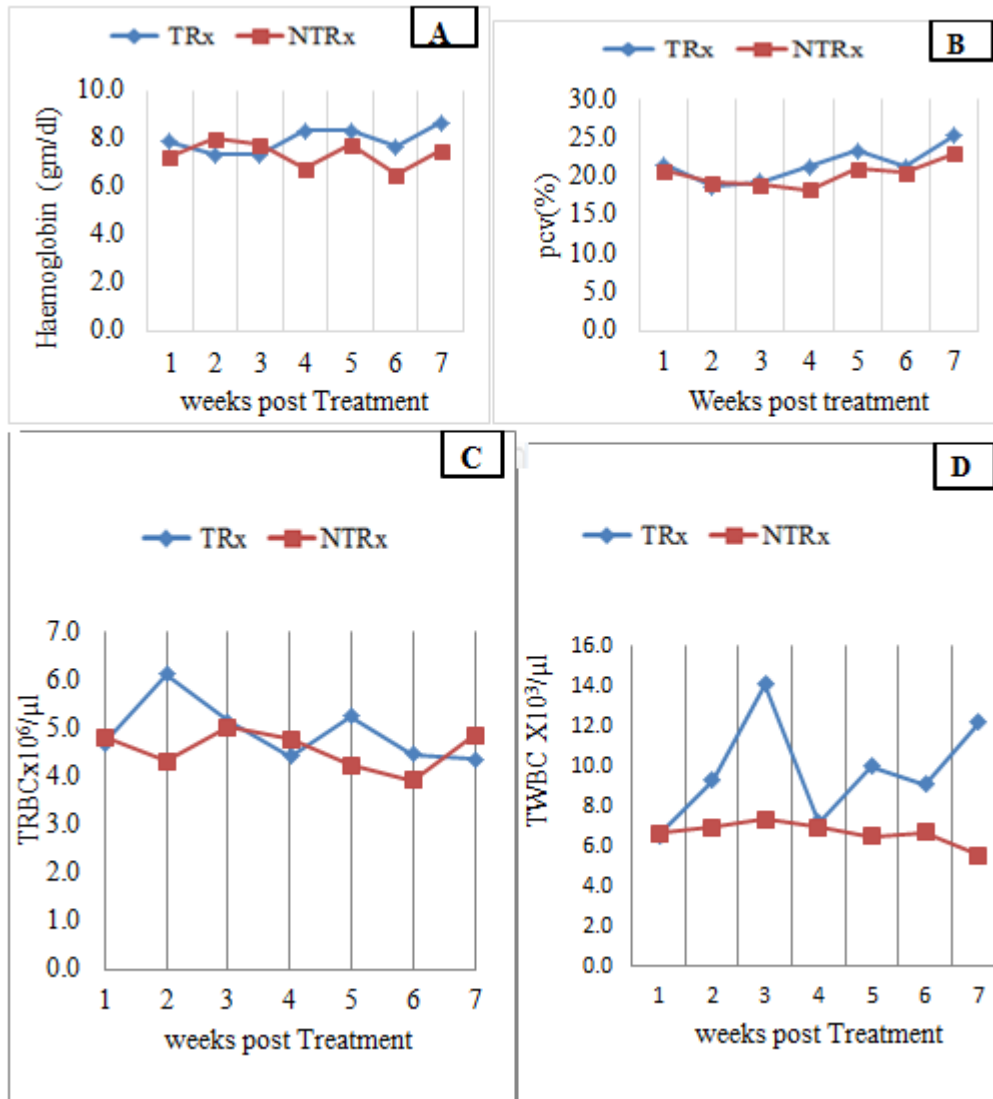


Figure 12: Mean haematological values comparison between treated (TRx) and non-treated (NTRx) horses after experimentally infected with *T. equiperdum* (Weeks 1 indicates the start of treatment with cymelarsan® after six (6) week of post infection). (A) Mean haemoglobin concentration in (g/dl), (B) Mean PCV values in percent, (C) Mean total RBC count in ($\times 10^6$ cells/ μ l), (D) Mean total WBC count in ($\times 10^3$ cells/ μ l).

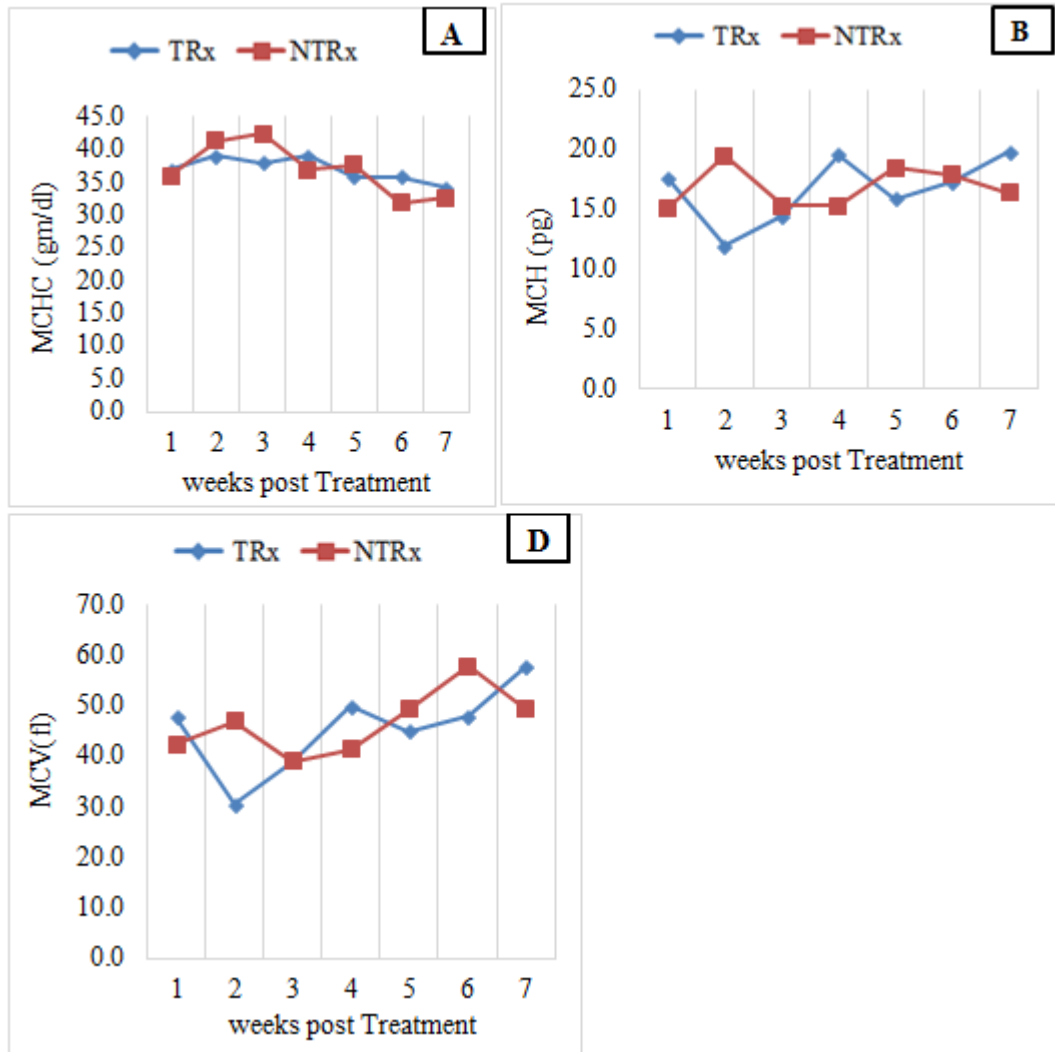


Figure 13: Mean red blood cell indices comparison between treated (TRx) and non-treated (NTRx) horses after experimentally infected with *T. equiperdum*. (Weeks 1 indicates the start of treatment with cymelarsan® after six (6) week of post infection), (a) mean MCHC in (gm/dl), (b) mean MCH in (pg), (c) mean in MCV(fl).

4.3. Venereal Transmission of *Trypanosoma equiperdum* in Mice

Parasite was established in mice at 3-4 days of post infection and peaked at swarming level (score +6) at 5-7 days of post infection. When the first appearances of parasite in male mice detected at level of parasitemia (10^1 - 10^3 trypanosome/ml); one *T. equiperdum* infected male mice and two healthy female mice in six replications were co-housed for

the purpose of copulation. Mating was observed in the follow up and the females gave birth indicating the presence of sexual intercourse. During the whole courses of the study period, parasite was not found in all of the females mice. No parasite was detected even in their offspring. In other groups as the parasitemia reached at peak level (score+6) in the male mice, they were also co-housed with healthy females. None of the females showed the presence of parasite in the blood as well as their offspring's. On the other hand, all of the infected male mice were died of parasitemia. In order to rule out the possibility of transmission occurring via any routes (other than sexual transmission), two infected female were co-housed with four non-infected females as a control, all the four non-infected females remained non-infected based on parasitological test for a period of three months. However the infected females were not survived more than ten days.

5. DISCUSSIONS

The current study revealed that the mean day of both stallion and mares infected with *T. equiperdum* becomes seropositive at 15 days of post infection irrespective of the route of entry and sex. In other findings Verloo *et al.* (2000) reported that in CATT RoTat 1.2 specific antibodies always appeared within one month after infection in rabbits experimentally infected with *T. evansi*. This finding was, however, in contrast to the findings of Borrowman (1976) who reported the mean days of horses became CFT Positive after 24 days of infection. The difference might be due to host variation, type of test used, type of strain used and route of inoculation. The present finding also noticed that the mean days of parasitemia onset was vary based on routes of infection, host difference, and concentration of inoculum used, so that, stallion infected via venous blood became parasitemic earlier than mares that infected via artificial insemination, which agrees with the finding of Pascucci *et al.* (2013) who described that after the infection of the genital mucosa via coitus, *T. equiperdum* invade the tissue and parasitize in the blood, lymph, CSF and sub-cutaneous lesions.

In the current study Significant higher in mean rectal temperature has been observed in all infected animals than pre-infection. Body temperature of all infected animals increased after infection and usually coincident with the appearance of parasitaemia, and then fluctuated throughout the study period. Fever has been reported in similar diseases (Caporale, 1946; Brun *et al.*, 1998; Claes *et al.*, 2005) that agree with the present finding. On the contrary, fever has not reported in similar disease by some authors (Barrowman, 1976; Coetzer, and Tustin, 2004). Possibly due to host response to infection and type of strain used. The Present study also agrees with other types of trypanosomiasis, the temperature of infected animals coincides with the appearance of parasitaemia, and then fluctuated throughout the study period (Adeiza *et al.*, 2008; Dagnachew *et al.*, 2015).

In the current study, all animals suffered from loss of body weight that leads to progressive emaciation, weakness, along with dull hair coat and loss of skin elasticity that were observed throughout the study period. However, appetites of animals remain

unchanged. This finding is in line with the previous reports of Caporale (1946); Alemu *et al.* (1997); Clausen *et al.* (1999); Hagos *et al.* (2010a); Lelli *et al.* (2012) and Vulpiani *et al.* (2013) in that all present observation have been registered by the authors.

The declines in mean body weight associated with high parasitemia which results in build-up of peroxides and free radicals in the body of infected animal and damage all components of the cell, including proteins and lipids (Saleh *et al.*, 2009). Uptake of host glucose by *T. evansi* for its metabolic activity (Hunt, 2010) and parasites induced metabolic enzymes insufficiencies in the host (Enwezor and Sackey, 2005). Since, *T. evansi* and *T. equiperdum* exhibited many similarities in biological, biochemical and molecular characteristics (Brun *et al.*, 1998), body weight loss of current study animals also might be attributed to the mechanism of *T. evansi*.

The depigmentation of skin observed in the present study on skin around anal region and under the tail of stallions and on skins of vulval, anal and under tail of mare was in line with previous finding (Claes *et al.*, 2003; Hagos *et al.*, 2010a and Pascucci *et al.*, 2013). Preputial swelling in stallions that gradually developed to scrotal areas and glans penis, as well as vulval edemas in mares was observed. Moreover, the mucopurulent vaginal discharge was also observed that adhered and dried on vulval lips is in agreement with previous reports (Hoare, 1972; Alemu *et al.*, 1997; Hagos *et al.*, 2010a; Lelli *et al.*, 2012; Pascucci *et al.*, 2013; Vulpiani *et al.*, 2013).

In the current study the edema of lower hind limb observed which is in agreement with previous reports (Puscussi *et al.*, 2013; Vulpiani *et al.*, 2013). In the current study edema of mammary gland was observed only in single mare that was not constant sign in other horses; possibly due to difference in the host response to infection. This finding is in line with Vulpiani *et al.* (2013) who reported edema of mammary gland in some mares. Skin plaques are regarded as important symptoms in cases of dourine. But, in certain surra infections these cutaneous plaques may be observed (Brun *et al.*, 1998). Hence, these plaques should not be considered to be pathognomonic for dourine. In this study, skin plaques were not observed in both mares and stallions throughout the study period *as has*

been reported previously (Alemu *et al.*, 1997; Clausen *et al.*, 1999; Hagos *et al.*, 2010a) in Ethiopia. However, these findings disagree with previous report in different countries (Barrowman *et al.*, 1976; Coetzer and Tustin, 2004; Claes *et al.*, 2005; Pascucci *et al.*, 2013 and Vulpiani *et al.*, 2013). The difference might be due to *T. equiperdum* strain used, breed of horse involved in experiment and host immune response to disease. This is supported with many authors that stated clinical signs and pathogenicity of dourine vary with breed of host, the virulence of the strain, the nutritional status of the horse, and stress factors (Hoare, 1972; OIE, 2008; Sidney *et al.*, 2013). Ventral edema was not manifested in current study both in mares and stallions. This finding was disagrees with previous reports Hagos *et al.* (2010a); Lelli *et al.* (2012); Vulpiani *et al.* (2013) and Puscussi *et al.* (2013) reported that the presence of ventral edema in some horses.

Nervous signs were manifested in all mares and stallions that were characterized by lameness in hind quarter, incoordination, difficulty in rising up and lying down and, asymmetrical posture on standing position and tendency to shift weight from one leg to another. The presence of neurological signs confirms the tropism of *T. equiperdum* for the peripheral nervous system and the lack of involvement of the central nervous system, in contrast with other trypanosomes (Barrowman, 1976; Clausen *et al.*, 1999; Puscussi *et al.*, 2013). Present finding noticed that stallions attempt to mount on mare for mating, but unable to mount due to hind quarter paralysis.

The present finding revealed that unilateral corneal opacity in two stallions and one mare were found. This finding was consistent with that of previous reports (Alemu *et al.*, 1997; Vulpiani *et al.*, 2013) in Ethiopia and Italy respectively. The use of Real-Time PCR enabled even low concentrations of DNA from the parasites to be detected in conjunctival swabs (Theis and Bolton, 1980).

In the present study, significant decrease in mean PCV, Hgb concentration and total RBC observed in animals during post-infection as compared with values of the pre-infection period. The decrease in PCV, RBC and Hgb parameter are indicators of anemia that persisted in course of study period. Anemia was moderate during early infection and

became severe as duration of infection progressed. In agreement with these findings, Sukanuma *et al.* (2016) described that slight decrease in PCV, Hgb concentration and total RBC in dourine infected horses in Mongolia. Present result was also in agreement with observation of Vulpiani *et al.* (2013) they recorded that anemia was moderate on onset and then became more severe in animals with chronic edematous lesions or nervous signs, with a hematocrit of 20% or less and fewer than 3 million erythrocytes/mm³ in some cases of dourine infected horses.

In other trypanosomosis, anemia is a consistent feature of infections caused by, amongst other factors, oxidative damage to erythrocyte membrane components. Reactive oxygen radicals generated during infections such as trypanosomosis can attack erythrocyte membrane, induce its oxidation and thus trigger haemolysis (Ngure *et al.*, 2009). Decrease in mean PCV values might be correlated with the decrease in total RBC count. A relative deficiency of blood cells occurs initially due to haemodilution and further exacerbated by hemolytic anaemia. Hemolysis could be caused by mechanical injury to erythrocytes by the lashing action of the powerful locomotory flagella and microtubule-reinforced bodies of the high number of the trypanosomes during parasitaemia (Vickerman and Tetley, 1978).

Erythrocyte membrane damage has also been associated with adhesion of erythrocytes and reticulocytes to trypanosome surfaces via sialic acid receptors leading to damages to erythrocyte cell membranes (Anosa and Kaneko, 1983). Furthermore, living and dead trypanosomes can produce various forms of active chemical substances, which can elicit erythrocyte injury (Tizard *et al.*, 1977; Naessens *et al.*, 2005).

Erythrocytic indices are used to determine the types of anaemia (Abenga *et al.*, 2005). In present study, insignificant difference was detected in mean MCV and MCH values and significant increase in MCHC was observed in pre-infection as compared to post-infection indicate the normocytic hypochromic type of anaemia in infected groups, insignificant difference in MCV and MCH values obtained agrees with the findings (Sukanuma *et al.*, 2016) and disagree in MCHC that was in normal range value.

In present findings overall mean of total WBC during pre-infection values had no significant difference as compare to that of post-infection. The mean total WBC gradually dropped on day 14 (3.3×10^3 cell/ μ l) which had significant difference with pre-infection value (9×10^3 cell/ μ l). Furthermore, the mean total WBC of infected animals also significantly higher than that of animals in pre-infection period specifically on PID 28. This was followed by an increase in WBC counts, principally associated with increased lymphocyte numbers and activity of the mononuclear phagocytic system during trypanosomiasis, in agreement with previous report (John *et al.*, 2006; Suganuma *et al.*, 2016).

The mean value of differential WBC after infection in eosinophil, basophil and monocyte significantly lower than before infection. The mean lymphocytes counts of animals after infection significantly remain elevated throughout the study period as compared with animals before infection, in accordance with the findings of Anosa *et al.* (1992) ascribed that lymphocytosis, is believed to result from trypanosome antigenic challenge leading to an increased proliferation of immuno-competent cells into antibody and or lymphokine producing cells of *T. vivax* infection in calves. In present study, the mean values of neutrophil after infection, fluctuates up and down without significant difference as compared to pre-infection values.

Treatment of cymelarsan® at standard dose of 0.25 mg/kg body weight was attempted on horses infected with *T. equiperdum*. No parasites were detected in blood of treated horses starting from 48 hours of post-treatment. However, this treatment with Cymelarsan was found to be ineffective because of relapse. Relapse was observed in blood of treated animals at 21 day of post-treatment. Similar result has been documented by cuachard *et al.* (2016) that the Cymelarsan® treatment failed to cure the two ponies at a single dosage and also this finding was in line with Beletu *et al.* (2015) also reported Cymelarsan® to be ineffective against *Trypanosoma equiperdum* in mice at doses of 0.25 mg/kg and 0.5 mg/kg body weight. However, this finding is inconsistent with the observation of Hagos *et al.* (2010b) who disclosed no relapses and parasites were found after revisiting and testing of the animals six and twelve months following initial treatment in chronic

clinical field cases. The difference could arise from field cases in previous study however experimental confined horses used for present study.

Treated group has shown significant improvement in mean PCV value as speculated with untreated group for few days. This result agrees with previous report by Hagos *et al.* (2010b) who ascribed that relative improvement in PCV levels of animals also appeared.

During at co-housing of infected male mice with un-infected female mice at level of parasitemia of 10^1 - 10^3 trypanosome/ml and 10^4 - 10^6 trypanosome/ml indicated that parasite was not detected in all female mice. Healthy female co-housed with infected male mice gave birth rather than being infected. In other group as parasitemia reached at peak level greater than (score+4) male mice co-housed with healthy female. This attempt also indicated that none of female mice did show presence of parasite in blood based on parasitological examination. Females that were co-housed with male mice in this group also gave birth. No parasite was detected even in new born mice by using parasitological methods (wet smear examination). None of offspring did manifest evidence of parasitemia until the end of experiment after a follow up of up to 3 months. However all infected male mice were died of infection. This finding was in agreement with previous report by Biteau *et al.* (2016) described that, in PCR test the presence of parasites in the uterus of one mouse was confirmed, even the blood of all mice being negative by PCR and LAMP. Subsequently, bioluminescent imaging of the offspring of infected female mice crossed with healthy males indicated the presence of parasites in the reproductive organs of both male (80%) and female (60%) offspring.

In present finding the vertical transmission of *T. b. gambiense* demonstrated in the previous study implies that it may be possible for a silent reservoir of parasites to exist, especially since the disease can progress differently in different hosts. Also, it should be considered that silent infections resulting in horizontal and vertical transmission may also occur in other Trypanozoon parasites (Biteau *et al.*, 2016). Thus, present finding was not conclusive yet, need DNA based technologies and other methods to verify horizontal and vertical transmission of *T. equiperdum* in mice.

To rule out the possibility of transmission occurring via any routes (other than sexually), infected four (4) female was co-housed with non-infected two (2) females. Subsequently, all four females remained non-infected based on parasitological test (wet smear) whereas infected females were died of infection. This result was consistent with Biteau *et al.* (2016) who observed that absence of transmission by co-housing infected and non-infected females, point towards sexual transmission as the most likely route of horizontal transmission in *T. b. gambiense*.

6. CONCLUSIONS AND RECCOMENDATIONS

The present study revealed that different types of clinical sign observed in experimental infected both sexes of 8 horses (4 mares and 4 stallions) with *T. equiperdum* at different stage of development course of the disease. The major notable clinical sign includes edema of limb, hind quarter paralysis and corneal opacity was commonly observed in some of both stallion and mares. Vulval edema, genital depigmentation and mammary gland edema noticed in mare whereas, prepucial and scrotal edema observed in stallion. The comparisons of haematological values of *T. equiperdum* infected to both sex of horses during pre and post-infection revealed haematological alteration in that the mean TRBC, PCV and Hgb values were decreased. In the infected animals normocytic hypochromic anemia was developed throughout the whole course of the study period. Cymelarsan treatment in the infected horses with *T. equiperdum* failed to bring complete restoration of hematological values and has no effect on the disappearance of the observed clinical signs. A single shot of cymelarsan[®] at a dose of 0.25 mg/kg body weight also failed to prevent the relapse of parasite in the blood. The subsequent attempt of venereal transmission of *T. equiperdum* to mice also failed to show production of positive of parasite as has been revealed by parasitological examination.

Based on the above conclusive remarks, the following recommendations are suggested:

- Further elucidative and detailed studies should be conducted on Hematological comparison of naturally and artificially infected horses with *T. equiperdum*.
- The present study showed possibility of dourine transmissions via semen during artificial breeding of equine to recommend that the stallions intended to be used for breeding purpose should be strictly tested.
- Parasitological examination of both vertical and horizontal (coital) transmissions of *T. equiperdum* in mice showed unsuccessful result to recommend that other confirmatory methods including DNA based technologies (PCR) and Bioluminescent Imaging (BLI) should be investigated.

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8. ANNEXES

Annex 1: Hemoglobin concentration determination using acid hematin method

Requirements: Sahlis instrument, blood sample

Procedures

- Take 0.1N HCl (1%) into central graduated tube up to mark 2.
- Suck the blood exactly up to mark 20 (20 μ l) with the help of sahlis pipette.
- Transfer the blood from pipette to central graduated tube of the hemometer.
- Mix it well with the help of stirrer or rod and allow it to react for two minute.
- Make up with distilled water by adding drop by drop until the color matches with the Standard comparator tube and mix well.
- When the color matches take out and record the values Normal value: horse 9 - 12gm/dl

Annex 2: Total red blood cell (RBC) count using Hemocytometer method

Requirements: Hemocytometer, cover slip, microscope, RBC diluting fluid, Haeyem's solution or Physiological saline 0.85% Nacl.

Procedures

- Take the blood in to RBC pipette up to 0.5 marks
- Immediately draw the RBC diluting fluid up to mark 101.
- Rotate the pipette between thumb and other fingers with finger eight (8) movements. This gives a dilution of 1:200.
- Clean the counting chamber of hemocytometer and cover slip
- Place the cover slip in position over the counting chamber by gentle pressure
- Expel a drop of blood on to the counting chamber by holding the pipette at an angle of 45°.
- Allow the hemocytometer for 2-3 min to settle down the RBC in counting chamber.

Calculation

Volume of one small square = $1/20\text{mm} \times 1/20\text{mm} \times 1/10\text{mm} = 1/4000\text{mm}^3$ Volume of 80 small square = $80 \times 1/4000\text{mm}^3 = 1/50\text{mm}^3$ Total number of RBC = Cells counted (N)/Volume of all squares \times dilution factor Total RBC = $N \text{ (cell counted)}/1/50\text{mm}^3 \times 1/200 = N \times 10,000$

Annex 3: Total white blood cell (WBC) Count using Hemocytometer method.

Requirements: Slides, microscope, hemocytometer, pipette , WBC diluent ,

Procedures

- Aspirate blood with pipette at 0.5 mark by avoiding air bubble
- Suck diluting fluids up to mark of 101
- Mix contents slowly and leave it for a minute
- Place cover slip on hemocytometer and place a drop of fluid4 angles 16x at the sides
- Start count at four corner at 10x/40x leave WBC touching the left upper part of the line
- Multiply the total number of WBC counted by 50 to come to the total WBC number.

Calculations

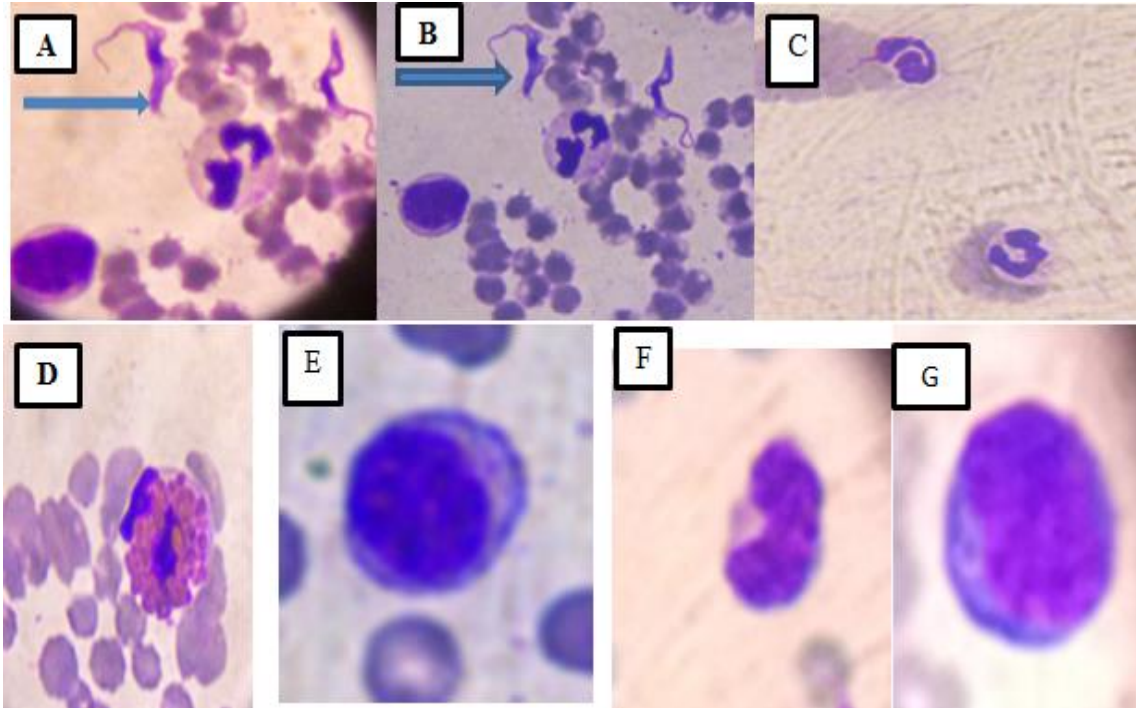
1. Area of one small square= $1/4 \times 1/4 \text{mm}^2 = 1/6 \text{mm}^2$
2. Depth of counting chamber= $1/10 \text{mm}$
3. Volume of one smallest square= $1/10 \text{mm} \times 1/6 \text{mm} = 1/160 \text{mm}^3$
4. The area 16 small square in one angle, thus the total square of found in 4 angles $16 \times 4 = 64$
5. Thus total volume= $1/160 \text{mm}^3 \times 64 = 64/160 \text{mm}^3 = 0.4 \text{mm}^3$
6. Dilution is 1:20.
7. No of cell counted/ dilution the volume $\text{mm}^3 = \text{NX } 50/0.4 \text{mm}^3 = \text{No } \times 50 \text{-----mm}^3$

Annex 4: Total differential WBC count

Materials: slides, microscope, distilled water, Diff Quick stain,

Methods

- Place a drop of blood near to the end of a clean slide
- Bring another slide and holding it at 45° attach it to the drop of blood
- Then push thin film of blood the slide slowly in forward direction to form a thin film of blood
- Dry the thin film of blood in the air and insert it into the differential rapid stain for 20 seconds
- Put the dry smear on the microscope and added a drop of immersion oil on the smear
- By using oil immersion objective count 100 WBC
- Identify each type of leukocytes and record your results as % of the total leukocyte count



A & B indicated by an arrow (*T. equiperdum*) (C) Neutrophil, (D) Eosinophil, (E) Lymphocyte, (F) Monocyte, (G) Basophil.

Annex 5: Card Agglutination for Trypanosomiasis Test (CATT/*T. evansi*)

The CATT/*T. evansi* is a rapid direct agglutination test, which uses formaldehyde fixed, Coomassie stained, freeze-dried trypanosomes of *T. evansi* VAT RoTat 1.2.

Reconstitution of the CATT antigens

- Using the syringe, add 2.5 ml of CATT buffer to vial of freeze dried CATT antigens.
- Immediately shake the vials for seconds so as to obtain homogenous suspension
- Put a dropper on the vials. Then after antigen suspension is ready to use.

Reconstitution of controls

- Using the syringe, add 0.5 ml of CATT buffer to vials of the positive and negative control.
- After reconstitution of each vials of CATT antigens test on drop of the positive control and one drop of the negative control to check the quality of the antigens.

Preparation of test samples

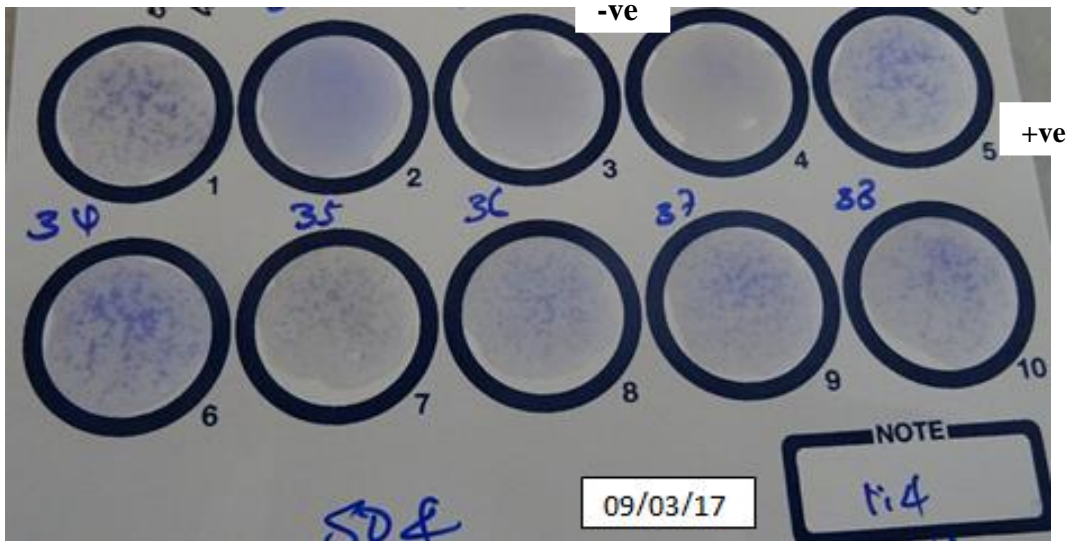
- Prepare serial twofold dilution 1:4, 1:8, 1:16, 1:32, and 1:64 of the test sample in CATT buffer.
- Using a micropipette put 25 micro liters of the serial twofold dilution on a test area of the card.
- Add one drop about 45 micro liter of the well homogenized CATT antigens in each test areas.
- Using stirring rod, mix and spread out the reaction mixture to about 1mm from the edge of the test areas. Wipe off the stirring rod after each use.
- Rotate the test card on a flatbed orbital for 5 minutes at 70 rpm.

Reading and interpretation

Result interpretation of CATT/ *T. evansi*

Agglutination	Test result
++++	Strongly positive(very strong agglutination)
++	Positive(strong agglutination)
+	Positive(moderate agglutination)
±	Weakly positive(weak agglutination)
-	Negative (Absence of agglutination)

Annex 6: CATT/ *T. evansi* test results



Annex 7: Wet film parasitaemia estimation

Number of trypanosomes	Score	Estimated parasitemia trypanosomes/ml
Swarming >100 per field	6+	$>5 \times 10^6$
>10 per field	5+	5×10^5
1-10 per field	4+	$10^4 - 5 \times 10^5$
1 per 2 field-1 per 10 fields	3+	$5 \times 10^3 - 5 \times 10^4$
1-10 per preparation	2+	$10^3 - 10^4$
1 per preparation	1+	$10^2 - 10^3$

(Source: Paris *et al.*, 1982)

Annex 8: Differential Quik Staining Procedure

Solutions required

1. Fixative (Fast green in methanol) - pale green colour
2. Stain solution 1 (Eosin G in phosphate buffer) - red colour
3. Stain solution 2 (Thiazine dye in phosphate buffer) - blue colour

Methods

1. Allow smears to dry
2. Dip slide or tape-strip five times, for one seconds each, into Fixative. Allow excess to drain after each dip.
3. Dip slide or tape-strip five times, for one seconds each, into Stain 1. Allow excess to drain after each dip.
4. Dip slide or tape-strip five times, for one seconds each, into Stain 2. Allow excess to drain after each dip.
5. Rinse slide or tape-strip in distilled water.
6. Blot or allow drying in air.

Examine at low power to identify structures and then under oil immersion.

Annex 9: During blood sample collection and mice inoculation in lab.



