Thesis Ref. No._____

# CHARACTERIZATION OF PATHOLOGICAL LESIONS ON THE NERVOUS SYSTEM OF HORSES NATURALLY AFFECTED BY DOURINE IN AND AROUND DODOLA WEREDA, ARSI-BALE HIGHLANDS OF OROMIA, ETHIOPIA

MSc Thesis


By

Melke Meseret Alemayehu

Department of Veterinary Pathology and Parasitology MSc Program in Tropical Veterinary Pathology

October, 2015

Bishoftu, Ethiopia

# CHARACTERIZATION OF PATHOLOGICAL LESIONS ON THE NERVOUS SYSTEM OF HORSES NATURALLY AFFECTED BY DOURINE IN AND AROUND DODOLA WEREDA, ARSI-BALE HIGHLANDS OF OROMIA, ETHIOPIA

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

By

Melke Meseret Alemayehu

October, 2015

Bishoftu, Ethiopia

Addis Ababa University

College of Veterinary Medicine and Agriculture

Department of Veterinary Pathology and Parasitology

---

As members of the Examining Board of the final MSc open defence, we certify that we have read and evaluated the Thesis prepared by Melke Meseret Alemayehu entitled as "Characterization of Pathological Lesions on The Nervous System of Horses Naturally Affected By Dourine in and Around Dodola Wereda, Arsi-Bale highlands of Oromia, Ethiopia". It is recommended that it is accepted as fulfilling the thesis requirement for the degree of Masters of Science in Tropical Veterinary Pathology.

Chairman

Dr. Yakob Hailu _____ _____

Signature Date

External Examiner

Dr. Getinet Abie _____ _____

Signature Date

Internal Examiner

Dr. Tilaye Demissie _____ _____

Signature Date

Advisors

1. Dr. Hagos Ashenafi _____ _____

   Signature Date

2. Prof. Merga Bekana _____ _____

   Signature Date

3. Dr. Bulto Giro _____ _____

   Signature Date

## SIGNED DECLARATION SHEET

Statement of author

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

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

Name: Melke Meseret Alemayehu Signature: _____

College of Veterinary Medicine and Agriculture, Bishoftu

Date of Submission: _____

## TABLE OF CONTENTS

**Table of Contents** **pages**

**TABLE OF CONTENTS (*continued*)**

## ACKNOWLEDGMENTS

I am highly grateful to the VLIR-UOS Ethio-Belgium team project for providing me research facilities like CATT test and post mortem kits, and also financial support for the accomplishment of this research work.

I am also highly grateful to the Alage ATVET College for granting me a study leave to pursue this study.

I feel great pleasure and honor to express my heartiest gratitude and deep sense of obligation to my advisor Dr. Hagos Ashenafi, Department head of Pathology and Parasitology, Addis Ababa University, College of Veterinary Medicine and Agriculture, for his keen interest, propitious guidance and valuable suggestions for the successful accomplishment of this study.

I express my deep sense of gratitude and profound indebtedness to my co-advisors; Dr. Bulto Giro and Professor Merga Bekana, for their valuable guidance, moral support, healthy criticism and inspiring guidance for the accomplishment of this manuscript.

With profound sense of gratitude, I am deeply thankful to Mr. Alemu Tola, Senior Lab. Technician, VLIR-UOS Ethio-Belgium team project laboratory, College of Veterinary Medicine and Agriculture, for his encouraging attitude, sympathetic and inspiring guidance, and moral support throughout the study periods.

I extend my heartiest thanks to all staff members of Dodola Veterinary clinic especially Mrs. Zertihune and Dr. Mohammad Hussein, for providing me helps in sampling during the conduct of research work. I am also very grateful to the laboratory staff of Histopathology Department, National Animal Health Diagnostic and Investigation Center, Sebeta, for extending help whenever needed.

## LIST OF FIGURES

## LIST OF APPENDICES

## ABBREVATIONS

| | |
|---|---|
| AFLP | Amplified Fragment Length Polymorphism |
| Ak | Akinetoplastid |
| BHK | Baby Hamster Kidney |
| BoTat | Bordeaux Trypanosoma antigen type strain |
| CATT | Card Agglutination Test for Trypanosomosis |
| cDNA | Complement DNA |
| CFT | Complement Fixation Test |
| CPE | cytopathic effect |
| CSA | Central Statistics Agency |
| CSF | Cerebrospinal Fluid |
| Dk | Dyskinetoplastid |
| DNA | Deoxyribo Nuclic Acid |
| EARO | Ethiopian Agricultural Research Organization |
| ELISA | Enzyme Linked Immunosorbent Assay |
| GMEM | Glasgow Minimum Essential Medium |
| HCT | Haematocrit Centrifugation Technique |
| H&E | haematoxylin and eosin |
| kDNA | kinetoplastic DNA |
| LAMP | Loop‐mediated isothermal amplification |
| mAECT | mini Anion Exchange Centrifugation Technique. |
| MEGA | Multiplex Endonuclease Genotyping Approach |
| MGE‐PCR | Mobile Genetic Elements PCR |
| NADH | Nicotinamide Adenine Dinucleotide Hydrogen |
| OIE | Organization for International Epizootics |
| OVI | Onderstepoort Veterinary Institute strain |
| PBS | Phosphate Buffered Saline |
| PCR | Polymerase Chain Reaction |
| RAPD | Random Amplification of Polymorphic DNA |
| RFLPs | Restriction Fragment Length Polymorphisms |

**ABBREVATIONS** (*Continued*)

| | |
|---|---|
| RNA | Ribonuclic Acid |
| RoTat 1.2 | Rhode Trypanosome antigenic type 1.2 |
| Rpm | revolution per minute |
| SSR‑PCR | Simple Sequence Repeat PCR |
| STIB–818 | Swiss Tropical Institute Basel strain 818 |
| USA | United States of America |

## ABSTRACT

A cross sectional study was undertaken in three purposively selected horse-breeding districts of the Arsi–Bale highlands of Ethiopia, namely Dodola, Assassa and Adaba from March 2015 to the end of June 2015. The aim of this study was to describe the neurologic signs and the pathologic findings in the nervous system of horses naturally affected by dourine; and to reveal the presence of the parasite in the nervous tissue or cerebrospinal fluid or in both. For this purpose, a total of 20 local breed of horses with naturally occurring neurologic signs of dourine were considered as study animals. Out of the 20 animals, 12 horses were clinically positive and then selected. From jugular vein of each animal, 7.5 ml and 10 ml of blood using heparinised and plain vacutainer tubes were collected for parasitological (Woo) test and serological (CATT) tests, respectively. The most common signs in the horses were notable weight loss, depigmentation on the vulva and perineal region, and hind legs paralysis. Out of the 12 horses, 2 mares revealed strong seropositive result in CATT test and were subjected for post mortem examination and followed by histopathological analysis. There were no clear gross pathological lesions in the brain, spinal cord and sciatic nerves of the two mares. None of the animals examined showed trypanosomes in Giemsa-stain as well as in blood samples by Woo test. Histopathological examinations of nervous tissue (cerebral cortex, cerebellum, spinal cord and sciatic nerve) showed lesions primarily on the lumbar and sacral regions of the spinal cord and the left sciatic nerve. The primary lesions were degenerative changes and neuronal necrosis of the spinal cord, as well as cellular infiltration and degeneration of the sciatic nerves. Therefore, the present study disclosed that clinically sick horses being seropositive without parasites in the blood as well as nervous tissues revealed moderate degree of pathological lesions. Further sensitive and specific diagnostic techniques need to be applied in order to detect parasitaemia. On the other hand immunological methods seem vital to determine the exact cause of the lesion.



**Key words**: Arsi–Bale highlands, CATT, Dourine, Ethiopia, Histopathology, Horses, Woo test

# 1. INTRODUCTION

Livestock production constitutes one of the principal means of achieving improved living standards in many regions of the developing world. In sub-Saharan Africa countries livestock plays a crucial role in both for the national economy and the livelihood of rural communities (CSA, 2008). Agriculture is the mainstay of the Ethiopian economy, and livestock is an integral part of the country's agricultural system. Despite its importance to a large part of the population and the economy at large, the livestock subsector has remained untapped (Asfaw, 1999). Ethiopia is believed to have the largest livestock population in Africa (CSA 2013). An estimate indicates that the country is a home for about 7 million horses, donkeys, and mules (CSA, 2008), 54 million cattle, 25.5 million sheep and 24.06 million goats (CSA 2013).

Equines have greatest contribution in agriculture and transport sector of the Ethiopian economy. They 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. Despite their tremendous contribution less attention has been paid to equines in terms of health care and husbandry managements (Maarten, 2009). Of the major causes of economic losses and low productivity of livestock, the prevalence of a large number of diseases in the country is considered to be the major one (EARO, 1999).

Of the various bottlenecks of equines in Ethiopia, the attribute due to trypanosomosis takes the lion share. The subgenus Trypanozoon has diverse means of transmission, which includes a number of Glossina species, where they undergo a complex mode of development, mechanical transmission by blood sucking flies in which there is no development and during coitus (Zablotskij *et al*., 2003). These flagellates can be found in virtually every warm-blooded vertebrate species (Stuart *et al*., 2008). According to Getachew *et al*. (2004), trypanosomosis is prevalent in two main regions of Ethiopia that is, the North West and the South West regions. Six species of trypanosomes are recorded in Ethiopia and the most important trypanosomes, in terms of economic loss in domestic livestock are the tsetse transmitted species: *T. congolense*, *T. vivax* and *T. brucei*. For the

closely related *T. brucei* subspecies, *T. b. rhodensiense*, which causes human sleeping sickness, cattle can be a reservoir host. The other trypanosome species of economic importance are *Trypanosoma evansi* of camels and *Trypanosoma equiperdum* of horses (Abebe, 2005).

Dourine caused by *Trypanosoma equiperdum*, a disease in equines, characterized by edematous lesions of the genitalia, fever, cutaneous eruptions, incoordination, facial paralysis, ocular lesions, anemia, and progressive emaciation (Barrowman, 1976; Luckins, 1994). Diagnosis of equine trypanosomosis caused by the subgenus Trypanozoon commence with the observation of clinical signs and symptoms however, further diagnosis requires demonstration of the parasite, serological, biochemical, and molecular tests. In Ethiopia little and fragmented studies were conducted with regard to Dourine based on clinical signs (Dagnachew *et al*., 1980), serologically by ELISA and CFT (Alemu *et al*., 1997), by ELISA, CATT/T. evansi and Latex/ T. evansi (Hagos *et al*., 2010) and molecular by PCR (Clausen *et al*., 1999).

Equines are considered to be the only natural host of *T. equiperdum* (Claes *el al.,* 2005). Horses are very susceptible to *T. equiperdum* and usually die at the end of a chronic disease that may last for 1–2 years. Occasionally, acute forms that lead to death in 2–3 months are seen in thoroughbred horses. Donkeys and mules, despite being susceptible to infection, develop a mild syndrome or remain asymptomatic. The incubation period in horses ranges from 1 week up to 6 months (Taylor and Authié, 2004). It is apparent that the disease, being transmitted in the act of coition, should show the principal lesions in the genital organs. But in all chronic cases of dourine, besides the lesions in the sexual organs pronounced derangements of peripheral nerves and the central nervous system are present, manifested by paralysis of nerves and atrophy of various groups of muscles. As trypanosomes can be found neither in the central nervous system nor in the peripheral nerves it must be assumed that the trypanosomes elaborate poisonous products or toxins which are responsible for the lesions (Robert, 1919).

The disease in horses is chronic, persists for one or two years and is generally divided into three phases, although the clinical course can vary considerably under different conditions. The first period is characterized by oedema, tumefaction and damage to the genitalia, and begins one to two weeks after infection. The second stage of disease is pathognomonic for dourine. In this period, typical cutaneous plaques or skin thicknesses can occur, with sizes ranging from extremely small to hand sized (Claes *el al.,* 2005). Interestingly, these plaques have also been observed sporadically in animals infected with *Trypanosoma evansi* (Brun, *et al*., 1998). The third phase of dourine is characterized by progressive anemia, disorders of the nervous system mainly paralysis of the hind legs and paraplegia and, finally, death (Claes *el al.,* 2005). Later in infection, the neurological signs worsen, leading to a paralytic syndrome. Polyneuritis and neuronal degeneration are the main microscopic lesions. Animals that die from dourine are usually severely emaciated and anemic (Taylor and Authié, 2004).

Pathology in tissues is associated with the relative ability of the parasites to invade extra-vascular spaces and organs. For example, *T. congolense* remains confined to the vascular system and *T. vivax*, although primarily a vascular parasite, has also been found in extravascular locations, but trypanosomes of the *Trypanozoon* group (*T. b. brucei, T. evansi* and *T. equiperdum*) are distributed in both the circulation and in the tissues (Taylor and Authié, 2004). *T. equiperdum* differs from other trypanosomes in that it is primarily a tissue parasite that rarely invades the blood; so it is extremely difficult to detect the parasite in the body fluids of infected horses (Claes *et al.*, 2005).

According to Gari *et al*. (2010), isolation of the trypanosomes causing dourine would give an opportunity to conduct sexual transmission study, pathological, and pharmacological study of the parasite giving bright hope for the control of the disease in the area of dourine suspected districts of Arsi-Bale highlands of Oromia Regional State, Ethiopia. A careful perusal of the literature on this disease reveals the fact that in most articles on dourine the clinical picture; etiology, symptoms, and treatment receives more attention than the pathological phase, the microscopic changes, in Ethiopia.

Therefore the objectives of this research are:

- to describe the neurologic signs and the pathologic findings in the nervous system of horses naturally affected by dourine; and
- to reveal the presence of the parasite in the nervous tissue or cerebrospinal fluid or in both.

## 2. LITERATURE REVIEW

## 2.1. Trypanosomes

### 2.1.1. General Morphological Characteristics

Trypanosomosis is a protozoan disease of both man and animals. The causative organisms' trypanosomes are strictly parasitic, flagellated and elongated spindle-shaped protozoa with an average length of 20-30 μm and a width of 1.5 to 3.5 μm. The single-copy organelles in the trypanosome cell (i.e. the flagellar pocket, flagellum, kinetoplast and nucleus) are concentrated between the posterior end and the centre of the cell. Morphological features like length, shape, presence or absence of free flagellum and position of kinetoplast are useful in the differentiation of the trypanosomes species (Matthews, 2005).

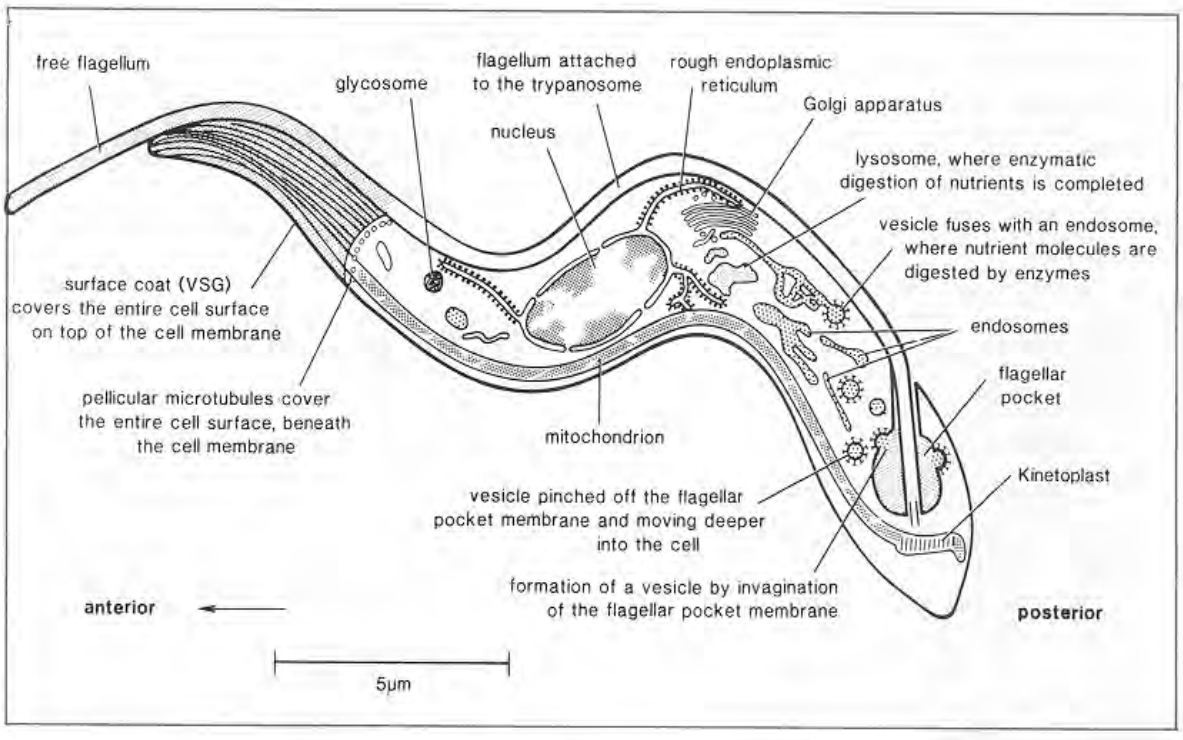


**Figure 1:** Schematic diagram of a trypanosome, illustrating the major organelles (https://www.ilri.org/InfoServ/Webpub/fulldocs/Ilrad88/Trypanosomiasis.htm)

All the species possess a flagellum, which arises at the posterior end of the trypanosome from a basal body at the foot of the flagellar pocket. The flagellum runs to the anterior end of the body and is attached along its length to the pellicle to form an undulating membrane. Thereafter the flagellum may or may not continue as a free flagellum (Mulligan, 1970; Hoare, 1972). The most posterior structure is the mouth of the flagellar pocket. A flagellar pocket is formed by an invagination of the plasma membrane at the position where the flagellum emerges from the cell. The flagellar pocket is the principal site of endocytosis and exocytosis. Therefore, most of the exchange processes with the host environment take place through the flagellar pocket (Overath and Engstler, 2004).

### 2.1.2. Classification and Taxonomy

Trypanosomes belong to the phylum of Sarcomastigophora, class Zoomastigophora, order of Kinetoplastidae, family of Trypanosomatidae and genus of *Trypanosoma*. The genus *Trypanosoma* is divided into two sections, the *Stercoraria* and *Salivaria* according to characteristics of their development in the insect vectors and mode of transmission (Hoare, 1972). The section *Stercoraria* contains genera in which the parasite completes its development in the "posterior station" of the gastrointestinal tract where trypanosome appears in the faeces of the vector, and its transmission is by contamination. The most important representative of the *Stercoraria* is *T. cruzi*, the causative agent of Chagas' disease in humans. In the section *Salivaria*, the development in the vector is completed in the "anterior station" and transmission is by inoculation of the trypanosomes. Trypanosomes in this group are usually pathogenic. *Salivarian* trypanosomes are thought to have originated from the tsetse (*Glossina* spp) belt in Africa with eventual spread to other continents accompanied by switching to other means of transmission (Stephen, 1986).

The main pathogens of the section *Salivaria* fall into three subgenera: *Duttonella*, *Nannomonas* and *Trypanozoon*. Subgenus *Trypanozoon* includes the pathogenic species *T. evansi*, *T. brucei* and *T. equiperdum*. *T. brucei* is further divided into three subspecies, the animal pathogen *T. b. brucei* and the trypanosomes responsible for human sleeping sickness *T. b. gambiense* and *T. b. rhodesiense* (Gibson, 2007). *T. b. rhodesiense* can be

distinguished from *T. b. brucei* by a single gene called serum resistant associated (SRA) gene which is found only in *T. b. rhodesiense* (Gibson, 2002). Subgenus *Trypanozoon* has diverse means of transmission, which includes "cyclical transmission" by blood-sucking tsetse flies where in they undergo a complex mode of biological development, "mechanical transmission" without biological development and sexual transmission during coitus (Stuart *et al*., 2008).

For more than a century *T. equiperdum*, *T. evansi* and *T. brucei* have been considered as separate species, based on differences in the mode of transmission, host range and pathogencity (Hoare, 1972). However, various studies indicated that *T. equiperdum* is closely related to *T. brucei* and *T. evansi*. Lun *et al*. (2004) indicated that *T. equiperdum* is more closely related to *T. evansi* based on Random Amplified Polymorphic DNA (RAPD) suggesting that the two parasites could be one species with different means of transmission. The first evidence of their relatedness is by some similarities in the clinical signs such as edematous swelling (Stephen, 1986). Li *et al* (2006) used amplification by PCR of a sequence of NADH dehydrogenase in the maxicircle kDNA to distinguish *T. b. brucei* and *T. equiperdum* from *T. evansi*. Nevertheless, his findings have been claimed not to differentiate between *T. b. brucei* and *T. equiperdum* as dyskinetoplastic strains of *T. equiperdum* exists. *Trypanosoma equiperdum* and *T. evansi* have been compared regarding their ultrastructure, mammalian hosts, way of transmission, pathogencity, diagnosis and treatment, biochemical and molecular characteristics. Electron microscopic examination revealed no ultra structural differences between the two species except that there were more coated vesicles in the flagellar pocket for *T. equiperdum* (Brun *et al*., 1998).

Biological, biochemical and molecular studies indicate many similarities between *T. evansi* and *T. equiperdum*. The most prominent differences between the two species are the presence of maxicircles in *T. equiperdum*, which are missing in *T. evansi*, and the route of transmission (Brun *et al*., 1998). Hence, the taxonomic classification of *T. evansi* and *T. equiperdum* remains unanswered. Thus, neither parasitological nor serological tests can make a clear distinction between *T. equiperdum*, *T. evansi T. b. brucei,* and

infections in solipeds. The clinical signs of dourine, chronic surra and chronic nagana are very similar and preclude correct differential diagnosis. Species-specific molecular tests to distinguish *T. equiperdum* are not available (Bishop *et al*, 1995; Claes, 2003). However, at least for *T. evansi* a species specific molecular detection technique RoTat 1.2 PCR exists (Holland *et al*., 2001; Claes, 2003).

**2.2. Equine Trypanosomes**

The equines are exposed to various bacterial, viral and parasitic diseases that may affect the health or even prove fatal. Of these, protozoan infections are important as these diseases affect the productivity and performance and even sometimes lead to death. One such important health hazard in the form of protozoan parasite is trypanosomes causing trypanosomosis in equines. Trypanosomosis, most widely distributed of the pathogenic animal protozoan infection, is caused by different species of trypanosomes that includes *Trypanosoma evansi, Trypanosoma brucei, Trypanosoma congolense, Trypanosoma vivax, Trypanosoma equiperdum* etc. Apart from horses, the disease affects a wide range of other principal hosts including, camels, buffaloes, cattle, dogs, elephants, pigs, cats, tapirs, capybaras, deer etc. The clinical picture of trypanosomosis is manifested as anaemia, fever, oedema of limbs and genitalia, petechial hemorrhages in conjunctiva (Urquhart, 1985).

Equines are particularly susceptible to infection with *T. evansi* and *T. brucei* but natural infection of *T. congolense* and *T. vivax* infection are rarely seen in horses (Kihurani *et al.* 1994). Traditionally, the subgenus of Trypanozoon comprises three socio‑economically important and highly pathogenic African trypanosomes, i.e., *Trypanosoma brucei*, *T. evansi* and *T. equiperdum* (Hoare, 1972). In horses, pathogenic trypanosomes like *T. evansi, T. equiperdum,* and *T. brucei* are considered to cause surra, dourine and nagana respectively (Claes *et al.,* 2003).


**Figure 2:** Distributions of Trypanozoon in Ethiopia (Abebe, 2005).

## 2.3. Dourine

### 2.3.1. Origin and Synonyms

Dourine, caused by *T. equiperdum* Doflein, 1901, has been recognized as a disease of breeding solipeds for many centuries (cited by Laveran and Mesnil, 1912). However, 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). 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).

### 2.3.2. Host Range, Distribution and Transmission

*Trypanosoma equiperdum* has been reported to infect horses, donkeys and mules. There is no natural reservoir of the parasite. Horses usually die from infection without treatment, whereas the infection may occur in donkeys and mules without obvious clinical signs. Zebras have tested positive by serology, but there is no conclusive evidence of infection. Various laboratory animals, including rats can also be infected. It is noted that murine-adapted clones of *T. equiperdum* can cause acute infection like *T. evansi* when passaged through mice, rats, rabbits, horses and dogs. Domestic animals such as sheep and goats infected with murineadapted strain of *T. equiperdum* produce the clinical manifestations of dourine (Brun *et al.*, 1998).

Dourine was once widespread during the times when the horse was militarily, economically and agriculturally important. It was of great concern in the United States and Canada at the beginning of 20th century. Nowadays, Western Europe, Australia and the United States are considered to be free from dourine (Claes, 2003). The latest official reports of dourine (i.e. CFT positive cases) were in China, Kazakhstan, Kyrgyzstan, Pakistan, Ethiopia, Botswana, Namibia, South Africa, Brazil, Italy and Germany. However, due to possible cross-reactions in the CFT it is difficult to conclude that seropositive animals are real *T. equiperdum* cases (Zablotskij *et al*., 2003).

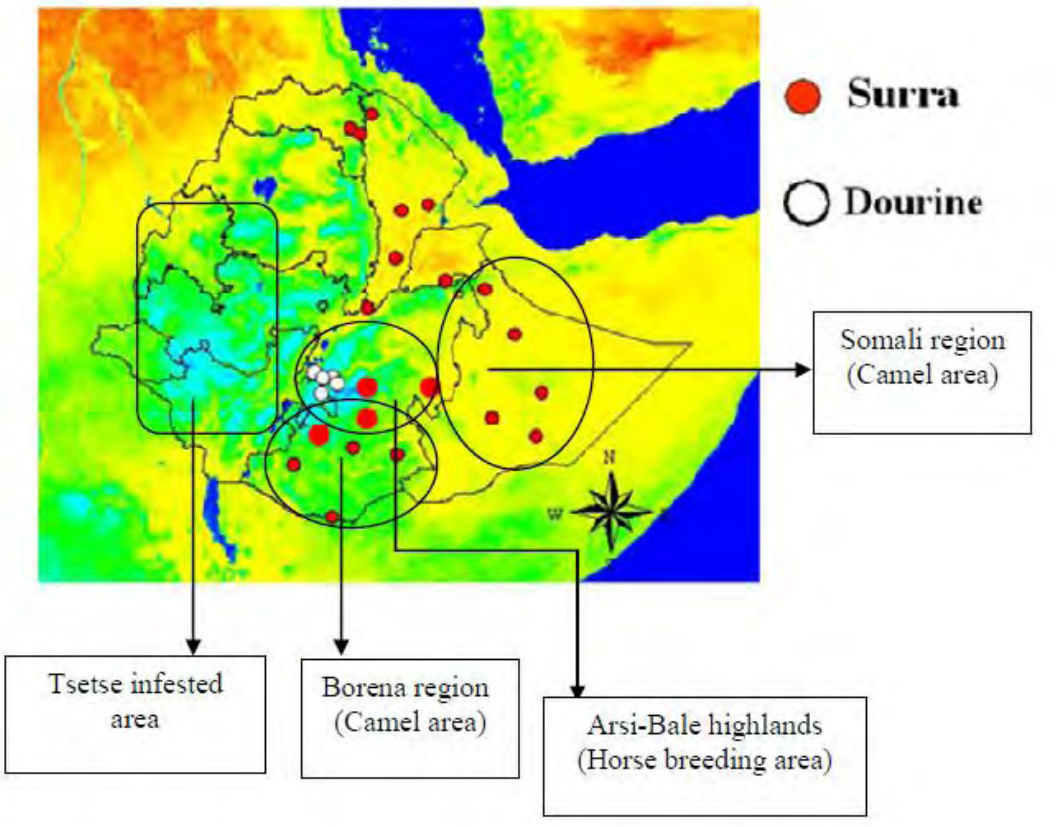


**Figure 3:** The distribution of dourine and surra in Ethiopia. The tsetse belt is localized in the south and south western parts of the country. Surra is a major disease in camel rearing areas of the south, east and north eastern parts of Ethiopia (Hagos *et al.*, 2010).

Unlike other trypanosomal infections, dourine is transmitted almost exclusively during coitus. The infection is more commonly transmitted from stallion to mare, facilitated by

the presence of the parasite in the seminal fluid and mucous exudates of the penis and its sheath. From the infected mare, the infection is transmitted to the stallion due to the presence of the parasite in the vaginal mucus. *T. equiperdum* can pass through intact mucous membranes and it is possible for foals to acquire infection by contamination of nasal or conjunctival membranes with the vaginal discharge. These infected foals can spread the organism when they mature. Other means of transmission may also be possible, but there is no evidence that arthropod vectors play any role in transmission. Intravenous or intraperitoneal experimental infections suggest that mechanical transmission by blood sucking flies cannot be excluded. However, the generally low number of parasites present in the blood for transient time does not favour this method to be main route of infection (Claes, 2003).

### 2.3.3. Course of Infection and Pathogenesis

The trypanosomes, which are present in the seminal fluid and mucous membranes of the genitalia of the infected donor animal, are transferred to the recipient during sexual intercourse. Parasites then may pass into the blood, where they are carried to other parts of the body. In typical cases, this metastatic invasion gives rise to characteristic cutaneous plaques. Dourine is often fatal, but spontaneous recoveries can occur (Hoare, 1972; Stephen, 1986). The incubation period, severity and duration of the disease vary considerably. In South Africa, the disease is typically chronic, usually mild and may persist for several years (Barrowman, 1976).

### 2.3.4. Clinical Signs

Generally the disease in horses is chronic, persists for one or two years and is generally divided into three phases, although the clinical course can vary considerably under different conditions: (genital oedema, plaques and skin eruptions, and neuropathological signs) (Claes *et al.*, 2005).

*Primary stage (Genital oedema)*

In stallions, the first symptoms are oedema of the prepuce and glans penis. The swelling may spread to the scrotum, perineum, ventral abdomen and thorax. Vesicles or ulcers may be seen on the genitalia, when they heal, these ulcers can leave permanent scars. Orchitis may occur and cause irritation, where the stallion constantly draws and retards the penis. Paraphimosis may also occur. Early symptoms in mares consist of vaginitis, with mucopurulent discharges. The vulva becomes oedematous; this swelling may extend along the perineum to the ventral abdomen and mammary gland. Vulvitis, vaginitis with polyuria and signs of discomfort may be seen. The genital region, perineum, and udder may become depigmented. Abortion can occur with more virulent strains (Hoare, 1972; OIE, 2008).

*Secondary stage (Plaques and skin eruptions)*

This stage, known as stage of Urticaria, is marked by distinct, raised round or oval shaped patchy eruptions called "plaques", that appear on the skin in both sexes. Oedematous patches, also called "silver dollar plaques", up to 5-8 cm diameter and 1 cm thick may appear on the skin, particularly over the neck, shoulders, ribs and thighs, and usually last for 3-7 days, and is considered to be pathognomonic for dourine (OIE, 2008).

*Tertiary stage (Neurological signs)*

The final phase known as stage of paralysis is characterized by disorders of the nervous system. Initially these signs consist of restlessness and the tendency to shift weight from one leg to another followed by progressive weakness and in coordination, and ultimately, paralysis (mainly of the hind legs) and death occur. Other clinical signs include progressive anaemia seen by increasing pallor of the mucous membranes of the eyes and mouth, conjunctivitis, keratitis, intermittent fever and emaciation (Hoare, 1972, Stephen, 1986, OIE, 2008).

2.3.5. Diagnosis

Diagnosis of equine trypanosomosis caused by the subgenus Trypanozoon commence with the observation of clinical signs and symptoms however, further diagnosis requires demonstration of the parasite, serological, biochemical, and molecular tests. In Ethiopia little and fragmented studies were conducted with regard to Dourine based on clinical signs (Dagnachew *et al*., 1980), serologically by ELISA and CFT (Alemu *et al*., 1997), by ELISA, CATT/T. evansi and Latex/ T. evansi (Hagos *et al*., 2010) and molecular by PCR (Clausen *et al*., 1999).

Clinical signs of dourine can provide a strong indication of the presence of the disease, as can its chronic evolution, but confirmatory diagnosis is needed. It is extremely difficult to detect the parasite in the body fluids of infected horses; therefore, diagnosis of *T. equiperdum* infection is based on serological evidence. Despite the development of antibody and antigen enzyme-linked immunosorbent assays for *T. equiperdum* (Katz *et al*., 2000), CFT remains the only internationally recommended test (Watson, 1915), although it does not distinguish clearly among *T. equiperdum, T. evansi* and *T. b. brucei* (Robinson, 1926). Indeed, because possible cross reactions with *T. evansi* and *T. b. brucei* might occur, these parasites cannot be distinguished from *T. equiperdum* unless the test samples originate from regions that are free from *T. evansi* and *T. b. brucei* (Claes *et al*., 2005).

Given that the morphology of the blood stream forms of *T. b. brucei*, *T. equiperdum* and *T. evansi* are so much alike and that it is difficult to differentiate them, great effort has been placed in biochemical and molecular approaches to differentiate their genetic characteristics. Originally, a wide range of parasitological and serological methods were employed to identify the infections caused by these three species. Parasitological ones mainly include classical direct microscopic detection of causative agents in the host blood, haematocrit centrifugation technique (also referred to as "WOO's technique" or "HCT") (Woo, 1969), mini‑anion exchange centrifugation technique (mAECT)

(Lumsden *et al*., 1979) and animal inoculation (xenodiagnosis) but which is not suitable for field work (Holland *et al*., 2001; Monzon *et al*., 1990).

Also, a series of techniques based on PCR have also been used, for example, minisatellite DNA analysis, (Biteau *et al*., 2000; MacLeod *et al*., 2000; MacLeod *et al*., 2001) amplified fragment length polymorphism (AFLP), (Agbo *et al*., 2002; Masiga *et al*., 2000) multiplex‐endonuclease genotyping (MEGA), (Agbo *et al*., 2003; Claes *et al*., 2003) mobile genetic elements (MGE)‐PCR, (Li *et al*. 2005) simple sequence repeat (SSR)‐PCR, (Li *et al*., 2005) and random amplification of polymorphic DNA (RAPD) (Li *et al*. 2005; Lun *et al*., 2004; Mathieu‐Daude *et al*., 1995). Although the simple and sensitive RAPD approach requires only very small sample amounts and no prior sequence information required, it is likely to create a good deal of characteristic and informative data (Lun *et al*., 2004). Combining RAPD analysis with MEGA technique, Claes *et al.* (2003) hypothesized that Chinese *T. equiperdum* stocks were much closer to *T. evansi* than to *T. brucei*, by analyzing the profiles of 10 *T. equiperdum*, 8 *T. evansi* and 4 *T. brucei* stocks, although they did not examine the maxicircle kDNA as it was probably inappropriate (Claes *et al*., 2003).

2.3.6. Pathology

Anaemia, cachexia and genital oedema are often seen at post-mortem. The oedema, which may be indurated, can extend to the ventral abdomen. Gelatinous exudates can often be seen under the skin. In stallions, the scrotum, sheath, and testicular tunica may be thickened due to the pervading exudates. In mares, exudates may thicken the vulva, vaginal mucosa, uterus, bladder, and mammary gland. The lymph nodes, particularly in the abdominal cavity are hypertrophied, softened and in some cases, haemorrhagic (OIE, 2008).

It is apparent that the disease, being transmitted in the act of coition, should show the principal lesions in the genital organs. But in all chronic cases of dourine, besides the lesions in the sexual organs pronounced derangements of peripheral nerves and the

central nervous system are present, manifested by paralysis of nerves and atrophy of various groups of muscles. As trypanosomes can be found neither in the central nervous system nor in the peripheral nerves it must be assumed that the trypanosomes elaborate poisonous products or toxins which are responsible for the lesions (Robert, 1919).

The clinical and pathological changes produced by strains of *T. equiperdum* maintained by serial passage or in the laboratory are different from those of the natural disease. Dourine in South Africa occurs as a sub-acute or chronic disease which frequently shows no clinical manifestations in affected horses. Passaged or laboratory maintained strains give rise, however, to an acute or peracute condition in horses or laboratory animals, the macroscopic lesions differing from those observed in naturally-infected horses (Parkin, 1948 ; Lavergne *et al*., 1969 ; Moulton, 1974).

The term "'oedematous" has been used to describe the clinical signs of the disease. Many horses, and especially mares, however, exhibit only transient oedema or none at all. In these cases the infection causes a progressive emaciation and, as the parasites cannot be demonstrated in the blood, the term “interstitial" seems more descriptive of this form (Barrowman, 1976). Most of the clinical signs and lesions confirmed literature description of the third phase of dourine (Caporale, 1946), with particular regard to the cachexia, anaemia, muscular hypotrophy, ataxia and lack of coordination of the hindquarters, ptosis of the lower lip, skin oedematous plaques and peripheral oedema. The presence of nervous signs without sensory alterations seems to confirm the tropism of *T. equiperdum* for the peripheral rather than the central nervous system, in contrast with other trypanosomes (Berlin *et al*., 2009; Barrowman, 1976).

The clinical examination of infected mare appeared cachexic with muscular hypotrophy, dehydration and general lymph node enlargement. The skin becames hypoelastic with numerous grazes around the iliac crest, and develop a depigmented area in the perineal zone, peripheral oedema and an oedematous plaque. Cutting of the oedematous plaque has been revealed a mild oedema underneath the derma. There will also be abundant synovial fluid in the tarsometatarsal joints. The udder of infected mare became warm and

hard, but not sore and also no lesions observed on cutting the udder. However, there might be a serum-like mammary secretion, which could not be sampled during autopsy (Pascucci *et al*., 2013). There is no lesions develop onto the parenchymatous organs, except the spleen, which is congested, with subcapsular blood suffusion and bloody nodules the size of a grain of rice in relief with respect to the capsule. On cutting of affected spleen, the connective tissue might be thickened due to hyperplasia of the white pulp. Neurological examination of the affected horse will reveal difficulty in walking, with marked ataxia of the hindquarters and spreading of the limbs. The body lymph nodes such as iliac, supramammary and popliteal lymph nodes become reactive (Scacchia *et al*., 2011). On histological examination, the affected spleen will reveal haemosiderin deposition; and the iliac, supramammary and popliteal lymph nodes show non-specific reactivity with hyperplasia of the plasma cells, a sign of increased haemolymphatic activity (Pascucci *et al*., 2013).

The oedematous plaque showed a characteristic picture of pustular dermatitis, particularly severe around the lesion, with severe inflammation and vacuolar degeneration extending to the deepest layers of the skin, with involvement of the cutaneous adnexa and perivascular plasma cell inflammation. There were exudates of cell detritus in the same area, mainly eosinophils and the bodies of free parasitic protozoa, in a picture described as “trypanosomal sand”. The udder was affected by interstitial plasma cell mastitis. There were microfoci of perivascular inflammation in the uterine submucosa. No dourine-specific lesions were found in the heart, bladder or other areas of the genital tract. No inflammatory lesions were found in the central nervous system, with the exception of the occasional eosinophil in the cerebellum, with only mild neuronal degeneration (Scacchia *et al*., 2011). The pustular dermatitis observed in skin lesions and corresponding histologically to “trypanosomal sand” just reported by Scacchia *et al*., (2011) is more deeply clarified here, and can be considered an original finding (Pascucci *et al*., 2011).

The occurrence of the nervous form of the disease, superimposed on the oedematous or interstitial form, appears to coincide with the presence of the parasite in the CSF. The

nervous symptoms were characterized by a short period of hyperaesthesia followed by a prolonged period of hypoaesthesia lasting until death. The motor affectation was one of incomplete ascending motor paralysis of variable duration. The symptoms originated in the perineum and hind-limbs and eventually involved the forelimbs (Barrowman, 1976).

The microscopic examination of the brain showed no appreciable changes in the nerve cells, the supporting tissue, or in the blood vessels. In the cervical, anterior, and middle dorsal portions of the spinal cord lesions could not be demonstrated even with the most sensitive methods of staining; and in the posterior dorsal portion the lesions were very slight, gradually increasing in the lumbar enlargement and becoming most marked in the sacral region. Degeneration in the sensory ganglion cells was present in all stages, varying from the beginning stage of chromatolysis that could barely be detected by the Nissel method alone to advanced degeneration and disintegration of plasmolysis that was brought out by less sensitive methods (Robert, 1919). The microscopic lesions of the nervous system were found to involve primarily the lumbar and sacral regions of the spinal cord and the sciatic and obturator nerves.

The histopathological change in these tissues primarily is one of radiculitis and polyneuritis, involving cellular infiltration and degenerative changes of the spinal nerves and spinal ganglia, extending along the larger peripheral nerves, notably the sciatic nerves. In contrast to the involvement of the central nervous system in *T. gambiense* infections in man (Van Boegart and Janssen, 1957) and experimentally prolonged *T. brucei* infections in horses (McCully and Neitz, 1971), histopathological changes in the central nervous system have not been recorded in natural *T. equiperdum* infections, except that Laveran and Mesnil (1907) found the lesion of the spinal nerves extending into the posterior columns of the spinal cord (Barrowman, 1976).

A possible explanation for the site of these nervous lesions in horses so affected is to be found in the mechanism of drainage of CSF which is known to contain trypanosomes and their extracellular products. If the trypanosomes, their extracellular products or their antigenic components in the CSF drain along the spinal nerves, they could produce a

direct effect or elicit a host response at the sites where the nerve tissue is no longer protected by the mechanism of the blood-brain-barrier. At this point the toxic or immunological stimulus would be at its greatest (Barrowman, 1976).

Within the central nervous system the host tissues are probably protected by the ependymal cells of the blood-brain-barrier. The alternative to this was given by Laveran and Mesnil (1907) in their suggestion that: " the change starts in one seat of primary infection, extends to the inguinal glands, thence presumably by the pelvic lymphatics to the lumbosacral plexus and the posterior lumbosacral roots to the central nervous system; consequently the lower part of the spinal cord and especially the posterior column is first and most affected." Although this route would be contrary to the flow of lymph, the motility of the parasites makes it feasible (Barrowman, 1976).

# 3. MATERIALS AND METHODS

## 3.1. Study Area

The study was conducted by visiting three purposively selected horse-breeding districts of the Arsi–Bale highlands of Ethiopia, namely Dodola, Assassa and Adaba. The Arsi–Bale highlands are found in the Oromiya Regional State southeast of the country where Asela and Robe, the capitals of Arsi and Bale zones, are located 175 and 430 km away from Addis Ababa respectively. Topographically, the altitude ranges from 500 to 4,130 meters above sea level, where a central plateau predominates with a narrow lowland area. Three climatic zones, including an arid, tropical highland and tropical forms, are known to exist. The area experiences a bimodal rainfall occurring from July to October and April to May. Average annual temperature of 20–25°C and rainfall of 200 mm in the lowlands as well as 10–15°C and a rainfall of 400 mm in the highlands are recorded. Agriculture is the mainstay of the livelihood of peoples and the leading economic activity of the area with a mixed farming system covering 90% of the total agricultural activities with crop–livestock production (Arsi-Bale Zone Agricultural and Rural Development Office, 2009).

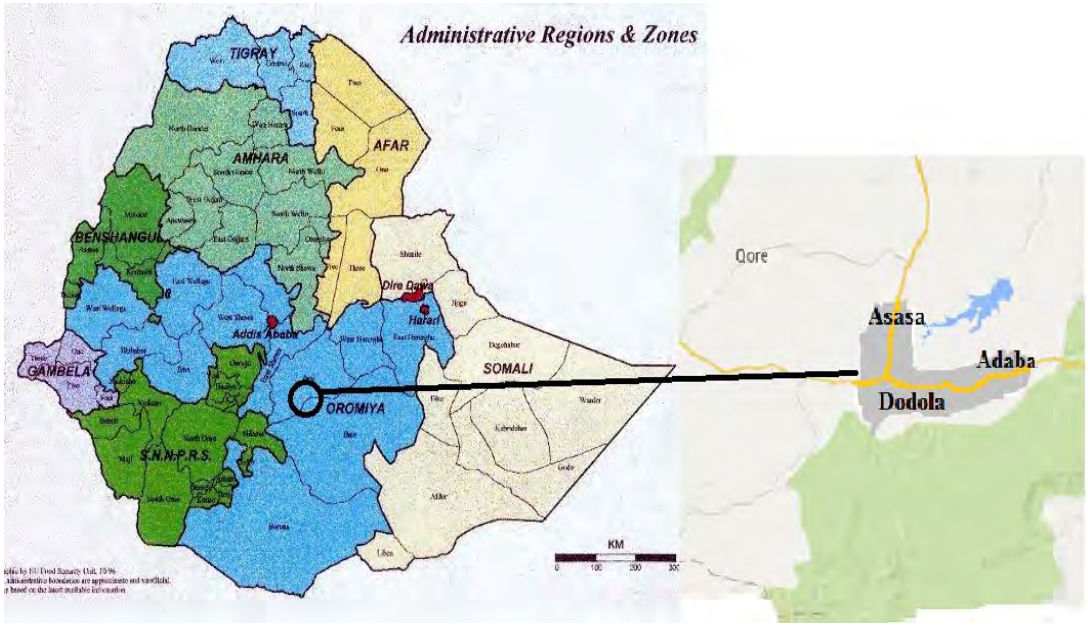


**Figure 4**: Map of Ethiopia showing study area (http://mapsof.net/map/ethiopia-regions-map)

### 3.2. Study Population

The study animals include sexually mature horses suspected with *T. equiperdum* infection under natural condition. Prior to the post mortem examination, the infected state of the animals were established by a series of parasitological and serological tests (see below) during a period of isolation.

### 3.3. Study Design

The study was cross sectional (observational) study that conducted from the beginning of March 2015 to the end of June 2015. A total of 20 local breed of horses, which were kept under traditional management system of free grazing and clinically positive for dourine, were considered as study animals and out of them two highly seroposetive mares purchased for detail and thorough post mortem examination and histopathology. Animals with history of recent trypanocidal treatment and castrated stallions were purposively excluded from sampling.

### 3.4. Methodology

#### 3.4.1. Clinical Examination

Clinical examination was performed on affected horses that were coming to the clinics of the three selected horse-breeding districts of the Arsi–Bale Highlands. According to Claes *el al.* (2005), the disease in horses is chronic, persists for one or two years and is characterized by oedema, tumefaction and damage to the genitalia, and begins one to two weeks after infection. The pathognomonic sign for dourine is typical cutaneous plaques, with sizes ranging from extremely small to hand sized. The last stage of dourine is characterized by progressive anemia, disorders of the nervous system mainly paralysis of the hind legs and paraplegia and, finally, death.

Based on the clinical signs, horses that showed the neurologic signs mainly paralysis of the hind legs, in addition to the pathognomonic signs in the genitalia, were selected for further diagnosis and histopathology.

3.4.2. Parasitological Tests

About 7.5 ml of blood samples were collected from 12 horses that were clinically suspected of dourine from their jugular vein using heparinized vacutainer tubes and venoject needles. The blood collection site was wiped with cotton wool soaked in alcohol. Capillary tube were filled up to three fourth volumes and centrifuged for 5 minutes in micro-centrifuges at 12,500 rpm, for parasitological Woo test. Subsequently, the capillary tubes were mounted in a special holder known as viewing chamber and examined microscopically at magnification of 10x at the buffy coat as described in (Woo, 1969; Reid *et al*., 2001) to look for live parasites. The tests were conducted at field level in Arsi-Bale highlands in Dodola veterinary clinic.

3.4.3. Serological Test

Whole blood samples collected for serological testing were allowed to clot over night at room temperature. Separated serum was filled in to serum cryogenic vials and stored at −20°C until testing. Card Agglutination Test for Trypanosomosis (CATT/ *Trypanosoma evansi*, which is a rapid direct agglutination test, that uses formaldehyde fixed, Coomassie stained, freeze-dried trypanosomes of *T. evansi* VAT RoTat 1.2) was employed to demonstrate the presence of antibodies in serum samples according to Claes *et al.* (2003). The tests were conducted at Dodola veterinary clinics. Positive results were determined at cut-off point dilutions 1:4 (Bajyana and Hamers, 1988; Verloo *et al*., 2000).

3.4.4. Post mortem Examination

Out of twelve horses thoroughly examined, two mares suffering from classical nervous signs and having strong positive for Card Agglutination Test for Trypanosomosis (CATT) were euthanized with sodium pentobarbital at 100 mg/kg dose through

intravenous injection for postmortem and Histopathological examination. The CSF (cerebrospinal fluid) was collected from the atlanto-occipital space as described by Furr and Andrews (2008) and stored at −20 °C until processing. Samples of CSF were centrifuged at 3,000 rpm for 15 min, where trypanosomes are detected at the bottom layer. Thick smears were prepared and stained with Giemsa solution for morphological examination under oil immersion. Gross lesions in the different parts of the nervous system were recorded. Histopathological samples were taken at thickness of 5-10mm from brain (cerebral cortex and cerebellum), spinal cord (cervical, thoracic, lumbar and sacral parts), and peripheral nerve (sciatic nerves). Tissue samples were fixed with buffered formalin, and transported to National Animal Health Diagnostic and Investigation Center (NAHDIC), for histopathology.

### 3.4.5. Histopathological Examination

Formalin fixed nerve tissues were trimmed at appropriate thickness. Tissues were dehydrated using ascending concentration of alcohols, cleansed by xylene and impregnated with molted paraffin wax. Following embedding with paraffin, tissues were sectioned at 5-µm thickness, (Lillie and Fulmer, 1976) and, stained with hematoxylin and eosin stains. Duplicate sections were stained with Giemsa for parasitological examination for the detection of the parasite in the nerve tissues. Slides were then examined under microscope.

### 3.4.6. Detection of Viral Growth in Cell Cultures

This has been performed to exclude viral diseases having similar neurological clinical signs such as equine infectious anemia and equine herpes virus 1 infection. A total of four spinal cord and sciatic nerve samples were collected from two mares and preserved in phosphate buffered saline (PBS) solution containing gelatin and antimicrobial agents and kept in refrigerator ($4^{o}$C) until and during transport in ice box to National Animal Health Diagnostic and Investigation Center (NAHDIC), for viral growth detection in cell culture.

*Viral culturing techniques*

Tissue samples were homogenized using mortar and pestle, centrifuged at 3400 rpm for 10 min and 0.5 ml of the supernatant was inoculated on the confluent BHK-21 cells and incubated at 37°C. Viral growth was recognized by the cytopathic effect (CPE), characterized by change in shape, cell detachment, fusion leading to syncytium formation, the presence of inclusion bodies and cell death (Gelagay *et al*., 2013).

### 3.5. Ethical Clearance

The study animals, horses, especially the two mares are euthanized based on the permission obtained from the Animal Research Ethical Review Committee, that provided the author an ethical clearance certificate with Ref. No. VM/ERC/004/03/07/2015, and Minutes No. and date of review: VM/ERC/004/07/015, 17/04/2015.

# 4. RESULTS

The present study was conducted on naturally infected mares of unknown duration.

## 4.1. Parasitological and Clinical Observations

A total of 20 naturally infected mares were studied. No rise in body temperature was observed in any of the infected animals. Different characteristic signs of dourine were observed in clinically sick horses (Figure 5). In four mares, vaginal discharge mainly of mucopurulent type with foul odour, oedema of the external genitalia and presence of depigmented scars over the external genitalia were the prominent signs observed in the genital form of the disease. In eight mares, frequent ulceration was observed, and there were ulcers on the labia and clitoris. Lameness in one or both legs, restlessness, partial dragging or stiffness of the hind legs, incoordination, asymmetrical posture and tendency to shift weight from one leg to another were the dominant signs observed in the nervous form of the disease. The left hind legs of the two slaughtered mares were often dragged on the ground. As the disease progressed, the difficulties in movement became prominent and the affected animals were not capable to move.

Both veterinarians and farmers observed that some horses with the nervous forms of the disease become paraplegic with marked muscular atrophy in the gluteal region followed by paralysis and finally death. In addition to all these clinical signs, dourine-suspected horses were frequently emaciated, weak and poor in body condition. The cutaneous form of the disease, which is mainly characterized by ‘urticarial plaques’, marked by distinct, raised round- or oval-shaped patchy eruptions were not observed in the present study. There was also no loss of appetite.


**Figure 5**: Weight loss (A) and oedema of the external genitalia (B) (Horse No. 1) and depigmented scar (C) (Horse No. 2)

### 4.2. Serological and Histopathological Examination

Out of the 12 sera tested with CATT 2 mares showed high positivity while 8 mares showed moderate seropositivity. Mares with high seropositive result were purchased for post mortem examination. There were no remarkable gross lesions in the brain, spinal cord and sciatic nerves in both mares slaughtered. No trypanosomes were detected in all Giemsa-stained smears (CSF) and tissue sections; and in blood samples under haematocrit centrifugation technique "WOO's technique".

Histopathological examinations of haematoxylin-eosin stained tissue sections of nervous tissue (cerebral cortex, cerebellum, spinal cord and sciatic nerve) showed microscopic lesions primarily on the lumbar and sacral regions of the spinal cord and the sciatic nerves. The primary lesions were degenerative changes (central chromatolysis) (figures 6, 7, 8, and 9) and neuronal necrosis (figure 6 and 8) of the spinal cord, and cellular infiltration (figure 10 and 11) and Wallerian degeneration (figure 10) of the sciatic nerve.

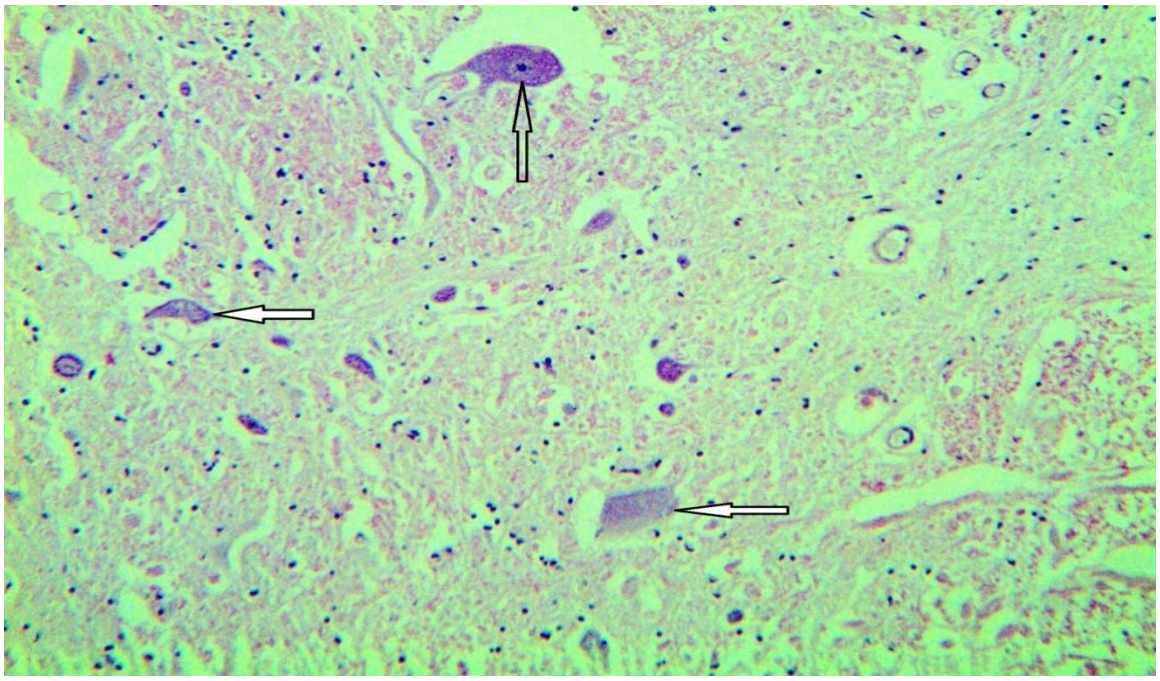

**Figure 6**: Lumbar region of spinal cord, Horse No. 2. Central chromatolysis (open arrows); shrunken, necrotic neuron (closed arrows). H&E stain 10x.

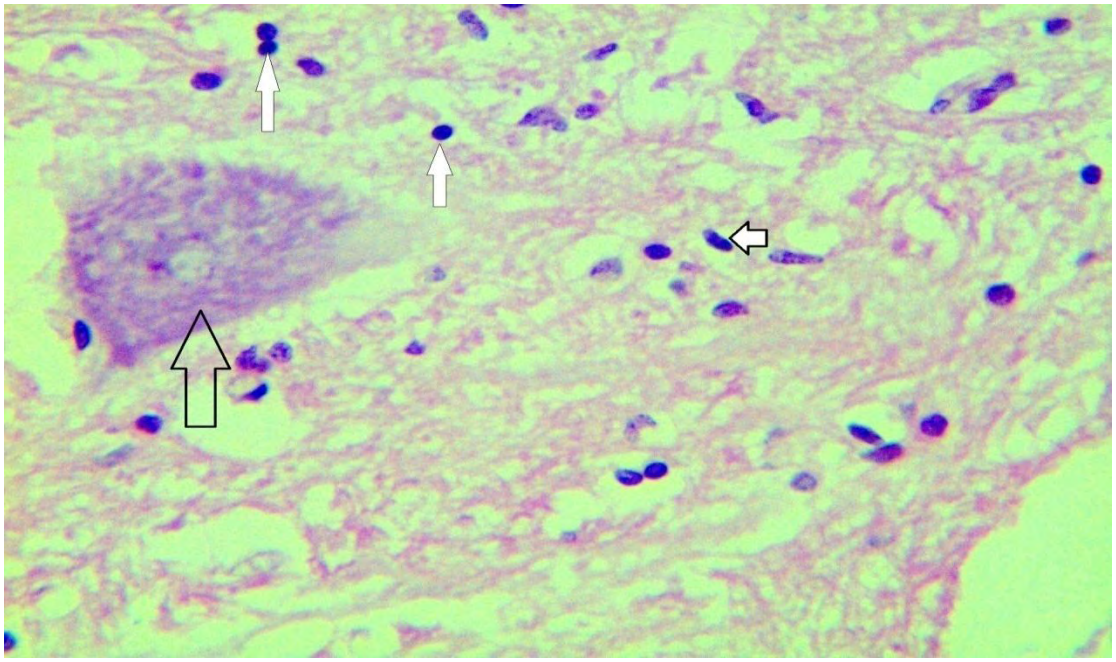

**Figure 7**: Lumbar region of spinal cord, Horse No. 1. Central chromatolysis (open arrow); Perineuronal satellite oligodendroglia (closed arrows) surround a degenerate neuron with condensed chromatin and little cytoplasm. H&E stain 40x.

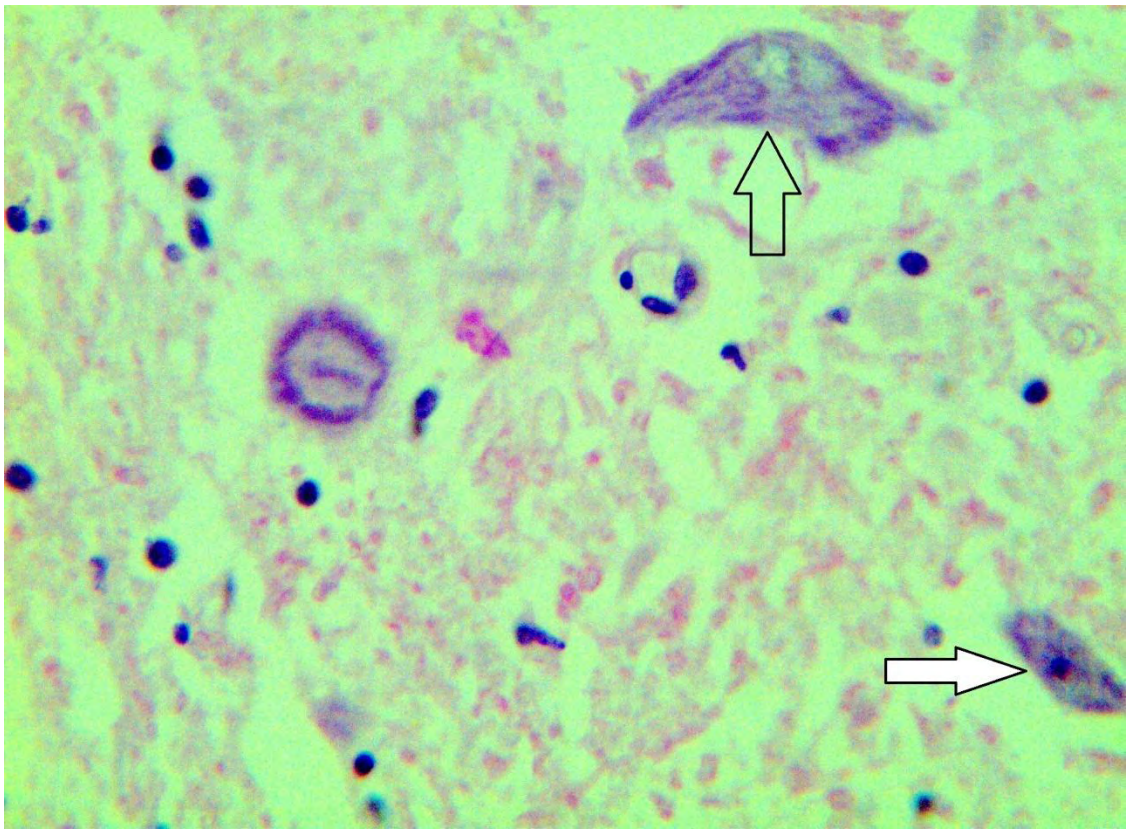

**Figure 8**: Sacral region of spinal cord, Horse No. 2. Central chromatolysis (open arrows); shrunken, necrotic neuron (closed arrows). H&E stain 40x.

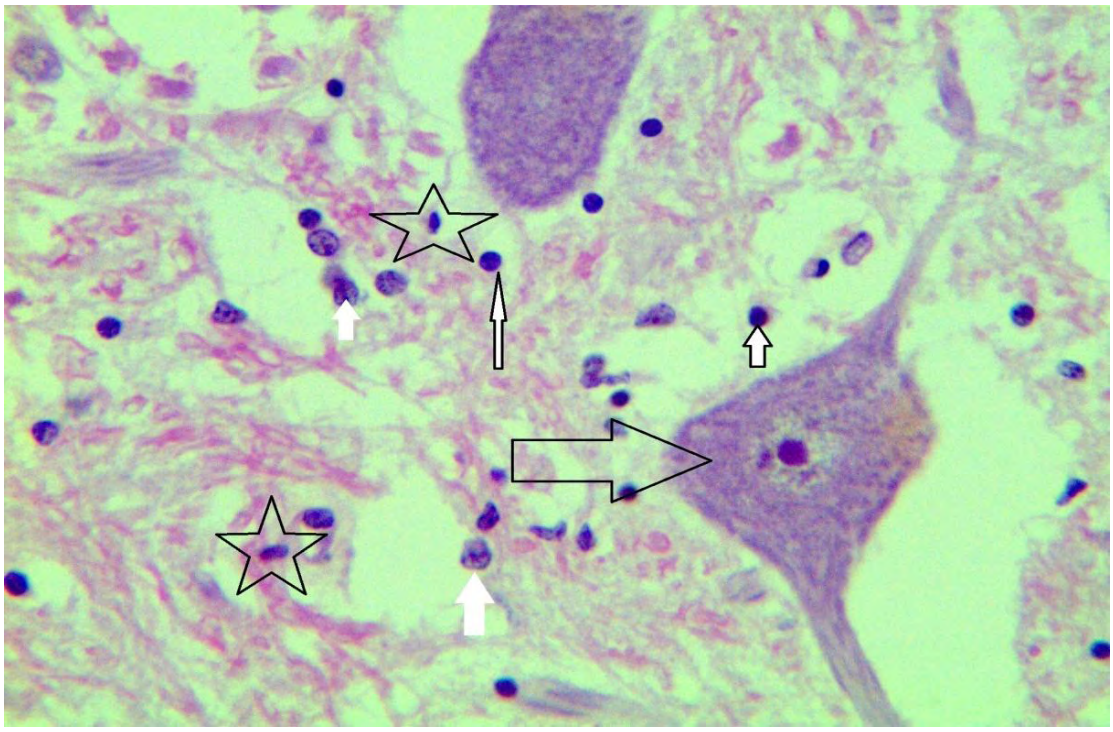

**Figure 9**: Sacral region of spinal cord, Horse No. 1. Central chromatolysis (open arrow); Perineuronal satellite oligodendroglia (closed arrows) surround a degenerate neuron with condensed chromatin and little cytoplasm; Astrocytes (white arrow) have larger nuclei (condensed chromatin) and the cell membrane and cytoplasm are rarely seen in non diseased conditions. Microglial cells with small, dense elongated nuclei *(inside star*). H&E stain 40x.

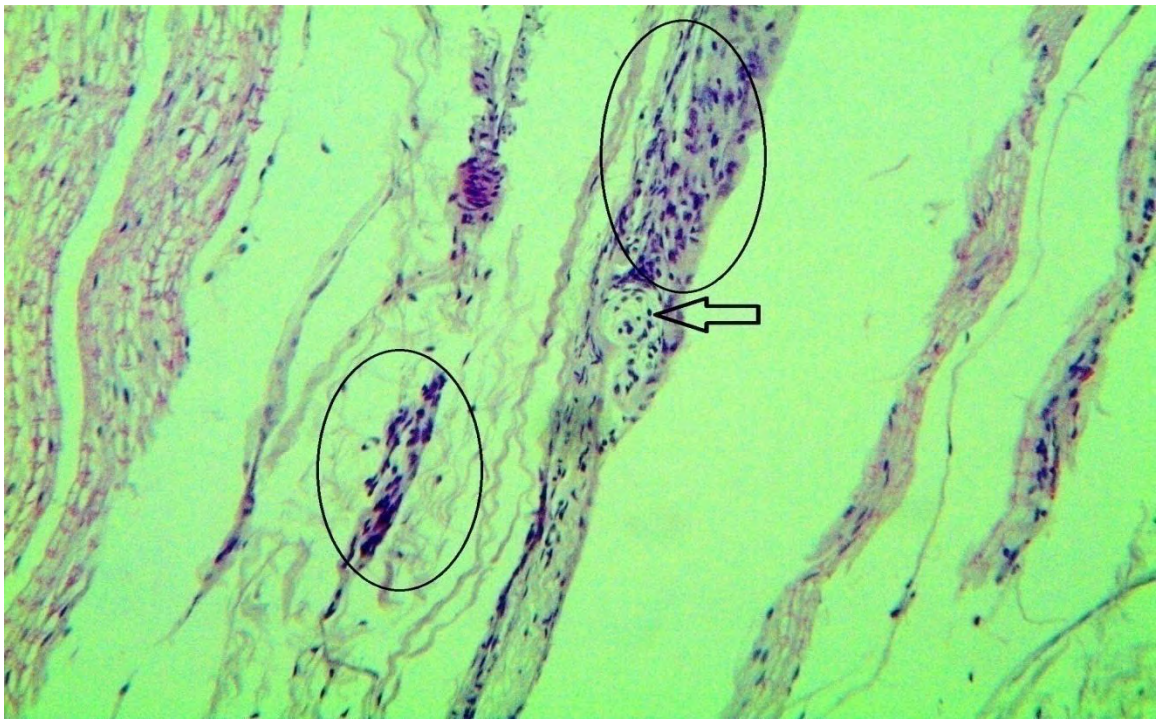

**Figure 10**: Left sciatic nerve, Horse No. 1. Wallerian degeneration with "digestion chamber" (open arrow) and cellular infiltrations (circled area). H&E stain 10x.

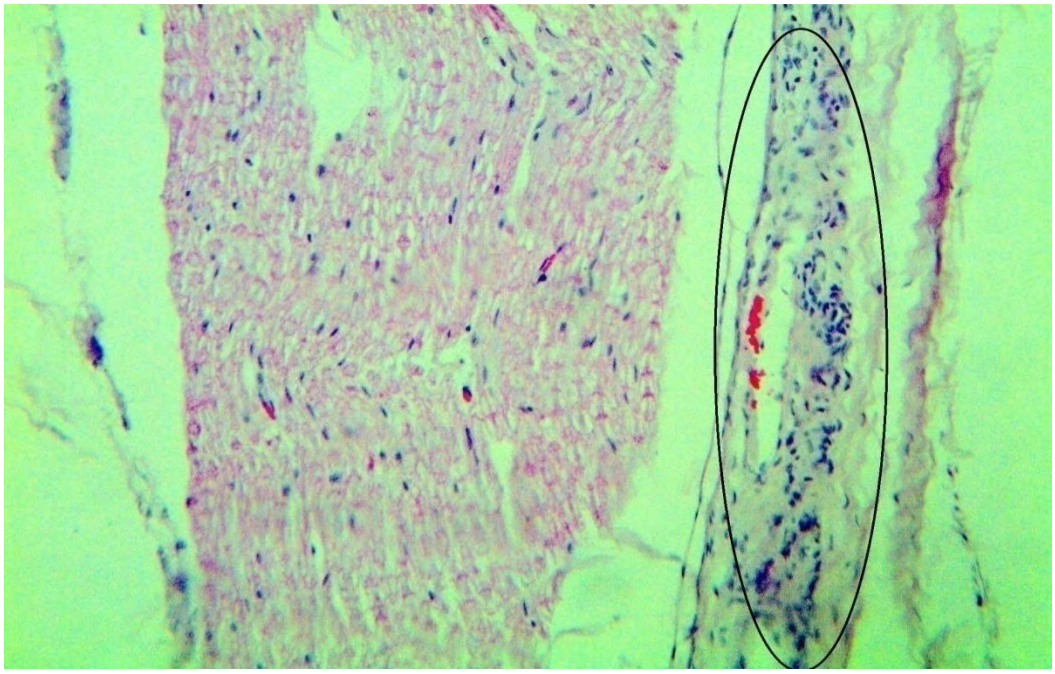

**Figure 11**: Left sciatic nerve, Horse No. 2. Cellular infiltrations in peripheral nerves (circled area). H&E stain 10x.

### 4.3. Detection of Viral Growth

Nervous tissues were cultured in the monolayer of BHK-21 cell line to detect the presence of viral growth; however, no cytopathogenic effects (CPE) were observed.

# 5. DISCUSSIONS

Data collected in this study demonstrate that most of the signs observed coincide with those reported in previous studies. The clinical picture of the disease corresponds closely to that described in earlier reports (Laveran and Mesnil, 1907; Walker, 1918; Watson, 1920; Claes *et al*., 2005).

Weight loss was observed in all animals, especially in 12 of the mares selected. There was no any appetite loss, in contrast with what would be expected from other debilitating diseases leading to the same degree of cachexia. It should be underlined that weight loss is one of the early signs that could lead the veterinarian or owner to suspect Dourine. No rise in body temperature was observed in any of the infected animals, which is in agreement with other studies (Barrowman, 1976; Coetzer and Tustin, 2004), but it is often reported in the literature (Brun *et al*., 1998; Claes *et al*., 2005).

Ventral oedemas were not seen in all observed mares and this may be attributed to the stage of the disease as most of them were in the third stage, but genital edemas were observed in few of animals. This could be explained because the horses were from natural infection and already showed signs of active disease on arrival at the veterinary clinic. Caporale (1946) suggested that the first stage of the disease was undetected or unreported at the holding. In addition, the route used for experimental infection was not the natural route of infection which is sexual transmission. Oedema of the mammary glands was not seen in all mares in the present study; however, Vulpiani *et al*. (2012) reported its persistence until the animal was euthanized when present.

The classic “silver dollar” plaque was not seen in the present study. According to Barrowman (1976), these lesions are rarely occur and transient in nature. These lesions are also difficult to differentiate from wheals triggered by other causes (allergic reactions to blood transfusion or insect bites) (Vulpiani *et al*., 2012), but they are considered pathognomonic by some authors (Brun *et al*., 1998; Claes *et al*., 2005), although their presence seems to be inconstant. Most authors report only the sporadic presence of this

sign (Walker, 1918; Watson, 1920; Barrowman, 1976; Claes *et al*., 2005), although it has been reported more frequently in outbreaks in Europe and North Africa (Coetzer and Tustin, 2004). Some attribute this difference to the *T. equiperdum* strain involved and others to the immune response. In any case, presence of wheals should lead to suspicion of Dourine infection (Vulpiani *et al*., 2012). Thus, although the plaque lesion may be pathognomonic, its rare occurrence, transient nature and possible confusion with other skin eruptions limit its diagnostic value.

Woo test showed no parasite in the blood samples taken from long-standing cases of unknown duration. This is supported by Barrowman (1976), in his report stated that an attempts to demonstrate the parasite in the blood of long-standing cases of unknown duration were unsuccessful; however, it has been possible to demonstrate the more prolonged presence of the trypanosomes in circulating plasma in horses whose actual infectious service date was known. Except Rouget (1896) and Gari *et al*. (2010) who reported the parasite in blood from horses suspected of dourine, most authors have experienced difficulty in demonstrating the parasite in naturally infected horses (Laveran and Mesnil, 1907; Watson, 1920; Khalilik, 1973) and have had only occasional success at the onset of the disease. Parkin (1948) recorded the more consistent finding of the parasite during the early stages of the disease in horses infected by sub-inoculation of blood.

In the present study, despite the absence of the parasite in the blood by parasitological (Woo test) test, out of 12 horses examined by serological test (CATT test) two mares' revealed strong seropositive results, but there was no any gross pathological lesions observed on the different part of spinal cord, brain and on the sciatic nerves.

There is very little published information on the pathology of dourine either natural or experimental infection in horses, especially on the nervous system of horses. In the current study 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 (particularly of the brain and most cranial part of spinal cord), in contrast with

other trypanozoon groups (Barrowman, 1976; Berlin *et al*., 2009). In contrast with *T. equiperdum*, McCully and Neitz (1971) reported that the most remarkable lesion in horses experimentally infected with *T. brucei* were found in the brain, and consisted of a diffuse meningoencephalitis characterized by massive perivascular cuffs of large lymphocytes, plasma cells, and a few typical cells of Mott.

In the current study, degenerative changes and neuronal necrosis were present in spinal cord, and cellular infiltration and degeneration in the sciatic nerves. As a result, from both slaughtered mares, the microscopic lesions of the nervous system were found to involve primarily the lumbar and sacral regions of the spinal cord and the left sciatic nerve.

Histopathological changes in these tissues have been recorded by Barrowman (1976) who reported radiculitis and polyneuritis, involving cellular infiltration and degenerative changes of the spinal nerves and spinal ganglia, extending along the larger sciatic nerves as the primary lesions. In contrast to the involvement of the central nervous system in *T. gambiense* infections in man (Van Boegart and Janssen, 1957) and experimentally prolonged *T. brucei* infections in horses (McCully and Neitz, 1971), histopathological changes have not been reported in the central nervous system of under natural *T. equiperdum* infections, except that Matt (1906, 1907, cited by Laveran and Mesnil 1907) found the lesion in the spinal nerves extending into the posterior columns of the spinal cord. According to Rodrigues *et. al*. (2009), *T. evansi* infection of horses, unlike to *T. equiperdum,* the pathological lesions involving the brain and characterized by moderate to severe perivascular lymphoplasmacytic meningoencephalitis, necrosis, edema and hemorrhage.

In this study an attempt has been made to demonstrate whether the parasite present in the cerebrospinal fluid or in the nervous tissue, or in both samples, but the trypanosome was not seen in either of the samples. This is contrary to Barrowman (1976) who reported that trypanosomes were shown to be present in the CSF of horses dying from the nervous form of the disease and in live, naturally-infected horses with nervous manifestations,

whereas they were not found in CSF from horses without these signs. According to Claes *et al*. (2005), the differences in pathology are remains unclear whether the differences are related to the *T. equiperdum* strain or differences in the host immune response, but according to Robert (1919), the natural route of infection is through coitus and the parasite normally localizes in the capillaries of the mucus membranes of the urogenital tract and from there the parasite may elaborate poisonous products or toxins which are responsible for the neuronal lesions.

No growth of virus was seen in the present study complementing that the slaughtered mares were not infected with viruses, which potentially confuse with the disease and thus helps the author to exclude viral diseases having similar neurological signs with dourine such as equine infectious anemia and equine herpes virus 1 infection.

# 6. CONCLUSIONS AND RECOMMENDATIONS

Dourine, venereally transmitted disease caused by *T. equiperdum,* is endemic in the study area in Arsi-Bale highlands, Oromia region, Ethiopia. Among the three known stages of the disease (genital, cutaneous and nervous), the genital and nervous signs were commonly observed in addition to loss of body weight. In the study area; horses naturally infected with *T. equiperdem*, especially once it reaches into third stage (nervous sign), it is difficult to found the parasite in the body fluids (blood and CSF) as well as in the nervous tissue. The current study revealed that even though the parasite is not found in the CSF and nervous tissues, it causes degenerative changes and neuronal necrosis in the affected parts of spinal cord, and cellular infiltration and degeneration of sciatic nerve. In nutshell, the disease revealed moderate degree of microscopic pathological lesions on the nervous tissues at the third stages of the disease.

Therefore, based on the above conclusion the following recommendations are forwarded:

- Absence of parasites in the blood, CSF as well as nervous tissue sections requires examination of chronically sick horses and the use of sensitive diagnostic tests. Probably, the moderate degree of gross and histopathological lesions is also attributed to the clinical stage of the disease. Further study should be conducted in horses with various stages of the disease.

- On the basis of the findings of the present study there is the need to correlate accurately defined infections caused by *T. equiperdum* with the immunological responses in order to establish the basic features of the host parasite relations that are responsible for disease. Because trypanosomes localize in tissues, the diagnosis of infection by direct examination of blood for parasites would appear to be an inefficient method, and the application of immunologic methods already in use in the other similar chronic diseases would be indicated.

- Further biochemical and molecular level analysis should be conducted to have a clear and full understanding of the pathology and pathophysiology of the disease.

## 7. REFERENCES


Abebe G., Malone J. B. and Thompson A. R. (2004): Geospital forecast model for tsetse transmitted animal trypanosomosis in Ethiopia. SINET, *Ethiopia J. Sci* ., **27**(1): 1-8.

Abebe G. (2005): Trypanosomosis in Ethiopia, Addis Ababa University, Faculty of Veterinary Medicine, Debre Zeit, pp. 18-21.

Agbo E. E., Majiwa P. A. and Claassen H. J. (2002): Molecular variation of *Trypanosoma brucei* subspecies as revealed by AFLP fingerprinting. *Parasitol.*; **124** (4):349‑358.

Agbo E. C., Duim B. and Majiwa P. A. (2003): Multiplex‑endonuclease genotyping approach (MEGA): a tool for the fine‑scale detection of unlinked polymorphic DNA markers. *Chromosoma*; **111**(8):518‑524.

Alemu T., Luckins A. G., Phipps L. P., Reid S. W. and Holmes P. H. (1997): The use of Enzyme Linked Immunosorbent Assays to investigate the prevalence of *Trypanosoma equiperdum* in Ethiopian horses, *Vet. Parasitol.*, **71**, 239-250.

Arsi-Bale Zone Agricultural and Rural Development Office (2009): Annual Meteorological and livestock report, Robe, Ethiopia.

Asfaw W. (1999): Message from Federal Veterinary Services Team. *Ethio. Vet. Epidemiol. Newslet*. **1**,1.

Bajyana Songa E. and Hamers R. (1988): A card agglutination test (CATT) for veterinary use based on an early VAT RoTat 1/2 of *Trypanosoma evansi*. *Ann. Soc. of Belgian Trop. Med.*, **68**, 233-240.

Barrowman P. R. (1976): Experimental intraspinal *Trypanosoma equiperdum* infection in a horse. *Onderstepoort J. of Vet. Res*. **43**, 201–202.

Berlin D., Loeb E. and Baneth G. (2009): Disseminated central nervous system disease caused by *Trypanosoma evansi* in a horse. *Vet. Parasitol*. **161**, 316–319.

Bishop P., Rae P. F., Philips L. P., Boid R. and Luckins A. G. (1995): *Trypanosoma equiperdum*: Detection of Trypanosomal antibodies and antigen by enzyme-linked immunosorbent assay. *Br. Vet. J*. **151**, 715-720.

Biteau N., Bringaud F. and Gibson W. (2000): Characterization of Trypanozoon isolates using a repeated coding sequence and microsatellite markers. *Mol. Bioch. and Parasitol.*; **105**(2):185‐201.

Boid R. (1988): Isoenzyme characterisation of 15 stocks of *Trypanosoma evansi* isolated from camels in the Sudan. *Trop. Med. Parasitol.* **39**(1):45‐50.

Borst P., Fase‐Fowler F. and Gibson W. C. (1981): Quantitation of genetic differences between *Trypanosoma brucei gambiense, rhodesiense* and *brucei* by restriction enzyme analysis of kinetoplast DNA. *Mol. Bioch. and Parasitol.* **3**(2):117‐131.

Brun R., Hermann H. and Zhao-Rong L. (1998): Trypanosoma evansi and Trypanosoma equiperdum: distribution, biology, treatment and phylogenetic relationship (a review). *Vet. Parasitol*. **79**, 95–107.

Buffard M. and Schneider G. (1900): Le trypanosome de la dourine. *Archives Parasitologie* **3**, 124–133. Cited in: Claes, F., Buscher, P., Touratier, L. and Goddeeris, B. M. (2005): *Trypanosoma equiperdum*: master of disguise or historical mistake? *Trends Parasitol*. **21**, 316–321.

Caporale G. (1946): Il Morbo coitale maligno. La Clinica Veterinaria **69**, 23–32. Cited in: Claes, F., Buscher, P., Touratier, L. and Goddeeris, B. M. (2005): *Trypanosoma equiperdum*: master of disguise or historical mistake? *Trends Parasitol.* **21**, 316–321.

Caporale V. and Semproni, G. (1980): Epidemiology of dourine in the equine population of the Abruzzi region. *Zentralbl Vet. Med. Biogra*. **27**, 489.

Caporale V. P., Biancifiori, F., Frescura, F., Di Matteo, A., Nannini, D. and Urbani, G. (1981): Comparison of various tests for the serological diagnosis of *Trypanosoma equiperdum* infection in the horse. *Comp. Immunol. Microbiol. Infect. Dis.* **4**, 243–246.

Carrington M., Carnall, N., Mandy, S. C., Anne, G., Maria, B. R., Christine, L. W. and Helena, W. (1998): The properties and function of the glycosylphosphatidylinositol-phospholipase C in *Trypanosoma brucei*. *Mol. and Biochem. Parasito.* **91**, 153–164.

Claes F., Verloo D., De Waal D., P.A.O. Majiwa, P., Baltz T., B. M. Goddeeris, B. and Büscher, P. (2002): The expression of RoTat 1.2 variable surface glycoprotein (VSG) in *Trypanosoma evansi* and *T. equiperdum*. *Vet. Parasitol*. **116**, 209–216.

Claes F., E. C. Agbo, M. Radwanska, M. F. Te-Pas, T. Baltz, D. T. De Waal, B. M. Goddeeris, E. Claassen, and P. Buscher (2003): How does *Trypanosoma equiperdum* fit into the Trypanozoon group, a cluster analysis by RAPD and multiplex endonuclease genotyping approach. *Parasitol*. **126**:425-31.

Claes F., Radwanska M., Urakawa T., Majiwa P.A., Goddeeris, B. and Büscher, P. (2004): Variable Surface Glycoprotein RoTat 1.2 PCR as a specific diagnostic tool for the detection of *Trypanosoma evansi* infections. *Kinetoplastid Biol Dis*, **3**(1):3.

Claes F., Buscher, P., Touratier, L. and Goddeeris, B. M. (2005): *Trypanosoma equiperdum*: master of disguise or historical mistake? *Trends Parasitol*. **21**, 316–321.

Clausen P. H., Gebreselassie, G., Abditcho, S., Mehlitz, D. and Staak, C. (1999): Detection of trypanosome DNA in serologically positive but aparasitaemic horses suspected of dourine in Ethiopia. *Tokai J. of exper. and Cli. Med.* **23** (6), 303-308.

Coetzer J. A. W. and Tustin R. C. (2004): Infectious disease of livestock. 2nd ed. Cape Town, Southern Africa: Oxford University Press.

CSA (2008): Agricultural Sample Survey, 2006/07 (1999 E.C.), Volume II: Report on Livestock and livestock characteristics (Private peasant holdings). Statistical Bulletin 417. Central Statistical Agency (CSA), Federal Democratic Republic of Ethiopia, Addis Ababa.

CSA (2013): Agricultural Sample Survey, 2012/13 (2005 E.C.), Volume II: Report on Livestock and livestock characteristics (Private peasant holdings). Statistical Bulletin 570. Central Statistical Agency (CSA), Federal Democratic Republic of Ethiopia, Addis Ababa.

Dagnachew Z., Shafo, K., Kelil, A. (1980): An investigation of dourine in Arsi administrative region. *Ethio. Med. J.* **4**, 3-19.

Dennis M. M. and Joanna M. B. (2006): Clinical textbook for veterinary technicians. Sixth edition. ELSEVIR SAUNDERS publisher. Pp 165-182.

Derbyshire J. B. and Nielsen N. O. (1997): Edward Watson and the eradication of dourine in Canada. *Can. vet. J.*, **38** (9), 582-586.

Desquesnes M., McLaughlin, G. and Davila, A. M. R. (2001): Detection and identification of Trypanosoma of African livestock through a single PCR based on internal transcribed space 1 of rDNA. *Int. J. Parasitol.* **31**, 610-614.

Desquesnes M. and Davila, A. M. R. (2002): Applications of PCR-based tools for detection and identification of animal trypanosomes. A review and perspectives. *Vet. Parasitol*. **109**, 213-231.

Ethiopian Agricultural Research Organization (EARO) (1999). – National Animal Health Research Program Strategy Document. EARO, Ministry of Agriculture, Addis Ababa, Ethiopia, Pp 1-46**.**

Furr M. and Andrews, F. (2008): Cerebrospinal fluid and the blood – brain barrier. In: Furr, M., and Reed, S., (eds.), Equine Neurology. Blackwell Publishing, Iowa, USA, pp. 33 – 46.

Gari F. R., Ashenafi, H., Tola, A., Goddeeris B. M., Claes F. (2010): Comparative diagnosis of parasitological, serological, and molecular tests in dourine-suspected horses. *Trop. Anim. Health Prod*. **42**, 1649–1654.

Gelagay A., Wosenne N., Tesfaye S., Shiferaw J. and Esayas G. (2013): Investigation of respiratory viruses in camel slaughtered at Addis Ababa Akaki Abattoir, Ethiopia. *Afri. J. of Agri. Resear. Academic Js.,* **8**: 4580-4587.

Giardina S., Paganico G., Urbani G. and Rossi M. (2003): A biochemical and immunological comparative study on *Trypanosoma equiperdum* and *Trypanosoma evansi. Vet. Res. Commun*. **27**, 289–300.

Gibson W. C., de T. F. and Godfrey D. G. (1980): Numerical analysis of enzyme polymorphism: a new approach to the epidemiology and taxonomy of trypanosomes of the subgenus Trypanozoon. *Adv Parasitol*; **18**:175‐246.

Gibson W., Borst P. and Fase‐Fowler F. (1985): Further analysis of intraspecific variation in *Trypanosoma brucei* using restriction site polymorphisms in the maxi‐circle of kinetoplast DNA. *Mol. Bioche. Parasitol.* **15**(1):21‐36.

Gibson W. (2002): Will the real *Trypanosoma brucei rhodesiense* please step forward? *Trends in Parasitol*. **18**, 486.

Gibson W. (2007): Resolution of the species problem in African trypanosomes. *Int J Parasitol*; **37**(8‑9):829‑838.

Hagebock J. M., Chieves L. and Frerichs W. M. (1993): Evaluation of agar gel immunodiffusion and indirect fluorescent antibody assays as supplemental tests for dourine in equids. *Am. J. Vet. Res.* **54**, 1201–1208.

Hagos A., Abebe, G., Buscher, P., Goddeeris, B. M. and Claes, F. (2010): Serological and parasitological survey of dourine in the Arsi–Bale highlands of Ethiopia. *Trop. Anim Health Prod.* **42** (4), 769.

Hide G, Buchanan N. and Welburn S. (1991): *Trypanosoma brucei rhodesiense*: characterisation of stocks from Zambia, Kenya, and Uganda using repetitive DNA probes. *Exp Parasitol*; **72**(4):430‑439.

Hoare C. A. (1972): The Trypanosomes of Mammals. Blackwell Scientific Publications, Oxford.

Holland W. G., Claes F. and My L. N. (2001): A comparative evaluation of parasitological tests and a PCR for *Trypanosoma evansi* diagnosis in experimentally infected water buffaloes. *Vet Parasitol*; **97**(1):23‑33.

Ijaz M. K., Nur-E-Kamal M. S., Mohamed A. I. and Dar F. K. (1998): Comparative studies on the sensitivity of polymerase chain reaction and microscopic examination for the detection of *Trypanosoma evansi* in experimentally infected mice. *Comp. Immunol. Microbiol. Infect. Dis.,* **21**: 215-23.

Kaminsky R., Schmid C. and Lun Z. R. (1997): Susceptibility of dyskinetoplastic *Trypanosoma evansi* and *T. equiperdum* to isometamidium chloride. *Parasitol Res*; **83**(8):816‑818.

Katz J., Richard D. and Judy N. (2000): Procedurally similar competitive immunoassay systems for the serodiagnosis of *Babesia equi, Babesia caballi, Trypanosoma*

*equiperdum,* and *Burkholderia mallei* infection in horses. *J. Vet. Diagn. Invest*. **12**, 46–50.

Khalilik K. (1973): An investigation of dourine and isolation of *Trypanosoma equiperdwn* in Iran. *Archives de I' lnstitut Razi,* **25**, 69- 72.

Kihurani D. O., V. M. Nantulya, S. M. Mbiuki, E. Mogoa, J. Nguhiu-Mwangi, P. M. Mbithi (1994). *T. brucei, T. congolense* and *T. vivax* infections in horses on a farm in Kenya. *Trop. Anim. Health Prod.* **26**: 95-101.

Kuboki N., Inoue N. and Sakurai T. (2003): Loop‑mediated isothermal amplification for detection of African trypanosomes. *J. Clin. Microbiol*; **41**(12):5517‑5524.

Lai D. H., Hashimi H. and Lun Z. R. (2008): Adaptations of *Trypanosoma brucei* to gradual loss of kinetoplast DNA: *Trypanosoma equiperdum* and *Trypanosoma evansi* are petite mutants of T. brucei. *Proc Natl Acad Sci*, USA; **105**(6):1999‑2004.

Laveran A. and Mesnil F. (1907): Trypanosomes and trypanosomiases. London: Ballii:re, Tindal and Cox.

Laveran A. and Mesnil F. (1912): Trypanosomes et Trypanosomiases, Masson et Co., Paris. Cited in: Claes, F., Buscher, P., Touratier, L. and Goddeeris, B. M. (2005): *Trypanosoma equiperdum*: master of disguise or historical mistake? *Trends Parasitol*. **21**, 316–321.

Lavergne M., Labert G. and Raynaud M. (1969): Macroblobulinemie chez le cheval au w urs de l'infection experimental a *Trypanosoma e<JUiperdum. Annates de 1'/nstitut Pasteur, Paris,* **116**, 781- 798. Cited in: Barrowman, P. R. (1976): Experimental intraspinal *Trypanosoma equiperdum* infection in a horse. *Onderstepoort J. of Vet. Res*. **43**, 201–202.

Li F. J., Gasser R. B. and Zheng J. Y. (2005): Application of multiple DNA fingerprinting techniques to study the genetic relationships among three members

of the subgenus Trypanozoon (Protozoa: Trypanosomatidae). *Mol. Cell Probes*; **19**(6):400‑407.

Li F. J., Lai D. H., Lukesˇ J., Chen X. G. and Lun Z. R. (2006): Doubts about *Trypanosoma equiperdum* strains classed as *Trypanosoma brucei* or *Trypanosoma evansi*. *Trends in Parasitol*. **22**, 55-56.

Li F. J., Gasser R. B. and Lai D. H. (2007): PCR approach for the detection of *Trypanosoma brucei* and *T. equiperdum* and their differentiation from *T. evansi* based on maxicircle kinetoplast DNA. *Mol Cell Probes*; **21**(1):1‑7.

Lillie R. D. and Fulmer H. M. (1976): Histopathologic techniques in practical histochemistry. McGraw Hill, New York.

Luckins A. G. (1994): Equine trypanosomiasis. Exotic disease series. *Equine Vet. Edu.* **6** (5), 259-262.

Lumsden W. H., Kimber C. D. and Evans D. A. (1979). *Trypanosoma brucei*: Miniature anion‑exchange centrifugation technique for detection of low parasitaemias: Adaptation for field use. *Trans R Soc Trop Med Hyg*; **73**(3):312‑317.

Lun Z. R., Allingham R. and Brun R. (1992): The isoenzyme characteristics of *Trypanosoma evansi* and *Trypanosoma equiperdum* isolated from domestic stocks in China. *Ann Trop Med Parasitol*; **86**(4):333‑340.

Lun Z. R., Brun R. and Gibson W. (1992): Kinetoplast DNA and molecular karyotypes of *Trypanosoma evansi* and *Trypanosoma equiperdum* from China. *Mol Biochem Parasitol*; **50**(2):189‑196.

Lun Z. R., Li A. X. and Chen X. G. (2004): Molecular profiles of *Trypanosoma brucei, T. evansi* and *T. equiperdum* stocks revealed by the random amplified polymorphic DNA method. *Parasitol Res*; **92**(4):335‑340.

Lun Z. R., Lai D. H. and Li F. J. (2010): *Trypanosoma brucei*: two steps to spread out from Africa. *Trends Parasitol*; **26**(9):424‑427.

Maarten P. (2009): Role and importance of equines and constraints of equine keeping in the Arsi-Bale Highlands of Ethiopia. MSc thesis, Catholic University Leuven.

MacLeod A., Tweedie A. and Welburn S. C. (2000): Minisatellite marker analysis of *Trypanosoma brucei*: reconciliation of clonal, panmictic, and epidemic population genetic structures. *Proc Natl Acad Sci* U S A; **97**(24):13442‑13447.

MacLeod A., Welburn S. and Maudlin I. (2001): Evidence for multiple origins of human infectivity in *Trypanosoma brucei* revealed by minisatellite variant repeat mapping. *J Mol Evol*; **52**(3):290‑301.

Masiga D. K. and Gibson W. C. (1990): Specific probes for *Trypanosoma* (*Trypanozoon*) *evansi* based on kinetoplast DNA minicircles. *Mol. Biochem. Parasitol*. **40**, 279-284.

Masiga D. K., Tait A. and Turner C. M. (2000): Amplified restriction fragment length polymorphism in parasite genetics. *Parasitol Today*; **16**(8):350‑353.

Mathieu‑Daude F., Stevens J. and Welsh J. (1995): Genetic diversity and population structure of *Trypanosoma brucei*: clonality versus sexuality. *Mol Biochem Parasitol*; **72**(1‑2):89‑101.

Matthews K. R. (2005): The developmental cell biology of *Trypanosoma brucei. J. Cell Sci.* **118**, 283-290.

McCully R. M. and Neitz W. O. (1971): Clinicopathological study on experimental *Trypanosoma brucei* infections in horses. Part 2. Histopathological findings in the nervous system and other organs of treated and untreated horses reacting to Nagana. *Onderstepoort J. of Vet. Res.* **38** (3), 141- 176.

Monzon C. M., Mancebo O. A. and Roux J. P. (1990): Comparison between six parasitological methods for diagnosis of *Trypanosoma evansi* in the subtropical area of Argentina. *Vet Parasitol*. **36**(1‐2):141‐146.

Moulton J. E. (1974): Pathogenesis of *Trypanosoma equiperdum* in deermice *(Peromyscus maniculatus). American J. of Vet. Res.* **35**, 961 - 976.

Mulligan H. W. (1970): The African Trypanosomosis. George Allen and Unwin Ltd, London. 950.

Ngaira J. M., Olembo N. K. and Njagi E. N. (2005): The detection of non RoTat 1.2 *Trypanosoma evansi*. *Exp Parasitol*; **110**(1):30‐38.

Njiru Z. K., Mikosza A. S. and Matovu E. (2008): African trypanosomiasis: sensitive and rapid detection of the sub‐genus Trypanozoon by loop‐mediated isothermal amplification (LAMP) of parasite DNA. *Int J Parasitol*; **38**(5):589‐599.

Nijru Z. K., Ouma J. O. and Enyaru J. C. (2010): Loop‐mediated isothermal amplification (LAMP) test for detection of *Trypanosoma evansi* strain B. *Exp Parasitol*; **125**(3):196‐201.

Nocard E. (1901): Sur les rapports qui existent entre la dourine et le surra ou le nagana. *C.R. Soc. Biol.*, Paris **64**, 464–466. Cited in: Claes, F., Buscher, P., Touratier, L. and Goddeeris, B. M. (2005): *Trypanosoma equiperdum*: master of disguise or historical mistake? *Trends Parasitol*. **21**, 316–321.

OIE (Organization for International Epizootics) (2000): Dourine. Part 2. Section 2.5. Chapter 2.5.2. *In* International animal health code: mammals, birds and bees, 9th Ed. OIE, Paris, 221-222.

OIE (Organization for International Epizootics) (2001): Dourine. Part 2. Section 2.5. Chapter 2.5.2. *In* Manual of standards for diagnostic tests and vaccines, 4th Ed., 2000. OIE, Paris, 528-534.

OIE (Organization for International Epizootics) (2008): Chapter 2, 5, 3: Dourine. In: Terrestrial manual of diagnostic tests and vaccines for terrestrial animals. www.oie.int/fileadmin/Home/eng/Health standards/tahm/2.05.03 DOURINE.pdf (accessed on 11. 11.14).

OIE (Organization for International Epizootics) (2010): Chapter 2.1.17 Trypanosoma evansi infection(surra). In Terrestrial Manual of Diagnostic Tests and Vaccines for Terrestrial Animals. www.oie.int/fileadmin/Home/eng/Health standards/tahm/2.01.17 TRYPANO.pdf (accessed on 08.11.14).

OIE (Organization for International Epizootics) (2012): *Trypanosoma Evansi* Infection (Surra). *Terrestrial Manual,* Chapter 2.1.17.

Overath P. and Engstler M. (2004): Endocytosis, membrane recycling and sorting of GPI-anchored proteins: T*rypanosoma brucei* as a model system. *Mol Microbio*. **53**, 735-44.

Parkin B. S. (1948): The demonstration and transmission of the South African strain of *Trypanosoma equiperdtmt* of horses. *Onderstepoort J. of Vet. Sci. and Anl Indu.* **23**, 41-57.

Pascucci I., Andrea D., Cesare C., G., Paolo C., Manuela T., Nicola F., Massimo S. and Vincenzo C. (2013): Diagnosis of dourine in outbreaks in Italy. *Vet. Parasitol.* **193** (30– 38).

Pays E. and Nolan D. P. (1998): Expression and function of surface proteins in *Trypanosoma brucei*. *Mol. Biochem. Parasitol*. **91**, 3-36.

Perrone T. M., Gonzatti M. I. and Villamizar G. (2009): Molecular profiles of Venezuelan isolates of Trypanosoma sp. by random amplified polymorphic DNA method. *Vet. Parasitol*. **161**(3‑4):194‑200.

Reid S. A., Husein A. and Copeman D. B. (2001): Evaluation and improvement of parasitological tests for Trypanosoma evansi infection. *Vet. Parasitol.* **102**(4), 291-297.

Robert J. F. (1919): Pathology of Dourine with Special Reference to the Microscopic Changes in Nerve Tissues and Other Structures. *J. of Agri. Res.* Vol. **XVIII**, (3), Washington, D. C.

Robinson E.M. (1926): Serological investigations into some diseases of domesticated animals in South Africa caused by trypanosomes. *Rep. Direct. Vet. Educ. Res., Dept. Agr. S. Afr*. **9**, 11–12.

Rodrigues A., Fighera R. A., Souza T. M., Schild A. L. and Barros C. S. L. (2009): Neuropathology of Naturally Occurring *Trypanosoma evansi* Infection of Horses. Departamento de Patologia, Universidade Federal de Santa Maria, Santa Maria, Brazil. Vet. Pathol. **46**: 251–258.

Rouget J. (1896): Contribution a l'etude du trypanosome de manniferes. *Annale de l'lnstitut Pasteur,* **10**, 716-728. Cited in: Barrowman P. R. (1976): Observation on the transmission, immunology, clinical signs and chemotherapy of dourine (*Trypanosoma equiperdum* infection) in horses, with special reference to cerebro-spinal fluid. *Onderstepoort J Vet Res* 1976; **43(2)**: 55-66.

Rouget J. (1986): Contribution a letude du trypanosome des mammifere. *Ann. Inst. Pasteur*. **10**, 716-728. Cited in: Barrowman, P. R. (1976): Experimental intraspinal *Trypanosoma equiperdum* infection in a horse. *Onderstepoort J. of Vet. Res*. **43**, 201–202.

Roy E. (2012): Giemsa's Staining Protocol for Tissue Sections. IMVS Division of Pathology. The Queen Elizabeth Hospital.

Scacchia M., Cammà, C., Di Francesco, G., Di Provvido, A., Giunta, R., Luciani, M., Marino, A.M.F., Pascucci, I. and Caporale, V. (2011): A clinical case of dourine in an outbreak in Italy. *Vet. Ital*. **47**, 473–475.

Shapiro T. A. and Englund P. T. (1995): The structure and replication of kinetoplast DNA. *Annu. Rev. Microbiol.* **49**, 117-143.

Stephen L. E. (1986): Trypanosomiasis. A veterinary perspective, Pergamon Press.

Stevens J. R., Nunes V. L. and Lanham S. M. (1989): Isoenzyme characterization of *Trypanosoma evansi* isolated from capybaras and dogs in Brazil. *Acta Trop*; **46**(4):213‐222.

Stuart K., Brun, R., Croft, S., Fairlam, A., Gürtler, R. E., McKerrow, J., Reed, S. and Tarleton, R. (2008): Kinetoplastids: related protozoan pathogens, different diseases. *J. of Clin. Invest*. **118**(4), 1301-1310.

Taylor K. and Authié E. M. L. (2004): Pathogenesis of animal trypanosomiasis. In *The Trypanosomiases* (eds. I. Maudlin, P.H. Holmes & M.A. Miles) CABI Publishing, 2004, pp. 331-353.

Theis J. H. and Bolton V. (1980): *Trypanosoma equiperdum*: movement from the dermis. *Exp. Parasitol*. **50**, 317–330.

Thekisoe O. M., Kuboki N. and Nambota A. (2007): Species‐specific loop‐mediated isothermal amplification (LAMP) for diagnosis of trypanosomosis. *Acta Trop*; **102**(3):182‐189.

Urquhart G. M. (1985): In: Veterinary Parasitology. Longman Scientific technical, UK.

Van Bogaert L. and Janssen P. (1957): Contribution a !'etude de Ia neurologic de Ia trypanosomiase humaine. *Annates r/e Ia Sacihe he/ge de Medecine tropicale,* **27**, 379- 426. Cited in: Barrowman P. R. (1976): Experimental intraspinal *Trypanosoma equiperdum* infection in a horse. *Onderstepoort J. of Vet. Res.* **43**, 201–202.

Van den Bossche P., De Deken R., Brandt J., Seibou B. and Geerts S. (2004): Recirculation of *Trypanosoma brucei brucei* in cattle after T. congolense challenge by tsetse flies. *Vet. Parasitol*. **121**, 79–85.

Ventura R. M., Takeda G. F. and Silva R. A. (2002): Genetic relatedness among *Trypanosoma evansi* stocks by random amplification of polymorphic DNA and evaluation of a synapomorphic DNA fragment for species‐specific diagnosis. *Int J Parasitol*; **32**(1):53‐63.

Verloo, D., Holland, W., My, L. N., Thanh, N. G., Tam, P. T., Goddeeris, B., Vercruysse, J. and Büscher, P. (2000): Comparison of serological tests for *Trypanosoma evansi* natural infections in water buffaloes from North Vietnam. *Vet. Parasitol.* **92**, 87-96.

Vulpiani M. P., Andrea C., Daniele G., Filomena I., Daniela P. and Nicola F. (2012): Reemergence of Dourine In Italy: Clinical Cases in Some Positive Horses. J. *of Equi. Vet. Sci.* **xxx** (2012) 1-7.

Waitumbi J. N. and Murphy N. B. (1993): Inter and intra-species differentiation of trypanosomes by genomic fingerprinting with arbitrary primers. *Mol Biochem Parasitol*; **58**(1):181‐185.

Walker J. (1918): The occurrence of dourine (slapziekte) in South Africa. In: Report of Director of Veterinary Research. Union of South Africa; **5**/6:189-206.

Wang Z. L. (1988): The similarities and differences of the characteristics between *T. equiperdum* and *T. evansi*. *Bul Vet Col (PLA*) (Chinese); 300‐303.

Watson E. A. (1915): Dourine and the complement fixation test. *Parasitology* **8**, 156–183.

Watson E. A. (1920): Dourine in Canada, 1904-1920. History, research, and suppression. Dominion of Canada: Department of Agriculture.

Woo P. T. (1969): The haematocrit centrifuge for the detection of trypanosomes in blood. *Can J Zool*; **47**(5):921‐923.

Ying W., Yan‐Zi W., Marc D. and Zhao‐Rong L. (2011): Molecular Epidemiology of *Trypanosoma Evansi* and *T. Equiperdum* and Atypical Human Infection by Animal Trypanosomes. *Madame Curie Report.* Landes Bioscience.

Zablotskij V. T., Georgiu C., De Waal T., Clausen P. H., Claes F. and Touratier L. (2003): The current challenges of dourine: difficulties in differentiating *Trypanosoma equiperdum* within the subgenus Trypanozoon. *Review Sci. Techn. O. Intl. Epizoo.* **22**, 1087-1096.

Zhang Z. Q. and Baltz T. (1994): Identification of *Trypanosoma evansi*, *Trypanosoma equiperdum* and *Trypanosoma brucei brucei* using repetitive DNA probes. *Vet Parasitol*; **53**(3‐4):197‐208.

Internet: Trypano - International Livestock Research Institute. https://www.ilri.org/InfoServ/Webpub/fulldocs/Ilrad88/Trypanosomiasis.htm. Accessed on 12/10/2014 at 11:13am.

# 8. APPENDICES

## Appendix 1. Preparation of fixative agents

1. Preparation of one liter of 10% buffered formalin:

- Formaldehyde Solution (37-40%) 100 ml
- Distilled Water 900 ml
- Monobasic Sodium Phosphate 4.0 gm
- Dibasic Sodium Phosphate 6.5 gm

## Appendix 2. Procedure of Haematoxylin and Eosin staining

1. Deparaffinize the embedded tissue in hot air oven.
2. Hydrate the section.
    - 3 dips in xylene (2 Min. each)
    - 3 dips in acetone / alcohol (2 Min. each)
    - In running tap water for 5 Minutes.
3. Mayer's haemotoxylin for 15 minutes.
4. Wash in running tap water for 20 minutes
5. Counter stain with eosin for 2 minutes
6. Dehydrate the section in 95% and absolute alcohol/ acetone 2 changes (2minutes each).
7. Clear in xylene 3 changes (2 minutes each)

8. Mount in Canada balsam or DPX

**Appendix 3. Ante Mortem and Post Mortem Findings Recording Sheet**

Date _____

Case origin _____

Owners name _____

Address _____

Animal spp. _____

Breed _____

Age _____

Sex _____

No. of sick animals _____

No. of dead animals _____

No. of animals at risk _____

**History and clinical signs**

---
---
---
---

**Ante mortem observation**

---
---
---
---

**Necropsy findings**

Skin

Mucus membrane

Natural orifice

Urogenital tract

Joint and muscle

Lymph nodes

Brain

Spinal cord

Peripheral nerves

Other findings

**Tissue specimens taken for histopathology:**

**Appendix 4. Giemsa's Staining Protocol for Tissue Sections; (**Roy, 2012)

**Method**

1. Bring sections to distilled water
2. Stain with diluted Giemsa's stain made up fresh
3. Rinse in distilled water
4. Differentiate with 0.5% aqueous acetic acid
5. Dehydrate rapidly
6. Clear and mount

**Results**

Bile pigments.....green

Collagen, muscle, bone.....pale pink

Micro-organisms, fungi, parasites.....purplish-blue

Starch granules, cellulose.....sky blue

Pigments (native colour is yellow/brown, or if fixed in dichromate containing fixative)...green

Nuclei.....dark blue to violet

Erythrocytes.....salmon pink

Cytoplasm…….....varying light blue shades

**Appendix 5. Necropsy procedure (Dennis and Joanna 2006)**

- The necropsy was beginning with the animal placed on its left side in lateral recumbency.
- The right inguinal area was incised, and the coxofemoral joint is penetrated. Muscles near the pelvis were severed, and the right hind limb is reflected away from the body.
- The spinal cord was transected by an incision into the ventral atlanto-occipital joint. Atlanto-occipital membranes, ligaments, and joint capsule were transected, disarticulating the head from the vertebral column.
- The skin was removed from the head by leaving it attached to the skin of the body and peeling the head forward out of the skin.
- The superficial muscles of the head were removed. External ears and temporal muscles were removed, exposing the calvaria (skull cap).
- The calvaria and caudal wall of the cranial cavity were removed from the skull as a unit, exposing the dorsum of the brain.
- Three cuts were made with a hacksaw or meat saw to accomplish this. The first was a transverse cut through the frontal bones immediately caudal to the orbits. Care was taken to make the cut just deep enough to transect bone but not deep enough to engage the brain beneath. The second and third cuts were made through the side walls and caudal wall of the cranial cavity.
- At 45-degree angles to the longitudinal axis of the skull, they were extending from the lateral ends of the transverse cut to the medial faces of the occipital condyles, progressing cranial from the foramen magnum with bone-cutting forceps.
- The calvaria and caudal wall, as a unit, were pried loose from surrounding bones and removed. The meninges (the three membranes that cover the brain) and the surface of the brain were examined in situ. The dorsal meninges were removed, and transected the cranial nerves, progressing rostrally from the foramen magnum for removal of the brain.
- The brain was examined for the presence of any gross lesions on the different parts, particularly on the cerebral cortex, cerebellum and medulla oblongata. A 1

cm thick suspected brain tissue (cerebral cortex and cerebellum) was taken by slicing with scalpel from each parts of the brain and then placed into universal bottle containing 10% neutral buffered formalin.

- Spinal cord removal was performed by a meat-cutter's band saw to extract the cervical cord by disarticulating the cervical vertebrae, one by one. Removal of the muscles before disarticulating at the facets and annulus fibrosus was done.
- The nerve roots were severed by advancing a pair of thin, long-handled scissors along the wall of the spinal canal. The spinal cord was removed using a saw to cut alongside the midline down the entire vertebral column and this will allow lateral removal of the spinal cord if the ipsilateral spinal nerves are cut as the cord is gradually removed.
- Then each part of the spinal cord (cervical, thoracic, lumbar and sacral) was examined for the presence of any gross lesion, and approximately 1 cm tissue sample including the gross lesions was taken for histopathology.

**Appendix 6. Viral culturing technique procedure** (Gelagay *et al*., 2013)

1. Four nervous tissue samples were processed and grown on monolayer of BHK-21 cells in flasks.
2. Briefly, 1 gm of each sample of nervous tissue was washed three times using sterile PBS on Petri dish, and then washed tissue was transferred to sterile mortar and cut into small pieces using scissor and minced by sterile scalpel blade.
3. The minced tissues were ground and homogenized using pestle.
4. Nine ml of PBS was added to the prepared nervous tissues and well mixed.
5. The homogenized tissues were transferred to test tube and centrifuged at 3400 rpm for 10 min and 0.5 ml of the supernatant was inoculated on the confluent BHK-21 cells and incubated at 37°C for 1 hr.
6. Following incubation, the inoculated cell lines were washed using PBS and 10ml complete Glasgow Minimum Essential Medium (GMEM) was added and incubated at 37°C to follow-up the development of cytopathogenic effect (CPE).