Thesis Ref. No. _____

CLINICAL SIGNS, SERUM BIOCHEMICAL PROFILES AND REPRODUCTIVE PATHOLOGICAL LESIONS IN HORSES NATURALLY INFECTED WITH DOURINE IN WESTERN ARSI ZONE, ETHIOPIA

MSc Thesis

By

Yonas Gizaw Habtemichael

Addis Ababa University College of Veterinary Medicine and Agriculture,

Department of Pathology and Parasitology

OCTOBER, 2015

BISHOFTU, ETHIOPIA

CLINICAL SIGNS, SERUM BIOCHEMICAL PROFILES AND REPRODUCTIVE PATHOLOGICAL LESIONS IN HORSES NATURALLY INFECTED WITH DOURINE IN WESTERN ARSI ZONE, 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

Yonas Gizaw Habtemichael

October, 2015

Bishoftu, Ethiopia

CLINICAL SIGNS, SERUM BIOCHEMICAL PROFILES AND REPRODUCTIVE PATHOLOGICAL LESIONS IN HORSES NATURALLY INFECTED WITH DOURINE IN WESTERN ARSI ZONE, ETHIOPIA

Submitted by: Yonas Gizaw	_____	_____
Name of Student	Signature	Date

Approved for submittal to thesis assessment committee

1. Dr. Hagos Ashenafi	_____	_____
Major Advisor	Signature	Date
2. Dr. Tilaye Demissie	_____	_____
Co- Advisor	Signature	Date
3. Prof. Merga Bekana	_____	_____
Co-Advisor	Signature	Date
4. Dr. Yacob Hailu	_____	_____
Department chairperson	Signature	Date

Addis Ababa University

College of Veterinary Medicine and Agriculture

Department of Pathology and Parasitology

As members of the Examining Board of the final MSc open defense, we certify that we have read and evaluated the Thesis prepared by: Yonas Gizaw entitled: Clinical Signs, Serum biochemical profiles and Reproductive pathological lesions in horses naturally infected with dourine in Western Arsi Zone, Ethiopia and recommend that it be accepted as fulfilling the thesis requirement for the degree of: Masters of Tropical Veterinary Pathology.

_____	_____	_____
Chairman (title and name)	Signature	Date
_____	_____	_____
External Examiner (title and name)	Signature	Date
_____	_____	_____
Internal Examiner (title and name)	Signature	Date
1. Dr. Hagos Ashenafi	_____	_____
Major Advisor	Signature	Date
2. Dr. Tilaye Demissie	_____	_____
Co- Advisor	Signature	Date
3. Prof. Merga bekana	_____	_____
Co-advisor	Signature	Date
4. Dr. Yacob Hailu	_____	_____
Department chairperson	Signature	Date

DEDICATION

This thesis manuscript is dedicated to my mother, Birtukan Tafes, my brother, Anteneh Gizaw for nursing me with affection and love and for their dedicated partnership in the success of my life and my father Gizaw Habtemichael who passed away when I was child for nursing me with affection, love and who helped me a lot in my childhood, wishing to be successful person.

STATEMENT OF AUTHOR

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

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: Yonas Gizaw Signature:_____

College of Veterinary Medicine and Agriculture, Bishoftu

Date of Submission: 5/10/2015

ACKNOWLEDGEMENTS

First and foremost, I would like to thank almighty God for giving me health, strength and in all aspects in accomplishing my works passing through a lot of obstacles.

I would like to acknowledge VLIR-UOS Ethio-Belgium team project for providing financial support, Postmortem Kit and CATT/*T.evansi* antigen with the buffer.

I am highly indebted to my advisors Dr. Tilaye Demissie, Dr. Hagos Ashenafi and Prof. Merga Bekana for their advice, guidance, constructive comments during the study period and paper writing up including their scientific inputs. I would like also to express my deepest appreciation and thanks to Dr. Getenet Abe for his incredible kindness, hospitality, allowing me to do histopathology without any cost in NAHDIC and for his constructive comments on the paper. Special thanks also go to Mr. Yosef Cherinet for his willingness, positivity and allowing to do the serum biochemical analysis.

I am great full to Mr. Tewdros Arega and Mr. Solomon Getachew in the department of veterinary pathology from National Animal Health and Diagnostic and Investigation center, for their technical assistance in the histopathology laboratory work.

My thanks also goes to Mr. Alemu Tola senior laboratory technician in the Ethio-Belgium project molecular biology laboratory for his constructive advices, demonstration of procedure of CATT test, kindness in providing equipments required for the research on time throughout the study period.

I would like to extend my sincere acknowledgment and words of thanks to Dr. Mohammed and Mrs. Zerithun field technicians in Dodola veterinary clinic for their support in investigation of the disease and postmortem examination.

My special thanks go to my mother Birtukan Tafes and my brother Anteneh Gizaw for their support throughout my academic time.

TABLE OF CONTENTS **PAGES**

TABLE OF CONTENTS (Continued)

LIST OF TABELS **PAGE**

LIST OF FIGURES **PAGE**

LIST OF ABBREVIATIONS

AAU-CVMA	Addis Ababa University College of Veterinary Medicine and Agriculture
AIDS	Acquired Immune Deficiency Syndrome
ALP	Alkaline Phosphatase
ALT	Alanine Amino Transferase
AST	Aspartate Amino Transferase
CATT	Card Agglutination Test for Trypanosomosis
CFT	Complement Fixation Test
DNA	Deoxyribo Nucleic Acid
ELISA	Enzyme Linked Immunosorbent Assay
FAO STAT	Food and Agriculture Statistical Database
KDNA	Kinitoplast DNA
NAHDIC	National Animal Health Diagnostic and Investigation Center
OIE	Office International des Epizootics
PCR	Polymerase Chain Reaction
RBC	Red Blood Cell
SPSS	Statistical package for Social Science
VSG	Variant Surface Glycoprotein

ABSTRACT

Dourine a venereal transmitted trypanosomosis is endemic in Ethiopia and is the major health problem threatening equines. Until recently, pathologic tissue changes associated with the disease are poorly described. The present study was conducted from November 2014 to June 2015 with an objective of investigating clinical signs; isolate *T. equiperdum* from blood, serum biochemical change and reproductive pathological lesions in dourine infected horses. A cross-sectional study design and purposive sampling were used to identify and select dourine infected horses. Twelve mares with typical signs of dourine and serologically positive with CATT/*T. evansi* were identified. The genital and nervous signs were most commonly observed in infected mares while the cutaneous signs were less prominent in the present study. Despite attempts made to isolate the parasite using woo test, no trypanosomes were detected in all of examined blood samples. There were no significant variation ($p>0.05$) in the mean values of total protein, albumin, AST, ALT and ALP between the infected and healthy groups but a relative increase were observed in all biochemicals analyzed except mean level of albumin which showed a relative decrease in infected group compared to healthy one. Gross lesions observed in the two euthanized infected mares includes, swollen vulva with visible areas of depigementation, congestion of the mucosa of vagina, thickened and congested mucosa of uterus, ovarian follicular cysts, slightly enlarged and congested spleen, enlarged and swollen liver with multiple necrotic foci. Microscopically, mononuclear cell infiltration mainly of lymphocytes and plasma cells and periglandular inflammation were observed in the vulva, vagina, cervix and uterus. In addition, interstitial mastitis, haemosidrine deposition in the spleen and liver and lymphcocyte depletion in the spleen were observed. The results of these gross and histological findings indicate the presence of various organs involvement with severe degree of lesions. Thus, dourine imposes further pathological studies in naturally and experimentally infected mare and stallions by increasing the sample size.

Key words: Ethiopia, Infiltration, Mare, Mononuclear cells, Serum biochemical, *Trypanosoma equiperdum*

1. INTRODUCTION

The world equine population is estimated at 44 million donkeys, 11 million mules and 59 million horses (FAOSTAT, 2012). More than 97% of the world's donkey and mule populations, and over 72 % of the world's horse population is found in developing countries specially kept for draft purpose (Swaan, 2006). Ethiopia has more than 6 million donkeys, the second largest donkey population in the world next to china, 1.9 million horses and over 350,000 mules (FAOSTAT, 2012) specifically kept for work. In Ethiopia, equines have their greatest contribution in agriculture and transport sector of the national economy. Equines are used for various works such as carting goods and people, carrying packs and bricks, and other construction materials, riding, tillage, weeding, and water carrying. Despite their tremendous contribution less attention has been paid to equines in terms of health care and husbandry managements (Maarten, 2009).

Throughout the world, the one common factor leading to the ill health, suffering and early demise of equines is the protozoan parasite, *Trypanosoma equiperdum*, causing dourine (Stephen, 1986). Dourine is a contagious disease of equids caused by the protozoan parasite *Trypanosoma equiperdum*. Once widespread, dourine has been eradicated from many countries but is still seen in horses in Asia, Africa, South America, southern and eastern Europe, Mexico and Russia and was reported in June 2011 in Sicily and then just north of Naples, on the Italian mainland (Sidney *et al*., 2013).

It is the only trypanosomosis that is not transmitted by blood-feeding vectors. Unlike other trypanosomal infections, dourine is transmitted almost exclusively during coitus (Claes *et al*., 2003). Dourine can affect horses, mules and donkeys. The latter are generally more resistant and often remain asymptomatic carriers.

Its course and clinical signs vary considerably depending on the virulence of the strain concerned. The course of the disease in horses is chronic, varying from a few months to 1-2 years. The clinical signs are marked by periodic exacerbation and relapse, ending in death or, possibly, recovery. Fever, local edema of the genitalia and

mammary glands, cutaneous eruptions, incoordination, facial paralysis, ocular lesions, anemia, and emaciation may all be observed. Edematous cutaneous plaques, 5–8 cm in diameter and 1 cm thick, are pathognomonic (Claes *et al*., 2005).

Trypanosoma equiperdum differs from other trypanosomes in that it is primarily a tissue parasite that rarely invades the blood. 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 (Hoare, 1972; Stephen, 1986).

The constant antigenic variations of the parasite results in the release of large amount of biological active products and the formation of immune complexes, which are certainly major factors in triggering a variety of clinical and pathological changes (Zwart, 1989).

During the course of trypanosomosis infection, trypanosomes cause specific and non specific damage to some of the organs involved in the reproductive process as well as the fetus. The organs include the pituitary gland, testis, epididymis, ovary and uterus. Lesions in the gonads lead to infertility while those in the fetus lead to foetal death, and/or neonatal death. Superimposed on these changes is damage to the pituitary gland (Ikede *et al*., 1988).

When animals become infected with trypanosomosis, their physiology alters (Biryomumaisho *et al*., 2003). This is due to the wide range of blood biochemical changes (Katunguka-rwakishaya, 1996). The evaluation of blood indices and parameters helps to determine the health status of animals and also to establish the degree of damage to hosts tissues as well as the severity of the infection (Otesile *et al*., 1991).

Dourine is endemic in Ethiopia with seroprevalence of greater than 28% and is considered the most important health problem threatening equines in most parts of the world (Clausen *et al*., 1999). Although dourine represents one of the most severe diseases of horses responsible for marked economic losses, few studies have been done on dourine in Ethiopia (Zeleke *et al*., 1980; Alemu *et al*., 1997; Clausen *et al*., 1999; Hagos *et al*., 2010a).

The literature on this topic is generally old and lacking graphic evidence, as this disease is not generally present in developed countries. Attempt for isolation of *T. equiperdum* is very difficult, as demonstrated by the low number of isolates in the past decades. Moreover, dourine is a chronic disease whose signs are not constantly present and whose pathogenicity can vary, depending on the strain concerned.

Though dourine still occurs in many parts of the world, since its eradication from North America and northern Europe, published research on the pathology, pathogenesis, immunology and chemotherapy of the disease has been neglected. The absence of published information on many aspects of the host's response to infection initiates observations should made on some of these. There is remarkable deficiency in our current knowledge of the pathology of the disease. Pathological tissue changes associated with the disease are poorly described and so far there is no published research works done on the pathological lesions of dourine in Ethiopia.

In line with the above background information and justifications, the main objectives of the present study include:

- to assess clinical signs, serum biochemical alteration and presence of *Trypanosoma equiperdum* in blood of dourine infected mares.
- to assess the pathological lesions of dourine in the reproductive organs of dourine infected mares.

2. LITERATURE REVIEW

2.1. Definition and Synonyms

Dourine is a chronic or acute contagious disease of equids that is transmitted directly from animal to animal during coitus (Rouget, 1986). The venereal disease of equines or dourine has been also known under different other names (Arabic "el Dourin", English "Covering disease", German "Beschalseuche", French "Mal de coit", Russian "Slucnaja Boleznj" or "Podsedal") (Hoare, 1972).

2.2. Etiology

Dourine is a protozoa parasitic disease of equids caused by *Trypanosoma equiperdum* of the subgenus Trypanozoon (Brun *et al*., 1998). This subgenus also includes the three subspecies of *Trypanosoma brucei* (*Trypanosoma brucei brucei*, *Trypanosoma brucei gambiense* and *Trypanosoma brucei* rhodesiense) and *Trypanosoma evansi. T. b. brucei* causing nagana in domestic animals and *T. b. rhodesiense* and *T .b. gambiense* causing sleeping sickness in human. Further, *T*. evansi causes Surra predominantly in livestock but also in other (Brun *et al*., 1998; Maudlin *et al*., 2004).

2.2.1. Identification of Trypanosoma equiperdum

It is difficult to distinguish *T. equiperdum* microscopically from other members of the subgenus Trypanozoon (*T. evansi*, *T. brucei*). In particular, *T. equiperdum* and *T. evansi* cannot be differentiated on the basis of morphological criteria (Claes *et al*., 2005). Like *T. evansi*, *T. equiperdum* is usually monomorphic (Figure 1). However, it sometimes exhibits pleomorphism like *T. evansi* during subpassages in rodents (Wei *et al*., 2011). At the fine structural level, there are relatively more coated vesicles in the flagellar pocket of *T. equiperdum*, compared with *T. evansi*. It becomes somewhat difficult to differentiate these two species with respect to the ultrastructural properties (Brun *et al*., 1998).

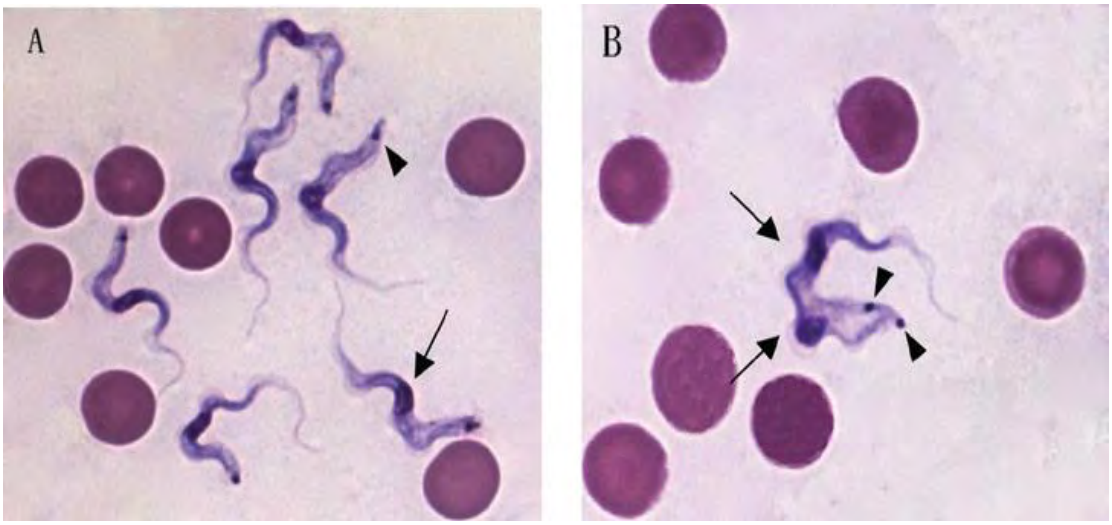


Figure 1: (A) Giemsa staining of *T. equiperdum* bloodstream stage of slender form and (B) division of slender form indicating the nucleus (arrow) and kinetoplast (arrow head) (Wei *et al*., 2011).

Neither parasitological nor serological tests are sensitive and specific enough, thus leading to various kinds of genetic and molecular methods which have been continually updated in order to enhance greater precision in diagnosis of *Trypanozoon* species and differentiation of these pathogens (Wei *et al*., 2011).

Accordingly, restriction fragment length polymorphisms (RFLPs) (Lun *et al*., 2004), genome fingerprinting (Waitumbis and Murphy, 1993) and repetitive DNA probes were used (Zhang and Baltz, 1994). A series of techniques based on PCR have also been used, for example, minisatellite DNA analysis, (Biteau *et al*., 2000) amplified fragment length polymorphism (AFLP), (Agbo *et al*., 2002) multiplex-endonuclease genotyping (MEGA), (Claes *et al*., 2003) mobile genetic elements (MGE)-PCR, simple sequence repeat (SSR)-PCR (Li *et al*., 2005) and random amplification of polymorphic DNA (RAPD). A PCR test based on the RoTat1.2 variable surface glycoprotein (VSG) cDNA sequence by (Claes *et al*., 2004). More ever, two kinds of techniques have been developed for detection and identification of African trypanosomes, i.e., fluorescence in situ hybridization with peptide nucleic acid probes (Radwanska *et al*., 2002) and the loop-mediated isothermal amplification (LAMP) reaction (Thekisoe *et al*., 2007; Njiru *et al*., 2008). However, despite the development of these, genetic and molecular techniques by different scholars clear species specific identification within subgenus trypanozoon remains difficult.

2.3. Epidemiology

2.3.1. Host range and geographical distribution

Trypanosoma equiperdum has been reported to infect horses, donkeys and mules. There is no known natural reservoir of the parasite other than infected equids (Brun *et al*., 1998). Donkeys and mules are more resistant than horses and may remain unapparent carriers. 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 (Brun *et al*., 1998).

Since *T. equiperdum* is a tissue parasite found in equines in nature, its establishment in the blood of laboratory animals is extremely difficult. However, once a strain becomes adapted to rodents, the parasites can be maintained by serial passages, in the same manner as *T. evansi*. 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 murine adapted strain of *T. equiperdum* produce the clinical manifestations of dourine (Wang, 1988).

Dourine has a worldwide distribution but few cases have been reported during the last three decades owing to the wide use of artificial fertilization technology (OIE, 2013). It 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 *et al*., 2003). The infection is endemic in many areas of Asia, Africa, Russia, Middle East and Eastern Europe (OIE, 2008). 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).

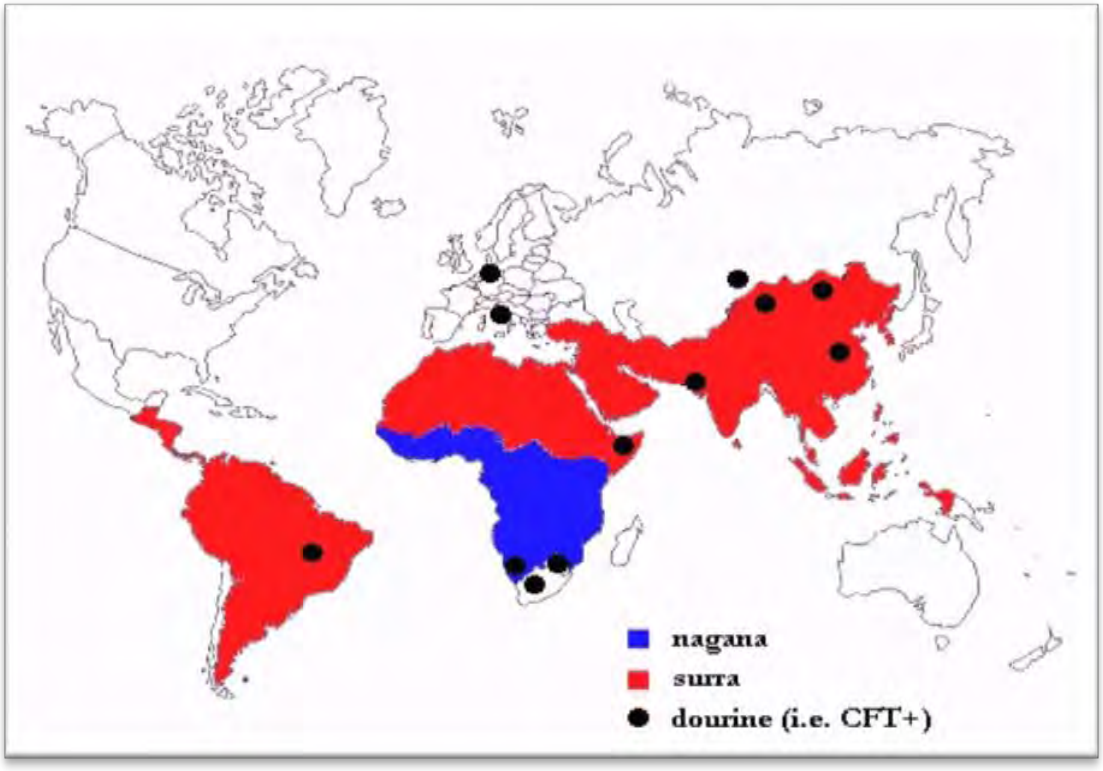


Figure 2: Distribution of Dourine in the world (Claes *et al*., 2003)

2.3.2. Occurrence and prevalence

Historically, dourine has been present in Europe, North America, Asia, and Africa. After World War I, the disease was eradicated from Western Europe by serologic screening, strict sanitation, and treatment of some horses with trypanocides (Claes *et al*., 2003). Dourine is considered a reportable disease by the World Organization for Animal Health (OIE) and is present in most of Asia, southeastern Europe, South America and Africa (Zablotskij *et al*., 2003). The prevalence of the disease in some countries is summarized in the table below (table 1).

Table 1: Prevalence of dourine in horses in some countries based on different testes.

Countries	Test employed	Prevalence (%)	Source
Botswana	CFT	9.0	(Masupu and Majok, 1998)
Ethiopia	ELISA	19.26	(Hagos *et al*., 2010a)
Kazakhstan	CFT	16.4	(Claes *et al*., 2005)
Mongolia	CFT and ELISA	7.6 and 6.7	(Clausen *et al*., 2003)
Nambia	CFT	8.33	(Kumba *et al*., 2002)
Italy	CFT	0.54	(Calistri *et al*., 2013)

2.3.3. Transmission

Unlike other trypanosomal infections, dourine is transmitted almost exclusively during coitus. Dourine is the only trypanosomosis that is not transmitted by an invertebrate vector. *T. equiperdum* differs from other trypanosomes in that it is primarily a tissue parasite that is rarely detected in the blood (OIE, 2013).

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. Trypanosomes are rarely observed in the bloodstream of the host because they are normally localized in the capillaries of the mucous membranes of the urogenital tract. However, a few trypanosomes occasionally appear in the peripheral blood of animals with chronic infection. This could provide the opportunity for bloodsucking insects to mechanically transmit this parasite, although this is considered to be very rare (Wang, 1988).

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 (OIE, 2013). Study conducted using clinical findings, laboratory and epidemiological analyses of the outbreaks in Italy,

based on features such as prevalence, age, reproductive activity and relationship between the affected animals indicated that the infection is transmitted directly from animal to animal during coitus (Calistri *et al*., 2013). As the disease progress, trypanosomes periodically disappear from urethra or vagina; during these periods, the animals are non infective. Non infective periods may last for weeks or months and are more likely to occur in the later stages of the disease. Thus, transmission is most likely in the early disease process (Wang, 1988).

An interesting finding in the literature was a positive PCR test result from a prepuce swab taken from a dourine-free stallion immediately after mounting an infected mare. The horse remained negative at all subsequent tests, supporting the theory that the parasite is present in the genital tissues but that sexual transmission is not constant (Vulpiani *et al*., 2013).

Trypanosoma 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. Foals born to mares infected with *T. equiperdum* may be infected in utero or may become infected during parturition. Transmission to foals by ingestion of infected colostrum or milk is considered rare (William and Steven, 2007). The presence of trypanosomes in the mammary secretions may support that the infection can occasionally pass to foals during suckling (Pascucci *et al*., 2013). Foals that ingest colostrum from infected mares will become seropositive due to passive transfer of antibodies; these foals are usually sero negative by 4 to 7 months of age (William and Steven, 2007).

2.4. Clinical Signs

The incubation period between exposure and initial clinical signs is highly variable; it may be as short as 1 to 2 weeks or as long as several years (William and Steven, 2007). Clinical signs of dourine are highly variable in manifestation and severity. The disease is characterized mainly by swelling of the genitalia, cutaneous plaques and neurological signs but severity varies with the virulence of the strain, the nutritional status of the horse, and stress factors. Clinical signs often develop over weeks or months, frequently waxing and waning with relapses, probably precipitated by stress. This can occur several times before the animal either dies or experiences an apparent recovery. The mortality rate is believed to be in excess of 50% (Sidney *et al*., 2013).

A number of authors have broken the course down into three stages: stage 1 (genital lesions), stage 2 (cutaneous signs) and stage 3 (nervous signs) (Claes *et al*., 2005). Stage 1 involves genital edema and swelling, manifesting 1-2 weeks after infection. In stage 2, typical cutaneous plaques ("silver dollar" plaques) appear, with thickening of the skin, considered pathognomonic by some authors. Stage 3 is characterized by progressive anemia, neurological disorders and paresis of the hindquarters, often ending in death.

A pathognomonic sign is the edematous plaque consisting of an elevated lesion in the skin, up to 5–8 cm in diameter and 1 cm thick. The plaques usually appear over the ribs, although they may occur anywhere on the body, and usually persist for between 3 and 7 days. They are not a constant feature. Pyrexia is intermittent; nervous signs include incoordination, mainly of the hind limbs, lips, nostrils, ears, and throat. Depigmentation of the genital area, perineum, and udder may occur. In the stallion, the first clinical sign is a variable swelling involving the glans penis and prepuce. The edema extends posteriorly to the scrotum, inguinal lymph nodes, and perineum, with an anterior extension along the inferior abdomen. In stallions of heavy breeds, the edema may extend over the whole floor of the abdomen (OIE, 2013).

Experimental infections in horses through infusion into the urogenital tract have been performed in South Africa using the OVI strain (Barrowman, 1976) in the USA using the American and Canadian strains (Hagebock, 1993) and in Kazakhstan using a wild-

type strain. In the US study, none of the 20 infected horses developed typical clinical signs; they showed only general signs of trypanosomosis. In South Africa and Kazakhstan, however, the animals showed typical signs of dourine, such as scrotal edema, emaciation and posterior paresis, but the presence of the pathognomonic dourine plaques were not reported by the authors. Apparently, differences in pathology are observed between animals in these experimental infections but it remains unclear whether the differences are related to the *T. equiperdum* strain that is used or whether they are caused by differences in the host immune response.

Observation made by Vulpiani *et al*. (2013) indicates that, infected stallions revealed mild signs than in the infected mares. Six months after infection, the stallions were almost asymptomatic. However, the differences with respect to sex cannot be statistically examined because of low number of considered cases in the study. Observation made by Watson (1920) indicates that, apart from the fact of increasing virulence resulting from continued passages, accords with general experience that the disease is usually more progressive in the stallion than in the mare.

2.5. Pathological Lesions of Dourine

The disease is characterized by edematous lesions of the genitalia, involvement of nervous system and progressive emaciation, and it is ultimately fatal in most cases. Typical cutaneous lesions, from which the disease derives its name "dourine," have been described as circular elevated plaques of thickened skin ranging in size from 1 to 10 cm in diameter, resembling money or "douros" (Claes *et al*., 2005).

2.5.1. Gross pathological lesion

Dourine is characterized by cachexia, anemia, muscular hypotrophy, ataxia and lack of coordination of the hindquarters, ptosis of the lower lip, genital lesions, skin edematous plaques and peripheral edema (Pascucci *et al*., 2013). 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).

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

The presence of dourine infection in the stallions did not appear to interfere with libido or the ability to achieve erection even where there is pronounced edema of the scrotum and sheath. Similarly the presence of infection did not appear to affect adversely the fertility of either stallions or mares. This study also reported, on five (5) occasions clean mares conceived to services by infected stallions and on three (3) occasions infected mares conceived to services by clean stallions. Two foals born to infected mares were normal and were reared to maturity (Barrowman, 1976).

2.5.2. Microscopic Lesions

On histological examination of tissue samples, the disease is characterized by haemosiderin deposition in the spleen, the iliac, supramammary and popliteal lymph nodes showed non-specific reactivity with hyperplasia of the plasma cells, a sign of increased haemolymphatic activity. The edematous 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 was an exudates of cell detritus in the same area, mainly eosinophils and the bodies of free parasitic protozoa, in a picture described as “trypanosomal sand” (Figure 2) (Pascucci *et al*., 2013). A similar finding has been reported by Scacchai *et al.* (2011).

In the nervous system of infected horses, neurodegenerative lesions and inflammatory vasculitis of the central nervous system with edematous infiltration in the facial and lingual nerves was reported. In the udders, there are histological lesions attributable to

severe interstitial inflammation accompanied by strong supra mammary lymph node reactivity and the presence of Russell's bodies. Multifocal areas of hepatitis in the liver, while the kidneys are affected by plasma cell inflammation of the renal pelvis. Periglandular inflammation in the vulva, vagina uterus and clitoris was also observed in infected mare (Pascucci *et al*., 2013). The constant finding of iliac and supramammary lymph node positivity and lymphatic activity on both macroscopic observation and histological examination seem to confirm that the parasite spreads mainly through the lymphatic system (Theis and Bolton, 1980; Pascucci *et al*., 2013).

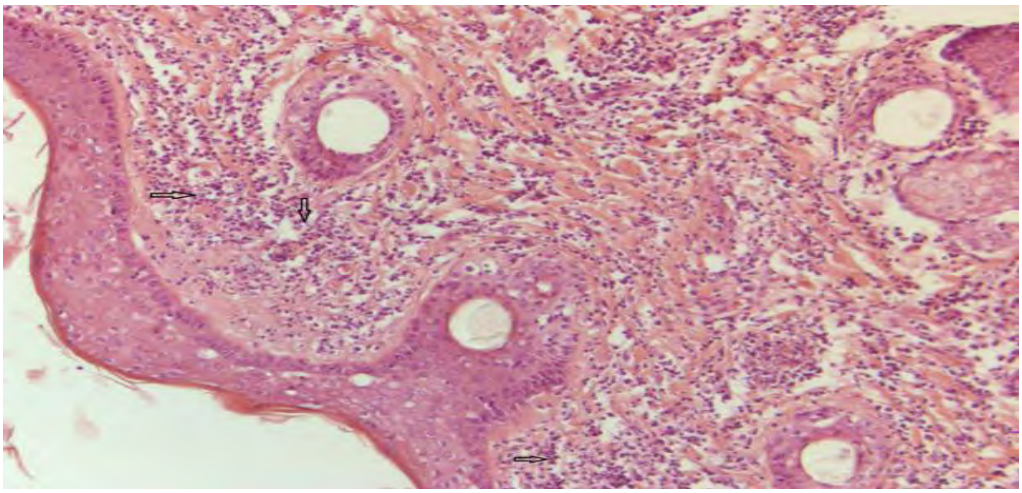


Figure 3: Section of skin of *Trypanosoma equiperdum* infected mare. Note the inflammatory cells in subcutaneous space and "trypanosomal sand" (arrow) area with pustular dermatitis and vacuolar degeneration of the basal cell layer HE 20× (Pascucci *et al*., 2013).

2.6. Diagnosis

Diagnosis of dourine is a challenge, due to limited knowledge about the parasite and host-parasite interaction following infection. In practice, diagnosis is based on clinical evidence supported by serology (Alemu *et al*., 1997; Hagos *et al*., 2010a). 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 (Claes *et al*., 2005). The incubation period may vary from few weeks to several years and some of the clinical signs, which include genital edema, weight loss, skin lesions known as "silver dollar plaques" and neurological signs, may be absent in the early stages or during latent infections (Luckins *et al*., 2004; Claes *et al*., 2005). Diagnosis of dourine therefore, requires confirmation by parasitological, serological and molecular techniques.

2.6.1. Parasitological diagnosis

Wet and thick blood films

In this test 5 to 10 µl of blood is placed on a slide and examined microscopically at x400 magnification under a cover slip. Trypanosomes are observed moving between the erythrocytes in infected animals. It has very low sensitivity, with a detection limit as high as 10,000 trypanosomes/ml, but it is still in use because of its low cost and simplicity. Giemsa or Field's-stained thin blood films have a similarly low sensitivity. It is time consuming (10-20 minutes per slide) and requires expertise to recognize the parasite.

Micro Hematocrit Centrifugation Technique (mHCT)

Micro Hematocrit Centrifugation Technique (mHCT) is a blood concentration technique (also called the capillary tube centrifugation technique or the Woo test) is the most frequently applied concentration technique with better sensitivity than direct microscopic examination. In this test, capillary tubes containing anticoagulant are filled three-quarters full with blood. The dry end is sealed with plasticine. By high-speed blood centrifugation in a hematocrit centrifuge for 6 to 8 minutes, trypanosomes are concentrated between the red blood cells and the plasma, together with the white blood cells. The capillary tubes mounted in a special viewing holder, can be directly examined at low magnification (x10 or x40) for motile parasites. The estimated detection threshold of mHCT is 500 trypanosomes/ml of blood sample (Woo, 1970; Reid *et al*., 2001).

Mini Anion-Exchange Centrifugation Technique (mAECT)

The mini Anion-Exchange Centrifugation Technique (mAECT) consists of separating the trypanosomes, which are less negatively charged than blood cellular components from venous blood via anion-exchange chromatography and finally concentrating them at the bottom of a plastic collector tube by low speed centrifugation. The tip of the glass tube is then examined in a special holder under the microscope for the presence of trypanosomes. The large blood volume up to 300 µl enables the detection

of less than 100 trypanosomes/ml, resulting in high sensitivity. However, the manipulations are quite tedious and time consuming (Reid *et al*., 2001; Buscher *et al*., 2009).

Apart from the above-mentioned parasitological diagnostic techniques examination of discharge from the genital tract and edematous fluids can be performed. Accordingly, the obtained discharge/fluid is microscopically examined as a fresh or fixed Giemsa stained preparation. However, direct parasitological demonstration of *T. equiperdum* is insensitive mainly due to the periodically cryptic nature of parasitemia that results in long periods during which the host has no detectable parasites in the blood (Hoare, 1972; Stephen, 1986; OIE, 2008).

Animal inoculation

Repeated attempts have been made by different workers (Alemu *et al*., 1997; Clausen *et al*., 1999, 2003) to demonstrate and isolate *T. equiperdum* in laboratory mice but all were unsuccessful. However, once a strain becomes adapted to rodents, the parasites can be maintained by serial passages, in the same manner as *T. evansi* (Luckins, 1994). Under laboratory conditions dogs can be infected with *T. equiperdum* as reported by Rouget (1896). In experimental infections carried out in the Institute for Tropical Medicine to raise antisera against VSGs, rabbits infected with the available laboratory strains developed clinical signs that could not be distinguished from those developed by rabbits infected with *T. evansi* (Verloo *et al*., 2001).

Owing to the marked predilection of *T. equiperdum* for the testicles of rabbits, some authors recommended intratesticular inoculation of these animals for the diagnosis of dourine in equines. Ruminants were refractory to infection with *T. equiperdum* (Hoare, 1972).

2.6.2. Serological techniques

It is extremely difficult to detect the parasite in the body fluids of infected horses Claes *et al*. (2005) therefore; diagnosis of *T. equiperdum* by standard parasitological techniques is difficult, owing to the low numbers of parasites in the blood or tissue

fluids. Consequently, the demonstration of trypanosomal antibodies in the serum has become the most important parameter determining the disease status of individual animals (Bishop *et al*., 1995). *Trypanozoon* group-specific trypanosomal antigen could be of use in an antibody assay for the diagnosis of *T. equiperdum* infections. However, based on anecdotal evidence, it appears that *T. equiperdum* infected laboratory animals and horses suspected of dourine also positively react in the CATT/ *T. evansi* and ELISA/ *T. evansi* prepared with fixed whole trypanosomes of the RoTat 1.2 VAT (Claes *et al*., 2003).

CATT/ *T. evansi* test is fast, uses a standardised antigen and can be performed in situ, i.e. without the need of a fully equipped laboratory. Recently, it has been proven that most so-called *T. equiperdum* strains also express isoVATs of *T. evansi* RoTat 1.2. Therefore, the CATT/*T. evansi* may prove to be a good test for equine trypanosomosis, regardless whether the causative agent is *T. evansi* (surra) or *T. equiperdum* (dourine) (Claes *et al*., 2003).

The complement fixation test is the most commonly used OIE prescribed serodiagnostic test developed for *T. equiperdum* and successfully used as part of a program to eliminate *T. equiperdum* from North America. It is still used for international trade in monitoring horses to export / import. Despite the usefulness and universal acceptance of the CFT for diagnosing dourine, some discrepancies have been recorded. The disadvantages of the CFT are that it requires careful continuous titration of numerous labile agents and that it does not function with sera having anti-complementary activity. CFT is not species specific, but only specific for the subgenus *Trypanozoon*. The drawback of the test is lower specificity where it cannot differentiate *T. equiperdum* from other similar trypanosomes. Hence, the diagnostic significance of CFT is therefore doubtful in countries where both *T. equiperdum* and *T. evansi* infection occur in equines (Luckins, 1994). Although the CFT has been in use for many years for diagnosis of dourine, it is considered to be less sensitive than ELISA and IFAT for the detection of the serum antibodies against *T. equiperdum* (Wassal *et al*., 1991; Bishop *et al*., 1995).

Indirect fluorescent antibody test is frequently used for the diagnosis of dourine, as a confirmatory test for CFT results, since immunofluorescence provides a reliable and sensitive technique. But its interpretation is both subjective and labour intensive and it is therefore more suited to the testing of small numbers of sera (Williamson *et al*., 1988).

The use of ELISA for routine diagnosis of dourine would provide a significant advantage over current serological tests if a defined antigen were used, since it would permit test standardization and more readily allow comparison of tests among laboratories. It additionally, lends itself to a considerable degree of automation, which makes it suitable for large number of samples (Wassal *et al*., 1991). Different workers have stated that the ELISA has a satisfactory concordance ratio with CFT and can be used to supplement CFT (Williamson *et al*., 1988; Alemu *et al*., 1997).

There are also several other alternative serological tests that are used, such as the agar gel immunodiffusion test, the arrayed immunodiffusion method (Hagebock *et al*., 1993) and the competitive immunoassay (cELISA). The cELISA method has several advantages over the CFT: it can be performed in less time than the corresponding CFT procedure, it is reproducible, results are objectively measured and calculated and the method is amenable to automation (Katz *et al*., 1999).

While serological tests can be the method of choice for mass screening of populations, their main limitation will remain the failure to demonstrate the parasite. Unfortunately, parasitological techniques are known to lack sensitivity, especially for the detection of *T. equiperdum*, which is considered to be a tissue parasite rather than a blood parasite (Brun *et al.,* 1998).

2.6.3. Molecular techniques

Although no *T. equiperdum* specific polymerase chain reaction (PCR) method is available, subgenus *Trypanozoon*-specific PCR can be used for detection of *T. equiperdum* DNA. Recently, a highly sensitive real-time PCR for *Trypanozoon* subgenus was applied on tissues and fluid samples from a naturally dourine-infected horse, enabling the detection of low numbers of parasites (Pascucci *et al*., 2013;

Scacchia *et al.,* 2011). PCR and other related DNA amplification methods have been used to examine exudates or tissue samples, taking into account their failure on blood samples after the initial phase of the infection (Calistri *et al*., 2013).

Direct diagnosis based on molecular techniques can be highly sensitive for parasite detection in body fluids such as blood (Becker *et al*., 2004). However, this approach is difficult to apply for mass screening and negative results do not exclude the possibility of infection. In fact *T. equiperdum* multiplies predominantly in extracellular tissue spaces and is seldom found in peripheral blood (Theis and Bolton, 1980). Diagnosis of *T. equiperdum* infection is thus still strongly based on serological evidence.

2.7. Treatment

Pharmaceutical therapy is not recommended because animals may improve clinically but remain carriers of the parasite (OIE, 2013). There are no officially approved drugs to treat horses suffering from dourine although some older publications mentioned experimental treatment of horses with suramin and neoarsphenamine (Ciuca, 1933) or quinapyramine sulphate (Vaysse and Zottner, 1950). Evidence from in vitro drug sensitivity determination of *T. equiperdum* (Zhang *et al*., 1992; Brun and Lun, 1994) indicates that suramin, diminazene, quinapyramine and cymelarsan are effective although no reports on clinical efficacy have been published.

Brun and Lun (1994) reported drug sensitivity of *T. equiperdum* isolates in vitro and found the isolate was highly sensitive to melarsorol, isometamidium and suramin; with regard to diminazene, *T. equiperdum* was not sensitive as the most sensitive *T. evansi* strains.

Hagos *et al*. (2010b) reported efficacy of Cymelarsan® and Diminasan® against *T. equiperdum* infections in mice and horses. Accordingly, Diminasan® in mice at doses ranging from 3.5 mg/kg to 28.0 mg/kg body weight failed to eliminate 713/943 or 834/940 Dodola strains in mice. In mice, Cymelarsan® failed to cure infection with *T. equiperdum* Dodola strains at 0.25 mg/kg and 0.5 mg/kg doses. However, at higher

doses of 1.0 mg/kg and 2.0 mg/kg body weight the drug effectively cleared the mice from parasites with no relapse for 60 days (Hagos *et al*., 2010b).

Cymelarsan® was found to be quite effective in curing horses at both 0.25 mg/kg and 0.5 mg/kg in acute as well as chronic form of dourine. Cymelarsan® at 0.25 mg/kg and 0.5 mg/kg body weight cleared parasitemia within 24 h post treatment and none of the animals were found to show relapse throughout the 320 days of observation. The sensitivity of the particular trypanosome strain to Cymelarsan® was also supported by the relative improvement in the mean PCV levels of horses following treatment. This is a first promising result in the possible cure of dourine infected horses, yet a thorough evaluation of Cymelarsan® in horses should be performed on a larger scale to validate these findings (Hagos *et al*., 2010b)

Diminasan® failure could be attributed to the relatively rapid excretion of the drug (Mulligan, 1970) or its pharmacokinetics. Diminasan® cannot cross the blood brain barrier and enter somatic tissues as a result it cannot be the curative drug for trypanosomes with tissue and central nervous system affinity. Indeed, Diminasan®, was found to be ineffective against infections involving central nervous system as parasitemia returned rapidly after a few days of post treatment and the central nervous system was demonstrated to be the source of relapsing parasitemia (Jennings *et al*., 1979, 1980). The relative efficacy of Cymelarsan® is associated with remarkable ability to cross the BBB (Pepin and Milord, 1994).

2.8. Prevention and Control

There is no vaccine available for dourine. As dourine is primarily a venereal disease, prevention of natural mating or artificial insemination with infected horses (stallions or mares) or infected stallion semen is the most important means of control. Prevention of dourine is therefore based on the establishment of freedom from infection and this is done by testing blood for the presence of antibodies against *T. equiperdum*, which is more reliable than testing for the presence of the protozoan parasite itself. Any introductions of horses from endemic areas or areas of incursion should be isolated and blood tested for antibodies by complement fixation test (CFT) (Sidney *et al*., 2013).

Control of the disease depends on compulsory notification, slaughter of infected animals and movement control enforced by legislation in most countries (OIE, 2013). Dourine should be eradicated from an incursion into a non endemic area by identification of the source, thorough tracing and testing of all in contacts and euthanasia of infected and seropositive horses (Sidney *et al*., 2013).

Currently an eradication strategy is imposed by the World Organization for Animal Health (OIE) with slaughtering of seropositive horses while treatment is prohibited (Zabotskij *et al*., 2003). However, it is not economically feasible to apply strict test and slaughter policy to control dourine in developing countries. Based on result of in vivo drug sensitivity study a revised strategy of the appropriate drug treatment in dourine endemic areas with instead of eradication could be recommended to the OIE (Hagos *et al*., 2010b).

It is important to note that castrating adult stallions does not always change the copulatory ability of such animals and it should be performed with caution when attempting an eradication programme. To prevent the introduction of dourine, serum samples should be taken following a period of isolation (quarantine) to ensure that the animals are not in the incubation period (Zablotskij *et al*., 2003).

The difficulty in the diagnosis of *T. equiperdum* has led to difficulties in obtaining reliable data on the prevalence and distribution of the disease, and for the implementation of monitoring, treatment and control programmes. Moreover, shortages of trypanocidal drugs and the absence of vaccines against trypanosomosis have hampered the control and prevention of the disease in endemic areas (Clausen *et al.*, 2003).

2.9. Status of Dourine in Ethiopia

Despite their important contribution to transport in rural and sub-rural parts of Ethiopia, little attention has been paid to equines in terms of health care and husbandry managements (Feseha *et al*., 1997).

The first official report of dourine in Ethiopia was made in 1980 when the Arsi Rural Development Unit asked the Tsetse and Trypanosomosis Survey and Control Department to investigate a persistent disease problem in horses in the administrative regions of Arsi and Bale (Zeleke *et al*., 1980). Since then, dourine has been found to be prevalent throughout the highlands of Ethiopia, particularly in the Arsi and Bale zones (Alemu *et al*., 1997). Because of diagnostic problem, unrestricted movement, uncontrolled breeding and lack of effective trypanocidal drugs, dourine remains a potential threat to the life and productivity of the high equine population in Ethiopia (Hagos *et al*., 2010b).

The problem of dourine in Ethiopia has been recognized by local farmers for many years and it has been found to be a threat to the life and productivity of the equine population in the Arsi-Bale highlands (Zeleke *et al*., 1980). According to this report, the disease was widely spread in Ethaya, Sagure, Bekoji and Koffle districts of Arsi-Bale highlands. The dourine endemic foci, the Arsi-Bale highlands of Ethiopia are situated outside the tsetse-infested belt (2400-3400 meters above sea level) (Abebe, 2005).

Figure 4: Distribution of dourine in Ethiopia (Abebe, 2005)

In those areas, the disease is known commonly as "Lappessa" or “Duda Kuta” which means emaciation or backbone breaker in the local language, respectively. There have been reports indicating the presence of *T. equiperdum* causing dourine in Ethiopia based on clinical signs (Zeleke *et al*., 1980), serological and molecular tests (Alemu *et al*., 1997; Clausen *et al*., 1999; Hagos *et al*., 2010a).

Since dourine is principally and only transmitted by coitus and due to the marked emaciation observed in late stages of the disease, some farmers used to call it ‘Horse's AIDS’. They associated the disease incidence with horses having sexual contact with so-called prostitute horses or diseased horses. Though dourine is a common clinical case throughout the year, it has a seasonal character, which most commonly occurs following the breeding season from June to late September. Sometimes a second peak is observed in the dry seasons of the year (March to May), which was probably associated with relapse of previously infected and recovered cases due to stressful conditions of feed shortage (Alemu *et al*., 1997; Hagos *et al*., 2010a)

Similarly the prevalence of dourine in the arsi-bale highlands has been reported by different researches. Accordingly, Clausen *et al.* (1999) reported 28.3% seroprevalence using CFT. Hagos *et al.* (2010a) reported seroprevalence of 28%, 24.81%, and 19.26% using CATT/*T. evansi*, Latex/*T. evansi* and ELISA/*T. evansi*, respectively. Fikru *et al.* (2010) reported the prevalence of dourine to be 4.6% (11 out of 237) parasitologically using Woo test and relatively higher prevalence using serology CATT/*T. evansi* test (27%) and molecular tests (36.7% RoTaT 1.2 PCR and 47.6% 18S PCR). Interestingly, this is for the first time that trypanosomes causing dourine were parasitologically demonstrated by Woo test in Arsi-Bale highlands from horses suspected of dourine and showing clinical signs. The findings of the study disclosed that dourine is highly prevalent and one of the major diseases of horses in the area (Fikru *et al*., 2010).

Isolation of *T. equiperdum*, the causative agent of dourine in horses, by standard parasitological techniques is usually difficult, due to low numbers of parasites in the blood or tissues fluids (Mulligan, 1970). However, the causative agent of dourine was isolated from two clinically sick horses in Dodola, Ethiopia. These horses were found to be positive in CATT/*T. evansi* (Bajyana and Hamers, 1988) and RoTat 1.2 PCR

(Claes *et al*., 2004) specifically developed for *T. evansi*. Yet, further analysis by RAPD (Claes *et al*., 2003) indicated that the Dodola strains have banding pattern similarity with *T. equiperdum* OVI strain, but not with the *T. evansi* strains tested. It can therefore be deduced that dourine in the Arsi–Bale highlands of Ethiopia is caused by *T. equiperdum*. 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 (Fikru *et al*., 2010).

Yet, a relative efficacy of Diminazene aceturate on *T. equiperdum* isolates was observed following in vitro drug sensitivity tests (Zhang *et al*., 1992; Brun and Lun, 1994). In contrast, it was shown by Tuntasuvan *et al.* (2003) that Diminazene aceturate was ineffective in curing and preventing relapses of *T. evansi* infections in horses and mules. Despite this knowledge, local veterinarians and veterinary assistants in the highlands of Ethiopia still use diminazene to treat suspected trypanosome infections.

Horses are treated against dourine only irregularly when trypanocidal drugs are available, but even such treated animals show frequent relapse and generally, treatment is not able to cure clinical cases. Some of the trypanocidal drugs used in the area, whenever available, include Veriben (diminazene aceturate) and quinapyramine sulphate (Triquin-S®,Wockhardt Veterinary Ltd., India) (Hagos *et al*., 2010b).

Ethiopia has a very large equine population and in view of unrestricted movement of horses and lack of adequate facilities for diagnosis and control of the disease in relation to breeding, dourine is potentially a very important disease (Alemu *et al*., 1997).

The Arsi-Bale highlands, the breeding area of horses in Ethiopia, are highly endemic for dourine and consequently need control in animal movement and care in breeding programme to restrain the disease (Hagos *et al*., 2010a).

3. MATERIALS AND METHODS

3.1. Study Area

The present study was carried out in two dourine prevalent or endemic foci districts namely Dodola and Assassa located in the Western Arsi highlands of Oromia regional state, Ethiopia. Dodola is located in the West Arsi Zone, 320 km away from the capital Addis Ababa, at 6.983°N latitude and 39.183°E longitude with an elevation ranging from 2,362 to 2,493 m above sea level. Assassa is also located in West Arsi Zone, about 300 km South from Addis Ababa with geographical coordinates are 7.2° N, 39.2° E, with an elevation 2,600 to 2,650 m above sea level. Agriculture is the mainstay of the livelihood of peoples and the leading economic activity of the area with a mixed farming system covering the highest percentage of the total agricultural activities with crop-livestock production (Arsi-Bale Zone Agricultural and Rural Development Office, 2009). Equine population is the highest in Oromia region mainly of the Arsi-Bale highlands (Arsi-Bale zone plan office, 2006).

3.2. Study Population

Animals considered in this study are sexually mature adult horses and which were suspected of naturally infected with *Trypanosoma equiperdum* showing typical clinical signs of dourine, living under a traditional management system of free grazing.

3.3. Study Design and Sampling Method

A cross-sectional study design and purposive sampling method were used to identify horses showing typical clinical signs of dourine from November 2014 to June 2015. Horses present in the study area were examined clinically for the presence of typical signs of dourine and were then followed by sample collection and laboratory examination.

3.4. Clinical Examination

Careful and systematic clinical examinations of sexually matured horses in the study area were done. Special attentions were given to the body condition, skin, external reproductive genitalia and nervous system. During field observation detail records about history, sex, age, observed signs and body condition of the animals were recorded. In addition, a detailed physical examination of horses including the measurement of the vital parameters (temperature, heart and respiratory rate) was conducted. Infected animals showing signs of dourine were tested parasitologically as well as, serologically using buffy coat examination and Card Agglutination Test for Trypanosomosis (CATT/*T.evansi*) respectively.

3.5. Blood Collection

Blood samples were collected from the jugular vein of horses showing sign of dourine twice using plain for serological and serum biochemical test and heparinized vacutainer tubes and needles for parasitological examination, after the site had been wiped with cotton wool soaked in alcohol.

3.6. Parasitological Examination

Haematocrit centrifugation technique (mHCT) was used to isolate the parasite from blood. Two capillary tubes with an internal diameter of 1 mm were filled with blood up to three-fourth (50 µl) of the capacity and centrifuged for 5 minutes in micro-centrifuges at maximum 12,000 rpm. The capillary tubes are then mounted in a viewing slide and the buffy coat plasma interface layers were examined at magnification of 10x under microscope as described by Woo (1970) and Reid *et al*. (2001) to look for live parasites.

3.7. Serological Examination

The CATT/RoTat 1.2 serological test was performed at field level which is considered as a rapid field screening tests in accordance with Claes *et al*. (2003). The antigen and buffer of CATT/*T. evansi* test was obtained from VLIR OUS Ethio-

Belgium Team Project laboratory. It is a rapid direct agglutination test, which uses formaldehyde fixed, Coomassie stained, freeze-dried trypanosomes of *T. evansi* VAT RoTat 1.2. In the CATT/ *T. evansi* test, 30 µl of sera diluted two-fold with PBS were mixed with 30 µl of reagent on a test card. The reagent and test serum were mixed, spread over approximately 1.5 cm. The tests were then checked with positive and negative controls before the whole samples were tested. The presence of antibodies was revealed by macroscopic agglutination (Bajyana and Hamers, 1988; Verloo *et al*., 2001).

3.8. Serum Biochemical Profile Analysis

Serum biochemical tests were performed on serum samples prepared from whole blood collected using plain vacutainer tubes, centrifuged at 3200rpm for 10-15 minutes after 1-2 hours of collection and stored at -20 ^{o}c until analysis is performed. Among the serum samples which were taken from clinically dourine suspected mares and tested positive using CATT/*T.evansi*, 10 positive serum samples were selected purposively. Similarly 10 CATT negative serum samples were taken purposively from horses which were apparently healthy and in good body condition as control group. In such a manner serum samples collected were divided into two groups containing 10 negative (apparently healthy group) and 10 positive serum samples. Serum samples were then tested using humaStar 80 clinical chemistry analyzer according to Reitman and Frankel (1957) and Human Gmbh (Wiesbaden, Germany) standard commercially available Kits according to the manufacturer's instruction to determine the concentration of total protein, albumin, aspartate aminotransferase (AST/GOT), (ALT/GPT) and alkaline phosphatase (ALP) in the two groups at Addis Ababa University College of Veterinary Medicine and Agriculture (AAU-CVMA) physiology and biochemistry laboratory (Annex 1).

3.9. Necropsy, Gross Lesion Description and Tissue Sample Collection

Two mares (M1 and M2) with suggestive clinical signs as well as serologically positive by CATT/RoTat 1.2 test were purchased and euthanized using sodium pentobarbital at a dose of 100 mg/kg through intravenous route. Necropsy of euthanized mares was performed according to the procedure by Dennis and Joanna

(2006) (Annex 2). Euthanized mares were then examined thoroughly for gross pathological lesions in various reproductive organs (udder, vulva, clitoris, vagina, uterus, oviduct, ovary and genital mucosa) as well as the skin, iliac, supramammary, popliteal lymph nodes, spleen, liver, kidney and lung according to VMTH (2009), which included lesion distribution, contour, texture, shape, size and color.

Tissues with lesions were sampled for histopathological examination. The lesion part of the tissue including the normal part is cut to the size of 1-2cm and put in the universal bottle containing 10% buffered formalin. The volume of the formalin was ten times larger than the size of the tissue samples according to Talukder (2007). The sampled tissues were then transported to the NAHDIC for histopathological processing.

3.10. Histopathological Examination

Tissue specimens were processed using standard methods of tissue processing procedure described by Talukder (2007) (Annex 3). Tissues were trimmed, fixed in 10% buffered neutral formalin, dehydrated in ascending grades of alcohol, cleared with xylene and impregnated with molten paraffin wax. Then tissues were sectioned at a thickness of five (5) micrometers, spread on warm water and mounted to frosted glass slides. Then the slides were incubated in incubator at $60^{o}C$ to molten paraffin wax. The sectioned tissues were then deparaffinized in three changes of xylene, rehydrated in descending grades of alcohol and stained with hematoxylin and eosin. Stained slides were examined under microscope using 4x, 10 x, 40x magnification and photomicrographs were taken for documentation (Talukder, 2007).

3.11. Ethical Statement

For the collection of blood specimens from horses and humane killing of horses (Euthanasia) for postmortem examination, ethical approval was obtained from the Ethical Committee of Addis Ababa University College of Veterinary Medicine and Agriculture (CVMA) (VM/ERC/004/03/07/2015).

3.12. Statistical Analysis

The data collected from the study animals were coded, entered and stored in MS excel spread sheet. The collected results of serum biochemical test were also entered into Microsoft excel spread sheet and the data was analyzed using SPSS version 20 software (SPSS, 2011). The mean values of the various serum biochemicals in the two groups were analyzed using independent student t-test. Statistically significant difference was said to exist if $p<0.05$. The gross as well as histopathological lesions and findings were described using qualitative methods.

4. RESULTS

4.1. Clinical Symptoms

Mares positive for *T. equiperdum* showed combinations of various characteristic clinical signs of dourine. The details of the combinations of the clinical signs were indicated in (Table 2). In table 2, a value (0 or 1) is attributed to a given sign in each mare on the basis of its presence (1) or absence (0). So that, it helps to establish which signs of the disease were most common and occur at the same time. The percentage of specific sign were calculated by dividing the total number of specific signs observed in each mare by total number of infected mares and multiplied by 100.

4.1.1. Emaciation or Weight loss

All animals with clinical signs of dourine were emaciated, weak and were in the state of poor body condition. Inelastic skin and a dull coat with numerous grazes around protruding bones were observed. There was no appetite loss in any of the cases during examination and as complained by the animal owners.

4.1.2. Genital signs

Edema of the external genitalia and depigmentation of skin commonly around the vulva but extending to the skin of tail in some cases (Figure 5A) and scars over the external genitalia, were the prominent signs observed in the genital form of the disease In some mares, there were ulcers on the labia and clitoris (Figure 5B) and vaginal discharges in some others (Figure 5C).

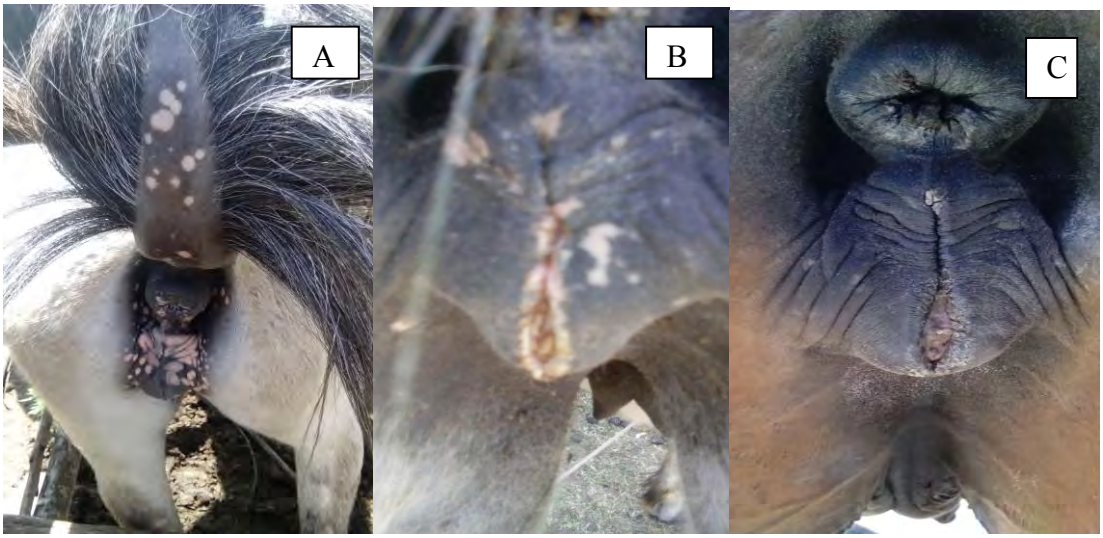


Figure 5: (A) Depigmentation in the perineal region and swollen vulva (B) Ulcerated lesion of labia and clitoris (C) Mucopurulent vaginal discharge

4.1.2. Edema of the mammary gland and ventral abdomen

Few of infected mares showed non-painful edema of the mammary glands and yellowish serum-like mammary secretion. Similarly, few mares showed ventral edema that extends from the sternum region to the umbilical area. In this study cutaneous wheals and plaques that are considered as important sign of cutaneous form of the disease were not observed in any of the positive mares.

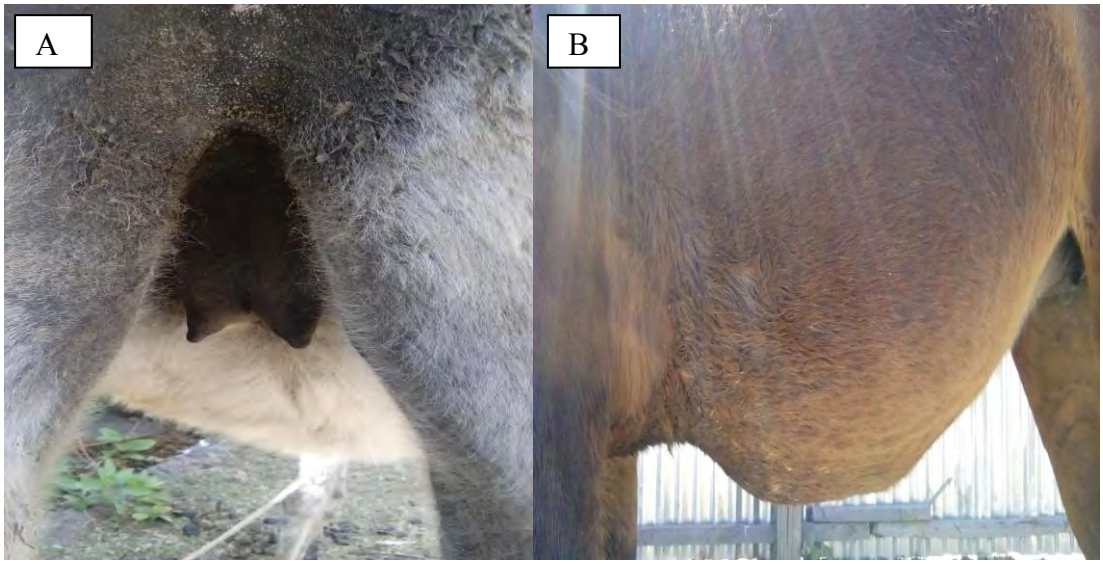


Figure 6: (A) Edema of the mammary gland (Non lactating mare) (B) Ventral edema (Mare)

4.1.3. Nervous signs

Neurological signs include difficulty in walking with marked ataxia of hindquarters and spreading of the limbs. The left hind leg of animals often dragged on the ground with straddle gait with the hind legs held apart, particularly when trying to walk forward.

Table 2: Clinical signs encountered in infected mares

	Genital signs			**Cutaneus signs**		**Nervous signs**
Naturally infected animals	Edema of the vulva	Emaciation	Depigementation around genitalia	Edema of mammary gland	Ventral edema	Difficulty in walking with straddle gait
H1 (M1)	0	1	1	1	0	1
H2 (M2)	1	1	1	0	0	1
H3	1	1	1	0	0	1
H4	1	1	0	1	1	1
H5	0	1	0	0	0	1
H6	1	1	1	0	1	1
H7	0	1	0	0	0	1
H8	1	1	1	1	0	1
H9	0	1	0	0	0	1
H10	1	1	0	0	0	1
H11	0	1	1	0	0	1
H12	0	1	0	0	0	1
Total	50%	100%	50%	25%	17%	100%

1, indicates clinical sign present; 0, indicates clinical sign absent.

4.2. Demonstration of the Parasite and Serology

Even though several attempts has been made to isolate the parasite in the buffy coat examination using Woo test of blood samples from clinically and serologically (CATT/*T.evansi*) positive horses, no trypanosomes detected in all examined blood samples. From the total of twenty (20) clinically suspected horses, twelve (12) mares were found positive serologically using CATT/*T. evansi* test.

4.3. Serum Biochemical Profile Changes

The mean serum level and significance (p-value) of difference for total protein, albumin, AST, ALT and ALP of the clinically negative and CATT negative and clinically positive and CATT positive serum samples are indicated in table 5. Even though, it is not significant (P> 0.05), it has been observed that there was a relative increase in the mean level of serum total protein, AST, ALT and ALP in clinical and serological positive serum samples when compared to the clinical and serological negative serum samples. However, the mean level of albumin is higher in the clinical and serological negative serum samples compared to positive serum samples, even though the difference was not statistically significant (P>0.05).

Table 3: Mean ± SD of serum biochemicals profiles of the clinically and CATT positive mares and clinically and CATT negative horses.

	Mean values ± SD		
Serum biochemical tests	CATT negative	CATT positive	P-value
Total protein (g/dl)	6.7 ±0.83	6.94 ±0.96	0.557
Albumin (g/dl)	2.81 ±0.32	2.74 ±0.45	0.694
AST (U/L)	273.47 ±60.9	320.68 ±135.36	0.338
ALT (U/L)	18.57 ±10.04	26.00 ±10.78	0.128
ALP (U/L)	503.86 ± 128.6	636.95± 177.45	0.071

4.4. Gross and Microscopic Lesions

4.4.1. Lesions encountered in reproductive organs

Mammary gland lesions

Grossly, the udder was slightly swollen and hard. Up on incising of the udder in the second mare (M2), calcified area of white chalky soft lesion was observed (Figure, 7A). Serum-like fluid was oozing from the base of udder up on incision in both euthanized mares. Microscopically, the mammary glands showed interstitial mastitis marked by infiltration of the interstitial space and peri glandular regions with mononuclear cells mainly of lymphocytes and plasma cells (Figure 7B). There were large aggregates of lymphocytes scattered in the mammary glands (Figure 7C). In addition, udder skin hyperplasia and areas of necrosis characterized by the loss of glands were observed (Figure, 7D).

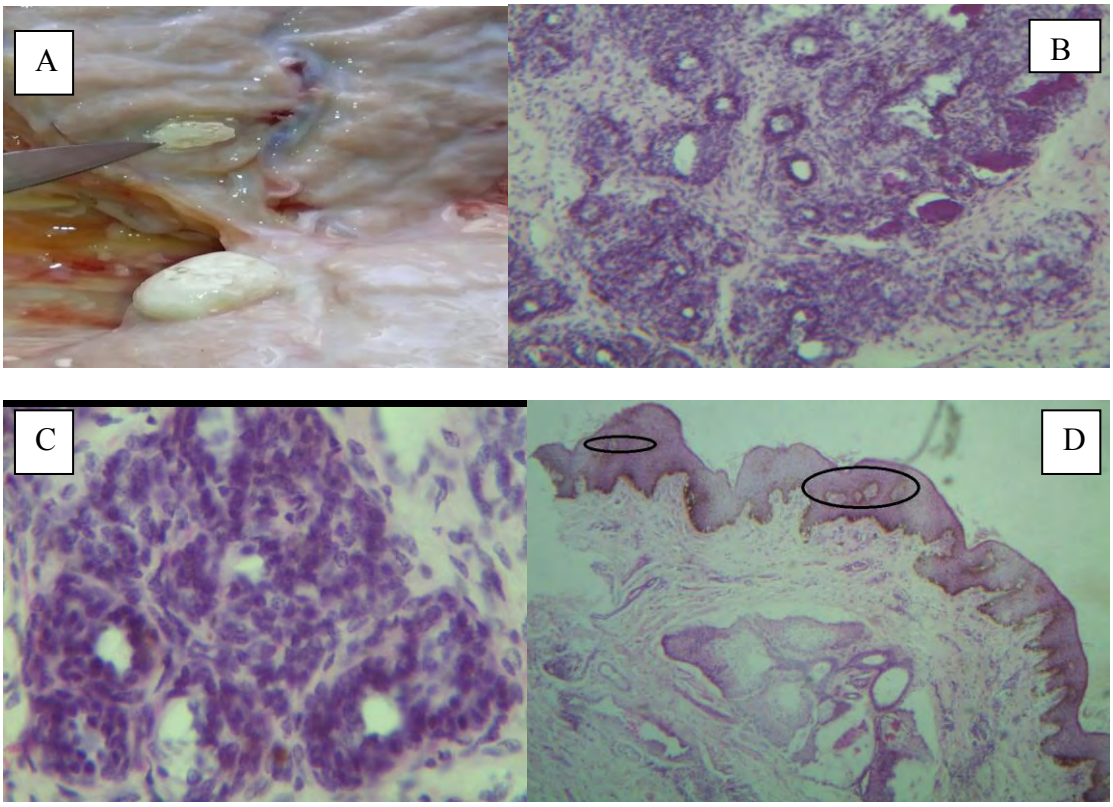
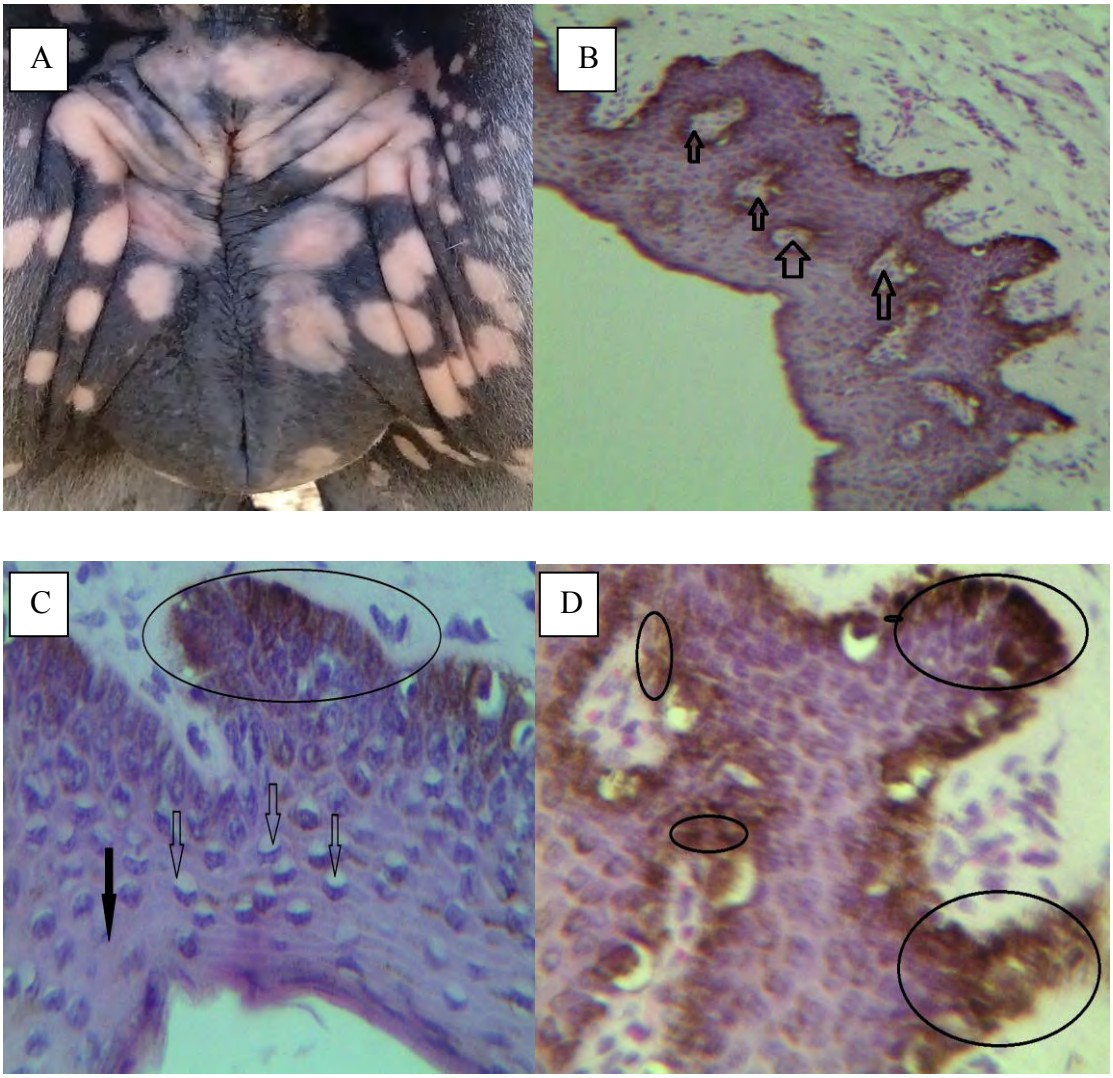


Figure 7: (A) Soft, white, chalky calcified substance within the mammary gland (gross), (B) Interstitial and periglandular infiltration of mammary gland with lymphocytes and plasma cells (10x), (C) Periglandular aggregates of lymphoctes (40 x), (D) hyperplasia of epidermis of skin part of the mammary gland (circled areas) (4x).

Vulval lesions

Grossly, the vulva was swollen and with visible wide spread areas of depigementation on the labia and thickening of the skin (Figure 8A). In addition to these, ulceration of vulva was also seen in one mare (M1). The mucosa of the vulva was normal grossly. Microscopically, the depigmented area of the vulval skin, were characterized by severe necrosis of keratinocytes that result in several cavities in the stratum spinosum (Figure, 8B), vacuolar degeneration of keratinocytes, presence of excessive free melanin in the stratum spinosum and basal layer. Similarly, degeneration of the basal cell layer including the melanocytes was also frequent lesion observed (Figure, 8C and D). A histologic feature of dermatitis marked by accumulation of mononuclear cells mainly of lymphocytes and plasma cells was evident in the dermis part of vulval skin (Figure, 8E). In addition, peri-glandular inflammation marked by infiltration of lymphocytes was also seen within the vulva (Figure, 8F).



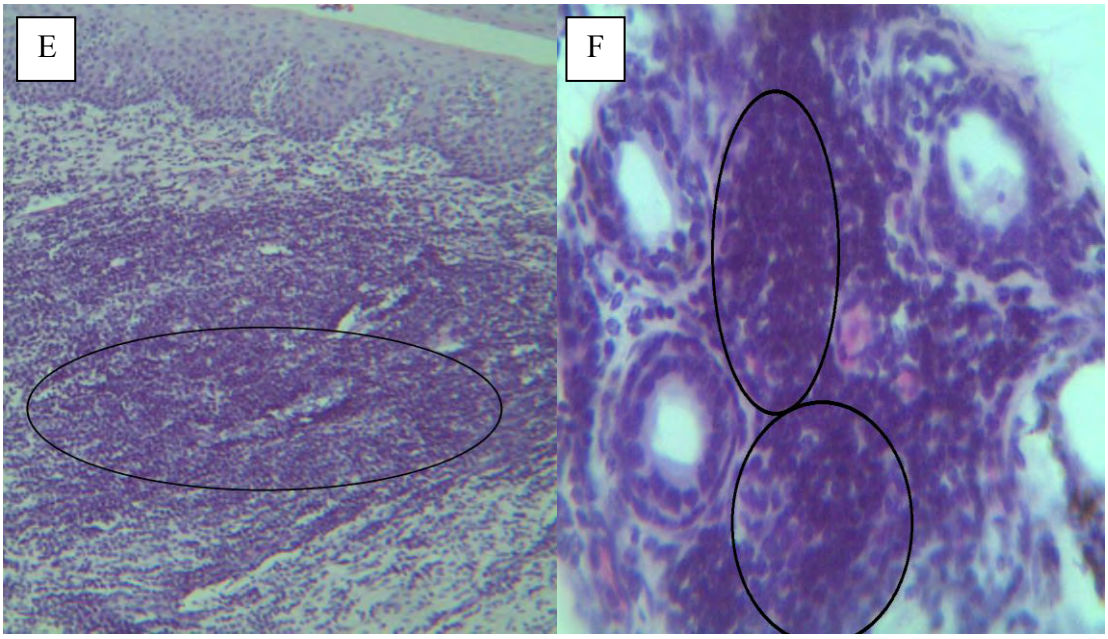


Figure 8: (A) Depigementation of the vulval lip (gross), (B) Necrotized areas in the stratum spinosum living cavity like structures (arrows), (C) Vacuolar degeneration of the cells (lighter arrows) and necrotized cell (darker arrow) in the stratum spinosum, degeneration and necrosis of the basal cells with melanin pigment were evident (circled areas) (D) shows excess free melanin in the stratum spinosum (small circles) and within basal layer (large circles), (E) Sever dermatitis with infiltration of lymphocytes and plasma cells in the epidermis and dermis (circled areas), (F) Periglandular aggregations of lymphocytes and plama cells within the vulva.

Vaginal lesions

Grossly, vaginal lesions were congestion and presence of frothy mucus on mucosal surface in one of the mare (M1) (Figure, 9A). However, there was no visible gross vaginal lesion in the other mare (M2). The microscopic vaginal lesion includes vaginitis with infiltration by mononuclear cell mainly of lymphocytes and plasma cells forming large aggregates or follicles in the mucosa and sub mucosa of vagina (Figure, 9B). The vaginal submucosal blood vessels were hyperemic, distended and are full of RBC were observed (Figure 9C). Periglandular and glandular (mural) inflammation characterized by infiltration of mononuclear cells mostly of lymphocytes were another vaginal lesions frequently observed.

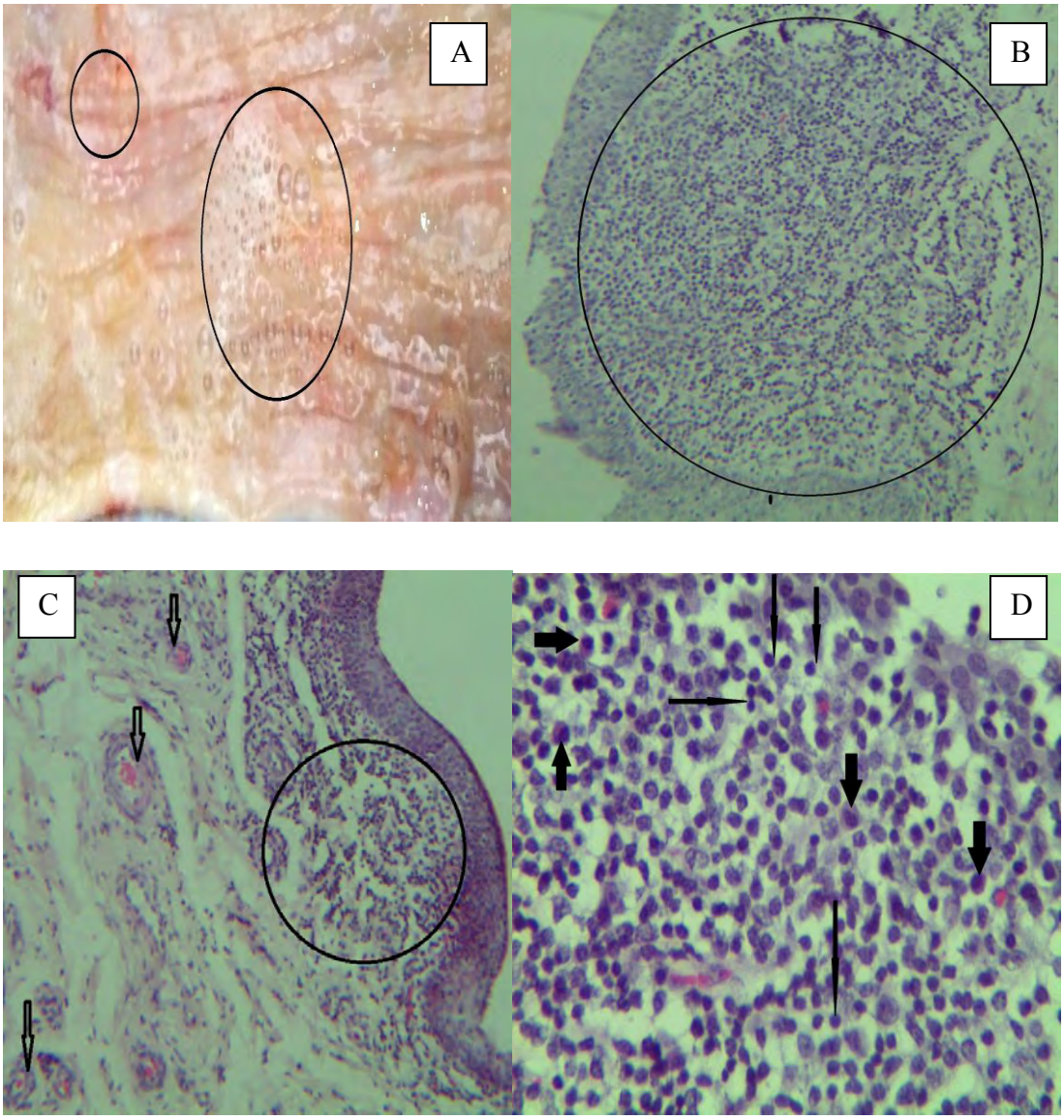


Figure 9: (A) Congestion (small circle) and frothy mucus on the mucosal surface of vagina (large circle) (gross), (B) Infiltration of the mucosa and sub mucosa with mononuclear cells mostly of lymphocytes and plasma cells (circled area), (C) infiltration of the sub mucosa (circled area) and congestion with evident of capillaries full of RBC (arrows), (D) Mononuclear cells in the mucosa of vagina showing lymphocytes (thinner arrows) and plasma cells (thicker arrows).

Uterine Cervical lesions

Grossly, there were no visible cervical lesions observed. However, microscopically infiltration with mononuclear cells (lymphocytes and plasma cells) in the mucosa and submucosa layer of cervix and peri glandular inflammation was observed (Figure, 10A and B).

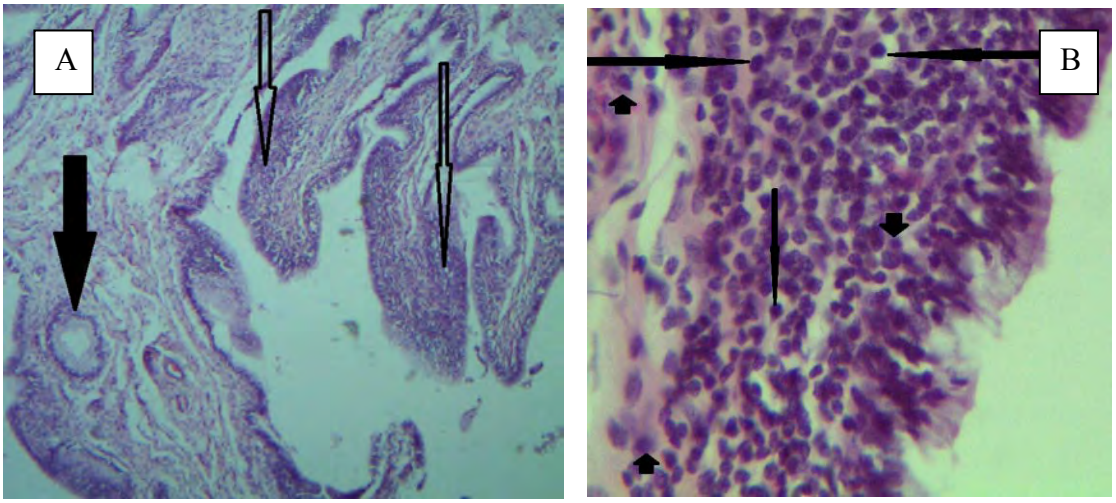
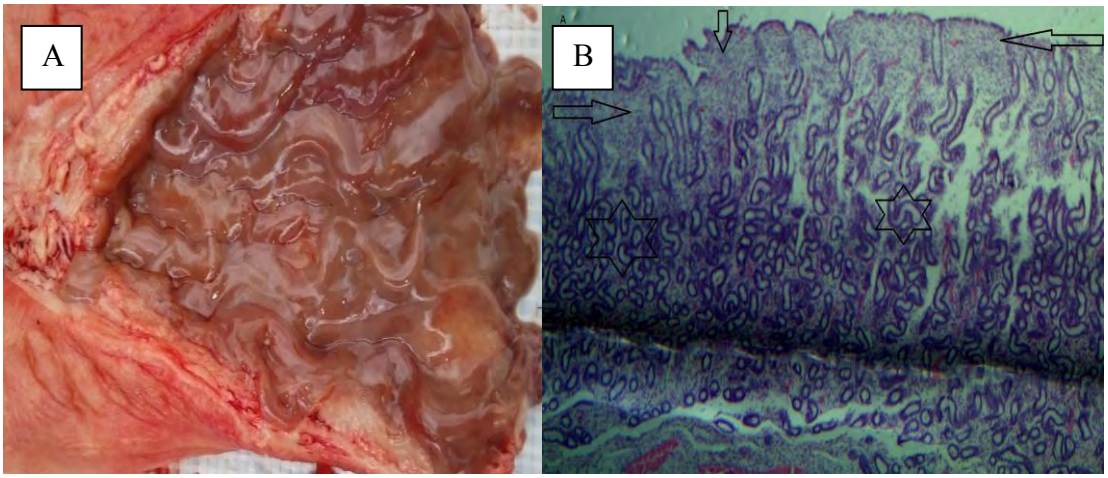


Figure 10: (A) Microscopically, mononuclear cells infiltration composed of lymphocytes and plasma cells of mucosa of the cervix (thinner arrows) and periglandular infiltration with lymphocytes and plasma cells (thicker arrow), (B) shows lymphocytes (thiner arrows) and plama cells (thicker arrows) in the mucosa of cervix.

Uterine lesions

Grossly, the entire mucosa of uterus was thickened and hyperemic in both mares (Figure, 11A). Microscopically, there were severe endometritis that were characterized by infiltration of mononuclear cells in the endometrium. Periglandular inflammations marked by infiltration of lymphocytes and plasma cells with hyperplasia of endometrial glands were also evident (Figure 11B, D and E). In the sub epithelial region, there were lymphocytic peri-vascular cuffing and blood vessels were hyperemic, dilated and full of RBCs (Figure, 11C). The myometrium was also infiltrated by lymphocytes (Figure 11F).



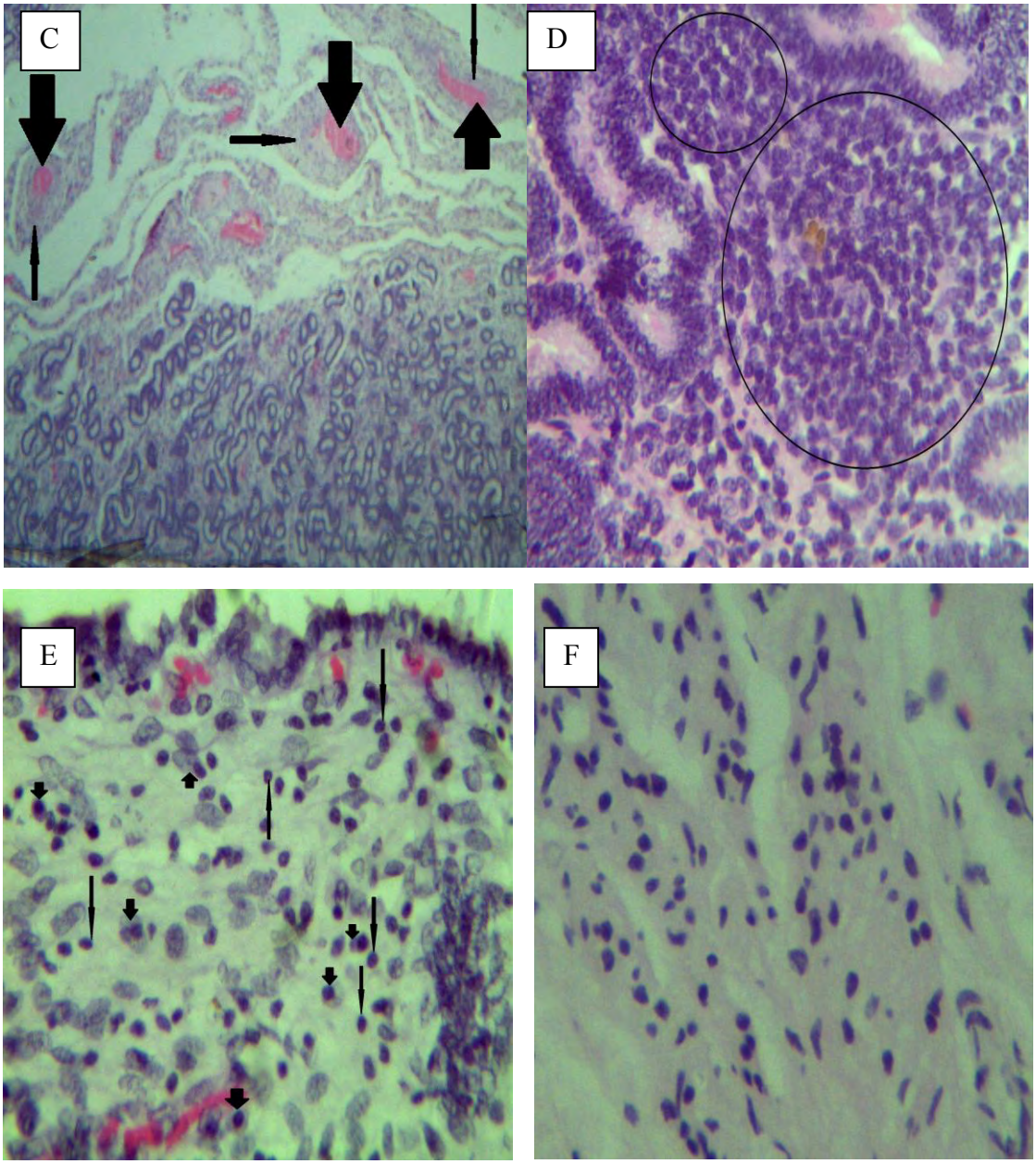


Figure 11: (A) Thickened and hyperemic uterus (gross), (B-F) shows microscopic features of uterus (B) infiltration of endometrium with mononuclear cells (arrows) and hyperplasia of endometrial glands (stars), (C) Congestion (thicker arrows) and perivascular cuffing of lymphocytes (thiner arrows), (D) Periglandular infiltration with lymphocytes and plasma cells (circled areas), (E) infiltration of endometrium with lymphocytes (thinner arrows) and plasma cells (thicker arrows), (F) mononuclear cells in the myometrium mostly of lymphocytes.

Ovarian and oviduct lesions

There were no gross and microscopic lesions within the ovary and oviduct in both mares (M1 and M2).

Ovarian follicular cysts

Ovarian follicular cysts were detected in both right and left ovaries of both mares. The cysts were multiple in number and the right ovaries contains more cyst compared to the left ovaries in both cases. Four follicular cysts were found in the right ovary of one mare (M1) and three in the other mare (M2) while left ovaries have two follicular cysts in both mares. These cysts contained clear light yellowish fluid, enclosed in a thin wall. Externally the cysts were transparent and vascularized with visible capillaries on their surface (Figure 12A). Microscopically, these cysts were lined by thin layer of granulosa cells, theca interna and externally theca externa and were fluid filled at the center (Figure 12B).

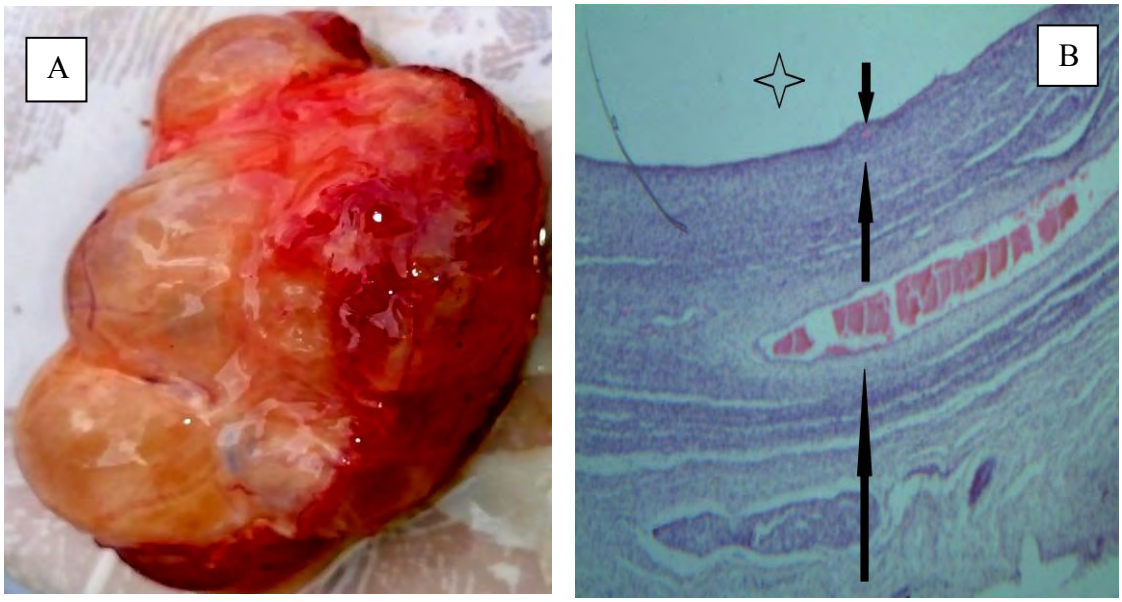


Figure 12: (A) Follicular cysts in the ovary (Gross) (B) Microscopic features of follicular cyst (H&E stain) (Star=cyst cavity, short arrow=thin layer of granulosa cell, medium arrow=theca enterna, long arrow=theca externa and congested blood vessel)

Paraovarian cyst

Paraovarian cyst attached to the mesovarium adjacent to the left ovary was seen in one mare (M2). The cyst was single, oval in shape, small in size (2 cm), transparent and thin wall containing clear watery fluid (Figure 13).

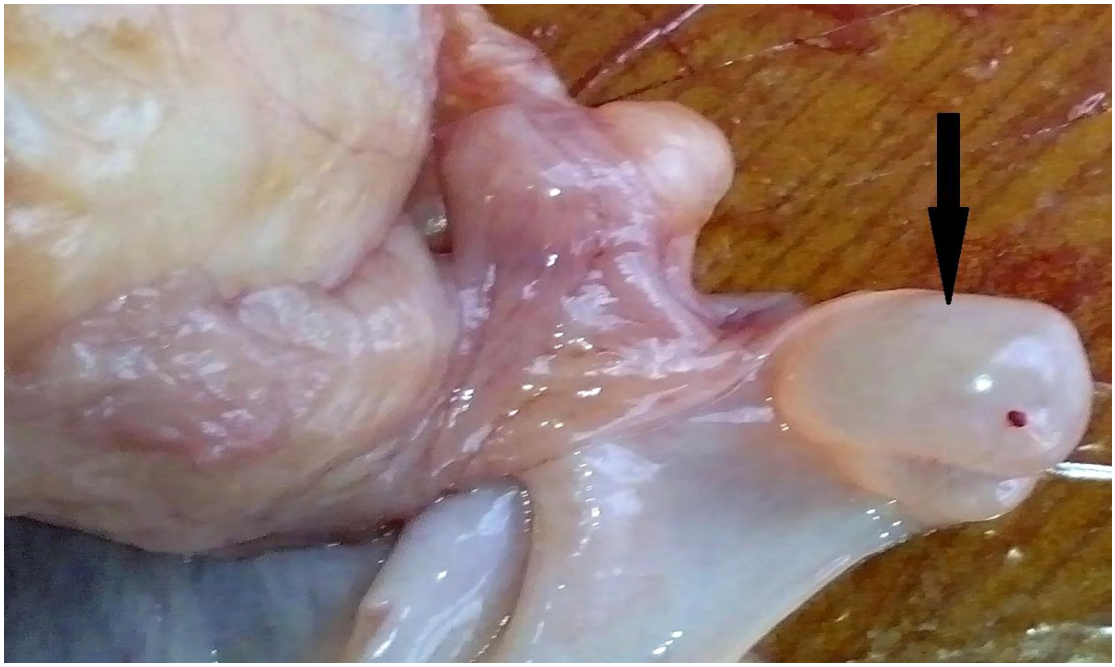


Figure 13: Paraovarian cyst (gross)

4.4.2. Lesions encountered in non reproductive organs

Spleen

Grossly, the spleens of both mares were slightly enlarged and congested (Figure 14A). Microscopically, there was depletion of lymphocytes in the white pulp and at the germinal centers, multifocal congestions marked by dilated and distended sinusoids with blood. Heamosiderin pigment deposition was evident in both the red and white pulp (Figure 14B and C). Heamosiderin laden macrophages was also evident (Figure D)

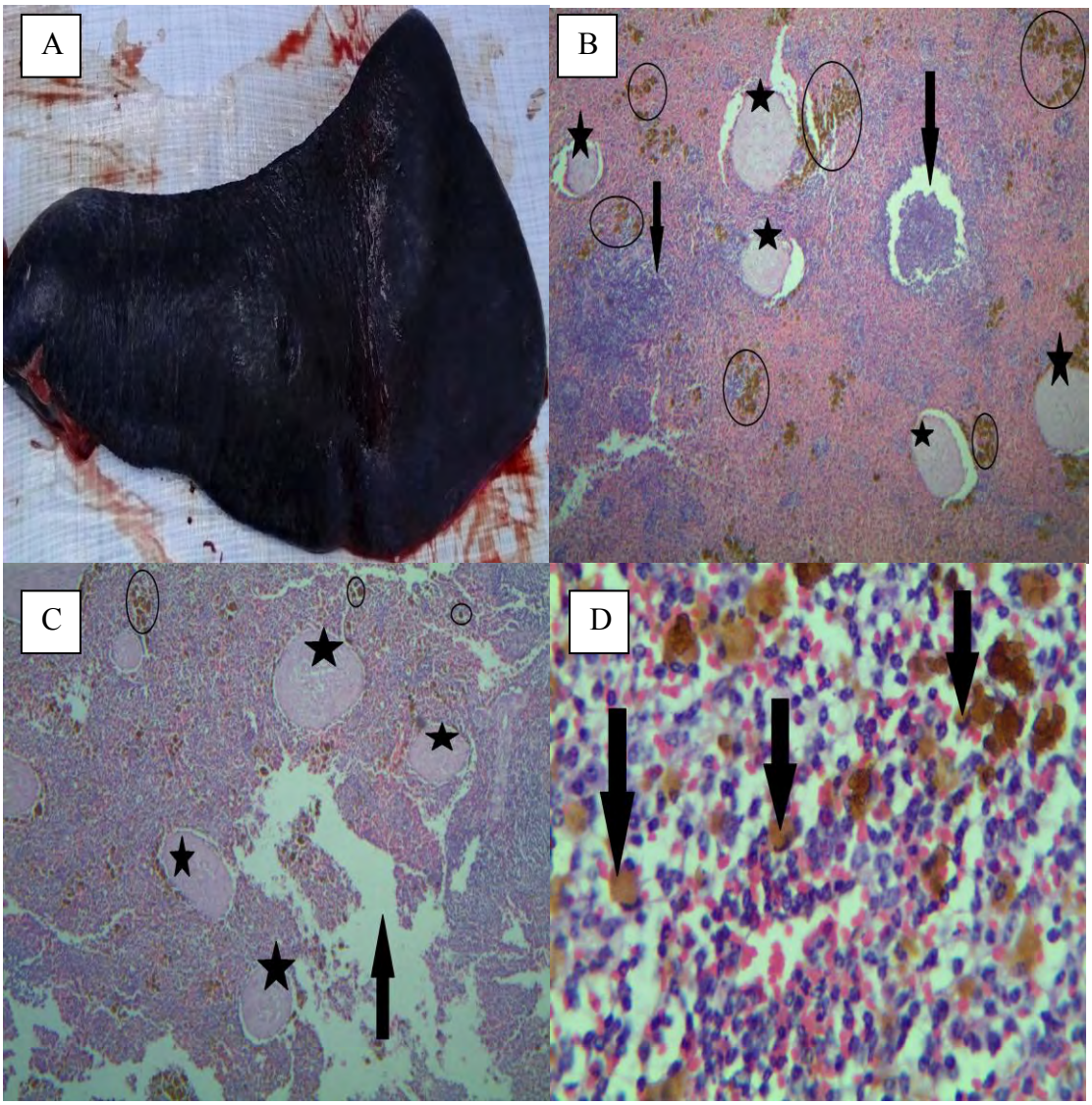


Figure 14: (A) Enlarged and congested spleen (gross) (B) and (C) Histopathologic features of spleen with depletion of lymphocytes (arrows), congestion (stars), heamosidren diposition (circled areas) (D) heamosidren containing macrophages (arrows).

Liver

Grossly, the liver was enlarged and swollen with multiple necrotic foci and patchy fibrinous material (Figure 15A). Accentuated lobular pattern and slight pale liver indicating cellular swelling was also clearly seen grossly (Figure 15B). Microscopically, in the liver there were periportal mononuclear cell infiltrations specifically lymphocytes, plasma cells and some macrophages. The portal areas were severely congested with widening of sinusoids and blood vessels (Figure 15C). Hemosiderin deposits were evident as golden-yellow globules. Areas of necrotic foci marked by degeneration (swelling of hepatocytes) and necrosis of hepatocyte were also observed microscopically (Figure 15D).

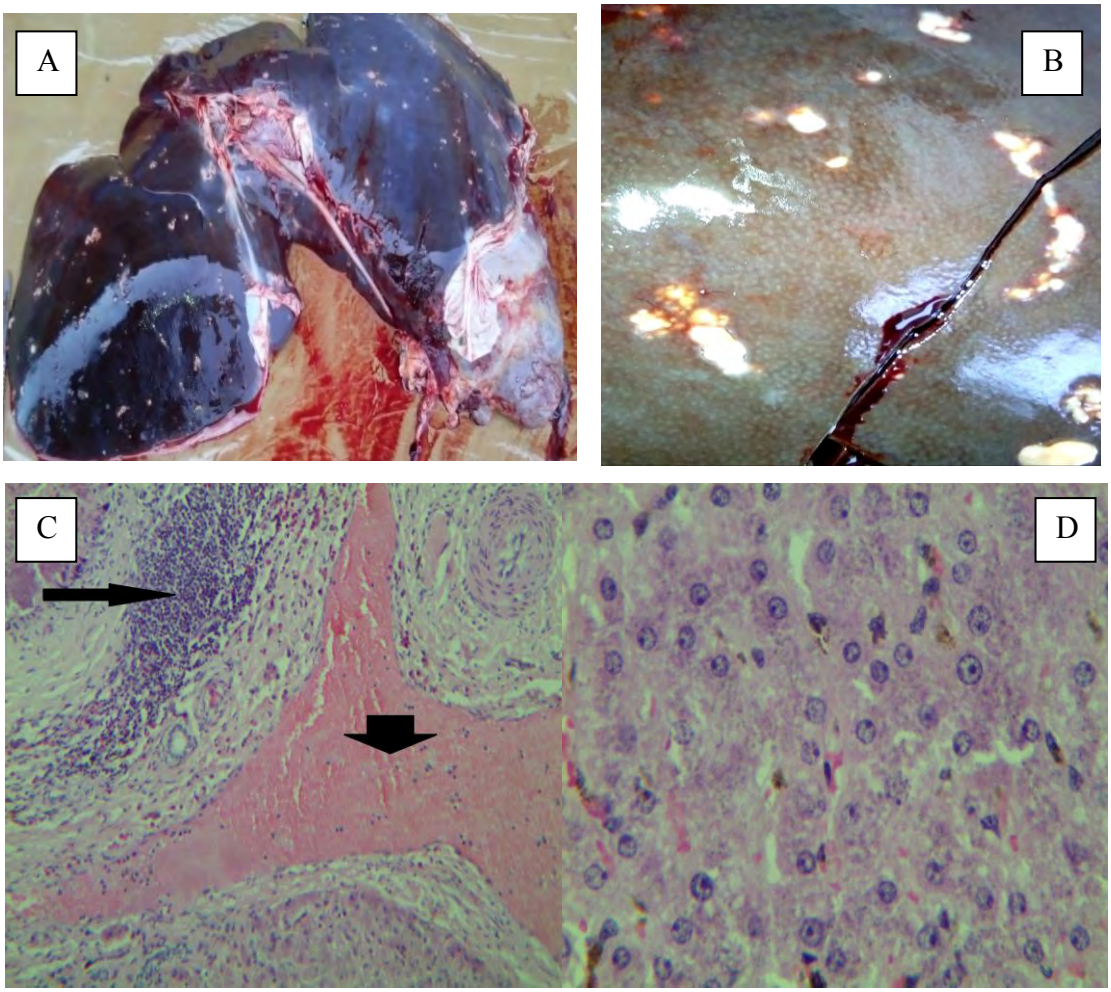


Figure 15: (A) Swollen liver with multiple necrotic foci and patchy fibrinous material (gross) (B) accentuated lobular pattern and slight pallor liver (gross) (C) periportal infiltration with mononuclear cells (thin arrow) and congestion of blood vessel (short thick arrow) (D) degeneration (swelling of hepatocytes) were the nucleus of hepatocytes are to the periphery and necrosis of hepatocyte, heamosidren pigment were also evident.

Kidney

Grossly, there was no lesion observed. Microscopically, in the kidney there were interstitial nephritis characterized by thickening of the wall of tubules with inflammatory cells mostly composed of lymphocytes and plasma cells.

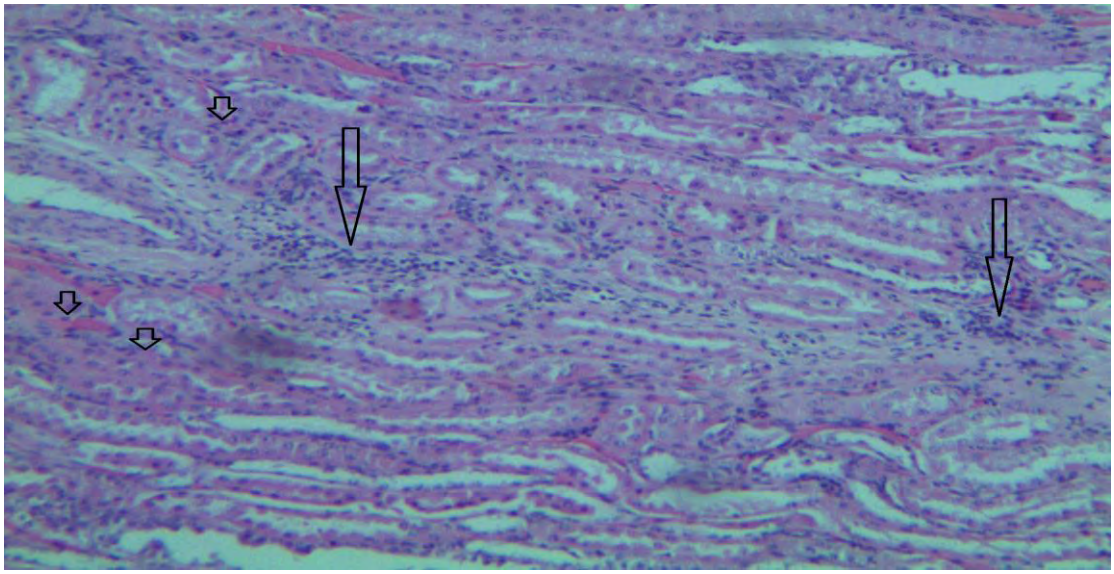


Figure 16: Kidney: Interstitial infiltration with mononuclear cells (long arrows) and thickening of the tubular lumen (short arrows).

Abdominal cavity

In one mare (M2), there was increased peritoneal fluid within the abdominal cavity but no visible lesions were observed in the mesothelium.

Figure 17: Increased peritoneal fluid within the abdominal cavity (gross)

5. DISCUSSION

The results of the present study indicate that most of the signs and lesions observed coincide with those reported in the few available literature studies. All mares with clinical sign of dourine were emaciated, weak and are in poor body condition even though, there was no appetite loss. This was in agreement with previous reports of Alemu *et al*. (1997) and Hagos *et al*. (2010a) from the study area and Pascucci *et al.* (2013) and Vulpiani *et al.* (2013) from Italy. Vulpiani *et al.* (2013) indicated that weight loss is one of the early signs that could lead the veterinarian or owner to suspect dourine.

Genital form of the disease which includes depigmentation of vulval skin, edema of the vulva, ulcer on labia and mucopurlent discharge were seen in some infected mares usually together with nervous sign. This was in agreement with previous reports (Alemu *et al*., 1997; Hagos *et al*., 2010a; Pascucci *et al*., 2013; Vulpiani *et al*., 2013).

Edema of the mammary glands and ventral region were seen in a relatively few cases. This sign was not constant sign observed, probably because some of the mares were in the first and third stage of the disease, when it is not present or might be due to difference in the host response to infection. Skin plaques or wheals, which are regarded as important symptoms in cases of dourine were not observed in this investigation. However, Claes *et al*. (2003) stated that plaques are a rare symptom and they can be observed in comparatively few cases. This finding was in line with Alemu *et al*. (1997) and Hagos *et al*. (2010a) who reported the presence of edema of mammary gland and ventral edema and absence of skin plaques in *T. equiperdum* infected horses in Ethiopia. However, Pascucci *et al*. (2013) stated the presence of skin wheals in few cases and Vulpiani *et al*. (2013) reported skin plaques in most of the infected horses studied.

The nervous signs such as difficulty in walking, marked ataxia of hindquarters and spreading of the limbs without sensory alterations in infected horses reported in the present study were in line with literatures and seems to confirm the tropism of *T. equiperdum* for the peripheral nervous system and lack of involvement of the central

nervous system, in contrast with other trypanosomes (Barrowman, 1976; Berlin *et al*., 2009; Pascucci *et al*., 2013).

The most commonly observed clinical signs in the present study are the genital and nervous signs of dourine while the cutaneous signs of the disease were not prominent. This is in argument with Vulpiani *et al.* (2013) and Pascucci *et al.* (2013) who reported the second phase (cutaneous signs) and third phase (nervous signs) are the common and prominent signs. This difference might be due to the difference in the strain of the parasite, breed of horse, nutrition and stress factor. This is supported with many authors that stated clinical signs and pathogenecity of dourine vary with breed of host, the virulence of the strain, the nutritional status of the horse, and stress factors (Hoare, 1972; Stephen, 1986; OIE, 2008; Sidney *et al*., 2013).

In this study no stallions showing typical sign of dourine were observed. This might be due to stallions were presented to the clinic by the owners in the late stage of the disease when the clinical signs disappeared and are mostly asymptomatic. This is supported with the observation of Vulpiani *et al.* (2013) that states the infected stallions revealed mild signs than in the infected mares. Six months after infection, the stallions were almost asymptomatic. However, the possibility of difference in susceptibility to infection with regard to sex is not described in the study due to small sample size used and yet not known.

It was not possible to isolate the parasite from the blood of infected horses showing clinical evidence of infection with *T. equiperdum* by buffy coat examination despite the several attempts have been made. This could be due to the low number of parasites normally present in infected tissues and the mild, short-lasting parasitaemia (Hoare, 1972; Stephen, 1986; OIE, 2008; Pascuccci *et al*., 2013). *T. equiperdum* is considered primarily a tissue parasite in nature and rarely found in the blood (Hoare, 1972; Stephen, 1986). According to literature, these diagnostic difficulties are typically due to disease caused by *T. equiperdum* (Zablotskij *et al*., 2003).

Despite these difficulties and failure to isolate *T. equiperdum* in the present and some previous studies, Fikru *et al.* (2010) isolated the parasite from the blood of two clinically sick horses in Dodola, Ethiopia. Over the past decades the difficulty of

isolating *T. equiperdum* from infected horses made its characterization and differentiation within the sub genus trypanozoon a problem, and still identification is made on those strains maintained in laboratory animals (Wei *et al*., 2011).

The mean values of the serum biochemical parameters analyzed in this study for both positive and non infected horses were within the range of normal reference values established for working equids of Ethiopia (Simenew *et al*., 2011). There was no significant difference in the total protein level observed between infected and healthy horses. Similar results were documented by Marques *et al.* (2000) in horse experimentally infected with *T. evansi*. Despite these, there is relative increase in total protein and decrease in albumin in infected horses compared to non-infected. The increase in protein levels during the chronic phase of the infection is usually due to the increase in globulin levels as a result of the immune response (humoral) by the animals to the infection (Orhue *et al*., 2005). Hyperglobulinemia coupled with by hypoalbuminemia in donkeys experimentally infected with *T. evansi* was also reported by Cadioli *et al.* (2006).

Increase in AST and ALT activity has been reported in *T. evansi* infected camels (De La Rue *et al*., 1997). The rise in AST activity can be attributed partly to cellular damage caused by the trypanosomes lysis, while the increase in ALT activity probably results from host destruction of trypanosomes (Enwezor and Sacky, 2005). Compared with the previous report in which, AST, ALT and ALP enzymes displayed significant increase during trypanosomosis (Rahman, 1992), in the present study, no significant changes were observed in these enzymes of infected horses despite the relative increase in infected horse compared to healthy group. These might occur due to the possibility that most of serologically positive mares included in the analysis might be in the early stage of the disease at time of examination when there is no sever organ damage as the CATT/*T.evansi* does not differentiate early and late infection. These findings are in concordance with the works of Chaudhary and Iqbal (2000) in camels naturally infected with trypanosomosis and Gutierrez *et al.* (2005) in camels infected with *T. evansi* that reported no significant changes in these enzymes level.

Literatures on pathological lesions caused by *T. equiperdum* in horses were very scanty and only Pascucci *et al.* (2013) tried to describe gross lesions in the reproductive organs caused by *T. equiperdum*, so that pathology of other trypanosomes might be used when comparison is needed. The gross reproductive pathological lesions such as lesion within the mammary gland, congestion and mucus on the surface of vagina, follicular cysts and paraovarian cyst encountered in this study in infected mares were not reported in the available literature, Pascucci *et al.* (2013) who reported absence of macroscopic lesions of the genital tract except congestion of uterine mucosa with widespread hemorrhages. However, congestion of the uterine mucosa observed in this study agrees with the report of Pascucci *et al.* (2013).

Ovarian follicular cysts which were observed in both infected mares in this study were not reported in literatures with infection of *T. equiperdum*. However, Vohradsky (1971) reported the presence of cystic ovaries and endometritis in cattle infected with *T. vivax*. Isoun and Anosa (1974) also observed numerous ovarian cysts containing trypanosomes in two sheep experimentally infected with *T. vivax.* Ovarian cysts occur due to ovulation failure. The cause of ovulation failure in mares has been suggested to be endocrine in nature, either from a lack of sufficient pituitary gonadotropin stimulation to induce ovulation or from insufficient estrogen production from the follicle itself (McCue, 1998).

Based on this, the occurrence of follicular cysts in the present study in infected mares might be suggested to occur due to damage to pituitary gland caused by infection with *T. equiperdum.* This is supported with researches done on other trypanosome species. Focal coagulative necrosis and interstitial mononuclear infiltration in pituitaries of sheep infected with *T. brucei* were reported by Ikede *et al.* (1975). Ikede *et al.* (1973) reported mononuclear infiltration of the *pars nervosa* and surrounding meninges of horse infected with *T. brucei.* Similarly, necrotizing adenohypophysitis characterized by widespread necrosis and disruption of the architecture of the adenohypophysis were observed in *T. brucei* infected dwarf does (Leigh *et al*., 2015). Degenerative changes in the secretory cells of the adenohypophyseal region were also reported in cattle experimentally infected with *T. congolense* (Abebe *et al*., 1993).

Paraovarian cyst observed in one of the mare in this study might not have any association with the disease and they are common in the mare and generally arise from structural remnants of the Mullerian or Wolffian ducts as indicated by McCue (1998).

Gross lesions in the non reproductive organs which include swollen liver and patchy fibrinous material in the surface of the liver with necrotic foci and accumulation of fluid in the abdominal cavity observed in this study are inconsistence with the report of Pascucci *et al*. (2013) who stated no lesions are observed in the parenchymatous organs except congestion of spleen. However, congestion of spleen observed in this study was in agreement with their observation.

The presence of increased amount of fluid in the abdominal cavity observed in one of the mare (M2) might occur due to hypoproteinemia mainly by low albumin level. Orhue *et al*. (2005) indicated that serum albumin levels decreased in trypanosomosis. The edema reported in the dependent parts of the body during the chronic stage of trypanosomosis could be due to a significant decrease in the albumin levels that possibly indicates great liver damage (Enwezor and Sacky, 2005).

Microscopic lesions observed in the present study such as severe interstitial mastitis with infiltration of mononuclear cells, periglandular inflammations with in vulva, vagina and uterus, infiltration of mononuclear cells within the kidney, heamosidren deposition within the spleen and periportal infiltration are in line with the observation of Pascucci *et al.* (2013) who reported similar finding in naturally infected mares with *T. equiperdum*. However, hyperplasia of skin part of the udder, severe infiltration of mucosa of vagina and cervix with lymphocytes and plasma cells, endometritis (infiltration of the endometrium with mononuclear cells) with hyperplasia of endometrial glands and infiltration of myometrium with lymphocytes observed in this study are not reported previously. This difference might be due to giving less attention to these lesions in previous studies or difference in the host response, strain of the parasite and stage of disease at the time of study.

Although, depigmentation around the perineum often described as characteristic of clinical cases of dourine (Stephen, 1986; Claes *et al*., 2003; Hagos *et al*., 2010a and Vulpiani *et al*., 2013), no microscopic description of such lesions were cited in previous literatures. Sever dermatitis with hydropic degeneration and necrosis of the keratinocytes of stratum spinosum, necrosis of basal cells including the melanocytes with excess free melanin pigment within the epidermis observed in this study were not characterized in previous literatures. The probable cause of depigmentation around the vulval skin of infected mares could be due to severe necrosis of melanocytes, as the depigmented areas were microscopically characterized by sever necrosis of cells, excess free melanin and formation of cystic structures in the epidermis. McGavin and James (2007) stated; melanin is stored in melanosomes in the cytoplasm of melanocytes. However, damage to cells which contain melanin (e.g., damage melanocytes and basal cells of the skin), causes loss of melanin pigment in the epidermis (leukoderma) resulting in depigmentation.

The depletion of lymphocytes observed in the germinal centers of the spleen in the present study could be due to the body requirement of this cell to combat the parasites in circulating blood and this agrees with the findings of Chaudhari and Iqbal (2000) in camels infected with *T. evansi*.

Heamosidren deposition in the spleen and liver of both mares observed in this study was in agreement with the finding of Pascucci *et al.* (2013) who reported heamosidren deposition in the spleen of mare naturally infected with *T. equiperdum*. The presence of increased heamosiderosis might be an indication of the major role the spleen plays in the destruction of red blood cells during trypanosomosis (Taylor and Authie, 2004).

Infiltration of tissues with mononuclear inflammatory cells especially lymphocytes, plasma cells and few macrophages is a hallmark of chronic inflammation (Jones *et al*., 1997). The microscopic findings of the present study, which were shown majority of mononuclear infiltration especially of lymphocytes, indicate the presence of chronic inflammatory process in several tissues.

6. CONCLUSION AND RECCOMENDATIONS

Due to the extreme variability of signs in infected horses, difficulties in clinical diagnosis and knowledge gap on the disease, it is important to observe and study new cases of dourine. The serum biochemical analysis in the present study indicates that *T. equiperdum* infection caused a statistically non significant alteration in the biochemical parameters in infected mares. The pathological study on naturally infected mares done in this study is more important in providing the real picture of disease than experimental infections. The results of gross and microscopic findings indicate that there is severe organ involvement with infiltration of organs with lymphocyte, plasma cells and few macrophages. Microscopic lesions in the reproductive organs of mares indicated massive infiltration of mononuclear cells mainly of lymphocytes, plasma cells and few activated macrophages revealed immunological response of the host to the parasite and parasite products. In addition it can prove the predilection site of the parasite to be in the reproductive tissues as indicated in the literature. So that, based on the gross and microscopic lesions, damage in tissues caused by *T. equiperdum* might occur due to direct damage by the parasite itself and or immune response by the host.

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

- Further pathological studies on the disease should be done in naturally infected horses (mare and stallions) by increasing the sample size by considering early/acute and chronic/advanced clinical cases.
- Experimental infections of natural hosts, experimental infections of unnatural hosts-all with trypanosome obtained direct from the natural host and then back again in the reversed order should be done in order to study pathology of dourine in detail in the future.
- Molecular pathology techniques should be implemented in various tissues to observe predilection sites of *Trypanosoma equiperdum*.

7. REFERENCES

Abebe G., Shaw M. K. and Eley R. M. (1993): *Trypanosoma congolense* in the microvasculature of the pituitary gland of experimentally infected Boran cattle *(Bos indicus). Vet Patho*., **30**:401-409.

Abebe G. (2005): Trypanosomosis in Ethiopia. *Ethiop. J. Bio. Sci.,* **4**:75-121.

Agbo E. E., Majiwa P. A. and Claussen H. J. (2002): Molecular variation of *T. brucei* subspecies as revealed by AFLP fingerprinting. *Parasitology.,* **124** (4):349-358.

Alemu T., Luckins A. G., Philips L. P., Reid S. W. J. and Holmes P. H. (1997): The use of ELISA to investigate the prevalence of *T. 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.

Arsi-Bale zone plan office (2009). Zonal Agricultural Compiled Report, Oromia region, Ethiopia, pp. 7-11.

Bajyana S. and Hamers R. (1988): A card agglutination test (CATT) for veterinary use based on an early VAT RoTat 1.2 of *T. evansi*. *Ann. Soc. Belge. Med. Trop*., **68**:233-240.

Barrowman, P. R. (1976): Experimental Intraspinal *T. equiperdum* Infection. In A Horse. *Orderstepoort J. vet. Res.,* **43** (4):201-202.

Becker S., Franco J. R., Simarro P. P., Stich A., Abel P. M. and Steverding D. (2004): Real-time PCR for detection of *T. brucei* in human blood samples. *Diagn. Microbiol. Infect. Dis.*, **50** (3):193–199.

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

Bishop P., Rae P. F., Philips L. P., Boid, R. and Luckins A. G. (1995): *T. 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 Biochem Parasitol*., **105** (2):185-201.

Brun R. and Lun Z. R. (1994): Drug sensitivity of Chinese *T. evansi* and *T. equiperdum* isolates. *Vet. Parasitol*., **52**:37-46.

Brun R., Hecker H. and Lun Z. R. (1998). *T. evansi* and *T. equiperdum*: distribution, biology, treatment and phylogenetic relationship (a review). *Vet Parasitol.,* **79**:95-107.

Buscher P., Ngoyi D. M., Kabore J., Lejon V., Robays J., Jamonneau V., Bebronne N.,Van der Veken W. and Bieler S. (2009): Improved Models of Mini Anion Exchange Centrifugation Technique (MAECT) and Modified Single Centrifugation (MSC) for Sleeping Sickness Diagnosis and Staging. *PLoSeg. Trop. Dis. Trop. Dis.*, **3**:471.

Biryomumaisho S., Katunguka-Rwakishaya E. and Rubaire-Akiiki C. M. (2003): Serum biochemical changes in experimental *T. congolencse* and *T. brucei* infection in small east African goats. *Vet. arhiv*., **73**:167-180.

Cadioli F. A., Marqus L. C., Machado R. Z., Alessi A. C., Aquino LP. CT. and Barnabe P. A. (2006): Experimental *T. evansi* infection in donkeys: hematological, biochemical and histopathological changes. *Arq Bras Med Vet Zootec*., **58**: 749-756.

Calistri P., Narcisi V., Atzeni M., De Massis F., Tittarelli M., Mercante M. T., Ruggieri E. and Scacchia M. (2013): Dourine Reemergence in Italy. *J. Equine Vet. Sci.,* **33***:*83-89.

Chaudhary Z. I. and Iqbal J. (2000): Incidence, biochemical and haematological alterations induced by natural trypanosomosis in racing dromendary camels. *Acta Trop.,* **77**: 209-213.

Ciuca A. (1933): La dourine. *Bull. Off. Int. Epiz*., **7**:168–172.

Claes F, Agbo E. C. and Radwanska M. (2003): How does *T. equiperdum* fit into the Trypanozoon group? A cluster analysis by RAPD and multiplex-endonuclease genotyping approach. *Parasitology*., **126** (5):425-431.

Claes F, Radwanska M. and Urakawa T. (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., Büscher P., Touratier L. and Goddeeris B. M. (2005): *Trypanosoma equiperdum*: master of disguise or historical mistake? *Trends Parasitol*., **21** (7):316–321.

Clausen P. H., Gebreselassie G., Abditcho S., Mehlitz D. and Staak C. (1999): Detection of Trypanosoma DNA in serological positive but aparasitemic horses suspected of dourine in Ethiopia. *Tokai. J. Exp., Clin. Med*., **23**:303-308.

Clausen P. H., Chulvun S., Sodnomdarjaa R., Greiner M., Noeckler K., Staak C., Zessin K. H. and Schein E. (2003): A field study to estimate the prevalence of *T. equiperdum* in Mongolian horses. *Vet. Parasitol.,* **115**:9-18.

De La Rue M. L., Carli GA. De., Herrera H. M. and Silva RAMS. (1997): Biochemical changes in acute infection of dogs with *T. evansi*. *J Protozool Res.*, **7**: 28–35.

Dennnis M. M. and Joanna M. B. (2006): Clinical textbook for veterinary technicians. 5th edition, Elseiver pub.pp 165-179.

Enwezor F. N. C and Sackey A. K. B. (2005): Camel trypanosomiasis - a review. *Vet Arch.*, **75**: 439–452.

Feseha G., Alemu G., Friew K., Abule I., Ketema Y. (1997): An overview of donkey utilization and management in Ethiopia, in: Starky, P., Fielding, D., (Eds.), Donkeys, people and development. ACP-EU Technical Centre for Agricultural and Rural Cooperation (CTA), Wageningen, pp. 46-52.

Fikru R., Hagos A., Alemu T., Bruno M. G. and Filip C. (2010). Comparative diagnosis of parasitological, serological, and molecular tests in dourine-suspected horses. *Trop Anim Health Prod.*, **42**:1649–1654.

Food and Agriculture statistical Database (FAOSTAT) (2012): Food and Agriculture Statistical data base: In: http://www.fao.org/corp/statistics/access online.

Gutierrez C., Corbera J. A., Juste M. C., Doreste F. and Morales I. (2005): An outbreak of abortions and high neonatal mortality associated with *T. evansi* infection in dromedary camels in the Canary Islands. *Vet Parasitol.*, **130**: 163–168.

Hagebock J. M., Chieves L., Frerichs W. M. and Miller C. D. (1993): Evaluation of agar gel immunodiffusion (AGID) and indirect fluorescent antibody (IFA) assays as supplemental tests for dourine in equids. *Am. J. vet. Res.*, **54** (8):1201-1208.

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

Hagos A., Goddeeris B. M., Yilkal K., Alemu T., Fikru R., Yacoba H. T., Feseha G. and Claes F. (2010b). Efficacy of Cymelarsan® and Diminasan® against *Trypanosoma equiperdum* infections in mice and horses. *Veterinary Parasitology*., **171**:200–206.

Hoare C. A. (1972). The trypanosomes of mammals. Blackwell scientific publications, Oxford, pp. 1-749.

Ikede B. O., Hill D. H., Akop okodji e J. U. (1973): Clinicopathological changes in a horse naturally infected with *T. brucei. Niger Vet J.*, **2**: 13-17.

Ikede B. O. and Losos G. J. (1975): Pathogenesis *of T. brucei* infection in sheep. II. Hypophyseal and other endocrine lesions. *J Comp Pathol*., **85**: 37-44.

Ikede B. O., Elizabeth E. and Akpavie S.O. (1988): Reproductive disorders in African trypanosomosis: A Review *Acta Tropica*., **45**:5-10

Isoun T. T. and Anosa V. O. (1974): Lesions in the reproductive organs of sheep and goats infected with *T. vivax*. *Z. Tropenmed. Parasit*., **25**:469-476.

Jennings F. W., Whitelaw D., Holmes P. H., Chizyuka H. G. B. and Urquhart G. B. (1979): The brain as source of relapsing of *T. brucei*. *Int. J. Parasitol*., **9**:81–384.

Jennings F. W., Urquhart G. M., Murray P. K. and Miller B. M. (1980): Berenil and nitroimidazole combinations in the treatment of *T. brucei* infection with central nervous system involvement. *Int. J. Parasitol*., **10**:27–32.

Jones T. C., Hunt R. D. and King N. M. (1997). Veterinary pathology, 6th ed., 351 West Camden street, Baltmore, USA, pp.586.

Katunguka-Rwakishaya E. (1996): The prevalence of trypanosomosis in small ruminants and pigs in a sleeping sickness endemic area of Buikwe country Mukono district, Uganda. *Revd' Elev. Me'd Vet.. Pays Trop*., **49**:56-58.

Katz J. B., Chieves L. P., Hennager S. G., Nicholson J. M., Fisher T. A. and Byers P. E. (1999): Serodiagnosis of equine piroplasmosis, dourine and glanders using an arraying immunoblotting method. *J. vet. diagn. Invest.*, **11** (3):292-294.

Kumba F. F., Claasen B. and Petrus P. (2002): Apparent Prevalence of Dourine in the Khomas region of Namibia. *Onderstepoort Journal of Veterinary Research.,* **69**:295-298.

Leigh O. O., Emikpe B. O. and Ogunsola J. O. (2015): Histopathological changes in some reproductive and endocrine organs of *Trypanosoma brucei* infected West African dwarf goat does. *Bulgarian J. Vet.Med.*, **18** (1):31-39.

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.

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

Luckins A. G., Barrowman P. R., Stoltsz W. H. and Van Der Lugt J. J. (2004). In: Coetzer, J. A. W., Tustin, R. C. (Eds.), Dourine. Infectious Disease of Livestock, 2nd ed. Oxford University Press, pp. 47–54.

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.

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.

Marques L. C., Machado R. Z., Alessi A. C., Aquino L.P.C.T and Pereira G. T. (2000): Experimental infection with *T. evansi* infection in horses: clinical and hematological observations. *Rev.Bras.Parasitol.Vet*, **9** (1):11-15.

Masupu, K. V. and Majok, A. A. (1998): Apparent prevalence of equine dourine in Kgalagadi district of Botswana. *Zimbabwe Vet. J.,* **29***:*113- 116.

Maudlin I., Holmes P. H. and Miles M. A. (2004). The trypanosomiasis. Oxfordshire: CABI Publishing.

McCue P. M. (1998). Review of Ovarian Abnormalities in the Mare, Proceedings of the Annual Convention of the American Association of Equine Practitioners. AAEP Proceedings,Vol. **44**.pp.127-131.

McGavin M. D. and James F. Z. (2008). Pathologic Basis of Veterinary Pathology. 4th ed. pp. 1135-1137.

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

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.

OIE (2008). Dourine. In: Manual of diagnostic tests and vaccines for terrestrial animals. Office International des Epizooties (OIE), Paris, France. pp. 845–851.

OIE (2013). Terrestrial Manual. Trypanosomosis (tsetse-transmitted). Office International des Epizooties (OIE), Paris, France. pp. 1-25.

Orhue N. EJ., Nwanze E. AC. and Okafor A. (2005): Serum total protein, albumin and globulin levels in *T. brucei*–infected rabbits: Effect of orally administered scoparia dulcis. *Afr J Biotech*, **4**: 1152– 1155.

Otesile E. B., Fagbemi B. O. and Adeyemo O. (1991): The effect of *T. brucei* infection on serum biochemical parameters in boars on different planes of dietary energy. *Vet. Parasitol*., **40**:207-216.

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

Pepin J. and Milord F. (1994): The treatment of human African trypanosomiasis. *Adv. Parasitol*., **33**:1–47.

Radwanska M., Magez S. and Perry-O'Keefe H. (2002): Direct detection and identification of African trypanosomes by fluorescence in situ hybridization with peptide nucleic acid probes. *J. Clin. Microbiol*., **40** (11):4295-4297.

Rahman Z. U. (1992). Serum biochemical, enzymes and haematological changes in one–humped camels infected with Surra. Proceedings of the first international camel conference, R &W Publications Limited, Dubai, pp. 405.

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

Reitman S. and Frankel S. (1957): A calometric method for the determination of serum glutamic oxaloacetic and glutamic pyruvic transaminase. *America j. Cl. pathology.,* **28**:56-62.

Rouget J. (1986): Contribution a l' étude du trypanosome des mammifere. *Ann. Inst. Pasteur.,* **10**:716–728.

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.

Sidney R., Andrew McG., James C. and Richard N. (2013). Dourine – an emerging venereal threat to European horses. *AHT / BEVA / DEFrA Equine Quarterly Disease Surveillance report*. **6**:7.

Simenew K., Gezahegne M., Getachew M., Wondyefraw M., Alemayehu L. and Eyob I. (2011): Reference values of clinically important physiological, hematological and serum biochemical parameters of apparently healthy working equids of Ethiopia. *Global Veterinaria*., **7** (1):1-6.

Statistical Package for Social Science, Inc. (SPSS). (2011). SPSS for window (Version 20.0) Chicago, ILLINOS, USA.

Stephen L. E. (1986). Trypanosomiasis, a veterinary perspective. Pergamon Press, Oxford, UK.

Swann W. J. (2006). Improving the welfare of working equine animals in developing countries. *Appl.Anim.Behav.sci*. **100**:148-151.

Talkuder S. (2007). Histopathological techniques: Tissue processing and staining. In: www. Talukderb.com.

Taylor K. and Authie M. L. (2004). Pathogenesis of animal trypanosomiasis. In *the trypanosomoses,* edited by I. Maudlin, P.H. Holmes & M.A. Miles. Oxfordshire: CABI Publishing. Pp.331–353.

Theis J. H. and Bolton V. (1980): *Trypanosoma equiperdum*: movement from the dermis. *Exp. Parasitol.,* **50** (3):317–330.

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

Tuntasuvan D., Jarabrum W., Viseshakul N., Mohkaew K., Borisutsuwan S., Theeraphan A. and Kongkanjana N. (2003): Chemotherapy of surra in horses and mules with diminazene aceturate. *Vet. Parasitol*., **110**: 227-33.

Vaysse J. and Zottner G. (1950). Contribution a letude de la chimiotherapie et de la chimioprevention de la dourine par l'antracyde. *Bull. Off. Int. Epiz.,* **34**:172–179.

Verloo D., Magnus E. and Buscher P. (2001): General expression of RoTat 1.2 variable antigen type in *Trypanosoma evansi* isolates from different origin. *Vet. Parasitol*., **97**:183-189.

VMTD (2009). Veterinary medical teaching hospital, anatomic pathology service, senior veterinary student rotaton handbook. *In: www.vetmed.ucdavis.edu.*

Vohradsky F. (1971): Clinical signs, daily rate of infection, physical changes of the blood and pathomorphological changes in cattle artificially infected by *Trypanosoma vivax. Rev. Elev. Méd. vét. Pays trop.,* **24**:251-263.

Vulpiani M. P., Carvelli A., Giansante D., Iannino F., Paganico, D. and Ferri N. (2013): Reemergence of dourine in Italy: clinical cases in some positive horses. *Journal of Equine Veterinary Science*., **33**:468-474.

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.

Wang Z. L. (1988). The similarities and differences of the characteristics between *T. equiperdum* and *T. evansi. Bul. Vet. Col. (PLA) (Chinese).,* **8**:300-303.

Wassal D. A., Gregory R. J. F. and Phipps L. P. (1991): Comparative evaluation of enzyme-linked immunosorbent assay (ELISA) for the serodiagnosis of dourine. *Vet Parasitol.*, **39**:233-239.

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

Wei Y., Wen Y., Desquesnes M. and Zhao-Rong Lun. (2011): Molecular epidemiology of *T. evansi* and *T. equiperdum* and atypical human infection by animal trypanosomes. *Madame Curie Report.* ©2011 Landes Bioscience.

William B. L and Steven H. S. (2007). Infectious diseases of breeding stallion in: Current Therapy in Large Animal Theriogenology (2nd Ed.), pp. 15-23.

Williamson C. C., Stoltz W. H., Mattheus A. and Schiele G. J. (1988). An investigation into alternative methods for the serodiagnosis of dourine. *Onderstepoort J. Vet. Res.,* **55**:117-119.

Woo P. T. K. (1970): The haematocrit centrifuge technique for the diagnosis of African Trypanosomasis. *Acta Trop.,* **27**:384-386.

Zablotskij V. T., Georgiu C., De Waal Th., Clausen P. H., Claes F. and Touratier L. (2003): The current challenges of dourine: difficulties in differentiating *T. equiperdum* within the subgenus *Trypanozoon*. *Rev. sci. tech. Off. int. Epiz.*, **22** (3):1087-1096.

Zeleke D., Ketema S. and Abdul S. (1980): An investigation of dourine in Arsi Administrative Region. *Ethiop. Vet. Bull***.,** **4**:3-19.

Zhang Z. Q., Giroud C. and Baltz T. (1992): *In vivo* and *in vitro* sensitivity of *T. evansi* and *T. equiperdum* to Diminazene, Suramine, Melcy, Quinapyramine and Isometamidium. *Acta Trop*., **50**:101-110.

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

Zwart D. (1989): Aspects of comparative pathology and pathogenesis of Trypanosomal infections in Africa. *Ann.Soc.belge Med. Trop*., **69**:105-112.

8. ANNEXES

Annex 1. Procedures and Principle of the biochemical tests

Procedure for instrument set up

Set the program o the instrument, type the individual's code number total protein, albumin AST, ALT and ALP tests are selected from test menu. After calibration adequate controls and serum was placed in sample cup by appropriate order and enough working reagents in reagent bottles were added. The instrument by itself pipettes programmed sample volume and working reagent and after incubation, the formed color absorbance read at wavelength and the results were displayed on screen.

Total protein

Principle

Peptide bonds of protein react with Cu ions in alkaline medium to form colored complex whose color intensity is directly proportional to the total protein concentration and measured at 540 nm.

Albumin

Principle

The method is based on the specific binding of bromocresol green (BSG), an anionic dye and the protein at acid pH with the resulting shift in the absorption wavelength of the complex. The intensity of the color formed is proportional to the concentration of albumin in the sample.

Aspartate amino transferase (AST/GOT)

Principle

Aspartate amino transferase catalyzes the transfer of the amino group from aspartate to oxoglutarate with the formation of glutamate and oxalacetate. The latter is reduced to malate by malate dehydrogenase (MDH) in the presence of reduced nicotinamide adenine dinucleotide (NADH).

The reaction is monitored kinetically at 340 nm by the rate of decrease in absorbance resulting from the oxidation of NADH to NAD^+, proportional to the activity of AST present in the sample.

Alanine Amino Transferase (ALT/GPT)

Principle

Alanine amino transferase catalyzes the transfer of the amino group from Alanine to oxoglutarate with the formation of glutamate and pyruvate. Pyruvate is then reduced to lactate by lactate dehydrogenase (LDH) in the presence of reduced nicotinamide adenine dineuclotide (NADH). The reaction is monitored kinetically at 340 nm by the rate of the decrease in absorbance resulting from the oxidation of NADH to NAD^+, proportional to the activity of ALT present in the sample.

Alkaline Phosphatase

Principle

Alkaline Phosphatase catalyzes the hydrolysis of 4-nitophenylphosphate (4-NPP) with the formation of free 4-nitrophenol and in organic phosphate, acting as the alanine buffer as a phosphate group acceptor. The reaction is monitored kinetically at 405 nm by the rate of the formation of 4-nitrophenol proportional to the activity of ALP present in the sample.

Annex 2. Necropsy procedure (Dennis and Joanna, 2006).

The mares were placed in left lateral recumbency. A midline incision is made beginning at the right axilla and extending cranially to the mandibular symphysis. The incision is continued in the opposite direction caudally as a median or paramedian incision passing between the mammary to the perineum. The upper forelimbs were reflected by dissection between the scapula and the ribs. Abdominal skin is reflected, and the hind limbs are reflected by extending the incision into the coxofemoral (hip) joints. The animals were then placed in dorsal recumbency (on its back). Skin incisions were extended down the cranial medial aspects of both rear legs, and the skin are reflected. As they are exposed in the dissection, superficial organs such as lymph nodes, mammary glands and skin were examined thoroughly.

Next, the three major body cavities (peritoneal, pleural, and pericardial) were opened. All organs are examined in situ, and any abnormalities were noted. The abdomen was opened by making a midline incision from the sternum to the symphysis pubis and making incisions laterally from the sternum along both caudal costal margins. The abdominal wall was then reflected laterally to expose the abdominal cavity. The diaphragm was punctured to check for negative pleural pressure, and the diaphragm was cutted away from the ventral and lateral rib cage. The ventral rib cage was removed by cutting the ribs bilaterally (on both sides) midway between the costochondral junction and the vertebral column.

The floor of the pelvis was removed to facilitate examination and removal of the urogenital tract. This is accomplished by making paramedian cuts through the obturator foramina on the floor of the pelvis. The mesovarium, mesosalpinx, and mesometrium are examined. Ovaries, oviducts, and uterus are freed from mesentery and reflected toward the pelvis. Ovaries, oviducts, uterus, cervix, vagina, and vulva are removed from the carcass as a unit. The ovaries are sliced longitudinally; oviducts are examined and palpated and uterus, cervix, vagina, and vulva are opened with scissors or a knife. Serosa, contents of the uterus, endometrium, cut surfaces, cervical folds, and luminal surface of vagina and vulva were examined.

Annex 3. Histopathological procedures (Takulder, 2007)

1. Fixation of tissue by 10% neutral buffered formaldehyde
2. Trimming part of the tissue in a way that the lesion we require be included or not missed and to fit standard histological processing tissue cassettes (5mm thickness).
3. Tissue specimen processing: fixation of tissue by formalin, Dehydrating tissue by increasing alcohols concentration, clearing of tissue by xylene, and impregnation of tissue by paraffin wax.

   Formalin-I 2hr → Formalin-II 2hr → 70% Alcohol 1hr → 95% Alcoho → 100% Alcohol-I 1hr → 100% Alcohol-II 2hrs → 100% Alcohol-III 2hrs → Xylene-I 1:30hrs → Xylene-II 1:30hrs → Xylene-III 1:30hrs → Paraffin-I 2hrs → Paraffin-II 3hrs.
4. Embedding of processed tissue: impregnated tissue is placed in a mould with their labels and then fresh melted wax (54-60c^{o}) is poured and allowed to settle and solidify.
5. Sectioninig: sectioning of tissue in 3-5 micron thickness and put on water bath to straighten the ribbon, and then adhere on the surface of frost ended and clear slide. Later label and put an incubator over night.
6. Staining: Hematoxyline eosine stain procedure
   a. Deparaffinize slides in 2 changes of xylene for 5minutes.
   b. Hydrate slides in 3 changes of 100% alcohol each for 3minutes and 1 changes of 95% alcohol for a minute and 1 change of 70% alcohol for 3minutes
   c. Rinse in distilled water until repples disappear from slides.
   d. Place in heamatoxyline (mayer's hematoxline) for 10-15 minutes
   e. Rinse in tap water until water runs clear
   f. Decolorize in 1% acid alcohol, 3-6 qiuck dips. Check differentiation microscopically: Nucleic should be distinct; cytoplasm should be uncolored.
   g. Rinse in tap water until ripples disappear from slides.
   h. Stain in eosin, 3 dips.
   i. Rinse in tap water until water runs clear.

j. Dehydrate in 95% alcohol of 3dips and 100% alcohol, 3 changes each for 3minutes.
k. Clear in 3 changes of xylene for 5 minutes each.
l. Mount cover glass with DPX.

Examination of the prepared slides under the microscope.