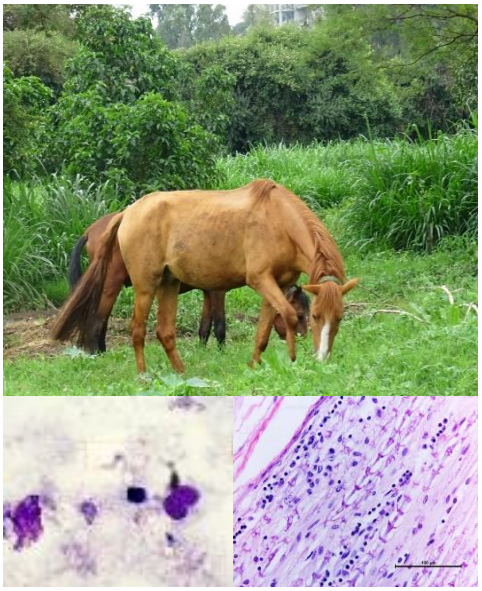
Dissertation Ref. No. 050/06/2019

Trypanosoma equiperdum: Venereal transmission and Pathogenesis



PhD Dissertation

By

Ahmed Yasine Ebrahim

Addis Ababa University, College of Veterinary Medicine and Agriculture
Department of Veterinary Pathology and Parasitology
PhD program in Tropical Veterinary Parasitology

June , 2019

Bishoftu, Ethiopia

Trypanosoma equiperdum: Venereal transmission and pathogenesis

A dissertation submitted to the College of Veterinary Medicine and Agriculture of Addis Ababa University in partial fulfillment of the requirements for the degree of Doctor of Philosophy in Veterinary Parasitology

By

Ahmed Yasine Ebrahim

Promoters

Prof. Dr. Hagos Ashenafi — Addis Ababa University, College of Veterinary Medicine

Dr. Jan Govaere — Ghent University, Faculty of Veterinary Medicine, Belgium

Co - Promoters

Prof. Dr. Bruno Goddeeris — KU Leuven, Faculty of Bioscience Engineering, Belgium

Prof. Dr. Luc Duchateau — Ghent University, Faculty of Veterinary Medicine, Belgium

Addis Ababa University
College of Veterinary Medicine and Agriculture
Department of Veterinary Pathology and Parasitology

As members of the Examining Board of the final **PhD** open defense, we certify that we have read and evaluated the **dissertation** prepared by **Ahmed Yasine Ebrahim** entitled "***Trypanosoma equiperdum*: Venereal transmission and pathogenesis**" and recommend that it be accepted as fulfilling the **dissertation** requirement for the degree of

Doctor of Philosophy in **Veterinary Parasitology.**

Prof. Yacob Hailu (AAU, CVMA) Chairman	Signature	Date
Dr. Shimelis Dagnachew (University of Gondar) External Examiner	Signature	Date
Dr. Bersisa Kumsa (AAU, CVMA) Internal Examiner	Signature	Date
Prof. Hagos Ashenafi (AAU, CVMA) Major Advisor	Signature	Date

I hereby certify that I have read the revised version of this dissertation prepared under my direction and recommend that it be accepted as fulfilling the dissertation requirement.

Prof. Hagos Ashenafi Major advisor	Signature	Date
Prof. Yacob Hailu Department chairperson	Signature	Date

Submitted by

Ahmed Yasine Name of student	Signature	Date

Table of contents

Acknowledgements/ ምሥጋና

First of all I would like to praise for the Almighty **ALLAH** (Subhanahu wa ta'ala) for giving me strength in every moment of my life. Alhamdulillah.

I am highly grateful to the ministry of education of Ethiopia and the Flemish government of Belgium VLIR-UOS TEAM Project for the funds in collaboration between AAU and Ghent University, sponsoring my PhD and making this scientific dream possible. I would like to express my deepest appreciation and thanks to my promoters Prof. Hagos Ashenafi, Dr. Jan Govaere, Prof. Bruno Goddeeris, and Prof. Luc Duchateau for their unreserved, continuous and all rounded support throughout my study, including their scientific inputs in all papers included in this thesis. Dear Prof. Hagos Ashenafi, you have been extremely kind and co-operative with me in all matters when I required your assistance. It was not like a boss but like elder brother to deal in any issues. **በጣም አመሠግናለሁ::** I would like to extend my special gratitude to my co-advisor Prof. Merga Bekana for his support to be engaged in this project from the beginning and show the road map of my research. Dear Prof. Merga Bekana, it's my pleasure to thank you for all the efforts you did to help me in the research on top of your national assignments.

I am grateful to the College of Veterinary Medicine of Addis Ababa University for the hospitality and all rounded support I received throughout my study. I would like to extend my special gratitude to Prof. Merga Bekana, Dr. Dinka Ayana, Dr. Fikru Regasa, Mr. Hika Waktole, and Mr. Alemu Tola for their concern, support and encouragement. Dear all, I would like to take this opportunity to sincerely appreciate your positive attitude towards the research. Dear Mr Alemu Tola, I don't have words powerful enough to express my deepest appreciation and thanks for your all rounded technical and moral support as well as for your father-son approach, advice and encouragement throughout my research work, including your scientific inputs. I am grateful to extend my appreciation and thanks to Dr. Tilaye Demise for his technical help in the post-mortem examination of the horses. It's a great honour for me to give in depth thanks to Prof. Alemayehu Lemma for his advice and technical help for semen collection from Horses. Merga Daba, Sisay Fanta and Alemitu Ararsa, your support during data collection was great. Tariku Zewde and Sisay Alemayehu, my animal attendants, I was happy during the stay with me in the experiment, the way you behave and respect to me and the way you handle the animals was very nice. You deserve appreciation. **በጣም አመሠግናለሁ::** I

would like to extend my sincere acknowledgement to Drs. Mohamed, Musa, Mesfin and Ermiyas (Dodola Vet. Clinic) for their unreserved support during my field studies.

It has been a great privilege to have all the Belgian partners in the project, Dr. Jan Govaere, Dr. Leen Van Brantegem, Dr. Griet Vercauteren and Prof. Peter Ghelhof, where their contribution to my scientific career has been immeasurable. Dear Dr. Jan, undeniably you deserve my deep and heartfelt respect and appreciation. Indeed, thank you very much. Dear Dr. Leen Van Brantegem and Prof. Peter Ghelhof it is my pleasure to sincerely acknowledge your incredible, kindness and hospitality in your laboratories besides the incredible scientific inputs in the study.

A countless thank goes to the people from the Faculty of Veterinary Medicine, Ghent University, laboratory of Parasitology, laboratory of Pathology and Department of Reproduction Obstetrics and Herd Health: Iris Peelaers (Parasitology); Christian Puttevils, Delphine Ameye, Sarah Loomans, Joachim Christiaens (Pathology); Cyrillus Ververs, Kim Roels, Margot VandeVelde, Maya Meesters, Petra Van Damme, Leila VandeVelde, Sandra Willaert, Ria Spiessens, Kenny Vandenbroucke and Marnik DeNorre (Reproduction, Obstetrics and Herd Health) for their overall hospitality, guidance, support and endless help. Thank you very much for making my studies and stay in Belgium comfortable.

I am highly proud to Wollo University, my employer for all rounded support during my study leave. My in depth thanks goes to Dr. Tarekegn Tintagu, Dr. Yalelet Worku, Dr. Awol Mohammed, Dr. Andualem Yimer and Dr. Belay Mulate for their unlimited support in facilitating administration issues at Wollo University and family support during my absence. I am happy to have you all as a colleague to work together.

My last but greatest debt is to my family: Yasine Ebrahim and Fatima Ali (Mom and Dad) thank you very much for your pray and encouragement. Jenet my beloved wife, Rushda my lovely sweet daughter, you suffer a lot in my absence from home for such an extended period of this PhD study. You deserve my greatest words of thanks of all. **እጅግ በጣም አመሰግናለሁ::** My brothers Toyib and Ousman with your families, my Sisters Zinet, Aminat, Zebiba, Nefisa and Firdews with your families, I am lucky to have you. Love you all.

Summary

Dourine, a venereal transmitted trypanosomosis caused by *Trypanosoma equiperdum*, has different clinical signs related to the reproductive and nervous system. It is one of the life-threatening venereal diseases in equidae. So far, there is no clear evidence on how and when stallions become infectious, nor which tissues are affected by the parasite in diseased animals. Post-infection, after a transient, temporary phase of parasitaemia, the parasite disperses to different tissues in an unknown distribution pattern. A review of the available literature was performed and knowledge of previous reports concerning dourine and *T. equiperdum* was gathered (Chapter 1).

T. equiperdum is very closely related to *T .evansi* (the causative agent of surra) in morphology, biochemical and molecular characteristics. Differentiation is possible by use of PCR targeting the kinetoplast DNA gene more specifically,the maxicircle and minicircle genes. Pathologic tissue changes associated with the disease are poorly described so far. The necessity to impose management strategies for the control and eradication of dourine from endemic areas is in demand of more evidence-based data about more accurate feasible diagnostic measures and a better understanding of possible pathways of disease transmission. The aim of this thesis was to get more insights into the pathogenesis and venereal transmission of *T. equiperdum* (chapter 2).

Histopathological lesions in four naturally *T. equiperdum*-infected horses in the chronical stage of dourine from Arsi-Bale highland of Ethiopia, exhibiting obvious clinical signs of dourine, were examined. Tissues collected at necropsy revealed on PCR test characteristics of *T. equiperdum.* A full post-mortem examination on the horses did not reveal typical gross lesions in the organs that were assumed to be responsible for the symptomatology. On histopathology, genital organs (especially testicles, uterus and vagina) were affected with mononuclear cell infiltration and erosions leading to degeneration of seminiferous tubules and perivascular lymphoplasmacytic cuffing in the uterus. In the nervous system, mononuclear cell infiltration was located in peripheral nerves and ganglia and in the white matter of the spinal cord, leading to axonal degeneration and fragmentation. Starting from tissue samples from the predilection sites of the parasite, real-time and conventional PCRs,

using (ITS, maxicircle, and RoTat 1.2 primers), the presence of *T. equiperdum* as causing agent and differentiation from *T. evansi,* was possible (chapter 3). The PCR test on samples from the predilection site of *T. equiperdum* such as the vagina, testicle, and caudal nerves (sciatic and obturator nerves) was first positive for ITS. To differentiate from *T. evansi*, PCRs targeting maxicircle genes (specific to *T. equiperdum*) and RoTat 1.2 (specific to *T. evansi*) were performed and found all samples to be maxicircle PCRs positive and RoTat 1.2 PCR negative indicating that the signs of dourine in these horses were caused by *T. equiperdum*.

T. equiperdum is known to be a tissue parasite as it invades the circulation for a brief period of time early in the infection. Post-infection, after a transient, temporary phase of parasitaemia, the parasite disperses to different tissues in an unknown distribution pattern. In this study, the distribution of the parasite after infection by artificial insemination (AI) or blood transfusion was described. Clinical and haematological parameters were recorded and finally the effect of Cymelarsan® treatment given at the chronic stage of the disease, was assessed. At the same time we looked whether this treatment could alleviate the clinical signs and health status of the animal and induces differences in the pathology and tissue distribution of the parasite or not (chapter 4).

Mares were artificially inseminated with *T. equiperdum* Dodola 943 spiked semen whereas stallions were infected by blood transfusion of the same strain. The course of the disease was monitored by parasitological (Woo), serological (CATT/*T. evansi*) and molecular (PCR) tests and clinical signs and haematological parameters were recorded. At 120 days post infection, they had a full necropsy for histopathology and PCR. A similar pattern of parasitaemia, disease progression and tissue distribution were seen in all horses with different routes of infection. Ejaculated semen in the preclinical stage and epididymal semen in the chronic stage of the disease was positive for *T. equiperdum* on PCR and caused infection in mice. Cymelarsan® treatment in the chronic stage did not result in a clinico-haematological or histopathological improvement. At necropsy, lesions were observed in the nervous and reproductive system. Histopathological lesions were most severe in the peripheral nerves and associated ganglia, the testicles and genital mucosae with multifocal infiltration of lymphocytes, plasma cells and

histocytes. The parasites disseminated to several tissues including the nervous system, testicles and semen.

Since treatment options for dourine are limited and results are disappointing, other measures should be taken into account in an attempt to eliminate dourine in endemic areas. Dourine is dealt with international legislative measures imposed by the World Organization for Animal Health (OIE) aimed at isolation, castration or slaughtering of positive horses. In developing countries like Ethiopia, it is economically and morally not feasible to impose strict slaughter policies to control dourine. When dealing with a disorder that is transmitted by venereal routes, it seems obvious that in an attempt to eliminate such a condition or even to diminish the morbidity of the disease, life cover should be replaced by artificial insemination. Purification of semen by single layer centrifugation (SLC) was attempted to remove trypanosomes from semen (Chapter 5).

SLC has been proven to be successful in reducing venereal transmitted diseases when dealing with other pathogens. For this hypothesis semen was spiked using cryopreserved *T. equiperdum* stabilates (Dodola 943) in varying concentrations, from 10^2 to $>5x10^6$ trypanosomes/ml. Subsequently, SLC was performed following standard procedures. The presence of the parasite in the purified semen was checked by wet smear examination, ITS1 PCR and *in vivo* inoculation in mice. Before SLC, all spiked semen samples, except the negative controls, were positive on PCR analysis. After SLC, all the pellets were found to be negative for *T. equiperdum* on microscopic examinations. PCR analysis also could not detect any parasite-DNA in the SLC-pellet of semen spiked with the lower concentration (10^2 to 10^4 trypanosomes/ml). However, in the SLC pellets spiked with higher concentrations (10^4 - $5x10^4$ trypanosomes/ml), only 1 out of the 4 replicates was negative for parasite DNA. All groups spiked with $>5x10^4$ trypanosomes were found to be positive on PCR. All mice in the positive controls exhibited parasitemia (5/5). Mice inoculated with SLC-purified semen that was spiked with lower than $5x10^4$ trypanosomes remained free of parasitemia, similar to the negative controls. However, inoculation with SLC-pellets from samples with a higher number of trypanosomes ($> 5x10^4$ - $5x10^6$ and $> 5x10^6$ /ml), induced parasitaemia in 2 out of 5 and 3 out of 5 mice, respectively. This study indicates that single layer centrifugation can be used to

clear *T. equiperdum* infected semen but that the success is dependent on the concentration of the trypanosomes in the semen.

Finally, all the obtained results were summarized and compared with the current knowledge and opinions and discussed in chapter 6.

In conclusion, our results indicate that transmission of *T. equiperdum* is possible through semen and semen from symptomless stallions, in the preclinical-, clinical- and in the post-treatment periods. The results of the treatment trial to manage dourine especially at the chronic stage discourages the use of Cymelarsan®. The observed histopathological lesions in the reproductive organs, distal spinal cord and peripheral nerves might give an explanation to the diagnostic clinical incoordination of the hind legs in *T. equiperdum*-infected horses and its presence in the reproductive tract can exemplify the venereal transmission.

Finally, our findings confirmed some of the assumptions about the pathogenesis and transmission of *T. equiperdum* and attributed to the growing knowledge about dourine in equines and trypanosomosis in general. When aiming to develop an all-encompassing eradication program, these insights may lead to a more efficient approach of dourine and thus add value to the agricultural and socio-economic situation in endemic areas

List of Abbreviations

AAT	African Animal Trypanosomosis
AI	Artificial Insemination
ATP	Adenosine Tri-Phosphate
BoTat	Bordeaux Trypanosome antigenic type
bp	Base pair
CFT	Complement Fixation Test
CIOMS	Council for International Organizations Of Medical Sciences
CSA	Central Statistics Authority
CSF	Cerebrospinal Fluid
DAPI	4',6-Diamidino-2-Phenyl Indole
DNA	Deoxyribonucleic Acid
dNTP	Deoxyribonuliotide Tri-Phosphate
eCATH	Equine Cathelicidin
EDTA	Ethylene Diamine Tetra-Acetic Acid
EHV	Equine Herpes Virus
ELISA	Enzyme-Linked Immunosorbent Assay
FAO	Food And Agriculture Organization
g	G-Force (Relative Centrifugal Force)
HAT	Human African Trypanosomiasis
HE	Haematoxylin-eosin
IAEA	International Atomic Energy Agency
ICLAS	International Council For Laboratory Animal Science
IFAT	Indirect Fluorescent Antibody Test
IHC	Immunohistochemistry
ITM	Institute of Tropical Medicine
ITS	Internal Transcribed Spacer
kDNA	Kinetoplast DNA
mAECT	Mini Anion Exchange Centrifugation Technique
mHCT	Micro Haematocrit Centrifugation Technique
NADH	Nicotinamide Adenine Dinucleotide

NTTAT	Non-Tsetse Transmitted Animal Trypanosomoses
OIE	World Organization For Animal Health
OVI	Onderstepoort Veterinary Institute
PCR	Polymerase Chain Reaction
qPCR	(Semi) Quantitative Polymerase Chain Reaction
RAPD	Random Amplification of Polymorphic DNA
RNA	Ribonucleic Acid
RoTat	Rhode Trypanosoma antigenic type
SIT	Sterile Insect Technique
SLC	Single Layer Centrifugation
SNP	Single Nucleotide Polymorphisms
VSG	Variable Surface Glycoprotein
WHO	World Health Organization

Chapter 1

General Introduction

Adapted from:

Ahmed, Y., Hagos, A., Merga, B., Van Soom, A., Duchateau, L., Goddeeris, B.M., Govaere, J., 2018. *Trypanosoma equiperdum* in the horse – a neglected threat?

Vlaams. Diergen. Tijds. 87, 66-75

1.1. Animal trypanosomosis

Trypanosomosis is a parasitic disease caused by different species of flagellated protozoa belonging to the genus *Trypanosoma,* which inhabit the blood and various body tissues and fluids of vertebrate hosts. Non-tsetse transmitted animal trypanosomosis (NTTAT) results from infection by *Trypanosoma (T.) evansi* and *T. equiperdum* where as the tsetse transmited animal trypanosomosis are caused by *T. congolense* and *T. brucei* . *T. vivax* can be transmitted both by tsetse flies and other biting flies (Touratier, 2000; Steverding, 2008). The extent of tissue invasion varies among the different species of the parasite (Igbokwe, 1994; Radostitis et al., 2006). The disease is frequently fatal and is a serious constraint to agricultural production in large parts of Africa, Middle East, Asia and Latin America exhibiting a direct impact on livestock productivity, livestock management and human settlement, and an indirect impact on crop agriculture (Swallow, 2000).

Trypanosomes species infect a wide range of wild animals and domestic animals such as cattle, horses, camels, donkeys, mules, water buffalo,pigs, goats, sheep and dogs (Nantuliya, 1990; Uilenberg et al 1998; Stevens and Brisse, 2004). *T. b. rhodesiense* and *T. b. gambiense* cause sleeping sickness in humans (Baltz et al 1985; Stevens and Brisse, 2004). The South American trypanosome *T. cruzi* also causes Chagas disease in humans (Cazulo et al., 1997). Classification of the different species of African trypanosomes and their hosts is presented in Table 1.1.

Table 1.1. Classification of the pathogenic African trypanosomes

Subgenus	Species/group	Definitive hosts	Reservoir hosts	Laboratory animals for isolation	Development/transmission
Duttonella	*T. vivax*	cattle, sheep, goats, buffalo, horses	wild mammals	usually none susceptible	
	T. uniforme	cattle, sheep, goats		none susceptible	mechanical transmission
Nannomonas	*T. congolense*	cattle, camels, horses, dogs, sheep, goats, pigs	wild mammals	rats, mice, guinea pigs, rabbits	in tsetse: mid gut and proboscis not known to maintain itself exclusively by mechanical transmission
	T. simiae	pigs	wart hog, bush pig	rabbits, monkeys	
	T. godfreyi	pigs	wart hog	none susceptible	
Trypanozoon	*T. brucei brucei*	horses, camels, dogs, sheep, goats, cattle, pigs	wild mammals	rats, mice, guinea pigs, rabbits	in tsetse: mid gut and salivary glands
	T. brucei rhodesiense *T. brucei gambiense*	human, affect domestic animals as *T. brucei brucei*	several groups of wild mammals (particularly for *T. brucei rhodesiense*)	as for *T. b. brucei* (after initial adaptation where *T. b. gambiense* is concerned)	
	T. evansi	camels, horses, dogs, buffalo, cattle	several wild mammals in Latin America	as for *T. brucei brucei*	mechanical transmission, oral transmission to carnivores
	T. equiperdum	equidae	Not known	as for *T. brucei brucei* (after initial adaptation)	venereal transmission

Source: FAO, 1998.

T. equiperdum, causing the sexually transmitted disease dourine in horses, belongs to the genus *Trypanosoma*, subgenus *Trypanozoon,* together with *T. evansi*, the causative agent of surra in Camels, *T. brucei* (*b.*) *brucei*, *T. b. rhodesiense* and *T. b. gambiense,* which cause nagana and human African trypanosomosis (sleeping sickness), respectively (Hoare 1972). *T. equiperdum* differs substantially from other trypanosome species that are transmitted by invertebrate vectors. Like all other Non-Tsetse Transmitted Animal Trypanosomoses (NTTAT) (e.g. *T. vivax* and *T. evansi*), *T. equiperdum* is not transmitted by tsetse flies (Touratier, 2000). *T. vivax* is mainly transmitted mechanically by a range of blood feeding insects including, *Tanabids, Stomxys* and *Cullicoides. T. evansi* also is mostly transmitted by biting flies of the genera *Tabanus, Stomoxys, Haematopota, Chrysops* and *Lyperosia* (Luckins, 1988; Desquesnes, 2004). *T. equiperdum* however is transmited venereally and directly from one infected horse to another. The trypanosomes, which are present in the seminal fluid and mucous membranes of the genitalia of the donor animal, are transferred to the recipient during sexual intercourse. Moreover, *T. equiperdum* further differs from other trypanosomes in that it is primarily a tissue parasite that rarely invades the blood (Barrowman, 1976; OIE, 2013; Vulpiani et al., 2013).

Dourine caused by *T. equiperdum* is characterized mainly by fever, oedematous swelling of the genitalia, cutaneous plaques and eruptions and neurological signs including incoordination, paralysis, ocular lesions (such as conjunctivitis, keratitis and mild corneal opacity), anaemia and progressive emaciation (Barrowman, 1976; Luckins, 1994; Vulpiani et al., 2013). Clinical signs related to dourine have been documented to be often obvious, but final diagnosis requires demonstration of the parasite through serological and molecular tests (OIE, 2013).

Among the various species of *Trypanosoma*, *T. equiperdum* and *T. evansi* cause similar signs. In view of this fact, the actual course of the disease caused by these two trypanosome species was uncertain due to the close similarity in their ultrastructure, genetic makeup and antigenic nature, as has been demonstrated by the fact that the two species have shown similar genetic and antigenic expression (Toutaier, 2000; Verloo et al., 2001; Claes et al., 2003a,b). However recent findings of whole genome analysis of *T. evansi* and *T. equiperdum* provided new insights of their distinction and their relation with the different *T. brucei* subspecies (Birhanu et al.,

2016; Cuypers et al., 2017). Both *T. evansi* and *T. equiperdum* evolved from *T. brucei* but with a different geographical origin. The *T. evansi* genomes are related to the *T. brucei* genomes from Western Africa whereas the *T. equiperdum* genomes related to the *T. brucei* genomes from Eastern Africa (Cuypers et al., 2017).

1.2. Appearance and importance of dourine

Dourine is known in most countries of the world as a notifiable disease (OIE 2013) and it threatens equidae around the globe. The disease is reported to be widespread in South America (Samper and Tibary, 2006; Sellon and Long, 2007; Perrone et al., 2009; Sanchez et al., 2015), Mongolia (Clausen et al., 2003), Namibia (Kumba et al., 2002), Eastern Europe (Discontools, 2011) and Ethiopia (Alemu et al., 1997; Clausen et al., 1999; Gari et al., 2010; Hagos et al., 2010a,b,c). Recently, the disease has also been observed in the south of Europe in Italy, after 30 to 40 years of a confirmed outbreak between the 1970s and 1980s (Caporale et al., 1980, Scacchia et al., 2011; Pascucci et al., 2013). The disease was introduced in Italy during the 1970s through the import of slaughter animals from an (unkown) endemic region. Despite the import regulation and veterinary border control, it was possible that some infected animals were introduced to the resident horse population either for breeding or for a period of conditioning before slaughter. The hypothesis is that an infected male was the main source of disease spread and the infection spread through the mountain pastures during mating season. In the end stallions of authorized studs became infected and after that dourine spread to the remaining of the population (Caporale et al., 1980). In the 2011 outbreak, a stallion purchased for stud purposes tested positive for dourine during a routine test procedure. The likely source of infection was a mare imported from the Netherlands (Scacchia et al., 2011). Apparentely, the main characteristics of the animals (gender, age and reproductive activity) and features of the outbreak (relationship between the affected animals and sequence of prevalence) confirmed that the infection was transmitted directly from animal to animal during coitus (Scacchia et al., 2011). Uncontrolled mating and ignoring veterinary obligations on animal identification and animal movement control were recognized as important factors in all outbreaks.

The disease can be carried to various parts of the world through the transportation of infected stallions, mares, donkeys and semen. The spread of the causal agent by artificial insemination (AI) has not been confirmed, though it could potentially occur since *T. equiperdum* is present in seminal fluid and genital tissues (Lelli et al., 2012). Besides venereal transmission, close contact between mare and foals (e.g. during nursing) (Brun et al., 1998; Lelli et al., 2012), contaminated equipment (such as an artificial vagina and breeding phantom) or contaminated personnel can also cause transmission of the disease (Metcalf, 2001; Samper and Tibary, 2006).

Today, despite the numerous benefits of shipping semen internationally, some serious threats remain unclear when the semen is contaminated with a communicable disease. Import regulations to prevent entry of the disease from endemic countries will require a negative dourine test for horses and horse gametes. The current regulations vary between countries (IHSES, 2007; Calistri et al., 2013), which makes it difficult to comply with all of them when dealing for instance with cryopreserved semen. The import regulations vary from requiring only a permit for importation to several months of quarantine of the stallion. Due to the lack of knowledge of the prepatent infectiousness of the semen of recently infected animals, the transmission of disease through the transport of either the breeding stock or their gametes is a threat for the equine industry (Metcalf, 2001).

Although dourine has a high mortality rate of up to 50%, some infected animals have been observed to recover spontaneously (Equimed, 2009; Ricketts et al., 2011; OIE, 2013). So far, there has been no known natural reservoir of the parasite other than infected equids. donkeys and mules are more resistant than horses and may therefore remain unapparent carriers in which the disease may often pass unperceived, even though their semen and vaginal secretions contain the infective trypanosomes (OIE, 2013).

1.3. Etiology

Trypanosomes are flagellated and elongated spindle-shaped protozoa with an average length of 20-30 µm and a width of 1.5 to 3.5 µm. The trypanosome cell has a blunted posterior end and a free flagellum at the anterior end (Figure 1) (Matthews, 2005).

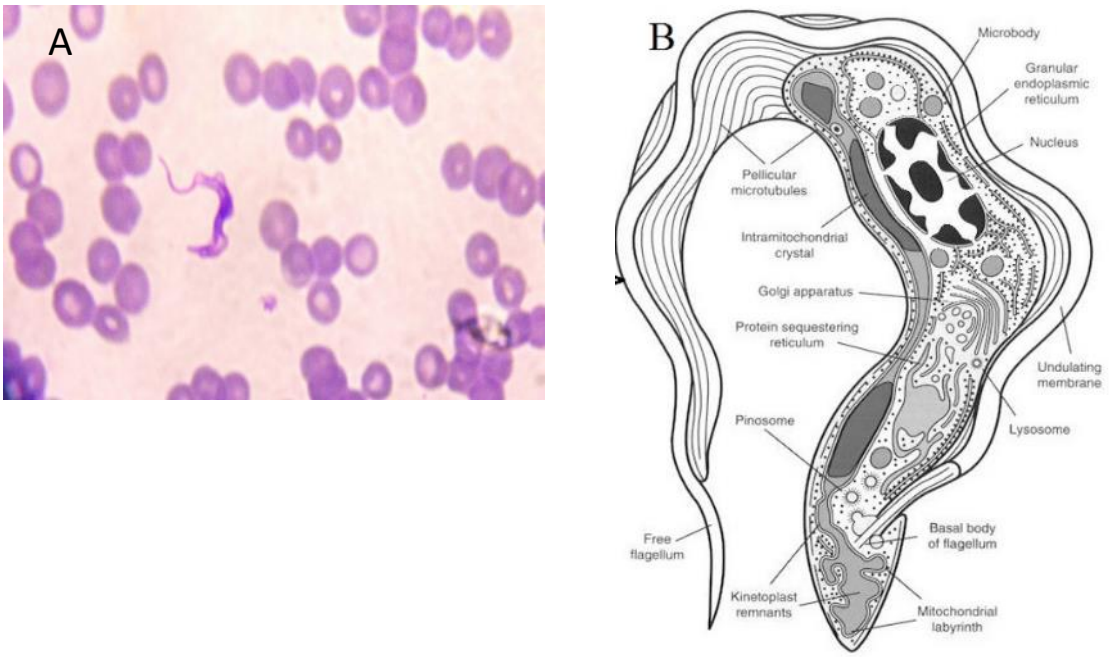


Figure 1.1. Gross and fine structure of Trypanosomes. (A) Dourine causing agent (*T. equiperdum*), Diff quick stain of thin blood smear at x 1000 microscopy from an experimentally infected and Dourine diseased horse, (B) Fine structure of *Typanosoma evansi*, as revealed by transmission electron microscopy of thin sections (Adapted from Vickerman, K. 1977 by Yaeger, R.G. 1996). https://www.ncbi.nlm.nih.gov/books/NBK8325/

The name *T. equiperdum* was postulated by Doflein in 1901 as cited in OIE (2013). *T. equiperdum* is a member of the non-tsetse-transmitted trypanosome group. The trypanosomes, which are strictly parasitic, are flagellar protozoa that belong to the phylum of Sarcomastigophora, the order of Kinetoplastidae, the family of Trypanosomatidae and the genus of *Trypanosoma,* under the Salivarian group. The subgenus Trypanozoon includes the pathogenic species *T. evansi*, *T. brucei* and *T. equiperdum. T. brucei* is further divided into three subspecies: the animal pathogen *T. b. brucei* (ruminants, equines), and the two human pathogens responsible for human sleeping sickness, *T. b. gambiense* and *T. b. rhodesiense* (Hoare, 1972). *T. equiperdum* has recently come to be considered a subspecies of *T. brucei* based on molecular analysis (Claes et al., 2003b; Li et al., 2005, Lun et al., 2010; Schnaufer, 2010; Carnes et al., 2015; Wen et al., 2016).

The members of the order Kinetoplastida contain a kinetoplast (k) DNA network, consisting of two types of interlocked circular DNA molecules: dozens of maxicircles, the equivalent of mitochondrial DNA in other organisms, and thousands of heterogeneous minicircles (Shlomai, 2004; Liu et al., 2005). The maxicircles have typical mitochondrial genes, most of which are

translatable only after RNA editing. The minicircles encode guide RNAs, which are required for decrypting the maxicircle transcripts (Lai et al., 2008).

The major difference at the molecular level among *T. brucei, T. equiperdum* and *T. evansi* is the absence of maxicircle kDNA from *T. evansi* and a partial deletion of maxicircle kDNA from some *T. equiperdum* strains (Frasch et al., 1980; Borst et al., 1987). All *T. equiperdum* and *T. evansi* strains show some degree of kDNA loss, ranging from intact networks with complete maxicircles, but minicircle homogenization to complete kDNA loss (Schnaufer et al., 2002; Lai et al., 2008). Originally, the terms dyskinetoplastic and akinetoplastic described cells completely lacking a kDNA structure. Therefore, most publications refer to all *T. equiperdum* and *T. evansi* strains as dyskinetoplastic, reserving the term akinetoplastic for strains completely lacking detectable kDNA (Schnaufer et al., 2002, Jensen et al., 2008; Lai et al., 2008; Schnaufer, 2010).

T. brucei is a kinetoplastid trypanosome whereas *T. evansi* and *T. equiperdum* are dyskinetoplastic trypanosomes. *T. evansi* lacks the maxicircle genes, but *T. equiperdum* does have the maxicircle genes, although with major deletions of some genes. So far, no akinetoplastic (lacking all kDNA) *T. equiperdum* strains have been observed (Birhanu et al., 2016). Two biological features differentiate *T. evansi* and *T. equiperdum* from *T. brucei*. First, they do not use tsetse flies as the vector for transmission, since *T. evansi* is transmitted by biting flies and *T. equiperdum* by sexual contact (Brun et al., 1998; Desquesnes et al., 2013). Secondly, *T. evansi* and *T. equiperdum* are dyskinetoplastic, lacking part of or all (akinetoplastic) of their kDNA (Lai et al., 2008). Partial dyskinetoplastidy or total akinetoplastidy locks the trypanosome in the blood stream form trypamastigotes in the host, and transmission between vertebrates becomes purely mechanical, without further development in a vector (Bringuard et al., 2006).

Trypanosomes of subspecies *T. equiperdum* 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 (Brun et al., 1998). However, a few trypanosomes occasionally appear in the peripheral blood of animals (Brun et al., 1998; Gari et al., 2010; Hagos et al., 2010c). The fact that foals have been found infected with *T. equiperdum* may indicate that the parasite can

also be directly transmitted through udder lesions or milk, or during passage through the birth canal upon parturition (Schultz, 1935; Wang, 1988; Brun et al., 1998). Vertical transmission of *T. evansi* has already been reported in camels (Narnaware et al., 2016).

1.4. Clinical signs

Clinical signs, although not pathognomonic, can be of great help in diagnosing the disease in endemic areas. The presence of oedematous swellings of the external genitalia, the development of plaques of the skin, and the appearance of nervous signs such as ataxia and incoordination are highly indicative for the disease (Vulpiani et al., 2013).

Substantial weight loss leading to severe emaciation, weakness and a generally poor condition has been reported in naturally infected as well as experimentally infected horses (Vulpiani et al., 2013). Hagos et al. (2010a, c) also reported marked muscular atrophy in the gluteal region, emaciation, weakness and poor body condition as important clinical signs in horses. However, the vulvar or preputial lesions of dourine might be difficult to clinically differentiate from other diseases such as Equine Coital Exanthema (EHV3) in the early stages and in latent cases (Barrowman, 1976). Equine Herpes Virus (EHV3) infection is manifested by the appearance of vesicles, ulcers and depigmented spots on the vaginal mucous membrane (Blanchard et al., 1992; Studdert, 1996; Allen and Umphenour, 2004), which is similar to the genital form of dourine. By contrast, however, EHV3 is self-limited and is not accompanied by emaciation, ataxia or incoordination.

Lesions in the genitalia are documented to be the first signs after *T. equiperdum* infection. In stallions, moderate oedema of the scrotum and preputial sheath (Figure 2E and F) is seen, accompanied by both preputial and urethral purulent discharge. In mares, oedema of the vulva, accompanied by ulcers along the rim of the vulva, vaginal discharge mainly of the mucopurulent type with foul odour, and oedema of the mammary gland and cloudy off-white mammary secretions have been documented as the prominent signs in the genital form of the disease. Later on in the progression of the disease, oedema of all ventral body parts, and cutaneous plaques all over the body can be seen. At this stage, the oedematous plaques, especially on the genitalia, will frequently ulcerate and become depigmented scars (Figure 2D)

(Hagos et al., 2010a; Vulpiani et al., 2013). This depigmentation has been indicated to be due to severe dermatitis with hydropic degeneration and necrosis of the keratinocytes and necrosis of basal cells including the melanocytes with excess free melanin pigment within the epidermis (Gizaw et al., 2017).

The next stage of dourine is characterized by progressive anaemia, with or without fever, and nervous disorders, mainly manifested as paraplegia and paralysis of the hind limbs, showing severe muscle atrophy and emaciation before death (Figure 2A, B and C) (Hoare, 1972; Stephen, 1986).

Nervous signs such as stiffness, weakness, lameness in one or both hind legs, staggering, lack of coordination, inability to stand upright after prolonged sternal or lateral recumbence, ataxia and facial paralysis have also been reported by Hagos et al. (2010a) and Vulpiani et al. (2013).

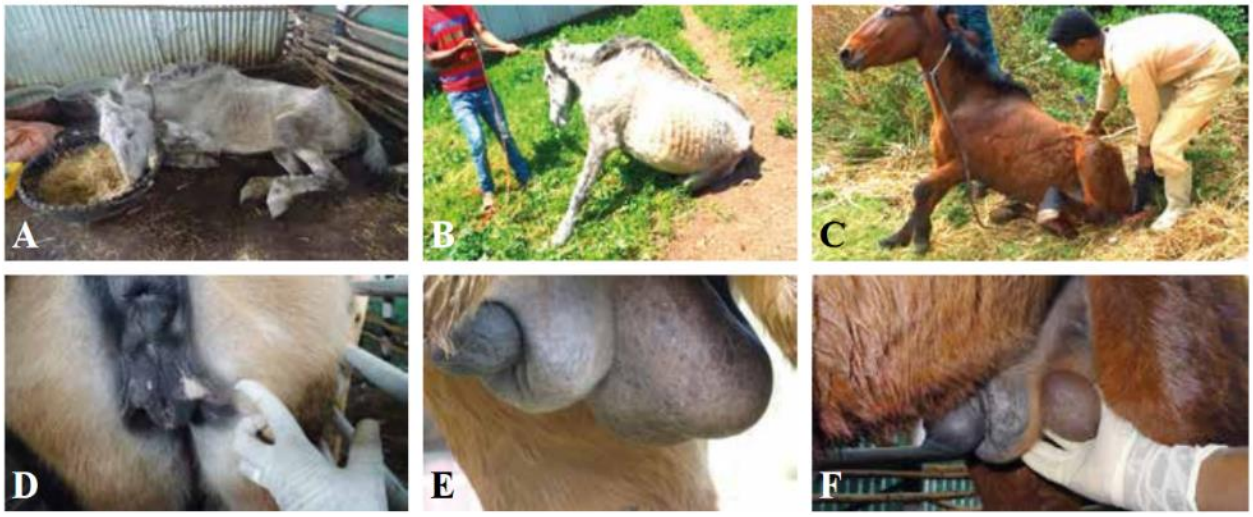


Figure 1.2. Symptomatology of dourine infected horses. (A) and (B) mare with weight loss and hind quarter paralysis. (C) stallion with hind quarter paralysis. (D) mare with a depigmented scar at the vulva. (E) and (F) swelling at the prepuce and scrotum from experimentally infected horses with *T. equiperdum* Dodola 943, (cfr chapter 4).

1.5. Pathology

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 have been described as circular elevated cutaneous plaques, 5–8 cm in diameter and 1 cm thick (OIE, 2013). Grossly dourine is characterized by cachexia, pale mucus membranes

due to anaemia, muscular atrophy, ataxia and incoordination of the hindquarters, ptosis of the lower lip and genital lesions (Barrowman, 1976; Pascucci et al., 2013). At post-mortem examination, gelatinous exudates are present under the skin. In stallions, the scrotum, preputial sheath, and testicular tunica are usually found thickened and in some cases the testes are embedded in a tough mass of sclerotic tissue. In mares, the vulva, vaginal mucosa, uterus and mammary glands might be thickened with gelatinous infiltration. Lymph nodes will be 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).

On histological examination of tissue samples, the disease is characterized by haemosiderin deposition in the spleen, lymph nodes usually show non-specific reactivity with infiltrations of the plasma cells. The edematous plaques show 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 (Scacchai et al., 2011; Pascucci et al., 2013). In the nervous system of infected horses, neurodegenerative lesions and inflammatory vasculitis with edematous infiltration in the facial 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 bodies. Russell bodies are eosinophilic, large, homogeneous inclusions usually found in a plasma cells undergoing excessive synthesis of immunoglobulin; the Russell body is a characteristic of the distended endoplasmic reticulum (Mossuto et al., 2015). Multifocal areas of hepatitis in the liver and plasma cell infltration of the renal pelvis in the kidneys have been reported. Periglandular inflammation in the vulva, vagina, uterus and clitoris was also observed in infected mare (Pascucci et al., 2013).

1.6. Diagnosis

Diagnosis has to be based on demonstrating the presence of the parasite itself or antibodies. However, there is no defined serological assay for T*. equiperdum* nor molecular markers that can be used for clinical use so far. The clinical signs and gross lesions in diseased animals are

suggestive, but cannot always be identified with certainty, especially in the early stages or in latent cases and cases of surra due to *T. evansi,* which exhibits similar clinical signs (OIE, 2013). Other conditions, such as equine coital exanthema or chronic irritation (e.g. urine scalding), might cause some similar clinical features (Blanchard et al., 1992; Studdert, 1996; Allen and Umphenour, 2004).

Differentiation between *T. equiperdum* and *T. evansi* based on parasite morphology is difficult in areas where both organisms are found (Brun et al., 1998; Sanchaz et al., 2015; Suganuma et al., 2016). So far there are no defined serological assays for the true *T. equiperdum,* and also no molecular markers for clinical use. Previously, Claes et al. (2004) and Gari et al. (2010) used a PCR to amplify a portion of the RoTat 1.2. VSG coding gene in order to detect *T. equiperdum*. More recently, it has been shown that RoTat 1.2 VSG is not present on *T. equiperdum* (Birhanu et al., 2016), thus making previously reported findings equivocal.

1.6.1. Direct diagnosis: parasitological techniques

Wet, thick blood films can be examined microscopically (x400) under a coverslip. Detection of trypanosomes moving in between the erythrocytes is a simple, although non-species specific, parasitological test. However, with a detection limit as high as 10,000 trypanosomes/ml, the technique has a very low sensitivity. Giemsa or stained thin blood films have a similarly low sensitivity. The sensitivity can be increased by applying concentration techniques such as used in the Woo test (Woo, 1970). This test includes a micro-centrifugation of 50µl whole blood at 13000 g for 5 minutes. Subsequently, the capillary tubes are mounted in a special holder and the buffy coat is examined microscopically at a magnification of ×100 to look for live parasites (Woo, 1970; Reid et al. 2001; Gari et al., 2010).

The mini Anion Exchange Centrifugation Technique (mAECT) separates trypanosomes from blood cells on an anion exchange chromatography gel, based on their differential surface charge in function of pH and ionic strength of the gel equilibration buffer. The buffer negatively charges the host blood cells, which are subsequently adsorbed onto the anion-exchange column, while the neutral or positively charged trypanosomes are eluted, retaining their viability (Lanham and Godfrey, 1970; Lumsden et al., 1979). Trypanosomes that are

eluted from the gel are taken up in a clear glass or plastic collector tube that is centrifuged at low speed (about 1,800 g) for 10 minutes to concentrate the parasites at the bottom of the tube where the trypanosomes can be microscopically observed under low magnification (x100). An improved model of mAECT column and collector tube has an analytical sensitivity of <30 trypanosomes/ml), is robust and avoids the need to mount the collector tube in water for microscopic examination (Büscher et al., 2009). mAECT is usually conducted on 0.5 ml of blood but prior centrifugation of a larger volume of blood (up to 5 ml) and loading the buffy coat on the mAECT column can lower the detection limit to 10 trypanosomes⁄ml (Camara et al., 2010). The mAECT is the most sensitive parasite detection technique with an analytical sensitivity that is similar or higher than that of of most molecular diagnostics for African trypanosomiasis but works only well on *T. brucei*, *T. evansi* and *T. equiperdum*. It is less performant for *T. vivax* and *T. congolense* (Büscher, 2014).

On microscopic evaluation, *T. equiperdum* and *T. evansi* cannot be differentiated from one another, a fact which makes the test less reliable in areas where both organisms are prevalent (OIE, 2012).

1.6.2. Immunohistochemistry and immunofluorescence

A more recent study managed to identify and localize trypanosome antigens directly in the tissue by immunochemistry, using goat anti-rabbit IgG conjugated in streptavidin peroxidase, visualized by 3-3 diaminobenzidine substrate and counter stained by Mayer's haematoxylin. In addition an immunofluorescence staining was successful using donkey anti-rabbit IgG and mounting the section with 4',6-diamidin -2- phenylindole (DAPI) (Pasucci et al., 2013).

1.6.3. Animal inoculation

Mice, rats, rabbits and dogs are susceptible to laboratory adapted *T. equiperdum* strains (Brun et al., 1998; Claes et al., 2005; Akhmetova et al., 2016). However, besides animal welfare issues, it is often difficult to obtain a first passage from samples obtained from the host, and animal inoculation is of little use as a routine method of diagnosis.

Blood from suspected animals can be used as inoculants for laboratory rodents. Under

laboratory conditions, dogs can develop dourine. Different routes of infection, such as subcutaneous, intraperitoneal, intravenous, intraurethral and intravaginal transmission, were tested and all gave rise to clinical signs of dourine. In dogs, inoculation of *T. equiperdum* produces the typical picture of dourine with trypanosomes present in the lesions, but not in the blood, and the infection may last from one to several months (Stephen, 1986).

Strains of *T. equiperdum* were successfully isolated after intratesticular injection in the rabbit with blood or material from infected horses (Claes, 2003; Claes et al., 2005). Similarly, after the injection of udder secretions from clinically diseased mares into the scrotum of rabbits, *T. equiperdum* could be isolated successfully from the scrotal tissue homogenate of the rabbit. The new isolate from that rabbit could also be re-isolated from scrotal oedema of another rabbit after successive inoculation, even after freezing of the inoculum in liquid nitrogen (Pascucci et al., 2013). However, blood and genital washes from serologically positive horses did not lead to infection when inoculated into mice and puppies (Alemu et al., 1997; Hagos et al., 2010a; Pascucci et al., 2013). Attempts to transmit the parasite to animals other than horses (rats by intraperitoneal way and dogs subcutaneously) were also unsuccessful with inocula of blood and cerebrospinalfluid (CSF) known to contain living trypanosomes (Barrowman, 1976).

1.6.4. DNA-based techniques

A highly sensitive method for detecting even a single parasite is the polymerase chain reaction (PCR) based on an amplification of trypanosomal DNA with a sensitivity of 0.5 pg of parasite DNA or one single parasite in 10µl blood (Wuyts et al., 1994). Molecular diagnostics have been introduced to avoid the low sensitivity of parasitological microscopic examinations. They are not used in routine diagnosis due to complexity and technical requirements. The PCR method for the amplification of a portion of the gene coding for a 135 bp portion of the 177 bp sequence of the highly repeated region within the Trypanozoon subgenus detected the organism from blood and other tissue fluids (Pascucci et al., 2013). The Onderstepoort Veterinary Institute (OVI) strain of *T. equiperdum* was used as positive control in this report.

Moreover, solid tissues from udder, mammary gland and iliac lymph nodes, vulva, clitoris and uterus showed positive results for the parasite in dourine affected animals.

In the diagnosis of animal trypanosomosis, Internal Transcribed Spacer regions(ITS) PCRs that allows discrimination of the trypanosome species in one single run of one single sample, based on the variability in length for the kinetoplastid between species, but being constant within a species, have been given a special interest (Desquesnes et al., 2001; Büscher, 2014). Several variants of PCRs that target the ITS1 sequence exist (Desquesnes et al., 2001; Fikru et al., 2012; Tran et al., 2014) and primer sequences have been modified to improve the sensitivity (Fikru et al., 2012). However this PCR test would not be able to distinguish *T. equiperdum* from *T. evansi* and from the other members of the *Trypanozoon* subgenus. A PCR targeting the RoTat 1.2 VSG gene was proposed to differentiate *T. evansi* (Urakawa et al., 2001). With this PCR, Claes and colleagues showed that most *T. equiperdum* isolates, except BoTat 1.1 and OVI, are in fact misclassified as *T. evansi* (Claes et al. 2004).

In the Kinetoplastids, the kDNA consists of interlocked circular DNA molecules organized in a huge network of two different types: maxicircles and minicircles (Lukes et al., 2005). *T. equiperdum* and *T. evansi* are dyskinetoplastic (kDNA) since they lack part of the kDNA (Claes et al., 2005; Lai et al., 2008; Schnaufer et al., 2002; Carnes et al., 2015). *T. equiperdum* typically has retained the maxicircles. *T. evansi* does not have maxicircles and either shows minicircle homogeneity or are akinetoplastic (Ou et al., 1991; Lun and Vickerman 1991; Ventura et al., 2000; Schnaufer et al., 2002). This maxicircle kDNA was considered to be a useful genetic marker to distinguish *T. brucei* and *T. equiperdum* from *T. evansi*, because of its presence in the former two species and absence from the latter species (Li et al., 2007; Domingo et al., 2003; Dean et al., 2013). Masiga and Gibson (1990) developed a kDNA probe based on minicircle kDNA sequence to detect *T. evansi*, but their method cannot be used to detect dyskinetoplastic strains of *T. evansi* and cannot be used to distinguish *T. evansi* from *T. equiperdum* and/or *T. brucei.*

1.6.5. Serology: Indirect diagnosis

From earlier studies, it was known that the diagnosis of *T. equiperdum* by standard parasitological techniques is difficult owing to the low numbers of parasites present in blood or tissue fluids and the frequent absence of clinical signs of disease in the prepatent and the chronic phases. Consequently, the demonstration of trypanosomal antibodies in the serum has become the most important diagnostic technique in determining whether an animal is currently infected or has been previously in contact with the parasite (OIE, 2013). Serological testing by the complement fixation test (CFT) has been widely used in health certification of horses for export (Wassal et al., 1991).

Antibodies are present in infected animals, whether they exhibit clinical signs or not. The CFT has been used to confirm clinical evidence and to detect latent infections (OIE, 2013). The reliability of CFT and IFAT for known *T. equiperdum* has been reported by Cauchard et al. (2014). However, uninfected equids, particularly donkeys and mules, often give inconsistent or nonspecific reactions because of the anti-complement effects of their sera. In this case, the indirect fluorescent antibody test (IFAT) is more adequate. Enzyme-linked immunosorbent assays (ELISAs) are also used (OIE, 2013).

However, the diagnostic antigens and antibodies currently available for use in sero-diagnostic tests are not specific for *T. equiperdum,* but react due to the cross-reactivity with the other Trypanosome spp. Significant improvements in dourine sero-diagnosis will require the development of more specific *T. equiperdum*-subunit antigens. A recent publication reported the successful *in vitro* cultivation of *T. equiperdum* OVI parasites that can be used in complement fixation tests (Bassarak et al., 2016). This might be of great help in obtaining a specific diagnosis for *T. equiperdum*, since the OVI strain is one of the few genuine *T. equiperdum* strains generally available in reference laboratories (Claes et al., 2003b). Until all of these requirements have been achieved, however, the diagnosis of dourine will of necessity involve the detailed history and the clinical and pathological findings, on top of the serology, to establish confirmation of the disease (Calistri et al., 2013).

Because of the lack of a specific antigenic marker to differentiate *T. equiperdum* from *T. evansi,* careful attention must be paid when choosing a strain to prepare such an antigen. According to recent data, true *T. equiperdum* needs to be differentiated from *T. evansi* (Claes et al., 2003a, b; 2004). The problem, however, is that many *T. equiperdum* strains have been found to be closely related to certain classes of *T. evansi* in cluster analysis by Random Amplified Polymorphic DNA (RAPD) and Multiplex-endonuclease genotyping (Claes et al., 2003b). With this in mind, the *T. equiperdum* OVI and BoTat 1.1 strains have been found to be the most suitable for use as antigen sources. Single Nucleotide Polymorphism (SNP) within the F1-ATP synthase γ subunit gene provided an identifying characteristic of *T. evansi* as distinct from *T. equiperdum* without relying on VSG genes or kinetoplast DNA (Birhanu et al., 2016). A recent finding of whole genome SNP analysis of *T. evansi* and *T. equiperdum* also provided new insights in the origin of both species and their relation with the different *T. brucei* subspecies (Cuypers et al., 2017). This method may be the future key for differentiation of the two species and then developing specific markers for diagnosis.

1.6.5.1. Agglutination Test for Trypanosomosis

Card and Latex agglutination tests developed for *T. evansi* from RoTat 1.2 antigen have been implemented for dourine diagnosis because of the cross-reactive nature of the antibodies of some strains of *T. equiperdum* (Claes 2002). Gari et al. (2010) and Hagos et al. (2010 a,b) have used the test to diagnose dourine in Ethiopia at the field level. However, there is no RoTat1.2 gene on true *T. equiperdum* strains (Birhanu, et al. 2016). Therefore, unless genuine antigens are identified from true strains of *T. equiperdum,* these agglutination tests will be no more functional to diagnose dourine.

1.6.5.2. Indirect Fluorescent Antibody Test (IFAT)

The Indirect Fluorescent Antibody Test (IFAT) is frequently used in the diagnosis of dourine as a confirmation test for a positive CFT result, since immunofluorescence is a more reliable and sensitive technique, though its interpretation is both subjective and labour intensive (Wiliamson et al., 1988). This test can be used in surveillance (prevalence of infection) and for

the purpose of declaring a population free of the disease (OIE, 2013). This test has been used with success to diagnose dourine in Italy (Pascucci et al., 2013).

1.6.5.3. Enzyme-Linked Immunosorbent Assay (ELISA) and Immunochromatographic test (ICT)

Although the CFT has been used for many years to diagnose dourine, it is considered to be less sensitive than ELISA and it has been suggested that ELISA could replace the CFT for animal health certification. ELISA is a very sensitive technique and its use for routine diagnostic serology of dourine would provide a significant advantage over current serological tests if a defined antigen were to be used, since it would permit test standardization and more readily allow comparison of the test results among the different laboratories. In addition, ELISA testing lends itself to a far greater degree of automation, which makes it suitable for large numbers of samples (Wassal et al., 1991; Bishop et al., 1995).

Different authors 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; Clausen et al., 2003, Bonfini et al., 2018). Similarly, Wasal et al. (1991) concluded that ELISA is a very sensitive test for dourine compared to the CFT and IFA tests.

Recently Davaasuren et al. (2017) have shown the feasibility of using an ELISA and Immunochromatographic test (ICT) to diagnose dourine based on recombinant GM6 antigen (rTeGM6-4r) derived from *T. equiperdum* isolated from the urethral mucosa of a clinically dourine diseased stallion in Mongolia (Suganuma et al., 2016). The result showed a good diagnostic value in testing the sera of *T. equiperdum*-infected horses. A study conducted to validate the ICT based on the rTeGM6-4r accurately diagnosed positive reference sera that had been prepared from dourine diseased horses in Mongolia. This field friendly test was found to be of equal value of the rTeGM6-4r ELISA (Mizushima et al., 2018). However, these methods have also been shown to diagnose *T. evansi*-infected water buffalo, cattle, goats and sheep (Nguyen et al., 2014; Nguyen et al., 2015). Due to this lack of specificity the test based on rTeGM6-4r antigen will be only helpful in the diagnosis of non-tsetse transmitted horse trypanosomiases in the field but couldn't be used to differentiate between the two trypanosome species (*T. evansi* and *T. equiperdum*).

Genotype analysis is able to differentiate *T. evansi* from T*. equiperdum* (Carnes et al., 2015; Birhanu et al., 2016; Cuypers et al., 2017). This may lead to the development of other antigen-specific markers for *T equiperdum* in future ELISA tests.

1.7. Treatment

The World Organization for Animal Health (OIE, 2013) currently imposes the slaughtering of CFT-positive horses as an effective control strategy. In general, treatment may result in asymptomatic carrier animals and is as such not recommended in a dourine-free territory because of fear for the continuing dissemination of the disease by the treated animals (Barrowman, 1976; Losos, 1986; OIE, 2000).

Evidence from *in vitro* drug sensitivity determination of *T. equiperdum* (Zhang et al., 1992; Brun and Lukins, 1994) indicates that Suramin, Diminazene, Quinapyramine and Cymelarsan® are effective against trypanosome species. Hagos et al. (2010c) carried out *in vivo* efficacy testing of Diminazene diaceturate (Diminasan®) and bis (aminoethylthio) 4-melaminophenylarsine dihydrochloride (Cymelarsan®) on mice, which demonstrated that Cymelarsan® is effective, but that Diminasan®, on the other hand, fails even at high doses of up to 28 mg/kg body weight (four times the recommended dose in cattle) to cure any of the mice infected with the *T. equiperdum* Dodola strain from Ethiopia.

Horses treated with Cymelarsan® at doses of 0.25 mg/kg and 0.5 mg/kg body weight showed no detectable parasitaemia 24 h after treatment. The mean PCV levels also improved after treatment, and seroreversion on card agglutination test for trypanosoma was observed starting from 150 and 170 days post treatment (Hagos et al., 2010c). This might have been due to the fact that the absence of the antigen source from the host system stopped the triggering of antibody production and consequently the antibodies were diluted in the serum. The study by Hagos et al. (2010c) showed improvement in body condition following the treatment of chronic infection and there was no relapse. Moreover, the clinical signs of incoordination of the hind legs, weakness and ventral oedema disappeared within 10 days, together with a progressive increase of the PCV. However, in recent reports, both Cymelarsan® and Diminasan® have been found to lead to relapse in treated mice (Habte et

al., 2014), and parasites can still be found in the CSF of horses treated with Cymelarsan® (Cauchard et al., 2016).

Recently, ex vivo trypanocidal activity of 1-(2-hydroxybenzylidene) thiosemicarbazide against Venezuelan *T. equiperdum* strain has been reported (Parra et al., 2017). The compound exhibits a greater inhibitory activity of the parasite in the culture medium. In another recent report, *in vitro* laboratory tests of equine antimicrobial peptide (eCATH1) showed promising results relating to its trypanocidal activity on Trypanozoon spp through plasma membrane permeabilization and mitochondrial alteration. The administration of eCATH1 at a dose of 10 mg/kg to *T. equiperdum*-infected mice diminished the mortality rate. This finding suggests that eCATH1 can be considered as a candidate for the development of new therapeutic agents for the treatment of trypanosomosis (Cauchard et al., 2016).

1.8. Prognosis

A treatment with Cymelarsan® eliminated the parasites from the horses's circulation within a short period of time (Hagos et al., 2010; Cauchard et al., 2016). However, since the parasite is a tissue parasite, it may hide in areas that cannot easily be reached by the drugs, thus resulting in relapse (Cauchard et al., 2016).

When horses are left untreated, the majority of cases will perish (Barrowman, 1976), or else they will develop a chronic form of dourine with clinical signs as described above (Barrowman, 1976; Hagos et al., 2010a, c; Vulpiani et al., 2013). Parasitaemia may disappear after 80 days of infection even though the general body condition continues to progressively deteriorate (Barrowman, 1976; Hagos et al., 2010c). Infected animals became aparasitaemic after 80 days post infection, though they expressed parasitaemia again when challenged with immunosuppressive drugs (Hagos et al., 2010c). Therefore it is assumed that the parasites can hide themselves from the immune system.

1.9. Current reports on *Trypanosoma equiperdum*

Most of the studies conducted on *T. equiperdum* focused on characterization of the trypanosome. In the literature many (sub)species characteristics of *T. equiperdum* are reported especially concerning antigenicity, structure, identification and diagnosis (Table 1.2). However very little is known about the transmission, pathology, tissue distribution of the parasite and treatment of dourine infections in horses.

Table 1.2. Summary of reports in the literature about T. equiperdum

Parameter	Study	References
Etiology	Molecular characteristics	Claes et al., 2003b Li et al., 2005 Lun et al., 2010 Schnaufer, 2010 Carnes et al., 2015 Sanchaz et al., 2015 Wen et al., 2016 Birhanu et al., 2016 Cuypers et al., 2017
	Study on target proteins for diagnosis and treatment	Bubis et al., 2018 Luciani et al., 2018 Ramírez-Iglesias et al., 2018
	Infectivity and virulence	Perrone et al., 2018
Diagnosis	Differentiation of *T. evansi* vs *T. equiperdum*	Brun et al., 1998
	Woo test	Gari et al., 2010 Hagos et al., 2010C
	Immunohistochemistry	Pasucci et al., 2013
	PCR	Pascucci et al., 2013 Claes et al. 2004 Gari et al., 2010 Fikru et al., 2012
	Serology - CFT	Barowman, 1976 Wassal et al., 1991 OIE, 2013 Cauchard et al., 2014 Bassarak et al., 2016
	- CATT	Claes et al., 2002 Claes et al., 2003a Gari et al., 2010 Hagos et al., 2010 a, b, C

	- ELISA	Williamson et al., 1988 Alemu et al., 1997 Clausen et al., 2003 Nguyen et al., 2014 Nguyen et al., 2015 Davaasuren et al., 2017 Bonfini et al., 2018
	- ICT	Davaasuren et al., 2017 Mizushima et al., 2018
Clinical signs	Clinical signs in cutis, reproductive and nervous system	Hoare, 1972 Barowman 1976 Stephen, 1986 Alemu et al 1997 Vulpiani et al., 2013 Hagos et al. (2010a, b, c)
Pathology	Only limited reports	Scacchai et al., 2011 Pascucci et al., 2013
Pathogenesis	Haematology during the progression of the infection. Pathology (full necropsy) of dourine diseased horses	So far not reported/unknown
Treatment	Cymelarsan® in the acute stage	Hagos et al., 2010c Herbert et al., 2018
	At chronic stage (remove the parasite from tissue and semen)	So far not reported/unknown
Transmission	Isolation from semen, infectivity of the semen post treatment	So far not reported/unknown
	Mating Isolated from urethra can *T. evansi* also be transmited venerealy	Barowman, 1976 Suganuma et al., 2016 So far not reported/unknown

Chapter 2

Aims and outline of the thesis

2. Aims of the study

Dourine is endemic in Ethiopia at Arsi-Bale highlands and is considered as one of the most important health problem threatening equines in most parts of the world. Specific knowledge about pathogenesis and transmission of the disease is limited. It is unclear when and how the affected animal is transmitting the disease. Pathologic tissue changes associated with the disease are poorly described. Since the parasite is a tissue parasite, it stays only for a brief period of time in the circulation. The distribution of the parasite in the different tissues of the host, once disappeared from the peripheral circulation, has not yet been fully clarified. A treatment with melarsamine hydrochloride (Cymelarsan®) at an early stage of the disease has been shown to clear off the parasite from the circulation, increases PCV and eliminates clinical signs. However, after treatment, the parasite can still be found in the cerebrospinal fluid (CSF). An efficient treatment to remove the parasite from the semen and genital secretions and the effect of treatment in a chronic stage of the disease is still unknown. The parasite is present in mucous tissues of the external genital tract, but transmission through artificial insemination (AI) is also possible. With limited treatment options, purification of infectious semen might be part of a prevention strategy in endemic regions. Purification of semen by single layer centrifugation (SLC) has been successful in reducing venereally transmitted diseases of bacterial and viral origin. However, when dealing with much larger particles such as *T. equiperdum*, successful semen purification by SLC is to our knowledge, not yet reported.

Therefore the general objective of the study was to obtain information on the transmission and pathogenesis of dourine caused by *T. equiperdum* in horses. Specifically the following aims have been set.

1. To assess the infectivity of semen and preputial fluid post controlled infection of naïve animals to gain better insights in the transmission of *T. equiperdum.*
2. To describe the clinical signs, haematology parameters, histopathological changes and tissue distribution of the parasite in the host.
3. To assess the possibilities of semen purification through single layer centrifugation (SLC) as a way to reduce the spreading of the disease by use of AI.

Histopathological changes associated with the disease in chronically diseased horses are poorly described. Thus, chapter 3 a description of the histopathological lesions of dourine diseased horses from natural infection at the chronic stage is given. In Chapter 4, the presence of the parasite in the preputial fluid, ejaculated and epididymal semen and its infectiousness, the clinical signs and haematological alterations in experimentally infected horses, the distribution of the parasite in the host tissues together with pathological lesions at post-mortem examination are described. In chapter 5 we evaluated single layer centrifugation as a possible means to remove *T. equiperdum* from infectious semen. Chapter 6 adresses the general discussion and perspectives for further research.

Chapter 3

Histopathological lesions in reproductive organs, distal spinal cord and peripheral nerves of horses infected with *Trypanosoma equiperdum*

Adapted from: Ahmed Yasine, Hagos Ashenafi, Peter Geldhof , Leen Van Brantegem, Griet Vercauteren, Merga Bekana, Alemu Tola, Ann Van Soom, Luc Duchateau, Bruno Goddeeris, Jan Govaere.

BMC Veterinary Research, 2019: In press

Abstract

Dourine, a venereal transmitted trypanosomosis caused by *Trypanosoma equiperdum*, has different clinical signs related to the reproductive and nervous system. Pathologic tissue changes associated with the disease are poorly described. The present study describes the histopathological lesions in four naturally *T. equiperdum*-infected horses in the chronical stage of dourine. The selected horses (2 mares and 2 stallions) were Woo negative, but CATT*/T. evansi* positive, having a low packed cell volume (PCV) and exhibiting obvious clinical signs of dourine. Different tissues were collected at necropsy for DNA extraction and PCR test for *T. equiperdum.* A full post-mortem examination on the horses did not reveal typical gross lesions in the organs that were assumed to be responsible for the symptomatology. On histopathology, genital organs (especially testicles, uterus and vagina) were affected, with mononuclear cell infiltration, and erosions leading to degeneration of seminiferous tubules and perivascular lymphoplasmatic cuffing in the uterus. In the nervous system, mononuclear cell infiltration was located in peripheral nerves and ganglia and in the white matter of the spinal cord, leading to axonal degeneration. Real-time PCR using ITS1 primers revealed the presence of Trypanosomes in these organs and conventional PCRs using maxicircle and RoTat1.2 primers confirmed the involvement of *T. equiperdum* since the DNAs from the vagina, testicle, distal spinal cord, sciatic and obturator nerves found to be positive for maxicircle and negative for RoTat 1.2. The observed histopathological lesions in the reproductive organs, distal spinal cord and peripheral nerves might give an explanation to the diagnostic clinical incoordination of the hind legs in *T. equiperdum*-infected horses.

Keywords: *Dourine, T. equiperdum, Horses, PCR, Histopathology*

3.1. Introduction

Diagnosis of dourine by standard parasitological techniques is difficult, due to the low parasitaemia in chronically infected animals. Therefore, in endemic areas, the demonstration of trypanosomal antibodies in the serum and the presence of clinical symptoms is used to determine the disease status of horses (Clausen et al., 1999,2003; Gari et al., 2010; Hagos et al., 2010a,b,c; Ahmed et al., 2018).

Post-mortem changes in dourine diseased horses were reported before (Barowman, 1976; Scacchia et al., 2011; Pascucci et al., 2013). However, most of them were focused on the isolation of the infectious agent and some organs with gross lesions only were considered for the pathology. Histopathologic changes from few organs were reported as additional information only. The pathology of *T. equiperdum* infection associated with the clinical signs has not yet been fully clarified and are poorly described. Therefore the objective of this study was to describe the histopathological lesions of horses naturally infected with dourine at the chronic stage of the disease.

3.2. Materials and methods

3.2.1. Study area

The study was carried out in the Arsi-Bale highlands (Dodola, Ethiopia), a dourine endemic area, located at 6.58°N latitude and 39.18°E longitude at 2,400 meters above sea level. Agriculture is the mainstay of the livelihood of people and the leading economic activity of the area.

3.2.2. Study animals and methodology

Animals considered in this study were sexually mature adult horses suspected of *T. equiperdum* infection by either exhibiting clinical signs and/or with a history of dourine, serologically positive by the Card Agglutination test for trypanosomes (CATT/*T. evansi*) and

with low packed cell volume (PCV) values. They were kept under a traditional extensive management system of free grazing in the communal lands.

Two mares and two stallions from the group of positive horses were purchased and euthanized by overdose intravenous administration of sodium pentobarbital after sedation with xylazine for necropsy according to the standard procedures (Whitwell, 2009). A full necropsy examination of the selected horses was performed including histopathologic and PCR examinations of a variety of tissue samples. Tissue specimens were collected and processed for histopathology using standard methods (Slaoui and Fiette, 2011) and stained with haematoxylin and eosin. Each tissue sampled for histopathology was also collected in separate Eppendorf tubes to extract DNA for PCR analysis .

3.2.3. Blood collection

Blood samples were collected from the jugular vein using plain vacutainer tubes for serological test and EDTA coated vacutainer tubes (Golden Vac^{TM}, Zhenjiang Gonggdong medical technology Co.Ltd.) for parasitological examination.

3.2.4. Parasitological examination

Haematocrit centrifugation was used to isolate the parasite from blood. Two capillary tubes were filled with blood up to three-fourth (50 µl) of the capacity, sealed and centrifuged for 5 minutes in micro-centrifuge at 13,000 g. The capillary tubes were then mounted in a viewing slide and the buffy coat plasma interface layers were examined at a magnification of x100 under the microscope as described by Woo (1970) and Reid et al. (2001) to look for live parasites. The PCV was measured using a microhaematocrit reader (Hawksley, UK).

3.2.5. Serological examination

The CATT/*T. evansi* serological test was performed as described previously (Claes et al., 2003a; Gari et al., 2010; Hagos et al., 2010a).

3.2.6. DNA extraction and PCR

DNA extraction was performed using a DNA extraction Kit for blood and tissues (Qiagen, Germany) following the protocol recommended to isolate DNA from animal tissue (Qiagen, 2006). After extraction, DNA was stored at −20°C until PCR analysis. The DNA concentrations were measured using the Nanodrop ND-2000 UV-Vis spectrophotometer (Nanodrop Technologies, USA). The quality was further checked by PCR using Cytochrome b primer (abundant in Vertebrates) targeting the host DNA.

The DNA samples were tested by real time PCR targeting the internal transcribed spacer-region (ITS1) of trypanosomes, with forward primer 5'TGTAGGTGAACCTGCAGCTGGATC3' and reverse primer 5'CCAAGTCATCCATCGCGACACGTT3' (Fikru et al., 2012) resulting in fragment of approximately 450 bp (Njiru et al., 2005; Fikru et al., 2012). The method was carried out on a Step One Plus Real-Time PCR System (Applied Biosystems, USA). The reaction mixture (20 µL total volume) contained 0.5µM of the two primers (ITS1F and ITS1R) each, 10µL of 2x Power SYBR®Green PCR Master Mix (Applied Biosystems) according to manufacture guidelines and 50ng of the extracted DNA and 6µL of nuclease-free water. Pure *T. equiperdum* DNA extracted from mini Anion-Exchange Centrifugation Technique (mAECT) purified trypanosome (*T. equiperdum* 943 Dodola) strain from ITM, Antwerp (Belgium) was used as a positive control, while blanks contained nuclease-free water and DNA extracted from tissues of Belgian horses from Ghent University, negative for *T. equiperdum,* as negative controls. The amplification protocol (Fikru et al., 2012) used was 10 min at 95°C followed by 40 cycles of denaturation at 95°C for 30 s, annealing temperature of 60°C for 30 s, elongation reaction at 72°C for 30 s and a final extension at 72°C for 5 min followed by 1min at 95°C and 1 min at 60°C. After the amplification, the samples underwent temperature ramping from 60°C to 95°C to calculate the PCR product dissociation curve. Samples were considered positive based on the observed amplification and melting curve in comparison to positive and negative control samples.

To fully differentiate *T. equiperdum* from *T. evansi* more PCRs were performed targeting the maxicircle of *T. equiperdum* which are not found in *T. evansi* (Brun et al., 1998). A set of PCRs targeting VSG genes (RoTat 1.2), maxicircle genes (ND4, ND5, ND7 and A6), with different sets of primers and reaction mixtures (Table 3.1) were used to differentiate *T. equiperdum* from *T.*

evansi (Birhanu et al., 2016). Amplifications in a conventional PCR were carried out in 200 µl thin-wall PCR tubes (Thermo Fisher Scientific, USA) in a Veriti thermal cycler 96 (Applied Biosystems, USA) with two kinds of reaction mixtures (Table 3.1). Where applicable, the published PCR protocols were adjusted to the requirements of the GoTaqG2 Flexi DNA polymerase (Promega, USA). Ten microliters of the amplified product was used for electrophoresis in 2% agarose gel at 85V for 35 min and stained with ethidium bromide for visualization under UV light.

Table 3.1. Molecular targets, primer name and sequences, length of expected amplicon, reaction mixtures and cycling conditions of the PCRs used to differentiate *T. equiperdum* and *T. evansi*

Target	Primers	Primer sequences	Amplicon length	Reaction mixture*	Cycling conditions	Adapted from
VSG RoTat 1.2	ILO7957 ILO8091	5'-GCC ACC ACG GCG AAA GAC-3' 5'-TAA TCA GTG TGG TGT GC-3'	488 bp	a	95°C for 5 min and 35 cycles of 30 sec at 94°C, 30 sec at 58°C, 30 sec at 72°C and final extension for 5 min at 72°C	Urakawa et al., 2001
VSG RoTat 1.2	RoTat1.2-F RoTat1.2-R	5'-GCGGGGTGTTTAAAGCAATA-3' 5'-ATTAGTGCTGCGTGTGTTCG-3'	205 bp	a	95 °C for 15 min and 40 cycles of 30 sec at 94 °C, 30 sec at 59 °C, 30 sec at 72 °C and final extension for 5 min at 72 °C.	Claes et al., 2004
Maxicircle A6	Forward Reverse	5'-AAAAATAAGTATTTTGATATTATTAAAG-3' 5'-TATTATTAACTTATTTGATC-3'	381 bp	b	95 °C for 5 min and 30 cycles of 94 °C for 1 min, 54 °C for 1 min and 72 °C for 30s followed by a final elongation step at 72 °C for 8 min	Domingo et al., 2003
Maxicircle ND4	Forward Reverse	5'-TGTGTGACTACCAGAGAT-3' 5'-ATCCTATACCCGTGTGTA-3	256 bp	b	Idem as above	Domingo et al.,2003
Maxicircle ND5	Forward Reverse	5'-TGGGTTTATATCAGGTTCATTTATG-3' 5'-CCCTAATAATCTCATCCGCAGTACG-3'	400 bp	b	Idem as above	Dean et al., 2013
Maxicircle ND7	Forward Reverse	5'-ATGACTACATGATAAGTA-3' 5'-CGGAAGACATTGTTCTACAC-3'	167 bp	b	Idem as above	Domingo et al.,2003

*Reaction mixture (a): 25 µl containing 25 ng DNA, 1x Green GoTaq G2 Flexi buffer, 2 mM of MgCl2, 0.2 mM of each dNTPs, 0.5 µM of each primer, 1.25U GoTaqG2 Flexi DNA polymerase. Reaction mixture (b): 25 µl containing 25 ng DNA, 1x Green GoTaq G2 Flexi buffer, 2 mM of MgCl2, 0.2 mM of each dNTPs, 1 µM of each primer, 1.25U GoTaqG2 Flexi DNA polymerase.

3.2.7. Ethical Statement

All procedures were approved by the Ethical Review Committee of Addis Ababa University, College of Veterinary Medicine and Agriculture (Permit No: VM/ERC/004/07/015).

3.3. RESULTS

3.3.1. Clinical signs

Horses positive for dourine exhibited a combination of various characteristic clinical signs. All animals were emaciated, had an inelastic skin and a dull hair coat with clearly pronounced and bulging tuber coxae, pelvic bones and ribs. According to the owners, horses did not suffer a loss of appetite but body weight loss aggravated slowly leading to cachexia. Oedema of the external genitalia with vaginal discharge (Figure 1A), ulceration (Figure 1B) and depigmentation of perineal skin were prominent signs observed in mares. One of the diseased mares showed oedema of the mammary glands (Figure 1B) with watery secretion in non-lactating physiological status. Nervous signs such as difficulty in walking with staggering movement from the hindquarter with one hind leg often dragged with straddle gait when trying to walk forward was observed in two of them. Clear clinical symptoms involving the genital system of the stallions at the time of examination were not noticed except depigmentation of the skin on the penis.

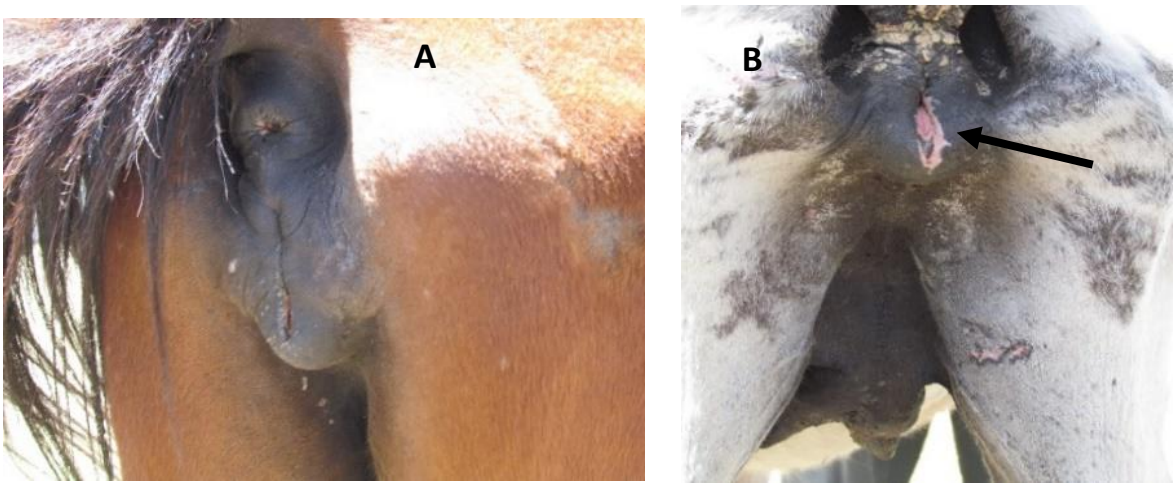


Figure 3.1. Clinical symptom in dourine diseased mares: a swollen vulva (A) and an ulcerated lesion of the labia (arrow) and oedema of the mammary gland in non-lactating mare (B)

3.3.2. Demonstration of the parasite and serology

During the selection process, several attempts were made to isolate the parasite in the buffy coat using the Woo test from clinically and serologically (CATT/*T. evansi*) positive horses and those with a history of dourine infection, but no trypanosomes were detected in any of the blood samples. The clinical signs observed in the selected horses are summarised in Table 3.2.

Table 3.2. Clinical signs, serology and PCV of selected horses

	Mares		**Stallions**	
Parameter used for selection	**N02**	**N59**	**N01**	**N6A**
Oedema of the vulva	+	+	NA	NA
Depigmentation around genitalia	+	-	+	+
Oedema of mammary gland	+	-	NA	NA
Difficulty in walking with straddle gait	+	+	+	-
Emaciation	+	+	+	+
CATT/*T. evansi*	+	+	+	+
PCV (%)	26	29	24	30

3.3.3. PCR detection of the parasite at necropsy

DNA extracted from tissues collected at necropsy was tested by real time (qPCR) and revealed the presence of the parasite in a number of organs (Table 3.3).

Table 3.3. Results of qPCR of necropsy samples by using ITS1 primer

	Mares		Stallions	
Name of Tissue	**N02**	**N59**	**N01**	**N6A**
Nervous system				
Brain	+	+	+	+
Spinal cord	+	+	+	+
Caudal nerve	+	-	+	-
Sciatic nerve	+	-	+	-
Cerebrospinal fluid	+	+	+	+
Reproductive system				
Penis	NA	NA	+	+
Testicle	NA	NA	+	+
Epididymis	NA	NA	+	+
Prostate gland	NA	NA	+	+
Vesicular gland	NA	NA	+	-
Bulbourethral gland	NA	NA	+	-
Ampulla	NA	NA	+	+
Urethra	+	-	+	+
Urinary bladder	-	+	ND	+
Vagina	-	+	NA	NA
Vestibule	+	+	NA	NA
Cervix	+	+	NA	NA
Uterus	+	+	NA	NA
Mammary gland	+	+	NA	NA
Others				
Kidney	ND	+	+	+
Heart	+	+	+	+
Spleen	+	-	-	+

(+)= PCR positive for trypanosomes, (-) = PCR negative, NA not applicable, ND= not done

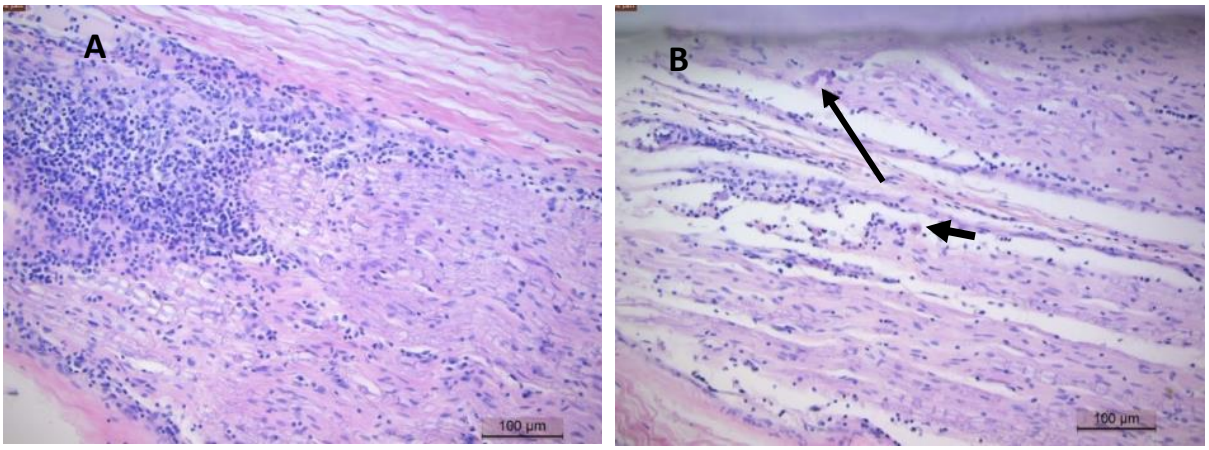
In order to make the difference between *T. equiperdum* and *T. evansi*, selected samples from nerve tissues, testicles and vestibules of all animals that were positive on RT- PCR using the ITS primer, were further checked by conventional PCR targeting the maxi-circle genes (unique for *T. equiperdum*) and VSG genes (RoTat 1.2) (typical for *T. evansi*) and were all found to be positive for the maxi-circle and negative for the RoTat 1.2.

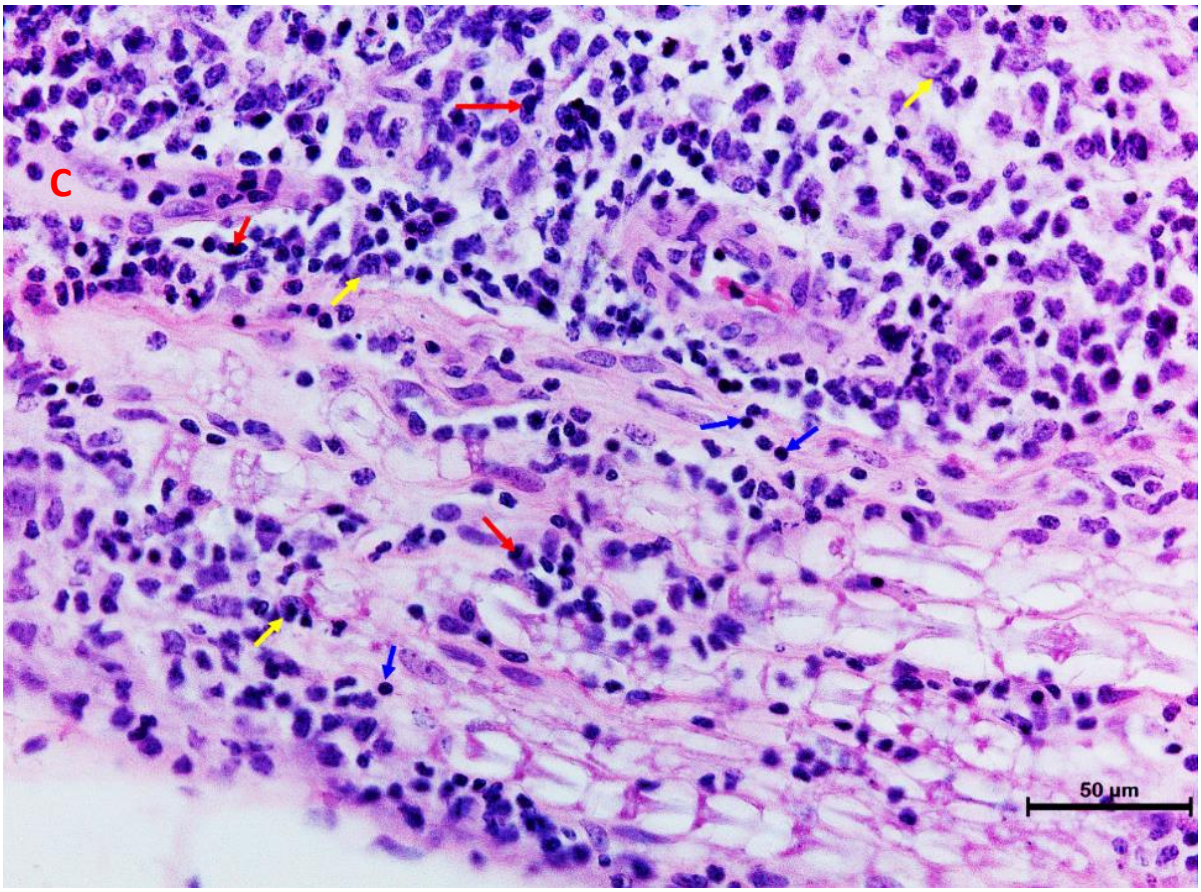
3.3.4. Post-mortem findings and histopathology

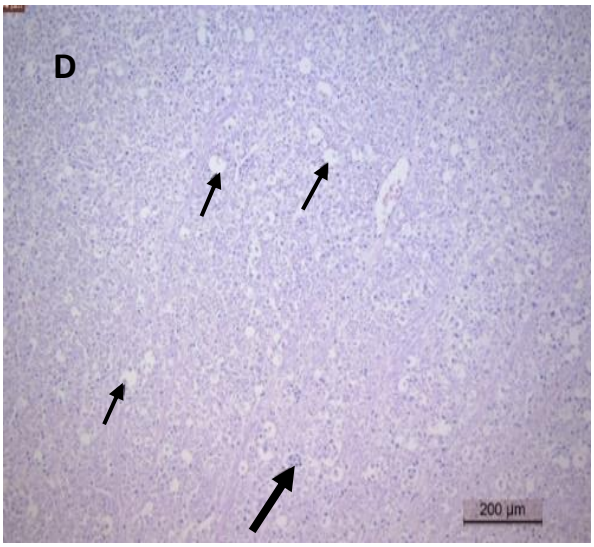
Necropsy of the naturally dourine diseased horses did not show significant gross lesions in most of the organs. A small haemorrhage in the spinal cord, fibrous adhesion between the cecum and right ventral colon, multifocal to coalescent haemorrhages and milliary brown pinpoint foci and some firm nodules and distended interlobular septa of the lung, moderate amount of thick mucous within the trachea, some pinpoint white zones in the liver were observed in some horses. There was some serous fluid accumulation in the pericardium in one horse. Nematod parasites in the intestine were found in all of the horses.

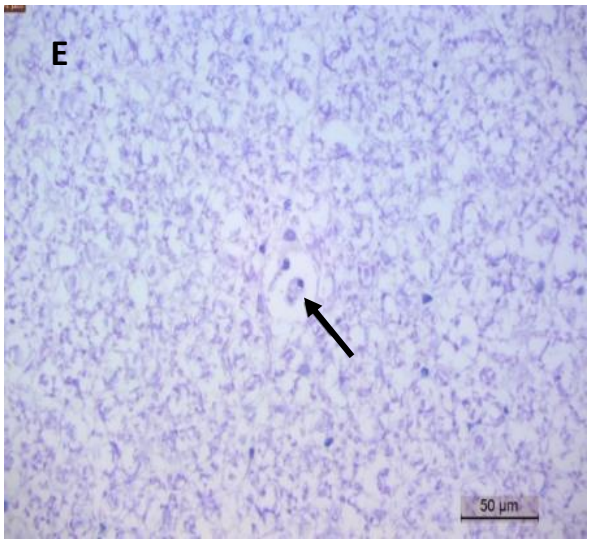
Histopathological lesions were most severe in the nervous system and consistent in all animals in the caudal peripheral (schiatic and obturator) nerves and associated ganglia. In the most severely affected nerves, multifocal infiltration of lymphocytes, plasma cells and histocytes were found between the axons of nerve fascicles, with variable axonal swelling and fragmentation (Figure 3.2A-C).

In the spinal cord, most prominent lesions were located in the white matter and consisted of multifocal axonal degeneration manifested by scattered empty myelin sheaths sometimes with infiltrated histiocytes and phagocytosis of debris, seldom with spheroids. Severe axonal degeneration occurred mainly in the dorsal funiculus in the posterior part of the spinal cord (Figure 3.2D-E). In the grey matter, histopathological lesions were minimal and confined to scattered rare neuronal degeneration (Figure 3.2F). No lesions were seen in the cerebrum, cerebellum or brain stem except for rare mild axonal degeneration.




C
50 µm


D
200 µm


E
50 µm

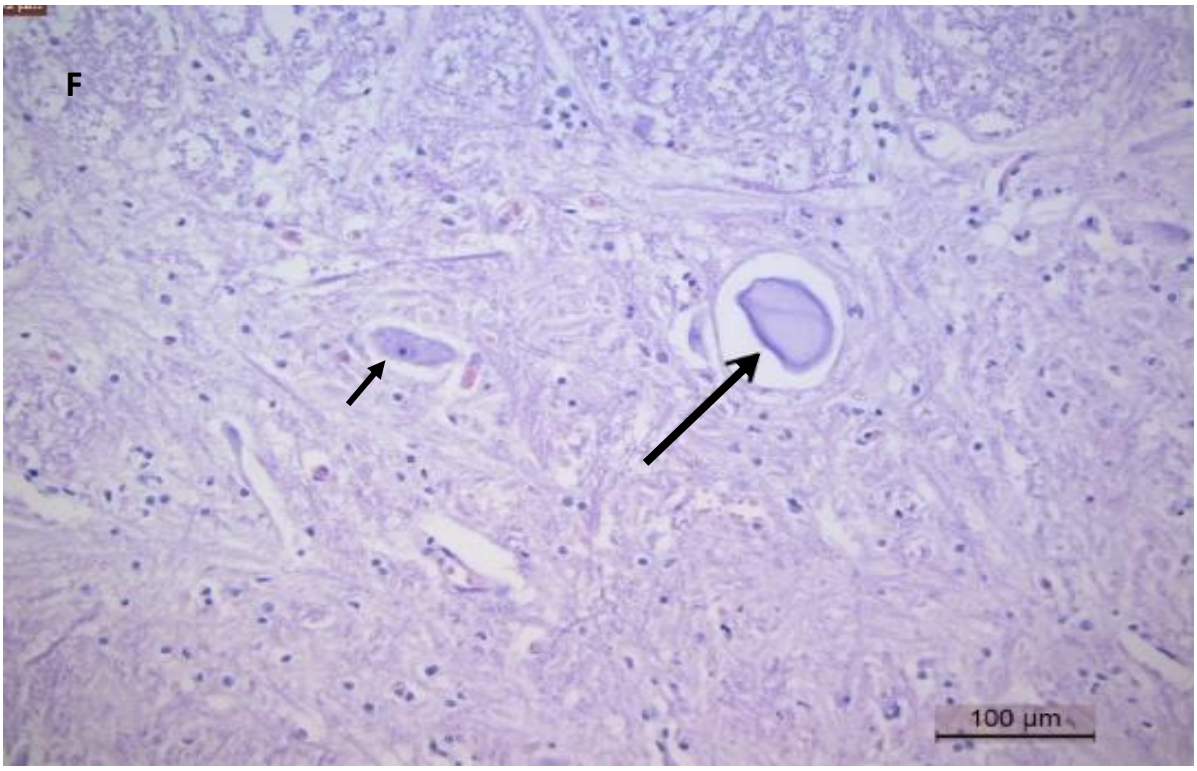
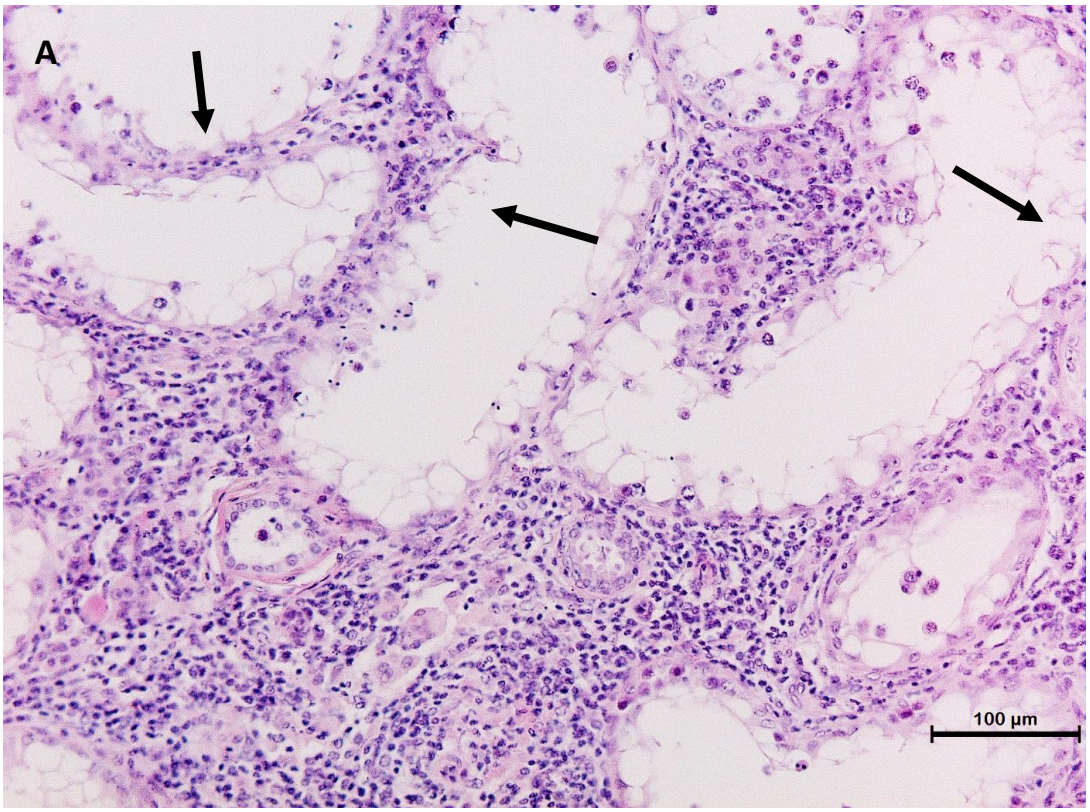
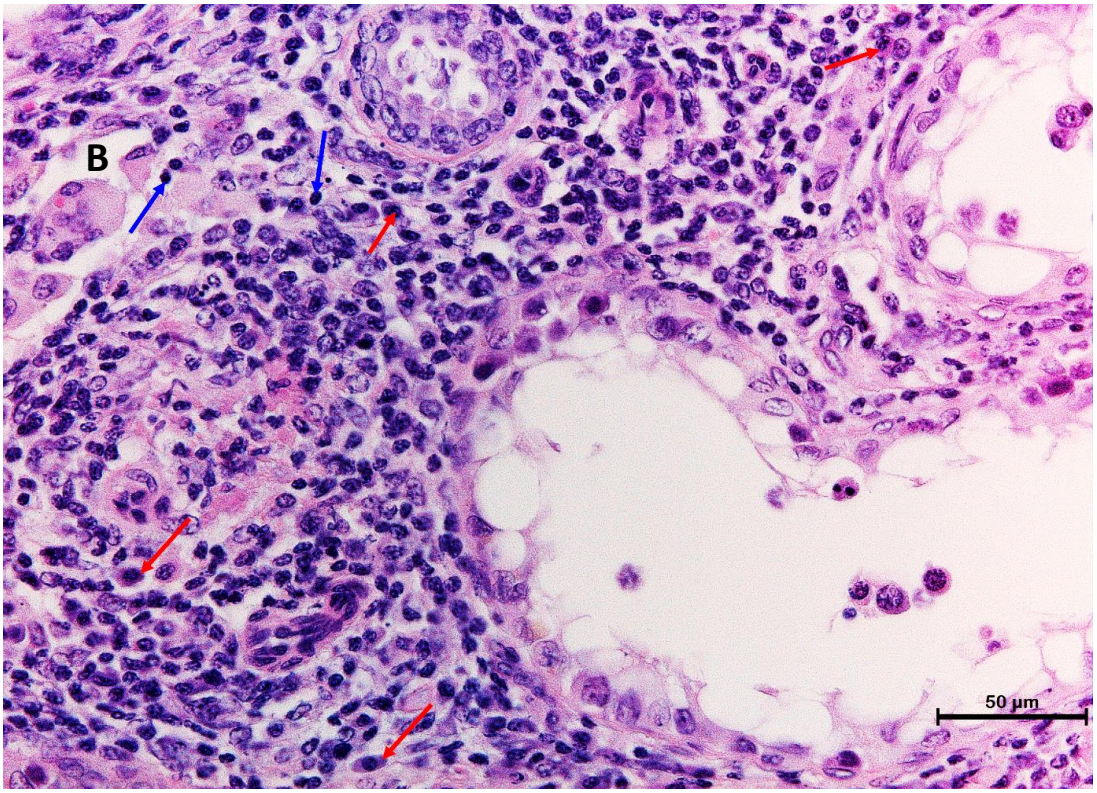


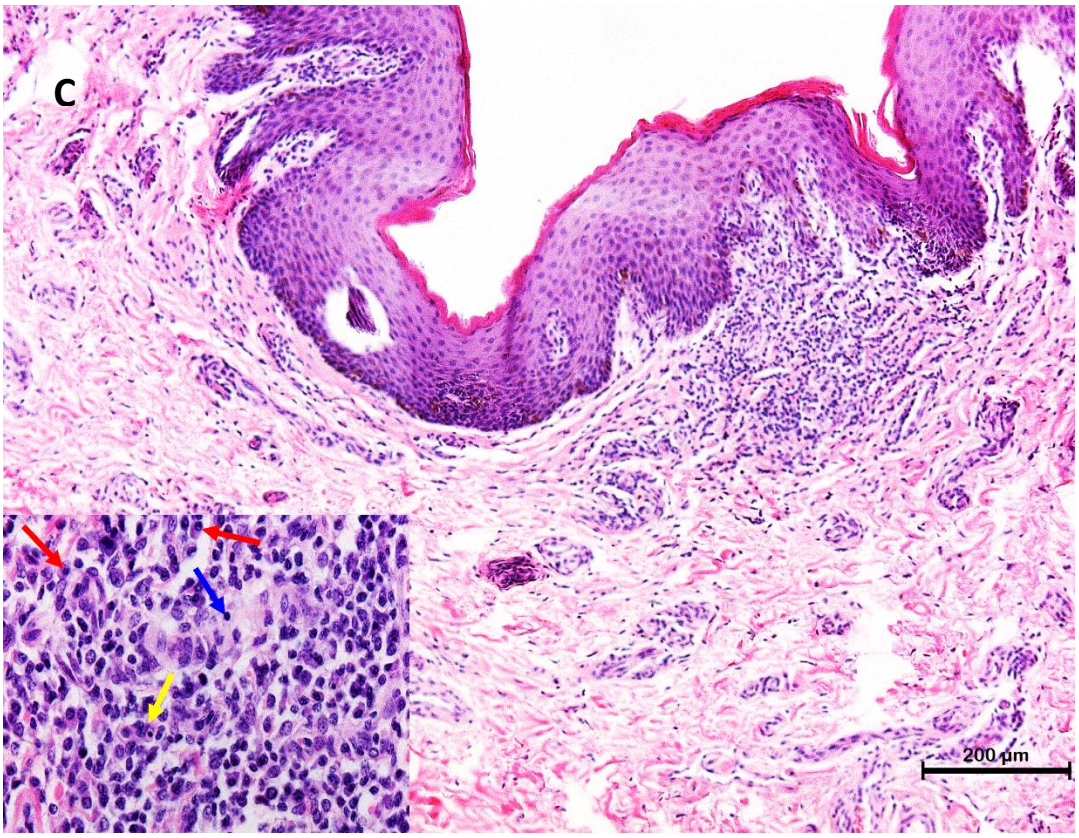
Figure 3.2. Photomicrograph of the histopathological lesions in the peripheral nerves (sciatic and obturator) and spinal cord of dourine diseased horses (Haematoxylin-eosin staining). (A) peripheral nerve with severe infiltration of inflammatory cells (B) peripheral nerve - infiltration of inflammatory cells with axonal swelling and fragmentation (large arrow) and phagocytosis of debris (small arrow) (C) peripheral nerve (details): lymphocytes (blue arrow), plasma cells (red arrow) and histocytes (yellow arrow) (D) spinal cord white matter - multiple empty myelin sheaths (small arrows), some with phagocytosis of debris (large arrow) (E) spinal cord white matter- swelling of myelin sheath with phagocytosis of debris (arrow) (F) spinal cord grey matter - focal neuronal degeneration (large arrow), normal neuronal cell body to the left (small arrow) for comparison

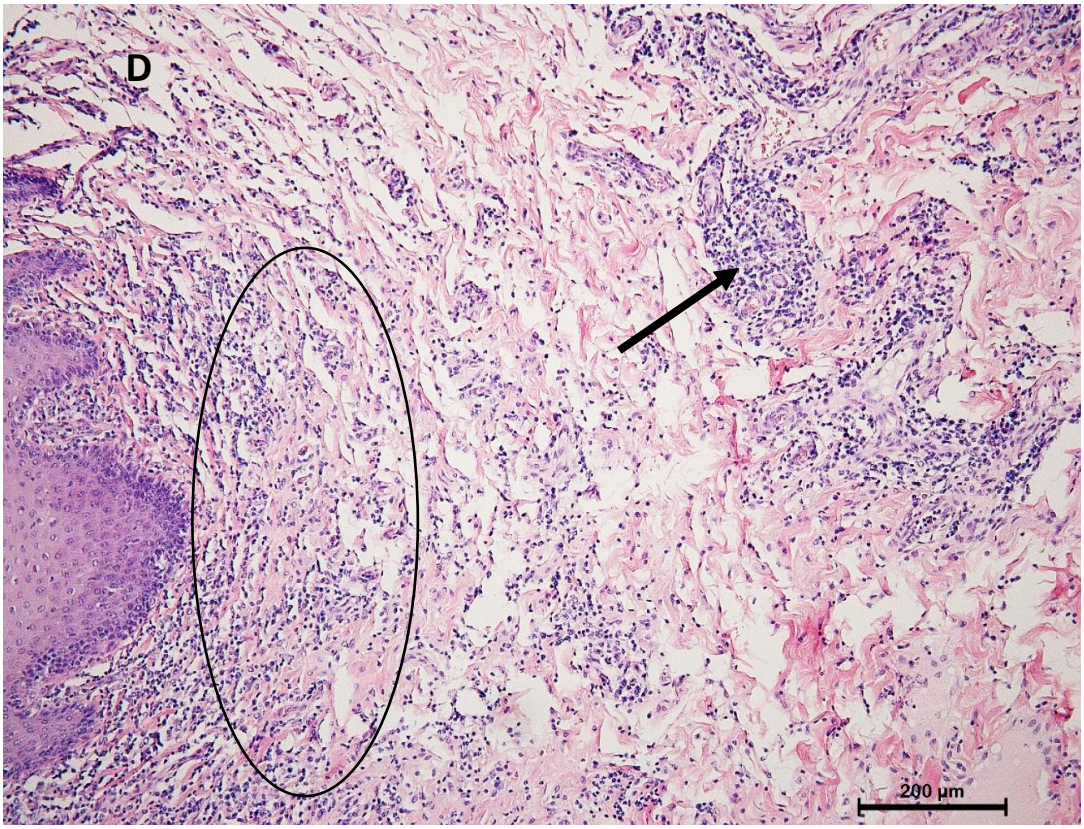
Histopathological examination of the male reproductive system showed severe lesions, diffuse orchitis characterized by moderate to severe interstitial infiltration of mainly lymphocytes, plasma cells and fewer histiocytes. Inflammation was associated with a mild to moderate increase in interstitial fibroblasts and diffuse severe degeneration of seminiferous tubules with absence of spermatogenesis (Figure 3.3A-B). Occasionally, also the interstitium of the epididymis was infiltrated by lymphocytes, plasma cells and histiocytes. In the superficial genital mucosa (glans penis), there was frequent lymphoplasmacytic and histiocytic infiltration (Figure 3.3C). No lesions were seen in the accessory sexual glands.

In the female reproductive system, the most severe lesions were found in the mucosa of the vagina and vestibule consisting of nodular infiltrations of lymphocytes, plasma cells and less histiocytes sometimes associated with small erosions (Figure 3.3D-E). In the uterus, there was mild lymphocytic inflammation confined to the endometrium (Figure 3.3F).


A
100 µm


B
50 µm


C
200 µm


D
200 µm

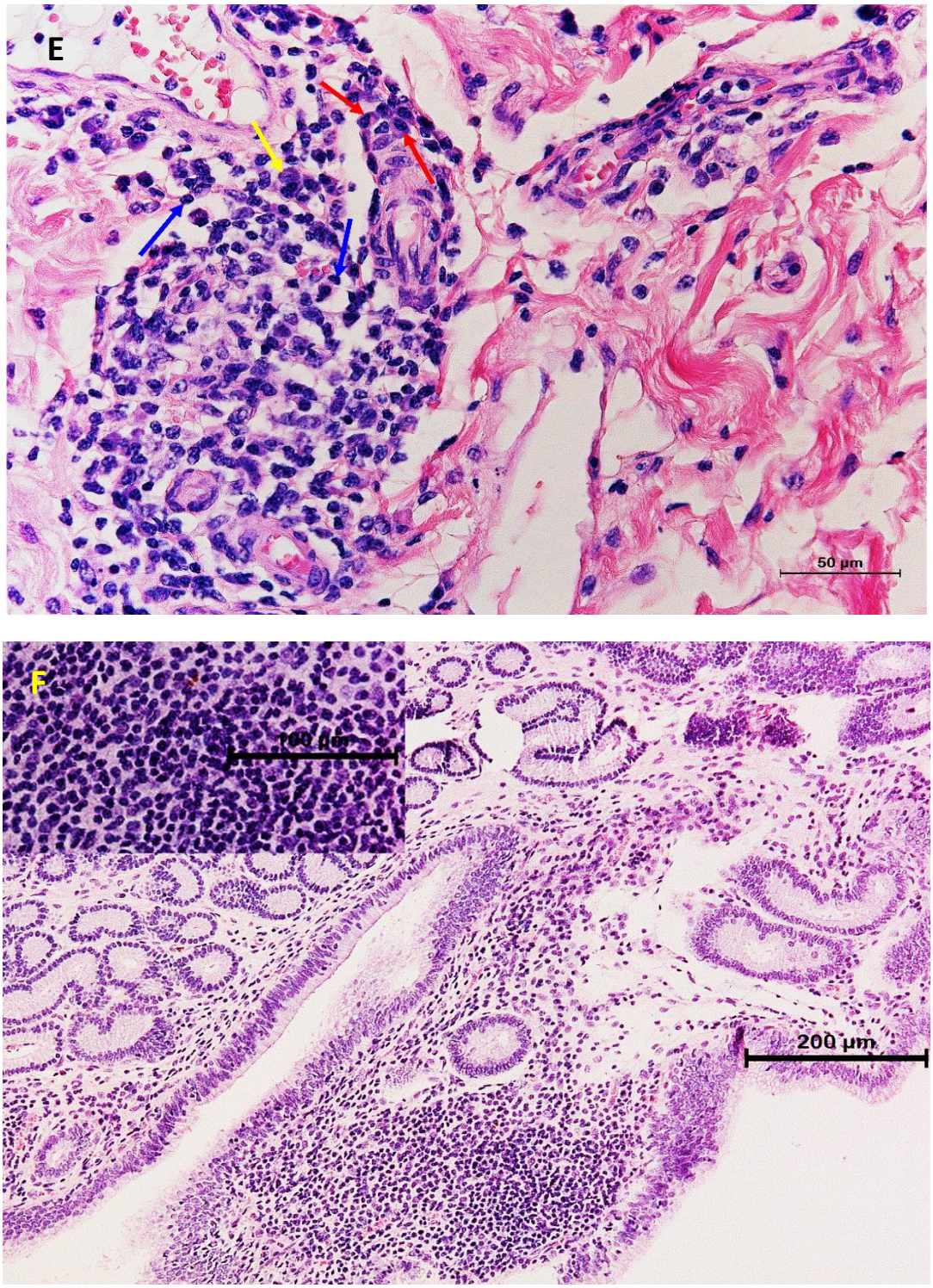


Figure 3.3. Photomicrograph of the histopathological lesions in the testicle, genital mucosa and uterus of dourine diseased horses (Haematoxylin-eosin staining). (A) Testicle – severe chronic orchitis with absence of spermatogenesis (open arrow) and severe infiltrations of lymphocytes and plasma cells in the interstitial space (B) testicle – severe chronic orchitis with severe infiltrations of lymphocytes (blue arrow) and plasma cells (red arrow) in the interstitial space (C) male genital mucosa (glans penis) – insert: diffuse infiltration of lymphocytes (blue arrow), plasma cells (red arrow) and histiocytes (yellow arrow) beneath the epithelium (D) genital mucosa (vestibule) – superficial diffuse (circle) and deep nodular (arrow) infiltration of lymphocytes, plasma cells and histiocytes (E) eenital mucosa (vestibule) – details of the nodular infiltrations of lymphocytes (blue arrow), plasma cells (red arrows) and histiocytes (yellow arrows) (F) uterus – insert: infiltration of lymphocytes in the endometrium

Kidneys showed no specific microscopic lesions except multifocal discrete foci of some mineralization at the level of the tubules in one horse (Figure 3.4A). In the spleen, there were many macrophages with intracytoplasmic brown granules (hemosiderin) and the white pulp was enlarged (Figure 3.4B). In the liver multifocal areas with necrotic cell debris surrounded by epithelial macrophages, lymphocytes and eosinophils and a fibrous capsule (granuloma) were observed. In the intestines, there was a moderate infiltration of lymphocytes and eosinophils in the lamina propria and submucosa and agrigates of macrophages in the lamina propria were seen. These macrophages were also found multifocal in the cranial mesenteric artery. The mesenteric lymph nodes were oedematous in subcortical and medullary sinuses with mild infiltration of macrophages. There were no specific microscopic changes in the heart, pancreas and adrenal glands.

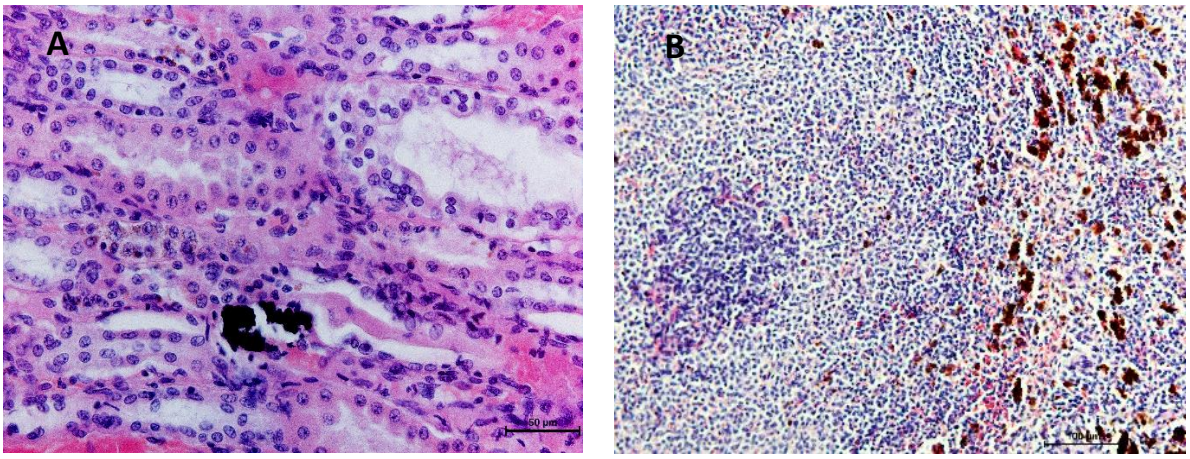


Figure 3.4. Photomicrograph of the histopathological lesions in the kidney and spleen of dourine diseased horses (Haematoxylin-eosin staining) (A) kidney - discrete foci of mineralization at the tubules (B) spleen - intracytoplasmic brown granules (hemosiderin) and enlarged white pulp

3.4. Discussion

During selection of the four dourine diseased horses for the necropsy study in Dodola, one hundred dourine suspected horses were examined. Most of the signs observed in the field survey were similar to those reported before (Alemu et al., 1997; Hagos et al., 2010a; Vulpiani et al., 2013). These signs include swelling of genitalia and udder, nervous signs, emaciation and depigmentation of the perineal skin. Skin plaques or wheals, which were previously regarded as important or pathognomonic symptoms in cases of dourine (OIE, 2013; Vulpiani et al., 2013) were not observed in in this study. Claes et al. (2003b) stated that plaques are a rare symptom and they can be observed only in a few cases. Alemu et al. (1997) and Hagos et

al. (2010a) also did not report these lesions in dourine affected horses in Ethiopia. However, Pascucci et al. (2013) and Vulpiani et al. (2013) reported the presence of skin wheals in most of the infected horses in Italy. The difference in symptomatology might be due to the difference in the clinical stage, strain of the parasite or breed of horse (Hoare, 1972; Stephen, 1986; Ricketts et al., 2011).

All the four selected horses in the current study had clinical signs of dourine involving the reproductive tract and the nervous system with emaciation, even though there was no appetite loss according to their owners.

It was not possible to isolate the parasite from the blood through buffy coat examination by the Woo test in any of the 4 horses in the current study. This could be due to the low parasitaemia at the chronic stage of the disease. *T. equiperdum* is considered primarily a tissue parasite and can rarely be found in the blood (Hoare, 1972; Stephen, 1986) which causes difficulties to diagnose the disease (Zablotskij et al., 2003). Despite these difficulties, Gari et al. (2010) and Hagos et al (2010c) isolated the parasite from blood and Pascucci et al. (2013) from the mammary secretions of clinically sick horses. Isolation of *T. equiperdum* from the urethral tract (Suganuma et al., 2016) and semen (Ahmed et al., 2017) of dourine diseased stallions was previously also reported.

Demonstration of *T. equiperdum* DNA in tissues by qPCR using the ITS1 primers revealed positive result for a number of tissues such as the brain, spinal cord, peripheral nerves of the hind quarter, uterus, ovary, vagina, testicles, kidney, heart and liver. This is in line with Pascucci et al. (2013) who reported better success in the detection of parasite DNA by RT- PCR in tissues other than blood such as vaginal swabs, joint fluid, lymph nodes and mammary secretions using a set of primers (Tb177F and Tb177R) directed for detection of a sequence common for the trypanozoon. Likewise, we have demonstrated the presence of *T. equiperdum* in several tissues from experimentally infected horses with a known strain of *T. equiperdum*. Demonstration of *T. equiperdum* DNA in tissues by PCR might significantly improve and facilitate the diagnosis of dourine in live animals by collecting biopsy samples from lymph nodes, skin lesions, vulva and uterus, or from aspirated epididymal semen.

To differentiate *T. equiperdum* from *T. evansi*, samples of DNA extracts from the vagina, testicle, caudal nerves, sacrococcygeal spinal cord and brain of these naturally infected horses, positive on RT- PCR using the ITS1 primers, directed towards a common gene of the trypanozoon, were run again over four more specific PCRs targeting maxicircle genes (specific to *T. equiperdum*) which include the ATPase subunit 6 (A6) and three NADH-dehydrogenase subunits (ND4, ND5, ND7) and two RoTat 1.2 PCRs targeting VSG genes (specific to *T. evansi*). All extracts were positive for all four maxicircle genes but negative for the RoTat 1.2 VSG genes. According to Birhanu et al. (2016) Ethiopian *T. evansi* stocks and *T. evansi* type A RoTat 1.2 and *T. evansi* type B KETRI 2479 were negative for all the four maxicircle genes, while *T. equiperdum* Dodola 940 which was isolated from dourine diseased horses in the study area (Hagos et al., 2010c) were positive for all four maxicircle genes similar to the present findings. This report is the first to differentiate *T. equiperdum* and *T. evansi* in the field based on PCRs using primers of the maxicircle genes. Moreover, diagnosis and differentiation was possible using extracts of tissue samples from the predilection sites of the parasite in dourine diseased horses. The lack of species-specificity of the CATT-test could also be demonstrated when Trypanosome free horses were infected experimentally with *T. equiperdum* Dodola 943 in another experiment and appeared to become positive when tested with the CATT/*T. evansi* test. This can be explained by the fact that CATT/*T. evansi* reagent consists of acetone/formaldehyde fixed coomassie-stained pure parasites (*T. evansi*) which may contain cross-reacting epitopes with other trypanosomes, in particular *T. equiperdum*. It is a crude antigen that has *T. evansi* specific (VSG RoTat 1.2) and nonspecific epitopes (shared by the trypanozoons) (Claes et al., 2002; Desquesnes et al*.,* 2007)

To exclude the Ethiopian *T. equiperdum* from tsetse transmitted *T. brucei* based on the presence of this target gene, Dodola (Ethiopia), where the current dourine diseased horses came from, is out of the tsetse belt (Abebe, 2005; Dagnachew et al., 2015). The trypanosomes responsible for AAT in Ethiopia are *T. vivax*, *T. congolense*, *T. brucei*, *T. evansi* and *T. equiperdum* (Dagnatchew, 1982). *T. congolense* and *T. brucei* are exclusively found in the tsetse-infested areas of Ethiopia while *T. evansi* and *T. equiperdum* occur in the tsetse-free areas. *T. vivax* can be found in both tsetse-infested and tsetse-free areas except in the highlands, which are >2500 meter above sea level (Dagnatchew, 1982; Abebe and Yilma 1996).

Literature on pathological lesions caused by *T. equiperdum* in horses is very scanty. In this study, no macroscopic lesions in the parenchymatous organs were seen except for small haemorrhages in the spinal cord and lungs and some pinpoint white zones in the liver. There was serous fluid accumulation in the pericardium in the current study. Scacchia et al. (2011) and Pascucci et al. (2013) reported that the spleen was congested with subcapsular blood suffusion and bloody nodules thickened connective tissue and abundant synovial fluid in the tarsometatarsal joints. The gross lesions in the non-reproductive organs and the nervous system, lungs, liver, intestines and mesenteric lymph nodes and accumulation of fluid in the pericardium observed in this study, were inconsistent with the report of Pascucci et al. (2013) that stated that no lesions were observed in the parenchymatous organs except the spleen. The lesions in the parenchyma of tissues other than the reproductive and nervous systems in this study were not reported as specific lesions to dourine. The histopathological changes, especially cellular infiltration and degenerative changes of the spinal nerves and spinal ganglia involving the obturator and sciatic nerves as seen on post mortem, were also reported by others (Barowman 1976) although no such inflammatory infiltration was found in other reports (Scacchia et al., 2011; Pascucci et al., 2013).

In contrast to the lesions in the central nervous system in horses infected by *T. evansi* (Seiler et al., 1981, Rodrigues et al., 2009) or *T. brucei* (Losos and Ikede, 1972; Kingston et al., 2016), histopathological changes in the brain induced by *T. equiperdum* in natural infected horses are minimal, notwithstanding our positive *T. equiperdum* PCR's for the brain. However, lesions of the spinal cord, especially in the distal part (lumbar and sacrococcygeal), are more severe than those of the brain. A possible explanation for the specific localisation of these lesions might be associated with the mechanism of cerebrospinal fluid (CSF) drainage. CSF contains many trypanosomes (and their extracellular products) and leaves the subarachnoid spaces through the arachnoid villi, reaching the dural sinuses and the venous blood stream. Absorption occurs mainly at the sub-arachnoid vessels. Finally, the CSF also flows along the sheaths of the spinal nerves oozing away into the lymphatic stream (Natalini, 2010). The trypanosomes, their extracellular products or their antigenic components will drain along the spinal nerves and might elicit a host response at these sites.

The microscopic lesions observed in the genital tract of mares in the present study, as infiltration of mononuclear cells, periglandular inflammation of vulva and vagina, were not reported earlier. However perivascular inflammation in the uterine submucosa and oedema of the skin overlying the udder are in line with the observations of Pascucci et al. (2013) in their report of natural *T. equiperdum* infected mares. Mild to severe lymphocyte and plasma cell infiltration of the mucosa of the vagina cervix and uterus (mononuclear cells infiltration) and infiltration of myometrium with lymphocytes found in this study were also observed in experimentally infected horses (Chapter 4). This difference might be due to the difference in the host response, the strain of the parasite and stage of disease at the time of the necropsy.

Depigmentation around the perineum is often described to be characteristic of clinical cases of dourine (Stephen, 1986; Hagos et al., 2010a; Vulpiani et al., 2013). In our study, this symptom was observed in one of the mares, microscopically characterized by mild lymphoplasmocytic inflammation. Pigmentary loss could be secondary to severe necrosis of the epidermis, containing the melanocytes. Gizaw et al.(2017) indicated that it was a sequel of a microscopically severe dermatitis with hydropic degeneration and necrosis of the keratinocytes in the stratum spinosum and necrosis of basal cells including the melanocytes within the epidermis. Since melanin is stored in melanosomes of melanocytes, damage to cells that contain melanin (e.g. melanocytes and basal cells of the skin) can cause loss of melanin pigment in the epidermis resulting in depigmentation (Myers et al., 2006).

There was no documentation whether or not the depigmented spots observed in the current study were already present before the *T. equiperdum* infection, for example after a (co-) infection with Equine Herpes Virus 3 (EHV3). Also transient clinical EHV3 infections are characterized by the appearance of depigmented spots on the vaginal mucous membrane (Blanchard et al., 1992; Studdert, 1996; Allen and Umphenour, 2004), which looks similar to those of dourine.

The involvement of intestine and mesenteric lymph nodes that showed non-specific reactivity with oedematous reaction and infiltration of eosinophils, plasma cells and macrophages might be the response to the intestinal parasites. Multifocal areas of hepatitis in the liver, plasma cell inflammation of the renal pelvis in the kidneys, oedematous lymph node reactivity were

also reported in dourine diseased horses similar to the current study (Pascucci et al., 2013). Haemosiderin deposition in the spleen observed in this study was in agreement with Pascucci et al. (2013) and might indicate a major role of the spleen in the destruction of red blood cells during trypanosomosis (Taylor and Authie, 2004).

Infiltration of mononuclear inflammatory cells, especially lymphocytes, plasma cells and some macrophages, in the nervous and reproductive tissues, is a hallmark of a chronic inflammation (Jones et al., 1997). The microscopic findings of the present study, which were shown in a majority of tissues examined, indicate the presence of a chronic inflammatory process. In conclusion, *T. equiperdum* was found to be distributed in a number of tissues with many histopathological features especially in the peripheral nerves and the genital organs. Lesions in the reproductive organs, the distal spinal cord and the peripheral nerves with massive infiltration of mononuclear cells mainly of lymphocytes, plasma cells and few macrophages revealed an immunological response of the host to the parasite (or products) and explains the clinical diagnostic observations of incoordination in the hind legs.

Chapter 4

Tissue (re)distribution of *Trypanosoma equiperdum* in venereal infected and blood transfused horses

Adapted From:

Ahmed Yasine, Merga Daba, Hagos Ashenafi, Peter Geldhof, Leen Van Brantegem, Griet Vercauteren, Tilaye Demissie, Merga Bekana, Alemu Tola, Ann Van Soom, Luc Duchateau, Bruno Goddeeris, Jan Govaere.

Veterinary Parasitology, 268, 2019, 87-97

ABSTRACT

Dourine, caused by *Trypanosoma equiperdum*, is a life-threatening venereal disease in equidae. So far, there is no clear evidence on how and when stallions become infectious, nor which tissues are affected by the parasite in diseased animals. Post-infection, after a transient, temporary phase of parasitaemia, the parasite disperses to different tissues in an unknown distribution pattern. This study describes the distribution of the parasite after infection by artificial insemination (AI) or blood transfusion. Mares (N=4) were artificially inseminated with *T. equiperdum* spiked semen whereas stallions (N=4) were infected by blood transfusion. The course of the disease was monitored by parasitological (Woo), serological (CATT/*T. evansi*) and molecular (PCR) tests and clinical signs and haematological parameters were recorded. At 120 days post infection, horses had a full necropsy, histopathology and PCR. A similar pattern of parasitaemia, disease progression and tissue distribution were seen in all horses. Ejaculated semen in the preclinical stage and epididymal semen in the chronic stage of the disease was positive on PCR and caused infection in mice. Cymelarsan® treatment in the chronic stage did not result in a clinico-haematological or histopathological improvement. At necropsy, lesions were observed in the nervous and reproductive system. Histopathological lesions were most severe in the peripheral nerves and associated ganglia, the testicles and genital mucosae with multifocal infiltration of lymphocytes, plasma cells and histocytes. The parasites disseminated to several tissues including the nervous system, testicles and semen. The results indicate that transmission of *T. equiperdum* is possible through semen even from symptomless stallions post-treatment.

Keywords: *Trypanosoma equiperdum, dourine, horse, venereal transmission, pathology, Cymelarsan, haematology*

4.1. Introduction

Dourine is a disease of equidae directly transmitted from animal to animal during mating (Barowman, 1976; Brun et al., 1998). An infected mare may also transmit the disease to her foal through milk or through udder lesions (Brun et al., 1998). *Trypanosoma equiperdum*, the causative agent of dourine, can be isolated directly from the urethral tract of an infected stallion (Suganuma et al., 2016). There is no clear evidence-based knowledge on how and when stallions become infectious. It is still unknown whether the transmission of the parasite occurs through the bruises and abrasions caused by intense contact during life covering (Blue, 1985) or by the presence of the parasite in the semen and genital secretions. Since the parasite is a tissue parasite it stays only for a brief period of time in circulation. It remains hitherto unknown how the parasite distributes into the different tissues of the host, once it has disappeared from the peripheral blood circulation. Treatment with melarsamine hydrochloride (Cymelarsan® MERIAL, France) at an early stage of the disease has been shown to clear off the parasite from the circulation, increased packed cell volume (PCV) and eliminate clinical signs (Hagos et al., 2010b). However, after treatment, the parasite can still be found in the cerebrospinal fluid (CSF) (Cauchard et al., 2016). An efficient treatment to remove the parasite from the semen and genital secretions of stallion and the effect of treatment in a chronic stage of the disease is currently not available.

This study was conducted (1) to examine the infectiousness of *T. equiperdum* spiked semen in the absence of intense physical contact or genital abrasions by use of artificial insemination, (2) examine the infectiousness of semen and preputial discharge of infected stallions in the preclinical period and after Cymelarsan® treatment, (3) describe the symptomatology and haematology post artificial infection in mares and stallions and, finally (4) examine the distribution of the parasite in the horse and the pathological lesions encountered at necropsy in artificially infected horses.

4.2. Material and methods

4.2.1. Study animals

Eight horses, four stallions (S01-S04) and four mares (M01-M04) of Selale horses (a local breed) were purchased from (Burayu District) in the Central Highlands of Ethiopia where dourine is not reported before and confirmed to be free of Trypanosome infection through parasitological (Woo) and serological (CATT/*T. evansi*) tests. They were housed in fly-proof stables supplemented with 2kg concentrated ration twice daily and grass hay ad libitum. Swiss albino mice (N=34), 8 weeks old from the National Veterinary Institute (NVI), Bishoftu, Ethiopia were used for the inoculation experiment. They were kept in plastic cages on wood shavings as bedding. Pelleted feed and water were given ad libitum.

All procedures were approved by the Ethical Review Committee of Addis Ababa University, College of Veterinary Medicine and Agriculture (Certificate Ref. No: VM/ERC/004/07/015).

4.2.2. Experimental infection procedures

The inoculum used in this trial was stabilate of *T. equiperdum* Dodola 943 originally isolated from horse in Dodola, Ethiopia and adapted in mice and cryopreserved in liquid nitrogen in Addis Ababa University College of Veterinary Medicine (Hagos et al., 2010c).The isolated *T. equiperdum* stabilate had maxicircle genes as been shown by PCR prior to the experimental infection (Domingo et al., 2003; Dean et al., 2013). Since the location of isolation (Dodola) is out of the tsetse belt, the presence of the tsetse transmitted *T. brucei* also could not be a confusing factor. To investigate the transmission of *T. equiperdum* through artificial insemination, *T. equiperdum* Dodola 943 cryostabilates (Hagos et al., 2010b) were thawed at room temperature for 3-5 minutes. Concentration was checked using the rapid matching technique (Herbert and Lumsden, 1976). Five ml of a fresh semen sample harvested from a non-infected stallion was spiked with the stabilate containing approximately 36,000 trypanosomes. Mares in natural oestrus with a preovulatory follicle close to ovulation were inseminated with this trypanosome-semen mixture in the uterus just cranial to the cervix with a routine AI. All clinical and haematology parameters of the inseminated mares were checked,

and the Woo tests and wet smear examinations were performed on a daily basis until parasitaemia was detected and weekly thereafter until the end of the study.

Stallions (N=4) were infected by blood transfusion. Each stallion received blood containing approximately 100,000 trypanosomes from donor mares which developed parasitaemia after infection by artificial insemination with *T. equiperdum* spiked semen. In total, 2ml blood was used with a parasite concentration of 50,000 trypanosomes/ml as assessed by a 'rapid matching technique' (Herbert and Lumsden, 1976). Stallions were monitored daily for clinical signs and weekly for haematology. On top of the clinical follow-up, ejaculated semen was collected every week by use of an artificial vagina. Stallions were allowed to mount a phantom and semen was collected with a Colorado-model artificial vagina (AV) with a disposable plastic inner liner and a collection bottle. When clinical symptoms were apparent, preputial fluid was also collected from the oedematous areas of the prepuce of the diseased stallions.

At post-mortem examination, epididymal semen was collected according to Roels et al. (2014). In short, testicles were collected via a routine castration procedure and a ligature of surgical material was placed proximally on the ductus deferens. After removal, the testicles were rinsed with a sterile saline solution, wrapped in a towel and placed in a box with ice and transported to the laboratory for semen collection. Upon arrival at the laboratory, care was taken to remove the surrounding fascia of the cauda and the ductus deferens to avoid blood contamination of the semen before collection. Semen was collected from the epididymis and vas deferens by retrograde flushing. A syringe connected to a pipette tip was inserted in the proximal part of the ductus deferens (Melo et al., 2008). An incision was made in the junction between the cauda and corpus of the epididymis. Thereafter, semen extender (INRA 96®) was passed through the cauda and ductus deferens under gentle pressure.

The presence of *T. equiperdum* in blood and semen was checked by wet smear examination, PCR and mice inoculation. Intraperitoneal inoculation of mice with the ejaculated semen obtained in the preclinical period of one of the stallions at day 13 post-inoculation was performed (N=7), as well as inoculation with preputial fluid from all the four stallions at day 63 post-infection (N=3) and of epididymal semen of three stallions (S02, S03, S04) at day 120 post-infection (N=5) of each stallion. Confirmation of infectivity of the inoculans (whether

ejaculated semen of a stallion in the preclinical phase, preputial fluid at day 63 post-infection or epididymal semen at post-mortem) was based upon a diagnosis of parasitaemia of the mice on wet smear samples.

4.2.3. Cymelarsan® treatment

The effect of Cymelarsan® treatment (bis-aminoethylthio-4-melaminophenylarsine dihydrochloride, MERIAL, France) in a chronic stage of the disease was evaluated. Three of the infected horses (M04, S02, and S03) were treated with Cymelarsan® while four (M01, M02, S01 and S04) were left untreated. Cymelarsan® was administered intramuscularly SID at a dose of 0.25 mg/kg body weight at day 60 post-infection when horses showed clear clinical signs of dourine such as swelling of the genitalia, staggering from the hindquarters in their movement and emaciation. Thereafter, changes in parasitaemia, clinical signs, and haematological values were recorded once per week for 4 consecutive weeks.

4.2.4. Clinical examination, Parasitological, Serological and PCR tests

4.2.4.1. Clinical examination

Animals were examined daily for clinical signs throughout the study period. Rectal temperature was taken on every other day basis in the morning, using a digital thermometer.

4.2.4.2. Parasitology

Blood samples were collected from the jugular vein on a daily basis until parasitaemia was detected and weekly thereafter until the end of the study. Parasitological examination was performed by wet smear examination and Haematocrit centrifugation (Woo) test. Wet blood films were examined under the microscope at x400 magnification to look for motile trypanosomes. The Woo test was performed on the negative samples of wet smear examination. Briefly, micro-hematocrit capillary tubes were filled with approximately 50µl of blood from the vacutainer tube, sealed and centrifuged for 5 minutes in micro-hematocrit centrifuge at 13,000 g. The tubes were then mounted in a specially designed viewing chamber and examined under the microscope at x100 magnification for the presence of motile trypanosomes at the interface of the buffy coat and plasma (Woo, 1970).

2.4.3. DNA extraction, conventional PCR and Real-time PCR

DNA extraction was performed using a DNA extraction kit for blood and tissues (DNeasy Blood and Tissue Kits, Qiagen, Germany) following the protocol recommended to isolate DNA from animal tissue (Qigen, 2006). After extraction, DNA was stored at −20°C until PCR analysis.

The DNA concentrations were measured using Nanodrop ND-2000 UV-Vis Spectrophotometer (Nanodrop Technologies, USA). The DNA samples from blood and semen were tested by conventional PCR using ITS1 primer with forward primer 5'TGTAGGTGAACCTGCAGCTGGATC3' and reverse primer 5'CCAAGTCATCCATCGCGACACGTT3' (Fikru et al., 2012). The cycling condition of ITS1 PCR was initial PCR reaction at 95°C for 5min, 34 cycles of denaturation at 94°C for 30 s, annealing temperature of 60°C for 30 s, and elongation reaction at 72°C for 30 s and a final extension at 72°C for 5min. All PCR amplifications were carried out in 200 µl thin-wall PCR tubes (Thermo Fisher Scientific, USA) in Veriti thermal cycler 96 wells (Applied Biosystems, USA). The reaction mixture was a 25 µl containing 50 ng DNA, 1x Green GoTaq G2 Flexi buffer, 2 mM of MgCl2, 0.2 mM of each dNTPs, 0.5 µM of each primer, 1.25 U GoTaqG2 Flexi DNA polymerase. Ten microliters of the amplified product were used for electrophoresis in 2% agarose gel at 85 V for 35 min and stained with ethidium bromide for visualization under UV light.

Trypanosome DNA testing from tissues collected at necropsy was performed using a Real-time PCR (RT- PCR) to amplify the internal transcribed spacer (ITS1) of the Trypanozoon subgenus and gave a constant product of approximately 450 bp (Njiru et al., 2005; Fikru et al., 2012). The method was carried out on a Step One Plus Real-Time PCR System (Applied Biosystems, USA) and output data were analysed by the Step one ™ version 2.3 software (Applied Biosystems). The reaction mixture (20 µL total volume) contained 0.5µM of the two primers (ITS1F and ITS1R) each, 2X Power SYBR®Green PCR Master Mix (Applied Biosystems) according to manufacture guidelines and 50ng of the extracted DNA and 6µL of nuclease-free water. Pure *T. equiperdum* DNA extracted from mini Anion-Exchange Centrifugation Technique (mAECT) purified trypanosome (*T. equiperdum* 943 Dodola) strain from ITM Antwerp (Belgium) was used as a positive control, while blanks contained nuclease-free water and DNA

extracted from tissues of Belgian horses, negative for *T. equiperdum* as negative controls. The application protocol (Fikru et al., 2012) was modified as follows: 10 min at 95°C followed by 40 cycles of denaturation at 95°C for 30s, annealing temperature of 60°C for 30 s, elongation reaction at 72°C for 30 s and a final extension at 72°C for 5 min followed by 1 min at 95°C and 1 min at 60°C. After amplification, the samples underwent temperature ramping from 60°C to 95°C to calculate the PCR product dissociation curve. Samples were considered positive based on the observed amplification and melting curve in comparison to positive and negative control samples.

4.2.5. Haematological Analysis

About 5ml of blood from the jugular vein of the horses was taken using ethylene diamine tetra-acetic acid (EDTA) coated vacutainers (Golden Vac™, Zhenjiang Gonggdong medical technology Co. Ltd.). The haematological analysis was performed on a weekly basis starting from one week prior to infection up to week 8 of the experiment to assess the impact of the infection on the haematological parameters. Haematology parameters were followed up in the same way in the treated horses (Cymelarsan® treatment) for four consecutive weeks.

The PCV, Haemoglobin concentration (Hgb), RBC count and total and differential white blood cell counts (WBC) were assessed. The PCV was measured by the haematocrit centrifugation technique using a microhaematocrit reader (Hawksley, UK). Total RBC and WBC counts were quantified using the improved Neubauer Haemocytometer. Haemoglobin concentration was determined using an acid haematin method. Thin blood smears were prepared and stained with differential quick stain (Diff-Quick stain) for differential leukocyte counts which were based on 100 cells per slide according to their characteristics, shape of the nucleus, and presence or absence of granules in their cytoplasm (Coles, 1986). The absolute numbers of leukocytes; eosinophils, lymphocytes, neutrophils, basophils and monocytes were obtained by using the differential white cell count percentages and the total leukocytes count.

4.2.6. Necropsy and histological observations

All horses were subjected to necropsy at the end of the experiment at 120 days post-infection. They were humanely euthanized by an intravenous administration of an overdose of sodium

pentobarbital after sedation with xylazine. Necropsy was performed according to standard procedures (Whitwell, 2009). They were examined thoroughly for gross pathological lesions in various organs. Impression smears were taken from different organs after lightly impressing a freshly cut surface of the organs on a slide, allowing it to dry at room temperature and staining it in Diff-Quick stain like a thin blood film (Swierczewski et al., 2013). Tissues samples from different organs were also collected for histopathology and DNA extraction. Tissue specimens for histopathology were processed using standard methods (Slaoui and Fiette, 2011) and stained with haematoxylin and eosin.

4.2.7. **Immunohistochemistry**

Immunohistochemistry was performed following standard procedures (Mungun-Ochir et al., 2019). Selected samples were assessed via immunohistochemistry using anti-CD3 polyclonal rabbit anti-human T-cell CD3 (Agilent, Ref. A0452) 1/100, polyclonal rabbit anti-CD20 (B-cell) (Thermo Scientific, Ref. RB-9013-P) 1/100, anti-CD45 monoclonal mouse anti-canine CD45 RA clone CA21.4B3 1/5 and monoclonal mouse antibody MAC387 (Abcam Ref.ab22506) 1/100.

Deparaffinised sections were immersed in 0.3% H_2O_2 (Agilent, S202386-2) for 5 minutes to block endogenous peroxidase, and then subjected to antigen retrieval with microwave treatment in citrate buffer (pH 6) for CD3, CD20 and CD45RA and proteinase K (Agilent, Ref.S300402-2) for MAC387. After pre-treatment, the sections were incubated overnight with the primary antibodies at 4°C.

Envision link rabbit (Agilent, Ref.K400311-2) were then applied for CD3 and CD20 where as Envision link mouse (Agilent, Ref.K400111-2) was applied for CD45RA and MAC387. The peroxidase was developed using liquid DAB+ (Agilent, Ref.K346811-2) and haematoxylin stain was used .

4.2.8. Data Analysis

Haematological data were entered into an Excel spreadsheet and analysed by R statistical software version 3.5.1 (R Core Team 2018). Descriptive statistics were used to describe the data. The analysis for the continuous response variables was based on a mixed model

including animal as random effect and time as categorical fixed effects factor. Each time point was compared with time 0 using the Dunnett's multiple comparisons technique. The significance level was set at $P< 0.05$.

4.3. Results

4.3.1. Venereal infection of mares by artificial insemination

4.3.1.1. Diagnosis of dourine

Mares inseminated with spiked semen all got dourine based on clinical symptoms visible from day 8 post-insemination/infection and parasites could be observed in Woo test starting from day 6 post-insemination. But later, once the parasitaemia was well established in the acute infection, trypanosomes were appreciated by wet smear as well. Blood collected during the prepatent period for DNA extraction and PCR test showed the presence of the parasite starting from day 5. The clinical signs of dourine such as swelling and depigmentation of the external genitalia, vaginal discharge, depigmentation of the perineal area and ataxia were observed (Table 4.1).

Table 4.1. Interval between *T. equiperdum* infection and positive test results in the various diagnostic tests and clinical symptoms in mares following artificial insemination with *T. equiperdum* spiked semen

HorseID	Positive for *T. equiperdum* (days post infection)		Symptoms of dourine noticed (days post infection)				
	Woo test	PCR	Oedema of the vulva	Oedema of mammary gland	Oedema of limbs	Depigmentation	Ataxia
M01	6	6	15	-	32	21	37
M02	15	12	9	-	24	37	75
M03	14	14	9	15	-	-	18
M04	8	5	8	-	-	56	-
Average	10.75	9.25	10.25	15	28	38	43.3

(-) indicates clinical signs absent

The level of parasitaemia was fluctuating for about 6-8 weeks of the study period. Later, animals became aparasitaemic at chronic stages. In the chronic stage, only a few trypanosomes were exceptionally visible by the Woo test. The body temperature increased after infection and thereafter oscillated usually in line with the waves of parasitaemia from an average of 36.5°C at day 0 to a maximum of 40°C at day 28 and then to 36.8°C at day 56. When parasitaemia was not apparent anymore, the rectal temperature remained around physiological values (37-38°C). Although the appetite remained unchanged, a gradual decline in body weight and condition leading to progressive emaciation and weakness, was obvious. Dull hair coat, muscular atrophy and loss of skin elasticity were observed throughout the study period. Depigmentation was observed on the skin around the vulvar areas. Swelling and mucopurulent vaginal discharge were seen. Oedema of lower hind limb below the stifle joint was observed in two of the four mares. Lameness and paralysis of the hindquarters were observed in all infected horses except one mare leading to partial dragging or stiffness of the hind legs, a staggering gait, posterior ataxia and an inability to stand upright after prolonged recumbency. In a standing position, the asymmetrical posture and tendency to shift weight from one leg to another was also observed. As the disease progressed horses were unable to stand up and remained in a recumbent position. Inflammation of the conjunctiva and lacrimal

discharge of the eyes was noticed in 1 of the 4 infected mares that eventually resulted in corneal opacity and led to permanent blindness.

4.3.1.2. Haematology

Three of the mares were included for haematological analysis since M03 was euthanized for animal welfare issue due to paralysis before the completion of the experiment. The weekly mean haematological changes during the study period are summarized in Figure 1A and B. A fluctuation in the haematological values throughout the study period was observed with a significant ($p<0.05$) decrease in mean RBC count and PCV starting from week 2. No significant variation ($P>0.05$) was observed in the in the Hgb concentration throughout the study period (Figure 4.1A). There was a significant decrease in the total WBC count and neutrophils count at week 2 and significantly increased by week 4 (Figure 4.1B).

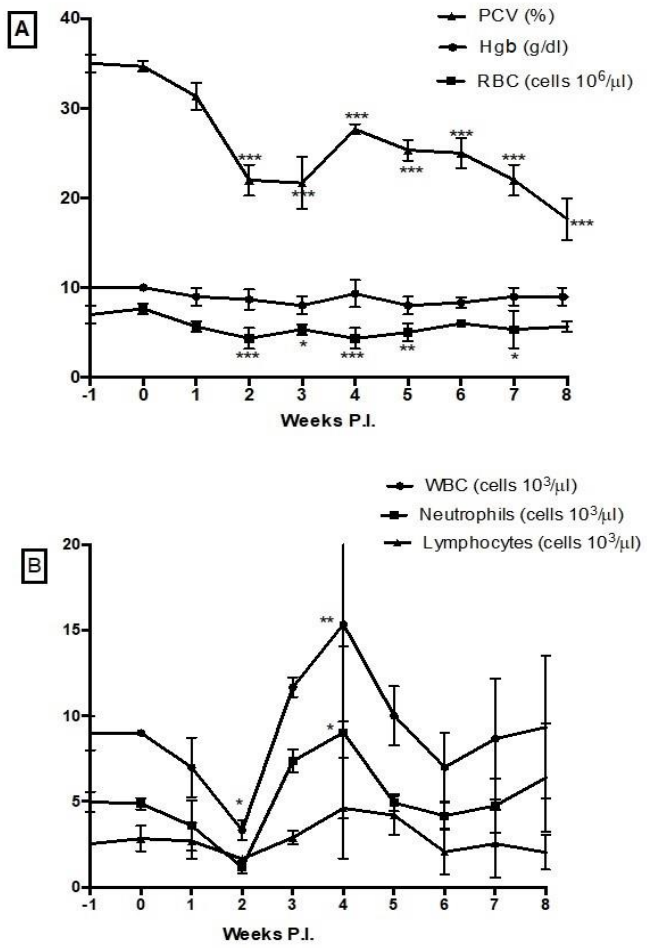


Figure 4.1. Mean haematological values in mares experimentally infected with *T. equiperdum* by artificial insemination (A) RBC count, PCV and Hgb concentration (B) total WBC, lymphocytes and neutrophils counts. (Significancy*** p=0.001, ** p=0.01, * p=0.05)

4.3.1.3. Tissue distribution of the parasite and pathological lesions on necropsy

The parasites were found in a number of organs such as the brain, uterus, ovary, heart, kidney and liver, as demonstrated from stained impression smears. The presence of the parasite in these organs was further confirmed by qPCR (Table 4.2).

Table 4.2. Results of impression smear and RT PCR analysis on tissue samples collected from mares at 120 days after an artificial *T. equiperdum* infection

Tissue sample	**M01**		**M02**		**M03***	**M04$_{Tx}$**	
Nervous system	**PCR**	**Smear**	**PCR**	**Smear**	**PCR**	**PCR**	**Smear**
Cerebrum	+	+	+	+	ND	+	+
Cerebellum	+	ND	+	+	+	+	+
Brain stem	+	ND	+	-	+	+	ND
Lumbar spinal cord	+	ND	+	ND	+	+	ND
Sacral spinal cord	+	ND	+	ND	+	+	ND
Obturator nerve	+	ND	+	ND	ND	+	ND
Reproductive system							
Uterus	+	-	+	+	+	+	+
Ovary	ND	-	+	+	+	+	-
Vagina	+	ND	+	ND	+	+	ND
Mammary gland	+	ND	+	ND	+	+	ND
Others							
Liver	-	-	+	+	+	+	-
Kidney	+	+	+	+	+	-	-
Heart	+	+	+	+	+	-	-
Pancreas	+	-	-	ND	-	-	-
Adrenal gland	+	-	ND	-	-	+	-
Spleen	-	-	-	-	ND	+	-

(+)=positive for trypanosomes, (-) = negative, ND= not done, Tx = Cymelarsan® treated, *Euthanized for the welfare of the animal since hind quarter paralysis occurred at day 18 post-infection and no impression smear was taken

Trypanosomes were demonstrated on impression smears on slides made at necropsy and stained by Diff-Quick stain from different organs (Figure 4.2). At necropsy, 120 days post-infection in the chronic stage of the disease, no clear visible gross lesions were observed in most organs except for the nervous system and the reproductive tract.

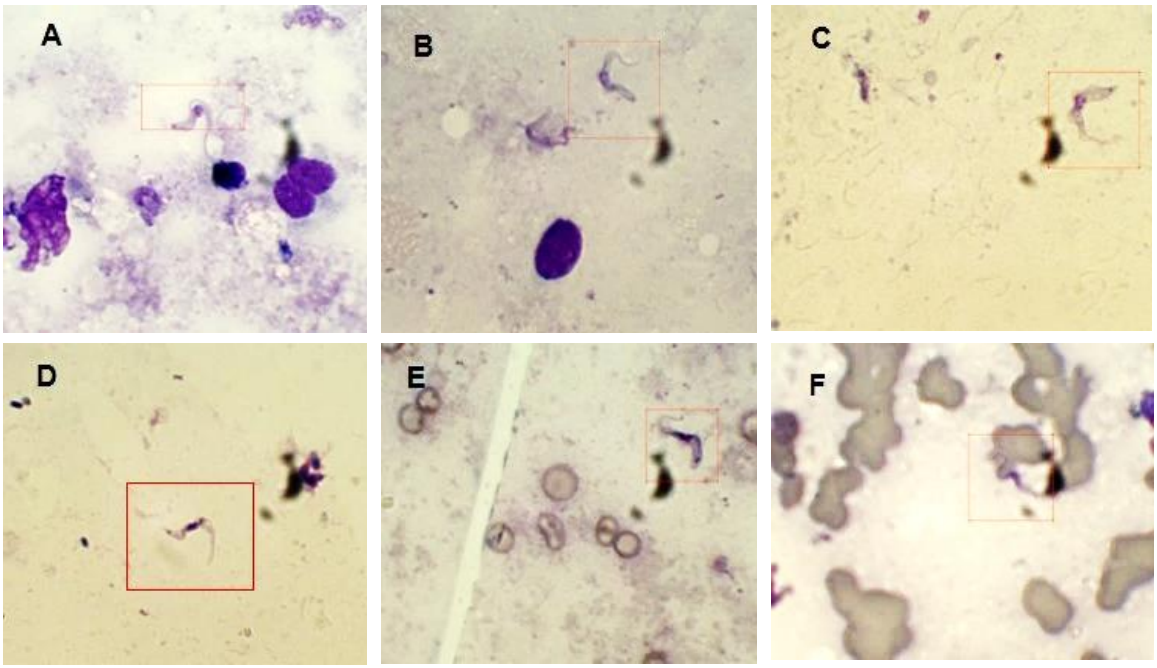


Figure 4.2. *T. equiperdum* from impression smears of the organs at necropsy (Diff-Quick stain) (A) cerebrum, (B) cerebellum (C) uterus, (D) ovary, (E) kidney and (F) testicle

Common gross lesions were yellowish fluid accumulations in the peritoneal and thoracic cavities, yellowish discoloration of the synovial and cerebrospinal fluids (sometimes), reduction of perirenal and pericardial adipose tissue with yellowish discoloration, oedema and yellowish discoloration and fluid accumulation at the base of the gluteal muscle following the caudal nerves (obturator and sciatic nerves). Congestion of the brain capillaries, oedema of the sacrococcygeal spinal cord and the caudal nerves were observed in the nervous system. In the reproductive tract, diffuse congestion was observed especially in the uterine horn, together with slight congestion in the vagina and cervix (in some animals), depigmentation of the vulva and perineal skin (Figure 4.3).

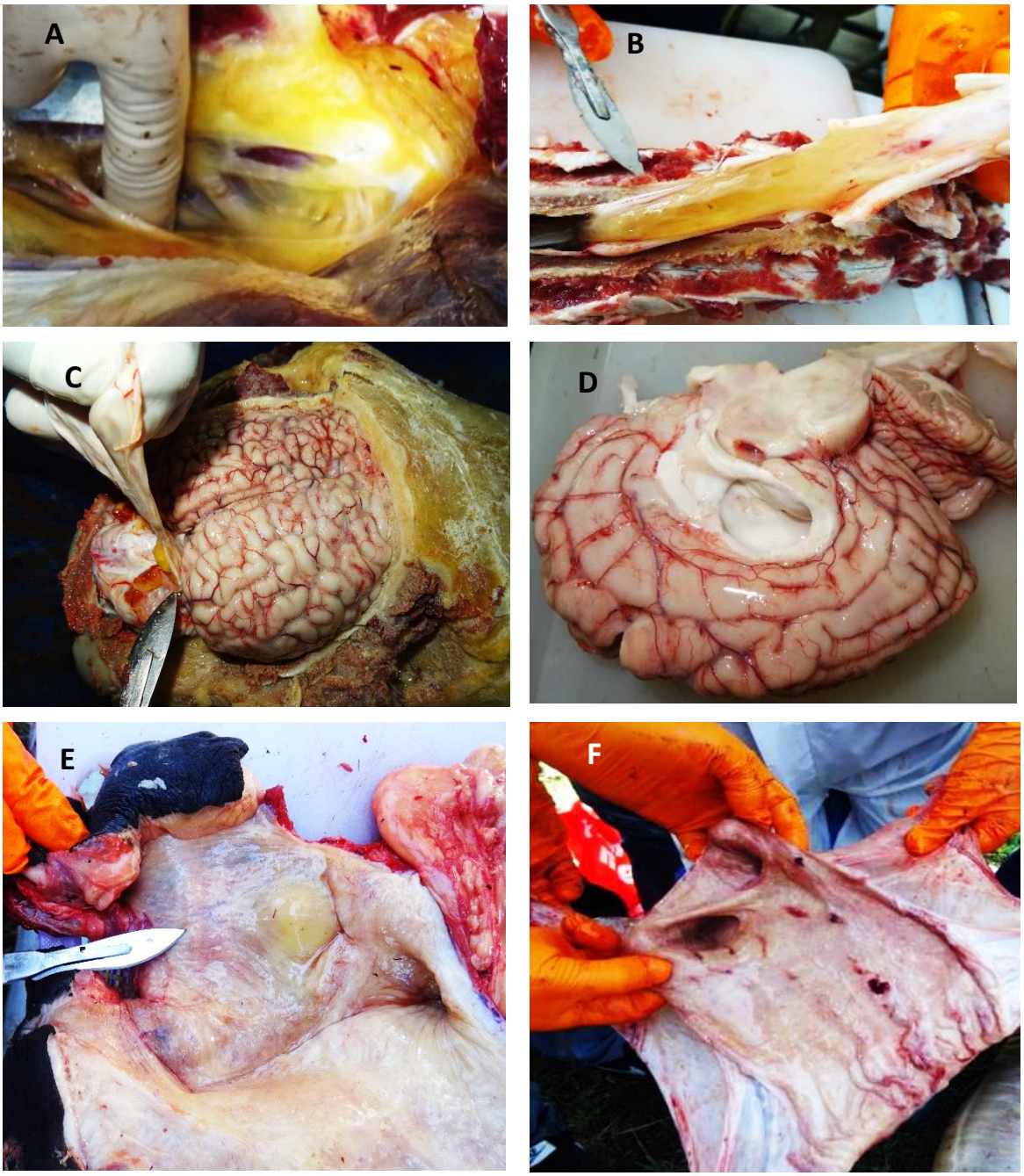
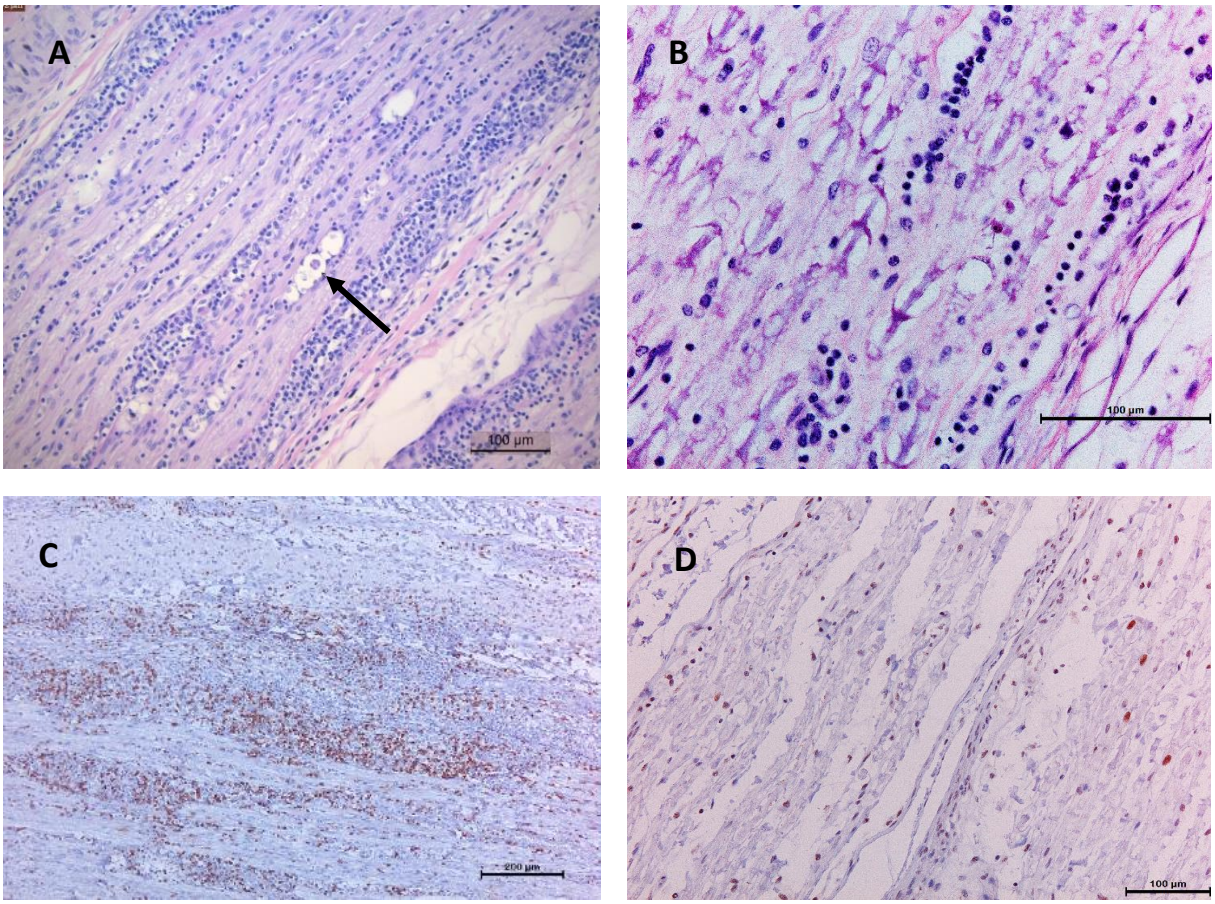


Figure 4.3. Some gross lesions observed at necropsy. (A) oedema and yellowish discoloration at the base of the gluteal muscle alongside the caudal nerves (B) oedema of the sacrococcygeal spinal cord (C) and (D) meningeal congestion of the brain (E) diffuse congestion in the vagina and cervix (F) diffuse congestion uterus

On histological examination of the nervous system, histopathological lesions were most severe and consistent in the peripheral nerves (and associated ganglia). The majority of the peripheral nerves showed multifocal infiltration of lymphocytes, plasma cells and fewer histocytes between the axons of almost all nerve fascicles sometimes associated with variable axonal swelling and fragmentation (Figure 4.4A-F). In the spinal cord, histopathological lesions were confined to the white matter and consisted of chronic axonal degeneration (as of few

numbers of empty myelin sheaths with infiltration of histiocytes and phagocytosis of debris, seldom with spheroids). In the lumbar spinal cord, mild meningeal perivascular infiltration of lymphocytes and histiocytes were found (Figure 4.4G). In the cerebrum, cerebellum and brain stem, lesions were mostly absent. Only in two animals, mild perivascular infiltration of mononuclear cells in the cerebellar meninges (mild non-suppurative meningitis) were present (Figure 4.4H). Cerebral meningitis was confined to only one animal. In another animal similar but moderate infiltrates were present in the meninges of the brain stem.



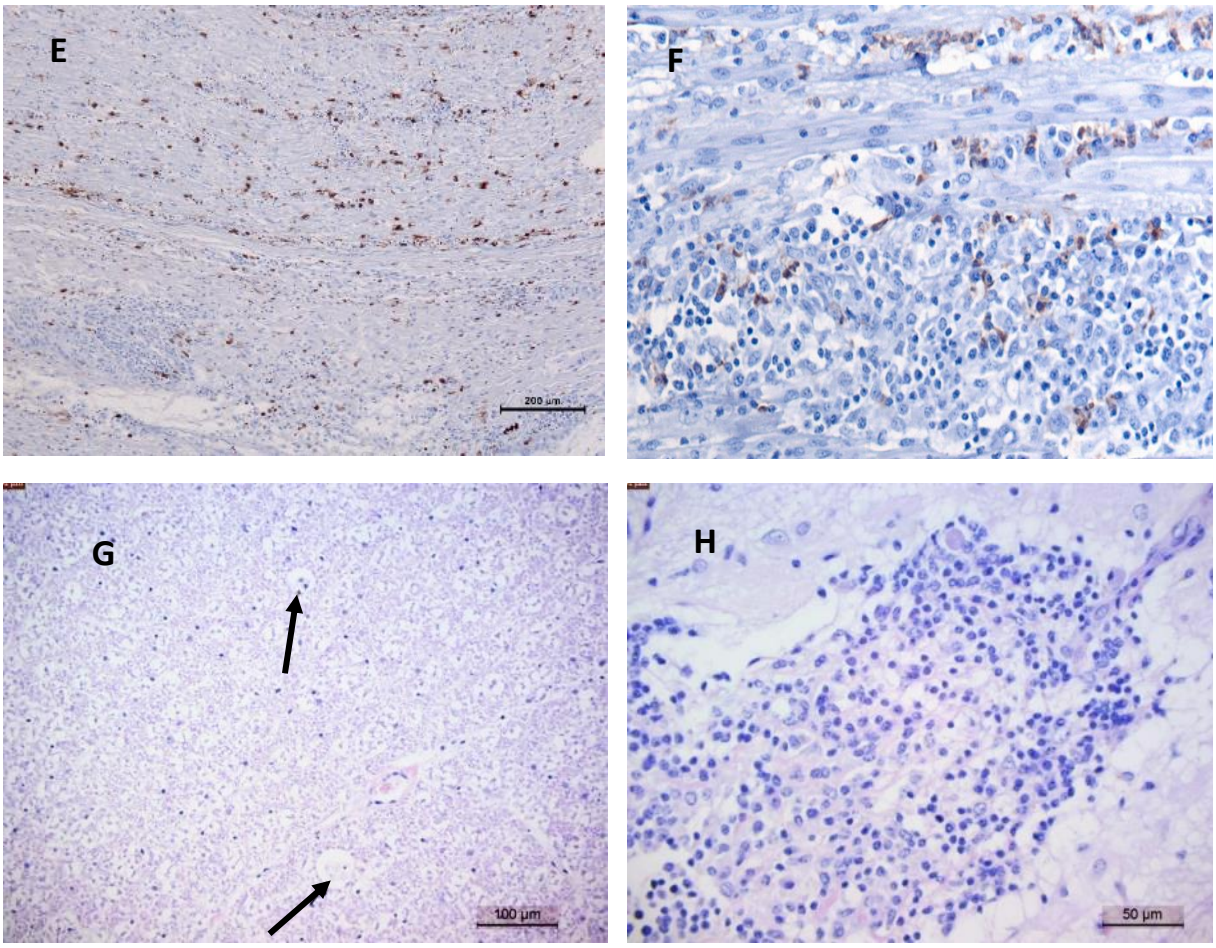
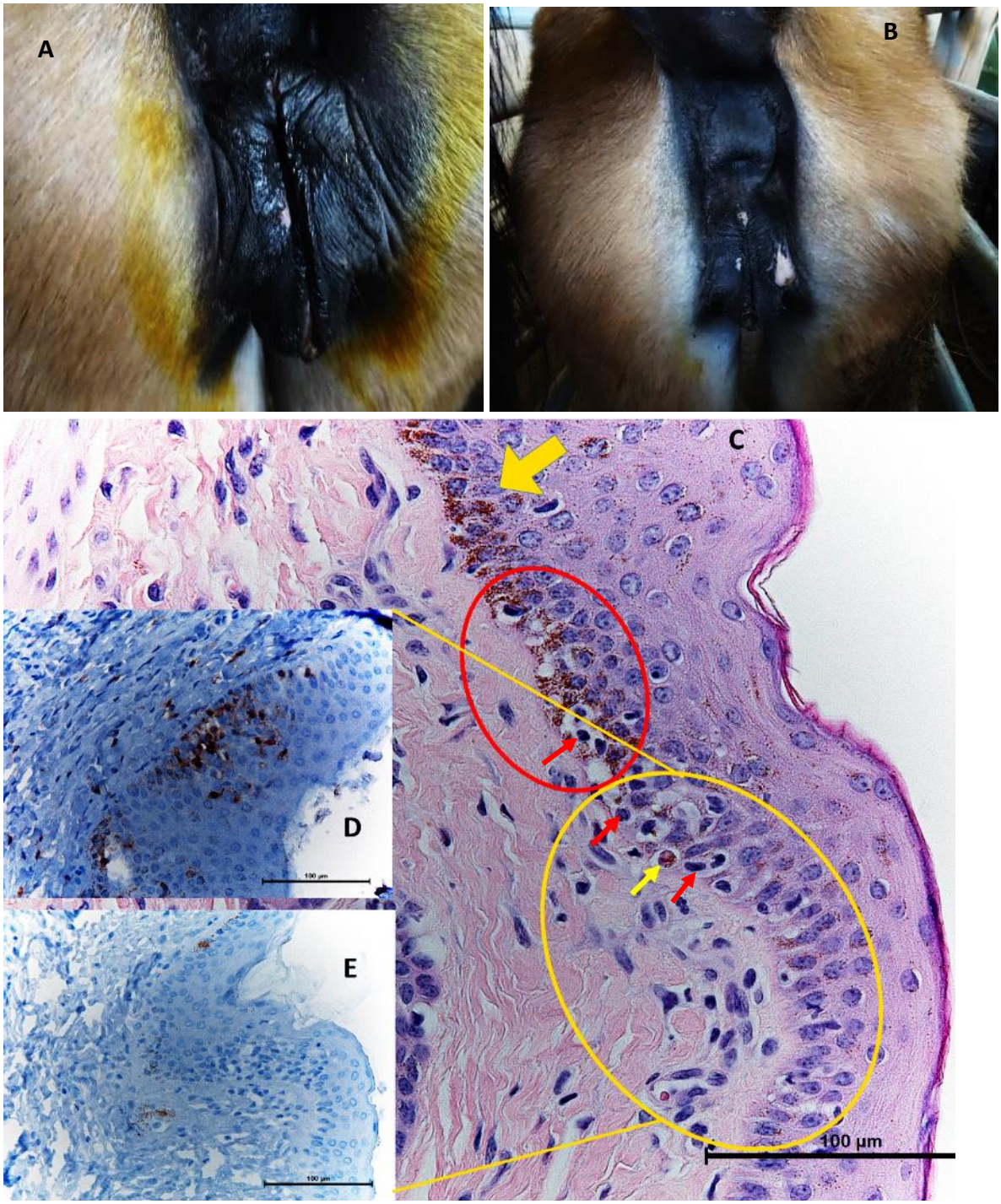


Figure 4.4. Photomicrograph of the histopathological lesions of some tissues of of nervous system of dourine diseased mares. HE staining of peripheral nerve shows intraneural infiltrations of inflammatory cells with axonal swelling and fragmentation (arrow) (A) and diffuse non-suppurative neuritis with detail infiltration of inflammatory cells and vaculation of the axons (B). IHC using anti-CD3 for T lymphocytes (C), CD20 for B lymphocytes (D), anti MAC387 for histocytes (E) and anti CD45 for plasma cells (F) revealed the specific cells involved in the cellular infiltration. HE staining of the spinal cord and brain indicate empty myelin sheaths with phagocytosis of debris (arrows) in the spinal cord (G) and meningeal infiltrates of inflammatory cells in the cerebrum (H).

After infection, focal depigmentation of the vulvar skin was seen. Histopathological sections of the depigmented skin showed infiltration of inflamatory cells at the basal layer of the epidermis and loss of melanocytes in the depigmented areas. Further CD3 immunohistochemistry staining determined the inflammatory cells as T-lymphocytes (Figure 4.5A-E).

In the reproductive tract, most severe lesions were found in the genital mucosa of the vestibule, vagina and cervix where they consisted of nodular infiltrations of lymphocytes, plasma cells and a few histiocytes sometimes with erosions of the epithelium (Figure 4.5F). In

the uterus, only minimal signs of inflammation were seen. Ovaries and mammary glands showed no histological abnormalities.



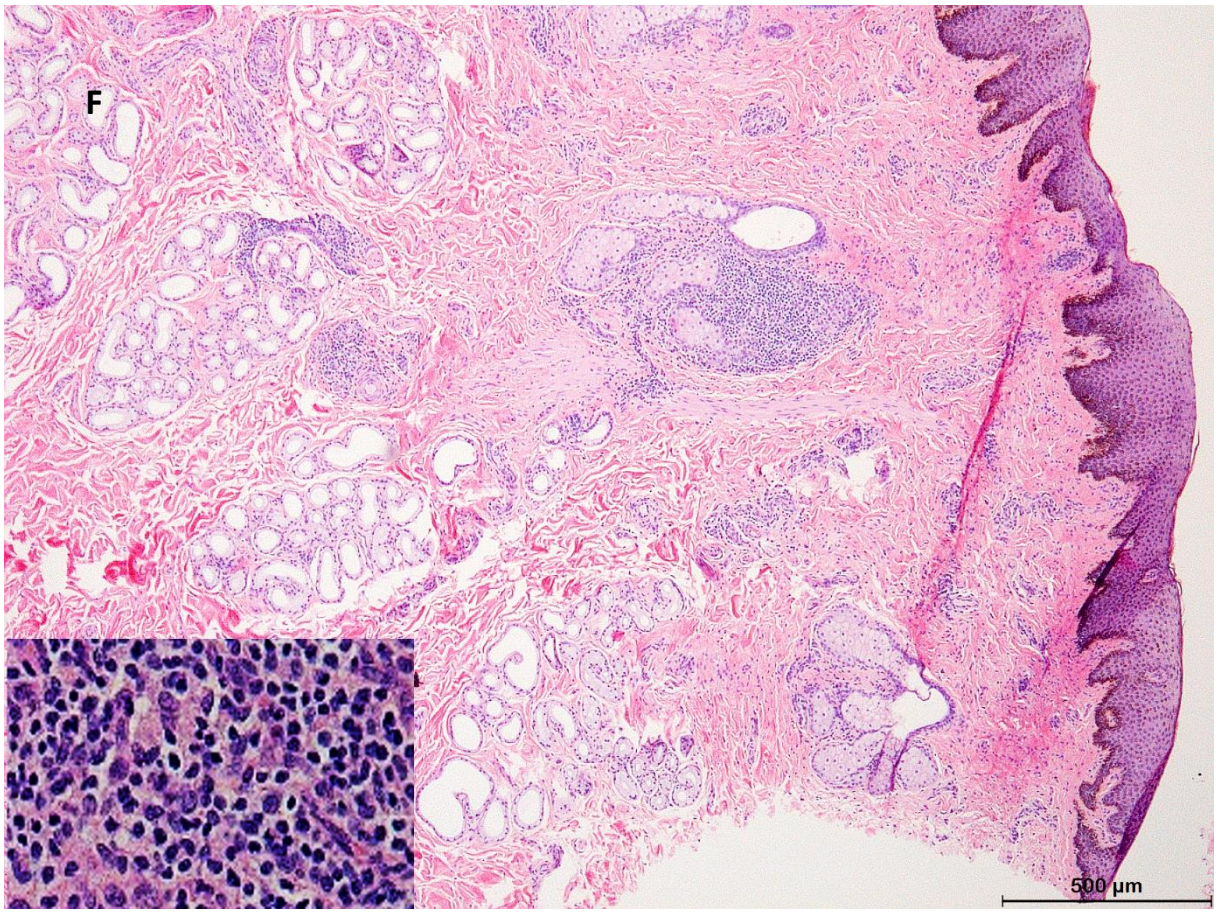


Figure 4.5. Photo micrograph of histopathological lesions in the depigmented skin areas and vaginal mucosa after *T. equiperdum* infection (A) Non depigmented perineal area before infection (B) depigmented skin of the vulva after infection (of the same mare) (C) depigmentated skin of the vulva (HE): normal part of the pigmented area (large yellow arrow) is with brown pigment. Melanocytes are being attacked by the inflammatory cells but not totally removed at the interface of the normal and depigmented part of the vulva (area of red circle). In the depigmented area (yellow circle) there are infiltrations of inflammatory cells (red arrow) at the basal layer of epidermis and melanocytes are lost. there are apoptotic cells of melanocytes after the attack of the inflammatory cells (small yellow arrow) (D) immunohistochemistry using anti-CD3 revealed infiltrating T lymphocytes as brown (IHC) (E) immunohistochemistry using anti-PNL2 reveraled no melanocytes (at the site of the inflammatory cells infiltration) (F) vaginal mucosa: nodular infiltrations of inflammatory cells (HE)

Kidneys in some animals showed multifocal, intraluminal eosinophilic, amorphous protein-rich material (protein-rich urinary filtration fluid) and cellular debris (Figure 4.6A). Occasionally, some tubules contained intraluminal degenerated epithelial cells and neutrophils. There was also basophilic amorphous material of intraluminal calcification (Figure 4.6B). There were some areas with small infiltrates of lymphocytes and plasma cells in the interstitium of the cortex (Figure 4.6C).

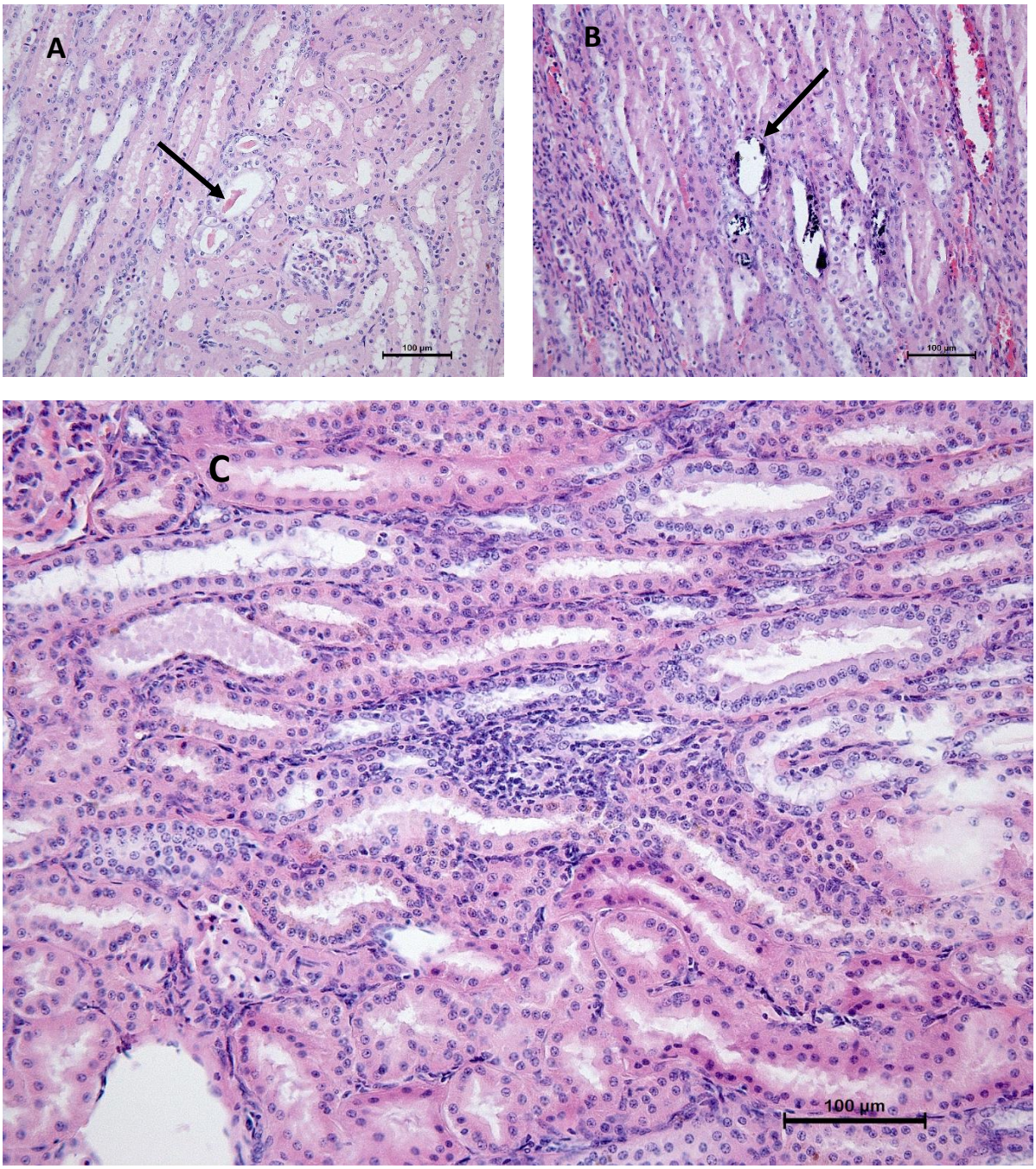


Figure 4.6. Photo micrograph of histopathological lesions of the kidneys after *T. equiperdum* infection (Haematoxylin-eosin staining). (A) intraluminal eosinophilic, amorphous protein-rich material (arrow) (B) intraluminal calcification (arrow) (C) infiltrates of lymphocytes and plasma cells in the interstitium

In the liver, the bile canaliculi were distended and filled with brown pigment (bile stasis). Many hepatocytes had intracytoplasmic brown granules and within the sinusoids and there were many Kupffer cells with brown granules, which stained blue with Berlin blue staining (hemosiderin) (Figure 4.7A). There was a mild portal infiltration of lymphocytes, plasma cells and a moderate infiltration of eosinophils in the liver (Figure 4.7B).

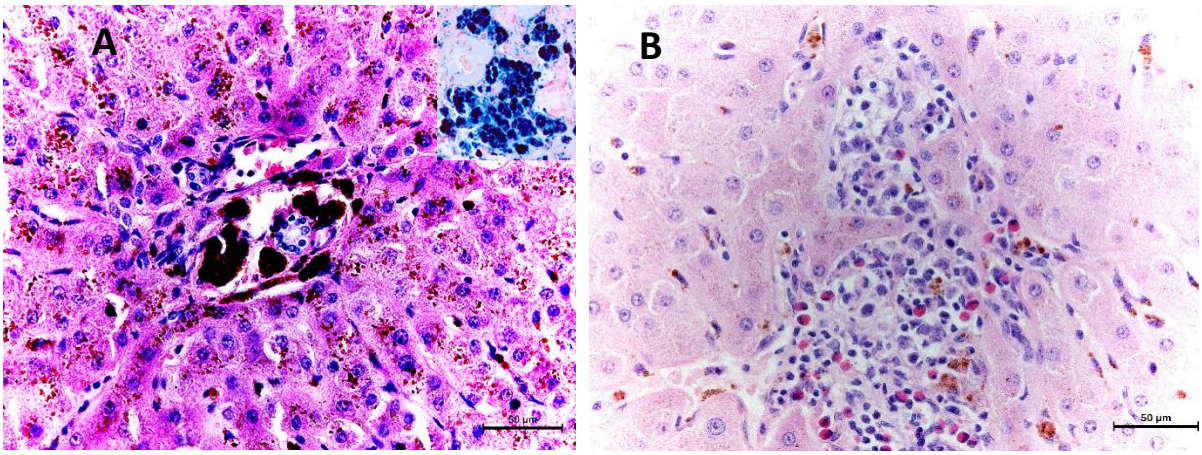


Figure 4.7. Photo micrograph of the histopathological lesion of the liver after *T. equiperdum* infection (A) Liver - Kupffer cells with pigment (brown granules) (HE), Insert: berlin blue stain (B) portal infiltrations of lymphocytes, plasma cells and eosinophils (HE)

In the lamina propria and, in a lesser amount, in the submucosa of the intestines, a mild to moderate infiltration of mainly lymphocytes and plasma cells and some eosinophils were observed. In the spleen, there were many macrophages with intracytoplasmic brown granules (haemosiderin). Some giant cells and many Russell bodies were observed. The white pulp was enlarged (reactive). In the red pulp, there was a mild infiltrate of eosinophils (Figure 4.8.A-B).

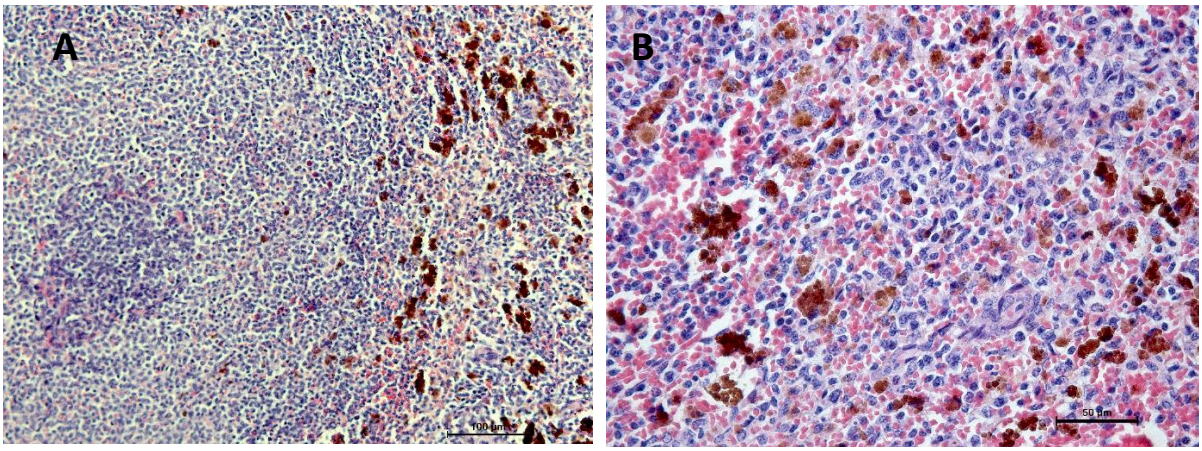


Figure 4.8. Photo micrograph of histopathological lesions of the spleen after *T. equiperdum* infection (Haematoxylin-eosin staining) (A) enlarged white pulp (reactive) (B) macrophages with intracytoplasmic brown granules

Different lymph nodes such as the prescapular, prefemoral, inguinal, popliteal, hepatic and renal lymph nodes showed similar microscopic features. The white pulp was enlarged and paler (reactive). In the medullary and interfollicular sinuses, there were some giant cells and a higher amount of macrophages. In the interalveolar septa of the lung, some macrophages with intracellular brown pigment were observed, as well as in the peribronchial areas moderate infiltrations of mainly lymphocytes and some macrophages containing brown

granules. The pigment was blue on Berlin blue staining indicating the presence of iron (hemosiderin). No specific microscopic changes were detected in the urinary bladder, stomach, heart, pancreas and adrenal glands of all horses. The eyes with corneal opacity contained microscopic small vessels with plump endothelial cells (neovascularization) in the superficial stroma of the cornea (Figure 4.9).

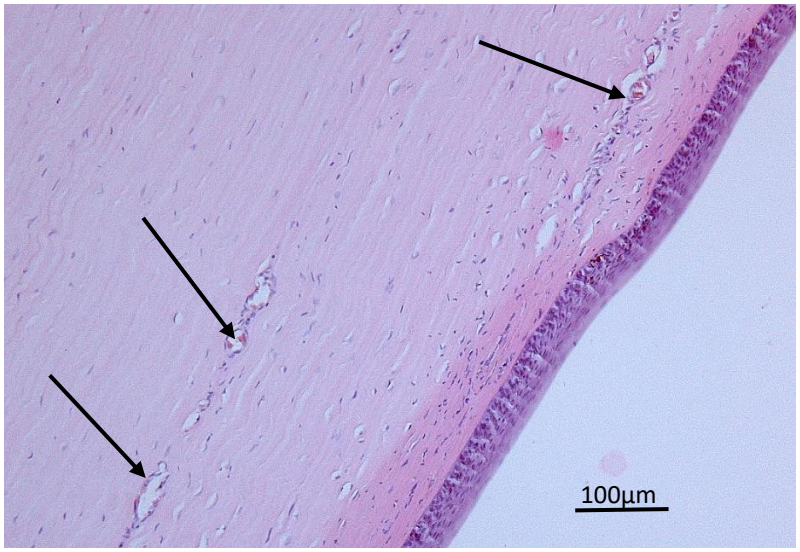


Figure 4.9. Photo micrograph of the histopathological lesion of the eye (cornea) after *T. equiperdum* infection (Haematoxylin-eosin staining). Cornea: small vessels with plump endothelial cells (neovascularization) (arrows)

4.3.2. Infection by blood transfusion in stallions

4.3.2.1. Diagnosis of dourine and infectiousness of semen and preputial discharge

Stallions, inoculated by blood transfusion, showed clinical symptoms of dourine such as swelling of the prepuce from day 10 post-infection (S01), day 19 (S04) and day 21 (S02 and S03), with intact libido but unable to mount due to back pain from day 13 (S01, S02, S03) and incoordination, ataxia and cachexia at later stages. Trypanosomes were observed in the blood from day 5 post-inoculation onwards by the Woo test. (Table 4.3). Semen could be collected successfully on day 7 post-inoculation from 3 of the four stallions (S02, S03, and S04) to check for the presence of the parasite by PCR. However, the result of the PCR on these semen samples for the parasite DNA was negative. Due to the difficulties to mount caused by the disease, no semen could be obtained anymore in 3 out of 4 stallions (S01, S02 and S03) later on. Chemical ejaculation, using Imipramine hydrochloride and xylazine and ground collection were not successful. Only one of the four stallions (S04) was able to mount the phantom at

day 13 post-infection. This ejaculated semen was used to inoculate mice (N=7) and DNA was extracted for PCR. Parasite DNA was detected by PCR test and mice inoculated by this ejaculated semen became parasitaemic (7/7).

Table 4.3. Interval between *T. equiperdum* infection and positive test results in the various diagnostic tests and clinical symptoms in stallions following artificial infection with *Trypanosoma* through blood transfusion

Horses ID	**Positive for *T. equiperdum* (days post infection)**	**Symptoms of dourine noticed (days post infection)**			
	Woo test	**Oedema of the prepuce**	**Oedema of limbs**	**Depigmentation**	**Ataxia**
S01	5	10	26	-	70
S02	5	21	-	43	102
S03	5	21	-	-	92
S04	5	19	-	-	55
Average	5	17.75	26	43	79

(-) indicates clinical signs absent

Since the route of infection in stallions was directly into the blood stream and the infection-dose was higher, the detection of trypanosomes by Woo test was earlier compared to that of the mares. Preputial swelling was manifested in all infected stallions and later extended to the scrotal area. Depigmentation was observed on the skin around the anal region in one of the stallions. Oedema of lower hind limbs below the stifle joint was observed in another stallion. Signs of dourine such as back pain, lameness and paralysis of the hindquarters leading to partial dragging or stiffness of the hind legs, a staggering gait, posterior ataxia and an inability to stand upright after prolonged recumbency were similar to what was observed in the mares. Stallions still exhibited libido but were unable to mount the mares nor the breeding phantom due to facing difficulties to jump. Inflammation of the conjunctiva and lacrimal discharge of the eyes was noticed in 2 of the 4 infected stallions that eventually resulted in corneal opacity and led to permanent blindness.

4.3.2.2. Haematology

In stallions after infection, a significant decrease in mean PCV, Hgb concentration, RBC and WBC counts compared to the values prior to infection was observed. There was a significant ($p<0.05$) decrease in the PCV, Hgb concentration starting from week 2 and RBC count from week 5 post-infection compared to the date of infection (week 0) (Figure 4.10A). The mean of total WBC count was significantly ($p<0.05$) reduced by week 2 post-infection. However, it quickly increased to the level above the pre-infection values and remained fluctuating during the study period. There was a slight increase in lymphocytes and neutrophils (Figure 4.10B).

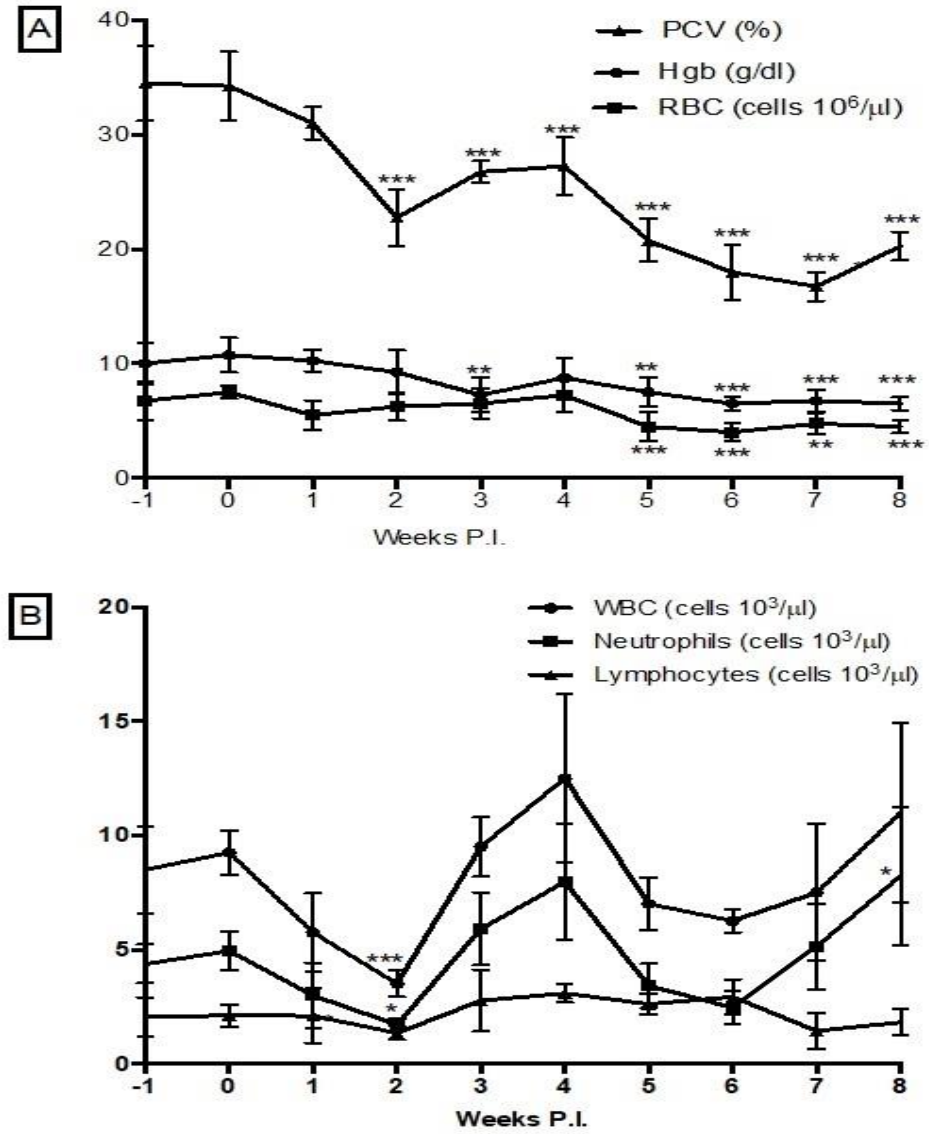


Figure 4.10. Mean haematological values in stallions experimentally infected with *T. equiperdum* by blood transfusion, (A) RBC count, PCV and Hgb concentration (B) total WBC, lymphocytes and neutrophils counts. (Significancy*** p=0.001, ** P=0.01, * p=0.05)

4.3.2.3. Tissue distribution of the parasite and pathological lesions

At necropsy, 120 days post-infection in the chronic stage of the disease, all gross and histopathological lesions of all organs of the nervous system, other vital organs such as the liver and spleen and the eye, with corneal opacity and distribution of the parasite (Table 4.4) were very similar to those of the mares. The impression smear from the brain, kidney and other vital organs showed the presence of the trypanosomes similar to the mares. Trypanosomes were also found in the testicles (Figure 4.2F).

Table 4.4. Results of impression smear and RT PCR analysis on tissue samples collected from stallions 120 days after an artificial *T. equiperdum* infection

Tissue sample	**S01**	**S02$_{Tx}$**		**S03$_{Tx}$**		**S04**	
Nervous system	**PCR**	**PCR**	**Smear**	**PCR**	**Smear**	**PCR**	**Smear**
Cerebrum	+	+	+	+	+	+	+
Cerebellum	+	+	-	+	-	+	+
Brain stem	ND	+	-	+	-	+	ND
Lumbar spinal cord	+	+	ND	+	ND	+	ND
Sacral spinal cord	+	+	ND	+	ND	+	ND
Obturator nerve	ND	+	ND	+	ND	+	ND
Reproductive system							
Epididymis	+	+	ND	+	ND	+	ND
Testicle	+	+	+	+	+	+	+
Prepuce	+	+	ND	ND	ND	+	ND
Vesicular gland	+	+	ND	+	ND	+	ND
Ampulla	+	-	ND	+	ND	+	ND
Prostate	+	+	-	+	-	-	ND
Others							
Lung	-	+	+	+	-	-	-
Liver	+	+	+	+	+	+	+
Kidney	-	-	-	+	+	+	+
Heart	-	+	+	+	-	+	-
Pancreas	-	-	-	+	-	+	-
Adrenal gland	+	+	-	+	-	-	-
Spleen	+	-	-	+	-	-	-

(+)= positive for trypanosomes, (-) = negative for trypanosomes, ND= not done, Tx = Cymelarsan® treated

On histological examination of the male reproductive tract, most severe lesions were found in the testicles (Figure 4.11 A- E). Testes showed diffuse orchitis characterized by moderate to severe interstitial infiltration of lymphocytes, plasma cells and few histocytes as well as a mild to moderate increase in interstitial fibroblasts (early fibrosis). Seminiferous tubules were severely degenerated and spermatogenesis was mostly absent. The epididymis was normal. The genital mucosae showed superficial lymphoplasmacytic infiltration of varying intensity. In the accessory glands, no histological changes could be noticed.

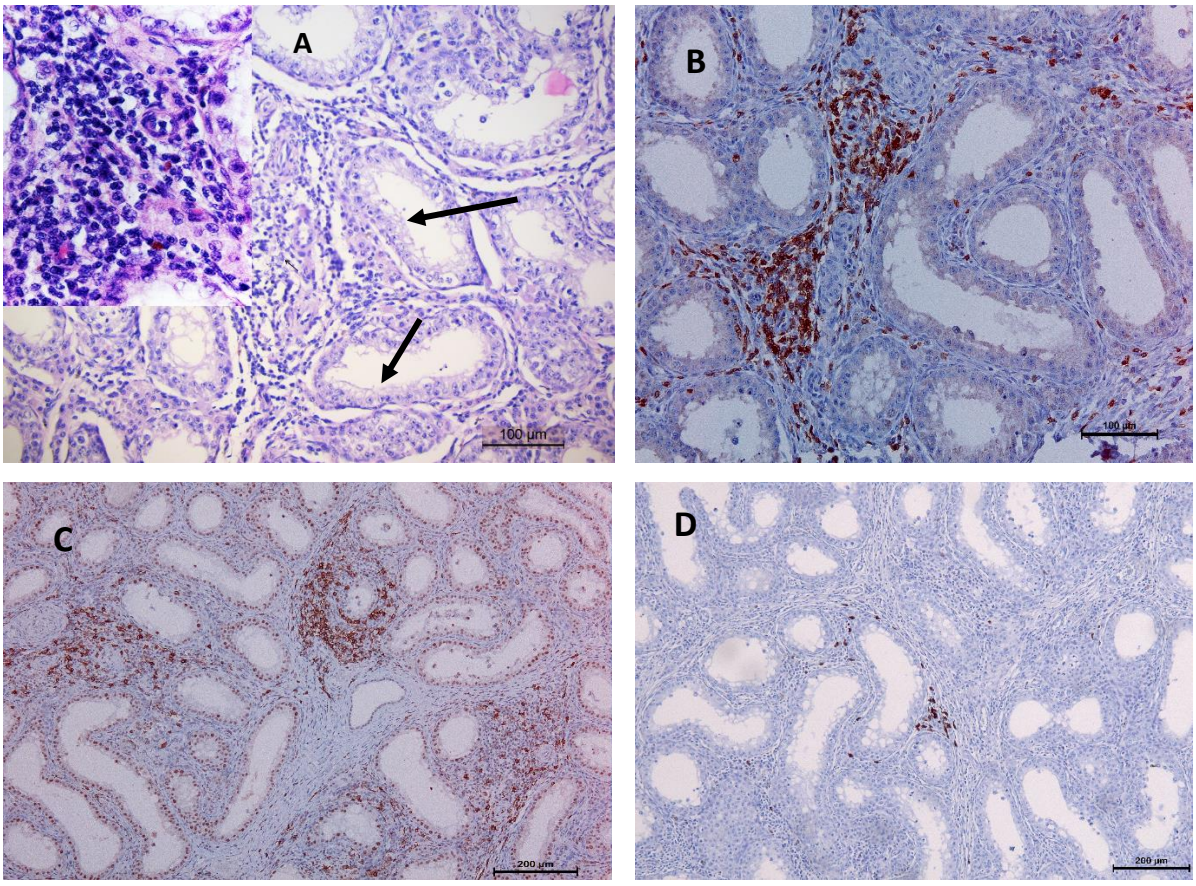


Figure 4.11. Photomicrograph of the histopathological lesions in the testicle of dourine diseased stallions (A) infiltration of lymphocytes and plasma cells (Insert) and severe degeneration of the tubuli (arrow). Immunohistochemistry using anti-CD3 (T-lymphocytes) (B), anti-CD20 (B-lymphocytes) (C) and anti-CD45RA (plasma cells) (D) reveald brown stained inflammatory cells respectively.

4.3.3. Cymelarsan® treatment

Cymelarsan® treatment (at day 60 post-*T. equiperdum* infection) failed to cure chronically infected horses. There was no improvement in clinical signs except a slight, transient increase of PCV for a few weeks. The presence of the parasite was demonstrated by impression smears at necropsy and further confirmed by qPCR from treated horses similar to the non-treated ones. Mice inoculated with preputial fluid of Cymelarsan® treated stallions at day 63 post-infection (n=2) were found to be aparasitaemic (0/3) for each stallion whereas those inoculated with preputial fluid from non-treated stallions (n=2) developed parasitaemia (3/3) each. Mice inoculated with epididymal semen after day 120 post-infection of the non-treated stallion all developed parasitaemia (5/5). However, when epididymal semen of Cymelarsan® treated stallions was used, parasitaemia developed only in mice inoculated with epididymal semen from one stallion (5/5) but not from the other one (0/5). All the preputial fluids and epididymal semen samples of both treated and non-treated stallions were found to be positive on PCR.

4.4. Discussion

Mares infected via artificial insemination (36,000 trypanosomes) developed parasitaemia starting from day 5 post-infection similar to horses infected by blood transfusion, regardless of the higher dose used (100,000 trypanosomes) in the transfusion infection. Dissemination of trypanosomes, even in testicular areas which are supposed to be delineated by a blood-testis barrier, was reported by Pascucci et al. (2013) where the intra-scrotal inoculation of rabbits with udder secretions containing 1,000 trypanosomes, resulted in invasion of all tissues.

In the current study, it is shown that ejaculated semen of infected stallions is already infectious to mice in the preclinical period and epididymal semen from Cymelarsan® treated stallions was still infectious. Moreover, it was shown that *T. equiperdum*-spiked semen was able to infect mares when used in artificial insemination. This clearly indicates that transmission of *T. equiperdum* is possible through semen itself and secondly, that semen from symptomless stallions post-treatment can transmit dourine. Although the presence of the parasite in the

semen was previously suggested by its isolation from the urethral tract of diseased horses (Suganuma et al., 2016) the current findings show the infectivity of the semen itself and confirm our previous hypothesis that the spread of dourine to various parts of the world might be enabled through the transportation of infected stallions, mares, donkeys and semen (Ahmed et al., 2018).

All infected animals exhibited a rise in temperature which coincides with the level of blood parasitaemia. The presence of fever is in accordance with previous findings (Brun et al., 1998; Claes et al., 2005) although others did not observe a temperature rise post *T. equiperdum* infection (Barrowman, 1976; Vulpiani et al., 2013). In other types of trypanosomosis (e.g. *T. vivax*) a rise in rectal temperature and temperature fluctuating with the level of parasitaemia is also a common finding (Delespaux, 2004; Adeiza et al., 2008; Dagnachew et al., 2015).

Weight loss, without a loss of appetite, was observed in all animals leading to a considerable degree of cachexia in accordance with previous reports (Barowman, 1976; Scacchia et al., 2011; Vulpiani et al., 2013). The decline in body condition is a sequel of the parasitaemia resulting in an accumulation of peroxides and free radicals in the body of infected animals causing severe cell damage (Saleh et al., 2009). Similar symptoms have been reported for *T. evansi* in camels (Enwezor and Sackey, 2005). Since, *T. evansi* and *T. equiperdum* exhibit many similarities in biological, biochemical and molecular characteristics (Brun et al., 1998, Ahmed et al., 2018), body weight loss in a *T. equiperdum* infected animal might be attributed to this same mechanism.

Depigmentation of skin around the perineal region observed in the present study has been reported previously (Claes et al., 2003; Hagos et al., 2010a; Pascucci et al., 2013). The probable cause of depigmentation around the vulvar skin of infected mares could be due to severe necrosis of the epidermis (which also affects the melanocytes). Gizaw et al. (2017) reported that the depigmented areas were microscopically characterized by severe necrosis of cells, excess free melanin and formation of cystic structures in the epidermis. According to Myers and McGavin (2006), after damage to cells which contain melanin (e.g. melanocytes and basal cells of the skin), the free melanin will be phagocytosed by macrophages which causes loss of melanin pigment in the epidermis resulting in depigmentation.

Preputial swelling in stallions gradually extending to the scrotal area and vulvar oedema and mucopurulent vaginal discharge in mares, were consistent findings in previous studies (Alemu et al., 1997; Hagos et al., 2010a; Lelli et al., 2012; Pascucci et al., 2013; Vulpiani et al., 2013). However, oedematous skin plaques, historically regarded as important or even pathognomonic symptoms in cases of dourine (Barrowman et al., 1976; Claes et al., 2005; Pascucci et al., 2013 and Vulpiani et al., 2013), were not observed in mares or stallions throughout the study period. Differences in *T. equiperdum* strain or breed, nutritional, stress factors and host immune response of horses involved in the experiment are possibly causing these differences in symptomatology (OIE, 2013; Ricketts, et al., 2011). The presence of ventral oedema in the experimental horses was restricted to the limbs (in only 3 out of 6 animals), prepuce and scrotum. However, previous reports describe ventral oedema extending to the belly region up to the sternum (Hagos et al., 2010a; Lelli et al., 2012; Pascucci et al., 2013; Vulpiani et al., 2013).

Nervous signs of dourine manifested themselves on average after 64 days in all infected horses and were characterized by hind quarter lameness, incoordination and ataxia. In most studies, nervous signs have been reported to occur only at later stages although not specified in days of occurrence (Scacchia et al., 2011; Vulpiani et al., 2013). In the present study, we noticed that stallions tried to mount mares or the breeding phantom thus exhibiting a normal libido but failed to do so due to back pain or hind quarter lameness at early stages of the disease i.e. 7-13 days post infection. In the clinical follow up and attempts to collect semen, stallions were teased to jump on a phantom and/or a mare by keeping an oestrous mare in front of the stallion (to tease the stallion to mount the phantom), stallions had an intact libido, they tried to mount several times, but were unable to jump and mount neither the phantom nor the mare. All stallions were used to this procedure since before the infection trial they all were trained to this way of semen collection. Moreover, dourine as disease is known by these typical signs of hind leg ataxia and is reflected in the local name of the disease as ‘Lapessa’ or ‘Kuta’ meaning a ‘back bone breaker’ (Affan Oromo)(Hagos et al., 2010). Conversely, these nerves signs were only observed very late in mares (after day 44). However a possible explanation might be that stallions are teased to mount and in this way, early symptoms become easy to notice whereas mares are left in peace at that time. The presence of

neurological signs confirms the tropism of *T. equiperdum* for the peripheral nervous system and the lack of involvement of the brain, in contrast with other trypanosomes.

Corneal opacity was found in two stallions and one mare and was consistent with previous reports (Alemu et al., 1997*;* Vulpiani et al., 2013). Aqueous intraocular fluid collected at necropsy from the eye suffering this corneal opacity was also positive for the parasite DNA in the qPCR. That way low concentrations of parasite DNA from conjunctival swabs were already detected in similar cases (Theis and Bolton, 1980, Pascussi et al., 2013).

Using a gentle pressure of a glass slide on a freshly cut surface of the tissues at necropsy and stained with Diff-quick was enough to demonstrate the presence of the parasite in different organs, more specifically in the nervous and reproductive tracts. This rather simple "impression smear" technique, as used to detect Leishmania and Toxoplasma (Swierczewski et al., 2013), has shown its value for quick screening of postmortem tissues for the presence of trypanosomes. In small mammals such as *Crocidura russula* and *Mus spretus* trypamastigote forms of trypanosomes (*Trypanosoma musculi* and *Trypanosoma microti)* from tissues such as heart, liver and kidney had also been previously identified by the use of this technique (Santos-Gomes et al., 1993). Based on the PCR results, trypanosome DNA could be extracted from most tissues of the infected animals (Table 4.4) and in several organs, the presence of the trypanosome itself could be demonstrated and made visible by use of impression smears, although blood contamination can't be excluded using this technique.

The literature on pathological lesions caused by *T. equiperdum* in horses is scarce. The gross pathological lesions in the reproductive tract, such as congestion of vaginal and uterine mucosa with widespread haemorrhage, are in agreement with the report of Pascucci et al. (2013).

Gross lesions in non-reproductive organs were not common except for abdominal fluid accumulation. This is in line with the report of Pascucci et al. (2013) who stated that no lesions are observed in the parenchymatous organs except for some congestion of the spleen. The presence of an increased amount of fluid in the abdominal cavity is a sequel of the hypoproteinaemia caused by the low albumin level. Orhue et al. (2005) indicated that serum

albumin levels decreased in trypanosomosis. The more general oedema during the chronic stage of trypanosomosis might be due to a significant decrease in the albumin levels (Enwezor and Sacky, 2005).

Previous attempts to use molecular techniques to investigate the presence of *T. equiperdum* involved analysis of blood samples (Clausen et al., 1999, 2003; Gari et al., 2010). Since *T. equiperdum* is a tissue parasite, blood might not be the most reliable matrix for the diagnosis of dourine, as parasitaemia is not constant (OIE, 2013). Pascucci et al. (2013) reported the detection of parasite DNA by qPCR in tissues other than blood such as vaginal swabs, joint fluid, lymph nodes and mammary secretions using a set of primers for detection of a gene specific for the trypanozoon group. In the current study, a number of tissues collected at necropsy were found to be positive for *T. equiperdum* by the use of real-time PCR using ITS1 primer. This might significantly improve the diagnosis of dourine in live animals by collecting biopsies from cerebrospinal fluid and lymph nodes. Also, uterine and vulvar biopsies collected in this study during the course of the disease, when parasites were not found in the blood, were positive for PCR (data not shown).

The common finding of a plasma cell infiltration, seen in a number of organs, seems to indicate that a diffuse immune-mediated inflammatory reaction could be the basis of the pathogenesis of the disease. Infiltration of tissues with mononuclear inflammatory cells, especially lymphocytes and plasma cells, is a hallmark of chronic inflammation (Jones et al., 1997). The microscopic findings of the present study, show an infiltration of mononuclear cells, especially lymphocytes, in several tissues (e.g. testicle, uterus, spinal cord) and indicate the presence of a chronic inflammatory process in accordance to previous reports (Morrison et al., 1981; Rodrigues et al., 2009; Gizaw et al., 2017).

Mammary glands had no histological abnormalities in the current study. However, trypanosome can be isolated from mammary gland secretions (Vulpiani et al., 2013). PCR test from mammary gland secretions of dourine diseased horses from natural infections in another study were also found positive. In the current study trypanosomes were detected from many more tissues without histological lesions. The same is true for the lesions in the mammary glands. The presence of the trypanosome in the mammary secretion may not end

up with lesions, so the absence of tissue lesions might not exclude infectivity of udder secretions

Starting from the first week post-infection anaemia with a decrease in mean PCV, Hgb concentration and RBC compared to pre-infection values was observed. The haemolysis of RBC induced by the trypanosomes might be responsible for the anaemia. Anaemia was moderate during early infection and became more severe as the disease progressed. Suganuma et al. (2016) described a slight decrease in PCV, Hgb concentration and RBC count in dourine infected stallions in Mongolia. Vulpiani et al. (2013) also reported that anaemia progressed from a moderate to a more severe state in dourine diseased horses with chronic oedematous lesions or nervous signs. A haemolytic reaction due to acute blood loss by haemolysis could be indicated by the reduction in the RBC count and PCV values. This is a normocytic normochromic type of anaemia (Weatherall, 2003).

The WBC counts of infected stallions decreased immediately one-week post infection and increased rapidly to the level above the pre-infection values starting two weeks later and remained fluctuating during the study. The fluctuating WBC is associated with increased numbers of lymphocytes and neutrophils. The increased activity of the mononuclear phagocytic system during trypanosome parasitaemia is an attempt to tackle the trypanosome distribution. In the first two weeks, this results in a decline in WBC numbers due to the massive use of circulating white blood cells more rapidly than they can be produced. The decline might be due to the usage of the circulating white blood cells more rapidly than they can be produced. The activity of the mononuclear phagocytic system during trypanosomosis is an attempt to tackle the trypanosomes. The neutrocytopenia observed during the course of infection is thought to increase the susceptibility of infected animals to concurrent infections (Stephen,1986; John et al., 2006; Suganuma et al., 2016).

The mean lymphocyte count post infection was elevated for some time as compared to values before infection and reduced to normal values after the parasitaemia and as such accompanying the numbers of trypanosomes in the circulation and when the challenge by the parasitaemia decreased, the immune response (WBC count) also diminished. Anosa et al. (1992) ascribed that lymphocytes react to trypanosome antigenic challenge by an increased

proliferation of immuno-competent cells into more specific antibody and/or lymphokine producing cells. The fluctuation in the neutrophil counts might be due to the phagocytosis of trypanosomes by the circulating neutrophil cells and their destruction in the spleen and lymph nodes (as been reported in mice infected with *T. brucei*, (Anosa and Kaneko, 1983) and an enhanced activation of neutrophil precursor cells in the bone marrow. The limitations of this study in haematology is that it didn't include a negative control.

Although Cymelarsan® treatment in the acute phase of the disease resulted in a significant improvement in mean PCV values and a cure rate of 100% (Hagos et al., 2010b), it was found to be ineffective in chronic cases of *T. equiperdum* diseased horses and no improvement of clinical signs was observed. On post-mortem examination, in both treated and non-treated animals, parasites could be found by impression smears and on qPCR.

After venereal transmission, *T. equiperdum* reaches the peripheral circulation and vice versa, as seen in stallions, semen contains trypanosomes early in the infection along with the development of clinical signs. However, in the chronic stage, parasites leave the circulation into tissues and cause severe tissue damages responsible for the signs of dourine. In conclusion, we were able to infect mares by AI with *T. equiperdum* spiked semen, resulting in all diagnostic clinical symptoms and changes in blood parameters. The parasites disseminated to blood and several tissues including the nervous system and reproductive organs. In stallions infected intravenously, parasites could be traced back in several organs, including the nervous system, reproductive organs and especially in semen. This study demonstrates and clears doubts to the venereal transmission and dangers for spread of *T. equiperdum* by AI.

Chapter 5

Removal of *Trypanosoma equiperdum* from equine semen by single layer centrifugation

Adapted from: Ahmed Yasine, Hagos Ashenafi, Peter Geldhof, Merga Bekana, Alemu Tola, Leen Van Brantegem, Ann Van Soom, Luc Duchateau, Bruno Goddeeris, Jan Govaere.

Experimental Parasitology, 2019, 200, 79-83

ABSTRACT

Trypanosoma equiperdum (T. equiperdum) causes dourine, a venereally transmitted infection in horses. Purification of semen by single layer centrifugation (SLC) has been proven to be successful in reducing venereally transmitted diseases when dealing with other pathogens. The objective of this study was to evaluate the purification of *T. equiperdum* spiked semen by SLC. Semen was spiked using cryopreserved *T. equiperdum* stabilates (Dodola strain isolate 943). In total, 6 concentrations, varying from 10^2 to >$5x10^6$ trypanosomes, were added to semen samples. Subsequently, SLC was performed following standard procedures. The presence of the parasite in the purified semen was checked by wet smear examination, ITS1 PCR and *in vivo* inoculation in mice. Before SLC, all spiked semen samples, except the negative controls, were positive on PCR analysis. After SLC, all the pellets were found to be negative for *T. equiperdum* on microscopic examinations. PCR analysis also could not detect any parasite-DNA in the SLC-pellet of semen spiked with the lower number of parasites (10^2 to 10^4 trypanosomes). However, in the SLC pellets spiked with 10^4 - $5x10^4$ trypanosomes, only 1 out of the 4 replicates was negative for parasite DNA. All groups spiked with >$5x10^4$ trypanosomes were found to be positive on PCR. All mice in the positive controls exhibited parasitemia (5/5). Mice inoculated with SLC-purified semen that was spiked with lower than $5x10^4$ trypanosomes, remained free of parasitemia, similar to the negative controls. However inoculation with SLC-pellets from samples with a higher number of trypanosomes (> $5x10^4$ - $5x10^6$ and > $5x10^6$), induced parasitaemia in 2 out of 5 and 3 out of 5 mice, respectively. This study indicates that single layer centrifugation can be used to clear *T. equiperdum* infected semen but that the success is dependent on the number of parasites.

Keywords: Mice Inoculation, PCR, Stallion Semen, Single Layer Centrifugation, *Trypanosoma equiperdium*

5.1. Introduction

Trypanosoma equiperdum is a flagellated protozoan parasite known to affect the nervous system and the reproductive tract of both stallions and mares. In horses, the morbidity post infection is high and mortality rates can mount up to 50% (Equimed, 2009; Ricketts et al., 2011; OIE, 2013). *T. equiperdum* can be isolated directly from the urethral tract of infected stallions (Suganuma et al., 2016) and from epididymal and ejaculated semen (Ahmed et al., 2017). Since the parasite is present in mucous tissues of the external genital tract and in semen, disease transmission through artificial insemination (AI) is also possible. With a high morbidity and mortality and limited treatment options, purification of infectious semen might be part of a treatment strategy to manage breeding activities in endemic regions.

Single layer centrifugation (SLC) of semen using a density gradient will, based on differences in isopycnic properties of the components, separate morphologically abnormal sperm cells from the normal ones (Pertoft, 2000; Morrell and Rodriguez-Martinez, 2009). Likewise, purification of semen by SLC has been successful in reducing venereally transmitted diseases of bacterial and viral origin (Blomqvist et al., 2011; Morrell and Wallgren, 2011; Morrell et al., 2013). Virus-free semen samples were obtained from an EVA (Equine Viral Arteritis) virus shedding stallion by SLC with Androcoll-E, i.e. the SLC step alone appeared to be sufficient to separate the virus from spermatozoa (Morrell et al., 2013). Similarly, aliquots of an ejaculate from an uninfected stallion, spiked with virus at 1, 10 and 100 times the infective dose of EVA and processed by density gradient centrifugation followed by swim-up technique, tested negative for EVA by PCR. The procedure was repeated using an ejaculate from a known EVA-shedding stallion and resulted in virus-free sperm (Morrell and Geraghty, 2006). Reducing viral titers of porcine circovirus (Blomqvist et al. 2011), and removal of bacteria from boar semen (Morrell and Wallgren, 2011) by SLC is also reported. Studies suggest that a double semen processing technique of density gradient centrifugation followed by a ‘swim-up’ can provide virus-free sperm preparations for assisted reproduction in human. Purification of hepatitis C infected semen by SLC-swim up, in an *in vitro* setting resulted in a pregnancy (Levy et al., 2002). However, when dealing with much larger particles such as *T. equiperdum* parasites, successful semen purification by SLC has to our knowledge, not yet been reported. Therefore, the

objective of this study was to evaluate the possibility to remove *T. equiperdum* from contaminated semen.

5.2. Material and methods

5.2.1. Experimental Animals

Four adult Selale stallions (a local breed) from dourine free areas in the Central Highlands of Ethiopia (Burayu District), free of Trypanozoon infection as shown by parasitological (Woo) and serological (CATT/*T.evansi*) tests were used as sperm donors in this study. They were housed in fly-proof stables, supplemented with 2 kg concentrates twice daily and were given grass hay and water ad libitum.

Swiss white mice (N=40), 8 weeks old, from the National Veterinary Institute (NVI) in Bishoftu, Ethiopia were used for the inoculation study. Mice were kept in plastic cages on wood shavings in a secluded environment. Pelleted feed and water were given ad libitum.

5.2.2. Preparations of Trypanosomes

Cryostabilates of *T. equiperdum* Dodola 943 (Hagos et al., 2010c) stored in liquid nitrogen were allowed to thaw at room temperature for a few minutes and examined under the microscope for viability and diluted in phosphate-buffered saline glucose to 1 trypanosome per field ($10^{5.7}$ trypanosomes/ml). Naïve mice were injected intraperitoneally with 200µl of this preparation containing approximately 100,000 trypanosomes. Parasitaemia in the mice was monitored daily by wet blood film examination using the matching technique (Herbert and Lumsden, 1976) and harvested at first peak parasitemia, typically at day 3-5 postinfection. Parasitaemia was checked in 5 µl of tail blood. As soon as trypanosomes were detected and reached swarming level i.e more than 100 trypanosomes per microscopic field (x400) the mice were anaesthetised and blood was collected in heparin-coated vacutainer tubes. Equal volumes of this blood and Triladyl-egg yolk-phosphate buffered saline glucose (PSG, 7.5 g/l $Na_2HPO_42H_2O$, 0.34 g/l NaH_2 PO_4H_2O, 2.12 g/l NaCl, 10 g/l D-glucose, pH 8) (Pyana et al., 2011) were mixed by vortexing for cryopreservation in liquid nitrogen until use. The concentration was then

adjusted by adding the appropriate volume of phosphate-buffered saline glucose before spiking (Paris et al., 1982) (Table 5.1).

Table 5.1. Number of Parasites used in the spiking experiment

No	No of trypanosomes	Replications*
0	0 (Negative control)	1
1	10^2 - 10^3	3
2	>10^3 - 10^4	3
3	>10^4 - 5x10^4	4
4	>5x10^4 - 5x10^5	4
5	>5x10^5- 5x10^6	4
6	>5x10^6	4

*Different samples of spiked semen

5.2.3. Semen collection, spiking and SLC

Stallions were allowed to mount a phantom and semen was collected with a Colorado-type artificial vagina (AV) with a disposable plastic inner liner using sterile, non-spermicidal lubricant. A total of 24 ejaculates obtained from 4 healthy stallions were obtained and diluted with equine semen extender (INRA 96®) to a concentration of 100x10^6sperm cells/ml and were spiked with *T. equiperdum* cryostabilates at different parasite concentrations.

SLC was performed on 23 ejaculates spiked with 6 different concentrations of trypanosomes in different replications (3 for the first 2 concentrations; and 4 for the others and one negative control, Table 5.1). In the different replications, semen from several different ejaculates were used to spike with the given numbers of parasites. Semen spiking was done by adding 1 ml of the different concentrations (10^2-10^3 to >5x10^6) of *T. equiperdum* (Dodola 943) in 5ml stallion semen. The viability of the parasite was checked in the spiked semen mixture using a wet smear examination. After spiking, 1 ml of the spiked semen was kept aside and served as pre-SLC positive control samples and the remaining 5 ml of the spiked semen was used for SLC (Table 5.1).

SLC was conducted following the procedures as previously described (Hoogewijs et al., 2011; Morrell et al., 2013). Briefly, 5 ml of the spiked semen was carefully layered in conical plastic tubes on top of 5 ml pre-warmed Androcoll-E®. The tubes were centrifuged at 300 g for 20 min at ambient temperature. The supernatant was separated by aspiration using an electrical pipette for PCR analysis. Then the sperm pellet was washed by centrifugation in 5ml of fresh semen extender at 500 g for 10min. Subsequently, the supernatant at this point was discarded and the sperm pellet was re-suspended in 1ml fresh extender. Both the SLC-pellet as well as the pre-SLC samples for each of the 6 different parasite concentrations of all replications were tested for the presence of *T. equiperdum* by wet smear examination and PCR. Non-spiked semen samples were used as negative controls. Aliquots of the spiked pre-SLC samples as well as the pellets and supernatants after SLC were used for DNA extraction and ITS1-PCR for the detection of trypanosome DNA.

5.2.4.DNA extraction and PCR

DNA extraction was performed using a DNA extraction Kit for blood and tissues (DNeasy Blood and Tissue Kits, Qiagen, Germany) following the protocol recommended to isolate DNA from animal tissue (Qiagen, 2006). After extraction, DNA was stored at −20°C until PCR analysis. The DNA concentrations were measured using the Nanodrop ND-2000 UV-Vis spectrophotometer (NanoDrop Technologies, USA) and all samples were checked by cytochrome b PCR (gene common to vertebrates).

The DNA samples were subjected to PCR targeting the internal transcribed spacer-regions (ITS1), with forward primer 5'TGTAGGTGAACCTGCAGCTGGATC-3' and reverse primer 5'-CCAAGTCATCCATCGCGACACGTT- 3' (Fikru et al., 2012). The cycling condition of ITS1 PCR was initial PCR reaction at 95°C for 5 min, 34 cycles of denaturation at 94°C for 30 s, annealing temperature of 60°C for 30 s, and elongation reaction at 72°C for 30 s and a final extension at 72°C for 5 min. All PCR amplifications were carried out in 200 µl thin-wall PCR tubes (Thermo Fisher Scientific, USA) in Veriti thermal cycler 96 wells (Applied Biosystems, USA). The reaction mixture was a 25 µl containing 50 ng DNA, 1x Green GoTaq G2 Flexi buffer, 2 mM of MgCl2, 0.2 mM of each dNTPs, 0.5 µM of each primer, 1.25U GoTaqG2 Flexi DNA polymerase. Ten

microliters of the amplified product was used for electrophoresis in 2% agarose gel at 85V for 35 min and stained with ethidium bromide for visualization under UV light.

5.2.5. Mice inoculation

Since the presence of the parasite DNA in the pellet is not necessarily an indication for its infectivity, mice inoculation was performed. In this case one ejaculate, spiked with the different number of trypanosomes (same wise above Table 5.1) was used. After SLC the pellet of each sample was inoculated into 5 mice. Pre SLC spiked mixture of each sample was also inoculated into 1 mice as a positive control. Parasitaemia in the mice was checked daily by wet smear examination of blood taken from the tip of the tail. The pellets were also tested by PCR. Ethical approval was obtained from the Ethical Review Committee of Addis Ababa University College of Veterinary Medicine and Agriculture (Certificate Ref. No: VM/ERC/004/07/015).

5.2.6. Statistical Analysis

The odds of finding trypanosomes after purification was modelled as a function of the spiking dose by exact logistic regression. The analysis was done by SAS Version 9.4 statistical software.

5.3. Results

5.3.1. Wet smear and PCR

After the SLC, the pellets from the sperm samples spiked with different numbers of parasites were all found to be negative for *T. equiperdum* by wet smear examinations at dark field microscopy (x400) (Table 5.2). Before SLC, all spiked semen mixtures, except the negative controls, were positive on PCR analysis. After SLC, PCR analysis could not detect any parasite-DNA in the SLC-pellet of semen spiked with the lower number of parasites 10^{2} - 10^{3} and >10^{3} – 10^{4} in none of the triplicates (N= 3). However, in the SLC pellets of the >10^{4} - 5x10^{4} trypanosomes, 3 out of 4 of the replicates were positive for parasite DNA and all replicates (N= 4) of the group spiked with a higher number of trypanosomes (>5x10^{4}), were found positive on PCR indicating the presence of parasite DNA in the pellet (Table 5.2). The odds of finding trypanosomes by PCR in the semen increased significantly with increasing spiking dose (odds ratio=2.82, P=0.0013).

Table 5.2. Wet smear examination and PCR results of SLC purified pellets of *T. equiperdum*-spiked semen

No of trypanosomes in the spiked semen	**Wet smear examination**	**PCR (positive/ replication)**
Negative control	-	0/1
10^2 - 10^3	0/3	0/3
$>10^3$ - 10^4	0/3	0/3
$>10^4$ - $5x10^4$	0/4	3/4
$>5x10^4$ - $5x10^5$	0/4	4/4
$>5x10^5$- $5x10^6$	0/4	4/4
$>5x10^6$	0/4	4/4

5.3.2. Mice inoculation

Mice inoculated with SLC-pellets from the negative control group or with SLC-pellets from semen spiked with low numbers of parasites (10^2 to $5x10^4$ trypanosomes) did not develop parasitaemia (0/20). However, when inoculated with SLC-pellets from semen initially spiked with the higher number of parasites ($>5x10^4$ trypanosomes), most of the mice were found to exhibit parasitaemia, as did all mice inoculated with the spiked pre-SLC samples (positive control). For these positive controls, all mice exhibited parasitaemia (5/5) whereas inoculation with SLC-purified semen initially spiked with $>5x10^4$ - $5x10^5$ and $>5x10^5$- $5x10^6$ trypanosomes lead to parasitaemia in 2 of the 5 mice and in the one spiked with $>5x10^6$ trypanosomes, in 3 of 5 mice (Table 5.3). The odds of mouse infection with spiked purified semen increased significantly with increasing spiking dose (odds ratio=8.82, $P<0.001$).

Table 5.3. Mice parasitaemia post inoculation using SLC purified pellets of *T. equiperdum*-spiked semen

No of trypanosomes in the spiked semen	**Number of mice positive/inoculated**	
	Pre-SLC sample (Pos control)	SLC purified pellet
Negative control	nd	0/5
10^2 - 10^3	1/1	0/5
>10^3 - 10^4	1/1	0/5
>10^4 - 5x10^4	1/1	0/5
>5x10^4 - 5x10^5	1/1	2/5
>5x10^5- 5x10^6	1/1	2/5
>5x10^6	nd	3/5

nd= not done

PCR analysis could not detect any parasite-DNA in the SLC-pellet of the semen spiked with the lower number of parasites of 10^2 - 10^3 to 5x10^4 trypanosomes (Figure 5.1).

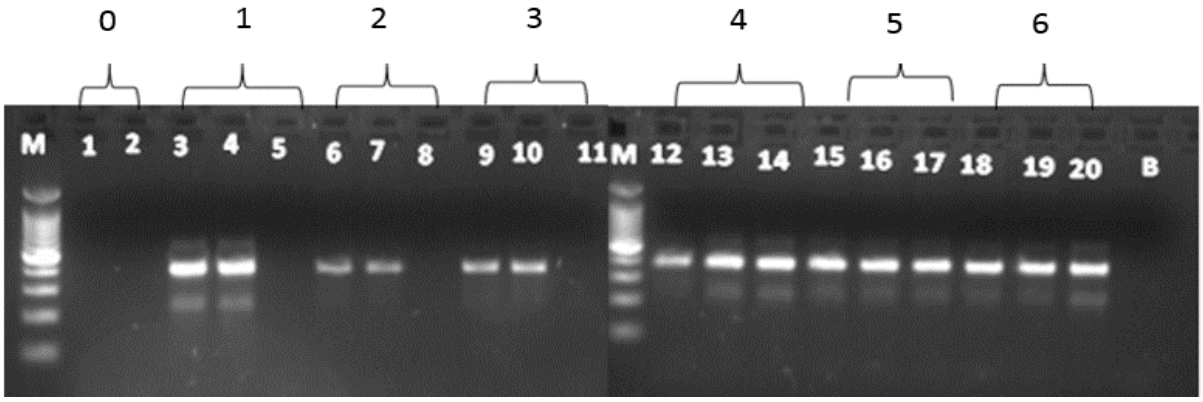


Figure 5.1. PCR profile of SLC-pellets spiked with different concentrations of *T. equiperdum*.Lane M: 100 bp plus marker, lanes 1&2: Supernatant and pellet of the negative control; lanes 3,6,9,12,15&18: the mixtures of spiked semen before centrifugation for each grade of parasitaemia; lanes 4,7,10,13,16&19: the supernatant after centrifugation; lanes 5,8,11,14,17&20: SLC-pellets after centrifugation and lane B: Blank. Numbers on top (0-6) are for samples refer to the increasing number of trypanosomes in the semen samples with 0: Non-spiked semen (negative control); 1-6: spiked semen (Table 5. 1).

5.4. Discussion

The outcome of this study shows that single layer centrifugation can be used to clear *T. equiperdum* infected semen but that the success of purifying the semen is dependent on the number of parasites. For semen spiked with low numbers of trypanosomes ($<5x10^4$), inoculation of the SLC-pellets in mice did not lead to parasitaemia and all pellets also proved to be negative on PCR. However, for samples spiked with more than $5x10^4$ Trypanosomes, SLC was not successful to clear the pellet, as some of the mice inoculated with the pellets developed parasitaemia. In addition, the samples also tested positive for the ITS1 PCR. Although the higher number of parasites used in this study ($>10^4$ Trypanosomes) are comparable to blood parasite concentrations in acutely infected animals, they most likely do not resemble the number of parasites present in the semen of infected animals. For example, semen collected from a stallion positive for *T. equiperdum* tested negative by wet smear examination but did induce parasitemia in mice following inoculation, suggesting that the number of parasites present in the semen was less than 10^2 (personal observation). This study is the first to describe the detection of *T. equiperdum* in semen by use of PCR and as such the sensitivity of the technique in this matrix is still to be determined. Nevertheless, the use of the qualitative statement of the PCR (on top of the mice inoculation test) preserves its added value in the current trial.

Since treatment options for dourine are limited and results are disappointing (Habte et al., 2015; Cauchard et al., 2016), other measures should be taken into account in an attempt to eliminate dourine in endemic areas. When dealing with a disorder that is transmitted by venereal routes, it seems obvious that in an attempt to eliminate such a condition or even to diminish the morbidity of the disease, life cover should be replaced by artificial insemination (AI). The intense contact during life covering, and the presence of the parasite in penile, scrotal, and urethral tissue (lesions) (Blue, 1985; Suganuma et al., 2016) will be avoided when AI is applicated. As shown in this study, SLC purification of trypanosome infected semen is feasible and by use of AI can be included in management measures in dourine endemic regions. However, until now, AI in horses is not commonly practiced and does not suit into the free pasture grazing extensive management system of horses practiced in Ethiopia. Since morbidity and mortality rates of dourine are quite high, and venereal transmission is the most

common route of spreading the disease, a more intensive management, with inclusion of AI, might be, at least for some populations, included in a strategy to eradicate dourine. Previously, dealing with contagious equine metritis, another venereal transmitted disease in equines, caused by *Taylorella equigenitalis*, the routine application of AI in an affected horse population was able to eradicate or at least diminish the prevalence of contagious equine metritis to only incidental findings in Europe (Timoney, 1996). Some nations reported to have eradicated contagious equine metritis including certain European countries, the U.S., Canada, Australia and Japan due to control programs of strict hygiene, integrated diagnosis and treatment and use of artificial insemination (Anzai et al., 2011; Schulman et al., 2013; CFSPH, 2015). The question, of course, remains at what concentration the parasite is typically present in an ejaculate and if a more persevering centrifugation protocol might be capable to remove also the higher number of parasites from the semen.

Chapter 6

General Discussion

6.1. Introduction

Dourine in Ethiopia is known by local farmers for many years and is considered a threat to the life and productivity of the equine population in the Arsi-Bale highlands (Zeleke et al., 1980). The reports of the presence of *T. equiperdum* causing dourine in Ethiopia are based on clinical signs and parasitological, serological and molecular tests (Zeleke et al., 1980; Alemu et al., 1997; Clausen et al., 1999; Gari et al., 2010; Hagos et al., 2010a,b). Nowadays animal import regulation and veterinary border control requires dourine free certificates in many countries in the world. For exmplein the case of Ethioia, Addis Ababa is the capital of the African Union and there are a lot of embassys of the United Nations and western world. Ambassadors from Europe and the United Nations are now a days requesting export certificates for their animals, especially pets and horses, when leaving Ethiopia after they completed their assignments. To enable export, horses should be free of dourine.

Literature is far more scarce and much less detailed when it comes to the description of the pathogenesis of *T. equiperdum* and its transmission, the distribution of the parasite in the host tissue once it escapes the periferal blood, the histopathological effects in the host and the treatment options of dourine affected animals. Furthermore, there were no published reports on the presence of the parasite in the semen. Likewise, the possibility to use AI to prevent dourine transmission of infected stallions after removal of the parasite from the semen by SLC is unknown. In an attempt to fill the gaps in the pathogenesis and the transmission of dourine in horses, this project tried to clarify the pathogenesis of naturally and artificially infected animals and to study the venereal transfer in more detail as well as the possibility to purify semen from infected animals. The first objective was achieved by carrying out descriptive studies of symptomatology, serology and haematological changes in natural- and artificial infected animals on the one hand, and by carrying out extensive post mortem examinations, histology and PCRs on tissues of these infected animals on the other hand (chapter 3 and 4). Furthermore, insemination trials were performed, infected semen was purified and the quality of purification was tested by PCR and the infectivity after purification of semen was evaluated by mice inoculation trials (chapter 5).

6.2. Clinical and histopathological features of *Trypanosoma equiperdum* infection

In this study we used dourine diseased horses both from natural infection from dourine endemic areas of the Arsi-Bale highland of Ethiopia and experimentally infected horses inoculated with a known *T. equiperdum* strain of Dodola 943 previously isolated and cryopreserved in liquid nitrogen (Hagos et al., 2010c).

The natural infected, dourine diseased horses were selected after screening more than 100 clinically suspected horses in Dodola (Fig 6.1), a dourine endemic region, by use of parasitological (Woo test) and serological tests (CATT/*T. evansi*) (Claes et al 2003b; Gari et al., 2010; Hagos et al., 2010a). Four horses (two stallions and two mares) were selected based on the clinical signs, serological result and a low PCV value and were subjected to necropsy. Tissue samples from different organs were collected for molecular (PCR) diagnosis of the parasite and for histopathological evaluation of the tissues for the presence of the parasite.

Figure 6.1. Field activities: screening of dourine diseased horses at Dodola using clinical, parasitological serological tests and necropsy for pathological examination (up left to right: clinical examination, blood collection, centrifugation for Woo test, bottom left to right: microscopic examination, serology, necropsy)

During the selection process, from the total of 100 suspected horses, 40% were found positive on CATT/*T.evansi* test and 22% were found to show at least one of the clinical signs of dourine. The mean PCV of serologically positive horses was significantly lower than in negative ones. Even though several attempts have been made to isolate the parasite in the buffy coat using the Woo test from clinically and serologically (CATT/ *T. evansi*) positive horses and those with

a history of a dourine infection, no trypanosomes were detected in examined blood samples. This could be due to the low number of parasites normally present in blood and the mild, short-lasting parasitaemia (Hoare, 1972; Stephen, 1986; Pascuccci et al., 2013). *T. equiperdum* is considered primarily a tissue parasite in nature and is rarely found in blood (Hoare, 1972; Stephen, 1986). The inability to find the parasite (using the Woo test) and the low sensitivity of the serological CATT test based on *T.evansi*, are typical challenges in the diagnostics of dourine caused by *T. equiperdum* in horses (Zablotskij et al., 2003).

Tissue samples at necropsy from the predilection places such as the reproductive organs and the nervous system were positive for *T. equiperdum* by real time PCR using the ITS1 primers with a base pair specific to the subgenus Trypanozoon.

In horses, the differentiation between an infection with *T. evansi* and the sexually transmitted *T. equiperdum* is challenging and is based on the absence of maxicircles in the kDNA of *T. evansi* while *T. equiperdum* can be revealed by maxicircle specific PCR (Claes et al. 2003b; Claes et al. 2004; Li et al. 2007a). Interestingly, DNA extracted from the vagina, testicles, sacrococegeal spinal cord and caudal nerves (sciatic and obturator nerves) of horses with clear signs of dourine were positive for *T. equiperdum* using maxicircle primers in the current study. Birhanu and colleagues (2016) confirmed that all *T. evansi* strains are negative but *T. equiperdum* strains are positive for the maxicircle PCR. Even though it remains impossible to separate the Ethiopian *T. equiperdum* from tsetse transmitted *T. brucei* based on the presence of this target gene, however, the Arsi-Bale region (Ethiopia), where the current dourine diseased horses came from (chapter 3), is out of the tsetse belt making differentiation between *T. bruci* and *T. equiperdum* more obvious. The trypanosomes responsible for AAT in Ethiopia are *T. vivax*, *T. congolense*, *T. brucei*, *T. evansi* and *T. equiperdum* (Dagnatchew, 1982). *T. congolense* and *T. brucei* are exclusively found in the tsetse-infested areas of Ethiopia while *T. evansi* and *T. equiperdum* occur in the tsetse-free areas. *T. vivax* can be found in both tsetse-infested and tsetse-free areas except in the highlands, which are >2500 meter above sea level (Dagnatchew, 1982; Abebe and Yilma 1996). This study is the first to differentiate *T. equiperdum* from *T. evansi* unequivocally in dourine diseased horses in the field. Previous studies (Alemu et al., 1997; Clausen et al., 1999; Gari et al., 2010; Hagos et al., 2010a,b) used

either PCR targetting genes of the subgenus Trypanozoon or serology to diagnose dourine in the area, resulting in uncertainty compared when using this more specific maxicircle PCR. Prospective studies in the area to determine the prevalence and some management aspects should base on this diagnostic approach.

There were no clear gross lesions in naturally dourine diseased horses at necropsy in the current study except a small haemorrhage in the spinal cord. Lesions not specifically related to dourine such as fibrous adhesion between the caecum and right ventral colon, multifocal to coalescent haemorrhages and milliary brown pinpoint foci and some firm nodules and distended interlobular septa of the lung, moderate amount of thick mucus within the trachea, some pinpoint white zones in the liver and nematode parasites such as *Oxyuris equi* in the intestestines were observed, which were not consistent to all horses. Some lesions in the liver were observed as granulomatous in the histopathology. This might be due to the migration of larvae of the nematode parasites. In the literature, reports of the pathological lesions caused by *T. equiperdum* in horses are very scanty. Gross reproductive pathological lesions such as congestion of uterine mucosa are reported by Pascucci et al. (2013) and Gizaw et al. (2017). They observed no lesions in the parenchymatous organs except congestion of the spleen. There was an increased amount of fluid in the abdominal cavity which might occur due to hypoproteinemia mainly caused by low albumin levels in trypanosomosis cases (Orhue et al. , 2005).

In the natural infected horses, microscopic lesions were observed in organs responsible for the clinical signs of the disease. These organs include the genital tract, the distal part of the spinal cord and caudal peripheral nerves such as the sciatic and obturator nerves. In the genital tract of mares, infiltration of mononuclear cells and periglandular inflammation in the vulva and vagina was observed. Mild to severe lymphocyte and plasma cell infiltration of the mucosa of vagina and cervix and uterus observed in this study were also seen in experimentally infected horses (chapter 4).

Depigmentation around the perineum, as explained in chapter 3 and 4, often described to be pathognomonic of clinical cases of dourine was seen in many cases in the screened population

(chapter 3) and artificial infected horses (chapter 4). Concerning the depigmentation spots observed in the naturally infected horses it was however not clearly known whether these were already present before the *T. equiperdum* infection. In theory, a preceeding- or co-infection with Equine Herpes Virus 3 (EHV3) infection might have caused similar lesions. EHV3 is manifested by the appearance of depigmented spots on the vaginal mucous membrane (Blanchard et al., 1992; Studdert, 1996; Allen and Umphenour, 2004), which is somewhat similar to the genital form of dourine. By contrast however, EHV3 is self-limited and is not accompanied by emaciation, ataxia or incoordination.

6.3. Transmission pathogenesis and tissue distribution of the parasite

Sexual transmission of *T. equiperdum* was already described a long time ago (Barrowman, 1976) but erratic in nature because a positive mare served by a negative stallion and viceversa did not always caused infection. Barrowman (1976) concluded that the animal is most likely to transmit the infection during the early stages of the disease and becomes later on a less active propagator of this disease. This may be dependent on the presence of active trypanosomes in the genital mucous membrane at the time of coitus. Parkin (1948) found that trypanosomes were demonstrable in the vaginal washings of recently infected mares but not in those with long standing infections. This was further confirmed by (Pascucci et al., 2013; Vulpiani et al., 2013). The presence of the trypanosome in the male reproductive tract was also shown by the isolation of the parasite from the urethral tract of a dourine diseased stallion (Suganuma et al., 2016) which can explain the transmission of the disease by coitus.

In the current study in chapter 4, mares were infected by artificial insemination with *T. equiperdum*-spiked semen, where upon the parasite reaches the peripheral circulation and exhibited a likewise pathogenesis as in stallions infected by blood transfusion. In stallions, trypanosomes could be detected in semen very early post infection preceeding the development of clinical signs. Even in the late chronic stage of the disease or post Cymelarsan® treatment, where parasitaemia was absent, parasites could be found in epididymal semen. This study completes and fills in the gaps in the venereal transmission pathway of dourine and co-occuring dangers for disease transmission by AI.

To describe the whole picture of the capacity and pathways of the venereal transmission of *T. equiperdum,* alternative experimental infections are needed. More specific experiments with negative stallions mounting infected mares in the preclinical, acute, chronic and post treatment stage, would complete the experiments as performed in chapter 4. Also direct vertical transmission to the offspring, i.e. intrauterine, or indirect, during parturition or suckling, should be given proper attention. Even more, since there is a similarity in pathology, symptology, parasite biology and molecular characterstics between *T. evansi* and *T. equiperdum* (Brun et al., 1998; Ahmed et al., 2018), it is recommended to confirm or deny scientifically the sexual transmission capacity of *T. evansi* in order to consider the veneral transmission route as a biological parameter to differentiate *T. evansi* from *T. equiperdum.* Thus controlled experimental studies as described in chapter 4 and discussed here above should be conducted with *T.evansi* as inoculans.

At necropsy the tissue distribution of the parasite was screened at first using gentle pressure of a glass slide on a freshly cut surface of the tissues of different organs where after the slides were stained with Diff-quick. Special attention was given to tissues of the nervous system and reproductive tract. This rather simple "impression smear" technique, as used to detect Leishmania and Toxoplasma (Swierczewski et al., 2013), was able to detect the parasite in easy to reach, voluminous organs (eg. testicle, uterus, ovary, brain) during necropsy, although further analysis by use of PCR (Table 4.4, chapter 4) could demonstrate *T. equiperdum* in several more organs (e.g. spinal cord, lymphnodes, spleen, heart). When comparing the results of the screening (impression smear) test with the PCR, no tissues appeared to be positive for *T. equiperdum* by impression smear and negative on PCR, but vice versa, several organs were positive on PCR where the impression smear technique couldn't be used (e.g. spinal cord, peripheral nerves) or was negative (e.g. lymphnodes, spleen). The distribution of *T. equiperdum* involves a variety of tissues, most commonly the reproductive organs and nervous system. Similar findings were reported earlier (Pascucci et al., 2013). The presence of the parasites in these tissues seems to confirm the specific tropism of *T. equiperdum* (Barrowman, 1976). In stallions, *T. equiperdum* inoculated by blood transfusion, caused dourine (chapter 4), disseminated to different tissues including the brain and the testicles, tissues that both are separated by a tissue-blood barrier. In another report, intratesticular

inoculation of the *T. equiperdum* leads to the development of orchitis in rabbits and isolation of the parasite from testicular oedema was possible (Pascucci et al., 2013) evincing the capacity of the parasite to distribute quite easily throughout the body regardless of any tissue barrier.

In the acute phase of the disease, Cymelarsan® treatment of dourine horses resulted in a significant increase in mean PCV values and a cure rate of 100% (Hagos et al., 2010). However, in the chronic stage, after day 60 post-infection of *T.equiperdum* infected horses, Cymelarsan® treatment was found to be ineffective (chapter 4), resulting in no improvement of clinical signs whatsoever, a relapse in parasitaemia and infectivity. On post-mortem examination, in both treated and non-treated animals, parasites could be found by impression smears and qPCR in testicles, uterus, brain and many other organs. Similar pathological lesions in both groups were found on histopathology.

The differences in cure rate between the current study (chapter 4) and the treatment in the acute phase (Hagos et al., 2010c) might find its explanation in the fact that once the parasites disseminate out of the peripheral circulation (chronic stage) they escape the treatment. This seems to be confirmed by Cauchard et al. (2016) and Hébert et al. (2018) who indicated a failure of Cymelarsan® treatment to clear of *T. equiperdum* OVI from the cerebrospinal fluid. Development of parasite resistance to anti trypanosomal drugs such as diminazine aceturate and isomethamidium chloride is reported in various *Trypanosoma* species in Ethiopia (Miruk et al., 2008; Moti et al., 2012; Dagnachew et al., 2015; Moti et al., 2015). Resistance of *T. evansi* to suramin has been observed experimentally in Sudan (El Rayah et al., 1999), China (Zhou et al., 2004) and Kenya (Otsyula et al., 1992). The hypothesis seems however less likely since, at least when dealing with *T. evansi*, there are no reports about development of resistance against Cymelarsan® in a study that analysed the resistance level of *T. evansi* isolated from the blood and CSF of relapsed horses after treatment with Cymelarsan® (Ravenborg, 1990). However *T. equiperdum* OVI has been reported to be less sensitive *in vitro* to Cymelarsan® than a panel of 19 other *T. equiperdum* and *T. evansi* strains, even though the *T. equiperdum* Dodola strain used by Hagos and colleagues (2010c) was not included in this study (Gillingwater et al., 2007). RAPD cluster analysis (Hagos, 2010) and genome-wide SNP

analysis (Cuypers et al., 2017) of many strains in the *Trypanozoon* subgenus assigned *T. equiperdum* Dodola in to *T. equiperdum* OVI group. Even though *T. equiperdum* Dodola is grouped to OVI, its known to be sensitive *in vitro* to Cymelarsan® (Büsher P., Personal communication, April 10, 2019). The limited numbers of horses included in our treatment study (N=3, chapter 4) is insufficient to conclude however the study of Hagos et al. (2010c) was as wel limited to 4 horses (i.e. 2 horses at 0.25mg/kg bw and 2 at a double doses) . Another study (Hébert et al., 2018) included 8 horses and saw as well no differences.

6.4. Semen purification and the use of AI

Since treatment options for dourine are limited and results are disappointing (Habte et al., 2015; Cauchard et al., 2016; Hébert et al., 2018), other measures should be taken into account in an attempt to eradicate dourine in endemic areas. When dealing with a disorder that is transmitted by venereal routes, it seems obvious that in an attempt to eradicate such a condition or even to diminish the morbidity of the disease, life cover should be replaced by artificial insemination (AI).

The intense contact during life covering and the presence of the parasite in penile, scrotal, and urethral tissue (lesions) (Blue, 1985; Suganuma et al., 2016) will be avoided when AI is applied. For example the Balkan countries in Europe such as Bulgaria, Czechoslovakia, Hungary, Rumania and Russia have implemented artificial insemination in horses in the 1950s and 1960s mainly for the control of dourine, and this aim has been achieved (Bowen, 1969). Importation of semen from non-endemic areas can be included in the management program. This might help to improve the genetic stock of the breeds as well. However, until now, AI in horses is not commonly practised and does not suit into the free pasture grazing extensive management system of horses practised in Ethiopia. Although the technique of equine AI as such is not difficult and AI is currently quite commonly practiced in Ethiopian dairy cattle practice. Since morbidity and mortality rates of dourine are quite high, and venereal transmission is the most common route of spreading the disease, a more intensive management, with the inclusion of AI, keeping selected breeding stallions and castrating the rest of the male population, might be, at least for some populations, included in a strategy to

eradicate dourine. However, there is a reluctance on the part of the local population to castrate stallions and thus lose the vigorous nature of the stallion which, scientifically or otherwise, is appreciated in sports and work performances. A side track in the venereal transmission story might be the custom of using donkeys to obtain mules which is still common practice and usually done intentionally and driven by deliberate action of the owners instead of free mating in the field.

In chapter 5, single layer centrifugation was applied to clear *T. equiperdum* from spiked semen but the success appeared to be dependent on the number of parasites present in the infected semen sample. Semen spiked with low numbers of trypanosomes ($<5x10^4$/ml) could be purified by the technique leading to a semen pellet that was not infective to mice when used in intraperitoneal inoculation and proved to be negative on PCR. When using higher numbers of parasites ($>10^4$ trypanosomes/ml), a concentration that is comparable to blood parasite concentrations in acutely infected animals, but much higher than numbers of parasites present in semen of infected animals, the SLC was partially successful (10^4 - $5x10^4$/ml) i.e. still positive on PCR however, not infectious anymore to mice or totally unsuccessful when concentrations over $5x10^4$ trypanosomes/ml were used as spike doses. Since this study is the first to describe the detection of *T. equiperdum* in semen by use of PCR, the sensitivity of the technique in this matrix is still to be determined. The question remains at what concentration the parasite is typically present in the ejaculate and if a more persevering centrifugation protocol might be capable to remove also the higher number of parasites from the semen sample.

In conclusion, *T. equiperdum* was found to be distributed in a number of tissues with many histopathological features especially in the spinal cord, peripheral nerves and the genital organs. Lesions involved include mainly massive infiltration of mononuclear cells especially lymphocytes, plasma cells and few macrophages. The lesions in the peripheral nerves and the spinal cord explain the clinical diagnostic observations of incoordination in the hind legs. In the experimental infection trial we were able to infect mares by AI with *T. equiperdum* spiked semen, resulting in diseased mares exhibiting all diagnostic clinical symptoms and changes in blood parameters. The parasites disseminated to blood and several tissues including the

nervous system and reproductive organs. Stallions infected intravenously were found to excrete the trypanosomes in the semen early before apparent clinical signs were observed and even after treatment with Cymelarsan®. Single layer centrifugation was able to clear *T. equiperdum* infected semen but the success is dependent on the number of trypanosomes.

References

Abebe, G., 2005. Trypanosomosis in Ethiopia. Ethiop.J.Biol.Sci. 4, 75-121.

Abebe, G., Yilma, J., 1996. Trypanosomosis: a threat to cattle production in Ethiopia. Rev. Med. Vet. 147, 897-902.

Adeiza, A.A., Maikai, V.A., Lawal, A.I., 2008. Comparative haematological changes in experimentally infected Savannah brown goats with *T. brucei* and *T. vivax*. Afr.J. Biotechnol. 7, 2295-2298.

Ahmed, Y., Hagos, A. Merga, B., Van Soom, A. Duchateau, L., Goddeeris, B.M., Govaere, J., 2018. *Trypanosoma equiperdum* in the horse – a neglected threat? Vlaams. Diergen. Tijds. 87, 66-75.

Ahmed, Y., Hagos, A., Merga, B., Alemu, T., Van Soom, A., Duchateau, L., Goddeeris, B., Govaere, J., 2017. Infectiousness of equine semen in the prepatent phase of dourine. Reprod. Dom. Anim. 52 (Suppl. 3), 66.

Akhmetova, G.D., Sabirovna, S.G., Eldesbaevna, T.G., Suttybaevna, U.S., Omirtaevna, N.B., Mikhdatovich, K.D., Bilalovich, A.K., Yergalievna, M.G., Ualievich, A.D., 2016. Isolation virulent strain of the *Trypanosoma equiperdum* for preparation of trypanosoma antigen. J. Biotechnol. 231S, S4–S109.

Alemu, T., Luckins, A.G., Phipps, L.P., Reid, S.W.J., Holmes, P.H., 1997. The use of enzyme linked immunosorbent assays to investigate the prevalence of *Trypanosoma equiperdum* in Ethiopian horses. Vet. Parasitol. 71, 239-250.

Allen, G., Umphenour, N., 2004. Equine coital exanthema. In: Coetzer, J., Tustin, R. (Eds.), Infectious Diseases of Livestock. Oxford Press, Cape Town, South Africa, pp. 860–867.

Anosa, V.O., Logan-Henfrey L.L., Shaw, M.K., 1992. Light and electron microscopic study of changes in blood and bone marrow in acute haemorrhagic *T. vivax* infection in calves. Vet. Pathol. 29, 33-35.

Anzai, T., Kamada, M., Niwa, H., Eguchi, M., Nishi, H., 2011. Contagious equine metritis eradicated from Japan. J. Vet. Med. Sci. 74, 519-522.

Barrett, M.P., 2018. The elimination of human African trypanosomeiasis is in sight: Report from the third WHO stakeholders meeting on elimination of gambiense human African trypanosomiasis. PLoS Negl. Trop. Dis., 12(12):e0006925.

Barrowman, P.R., 1976. Observations on the Transmission, Immunology, Clinical Signs and Chemotherapy of dourine (*Trypanosoma equiperdum* infection) in horses, with special reference to Cerebro-Spinal Fluid. Onderstepoort J. Vet. Res. 43, 55-66.

Bassarak, B., Moser, I., Menge, C., 2016. In vitro production of *Trypanosoma equiperdum* antigen and its evaluation for use in serodiagnosis of dourine. Vet. Parasitol. 223, 133–140.

Birhanu, H., Gebrehiwot, T., Goddeeris, B.M., Büscher, P., Van Reet, N., 2016. New *Trypanosoma evansi* type B isolates from Ethiopian dromedary camels. PLoS Negl. Trop. Dis. 10, e0004556.

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

Blanchard, T., Kenney, R., Timoney, P., 1992. Venereal diseases. Vet. Clin. North Am. Equine Pract. 8, 191–203.

Blomqvist, G., Persson, M., Wallgren, M., Wallgren, P., Morrell, J.M., 2011. Removal of virus from boar semen spiked with porcine circovirus type 2. Anim. Reprod. Sci. 126, 108–114.

Blue, M.G., 1985. Genital injuries from mating in the mare. Equine Vet. J. 17, 297-299.

Bonfini, B., Tittarelli, M., Luciani, M., Di Pancrazio, C., Rodomonti, D., Iannetti, L., Vulpiani, M.P. and Di Febo, T., 2018. Development of an indirect ELISA for the serological diagnosis of dourine. Vet. Parasitol. 261, 86-90.

Borst, P., Fase-Fowler, F., Gibson, WC., 1987. Kinetoplast DNA of *Trypanosoma evansi*. Mol. Biochem. Parasitol. 23,31–8.

Bowen, J.M., 1969. Artificial insemination in the horse. Equine Vet. J. 1(3), 98-110.

Bringaud, F., Riviere, L., Coustou, V., 2006. Energy metabolism of trypanosomatids: adaptation to available carbon sources. Mol. Biochem. Parasitol. 149,1-9.

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

Bubis, J., Martínez, J.C., Calabokis, M., Ferreira, J., Sanz-Rodríguez, C.E., Navas, V., Escalona, J.L., Guo, Y. and Taylor, S.S., 2018. The gene product of a *Trypanosoma equiperdum* ortholog of the cAMP-dependent protein kinase regulatory subunit is a monomeric protein that is not capable of binding cyclic nucleotides. *Biochimie*, 146,166-180.

Büscher, P., 2014. Diagnosis of African trypanosomiasis. In S. Magez & M. Radwanska (Eds) Trypanosomes and trypanosomiasis, Wien: Springer-Verlag. pp. 189-216.

Büscher, P., Mumba-Ngoyi, D., Kabore, J., Lejon, V., Robays, J., Jamonneau, V., Bebronne, N., Van der Veken, W., Biéler, S., 2009. Improved models of mini Anion Exchange Centrifugation Technique (mAECT) and Modified Single Centrifugation (MSC) for sleeping sickness diagnosis and staging. PLoS Negl.Trop.Dis. 3, e471.

Calistri, P., Valeria, N., Marcello, A., Fabrizio, D.M., Manuela, T., Mercante, M.T., Enzo, R., Scacchia, M., 2013. Dourine Reemergence in Italy. J. Equine Vet. Sci. 33, 83-89.

Camara, M., Camara, O., Ilboudo, H., Sakande, H., Kabore, J., N'Dri, L., Jamonneau, V., Bucheton, B., 2010. Sleeping sickness diagnosis: use of buffy coats improves the sensitivity of the mini anion exchange centrifugation test. Trop. Med. Int. Health 15, 796-799.

Caporale, V.P., Battelli, G. and Semproni, G., 1980. Epidemiology of Dourine in the Equine Population of the Abruzzi Region. Zentralblatt Veterinarmedizin Reihe B 27(6), 489-498.

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

Carnes, J., Anupama, A., Balmer, O., Jackson, A., Lewis, M., Brown, R., Cestari, I., Desquesnes, M., Gendrin, C., Hertz-Fowler, C., Imamura, H., Ivens, A., Koreny, L., Lai, D.H., MacLeod, A., McDermott, S.M., Merritt, C., Monnerat, S., Moon, W., Myler, P., Phan, I., Ramasamy, G., Sivam, D., Lun, Z.R., Luke, J., Stuart, K., Schnaufer, A., 2015. Genome and phylogenetic analyses of *Trypanosoma evansi* reveal extensive similarity to *T. brucei* and multiple independent origins for dyskinetoplasty. PLoS Negl. Trop. Dis. 9 (1), e3404.

Cauchard, J., Carnicer, D., Madeline, A., Guitton, E., Giraudet, A., Büscher, P., Hebert, L., Laugier, C., 2016. Evaluation of Melarsamine hydrochloride (Cymelarsan®) efficacy for the treatment of dourine nervous form on experimentally infected ponies. J. Equine Vet. Sci. 39, S45eS55.

Cauchard, J., Soldan, A., Madeline, A., Johnson, P., Buscher, P., Petry, S., 2014. Inter-laboratory ring trials to evaluate serological methods for dourine diagnosis. Vet. Parasitol. 205, 70-76.

Cauchard, S., Van Reet, N., Büscher, P., Goux, D., Grötzinger, J., Leippe, M., Cattoir, V., Laugier, C., Cauchard, J., 2016. Killing of trypanozoon parasites by the Equine cathelicidin eCATH1. Antimicrob. Agents Chemother. 60, 2610-2619.

CFSPH, 2015. Center for Food Security and Public Health: Contagious Equine Metritis. Iowa state university, institute for internal cooperation in animal biologics accessed http://www.cfsph.iastate.edu/Factsheets/pdfs/contagious_equine_metritis.pdf

Claes, F., 2003. Characterization of pathogenic Trypanosma spp. how does *Trypanosoma equiperdum* fit into the Trypanozoon group. Katholic University of Leuven, Belgium, PhD. Thesis.

Claes, F., Agbo, E.C., Radwanska, M., Te Pas, M.F., Baltz, T., De Waal, D.T., Goddeeris, B.M., Claassen, E., Buscher, P., 2003b. How does *T. equperdum* fit into the Trypanozooan genus? A cluster analysis and multiplex genotyping approach. Parasitol. 126, 425-431.

Claes, F., Büscher, P., Touratier, L. Goddeeris B.M., 2005. *Trypanosoma equiperdum*: Master of disguise or historical mistake? Trends Parasitol. 21, 316–321.

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

Claes, F., Verloo, D., De Waal, D.T., Majiwa, P.A.O., Baltz, T., Goddeeris, B.M. Büscher, P., 2003a. The expression of RoTat 1.2 variable surface glycoprotein (VSG) in *Trypanosoma evansi* and *T. equiperdum*. Vet. Parasitol. 116, 209–216.

Claes, F., Verloo, D., De Waal, D.T., Urakawa, T., Majiwa, P., Goddeeris, B.M. and Büscher, P., 2002. Expression of RoTat 1.2 Cross-reactive Variable Antigen Type in *Trypanosoma evansi* and *T. equiperdum*. Ann. N. Y. Acad. Sci. 969(1), 174-179.

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

Clausen, P.H., Gebreselassie, G., Abditcho, S., Mehlitz, D., 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.

Coles, E.H., 1986. Veterinary clinical Pathology 4th ed. WB Saunders company, Philadelphia. London, pp.136-170.

Cuypers, B., Van den Broeck, F., Van Reet, N., Meehan, C.J., Cauchard, J., Wilkes, J.M., Claes, F., Goddeeris, B., Birhanu, H., Dujardin, J.C., Laukens, K., 2017. Genome-wide SNP analysis reveals distinct origins of *Trypanosoma evansi* and *Trypanosoma equiperdum*. Genome Biol. Evol. 9(8), 1990-1997.

Dagnachew, S., Bezie, M., Terefe, G., Abebe, G., Barry, J.D., Goddeeris, B.M., 2015a. Comparative clinico-haematological analysis in young Zebu cattle experimentally infected with *Trypanosoma vivax* isolates from tsetse infested and non-tsetse infested areas of Northwest Ethiopia. Acta Vet. Scand. 57, 24.

Dagnachew, S., Terefe, G., Abebe, G., Barry, D., McCulloch, R., Goddeeris, B.M., 2015b. *In vivo* experimental drug resistance study in *Trypanosoma vivax* isolates from tsetse infested and non-tsetse infested areas of Northwest Ethiopia. Acta Trop*.,* 146, 95-100.

Dagnatchew, Z., 1982. Trypanosomiasis in Ethiopia. International Symposia on Veterinary Epidemiology and Economics proceedings, ISVEE 3: Veterinary Epidemiology and Economics, Proceedings of the 3rd International Symposium, Arlington, Virginia, USA, pp. 467-473, Sep 1982.

Davaasurena, B., Amgalanbaatar, T., Musinguzi, S.P., Suganuma, K., Otgonsuren, D., Mossaada, E., Narantsatsral, S., Battur, B., Battsetseg, B., Xuan, X., Inouee, N., 2017. The evaluation of GM6-based ELISA and ICT as diagnostic methods on a Mongolian farm with an outbreak of non-tsetse transmitted horse Trypanosomosis. Vet. Parasitol. 244, 123–128.

Dean, S., Gould, M.K., Dewar, C. E., Schnaufer, A.C., 2013. Single point mutations in ATP synthase compensate for mitochondrial genome loss in trypanosomes. Proc. Natl. Acad. Sci. USA, 110, 14741-14746.

Delespaux, V., Adapted from: R.J Connor and P. Van Den Bossche, 2004, African animal trypanosomoses, In: Coetzer, J.A.W. and Tustin, R.C. (eds.) Infectious diseases of livestock, Oxford University Press, Cape Town, 12, 251–295.

Desquesnes, M., 2004. Livestock trypanosomoses and their vectors in Latin America. OIE (World Organisation for Animal Health). Paris, France, pp, 15-21. Accssed on 10/01/2019 from https://www.oie.int/doc/ged/D9818.PDF

Desquesnes, M., Bosseno, M.F., Brenière, S.F., 2007. Detection of Chagas infections using *Trypanosoma evansi* crude antigen demonstrates high cross-reactions with *Trypanosoma cruzi*. Infect. Genet. Evol. 7, 457-462.

Desquesnes, M., Holzmuller, P., Lai, D.H., Dargantes, A., Lun, Z.R., Jittaplapong, S., 2013. *Trypanosoma evansi* and Surra: A Review and Perspectives on Origin, History, Distribution, Taxonomy, Morphology, Hosts, and Pathogenic Effects. Biomed. Res. Int. 2013, 1–20. doi:10.1155/2013/194176.

Desquesnes, M., McLaughlin, G., Zoungrana, A., Dávila, A.M., 2001. Detection and identification of Trypanosomes of African livestock through a single PCR based on internal transcribed spacer 1 of rDNA. Int.J.Parasitol. 31, 610-614.

Discontools, 2011. Trypanosomiasis (African) Scores for None Tsetse transmitted. http://www.discontools.eu/Diseases/GenerateDiseaseCategories/61?type=pdf accessed on 1 November, 2015.

Domingo, G.J., Palazzo, S.S., Wang, B., Pannicucci, B., Salavati, R., Stuart, K.D., 2003. Dyskinetoplastic *Trypanosoma brucei* contains functional editing complexes. Eukaryotic Cell, 2, 569-577.

El Rayah, I.E., Kaminsky, R., Schmid, C., El Malik, K.H., 1999. Drug resistance in Sudanese *Trypanosoma evansi*. Vet. Parasitol. 80, 281-287.

Enwezor, F.N.C., Sackey, A.K.B., 2005. Camel Trypanosomosis, A Review. Vet. Arhiv. 75, 439-452.

Equimed, 2009. Dourine, Protozoal-based venereal disease. Accessed on 24/06/2017 from http://equimed.com/diseases-and-conditions/reference/dourine

FAO, 1998. A field guide for the diagnosis, treatment and prevention of African animal trypanosomosis. Uilenberg, G. and Boyt, W.P. (eds). Food and Agriculture Organization, Rome, Italy. http://www.fao.org/3/X0413E/X0413E02.htm#note1.3

Fikru, R., Goddeeris, B.M., Delespaux, V., Moti, Y., Tadesse, A., Bekana, M., Claes, F., De Deken, R. Büscher, P., 2012. Widespread occurrence of *Trypanosoma vivax* in bovines of tsetse-as well as non-tsetse-infested regions of Ethiopia: a reason for concern? Vet. Parasitol. 190, 355-361.

Fikru, R., Hagos, A., Rogé, S., Reyna-Bello, A., Gonzatti, M.I., Merga, B., Goddeeris, B.M., Büscher, P. 2014. A proline racemase based PCR for identification of *Trypanosoma vivax* in cattle blood. PLoS One, 9, e84819

Frasch, A.C., Hajduk, S.L., Hoeijmakers, J.H., Borst, P., Brunel, F., Davidson, J., 1980. The kinetoplast DNA of *Trypanosoma equiperdum*. Biochim. Biophys. Acta 607(3),397-410.

Gari, F.R., Hagos, A., Alemu, T., Goddeeris, B.M., Claes, F., 2010. Comparative diagnosis of parasitological, serological, and molecular tests in dourine-suspected horses. Trop. Anim. Health Prod. 42, 1649–1654.

Gillingwater, K., Büscher, P., Brun, R., 2007. Establishment of a panel of reference *Trypanosoma evansi* and *Trypanosoma equiperdum* strains for drug screening. Vet. Parasitol. 148, 114-121.

Gizaw, Y., Megersa, M., Fayera, T., 2017. Dourine: a neglected disease of equids. Trop. Anim. Health Prod. 49, 887–897.

Habte, B., Bsrat, A., Ashenafi, H., Regassa, F., 2014. Efficacy of Some Trypanocidal Drug against *Trypanosoma equiperdum* OVI in Experimentally Infected Mice in Debre Zeit, Ethiopia. Eur. J. Biol. Sci. 7 (1), 7-13.

Hagos A., Goddeeris B.M., Yilkal K., Alemu T., Fikru R., Yacob H.T., Feseha G., Claes F., 2010c. Efficacy of Cymelarsan® and Diminasan® against *Trypanosoma equiperdum* infections in mice and horses. Vet. Parasitol. 171, 200–206.

Hagos, A., 2010. Isolates of Trypanosomes from Ethiopian Horses. Katholic University of Leuven, Belgium, PhD. Thesis.

Hagos, A., Abebe, G., Büscher, P., Goddeeris, B.M., Claes, F., 2010a. Serological and parasitological survey of dourine in the Arsi–Bale highlands of Ethiopia. Trop. Anim. Health Prod. 42, 769-776.

Hagos, A., Degefa, G., Yacob, H., Fikru, R., Alemu, T., Feseha, G., Claes, F., Goddeeris, B.M., 2010b. Seroepidemiological survey of trypanozoon infection in horses in the suspected dourine-infected Bale highlands of the Oromia region, Ethiopia. Rev. Off. Int. Epizoot. 29(3), 649-654.

Hébert, L., Guitton, E., Madeline, A., Géraud, T., Zientara, S., Laugier, C., Hans, A., Büscher, P., Cauchard, J., Petry, S., 2018. Melarsomine hydrochloride (Cymelarsan®) fails to cure

horses with *Trypanosoma equiperdum* OVI parasites in their cerebrospinal fluid. Vet. Parasitol., 264, 47-51.

Herbert, W.J., Lumsden, W.H.R., 1976. *Trypanosoma brucei*: a rapid "matching" method for estimating the host's parasitemia. Exp. Parasitol. 40, 427-431.

Hoare, C.A., 1972. The trypanosomes of mammals. A zoological monograph. The trypanosomes of mammals. A zoological monograph. Blackwell Scientific Publications, Oxford, United Kingdom, pp. 593-604.

Hoogewijs, M., Morrell, J., Van Soom, A., Govaere, J., Johannisson, A., Piepers, S., De Schauwer, C., De Kruif, A., De Vliegher, S., 2011. Sperm selection using single layer centrifugation prior to cryopreservation can increase thawed sperm quality in stallions. Equine Vet. J. 43, 35-41.

Igbokwe, I.O., Umar, I.A., Omage, J.J., Ibrahim, N.D.G., Kadima, K.B., Obagaiye, O.K., 1996. Effect of acute *Trypanosoma vivax* infection on cattle erythrocyte glutathione and susceptibility to in vitro peroxidation. Vet. Parasitol., 63, 215-224.

IHSES, 2007. Import Health Standard For Equine Semen From The European Union Issued pursuant to Section 22 of the Biosecurity Act 1993 Dated: 01 August 2007 Available http://mpi.govt.nz/document-vault/1760 Online source Accessed 5 November 2015.

Jensen, R.E., Simpson, L., Englund, P.T., 2008. What happens when *Trypanosoma brucei* leaves Africa. Trends parasitol. 24(10),428-431.

John, M.K., John, K.T., Maina, N., Raymond, M., David, M.M., Joseph, M.N., 2006. Haematology of experimental *Trypanosoma brucei rhodesiense* infection in vervet monkeys. Afr. J. Health Sci. 13, 59-65.

Jones, T.C., Hunt, R.D., King, N.W., 1997. Veterinary pathology. 6th Ed. Baltmore (Md.): Williams and Wilkins, London, Philadelphia. Pp 177-196.

Kingston, D., Rodgers, J., Sharpe, S., Berman, K., Morrison, L., Kennedy, P., Bradley, B., Sutton, D.G.M., 2016. Equine central nervous system trypanosomosis in the Gambia is caused by genetically diverse populations of *Trypanosoma brucei* parasites. J. Equine Vet. Sci. 39, S100-S101.

Kumba, F.F., Claasen, B., Petrus, P., 2002. Apparent prevalence of dourine in the Khomas region of Namibia. Onderstepoort J. Vet. Res. 69, 295-298.

Lai, D.H., Hashimi, H., Lun, Z.R., Ayala, F.J., Lukeš, J., 2008. Adaptations of *Trypanosoma brucei* to gradual loss of kinetoplast DNA: *Trypanosoma equiperdum* and *Trypanosoma evansi* are petite mutants of *T. brucei*. Proceedings of the National Academy of Sciences, 105(6), 1999-2004.

Lanham, S.M., Godfrey, D.G., 1970. Isolation of salivarian trypanosomes from man and other mammals using DEAE-cellulose. Exp. Parasitol. 28, 521-534.

Lelli, R., Calistri, P., Giovannini, A., Caporale, V., 2012. Evidence of *T. equiperdum* infection in the Italian dourine outbreaks. J. Equine Vet. 32(10), S70-S71.

Levy, R., Bourlet, T., Maertens, A., Salle, B., Lornage, J., Laurent, J.L., Pozzetto, B., Guerin, J.F., 2002. Pregnancy after safe IVF with hepatitis C virus RNA-positive sperm**.** Hum. Reprod. 10, 2650-3.

Li, F.J., Gasser, R.B., Lai, D.H., Claes, F., Zhu, X.Q., Lun, Z.R., 2007. PCR approach for the detection of *Trypanosoma brucei* and *T. equiperdum* and their differentiation from *T. evansi* based on maxicircle kinetoplast DNA. Mol.Cell Probes 21, 1-7.

Li, F.J., Gasser, R.B., Zheng, J.Y., Claes, F., Zhu, X.Q., Lun, Z.R., 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.

Liu, B., Liu, Y., Motyka, S.A., Agbo, E.E., Englund, P.T., 2005. Fellowship of the rings: the replication of kinetoplast DNA. Trends parasitol. 21(8),363-369.

Losos, G. J., Ikede, B.O., 1972. Review of pathology of diseases in domestic and laboratory animals caused by *Trypanosoma congolense*, *T. vivax, T. brucei, T. rhodesiense* and *T. gambiense.* Vet. Pathol. 9(1_suppl), 1-79.

Losos, G.J., 1986. Infectious Tropical Disease of Domestic Animals. New York: Churchill Livingstone, Inc., Pp 182-318.

Luciani, M., Di Febo, T., Orsini, M., Krasteva, I., Cattaneo, A., Podaliri Vulpiani, M., Di Pancrazio, C., Bachi, A. and Tittarelli, M., 2018. *Trypanosoma equiperdum* low Molecular Weight Proteins as candidates for specific serological Diagnosis of Dourine. Front. Vet. Sci. *5*, 40.

Luckins, A.G., 1994. Equine Trypanosomosis. Equine Vet. Educ. 6, 259-262.

Luckins, A.G., 1988. *Trypanosoma evansi* in Asia. Parasitol. Today 4(5), pp.137-142.

Lukes, J., Hashimi, H. & Zíková, A. (2005). Unexplained complexity of the mitochondrial genome and transcriptome in kinetoplastid flagellates. Curr.Genet., 48, 277-299.

Lumsden, W.H., Kimber, C.D., Evans, D.A., Doig, S.J., 1979. *Trypanosoma brucei*: miniature anion-exchange centrifugation technique for detection of low parasitaemias: Adaptation for field use. Trans. R. Soc. Trop. Med. Hyg, 73, 312-317.

Lun, Z.R., Lai, D.H., Li, F.J., Lukes, J., Ayala, F.J., 2010. *Trypanosoma brucei*: Two steps to spread out from Africa. Trends Parasitol. 26, 424–427.

Lun, Z.R., Vickerman, K., 1991. Multinuclear forms in a dyskinetoplastic strain of *Trypanosoma evansi* in mice. Annales de parasitologie humaine et compare´e, 66, 51-53.

Masiga, D.K., Gibson, W.C., 1990. Specific probes for *Trypanosoma (Trypanozoon) evansi* based on kinetoplast DNA minicircles. Mol. Biochem. Parasitol. 40(2), 279-283.

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

Melo, C.M., Papa, F.O., Fioratti, E.G., Villaverde, A.I.S.B., Avanzi, B.R., Monteiro, G., Dell'aqua Junior, J.A., Pasquini, D.F., Alvarenga, M.A., 2008. Comparison of three different extenders for freezing epididymal stallion sperm. Anim. Reprod. Sci. 107, 331-331.

Metcalf, E.S., 2001. The role of international transport of equine semen on disease transmission. Anim. Reprod. Sci. 68, 229–237.

Miruk, A., Hagos, A., Yacob, H.T., Asnake, F., Basu, A.K., 2008. Prevalence of bovine trypanosomosis and trypanocidal drug sensitivity studies on *Trypanosoma congolense* in Wolyta and Dawero zones of southern Ethiopia. Vet. Parasitol., 152, 141-147.

Mizushima, D., Amgalanbaatar, T., Davaasuren, B., Molefe, N.I., Battur, B., Battsetseg, B., Inoue, N., Yokoyama, N., Suganuma, K., 2018. The utility of an rTeGM6-4r-based immunochromatographic test for the serological diagnosis of non-tsetse-transmitted equine trypanosomosis in rural areas of Mongolia. Parasitol. Res. 117(9), 2913-2919.

Morrell, J.M., Geraghty, R.M., 2006. Effective removal of equine arteritis virus from stallion semen. Equine Vet. J. 38, 224-229.

Morrell, J.M., Rodriguez-Martinez, H., 2009. Biomimetic Techniques for Improving Sperm Quality in Animal Breeding: A Review. Open Andrology J. 1, 1-9.

Morrell, J.M., Timoney, P., Klein, C., Shuck, K., Campos, J., Troedsson, M., 2013. Single-Layer Centrifugation Reduces Equine Arteritis Virus titer in the Semen of Shedding Stallions. Reprod. Dom. Anim. 48, 604–612.

Morrell, J.M., Wallgren, M., 2011. Removal of bacteria from boar ejaculates by Single Layer Centrifugation can reduce the use of antibiotics in semen extenders. Anim. Reprod. Sci. 123(1-2), 64-9.

Morrison, W.I., Murray, M., Sayer, P.D., Preston, J.M., 1981. The pathogenesis of experimentally induced *Trypanosoma brucei* infection in the dog. I. Tissue and organ damage. Am. J. Pathol. 102, 168.

Mossuto, M.F., Ami, D., Anelli, T., Fagioli, C., Doglia, S.M., Sitia, R., 2015. Biochemical nature of Russell bodies. Scientific reports, *5*, 12585.

Moti, Y., De Deken, R., Thys, E., Van den Abbeele, J., Duchateau, L., Delespaux, V., 2015. PCR and microsatellite analysis of diminazene aceturate resistance of bovine trypanosomes correlated to knowledge, attitude and practice of livestock keepers in South-Western Ethiopia. Acta Trop. 146, 45-52.

Moti, Y., Fikru, R., Van Den Abbeele, J., Büscher, P., Van den Bossche, P., Duchateau, L., Delespaux, V., 2012. Ghibe river basin in Ethiopia: present situation of trypanocidal drug resistance in *Trypanosoma congolense* using tests in mice and PCR-RFLP. Vet. Parasitol. 189, 197-203.

Musa, M.M., Abdoon, A.M.O., Nasir, B.T., Salim, Y.I., Abdel-Rahman, A.Y. and Shommein, A.M., 1994. Efficacy of Cymelarsan® in the treatment of natural chronic *Trypanosoma evansi* infection in camels in the Sudan. Rev. élev. méd. vét. pays trop. 47(4), 397-400.

Mungun-Ochir, B., Horiuchi, N., Altanchimeg, A., Koyama, K., Suganuma, K., Nyamdolgor, U., Watanabe, K.I., Baatarjargal, P., Mizushima, D., Battur, B. and Yokoyama, N., 2019. Polyradiculoneuropathy in dourine-affected horses. *Neuromuscular Disorders*. (2019), doi: https://doi.org/10.1016/j.nmd.2019.03.005. In Press.

Myers, R.K. McGavin, M.D., 2006. Cellular and Tissue Responses to Injury In: McGavin M.D., Zachary J.F.(eds.) Pathologic Basis of Veterinary Disease. Fourth ed. Mosby Elsevier, St. Louis, pp. 3-59.

Natalini, C.C., 2010. Spinal anesthetics and analgesics in the horse. Veterinary Clinics: Equine Practice 26, 551-564.

Nguyen, T.T., Motsiri, M.S., Taioe, M.O., Mtshali, M.S., Goto, Y., Kawazu, S., Thekisoe, OM., Inoue, N., 2015. Application of crude and recombinant ELISAs and immunochromatographic test for serodiagnosis of animal trypanosomosis in the Umkhanyakude district of KwaZulu-Natal province, South Africa. J. Vet. Med. Sci. 77, 217–220.

Nguyen, T.T., Zhou, M., Ruttayaporn, N., Nguyen, Q.D., Nguyen, V.K., Goto, Y., Suzuki, Y., Kawazu, S., Inoue, N., 2014. Diagnostic value of the recombinant tandem repeat antigen TeGM6-4r for surra in water buffaloes. Vet. Parasitol. 201, 18–23.

Njiru, Z.K, Constantine, C.C., Guya, S., Crowther, J., Kiragu, J.M., Thompson, R.C., Dávila, A.M., 2005. The use of ITS1 rDNA PCR in detecting pathogenic African trypanosomes. Parasitol. Res. 95(3), 186-92.

OIE 2013. Dourine, Chapter 2.5.3. OIE Terrestrial Manual Version adopted by the World Assembly of Delegates of the OIE in May 2013, Paris, Pp. 1-6.

OIE, 2000. Report of the meeting of the OIE Ad Hoc Group on Non-Tsetse Transmitted Animal Trypanosomosis (NTTAT), 68/SG/18, 19 May 1999, Paris, Pp 14.

OIE, 2012. Chapter 2.1.17 *Trypanosoma evansi* infection (surra). In Terrestrial Manual of Diagnostic Tests and Vaccines for Terrestrial Animals.

Orhue, N.E.J., Nwanze, E.A.C., 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.

Otsyula, M., Kamar, K., Mutugi, M.W., Njogu, A.R., 1992. Preliminary efficacy trial of Cymelarsan, a novel trypanocide, in camels naturally infected with *Trypanosoma evansi* in Kenya. Acta Trop. 50, 271-273.

Ou, Y.C., Giroud, C., Baltz, T. 1991. Kinetoplast DNA analysis of four *Trypanosoma evansi* strains. Mol. Biochem. Parasitol, 46, 97-102.

Paris, J., Max Murray, F., Mc Odimba, F., 1982. Comparative evaluation of the parasitological techniques currently available for the diagnosis of African trypanosomiasis in cattle. Acta Trop. 39, 307-316.

Parkin, B.S., 1948. The demonstration and transmission of the South African strain of *Trypanosoma equiperdtmt* of horses. Onderstepoort J. Vet. Sci. Anim. Industry 23, 41-57.

Parra, N., Jaume, M., Boscán, K., Hernández, A., Mijares, A., González, M., Alvarado, Y., Restrepo, J., 2017. Ex vivo trypanocidal activity of 1-(2-hydroxybenzylidene) thiosemicarbazide against *Trypanosoma equiperdum*. Vet. Parasitol. 245, 163–167.

Pascucci, I., Di Provvido A., Cammà C., Di Francesco G., Calistri P., Tittarelli M., Ferri N., Scacchia M., Caporale V., 2013. Diagnosis of dourine in outbreaks in Italy. Vet. Parasitol. 193, 30– 38.

Pepin, J., Milord, F., 1994. The treatment of human African trypanosomiasis. Adv. Parasitol. 33, 1–47.

Perrone, T., Aso, P.M., Mijares, A., Holzmuller, P., Gonzatti, M. and Parra, N., 2018. Comparison of infectivity and virulence of clones of *Trypanosoma evansi* and *Trypanosoma equiperdum* Venezuelan strains in mice. Vet. Parasitol. 253, 60-64.

Perrone, T.M., Gonzatti, M.I., Villamizar, G., Escalante, A., Aso, P.M., 2009. Molecular profiles of Venezuelan isolates of *Trypanosoma* spp. by random amplified polymorphic DNA method. Vet. Parasitol. 161(3-4), 194-200.

Pertoft, H., 2000. Fractionation of cells and subcellular particles with Percoll. J. Biochem.

Pyana, P.P., Ngay, Lukusa, I., Mumba Ngoyi, D., Van Reet, N., Kaiser, M., Karhemere Bin Shamamba, S., Büscher, P., 2011. Isolation of *Trypanosoma brucei gambiense* from cured and relapsed sleeping sickness patients and adaptation to laboratory mice. PLoS Negl. Trop. Dis. 5, e1025.

Qiagen, 2006. DNeasy Blood and Tissue Handbook 07/2006. QIAGEN GmbH, QIAGEN Strasse 1. 40724. Hilden, Germany.

R Core Team, 2018. A Language and Environment for Statistical Computing. R Foundation for Statistical Computing, Vienna. https://www.R-project.org

Radostitis, D.M., Gay, C.C., Hinchelift, K.W., Constable P.C., 2006. Veterinary medicine: a textbook of the diseases of cattle, sheep, goats, pigs and horses. 10th ed. London: W.B. Saunders Co. Ltd. pp. 1531–40.

Ramírez-Iglesias, J.R., Pérez-Gordones, M.C., del Castillo, J.R., Mijares, A., Benaim, G. and Mendoza, M., 2018. Identification and characterization of a calmodulin binding domain in the plasma membrane Ca2+-ATPase from *Trypanosoma equiperdum*. Mol. Biochem. Parasitol. 222,51-60.

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

Ricketts, S., McGladdery, A., Crowhurst, J., Newton, R., 2011. Dourine, an emerging venereal threat to European horses. Equine Quarterly Disease Surveillance report, 6, 7(2), 15-18. Accessed on 24/06/2017 https://www.aht.org.uk/skins/Default/pdfs/DefraApr-Jun11_Focus2.pdf

Rodrigues, A., Fighera, R.A., Souza, T.M., Schild, A.L., Barros, C.S.L., 2009. Neuropathology of naturally occurring *Trypanosoma evansi* infection of horses. Vet. Pathol. 46, 251-258.

Roels, K., Leemans, B., Ververs, C., Govaere, J., Hoogewijs, M., Van Soom, A., 2014. Collection and freezing of equine epididymal spermatozoa. Vlaams. Diergen. Tijds. 83, 321-325.

Saleh, M.A., Al-Salhy, B.M., Sanousi, S.A., 2009. Oxidative stress in blood of camels naturally infected with *Trypanosoma evansi*. Vet. Parasitol. 162,192-199.

Samper, J.C., Tibary, A., 2006. Disease transmission in horses. Theriogenology, 66, 551–559.

Sánchez, E., Perrone, T., Recchimuzzi, G., Cardozo, I., Biteau, N., Aso, P.M., Mijares, A., Baltz, T., Berthier, D., Balzano-Nogueira, L., Gonzatti, M.I., 2015. Molecular characterization and classification of *Trypanosoma* spp. Venezuelan isolates based on microsatellite markers and kinetoplast maxicircle genes. Parasit. vector. 8(1),536.

Santos-Gomes, G.M., Abranches, P., Maraghi, S., Dirie, M.F., Silva-Pereira, M.C., Valverde, D., Molyneux, D.H., 1993. Laboratory and field studies on Herpetosoma trypanosomes from Portugal. Ann. Parasitolo. Hum. Com. 68, 163-168.

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

Schnaufer, A., 2010. Evolution of dyskinetoplastic trypanosomes: how, and how often? Trends Parasitol. 26(12), 557–558.

Schnaufer, A., Domingo, G.J., Stuart, K., 2002. Natural and induced dyskinetoplastic trypanosomatids: how to live without mitochondrial DNA. Int. J. Parasitol. 32(9),1071-1084.

Schulman, M.L., May, C.E., Keys, B., Guthrie, A.J., 2013. Contagious Equine Metritis: Artificial reproduction changes the epidemiologic paradigm. Vet. Microbiol. 167, 2-8.

Schulz, K., 1935. 'Dourine or slapsiekte', J. Sth. Afr. Vet. Med Ass., 6, 4-15, cited in Barrowman et al. 1994.

Seiler, R.J., Omar, S., Jackson, A.R.B., 1981. Meningoencephalitis in naturally occurring *Trypanosoma evansi* infection (surra) of horses. Vet. Pathol. 18, 120-122.

Sellon, D.C., Long, M.T., 2007. Equine Infectious Diseases, 2nd Edition, Saunders ELSEVIER publishing, Pp. 473-479.

Shlomai, J., 2004. The structure and replication of kinetoplast DNA. Curr. Mol. Med. 4,623–47.

Slaoui, M., Fiette L., 2011. Histopathology procedures: from tissue sampling to histopathological evaluation. Methods Mol. Biol. 691, 69-82.

Stephen, L.E., 1986. Trypanosomiasis: A Veterinary Perspective. Pergamon Press, New York, pp. 351-420.

Studdert, M., 1996. Equine coital exanthema (Equine Herpesvirus 3). In: Studdert, M. (Ed.), Virus Infections of Equines. Elsevier, Amsterdam, pp. 39–46.

Suganuma, K., Narantsatsral, S., Battur, B., Yamasaki, S., Otgonsuren, D., Musinguzi, P.S., Davaasuren, B., Battsetseg, B., Inoue, N., 2016. Isolation, cultivation and molecular characterization of a new *Trypanosoma equiperdum* strain in Mongolia. Parasit. Vector. 9, 481.

Swallow, B.M., 2000. Impacts of trypanosomiasis on African agriculture. Nairobi, Kenya: ILRI (International Livestock Research Institute), pp. 1–46.

Swierczewski, B.E., John C.H., 2013. Examination of Blood, Other Body Fluids, Tissues, and Sputum In: Magill, Alan J., Hill, David R., Solomon, Tom., Ryan, Edward T. (eds.) Hunter's Tropical Medicine and Emerging Infectious Disease (Ninth Edition) W.B. Saunders Elsevier, London. pp. 1082-1083

Taylor, K., Authie, E.M.L., 2004. Pathogenesis of animal trypanosomiasis. In: Maudlin, I., Holmes, P.H., and Miles, M.A. (eds.) The Trypanosomoses. CABI Publishing, Wallingford, Oxfordshire, UK. Pp. 331–353.

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

Timoney, P.J. 1996. Contagious Equine Metritis. Comp. Immun. Microbiol. Infect. Dis. 19, 199-204.

Touratier, L., 2000. Challenges of Non-Tsetse Transmitted Animal Trypanosomoses (NTTAT): An Outline and Some Perspectives. Ann. N. Y. Acad. Sci. 916(1), 237-239.

Tran, T., Napier, G.B., Rowan, T., Cordel, C., Labuschagne, M., Delespaux, V., Van Reet, N., Erasmus, H., Joubert, A. Büscher, P., 2014. Development and evaluation of an ITS1 "Touchdown" PCR for assessment of drug efficacy against animal African trypanosomosis. Vet. Parasitol., 202, 164-170.

Urakawa, T., Verloo, D., Moens, L., Büscher, P., Majiwa, P.A., 2001. *Trypanosoma evansi*: cloning and expression in *Spodoptera fugiperda* insect cells of the diagnostic antigen RoTat1. 2. Exp. parasitol. 99, 181-189.

Ventura, R.M., Takata, C.S., Silva, R.A., Nunes, V.L., Takeda, G.F., Teixeira, M.M., 2000. Molecular and morphological studies of Brazilian *Trypanosoma evansi* stocks: the total absence of kDNA in trypanosomes from both laboratory stocks and naturally infected domestic and wild mammals. J. Parasitol. 86, 1289-1298.

Verloo, D., Magnus, E., Büscher, P., 2001. General expression of RoTat 1.2 variable antigen type in *Trypanosoma evansi* isolates from different origin. Vet. Parasitol. 97(3), 185-191.

Vulpiani, M.P., Carvelli, A., Giansante, D., Iannino, F., Paganico, D., Ferri, N., 2013. Re-emergence of dourine in Italy: clinical cases in some positive horses. J. Equine Vet. Sci. 33, 468-474.

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., Phipps, L.P., 1991. Comparative evaluation of enzyme-linked immunosorbent assay (ELISA) for the sero diagnosis of dourine. Vet. Parasitol. 39, 233-239.

Weatherall, D.J., 2003. Normochromic, normocytic anaemia In: David A. Warrell, Timothy M. Cox, and John D. Firth (eds). Oxford Textbook of Medicine, 4th edition, Oxford press, UK.

Wei, Y.**,** Wen Y.Z.**,** Desquesnes, M.**,** Lun, Z.R.**,** 2011. Molecular epidemiology of *Trypanosoma evansi* and *T. equiperdum* and atypical human infection by animal trypanosomes In: The molecular epidemiology of Trypanosomes and *Leishmania*. Geoff Hide. USA, Landes Bioscience and Springer Science, pp.14

Wen, Y.Z., Lun, Z.R., Zhu, X.Q., Hide, G., Lai, D.H., 2016. Further evidence from SSCP and ITS DNA sequencing support *Trypanosoma evansi* and *Trypanosoma equiperdum* as subspecies or even strains of *Trypanosoma brucei*. Infect. Genet. Evol. 41,56-62.

Whitwell, K., 2009. Post-mortem examination of horses. In Practice 31, 104-113.

Williamson, C.C., Stoltsz, W.H., Mattheus, A., Schiele, G.T., 1988. An investigation into alternative methods for the serodiagnosis of dourine. Onderstepoort J. Vet. Res. 55(2), 117-119.

Woo, P.T.K., 1970. The Haematocrit centrifuge technique for the diagnosis of African Trypanosomoses. Acta Trop. 27, 384-386.

Wuyts, N., Chokesajjawatee, N., Panyim, S., 1994. A simple and highly sensitivity detection of *Trypanosoma evansi* by DNA amplification. Southeast Asian J. Trop. Med. Public Health 25, 266-271.

Yaeger, RG., 1996. Protozoa: Structure, Classification, Growth, and Development. In: Baron S, editor. Medical Microbiology. 4th edition. Galveston (TX): University of Texas Medical Branch at Galveston. Chapter 77. https://www.ncbi.nlm.nih.gov/books/NBK8325/

Zablotskij, V.T., Georgiu, C., De Waal, T., Clausen, P.H., Claes, F., Touratier, L., 2003. The current challenges of dourine: difficulties in differentiating *Trypanosoma equiperdum* within the subgenus *Trypanozoon*. Rev. Sci. Tech. Off. Int. Epizoot. 22, 1087-1096.

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

Zhang, Z.Q., Giroud, C., 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.

Zhou, J., Shen, J., Liao, D., Zhou, Y., Lin, J., 2004. Resistance to drug by different isolates *Trypanosoma evansi* in China. Acta Trop. 90, 271-275.

Curriculum vitae and publications

Ahmed Yasine Ebrahim was born on 11th May 1981 in Haik, South Wollo, Ethiopia. He completed highschool in 2000 and passed the Ethiopian School Leaving Certificate Examination (ESLCE) with distinction. In 2001, he joined Addis Ababa University Faculty of Veterinary Medicine, Debre Zeit, Ethiopia. He graduated in September 2006 as Doctor of Veterinary Medicine and then he worked as field veterinarian in Dewachefa district, Kemise, Ethiopia from October 2006 to August 2008.

In September 2008, he joined Addis Ababa University College of Veterinary Medicine, to continue his Masters in Veterinary Parasitology and graduated in July, 2010. During his MSc research, he was member of the research team of Ethiopian Public Health Institute, Directorate of Traditional and Modern Medicine. He involved in the research of traditional herbal medicine for the control of ectoparasites in animals underwent by the collaboration of the College of Veterinary Medicine, Addis Ababa University, Ethiopian Institute of Agricultural Research, Adami-Tulu Pesticide Processing Share Company and the Ethiopian Public Health Institute. At the end of the research the institutions were granted a Utility Model Certificate of the intellectual Property Right from the Ethiopian intellecual property office in accordance with article 42 of the proclamation concerning inventions No. 123/1995 entitled ***"Herbal composition for controlling ectoparasites in Ruminants"*** certificate number 713 dated 09 Dec 2015. The Ethiopian intellecual property office acknowledge the individual inventors for their outstanding inventions by a letter dated 18/4/08 EC, No. EIPO/CON/3-1/08 of which Ahmed Yasine was one of them.

In August 2010, he joined Kombolcha College of Agriculture, Kombolcha, Ethiopia as academic staff in the Department of Animal Health. In October 2011, he joined Wollo University, School of Veterinary Medicine Dessie, Ethiopia as Assistant Professor.

In December, 2013, he started with his PhD studies sponsored by Ethiopian-Belgian VLIR-UOS TEAM project "*Trypanosoma equiperdum*: Venereal Transmission and Pathogenesis" at the Faculty of Veterinary Medicine, Ghent University, Belgium and Addis Ababa University, College of Veterinary Medicine, Ethiopia under the supervision of Dr. Jan Govaere, Prof. Hagos

Ashenafi, Prof. Bruno Goddeeris Prof. Luc Duchateau and Prof. Ann Van Soom. The results obtained in this PhD research project are described in this thesis.

List of publications

Ahmed Yasine, Hagos Ashenafi, Peter Geldhof , Leen Van Brantegem, Griet Vercauteren, Merga Bekana, Alemu Tola, Ann Van Soom, Luc Duchateau, Bruno Goddeeris, Jan Govaere (2019). Histopathological lesions in reproductive organs, distal spinal cord and peripheral nerves of horses infected with *Trypanosoma equiperdum.* **BMC Veterinary Research**, 2019, In press.

Ahmed Yasine, Hagos Ashenafi, Peter Geldhof, Merga Bekana, Alemu Tola, Leen Van Brantegem, Ann Van Soom, Luc Duchateau, Bruno Goddeeris, Jan Govaere, 2019. Reduction of *Trypanosoma equiperdum* from equine semen by single layer centrifugation. **Experimental Parasitology**, 200, 79-83.

Ahmed Yasine, Merga Daba, Hagos Ashenafi, Peter Geldhof, Leen Van Brantegem, Griet Vercauteren, Tilaye Demissie, Merga Bekana, Alemu Tola, Ann Van Soom, Luc Duchateau, Bruno Goddeeris, Jan Govaere, 2019. Tissue (re)distribution of *Trypanosoma equiperdum* in venereal infected and blood transfused horses. **Veterinary Parasitology**, 268, 87-97.

Ahmed Y., Hagos, A., Merga, B., Van Soom, A., Duchateau, L., Goddeeris, B.M., Govaere, J., 2018. *Trypanosoma equiperdum* in the horse – a neglected threat? **Vlaams Diergeneeskd Tijdschr** 87, 66-75.

Mensur, S., Ansuar, I., Tesfaye, A., Abdulkaf, K., **Ahmed, Yasine,** 2016. Small ruminant fasciolosis and its economic impact in an export abattoir of Ethiopia. **Livestock Research for Rural Development.** Volume *28,* Article #161. http://www.lrrd.org/lrrd28/9/mens28161.html.

Ahmed Yasine., 2015. Biological Control of vector borne Protozoan Parasites of Veterinary Importance**.** ***Applied Science Reports****,* 10 (3), 122-127.

Ahmed Yasine., Bersissa Kumsa., Yacob Hailu, Dinka Ayana. 2015. Mites of sheep and goats in Oromia Zone of Amhara Region, North Eastern Ethiopia: Species, prevalence and farmers awareness. **BMC Veterinary Research***,* 11**,**122, DOI 10.1186/s12917-015-0433-6

Habtamu Tesfaye, Abadi Amare, Shahid Nazir, **Ahmed Yasine,** 2015. Major Metacestodes in small ruminants slaughtered at Dessie municipal abattoir, Eastern Ethiopia: prevalence, cyst viability, organ distribution and economic implications. Comparative Clinical Pathology, 24, 217 erratum to **Comparative Clinical Pathology**, 24,659–668, DOI 10.1007/s00580-014-1964-0.

Nazir, S., Kamil, S.A., Riyaz, A., Mir, M.S., Darzi, M.M., **Yasine, A.,** Goudar K.S., 2014. Pathology and colonization of internal organs after experimental infection of broilers by Salmonella enterica serovar Gallinarum oral and intraperitoneal. ***Revue d'élevage et de médecine vétérinaire des pays tropicaux,*** 67(2): 53-60.

Eyasu Adale, **Ahmed Yasine,** 2013. Prevalence of bovine trypanosomosis in Wolaita Zone Kindo Koish District of Ethiopia. ***African Journal of Agricultural Research,*** 8(49), 6383-6387.

Ahmed Yasine, 2012. Helminths and anthelminthic resistance of small ruminants in Ethiopia, Lambert Academic publishing, ISBN: 978-3—659-14520-9**.**

Abstract

Ahmed Y., Hagos, A., Merga, B., Alemu, T., Van Soom, A., Duchateau, L., Goddeeris, B., Govaere, J., 2017. Infectiousness of equine semen in prepatent phase of Dourine. ***Reproduction in Domestic Animals****.* 52. p.66-66.

Conference papers

Ahmed, Y., Hagos, A., Geldhof, P., Merga, B., Alemu, T., Van Brantegem, L., Van Soom A., Duchateau L., Goddeeris B., Govaere J., 2018. Symptomatology and diagnosis of Dourine in horses infected by insemination or transfusion. 3rd International Conference on Non Tsetse Transmitted Animal Trypanosomosis, 2-4 December 2018, North-West University, Potchefstroom, South Africa.

Ahmed, Y., Hagos, A., Geldhof, P., Merga, B., Tola, A., Van Brantegem, L., Vercauteren, G., Van Soom, A., Duchateau, L., Goddeeris, B., Govaere, J., 2018. Pathology in dourine diseased horses. OIE Non Tsetse Transmitted Animal Trypanosomoses Network. Minutes of the 4th annual meeting, 29 June 2018, Paris, France.

Ahmed, Y., Hagos, A., Merga, B., Alemu,T., Van Brantegem L., Van Soom, A., Duchateau, L., Goddeeris B., Govaere, J., 2017. Purification of *Trypanosoma equiperdum* spiked equine semen by single layer centrifugation. 2nd International Conference on Non Tsetse Transmitted Animal Trypanosomosis, 18-19 December 2017, Antwerp, Belgium.

Ahmed, Y., Hagos, A., Merga, B., Alemu, T., Van Soom, A., Duchateau, L., Goddeeris, B., Govaere, J., 2017. Infectiousness of equine semen in prepatent phase of Dourine. 21st Annual Conference of the European Society for Domestic Animal Reproduction (ESDAR), Bern, Switzerland, 24 - 26 August 2017.

Thesis

- *In vitro* and *in vivo* acaricidal efficacy evaluation of selected medicinal plants against mange mites of Goats (*Sarcoptes scabiei* var. *caprae*). MSc Thesis AAU, Faculty of Veterinary Medicine, Debre Zeit, Ethiopia, 2010.
- Sheep and Goat Dermatophilosis in and around Kombolcha, South Wollo, North East Ethiopia. DVM thesis, AAU, Faculty of Veterinary Medicine, Debre Zeit, Ethiopia, 2006.

Seminars

- Dourine in horse: appearance and diagnostic challenges. Seminar on current Parsitology topics in PhD course work. AAU, College of Veterinary Medicine, Debre Zeit, Ethiopia, 2014.
- Antihelminthic Resistance in Small Ruminants. Seminar on current topics on basic Scinces in MSc course work. AAU, Faculty of Veterinary Medicine, Debre Zeit, Ethiopia, 2009.
- Socioeconomic importance and major health problems of Donkeys in Ethiopia. Seminar on current topics in livestock Production and development in DVM course work, AAU, Faculty of Veterinary Medicine, Debre Zeit, Ethiopia, 2005.