ASSESSMENT OF TRYPANOCIDAL DRUG RESISTANT *TRYPANOSOMA CONGOLENSE* ISOLATES AND SURVEY ON TRYPANOCIDAL DRUG MANAGEMENT PRACTICES IN SELECTED DISTRICTS OF WESTERN AMHARA REGIONAL STATE, ETHIOPIA

BY

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A THESIS SUBMITTED TO THE SCHOOL OF GRADUATE STUDIES, ADDIS ABABA UNIVERSITY IN PARTIAL FULFILLMENT OF THE MASTER OF SCIENCE DEGREE IN EXPERIMENTAL PHARMACOLOGY

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Assessment of trypanocidal Drug Resistant *Trypanosoma congoense* Isolates and Survey on Trypanocidal Drug Management Practices in Western Amhara Regional State, Ethiopia

By

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A Thesis Submitted to the School of Graduate Studies, Addis Ababa University in Partial Fulfillment of the Master of Science Degree in Experimental Pharmacology

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My special thank is also for the Drug Administration and Control Authority of Ethiopia for their cooperation in analysis of drug samples used for experimental studies.
### LIST OF ABBREVIATIONS

<table>
<thead>
<tr>
<th>Acronym</th>
<th>Description</th>
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<tbody>
<tr>
<td>AFLP</td>
<td>Amplified Fragment Length Polymorphism</td>
</tr>
<tr>
<td>ANRS</td>
<td>Amhara National Regional State</td>
</tr>
<tr>
<td>D</td>
<td>Diminazene</td>
</tr>
<tr>
<td>DACA</td>
<td>Drug Administration and Control Authority</td>
</tr>
<tr>
<td>DA</td>
<td>Diminazene aceturate</td>
</tr>
<tr>
<td>DEAE</td>
<td>Diethyl amino-ethyl</td>
</tr>
<tr>
<td>H</td>
<td>Homidium bromide (ethidium)</td>
</tr>
<tr>
<td>I</td>
<td>Isometamidium</td>
</tr>
<tr>
<td>IFAT</td>
<td>Indirect Flourescent Antibody Test</td>
</tr>
<tr>
<td>ILRI</td>
<td>International Livestock Research Institute</td>
</tr>
<tr>
<td>ISMM</td>
<td>Isometamidium chloride</td>
</tr>
<tr>
<td>MEP</td>
<td>Mitochondrial electrical potential</td>
</tr>
<tr>
<td>MoARD</td>
<td>Ministry of Agriculture and Rural Development</td>
</tr>
<tr>
<td>Q</td>
<td>Quinapyramine</td>
</tr>
<tr>
<td>RFLP</td>
<td>Restriction Fragment Length Polymorphism</td>
</tr>
<tr>
<td>PA</td>
<td>Peasant Association</td>
</tr>
<tr>
<td>PCV</td>
<td>Packed Cell Volume</td>
</tr>
<tr>
<td>SHAM</td>
<td>Salicylhydroxamic acid</td>
</tr>
<tr>
<td>SRD</td>
<td>Slow Release Device</td>
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</tbody>
</table>
# TABLE OF CONTENTS

ACKNOWLEDGEMENTS ........................................................................................................... I

LIST OF ABBREVIATIONS ...................................................................................................... II

LIST OF TABLES ..................................................................................................................... V

LIST OF FIGURES .................................................................................................................. VI

LIST OF ANNEXES ................................................................................................................ VII

ABSTRACT ............................................................................................................................... VIII

1. INTRODUCTION ................................................................................................................... 1

2. LITERATURE REVIEW ......................................................................................................... 3
   2.1. Epidemiology of African Bovine Trypanosomosis ......................................................... 3
   2.2. Diagnosis of Cattle Trypanosomosis ............................................................................. 3
       2.2.1. Clinical Diagnosis .................................................................................................. 3
       2.2.2. Identification of the agent .................................................................................... 4
   2.3. Treatment and Control of African Bovine Trypanosomosis ......................................... 6
       2.3.1. Chemotherapy and chemoprophylaxis ................................................................. 7
       2.3.2. Vector Control ..................................................................................................... 8
       2.3.3. Trypanotolerant Cattle Breeding ......................................................................... 9
   2.4. Chemoresistance: control and prevention ................................................................. 10
       2.4.1. Mechanisms and genetics of resistance to trypanocides ..................................... 10
       2.4.2. Detection of Drug Resistance ............................................................................. 13
       2.4.3. Control and prevention of chemoresistance ......................................................... 15
   2.5. Drug Resistant Parasites and Impact on Productivity ................................................. 16

3. OBJECTIVES .......................................................................................................................... 17
   3.1. General Objectives ....................................................................................................... 17
   3.2. Specific Objectives ...................................................................................................... 17

4. MATERIALS AND METHODS ............................................................................................ 18
   4.1. Study Area .................................................................................................................... 18
   4.2. Materials ...................................................................................................................... 20
       4.2.1. Drugs .................................................................................................................. 20
       4.2.2. Experimental animals ....................................................................................... 20
   4.3. Sampling ...................................................................................................................... 20
LIST OF TABLES

Table 1: Published survey results for drug resistance in African Bovine trypanosomosis………10
Table 2: Presampling considerations before trypanosomal isolation in study sites……………….22
Table 3 : Random allocation of mice for the experimental study…………………………………….25
Table 4: Respondents on the trypanocidal drug use and type of drugs used in the study sites….28
Table 5: Average PCV of parasitologically positive and negative animals in the study sites…..32
Table 6: Results of parasitological trypanosomosis survey conducted in the study areas………32
Table 7: Treatment of the single pool derived from Debreelias, Dembecha and Jabitahnan…..34
Table 8: Treatment of three pools separately taken from Debreelias, Dembecha and
Jabitahnan……………………………………………………………………………………………….36
Table 9: Physicochemical analysis of trypanocidal drugs according to DACA…………………..37
LIST OF FIGURES

Figure 1: Map of Ethiopia indicating different Administrative Regions…………………………19
Figure 2: Map of Amhara Region indicating study sites…………………………………………19
Figure 3: Locally prepared metal piece used for measuring isometamidium chloride…………..27
Figure 4: Underdosing practices of diminazene aceturate, homidium bromide and isometamidium
   chloride indicated by respondents in questionnaire survey in three PA’s of study areas..29
Figure 5: Diminazene aceturate (A), Homidium bromide (B) and Isometamidium chloride (C)
   dosage practices by peasants in Jabigenet, G/kendamu and Woinma peasant associations
   as indicated by respondents during questionnaire survey (N=91)……………………………30
Figure 6: Different sources of trypanocidal drugs in Jabigenet, G/kendamu and Woinma
   (N=127)…………………………………………………………………………………………………31
Figure 7: Parasitaemia exhibited in experimental mice with three trypanocidals and sanative
   combinations s1 and s2…………………………………………………………………………………35
LIST OF ANNEXES

Annex 1: Wet film parasitaemia estimation.................................................................62
Annex 2: Dosage Preparation.................................................................63
Annex 3: Questionnaire survey format.................................................................64
A study was conducted from December 2006 to July 2007 in trypanosomosis endemic areas of western Amhara with the objective of assessing trypanocidal drug resistant *Trypanosoma congolense* isolates and current status of trypanocidal drug management practices. Experimental trypanocidal drug sensitivity test was performed in experimentally infected mice using combined and separately pooled trypanosomal isolates obtained from different districts. This was complimented by a survey that targeted farmers and health professionals involved in veterinary service aiming at current status of trypanocidal drugs. Questionnaire survey on farmers revealed that 71.7% of the respondents had the experience of using trypanocidal drugs by themselves and at least 54% of them practiced underdosing with any of the three trypanocidal drugs (diminazene aceturate, isometamidium chloride and homidium bromide). Furthermore, from questionnaire survey on professionals, 55.1% of the respondents in veterinary service (MoARD) indicated the use of increased dosages as a measure to avert treatment failures. Point prevalence of trypanosomosis obtained from cattle screened for isolation was found to be 12.4% in Debreelias, 20% in Dembecha and 27.2% for Jabitahnan. *T. congolense* accounted for 72.9% the overall trypanosomosis cases. The drugs tested failed to permanently clear the parasite, being relapsing at 5.8±0.96 days with 70 mg/kg diminazene aceturate; 14.2±1.99 days with 10 mg/kg isometamidium chloride and 4.6±3.75 days with 10 mg/kg homidium bromide. The difference in the relapse duration was found to be statistically significant among drugs, being longer with isometamidium (p<0.001) than others. The drugs were also unable to permanently clear trypanosomes from the combined pool when given in combination at intervals, as there was a 40% relapse with 70 mg/kg diminazene aceturate initial treatment followed by isometamidium 5 mg/kg at 17±3.92 days. However sanative pairs appeared to be relatively effective than single drug alone, as relapse duration was significantly longer (P<0.001) in the former than the latter. Separate pools from each district was also used to compare the relative contribution of drug resistance phenotype in combined pool and similar results were observed (relapses at 7.8±1.57 days; 7.0±1.24 days and 5.8±0.96 days with 70 mg/kg diminazene aceturate; 17.50±1.88 days; 18.20±2.66 days and 11.00±2.15 days with 10 mg/kg isometamidium chloride in pools from Debreelias, Dembecha and Jabitahnan, respectively). The present study revealed the presence of multidrug resistance that could most probably be attributed to the drug-use practices exercis
the livestock production system. Wise use of currently available trypanocidals in conjunction with other alternative control strategies should be adopted as timely solution for the current problem of trypanosomosis in the region.

*Keywords:* Bovine/Relapse Duration /Trypanosoma congolense / Trypanocidal Drugs/Western Amhara
1. INTRODUCTION

Livestock are of enormous importance in Africa, economically for nutritional and agricultural purposes as well as socially. The problem of African animal trypanosomosis, also called “Nagana”, was recognized by African stockmen long before the cause of the disease was known. African bovine trypanosomosis is a collective term for a group of diseases brought about by one or more of the pathogenic trypanosome species namely: *T. vivax*, *T. congolense* and *T. brucei*. It is a wasting disease in which there is a slow progressive loss of condition accompanied by increasing anemia and weakness to the point of extreme emaciation, collapse and death (Uilenburg, 1998).

Treatment and prevention of African animal trypanosomosis nowadays relies essentially on three drugs namely: Homidium chloride/homidium bromide diminazene aceturate and isometamidium chloride. However, resistance to one or more of the three-trypanocidal drugs used in cattle has been reported in many sub-Saharan Africa countries (FAO, 1998). Out of these countries, Burkina Faso, Ethiopia, Kenya, Tanzania, Somalia, Uganda, and Zimbabwe are included (Peregrine, 1994). Published experimental studies have reported the occurrence of resistance in population of trypanosomes to diminazene and/or isometamidium as reviewed in Table 1 below. However, trypanosomes are usually not resistant to both diminazene and isometamidium at the same time. Thus, these compounds have been termed as a sanative pair for the control of bovine trypanosomosis (Whiteside, 1960). However, reports are still documented for the expression of multiple drug resistance phenotype at the same time in Burkina Faso (Sones *et al.*, 1988; Clausen *et al.*, 1992), suggesting that the concept of sanative pairs might no longer be relied.

At least 6 million of the 45 million heads of cattle that are raised under trypanosomosis risk in Africa are now found in the West and Southwest Ethiopia. The northwest region of Ethiopia is also affected by tsetse and non-tsetse transmitted trypanosomosis (Abebe and Jobre, 1996; Afewerk *et al.*, 2000; Sinishaw *et al.*, 2005). In Ethiopia, the occurrence of drug resistant population of trypanosomes has also been confirmed by earlier studies reported in different regions of the country such as Diddessa and Angar valleys (Scott and Pegram, 1974), Benishangul Gumuz (Afewerk *et al.*, 2000), North Omo (Ademe and Abebe, 2000) and Ghibe valley, Ethiopia (Codjia *et al.*, 1993; Leak *et al.*, 1993; Peregrine *et al.*, 1994). Multi drug resistance in population of trypanosomes particularly *T. congolense* has also been reported in...
Ghibe valley and Benishangul Gumuz region of Ethiopia (Codja et al., 1993; Mulugeta et al., 1997; Afewerk et al., 2000).

In Western Amhara, the presence of animal trypanosomosis caused by *T. congolense*, *T. vivax*, and *T. brucei* in North Gondar, West Gojam and East Gojam Zones has been demonstrated (BRLR, 2006; Shimelis, 2003; Cherenet et al., 2006). The regional MoARD disclosed that in districts like Metema, Quara, Tach Armachiho, Jawi and Jabitahnan where the region has been launching settlement programmes; *T. congolense* and *T. vivax* have been serious impediments inflicting the process of providing ploughing oxen to the settlers (Personal communication). *T. vivax* was indicated as having wide area coverage unlike its lesser pathogenicity and complaint of chemotherapeutic failures compared to *T. congolense*. Despite informal complaints by professionals in the region about chemotherapeutic failures, the regional BoARD has been insisted largely on chemotherapeutics (diminazene aceturate and isometamidium chloride) as a means to treat and control cattle trypanosomosis (personal communication). Despite the enormous threat looming in the region no well defined research has been undertaken on the current status of trypanocidal drugs and their management practices for designing strategies to be followed by the regional veterinary service in control of trypanosomosis, forming the rationale for initiating the present study.
2. LITERATURE REVIEW

2.1. Epidemiology of African Bovine Trypanosomosis

The impact of the tsetse-associated disease extends in sub-Saharan Africa over some 10 million km² (a third of the continent). Direct losses from mortality and morbidity of trypanosomosis and the costs of programmes in attempt to control it are estimated to amount between US$600 million and $1.2 billion each year (FAO, 1994). The total expenditure on trypanocides in sub-Saharan Africa has been estimated with the total value of trypanocidal drug sales at US$ 20 million, or approximately 50 million doses/year (Sones, 1999). Trypanosoma congolense is attributed as a major cause of disease in African cattle, as a result, is of major economic importance (Kristjanson et al., 1999).

Trypanosoma congolense has been classified into three different types; savannah, forest and Kilifi (Young and Godfrey, 1983; Knowles et al., 1988). The pathogenicity appears to vary depending on which type or strain of Trypanosoma congolense is involved (Bengaly et al., 2002). Strains within the savannah type are regarded as the most pathogenic to mice and cattle (Gow et al., 2007). However, certain breeds of African cattle have been shown to exhibit a level of tolerance to trypanosome infection (Naessens, 2006), which will be discussed later in control section. T. congolense is not only cyclically transmitted by tsetse flies (members of the genus Glossina spp.) but also mechanical transmission with needles and other species of insect have been indicated under experimental and field conditions (Sumba et al., 1998; Desquesnes and Dia, 2003).

2.2. Diagnosis of Cattle Trypanosomosis

2.2.1. Clinical Diagnosis

The disease is characterized by intermittent fever, anemia, lymphadenopathy, splenomegaly and cachexia often followed by death in untreated cases (Mulligan, 1970). Loss of condition will soon become obvious as first the fat beneath the skin and then the muscles themselves are greatly reduced and the underlying bones become apparent. The skin often loses its suppleness (“turgor”) because of dehydration, the eyes are sunken and at this stage the classical signs of anemia are obvious, the visible mucous membranes are pale and the blood is watery in appearance. The
emaciation is associated with weakness and in the final stages results in inability to stand, and in pressure sores and ulceration of the skin over the bony prominences. There is very often an increased secretion of tears (lachrymation) (Uilenberg, 1998).

2.2.2. Identification of the agent

Trypanosomes are unicellular protozoal parasites thoroughly adapted to living and moving in the blood plasma or tissue fluid of the host. They are elongated, streamlined and tapered at both ends. Size varies from 8 to over 50 μm. The pellicle, the outer layer of the cytoplasm, is flexible enough to permit a degree of body movement, while retaining a definite shape. A flagellum arises near to the posterior end from a parabasal body, and runs the length of the trypanosome. Along the length of the body, the pellicle and cytoplasm are pinched up into a thin sheet of tissue called the undulating membrane, through the outer margin of which runs the flagellum. Among other basic morphological features, a distinct well-defined body, the kinetoplast, is seen near to the posterior end of the trypanosome and differs in size and position according to the species. The extent of the undulating membrane and the absence or presence of the free flagellum serves as tools in specific identification of each trypanosomes species (Uilenburg, 1998).

Parasite detection techniques are highly specific, but their sensitivity is relatively low. Due to this low sensitivity, the apparent parasitological prevalence of trypanosomosis is generally lower than the true parasitological prevalence. Moreover, in areas where trypanocidal drugs are used extensively, parasites may not be detected (Paris et al., 1982).

I. Direct examination techniques

Wet films of fresh blood, usually obtained from the ear vein, jugular vein or the tail constitute the simple, inexpensive and rapid method. Trypanosomes can be recognised by their movement among the red blood cells. Depending on the size and movement of the trypanosome a presumptive diagnosis can be made of the trypanosome species. The diagnostic sensitivity of the method is generally low but depends on the examiner's experience and the level of parasitaemia. Sensitivity can be improved significantly by lysing the RBCs before examination using a haemolytic agent such as sodium dodecyl sulfate (SDS) (OIE, 2004).
Thin and thick blood smear films are also simple and relatively inexpensive methods like wet blood films, but results are delayed because of the staining process. Trypanosomes are easily recognized by their general morphology, but may be damaged during the staining process. This may make it difficult to identify the species. Usually, both thin and thick smear is made from the same sample. Thick smears contain more blood than thin smears and, hence, have a higher diagnostic sensitivity. Thin smears on the other hand allow trypanosome species identification (OIE, 2004).

**II. Parasite concentration techniques**

Microhaematocrit centrifugation technique/Woo method (Woo, 1970) and Buffy coat technique/Murray method (Murray et al., 1977) are commonly used concentration techniques. Identification of trypanosome species is difficult as the specific gravity of *T. congolense* is similar to that of RBCs, parasites are often found below the Buffy coat in the RBC layer. To improve the separation of RBCs and parasites, and increase the sensitivity for *T. congolense*, the specific gravity of RBCs can be increased by the addition of glycerol (Murray et al., 1977). The plasma/white blood cell interface (Buffy coat) is examined by slowly rotating the tube. Trypanosome movement can first be detected at lower objective then increased (Desquesnes and Tresse, 1996).

The buffy coat technique represents another improved and widely used technique for the detection of trypanosomes where the Buffy coat and the uppermost layer of RBCs taken and extruded on to a clean microscope slide and examined for the presence of motile trypanosomes. The sensitivity of the buffy coat method can be improved by using the buffy coat double-centrifugation technique. Unlike microhaematocrit centrifugation technique, the buffy coat technique has the added advantage that preparations can be fixed and stained for more accurate identification of species and for retention as a permanent record (Kratzer and Ondiek, 1989).

**III. Animal inoculation**

The sub inoculation of blood into rodents, usually mice or rats, is particularly useful in revealing subpatent infections. The laboratory animals are injected intraperitoneally with 0.2-5 ml (depending on the size) of freshly collected blood. They are bled three times a week for at least 2
months. Collected blood is examined using the wet film method. Nevertheless, the method is not practical; it is expensive and diagnosis is not immediate. The method is highly sensitive in detecting *T. brucei* infections. However, some *T. congolense* strains are not easily transmitted and *T. vivax* rarely infects laboratory rodents. Also animal inoculation should be avoided as it raises serious animal welfare concerns (Schlater and Bossche, 2004).

**IV. Tests to detect trypanosomal antigen**

A PCR method has been developed as a tool for the diagnosis of infections with African animal trypanosomes. Specific repetitive nuclear DNA sequences can be amplified for the three types of *T. congolense* (Masiga et al., 1992; Desquesnes, 1997; Desquesnes and Davila, 2002). Unlike their prohibitive cost for routine use, PCR restriction fragment length polymorphism (RFLP) assays have been recently developed that allow the identification of all *Trypanosoma* species as single or mixed infections using one single test (Delespaux et al., 2003; Desquesnes et al., 2001; Geysen et al., 2003).

Several antibody detection techniques have also been developed to detect trypanosomal antibodies for the diagnosis of animal trypanosomosis. The methods of choice are the indirect fluorescent antibody test (IFAT) (Katende et al., 1987; Greiner et al., 1997) and the trypanosomal antibody-detection ELISA (Luckins, 1977; Hopkins et al., 1998). ELISAs using *T. congolense* precoated microtitre plates have been developed (Rebeski et al., 2000). They detect immune responses to current and past infections and can, therefore, only provide a presumptive diagnosis of active infection. Sample collection and storage is made easy through the use of filter papers. All of these factors make the antibody ELISA a very useful test for large-scale surveys to determine the distribution of tsetse-transmitted trypanosomosis (Schlater and Bossche, 2004).

**2.3. Treatment and Control of African Bovine Trypanosomosis**

Historically sodium antimony tartrate has been the only relatively successful remedy for African cattle trypanosomosis between the two world wars. It was, however, difficult to use, the tissue irritation attendant upon its injection required that it be administered intravenously and a complete cure could, at best, be assured only by repeated treatments. Control became widespread with the arrival of synthetic insecticides in the 1940s. Selective spraying of the vegetation
support of the flies and later the application of insecticides by aircraft opened the way for large-scale tsetse eradication (Uilenberg, 1998). Currently, there are three principal control strategies for tsetse-transmitted trypanosomosis in Africa: selection and breeding of trypanotolerant cattle; chemotherapy and chemoprophylaxis using trypanocidal drugs and vector (mainly of tsetse fly) control/eradication (insecticidal spraying, insecticidal targets, traps, and the sterile insect technique) (McDermott and Coleman, 2001).

2.3.1. Chemotherapy and chemoprophylaxis

Certain compounds have specific effects on some enzyme system or block essential metabolic pathways, but the exact way in which they work is often not known or only incompletely understood which is true of most of the trypanocides. Curative drugs are mainly used where disease incidence is low and only a limited number of animals in a herd contract the disease during the course of a year. All curative drugs also possess some residual effect, but in the case of diminazene aceturate and melcy this is practically negligible being rapidly excreted. Thus, its preventive effect is short (Uilenberg, 1998).

Chemoprophylaxis imply a residual effect, as prevention depends on the persistence of the drug in the system of the animal. Often, a depot of the drug is formed at the site of the injection where it is retained and slowly released into the circulation to maintain a concentration in the blood at a level at which no trypanosomes can exist. Other drugs are instead found loosely attached to blood proteins and become slowly available to act on the parasite. The other way to create a drug depot is by incorporating it into a suitable support so that after subcutaneous implantation the drug will be gradually released so that an adequate prophylactic (and curative) level is maintained in the blood and tissue fluid. The availability of biodegradable polymers provides a good opportunity to develop a slow release system by means of an implantable device consisting of a drug dispersed in a polymer matrix allowing a slow release of the drug and a prolongation of the prophylactic period. Recently, promising results were obtained using subcutaneously implanted SRD of biodegradable polyesters/trypanocidal drugs. A significant extension of the prophylactic period was obtained using SRD in comparison with the intramuscularly injected drug (LemmouChi and SChaCht, 1997). Isometamidium chloride has been used in the field for several decades prophylactically or therapeutically for livestock suffering from trypanosomosis due to infection with T. congolense and others (Leach and Roberts, 1981).
Treatment and prevention of African animal trypanosomosis nowadays relies essentially on three drugs namely: homidium salts, diminazene aceturate and isometamidium chloride. Salts of these drugs have been in use for more than 45 years in Africa. However, almost all of these trypanocidals are gradually losing their efficacy due to drug resistance (Delespaux and Koning, 2007; Williamson, 1970).

### 2.3.2. Vector Control

Each of the available options for tsetse control or eradication has its own advantages and specific limitations. The strategies for using these options may vary considerably depending on the specific objective, technical and logistical feasibilities and cost requirements. However in most circumstances, viable agricultural systems can be established effectively only when several methods are combined (FAO, 2001).

#### I. Ecological control

The reproduction of tsetse flies is unique among insects and their peculiar life cycle has important implications for control or eradication efforts. Tsetse reproduce by adenotrophic viviparity i.e. the egg contains sufficient yolk to sustain the entire embryonic development and the larva is nourished in the female by special maternal organs (Vreysen, 2001). The absence of eggs and a free larval stage in nature and the fact that the pupal development occurs in the soil makes the adult fly the only phase easily accessible for control purposes. Early methods to control the tsetse fly such as the removal of vegetation or the destruction of game animals may have been very effective, but have become environmentally unacceptable (Dransfield et al, 1991).

#### II. Use of Insecticides

Tsetse flies are highly susceptible to the action of insecticides, and many different products, starting with DDT (previously banned but now re-emerging) and dieldrin up to the more recently introduced and less harmful pyrethroids, have been used over the past 50 years to control and eradicate tsetse (Uilenberg, 1998). The selective spraying of residual insecticides on resting sites of tsetse from the ground or with helicopters in the dry season was used to eliminate tsetse from 200,000 km² in Northern Nigeria (Vreysen, 2001).
III. Sterile male technique

The sterile insect technique (SIT) is one area-wide insect pest management method where the insect pest is controlled or eradicated by affecting its reproductive capacity. It relies on the production of sterile males (target insect) in mass-rearing facilities and release in sustained numbers in the natural habitat large enough to outnumber the wild pest population (Vreysen, 2001). Males are sterilized by radiation at the appropriate stage and then taken to the selected area and released. Eventually, so few fertile insects remain that fertile matings do not occur and the population is eliminated (Feldmann and Hendrichs, 2001).

IV. Use of traps or screens

The use of traps and targets has largely replaced the spraying of insecticides. The goal is to exert a modest daily mortality of 2-3 % on the female tsetse fly population by attracting them to a device and inducing a landing response. The flies are killed by the contact with a lethal dose of insecticides applied to the surface of the target or by heat or starvation after being guided to a non-return cage of a trap (Dransfield et al., 1990; Willemse, 1991). Because of the relatively low cost of the materials and the apparently unsophisticated technology, the use of traps and targets has been promoted as the most sustainable method of tsetse control, especially in the context of community participation (Vreysen, 2001).

2.3.3. Trypanotolerant Cattle Breeding

Certain local breeds have developed a tolerance to trypanosome infections during the centuries spent in areas strongly infested by glossines. This ability, named trypanotolerance, results from several biological mechanisms under multigenic control (Hanotte et al., 2003). Indeed, some breeds present the remarkable capacity to control their level of parasitemia, to resist the development of severe anemia during the infection, and to remain productive in a zone strongly infested by tsetse flies. These two characteristics (limited parasitemia and anemia) are known to be highly heritable and genetically linked to cattle productivity (Hill et al., 2005). More than one single gene, it seems probable that two pools of genes, are involved but all the techniques used up to now have failed to identify them (Berthier et al., 2006).
2.4. Chemoresistance: control and prevention

Drug resistance, also called drug fastness, may be defined as a loss of sensitivity by a strain of an organism to a compound to which it had previously been susceptible. It implies failure of treatment or prevention, and if no other active drugs are available the animal has to rely on its immune defences alone to combat the disease. Drug resistance is known in many pathogenic micro-organisms, as well as in larger parasites. The discovery of the first compound active against *T. brucei rhodesiense* was rapidly followed by reports of resistance to that drug. Sooner or later, newly developed trypanocidal drugs have failed to cure some cases of human or animal trypanosomosis after a period of use (Uilenberg, 1998). A drug resistance study done in different African countries is reviewed as follows in Table 1.

Table 1: Published survey results for drug resistance in African Bovine trypanosomosis.

<table>
<thead>
<tr>
<th>Country</th>
<th>Species</th>
<th>No. of isolates.</th>
<th>% of Resistant isolates</th>
<th>Resistant to</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Examined</td>
<td>Resistant</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Zambia</td>
<td>Tc</td>
<td>71</td>
<td>24</td>
<td>33.8</td>
<td>I</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>8</td>
<td>11.3</td>
<td>D</td>
</tr>
<tr>
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<td>I,D</td>
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<td>17</td>
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<tr>
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</tr>
<tr>
<td></td>
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<td>10</td>
<td>100</td>
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<td>I</td>
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<tr>
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<td>19</td>
<td>12</td>
<td>63</td>
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<tr>
<td></td>
<td>Tb</td>
<td>12</td>
<td>2</td>
<td>17</td>
<td>D, I</td>
</tr>
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<td></td>
<td>1</td>
<td>8</td>
<td>I</td>
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<tr>
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<td>Tc, Tv,</td>
<td>12</td>
<td>5</td>
<td>42</td>
<td>H</td>
</tr>
<tr>
<td></td>
<td>Tb</td>
<td></td>
<td></td>
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<td></td>
</tr>
<tr>
<td>Uganda</td>
<td>Tb</td>
<td>36</td>
<td>1</td>
<td>3</td>
<td>D, I</td>
</tr>
<tr>
<td>Zimbabwe</td>
<td>Tc</td>
<td>14</td>
<td>6</td>
<td>43</td>
<td>D</td>
</tr>
</tbody>
</table>

D = diminazene; H = homidium bromide (ethidium); I = isometamidium Q=Quinapyramine
2.4.1. Mechanisms and genetics of resistance to trypanocides

I. Isometamidium chloride and Homidium bromide/chloride

The first reports of isometamidium use date from 1963 and the first case of resistance to homidium and cross-resistance between homidium bromide/chloride and isometamidium chloride was reported in 1967 (Delespaux and Koning, 2007). The main mode of action of isometamidium chloride was suggested to be the cleavage of kDNA-topoisomerase complexes, causing the desegregation of the minicircle network within the kinetoplast (Shapiro and Englund, 1990), though findings later shown dyskinetoplastic trypanosomes observed at least as sensitive to isometamidium chloride as kinetoplastic lines (Kaminsky et al., 1997). This explanation was supported by observations from later studies who showed that the trypanosome kinetoplast as the primary site of isometamidium accumulation in T. congolense (Wilkes et al., 1995) which was later confirmed for the hemoflagellate Cryptobia salmositica (Kinetoplastida, bodonina) (Ardelli and Woo, 2001; Woo, 2003) and for T. brucei (Boibessot et al., 2002), using more sophisticated chromatographical and microscopical techniques.

The mechanism of resistance to isometamidium chloride (ISMM), however, is less clear despite, certain suggested mechanisms from experimental finding results. 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). Later on, it was shown that the maximal uptake rates (Vmax) of ISMM in resistant T. congolense were significantly lower than in sensitive populations (Mulugeta et al., 1997). It remains to be shown whether this is caused by a decreased number of protein transporters of ISMM in the plasma membrane and/or by changes in the balance between influx and efflux. The role of nucleoside transporters in resistance to ISMM by T. congolense remains to be examined, although changes in these transporters have been associated with resistance to arsenical drugs in T. brucei (Carter and Fairlamb, 1993; Carter et al., 1995; Ross and Barns, 1996). From further studies, changes in mitochondrial electrical potential have been demonstrated in ISMM resistant T. congolense (Wilkes et al. (1997).

Resistance to isometamidium is mostly associated with cross-resistance to homidium (Peregrine et al., 1997), and it could be speculated that these structurally related compounds might share the
same uptake mechanism albeit that their distributions within the trypanosome are slightly different. Isometamidium is mainly concentrated in the kinetoplast, whereas homidium spread much more diffuse throughout the trypanosome (Boibessot et al., 2002). The transport of isometamidium was known to be energy dependent, as it was reduced in the presence of metabolic inhibitors such as SHAM/glycerol (Sutherland et al., 1992; Sutherland and Holmes, 1993).

Observations by keeping in isometamidium free medium, no difference in isometamidium diffusion out of the cell was observed between sensitive and resistant strains, but a large proportion of the drug, sequestered within the mitochondrion of the sensitive strains, is retained (Wilkes et al., 1997). In T. b. brucei, the P2 adenosine transporter may be responsible for part of the isometamidium uptake as the drug inhibits P2-mediated adenosine uptake (De Koning, 2001) but the low level of cross resistance between diminazene and isometamidium suggests that this contribution is not an essential one. In T. evansi, RNAi of the TevAT1 gene conferred only low levels of isometamidium resistance (Witola et al., 2004) while loss of TbAT1/P2 leads to almost 20-fold resistance to DA (Matovu et al., 2003; De Koning et al., 2004). Recent Amplified Fragment Length Polymorphism (AFLP) studies on two isogenic clones of T. congolense, the parent clone being sensitive to isometamidium (CD50: 0.018 mg/kg in mice) and the derived one presenting a CD50 94-fold higher, showed at least 58 polymorphic fragments only present in the derived resistant clone (Delespaux et al., 2005) suggesting isometamidium resistance may well be the result of changes in several genes (Matovu et al., 2003).

Nevertheless, the AFLP study identified a putative protein that could be involved in the transport of isometamidium and interestingly, resistance appeared to be determined by a GAA codon insertion in this protein (Delespaux et al., 2005) most resistant strains were shown homozygous for the specified insertion. Although contradictory observations have been reported on the genetic stability of ISMM resistance, from field observations in Ethiopia, based on cloned populations, showed that the drug-resistant phenotype of T. congolense had not altered over a period of four years (Mulugeta et al., 1997).

Although their mutagenic activity has been known for a long time (MacGregor and Johnson, 1977), homidium chloride and especially homidium bromide or ethidium are still widely used as
trypanocidal drugs. The mechanism of their antitrypanosomal action is also not well understood. However, it has been shown that the drugs interfere with glycosomal functions, the function of an unusual adenosine monophosphate-(AMP) binding protein, trypanothione metabolism and the replication of kinetoplast minicircles (Wang, 1995). The mechanism of resistance by trypanosomes to these drugs is unknown. As mentioned previously there are indications, however, that it is similar to that described for isometamidium (Peregrine et al., 1997).

II. Diminazene aceturate

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 (Peregrine and Mamman, 1993). Similarly the molecular basis of resistance to diminazene in trypanosomes is not clear. It has been shown that the accumulation of diminazene was markedly reduced in arsenical-resistant *T. brucei* owing to alterations in the nucleoside transporter system (P2) (Carter and Fairlamb, 1993; Carter et al., 1995). However, there might be other resistance mechanisms which cannot be excluded (FAO, 1998). However, it was shown that the phenotype of multiple drug-resistant (including diminazene) *T. congolense* remained stable over a period of four years (Mulugeta et al., 1997). RNA-interference silencing of the adenosine transporter-1 gene in *Trypanosoma evansi* confers resistance to diminazene aceturate (Witola et al., 2004).

2.4.2. Detection of Drug Resistance

Several methods have been described to identify drug resistance in trypanosomes. At present, three types of technique are commonly used to identify drug resistance: tests in ruminants; tests in mice; and in vitro assays. None of these is, however, an ideal test and other tests are still in the phase of development or validation (Peregrine, 1994).

1. Tests in ruminants

Tests in ruminants provide direct information from studies in ruminants using recommended doses of trypanocide. It is done by infecting a group of cattle or small ruminants with the isolate under investigation and later, when the animals are parasitaemic, treating them with various levels of trypanocide. The animals are then regularly monitored over a prolonged period (up to 100 days) to determine the effective dose (ED) and curative dose (CD) (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 reinfection during the study (Sones et al., 1989).

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 (FAO, 1998).

II. Tests in mice

After expansion of an isolate in a donor mouse, groups of five or six mice are inoculated with trypanosomes. Twenty-four 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 advantage of the mouse assay is that it is cheaper than the test in cattle. There are several disadvantages, however: (i) most T. vivax isolates, and also some T. congolense isolates, do not grow in mice; (ii) although there is reasonable correlation between drug sensitivity data in mice and in cattle, higher doses of drug must be used in mice (normally ten times higher) in order to obtain comparable results to those obtained in cattle because of the vast difference in metabolic size; (iii) precise assessment of the degree of resistance needs a large number of mice per isolate, which makes it a labour-intensive test - identification of a discriminatory dose, above which an isolate should be considered as resistant, could drastically reduce the number of mice and the amount of work to be carried out; and iv) it takes as long as 60 days to evaluate the drug sensitivity of an isolate (FAO, 1998).

V. Trypanocidal drug ELISAs

As an alternative to the tests mentioned above, the use of trypanocidal drug enzyme-linked immunosorbent assays (ELISAs) in combination with parasite detection tests has given
promising results for the detection of resistant trypanosomes. A competitive ELISA which allowed the detection of small amounts of isometamidium in serum of cattle was first described by Whitelaw et al (1991). This technique was further improved and has been validated in cattle under experimental and field conditions (Eisler et al., 1997). The test is both sensitive, detecting subnanogram 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 (FAO, 1998). Observations showed that the presence of trypanosomes in animals with an Isometamidium chloride concentration of > 0.4 ng/ml suggests resistance; the higher the drug level detected the greater the degree of resistance that could be inferred (Eisler et al., 1993). Similar test for diminazene is in development (Murilla, 1996).

2.4.3. Control and prevention of chemoresistance

Although there is still a lack of knowledge about the exact mechanisms of resistance and the factors responsible for the development of drug resistance, urgent measures are needed to maintain the efficacy of the existing drugs. Based on experiences of the control of resistance to other drugs such as antimalarials, antibiotics and anthelmintics it is suggested that reliance on the “sanative pair” guideline might not be sufficient to control resistance to trypanocides. This guideline needs to be accompanied by the following additional measures:

- **Reduction in the number of treatments.** The most efficient way to delay the development of drug resistance is to reduce the selection pressure caused by these drugs. Reduction of the number of trypanocidal treatments by integrating drug treatment with other control measures may help alleviate the problem (Geerts and Holmes, 1998).

- **Avoidance of underdosing.** Underdosing commonly occurs in the field and is an important cause of resistance development. Measures should be adopted to minimize underdosing. Better formulations of the existing prophylactic drugs may help to avoid subtherapeutic concentrations, which exert a strong selection pressure for resistant clones.

- **Quinapyramine should no longer be used in cattle.** Cross-resistance with the other available trypanocides has now been clearly demonstrated at the level of individual trypanosomes. The use of this drug in cattle is therefore contraindicated (FAO, 1998).
2.5. Drug Resistant Parasites and Impact on Productivity

The fact that whether or not drug-resistant trypanosomes are less pathogenic than susceptible ones remains a controversial issue. However, several observations have claimed loss of virulence and/or a loss of fitness in drug-resistant trypanosomes (Silayo and Marandu, 1989; Berger et al., 1995; Mutugi et al., 1995). Transmission by tsetse flies, however, does not appear to affect the drug sensitivity of trypanosomes and drug-resistant strains remain resistant after passage through tsetse flies. This was verified by results of different studies in the past (Moloo and Kutuza, 1990; Peregrine et al., 1997). However, studies using four populations of *T. congolense*, ranging from extremely sensitive to strongly resistant to iometamidium chloride, found no differences in virulence between them. Only the most resistant one showed a reduced viability, *i.e.* it took longer to establish parasitaemia than the other three (ILRI, 1996). In studies undertaken to associate transmissibility of resistant strains and the infection rate in Glossina morsitans, morsitans differed significantly between clones and was significantly higher in tsetse flies infected with the *T. congolense* clone with the highest level of drug resistance (Van den Bossche et al., 2006).
3. OBJECTIVES

3.1. General Objectives

As part of a contribution to the regional agriculture and rural development through alleviation of existing livestock impediments, the present study was undertaken with the following objectives:

- Assessment of current status of trypanocidal drug management practices in trypanosomosis areas.
- Assessment of trypanocidal drug resistance in selected *T. congolense* endemic districts of the region.

3.2. Specific Objectives

The specific objectives of the present study were:

- Assessment of risk factors at handling, distribution and administration of trypanocidal drugs as well as disease management in related stakeholders responsible for promoting expression of drug resistance,
- Determine the point prevalence of cattle trypanosomosis in selected districts of western Amhara Region,
- Assessment of relapses in mice inoculated with *T. congolense* isolates after treatment with diminazine aceturate, isometamidium chloride and homidium bromide and comparison of drug resistance using relapse intervals,
- Assessment of sanative effects of routinely used trypanocidal drugs and thus presence of isolates not sensitive to trypanocidal drugs in combination in any of the study sites,
- Forward suggestions on possible options for the existing problems as well further studies to be conducted in the region.
4. MATERIALS AND METHODS

4.1. Study Area

The Amhara National Regional State (ANRS) covers an area of approximately 170,000 km² and is divided into 11 Administrative Zones, each consisting of several districts (shown in Figure1). It is characterized by plateau, mountains and broad valleys. The elevation varies from about 500 (around Metema) to 4600 metres above sea level around Semein mountains/Ras Dashen. The climate can be divided into a rainy season (between late February and early May), a short dry period in May and a longer rainy season (from June to October). In both areas of the region, about 85% of the population is dependent on mixed farming.

Trypanosomosis affected area of the region where the present study was undertaken is located in the densely populated Western Amhara National Regional State. It is demarcated at approximately about 9°.48′-14°02′ N and 35°13′-38°31′ E. It has a total gross land area of 5,558,600 hectares and it is an area where lowlands with great agricultural potential in the region are mainly located (BoFED, 2006; Encarta, 2006).
Figure 1: Map of Ethiopia indicating different administrative regions
(Source: UNDP, 2000)

Figure 2: Map of Amhara Region indicating study sites
(Map frame adopted from Amhara Region BoFED, 2006).
Cattle reared in trypanosomosis endemic areas of the region include Fogera, Arsi breed typically of the small east African zebu, Horo/wollega/ and Mahberesilassie composite breeds with relative importance of disease resistance.

4.2. Materials

4.2.1. Drugs

Diminazine aceturate (Verevin®), Batch No. ALS-510, Ashis Life Science, Mumbai, India), Isometamidium chloride (Veridium®), Batch No. 92A1, Laprovet 2, Lachemin De La Milletiero Tours Cedex, France) were officially requested and donated from the regional MoARD and homidium bromide (Ethidium®, Batch No. 35A1, Laprovet, Lachemin De La Milletiero Tours Cedex, France) was purchased from genuine veterinary drug supplier in the region.

4.2.2. Experimental animals

Swiss Albino mice 12-13 weeks old and weighing 31–34 gram were obtained from the breeding colony of the Ethiopian Health and Nutrition Research Institute. Mice were maintained under standard conditions with access to pelleted feed and water ad libitum. Caging was kept so as to prevent entrance of insects.

4.3. Sampling

4.3.1. Sampling for the survey

Households involved in primary trypanosomal isolation were used for individual questionnaire data collection. A total of 49 individuals engaged in veterinary service (employed by the regional MoARD) and 43 individuals involved in 43 drug retail outlets, which were accessible in the 23 districts were also included in the questionnaire and field observationl studies, respectively.

4.3.2. Sampling for primary T. congolense isolation

Three peasant associations (PA’s) found within the three districts (one from each) were considered for T. congolense isolation for experimental sensitivity study. Tsetse infested districts namely: Debreelias, Dembecha and Jabitahnan were purposivey selected based on high challenge of trypanosomosis and access to vehicle transport (BRLR, 2006). One PA from each selected
district namely; Jabigenet PA from Debreelias, Gedebkendamu PA from Dembecha and Woinma PA from Jabitahnan were again selected purposively based on accessibility for vehicle and level of *T. congolense* local prevalence (8% and above) based on the information obtained from veterinary service team from the respective districts and regional laboratory.

With the objective of targeting cattle harbouring trypanosomosis among the entire population within the selected PA sites, Thrusfield's derivation was used (Thrusfield, 2005).

\[
n = \left\{1 - (1 - P_1)^{1/d}\right\} \left\{N - d/2\right\} + 1
\]

Where \(N\) = total estimated population

\(n\) = required sample size

\(d\) = minimum number of expected animals diseased

\(P_1\) = Probability of finding at least 1 case in a sample (mostly 95%)

Different epidemiological study reports from regional laboratory and published studies on prevalence of trypanosomosis (*T. congolense*) have indicated prevalence of 8-24% in different tsetse infested areas (Shimelis, 2003; BRLR, 2006; Cherenet, 2006). To increase sample size for reliability of including diseased animals, 8% prevalence was taken. As shown in Table 2, sampling households rather than individual animals were considered as sampling units for simplicity. Furthermore on the assumption that at least one cattle is brought from each household, cattle belonging to 37 households were selected using systematic random sampling technique (in cases where more than one cattle are brought all animals were included). Using the minimum required number as basis, every \(N/n^{th}\) owner’s cattle coming for the appointed free service was included in primary trypanosome screening until the required positive cases were attained. From personal observation, 85% of cattle owners were expected to be involved in health service. Socioeconomic data was taken from development agents in respective PA’s.

The approach for selection of trypanosome isolates for mice drug-sensitivity studies involved a random selection of three from all the viable field isolates that were collected in each district. Nine isolates were included from three study sites (three from each) for combined pool (Eisler *et al.*, 2001). Two step combination was undertaken; one of the pools containing nine isolates taken from three study sites in single pool and the other three pools of *T. congolense* isolates being constituted from each district separately.
Table 2: Presampling considerations before trypanosomal isolation in study sites

<table>
<thead>
<tr>
<th>Peasant association</th>
<th>District</th>
<th>Household expected (N)</th>
<th>Cattle population (estimated)</th>
<th>Expected <em>T. congoense</em> harbouring cattle (d)</th>
<th>Minimum sample size required (n)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Jabigenet</td>
<td>Debreelias</td>
<td>1040</td>
<td>3750</td>
<td>300</td>
<td>37 household</td>
</tr>
<tr>
<td>G/kendamu</td>
<td>Dembecha</td>
<td>950</td>
<td>3290</td>
<td>263</td>
<td>37 households</td>
</tr>
<tr>
<td>Woinma</td>
<td>Jabitahnan</td>
<td>680</td>
<td>1950</td>
<td>156</td>
<td>37 households</td>
</tr>
</tbody>
</table>

4.4. Sample and Data Collection

4.4.1. Field survey data collection

i) Questionnaire

Field investigations were carried out with major objective of assessing type of drugs and drug management practices employed as a means to combat trypanosomosis common in the study areas. A total of 127 farmers (44 from Debreelias; 43 from Dembecha and 40 from Jabitahnan) were interviewed. Structured questionnaire (Annex-I) designed to know perception about the disease, history of disease occurrence, seasonality, measures accustomed to treat their animals and incidence of relapses experienced after treatment was used for interview of each individual. In addition, questionnaire survey was done for professionals working on animal health in trypanosomosis areas of the region (under MoARD) to obtain information on current status of trypanocidal drugs and their routine management practices. A total of 49 animal health workers comprising veterinarians, animal health assistants as well as animal health technicians were interviewed directly with aid of simplified questionnaire on general therapeutic procedures, frequencies of relapses/failures and local measures undertaken to avoid resistance.

ii) Focus group discussion

Groups comprising 8-10 individual cattle owners (Catley and Mariner, 2002) were also invited to attend a discussion so as to have a better idea about the general situation in the area and
triangulate information obtained from the individual interview. A total of nine groups, of which, three with in each study site were involved in focal group discussions.

**4.4.2. Blood sample collection**

Blood samples were taken from auricular vein of animals brought from each selected household using hamatocrit capillary tubes. Three fourth (3/4) filled hamatocrit capillary tubes were sealed by critoseal to avoid spilling out on centrifugation. Sealed tubes were then centrifuged in Microhematocrit centrifuge adjusted at 12, 000 rpm for 5 minutes. Tubes taken out and kept in labelled track to maintain identity of each sample. The contents of the capillary tube (including about 1 mm above and below the buffy coat) was expelled by cutting with diamond tipped pen, mixed and spread on a clean glass slide and covered with coverslip. The preparation was then examined under 40 x magnifications (OIE, 2004). The blood filled capillary tubes reserved from primary screening procedures was used for further species identification. Each positive blood sample was traced back to be rescreened by tail vein wet film before fresh blood sample was collected from jugular vein. Nearly 5 ml of blood sample was collected from jugular vein using heparinized vacutainer tubes from both positive cases.

**4.5. Experimental Sensitivity Trial**

**4.5.1. Maintenance and amplification of field isolates**

Blood samples containing three isolates selected from of known *T. congolense* positive animals were mixed using equal 2 ml volumes. Out of the 6 ml mixed blood, 0.2 ml was then inoculated intra peritoneally to replicates of mice. Male goat was also used for maintenance of isolates to avoid loss due to death of mice. Nearly 3ml of mixed blood was infused intravenously for maintenance goat from both districts. Similarly the same procedure was repeated for the other two study sites. Once mice used for maintenance of the field isolates become uniformly parasitaemic, blood was taken from retro-orbital venous sinus (IACUC, 2003) and equal (0.3 ml) volume of blood was mixed together in heparinized vacutainer tube. The mixed inoculum was inoculated to group of replicates of mice to amplify and expand for actual experimental trials. This was done to assess existence of isolates refractory to avialable trypanocidal drugs. Mice infected with isolates obtained from each district were maintained for separate studies to look for the contribution of each district for the drug resistance phenotype observed in the single pool.
derived from three districts. The whole procedure was the same except the source and number of isolate included.

4.5.2. Infection of experimental mice

After mice inoculated for amplification became optimally parasitaemic (estimated concentration of $10^6$/ml), blood from retro-orbital venous sinus was taken. It was expanded by diluting at 1 to 4 ratios with 0.85% physiological saline to attain the recommended parasite concentration ($10^5$ in the inoculum) (Eisler et al., 2001). It was then inoculated to experimental group of mice intended for the sensitivity study.

4.5.3. Dosing and monitoring of experimental mice

The drugs were dissolved in an appropriate quantity of sterile distilled water such that the required dose to be tested was contained in 0.2 ml (see Annex II) (OIE, 2004). Mice were randomly divided into experimental groups after experimental infection and were labeled as indicated in Table 3. All experimental groups were then treated after parasitaemia with ascending doses of three trypanocidal drugs starting from lower ones (0.5 mg/kg for isometamidium chloride and homidium bromide, and 3.5 mg/kg for diminazene aceturate only used for comparison) and continued to comparative recommended dosages (5 mg/kg and 10 mg/kg for isometamidium chloride and homidium bromide; 35 mg/kg and 70 mg/kg for diminazene aceturate) (FAO, 1998).

A separate study aimed at tracing the relative contribution of isolates in each study site was undertaken using pool from each district alone. In this trial, only comparative recommended dosages in mice were used with only two of the trypanocidal drugs (isometamidium and diminazene aceturate). Homidium bromide was omitted due to failure to clear parasitaemia and existence of signs of toxicity in comparative dosages used in the single pool derived from the three districts (Results of combined pool trial). After treatment, mice were monitored every other day for relapses. Trypanosomes were considered sensitive for specific treatment if 80% of mice in a group show no apparent parasitemia during the two month observation period (FAO, 1998). In relapsing cases specifically in combined pool experimental trial, drugs with sanative effects were tested. Level of parasitaemia was estimated using number of trypanosomes per microscopic field (Murray et al., 1977).
Table 3: Random allocation of mice for the experimental study.

<table>
<thead>
<tr>
<th>Group</th>
<th>Number of mice</th>
<th>Isometamidium chloride</th>
<th>Diminazene aceturate</th>
<th>Homidium bromide</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td>5</td>
<td>0.5 mg/kg Bw</td>
<td>-</td>
<td>0.5 mg/kg Bw</td>
</tr>
<tr>
<td>II</td>
<td>5</td>
<td>1 mg/kg Bw</td>
<td>3.5 mg/kg Bw</td>
<td>1 mg/kg Bw</td>
</tr>
<tr>
<td>III</td>
<td>5</td>
<td>5 mg/kg Bw</td>
<td>35 mg/kg Bw</td>
<td>5 mg/kg Bw</td>
</tr>
<tr>
<td>IV</td>
<td>5</td>
<td>10 mg/kg Bw</td>
<td>70 mg/kg Bw</td>
<td>10 mg/kg Bw</td>
</tr>
<tr>
<td>V</td>
<td>5</td>
<td>Control</td>
<td>Control</td>
<td>Control</td>
</tr>
</tbody>
</table>

4.5.4. Drug quality assurance

Representative samples of drugs were submitted to Drug Administration and Control Authority of Ethiopia for assessment of certain critical analytical values like identity of drug material and content assay which was assumed to affect the overall outcome of the current experimental trial. The details of analytic methods followed could not be included due to confidentiality of the procedure.

4.6. Statistical Analyses

All information collected from field and laboratory observations was entered and stored in Microsoft office excel data sheet. Data collected from field observation and questionnaire was analysed using SPSS 11.5 and was presented using percentages, mean ± standard deviation, charts graphs and frequency tables. Statistical significance of differences between levels and drug combinations in relapse durations for experimental studies was analysed using analysis of variance (one way ANOVA). Variation in relapse duration was compared between different groups and variation among each separate group was compared using multiple comparisons on Tukey HSD post hoc tests. P-values less than 0.05 were considered as a statistically significant difference between considered variables. Winepiscope version 2.0 for windows was used for comparing significance of difference between proportion of respondents in tsetse infested and nontsetse district professionals.
5. RESULTS

5.1. Field Surveys

5.1.1. Disease perception

Among livestock diseases affecting livestock production in the area, trypanosomosis was indicated as the most important health problem of cattle in the group discussion. In group discussion, the farmers claimed trypanosomosis to cause reduced appetite, lacrimation, rough hair coat, diarrhoea, emaciation, weakness and as a result reduction in the working power of their oxen for tillage. All individuals in group discussion also agreed with the fact that the recurrence of the disease is pronounced in rainy season due to fly population. However, severity of the disease is more pronounced during dry season due to poor feed availability. All groups in Woinma PA of Jabitahnan agreed that the disease is getting worse in the area due to failure of drugs to treat trypanosomosis. However, groups in Jabigenet of Debreelias agreed reduction in disease threat due to increased access to trypanocidal drugs. Only one of the groups in Gedebkendamu PA of Dembecha agreed the decreased pattern due to a similar reason to that of Debreelias. The remaining two groups in Gedebkendamu PA of Dembecha indicated that they had observed reduction only during trap implementation trial undertaken about two years ago. All groups in Woinma PA of Jabitahnan claimed the disease as increased threat to their livestock production and tend to abandon the existing fertile land desperately because of increased challenge to their animals. Group discussion in Woinma PA of Jabitahnan indicated high mortality of the disease when animals are imported from highlands.

The significance of the disease in the area was underscored by the individual interview as well. In individual interview, 96.80 % of farmers were well aware of the common clinical signs and 69.3% knew the mechanism of transmission of the disease. Similarly, 48 % of the respondents affirmed the presence of the disease for over 20 years.
5.1.2. Drug handling and therapeutic practices

i) Farmers

It was noted in the group discussion that farmers in the Woinma and Gedebkendamu PAs are well acquainted with the locally prepared metallic material with its importance in trypanocidal drug use (Figure 3). The farmers indicated the use of two units (using local measuring metallic piece) of Isometamidium chloride for one adult cattle of normal tropical live weight of 250 kg. All nine groups from both study sites indicated previous use of a fraction of single Diminazene aceturate sachet for more than one adult cattle in fear of toxicity. However, currently due to observation of treatment failures, farmers started giving more than one sachet (recommended for 300 kg live weight). Although group discussion in Dembecha and Debreelias indicated that Diminazene aceturate relieve their cattle from trypanosomosis for less than a week, this notion was not shared by farmers in Wonima, where Diminazene aceturate is said to be ineffective or produce relief only for about two days. It was also stated that Isometamidium chloride is used there and produce relief for two to three weeks and indeed administration of Isometamidium chloride at the end of each month is a common practice in Woinma PA.

Figure 3: Locally prepared metal piece used for measuring Isometamidium chloride. (Metallic piece seen in figure 2 is also used in many rural areas of Amhara region for removing excess wax from the ear “kuke mawcha”).

The responses obtained from individual interviews were mostly direct extensions of general ideas raised in the group discussions. Out of the total respondents, 29.9 % were familiar in the use of
locally adopted material for measuring Isometamidium chloride and this accounted for 84.4% of Isometamidium chloride users indicated in Table 9. A higher proportion of respondents (77.5%) which use isometamidium chloride were found in Woinma kebele of Jabithahn, which appeared to be due to higher trypanosomosis challenge. On the other hand, 71.7 % of the respondents indicated the experience of using trypanocidal drugs to treat their cattle when trypanosomosis is suspected and all of them were found to be familiar with use of diminazene aceturate (indicated in Table 4). The interview also revealed that 15.7% of respondents had the experience of using traditional medicament and indigenous knowledge when initial signs like inappetence exist in their cattle. These cattle owners use egg, mustard, pepper, salt and oil mixture. About 1.65% of the respondents indicated the experience of mixing antibiotics like penicillin when constituting injectable solution thinking better response from treatment.

Table 4: Respondents on the trypanocidal drug use and type of drugs used in the study sites.

<table>
<thead>
<tr>
<th>Issue</th>
<th>Jabigenet</th>
<th>Gedebkendamu</th>
<th>Woinma</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>Trypanocidal drug users</td>
<td>26 (59.1%)</td>
<td>29 (67.5%)</td>
<td>36 (90%)</td>
<td>91 (71.7%)</td>
</tr>
<tr>
<td>Diminazene only</td>
<td>17 (38.6%)</td>
<td>24 (55.8%)</td>
<td>5 (12.5%)</td>
<td>46 (36.2%)</td>
</tr>
<tr>
<td>Diminazene and isometamidium</td>
<td>7 (15.9%)</td>
<td>4 (9.3%)</td>
<td>17 (42.5%)</td>
<td>28 (22.1%)</td>
</tr>
<tr>
<td>Dimin.,isometam.and ethidium</td>
<td>2 (4.6%)</td>
<td>1 (2.4%)</td>
<td>14 (35%)</td>
<td>17 (13.4%)</td>
</tr>
<tr>
<td>Total respondents #</td>
<td>44</td>
<td>43</td>
<td>40</td>
<td>127 (100%)</td>
</tr>
</tbody>
</table>

# Non-drug users are not displayed.

Among the overall trypanocidal drug users, 54 % practice underdosing of drugs that are commonly used in their locality. The Relative significance of responses for underdosing practices in both the study areas was pronounced for Diminazene aceturate (51.9 %) followed by isometamidium chloride (44.5 %) and homidium bromide 3.6 % (indicated in Figure 4). Out of the total responses for underdosing practices in both study sites, 40.96 % was in G/kendamu, followed by Woinma (33.7 %) then Jabigenet 25.3 %. Dosing practice by study area was depicted in Figure 5.
Out of the total respondents that use trypanocidal drugs, 12% indicated the administration of trypanocidal injectable solutions via uncommon routes like intraperitoneally at the paralumbar fossa and intrathoracic cavity between thoracic vertebrae. However, the remaining 88% of respondents using trypanocidals accustomed to use intramuscular route through neck and gluteal muscles.
Figure 5: Diminazene aceturate (A), Homidium bromide (B) and Isometamidium chloride (C) dosage practices by peasants in Jabigenet, G/kendamu and Woinma peasant associations as indicated by respondents during questionnaire survey (N=91).

Although there appeared to be a widespread use of trypanocidals by farmers, only <11% of the respondents had the experience of purchasing drugs from the open market/illegal shops as indicated in Figure 6.
**ii) Individuals in veterinary service**

All 100% of the interviewed professionals practicing in tsetse infested areas and 46.4% of those in nontsetse infested areas indicated treatment failure, which might be associated to drug resistance to either of the trypanocidals provided through MoARD. Significant difference was observed in proportion of respondents indicating treatment failure (P<0.01) between tsetse infested and tsetse free areas. Moreover, 79.6% of the (MoARD worker) respondents relied on general clinical signs and history from the owner due to lack of diagnostic facilities. Only 20.4% of them use laboratory confirmation as routine procedure before treating suspected trypanosomosis cases. Interestingly, 55.1% of the respondents stated the use of increased dosages than recommended as a measure to avert chemotherapeutic failures.
5.2. Cross-sectional Surveys

5.2.1. Hematological findings

The mean PCV of infected cattle (21.2±5.12) was significantly lower (P < 0.001) than the mean PCV of non-infected cattle (26.34±5.71%) of both sites indicating anaemic state as shown in Table 5.

Table 5: Average PCV of parasitologically positive and negative animals in the study sites.

<table>
<thead>
<tr>
<th>District</th>
<th>Location</th>
<th>Tryps. Positive</th>
<th>Tryps. Negative</th>
</tr>
</thead>
<tbody>
<tr>
<td>Debreelias</td>
<td>Jabigenet</td>
<td>25.5±7.00</td>
<td>29.11±5.58</td>
</tr>
<tr>
<td>Dembecha</td>
<td>G/Kendamu</td>
<td>19.28±3.47*1</td>
<td>23.82±4.33</td>
</tr>
<tr>
<td>Jabitahnan</td>
<td>Woinma</td>
<td>19.73±3.66*2</td>
<td>25.16±5.74</td>
</tr>
</tbody>
</table>

*1 minimum and maximum pcv 11, 26 *2 minimum and maximum pcv 14, 26

5.2.2. Parasitological Findings

During the primary parasitological screening, three species of trypanosomes namely: *T. congolense*, *T. vivax* and *T. brucei* were detected. Point prevalence of trypanosomosis obtained from cattle screened for isolation was found to be 12.4% in Debreelias, 20% in Dembecha and 27.2% for Jabitahnan. Proportional wise 72.9% accounted for *T. congolense*, 15.6% for *T. vivax*, 4.2% for *T. brucei* and remaining 8.3% for mixed infections (Table 6).

Table 6: Results of parasitological trypanosomosis survey conducted in the study areas
All trypanosomosis positive cases in Woinma (Jabitahnan district) treated with Isometamidium chloride (1 mg/kg) were visited after 4 weeks to assess prophylactic coverage of 6 weeks in greater challenge areas indicated by the manufacturer. Despite of efforts advised to consult and get treatment by assigned personnel until subsequent visit, all cattle considered were treated by farmers with Isometamidium chloride and still 31.8% were positive for *T. congolense*.

5.3. Experimental Studies

All mice inoculated with fresh blood containing *T. congolense* isolates taken from field were parasitologically positive. Parasitaemia was demonstrated after 12 days of inoculation in mice used for primary isolation and maintenance. However, parasitaemia was demonstrated 7 days post-inoculation in mice used for amplification. The goat which was used for maintenance of field isolates parallel to mice was treated with diminazene aceturate after 12 days post-infection when parasitaemic (+4) and relapsed after 6 days, retreated with isometamidium chloride (1 mg/kg) and dead after 13 days post-treatment. In the current study, alopecia and bleeding lesions were eminent on hind quarters of mice at third day of homidium bromide treatment (10 mg/kg and 5 mg/kg). During experimental period, highest mortality of mice (15% of the total) happened with in period of 6 hours post-inoculation with infective blood or trypanocidal treatment.

5.3.1. Sensitivity status of combined pool

Dosages of both trypanocidal drugs used for comparison of cattle doses in mice have totally failed to clear initial level of parasitaemia (data not shown). Moreover, all treatments had failed to clear trypanosomes permanently from experimentally infected mice as shown in Table 6. Homidium bromide was also not effective in initial clearance of trypanosomes at comparative mice doses. Although the drugs failed to confer complete clearance, there was a significant difference in relapse duration, i.e., isometamidium chloride vs. diminazene aceturate (P<0.005) and isomethamidium chloride and homidium bromide (P<0.001) being longer for Isomethamidium chloride. Fast apparent clearance was observed for diminazene aceturate (with in 48 hours) unlike isometamidium chloride where clearance was seen after 96 hours post-treatment (Figure 7). However, for diminazene aceturate and homidium bromide, with
comparative therapeutic and prophylactic doses in mice, there was a statistically significant difference (P<0.05) between mean relapse durations for increasing dosages used.

Although none of sanative combinations could have resulted complete clearance of trypanosomes from mice, a significant difference (P<0.01) in relapse durations between single and combined treatment existed when given with in 10 days interval. This was the only combination that resulted clearance in 60% of mice in the group. However the group was not considered sensitive as 80% clearance was not achieved (FAO, 1998). It was also observed that initial diminazene aceturate treatement followed by isometamidium chloride had longer relapse duration (P<0.01) and 60% clearance resulted as compared to isometamidium chloride followed by diminazene aceturate as sanative pair.

Table 7: Treatement of the single pool derived from Debreelias, Dembecha and Jabitahnan.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Group</th>
<th>Relapse</th>
<th>Relapse duration</th>
<th>Remark</th>
</tr>
</thead>
<tbody>
<tr>
<td>Diminazene aceturate</td>
<td>35 mg/kg</td>
<td>2/2</td>
<td>2.4±2.95</td>
<td>Only two cleared temporarily after 48 hours post-treatment</td>
</tr>
<tr>
<td></td>
<td>70 mg/kg</td>
<td>5/5</td>
<td>5.8±0.96</td>
<td>All cleared temporarily after 48 hours post-treatment</td>
</tr>
<tr>
<td>Isometamidium chloride</td>
<td>5mg/kg</td>
<td>5/5</td>
<td>12.60±1.47</td>
<td>All cleared temporarily after 96 hours post-treatment</td>
</tr>
<tr>
<td></td>
<td>10mg/kg</td>
<td>5/5</td>
<td>14.20±1.99</td>
<td>All cleared temporarily after 96 hours post-treatment</td>
</tr>
<tr>
<td>Homidium bromide</td>
<td>5mg/kg</td>
<td>NPC</td>
<td>NPC</td>
<td>No post-treatment clearance (NPC)</td>
</tr>
<tr>
<td></td>
<td>10mg/kg</td>
<td>3/3</td>
<td>4.6±3.75</td>
<td>Only three cleared temporarily after 96 hours post-treatment</td>
</tr>
<tr>
<td>Controls (saline treated)</td>
<td>Positive controls remained with +6 level of parasitaemia (15/15)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Negative controls remained healthy (non parasitaemic) until end of the experimental study (15/15)</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
5.3.2. Sensitivity status of separate pools

The result obtained from experimental trial on isolates of each district separately revealed similar pattern of resistance to trypanocidal drugs. Complete clearance was observed only in 40 % of mice used for Debreelias isolate with isometamidium chloride 10 mg/kg as shown in Table 7. Similarly comparison of similar treatement groups in single pool from three districts and three pools from isolates in each district seen separately showed no significant difference in their mean relapse durations (P>0.05).
Table 8: Treatment of three pools separately taken from Debreelias, Dembecha and Jabitahnan.

<table>
<thead>
<tr>
<th>Pool origin</th>
<th>Treatment</th>
<th>Group</th>
<th>Relapse number</th>
<th>Relapse Duration</th>
<th>Remark</th>
</tr>
</thead>
<tbody>
<tr>
<td>Debreelias</td>
<td>Isomethamidium chloride 10 mg/kg</td>
<td>3/5</td>
<td>18.33±1.31</td>
<td>2 mice have cleared</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Diminazene aceturate 70 mg/kg</td>
<td>5/5</td>
<td>7.81±1.56</td>
<td>No complete clearance</td>
<td></td>
</tr>
<tr>
<td>Dembecha</td>
<td>Isomethamidium chloride 10 mg/kg</td>
<td>5/5</td>
<td>18.20±2.66</td>
<td>No complete clearance</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Diminazene aceturate 70 mg/kg</td>
<td>5/5</td>
<td>7.00±1.24</td>
<td>No complete clearance</td>
<td></td>
</tr>
<tr>
<td>Jabitahnan</td>
<td>Isomethamidium chloride 10 mg/kg</td>
<td>5/5</td>
<td>11.00±2.15</td>
<td>No complete clearance</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Diminazene aceturate 70 mg/kg</td>
<td>5/5</td>
<td>5.80±0.96</td>
<td>No complete clearance</td>
<td></td>
</tr>
</tbody>
</table>

5.3.3. Drug Quality Assurance

Diminazene aceturate and Homidium bromide used in the drug sensitivity studies were analysed by DACA for minimum requirements. The subjected drugs were found to meet the requirements for identity and content assay as shown in Table 9. However, isometamidium chloride could not be analysed due to lack of availability of the standard chemical to compare the sample specimen provided.
Table 9: Physicochemical analysis of trypanocidal drugs according to DACA.

<table>
<thead>
<tr>
<th>Drug</th>
<th>Test parameters</th>
<th>Physicochemical result</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Specification</td>
</tr>
<tr>
<td>Homidium bromide</td>
<td>Appearance</td>
<td>Reddish purple, plain, biconvex, round tablets</td>
</tr>
<tr>
<td></td>
<td>Identification</td>
<td>UV scan comparison against working standard and reference scan</td>
</tr>
<tr>
<td></td>
<td>Assay</td>
<td>225 mg to 275 mg/tablet</td>
</tr>
<tr>
<td>Diminazene aceturate</td>
<td>Appearance</td>
<td>Yellow coloured granules</td>
</tr>
<tr>
<td></td>
<td>Identification</td>
<td>DA - UV scan Phenazine - color reaction</td>
</tr>
<tr>
<td></td>
<td>Assay</td>
<td>90-110 % of labeled amount</td>
</tr>
</tbody>
</table>
6. DISCUSSION

*Questionnaire survey and field observation*

The study was conducted from December 2006 to July 2007 to assess current status of trypanocidal drugs in selected trypanosomosis districts of western Amhara region. The results of questionnaire survey indicated that trypanosomosis has been occurring in the area before many years causing a considerable loss either due to cost of treatment or mortality and morbidity. The present complaint of farmers is also frustrating in such a way that there may be abandonment of tsetse infested areas in the future provided that the problem persisted. The survey has also shown the presence of indiscriminate use of trypanocidal drugs by peasants to treat their animals. Treatment practice by farmers themselves seems routine activity. Most of the farmers in focal group discussion indicated underdosing trypanocidals especially isometamidium chloride as described by the use of locally prepared metallic material that practically could measure less than 30 mg of isometamidium powder. From personal observation and group discussion results, underdosing was ascribed as lack of knowledge about trypanocidal use, insufficient and/or absence of veterinary service and even type of drug dosage forms /one sachet isometamidium chloride (1gm for 8 adult cattle).

Response obtained from questionnaire survey on individual farmers was in agreement with the information obtained in focal group discussion. A very significant proportion of farmers (71.7%) treat their animals by themselves. For instance, 5.5 %, 100 % and 46.2 % of the respondents were using diminazene aceturate with dosage practices below the recommended level in Woinma, G/kendamu and Jabigenet, respectively. Furthermore, 100 %, 100 % and 77.42 % of the respondents were using isometamidium chloride with dosage practices below the recommended level in Jabigenet, G/kendamu and Woinma, respectively. Isometamidium chloride underdosing was relatively lower in Woinma due to increased challenge of trypanosomosis and resistance expression that forced farmers to use increased drug amounts. However homidium bromide underdosing was relatively of minor importance which could be attributable to dosage form and lower overall use as treatement of bovine trypanosomosis.

Underdosing is indicated as one of the major causes of development of resistance. Subtherapeutic drug concentrations exert a strong selective pressure for the emergence of resistance clones that
pre-exist in the trypanosome population. This fact was substantiated by studies at different time periods. The level of resistance of the laboratory clones of *T. congolense* to isometamidium chloride has shown to be increased 94-fold over an 11-month period by repeatedly treating infected mice with subcurative doses of isometamidium chloride and on the basis of CD50s in mice. The 94-fold increase in resistance was shown to be associated with a 3.4-fold increase in resistance to diminazene aceturate, a 33-fold increase in resistance to homidium bromide, and a 4.2-fold increase in resistance to quinapyramine sulphate in Kenya (Peregrine *et al.*, 1997). Similar results were seen *in vitro* sensitivity trials in Serengeti, Tanzania (Gray and Peregrine, 1993). Exposure of parasites to subtherapeutic drug concentrations, either due to underdosing and uncontrolled use of trypanocidals in lack of proper diagnosis were also described as a major reason for increasing resistance throughout Africa. However, it was also stressed that prolonged and frequent use of trypanocidals in high challenge area, even when well applied, is likely to select for resistance as well (Clausen *et al.*, 1992; Geerts and Holmes, 1998).

Similar trend of trypanocidal drug use was documented from survey studies conducted in Zambia, where bovine owners administer most of available trypanocides themselves. However, evidence from the survey indicated as most of the farmers did not under-dose with either diminazene aceturate or isometamidium chloride. Moreover, other factors enhancing the development of resistance to trypanocides in trypanosomes were not present in the areas surveyed (Van den Bossche *et al.*, 2000). From the result obtained on separate questionnaire survey in Eastern Province of Zambia by interviewing 122 farmers and 50 veterinary assistants having bovine, all were familiar and know isometamidium injectable solution preparation appropriately for adult bovine of 250 kg; still 76% of the farmers check the expiry date of the package before use and remaining 24% of the farmers trusted the veterinary assistant about the quality of the drug (Delespaux *et al.*, 2002). All the above situations could able to indicate gaps in creating awareness pointing to the urgency of intervening on trypanocidal drug use in trypanosomosis endemic areas of country as a whole.

In the present study, all cattle owners (100 %) were using diminazene aceturate where as 49.5% and 18.7% of drug users had the experience of using isometamidium chloride and homidium bromide, respectively. From personal communication with professionals in veterinary service under MoARD, diminazene aceturate is also employed as a treatment for babesiosis and
anaplasmosis in many parts of the study area contributing to availability in sub curative blood levels for trypanosomes. Owing to its very rapid clearance, there is reduced risk of exposure to subcurative levels resulting in development of resistance less than the expected rate. However, the relatively cheaper price, very wide availability and activity against various tick-borne diseases are described as most important features contributing to wide spread resistance to diminazene (Delespaux and Koning, 2007).

A higher proportion of respondents (p <0.01) indicated therapeutic failures in tsetse infested districts of the study areas which may indicate the relative importance of drug resistance. This could be verified by the fact that selection by drugs takes place during asexual multiplication in the animal or human host. During the passage through the tsetse fly genetic exchange (sexual recombination) may occur, at least in T. brucei, and is strongly suspected in T. congolense given the high degree of genetic diversity observed (Delespaux et al., 2007).

Although all trypanocidal drugs are initially imported through legal and standard import agents, 95.34 % lower level drug outlets were seen selling directly to farmers as like any commercial item. About 11.62 % of private firms were seen to be managed by non-professionals at time of observation. Only 11 % of the respondents indicated their trypanocidal source from open market (illegal) shops. According to personal observations and information obtained from group discussion, there seems to exist a flourishing black market and farmers can purchase varieties of trypanocidal drugs in village markets. The actual source of drugs for farmers is expected to be underestimated in results of questionnaire survey due to fear of responsibility by respondents. In study reports in eastern province of Zambia, all farmers bought isometamidium chloride from veterinary assistants or directly from the Veterinary offices. Veterinary assistants did buy from the veterinary offices (Delespaux et al., 2002) which is indicator of better situation in delaying the development of trypanocidal drug resistance. The same trend should be adopted at least for the present study sites.

The epidemiology of drug resistant populations of trypanosomes is changing time to time. Once established the incidence is progressively spread within the population. The incidence of recurrent infection was 7 % in 1986 and increased to 14 % in 1989 in Ghibe valley of Ethiopia (Rowlands et al., 1993). Transmission by tsetse flies do not appear to affect the drug sensitivity
of trypanosomes and drug resistance strains remain to have unchanged resistance profile after passage through tsetse flies (Moloo and Kutuza, 1990). Therefore this trait is capable of spreading through wider area with animal movement and/or spread of tse tse population. Because of these reasons there is an urgent need for designing strategies to restrict/control animal movement and monitoring of trypanocidal drug use so as to delay the dissemination of drug resistance, thus minimizing negative impact to be posed on livestock productivity as whole in trypanosomosis endemic areas of the region.

The existing multifaceted problems were further exacerbated by farmers’ level of perception in the use of trypanocidal drugs. The current implication of multidrug resistance is not practically far from what is happening in actual field conditions. However, the paradox exists, as to how the animals existed in the area with no complaints of equivalent mortality relative to intensity of problem. Still some of the cattle observed in field surveys were with good body condition /or normal PCV range and having past history of recurrent therapeutic failures at least witnessed by professionals engaged in veterinary service. In focal group discussion, farmers indicated the existence of high mortality rates whenever animals are brought from high land areas for ploughing purposes. It could be deduced on the possible adaptive mechanisms where by the local breed types have evolved be it physiological or genotypic mechanisms indicating a state of trypanotolerance. It can also be explained as the trypanosomal agent might have engaged in adaptive mechanisms in enhancing of drug resistance rendering it to lesser virulence. Out of the total of animals sampled and positive for *T. congolense* 41 % in Woinma and 55% in G/kendamu were with PCV values greater than or equal to 20 %. Similarly 43 % of bovine sampled and positive for *T. congolense* in Debreelias were with PCV values greater than 25 %.

Similar observations were reported on the responses of four indigenous bovine breeds of Ethiopia, namely Abigar, Horro, Sheko and Gurage, to natural challenge of trypanosomosis in the Tolley–Gullele area of the Ghibe valley. Accordingly, the Sheko breed showed a significant degree of resistance measured in terms of PCV values and mortality (Lemecha et al., 2006). A research out put by ILRI (1996) has indicated loss of fitness in drug resistance strains. Therefore, further studies for exploitation of the remaining hopes in down regulation of existing problems of trypanosomosis like characterization of trypanotolerance and vector control should be overstressed.
Trypanosomosis prevalence

Results from the trypanosomosis parasitological survey indicated that bovine trypanosomosis is prevalent in the three districts considered for *T. congolense* isolation. The observed 19.6 % point prevalence of trypanosome infection in bovine seems to be relatively lower. This is may be due to the fact that the lower sensitivity of diagnostic techniques as well as the small sample size considered as compared to the sample size to be followed for actual prevalence study. Parasitological prevalence was highest in Jabitahnan district and lowest in Debreelias. This difference could be attributed to greater tsetse fly challenge existing around Bir River in Woinma that join finally to the Abay basin. A high proportion of the infections were due to *T. congolense*, although *T. vivax* and *T. brucei* infections were also detected in all the three districts. The presently observed high prevalence of *T. congolense* was comparable with previous prevalence observations in tsetse infested areas of the same region (Cherenet *et al.*, 2006) as well as other African countries, such as Malawi, Mozambique, Namibia, Zambia, Zimbabwe and Cameroon (Joshua *et al.*, 1995; Van den Bossche, 2001; Sinyangwe *et al.*, 2004; Mamoudou *et al.*, 2006). Abay (Blue Nile) basin was indicated as being among the main river basins infested with tsetse fly, vector of cyclical trypanosomosis, in Ethiopia (Abebe, 2005).

Experimental sensitivity trials

Experimental trials conducted to assess the sensitivity status of *T. congolense* pooled isolates from three selected study sites showed that none of the trypanocidals commonly employed in the area were effective in clearing trypanosomes from mice upto the maximum comparative dosage levels. Bovine doses were also used in mice for simple comparison purposes and failed to cause apparent clearance of trypanosomes from mice. Experimental trials aimed at tracing origins of drug resistant isolates among three study sites using diminazene aceturate and isometamidium chloride also revealed similar resistance patterns with isolate pooled from three districts in terms of relapse durations indicating wider spatial distribution of drug resistant *T. congolense* isolates at least in three districts. Furthermore, experimental trials undertaken to see sanative (combined) effects of trypanocidals were not effective in clearing trypanosomes from mice under combinations of isometamidium chloride with diminazene aceturate given at intervals.
Experimental sensitivity trials conducted on mice using isolates of *T. congolense* taken from Ghibe, Bedele, Sodo and Arbaminch reported failure of trypanocidals to ensure complete clearance using bovine doses (Chaka and Abebe, 2003). Other studies made in Benishangul Gumuz region of North West Ethiopia reported *T. congolense* isolates refractory to treatments up to 28 mg/kg diminazene aceturate and 4 mg/kg of isometamidium chloride in mice (Afewerk *et al.*, 2000). Despite tenfold comparative dosages (FAO, 1998) were not achieved in mice to conclude resistance at therapeutic levels in bovine, level of drug sensitivity of trypanosomes at that specific study period could be an indicator for greater tendency of refractoriness to available trypanocidals at earlier time period.

Trypanosomes are usually not resistant to both diminazene aceturate and isometamidium chloride or homidium salts at the same time and thus these compounds have been termed as a sanative pairs for the control of bovine trypanosomosis since the phenomenon described in 1960 (Whiteside, 1960). Studies with stock of *T. congolense* isolated from Benishangul Gumuz region of Ethiopia reported clearance of trypanosomes from mice using sanative doses less than recommended ten fold dosages (Afewerk and Abebe, 1998). However after further cloning of the same source stabilate, strains refractory to sanative combinations was later reported (Afewerk *et al.*, 2000). Similarly, many study results either in Africa or other parts of Ethiopia had reported observations with development of multidrug resistance. In Burkina Faso (Sones *et al.*, 1988; Clausen *et al.*, 1992) and in Ethiopia (Codjia *et al.*, 1993; Mulugeta *et al.*, 1997) resistance to sanative combinations of trypanocidal drugs was mentioned for *T. congolense*. Infact cloning of trypanosome population may help to amplify resistant strain for screening existence of resistant strains within a stabilate. However in present study, an abundance pool of resistance strains existed which showed refractoriness to combined treatments before cloning.

In contrary to the present experimental trial results, studies conducted elsewhere in Africa have reported the effectiveness of trypanocidals in mice. Using discriminatory doses of 1.0 mg/kg isometamidium chloride and 20 mg/kg diminazene aceturate, 53.5% of isolates were sensitive to both drugs in experimental mice in Zambia (Sinyangwe *et al.*, 2004). In Burkinafaso, clone of identity IL 3000 resistant to 1 mg/kg isometamidium was successfully treated with a dose of 5 mg/kg isometamidium chloride in mice (Knoppea *et al.*, 2006). Results of successful *T. congolense* clearance in experimental rats, at 10.5 mg/kg diminazene aceturate were documented
from Nigeria (Egbe-Nwiyi et al., 2006). Perhaps better sensitivity could have been resulted if the recommended comparative dosages were maintained.

Collateral efficacy trial on bovine screened in Woinma field $T. congolense$ isolation revealed prophylactic coverage of isometamidium chloride to be less than four weeks unlike manufacturer’s advocation of 6-16 weeks. Field observations made in other regions of Ethiopia were in agreement with the present findings. Isometamidium chloride prophylactic efficacy less than 30 days was documented on bovine naturally infected with $T. congolense$ in three villages of Kindo Koysha, Southern Ethiopia (Ademe and Abebe, 2000). In contrary to the present observation, studies carried out elsewhere in Africa have reported the effectiveness of trypanocidals in field studies. In Kenya, isometamidium chloride based products Samorin and Veridium were found effective in prophylaxis against bovine trypanosomoses when administered at 0.5 mg/kg for about 70 days (Stevenson et al., 2000).

The outcome of the present trypanocidal drug sensitivity / resistance test in mice clearly shows the presence of trypanosomal isolates that have developed drug resistance phenotype to the currently available trypanocides. Regional trypanosomosis control schemes through increasing provision of trypanocidals may not be reliable and effective. In areas where multiple trypanocidal resistance is expressed at the level of the individual trypanosome, chemotherapy becomes increasingly ineffectual and thus intervention at the level of the vector is described (Peregrine et al., 1994).

In the present experimental trial, initial and peak parasitaemia were demonstrated 72 hours and 120 hours post-inoculation, respectively. The mice were treated at first peak of parasitemia (120 hours post inoculation). Drug administration through intraperitoneal route is recommended at least after 24 hours post inoculation of mice to avoid misdiagnosis of resistant strains as sensitive (Eisler et al., 2001). Other authors also recommended treatment of experimental mice at the first peak of parasitaemia (FAO, 1998). In the present experimental trial, parasitaemia was demonstrated after 12 and 7 days in mice used for isolation and amplification, respectively. Similar observation with first peak of parasitaemia 5-7 days post-infection using primary isolate of $T. brucei$ was reported (Olila et al., 2002). The prepatent period in $T. congolense$ infected rats was reported to be longer than in $T. brucei$ infected rats. Procedures of treating experimental rats
after 14 days of infection at first peak of parasitaemia was followed in more recent experimental trials in Nigeria (Egbe-Nwiyi et al., 2006). Mutant forms of an organism are likely to be less fit than their wild-type strains in the absence of selection which is substantiated by researches that demonstrate existence of drug resistance phenotypes reducing infectivity (Coleman and McDermott, 2000).

In the present study, at third day of homidium bromide treatment (10 mg/kg and 5 mg/kg), alopecia and bleeding lesions were eminent on hind quarters of mice. Photoconversion of dihydroethidium has been described following exposure to short wave length illumination and long exposure to full spectrum UV-light. An exposure to high concentrations of homidium bromide in laboratory mice results in acute cell toxicity by interfering with mitochondrial respiration. It is mutagen, teratogenic and irritant to the eye, respiratory system and skin (Zanetti et al., 2005). Acute toxicity due to homidium bromide was also described in bovine as an acquired sensitivity to light (photosensitivity), causing necrosis and sloughing of extensive areas of unpigmented skin, later on followed by infection and death (Uilenberg, 1998). In the present experimental sensitivity trial, highest mortality of mice (15% of total) happened with in a period of 6 hours following either inoculating with infective blood or treatment with trypanocidals. Administration of infective inoculum or trypanocidals could be attributed for mortality due to injury of internal organs by needle or injection solution when performing intraperitoneal inoculation.

Drug quality assurance

The quality of trypanocidals to be used in the control of trypanosomosis determines the therapeutic efficacy. However, in the present study homidium bromide and diminazene aceturate were found to be less effective in the experimental sensitivity/resistance trial. Both of these drugs were found to have fulfilled the minimum standard for inland use. Although isometamidium chloride could not be verified due to inavialability of working standard in quality certification laboratory, it seems unlikely for great variation to occur due to initial procedures followed during importation by the Ministry of Agriculture and Drug Administration and Control Authority of Ethiopia. None of the encountered studies undertaken in trypanosomal sensitivity experiments have validated drug quality as an important component misleading of drug
resistance. Counterfeit drugs are currently described as highest weapon of terrorism against public health, as well as an act of economic sabotage.

Although no published articles are available on Ethiopian scenario, studies in Nigeria reported 25 % to 54 % of samples taken from pharmacy shop at different times to be counterfeit drugs (Akunyili, 2005). A huge economic loss of 32 billion dollars was also recently reported globally (Akunyili, 2008). Perhaps what has been worried seems drugs of medical importance as reflected practically in actual field situations where some shop retailers defended as they used to sell some of veterinary products like albendazole for long and underestimating their illegacy. Veterinary service neglected and being thin on the ground under the prevailing situations, prone to risk of increased prevalence of counterfeited drugs. Therefore drug quality should be emphasized in sensitivity trials. From personal communication with concerned bodies, a single drug identity is admitted for importation/or inland use for five successive years after quality certification and/or registration procedure. In Amhara region, about 120 batches of isometamidium chloride (product veridium®) were encountered necessitating the curiosity to prevent counterfeiting.
7. CONCLUSIONS AND RECOMMENDATIONS

Trypanosomosis is shown to be the most important constraint for cattle production in western districts of Amhara Region. The result of the present study has indicated the existence of *T. congolense* isolates that have developed resistance to one or more of the currently available trypanocides (diminazene aceturate, isometamidnun chloride and homihum bromide) both separately and/or in combination. Management practices of trypanocidals observed in both study areas was self-explanatory for the outcomes obtained in experimental trials. Experimental trial so far undertaken in both study areas is a very important cue for the future prospects in the exclusive use of trypanocidals in tsetse infested areas for the control of trypanosomosis. Although claims against trypanocidal drugs in non-tsetse infested districts seem to be relatively of minor importance, ill-defined management practices observed in visited areas were also potential indicators for a similar problem to happen in near future.

Based on observations and findings obtained, regional control of trypanosomosis needs to focus on the proper use of the available trypanocidals in conjunction with other alternative control strategies. In this essence, the following recommendations are forwarded:

- Adoption of integrated control strategies apart from exclusive use of trypanocidals through MoARD has to be stressed before the existing problems of resistance further deteriorated regionally in future.
- Proper and strict follow up of trypanocidal drug use should be implimented by concerned stakeholders like DACA and MoARD.
- Chemotherapeutic as well as chemoprophylactic schemes have to follow appropriate diagnostic procedures in order to minimize exposure of trypanosomes to subcurative level of trypanocidals hastening expression of drug resistance phenotype.
- Extension services implemented in MoARD should have to incorporate participatory packages on public awareness creation in disease control stratagies specially risk of misuse of trypanocidals.
- Admission of specific trypanocidal drug for successive years after single initial quality certification has to be seen curiously or else a means has to be devised to check post market quality of successively imported batches.
8. SUGGESTIONS

The following issues are suggested as important gaps to be considered and studied as a tool to fight against trypanosomosis in future.

- Further experimental studies have to be mounted so as to see the current picture of trypanocidal drug resistance in non-tsetse infested areas of the region.
- Molecular and genetic characterization of each trypanosomal spp existing in the region should have to be undertaken so that further studies on genetic basis of drug resistance can be facilitated.
- Any existing ethnoveterinary practices as treatment to trypanosomosis should be assessed.
9. REFERENCES


tsetse-infested zones of the Amhara Region, northwest Ethiopia. *Veterinary Parasitology*, **140**:251–258


trypanosome populations from bovine in a peri-urban dairy production system in Uganda. 
*Acta Tropica*, **84**:19/-30.


58


59


Annex 1: Wet film parasitaemia estimation

<table>
<thead>
<tr>
<th>Number of trypanosomes</th>
<th>Score</th>
<th>Estimated parasitaemia</th>
</tr>
</thead>
<tbody>
<tr>
<td>Swarming &gt;100 per field</td>
<td>6+</td>
<td>&gt; 5 x10^6</td>
</tr>
<tr>
<td>&gt;10 per field</td>
<td>5+</td>
<td>&gt; 5x10^5</td>
</tr>
<tr>
<td>1-10 per field</td>
<td>4+</td>
<td>10^4 - 5x10^5</td>
</tr>
<tr>
<td>1 per 2 fields - 1 per 10 fields</td>
<td>3+</td>
<td>5x10^3 - 5x10^4</td>
</tr>
<tr>
<td>1-10 per preparation</td>
<td>2+</td>
<td>10^3 - 10^4</td>
</tr>
<tr>
<td>1 per preparation</td>
<td>1+</td>
<td>10^2 - 10^3</td>
</tr>
</tbody>
</table>

(Source: Paris et al., 1982)
Annex 2: Dosage Preparation

Drug dosage needed to be given (Drug “X” at “Y” mg/kg)

Maximum recommended volume = 0.2 ml IP (FAO, 1998; OIE, 2004)

Dosage rate needed to be inoculated = “Y” mg/kg

Average weight of mice = 0.0325 kg (measurement using digital balance)

Total dose to be given = “Y” mg/kg x 0.0325 kg = 0.0325 “Y” mg.

Required concentration of injectable solution = 0.0325 “Y” mg/0.2 ml = 0.1625 “Y” mg/ml

To have manageable volumes = 100 x 0.1625 “Y” mg/100 ml = 16.25 “Y” mg of Drug “X” in 100 ml volume.

1 unit of purchased formulation/sachet/tablet/any contains “Z” mg of bioactive drug

By computing, for required concentration.

“Z” mg of Drug “X” should be dissolved in Z x 100/[16.25 “Y”] ml of distilled water.

For X = Diminazene, “Z” represent 1050 mg, Y represent 70, 35, 3.5

X = Isometamidium, “Z” represent 1000 mg, Y represent 10, 5, 1.0, 0.5

X = Ethidium, “Z” represent 250 mg, Y represent 10, 5, 1.0, 0.5
Annex 3: Questionnaire survey format

I. Questionnaire for group discussion (participatory approach)

Points of Discussion

- General Animal Health
- List of animal diseases in the area
- Trypanosomosis epidemiology and impacts on farmers' livelihood
- Trypanocidal drugs and therapeutic management practices commonly known in the area

II. Questionnaire for individual cattle owners

District------------- Kebele------Village--------Date-------------------

1. General Animal Health

1.1. List of animal diseases in your locality in their order of importance

______________________________________________________________________________
______________________________________________________________________________
______________________________________________________________________________

2. Perception and incidence of trypanosomosis

2.1. Is there trypanosomosis in your locality?

1. Yes ■ 2. No ■

2.2. What are the clinical signs to suspect trypanosomosis in your locality?

________________________________________________________________________

2.3. When did the first case of trypanosomosis occur in your locality?

________________________________________________________________________

2.4. How is the prevalence of trypanosomosis hitherto?

1. Increased ■ 2. Decreased ■ why? -----------------------------------

2.5. How is the mechanism of transmission among bovine?

________________________________________________________________________
2.6. Is there seasonal variation in prevalence of trypanosomosis?

1. Yes  
2. No  If yes, when?________________________

3. Therapeutic Practices

3.1. How do you combat trypanosomosis when it exists in your herd?

1. Traditional medicine locally available
2. Buying and adminstration of veterinary drugs by their own
3. Travelling to nearby veterinary clinic
4. All alternatives are employed

3.2. Do you use traditional medicine to treat your animals?

3.2.1. What type of traditional medicine you use for your bovine when affected by trypanosomosis?____________________________________________________

3.2.2. How do you prepare and administer to your animals?________________________

3.2.3. Does it cure the disease or give sort of relief?______________________________

3.3. Do you use veterinary drugs to treat trypanosomosis? _______________________

3.3.1. Which type of veterinary drugs you know and experienced as treatment for your animals?

1. Diminazene/yellow powder/Bicha
2. Isometamidium/coffee/Buna
3. Ethidium (red tablet)
4. Others (specify)

3.3.2. Who administer drugs to your animals?

1. Yourself  2. Experienced villeger
3. Anyother professional

65
3.3.3. How do you apply / prepare injectable solutions?

1. Measurement
   A. Standard measurement / Gramm Other local measurement
   B. Locally adopted measurement / metallic probe
   C. Simple guess

2. Solvent used (water, oil, etc)

3. Other substances added
   A. antibiotics
   B. Oil/salt

3.3.4. How do you administer trypanocidal drugs to your animals?

1. Route of administration

2. Site of administration

3.3.5. What amount of injectable solution do you give for each of your animals with trypanosomosis?

1. For calves
   2. For heifers
   3. For adults

3.3.6. How many of your animals get cured after your treatment?
   ¼
   ½
   ¾
   ¹⁄₁₀

3.3.7. How many times you treat your animals for trypanosomosis per year?
   2X
   3X
   4X
   5-10X
   >10

3.4. Where do you get trypanocidal drugs?

1. Pharmacy/Vet. or human

2. Shop/Others

3.5. Can you show one sample drug or its container left?
3.5. What is common price for each drug sample?

3.3. Questionnaire (for professionals engaged in veterinary service)

District………Place of Work…………Status/DVM/AHA/AHT

1. Is there trypanosomosis in your area? Yes ☐  No ☐

2. What general procedures are used before treatment of trypanosomosis?

_________________________________________________________________________

3. What type of drugs have you been using?

1. Trade mark/active ingredient

2. Price per dose

4. At what dosage?

Normal

Extralabel

5. Are there relapses/therapeutic failures encountered? Yes ☐  No ☐

At what time duration?

6. What measures taken to overcome relapses?

7. Can you estimate relapse rates?
SIGNED DECLARATION

This thesis is my original work, has not been presented for a degree in any other university and all sources of material used for the thesis have been duly acknowledged.

Name

________________________________________________________________________

Signature

________________________________________________________________________

Date of submission Addis Ababa, Ethiopia, April 2008

This thesis has been submitted for examination with our approval as university advisors.

1. Dr. Ephrem Engidawork

______________________________

Signature

______________________________

2. Dr. Hagos Ashenafi

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Signature

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