ADDIS ABABA UNIVERSITY
FACULTY OF VETERINARY MEDICINE

SEROLOGICAL & PARASITOLOGICAL SURVEY OF DOURIN (TRYPANOSOMA EQUIPERDUM) IN SELECTED SITES OF ETHIOPIA

HAGOS ASHENAFI

JUNE 2005
DEBRE ZEIT

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MSc Thesis

By
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A thesis submitted to the School of Graduate Studies of Addis Ababa University
in partial fulfilment for the requirements for the
Degree of Master of Science in Tropical Veterinary Medicine

By

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Board of External Examiners	**Signature**
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TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF ANNEXES

LIST OF ABBREVIATIONS

AGID	Agar Gel Immuno Diffusion
ARDU	Arsi Rural Development Unit
BW	Body Weight
BoTat	Bordeaux Trypanosoma Antigenic Type
CATT	Card Agglutination Test for Trypanosomosis
CFT	Complement Fixation Test
DIM	Diminazene Aceturate
ELISA	Enzyme Linked Immunosorbent Assay
FAO	Food and Agricultural Organization
IFAT	Indirect Fluorescent Antibody Test
IgG	Immunoglobulin G
ISMM	Isometamidium Chloride
Kg	Kilogram
mAECT	Mini Anion Exchange Centrifugation Technique
mHCT	Mini Haematocrit Centrifugation Technique
MEGA	Multiplex - Endonuclease Genotyping Approach
OIE	Office International des Epizooties
OVI	Onderstepoort Veterinary Institute
PBS	Phosphate Buffered Saline
PCR	Polymerase Chain Reaction
PCV	Packed Cell Volume
PP	Percent Positivity
RoTat	Rhode Trypanosoma Antigenic Type
SNNPRS	Southern Nation, Nationalities and People Regional State
VAT	Variable Antigenic Type
VSG	Variable Surface Glycoproteins

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ABSTRACT

The present study was conducted from August 2004 to April 2005 with the objectives of determining the prevalence, extent and distribution of dourine in horses and investigating occurrence of clinical cases as well as carrier states of donkeys and mules in the Arsi-Bale highlands. At the same time a longitudinal study was conducted to assess the efficacy of the Isometamidium chloride (ISMM) and Diminazene aceturate (DIM) in the treatment of clinical cases of dourine. An investigation was also conducted encompassing questionnaire and serological survey on the presence of dourine in adjacent geographical areas of the Arsi-Bale highlands and other selected parts of the country with high equine population. The study methodology was based on questionnaire, serological, clinical and parasitological survey. The questionnaire survey indicated that dourine, locally known, as "Lappessa" or "Dirressa" is a major health problem of equines in the Arsi-Bale highlands. Though, dourine is a common clinical case through out the year, it has a seasonal character, which most commonly occurs following the breeding season from June to late September. Sometimes a second peak is observed in the dry seasons of the year (March to May), which probably associated with relapse of previously infected and recovered cases due to stressful conditions of feed shortage. Of 649 horse sera tested for the detection of antibodies against *T. equiperdum*, revealed a seropositive of 184 (28.35%), 161 (24.81%) and 125 (19.26%) for CATT, LATEX and ELISA tests, respectively. Risk factors with significant association to dourine were parity number, previous history of abortion and body condition score. No trypanosomes or any other haemoparasites could be detected in all examined Giemsa stained smears (thin blood, genital discharge and tissue fluids) as well as in blood samples by mHCT. All the puppies inoculated with blood samples, genital washes and oedematous fluids remained parasitologically negative. Different characteristic clinical signs of dourine were observed in clinically sick horses of both sexes. The prominent clinical signs observed as genital form of the disease include vaginal discharge mainly of mucopurulent type with foul odour, oedema and presence of depigmented scars over the external genitalia, oedema of the scrotum and prepuce accompanied by prepucial as well as urethral discharge and ulceration of the genital mucosae mainly of the penile. In both sexes, lameness in one or both legs, partial dragging and stiffness of the hind legs and incoordination. were the dominant signs observed as nervous form of the disease.

Horses with different clinical signs of dourine were categorised into one of the following five groups based on the clinical findings: Apparently healthy (468); non-specific signs like emaciation and weakness (69); genital form (30); nervous form of (15) and both genital and nervous forms of the disease (67). Fifty-four horses with high percentage positivity for the indirect antibody ELISA and nineteen horses with strong agglutination reactions having end titre of 1:32 for both CATT and LATEX tests belonged to the five of the different clinical groups. Chi-square analysis of the distribution of animals, classified as either positive or negative on the basis of all the three tests, in the clinical groups demonstrated a statistically significant difference between groups, with stratum specific prevalence of positive animals increasing with increasing severity of clinical signs ($p<0.001$). The concordance between (CATT and ELISA) and (LATEX and ELISA) test results were 64.80% and 69.60%, respectively. The kappa statistic between the tests indicated substantial agreement where 125 sera tested positive and 435 sera tested negative in CATT and ELISA tests and with 125 sera tested positive and 450 sera tested negative in LATEX and ELISA tests. A longitudinal study was carried out to assess the therapeutic activity of ISMM and DIM in selected serologically positive clinical cases of dourine in Asassa district of the Arsi highlands. A total of 24 adult local horses were divided into 3 groups of equal number. PCV level, eosinophil count, body temperature, serological status, improvement of the existing clinical signs and body condition score were monitored twice in 20 days interval for a period of 40 days. The results indicated that mean PCV, eosinophil counts and body temperature improved in both ISMM and DIM treated group during the 40 days post treatment period. A statistically significant increase in PCV and body temperature was observed starting from the day 20 ($P<0.01$) and a highly significant variation ($P<0.001$) was observed at the end of the experiment. There was a marked improvement in the existing clinical signs and body condition score of the study animals under the ISMM and DIM groups as compared to control animals within the 40 days post treatment follow up time. All the study animals remained serologically positive for both CATT and LATEX tests throughout the 40 days of the study period. The occurrence of active clinical cases of dourine in two donkeys as well as serological carrier states in 12 donkeys and 5 mules were also observed. Serological as well as questionnaire based survey conducted in various representative sites of the country as to the occurrence of dourine revealed the presence of the disease in Uraga and Shashemene districts of the Oromiya Region and Arbegona district of the SNNPRS (Southern Nation, Nationalities and People Regional State).

This is the first report to establish the occurrence of dourine outside of the previously known endemic foci of the Arsi-Bale highlands of Ethiopia. Although there was no direct detection of *T. equiperdum*, there is strong evidence that dourine is highly prevalent, most important problem in the Arsi-Bale highlands, where the situation is getting worse with the present spreading trend to areas previously known as free of the disease, necessitates urgent national control intervention.

Keywords: Dourine, *Trypanosoma equiperdum*, CATT, LATEX, ELISA, Ethiopia.

1. INTRODUCTION

Ethiopia possesses 2.75 million horses, 5.02 million donkeys and 0.63 million mules (CSA, 2003). Horses have a prominent position in the agricultural and transport systems as draft, pack and riding animals. In a country where there is less developed modern transport and communication service, the natural choice rests on the use of human and pack animals mode of transport, as it has been the case in some parts of the world. Thus, in a developing country like Ethiopia, the contribution of equines in the energy scenario is of considerable significance. The provisions of transport through pack animals, drawing carts, as riding animals or taxi operations, almost certainly contributes more to the national economy. The use of equines is limited to their power as transportation (saddle type, cart pulling and packing). Only few regions in northwestern and south eastern (Arsi-Bale highlands) of Ethiopia use equines for ploughing (tillage) and threshing of crops is practised.

Despite the significance of horses in the sector of transportation and agriculture to the economy of the nation, the treatment accorded to these species of animals has been far below than that given to other species of animals. This can partly be due to the age-old erroneous concept that these species are hardy, tolerant and probably because they are not providers of meat and milk (Feseha, 1993). African horse sickness, anthrax, epizootic lymphangitis, dourine, equine piroplasmosis, horse mange, rabies, glanders and ulcerative lymphangitis are among the major diseases that effect horses in Ethiopia (FAO, 1996).

Throughout the world, the one common factor leading to the ill health, suffering and early demise of equines is the protozoan parasite, *Trypanosoma equiperdum*, causing dourine (Stephen, 1986). Dourine is a chronic or acute contagious disease of breeding solipeds that is transmitted directly from animal to animal during coitus. Among the Non-Tsetse Transmitted Trypanosomoses (NTTAT), dourine is included in list B of the OIE notifiable diseases list (OIE, 2001). Dourine is the only trypanosomosis that is not transmitted by an invertebrate vector. *T. equiperdum* differs from other trypanosomes in that it is primarily a tissue parasite that rarely invades the blood

There is no known natural reservoir of the parasite other than infected equids. It is present in the genital secretions of both infected males and females. The incubation period, severity and duration of the disease vary considerably; it is often fatal, but spontaneous recoveries do occur. Sub clinical infections occur, and donkeys and mules are more resistant than horses and may remain inapparent carriers. *T. equiperdum* is a cosmopolitan, but due to the strict enforcement of control measures, dourine declined quickly in most parts of the world at the beginning of the 20^{th} century, particularly from the 1950s onwards and currently Western Europe, Australia and the United States are considered to be free from dourine (OIE, 2001).

The problem of dourine in Ethiopia has been recognized by local farmers for many years and it has been found to be a threat to the life and productivity of the equine population in the endemic areas. However, the first official report of the disease was made in 1980 when the Arsi Rural Development Unit (ARDU) requested the Tsetse and Trypanosomosis Survey and Control Department to investigate a persistent disease problem in horses in the administrative regions of Arsi and Bale (Zeleke *et al.*, 1980).

According to this report, the disease was widely spread in Ethaya, Sagure, Bekoji and Koffle districts of Arsi-Bale highlands. In those areas, the disease is known commonly as "Lappessa Hidakuta" "Lappessa Duda Kuta" which means back bone breaker in the local language or simply "Kuta" which means breaker. Since then dourine was found to be prevalent in the highlands of Ethiopia particularly in Arsi and Bale zones (Alemu *et al.*, 1997). Similarly, multiple cases of serological complement fixation test (CFT) and enzyme linked immunosorbent assay (ELISA) and Trypanozoon polymerase chain reaction (PCR) positive, yet aparasitemic horses were reported in Arsi and Bale zones in Ethiopia (Clausen *et al.*, 1999). However, the presence or absence of dourine in adjacent geographical areas to the Arsi-Bale highlands and other parts of the country where there is high equine population, unrestricted mobility and uncontrolled breeding, remained unknown. Diagnosis of *T. equiperdum*, the causative organism of dourine in horses, by standard parasitological techniques is difficult, owing to the low numbers of parasites present in the blood or tissue fluids and the frequent absence of clinical signs of disease. Consequently, the demonstration of trypanosomal antibodies in the serum has become the most important parameter in determining the disease status of individual animals (Bishop *et al.*, 1995).

The principal reason for using serological tests for the diagnosis of trypanosomosis is to overcome the low level of sensitivity of parasitological tests in detecting chronic infections. Alternative strategies, utilizing nucleic acid technologies, such as the PCR also offer high sensitivity and might be of use in the diagnosis of *T. equiperdum* (Luckins, 1992). For the isolation of such parasites, potential loci are Mongolia and Ethiopia (OIE, 1999). In Mongolia, reports of clinical cases of dourine as well as cases of CFT-positive and Trypanozoon PCR-positive horses have been reported. Attempts to isolate the parasite from blood, however, were not successful (Clausen *et al.*, 2003). In Ethiopia multiple cases of serological (CFT and ELISA) and Trypanozoon PCR-positive yet aparasitemic horses are reported (Clausen *et al.*, 1999). No parasites could be isolated from these PCR-positive horses. Based on the observation that trypanosomal DNA has been detected in the blood samples from Mongolia and Ethiopia, it looks encouraging enough to believe that it could be possible to isolate a parasite using different parasitological techniques. The difficulty in the diagnosis of *T. equiperdum* lead to difficulties in achieving reliable data on the prevalence, and distribution and in the implementation and monitoring of the disease control programmes. Therefore, the specific objectives of the present study include:

- To determine the prevalence, extent and distribution of dourine in horses of Arsi-Bale highlands based on questionnaire, serological and parasitological techniques.
- To investigate clinical cases and observe the serological status of donkeys and mules in the Arsi-Bale highlands.
- To conduct and assess the efficacy of commonly available trypanocidal drugs Berenil and Trypamidium in the treatment of clinical cases of dourine in the Arsi-Bale highlands.
- If possible to isolate *T. equiperdum* strains from clinical cases and to further investigate for future comparison between *T. equiperdum* and *T. evansi* strains at the molecular level with the existing stocks from specialized laboratories.
- To investigate the presence of dourine in adjacent geographical areas of the Arsi-Bale highlands and other selected parts of the country where there is high equine population.
- To institute practical recommendations and control options in order to reduce the impact of the disease and to further limit its distribution in the equine population of the country.

2. LITREATURE REIVEW

2.1. The disease

2.1.1 The history of *Trypanosoma equiperdum*

Dourine, caused by *Trypanosoma equiperdum* Dofelin, 1901, has been recognized as a disease of breeding solipeds for many centuries (Hoare, 1972). Although the disease has been known since ancient times, its nature was established only in 1896 when Rouget discovered trypanosomes in the blood of infected Algerian horses. However, the parasite got lost before Rouget (1896) could reproduce the disease in horses. It was only several years later that Buffard and Schneider in 1900 were able to reproduce dourine in a horse following the subcutaneous inoculation of a parasite isolated from a naturally infected Algerian horse that was maintained through several passages in experimentally infected dogs. The disease has been also observed in France, Hungary and Germany. Since the 19th century, dourine occurred only sporadically in Europe. During World War II, however, *T. equiperdum* was re-introduced into Western Europe with Russian and Algerian horses, used in the German army and in France, respectively. After the war, the disease was eradicated in Western Europe by a systematic screening and control: Clinical examination, confirmatory diagnosis by the complement fixation test (CFT), enforcement of zoo-sanitary measures including stamping out and in some cases treatment with high dosages of neoarsphenamine (Hoare, 1972). In the Western hemisphere, dourine existed in USA since 1886 and in Canada since 1904, but while it was eradicated in the latter country some 15 years later (Watson, 1920). The disease also occurred in Chile, Venezuela and Brazil (Hoare, 1972).

Zeleke *et al.*, (1980) first reported the presence of dourine in Ethiopia. According to this report, *T. equiperdum* was widely spread in the districts of Ethaya, Sagure, Bekoji and Koffle in the former Arsi-Bale administrative regions. Horses suffering from clinical symptoms of dourine have also been recently reported in Ethiopia (Alemu *et al.*, 1997; Clausen *et al.*, 1999), particularly from the Arsi-Bale highlands, unfortunately without success of parasite isolation.

2.1.2. Synonyms

The venereal disease of equines or dourine has been known under other names (Arabic "el Dourin", English "Covering disease", German "Beschalseuche", French "Mal de coit", Russian "Slucnaja Boleznj" or "Podsedal") (Hoare, 1972). In Ethiopia, the disease is known commonly as "Lappessa Dudakuta" or "Lappessa Hidakuta" which means back bone breaker in the local language or simply "kuta" which means breaker (Zeleke *et al.*, 1980).

2.2. Aetiology

2.2.1. Taxonomy

The causative organisms, Trypanosomes, which are strictly parasitic, are flagellar protozoa; 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* as indicated below in Table 1. *T. brucei* is further divided into three subspecies, the animal pathogen *T. b. brucei* and the trypanosomes responsible for human sleeping sickness *T. b. gambiense* and *T. b. rhodesiense* (Hoare, 1972).

Table 1: Taxonomy of *Trypanosoma equiperdum*

Phylum:	Protozoa
Class:	Zoomastigophora
Order:	Kinetoplastida
Family:	Trypanospomatidae
Genus:	*Trypanosoma*
Subgenus:	Trypanozoon (Brucei group)
Species:	*T. brucei, T. evansi, T. equiperdum*
Subspecies:	*T. b. brucei. T. b. gambiense, T. b. rhodesiense*

(Source: Hoare, 1972)

2.2.3. Morphology

Trypanosoma equiperdum, T. b. brucei and *T. evansi*, the three-trypanosome subspecies in the Trypanozoon group are animal pathogens highly virulent in horses, and morphologically almost indistinguishable. The morphology of *T. equiperdum* is identical to the blood stream slender form of *T. b. brucei*, having a single free flagellum with long slender trypomastigote forms. *T. equiperdum* is also morphologically indistinguishable from *T. evansi*, like the latter species, it is typically monomorphic, being represented by thin (slender and intermediate) trypomastigotes possessing a free flagellum, although pleomorphic, stumpy, protonuclear forms are recognized.

As in *T. evansi*, the nucleus lies in the centre of the body. The kinetoplast is very distinct and terminal or sub-terminal in position, with well-developed undulating membrane and free flagellum. The size of *T. equiperdum* is like wise within the range of that of *T. evansi*, with the length of different strains varying from 15.6 - 31.3 µm and 1.5 - 2.2. µm width. The other characteristic which *T. equiperdum* shares with *T. evansi* is a tendency to produce dyskinetoplastic strains (Hoare, 1972).

2.3. Epidemiology

2.3.1. Host range and distribution

Dourine mainly affects horses, donkeys and mules. The disease is generally more severe in improved breeds of horses and milder in native ponies, donkeys and mules. Various laboratory animals, including rats can also be infected. Zebras have tested positive by serology, but there is no conclusive evidence of infection. Horses and donkeys appear to be the only natural reservoirs of *T. equiperdum*. Male donkeys can be asymptomatic carriers. Since the transmission depends on sexual compatibility the host range is limited to equidae and no other reservoir host exists (Luckins, 1994).

Dourine was once widespread, but has been eradicated from a number of countries. Currently the disease is endemic in most parts of Northern and Southern Africa, Asia, Southern Europe and America. Nowadays, Western Europe, Australia and the United States are considered to be free from dourine (OIE, 2001). However, sporadic cases of CFT positive animals occur in dourine free countries (e.g. Italy and recently in Germany).

The latest official reports of dourine (i.e. CFT positive cases) were in China, Kazakhstan, Kyrgyzstan, Pakistan, Ethiopia, Botswana, Namibia, South Africa, Brazil, Italy and Germany Unfortunately, countries where dourine is currently reported often lie within the distribution area of *T. evansi*, except for South Africa, Mongolia and parts of Russia where *T. evansi* has not been described yet (Zablotskij *et al.*, 2003).

Unlike the case of insect borne trypanosomosis the epidemiology of dourine is simpler as it does not depend on vectors and their ecology. The propagation of dourine depends primarily on the conditions favouring the transfer of *T. equiperdum* from one equine host to another, e.g. among breeding animals in studs and among those in free-range herds. As it so not dependent on insect vectors, it has been able to spread as far north as Canada, Russia and other European countries, as far south as Chile and South Africa (Hoare, 1972).

Dourine was found to be prevalent in the highlands of Ethiopia particularly in Arsi-Bale highlands and a potential threat to the equine population in these areas (Zeleke *et al*., 1980, Alemu *et al*., 1997; Clausen *et al*., 1999). Its presence was first reported in 1980, in the former Arsi and Bale administrative regions and the disease is known commonly as "Lappessa Hidakuta" or "Lappessa Duda Kuta" which means back bone breaker in the local language or simply "Kuta" which means breaker. In view of the large number of horses in Ethiopia, the unrestricted movement of animals through out the country for trade and transport purpose, lack of adequate facilities for diagnosis and control of the disease in relation to breeding, it is likely that dourine may have a much wider distribution than the Arsi-Bale highlands, especially in places where there are high equine population.

2.3.2. Transmission

Unlike other trypanosomal infections, dourine is transmitted almost exclusively during coitus. The infection is more commonly transmitted from stallion to mare, facilitated by the presence of the parasite in the seminal fluid and mucous exudate of the penis and its sheath. From the infected mare, the infection is transmitted to the stallion due to the presence of the parasite in the vaginal mucus. As trypanosomes are not continually present in the genital tract throughout the course of the disease, transmission of infection does not necessarily take place at every copulation involving an infected animal. *T. equiperdum* can pass through intact mucous membranes and it is possible for foals to acquire infection by contamination of nasal or conjunctival membranes with the vaginal discharge. These infected foals can spread the organism when they mature.

Other means of transmission may also be possible, but there is no evidence that arthropod vectors play any role in transmission. Intravenous or intraperitoneal experimental infections indicate that mechanical transmission by blood sucking flies cannot be excluded. However, the generally low number of parasites present in the blood does not favour this method to be main route of infection (Claes, 2003). Human carelessness may also be responsible for conveying the infection, e.g. when contaminated utensils are used for grooming the horses or unsterilized instruments for artificial insemination. Animals other than equids can be infected experimentally (OIE, 2001).

2.3.3. Course of infection and pathogenesis

The trypanosomes, which are present in the seminal fluid and mucous membranes of the genitalia of the infected donor animal, are transferred to the recipient during sexual intercourse. It is considered that transferred trypanosomes penetrate the intact mucous membranes and initiate an infection in the recipient animal. Trypanosomes are rarely observed in the blood stream of the host because they are normally localized in the capillaries of the mucous membranes of the urogenital tract (Hoare, 1972; Brun *et al.*, 1998). The first symptoms may appear in the genitalia between one week and 3-4 months after infection, when the parasites are localized in the mucous membranes and oedemata of equines of both sexes, as well as in the vaginal mucus of mares. About 30 days later the trypanosomes find their way into the blood stream, which they are carried to various parts of the body and invade the skin, giving rise about 40-60 days after the onset of the infection to the characteristic urticarial plaques, which may appear and disappear at irregular intervals. During the first few days the trypanosomes proliferate in the sero-sanguinous exudate of the plaques but, as the latter are reduced in size and absorbed most of the trypanosomes degenerate and eventually perish, those that survive are lodged in the subcutaneous tissues and may give rise to new crops of plaques. The latter stages of dourine are characterized by anemia and nervous disorders, manifested chiefly by paralysis of the hind limbs (Hoare, 1972). Most of the clinical manifestations of dourine are the result of the histotropism of *T. equiperdum*, especially for the mucosa of the genital organs and the cutaneous tissues.

The pathological effect has been attributed to the secretion of a toxin by the parasite. It is thought that when the parasites invade the tissue they cause vasomotor disturbances with exudation of the plasma and an inflammatory reaction at the sites of irritation, giving rise to the oedematous swellings and plaques. The toxin elaborated in these lesions is carried away through the blood stream, causing inflammation and degeneration of the peripheral nerves. The motor and sensory disturbances in the later phases of the disease are direct result of these inflammatory changes, while the emaciation of the animals is due to atrophy of the muscles served by the damaged nerves (Hoare, 1972). Like wise, Waston (1920) believed that the sudden death of infected rodents during high parasitaemia was due to the release of toxins into the circulation. Though as a rule dourine is a fatal disease, with an average mortality rate of 50 percent, especially in stallions, infected animals sometimes recover spontaneously (OIE, 2001).

2.3.4. Immunity

Infection with *T. equiperdum* may run a symptomless course in native free-range horse, and horses of different breeds, as well as individual animals, vary in their susceptibility to the disease. It is also known that donkeys and mules are more resistant to the infection than horses. Infected animals produce antibodies to successive antigenic variants (OIE, 2001). Some foals may acquire passive immunity from colostrum of infected mares without becoming actively infected; in such foals, the antibody titer declines, and the animal becomes seronegative by 4 to 7 months of age (Barrowman, 1976).

It was also demonstrated that considerable degree of immunity conferred inutero during the last 3 months of gestation, protected a donkey foal born of an infected mother from infection with *T. equiperdum* at 8 months of age (Hoare, 1972). The chronic and often prolonged courses of the disease, as well as cases of spontaneous recovery, provide evidence of acquired immunity (Watson, 1920).

2.4. Clinical signs

Generally the disease is divided into three phases (genital oedema, plaques and skin eruptions, neuropathological signs). The symptoms of dourine vary with the virulence of the strain, the nutritional status of the horse, and stress factors. Stages of exacerbation, tolerance and relapse can occur several times before the animal either recovers or dies (Hoare, 1972).

2.4.1. Primary stage (Genital oedema)

In stallions, the first symptoms are oedema of the prepuce and glans penis. The swelling may spread to the scrotum, perineum, ventral abdomen and thorax. Vesicles or ulcers may be seen on the genitalia, when they heal, these ulcers can leave permanent scars (leukodermic patches). Orchitis may occur and cause irritation, where the stallion constantly draws and retards the penis. Paraphimosis may also occur. Early symptoms in mares consist of vaginitis, with mucopurulent discharges. The vulva becomes oedematous; this swelling may extend along the perineum to the ventral abdomen and mammary gland. Vulvitis, vaginitis with polyuria and signs of discomfort may be seen. The genital region, perineum, and udder may become depigmented. Abortion can occur with more virulent strains (Hoare, 1972; OIE, 2001).

2.4.2. Secondary stage (Plaques and skin eruptions)

This stage, known as stage of Urticaria, is marked by distinct, raised round or oval shaped patchy eruptions called "plaques", that appear on the skin in both sexes. Oedematous patches, also called "silver dollar plaques", up to 5-8 cm diameter and 1 cm thick may appear on the skin, particularly over the neck, shoulders, ribs and thighs, and usually last for 3-7 days, and is considered to be pathognomonic for dourine (OIE, 2001). These plaques do not occur with all strains and have also been observed sporadically in animals infected with *T. evansi* (Brun *et al.*, 1998).

2.4.3. Tertiary stage (Neurological signs)

The final phase known as stage of paralysis is characterized by disorders of the nervous system. Initially these signs consist of restlessness and the tendency to shift weight from one leg to another followed by progressive weakness and in coordination, and ultimately, paralysis (mainly of the hind legs), paraplegia and death occur. Marked atrophy of the hindquarters is a common finding and in all animals there is loss of condition. Other clinical signs include progressive anemia may be seen by increasing pallor of the mucous membranes of the eyes and mouth, conjunctivitis, keratitis, intermittent fever and emaciation (Hoare, 1972; Stephen, 1986).

2.5. Pathology

Anemia, cachexia and genital oedema are often seen at post-mortem. The oedema, which may be indurated, can extend to the ventral abdomen. Gelatinous exudates can often be seen under the skin. In stallions, the scrotum, sheath, and testicular tunica may be thickened and infiltrated. The testes may be embedded in sclerotic tissue and may not be recognizable. In mares, a gelatinous infiltrate may thicken the vulva, vaginal mucosa, uterus, bladder, and mammary gland. The lymph nodes, particularly in the abdominal cavity, are hyperthrophied, softened and in some cases, haemorrhagic. The spinal cord of animals with paraplegia is often soft, pulpy and discoloured, particularly in the lumbar and sacral regions (OIE, 2001).

2.6. Diagnosis

2.6.1. Symptomatology

In places where the disease is known to exist or when a suspected animal has been exposed, the symptoms of oedematous swelling of external genitalia, eruption of cutaneous plaques or dollar spots, locomotor disturbances of hind limbs and facial paralysis are sufficiently diagnostic.

Although the clinical manifestations and lesions are pathognomonic of dourine, these features cannot be definitely identified in the early stages of the infection and in latent cases (Hoare, 1972). Dourine has to be differentiated from coital exanthema, which is characterized by the appearance of vesicles, ulcers and depigmented spots on vaginal mucus membrane. In dourine, vesicles and ulcers are not important. Moreover, in some countries (e.g. in S. America) *T. evansi* infections give rise to similar clinical signs. Dourine should also be differentiated from equine viral arteritis and purulent endometritis such as contagious equine metritis (OIE, 2001).

2.6.2. Parasitology

A definitive diagnosis depends on the recognition of the clinical signs followed by demonstration of the parasite. Direct parasitological demonstration of *T. equiperdum* is unusual as it was always very difficult to isolate the organism directly from either the blood or pathological secretions (oedemas, plaques) of infected horses, even at the earliest stages of the infection (Rouget, 1896; Schneider and Buffard, 1900). This is possibly because on the one hand trypanosomes are only sparsely present and extremely difficult to find, even in oedematous areas. On the other hand, the trypanosomes are only fleetingly present in the blood, and in small numbers that defy detection (OIE, 2001).

In the early stages of the disease the parasites are sought for. In the vaginal tract of mare, by microscopical examination of the vaginal washings or of the fluid exuding after scarification of the mucosa with the aid of spatula, and in the scrapings of the urethra in the stallion obtained with the aid of a probe. In the later stages of the disease the parasites may be found in the fluid contents of the oedemata and plaques, especially shortly after their eruption. The skin over the swelling is washed, shaved and dried, after which punctures are made through its border and the exuding fluid is examined microscopically in fresh preparations. As a rule, trypanosomes are present there only for a few days; therefore, the lesions should be examined repeatedly. In the blood the parasites are practically undetectable, even in thick films, but they are sometimes revealed by concentration (buffy coat) method (Hoare, 1972).

However, several authors succeeded in isolating strains either directly from the blood of equines thought to be infected (Schneider and Buffard, 1900, 1903; Laveran and Mesnil, 1912; Barrowman, 1977), from laboratory animals (rabbits and mice) injected intraperitoneally (Lun, 1995) and subcutaneously (Laveran and Mesnil, 1912). As dourine is the only trypanosome to affect horses in temperate climates, the observation of trypanosomes in thick blood films is sufficient for a positive diagnosis. However, in countries where nagana or surra occur, it is difficult to distinguish *T. equiperdum* microscopically (morphology, motility) from other members of the subgenus Trypanozoon, especially from *T. evansi*. In particular, *T. evansi* and *T. equiperdum* cannot be differentiated on the basis of morphological criteria. Both are monomorphic, slender trypomastigotes with a free flagellum although pleomorphic, stumpy, proteonuclear forms are recognized. Typical strains of *T. equiperdum* range in the length from 15.6 to 31.3 μm (Brun *et al.*, 1998).

2.6.3. Serology

Diagnosis of *T. equiperdum* by standard parasitological techniques is difficult, owing to the low numbers of parasites in the blood or tissue fluids. Consequently, the demonstration of trypanosomal antibodies in the serum has become the most important parameter determining the disease status of individual animals (Bishop *et al.*, 1995). A range of techniques for serological diagnosis of dourine has been attempted and available: complement fixation test, indirect immunofluorscent antibody test, enzyme linked immunosorbent assays, card agglutination test for trypanosomosis / *T. evansi* RoTat 1.2 and LATEX/ *T. evansi* Ro Tat 1.2 (Bajyana and Hamers, 1988; Wassal *et al.*, 1991; Bishop *et al.*, 1995; Touratier, 2000; Claes, 2003).

2.6.3.1. Complement fixation test (CFT)

The complement fixation test (CFT) is the most commonly used OIE - prescribed serodiagnostic test for international trade for dourine. Despite the usefulness and universal acceptance of the CFT for diagnosing dourine, some discrepancies have been recorded. The disadvantages of the CFT are that it requires careful continuous titration of numerous labile agents and that it does not function with sera having anticomplementary activity (OIE, 2001).

CFT is not species specific, but only specific for the genus Trypanosoma. The diagnostic significance of this test is therefore doubtful in countries where both *T. equiperdum* and *T. evansi* infection occur in equines. The test interpretation is often subjective, test sensitivity is relatively low compared with more modern assay methods, and the sensitivity of the CFT declines as the serologic responses of exposed animals shifts from initial Ig M based reactions to those of other immunoglobulin classes and subclasses (Katz *et al.*, 1999). Although the CFT has been in use for many years for diagnosis of dourine, it is considered to be less sensitive than enzyme linked immunosorbent assays (ELISAs) and indirect fluorescent antibody test (IFAT) for the detection of the serum antibodies against *T. equiperdum* (Wassal *et al.*, 1991; Bishop *et al.*, 1995).

2.6.3.2. Enzyme - Linked Immunosorbent Assay (ELISA)

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

Trypanozoon group - specific trypanosomal antigen could be of use in an antibody assay for the diagnosis of *T. equiperdum* infections. Characterization of the group - specific antigen would allow identification of the peptide sequence in the epitope, which could be synthesized and used in a standardized ELISA for serological testing of dourine. In addition, an antigen detecting ELISA using monoclonal antibody directed against the same group - specific antigen might also be of use in identifying animals with active infections. To ascertain what is the likelihood that an infection with *T. equiperdum* is present when a test result is positive or that *T. equiperdum* is absent when a test result is negative, requires information on the predictive values of the assays (Bishop *et al.*, 1995).

2.6.3.3. Card Agglutination Test for Trypanosomosis CATT/ T. evansi

The monolayer of variable surface glycoproteins (VSG) determines the variable antigenic type (VAT) of an individual trypanosome is, highly immunogenic and elicits VAT specific antibodies with agglutinating and lytic activities (Van Meirvenne *et al.*, 1995). The variable antigen type (VAT) Rhode Trypanosoma Antigenic Type (RoTat 1.2) has been cloned from a *T. evansi* strain isolated in 1982 from a water buffalo in Indonesia. Based on the RoTat 1.2 VAT, different diagnostic antibody detection tests for *T. evansi* have been developed, namely CATT/ *T. evansi*, a direct agglutination test (Bajyana and Hamers, 1988), an indirect agglutination test LATEX/ *T. evansi* (Verloo *et al.*, 2001) and Immune Trypanolysis (Van Meirvenne *et al.*, 1995).

However, based on anecdotal evidence, it appears that *T. equiperdum* infected laboratory animals and horses suspected of dourine also positively react in the CATT/ *T. evansi* and ELISA/ *T. evansi* prepared with fixed whole trypanosomes of the RoTat 1.2 VAT. The CATT/ *T. evansi* developed for *T. evansi* infection is also recommended as a field-screening test for *T. equiperdum* diagnosis (Touratier, 2000). The CATT - antigen is a freeze-dried purified suspension of purified, fixed and stained blood stream from trypanosomes expressing a predominant variable antigen type of *T. evansi* (RoTat 1.2) (Bajyana and Hamers, 1988). The CATT has its advantage in its simplicity, although the test interpretation is subjective. It was observed that the CATT is not as sensitive as ELISA, CFT and IFAT, but able to detect all animals with clinical signs of dourine. It could be usefully employed especially as a field-screening test (Williamson *et al.*, 1988).

2.6.3.4. LATEX Agglutination / *T. evansi* Test

LATEX agglutination / *T. evansi* is a rapid antibody detecting indirect agglutination test, in which the antigen consists of purified variable surface glycoproteins (VSG) of *T. evansi* Vat RoTat 1.2 covalently coupled to latex particles (0.9 micron in diameter). The reagent is stabilized by lyophilisation and rehydrated with deionized water before use. Serum dilutions are prepared both for CATT/ and LATEX as two fold dilutions with PBS. Twenty micro litres of diluted sera are mixed with 20 micro litre of reagent on a test card. This method is more specific in testing for *T. evansi* than the CATT method (Verloo *et al.*, 2001).

2.6.3.5. Other serological tests

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

The AGID has been used to confirm positive test and to test anticomplementary sera. A seven - well pattern in 0.8% agarose in Tris buffer is used, with the CFT antigen in the centre well and positive control sera and unknown sera in alternate peripheral wells. AGID test is more specific than CFT and IFAT and can be used to confirm false positive results by these tests. AGID is easier to perform, that makes it suitable for large numbers of samples but the results would be obtained after 24 hours (Joyce *et al.*, 1993).

2.6.4. Animal inoculation

Under laboratory conditions, dogs develop dourine as reported by Rouget (1896). Different routes of infection such as subcutaneous, intraperitoneal, intravenous, intra-urethral and intra vaginal transmission were tested and all gave rise to obvious clinical signs of dourine. The susceptibility of dogs to *T. equiperdum* is generally high (Rouget, 1896; Schneider and Buffard, 1900) and this means that strains can be sent from remote countries after the animals have been experimentally infected. However, some breeds, like the parish dogs in India, are less susceptible or sometimes even completely resistant. In dogs, inoculation of *T. equiperdum* produces the typical picture of dourine with trypanosomes present in the lesions but not in the blood; the infection may last from one to several months Rouget (1896). Early experiments with rabbits reported specific clinical signs of dourine. In contrast, in recent experimental infections carried out in the tropical institute of medicine to raise anti sera against variable surface antigens, rabbits infected with the available laboratory strains developed clinical signs that could not be distinguished from those developed by rabbits infected with *T. evansi* (Claes, 2003).

Owing to the marked predilection of *T. equiperdum* for the testicles of rabbits, some authors recommended intra-testicular inoculation of these animals for the diagnosis of dourine in equines (Hoare, 1972). Mice and rats can be infected with the parasite, but do not develop the normal form of dourine although all existing and available laboratory strains of the parasite grow easily in these animals (Baltz *et al.*, 1986). However, laboratory rats are refractory to initial inoculation of the suspected material of *T. equiperdum* from equines. For the same reason, laboratory rodents can be used for the detection of *T. equiperdum* (Hoare, 1972).

Several and repeated attempts has been made so far by different workers (Barrowman, 1976; Alemu *et al.*, 1997; Clausen *et al.*, 1999; Clausen *et al.*, 2003) to demonstrate and isolate *T. equiperdum* in laboratory mice but all were unsuccessful. Ruminants seen to be refractory to infection with *T. equiperdum* (Hoare, 1972); but Wang (1988) was able to produce clinical manifestations of dourine in sheep and goats following the inoculation of mice adapted strain of *T. equiperdum*.

2.7. Treatment

There are no officially approved drugs to treat horses suffering from dourine although some older publications mentioned experimental treatment of horses with suramin and neoarsphenamine (Novarserobezol; Ciuca, 1993) or quinapyramine sulphate (Vaysse and Zottner, 1950). Nowadays, dourine is dealt with international legislative measures aimed at isolation, castration or slaughtering of CFT positive horses. It is important to note that castrating adult stallions does not always change the copulatory ability of such animals and it should be performed with caution when attempting an eradication programme. To prevent the introduction of dourine, semen samples should be taken following a period of isolation (quarantine) to ensure that the animals are not in the incubation period (Zablotskij *et al.*, 2003). In the laboratory, *T. equiperdum* has proved sensitivity to trypanocidal drugs diminazene aceturate, melarsomine, isometamidium, suramin and neoarsphenamine (Zhang *et al.*, 1992, Brun and Lun, 1994, Kaminsky *et al.*, 1997, Touratier, 2000). However, only neoarsphenamine and suramin have been used in large dourine eradication programmes.

The treatment schedule for neoarsphenamine was to be administered twice in high doses of 40 gram to 50 gram per adult horse. This treatment should be repeated once a year. In donkeys, neoarsphenamine was found to be toxic. For horses, even if high doses are debilitating, such doses are needed to prevent the appearance of *T. equiperdum* chemo-resistant carriers (Eyraud, 1934, Saurat, 1946, Wargacki, 1949, Kazansky, 1958). However, therapy is not recommended as infections may not respond to the drugs and animals could become carriers. Similarly, Luckins (1994) reported that *T. equiperdum* to be susceptible to quinapyramine sulphate (Trypacide, Rhone Merieux, Harlow, Essex, UK) and Suramin (Neganol Bayer, Bury St. Edmunds, Suffolk, UK).

The only effective control of dourine remains strict control of breeding; good hygiene is essential during assisted mating because infection may be transmitted through contaminated fomites. Serological testing ensures that infected animals are not used for breeding and legislation requiring testing of horses for export prevents the movement of infected animals. In Europe and in Asian part of Russia, dourine is strictly controlled by measures put in place after CFT testing. These measures include the segregation and quarantine of reactors, treatment with high doses of trypanocides by close surveillance for several months and the slaughtering of the reactors (Zablotskij *et al.*, 2003).

2.8. Current diagnostic challenges

Currently, neither parasitological nor serological tests can make a clear distinction between *T. b. brucei, T. equiperdum* and *T. evansi* infections in solipeds. The clinical signs of dourine, chronic surra and chronic nagana are very similar and prohibit correct differential diagnosis (Zhang *et al.*, 1994; Bishop *et al*, 1995). Species - specific molecular tests to distinguish these three trypanosomes are not available and the existing molecular trypanosome detection techniques have not yet been validated for diagnostic purposes (Zhang *et al.*, 1994). An overview of the biological parameters currently used to distinguish between *T. b. brucei, T. evansi* and *T equiperdum* and their respective diseases is given in Table 2.

Table 2. Classification of the Trypanozoon subgenus according to the current biological parameters.

Parameters		***T. b. brucei***	***T. evansi***	***T. equiperdum***
Disease		Nagana	Surra	Dourine
Course		Acute to chronic	Acute to chronic	Acute to chronic
Clinical signs		Anaemia, fever, oedema, urticarial plaques	Anaemia, fever, oedema, urticarial plaques	Anaemia, fever, oedema, paralysis and oedematous cutaneous plaques
Transmission		Cyclical (Tsetse fly)	Mechanical (Biting flies)	Sexual
Host		Multiple	Multiple	Equine
Diagnosis				
	Parasitology	mHCT	MAECT	None
	Serology	IFAT, ELISA	CATT/T. evansi, LATEX/T.evansi ELISA, Trypanolysis RoTat 1.2	CFT
	PCR	PCR/Trypanozoon	PCR/Trypanozoon	PCR/Trypanozoon
Treatment		Various drugs	Various drugs	None

Source: (Claes, 2003).

Trypanosoma evansi and *T. equiperdum* were compared regarding their ultra structure, mammalian hosts, way of transmission, pathogencity, diagnosis and treatment, biochemical and molecular characteristics (Brun *et al*., 1998). Electron microscopic examination revealed no ultra structural differences between the two species except that there were more coated vesicles in the flagellar pocket for *T. equiperdum*. Biological, biochemical and molecular studies indicated many similarities between *T. evansi* and *T. equiperdum* as indicated below in Table 3.

The most prominent differences between the two species are the presence of maxicircles in *T. equiperdum*, which are missing in *T. evansi*, and the route of transmission. While biting flies transmit *T. evansi*, *T. equiperdum* is transmitted from one equine host to another during copulation when mucous membranes come into contact. Otherwise the two species are remarkably similar. In Ethiopia, the distribution of dourine and surra are in close proximities and within a range of 100 km distance from each other (Figure 1).

Table 3: Comparison of the characteristics of *T. evansi* and *T. equiperdum*.

Character	***T. evansi***	***T. equiperdum***
Size	15-36μm with free flagellum	15.6-31.3μm
Morphology	Typically monomorphic, pleomorphic forms occur in some strains	Typically monomorphic, pleomorphic forms occur in some strains
Natural hosts	Equines, camels, cattle, buffalo, deer, Asian elephants, tigers, vampire bats etc	Equines only
Vectors and transmission	Tabanus spp. Stomoxys spp (Mechanical transmission)	Direct transmission during coitus
Drugs used for treatment	Diminazene, Suramin, Quinapyramine and Cymelarsan	Identical to *T. evansi*, usually not recommended
Isoenzymes	Differences were found only in two (MDH and ALAT) of 16 enzymes between strains of *T. evansi* and *T. equiperdum*	
KDNA and hybridization with PTK 420	Minicircles only	Minicircles hybridized with PTK 420, Maxicircles also present
RFLPs in rDNAs and VSG genes	No differences was found	

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

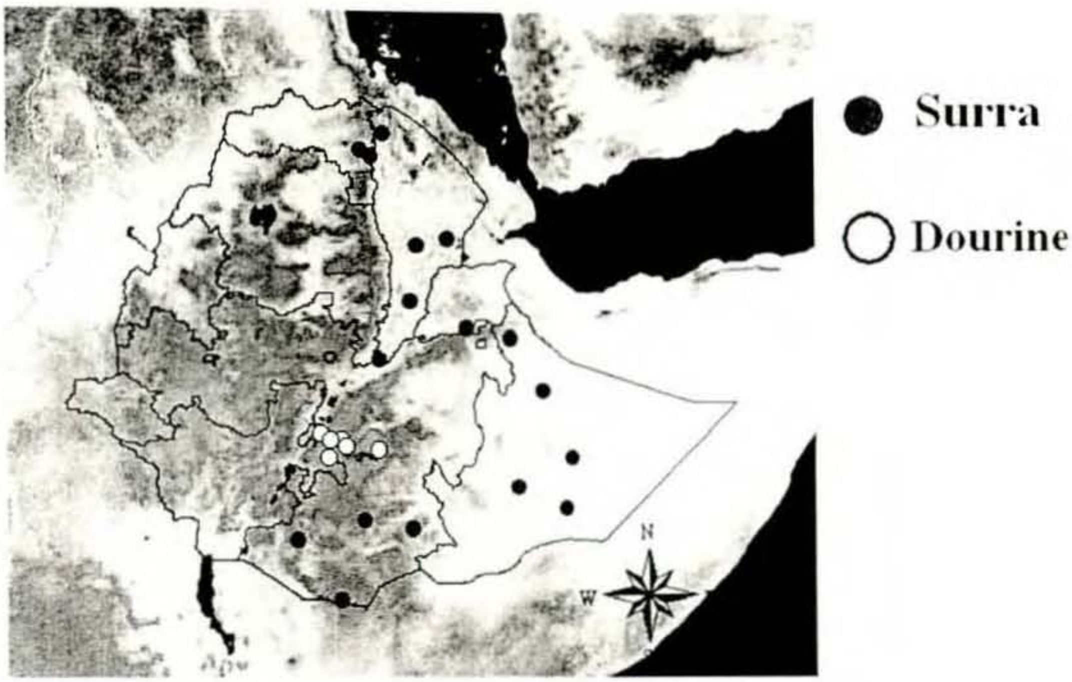


Figure 1: Distribution of dourine and surra in Ethiopia

For *T. evansi* infections, the only specific test available so far is based on the detection of a kinetoplast DNA sequence (Masiga and Gibson, 1990; Viseshakul and Paynim, 1990). However, the existence of dyskinetoplastic trypanosomes such as *T. evansi* RoTat 5.1 and E152 (Masiga and Gibson, 1990) casts doubt about the diagnostic potential of such tests to detect all infections caused by *T. evansi* parasites. According to recent studies by Claes (2003), based on the expression of the RoTat 1.2 VSG in different *T. evansi* and *T. equiperdum* strains examined using the antibody detection tests revealed that RoTat 1.2 is not strictly *T. evansi* specific or RoTat 1.2 expressing putative *T. equiperdum* strains are in fact misidentified *T. evansi* and BoTat 1.1 and OVI are the only genuine *T. equiperdum* strains in the tested collection. Based on the previously disclosed DNA sequence of the RoTat 1.2 gene (Urakawa *et al.*, 2001), a RoTat 1.2 specific PCR has been developed and this PCR confirmed the above-mentioned hypothesis. Molecular and immunological research studies are currently in development could give rise to promising findings in the near future (Touratier, 2000; Claes *et al.*, 2003; Lun, *et al.*, 2003).

3. MATERIALS AND METHODS

3.1. Study area

The present study was conducted in the Oromiya Regional State in Arsi-Bale highlands and eight selected sites of the country where occurrence of dourine is not previously reported and known. Investigation for the presence of dourine (*T. equiperdum*) outside of the endemic foci was conducted in eight selected representative areas of the country: from the central highlands of Ethiopia, Selale zone, Fitche district and East Shoa zone, Shashemene district; Eastern Ethiopia, Shinelle zone, Shinelle district; Western Ethiopia, Jimma zone, Dedo district; Southern Ethiopia, Sidama zone, Arbegona district and Gurji zone, Uraga district; North western Ethiopia, Awi zone, Enjebara district and North eastern Ethiopia, South Wollo zone, Kutaber district (Figure 2).

Arsi-Bale highlands are found in the Oromiya Regional State southeast of the country where Asela and Robe the capitals of Arsi and Bale zones are located 175 Kms and 430 Kms away from Addis Ababa. Topographically, the altitude ranges from 500 to 4130 m.a.s.l. where a central plateau (2000-2500 m.a.s.l.) predominates with a narrow lowland area. Three climatic zones, including an arid, tropical highland and tropical forms are known to exist. The area experiences a bimodal rainfall occurring from July to October and April to May. An average annual temperature of 20- 25 ^{0} C and rainfall of 200 mm in the lowlands whereas 10- 15 ^{0}C with a rainfall of 400 mm in the highlands are recorded. Vegetation of the area change with altitude and rainfall ranging from scattered trees and bushes to dense shrubs in different altitude and from thorny and fibrous grass of dry season to bushy and soft grass of rainy season. From the total area 43% is used for grazing, 35% for cultivation, 8.6% for forestland, 2.7% unproductive and 10.7% productive but unutilised land. The major land cover is thus used for grazing which support on average 27 livestock per hectare (Arsi Plan and Economic Development Office, 1999). Agriculture is the mainstay of the livelihood of peoples and the leading economic activity of the area with a mixed farming system covering 90% of the total agricultural activities with crop-livestock production.

Livestock plays an integral role for agricultural activity, which also provides meat, milk, cash income and transportation purposes. The livestock species reared are cattle, sheep, goat, horses, donkeys, mules, camels and poultry. Equine population is the highest in the Oromiya Regional State mainly of the Arsi-Bale highlands (CSA, 2003). Equines have a prominent position in the agricultural and transport systems as draft, pack and riding animals. In the Arsi-Bale highlands where there is less developed modern transport and communication service, the natural choice rests on the use of human and pack animals mode of transport, as it has been the case in some parts of the world. Thus, the contribution of equines in the energy scenario is of considerable significance. Communal grazing is the traditional way of feeding animals and crop residue used extensively during the dry period in the study area. In the Arsi-Bale highlands, infectious diseases like anthrax, black leg, pasteurolosis, lumpy skin disease, African horse sickness, and parasitic diseases such as fasciolosis, dictyocaulosis, trichostrongylosis, protozoan diseases primarily trypanosomosis (dourine) and coccidiosis etc. are the main constraints of livestock production. Lack of grazing land due to expansion of cultivated land also exacerbates the livestock to be affected by the above-mentioned problems.

3.2. Study design and sampling strategies

3.2.1. Arsi-Bale highlands

3.2.1.1. Cross sectional study

A Cross-sectional study design based on serological, parasitological and questionnaire survey was conducted in six selected horse-breeding districts of the Arsi-Bale highlands. A combination of multistage stratified and purposive sampling methods were applied according to Thrusfield, (1995). First the six discrete study districts were selected from Arsi and Bale zones (first stage) to represent the Arsi-Bale highlands. Then lists of PA's (Peasant association) within districts were complied from data obtained in the districts agricultural office (second stage) and sampling PA's were selected based on representation of the respective districts and accessibility. Villages were selected in collaboration with the respective district's animal health personnel, selected by purposive sampling on the basis of farmer's cooperation, logistics, share of communal grazing land and accessibility (third stage). Selected villages and herds grazing within the same grazing land were considered as strata. With in each stratum, sampling was performed irrespective of the other strata.

Taking average prevalence of 30% based on previous reports (Alemu *et al.*, 1997; Clausen *et al.*, 1999), absolute desired precision of 5% and confidence level of 95% for estimating prevalence in simple random sampling according to Thrusfield, (1995) the sample size was determined as follows:

$$n = \frac{1.96^2 P_{exp} (1 - P_{exp})}{d^2}$$

Where:

n = required sample size

P_{exp} = expected prevalence

d = desired absolute precision

The calculated sample size for estimating prevalence in simple random sampling was 323. In order to adjust the sample size required for the present multi stage stratified random sampling method for an estimate of the disease prevalence at a 5% level of precision, the sample size was inflated two times than in simple random sampling. Therefore, a total of 646 animals were sampled proportionally from the Arsi-Bale highlands, 388 (60% from the Arsi zone) and 258 (40% from the Bale zone) based on horse population size. In order to investigate clinical cases as well as observe serological status of donkeys and mules in the Arsi-Bale highlands, 10 animals for each species (a total of 60 donkeys and 60 mules) were considered uniformly in all of the study districts. On the other hand, purposive sampling was employed for the parasitological investigation from selected clinical cases to increase chance of isolation of the parasite. There was no evidence of *T. evansi* infections in the Arsi-Bale highlands and moreover the study sites were at altitudes outside of the known tsetse infested areas. Animals considered in this study were sexually mature adults of varying ages primarily horses and certain numbers of donkeys and mules living under a traditional management system of free grazing. Important study variables including age, sex, body condition, parity number, history of previous abortion, castrated or non-castrated, born on farm/bought outside Arsi-Bale local markets, clinical stage and equine species were considered and recorded in pre designed data collection format (Annex 1).

Aging of horses and donkeys was made based on the description provided by Coombs, (2002). Animals between 3 and 6 years of age were considered as young adults while more than 6 years old as adults. Body condition scoring and live weight estimation of horses was made based on the description provided by Coombs, (2002). Details are attached in Annex 2.

A. Questionnaire survey

A pre-tested structured questionnaire, designed to include questions about factors either known or thought to influence the spread of dourine, was given in person. The format was filled by directly interviewing randomly selected animal owners (n= 60), professionals and paraprofessionals (Community Animal Health Workers) working in the areas (Annex 3). The questionnaire was pre-tested before the actual survey for time, resource and relevance of type of questions included. As the researcher was able to speak and hear the local language (Afan Oromo) the effect of interpreter was reduced.

B. Parasitological survey

Blood examination

Following collection of blood (about 10 ml) from the jugular vein of horses, mini Haematocrit Centrifugation Technique (mHCT) according to Woo (1970) was conducted for parasitological diagnosis of dourine. A capillary tube containing anticoagulant (Heparin / EDTA) was filled with blood for three quarters and sealed on the dry end with plasticine. The capillary tube was centrifuged at high-speed 12,000 rpm for 5 minutes in haematocrit centrifuge. By high-speed centrifugation of blood in haematocrit centrifuge, trypanosomes are concentrated at the level of the white cells, between the plasma and the erythrocytes and screened for under microscope. Each microhaematocrit tube was cut 1 cm above and 1 mm below the buffy coat layer. The content then expressed on clean slide, examined for motile parasites under a 40 X objective microscope. The buffy coat contents were directly inoculated into dogs subcutaneously within 30 minutes of collection with 1-2 ml of total volume.

Smear examination

By washing out vaginal discharge using vaginal speculum and penile urethra using a catheter and 20 ml syringe with sterile normal saline, the genital wash from clinically suspected cases of dourine was collected in sterile tube. Then the wash centrifuged at 3000 rpm and the deposit examined as a wet smear preparation under a cover slip with a magnification of 300. At the same time thin smears were prepared (dried, fixed, stained with Giemsa) and microscopically examined under oil immersion. The swollen clitoris or prepuce as well as glans penis were cleaned with a swab soaked with ethyl alcohol and incised with sterile scalpel blade and as blood/ oedematous fluid oozes out, thin smears were prepared (dried, fixed, stained with Giemsa) and microscopically examined under oil immersion.

Animal inoculation

On the basis of previous works conducted by (Rouget, 1896; Schneider and Buffard, 1900) young dogs (puppies) up to two months of age were used as laboratory animal for the isolation of *T. equiperdum*. Specimens obtained from clinical cases of dourine such as vaginal or prepucial flush, oedematous fluids and buffy coat contents were inoculated into dogs subcutaneously within 30 minutes of collection with 1-2 ml of total volume. An attempt also made to increase the possibility of isolating trypanosomes by inoculating dogs with different specimens from multiple horses. Parasitemia were monitored weekly by the mHCT according to Woo, 1970 and animals followed for three months. Details are attached in Annex 9.

C. Serological survey

Approximately, 7-10 ml of blood samples were collected from the jugular vein of each animal using plain vacutainer tubes and needles, after the site is wiped with cotton wool soaked in alcohol. The vacutainer tubes were labelled and set tilted on a table overnight at room temperature to allow clotting. Then the serum samples were filled into serum storage (Polypropylene sterile cryogenic) vials and stored at -20^0 C until the serological tests were performed.

Study on the seroprevalence of *T. equiperdum* in Arsi-Bale highlands from a total of 646 sera samples was conducted using the card agglutination for trypanosomosis test (CATT/ *T. evansi*), and LATEX/ *T. evansi*. For both tests (CATT and LATEX) the end point titre was defined as the highest dilution of the test serum still showing a single a positive result and positive samples were determined at cut-off point dilutions 1:8 and above. Moreover, those positive as well as negative sera for both tests were subjected for examination by the ELISA Ro Tat 1.2 VSG of *T. evansi*, at the Prince Leopold Institute of Tropical Veterinary Medicine (ITM), Antwerp, Belgium. The test procedures followed and details of the diagnostic steps applied were those described in the bench protocol manual of the Prince Leopold Institute of Tropical Veterinary Medicine (ITM), Antwerp, Belgium (Annex 4 and 5). Each sample will be measured twice to find an average OD. Results may plus or minus number depend on the OD of control sample. The positive sample is the one yielding an OD over 50% of the control one.

3.2.1.2. Longitudinal study: Assessment of the therapeutic activity of Isometamidium Chloride and Diminazene Aceturate in the field

A. Study animals and management

Selected 24 adult local horses with clinical dourine cases and confirmed serological (CATT and LATEX) test results were used in the present field treatment trial. Additional criteria of selection of the study animals were their relative uniformity in their initial body conditions, size, age, sex, reproductive status and PCV. All the experiment animals were kept under traditional village management and were regularly monitored once in 20 days, body condition scores evaluated, blood and serum samples collected (Annex 7 and 8).

B. Experimental Protocol

Treatment groups

The selected 24 animals were divided into two treatment groups of equal number (8) designated as Group I and II and one control group consisting of eight animals (Table 4).

Table 4: Repartition of experimental animals and description of the treatment administration.

Groups	No. of animals	Treatment groups		Control group
		ISMM	DIM	
I	8	0.5 mg/kg bw	-	-
II	8	-	3.5 mg/kg bw	-
III	8	-	-	Untreated

Every animal in group I and II were treated once with Isometamidium chloride at dose rate of 0.5 mg/kg BW as 2 % solution (TRYPAMIDIUM-SAMORIN ® Manufactured by Merial- 17, rue Bourgelat 69002 Lyon France. DOM: 25/06/2003, Batch: w 391971, Exp: 06/2008) and Diminazene aceturate at dose rate of 3.5 mg/kg BW as 7% solution (DIMINAZENE ACETURATE-BERENIL® Norotryp, Norbrook Laboratories Limited, Station Works, Newry, Co., Down, N. Ireland, U.K., B.N: 34441, DOM: 21/010/2003, Batch: G 002, Exp: Oct- 2007), respectively. Animals in the control group were remained untreated, hence served as positive controls.

For calculating the treatment dose, the body weights of each of the animals were estimated before treatment, using tapes for measuring heart girth and body condition scoring methods (Coombs, 2002). Both Diminazene aceturate and Isometamidium chloride were administered by deep intramuscular route in to the muscles of the middle third of the neck and volumes exceeding 20 ml were applied at two different locations in order to avoid local pain.

Measured Parameters

All the experimental animals were monitored initially and then after once in 20 days to determine PCV, body temperature, leukocyte differential count, CATT LATEX and ELISA serological tests, evaluate body condition score and assess improvement of the previously existed clinical signs.

Sampling and Examination Procedures

A. Packed Cell Volume

Blood samples were collected by bleeding animals from marginal ear veins into paired heparinized microhaematocrit capillary tubes up to ¾ of their length. One end of the tubes were then sealed with cristaseal (Hawaksly, England). The tubes were symmetrically loaded in the haematocrit centrifuge, with the sealed end outwards, centrifuged at 1200 rpm for 5 minutes. PCV levels of individual samples were determined on haematocrit reader (Hawaksly, England) and the values were expressed in percentages (Woo, 1970).

B. Differential Leukocyte Count

During blood sampling for PCV, thin blood samples were also prepared for the purpose of carrying out differential leukocyte count. Thin smears were first air dried and fixed with methanol for 3-5 minutes, and stained with Giemsa for 30 minutes, washed with distilled water and dried on the air. Thin smears were microscopically examined under oil immersion magnification (X100) and counting and classifying of 200 leukocytes were made using Battlement method and finally values were expressed in percentage (Dacie and Lewis, 1991). For the purpose of this study, the proportional percentage of eosinophil count was only considered.

C. **Body temperature determination**

Body temperature of the study animals was recorded by inserting digital thermometer through the rectal mucosae and keeping for 2-3 minutes.

D. **Serological (CATT, LATEX and ELISA) tests**

Study on the serological status of the study animals was conducted using the card agglutination for trypanosomosis test (CATT/ *T. evansi*), LATEX/ *T. evansi* and ELISA/ *T. evansi* tests. The test procedures followed and details of the diagnostic steps applied were those described in the bench protocol manual of the Prince Leopold Institute of Tropical Veterinary Medicine (ITM), Antwerp, Belgium (Annex 4).

E. **Body condition scoring**

Body condition scoring of the study animals was estimated according to the description provided by Coombs, 2002.

3.2.2. Investigation of dourine outside the endemic foci

Investigation for the presence of dourine outside the endemic area of the Arsi-Bale highlands was conducted using questionnaire and serological survey. Using a pre-tested and semi-structured questionnaire format, an attempt was made to generate information on the general aspects of dourine. The format was filled by directly interviewing randomly selected animal owners and professionals working in the areas (Annex 6). The questionnaire was pre-tested before the actual survey for time, resource and relevance of type of questions included. Study on the serological investigation of dourine (*T. equiperdum*) in areas outside of the endemic foci in selected parts of the country, was conducted using the CATT, LATEX and ELISA tests. For both CATT and LATEX tests the end point titre was defined as the highest dilution of the test serum still showing a single a positive result.

Moreover, those positive as well as negative sera for both tests were subjected for examination by the ELISA Ro Tat 1.2 VSG of *T. evansi*, at the Prince Leopold Institute of Tropical Veterinary Medicine (ITM), Antwerp, Belgium. The test procedures followed and details of the diagnostic steps applied were those described in the bench protocol manual of the Prince Leopold Institute of Tropical Veterinary Medicine (ITM), Antwerp, Belgium (Annex 4 and 5).

The study design to undertake the present dourine investigation outside of the endemic foci (Arsi-Bale highlands) was cross sectional type involving systematic random sampling method. In order to ensure representativeness and randomness with regard to the study animals, horses sampled were from one main market place during the main market day of the respective area. The study animals from the market places were selected systematically by taking every fifth animal or otherwise. The starting point for the first interval was selected on a formal random basis. The minimum sample size, which enables us to detect at least one diseased animal in the population, was calculated as follows (Thrusfield, 1995):

$$n = (1-(1-\alpha)^{1/d}) \times [(N-d/2)+1]$$

Where:

n = required sample size

α = is the desired confidence level (that is the probability of finding at least one positive in the sample)

N= Population size of equines in a given district

d = Prevalence of the disease when it exists in a population x N (population size)

Considering α (confidence level) to be 99%, an average of total population of 5,000 horses in a district and 10% prevalence of the disease when it exists in a population, the minimum sample size which enable us to detect at least one diseased animal in the population was 44 and therefore a total of 352 animals were examined from the eight different sites of the country. The study sites were adjacent geographical areas of the Arsi-Bale highlands and other selected parts of the country where there is high equine population, unrestricted mobility, and uncontrolled breeding.

Based on high horse population size, particularly being horse-rearing areas of the country and suspect for the possible presence of the disease, eight representative sites were considered for the questionnaire and serological survey of dourine. From the central highlands of Ethiopia, Selale zone, Fitche district and East Shoa zone, Shashemene district; Eastern Ethiopia, Shinelle zone, Shinelle district; Western Ethiopia, Jimma zone, Dedo district; Southern Ethiopia, Sidama zone, Arbegona district and Gurji zone, Uraga district; North western Ethiopia, Awi zone, Enjebara district and North eastern Ethiopia, South Wollo zone, Kutaber district (Figure 2).

For all study sites mentioned above, an accurate on site 3D GPS location, latitude -longitude coordinates were taken using GPS 76, GARMIN device to specifically indicate the respective study sites (Annex 10). The GIS databases were developed using Arc View 3.2 GIS (ESRI, Redlands, CA) and ERDAS imagine 8.3.1 (ERDAS, Atlanta, GA) softwares. The GPS locations of all the study sites included in the GIS were transformed to geographic, latitude-longitude, decimal degree format using the ESRI Digital Chart of the World (DCW) as a base map standard for GIS construction. The food and Agriculture (FAO) Crop Production System Zone (CPSZ) database was originally developed for predicting a relative ecological suitability of an area for various crops (Abebe *et al.*, 2004).

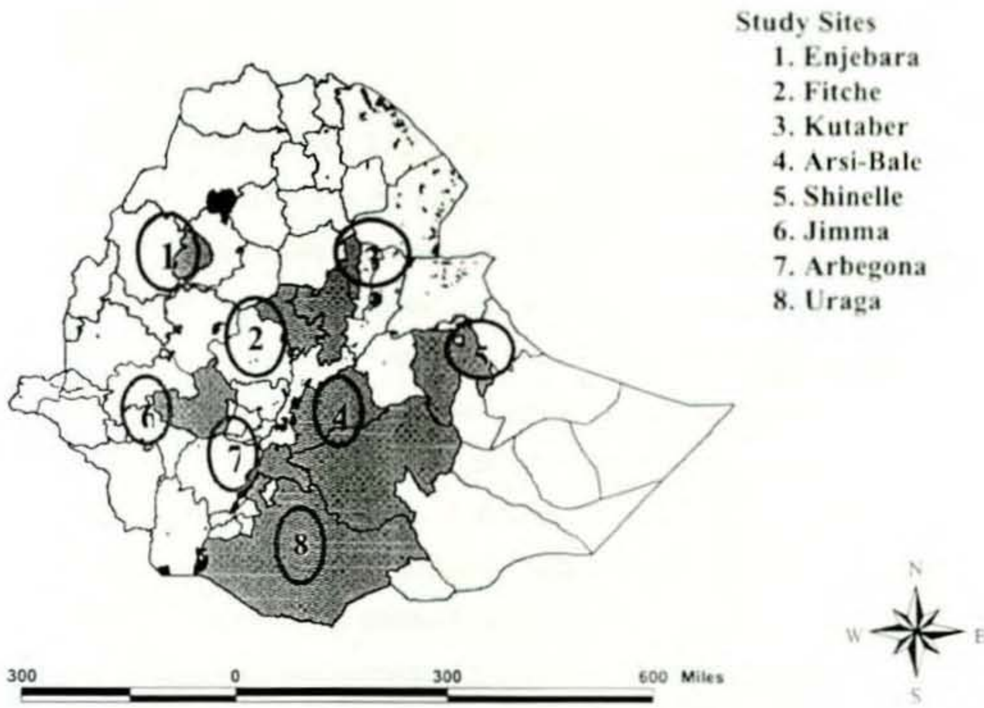


Figure 2: Map showing selected study areas in Ethiopia.

3.2.3. Data Analysis

3.2.3.1. Data management

Data obtained by means of questionnaire survey, treatment trial and parasitological and serological of individual animals was inserted into MS Excel Spread Sheets (Microsoft Corp.) to create a database and transferred to the SPSS software programme of the computer before analysis.

3.2.3.2. Statistical analysis

Collected data was subjected to SPSS, 2002 software of the computer program for the statistical analysis. The prevalence is defined as the proportion of the number of positive animals to the total number of animals examined and expressed in percent. Data on the questionnaire was summarised using frequency distribution and percentages. The impact of explanatory variables (sex, age, animal origin, parity number, history of previous abortion, effect of castration and body condition) on the serological test results was assessed by logistic regression models. The exponentiated estimates of the coefficients of the models were interpreted as odds ratio (OR). Three separate regression models were built. In the first model, risk factors associated with male animals like effect of castration were considered. In the second model, risk factors associated with female animals namely history of previous abortion and parity number were included. In the third model, risk factors associated commonly for both male and female animals such as age, sex and animal origin were included. Univariable logistic regression analysis was employed to determine the association of risk factors with the seroprevalence of dourine. Only risk factors of high biological relevance or variables with a p-value < 0.20 on univariate analysis were subjected to multivariate analysis to determine major risk factors. Variation among and within the clinical groupings was determined by ANOVA and the distribution of animals among the clinical groups, as classified either positive or negative by the CATT, LATEX and ELISA test results was assessed by the Chi-square (χ^2). Kappa statistical test was employed to determine the test agreement of the different serological tests. Least Square Repeated measure analysis of variance was used to show the effect of ISMM and DIM therapy on PCV, body temperature and eosinophil counts on 0, 20 and 40 days after treatment (SPSS, 2002).

4. RESULTS

4.1. Arsi-Bale highlands

4.1.1. Cross sectional study

4.1.1.1. Parasitological study

No trypanosomes or any other haemoparasites could be detected in all examined Giemsa stained smears (thin blood, genital discharge and tissue fluids) as well as in blood samples by mHCT. All the puppies inoculated with blood samples, genital washes and oedematous fluids remained parasitologically negative.

4.1.1.2. Questionnaire survey

The results of the questionnaire survey revealed the presence of major diseases of horses such as: Dourine, Epizootic lymphangitis, African Horse Sickness, Anthrax and Ecto-Endo parasitism in the Arsi-Bale highlands of Ethiopia. Both animal owners and professionals interviewed reported that dourine is a major health problem of horses causing high mortality and economic loss (Table 5). The farmers reported that the first sign of the disease in clinically affected horses is incoordination, especially of the hindquarters and swelling of the external genitalia. The disease is locally known by different names as "Lappessa" mainly by the Arsi farmers, which refers to the extreme emaciation of affected cases, "Duda Kuta", "Kuta" or "Diressa" by the Bale people which refers to the hind leg paralysis (in English it means back bone breaker).

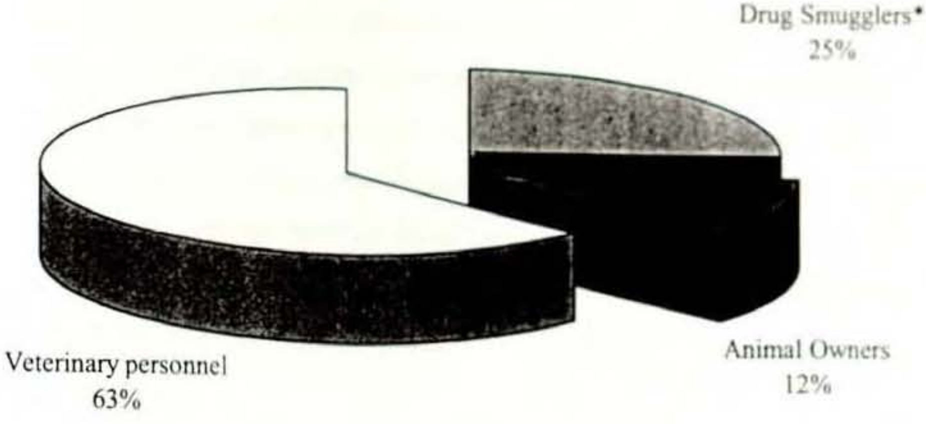
Since dourine is principally and only transmitted by coitus and moreover, attributed to the absence of curative treatment nor prophylactic vaccine and the marked emaciation observed in late stages of the disease, some farmers used to call it "Horse's AIDS". Even other farmers associated the disease incidence with horses having sexual contact with the so-called "Prostitute horses" or diseased horses.

It was also emphasised by the farmers as well as professionals that the problem of dourine is becoming more and more severe and increasing its extent and magnitude, as there is no effective curative or prophylactic therapeutic agents. Though, dourine is a common clinical case through out the year, it has a seasonal character, which most commonly occurs following the breeding season from June to late September. Sometimes a second peak is observed in the dry seasons of the year (March to May), which probably associated with relapse of previously infected and recovered cases due to stressful conditions of feed shortage (Table 5).

Horses are treated against dourine only irregularly, when trypanocidal drugs are available and even such treated animals show frequent relapse and generally treatment is not effective enough to cure clinical cases. Some of the trypanocidal drugs used in the area, whenever available include Verbien (Diminazene aceturate) of Pakistan and Korea products and in the past before ten years time Quinapyramine sulphate (Triquin- S®, Wockhardt Veterinary Ltd., India). According to the questionnaire survey smugglers, owners and veterinary personnel involved in the treatment of dourine infected animals are indicated in figure 3.

Table 5: Interview results of individual farmers from the questionnaire survey.

Interview (Points of focus)	No. of respondents	Proportion	95 % CI
Previous problem of dourine	60	32 (53.33%)	(66.06, 40.60)
Dourine a major disease of horses	60	41 (68.33%)	(80.20, 56.46)
Mares with previous history of abortion	60	12 (20%)	(30.20, 9.80)
Animal history (Origin)	60		
Born on farm		38 (63.33%)	(75.63, 51.04)
Bought outside Arsi-Bale local markets		22 (36.67%)	(48.96, 24.38)
Seasonality of the disease occurs	60		
Yes		51 (85%)	(94.11, 75.89)
No		9 (15%)	(24.11, 5.89)
Status of the disease	60		
Getting worse		36 (60%)	(72.50, 47.5)
Getting better		6 (10%)	(17.66, 2.34)
No change		18 (30%)	(41.69, 18.31)
Treatment of clinical cases	60		
Cases cure		7 (11.67%)	(19.86, 3.48)
Cases relapse		53 (88.33%)	(97.84, 78.8)



*Drug smugglers are non-professional people who bring trypanocidal and other drugs from towns and sell them to farmers in the area illegally.

Figure 3: Personnel involved in the treatment of dourine in the Arsi-Bale highlands of Ethiopia

4.1.1.3. Clinical examination

Different characteristic clinical signs of dourine were observed in clinically sick horses of both sexes. In mares, vaginal discharge mainly of mucopurulent type with foul odour, oedema of the external genitalia and presence of depigmented scars over the external genitalia were the prominent signs observed as genital form of the disease. In some mares frequent ulceration was observed and there were ulcers on the labia and clitoris. In stallions, oedema of the scrotum and prepuce accompanied by prepucial as well as urethral discharge and ulceration of the genital mucosae mainly of the penile tissue were the frequently observed signs as genital form of the disease.

In both sexes, lameness in one or both legs, restlessness, partial dragging or stiffness of the hind legs, incoordination, asymmetrical posture and tendency to shift weight from one leg to another were the dominant signs observed as nervous form of the disease. Both veterinarians and farmers indicated that some of these nervous forms of the disease become paraplegic with marked muscular atrophy in the gluteal region followed by paralysis and finally death. Interestingly, in addition to all these clinical signs, dourine suspected horses were frequently emaciated, weak and in poor body condition. The cutaneous form of the disease, which mainly characterised by 'urticarial plaques', marked by distinct, raised round or oval shaped patchy eruptions that appear on the skin in both sexes was not observed. Based on the clinical signs indicated above, horses were categorised into one of the following five groups (Figure 4): Some selected pictures taken during the fieldwork are presented below in figure 5.

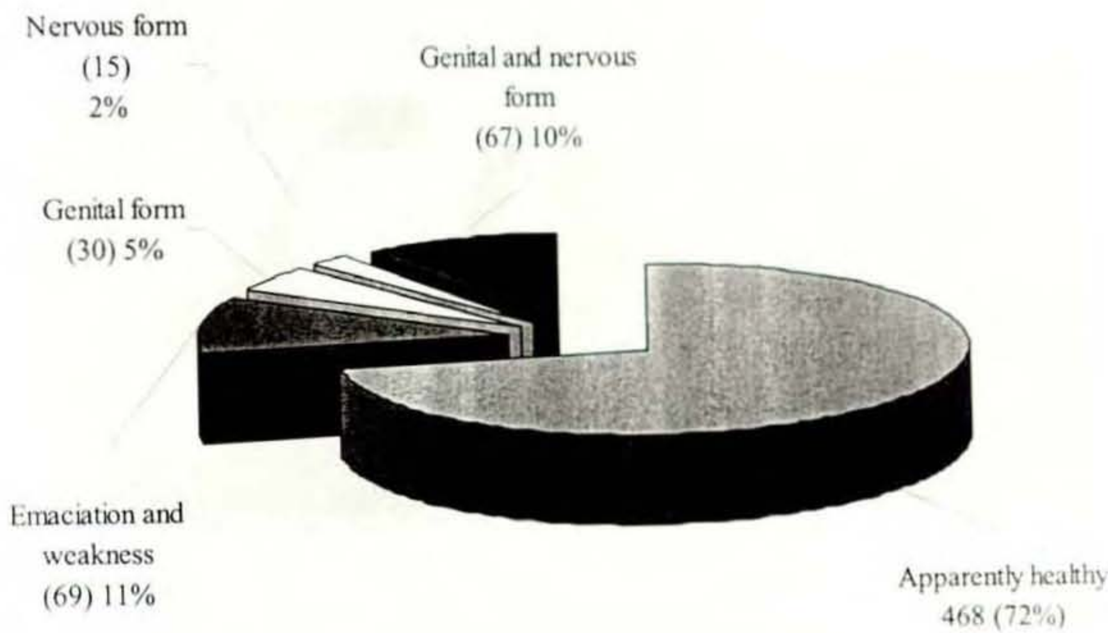
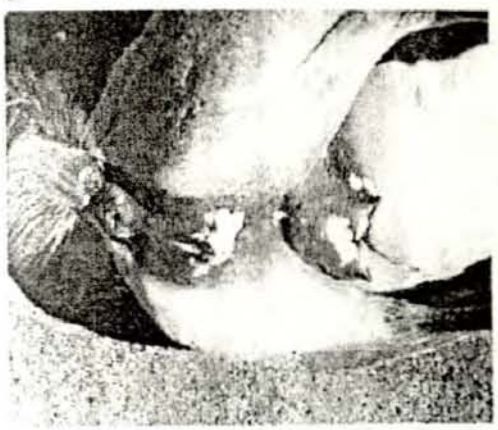


Figure 4: Repartition of the study animals based on clinical findings.

A

B

C



D

A- Photo showing oedematous swelling over the ventral abdomen and penis

B- Photo showing depigmentation over the external genitalia

C - Photo showing depigmentation over the external genitalia and udder

D- Photo showing poor body condition

Figure 5: Selected serologically positive clinical cases of dourine in horses from the Arsi-Bale highlands.

4.1.1.4. Serological study

Out of a total of 649 horse sera tested for the detection of antibodies against *T. equiperdum* in the Arsi-Bale highlands, 184 (28.35%), 161 (24.81%) and 125 (19.26%) samples were found to be seropositive for CATT, LATEX and ELISA tests, respectively (Table 6).

Table 6: Seroprevalence of dourine (*T. equiperdum*) in horses of the Arsi-Bale highlands based on CATT, LATEX and ELISA tests.

Study districts	Sample size	Seroprevalence		
		CATT	LATEX	ELISA
Arsi-Robe	128	20 (15.63%)	17 (13.28%)	13 (10.16%)
Asassa	130	36 (27.69%)	34 (26.15%)	26 (20%)
Koffle	128	49 (38.28%)	46 (35.94%)	36 (28.13%)
Dodola	91	30 (32.97%)	26 (28.57%)	22 (24.18%)
Goba	86	27 (31.39%)	18 (20.93%)	14 (16.28%)
Kokosa	86	22 (25.58%)	20 (23.26%)	14 (16.28%)
Over all prevalence	649	184 (28.35%)	161 (24.81%)	125 (19.26%)

Of the 649 horse sera tested for the detection of anti-*T. equiperdum* antibody using indirect antibody ELISA, 125 samples had percentage positivity values over 2.1-fold the negative PP value, of which 66 (52.8%) were female and 59 (47.2%) were male. The frequency distribution of the test sera showed a clear demarcation of the positive/negative threshold (Figure 6).

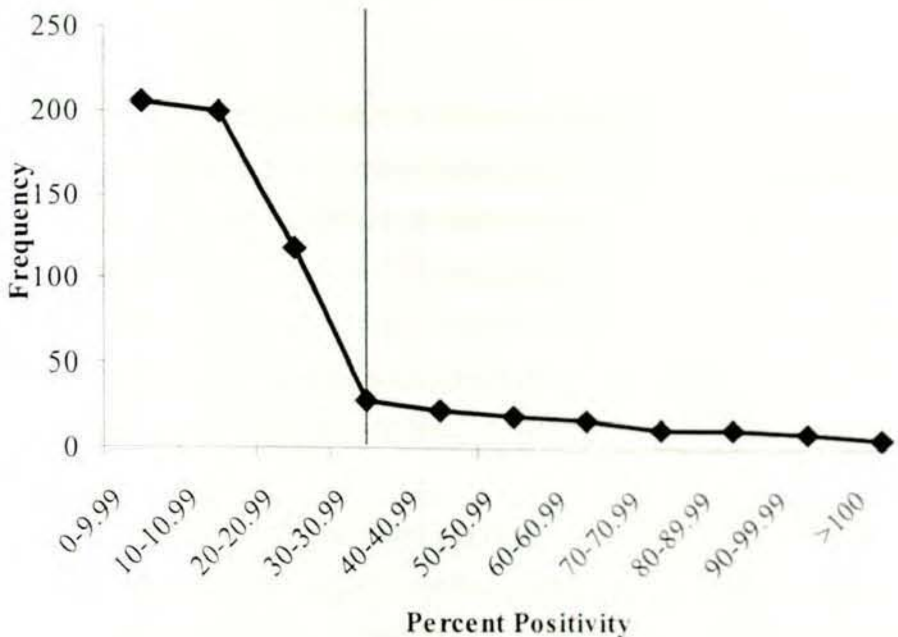


Figure 6: Frequency distribution of horse sera tested for indirect ELISA antibody detection. The dotted line denotes the cut-off point.

Fifty four horses with high percentage positivity for the indirect antibody ELISA test belonged to the five of the different clinical groups: Fourteen animals with out obvious clinical signs (group1), Ten animals with non-specific signs for dourine like emaciation and weakness (group 2), six animals with typical nervous form of the disease (group 4) and twenty four animals with clinical signs of dourine including both genital and nervous forms (group 5).

Similarly, 19 horses with strong agglutination reactions having end titre of 1:32 for both CATT and LATEX tests belonged to the five of the different clinical groups: three animals with out obvious clinical signs (group1), one animal with non-specific signs for dourine like emaciation and weakness (group 2), one animal with typical nervous form of the disease (group 4) and eleven animals with clinical signs of dourine including both genital and nervous forms (group 5). For the indirect antibody ELISA test, there was a significant variation among the clinical groups (ANOVA $F=14.83$, $P<0.001$) with increasing test result readings corresponding to increasing clinical score.

Chi-square analysis of the distribution of animals, classified as either positive or negative on the basis of all the three tests, in the clinical groups demonstrated a statistically significant difference between groups, with stratum specific prevalence of positive animals increasing with increasing severity of clinical signs ($p<0.001$). The seroprevalence on the basis of the ELISA test for the five clinical groups 1, 2, 3, 4 and 5 were, 4.10%, 17.39%, 76.67%, 80% and 88.06%, respectively. Similarly, the seroprevalence based on CATT and LATEX tests for the five clinical groups 1, 2, 3, 4 and 5 were 5.98%, 46.38%, 73.33%, 80% and 92.54%, respectively.

Using logistic regression, the OR (95% confidence interval, CI) for males versus females was 0.808 (0.524, 1.091) and 0.771(0.421, 1.420); young adults horses (up to 6 years) versus adults horses (older than 6 years) was 0.992 (0.631, 1.353) and 0.792 (0.431, 1.153) horses born on farm versus bought outside Arsi-Bale local markets was for 0.921 (0.461, 1.381) and 0.508 (0.324, 0.791) for the outcome variable CATT and LATEX tests, respectively (Table 7). A similar model for the outcome variable ELISA revealed an OR of 0.763 (0.324, 1.201) for sex, 0.492 (0.231, 0.753) for age and 0.592 (0.362, 0.821) for animal origin (Table 8). All the explanatory variables indicated above: sex, age and animal origin were not significantly correlated with positive serological status in CATT, LATEX and ELISA tests.

Separate logistic regression analysis for the independent variable effect of castration, uncastrated horses versus castrated horses revealed an OR of 0.256 (0.256, 0.958) and 0.701 (0.241, 1.161) for the outcome variable CATT and LATEX tests, respectively (Table 7). A similar model for the outcome variable ELISA revealed an OR of 0.529 (0.201, 0.857) for effect of castration (Table

8). Hence, the explanatory variable effect of castration was not significantly correlated with positive serological status in CATT, LATEX and ELISA tests.

Mares with previous abortion history had an OR of 4.362 (2.208, 6.516) and 3.50 (2.180, 5.590) positivity compared to horses without previous abortion history for CATT and LATEX tests, respectively (Table 7). The OR for the same factor ELISA positivity was 3.231 (2.168, 4.294) (Table 8). The chance of seropositivity for mares with an abortion history was about more than three times the chance of mares without this history. Horses with higher parity number (>6) had an OR of 5.156 (2.356, 7.956) positivity compared to horses with lower parity number (0-5) for CATT and LATEX tests, respectively (Table 7). The OR for the same factor ELISA positivity was 3.445 (1.365, 5.525) (Table 8).

Horses with poor body condition (very thin and thin) had an OR of 3.159 (1.254, 5.065) and 3.560 (2.220, 5.710) positivity compared to horses relatively good body condition (less thin, less than moderate and moderate) scores for CATT and LATEX tests, respectively (Table 7). The OR for the same factor ELISA positivity was 2.698 (1.014, 4.383) (Table 8). Hence, a higher risk of seropositivity for horses with previous history of abortion, higher parity number and poor body condition could be identified with CATT, LATEX and ELISA tests (Table 7 and 8).

Table 7: Odds ratio comparison of the sero-prevalence of dourine based on CATT and LATEX tests by major risk factors, in the Arsi-Bale highlands of Ethiopia.

Factors	CATT		LATEX	
	OR	95% C.I and P-value	OR	95% C.I and P-value
Sex				
Male vs Female	0.808	(0.524, 1.091) 0.025	0.771	(0.421, 1.420) 0.035
Age				
Young adults vs Adults	0.992	(0.631, 1.353) 0.019	0.792	(0.431, 1.153) 0.031
Animal origin				
Born on farm vs Bought outside Arsi-Bale local markets	0.921	(0.461, 1.381) 0.021	0.508	(0.324, 0.791) 0.055
Parity number				
0-5 vs >6	5.156	(2.356, 7.956) 0.000	6.220	(5.180, 8.160) 0.000
Previous abortion				
Yes vs No	4.362	(2.208, 6.516) 0.000	3.50	(2.180, 5.590) 0.000
Castration				
Castrated vs Uncastrated	0.256	(0.256, 0.958) 0.007	0.701	(0.241, 1.161) 0.009
Body condition score				
Poor* vs Moderate**	3.159	(1.254,5.065) 0.000	3.560	(2.220, 5.710) 0.000

Poor* refers to very thin and thin while moderate** refers to less thin, less than moderate and moderate body condition scales

Table 8: Odds ratio comparison of the sero-prevalence of dourine based on ELISA test by major risk factors, in the Arsi-Bale highlands of Ethiopia.

Factors	ELISA	
	OR	95% C.I and P-value
Sex		
Male vs Female	0.765	(0.324, 1.261) 0.045
Age		
Young adults vs Adults	0.492	(0.231, 0.753) 0.065
Animal origin		
Born on farm vs Bought outside Arsi-Bale local markets	0.592	(0.362, 0.821) 0.054
Parity number		
0-5 vs >6	3.445	(1.365, 5.525) 0.000
Previous abortion		
Yes vs No	3.231	(2.168, 4.294) 0.000
Castration		
Castrated vs Uncastrated	0.529	(0.201, 0.857) 0.052
Body condition score		
Poor** vs Moderate***	2.698	(1.014, 4.383) 0.000

Poor* refers to very thin and thin while moderate** refers to less thin, less than moderate and moderate body condition scales.

The concordance between (CATT and ELISA) and (LATEX and ELISA) test results were 64.80% and 69.60%, respectively. The kappa statistic between the tests indicated substantial agreement (Thrusfield, 1995) where 125 sera tested positive and 435 sera tested negative in CATT and ELISA tests and with 125 sera tested positive and 450 sera tested negative in LATEX and ELISA tests (Tables 9 and 10).

Table 9: Cross tabulation of CATT and ELISA test results with sera of 649 horses from the Arsi-Bale highlands of Ethiopia.

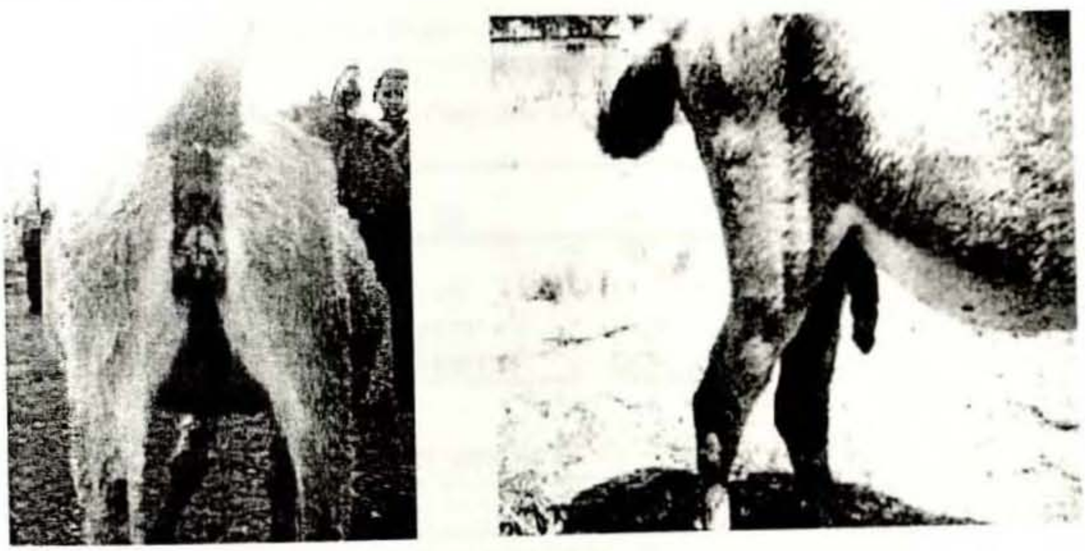
Test		ELISA		
		Positive	Negative	Total
CATT	Positive	125	18	143
	Negative	71	435	506
	Total	196	453	649

Table 10: Cross tabulation of LATEX and ELISA test results with sera of 649 horses from the Arsi-Bale highlands of Ethiopia.

Test		ELISA		
		Positive	Negative	Total
LATEX	Positive	125	23	148
	Negative	21	450	471
	Total	176	473	649

4.1.1.5. The Status of dourine in donkeys and mules of the Arsi-Bale highlands

An attempt has been made to investigate the occurrence of clinical cases of dourine in donkeys and mules as well as assess serological status in each of the study districts in the Arsi-Bale highlands. Out of a total of 120 apparently healthy (60 donkeys and 60 mules) randomly selected from the six study districts of the Arsi-Bale highlands, 12 donkeys and 5 mules were found to be seropositive to CATT, LATEX and ELISA/*T. evansi* tests. It was also observed that two active clinical cases of dourine in one male and female donkeys at the Dodola Veterinary Clinic of the Bale highlands. The clinical cases presented demonstrated genital as well as nervous forms of the disease such as mucoid vaginal discharge, oedema of the external genitalia, swelling of the vulva, depigmented scars over the external genitalia, swelling of the scrotum, ulceration of the penile tissue, emaciation, weakness and hind leg incoordination. Moreover, the female donkey had a previous history of abortion. The picture of the donkeys with typical depigmented scars over the external genitalia, swelling of the vulva and ulceration of the penile tissue is shown below (Figure 7).



A B

Figure 7: Serologically positive donkeys with genital form of dourine, (A) depigmented scars over the external genitalia and (B) ulceration of the penile tissue.

4.1.2. Longitudinal study: Assessment of the therapeutic activity of Isometamidium chloride and Diminazene aceturate in the field

4.1.2.1. Packed Cell Volume (PCV) readings

None of the ISMM (0.5 mg/kg BW) and DIM (3.5 mg/kg BW) treated horses were found to show relapse through out the post treatment period of follow up. The mean PCV of the ISMM and DIM treated animals has improved during the 40 days post treatment period. A statistically significant increase in PCV was observed starting from the day 20 (Repeated measure of ANOVA, $P<0.01$) and a highly significant variation (Repeated measure of ANOVA, $P<0.001$) was observed at the end of the experiment (day 40 post treatment) and PCV has significantly differed between each 20 days of post treatment period (Table 11).

Table 11: Average PCV (%) readings* over the study period.

Group	Mean PCV levels			Change in PCV level between day 0 and day 40	PCV change %
	Day before treatment	Days after treatment			
	0	20	40		
I	25.75^a	30^b	31.5^b	5.5	22.33
II	25^a	28.25^b	30.25^b	5.25	21
III	24.5^a	24^a	23.5^a	-1	-4.08

a, b Means followed by different superscripts differ significantly (Repeated measure of ANOVA, $P< 0.001$)

* Mean PCV improvement was calculated using the formula:

$$\chi \text{ PCV Improvement } \% = \frac{(PCV_f - PCV_i)}{PCV_i} \times 100$$

Where PCV_f = PCV level at day 40 and PCV_i = PCV level at day 0

4.1.2.2. Differential leukocyte count

In the present study, although differential leukocyte counts was carried out to estimate the different leukocytes proportion in the circulation, emphasis was given only to the eosinophils. The mean eosinophil count of animals in Group III was the highest (19.5 %). Eosinophil counts for animals in Group I and II were relatively lower, 14.17 % and 16.25 %, respectively (Table 12). The mean eosinophil count of animals in Group I and II was significantly lower (Repeated measure of ANOVA, P<0.01) than Group III.

Table 12: Mean eosinophil count in the 40 days study period.

Group	Eosinophil count			Mean eosinophil count
	Day before treatment	Days after treatment		
	0	20	40	
I	16.5 ± 1.2^{a}	13.5 ± 1.31^{b}	12.5 ± 1.29^{b}	14.17 ± 1.73
II	19.25 ± 1.06^{a}	16.25 ± 0.83^{b}	13.25 ± 0.93^{b}	16.25 ± 1.96
III	19.5 ± 1.96^{a}	19 ± 1.31^{a}	20 ± 0^{a}	19.5 ± 0.57

a, b, figures followed by different superscript differ significantly (Repeated measure of ANOVA, P<0.01).

4.1.2.3. Body temperature measurements

All of the study animals showed febrile reaction with raised body temperature above $38^{0}C$ at day 0 of the experiment and none in ISMM and DIM groups and all in the control group revealed fever at day 40 of the study period. The mean body temperature of the ISMM and DIM treated study animals has improved during the 40 days post treatment period.

A statistically significant change in body temperature was observed starting from the day 20 (Repeated measure of ANOVA, $P<0.01$) and a highly significant variation (Repeated measure of ANOVA, $P<0.001$) was observed at the end of the experiment (day 40 post treatment) and body temperature has significantly differed between each 20 days of post treatment period for ISMM and DIM treatment groups (Table 13).

Table 13: Mean body temperature (^{0}C) profile over the study period.

	Mean body temperature in ^{0}C		
Group	Day before treatment	Days after treatment	
	Day 0	Day 20	Day 40
I	38.725 ± 0.64^a	37.725 ± 0.39^b	37.275 ± 0.29^b
II	38.275 ± 0.97^a	37.725 ± 0.38^b	37.35 ± 0.25^b
III	38.625 ± 0.34^a	38.35 ± 0.19^a	38.05 ± 0.13^a

$^{a, b}$, figures followed by different superscript differ significantly (Repeated measure of ANOVA, $P<0.01$).

4.1.2.4. Serological status

All the study animals (treated and untreated) remained serologically positive for all CATT LATEX and ELISA tests throughout the 40 days of the study period. However, there was a decrease in seropositivity pattern in both ISMM and DIM treated groups starting from day 20 post treatment.

4.1.2.4. Body condition score evaluation

There was a marked improvement in body condition score of the study animals under the ISMM and DIM groups as compared to control animals within the 40 days post treatment follow up time. All the study animals considered in both treatment and control groups were with very thin body condition score at the initial day of treatment and only three body condition scores namely very thin, thin and less thin were observed throughout the study period. However, following treatment with ISMM and DIM resulted in relative improvement of the body condition starting from day 20 of the study period (Figure 7).

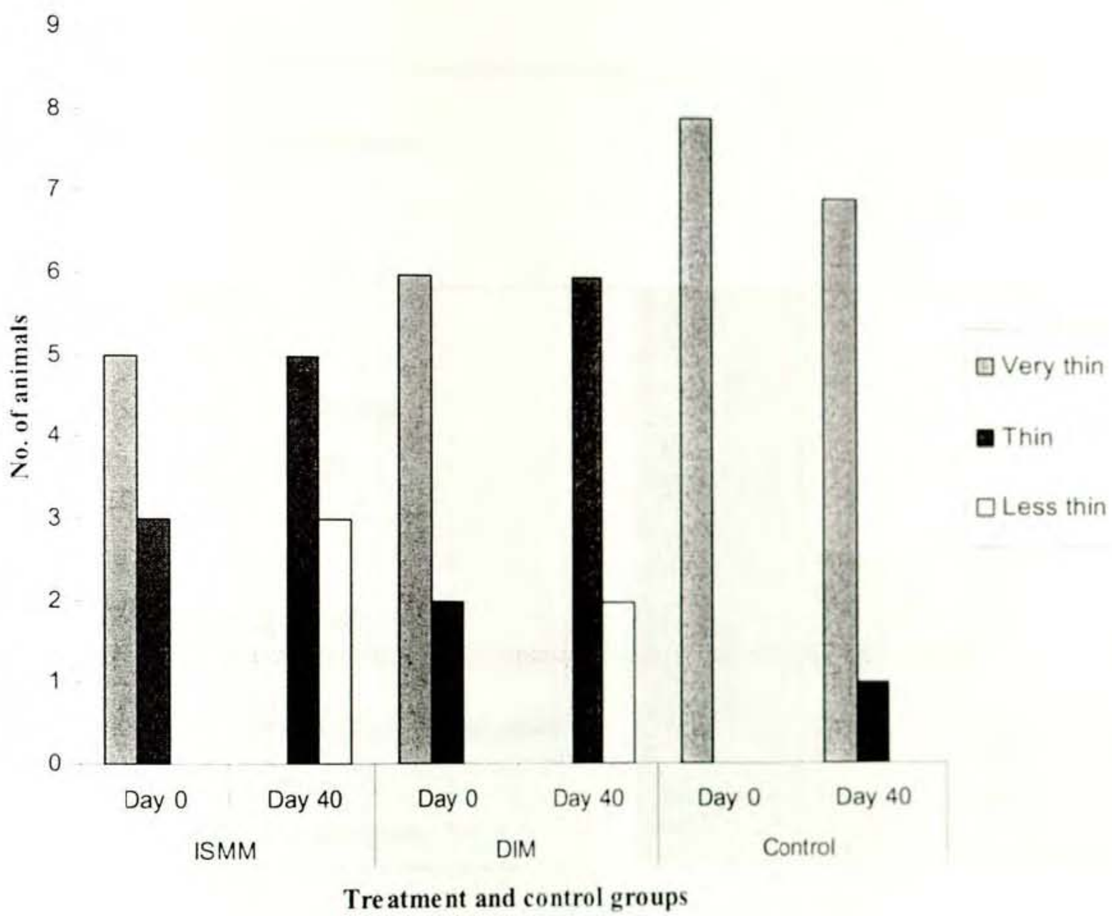


Figure 7: Body condition score evaluation of the study animals during the treatment trial.

4.1.2.4. Improvement of the existing clinical signs

There was marked improvement of most of the existing clinical signs of dourine in both ISMM and DIM treated groups than the control groups. The observed clinical signs of the disease started to disappear step-by-step following treatment, while others persisted beyond day 40 post treatment as indicated below in Table 14. In three of the animals treated with ISMM and two animals treated with DIM, a severe swelling reaction at the site of injection was evident.

Table 14: List of the clinical signs of dourine persisted in treatment groups during the study period.

List of clinical signs	Days post treatment		
	Day 0 to 20	Day 20 to 40	Beyond Day 40
Oedema of the genitalia			
Swelling of the vulva			
Mucoid vaginal or urethral discharge			
Oedema of the ventral abdomen			
Fever			
Weakness			
Ulceration of the genital mucosa			
Incoordination			
Depigmentation of the external genitalia and udder			
Lameness in one or both limbs			
Muscular atrophy in the gluteal region			

4.2. Investigation of dourine outside of the endemic foci

Serological as well as questionnaire-based survey conducted in various representative sites of the country as to the occurrence of dourine outside of its endemic foci revealed the presence of the disease in Uraga, Shashemene and Arbegona districts of the Gurji, East Shoa and Sidama zones, respectively. This is the first report to establish the occurrence of dourine outside of the previously known endemic foci-the Arsi-Bale highlands of Ethiopia. Though questionnaire survey do not indicated the occurrence of dourine, all the serological tests employed (CATT, LATEX and ELISA) revealed the presence of antibodies against *T. equiperdum* at least in one of the examined animals in Enjebara, Kutaber, Dedo and Shinelle districts. Dourine is not known both by the farmers as well as veterinarians interviewed in the Enjebara, Kutaber, Dedo and Shinelle districts. Veterinarians in all of the study districts explained that such disease has not been so far encountered among equines. However, in Fitche district of the Selale zone neither questionnaire survey nor the serological tests employed revealed the presence of antibodies against *T. equiperdum* and indicated the possible absence of the disease in the area (Table 15).

The occurrence of dourine in Uraga district was in the form of outbreak, where it was found to be a newly introduced disease of horses mainly observed in one of the PA's namely in Raro. Unlike other diseases of equines it has no local name. However, farmers of the area were describing and relating the new disease like that of AIDS in human beings. A number of horses and one donkey were dead after manifesting typical clinical signs. As the veterinary professionals and farmers at the very beginning were not aware of the nature of new disease, the disease resulted in death of a number of animals and gets the chance of spreading in the PA very easily in a short period of time. The first cases of dourine were observed at the very beginning of the long rainy season. The new disease was diagnosed as dourine based on the clinical findings with the existence of the following signs: mucoid vaginal discharge with foul odour, oedema of the external genitalia, depigmented scars over the external genitalia, oedema of the ventral surface of abdomen, oedema of the scrotum and prepuce, ulceration of the genital mucosae mainly of the penile tissue, emaciation and weakness, muscular atrophy in the gluteal region, hind leg incoordination and paralysis.

Smears were prepared from vaginal discharge; oedematous fluids and peripheral ear vein blood and stained then with Giemsa from apparent clinical cases of dourine. No trypanosomes or any other haemoparasites could be detected in all examined Giemsa stained smears (thin blood, genital discharge and tissue fluids) as well as in blood samples by mHCT. Out of 44 serum samples collected from horses showing clinical signs of dourine and apparently healthy subjected to serological tests and revealed the presence of antibodies against *T. equiperdum* in 18,12 and 14 animals, in CATT, LATEX and ELISA tests, respectively.

Though no trypanosomes could be detected from the examined Giemsa stained smears, it can be concluded on the basis of the clinical manifestations and serological tests (demonstration of antibodies against *T. equiperdum*), the new disease to be dourine. According to the information collected from farmers of the area it looks the disease is occurring in the equine population of Uraga for the first time, most likely as a result of newly introduced horses from dourine endemic areas, from Kokosa district of the Bale highlands.

Table 15: Results of dourine investigation on the basis serological and questionnaire survey in horses from different selected representative parts of Ethiopia.

Region	Zone/district	No. of seropositive animals			Serological survey	Questionnaire survey
		CATT	LATEX	ELISA		
Amhara	Awi/Enjebara	1	2	2	Suspected	Absent
Amhara	South Wollo/Kutaber	4	3	3	Suspected	Absent
Oromiya	Gurji/Uraga	18	12	14	Present	Present
Oromiya	Jimma/Dedo	4	2	2	Suspected	Absent
Oromiya	East Shoa/ Shashemene	6	5	1	Present	Present
Oromiya	Selale/Fitche	0	0	0	Absent	Absent
Somali	Shinelle/Shinelle	3	2	0	Suspected	Absent
SNNPRS	Sidama/ Arbegona	9	6	1	Present	Present

5. DISCUSSION

No trypanosomes or any other haemoparasites could be detected in all examined Giemsa stained smears (thin blood, genital discharge and tissue fluids) as well as in blood samples by mHCT. Attempts made to isolate the causative agent of dourine following animal inoculation with blood and genital discharge from clinically dourine infected horses remained aparasitemic during the study which was terminated 8 weeks post inoculation. This result is consistent with those of previous reports, which showed that *T. equiperdum* is not readily transmitted from equines to laboratory animals like mice and rabbits (Watson, 1920; Alemu *et al.*, 1997; Clausen *et al.*, 1999). Also several attempts to isolate *T. equiperdum* in Eastern Europe were unsuccessful (Touratier, 2001). As *T. equiperdum* is primarily a tissue parasite, its establishment in the blood of laboratory animals presents the greatest difficulty (Hoare, 1972). Unfortunately, parasitological techniques can not always detect ongoing infections as level of parasitaemia is often low and fluctuating, particularly during chronic stage of the disease (Nantulya, 1990). As sub inoculation of blood in laboratory animals is hardly successful, an alternative approach, not used in the present studies, is to transfuse blood from clinical cases in to susceptible, disease free horses (Parkin, 1949). This could enable transfer of the disease and a greater chance of detecting trypanosomes early in infection.

Although it was not possible to trace the origin of the spread of the disease or to associate the first occurrence of the disease with any particular event in the past, farmers consider dourine to be introduced in to the country at times of colonization attempt by Italy. It was emphasised by the farmers as well as professionals that the problem of dourine is becoming more and more severe and increasing its extent and magnitude, as there is no effective curative or prophylactic therapeutic agents and treatment take place whenever trypanocidal drugs are available.

They were aware also that the disease has a seasonal character, which mainly coincides with the breeding season. Professionals reported that relapses are common in previously treated animals usually at times of feed shortage and stress in the dry seasons of the year. A prominent feature of trypanosomosis is the relapsing nature of the disease where there is periodical expression of surface coat glycoproteins of a differing antigenic nature (Hoare, 1972).

Equines particularly horses in the Arsi-Bale highlands travel long distances towards the neighbouring lowland districts of the East Shoa zone (namely Fentale and Boset), Arsi zone (namely Merti and Jeju), Bale zone (namely Mena and Ghinir), and adjacent camel rearing areas for trade purpose both as a pack and transport animals, where camels are the dominant livestock species and *T. evansi* (surra) is common problem.

Camel trypanosomosis locally known as "Dhukane" is one of the major problems prevailing in the areas (Elias, 2003). According to recent survey conducted on camel trypanosomosis in selected lowland areas of Arsi, Bale and East Shoa zones, parasitological examination of blood samples from 347 animals revealed the presence of *T. evansi* in 19 (5.5%) (Elias, 2003). Camels also move long distances during dry period (September to February) in all the study sites in search of feed and water. In Ethiopia, little research was done on camel trypanosomosis. According to different investigators prevalence ranging from 0.3% to 31.9% was recorded in different camel rearing areas of the country (Annex 11). *T. evansi* is a widely distributed haemoflagellate of veterinary importance that infects a variety of larger mammals including horses, mules, camels, buffalo, cattle and others.

In Africa (Somali, Kenya, Ethiopia, Sudan and Chad) camels are most affected. In spite of their wider distribution throughout the world some animal trypanosomes and mainly *T. evansi* infections seemed considerably less investigated than the African trypanosomosis (human and animal). Correspondingly there was less information available on the incidence and economic significance of *T. evansi* infection on basis of the affected animal species (camel, cattle, buffaloes, horses...) and respective regions where the disease occurs (OIE, 1999). Though the present study was conducted outside of tsetse-infested belt (2400- 3400 m.a.s.l), the area is in close proximity to the camel rearing lowlands of the Borena (in the south) and Somali region (in south east) than any other highlands of the country. In those camel rearing areas of the country, *T. evansi*, which is the causative agent of surra in camels, is a major problem (Tekle and Abebe 2003).

The postulated phylogenetic relationship among the three closely related trypanosome species of the subgenus Trypanozoon indicated close relationship between *T. evansi* and *T. equiperdum* (Hoare, 1972, Zhang and Baltz, 1994, Brun *et al.*, 1998 and Claes, 2003). The present situation is admirably summed up by Hoare (1972), which speculated that it is probable that *T. evansi* was originally a parasite of camels and only later spread to strain of horses, it is conceivable that *T. equiperdum* had evolved from an equine strain of *T. evansi* in enzootic areas of surra. For centuries dourine was co-existent with surra throughout many subtropical countries of northern Africa and southern Asia and it is significant that even at present the areas of distribution of surra and dourine coincide in some places, e.g. in north Africa, Soviet Middle Asia and India. Like surra, dourine was spread by free transport of horses to other continents, going even further than surra since its transmission freed it from the restricting effects of the intermediate insect vectors.

Presumably, in the course of its differentiation from *T. evansi, T. equiperdum* gradually adapted itself to tissue parasitism, which did not lend itself to transmission by blood sucking insects, with the result that this parasite emancipated itself completely from any invertebrate vectors. In earlier publications, Hoare (1956, 1957) suggested that *T. evansi* might have arisen directly from *T. brucei*, being introduced into camels when they entered the tsetse fly belt, and then becoming adapted to mechanical transmission by tabanids.

Isoenzyme analysis has widely been used to group morphologically similar or identical forms in a variety of parasitic protozoa, especially among trypanosomes (Brun *et al*, 1998). Results from biochemical and molecular studies on *T. evansi* and *T. equiperdum* strains isolated from Asia, West Africa and South America may belong to the same species. Twelve strains of *T. evansi* and one recently isolated strains of *T. equiperdum* from China were investigated for 16 enzymes using cellulose and thin-layer starch gel electrophoresis. Differences between strains were observed in only two of the sixteen enzymes, i.e in malate dehydrogenase (MDH) and alanine amino transferase (ALAT). These results indicate that both *T. evansi* and *T. equiperdum* strains from China form a homogenous group with *T. evansi* strains from other parts of the world. Differences were not found between strains of *T. evansi* and *T. equiperdum* using RFLPs in kDNA, genes for ribosomal RNAs and for variant surface glycoproteins, though differences in molecular karyotypes were observed between *T. evansi* and *T. equiperdum* (Brun *et al.*, 1998).

In search of new markers that could possibly differentiate both parasites, Claes, 2003, looked at the internal transcribed spacer region 1 (ITS-1). Though this spacer is known to be useful to determine genetic diversity between closely related species or subspecies, no significant differences can be found within the subgenus Trypanozoon using this approach. More recently an attempt was made to determine the characteristics of several *T. equiperdum*, *T. evansi* and *T. b. brucei* populations using two other molecular techniques; Random Amplified Polymorphic DNA (RAPD) and Multiplex - Endonuclease Genotyping Approach (MEGA) (Urakawa *et al.*, 2001; Ventura *et al.*, 2001; Verloo *et al.*, 2001; Claes, 2003). The obtained results showed that most *T. equiperdum* strains are very similar if not identical to *T. evansi* while only *two T. equiperdum* strains; BoTat 1 and OVI differ and were more identical to *T. b. brucei*. Interestingly, these two peculiar *T. equiperdum* strains are the same ones that lack the RoTat 1.2 VSG gene.

Claes, 2003 formulated a new hypothesis that the species and OVI are particular strains of *T. b. brucei*, while all other *T. equiperdum*, containing the RoTat 1.2 VSG gene, are in fact misidentified *T. evansi* strains and are actually *T. evansi*. However, to confirm the above hypothesis, new strains isolated from horses with apparent clinical signs of dourine should be submitted to the same characterization tests (ITS 1, RAPD and MEGA) and the results compared.

What, then, are the phylogenetic relationships between these two species, *T. evansi* and *T. equiperdum* and the true situation in Ethiopia, which actually vary in the mode of transmission, life cycle and the presence or absence of maxicircles and the evident overlapping distribution of surra and dourine? From the foregoing account all the biological, clinical, morphological and molecular evidence (as summarized in Table 3, Brun *et al.*, 1998) strongly supports the notion that *T. evansi* and *T. equiperdum* are sister taxa that arose from a common ancestor that descended from *T. brucei*. If this represents the real situation or is more the result of the tissue tropism which makes detection of trypanosomes (especially in the blood) unlikely remains an open question. Recent studies by Alemu *et al.*, 1997 and Clausen *et al.*, 1999 in the same study areas in the Arsi-Bale highlands of Ethiopia, indicated the prevalence of *T. equiperdum* being significant based on indirect methods (antibody and antigen detection), clinical signs and PCR (detection of trypanosome DNA) although no parasites could be found.

The results of the logistic regression models suggested that all the serological tests employed CATT, LATEX and ELISA results were affected by the explanatory variables specifically history of previous abortion, higher parity number and poor body condition. The possible explanation for the marked correlation between higher seroprevalence and independent variables like previous history of abortion and higher parity number is associated with the number of previous matings and genital contacts, which possibly increases chance of acquiring the infection from an infected or carrier host. On the contrary all the serological results were not affected by the sex, age, animal origin and castration status. This might be attributed to the fact that animals in all sex and age groups and regardless of their origin were equally exposed to the disease.

The ELISA results substantially correlated with the CATT and LATEX tests, where more than 435 sera tested negative and 125 sera tested positive in all tests. However, 59 positive and 59 negative CATT reactors and 36 positive and 36 negative LATEX reactors did not agree with the ELISA. Discrepancies encountered between (CATT and ELISA) and (LATEX and ELISA) results could be due to the differences in sensitivity and specificity of the tests and partly different Immunoglobulin isotypes targeted by the tests. Caporale *et al.*, 1981 and Clausen *et al.*, 2003 reported a high concordance rate 97.6% and 95.6% between CFT and ELISA tests.

The clinical signs common to dourine, such as incoordination, especially of the hindquarters and oedematous swelling of the external genitalia (Hoare, 1972) were observed in horses in this study. There is evidence that the occurrence of nervous symptoms in dourine-infected horses is associated with the presence of *T. equiperdum* parasites in cerebrospinal fluid (Barrowman, 1976). Skin plaques, which are regarded, as important symptoms in cases of dourine were not observed during this study, although skin eruptions and poor body conditions were prominent. However, in recent infections oedematous plaques have not been observed (Alemu *et al.*, 1997) and in *T. evansi* infections cutaneous plaques have been observed (Brun *et al.*, 1998); so these plaques cannot be considered to be pathognomonic. These plaques do not occur with all strains and have also been observed sporadically in animals infected with *T. evansi*. Similarly, Watson (1920) stated that 'plaques' are rare symptom and they can be observed in comparatively few cases. In the early stages of the infection, oedema of the genital organs and fever are the rule

As *T. equiperdum* is the only trypanosome to affect horses in temperate climates, the isolation of the parasite or the detection of trypanosomal antibodies is sufficient for a positive diagnosis. On the contrary, in countries of Africa, where overlap of *T. equiperdum* and other members of the subgenus Trypanozoon exist, it is difficult to distinguish *T. equiperdum* microscopically or serologically (OIE, 2001). In addition, as the detection of antibodies indicates that there has been infection, but as antibodies tend to remain detectable for some time (weeks, sometimes months) after all trypanosomes have disappeared from the organism (either by drug treatment or self cure) a positive result is no proof of active infection which make antibody detection tests useless for follow up studies (Buscher, 2001). Indirect tests based on antibody detection may be poor due to cross-reaction with other infections, depending on the purity of the antigen applied. It appears that the only officially approved test by the OIE is the complement fixation test (CFT), although it is generally accepted that this test cannot discriminate between *T. evansi* and *T. equiperdum*. Currently neither serological, parasitological nor DNA based tests (Clausen, 1999; Clausen, 2003) allow a subspecies identification within the subgenus Trypanozoon, no definitive diagnosis can be given for *T. equiperdum*. Whether the examined animals are infected with *T. equiperdum* (the causative agent of dourine) or with *T. evansi* (the causative agent of surra) remains an open question.

However, based on the clinical findings, the negative parasitological results and strong association between seropositivity and clinical cases, circumstantial evidence supports the existence of infections with the causative agent of dourine. On the other hand results from biochemical and molecular studies on *T. equiperdum* and *T. evansi* may belong to the same species (Brun *et al.*, 1998). The major difference is that *T. evansi* is a blood parasite which spreads throughout the whole body, while *T. equiperdum* usually parasitises tissue. Until DNA sequence differences between these species are known, the diagnosis of dourine will rely on circumstantial evidence. The need for new diagnostic tools for differential diagnosis of both parasites, has been emphasised but, for the time being, still remains unmet (Touratier, 1999).

The *T. evansi* RoTat 1.2 antigen which was not species-specific used in this study for the detection of circulating antibodies against *T. equiperdum* performed well and a number of test sera showed high antibody positivity in comparison with the threshold value.

Diminazene aceturate is less effective against trypanosomes of the subgenus Trypanozoon than against *T. congolense* and *T. vivax* and rapidly excreted (Mulligan, 1970). Moreover, both Diminazene aceturate and Isometamidium chloride are unable cross the Blood Brain Barrier and somatic tissue and obviously cannot be curative drug for *T. equiperdium*, which is mainly a tissue parasite.

Accurate interpretations of differential leukocyte count require the value of total WBC count. In this study, however, it was not possible to perform total WBC count in the field due to the absence of WBC diluents and materials. Therefore, the results obtained from differential WBC counts alone were used for the interpretation provided. The highest mean eosinophil count (19.5 %) was recorded in untreated group as compared to the ISMM and DIM treated animals. Significant difference ($P<0.05$) in mean eosinophil counts between ISMM treated; DIM treated on the one hand and untreated animals on the other hand indicated the direct relationship between Trypanosomes infection and higher eosinophil counts.

Increased eosinophil count may be ascribed to the response of the host's effector mechanism to parasitic infection in those that involve tissue migration (Fraser *et al*., 1991). Though there was an apparent relative decrease in the mean eosinophil count in the ISMM and DIM treated animals as compared to those of the untreated group, the level of mean eosinophil count was still higher. The possible explanation for the observed persistent eosinophila among the trypanocidal treated groups might be attributed to other concurrent parasitic helminth infections apart from Trypanosomes. This finding agrees with results of Hendy, 1988, Kaufman *et al*., 1992, Dwinger *et al*., 1993 and Zewde, *et al*., 2001 in which they reported an increased pathogencity of helminth infection superimposed on Trypanosomes, where the effect of antihelminthic treatment and trypanosomosis prophylaxis were additive. On the other hand it be explained that the persistent eosinophila observed among the trypanocidal treated groups is due the fact that *T. equiperdum* is essentially a tissue parasite and causes at most very low parasitaemias in the peripheral circulating blood and neither of the trypanocidal drugs used in this study were not effective enough to eliminate the parasite from its predilection sites.

There was a relative improvement in the body condition score of animals in both ISMM and DIM treatment groups as compared to that of the untreated groups. However, it is important to realize that drugs alone will not cure trypanosomosis and result in improved body condition. Trypanosomes overwhelm the immune system of the host; they are immunosuppressive. Chemotherapy by stopping the multiplication of the trypanosomes helps the immune system to overcome the infection. Typically, trypanosomosis is a wasting disease in which there is a slow progressive loss of condition accompanied by increasing anemia and weakness to the point of extreme emaciation, collapse and death (Mulligan, 1970).

Body temperature of an animal is an extremely useful guide to the presence of infectious disease, including trypanosomosis. The animal reacts to an infection by fever, which is a sign of the response of the bodily defences to the invasion by the infective agent. Likewise, in our case the body temperature of the experimental animals in the control group as compared to the treatment groups fluctuated around average, which is higher than normal (38.0 ± 0.5^0 C).

All the study animals in the experimental groups remained serologically positive post treatment for up to 40 days. The possible explanation could be due to the fact that the study period elapsed for short duration and moreover, both of the drugs used might not be quite effective to eliminate the parasites residing at the tissue level so as to reduce the continuous antigenic stimulation and thereby lower antibody response.

In view of the frequent occurrence of relapses where one time treatment of clinical cases is not curative enough it can possibly be recommended that an animal should get on average two treatments per year and treatment should be repeated. Some professionals based on several years of working experience recommended a treatment schedule, which they found to be effective as providing double dose in the first day and after 24 hours single dose followed by third treatment in the dry seasons of the year. As no method of immunization against dourine exist at present and moreover, treatment of clinical cases with the available trypanocidal drugs may result in inapparent disease carriers and is not recommended in a dourine free territory (OIE, 2001).

However, treatment of clinical cases in such endemic areas seems to be beneficial taking in to account the indispensable role played by horses in the Arsi-Bale highlands due to the rugged mountainous terrain of the area where these animals are still the main method of transporting both people and agricultural products and as treatment reduces mortality and results in marked improvement of clinical signs. This is due to the fact that whether clinical cases are treated or not, the animals will tend to be carriers. Therefore, in such an endemic area, where it is difficult to effectively control the disease, it will be worth treating clinical cases to alleviate the disease, enable animals to perform well and thereby reduce mortality.

Both farmers and professionals stressed the issue of lack of availability of trypanocidal drugs for the treatment of clinical cases of dourine. Further more, the presence of drug smugglers, retailers in the local open-air markets and the practice of self-treatment by the farmers, has raised the fear of drug resistance for dourine, as it has been the case for other tsetse transmitted trypanosomosis in other parts of the country. Drug therapy has been the main strategy used in the past to control trypanosomosis throughout Ethiopia (Abebe and Yilma, 1996). There is a flourishing black market and farmers can purchase a variety of trypanocidal drugs in most village markets (Questionnaire result), although all trypanocidal drugs are supposed to be imported through the Federal Drug Administration Authority. Such widespread use and misuses of trypanocidal drugs in the Arsi-Bale highlands will definitely results in the development of drug resistant population of *T. equiperdum*. Apart from the question of clinical efficacy of the available drugs, the problem with trypanocidal drugs become exacerbated, as there is no regular as well as sufficient supply to the veterinary clinics in the endemic areas of the Arsi-Bale highlands.

The Oromiya Agricultural Office that is responsible to supply veterinary drugs, vaccines, diagnostic tools, chemicals, reagents and other items to the respective zones and districts found in the regional state, seems to be not aware of the problem of dourine in the Arsi-Bale highlands given the indispensable role played by equines. It is surprising that some districts even do not receive any trypanocidal drugs. Probably the main reason for the lack of sufficient and regular supply of trypanocidal drugs to the dourine endemic areas of the Arsi-Bale highlands is that most part of western Oromiya Regional State is affected with tsetse transmitted trypanosomosis and found within the tsetse belt.

As a result more attention and emphasis is drawn towards the supply of trypanocidal drugs to the western Oromiya. Equines in Ethiopia, both in vast territories of the nation and large cities or town remained the most utilized means of transportation. Their contribution to the economy of the nation is both as a pack and riding animals. This is probably due to the mountainous nature of the country that made construction of roads very difficult and the meagre income of the peasants. Therefore, the use of equines probably remains to be the only means of transport in the rural area for the coming several decades (Wilson, 1990). Generally speaking the socio-economic significance as well as importance attached to equines by both professionals and farmers in this country is totally poor and therefore, attributed to such professional and social negligence and lack of knowledge, equines are not well treated and given due consideration to their well-being and productivity.

Results of the present field treatment trail with the commercially available trypanocidal drugs indicated marked improvement in the clinical signs, body condition score and better performance, at day 20 and 40 post treatment. This study provided evidence from the present *in vivo* experiment that treatment of clinical cases of dourine with some of the trypanocidal drugs Isometamidium chloride and Diminazene aceturate was quite effective. Though for the treatment of dourine the same drugs that are used for *T. evansi*, are available so far no reports on clinical efficacy have been published. However, some older publications mentioned experimental treatment of horses with suramin and neoarsphenamine (Novarserobezol; Ciuca, 1993) or quinapyramine sulphate (Vaysse and Zottner, 1950). Evidence from *in vitro* drug sensitivity determination of *T. equiperdum* (Zhang *et al*., 1992; Brun and Lun, 1994) indicated that suramin, diminazene, quinapyramine and cymelarsan are effective against this trypanosome species. Similarly, Luckins (1994) reported that *T. equiperdum* to be susceptible to quinapyramine sulphate (Trypacide, Rhone Merieux, Harlow, Essex, UK) and Suramin (Neganol Bayer, Bury St. Edmunds, Suffolk, UK). There are no officially approved drugs to treat horses suffering from dourine (OIE 2001, Zablotskij *et al*., 2003). However, therapy is not recommended, as infections may not respond to the drugs and result in inapparent disease carriers. For this reason treatment is not recommended in a dourine free territory (Barrowman, 1976).

The occurrence of dourine for the first time has been established in areas outside of the endemic foci, in Uraga, Arbegona and Shashemene districts through purchase and unrestricted movement of animals from the Arsi-Bale highlands for trade purpose. As the distribution of dourine is not restricted by environmental factors and it is possible through unrestricted movement of infected animals for the disease to be become established almost anywhere (Luckins, 1994). A large number of horses are constantly purchased from the neighbouring districts of Koffle and Asassa (Arsi highlands) to the eastern Shoa zone mainly for the purpose of cart pulling and it was through such unrestricted movement of animals that the disease get introduced in Shashemene district. Even horses of Koffle and Asassa origin, move up to Awassa town and its surroundings (in the south), Arsi-Negelle and Meki (in the north) and Aje town (in the south west) directions by local merchants for trade purpose. This a potential threat to the horses of central highlands of Ethiopia, where there is huge population.

Dourine is well known by the farmers as well as veterinarians in the Shashemene and Arbegona districts. Locally the disease is known as "Kuta" referring to the hind leg paralysis and incoordination in the local language. Veterinarians of both districts explained dourine being one of diseases of horses occurring in the areas and they do treat the rarely appearing clinical cases using trypanocidal drugs like Veriben and Berenil. The main market places for equines (mainly horses) are the Kokosa and Koffle highlands of the Arsi-Bale zones, where dourine is endemic. The market route extends from the Oromiya region (Kokosa of Bale zone and Koffle of Arsi zone) to the SPNNRS (Arbegona to Bore to Aleta Wondo districts) and finally to the Oromiya region (Uraga district of the Gurji zone).

Currently, neither parasitological nor serological tests can make a clear distinction between *T. b. brucei*, *T. equiperdum* and *T. evansi* infections in solipeds (Zablotskij *et al.*, 2003). Similarly, all the serological tests employed in the present study are not capable of differentiating antibodies against the three species of the subgenus Trypanozoon, and therefore it is seems logical to draw the following possible explanations as to the existence of seropositive animals in the absence of clinical dourine cases in Enjebara, Kutaber, Dedo, and Shinelle districts of the country.

As there is no concrete information available on the prevalence, extent as well as distribution of either *T. evansi* or *T. brucei* in horses in different parts of the country, it possible that the existence of the present seropositive cases in Enjebara, Kutaber, Dedo and Shinelle districts might be cross reactions with other members of the subgenus Trypanozoon. Those seropositive animals might be carrier ones (previously infected and recovered) possibly originated from the dourine endemic foci through purchase or for trade and transport purposes. However, the probability of finding such animals at a particular time of sampling is very low. Attributed to the limitation of the serological tests, it was not possible to conclude that the demonstrated antibodies were actually against *T. equiperdum* or *T. b. brucei*, or *T. evansi*.

In view of the large number of horses in Ethiopia, as the disease transmission is not dependent on insect vectors, the unrestricted movement of animals through out the country for trade and transport purposes, uncontrolled breeding, lack of adequate facilities for diagnosis and control of the disease in relation to breeding, it is likely that dourine may have a much wider distribution than presently realised.

6. CONCLUSION AND RECOMMENDATIONS

Dourine is often described as a slowly disappearing disease of horses and other equines, which is true for most developed countries. On the other hand in African countries mainly in Ethiopia where the disease is endemic and of significant importance given the indispensable role played by equines, the disease deserves more attention. The great agricultural potential of the Arsi-Bale highlands of Ethiopia can only be exploited if dourine, which has been found to be a threat to the life and productivity of the equine population, is well controlled. Diagnosis of *T. equiperdum*, the causative agent of dourine in horses, by standard parasitological techniques is difficult owing to the low numbers of the parasites present in blood or tissue fluids. Consequently, the demonstration of trypanosomal antibodies in the serum has become the most important parameter in determining the disease status of individual animals. Neither parasitological nor serological tests could make a clear distinction between *T. b. brucei*, *T. evansi* and *T. equiperdum* infections in solipeds. Morphologically the three parasites are identical and the current parasitological techniques are the same for all the three species while for *T. equiperdum* no parasitological test seems to be sensitive enough. In many instances, the parasite load is extremely low which makes the detection of trypanosomes rather cumbersome and poorly sensitive. The situation is even worse for *T. equiperdum* in horses where parasite detection sensitivity is almost zero. Although it was not possible to obtain direct parasitological evidence for infection, the results of the serological assays, together with the clinical signs of the disease observed in a number of animals, provide strong circumstantial evidence that *T. equiperdum* occurs mainly in the Arsi-Bale highlands of Ethiopia. For the first time the occurrence of dourine outside of the previously known endemic foci of the Arsi-Bale highlands, was established and confirmed in neighbouring areas namely in Sidama zone of the Arbegona district, East Shoa zone of the Shashemene district and Gurji zone of the Uraga district. Dourine, which is by default known, as a disease occurring only in the Arsi-Bale highlands is getting spreading and becoming a potential threat to equines in the geographically adjacent areas through trade and unrestricted movement of animals outside of the endemic foci.

In view of difficulties attached to diagnosis, irregularities of therapeutic outcome, lack of vaccine or chemoprophylaxis, effective means of transmission of the parasite from animal to animal, and most of all owing to the current spreading trend of the disease outside of its endemic foci being a potential threat to the high population of horses in central highlands of the country, the following recommendations and alternative control options are suggested:

- In countries where dourine is a major problem strict control of breeding, of movement of horses, and quarantine and slaughter in clinical outbreaks has a marked effect on the incidence of the disease. Detection of carrier animals by complement fixation tests and slaughter or castration leads to eventual eradication. In-contact animals are declared free after three consecutive monthly negative complement fixation tests. However, in countries like Ethiopia where tsetse and non-tsetse transmitted trypanosomosis occur and the possibility of cross reaction with dourine present, it is practically impossible to implement the above control measures and therefore, the relevant body need to consider the following points seriously: stop purchasing horses from Arsi-Bale highlands, stop using Arsi-Bale highland horses for breeding purpose, castrate all male horses that are serologically positive for dourine and apply strict movement and quarantine of horses.
- Taking in to account, the previous detection of trypanosomal DNA from dourine infected horses of the Arsi-Bale highlands, further detailed studies need to be conducted to isolate parasites involving particular activities like experimental infection of horses, post-mortem examination of classical cases, use of sensitive parasitological techniques such as mAECT.
- Due to the lack of information as to the incidence, distribution and economic significance of *T. evansi* and *T. brucei* infection in domestic animals, an epidemiological study encompassing those trypanosome species should be conducted involving various hosts in different parts of the country.
- To further clarify the confusion about *T. equiperdum*, we propose to isolate new trypanosome strains from well-defined dourine, surra and nagana cases in horses, to analyse them with the most sensitive and specific serological and molecular techniques.

- The present *in vivo* drug sensitivity study indicated that significant numbers of dourine-infected animals regardless of the clinical stage were cured and it can be recommended to the OIE to replace the current strategy of eradication by an appropriate drug treatment. Regarding animal welfare this would be a big step forward. Moreover, owing to the difficulties and challenges attached to the diagnostic techniques of the disease, it can be recommended to the OIE to further consolidate the research and experimental studies on dourine.
- In areas like the Arsi-Bale highlands where the disease is endemic and equines are allowed to run in open range, stopping of natural breeding practices and application of controlled breeding would be practically difficult. In view of the availability of artificial insemination centres throughout the country, which is mainly used for upgrading genetic makeup of the indigenous cattle, at least theoretically artificial insemination would appear more appropriate to practice in equines too.

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8. ANNEXES

Annex 1: Sample collection format used in the present dourine study in the Arsi-Bale highlands of Ethiopia.

Date of sampling: ....

Contact details:

Farmer/Owner name:...

Address (Zone/District/PA):..

Animal identification and history:

- Animal ID/Code..................................
- Species..
- Sex..
- Age...
- Bought outside Arsi-Bale local markets or born on farm........................
- Parity number.......................................
- Previous history of abortion.............yes/ no
- Body condition score...........................
- Castrated/ Uncastrated..........................
- Clinical signs and stage observed...
....

Samples collected

- Blood (heparin) ....yes/no
- Serum........................yes/no
- Oedematous fluid (Smear)........................yes/no
- Vaginal / Prepucial fluid (Smear)...................yes/no
- Others ....
- Dog inoculation...................................yes/no

Annex 2: A Guide to live weight estimation and body condition scoring of equines.

A) Live weight estimation

The girth (Heart girth): The girth is the measurement around the body just behind the front legs, in centimeters (cm).

The length: The length is the distance from the pin bone (tuber ischii) to the elbow in a straight line in centimeters (cm).

Having obtained the measurements the next stage is to estimate the live weight. This can be done as follows:

The best equation to use to estimate live weight of an adult donkeys and horses is one which involves taking two measurements on the body- the girth and the length. Then live weight will be estimated from the following formula:

$$\text{Live weight (Kg)} = (\text{Heart girth [cm]}^{2.12}) \times (\text{Length [cm]}^{0.668}) / 3801$$

B) **Body condition scoring**

Table 1: Estimation of body condition score in the horse and donkey on a scale from 1 to 5.

Body Condition Score	Description of animal
1. Very thin (Emaciated)	Animal emaciated, weak and lethargic with very little muscle covering bones. Bony structures easily seen over entire body.
2. Thin	Individual ribs, dorsal spinous processes of vertebrae and spine of scapula are sharply defined. Thin neck, sharply angled shoulders. Very little muscle.
3. Less thin	Vertebral column prominent and individual spinous processes can be felt, but some muscle-covering spine. Ribs and bony prominences of rump prominent. Loin and rump areas concave. Little fat or muscle over withers or shoulder.
4. Less than moderate	Bony prominences are palpable but less obvious. Vertebral column visible. Rump flat but not concave. Some muscle and fat over withers shoulder and neck.
5. Moderate	Muscles over dorsal of spine developed, can still palpate spinal column. Rump rounded with tuber coxae and tuber ischii no longer visible. Ribs can be felt but are not visible. Some fat at base of neck and front of chest.

Annex 3: Questionnaire survey to assess the overall situation of dourine in the Arsi-Bale highlands of Ethiopia.

Date of interview : ……………………

Contact details:

Farmer/Owner name:……………………………………………………………………

Address: ……………………………………………………………………………

Region……Zone………District…………Peasant Association……………………

Animal information

- Species: …………………………………
- Sex: ……………………………………
- Age: ……………………………………

Does the animal have clinical signs of dourine: yes/no

If yes,

Which signs: ……………………………………………………………………………

Had the animal a problem of dourine previously: yes/no

If yes,

- When? ……………………………………………………………
- Has the animal been treated? ……………………………………

If yes,

- When? ……………………………………………………………
- Which drug was used? ……………………………………………
- Who treated the animal? …………………………………………
- Which dose? ……………………………………………………
- Was the animal cured? ……………………………………………

Yes/ no/ relapse/…………………………………………………………………

Had the animals previously any other problems/diseases? Yes/no

If yes,

-Abortion: yes/no……………………………………………………

- Diseases: ……………………/…………………………/……………………/…………

- Was treated yes/no with……………………………………………………………

Did the animals have any problem with surra? Yes/no

……………………………………………………………………………………………………

Animal history:

Animal born on farm: yes/no ……………………………………………………………

Bought outside Arsi-Bale local markets from: yes/no……………………………………

Which livestock species reared in the area and commonly found? ……………………

What do you call the disease (dourine) by local name?

……………………………………………………………………………………………………

What is the importance of this disease compared to other diseases?

……………………………………………………………………………………………………

What are the main clinical signs observed when an animal affected by trypanosomosis?

……………………………………………………………………………………………………

……………………………………………………………………………………………………

…….

In which season does the disease occur commonly?

……………………………………………………………………………………………………

When did you know the problem of trypanosomosis in the area?

……………………………………………………………………………………………………

What is the status of the disease once you know in this area? It is getting better/ It is getting worse/ Nothing is changed/ I don't know

……………………………………………………………………………………………………

Are there traditional method of treatment and management practices for controlling and prevention of trypanosomosis? Or what are the main control measures of dourine in your area?

……………………………………………………………………………………………………

……………………………………………………………………………………………………

Other remarks and personal observations:

……………………………………………………………………………………………………

……………………………………………………………………………………………………

Annex 4: Details of the Serological tests and laboratory procedures followed in the present dourine study.

1. Card Agglutination for Trypanosomosis Test (CATT/ *T. evansi*)

The CATT/ *T. evansi* is a direct rapid card agglutination test which uses formaldehyde fixed, freeze- dried trypanosomes expressing a predominant variable antigen type of *T. evansi* (RoTat 1.2) stained with Coomassie blue.

Reconstitution of the CATT antigen

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

Reconstitution of the controls

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

Preparation of test samples

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

Reading and interpretation

Evaluate the agglutination reaction as indicated below in Table 2 as follows:

Table 2: Result interpretation of CATT/ *T. evansi* test.

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

Table 3: CATT/*T. evansi* test result recording format used in the present study.

No.	Name (Sample code)	CATT/ *T. evansi* test						End titre
		1:2	1:4	1:8	1:16	1:32	1:64	
1								
2								
3								
4								
5								
6								
7								
8								

2. LATEX (LATEX/ *T. evansi*) test

LATEX agglutination/*T. evansi* is a rapid antibody detecting indirect agglutination test, in which the antigen consists of purified variable surface glycoproteins (VSG) of *T. evansi* Vat RoTat 1.2 covalently coupled to latex particles (0.9 micron in diameter). This method is more specific in testing for *T. evansi* than the CATT method (Verloo *et al.*, 2001).

Reagents

Latex: Lyophilized latex suspension coated with semi-purified variable surface antigens from *T. evansi* VSG Ro Tat 1.2 trypanosomes.

Buffer: Phosphate buffered saline with 0.02 % sodium azide (PBS) for negative control, reconstitution of the positive control and dilution of the test sera.

Positive control: Freeze-dried goat immnunization serum.

Reconstitution of Lyophilized latex reagent

- Resuspend the latex reagent with 1 ml of buffer (PBS). Mix gently for 30 seconds. Use the same day.

Reconstitution of the positive control

- Dissolve the content of the positive control vial in 0.5 ml of PBS. No further dilution is needed. If not used the same day, store at – 20^0 C.

Dilution of the test samples

- Prepare serum dilutions 1:2, 1:4, 1: 8, 1:16, 1: 32 and 1: 64 in buffer (PBS) in a microplate as follows. Dispense 40 µl of PBS buffer in each well of columns 1 to 4.
- In well A1, dispense 30 µl of serum to dilute, mix properly and transfer 30 µl to well A2, mix and transfer 30 µl in well A3, mix and transfer 30 µl in well A4. Dilute 7 other blood samples in the column B to H in the same way. Use the other half of the microplate, from column 6 to, to dilute 8 other blood samples.

Execution of the test

- The test on serum is performed with 20 µl of dilutions 1:2, 1:4, 1:8 and1:16.
- Adjust the speed of the rotator at 70 rpm.
- Dispense 20 µl well mixed latex suspension onto a spot of a test card. Add 20 µl of test sample, positive control or negative control (PBS). With a plastic stick, mix well and spread over ± 1cm diameter. Wipe the stick with paper between each sample.
- Rock the card on a rotator for 5 minutes. To prevent evaporation put the cover over the card.

Reading the test result

Evaluate the agglutination reaction as indicated below in Table 4 as follows:

Table 4: Result interpretation of LATEX/ *T. evansi* test.

Code	Agglutination	Result
0	Absent	Negative
1	Hardly visible	Negative
2	Manifest	Weakly positive
3	Intense	Positive
4	Almost complete	Strongly Positive

Table 5: LATEX/*T. evansi* test result recording format used in the present study.

No.	Name (Sample code)	LATEX/ *T. evansi* test						End titre
		1:2	1:4	1:8	1:16	1:32	1:64	
1								
2								
3								
4								
5								
6								
7								
8								

3. ELISA Ro Tat1.2 VSG of *T. evansi* test

The principle of ELISA test is an agglutination reaction between antigen and antibody.

Materials:

Antigen: RoTat 1.2 VSG of *T. evansi*.

Sample: Diluted horse serum.

Conjugate: Prot A-PO 1 mg SIGMA Lot 106H8280. The conjugate is rehydrated with 1 ml H_2O and aliquoted in 10 µl.

Method:

Coating:

- The VSG is diluted in PBS to a concentration of 2 μg/ml.
- 150 μl/well, incubate overnight at 4^0C. Coating can also be achieved by incubating at 37^0C for 1 hr
- Wells are left dry for the antigen negative control.

Saturation:

- Remove antigen solution, add 350 μl PBS-Blotto in each well, incubate for 1 hour at room temperature.
- Remove PBS-Blotto, add 350 μl PBS-Sucrose in each well, incubate for 30 minutes at room temperature.
- Remove PBS-Sucrose, cover the plate and keep at - 70^0C.

ELISA procedure:

Washing:

- Thaw the plate and wash 3x1 sec with 350 μl PBS- Tween.

Sample incubation:

- Serum is diluted 1:100 in PBS-Blotto.
- 50 μl of sample is applied in Antigen coated and control wells.
- The plate is shaken for 30 minutes on an ELISA-shaker at room temperature.

Washing:

- Wash 3x1 sec with 350 μl PBS- Tween.

Conjugate incubation:

- Dilute the Prot A-PO 1:5000 in PBS-Tween.
- 150 μl/well, incubate for 1 hour at room temperature.

Washing:

- Wash 5x 1 sec with 350 μl PBS- Tween.

Substrate incubation:

- 150μl substrate solution/ well, incubate for 1 hour at room temperature.

Photometric reading:

- Read absorption at 415 nm.

Interpretation:

1. OD cut off: OD values of the samples will be deducted from the OD of an antigen negative plate. Any sample giving the OD more than 0.5 will be a positive one.

2. Percent Positivity (PP): The sample OD will be compared with the OD (100%) of positive control serum and are calculated in a formula as following:

$$PP = \frac{\dfrac{TA1 + TB1}{2} - \dfrac{NA1 + NB1}{2}}{\dfrac{PC1 + PC2}{2} - \dfrac{NC1 + NC2}{2}} \times 100\%$$

PP	=	Percent Positivity
TA1	=	Well 1 of sample 1
TB1	=	Well 2 of sample 1
NA1	=	Well 1 of a negative plate
NB1	=	Well 2 of a negative plate
PC1	=	Well 1 of a positive sample
PC2	=	Well 2 of a positive sample
NC1	=	Well 1 of a positive control serum in a negative plate
NC2	=	Well 2 of a positive control serum in a negative plate

Each sample will be measured twice to find an average OD. Results may plus or minus number depend on the OD of control sample. The positive sample is the one yielding an OD over 50% of the control one.

Table 6: ELISA/*T. evansi* test result recording format used in the present study.

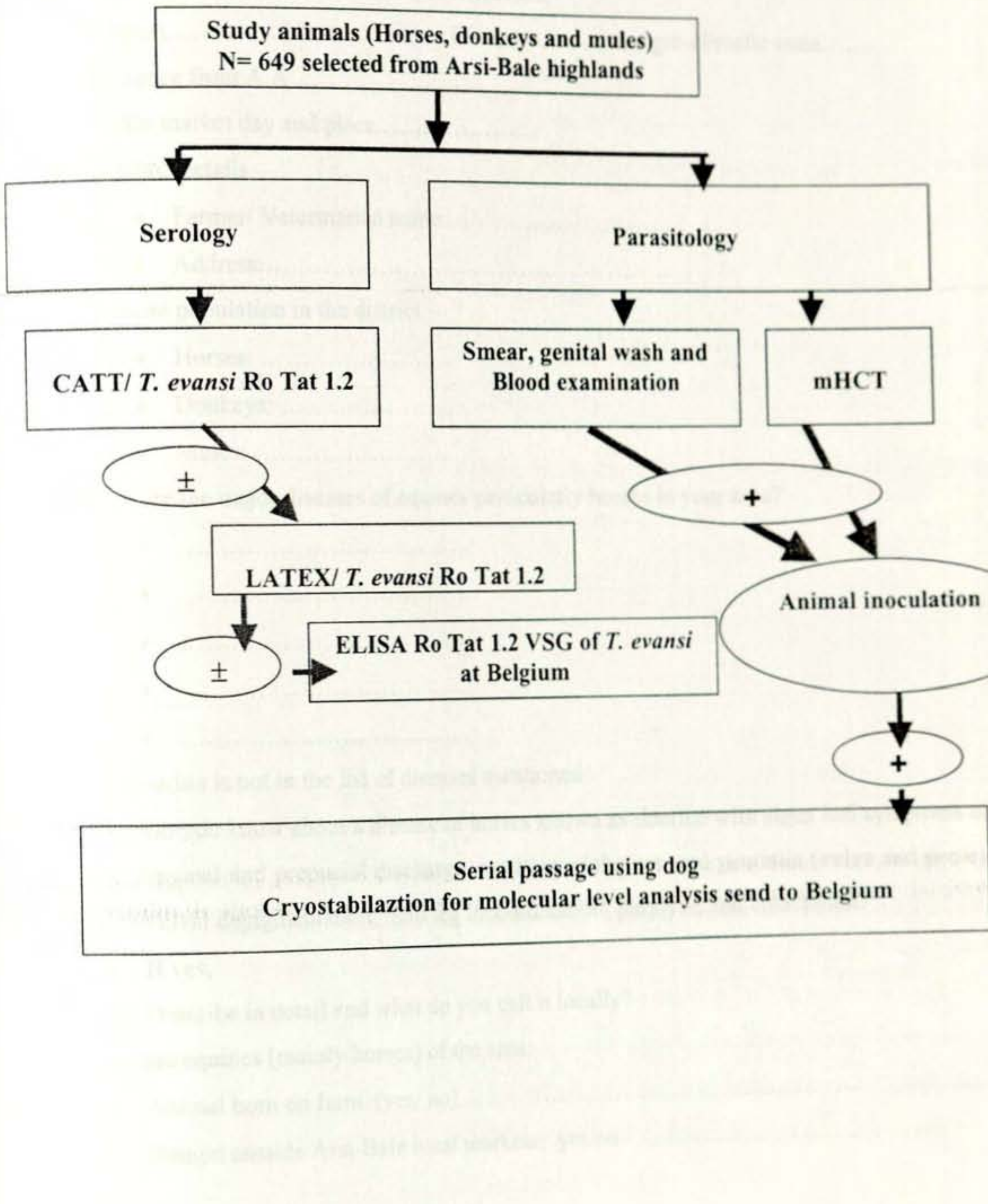
Date: ....

Technician/operator: ....

Comment: ....

A			1	9	17	25	33	41	49	57	65	73
	C++	C++										
B			2	10	18	26	34	42	50	58	66	74
	C++	C++										
C			3	11	19	27	35	43	51	59	67	75
	C+	C+										
D			4	12	20	28	36	44	52	60	68	76
	C+	C+										
E			5	13	21	29	37	45	53	61	69	77
	C-	C-										
F			6	14	22	30	38	46	54	62	70	78
	C-	C-										
G			7	15	23	31	39	47	55	63	71	79
	C_0	C_0										
H			8	16	24	32	40	48	56	64	72	80
	C_0	C_0										
	1	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**

Annex 5: Diagnostic flow-chart, dourine (*T. equiperdum*) in the Arsi-Bale highlands of Ethiopia.



Annex 6: Questionnaire survey used to investigate the occurrence of dourine (*T. equiperdum*) outside of the endemic foci in selected areas of Ethiopia.

1. Date of interview : ....
2. Region...............Zone.............District............. Agro-climatic zone...............
   Distance from A.A ....
   Main market day and place...................
3. Contact details
   - Farmer/ Veterinarian name:............................
   - Address:...
4. Equine population in the district
   - Horses: ....
   - Donkeys: ....
   - Mules: ....
5. What are the major diseases of equines particularly horses in your area?
   - ....
   - ....
   - ....
   - ....
   - ....
6. If dourine is not in the list of diseases mentioned:
   - Do you know about a disease of horses known as dourine with signs and symptoms of vaginal and prepucial discharge, swelling of the external gentialia (vulva and penis), vulval depigmentation, hind leg incoordination, paralysis and emaciation?
   - **If yes,**
     Describe in detail and what do you call it locally?
7. Origin equines (mainly horses) of the area:
   - Animal born on farm (yes/ no) ....
   - Bought outside Arsi-Bale local markets: yes/no...

Annex 7: Format used for the field treatment trial of dourine in Asassa district of the Arsi highlands, Ethiopia.

Animal identification: Animal code: ____Age: _____Sex: ____ Parity number: _____

Drug: ISSM (0.5 mg/kg BW) / DIM (3.5 mg/kg BW)

Parameters	Day 0	Day 20	Day 40
PCV			
Diff. count			
Lymphocyte			
Monocytes			
Neutrophils			
Eosinophils			
Basophils			
Body Condition			
Very thin			
Thin			
Less thin			
Less than moderate			
Moderate			
Serology			
CATT			
LATEX			
ELISA			

Annex 8: Format used for the field treatment trial of dourine in Asassa district of the Arsi highlands, Ethiopia.

Clinical Signs	Day 0	Day 20	Day 40
Fever			
Mucoid vaginal or urethral discharge			
Oedema of the genitalia			
Swelling of the vulva			
Swelling of the udder			
Oedema of the scrotum			
Oedema of the prepuce			
Oedema of the ventral surface of the abdomen			
Ulceration of the genital mucosae			
Depigmented scars			
Urticarial plaques on the flanks			
Weakness			
Lame in one or both hind limbs			
Muscular atrophy in the gluteal region			
Incoordination			
Ataxia			
Paralysis			

Annex 9: Follow up format used for cultivation of trypanosomes from dogs inoculated with specimens from field clinical cases of dourine.

Dog Inoculation

Date (Day 0): ....

Dose	No. of dogs	Route of inoculation	Types of specimen inoculated
____ml	________	I.P S.C I.V	________
____ml	________	I.P S.C I.V	________
____ml	________	I.P S.C I.V	________
____ml	________	I.P S.C I.V	________

Follow up of Parasitemia

Date			
Dog 1			
Dog 2			
Dog 3			
Dog 4			

Sub inoculation

Dog No.________________

Dose	Number of dogs	Route of infection
____ml	________	I.P S.C I.V
____ml	________	I.P S.C I.V
____ml	________	I.P S.C I.V
____ml	________	I.P S.C I.V

Annex 10: Locations of the different sites in the present dourine study in terms of 3D GPS and distance from the Capital (Addis Ababa).

Study sites (Zone/District)	Latitude	Longitude	Distance from A.A (Kms)	Round trip distance from A.A (Kms)
Arsi/ Asela	7.9395	39.1330	175	350
Arsi/ Arsi-Robe	7.2627	38.6219	225	450
Arsi/ Asassa	7.1621	39.0865	285	570
Arsi/ Koffle	7.1345	38.8328	275	550
Bale/ Dodola	6.9774	39.1838	321	642
Bale/ Goba	6.9322	39.9488	445	950
Bale/ Kokosa	6.7484	38.7930	359	718
Selale/ Fitche	9.1800	38.7500	110	220
East Shoa/ Shashemene	7.2285	38.6399	250	450
Jimma/ Dedo	7.6666	36.8333	367	734
Gurji/ Uraga	5.9400	38.4700	430	860
Awi/ Enjebara	10.9700	36.8400	452	904
South Wollo/ Kutaber	11.4700	39.5200	425	850
Sidama/ Arbegona	6.5167	38.8500	368	736
Shinelle/ Shinelle	9.8400	41.8300	535	1,070
Total				**10,054**

- Where 1 mile = 1.60934 Kms; 10,054 Kms = **6,247.28 miles**
- A.A = Addis Ababa (Capital city)

Annex 11: Retrospective data on the prevalence of camel trypanosomosis in various parts of Ethiopia.

Authors and year	Study areas	No. of camels examined	No. Positive	Prevalence (%)	Trypanosoma species identified
Richard (1979)	Borena	88	11	12.5	*T. evansi*
Zeleke (1982)	Ogaden	226	20	8.8	Mixed *T. evansi* and *T. vivax*
Melaku (1985)	Dire Dawa, Issa and Guragura zones of Eastern Ethiopia	327	1	0.3	*T. evansi*
Ketema. (1990)	Borena	1100	237	21.54	*T. evansi*
Abebe (1991)	Ogaden	321	21	6.54	*T. evansi*
Tenaye (1993)	Borena	294	94	31.9	*T. evansi*
Tekele and Abebe (2001)	Borena	391	43	10.9	*T. evansi*
Getahun (1998)	Leben, Borena	324 Wet season	33	10.2	*T. evansi*
		258 Dry season	8	2.8	(Mixed) *T. evansi* and *T. vivax*
Ahmed (1998)	Dire Dawa, Jijiga East Hararghe	336	26	7.7	*T. evansi*
Reshad (1999)	Dire Dawa	248	12	4.8	*T. evansi*
Demeke (2000)	Afar and Tigray	280	14	5	*T. evansi*
Elias (2003)	Arsi, Bale and East Shoa	347	19	5.5	*T. evansi*

9. CURRICULUM VITAE

Personal Information

Name: Hagos Ashenafi Tafesse

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Sex: Male

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Date of birth: 27 January 1976

Marital status: Single

Academic qualification: Doctor of Veterinary Medicine (DVM)

Scientific Membership: Member of the Ethiopian Veterinary Association (EVA)

Educational Background

- 1983-1991: Grade 1- 8. Hibret ferie elementary and junior secondary school (Addis Ababa).
- 1991-1994: Grade 9-12. Yekatit 12 comprehensive secondary school (Addis Ababa). Award: Ethiopian School Leaving Certificate Examination (ESLCE) GPA - 3.60 (Distinction).
- 1994-2000: Addis Ababa University, Faculty of Veterinary Medicine, Debre Zeit. Award: DVM degree, cGPA- 3.33 (Distinction).
- 2004 to the present: Addis Ababa University, Faculty of Veterinary Medicine, Debre Zeit. Postgraduate MSc Student in Tropical Veterinary Medicine.

Work Experience

- From April, 2001 to May 9 2003, Addis Ababa University, Faculty of Veterinary Medicine, Debre Zeit, **Assistant Lecturer**, at the department of Pathology and Parasitology.
- From May, 9 2003 to the present, Addis Ababa University, Faculty of Veterinary Medicine, Debre Zeit, **Lecturer**, at the department of Pathology and Parasitology.

- Participated on Workshop held at NAHRC (National Animal Health Research Center), Sebeta from July 21- 23/ 2003 on **Laboratory Quality Assurance** organized by Ethio-French Project and CIRAD/EMVT.
- Certificate of completion on **Computer Computing** from African Virtual University, Science Faculty, Addis Ababa University (January 2002 – May 2002).
- Attended training on the **Diagnostic Techniques of Dourine (*Trypanosoma equiperdum*)** in Belgium at Institute of Tropical Medicine Antwerp, from Jan. 28 – Feb. 19/ 2004.

Research and Publications

- **Ashenafi, H.** Epizootic lymphangitis: A major disease of horses in Ethiopia. Seminar on current topics in livestock production and development. In DVM course work. Addis Ababa University, Faculty of Veterinary Medicine, Debre Zeit, 1999.
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10. SIGNED DECLARATION SHEET

I, the undersigned, declare that the thesis is my original work and has not been presented for a degree in any university.

Name: ____________________

Signature: ____________________

Date of submission: ____________________

This thesis has been submitted for examination with my approval as University advisor.

Advisor:

Professor Getachew Abebe ____________________