Thesis Ref No._____

IN VIVO DRUG SENSITIVITY TESTS OF *TRYPANOSOMA VIVAX* ISOLATES FROM SELECTED TSETSE INFESTED AND TSETSE FREE AREAS OF NORTH WEST, ETHIOPIA

MSc Thesis

By

BINIAM TSEGAYE

Addis Ababa University, College of Veterinary Medicine and Agriculture,

Department of Pathology and Parasitology

JUNE, 2014

BISHOFTU, ETHIOPIA

IN VIVO DRUG SENSITIVITY TESTS OF *TRYPANOSOMA VIVAX* ISOLATES FROM SELECTED TSETSE INFESTED AND TSETSE FREE AREAS OF NORTH WEST, ETHIOPIA

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

By

BINIAM TSEGAYE

JUNE, 2014

BISHOFTU, ETHIOPIA

Addis Ababa University

College of Veterinary Medicine and Agriculture

Department of Pathology and Parasitology

As members of the examining board of the final MSc open defense, we certify that we have read and evaluated the thesis prepared by **Biniam Tsegaye** entitled ***In vivo* drug sensitivity tests of *Trypanosoma vivax* isolates from selected tsetse infested and tsetse free areas of Northwest, Ethiopia** and recommend it to be accepted as fulfilling the thesis requirement for the degree of Masters of Science in Tropical Veterinary Parasitology.

Dr.Tilaye Demisse		
Chairman	**Signature**	**Date**
Prof.Getachew Tilahun		
External Examiner	**Signature**	**Date**
Dr.Yacob Hailu		
Internal Examiner	**Signature**	**Date**

1. Dr.Shimelis Dagnachew		
Major Advisor	**Signature**	**Date**
2. Dr.Getachew Terefe		14/07/2014
Co-Advisor	**Signature**	**Date**
3. Dr.Hagos Ashenafi.		14/07/2014
Co-Advisor	**Signature**	**Date**
4. Prof.Gooddeeris B. M.		
Co-Advisor	**Signature**	**Date**
5. Dr.Hagos Ashenafi		14/07/2014
Department chairperson	**Signature**	**Date**

DECLARATION

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

Name: Biniam Tsegaye

Signature: _____

College of Veterinary Medicine and Agriculture, Bishoftu

Date of Submission: 10/06/2014

ACKNOWLEGEMENTS

I would like to express my heartfelt thanks to my advisor Dr. Shimelis Dagnachew for his intellectual guidance, devotion of time in the data analysis, critical correction of this paper and generally for his good approach and advise. I also like to extend my thanks to Dr.Getachew Terefe and Dr.Hagos Ashenafi for their advises in the preparation of this thesis.

I would like to extend my acknowledgment to the Ethio-Belgium VLIR-OUS funded PhD laboratory for provision of materials during the laboratory work and also the GALVMed project for their undeniable support. My appreciation also goes to Ato Alemu Tola who is a senior laboratory technician working at the laboratory for his keen support during the experiment, and also to Drs. Addisu Awoke, Frehiwot Tesfu, and Melkamu Bezie who worked together with me during the experiment.

The last but not the least, this paper would not be successful without the moral contribution from my mother W/o Hirut Estifanos and also the rest of my family.

TABLE OF CONTENTS PAGES

Table of contents (Continued)

LIST OF TABLES

PAGES

LIST OF FIGURES

PAGES

LIST OF ANNEXES

PAGES

LIST OF ABBREVIATIONS

AAT	African Animal Trypanosomosis
CD	Curative Dose
DA	Diminazene Aceturate
DIGIT	Drug Incubation Glossina Infectivity Test
DIIT	Drug Incubation Infectivity Test
DNA	Deoxyribonucleic acid
ED	Effective Dose
EDTA	Ethylene diamine tetra acetic acid
ELISA	Enzyme Linked Immuno Sorbent Assay
FAO	Food and Agricultural Organization
FITCA	Farming In Tsetse Controlled Area
GALVMed	Global Alliance for Livestock and Veterinary Medicine
ISM	Isometamidium Chloride
MEP	Mitochondrial Electric Potential
PCR	Polymerase Chain Reaction
PCV	Packed Cell Volume
PTM	Peritrophic membrane
RNA	Ribonucleic Acid
rpm	revolutions per minute
SPSS	Statistical Packages for Social Science
SSA	Sub-Saharan Africa

ABSTRUCT

Tsetse transmitted animal trypanosomosis is an economically devastating disease in Sub-Saharan Africa where trypanocidal drug treatment is the mainstay of control but their efficacy is hampered because of the widespread occurrence of drug resistance. The present study conducted from March to June of 2014 in young zebu cattle was to determine the drug sensitivity of *T. vivax* isolates from selected tsetse infested and tsetse free areas of Northwest Ethiopia and also to look for hematological profiles improvement after treatment. A total of three isolates one from tsetse infested and two from tsetse free areas were used. A prospective study and randomized controlled block design was applied to the experimental animals together with preliminary questionnaire survey on drug usages. The study revealed that one isolate from tsetse free area was resistant to 7mg/kg of DA. The finding of one relapse from the tsetse isolate to 1mg/kg of ISM is highly indicative that more relapses could be found if more isolates were used. The decrease in packed cell volume and hemoglobin concentration improvements was more common in breakthrough infections. Results of the experiment on the low efficacy of the drugs were supported by respondents of questionnaire survey. In conclusion, drug resistance is a threat in both areas and the situation is magnified in the non-tsetse areas so that control of the disease should be an integrated approach. Moreover, the use of sanative pair with DA and ISM in accordance with the reported drug resistance is important to reduce the problems in the study sites. Furthermore, extensive data on trypanocidal drug sensitivity tests on more *T. vivax* isolates using advanced molecular techniques is essential.

Key words: *Trypanosoma vivax, diminazene aceturate, isometamidium chloride, young zebu cattle, North west Ethiopia.*

1. INTRODUCTION

African animal trypanosomosis (AAT) or nagana is caused by *Trypanosoma congolense*, *T. vivax* and *T. brucei* species while *T. evansi* causes Surra in camels (Mbaya *et al.*, 2010). Most African trypanosomes are transmitted cyclically by tsetse flies, which inhabit many parts of the continent that are restricted to latitude of about 15°N and 29°S of the equator (Delespaux, 2004). In Ethiopia, they are confined to southwestern and northwestern regions between longitude 33°and 38°E and latitude 5°and 12°N an area that covers 220,000 km² (Getachew, 2005). Tsetse-transmitted trypanosomosis affects cattle production over approximately 9 million km^2 of sub-Saharan Africa (Mattioli *et al.*, 2004). However, *T. vivax* is exceptionally important in Africa and in South America because the parasite can be mechanically transmitted by other biting flies (Kristjanson *et al.*, 1999). In Ethiopia, particularly of the northwest region is affected by both tsetse and non-tsetse transmitted trypanosomosis (Getachew and Yilma, 1996; Cherenet *et al.*, 2006; Sinshaw *et al.*, 2006; Shimelis *et al.*, 2007). In tsetse infested areas of Dembecha and Jabitehenan districts of northwest Ethiopia, the prevalence of trypanosomosis was 17.07% of which 26% of the infection was due to *T. vivax* (Shimelis *et al.*, 2005). Epidemiological investigation by Cherenet *et al.* (2006), on bovine trypanosomosis in tsetse-infested and tsetse-free areas of northwest Ethiopia indicated 15.6% and the dominant species was *T. vivax*. In other studies of mechanically transmitted trypanosomosis bordering Lake Tana of northwest Ethiopia the prevalence of trypanosomosis in cattle was 9.1% with a *T. vivax* infection (Sinshaw *et al.*, 2006).

African animal trypanosomosis is most important in cattle but small ruminants are also fully susceptible. Infection of cattle by one or more of the three species results in sub-acute, acute or chronic disease. The cardinal clinical sign observed in AAT is anemia, within a week of infection with *T. congolense* and *T. vivax*. There is a pronounced decrease in packed cell volume (PCV), hemoglobin and red blood cells as was observed in goats experimentally infected with *T. vivax* (Osman *et al.*, 2012). A field study in West Africa investigating the usefulness of anemia in the diagnosis of trypanosomosis showed that anemia can be considered as a reasonably accurate indicator of the disease with a

sensitivity of 56% and a specificity of 80% than other signs as emaciation, staring coat, lymphadenopathy, fever, lacrimal and salivary or nasal discharge (Schad *et al.,* 2008). So, knowing of the hematological profiles of animals infected with trypanosomes has a paramount importance in the detection of infection and treatment. In trypanosome endemic areas, trypanocidal drugs; both prophylactic and curative, are the most widely used methods of trypanosomosis control (Clausen *et al.,* 2010).

AAT control measures are mainly targeted towards elimination of the parasite from host blood and prevention of tsetse bites through vector control. Parasite elimination is mainly achieved through use of trypanocides which can be either curative or prophylactic; of which diminazene aceturate (DA) and isometamidium chloride (ISM) are commonly used to achieve the respective goals. However, this control arsenal is now hampered by increasing reports of drug resistance (Delespaux *et al.*, 2008). At present, there are twenty-one African countries in which trypanocidal drug resistance has been reported (Delespaux *et al.,* 2008; Chitanga *et al.,* 2011). In addition, the occurrence of multiple drug resistance to diminazene, isometamidium and homidium has been reported in trypanosome populations in ten African countries (Delespaux *et al.*, 2008) where this threatens the last stand to overcome drug resistance through the use of the sanative pair (Mamoudou *et al.,* 2008). Despite the high usage of these veterinary trypanocides, the interest of pharmaceutical industries to invest in research for developing new products remains low, leaving farmers to rely on the existing drugs for many decades. Because of the privatization of veterinary services in most parts of Africa, farmers have easy access to these trypanocides and this has resulted in rampant misuse and under-dosage of the medications, actions which have been blamed for the emergence of trypanocidal drug resistance. In addition, drugs used in animal disease are generally subjected to lower standards of quality control than those used in human disease (Chitanga *et al.*, 2011). Thus, drug resistance in trypanosomes poses a serious problem to livestock productivity unless checked and brought under control (Holmes *et al.,* 2004).

The control of trypanosomosis in Ethiopia relies on trypanocidal drugs with DA and ISM being frequently employed and resistance by *T. congolense* has been reported in some

areas (Afework *et al.,* 2000; Tewolde *et al.,* 2004; Shimelis *et al.*, 2008; Moti *et al.*, 2012). In general, most of the studies conducted in Ethiopia to assess the therapeutic and prophylactic efficacy of trypanocidal drugs have involved *T. congolense* in experimentally infected mice where it was possible to demonstrate the general status of resistance to the drugs by using ruminants. However, in tsetse free area of Tselemti Woreda of Tigray region, resistance of *T. vivax* to DA and ISM has been reported in goats (W/yohannes *et al.*, 2010). A recent study indicted the occurrence of resistance for *T. vivax* isolates from the northwest part of the country using the recommended curative doses DA and ISM (Shimelis, unpublished data). In both tsetse and tsetse free areas of the study sites, extensive trypanocidal drug sensitivity status is not established for *T. vivax* isolates.

When considering *T. vivax*; albeit case reports of resistance to ISM and DA has been described in East and West Africa (Schönefeld *et al.,* 1987) and the information is scarcer especially in studies conducted to see whether there is a difference in resistance between *T. vivax* from tsetse infested and tsetse free areas at the same time. Due to the presence of other trypanosomes in tsetse infested areas as compared to tsetse free areas of northwest Ethiopia, there could be a difference in the frequency trypanocidal drugs usage because of the pathogenic effect of *T. congolense*. Therefore, it is important to see whether there is a difference or not between *T. vivax* isolates in tsetse infested and tsetse free areas of northwest Ethiopia.

Therefore, the objectives of the present research work are:

- To determine and compare the drug sensitivity of *T. vivax* isolates from tsetse infested and tsetse free areas of North West Ethiopia in young Zebu cattle.

- To look for the associations of the hematological profiles with the occurrence of drug resistance in young zebu cattle infected with *T. vivax* isolates from tsetse infested and free areas of the North West Ethiopia.

2. LITERATURE REVIEW

2.1. General overview on African animal trypanosomosis

Trypanosomosis remains a major constraint to the development of livestock in Sub-Saharan Africa (SSA). About 80% of land is tilled by hand due to the high risk of AAT that threatens the survival and use of draught animals (Jamal *et al.,* 2005). It is estimated that about 50 million cattle are exposed to the disease and about 35 million doses of trypanocides are used per year (Mattioli *et al.,* 2004). It causes serious economic losses and an estimated annual loss in cattle production alone has been reported to be in the range of 1.0–1.2 billion dollars (FAO, 2002). Due to environmental changes (land use changes due to the increasing population and deforestation) the epidemiology of animal trypanosomosis is changing (Van den Bossche *et al.*, 2011). Generally, the fight against the disease is either managed by the control of the vector or of the parasite or a combination of both. However, in poor rural communities, which are mostly affected by the disease, control is mainly relying on the use of trypanocidal drugs (Jamal *et al.,* 2005). The inevitable outcome of continued use of the same compounds for decades has resulted in drug resistance that has been largely responsible for the frequently observed chemotherapeutic failures (Delespaux *et al.,* 2008).

2.2. The parasite

Trypanosomes are unicellular protozoan parasites of the phylum *Sarcomastigophora*, order *Kinetoplastida*, family *Trypanosomatidae*, and genus *Trypanosoma* (Levine *et al.,* 1980). They are haemoflagellated parasites characterized by one nucleus and one flagellum, either free or attached to the parasites body by means of an undulating membrane. They also usually contain a small compact kinetoplast, a disc-shaped DNA-containing organelle, situated within a large mitochondrion (Brun *et al.,* 1998). Kinetoplast DNA is arranged into a network of linked circles, grouped into minicircles and maxicircles. Within the kinetoplast network, there are around 20,000 minicircles and 20-50 maxicircles. Three principal parasites namely, *T. congolense, T. vivax* and *T. b.*

brucei are known to transmit trypanosomosis in bovines normally via the bite of an infected tsetse. In some rare instances, trypanosome species like *T. vivax* and *T. congolense* are transmitted mechanically (Desquesnes and Dia, 2003). The different trypanosome species differ in morphological characteristics as described by Maudlin *et al.*, (2004).

Trypanosoma congolense is divided into subtypes, with different distributions and pathogenicity: Savannah type, Forest type, Tsavo type, and Kilifi type (Majiwa *et al.*, 1993). *T.congolense* savannah type is the most pathogenic of the four and is capable of causing severe anemia and even death of infected cattle (Bengaly *et al.*, 2002). *T. vivax* shows variable levels of virulence and distinct pathogenicity in West African isolates, whereas the East African isolates largely cause chronic infection (Gardiner and Mahmoud, 1992). In East Africa, there are two types of *T. vivax* isolates: the hemorrhagic *T. vivax* that causes an acute hemorrhagic syndrome and the mild strain (Magona *et al.*, 2008). Infections with *T. brucei*, on the other hand, have been described as being chronic and sub patent, where cattle may act as important reservoirs of human pathogenic *T. brucei* species and can play an important role in the epidemiology of human sleeping sickness (Fevre *et al.*, 2001).

2.3. Life cycle of trypanosomes in the tsetse fly

Bloodstream African trypanosomes ingested by the tsetse fly embark upon a journey fraught with hazards. In order to complete the journey, multiplication and change of parasite form must occur along the route as different environments are encountered. The exact pathway taken by the trypanosomes in the vector depends upon the species. *Trypanosoma brucei* bloodstream forms on entering the fly undergo active division in the midgut as large procyclic trypomastigotes. These penetrate the peritrophic membrane (PTM) of the gut to reach the ectoperitrophic space where they migrate forward to the proventriculus and cease dividing to become elongate mesocyclic trypomastigotes. In this form the parasites retraverse the PTM and migrate *via* the esophagus, proboscis lumen, and hypopharynx to the vector's salivary glands. Here a second bout of multiplication

occurs during which the trypanosomes are anchored by their flagella to the vector's salivary gland epithelium after assuming the epimastigote form. The epimastigotes differentiate into the free, non-dividing metacyclic trypomastigotes that alone among the fly developmental forms can infect a mammal (figure 1). The entire developmental cycle takes 3-5 wks. *Trypanosoma congolense* has a similar developmental cycle except that the epimastigotes multiply attached to the chitinous wall of the proboscis (labrum) and the premetacyclic trypomastigotes swim to the hypopharynx where they mature into metacyclics. *T. vivax,* the third major African trypanosome, omits the fly midgut phase altogether, its vector procyclic stage possibly occurring deep in the foregut (cibarium) and quickly transforming to epimastigotes which invade the proboscis to generate metacyclics in the same manner *as T. congolense.* The time elapsing between trypanosome ingestion and extrusion of metacyclics may be as short as 10 days in this species. Not all flies ingesting trypanosomes produce metacyclics: in many the infection aborts. *T. brucei,* with the longest cycle, may produce metacyclics in only 2-5% of flies (Vickerman *et al.,* 1988).

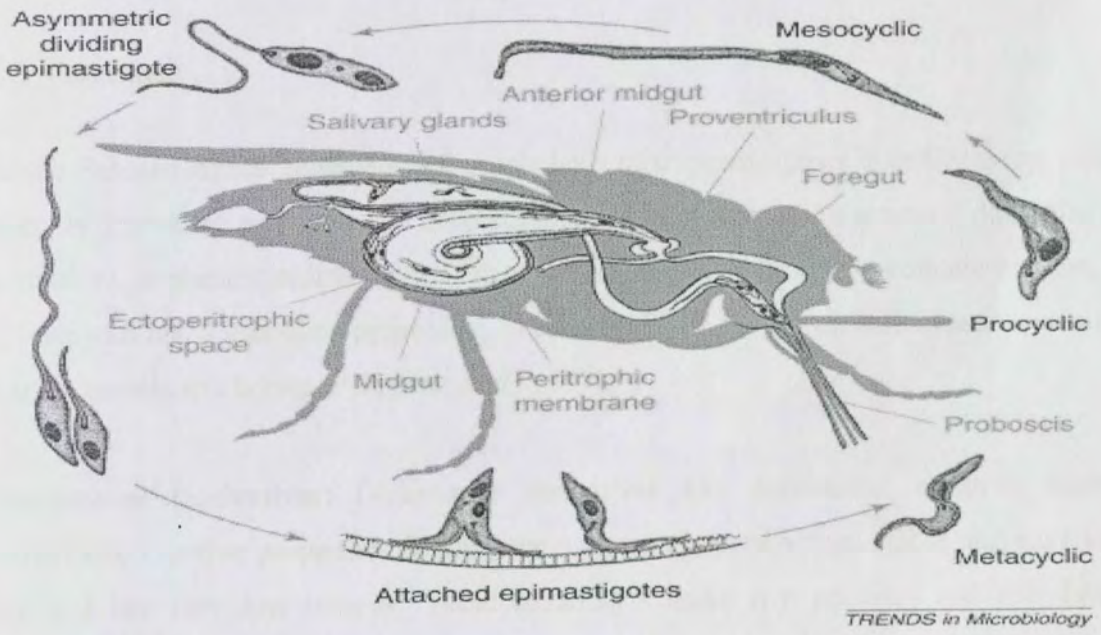


Figure.1. Schematic representations of trypanosomes in the tsetse fly (Vickerman *et al.,* 1988).

2.4. Transmission

Tsetse-transmitted trypanosomes occur in Africa according to the distribution of the vector. Mechanically transmitted trypanosomes can occur elsewhere in Africa, large areas of Asia, Middle East and South America (ILRAD, 1987). Among the Salivarian group only *T.vivax* is considered to be spread beyond the confines of tsetse fly belt by mechanical transmission (Hall, 1985). The infection with *T. vivax* is widespread: if transmitted by *G. palpalis*, it shows a light and chronic course, but if as in east Africa, *G. pallidipes* becomes the vector or if it is *G. morsitans* or *G. tachinoides* as in West Africa, the disease occurs with high fever, oedemas in the subcutis and causes death after 3-4 weeks. Multiple infections are also of the most important features of trypanosomosis in cattle (Losos, 1986). *T. vivax* causes major losses in cattle in West Africa; whereas in east Africa, the disease is usually characterized by low mortality and morbidity (Hoare, 1972). However, there have been outbreaks of hemorrhagic disease caused by *T. vivax* in Kenya (Olubayo *et al.,* 1985). On the other hand, *T. congolense* is most important in east Africa causing serious economic loss (Losos, 1986).

2.5. Treatment

In sub-Saharan Africa, treatment and prophylaxis of trypanosomosis in cattle, sheep, and goats is dependent on the use of three compounds: diminazene, an aromatic diamidine; homidium, a phenanthridine; and isometamidium, a phenanthridine- aromatic amidine. Quinapyramine, a quinoline pyrimidine, is recommended for use against trypanosomosis only in camels and horses (Peregrine *et al.,* 1997).

Diminazene Derivatives: Diminazene derivatives like diminazene aceturate have remarkable curative properties. Diminazene aceturate is very active, stable and easy to use and has very low toxicity. These advantages make it a practical and risk free trypanocides at least for cattle. It is prepared as a yellow powder and easily soluble in water. This solution can only be kept for two to three days. It is injected subcutaneously in cattle (slight local reactions possible) or intramuscularly (very rapid absorption) at a

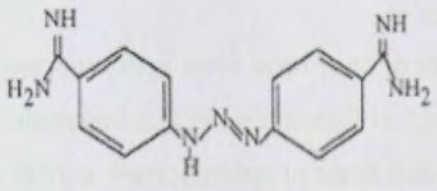
dose of 3.5 mg/kg live weight for treating *T. vivax* and *T. congolense* infections. Infections due to *T. brucei* can be treated in horse and cattle with the dose of 7mg/kg (Whiteside, 1962). Diminazene derivatives bind to DNA and interfere with parasite replications. This class of drugs has tendency to accumulate in tissue, therefore half life is very long, which may lead to residual problems in food producing animals (Riviere and Popich, 2009).

Homidium Salts: Homidium salts are effective against *T. vivax* infections in cattle but less so against *T. congolense* and *T. brucei*. Their limited and protective activity in cattle depends on severity challenge and may last three to five weeks. Homidium resistant trypanosome can be controlled by diminazene or isometamidium (Taylor *et al.*, 2007). It is given to cattle in one or 2.5% solutions at the rate of 1mg/kg. Novidium, which is a mixture of homidium chloride and bromide, has the same action as ethidium. It can also be used in *T. brucei* infections in dogs at the rate of 3-5mg/kg (Mira and Ralph, 1989).

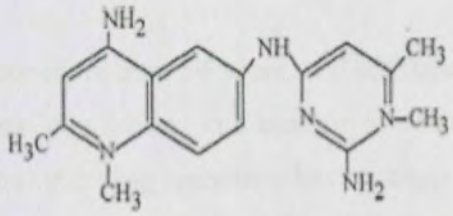
Isometamidium: Isometamidium is a phenanthridine aromatic amidine with a narrow therapeutic index which has been marketed for both a prophylactic and a therapeutic trypanocidal agent. Isometamidium chloride is used as curatively at lower dosage rates and prophylactically at higher dosage rates. It is usually prepared as red powder easily soluble in water. It is used in a one or two percent aqueous solution and administered by deep intramuscular injection at the rate of 0.25-1mg/kg, depend on drugs resistant risk. Strain of trypanosomes resistant to isometamidium and other phenanthridine appear frequently, but they remain susceptible to diminazene aceturate. It is given to the animal at dose rate of 0.51mg/kg and it will be protected for two to four months depending on the extent infections risk. Dromedaries appear to be more sensitive to this drug than other animals (Mira and Ralph, 1989).

Quinapyramine Sulphate: Quinapyramine methyl sulphate is sold in the form of white powder that dissolves easily in water. It is prescribed as a curative drug for cattle and small ruminants and is given subcutaneously as a 10% aqueous solution at dose 5mg/kg. It is used to treat *T. evansi* infectious in dromedaries at a standard dose of 2gm/ adult.

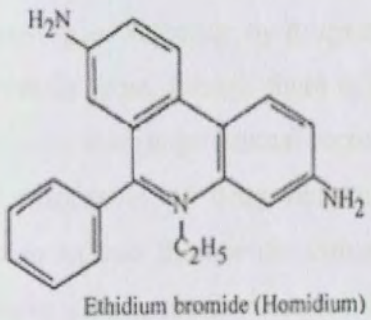
From 1950 until recently it was used in all the African countries giving excellent result for cattle trypanosomosis (especially *T. congolense*); it was slightly less successful against *T. vivax*. It causes appreciable systematic reactions and intramuscular injection cause painful local reactions leading to discomfort or lameness. In pig with *T. simiae* infections heavy doses (12.5-35mg/kg) can be used as curative treatment. Trypanosomes resistant to this compound should be treated with diminazene (Mira and Ralph, 1989).



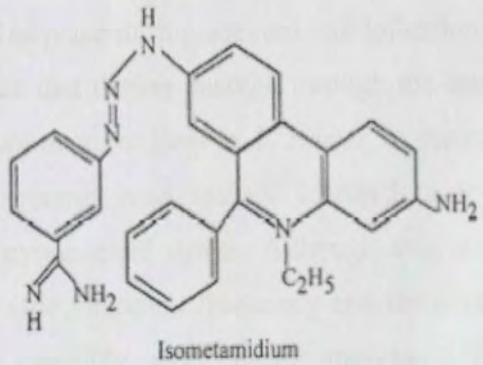
Diminazene aceturate



Quinapyramine



Ethidium bromide (Homidium)



Isometamidium

Figure.2 The structures of commonly used drugs in Africa.

A recent research conducted by Weiss et al (2013) showed that Wigglesworthia free (those flies that still house commensal Sodalis and parasitic Wolbachia) and flies that lacked all of their symbiotic microbes throughout their entire lifecycle flies exhibit a similarly high susceptibility to infection with trypanosomes indicating that obligate Wigglesworthia, as opposed to Sodalis or Wolbachia, is the primary modulator of tsetse's immune response following challenge with pathogenic trypanosomes. Extensive knowledge accumulated on tsetse and its symbionts now provides unique disease

management opportunities based on the control of parasite development in its invertebrate host. Their close proximity to the developing trypanosomes in the gut, the ease of prokaryotic transformation systems and gene expression, as well as the soon available genome sequence information make these symbionts good candidates for delivery of foreign gene products (Aksoy and Rio, 2005).

2.6. Trypanocidal Drug Resistance

Trypanocidal drugs have been used in the field for more than 30 years, and resistance to each compound has been reported in trypanosome populations in a number of countries across Africa. Furthermore, in some instances, multiple drug resistance has been reported and is a particular threat to livestock production (Peregrine *et al.*, 1997; Holmes *et al.*, 2004). It is important to stress that drug resistance is not an "all or nothing" phenomenon and the degree of drug sensitivity and resistance varies considerably between individual trypanosomes. Selection by drugs essentially takes place during asexual multiplication in the animals' host, though there is some evidence that during passage through the tsetse fly, genetic exchange (sexual recombination) may occur at least in *T. brucei*. In the past the development of drug resistance in trypanosomes was mainly ascribed to their exposure to sub therapeutic concentration of trypanocidal drugs. Although this is an important aspect, the intensity of drug pressure (the treatment frequency and the degree of exposure of the parasite population) is probably even more important. The immunocompetance of the host also appears to play an important role (McDermott *et al.*, 2003).

2.6.1. Mechanisms of trypanocidal drug resistance

Trypanosome kinetoplast is the primary site of ISM accumulation and decreased levels of drug accumulation have been observed in drug resistant populations of *T. congolense* (Sutherland *et al.*, 1991) and later work found indirect evidence of an increased efflux of drug from resistant trypanosomes (Sutherland and Holmes, 1993). Mulugeta et al. (1997) showed that the maximal uptake rates (V_{max}) of ISM in resistant *T. congolense* were

significantly lower than in sensitive populations. It remains to be shown whether this is caused by a decreased number of protein transporters of ISM in the plasma membrane and/or by changes in the balance between influx and efflux. The role of nucleoside transporters in resistance to ISM by *T. congolense* remains to be examined, although changes in these transporters have been associated with resistance to arsenical drugs in *T. brucei* (Ross and Barns, 1996). More recently, changes in mitochondrial electrical potential have been demonstrated in ISM resistant *T. congolense* by Wilkes *et al.,* (1997). Carter *et al.,* (1995) showed that the accumulation of diminazene was markedly reduced in arsenical-resistant *T. b. brucei* owing to alterations in the nucleoside transporter system. The mechanisms of ISM of resistance by trypanosomes to these drugs are unknown. There are indications, however, that it is similar to that described for ISM (Peregrine *et al.,* 1997).

Although diminazene probably exerts its action at the level of the kinetoplast DNA, this has not been proven *in vivo,* and other mechanisms of action cannot be excluded (Delespaux, 2004). Berger *et al.* (1993) showed that the accumulation of diminazene was markedly reduced in arsenical-resistant *T. brucei* owing to alterations in the nucleoside transporter system (P2). Increased resistance to diminazene was also observed in P2 deficient mutant of *T. brucei* (Matovu, 2003) and recently, RNA interference silencing the adenosine transporter-1 gene in *T. evansi* conferred resistance to diminazene aceturate (Witola *et al.*, 2004). Those results are confirmed by De Koning *et al.,* (2004) who conclude that the P2/TbAT1 gene mediates diminazene transport almost exclusively explaining the observed diminazene resistance phenotypes of TbAT1-null mutants and field isolates.

2.7. Detection of trypanocidal drug resistance

2.7.1. In vivo tests

A standardized protocol for the assessment of susceptibility and resistance to trypanocidal drugs in mice or in ruminants has been described by Eisler *et al.*, 2001.

Although there is a good correlation between the tests in mice and in ruminants, the curative dose that must be used in ruminants cannot be extrapolated from the results in mice. Another disadvantage of the mouse test is the fact that *T. vivax* and also some *T. congolense* isolates do not develop in mice (Geerts *et al.*, 2000).

Test in ruminants: The tests consist of infecting a group of cattle or small ruminants with the isolate under investigation and later, when the animals are parasitaemic, treating them with various dosages of trypanocides or following a simplified procedure with a single discriminatory dose as described by Eisler *et al.,* 2001.The animals are then regularly monitored over a prolonged period (up to 100 days) to determine the effective dose (ED), i.e. the dose that clears the parasites from the circulation, and the curative dose (CD), i.e. the dose that provides a permanent cure (Sones *et al.,* 1988). For these studies, the cattle or small ruminants must be kept in fly-proof accommodation or in a non-tsetse area in order to eliminate the risk of re-infection during the study. A variation of this technique was used by Ainanshe *et al.,* (1992) in Somalia to examine a group of isolates from a district. Blood from a group of infected cattle was inoculated into a single recipient calf, which was monitored, and later, when it was parasitaemic, treated with trypanocides at the recommended dose. A breakthrough infection, indicative that one of the inoculated trypanosome populations was drug resistant, was inoculated into groups of calves and mice to determine the level of drug resistance. This technique is useful in situations where laboratory facilities are very limited but it only allow a qualitative assessment and does not indicate how many of the isolates inoculated into a single calf were resistant. Further constraints to this technique are that not all populations might grow equally well and that sensitive isolates might overgrow resistant ones when inoculated together (Sones *et al.,* 1989). However this is not a consistent observation. A useful indication of the level of resistance can be obtained from studies in ruminants (and mice) by recording the length of time between treatment and the detection of breakthrough populations of trypanosomes: the shorter the period, the greater the level of resistance (Ainanshe *et al.,* 1992). The advantages of studies in ruminants are that most trypanosome isolates of cattle are able to grow in these hosts and that the data obtained are directly applicable to the field. The disadvantages are the long duration (a follow-up

of 100 days is necessary to allow the detection of relapses) and the cost (purchase and maintenance of the animals are expensive). Furthermore, if only one isolate per animal is tested, it is usually impractical and too expensive to examine a large number of isolates.

Test in mice: After expansion of an isolate in a donor mouse, groups of 5 or 6 mice are inoculated with trypanosomes, 24 hours later or at the first peak of parasitaemia each group except the control group is treated with a range of drug doses. Thereafter, the mice should be monitored three times a week for 60 days. The ED50 or ED95 (the effective dose that gives temporary clearance of the parasites in 50 or 95 percent of the animals, respectively) can be calculated, as can the CD50 or CD95 (the curative dose that gives complete cure in 50 or 95 percent of animals, respectively). Sones *et al.,* (1988) used groups of five mice, which allowed an easy calculation of ED80 and CD80 values (1 out of 5 mice was not cleared or cured). These figures should be compared with those obtained using reference sensitive trypanosome strains. The advantage of mouse assay is that it is cheaper than the test in cattle. There are several disadvantages, however: 1) most *T. vivax* isolates, and also some *T. congolense* isolates, do not grow in mice; 2) higher doses of drug must be used in mice (normally 10x higher) in order to obtain comparable results to those obtained in cattle because of the vast difference in metabolic size; 3) precise assessment of the degree of resistance needs a large number of mice per isolate, which makes it a labor intensive test identification of a discriminatory dose, and 4) it takes as long as 60 days to evaluate the drug sensitivity of an isolate (Sones *et al.,* 1988).

2.7.2. In vitro tests

In vitro tests using bloodstream or metacyclic trypanosomes can be used to detect resistance in *T. brucei* and *T.congolense* (Gray *et al.,* 1993). A major disadvantage of these tests is the slow adaptation of the trypanosomes to the culture conditions. Furthermore, it is difficult to maintain *T. congolense* in vitro (Clausen *et al.,* 2000).Two alternative approaches for *T. congolense* have been evaluated in which a short in vitro incubation in the presence of various drug concentrations is sufficient. The first approach is the drug incubation infectivity test (DIIT) where infectivity of the trypanosome is

evaluated after incubation for 4 hrs in plasma samples derived from cattle treated with trypanocidal drugs. The second approach is the drug incubation Glossina infectivity test (DIGIT), and in this case the main limiting factor being the availability of tsetse flies (Knoppe *et al.*, 2006).

2.7.3. Trypanocidal drug ELISA

The use of trypanocidal drug ELISA in combination with parasite detection tests has given promising results for the detection of resistant trypanosomes. The test is both sensitive, detecting subnanogramme concentrations, and specific. It allows the monitoring of drug levels over extended periods and the evaluation of factors influencing drug disappearance rates from the plasma. One interesting finding has been that the drug disappears more rapidly in animals challenged and becoming infected with drug-resistant trypanosome isolates than in those challenged but protected against infection with sensitive trypanosomes (Eisler *et al.*, 1994).

2.7.4. Conventional field tests for the detection of trypanocidal drug resistance

Resistance to ISM can be assessed under natural Trypanosoma challenge in the field using the 'block treatment' approach (Eisler *et al.*, 2000). Two groups of infected cattle, either treated with 1 mg kg^{-1} ISM or untreated (each group consisting of 30 to 80 animals) are exposed to natural challenge and tested for the presence of trypanosomes in the blood using the phase contrast Buffy coat technique every two weeks for two to three months. If >25% of ISM-treated cattle become infected within eight weeks of exposure, drug resistance is strongly suspected. This approach can also be used for assessing whether there is suspected resistance to DA by treating the control group at the start of the experiment, and all animals that become infected during the trial, with DA and checking for the presence of parasites two weeks after treatment (McDermott *et al.*, 2003). Furthermore, longitudinal parasitological field data can be suitably analyzed using appropriate statistical techniques to detect problems of resistance to DA (Rowlands *et al.*, 1993).

2.7.5. Genetic markers for drug resistance in trypanosomes

Due to the vast limitations of the currently available tests to validate drug resistance in trypanosomes, an alternative approach in the future may be made to identify genetic markers for drug resistance, which might be developed into reagents for the identification of resistant trypanosomes using the polymerase chain reaction (PCR). A PCR-based test could provide a rapid and convenient tool, suitable for large-scale epidemiological surveys of livestock. Developments of such tests require the identification of genetic mutations that may be associated with drug resistance in livestock-infective trypanosomes (Vitouley *et al.*, 2011).

2.7.6. Potential new tests for detection of resistance to isometamidium

Other tests that are still in the experimental stage or that are not used frequently are the tests based on the mitochondrial electrical potential (MEP) and the ISM-ELISA technique. It has been suggested that variation of the MEP might be the primary factor determining the rate of ISM accumulation in the trypanosome kinetoplast. Initial studies using a limited number of *T. congolense* populations have shown that an increased or decreased MEP might be a candidate quantitative marker for ISM susceptibility or resistance, respectively. The use of an ELISA for the detection of ISM in the serum can be combined with the 'block treatment' or individual treatment of ruminants to detect resistant trypanosomes. The presence of trypanosomes in animals with an ISM serum concentration >0.4 ngm ml^{-1} suggests that parasites are resistant (Holmes *et al.*, 2004).

2.8. Distribution of Trypanocidal Drug Resistance

The first case of drug resistance in trypanosomes was reported in 1967 in northern Nigeria (Na'isa, 1967). At present, there are twenty-one African countries (e.g. Burkina Faso, Chad, Côte d'Ivoire, Ethiopia, Kenya, Nigeria, Somalia, Sudan, the United Republic of Tanzania, Uganda, Zimbabwe, the Central African Republic, Zambia, Cameroun, Mozambique, Benin, Ghana and Togo) in which trypanocidal drug resistance

has been reported (Delespaux *et al.,* 2008; Chitanga *et al.,* 2011). In addition, the occurrence of multiple drug resistance to diminazene, isometamidium and homidium has been reported in trypanosome populations in ten African countries (Delespaux *et al.*, 2008), some of them include Nigeria, Kenya, Burkina Faso, Sudan and Ethiopia. Even more worrying are the recent reports of multiple drug resistance (to ISM and DA) because this is threatening the last stand to overcome drug resistance through the use of the sanative pair (Mulugeta *et al.,* 1997).

Table. 1. Summary on drug resistant trypanosomes.

Country	Trypanosome species	Resist to (*)	References
Burkina Faso	*Tc*	I	Pinder and Authié, 1984
		I,D,H	Clausen *et al.*,1992
	Tv	I,D	McDermott *et al.*, 2003; Sow *et al.*, 2012
Mali	*Tc*	I,D	Mungube *et al.*, 2012
	Tv	I	Mungube *et al.*, 2012
Mozambique	*Tc*	I,D	Jamal *et al.*, 2005
Kenya	*Tc*	I	Gray *et al.*, 1993
	Tc	I,D,Q	Peregrine,1997
Zambia	*Tc*	I,D	Chitanga *et al.*, 2011
Zimbabwe	*Tc*	I,D	Joshua *et al.*,1995
Kenya/Somalia	*Tv*	I	Schönefeld *et al.*, 1987
		H	Ainanshe *et al.*, 1992
Nigeria	*Tv*	D, H, I	Ilemobade, 1979
	Tb	D, I	Kalu, 1995
Sudan	*Tc, Tv, Tb*	H	Abdel Gadir *et al.*, 1981
Uganda	*Tb*	D, I	Matovu *et al.*, 1997
Ethiopia	*Tc*	D,H,I	Mulugeta etal.,1997
	Tc	I,D	Afework *et al.*, 2000
	Tc, Tv, Tb	I	Tewolde *et al.*, 2004
	Tv	I,D	W/yohannes *et al.*, 2010
	Tc	D,I	Moti *et al.*,2012
South America	*Tv*	D	Jones and Davila, 2001
China	*Tb*	Q	Liao and Shen, 2010

(*) D = Diminazene Aceturate; H = Homidium Bromide (Ethidium); I = Isometamidium Chloride; Q = Quinapyramine; Tc = *T. congolense*; Tv = *T. vivax*; Tb = *T. brucei*, *(Compiled data)*

3. MATERIALS AND METHODS

3.1. Study areas

3.1.1. Description of the sampling sites

Trypanosoma vivax isolates were originally collected from tsetse infested and tsetse free areas of Northwest Ethiopia. *Trypanosoma vivax* isolates for tsetse infested areas were collected from Jabitehenan districts of Birsheleko area and for tsetse free areas from Bahir Dar Zuria district of northwest Ethiopia (Fig 3).

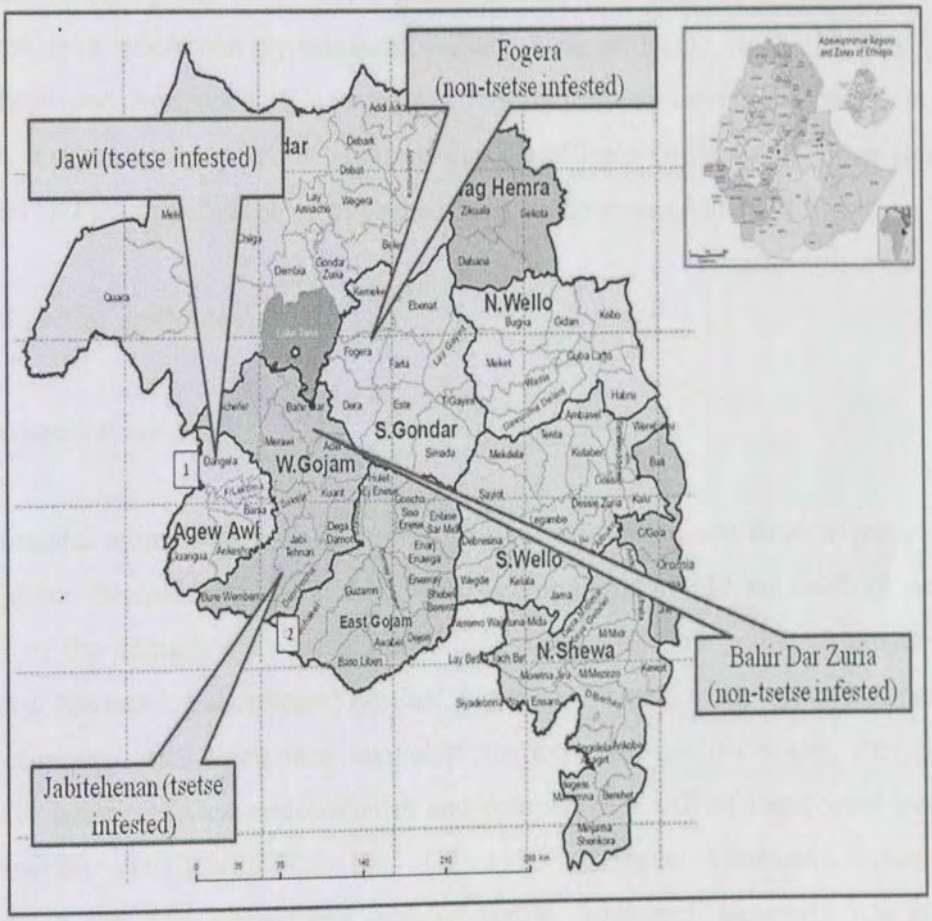


Figure.3. Map of the Amhara Region showing the sampling sites (northwest Ethiopia)

3.1.2. Experimental study site

The experimental study was undertaken in the premises of the College of Veterinary Medicine and Agriculture of Addis Ababa University at Bishoftu. The experimental fly-proof house which is found in the compound and used for the study was constructed by the GALVMed project. Bishoftu is located 45 Kms east of Addis Ababa and the area possess midland agro ecology with rainy season extends from June to September and followed by long dry season.

3.2. Questionnaire survey

A questionnaire survey was conducted to gather data on the history of trypanocidal drugs usage in the area where the trypanosome isolates were collected (including the type, duration, dose and frequency of treatments). It also focused on who administers the drugs, their sources, and perception of farmers on the efficacy of the drugs, other control options used and the significance of trypanosomosis in the areas (Annex 1).

3.3. Experimental methodology

3.3.1. Experimental animals

The experimental animals of 9 to 12 months of age were purchased from trypanosome free area (Debre Berhan; north central highland Ethiopia) about 130 km north of Addis Ababa. All of the animals were treated with long acting oxytetracycline (Alamycin10, 1.5-4.0mg/kg, Norbrook Laboratories Limited, Northern Ireland) while being transported to the experimental site. Upon their arrival to the experimental study site; they were examined for hemoparasites, endoparasites and ectoparasites. All of them were treated with Ivermectin (IVERMECTIN 1%, 0.2ml/kg, Chengdu Quinkun Veterinary Pharmaceuticals Co. Ltd, China) and triclabendazole. Additional treatment was given with albendazole (ALBENDA-QK, 15mg/kg, Chengdu Quinkun Veterinary Pharmaceuticals Co. Ltd, China) after a week. One month prior to the challenge, animals were housed in fly proof compartments.

3.3.2. Trypanosoma vivax stocks

The trypanosomes were collected from naturally *T. vivax* mono-infected cattle using parasitological techniques from the sampling sites by Dr.Shimelis Dagnachew (PhD Fellow). The isolates were confirmed by PCR as pure *T. vivax*. Stabilates of *T. vivax* isolates were prepared and cryopreserved in liquid nitrogen tank (-196 ^{o}c) at Ethio-Belgium project laboratory which is found in the College. For this experiment the following stabilates were used; ETBS 2 (Ethiopia-Birsheleko isolate 2) from tsetse infested area and ETBD 2 and 3 (Ethiopia- Bahir Dar isolate 2 and 3) from tsetse free areas.

3.3.3. Experimental design and trypanocidal drugs

Prospective experimental study type was conducted from March to June of 2014 for the investigation of drug sensitivity. The trypanocidal drugs used in the experiment were diminazene aceturate (BERENIL R.T.U, Lot No.A189A01, Exp.03-2015, 20 Spartan Rd., Spartan, Republic of South Africa) and Isometamidium chloride (Veridium.T.M, Lot No.198A1, Exp. 06-2015, CEVA SANTE ANIMALE, Libourne-France) where Both of them were tested for their right composition. Diminazene aceturate was injected as a 7% solution at dose of 7 mg/kg body weight and Isometamidium chloride was injected as 1% solution at dose of 1 mg/kg of body weight. Distilled water was used to dissolve appropriate quàntities of ISM before it was administered to the animals. The drugs were administered through intramuscular route to animals on the basis of accurate body weight measurement taken immediately before treatment using digital weighing machine (TAL-TEK Livestock Scale, South Africa). Randomized controlled block design was used where thirty six of the experimental animals were randomly divided into six blocks on the basis of *T. vivax* isolates and trypanocidal drugs as indicated below.

Group1 (TT+DA) = infected with *T. vivax* isolate from tsetse area (TT) and treated with DA.

Group 2 (TT+ISM) = infected with *T. vivax* isolate from tsetse area (TT) and treated with ISM.

Group 3 (NT1+DA) = infected with *T. vivax* isolate from tsetse free area (NT1) and treated with DA.

Group 4 (NT1+ISM) =infected with *T. vivax* isolate from tsetse free area (NT1) and treated with ISM.

Group 5 (NT2+DA) = infected with *T. vivax* isolate from tsetse free area (NT2) and treated with DA

Group 6 (NT2+ISM) =infected with *T. vivax* isolate from tsetse free area (NT2) and treated with ISM.

3.3.4. Artificial inoculation of experimental animals

After *T. vivax* stabilates were propagated on the donor calves, determination of the level of parasitaemia was calculated using "rapid matching method" (Herbert and Lumsden, 1976) (Annex 2). Then, all the experimental animals were inoculated with 2ml of blood at optimum infective dose of $1x10^6$ trypanosomes/ml.

3.3.5. Trypanocidal drugs sensitivity test

Parasitological examination using the Buffy coat technique was done daily until detection of peak parasitaemia (Murray *et al.,* 1977) and score of parasitemia was done according to Paris, et al (1982). Then, all infected animals were treated with 7mg/kg b.wt of DA and 1mg/kg b.wt of ISM according to their respective groups. All animals were followed for a fall in parasitaemia using the rapid matching technique and changes in PCV first twice a week for two weeks post treatment (PT) and then weekly until the end of the experiment. All relapses after two weeks PT were registered. Relapse infections detected within 100 days of administration of a trypanocidal drug were indicative of resistance. If relapse occurs in more than 20% or more of the calves tested (i.e. for a total of between one and four calves, at least 1 relapse; for a total of 5 or 6 calves, at least 2 relapses), the isolate was said to exhibit resistance to the dose of drug used (Eisler *et al.*, 2000 and 2001; Delespaux *et al.*, 2009) (Annex 3).

3.3.6. Hematological examinations

With regard to hematological parameters, PCV measurement was done daily before treatment, twice a week for two weeks and once till the end of the experiment. Hemoglobin concentration and total erythrocyte counts were done on weekly basis up to eight weeks of the experiment. Blood was taken from the jugular vein using EDTA coated vacuetainer tubes. For PCV determination, the capillary tubes were centrifuged at 12000 rpm for 5 minutes and then read on the scaling instrument (Murray *et al.*, 1983). Hemoglobin concentration was determined using acid hematin method where the values were expressed in g/dl. Total erythrocyte counts were done using an improved Neubauer hemocytometer and the values were expressed in million cell/ mm^3 blood (Kemal, 2014) (Annex 4).

3.4. Animal feeding and management

Throughout the experiment, animals were fed *ad libitum* with grass hay and water. Supplemental feeds with concentrates and mineral lick was provided once per day. Animal welfare was obeyed and all protocols and procedures used in the study were according to the guideline set by the animal research and ethics review committee of the College of Veterinary Medicine and Agriculture of Addis Ababa University. At the end of the experiment, all of the infected animals were euthanized using high dose sodium phenobarbitol injection in their jugular vein.

3.5. Data management and analysis

The entire data source was recorded into Microsoft Excel 2007 spread sheets. Data management and analysis was done using SPSS version 20. The statistical tests that were used include: One Way-ANOVA, descriptive statistics and graphs. The test was considered significant at $p < 0.05$.

4. RESULTS

4.1. Questionnaire survey

A total of 100 respondents consisting of 50 each from both of the sampling sites were included in the questionnaire survey. Half of the respondents (50%) from Bahir Dar Zuria and only 22% of the respondents from Jabitehenan areas rear only cattle for draught purpose and as a source of income. In both situations communal or free grazing is the major livestock management practice. When considering the importance of trypanosomosis in the sampling sites, 84% and 100% of the respondents from tsetse free and tsetse infested areas respectively ranked trypanosomosis first and known the disease for the last twenty years.

When considering the control of trypanosomosis in both sites, all of the respondents preferred treatment of their animals with trypanocidal drugs rather than other control options. The sources of drugs used by the respondents from tsetse free areas includes, 56% (n=28) from veterinary clinics, 34% (n=17) from private drug shop and 10% (n=5) from illegal drug markets. In the case of tsetse infested areas the sources of the drugs 0were, 18% (n=9) from veterinary clinics, 48% (n=24) from private drug shops and 34% (n=17) from illegal drug markets (Fig 4). Most of the cattle owners from the tsetse free areas, 66% (n=33) got their cattle treated by veterinary professionals as compared to 68% (n=34) of the farmers from tsetse infested areas which treat their animals by themselves (Fig 5).

Figure. 4. The sources of trypanocidal drugs from the sampling sites.

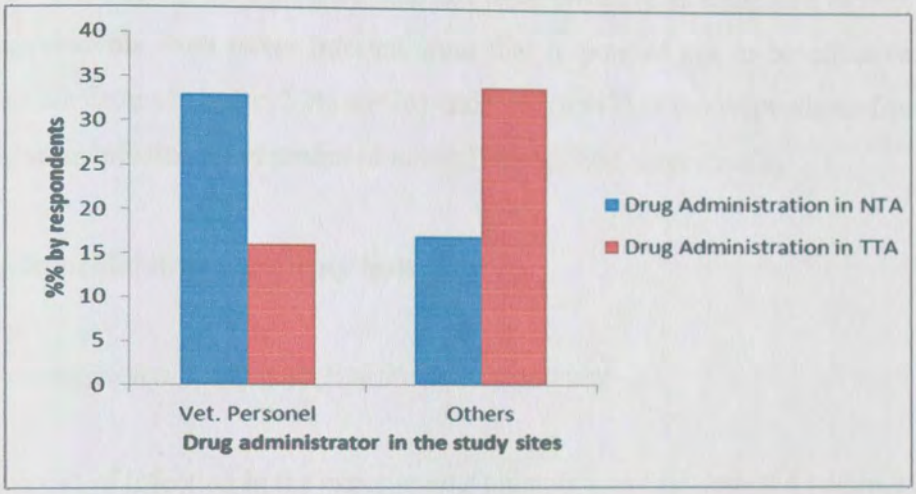


Figure.5. Drug administrator from the sampling sites.

Out of the fifty respondents from tsetse free areas, 40% (n=20), 20% (n=10) and 40% (n=20) preferred to use DA, ISM and both as common trypanocidal drugs as compared to 30% (n=15), 2% (n=1) and 68% (n=34) from tsetse infested areas respectively (Fig 6A). When considering treatment frequencies with trypanocidal drugs, 80% (n=40) of the respondents from tsetse free areas got their animals treated at least one to three times a year as compared to 68% (n=34) of the respondents from tsetse infested areas that got their cattle treated at least more than ten times a year (Fig 6B).

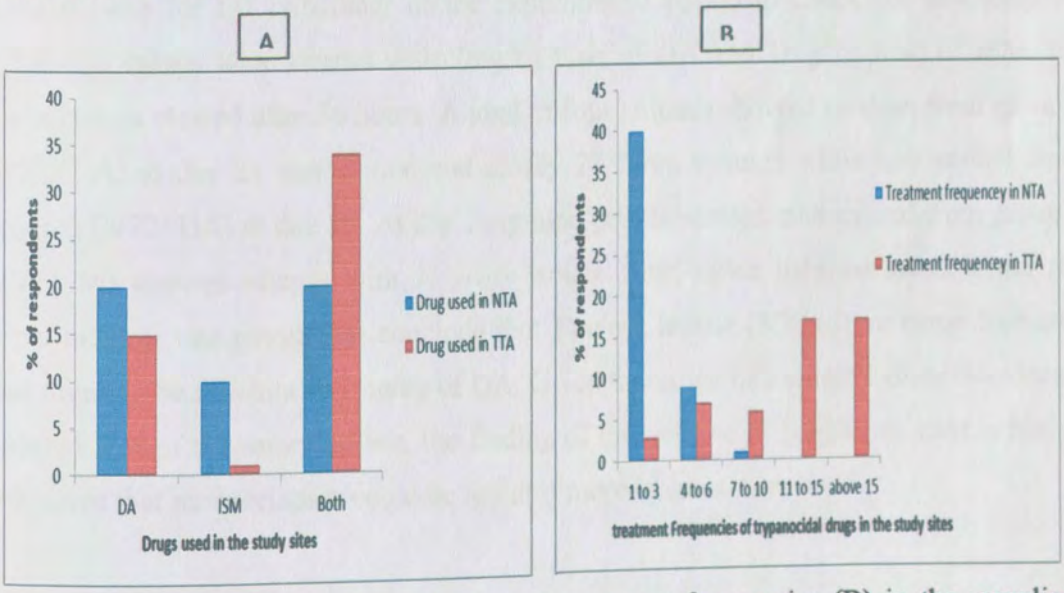


Figure.6. Trypanocidal drugs used (A) and treatment frequencies (B) in the sampling sites

Considering the drug efficacy, 66% (n=33) of the respondents from tsetse free areas responded treatment of their animals with DA to be effective as compared to 94% (n=47) of the respondents from tsetse infested areas that responded not to be effective. With regards to the drug of choice, 52% (n=26) and 94% (n=47) of the respondents from tsetse free and tsetse infested areas preferred to use DA and ISM respectively.

4.2. Trypanocidal drug sensitivity tests

4.2.1. Parasitological findings before and after treatment

Establishment of infection in the experimental animals was first detected within four days of challenge in animals belonging to the non-tsetse isolates while on day seven for tsetse isolate. On clinical examination, the animals were febrile with rough hair coat, pale mucus membrane and enlarged superficial lymph nodes. On day seven of post infection, all of the animals become parasitaemic and treatment was given on day fourteen when they reached peak parasitaemia as shown below (Fig 7). Generally, isolates from tsetse free areas established quickly as compared to the isolate from tsetse infested area.

Experimental animals were followed twice a week for two weeks post treatment and on weekly basis for the remainder of the experimental period to check for any relapses. When the calves were treated with 7mg/kg b.wt of DA and 1mg/kg b.wt of ISM, the parasitaemia cleared after 36 hours. A total of four animals showed relapse; from group 3 (NT1+DA) at day 21 one animal and at day 28 three animals while one animal from group 5 (NT2+DA) at day 35. At day forty nine post treatment, one animal from group 2 (TT+ISM) showed relapse with *T. vivax* isolate from tsetse infested areas. From the experiment, it was possible to conclude that *T. vivax* isolate (NT1) from tsetse free area was found to be resistant to 7mg/kg of DA. Given the usage of a single isolate from tsetse infested area of the sampling site, the finding of one relapse to 1mg/kg of ISM is highly indicative that more relapses could be found if more isolates were used.

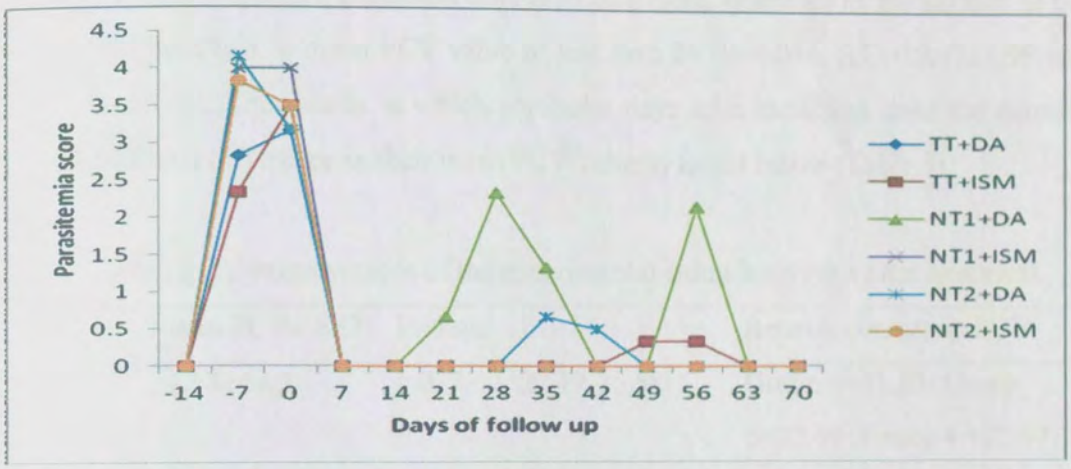


Figure.7. Waves of parasitaemia for the different isolates (day -14 represents day of infection, day 0 represents treatment day and starting from day 7 are days after treatment)

4.2.2. Hematological findings

Comparison of the packed cell volume using paired sample t-test showed that the mean PCV values of the experimental groups before and after treatment to be significant (P=0.027). One-way ANOVA was further used to compare within groups PCV improvement and the result showed that animals in Group 3 (NT1+DA) and 6 (NT2+ISM) were found to had the least PCV improvement (P<0.001) after being treated with trypanocidal drugs as shown below (Table 2)

Table.2 Mean PCV comparison of the experimental groups before and after treatment

T. v isolates	Mean PCV± SD BT*	Mean PCV± SD AT*	P-value
TT1+DA	23.33±3.8	26.20±5.5	0.027
TT1+ISM	20.42±3.7	25.51±2.4	
NT1+DA	19.33±3.9	23.14±3.6	
NT1+ISM	20.42±4.4	23.79±3.2	
NT2+DA	23.83±1.6	25.48±2.1	
NT2+ISM	17.5±3.3	22.7±2.6	

T. v= Trypanosoma vivax, BT=before treatment and AT*= after treatment*

One way ANOVA was used to compare the mean PCV improvement of the experimental animals in their respective groups on days after treatment; where all of the animals at the day of treatment had a mean PCV value of less than 24 (P=0.046, S.D=19.42±4.98 and CI=17.73-21.10). The details at which particular days after treatment does the animals had a significant difference in their mean PCV value is found below (Table 3).

Table.3 Mean PCV improvement of the experimental animals on days after treatment.

Days	Mean PCV± S.D	P-value	C.I	Remarks on group PCV
42	24.18±4.03	0.037	22.77-25.58	Group 3=21.83; Group 6=22.40; Group 4 =22.67
56	25.91±4.65	0.031	24.26-27.56	Group3= 20.20

Comparison of mean hemoglobin concentration before and after treatment was found to be not significant (P=0.345). However, results of one-way ANOVA revealed that animals in Group 3 (7.5 g/dl), 4 (7.7g/dl) and 6 (7.12g/dl) were found to had the least hemoglobin concentration improvement (P=0.001, S.D= 8.1±2.28, CI=7.82-8.37) after being treated with trypanocidal drugs.

One way ANOVA was further used to see the mean hemoglobin concentration improvement on days after treatment; where all of the animals at the day of treatment had a mean hemoglobin concentration value of less than 8 g/dl (P=0.003, S.D=6.38±2.06 and CI=5.50-7.24). On day seven post treatment, all of the experimental animals within their respective groups had a mean hemoglobin concentration value of less than the normal range and the difference is significant (P=0.005, S.D=6.13±1.49 and CI=5.48-6.77). Similarly on day 42 post treatment, except from animals form group 1 (TT+DA) all of the animals in the rest groups had a mean hemoglobin concentration value of less than the normal range and the difference is significant (P=0.014, S.D=7.35±1.37 and C.I=6.76-7.94) (Fig 8).

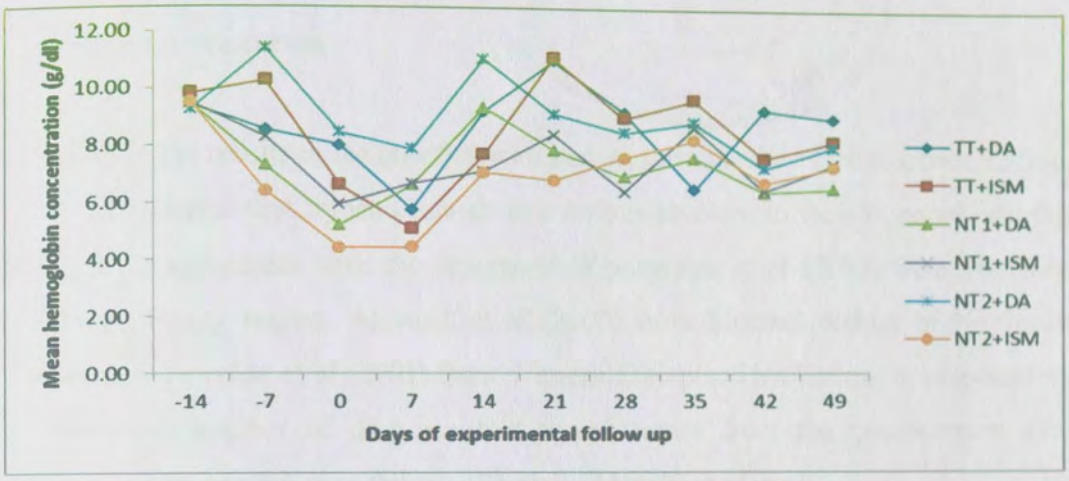


Figure.8. Mean hemoglobin concentration improvement on days after treatment (day -14 represents day of infection, day 0 represents treatment day and starting from day 7 are days after treatment).

Comparison of the mean erythrocyte count for the experimental animals before and after treatment was found to be significant (P=0.005). Furthermore, one-way ANOVA revealed that animals in all groups had a mean erythrocyte count of less than the normal range and the differences was found to be significant (P<0.01, S.D= 5.9±1.62, CI=5.71-6.10). Mean erythrocyte count improvement of days after treatment for the experimental animals shown below (Fig 9).

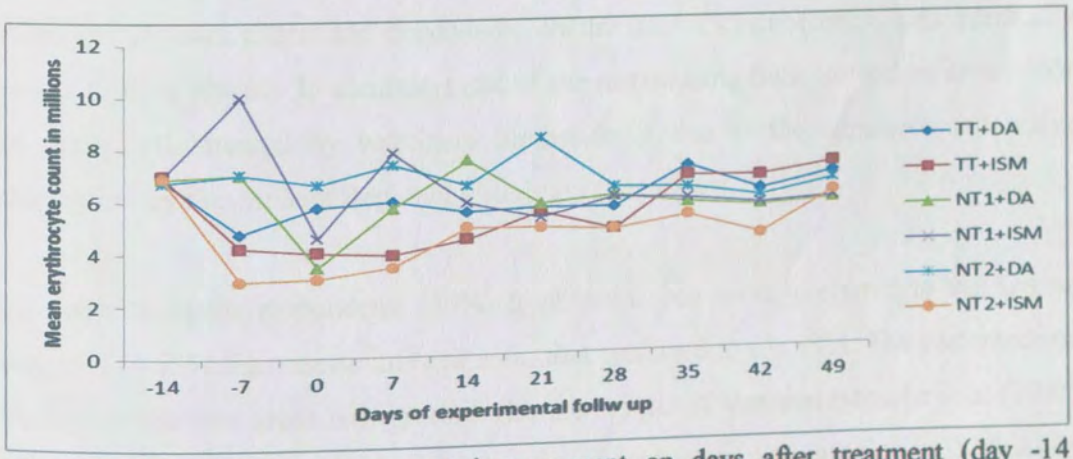


Figure.9. Mean erythrocyte count improvement on days after treatment (day -14 represents day of infection, day 0 represents treatment day and starting from day 7 are days after treatment)

5. DISCUSSION

5.1. Questionnaire survey

According to the results of the questionnaire survey, the majority of the respondents from both areas reported that trypanosomosis is a serious problem to their livestock and this finding is in agreement with the reports of W/yohannes et al (2010) from Tselemeti Woreda of Tigray region, Afework et al (2000) from Metekel district of Northwest Ethiopia and Tewolde et al (2001) from Western Ethiopia. This finding is supported by the increasing number of drug resistant trypanosomes from the questionnaire sites resulting in trypanocidal drug failures (Shimelis, Unpublished data).

Most of the cattle owners (68%) from tsetse infested areas treated their animals by themselves or by uncertified individuals. This finding is in agreement with the report of about 57% by Tewolde et al (2001) from Western Ethiopia, 43% by Afework et al (1998) from Metekel district, Northwest Ethiopia and 30% by W/yohannes et al (2010), from Tselemeti Woreda of Tigray region. Reasons for trypanocidal drugs misusages could be related to the inadequacies of veterinary services in their vicinity, the ease in the availability of trypanocidal drugs in illegal markets, the high number of treatment failures that would force them to have the drugs at their back yard and recent failures in sustaining tsetse control activity by the Amhara Regional Veterinary Laboratory that makes the farmers prone and dependable on the use of chemotherapy and eventually leading to drug abuses. In contrast, most of the respondents from tsetse free areas, 66% got their cattle treated by veterinary professionals due to the closeness and active intervention by the Amhara Regional Veterinary Laboratory.

The majority of the respondents (30%) from tsetse free areas, preferred to use DA as compared to 68% from tsetse infested areas that preferred to use ISM. The preference of DA from tsetse free areas is supported with the reports of Van den Bossche et al (2000) which indicated that the majority of farmers preferred to use DA rather than ISM. As in the case of tsetse infested areas, the drug preference of ISM by the respondents was due

to its prophylactic effect. When considering treatment frequencies, most of the respondents (68%) from tsetse infested areas treated their animals on average more than fifteen times a year (2-3 times a month) as compared to the respondents (80%) from tsetse free areas with few treatment frequencies. The high frequency of treatment in tsetse infested areas is comparable with the reports of Shimelis (2004) with treatment frequencies of 3.0-4.0 per month; Tewolde et al (2001) reported 2.5-3.5 per month. This could be explained by the presence of other species of trypanosomes as Uilenberg (1998) who reported that the number of treatments over a year reflects the magnitude of trypanosome challenge in an area.

5.2. Trypanocidal drug sensitivity test

5.2.1. Parasitological findings

The early detection of parasitaemia from the tsetse free isolate infected groups as compared to the tsetse isolate infected group could be associated with the ease in the adaptation of trypanosomes to mechanical way of transmission. It is supported by Langridge (1976) who stated that trypanosomes from non-tsetse areas are adapted to mechanical ways of transmission from animal to animal through the use of syringes.

In the present experiment, one of the *T. vivax* isolate (NT1+DA) from tsetse free areas of the northwest Ethiopia was found to be resistant to diminazene aceturate at the dose of 7mg/kg of body weight. Recent reports from the same study areas of northwest Ethiopia showed the occurrence of *T. vivax* resistant isolates at 3.5 mg/kg body weight (unpublished data) in cattle. Similarly W/yohannes et al (2010) reported diminazene resistant *T. vivax* isolates at the dose of 3.5 mg/kg body weight from Tselemeti Woreda of Tigray region in goats. On the other hand, Terfa (2008) reported diminazene resistant *T. congolense* isolates from Birbir valley Gawo Dalle district of Western Ethiopia at the same dose we used in the present study. Codjia et al (1993) collected 12 *T. congolense* isolates from cattle bred in the Ghibe valley and reported resistance to the maximum recommended dose of DA (7mg/kg B.W.). Most of the drug sensitivity tests conducted so

far had focused on *T. congolense* even with few drug sensitivity tests on *T. vivax* using DA at the dose of 3.5 mg/kg body weight. Moreover in the present study the finding of one relapse (NT2+DA) in the other isolate from non-tsetse areas gives an indication of the occurrence of resistance. The reason for the high number of DA resistant *T. vivax* from tsetse free areas might be on the quality of the drugs used as evidenced by the 10% respondents which used drugs from illegal sources. In addition the frequent usage of DA in these areas favors selection pressure of the drugs by resistant trypanosomes and under dosing which are the root causes to the development of drug resistance (Whiteside, 1960). This is in agreement with recent studies on the quality of the DA and ISM sold in sub-Saharan Africa, stating that a great majority of these products do not respect the standards established by the original producers (Schad *et al.*, 2008; Tettey *et al.*, 2002). Results on the sensitivity of *T. vivax* isolated from tsetse areas using DA at the dose of 7mg/kg of body weight showed no relapse and could be explained by the sensitivity of the isolate for the dose used.

In the present experiment, none of the *T. vivax* isolates from both of the sampling sites were found to be resistant to ISM at the dose of 1mg/kg body weight, however, the finding of one relapse in TT+ISM group at 1mg/kg of ISM is indicative for resistance if more isolates were used. The occurrence of relapse in ISM treated groups for the tsetse area might be associated to the frequent application of this drug compared to DA as evidenced by the respondents while this practice is not commonly used for the tsetse free areas. This finding is roughly in agreement with the recommendations by Eisler et al (2000) which is to use ISM at a dose of 1 mg/kg body weight rather than the dosage commonly used which is 0.5 mg/kg body weight. However, the present finding is in contrast to the reports by Sow et al (2012) indicating that *T. vivax* isolated from Burkina Faso and tested at a dose of 1mg/kg b.wt was found to be resistant. Correspondingly, Chaka and Abebe (2003) reported that using of ISM at the dose up to 1 mg/kg body weight failed to clear the infection for *T. congolense* isolates. This disagreement could be explained by the less number of *T. vivax* isolates used in the experiment as compared to other studies.

5.2.2. Hematological findings

With regard to the PCV improvement, results of the experiment showed that the mean PCV values of less than the normal range was associated with break through infections as in the case of animals in group three but was not the same as to the animals in group six. This finding is supported by Holmes and Jenning (1976), stating that PCV improvements lower than the normal value is associated with the relapses and/or re-emerging of trypanosomes from sites which are inaccessible to trypanocides. The reduction of PCV values which could be associated to the decrease in total RBC count in the present finding is in agreement to reports done by (Osman *et al.,* 2012) who indicated that the decrease in PCV could also be explained by a decrease in total erythrocyte count or due to hemodilution. As to the case of the animals in group six, the finding of PCV value of less than the normal range could be explained by the less sensitivity of the diagnostic techniques used and also with the chronic stage of the disease characterized by a fall in PCV value with little detection of parasitaemia.

With regard to the hemoglobin concentration, results of the experiment on mean hemoglobin concentration values after treatment to be not significant with animals from group three, four and six having the least hemoglobin concentration improvement. The decrease in hemoglobin concentration as for animals in group three is explained by the break through infections and is supported by the reports of Osman et al (2012), stating that the mean hemoglobin concentration significantly reduced upon being infected with *T. vivax* whereas for the animals in group four and six, may be associated with the chronic nature of the disease.

6. CONCLUSION AND RECOMMENDATIONS

The findings of the present study revealed that the use and management of trypanocidal drug is in discriminatory and farmers were not reliable on the efficacy of the available drugs mainly in the tsetse infested areas. The experimental drug sensitivity study on *T. vivax* isolates confirm the presences of one resistant strain from non-tsetse areas against DA at 7mg/kg body weight. Moreover one relapse was detected from the other non-tsetse isolate against DA and one relapse from tsetse isolate against ISM at 1mg/kg body weight. The occurrence of relapses is associated with reduction in the improvement of hematological profile. Generally, when there are indications of drug resistance in a certain area, it is essential to try to maintain the efficacy of the currently available drugs.

So, based on the above remarks the following points are forwarded:

- Sustaining an integrated disease management strategy with legislative reinforcement by way of elaborating a national drug use policy is required to address the indiscriminate drug usage and also to stop illegal traders in the sampling sites.

- Further studies should be conducted in Ethiopia in particular and across the tsetse and biting flies infested areas of Africa in general using a more advanced detection tests to further elaborate the problem of drug resistance.

- The use of sanative pair treatment with DA and ISM in relation to the occurrence of drug resistance is essential to resolve the problem in the study sites.

7. REFERENCES

Abdel Gadir, F., Osman, O.M., Abdella, H.S., Abdel Razig, M.T. (1981). Ethidium bromide resistant trypanosomes in southern Darfur. *Sudan J. Vet. Res.* **3**:63-65.

Afewerk, Y., Clausen, P.H., Abebe, G., Tilahun, G., and Mehlitz, D. (2000). Multiple-drug resistant *T. congolense* populations in village cattle of Metekel district, north-west Ethiopia. *Acta Tropica.* **76**: 231-238.

Ainanshe, O.A., Jennings, F.W. and Holmes, P.H. (1992). Isolation of drug-resistant strains of *Trypanosoma congolense* from the lower Shebelle region of southern Somalia. *Trop Anim Health Prod.* **24**: 65-73.

Aksoy, S., and Rio, V.M. R. (2005). Interactions among multiple genomes: Tsetse, its symbionts and trypanosomes. *Ins. Biochem. Mol. Bio.* **35**: 691–698.

Bengaly, Z., Sidibe, I., Ganaba, R., Desquesnes, M., Boly H., Sawadogo, L. (2002). Comparative pathogenicity of three genetically distinct types of *Trypanosoma congolense* in cattle: clinical observations and hematological changes. *Vet. Parasitol.* **108**: 1–19.

Berger, B.J., Carter, N.S. and Fairlamb, A.H. (1993). Polyamine and Pentamidine Metabolism in African Trypanosomes. *Acta Tropica.* **54**: 215-224.

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

Carter, N.S., Berger, B.J., Fairlamb, A.H. (1995). Uptake of diamidine drugs by the P2 nucleoside transporter in melarsen-sensitive and resistant *Trypanosoma brucei brucei. J.Biol. Chem.* **270**: 28153-28157.

Chaka, H., Abebe, G. (2003). Drug resistant trypanosomes: a threat to cattle production in the Southwest of Ethiopia. *Rev. Elev. Méd. Vét. Pays Trop.* **56**:33-36.

Cherenet, T., Sani, R.A., Speybroeck, N., Panandam, J.M., Nadzr, S., Van den Bossche, P. (2006). A comparative longitudinal study of bovine trypanosomosis in tsetse-free and tsetse-infested zones of the Amhara region, northwest Ethiopia. *Vet. Parasitol.* **140**:251-258.

Chitanga, S., Marcotty, T., Namangala, B., Van den Bossche, P., Van Den Abbeele, J, et al. (2011). High Prevalence of Drug Resistance in Animal Trypanosomes without a History of Drug Exposure. *PLoS Negl Trop Dis.* **5**:1.

Clausen, P.H. *et al,* (2000). Application of *in vitro* methods for the detection of drug resistance in trypanosome field isolates, *ICPTV Newsletter.***2**: 9-12.

Clausen, P.H., Bauer, B., Zessin, K.H., Diall, O. *et al.*, (2010). Preventing and Containing Trypanocide Resistance in the Cotton Zone of West Africa. *Transboundary and Emerging Diseases.* Blackwell Verlag GmbH. **57**:28-32.

Clausen, P.H., Sidibe, I., Kabore, I., Bauer, B. (1992). Development of multiple drug resistance of *Trypanosoma congolense* in Zebu cattle under high natural tsetse fly challenge in the pastoral zone of Samorogouan, Burkina Faso. *Acta Trop.* **51**:229-236.

Codjia, V., Mulatu, W., Majiwa, P.A.O., Leak, S.G.A., Rowlands, G.J., Authie, E., Dieteren, G.D.M., Peregrine, A.S., (1993). Epidemiology of bovine trypanosomosis in the Ghibe Valley Southwest Ethiopia. Occurrence of populations of Trypanosoma congolense resistant to diminazene, isometamidium and homidium. *Acta Trop.* **53**:151-163.

De Koning, H.P., Anderson, L.F., Stewart, M., Burchmore, R.J., Wallace, L.J., Barrett, M.P. (2004). The trypanocide diminazene aceturate is accumulated predominantly through the TbAT1 purine transporter: additional insight on diamidine resistance in African trypanosomes. *Antimicrob. Agents Chemother.* **48**: 1515-1519.

Delespaux, V. (2004). Improved diagnostic of trypanosome infection and drug resistant *Trypanosoma congolense* in livestock. PhD thesis, Université Libre de Bruxelles. p: 135.

Delespaux, V., Geysen, D., Van den Bossche, P., Geerts, S. (2008). Molecular tools for the rapid detection of drug resistance in animal trypanosomes. *Trends Parasitol.* **24**: 236–242.

Delespaux,V., Geysen, D., Van den Bossche, P., Geerts, S. (2009). Molecular tools for the rapid detection of drug resistance in animal trypanosomes. *Trends Parasitol.* **24** No.5.

Desquesnes, M., Dia, M.L. (2003). *Trypanosoma vivax* mechanical transmission in cattle by one of the most common African tabanids, *Atylotus agrestis*. *Exp. Parasitol.* **103** (1-2): 35-43.

Eisler, M.C., Arowolo, R.O., Gault, E.A., Moloo, S.K., Holmes, P.H., Peregrine, A.S. (1994). Isometamidium concentrations in the sera of Boran cattle: correlation with prophylaxis against tsetse-transmitted *Trypanosoma congolense*. *Acta Trop.* **56**:39-50.

Eisler, M.C., Brandt, J., Bauer, B., Clausen, P.H., Delespaux, V., Holmes, P.H., Ilemobade,A., Machila, N., Mbwambo, H., McDermott, J., Mehlitz, D., Murilla, G., Ndung´u, J.M.,Peregrine, A.S., Sidibe, I., Sinyangwe, L., and Geerts, S. (2001). Standardized tests in mice and cattle for the detection of drug resistance in tsetse-transmitted trypanosomes of African domestic cattle. *Vet. Parasitol.* **97**:171-182.

Eisler, M.C., McDermott J., Mdachi, R., Brandt J., Murilla G.A, Sinyangwe L., Mubanga, J., Machila N., Mbody Weightambo, H., Coleman, P.G., Clausen P.-H., Bauer, B., Sidibe, I.., Geerts, S., Peregrine, A.S. (2000). Rapid method for the assessment of trypanocidal drug resistance in the field. In: The proceeding of the 9th Symposium of the International Society for Veterinary Epidemiology and Economics (ISVEE9). Breckenridge, Colorado, USA, 6-11.

FAO, (2002). Program Against African Trypanosomosis (PAAT)-Twenty second Regional Conference for Africa, Cairo, Egypt, 4–8 February.

Fevre, E.M., Coleman, P., Odiit, M., Magona, J.W., Welburn, S.C., Woolhouse, M.E.J. (2001). The origins of a new *Trypanosoma brucei rhodesiense* sleeping sickness outbreak in eastern Uganda. *Lancet.* **358**: 625–628.

Gardiner, P.R., Mahmoud, M.M. (1992). Salivarian trypanosomes causing disease in livestock outside sub-Saharan Africa. In: Kreier, J.P., Baker, J.R. (Eds.), *Parasitic Protozoa. Academic Press*, London, 277–313.

Geerts, *et al.* (2000). *In vivo* tests for the detection of resistance to trypanocidal drugs: tests in mice and in ruminants, *ICPTV Newsletter.***2**: 6–7.

Getachew, A. (2005). Trypanosomosis in Ethiopia. *Ethiop. J. Biol. Sci.* **4**:75-121.

Getachew, A. and Yilma, J. (1996). Trypanosomosis: A threat to cattle production in Ethiopia. *Revue Méd. vét.*, **147**: 897-902.

Gray, M.A. *et al.* (1993). Drug-sensitivity screening in-vitro of populations of *Trypanosoma congolense* originating from cattle and Tsetse-flies at Nguruman, *Kenya. Acta Trop.* **55**:1–9.

Hall, H.T.B. (1985). Diseases and Parasites of Livestock in the Tropics. 2^{edn}. Intermediate Tropical Agriculture Series, Longman. London and New York.

Herbert, W.J. and Lumsden, W.H.R. (1976). *Trypanosoma brucei:* A rapid "matching" method for estimating the host's parasitaemia. *Exp. Parasitol.* **40** (3): 427-431.

Hoare, C. (1972). The Salivarian. In: The trypanosomes of mammals. A zoological monograph. Blackwell Scientific Publications. Oxford and Edinburgh. pp. 401-609.

Holmes, P.H. and Jennings, F.W. (1976). Pathophysiology of parasitic infection (ed, E.J.L souls by); Academic Press, New York, 199-210.

Holmes, P.H., Eisler, M.C., Geerts, S. (2004). Current Chemotherapy of Animal Trypanosomiasis. In: Ian Maudlin, Peter H. Holmes and Michael A. Miles (eds.). The Trypanosomiasis. CABI International. Wallingford, UK. pp. 431-444.

Ilemobade, A.A. (1979). Drug sensitivity of mouse infective *T. vivax* isolates in cattle and sheep. In: Proceedings of the 16th meeting of International Scientific Research Council for Trypanosomiasis Research and Control ISCTRC. Yaounde, Cameroun. No.111. pp. 251-253.

ILRAD, (1987). Report: improved trypanosomosis survey of Ethiopia Ministry of Oversea Development, Britain

Jamal, S., Sigauque, I., Macuamule, C., Neves, L., Penzhorn, B.L., Marcotty,T. And Van den bossche, P. (2005).The susceptibility of *T.congolense* isolated in Zambézia Province, Mozambique, to isometamidium chloride, diminazene aceturate and homidium chloride. *Journal of Veterinary Research.***72**:333-338.

Jones, T.W., Davila, A.M.R. (2001). *Trypanosoma vivax*-Out of Africa. *Trends Parasitol.* **17** (2):101.

oshua, R.A., Obwolo, M.J., Bwangamoi, O., Mandebvu, E. (1995). Resistance to diminazene aceturate by *T. congolense* from cattle in the Zambezi Valley of Zimbabwe. *Vet. Parasitol.***60**: 1-6.

Kalu, A.U. (1995). Sensitivity of animal-derived Trypanozoon stocks from sleeping sickness endemic foci of Nigeria to trypanocides and human plasma. *Rev. Élev. Méd. Vét. Pays Trop.* **48**:139-144.

Knoppe, T.N. *et al.,* (2006). Isometamidium sensitivity of *Trypanosoma congolense* stocks from cattle in West Africa tested in mice and the drug incubation infectivity test, *Acta Trop.* **97**:108–116.

Kristjanson, P.M., Swallow, B.M., Rowlands, G.J., Kruska, R.L., De Leeuw, P.N. (1999). Measuring the costs of African animal trypanosomosis, the potential benefits of control and returns to research. Agric. Systems, **59**:79-98.

Langridge, P.W. (1976). A Tsetse and Trypanosomosis Survey of Ethiopia. Ministry of Overseas Development (UK) and Ministry of Agriculture of the Ethiopian Government.

Levine, N.D., Corliss, J.O., Cox, F.E.G., Deroux, G., Grain, J., Honigberg, B.M., Leedale, G.F., Loeblich, A.R., Lom, J., Lynn, D., Merinfeld, E.G., Page, F.C., Poljansky, G., Sprague, V., Vavra J., Wallace, F.G. (1980). A newly revised classification of the protozoa. *J. Protozool.* **27**: 37-58.

Liao, D., and Shen, J. (2010). Studies of quinapyramine-resistance of Trypanosoma brucei evansi in China. *Acta Tropica.* **116**:173–177.

Losos, G. J. (1986). Infectious Tropical Diseases of Domestic Animals. International Development Research Centre, New York.

Magona, J.W., Walubengo, J., Odimin, J.T. (2008). Acute hemorrhagic syndrome of bovine trypanosomosis in Uganda. *Acta Trop.* **107**: 186-191.

Majiwa, P.A.O., Maina, M., Waitumbi, J.N., Mihok, S., Zweygarth, E. (1993). *Trypanosoma* (Nannomonas) *congolense*: molecular characterization of a new genotype from Tsavo, Kenya. *Vet. Parasitol.* **106**: 151-162.

Jamoudou, A., Delespaux, V., Chepnda, V., Hachimou, Z., Andrikaye, J.P., Zoli, A., Geerts, S. (2008). Assessment of the occurrence of trypanocidal drug resistance in trypanosomes of naturally infected cattle in the Adamaoua region of Cameroon using the standard mouse test and molecular tools. *Acta Trop.* 106:115-118.

Jatovu, E., Iten, M., Enyaru, J.C.K., Schmid, C., Lubega, G.W., Brun, R. and Kaminsky, R. (1997). Susceptibility of *Trypanosoma brucei rhodesiense* isolated from man and animal reservoirs to diminazene, isometamidium and melarsoprol. *Tropical Medicine and International Health.* **2**:13-18.

Jatovu, E., Stewart, M. L., Geiser, F., Brun, R., Mäser, P., Wallace, L. J. M., Burchmore, R. J., Enyaru, J. C. K., Barrett, M. P., Kaminsky, R., Seebeck, T., de Koning, H. P. (2003). Mechanisms of arsenical and Diamidine uptake and resistance in *Trypanosoma brucei. Eukaryot. Cell.***2**:1003-1008.

Jattioli, R.C., Feldman, U., Hendrickx, G., Wint, W., Jannin, J., Slingenbergh, J. (2004). Tsetse and Trypanosomosis intervention policies supporting sustainable animal agricultural development. *Food. Agr. Env.* **2**:310-314.

Jaudlin, I., Holmes, P.H., Miles, M.A. (2004). Trypanosomes. In: The Trypanosomiasis. CABI International Wallingford, UK. pp 1-25.

Jbaya, A.W., Ibrahim, U.I. and Apagu, S.T. (2010). Trypanosomosis of the dromedary camel (*Camelus dromedarius)* and its vectors in the tsetse-free arid zone of northeastern, Nigeria, *Nigerian Veterinary Journal.* **31**(3): 195-200.

JcDermott, J. *et al.*, (2003). Field studies of drug-resistant cattle trypanosomes in Kenedougou Province, Burkina Faso, *Acta. Trop.* **86**:93–103.

Jira, S.F. and Ralph, R. (1989. Manual of Tropical Veterinary Parasitology. 1st ed. England C.A.B, pp: 181-260.

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

ulugeta, W., Wilkes, J.M., Mulatu, W., Majiwa, P.A.O., Masake, R., Peregrine, A.S. (1997). Long-term occurrence of *T. congolense* resistant to diminazene, isometamidium and homidium in cattle at Ghibe, Ethiopia. *Acta. Trop.* **64**:205–217.

ungube, *et al.,* (2012). Detection of multiple drug-resistant Trypanosoma congolense populations in village cattle of south-east Mali. *Parasites & Vectors*.**5**:155.

urray, M., Murray, P. K., Mcintyre, W.I.M. (1977). An improved parasitological technique for the diagnosis of African trypanosomosis. *Trans. R. Soc. trop. Med. Hyg.,* **71**: 325-326.

urray, M.; Truil, J.C.M; Turner, D.A. and Wissocg, (1983). Animal Health Livestock Productivity and Trypanotolerance Network Training Manual. 4-16 ILCA – ILRI.

a'isa, B.K. (1967). Follow-up of a survey on the prevalence of homidium resistant strains of trypanosomes in cattle in Northern Nigeria and drug cross-resistance tests on the strains with Samorin and Berenil. *Bull. Epizoot. Dis. Afr.* **15**: 231–241.

ubayo, R. O. and Mugera G.M. (1985). Pathogenesis of Hemorrhages in *T. vivax* Infection in Cattle. Disseminated Intravascular Coagulation. *Bull. Anim. Hlth. Prod. Afri.* **33**:211-217.

sman,N.M., Fadl,M. andA/Rahman,A.H. (2012). Hematological profile and parasitological diagnosis of Trypanosoma vivax infection in Sudanese Nubian goats. *U of K. J. Vet. Med. & Anim. Prod.* **3**(1):28-45.

ris, J., Murray, M. and Mc odimba, E.A (1982). A comparative evaluation of the parasitological techniques currently available for the diagnosis of African trypanosomosis in cattle. *Acta Trop.* **39**:307-316.

regrine, A.S., Gray, M.A., Moloo, S.K. (1997). Cross-resistance associated with development of resistance to isometamidium in a clone of *Trypanosoma congolense*. *Antimicrob. Agents Chemother.* **41**: 1604-1606.

nder, M., and Authié E. (1984). The appearance of isometamidium resistant *Trypanosoma congolense* in West Africa. *Acta. Trop.* **41**:247-252.

viere, J.E. and Popich M.G.(2009). Veterinary Pharmacology and therapeutics. 9th ed. Blackwell. pp: 1174-1175.

ss, C.A., Barns A.M. (1996). Alteration to one of three adenosine transporters is associated with resistance to Cymelarsan in *Trypanosoma evansi. Parasitol. Res.* **82**: 183-188.

wlands, G.J. *et al.,* (1993). Epidemiology of bovine trypanosomiasis in the Ghibe Valley, Southwest Ethiopia: factors associated with variations in trypanosome prevalence, incidence of new infections and prevalence of recurrent infections, *Acta. Trop.***53**:135–150.

ıad, G.J., Allanson, A., Mackay, S.P., Cannavan, A., Tettey, J.N.A. (2008). Development and validation of an improved HPLC method for the control of potentially counter feit isometamidium products. *J. Pharm. Biomed. Anal.* **46**:45-51.

ıönefeld, A., Röttcher, D., Moloo, S.K. (1987). The sensitivity to trypanocidal drugs of *Trypanosoma vivax* isolated in Kenya and Somalia. *Trop.Med.Parasitol.* **38**:177-180.

ımelis, D. and Getachew, A. (2007). Current status of tsetse transmitted trypanosomosis in new settlement areas of Amhara region northwest Ethiopia. In: Proceedings of the 29th meeting of the International Scientific Council for Trypanosomiasis Research and Control (ISCTRC), held in Futungu Conference Centre, Luanda, Angola, October 1-5.

ımelis, D., Arun, K.S. and Getachew, A. (2008). Assessment of Trypanocidal Drug Resistance in Cattle of the Abay (Blue Nile) Basin Areas of Northwest Ethiopia. *Ethiop.Vet.J.* **12**(1): 45-59.

ımelis, D., Arun, K.S., and Getachew, A. (2005). Epidemiology of Bovine Trypanosomosis in the Abay (Blue Nile) Basin Areas of Northwest Ethiopia. *Rev. Élev. Méd. Vét. Pays Trop.* **58**:151-157.

ımelis, D. (2004). Epidemiology of bovine trypanosomosis in the abay basin areas of northwestern Ethiopia.MSc thesis, AAU, Debre Zeit, Ethiopia

ıshaw, A., Abebe, G., Desquesnes, M., Yon, W. (2006). Biting flies and *Trypanosoma vivax* infection in three highland districts bordering Lake Tana, Ethiopia. *Vet. Parasitol.* **142**:35–46.

ıes, K.R., Holmes, P.H., Urquhart, G.M. (1989). Interference between drug-resistant and drug-sensitive stocks of Trypanosoma congolense in goats. *Res. Vet. Sci.* **47**:75-77.

ıes, K.R., Njogu, A.R., Holmes, P.H. (1988). Assessment of sensitivity of Trypanosoma congolense to isometamidium chloride: a comparison of tests using cattle and mice. *Acta Trop.* **45**:153-164.

w, A., Sidibé, I., Bengaly, Z., Marcotty, T., Séré,M. et al.,(2012). Field detection of resistance to isometamidium chloride and diminazene aceturate in *Trypanosoma vivax* from the region of the Boucle du Mouhoun in Burkina Faso. *Vet. Parasitol.* **187**: 105-111.

therland, I.A., Holmes, P.H. (1993). Alterations in drug transport in resistant *T. congolense*. *Acta Trop.* **54**: 271-278.

therland, I.A., Peregrine, A.S., Lonsdale-Eccles, J.D., Holmes, P.H. (1991). Reduced accumulation of isometamidium by drug-resistant *T. congolense*. *Parasitol.* **103**:245-251.

ylor, M.A., Coop, R.L. and Wall, R.L. (2007).Veterinary Parasitology. 3rd ed. UK: Blackwell publishing, pp: 787-788.

rfa, W.(2008).Studies on bovine trypanosomosis and therapeutic efficacy of selected trypanocidal drugs in Birbir Valley of Gawo-Dalle district, West Oromia, MSc thesis, FVM, AAU, Debre zeit ,Ethiopia.

ttey, J.N.A., Astriku, C., Chizyuka, G., Slingenbergh, J. (2002). Nonconformance of diminazene preparations to manufacturers' label claims:an extra factor in the development of chemoresistance? Newsletter Integrated Control Pathogenic Trypanosomes Vectors (ICPTV), 24–26.

welde, N., Abebe, G., Eisler, M.C., McDermott, J.J., Greiner, M., Afework, Y., Kyule, M., Munstermann, S., Zessin, K.H., Clausen, P.H. (2004). Application of field methods to assess isometamidium resistance of trypanosomes in cattle in western Ethiopia. *Acta. Trop.* **90**:163-170.

lenberg, G. (1998). A Field Guide for the Diagnosis, Treatment and Prevention of African Animal Trypanosomosis. FAO, Rome, Italy.

den Bossche, P., Delespaux, V. (2011). Options for the control of tsetse-transmitted livestock trypanosomosis. An epidemiological perspective. *Vet. Parasitol.* **181**:37–42.

den Bossche, P., Doran, M., Connor, R.J. (2000). An analysis of trypanocidal drug use in the Eastern Province of Zambia. *Acta Trop.* **75**:247-258.

kerman, K., Tetley, L., Hendry, K. A.K. and Turner, C.M.R. (1988). Biology of African trypanosomes in the tsetse fly. *Biology of the Cell.* **64**:109-119.

ouley, H.S., Mungube, E.O., Allegye-Cudjoe, E., Diall, O., Bocoum, Z., et al., (2011). Improved PCR-RFLP for the Detection of Diminazene Resistance in Trypanosoma congolense under Field Conditions Using Filter Papers for Sample Storage. *PLoS Negl Trop Dis.* **5**(7).

yohannes, D., Etsay, K. and Getachew, A. (2010). Study on the assessment of drug resistance on *Trypanosoma vivax* in Tselemti woreda , Tigray, Ethiopia. *Ethiop. Vet. J.***14** (1): 15-30.

iss, B.L., Wang, J., Maltz, M.A., Wu, Y., Aksoy, S. (2013). Trypanosome Infection Establishment in the Tsetse Fly Gut Is Influenced by Microbiome-Regulated Host Immune Barriers. *PLoS Pathog* **9**(4).

iteside, E.F. (1960). A strain of T. congolense directly resistant to berenil. *J. Comp.Pathol.* **73**: 167-175.

iteside, E.F. (1962). Interactions between drugs, trypanosomes and cattle in the field. In Goodwin &Nimmo-Smith, eds. Drugs, parasites and Hosts, pp: 116-141

lkes, J.M., Mulugeta, W., Wells, C., Peregrine, A.S. (1997). Modulation of mitochondrial electrical potential: a candidate mechanism for drug resistance in African trypanosomes. *Biochem. J.* **326**: 755-761.

tola, W.H, Inoue, N., Ohashi, K. and Onuma, M. (2004).RNA-interference silencing of the adenosine transporter-1 gene in Trypanosoma evansi confers resistance to diminazene aceturate. *Exp Parasitol,* **107**: 47-57.

IST OF ANNEXES

ıex.1 Questionnaire format developed for the study

santAssociation.....

age.....Date.....Code.....

Livestock management

Which livestock species are kept?

tle Sheep and goat Equines Others

What is the grazing management of your animals?

mmunal and free grazing; live at the outside of the farmers' house in beret system

vate and free grazing; live at the outside of farmers' house

ıer

l feed

f management is based on free grazing system, are they in herd or in small groups?

.....

.....

Where do animals graze?

.....

.....

Have you ever purchased cattle from other areas and introduced into your herd? ___ If , from where? _____

Have you ever sold your cattle to customers outside your Woreda? _____If yes, to ere_____

Major diseases of livestock

What are the most common diseases affecting your livestock?

.....

.....

Does trypanosomosis occur in this area? (Yes, no, other)?

, what is the rank of trypanosomosis with regard to animal losses compared to other ses?

.....

nich livestock does trypanosomosis most affect?

e (yes, no, other) Sheep (yes, no, other)

(yes, no, other) Equine (yes, no, other) Others(specify) _____

What signs do you commonly observe when your animals get sick with nosomosis?

.....

Does the disease problem A) kill the animal B) cause production loss C) cause loss of k efficiency of oxen?

n which season/month do livestock most often get the disease (trypanosomosis)?

.....

How is the disease transmitted?

By Flies B) by Ticks C) by contact D) I don't

w E) Others specify_____

Management of trypanosomosis

Is there any trypanosomosis and fly control operation in this area? Yes----, No-----

If no, do you want a control program to be established in your area?----------, you willing to support the program if established----------

If yes to Q14, what method (s) is employed? A) Treatment of affected animals Fly control C) resting animals from work D) Feeding well of affected animals E. ers specify----------

If treatments are employed, where are the common drug sources? A) Veterinary ics B) Drug stores C) Open markets and informal sources D) others--------

Who are giving the treatments? A) Myself/family member B) Animal health sonnel C) Other people specify_____

Which drugs are most commonly used in the area (Name, type, color etc)?

Modern, specify----------,

onal, specify----------

Are the treatments effective or not effective? ----------

If yes, how do you describe the effectiveness? A) Reduction in clinical signs B) ng efficiency C) Milk production D) Body condition E) reduced mortality F) pecify-----

What is the cost of treatment/animal/year in the area?----------and for the nimals/house hold----------

Since when have you been using each of the drugs you oned?_____

When was your animals lastly treated against trypanosomiasis? _____Did e any improvement? .....

Which drug do you think is most effective to treat your animals against nosomosis? _____

If treatment is not effective, what do you think is the reason? A) misdiagnosis B) ctive drug C) other specify_____

If you think treatment failure is due to ineffective drug, what solution do you st? A) new drug B) previously effective drugs (mention_____) C) other fy_____

If you treat the animal by yourself or by a family member, how much do you give n animal? _____

If you treat the animal by yourself or by a family member, what are your criteria termine the dose? A) based on price affordability B) reading the dosage prescribed ive for all animals the same dose D) as advised by a professional E) arbitrary _____

What is the frequency of treatment per animal in a year? _____

If fly control operations are going on in your area, did you see any change since operation started?----------, If yes describe the changes in terms of the: No. of nals you have------, condition of your animals-------, money invested on treatment-----

rking efficiency of your animals--------, productivity of your animals-------, selling of your animals--------, mortality----------

What is the situation of trypanosomosis from your experience? A) improving B) nge C) increasing D) do not know

Are there traditional method of treatment and management practices for lling and prevention of trypanosomosis? ----------

k You!

of interviewer .....

..... Signature.....

x.2. Principles of the Rapid matching method

hing chart and tables: The chart and tables required for rapid matching methods nown below.

nisms: *Trypanosoma vivax* isolated of tsetse infested and tsetse free areas of west Ethiopia will be used for calibration.

d film: A wet film of the blood of the infected animals will be made under a 7 x 22-cover glass. The quantity of blood should be just insufficient to fill the whole space r the cover glass when this is pressed down gently. The film is examined under x400 nification, and a field is chosen in which the cells are evenly distributed. Rouleaux be present, but otherwise the cells should not form more than one layer.

hods

e than one organism per microscope field: The best match should be chosen quickly out attempting to count the organisms, most attention being given to their spacing. en large numbers are present it is best to compare a section of the field, say a quarter nent, with a similar portion of the chart. It has been noticed that with a parasitaemia ntilog 8.7 organisms/ml the trypanosomes often form a reticulated pattern with nps of red cells between them; at antilog 9.0 organisms/ml the distribution is more

he trypanosomes swarming round and over every erythrocyte. parasitemias
ng that in the circle marked 9.0 are recorded as >9.0.

rganism per field or fewer: If, when the selected field is examined, no
somes, or only a single one, are seen, a count is made in 5, 10, or 20 fields. The
s first made of 5 fields. If 2 or more organisms are seen, then equivalences are read
n the "5 field" section of Figure. If fewer, recourse is had to counting 10 or 20
referring in each case to the appropriate section of Figure. When no organisms are
20 fields, parasitemia is recorded as <antilog 5.4 organisms/ml.

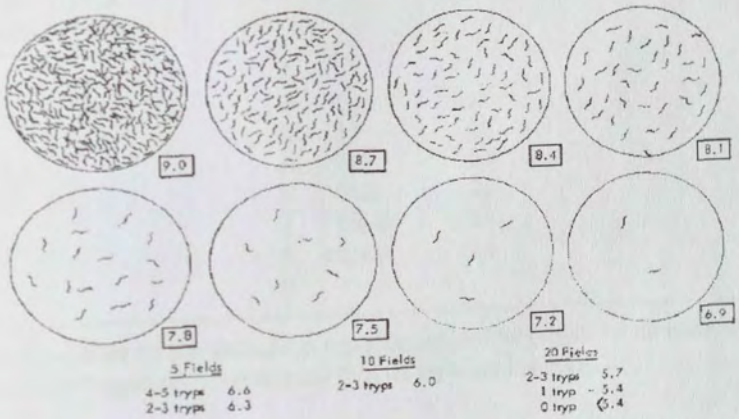


FIG. 1. Chart and table for estimating trypanosome parasitemias. The circles are used for
itching when more than one organism per microscope field is present, the tables for lower
ncentrations. The values in the boxes in the charts and in the tables indicate the logarithm
the number of trypanosomes per milliliter as computed for *Trypanosoma brucei* infections
mouse blood inspected under ×400 magnification. For viewing at 25 cm, the circles are
awn with a diameter of 6.5 cm. They contain representations of trypanosomes (6 mm) that
crease in number by twofold steps.

howing Rationale of Computation of Values for the Circles and Tables in Fig. 1 for Wet Films of Blood from Mice Infected with *Trypanosoma brucei* Viewed under ×400 Magnification[a]

Organisms in 20 fields	Organisms in 10 fields	Organisms in 5 fields		Organisms per field	Equivalent log number of organisms per milliliter of blood
				>256	>9.0
			M	256	9.0
			A	128	8.7
			T	64	8.4
			C	32	8.1 Reference point
			H	16	7.8
			I	8	7.5
			N	4	7.2
			G	2	6.9
			C		
		4–5	O	1	6.6
		2–3	U	0.5	6.3
	2–3		N	0.25	6.0
			T	0.125	5.7
2–3			I	0.0625	5.4
1			N	<0.0625	<5.4
0			G		

he base 10 logarithm value for the concentration of organisms per milliliter of blood for the reference (the circle containing 32 "organisms") was obtained from the regression line in Fig. 2.

x. 3. Details on the experimental test protocol:

out 1 month prior to experimental work, the animals will be moved to a fly-proof purchased from trypanosome free areas and will be tested for the trypanosome tions.

order to remove existing parasite burdens, all animals will be treated with nectin. Before inoculation of the trypanosomes, animals will be examined for two s for absence of parasite infections using PCV and clinical parameters as indicators. fter confirming the viability and estimated infective doses of the trypanosomes oscopically, inoculation of fresh infected blood into a jugular vein of experimental als will be done with continuation of the clinical and parasitological monitoring times per week. During a significant deterioration in clinical condition observed, and parasitaemia status will be monitored the same day.

ıe first peak of parasitaemia, the animal will be weighed and treatment will be ıtramuscularly on the same day, with one of the following: a 2% w/v solution of ımidium chloride at a dose of 0.5 mg/kg BW and a 7% w/v solution of zene aceturate at 3.5 mg/kg BW irrespective of the group.

ıpsed animals will be re-treated when the PCV falls by one-fifth of the value ed at the time relapse will be first detected, or if the PCV falls below 15%, or if ered necessary on the basis of clinical examination (for the welfare of the animal). ne between treatment and relapse will be recorded. Re-treatment will be done ıscularly on the same day, with the second most commonly used drug, using the regimens given above. The experiment will be terminated when no relapse will be d following administration of the first or second trypanocidal drug.

.4 Details on Procedures used for hematological examinations

cedures on hemoglobin determination using acid hematin method

rements: Sahlis instrument, blood sample

dure

0.1N HCl (1%) into central graduated tube up to mark 2.

the blood exactly up to mark 20 (20 µl) with the help of sahlis pipette.

sfer the blood from pipette to central graduated tube of the hemometer.

it well with the help of stirrer or rod and allow it to react for two minute.

e up with distilled water by adding drop by drop until the color matches with the ard comparator tube and mix well.

en the color matches take out and record the values

al value: Bovine 8 - 15gm/dl

ocedures on Total RBC Count using Hemocytometer method

irements: Haemocytometer, cover slip, microscope, RBC diluting fluid, Haeyem's ion or Physiological saline 0.85% Nacl.

ure

he blood in to RBC pipette up to 0.5 marks

diately draw the RBC diluting fluid up to mark 101.

e the pipette between thumb and other fingers with finger eight (8) movements.

ves a dilution of 1:200.

the counting chamber of haemocytometer and cover slip

the cover slip in position over the counting chamber by gentle pressure

a drop of blood on to the counting chamber by holding the pipette at an angle of

v the hemocytometer for 2-3 min to settle down the RBC in counting chamber

ation

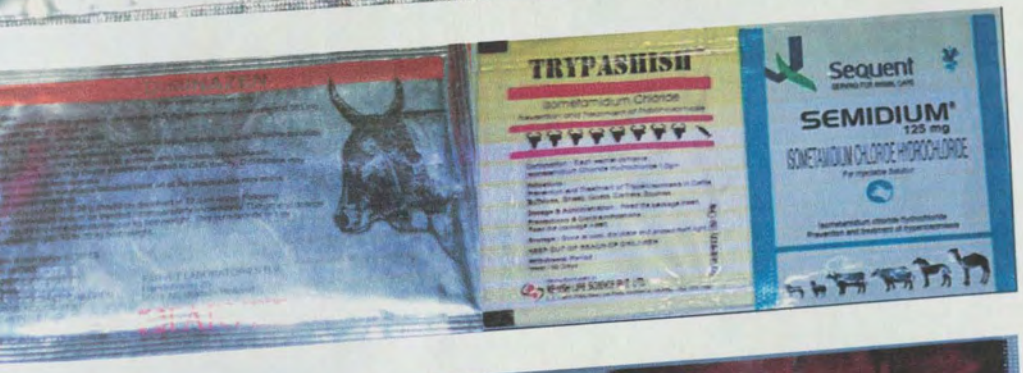
e of one small square = 1/20mm × 1/20mm × 1/10mm = 1/4000mm3

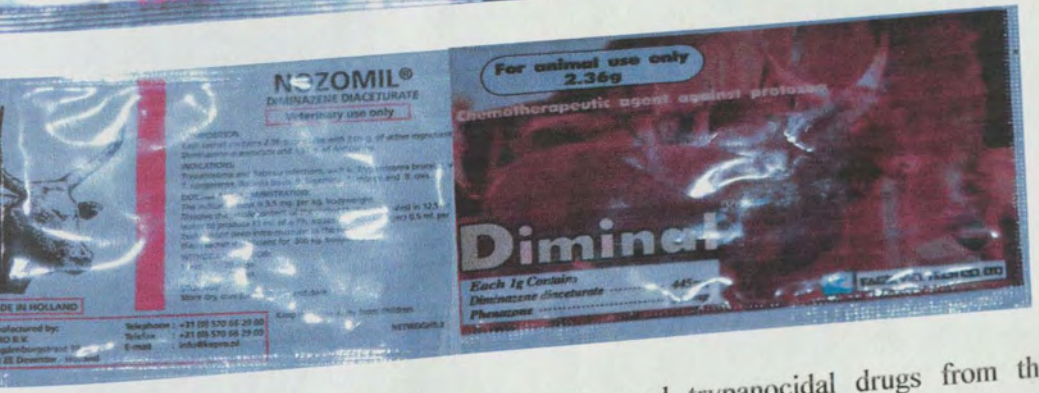
e of 80 small square = 80 × 1/4000mm3 = 1/50mm3

umber of RBC = Cells counted (N)/Volume of all squares × dilution factor

RBC = N (cell counted)/1/50mm3 × 1/200 = N × 10,000

tos taken during A. Questionnaire survey, B. Trypanocidal drug treatment, C. erimental animals in the fly proof house and D. Animals with relapses.





notos on the trade names of the commonly used trypanocidal drugs from the uestionnaire survey sites.