


Detection and magnitude of *Trypanosoma congolense* chemo-resistance associated with livestock owners drug use perception in Ghibe valley of Southwestern Ethiopia

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There is an appointed time for everything.

And there is a time for every event under heaven ...

Ecclesiastes 3:1

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Table of contents

Acknowledgments ..... **i**

Table of contents ..... **v**

List of abbreviations ..... **xi**

List of figures ..... **xiii**

List of tables ..... **xv**

Chapter 1 ..... **1**

General introduction **1**

1.1 Trypanosomosis 3

1.2 Etiology 3

1.3 *Trypanosoma* life cycle 4

1.4 Pathology and pathogenesis 6

1.5 Clinical signs of trypanosome infections 8

1.6 Epidemiology 9

1.6.1 Hosts 9

1.6.2 Transmission of trypanosome infection 9

1.7 Occurrence of the disease 10

1.8 Development activities versus tsetse and trypanosomes dynamics 11

1.9 Current trypanosomosis situation in Ethiopia 13

1.10 Diagnostic methods 13

1.10.1 Clinical diagnosis 13

1.10.2 Post-mortem lesions 14

1.10.3 Laboratory diagnosis 14

1.10.4 Parasitological methods 15

1.10.5 Serological tests 16

1.10.6 Molecular Tests 16

1.11 Treatment of trypanosomosis 18

1.12 Prevention and control of trypanosomosis 19

1.12.1 Sanitary prophylaxis 19

1.12.2 Chemoprophylaxis 20
1.13 References 20
General and specific objectives 29
Chapter 2 35
Trypanocidal drug resistance in East Africa: A review 35
2.1 Background and justification 37
2.2 Trypanocidal drugs and their use in East Africa 39
2.2.1 The livestock owner 41
2.2.2 The political level 42
2.3 Trypanocidal drug resistance reports from various parts in East Africa 42
2.3.1 Drug resistance in Ethiopia 43
2.3.2 Drug resistance in Tanzania 44
2.3.3 Drug resistance in Kenya 45
2.3.4 Drug resistance in Uganda 45
2.4 Socio-economic implication of drug resistance in East Africa 46
2.4.1 Crisis of professional enthusiasm 46
2.4.2 Livestock farmer and the farming system 47
2.4.3 Impact on pharmaceutical industries 47
2.5 Detection methods of trypanocidal drug resistance 48
2.6 Conclusion 53
2.7 References 54
Chapter 3 63
Ghibe river basin in Ethiopia: Present situation of trypanocidal drug resistance in *Trypanosoma congolense* using tests in mice and PCR-RFLP 63
3.1 Abstract 65
3.2 Introduction 66
3.3 Materials and Methods 67
3.3.1 Study area 67
3.3.2 Cross-sectional study 68

3.3.3 Animals 68
3.3.4 Blood collection 68
3.3.5 Parasitological tests 69
3.3.6 Molecular assays 70
3.3.7 Data analysis 71
3.4 Results 72
3.4.1 Prevalence and species identification 72
3.4.2 *Dpn*II-PCR-RFLP resistance study 73
3.4.3 *T. congolense* isolation 73
3.4.4 Resistance tests in mice 74
3.4.5 18S and *Dpn*II-PCR-RFLP on samples from mice 74
3.4.6 Mice isolation versus BCE and 18S-PCR-RFLP on DNA from filter papers 75
3.5 Discussion - conclusions 75
3.6 Acknowledgements 79
3.7 References 79
Chapter 4 85
Detection of African animal trypanosomes: The haematocrit centrifugation technique compared to PCR with samples stored on filter paper or in DNA protecting buffer 85
4.1 Abstract 87
4.2 Introduction 87
4.3 Materials and Methods 90
4.3.1 Study animals and site 90
4.3.2 Protocol 90
4.3.3 Data analysis 92
4.4 Results 92
4.4.1 Percentage positivity using different sampling methods 92
4.4.2 Comparison of HCT and PCR-RFLP-fp 92
4.4.3 Comparison of HCT versus PCR-RFLP-pb 94
4.4.4 Comparison of PCR-RFLP-pb and PCR-RFLP-fp 94

4.4.5 Relationship of PCV value with HCT and PCR-RFLP-fp or -pb 95
4.5 Discussion 96
4.6 Conclusion 98
4.7 References 99
Chapter 5 103
Close-up on diminazene aceturate resistance by PCR and microsatellite analysis correlated to knowledge, attitude and practice of livestock keepers: the case of Ghibe river basin in South-western Ethiopia 103
5.1 Abstract 105
5.2 Introduction 106
5.3 Materials and Methods 108
5.3.1 Study site description 108
5.3.2 Longitudinal study 108
5.3.3 Questionnaire Survey 113
5.3.4 Statistical analysis 114
5.4 Results 115
5.4.1 Prevalence and diminazene aceturate resistance 115
5.4.2 Longitudinal study 116
5.4.3 Questionnaire survey 118
5.5 Discussion 120
5.5.1 Prevalence and resistance 120
5.5.2 Longitudinal study 122
5.5.3 Questionnaire survey 122
5.6 Conclusions and implications 123
5.7 Acknowledgement 124
5.8 References 125
Chapter 6 137
General discussion and future perspectives 137
6.1 General Discussion 139

6.1.1 High prevalence of *T. congolense* savannah infections in the Ghibe river basin . 139

6.1.2 The magnitude of DA and ISM resistance in the Ghibe valley, South-western Ethiopia 142

6.1.3 Farmer's perception/knowledge about trypanocidal drugs..... 143

6.1.4 Recommendations for trypanosomosis control in the Ghibe valley, South-western Ethiopia 144

6.1.5 Conclusion..... 146

6.2 Future Perspectives..... 147

6.3 References..... 148

Summary..... 155

Samenvatting..... 159

Curriculum vitae..... 165

Bibliography..... 169

List of abbreviations

a.o.	Among others
AAT	African Animal Trypanosomosis
ADI	Acceptable daily intake
AFLP	Amplified fragment length polymorphism
AS1	A special stabilizing reagent
B.W.	Body weight
BCE	Buffy Coat Examination
bp	Base pairs
BSF	Bloodstream forms
CI	Confidence Interval
DA	Diminazene Aceturate
DEAE	Diethylamioethyl
DIIT	Drug incubation infectivity test
DNA	Deoxyribonucleic acid
EDTA	Ethylene diamine tetra acetic acid
ELISA	Enzyme-linked immunosorbent assay
FAO	Food and Agriculture Organization of the United Nations
fp	Filter paper
FTA	Fast Technology for Analysis of nucleic acids
GALVmed	Global Alliance for Livestock Veterinary Medicine
GIS	Geographic Information System
HAT	Human African Trypanosomosis
HCT	Haematocrite Centrifugation Technique
i.m.	Intramuscular
IAEA	International Atomic Energy Agency
IFAT	Indirect fluorescent anibody test
ILRI	International Livestock Research Institute

ISM	Isometamidium Chloride
ITS-1 rDNA	Internal Transcribed Space - 1 ribosomal DNA
Kg	Kilogram
µg	Microgram
mg	Milligram
ml	Milliliter
ng	Nanogram
OIE	Office International des Epizooties
PAs	Peasant Associations
pb	Protection buffer
PCR	Polymerase Chain Reaction
PCV	Packed Cell Volume
PSG	Phosphate saline glucose
RFLP	Restriction Fragment Length Polymorphism
SSA	Sub-Saharan Africa
SSCP	Single Strand Conformation Polymorphism
Ssu-rDNA	Small Sub-Unit Ribosomal DNA
T and T	Tsetse and trypanosomosis
T. brucei	*Trypanosoma brucei*
T. congolense	*Trypanosoma congolense*
T. vivax	*Trypanosoma vivax*
TbAT1	*Trypanosoma brucei* Adenosine Transporter 1
TCM	*Trypanosoma congolense* microsatellite
TcoAT1	*Trypanosoma congolense* Adenosine Transporter 1
TLU	Tropical Livestock Unit
UV	Ultraviolet
WHO	World Health Organization

List of figures

Figure 1.1 Life cycle of trypanosome. 7

Figure 2.1 Map of East African countries..... 38

Figure 5.1 Map of Ethiopia depicting sites of questionnaire survey and longitudinal study (left) in the Ghibe..... 110

Figure 5.2 Sketch of longitudinal study protocol followed during DA resistance study in the Ghibe valley..... 111

Figure 5.3 PCV evolution for the two groups of animals (HCT negative or positive at t_0) followed by longitudinal study in the Ghibe valley. 117

List of tables

Table 1.1 Outline of classification 5

Table 2.1 Trypanocidal drugs in use. 40

Table 3.1 Summary of *T. congolense* infections based on different test methods 72

Table 3.2 Diminazene resistance results of *T. congolense* using *Dpn*II-PCR-RFLP according to study sites 73

Table 3.3 Median prepatent periods according to doses and treatments of *T. congolense* infections in mice 74

Table 3.4 Summary of discrepancies between 18S-PCR-RFLP and BCE for 24 mice injected samples 76

Table 4.1 The number and percentage of positivity of trypanosome infections detected by HCT, PCR-RFLP-fp and PCR-RFLP-pb in cattle blood samples from Ethiopia 93

Table 4.2 Comparison of the detection of trypanosome infection using HCT, PCR-RFLP-fp and PCR-RFLP-pb on cattle blood samples from Ethiopia 93

Table 4.3 Relationship between PCV category and presence of trypanosomes infection with the three different tests used on cattle blood from Ethiopia 95

Table 5.1 Infection rate per species of trypanosomes (HCT for the 587 animals of the study) in the Ghibe valley 115

Table 5.2 Association of trypanosomosis in the Ghibe valley with study sites, age, sex and body condition 116

Table 5.3 Microsatellite loci analysis results depicting infection events in cattle from the Ghibe valley as a function of the number of months after treatment 118

Table 5.4 Average herd Tropical Livestock Unit (TLU) profiles of different PAs in the Ghibe valley 119

Table 5.5 Different practices of DA and ISM use by farmers in the Ghibe valley 120

Table 5.6 Reported DA and ISM treatments by farmers in the Ghibe valley 121

Chapter 1

General introduction

1.1 Trypanosomosis

Trypanosomosis is a haemoprotozoan disease of humans and animals caused by several species of parasites of the genus *Trypanosoma* (CFSPH, 2009; FAO/IAEA, 1993; Nakayima et al., 2012). The parasite is mainly transmitted cyclically by the genus *Glossina* (tsetse flies), but also mechanically by several biting flies such as *Tabanus* and *Stomoxys* (Nakayima et al., 2012; OIE, 2013a). The disease can affect various species of mammals but, from an economic point of view, tsetse-transmitted trypanosomosis, is particularly important in cattle. It is characterized by intermittent fever, parasitaemia, anaemia, lymphadenopathy, jaundice, progressive emaciation, weakness and reduced productivity. The transmission of this debilitating and fatal disease not only prevents the rearing of livestock for meat or as banking system, but also their use for traction in resource-poor regions like Ethiopia (Davila et al., 2003).

1.2 Etiology

Trypanosomes are flagellate protozoans that inhabit the blood plasma, the lymph and various tissues of their hosts (OIE, 2013a; Steverding, 2008). The genus *Trypanosoma* belongs to the protozoan branch, order Kinetoplastida and family Trypanosomatidae (CFSPH, 2009; OIE, 2013a). Tsetse-transmitted trypanosomes belong to the salivarian section, subgenus Nannomonas for *T. congolense*, Duttonella for *T. vivax* and *Trypanozoon* for *T. brucei* ssp. (OIE, 2013a). Six species of trypanosomes have been known to exist in Ethiopia and the most important trypanosomes, in terms of economic loss in domestic livestock are reported to be the tsetse transmitted species such as *T. congolense*, *T. vivax* and *T. brucei*. In addition, the closely related *T. brucei* subspecies, *T. b. rhodesiense* and *gambiense* cause human sleeping sickness. The other *Trypanosoma* species of importance are *T. evansi* of camels and *T. equiperdum* of horses (Getachew, 2005).

The discriminatory power of molecular techniques reveals far greater levels of genetic diversity than acknowledged by current nomenclature (Adams et al., 2010). Recently, the application of

molecular and biochemical analysis such as isoenzyme profiles, DNA and RNA technology and molecular karyotyping have been used for the classification of the parasitic protozoa (Imam, 2009). Table 1.1 below depicts details of the classification.

In addition, several types of *T. congolense* have been distinguished by molecular biology; the most common and pathogenic one in cattle has been reported as the savannah type (large variation in pathogenicity within the savannah subgroup), the other ones (type forest and Kilifi or Kenya coast) have been found less pathogenic and have different host affinity (OIE, 2013b).

1.3 *Trypanosoma* life cycle

African trypanosomes alternate their life cycle between a vertebrate host and tsetse fly (a.o. *Glossina* spp.). These parasites cause human African trypanosomosis (HAT or sleeping sickness) and animal African trypanosomosis (AAT) (Dyer et al., 2013). The life cycle of *T. congolense* and *T. brucei* in the fly were described in the last century, but comparatively few details are available for *Trypanosoma* (*Nannomonas*) *congolense*, despite the fact that it is the most prevalent and widespread pathogenic species for livestock in tropical Africa (Peacock et al., 2012). *T. congolense* has a complex life cycle: bloodstream forms (BSF) proliferate in the blood of the infected mammalian host and are ingested by tsetse during the blood meal, procyclic forms (PCF) differentiate in the insect midgut, migrate to the proboscis (mouth parts) where they attach as epimastigote forms (EMF) and finally differentiate into infective metacyclic forms (MCF) that are transmitted to a new mammalian host during the next blood meal (Coustou et al., 2010). When the fly takes up bloodstream form trypanosomes, the initial establishment of mid-gut infection and invasion of the proventriculus is much the same in *T. congolense* and *T. brucei*.

Table 1.1 Outline of classification as adapted from (Corliss, 1994; Imam, 2009)

Kingdom
Protista

Subkingdom
Protozoa

Phylum
Sarcomastigophora

Subphylum
Mastigophora

Order
Kinetoplastida

Family
Trypanosomatidae

Genus
Trypanosoma

Section	**Subgenus**	**Species**
Salivaria	Nannomonas	*T. (N.) congolense, T. (N.) simiae, T. (N.) godfreyi*
	Duttonella	*T.(D.) vivax*
	Trypanozoon	*T.(T.) brucei*
		T.(T.) b. brucei
		T.(T.) b. gambiense
		T.(T.) b. rhodesiense
		T.(T.) equiperdum
		T.(T.) evansi
	Pycnomonas	*T.(P.) suis*
Stercoraria	Schizotrypanum	*T.(S.) cruzi*
	Megatrypanum	*T.(M.) theileri*

However, the developmental pathways subsequently diverge, with production of infective metacyclics in the proboscis for *T. congolense* and in the salivary glands for *T. brucei* (Peacock et al., 2012) (Figure 1.1). Coustou et al. (2010) recently devised a complete in-vitro life cycle model of *T. congolense* which offers the opportunity to perform functional genomics analysis like RNA interference (RNAi) throughout the whole life cycle.

1.4 Pathology and pathogenesis

Most trypanosomatid parasites including *T. congolense* have both arthropod and mammalian hosts. In addition, the ability to survive and complete a developmental cycle in each of these very different environments is essential for life cycle progression and hence for being a successful pathogen. For African trypanosomes, where the mammalian stage is exclusively extracellular, this presents specific challenges and requires evasion of both the acquired and innate immune systems, together with adaptation to a specific nutritional environment and resistance to mechanical and biochemical stresses (Gadelha et al., 2011).

Following the successful feeding of an infected tsetse fly, metacyclic trypanosomes become established in the skin. Initial replication of trypanosomes is at the site of inoculation in the skin; this causes a swelling or chancre (FAO, 1998). Local skin reaction can develop in human as well as domestic animals. Trypanosomes then spread via draining lymphatics to the lymph nodes and blood and continue to replicate. During the acute phase of trypanosomosis, changes in vascular bed are the most striking. These changes in vascular bed include thrombi formation, thrombocytopenia, vasculitis in varying degrees and formation of vasoactive substances. Antibodies elicited by the glycoprotein coat of the trypanosome kill the trypanosome and result in the development of immune complexes.

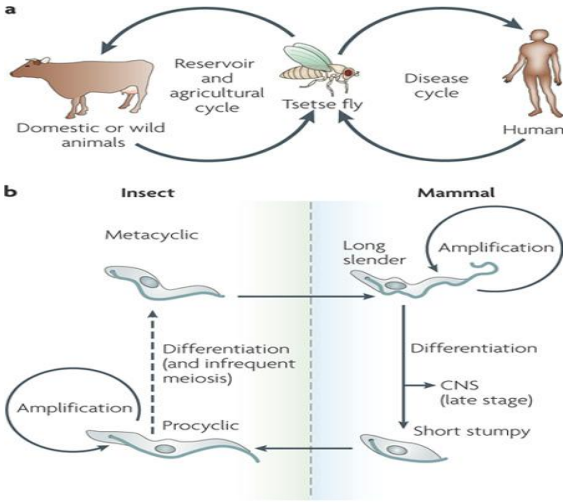

Figure 1.1 Life cycle of trypanosome.

a) Dual cycle between the tsetse fly, humans and animal reservoirs. The animal reservoirs encompass both sylvatic (native wild and domesticated agricultural animals, providing a double challenge to control. b) Simplified life cycle emphasizing the alteration between amplification and differentiation. Infected mammals harbour high numbers of dividing long slender forms of the parasites in their bloodstream and tissue spaces. Long slender forms differentiate to a short stumpy form, which is pre-adapted to the insect vector and is growth arrested. At some point invasion of the central nervous system (CNS) occurs, with serious clinical implications. Transfer of the short stumpy form to the insect vector in a blood meal is accompanied by rapid differentiation to the procyclic form, which re-enter the cell cycle. Multiple differentiation stages, with a non-obligatory meiotic and sexual phase, are required to generate the mammalian infective metacyclic forms (not shown). Metacyclic forms reside in the salivary glands and are also growth arrested. Transfer of these parasites back to a mammalian host during feeding completes the life cycle. Adapted from Field and Carrington (2009).

Inflammatory processes occur in various organs with meningitis as the first lesion in the central nervous system, which can gradually result in meningo-encephalitis. Other organs which can be involved during trypanosomosis are the eye, endocrine organs and pituitary gland which can lead to severe hormonal imbalances in human and animals of both sexes (Zwart, 1989). In calves experimentally infected with *T. congolense* the most significant findings were enlargement of the liver, kidneys and spleen and increased volume of hematopoietic marrow at postmortem examination (Valli et al., 1978). Anaemia in human and animals is of major clinical importance in trypanosomosis. Moreover, glomerulonephritis and immunosuppression have been well documented in the pathogenesis of trypanosomosis. Immunologic lesions are significant in trypanosomosis and it has been suggested that many of the lesions in these diseases may be the result of the deposition of immune complexes that interfere with or prevent, normal organ function (Zwart, 1989).

1.5 Clinical signs of trypanosome infections

Most cases of trypanosomosis are chronic, but acute disease, which may be fatal within a week, can also occur. The first sign of trypanosomosis may be a localized swelling (chancre) at the site of the fly bite, but this usually remains unnoticed. The primary clinical signs are intermittent fever, signs of anemia, lymphadenopathy and weight loss. Animals lose condition and become progressively emaciated. Milk yield may be decreased in dairy animals. Neurological signs, dependent edema, cardiac lesions, diarrhea, keratitis, lacrimation, appetite loss and other clinical signs have also been reported. Effects on reproduction include abortions, premature births and perinatal losses, as well as testicular damage in males. Deaths could be possible among untreated chronically infected animals and animals that recover clinically may relapse when stressed. Sudden deaths have been reported in small ruminants infected with *T. vivax*. Trypanosomes can cause immunosuppression and concurrent infections may complicate the disease (CFSPH, 2009).

1.6 Epidemiology

1.6.1 Hosts

The tsetse fly is unique to the African continent and transmits a parasite harmful to humans and lethal to livestock (Alsan, 2013). The Tsetse fly (*Glossina*) is the biological vector and wild animals are natural hosts. Wild animals susceptible to trypanosome infection include deer, antelope, African buffalo, wild Equidae, lions, leopards, warthogs, elephants, nonhuman primates and various rodents (CFSPH, 2009). Domestic animals are incidental hosts and cattle are the most important economically. However, anthropogenic changes (e.g. clearing of vegetation for settlements and agriculture, habitat fragmentation) can alter the interaction between the host, the parasite and the vector (Van den Bossche et al., 2010). In humans the disease is termed sleeping sickness and is caused by *T. brucei gambiense* and *T. brucei rhodesiense*. The animal trypanosomes very rarely cause human infection, but they do share animal reservoirs (wild and domestic) and tsetse vectors (OIE, 2013b). However, Wissmann et al. (2014) reported an association between the presence of HAT in a village and infection of cattle village with human infective parasites. In Tanzania, Hamill et al. (2013) documented pigs carrying the human infective sub-species *T. b. rhodesiense* using PCR based method. Furthermore, Desquesnes et al. (2013a) reviewed zoonotic aspects of *T. evansi*.

1.6.2 Transmission of trypanosome infection

Cyclical transmission: trypanosomes are transmitted through the bite of an infected tsetse fly. Tsetse flies get the infection when feeding on an infected animal; after implementation of the parasitic cycle during which the trypanosomes actively multiply in the fly (15–21 days) it becomes infective and may remain infective for the rest of its life (FAO, 1983). Transmission occurs in the early stage of the blood feeding, when the fly injects some saliva before sucking the blood of its host (CFSPH, 2009). This form of transmission occurs with *T congolense*, *T. vivax*, *T. simiae*, *T. brucei* and the trypanosomes responsible for human sleeping sickness, *T. brucei gambiense* and *T. brucei rhodesiense* (Morlais et al., 1998). *Glossina* spp. are strictly blood feeders living exclusively in tropical Africa. There are about thirty species or subspecies,

classified in three groups: palpalis, morsitans and fusca. Each species has distinct biological characteristics, but in general it may be said that the palpalis group consists basically of the species living in forest galleries or in the marginal areas of forests; the fusca group consists of large-sized species whose habitat is generally associated with equatorial forests; and the morsitans group consists mainly of species living in wooded savanna (FAO, 1983; Rogers and Randolph, 1993).

Mechanical transmission: Biting flies, especially tabanids and stomoxes, but possibly other biting insects (including tsetse flies) are the mechanical vectors of *T. vivax*, *T. congolense* and *T. evansi*. Mechanical transmission can occur when interrupted feeding is re-started on a new host; thus it is efficient inside a group of animals but has little chance to occur at distance. Transmission of trypanosomes is effected provided that the interval between the two meals is short (Desquesnes and Dia, 2003; FAO, 1983). Vertical transmission can occur intra-utero and during partum. For *T. evansi*, contamination of wound and iatrogenic transmission caused by use of non-sterile surgical instruments could spread the disease (Desquesnes et al., 2013a).

1.7 Occurrence of the disease

African animal trypanosomosis occurs where the tsetse fly vector exists in Africa, between latitude 15°N and 29°S. *T. vivax* can also be transmitted mechanically by biting flies and thus is also found in parts of Africa free or cleared of tsetse and parts of Central and South America (CFSPH, 2009). *T. evansi* is found in Africa only in the Saharan and Sahelian regions where it is primarily a camel parasite, but it may be a parasite of horses, cattle and dogs as well. It also occurs in Asia, where it commonly causes disease in camels and horses. In Central and South America, it causes disease less commonly in cattle, water buffaloes, elephants and dogs. Thus it has a very wide distribution (Desquesnes et al., 2013b; FAO, 1983).

1.8 Development activities versus tsetse and trypanosomes dynamics

The distribution and incidence of vector-borne diseases are affected by complex interactions between pathogen(s), vector(s) and host(s). In addition to genetic and phenotypic interactions between these factors, the population of vectors and hosts are also modulated by local environmental factors that can substantially affect their dynamics and influence disease transmission patterns and disease impact (Van den Bossche et al., 2010). Furthermore, changes in land cover and land use can also have a direct or indirect impact on the biodiversity of hosts and vectors and on the various interfaces between vectors and hosts including wildlife, livestock and humans (Reid et al., 2000; Van den Bossche et al., 2010). Briefly, anthropogenic land use changes can cause infectious disease outbreaks and re-emergence of the disease, and also modify the transmission dynamics in endemic regions. These changes include agricultural encroachment, deforestation, road construction, dam building, irrigation, wetland modification, mining, the concentration or expansion of urban environments and coastal zone degradation. Such changes in turn cause a cascade of factors that exacerbate the infectious disease burden (Patz et al., 2004).

Countries in East Africa in an early stage of development depend heavily on the agricultural sector for the increase in employment, foreign exchange, government revenue and food (Lele, 1989). Thus, the continuous development pattern in East Africa directly and/or indirectly affects trypanosomosis dynamics in many ways. Massive activities in the area of agricultural land expansion and development of infrastructures like road and residence are all happening at the expense of environmental destruction. Population growth, loss of herding lands to farmers, ranchers, game parks and urban growth, increased commoditization of the livestock economy, out-migration by poor pastoralists and dislocations brought about by drought, famine and civil war are increasing throughout East Africa. These problems are intensified as international development programs encourage privatization and individualization of formerly communally held resources (Fratkin, 2001).

Habitat fragmentation is one repercussion of development activities in individual countries and across the region. It has been indicated that habitat loss has a pervasive and disruptive impact on biodiversity in habitat remnants. The magnitude of the ecological impacts of habitat loss can be exacerbated by the spatial arrangement or fragmentation of the remaining habitat. Fragmentation in se is a landscape-level phenomenon in which species that survive in habitat remnants are confronted with a modified environment of reduced area, increased isolation and novel ecological boundaries. The implications of this on individual organisms are many and various, because species with differing life history strategies are differentially affected by habitat fragmentation (Ewers and Didham, 2006). Changes in agriculture, destruction of rain forest by building roads, clearing areas for commercial ventures further enhance peoples' encounter with the vectors and other animals harboring trypanosomes thereby increasing the rate of transmission of the disease (Samdi et al., 2012). In eastern Zambia Ducheyne et al. (2009) reported that heavily fragmented areas have lower numbers of tsetse flies, but when the fragmentation of natural vegetation decreased, the number of tsetse flies increased following a sigmoidal-like curve. In another study in Panama, Cottontail et al. (2009) stated that anthropogenic influence on ecosystems such as habitat fragmentation has impact on species diversity and interactions. In their study trypanosome prevalence was significantly higher in bats from forest fragments, than in bats captured in continuous forest.

Fly habitat reduction by anthropogenic factors is sometimes found in situations where pressure on land use is high (Mattioli et al., 2004). For instance, gradual encroachment of people and their livestock into inhabited areas and the subsequent alteration of the landscapes have been explained to be the major drivers of change in the development of an endemic livestock trypanosomosis situation (Van den Bossche et al., 2010). However, even in these scenarios tsetse encroachment often continues to curtail crop-livestock integration. This has been particularly the case for riverine tsetse fly species that often are able to become peri-domestic and accept irrigation schemes as new habitat and change host preference from reptiles to livestock and man (Mattioli et al., 2004). Because of this increasing host and reservoir role of

livestock, parasite transmission is gradually changing from a dominant 'sylvatic' to a dominant 'domestic' cycle (Van den Bossche et al., 2010).

1.9 Current trypanosomosis situation in Ethiopia

Trypanosomosis in cattle, locally referred as "Gendi", is a serious constraint to livestock in areas of northwest and southwest Ethiopia at an altitude of below 2000 meters above sea level (m.a.s.l.) (Getachew, 2005). This disease has been reported in different animals from different parts of Ethiopia. For instance, typanosomosis prevalence was 6.3% in donkeys from Assosa and Homosha districts in Benishangul Gumuz Regional state (Abebe and Wolde, 2010), 5.85% in cattle from Western region (Dinsa et al., 2013), 5.6% in small ruminants from Awi zone (Kebede et al., 2009), 12.42% in cattle from Metekel and Awi zones (Mekuria and Gadissa, 2011), 8.2% in cattle from East Gojam zone (Mihret and Mamo, 2007), 4.4% in cattle from two districts of Bench Maji zone (Tadesse and Tsegaye, 2010), 17.2% in cattle from Metekel district of Northwest Ethiopia (Afewerk et al., 2000) and 8.57% in cattle from three districts of Western Oromia (Tasew and Duguma, 2012).

1.10 Diagnostic methods

The incubation period is 1-4 weeks and the disease in cattle usually has a chronic course with possible mortality, especially if there is poor nutrition or other stress factors (Aiello and Moses, 2012). There are a number of techniques available for the diagnosis of animal trypanosomosis and each has its own limitations and advantages. The diagnosis is made through laboratory methods, because the clinical features of infection are not sufficiently specific (CDC, 2012).

1.10.1 Clinical diagnosis

The clinical signs of disease caused by these organisms vary, depending on the trypanosome species, the virulence of the particular isolate, age, and the species of host infected (Aiello and Moses, 2012; FAO/IAEA, 1993). The disease is classically acute or chronic and is affected by

poor nutrition, concurrent diseases and other stressors. Some animals may slowly recover but usually relapse when stressed for example by work, pregnancy, milking or adverse environmental conditions (FAO/IAEA, 1993). Thus, trypanosomosis is generally a chronic evolving disease which could be fatal if appropriate treatment is not established. The most important clinical sign is non-regenerative anaemia which is the most common why reason animals are unable to function normally. The major clinical signs are: intermittent fever, anaemia, oedema, lacrimation, enlarged lymph nodes, abortion, decreased fertility, loss of appetite, body condition and productivity, early death in acute forms, emaciation and eventual death in chronic forms often after digestive and/or nervous signs (Eisler et al., 2004; FAO, 1983; Getachew, 2005).

1.10.2 Post-mortem lesions

Necropsy findings vary and are non-specific (Aiello and Moses, 2012; CFSPH, 2009). Acute cases are usually related to anaemia and prolonged antigen-antibody response: emaciation and serous atrophy of fat, enlarged lymph nodes, liver and spleen, excessive fluid in the body cavities and subcutaneous oedema and petechial haemorrhages. In chronic cases, lymphoid tissue may be atrophic or swollen with serous atrophy of fat and anemia. In the terminal phases, severe myocarditis is common as the animal is too debilitated to mount an immune response (Aiello and Moses, 2012; CFSPH, 2009).

1.10.3 Laboratory diagnosis

The choice of which test to use for the diagnosis of trypanosomosis depends first of all on the level at which the test is to be used (farmer, veterinary diagnostic laboratory, research institute) and the purpose for which the results obtained will be used (FAO/IAEA, 1993). Definitive diagnosis of the disease is dependent on the detection of the trypanosome in blood samples (Getachew, 2005). The more accurate the diagnosis of infections can be, the more targeted interventions can become and the greater the reduction in the risk of human disease. Selective treatment of infected animals in this way results in a holistic approach to disease

control, concurrently combating livestock and human disease agents and resulting in an overall improvement in the livestock and human health of rural populations (Picozzi et al., 2002).

1.10.4 Parasitological methods

The diagnosis is based on finding the parasite in body fluid or tissue by microscopy (CDC, 2012). Parasitological tests such as wet blood smears, Giemsa stained thin and thick blood smears, the micro haematocrit centrifuge technique (Woo method) (Woo, 1970), the buffy coat darkground-phase contrast technique (Murray et al., 1977) and miniature-anion exchange centrifugation technique (Lumsden et al., 1977) can be used to confirm a clinical diagnosis by demonstrating the presence of trypanosomes. Freshly collected blood can also be inoculated into laboratory mice which can then be examined for periods of up to 30 to 60 days to determine if trypanosome infections develop in them (FAO/IAEA, 1993).

The examination of wet blood films and Giemsa stained thick and thin fixed blood films with the aid of the light microscope have been used as diagnostic methods ever since they were first used to identify the aetiological agents of trypanosomosis. In wet preparations, a drop of fresh blood is placed on a slide and covered with a glass slip. The slide is examined under a microscope and the trypanosome species is determined by the type of movement they produce among the erythrocytes. The detection limit of this technique is $8.3x10^3$ trypanosomes per ml of blood (Eisler et al., 2004; Getachew, 2005; Paris et al., 1982).

The microhaematocrit centrifuge technique (HCT), about 70µl of blood is collected in a microhaematocrit capillary tube and sealed on one end. The capillary tube is centrifuged at 15,000 rpm for 5 minutes to concentrate the trypanosomes in the buffy coat layer. Then, the capillary tube is placed in a Woo viewing chamber and a cover slip of 24x24 mm is placed on the capillary tube. The space between the tubes and the cover slip is filled with a drop of water to reduce light diffraction. The buffy-coat plasma junction is examined for the presence of trypanosomes and species is identified through the type of their motility. The detection limit of

this technique is $5.2x10^2$ trypanosomes per ml of blood (Eisler et al., 2004; Paris et al., 1982; Woo, 1970).

Each test has been reported to have its own merits and demerits. According to Marcotty et al. (2008), the buffy coat method fails to detect 66% of the infected animals despite the sometimes high concentration of the parasite. However, in the absence of other diseases causing anaemia in individual animals, the findings of their study also indicated that the PCV-value of an individual animal is a good indicator of the presence of a trypanosomes infection. Hence, in the absence of other factors causing anaemia, the use of PCV and its parallel combination with the parasitological diagnosis could improve the detection of trypanosome-infected animals and ease the decision to treat them with a trypanocidal drug (Marcotty et al., 2008).

1.10.5 Serological tests

Most serological tests offer an increased diagnostic sensitivity and specificity. Such tests are either capable of detecting anti-trypanosome antibodies, such as the indirect fluorescent antibody test (IFAT) and the antibody-detection enzyme-linked immunosorbent assay (Ab-ELISA), or capable of detecting antigen fractions of the parasites (Ag-ELISA) (Eisler et al., 2004; FAO/IAEA, 1993). However, serological tests have no use in the early stages of an infection, before the rise of specific antibodies. There is thus a need for simple tools that improve the sensitivity of the parasitological diagnosis, particularly in chronic clinical cases and hence support the management of trypanosomosis (Marcotty et al., 2008). Serological tests based on antibody detection cannot differentiate between an active and a cured infection.

1.10.6 Molecular Tests

Diagnostic problems could be resolved by using molecular diagnostic tests though it has limitations such as the need for sophisticated and expensive infrastructure, and results become available only after a considerable delay (Eisler et al., 2004; Marcotty et al., 2008). However,

DNA based methodologies have been in use in different parts of the world for diagnosis of diseases (Picozzi et al., 2002). Detection of trypanosome DNA by PCR has resulted in a considerable improvement of species-specificity in the diagnosis of these parasites, in particular when compared to parasitological examination, which is limited in its specificity to the sub-genus level. In addition, it has increased the sensitivity of diagnosis which is generally two to three times higher than direct parasitological techniques (Desquesnes and Dávila, 2002; Desquesnes et al., 2001).

Rudramurthy et al. (2013) developed an invariant surface glycoprotein-75 (ISG-75) gene based PCR assay that amplifies a 407 base pairs (bp) product specifically from the different *T. evansi* isolates. Cox et al. (2005) reported a PCR technique that targets the gene encoding the small ribosomal subunit in order to identify and differentiate all clinically important African trypanosome species and some subspecies. Desquesnes et al. (2001) successfully used a single PCR based on internal transcribed spacer 1 (ITS-1) of rDNA for detection and identification of *Trypanosoma* of African livestock including *T. congolense* types. Furthermore, Geysen et al. (2003) developed a semi-nested PCR-RFLP approach using 18S small sub-unit ribosomal (Ssu-rDNA) gene amplification as an easy method for species-specific diagnosis of *Trypanosoma* species in cattle. Recently, *Trypanosoma vivax* specific PCR based on *T. vivax* proline recemase (TvPRAC) gene was developed and proved to be specific for *T. vivax*, irrespective of its geographical origin. However, its analytical sensitivity was reported to be lower than that of ITS-1 PCR (Fikru et al., 2014).

DNA techniques provide tests with high sensitivity to such a level that one single trypanosome can be detected in samples of whole blood. Nevertheless, microscopy remains useful particularly as it can be carried out directly in the field level and gives immediate results. The original molecular protocols were not immediately applicable to conditions in the field, due to the amount of processing required and the time involved prior to the testing of the samples themselves. However, developments in methods of extracting and/or preserving DNA have changed this, making sample collection with a view to diagnosis by PCR at the field level more

realistic (Picozzi et al., 2002). For instance, easy collection, storage or transfer of blood spots or buffy coats on filter paper has been successfully used for PCR-RFLP detection of *T. congolense* (Vitouley et al., 2011).

1.11 Treatment of trypanosomosis

It has been indicated that in the absence of an effective vaccine, the control of trypanosomosis has to depend on the use of trypanocidal drugs, trypanotolerant breeds of livestock, tsetse traps and insecticides (Holmes, 1997). However, the use of trypanocidal drugs to treat trypanosomosis in livestock has been and still is the main approach in controlling this disease in most African countries. The method is complicated by the fact that the efficacy of the currently available drugs is diminishing fast in some areas due to the development of drug resistance (FAO/IAEA, 1993; Holmes et al., 2004).

Since 1938, the date of the discovery of the trypanocidal properties of the phenanthridines, trypanocidal drugs remained popular with farmers because they allow treatment of individual animals, they are relatively cheap and commonly available. Reports indicate that approximately 35 million doses of trypanocidal treatments are used each year in Africa (Geerts and Holmes, 1998; Holmes and Tort, 1988). Currently available trypanocidal drugs for use in domestic livestock are: homidium salts (Ethidium-Novidium), diminazene aceturate (Berenil), isometamidium (Samorin, Trypamidium) (Aiello and Moses, 2012; FAO, 1983; Geerts et al., 2001; Holmes et al., 2004), quinapyramine sulfate (Antrycide), suramin sodium (Aiello and Moses, 2012; FAO, 1983) and melarsomine (Cymelarsan) (Holmes et al., 2004). The use of quinapyramine in cattle is discontinued due to its capacity to induce multi-drug resistance (Holmes et al., 2004). Livestock farmers in Ghibe valley most commonly use the trypanocidal drugs DA (Diminazene aceturate) and ISM (Isometamidium chloride) of diverse brands to control trypanosomosis in almost all sick animals without any clinical examination.

Against this widespread use of chemotherapy for control of trypanosomosis, trypanocidal drug resistance has been reported from different parts of Ethiopia. Multiple drug resistant *T. congolense* populations to DA and ISM were reported in naturally infected cattle of Metekel district (Afewerk et al., 2000). *T. congolense* populations resistant to diminazene aceturate (Berenil®), isometamidium chloride (Samorin®) and homidium chloride (Novidium®) were also reported in cattle from Ghibe (Mulugeta et al., 1997). *T. congolense* isolates resistant to DA and ISM were found in Ghibe, Bedelle and Sodo (Chaka and Abebe, 2003). Tewelde et al. (2004) reported isometamidium resistance in cattle from upper Didessa valley of western Ethiopia. All of these reports employed either a mice experiment model or block treatment in field conditions for the diagnosis of trypanocidal drug resistance.

1.12 Prevention and control of trypanosomosis

Control and prevention of trypanosomosis involves different techniques in different parts of the world. The selection of techniques and the sequence in which they should be applied, however, depends on the prevailing conditions. Some of these conditions include the size of the area, tsetse distribution patterns and abundance, tsetse vulnerability, livestock systems, agricultural practices, livestock breeds, availability of infrastructure and capacity; availability of and access to intervention technologies and market demands and trade opportunities (Ilemobade, 2009).

1.12.1 Sanitary prophylaxis

African Animal Trypanosomosis (AAT) can be controlled by reducing or eliminating tsetse fly populations by traps, insecticides applied directly on the animal as a spray or pour-on (CFSPH, 2009). The sterile male technique does not require insecticide and is environmentally benign (Allsopp, 2001). This technique is potentially valuable since females mate only once in a lifetime but production facilities are expensive and can only be applied at the end of the eradication campaign, when the density of remaining flies is very low. In addition, good husbandry of animals at risk and avoiding contact with tsetse flies as much as possible are common practices.

Introduction and selection of trypanotolerant breeds like the N'Dama and West African Shorthorn are also advocated (OIE, 2013b).

1.12.2 Chemoprophylaxis

Trypanocidal drugs are a widespread method of control in most African countries. However only three drugs are available for use against tsetse transmitted trypanosomosis, principally in cattle. They are diminazene, isometamidium and homidium (Holmes, 1997). Isometamidium chloride can be used as both curative and prophylactic drug whereas diminazene aceturate as curative. In addition, quinapyramine sulphate and chloride are available on market, but should not be used in ruminants as it causes cross-resistance to all available trypanocidal drugs (Geerts and Holmes, 1998). Chemoresistance may occur and care must be taken due to the presence of fake drugs on some markets. No vaccines are available nor likely to be there in the near future because of the ability of trypanosomes to rapidly change variable surface glycoproteins (VSG) in their coats to avoid an effective Immune response (antigenic variation) (Davila et al., 2003).

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General and specific objectives

Ghibe/Omo is one of the major river basins in Ethiopia. The Ghibe River flows in a southerly direction in this valley and is a tributary of the Omo River, flowing into Lake Turkana on the Ethiopia/ Kenya border. The valley base lies at an altitude of approximately 1100 m (Leak et al., 1996). In and around the valley a significant number of farmers make their living by a mixed crop-livestock production system. Trypanosomosis and land use are closely inter-related (Holmes, 1997). Evidently in trypanosomosis endemic areas like Ghibe, the most important indirect impact of trypanosomosis on crop production is through the availability and health of animals that provide animal traction. Because additional traction capacity allows farmers to expand the area that they cultivate, they can thus increase yields of existing crops, grow a different mix of crops or allocate labour, land and fertilizer more efficiently. Obviously, without effective drug therapy, the incidence of trypanosomosis increases and can thus devastate herds of trypano-susceptible cattle and the farming systems in which those cattle are integrated (Swallow, 1999).

Tsetse and trypanosomosis research and intervention in Ghibe Valley of South-western Ethiopia has started long time back (Codjia et al., 1993; Leak et al., 1996; Rowlands et al., 1999) and yet the disease continues to have an impact on both the agricultural and animal production sectors. In this regard, the development of drug resistance takes the lion's share among factors contributing to reduced efficiency of ongoing control and prevention programs because control and prevention has been almost entirely dependent on chemotherapy and chemoprophylaxis. In view of today's trypanocidal drug resistance reports from various parts of the tsetse belt in Ethiopia, there is an urgent need for an in-depth study of trypanocidal drug resistance development. Furthermore, resistant *T. congolense* strains circulating in indigenous cattle should be characterized. Insights in the resistance pattern in Ghibe river basin of Ethiopia are required; this will be helpful to formulate effective intervention protocols to be applied on a large scale.

General objective

The general objective of this PhD dissertation is to assess the magnitude of *T. congolense* trypanocidal drug resistance using different tests and survey the perception of drug use by farmers in Ghibe valley of Ethiopia.

Specific objectives

This study was undertaken for the following specific objectives:

i. To review literature on trypanocidal drug resistance in east African countries mainly Ethiopia, Kenya, Uganda and Tanzania (**Chapter 2)**.

ii. To assess the occurrence of DA and ISM resistance on *T. congolense* isolates using a mice-based experimental model and to compare it with a PCR-RFLP method (**Chapter 3**).

iii. To compare the conventional haematocrit centrifugation technique with PCR on different sample storage conditions (**Chapter 4**).

iv. To study *T. congolense* infection dynamics over a period of 6 months (longitudinal study) in high prevalence areas of Ghibe valley using microsatellite markers in light of drug resistance (**Chapter 5**).

v. To conduct a questionnaire survey that generates information on knowledge, attitude and practice of livestock owners towards trypanocidal drug use (**Chapter 5**).

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Chapter 2

Trypanocidal drug resistance in East Africa: A review

2.1 Background and justification

Botswana, Ethiopia, Eritrea, Kenya, Malawi, Swaziland, Madagascar, Mozambique, Sudan, Tanzania, Uganda, Zambia and Zimbabwe form together East Africa (Figure 2.1). Arable areas in the region are under severe pressure to increase their productivity in order to feed a rapidly increasing human population (IFPRI, 2013). In fact the potential for increasing livestock production can only be fully realized if the animals are adequately protected against the adverse effects of periodic stresses and diseases (Samdi et al., 2010).

Trypanosomosis in domestic livestock negatively impacts food production and economic growth in many parts of the world, particularly in sub-Saharan Africa (SSA) (Taylor, 1998). It has been said that a large proportion of the world's poorest people struggles to exist in tsetse-infested SSA (Allsopp, 2001) which encompass East Africa. In the tsetse-infested countries of East Africa, half of the population suffers from food insecurity. It has been documented that approximately 85% of the poor are located in rural areas out of which over 80% relies on agriculture for their livelihood (Mattioli et al., 2004). For obvious reasons, the agricultural segment in this region is dependent on animal traction power.

Endemic animal diseases such as tsetse-transmitted trypanosomosis are a constant drain on the financial resources of African livestock keepers and on the productivity of their livestock (Shaw et al., 2014). African trypanosomosis is a disease complex unique to SSA, affecting both humans (sleeping sickness) and animals (nagana or African animal trypanosomosis (AAT). It is caused by a protozoan blood parasite, the trypanosome, and is transmitted by the tsetse fly (Glossina) which is confined to SSA (Ilemobade, 2009). The causative agent, the trypanosome, can be found wherever the tsetse fly vector exists. Tsetse flies have been reported to be endemic in Africa between latitude 15° N and 29° S, from the southern edge of the Sahara desert to Zimbabwe, Angola and Mozambique.

Figure 2.1 Map of East African countries. (Source: http://wikitravel.org/upload/shared//3/3f/East_Africa_regions_map.png).

Trypanosomes, particularly *T. vivax,* can spread beyond the "tsetse fly belt" by transmission through mechanical vectors such as *Tabanus spp* and other biting flies. For instance, *T. vivax* is found in South and Central America and in the Caribbean, areas free of the tsetse fly (Aiello and Moses, 2012; CFSPH, 2009). In Africa, the most important trypanosome species from both medical and veterinary point of view are those transmitted by tsetse flies of the African genus Glossina (FAO/IAEA, 1993).

African trypanosomosis has been considered as a re-emerging infection of epidemic proportion as the necessary control programs have not been maintained due to a variety of social, political and economic reasons. It has not been possible to use novel control tools in these regions because of political unrest and economic constraints (Seed, 2001). Though Tsetse and Trypanosomosis (T and T) is one of the trans-boundary insect pest and disease problems that constitute a key bottleneck to sustainable agriculture and rural development (SARD) in SSA, including East Africa (Ilemobade, 2009), no strong regional collaboration exists to implement available control strategies. In addition, there are fragmented reports of trypanocidal drug resistance development in different areas across the region. Therefore the objective of this review paper is to discuss the situation of trypanocidal drug resistance in East African countries with emphasis on Ethiopia, Kenya, Uganda and Tanzania.

2.2 Trypanocidal drugs and their use in East Africa

Currently, only three trypanocides are available for controlling tsetse-transmitted trypanosomosis in domestic ruminants and these are isometamidium (ISM) and homidium, which have both prophylactic and therapeutic effects, and diminazene, which has only therapeutic properties (Geerts et al., 2001). Of these compounds, the most widely used therapeutic agent in cattle, sheep and goats is diminazene aceturate (DA). In camels, melarsomine is used for treatment of *T. evansi* infection (Holmes et al., 2004). Details of these trypanocidal drugs are summarized in Table 2.1.

Diminazene was first described in 1955 (Peregrine and Mamman, 1993). In addition to its trypanolytic effects, recently it has been shown that it also modulates the host immune response by inhibiting key signaling molecules and transcription factors associated with pro-inflammatory cytokine production (Kuriakose and Uzonna, 2014).

Table 2.1 Trypanocidal drugs in use. Adapted from Desquesnes et al. (2013) and Holmes et al. (2004)

Trypanocidal drugs	Trade name	Method of treatment		Indications		Toxic effects			Treatment of relapses
		Dosage (mg/kg)	Injection*	Highly active on**	Less active on	Good tolerance	Possible local reactions	Possible general reactions	
Diminazene aceturate***	Berenil	3.5	SC or IM	*T.c.* *T.v.*	*T.b.* *T.e.*	Cattle Sheep Goats	Horses	Horses Camels	Isometamidium
Quinapyramine sulfate	Antrycide (sulfate)	5	SC	*T.c.* *T.v.* *T.b.* *T.e.*		Camels	Horses		Isometamidium
Isometamidium chloride	Samorin, Trypamidium	0.25 to 1	IM (deep)	*T.v.* *T.c.*	*T.b.*	Cattle Sheep Goats Horses	Cattle		Diminazene
Suramin sodium		10	IM	*T.e.* *T.b.*		Camels Horses			Quinapyramine
Melarsomine dihydrochloride	Cymelarsan	0.25 0.25-0.5	IM	*T.e.*		Camels Horses			

*im = intramuscular injection: sc = subcutaneous injection; ** T.c. as *T. congolense*; T.v. as *T. vivax*; T.b. as *T. brucei* and T.e. as *T. evansi*; *** The recommended dose for T.b. and T.e. is 7 mg/Kg

Diminazene aceturate is marketed a.o. under the trade names Azidine, Berenil, Ganaseg, Ganasegur and Veriben as both a trypanocide and babesiacide for domestic livestock (Peregrine and Mamman, 1993).

Isometamidium (Samorin, Trypamidium) was introduced on the market in 1961 and little is known about its mechanism of action (Boibessot et al., 2006) except for its DNA intercalating properties (Wainwright, 2010) and thus mutagenic effects. Its main advantage is its effectiveness on trypanosomes resistant to other drugs. At the same time it has the disadvantage of easily creating drug-resistant strains itself; however, these trypanosomes show no cross-resistance with DA, which therefore retains its effectiveness on such strains. DA and ISM constitute a "sanative pair" which means that once resistance develops to one of the drugs, the other drug should be used to control the infection (Desquesnes et al., 2013). ISM can provide up to 6 months protection against tsetse challenge. The ISM deposit at the injection site can cause a persistent local reaction which may be invisible from the outside if a deep intramuscular injection has been given, as is recommended (FAO, 1983).

The appropriate use of the few available trypanocidal drugs is the coordinated effort of livestock owner, private and government bodies. All of them have their role to play in avoiding drug resistance, as is described below.

2.2.1 The livestock owner

Trypanocidal drugs are freely available in different shops of rural Africa and farmers have full access to get them for treatment of their animals. As there has been neither guideline nor awareness on when to use, to which animal, how much to use and how to administer, treatment failure is likely to occur in a high proportion of animals. The failure of drug treatment might have causes other than drug resistance such as significant under-dosing, treatment with sub-standard quality drugs, or rapid re-infection after treatment (Geerts et al., 2001). A study indicated that giving rational drug use information to farmers improved their knowledge and

management of trypanosomosis as well as the clinical outcome in cattle they treated and had no discernible negative impacts (Grace et al., 2008).

2.2.2 The political level

In the past, the availability of trypanocidal drugs was strictly controlled in most African countries by Government Veterinary Departments. However, in recent times, with the privatization of veterinary services and a general trend towards deregulation of markets, trypanocides, along with many other pharmaceutical products, have become more freely available through local pharmacists, agro-veterinary suppliers and the informal sector and many are now purchased directly by farmers (Geerts et al., 2001).

In principle, government regulatory agencies have the important responsibility to ensure that only safe, effective, high-quality, well-manufactured and properly labeled animal drugs and biologicals are available on the market and that unsafe and ineffective products, such as counterfeit and illegally compounded medicines are not available on the market (Smith, 2013). Currently, however, no effective regulatory programs seem to be in place in East Africa. Because of this inefficient or absent regulatory program, trypanocidal drugs are misused by farmers and even paraprofessionals. It also paved the way for some illegal production units to flourish and produce counterfeit and substandard drugs. Counterfeit drugs are those that mimic authentic drugs; substandard drugs are those produced with little or no attention to good manufacturing practices (Pecoul et al., 1999). Treatment failure and drug-resistance are thought to be possible consequences of the use of substandard drugs (Shakoor et al., 1997).

2.3 Trypanocidal drug resistance reports from various parts in East Africa

The widespread and often unregulated use of currently available drugs, both as treatment and in prophylaxis, has inevitably led to the development of drug resistance (Geerts et al., 2001). Some cases of resistance were observed early in the use of DA. It was the accepted view at the time that this was the result of cross-resistance with quinapyramine and that DA did not

directly cause resistance because of its rapid elimination through the kidneys, which prevents accumulation of residual sub-curative doses. Since 1967, however, strains of trypanosomes directly resistant to DA have been found in various countries, notably in the Central African Republic, Chad, Kenya, Nigeria and Uganda, primarily with regard to *T. vivax* but also to *T. congolense*. These strains are fortunately still vulnerable to the phenanthridine group of trypanocides, particularly ISM, leading to the conclusion that in case of failure of a DA treatment it is preferable to use another trypanocide such as ISM rather than giving further treatment with an increased dose of DA (FAO, 1983). Various researchers across East Africa reported resistance to currently available trypanocidal drugs and the approaches to the mechanisms of drug resistance in trypanosomes have been extensively reviewed by Alsford et al. (2013) and Delespaux et al. (2008b).

Drug resistant trypanosomes have been claimed to be less virulent. For instance, Egbe-Nwiyi et al. (2005) demonstrated that DA resistance appeared to reduce the virulence of *T. brucei* in naive rats used in their study. However, loss of fitness in drug resistant trypanosomes has not been a consistent finding from the limited number of studies that have been conducted (Holmes et al., 2004). This was never confirmed in cattle but would have important epidemiological repercussions in the field.

2.3.1 Drug resistance in Ethiopia

Trypanocidal drug resistance studies in different areas of Ethiopia used methods such as mice based tests (Afewerk et al., 2000; Chaka and Abebe, 2003; Codjia et al., 1993; Mulugeta et al., 1997), tests in rats (Mulugeta et al., 1997), sensitivity tests in cattle (Afewerk et al., 2000; Chaka and Abebe, 2003; Codjia et al., 1993; Mulugeta et al., 1997; Tewelde et al., 2004) and test in goats (Codjia et al., 1993). Apart from these *in vivo* and field based techniques, Moti et al. (2012) reported high prevalence of DA resistance using the *Dpn*II-PCR-RFLP (see further section 3.5).

Chaka and Abebe (2003) reported that isolates of *T. congolense* from Ghibe, Bedelle and Sodo were resistant to a therapeutic dose of DA (3.5 mg/kg) and to a standard therapeutic and prophylactic doses of ISM (0.5 and 1 mg/kg). However, all three stocks were found to be sensitive to 7 mg/kg DA. The fourth, the Arba Minch stock, was reported resistant to the manufacturers' recommended doses of DA and ISM.

According to Codjia et al. (1993), 12 trypanosome isolates collected from cattle in Ghibe were reported to be resistant to 7.0 mg/kg, 0.5 mg/kg and 1.0 mg/kg body weight of diminazene, isometamidium and homidium chloride, respectively, except for one isolate which was sensitive to 0.5 mg/kg b.w. ISM. In another study, Mulugeta et al. (1997) indicated a long-term occurrence of *T. congolense* resistant isolates to diminazene, ISM and homidium at doses of 7.0 mg/kg, 0.5 mg/kg and 1.0 mg/kg, respectively in cattle from Ghibe valley. Recently, Afewerk et al. (2000) showed the presence of multiple-drug-resistant *T. congolense* in village cattle of Metekel district, northwest Ethiopia. In addition, Tewelde et al. (2004) documented the presence of ISM resistance of trypanosomes infecting cattle in the upper Didessa valley and ISM resistant *T. congolense* has also been reported in naturally infected donkeys (Assefa and Abebe, 2001). Moti et al. (2012) also reported *T. congolense* DA and ISM resistance using a mice model and DA resistance using *Dpn*II-PCR-RFLP in the Ghibe valley of Ethiopia. Besides, there are reports of multiple drug resistance (Afewerk et al., 2000; Codjia et al., 1993; Mulugeta et al., 1997; Tewelde et al., 2004) which need to be taken into consideration during control and prevention using drugs.

2.3.2 Drug resistance in Tanzania

Mbwambo et al. (1988) subjected cattle naturally infected with *T. congolense* to DA treatment and reported *T. congolense* refractory to treatment at 7.0 to 14.0 mg/kg at Kibaha, Tanzania. *T. brucei rhodesiense* strains isolated from sleeping sickness patients in Tanzania showed resistance to melarsoprol at 5 mg/kg and at 10 mg/kg; diminazene at both 14 and 28 mg/kg;

suramin at a dose of 5 mg/kg; ISM at 1.0 mg/kg and one isolate was cross-resistant to suramin and ISM (Kibona et al., 2006).

2.3.3 Drug resistance in Kenya

Multiple drug resistance was reported in *T. congolense* isolated from parasitaemic local Zebu cattle in Transmara District, Kenya, to therapeutic doses of both DA and ISM, 10 mg/kg and 1 mg/kg, respectively (Mapenay and Maichamo, 2008). In another study, seven *T. vivax* isolates from Kenya and Somalia were found resistant to the recommended dose rates of ISM, homidium and quinapyramine but all were sensitive to DA (Schönefeld et al., 1987). Field survey on drug use in Kwale district of Kenya documented cases of incorrect drug use and suggested this to be one of the reasons of drug resistance (Mugunieri and Murilla, 2003). Gray et al. (1993) used an in vitro assay to determine the drug sensitivity in *T. congolense* and reported high levels of resistance in KE 3302 and KE 3303 to ISM at doses of 100 ng/ml and 1000 ng/ml, respectively at Nguruman, Kenya.

2.3.4 Drug resistance in Uganda

In Mukono County farms, eighteen trypanosome isolates were collected from cattle and were characterized in cattle, goats and mice for their sensitivity to homidium, ISM and diminazene. In this study, 4 populations were reported refractory to treatment with ISM at 0.5 mg/kg bw in at least 1 out of 3 goats each. Furthermore, 5 populations were documented refractory to treatment with homidium chloride at 1.0 mg/kg b.w. in a minimum of 2 out of 3 goats each (Olila et al., 2002). In vivo drug sensitivity study of *Trypanosoma evansi* isolates from camel in Moroto revealed relapses when half the recommended doses of Triquin-S® (quinapyramine sulphate) and Cymelarsan® (melarsenoxyde cysteamine) were used and Trypan® (diminazene aceturate) up to 14mg/kg was not able to clear the infection in mice (Kabi et al., 2009).

As cross-resistance between currently available trypanocidal drugs has been reported in many countries in East Africa, which makes that the concept of sanative pairs may no longer work

(Getachew, 2005). These difficulties and the apparent impossibility to develop a vaccine and the logistical problems of restocking with the few trypanotolerant breeds of cattle, means that trypanosomosis is best tackled by controlling tsetse, with drugs as temporary expedients (Vale, 2009).

2.4 Socio-economic implication of drug resistance in East Africa

AAT is a dynamic disease, both in space and time. The most important factor affecting the disease today is the rapidly expanding human population complicated by differing local situations (Ilemobade, 2009). The functions of livestock in Africa are multiple with their economic and social importance depending on the production system (FAO/IAEA, 1993). Domestic animals enhance the economic viability and sustainability of farming systems. They diversify production and management options, increase total farm production and income, provide year-round employment and provide insurance in times of need. Sales of livestock products provide funds for purchasing critically needed crop inputs and for financing farm investments. Livestock often forms the major capital reserve of farming households (FAO, 1992). Since the socio-economic impact of AAT varies within and between countries, regions and agro-ecological zones, a proper appreciation of the causal relationship between poverty and tsetse related development constraints is crucial (Mattioli et al., 2004). The socio-economic implications of drug resistance development are also diverse and difficult to quantify.

2.4.1 Crisis of professional enthusiasm

Veterinary professionals diagnose sick animals and treat them accordingly. In this activity, one form of professional satisfaction is observing animals recovering because of the treatment that was administered. In contrast, a failure of the treatment because of conditions like drug resistance inevitably makes the clinician less confident in his day to day professional practice. This condition might even be more pronounced in the African context where most of the communities are poorly aware of good veterinary practices.

2.4.2 Livestock farmer and the farming system

Clinical diagnosis of sick animals is mostly followed by the right treatment, but treatment failure is sometimes inevitable because of the presence of drug resistance in trypanosomosis endemic areas. If treated animals do not recover, livestock farmers are not motivated to present their animals to a veterinary "hospital" or clinic in the rural areas of East Africa. Broadly, because of resistance development, the use of chemotherapy and chemoprophylaxis is in question, thus so is the control of the disease. From the point of view of producers, livestock diseases including trypanosomosis are essentially an economic problem. Diseases that reduce production, productivity and profitability are associated with the cost of their treatment, disruption of local markets, international trade and exacerbate poverty on rural, local, and regional communities (Lammy et al., 2012). It can cause direct losses (deaths, stunting, reduced fertility, and changes in herd structure) and indirect losses (additional costs for drugs, added labor costs and profit losses due to denied access to better markets and use of suboptimal production technology) (Lammy et al., 2012; Rushton, 2009). Apart from the above direct economic loss, a vast area of arable land cannot be utilized for agricultural purposes due to the presence of trypanosomosis. In addition, trypanosomosis is also partially responsible for the shortage of animal protein in the world (Dagnatchew, 1982). In terms of agricultural Gross Domestic Product (GDP) the total loss has been reported as US$ 4.75 billion per year in Africa (FAO, 2014).

2.4.3 Impact on pharmaceutical industries

All three drugs (ISM, DA, Homidium) have been on the market for more than 50 years and, for much of this time, they have been provided by a few European manufacturers (Geerts et al., 2001). However, the absence of new and efficient veterinary drugs has contributed to the appearance of counterfeit drugs on the market in East Africa (Pecoul et al., 1999; WHO, 2012, 2010) made by local companies that now compete with imported standard drugs from licensed companies. Obviously, this market competition discourages the few pharmaceutical industries to maintain the production and invest in the search for new drugs. In addition, a lack of quality

control on production of generic forms of the drug contribute to treatment failure and development of drug resistance (Pecoul et al., 1999).

2.5 Detection methods of trypanocidal drug resistance

The trypanosomes infective to livestock in Africa have not been studied in detail at the molecular level as compared to those that are infective to humans (Delespaux et al., 2005). Several methods have been developed during recent years for the detection of drug resistance in trypanosomes. Most of the *in vivo* and *in vitro* assays for this purpose are better suited for the determination of drug resistance of a small number of isolates, than for large-scale screening (Gall et al., 2004).

In vivo assays

The most commonly used *in vivo* assays include tests in mice and ruminants (Eisler et al., 2001). Guidelines for standardized testing of trypanocidal drugs *in vivo* are well described in Eisler et al. (2001).

The tests in mice are primarily applicable to those trypanosome species that are readily infective for mice, i.e. *T. brucei* and *T. congolense*. The single-dose test in mice has been conveniently used in areas with limited laboratory facilities and was found appropriate for the characterization of geographic areas in terms of trypanocidal drug resistance using a large number of trypanosome isolates compared to tests in cattle (Eisler et al., 2001). A group of six mice is required for each drug to be tested and an additional control group of 6 mice. ISM and DA should be used at the dose of 1.0 and 20.0 mg/Kg B.W., respectively. In contrast, a multi-dose mice test requires at least 6 groups of six mice for each drug to be tested. The dose range varies between 0.01 and 20 mg/Kg B.W. for ISM and 1-60 mg/Kg B.W. for DA. After treatment, a parasitological examination of the mice should be conducted twice a week using phase-contrast microscopy of a wet smear of tail blood. The treated groups should be followed until relapse occurs or until 60 days post treatment (Eisler et al., 2001).

The tests in ruminants consist of infecting a group of cattle (mainly calves) or small ruminants with the isolate under investigation and to treat them with various dosages of trypanocide at the first appearance of parasites. The test in calves is mostly used for the investigation of drug resistance in *T. vivax* which is not usually infective for mice. Calves used should be about 3-6 months old, of a genotype similar to the cattle in the area under study and without prior exposure to tsetse or trypanosomosis. It is advisable to use a minimum of three and preferably six animals per isolate and the animals must be kept in fly-proof accommodation or in a non-tsetse area. The animals are regularly monitored over a period of 100 days to determine the efficacy of standard drug doses in terms of their ability to provide a permanent cure (Eisler et al., 2001; Holmes et al., 2004).

The disadvantages of these in vivo assays are the requirement of a long duration of follow-up (60 days in mice and up to 100 days in cattle), curative doses in ruminants that cannot be fully extrapolated from the results in mice and the fact that *T. vivax* and some *T. congolense* strains do not grow in mice (Delespaux et al., 2008b; Eisler et al., 2000, 2001). The tests in ruminants are useful when laboratory facilities are limited, but they only allow the qualitative assessment of the inoculated isolate. Furthermore, it must be taking into account that not all populations of trypanosomes that are inoculated grow equally well (Holmes et al., 2004).

In vitro assays

An *in vitro* assay utilizes procyclic, metacyclic or bloodstream forms of trypanosomes for evaluation of drug sensitivity (Holmes et al., 2004). In another study, metacyclic forms have been used for sensitivity study of ISM, DA and homidium salts at various drug concentrations (0.5 ng - 50 µg/ml). The results of this *in vitro* assay has been found well correlated with field observations (Gray et al., 1993). *In vitro* assays are expensive to perform and require good laboratory facilities and well-trained staff (Holmes et al., 2004). As an alternative to *in vitro* cultivation of bloodstream forms, Clausen et al. (1999) used a drug incubation *Glossina* infectivity test (DIGIT) to assess the susceptibility of *T. congolense* bloodstream forms to

trypanocidal drugs. Briefly, blood infected with *T. congolense* is incubated at 37°C for 30 minutes and 12 hours, respectively, in the presence of different drug concentrations (0.5, 1,10 and 100 µg/ml blood) of DA or ISM. After that, the trypanosome/blood/drug suspensions are fed to tsetse flies through an in vitro feeding system, using a silicon membrane. All tsetse flies are then dissected and examined for presence of trypanosomes in labrum, hypopharynx and midgut 20 days after their infective blood meals. This technique distinguish resistant from sensitive isolates and does not require experimental animals, but it does require a supply of teneral tsetse flies from an artificially reared colony (Holmes et al., 2004). In another study, Knoppe et al. (2006) used a drug incubation infectivity test (DIIT) to confirm all field stocks that were resistant to 1.0 mg/kg B.W. isometamidium in the standard mouse test (SMT). Briefly, non- immunosuppressed donor mice are infected with trypanosomes by intraperitoneal inoculation. Trypanosomes are harvested from anesthetized mice and separated from blood cells using DEAE cellulose. A sample of 0.5 ml of 1-2 x 10^5 viable trypanosomes per ml PSG, pH 8.0 containing 20% goat sera are incubated in ISM concentrations 0, 0.5, 5, 50 or 500 ng/ml at 35°C, 5% CO_2 in air for 10 minutes in 24 well culture plates. The tests are carried out in five replicates. After incubation, 0.5 ml of each well's contents are injected intraperitoneally into one mouse each. Mice are screened three to five times per week using HCT for 30 days for trypanosome infections. When found positive on two consequetive days, mice are considered infected. Those *in vitro* techniques are not routinely used and no recent publication is reporting their use.

Field observational method

Longitudinal block treatment is an observational methods developed to overcome the expensive and time consuming constraints of both *in-vivo* and *in-vitro* tests. It is conducted without the need for isolation of trypanosomes under natural challenge in the field and appropriate for areas without laboratory facilities (Eisler et al., 2000, 2001). Cattle are randomized in a treatment and a sentinel groups. All animals are given a dose of 7 mg/Kg DA before the start of the experiment. The treatment group is given ISM at 1 mg/Kg B.W. and all

treatment and sentinel cattle are followed for presence of trypanosomes every two weeks using the phase-contrast buffy coat technique (Murray et al., 1977) over a three months period. If more than 25% of ISM treated cattle become infected within 8 weeks of exposure, this strongly leads to the suspicion of the occurrence of drug resistant trypanosomes (Eisler et al., 2000). If less than 25% of the sentinel group is becoming positive, it indicates a low challenge of the disease. Prophylaxis is thus not advised in such a context. Mungube et al. (2012) are reporting a shorter and less labor intensive method of block treatment and the observation period is limited to 1 month. Alternatively to the block treatment methods, longitudinal parasitological data has been used to compare trypanosome incidence and prevalence in herds under therapeutic drug regimen, using PCV to distinguish new from recurrent infections (Holmes et al., 2004). Rowlands et al. (2001) described and used a method for calculating new infection incidence from monthly field data in cattle in southwest Ethiopia. In their study, an infection was defined as a new infection if it was preceded by two previous months in which both samples had a PCV ≥ 26% and were not detected with trypanosomes. This method requires a huge dataset covering a long period of time and is not used in practice.

ISM-ELISA and the mitochondrial electrical potential

ISM resistance can also be detected using an ELISA technique in which ISM serum concentration of at least 0.4 ng/ml with the concomitant presence of trypanosomes has been documented to suggest that the parasites are resistant (Delespaux et al., 2008b; Eisler et al., 1997). In another study, in a drug sensitive population of *T. congolense*, agents affecting mitochondrial function were shown to produce dose dependent inhibition of mitochondrial membrane potential, as measured by the accumulation of the lipophilic cations methyltriphenylphosphonium iodide or rhodamine 123. It was also reported that the agents produced parallel inhibition of isometamidium uptake, suggesting an involvement of mitochondrial membrane potential in the accumulation of the drug which could be used as a candidate marker for ISM sensitivity study (Delespaux et al., 2008b; Wilkes et al., 1997). This method has not been developed up to now (17 years later).

Molecular methods for detection of drug resistance

A simple PCR-RFLP test for DA resistance detection was developed by means of the Single Strand Conformation Polymorphism technique (SSCP) based on a *T. congolense* gene (wrongly called *TcoAT1)* presenting a high blast similarity with the adenosine transporter 1 gene (*TbAT1*) of *T. brucei* coding for a P2 type nucleoside transporter (Delespaux et al., 2006). Later on, this PCR-RFLP test's ability to amplify low concentrations of DNA was enhanced by adding a step of whole genome amplification using the QIAGEN REPLI-g UltraFast Mini Kit. Its specificity was improved by replacing the BclI (T^GATCA) enzyme by *Dpn*II (^GATC) (Vitouley et al., 2011). Recently this test was questioned by Munday et al. (2013), who reported that the wrongly called *TcoAT1* gene was coding for a P1 type purine nucleoside transporter (TcoNT10) that does not mediate DA uptake. Based on the available data, there was a 100% correlation between gold standard (test in mice) and the *Dpn*II-PCR-RFLP (Moti et al., 2012). Delespaux and de Koning (2013) argue that due to this large correlation the test remains valid until additional experimental data further validate or invalidate it.

Amplified fragment-length polymorphism (AFLP) was also used for the study of ISM resistance in two isogenic clones of *T. congolense* differing in the level of resistance by a 96 fold factor (Delespaux et al., 2005). The procedure identified a gene coding for an 854 amino acid protein (presenting some signatures of an ABC (ATP binding cassette) transporter (Delespaux et al., 2008a, 2005). A triplet insertion coding for an extra lysine has been found in the genes of the *T. congolense* resistant strains. The presence of the insertion was found to be consistently present in the genomes of the *T. congolense* clones that were resistant to ISM (Delespaux et al., 2005). However, this test was abandoned as the correlation was consistent with Zambian isolates but fell down to 30% with isolates from Burkina Faso (Delespaux personal communication). In another study, Afework et al. (2006) used PCR-RFLP based on the adenosine transporter-1 gene in *T. brucei* (*TbAT1*), encoding a P2-type nucleoside transporter, from *T. brucei brucei* field stocks to investigate a possible link between the presence of mutations in this gene and ISM resistance. This *Sfa*NI-PCR-RFLP test is based on the polymorphism of a 677 bp fragment of the

TbAT1 gene (Afework et al., 2006; Delespaux et al., 2008b). *T. brucei* being not virulent for livestock, a diagnostic test for this species is of limited interest. Furthermore, the authors used strains that were resistant to both DA and ISM for the validation of their test rendering any interpretation of the results impossible. There is currently no satisfactory molecular test for the detection of ISM resistance in trypanosomes.

2.6 Conclusion

Chemotherapy and chemoprophylaxis have been the main methods of control and prevention of trypanosomosis. Nevertheless, the development of drug resistance has certainly brought doubt in their future application. The development of drug resistance is multi-factorial and has serious implication for countries that are almost entirely dependent on agriculture. Therefore, it is advisable to bring together all the stakeholders involved in the fight against the development of resistance and share the good practices across East Africa. Beyond the fragmented approach of control and prevention, the establishment of short and long term partnership with international organizations would certainly leverage the national and regional effort. Geospatial mapping of drug resistance nationally and in the region is also worth considering in order to identify drug resistance hot spot areas and tackling them subsequently. Such a map would also be helpful in establishing a field monitoring system that could be used to target field interventions more effectively and efficiently. Mapping the market chain for trypanocidal drugs both nationally and in the region would help to establish a regulatory mechanism for the marketing of trypanocidal drugs. Since the community should also be part of the drug resistance fighting program, extension education for livestock owners on different topics is required. This would involve preparation of generic booklets that contain information on the whole package of trypanocidal drug use which can be translated to respective countries local languages for subsequent use by livestock owners.

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Chapter 3

Ghibe river basin in Ethiopia: Present situation of trypanocidal drug resistance in *Trypanosoma congolense* using tests in mice and PCR-RFLP

Adapted from:

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

3.1 Abstract

A cross-sectional study was carried out in the Ghibe valley from August to October 2010. A total of 411 head of cattle were sampled in eight villages for buffy coat examination (BCE) and blood spots were collected from each animal for trypanosomes diagnosis by 18S-PCR-RFLP and diminazene aceturate (DA) resistance by *Dpn*II-PCR-RFLP. Three villages were selected from ILRI-priority zones (Abelti, Yatu and Keta Bosso where disease control is implemented) whereas the other five from non-priority zones (Weira, Jatu, Wedesa, Shumoro and Gomsho where no organized disease control is happening). Twenty-four samples (5.84%) were diagnosed positive for *T. congolense* by BCE and injected in mice for further characterization. Twelve of those isolates successfully multiplied in mice and were tested by an *in vivo* mouse test for diminazene (DA) (10 and 20 mg/kg B.W.) and isometamidium (ISM) (1mg/kg B.W.) resistance. All were shown to be resistant to both drugs at all doses. The use of the *Dpn*II-PCR-RFLP on these isolates confirmed their DA-resistance profile. Seventy-three of the collected blood spots (17.8%) were diagnosed positive for *T. congolense* by 18S-PCR-RFLP of which 37 (50.7%) gave amplification products with the *Dpn*II-PCR-RFLP. Here, 35 (94.6%) showed a resistant profile, 1 (2.7%) a sensitive profile and 1 (2.7%) a mixed profile. The data were analysed by logistic regression model and the relapsing time in mice tests was assessed using the Cox regression model. There was no significant intervention effect (P=0.83) with odds ratio equal to 1.21 when using the BCE data. The *18S*-PCR-RFLP test also showed no significant intervention effect (P=0.60) with odds ratio equal to 1.43. The hazard ratio of getting parasitaemic after treatment with DA at 20 mg/kg B.W. compared to the control group was 0.38 which differs significantly from one (P<0.001). Relapsing time after treatment with DA 10 mg/kg B.W. or ISM 1mg/kg B.W. was also significantly longer than the prepatent period of the control group. The situation of drug resistance in the Ghibe valley is further discussed.

Keywords: Trypanosoma congolense, isometamidium, diminazene, Ghibe, Ethiopia, drug resistance

3.2 Introduction

Tsetse-transmitted trypanosomes are responsible for diseases unique to Africa affecting both humans and animals. They occur in about 10 million km^2 in 37 sub-Saharan countries (FAO, 2002) and constitute a major threat to the survival and productivity of domestic livestock in sub-Saharan Africa (Holmes, 1997). Trypanosomes cause serious economic losses and an estimated annual loss in cattle production alone has been reported to be in the range of 1.0-1.2 billion dollars (FAO, 2002).

The situation of African Animal Trypanosomosis (AAT) has become even worse due to the fact that some drugs have been abandoned due to resistance (Matovu and Mäser, 2010). Practically, only ethidium bromide (Homidium®) and DA (Berenil®) are still available for therapy and ISM (Samorin®, Trypamidium®) for prophylaxis (Holmes, 1997; Geerts et al., 2001; Matovu and Mäser, 2010). The inevitable outcome of continued use of the same compounds for decades has resulted in drug resistance that has been largely considered responsible for the frequently observed chemotherapeutic failures. At present, trypanocidal drug resistance is reported in 18 countries of sub-Saharan Africa (Delespaux et al., 2008). Although tsetse and trypanosomosis research and intervention in the Ghibe Valley of Ethiopia has started a long time ago (Rowlands et al., 1993; Codjia et al., 1993; Mulugeta et al., 1997), the disease persists to exert its deleterious effects on the most vulnerable segment of the society. Resistance to commonly used drugs has been reported from different parts of Ethiopia including Ghibe Valley. For instance, Tewelde *et al.* (2004) reported ISM resistance of trypanosomes (*T. congolense*, *vivax* and *brucei*) infecting cattle in the upper Didessa valley of Western Ethiopia. Mulugeta *et al.*(1997) documented multiple drug resistance in *T. congolense* at the Ghibe river basin. Chaka and Abebe (2003) reported the existence of *T. congolense* resistance originally isolated from cattle in the Southwest of Ethiopia, namely, Ghibe, Bedelle, Sodo and Arbaminch. In view of today's resistance reports from many parts of Ethiopia including Ghibe valley, intervention needs to be complemented with regular surveillance of the evolution of the trypanocidal drug resistance.

The aim of this study was to explore an easier and faster method of DA resistance diagnosis based on the *Dpn*II-PCR-RFLP (Vitouley et al., 2011). Results from this *Dpn*II-PCR-RFLP DA resistance test were compared with the gold standard, i.e., the mice test (Eisler et al., 2001), to evaluate the newly proposed molecular test. The mice test was also used to diagnose ISM resistance. Based on the comparative results of the two drug resistance tests, the actual situation of drug resistance in the Ghibe Valley is discussed.

3.3 Materials and Methods

3.3.1 Study area

The Ghibe valley is 183 km away from Addis Ababa and located in Southwest Ethiopia. Farmers in and around the valley make their living by mixed farming systems and cattle production using traditional village management for subsistence (Leak et al., 1993). The area receives a mean annual rainfall of 900 mm and the mean monthly maximum temperature ranges from 29.8 to 44.0^{o}C (Leak et al., 1993; Rowlands et al., 2001). The Livestock Research Institute (ILRI) is running interventions against trypanosomosis using monthly DA (Vetiben®) administrations (3.5mg/KG B.W.) for all animals found infected and monthly deltamethrin (cypermethrin®) pour-on application (20 ml/adult animal) at the expenses of the community since 1990. The intervention covers two sites namely: upper and lower Ghibe and more than 20 villages benefit from this activity. Our study involved both ILRI-intervention and non-intervention sites around the Ghibe river valley. Intervention was decided in priority zones were the impact of the disease was considered by the farmers as the highest based on their own perceptions. A total of eight kebeles (smallest administrative unit of Ethiopia) were selected to include three from ILRI-priority zones (Abelti, Yatu and Keta Bosso) and the other five from non-priority zones (Weira, Jatu, Wedesa, Shumoro and Gomsho). The vegetation coverage and thus, the tsetse habitat in the entire region are spotty and limited to the vegetation of the river banks or to protected forest zones because of the intensive agricultural expansion in the area. The choice of the kebeles was based on expert opinion taking into account a comparable vegetation of the river banks and equal distance between grazing zones and rivers to harmonize the tsetse challenge

between the 8 study sites. In the non-priority zones, veterinary drugs including trypanocidal drugs are available freely in all rural shops or local markets and the farmers don't have the habit of presenting sick animals to veterinarians for proper diagnosis of diseases. Thus, the administration of drugs is usually done by the breeders often without veterinary advice. No information is available on the treatment frequencies, type of trypanocide, doses injected due to this unsupervised use of the drugs out of any legal sanitary framework.

3.3.2 Cross-sectional study

The cross-sectional study was carried out from August to October 2010. To obtain a 95% confidence interval for the prevalence of trypanosomosis that has a width equal to 10%, a minimum sample size of 384 animals was required, assuming that the real prevalence equals 50% to plan for the maximum possible sample size (Thrusfield, 1995). Based on the sampling size pre-requisite, a total of 411 whole blood samples were collected from East African Zebu cattle in the study area.

3.3.3 Animals

A heterogeneous group of East African Zebu cattle in different age and sex categories was used for this study (198 males, 213 females, aged between 1 and 15 years old). Accordingly, all owners in every kebeles were informed to gather their animals the following day at the proposed sampling site. These 411 Zebu cattle were selected randomly using the lottery method.

3.3.4 Blood collection

From each cattle, 9 ml of whole blood was collected from the jugular vein into heparinised Venosafe tubes (Terumo) for further analysis.

3.3.5 Parasitological tests

3.3.5.1 Buffy coat examination (BCE)

The BCE was performed as described by Woo (Woo, 1970) immediately after blood collection in the field. Briefly, about 70µl of blood was collected in a microhaematocrit capillary tube and sealed on one end. The capillary tube was centrifuged at 15,000 rpm for 5 minutes to concentrate the trypanosomes in the buffy coat layer. Then, the capillary tube was placed in a Woo viewing chamber and a cover slip of 24x24 mm was placed on the capillary tube hereafter the space between the tubes and the cover slip was filled with a drop of water to reduce light diffraction. The buffy-coat plasma junction was examined for the presence of trypanosomes and the Packed Cell Volume (PCV) was measured.

3.3.5.2 Isolation of Trypanosomes

In the field, about 0.5 ml of blood samples BCE-positive for the presence of trypanosomes (single or mixed infections) were injected intraperitoneally into three mice aged from 5 to 8 weeks and weighing on average 30 g each. When the parasitaemia reached a minimum of 7.1 on the Herbert and Lumsden scale (Herbert and Lumsden, 1976), the blood was collected by cardiac puncture under final anaesthesia. This blood from mice was used to prepare cryostabilates stored in liquid nitrogen until further inoculation.

3.3.5.3 Stabilate preparation

The cryomedium was prepared in advance by mixing one part of egg yolk to three parts of phosphate saline glucose buffer (PSG) and to three parts of Triladyl® (Minitube). The ingredients were carefully mixed and divided in aliquots of 0.5 ml in Sarstedt microtubes (Sarstedt) and stored at -20°C until use. Stabilates were prepared by gently mixing 0.5 ml volumes of mice blood with confirmed presence of trypanosomes with an equal volume of thawed cryomedium in a Sarstedt microtube that was suspended in the vapor phase of liquid nitrogen for 1 hour before being immersed in the liquid phase for long term storage.

3.3.5.4 Drug resistance tests in mice

This test was conducted after stabilate preparation in the laboratory and the protocol described by Eisler *et al*. (2001) was used with minor modifications. In this study, male and female Swiss-white mice aged between 8 and 10 weeks and weighing between 25 to 30g were used. They were housed in clean cages at room temperature in fly-proof conditions, fed with commercial pellets and water *ad libitum*. The mice were screened for the presence of blood parasites using wet and Giemsa-stained thin blood films prior to the beginning of the experiment.

Parasitaemic blood of each *Trypanosoma* isolate was diluted in PSG to obtain a final concentration of 10^5 trypanosomes per ml and inoculated into 4 groups of 6 mice each. Twenty four hours after inoculation, the mice of three groups were treated with the trypanocidal drugs. The first group was treated intraperitoneally with 1mg/kg B.W. ISM (Veridium®, Lot No. 173A1), the second and third groups with 10 and 20mg/kg B.W. DA (Veriben®, Lot No. 500A1), respectively. The control group was injected with distilled water. Mice were monitored twice a week for a period of two months for the presence of trypanosomes by the examination of wet smears of tail blood. An isolate was considered as resistant when more than one out of the 6 treated mice became positive within the observation period. Two reference strains TRT8 and IL1180 sensitive to DA and ISM, respectively were added as quality control for the trypanocides used. The TRT8 isolate originates from cattle in the Eastern Province of Zambia and was then cloned visually from a single trypanosome. It shows a *T. congolense* savannah type profile with the 18S-PCR-RFLP and a DA-sensitive profile with *Dpn*II-PCR-RFLP. The IL1180 isolate was described by Peregrine et al. (1997).

3.3.6 Molecular assays

3.3.6.1 Field Samples

The buffy coat of 411 cattle were spotted on filter paper Whatman 4 (Whatman) in the field, dried protected from UV light and stored in plastic bags containing silica gel until further processing less than 4 months after preparation.

3.3.6.2 Samples from mice test

Both treated and untreated mice showing trypanosomes circulating in peripheral blood were bled from the tail and blood was spotted on filter paper Whatman 4 (Whatman) dried protected from UV light and stored in plastic bags containing silica gel until further processing.

3.3.6.3 Trypanosome species identification (18S-PCR-RFLP)

DNA was extracted using the saponine-PBS method described by de Almeida *et al.*(1998). For species identification, DNA amplifications were performed using three primers targeting the 18S small ribosomal subunit gene sequence and followed by digestion of the products using *Msp*I enzyme as described by Geysen *et al.* (2003).

3.3.6.4 Diminazene resistance test (DpnII-PCR-RFLP)

Primers targeting the TcoNT10 gene coding for a P1-type purine transporter and the *Dpn*II restriction enzyme were used as described by Delespaux *et al*. (2006) and modified by Vitouley et al. (2011) but without the described whole genome amplification step. The accession number of TcoNT10 gene is TcIL3000_9_2500 (http://www.genedb.org).

3.3.7 Data analysis

Prevalence and resistance, both binary response variables, were analysed by the logistic regression model introducing intervention as fixed effect and kebele as random effect. When the variation due to kebele is significantly different from zero, the prevalence (of resistance) varies significantly from kebele to kebele. The relapsing time according to drugs and doses was assessed using the Cox regression model with strain as stratum and drug regimen as categorical fixed effect. All tests were done at the 5% global significance level. All analyses were performed using SAS Version 9.2.

3.4 Results

3.4.1 Prevalence and species identification

Among the 411 samples tested, *T. congolense* was diagnosed in 24 (5.8%) and 73 (17.8%) blood samples using BCE and *18S*-PCR-RFLP respectively. Nine (2.2%) samples were positive for both tests (Table 3.1). The *18S*-PCR-RFLP confirmed that all the *T. congolense* were savannah type. A few *T. vivax* were observed in single or mixed infection with *T. congolense* but were not further considered in this study.

Table 3.1 Summary of *T. congolense* infections based on different test methods

Kebele	Intervention	Cattle sampled	BCE	18S-PCR-RFLP	Both tests positive
Wedesa	No	38	2	11	1
Shumoro	No	63	3	19	2
Gomsha	No	14	0	0	0
Jatu	No	34	5	4	3
Weira	No	50	0	1	0
Yatu	Yes	75	11	17	3
Keta Bosso	Yes	76	2	8	0
Abelti	Yes	61	1	13	0
Total		**411**	**24 (5.8%)**	**73 (17.8%)**	**9 (2.2%)**

The prevalences observed in priority zones and non-priority zones were comparable (P=0.83) with odds ratio equal to 1.21 (95% CI: 0.24 - 6.02) when using the BCE data. The variance of the random kebele effects was equal to 0.77 (SE=0.71) and did not differ significantly from zero (P=0.31). Similarly, *18S*-PCR-RFLP test showed similar prevalences in priority and non-priority zones (P=0.60) with odds ratio equal to 1.43 (95% CI: 0.40 - 5.23). The variance of the random kebele effects was equal to 0.59 (SE=0.49) and did not differ significantly from zero (P=0.27). Therefore, in both test methods, we cannot conclude that there was over-dispersion rather the prevalence was uniform over kebeles.

3.4.2 *Dpn*II-PCR-RFLP resistance study

All 73 samples positive for *T. congolense* savannah type using the 18S-PCR-RFLP were tested for DA susceptibility using the *Dpn*II-PCR-RFLP (Table 3.2). Among the 73 samples, 37 (50.7%) gave amplification products. Among the 37 positive, 35 (94.6%) showed resistant profiles, 1 (2.7%) sensitive and 1 (2.7%) mixed profiles. There was almost no variation in resistance over the different kebeles, as almost all the strains were found to be resistant.

Table 3.2 Diminazene resistance results of *T. congolense* using *Dpn*II-PCR-RFLP according to study sites

Kebele	Intervention	18S-PCR-RFLP	*Dpn*II-PCR-RFLP			
		Positive	**Amplified**	**Resistant**	**Sensitive**	**Mixed**
Wedesa	No	11	8	8	0	0
Shumoro	No	19	8	8	0	0
Gomsha	No	0	0	0	0	0
Jatu	No	4	2	2	0	0
Weira	No	1	0	0	0	0
Yatu	Yes	17	8	7	0	1
Keta	Yes	8	5	4	1	0
Bosso						
Abelti	Yes	13	6	6	0	0
Total		**73**	**37**	**35**	**1**	**1**
		(17.8%)	**(50.7%)[a]**	**(94.6%)[b]**	**(2.7%)[b]**	**(2.7%)[b]**

[a] % of the 18S-PCR-RFLP positive
[b] % of the 18S-PCR-RFLP positive that also amplified using *Dpn*II-PCR-RFLP

3.4.3 *T. congolense* isolation

Among the 24 fresh blood samples that were diagnosed as *T. congolense* by BCE and injected into mice, 12 (50%) were successfully isolated and preserved in liquid nitrogen.

3.4.4 Resistance tests in mice

The 12 *T. congolense* isolates were found resistant to DA at 10 and 20 mg/kg B.W. The median prepatent period was 9 and 12 days for 10 and 20 mg/kg B.W. DA treatment groups respectively. The same isolates were found resistant to ISM at the dose of 1 mg/kg B.W. and the median prepatent period was 8.8 days (Table 3.3).

Table 3.3 Median prepatent periods according to doses and treatments of *T. congolense* infections in mice

Group	Median PP[a] (days)	Minimum PP[a] (days)	Maximum PP[a] (days)
Control	8	6	13
ISM 1 mg	8.75	6	60
DA 10 mg	9	6	25.5
DA 20 mg	12	8.5	20

[a]Prepatent period

The hazard ratio of getting parasitaemic after treatment with DA at 20 mg/kg B.W. compared to the control group was 0.38 (95%, CI: 0.26 - 0.56) which differs significantly from one ($P<0.001$). Relapsing time after treatment with DA 10 mg/kg B.W. or ISM 1mg/kg B.W. was also significantly longer than the appearance of parasites in the control group. As expected, the sensitive strain TRT8 did not relapse in mice after treatment as confirmed by negative microscopical examination and 18S-PCR-RFLP during the two months of observation.

3.4.5 18S and *Dpn*II-PCR-RFLP on samples from mice

All 12 isolates were confirmed to be *T. congolense* savannah type and resistant to DA using *Dpn*II-PCR-RFLP with blood spotted on filter papers from mice. The samples collected in the field and spotted on filter paper corresponding to those 12 isolates were also checked with the

two PCR-RFLP's. Only six (50%) of those field samples gave PCR amplification products. All were confirmed to have a DA-resistant profile.

3.4.6 Mice isolation versus BCE and 18S-PCR-RFLP on DNA from filter papers

For the 24 samples that were diagnosed in the field as *T. congolense* by BCE and inoculated in mice, the similarities and discrepancies between the BCE and molecular identification techniques are summarized in table 3.4.

From the 24 samples, 9 were diagnosed correctly by both tests, 12 were negative with the 18S-PCR-RFLP, 1 was characterized as *T. theileri* by 18S-PCR-RFLP, 1 mixed infection *T. congolense-vivax* was found to be *T. theileri* by 18S-PCR-RFLP, another mixed infection *T. congolense-T. vivax* was diagnosed as *T. vivax* by 18S-PCR-RFLP. More importantly, the 18S-PCR-RFLP detected three times more positives than the microscopic examination.

3.5 Discussion - conclusions

The aims of this survey were to evaluate the current situation of trypanocidal drug resistance in the Ghibe valley using fast and easy molecular methods and validating those results by tests in mice. To achieving this, the molecular tests were performed on 411 samples from which a subset (24 samples that were microscopically trypanosome positive in BCE) was also tested in an *in vivo* mouse test for drug resistance. When examining the discrepancies between the BCE and 18S-PCR-RFLP (Table 3.4), it might seem intriguing that positive samples by microscopical examination were found negative by PCR-RFLP. This is probably due to the low concentration of parasites and the random effect created by the low volume of blood (50µl – the content of a capillary tube) examined by both methods. A parasitaemia of 20 trypanosomes/ml blood corresponds to 1 single trypanosome in 50µl.

Table 3.4 Summary of discrepancies between 18S-PCR-RFLP and BCE for 24 mice injected samples

Isolates Ref No	Kebele	BCE	18S-PCR-RFLP	*Dpn*II-PCR-RFLP	Isolated
F042	Shumoro	T.c.	N	N	No
F072	Shumoro	T.c.-T.v.	T.c.	N	No
F127	Jatu	T.c.	N	N	No
F140	Jatu	T.c.	T.c.	N	No
F153	Yatu	T.c.+T.v.	N	N	No
F159	Yatu	T.c.+T.v.	T.v.	N	No
F165	Yatu	T.c.	N	N	No
F171	Yatu	T.c.	N	N	No
F178	Yatu	T.c.	N	N	No
F189	Yatu	T.c.+T.v.	T.th.	N	No
F244	Keta Bosso	T.c.	T.th.	N	No
F246	Keta Bosso	T.c.+T.v.	N	N	No
F015	Wedessa	T.c.	T.c.	R	Yes
F018	Wedessa	T.c.	N	N	Yes
F058	Shumoro	T.c.	T.c.	R	Yes
F119	Jatu	T.c.	N	N	Yes
F125	Jatu	T.c.	T.c.	R	Yes
F148	Jatu	T.c.	T.c.	R	Yes
F164	Yatu	T.c.+T.v.	N	N	Yes
F166	Yatu	T.c.	U	N	Yes
F172	Yatu	T.c.+T.v.	T.c.	N	Yes
F187	Yatu	T.c.+T.v.	T.c.	R	Yes
F203	Yatu	T.c.	T.c.	R	Yes
F393	Abelti	T.c.	N	N	Yes

With T. c. as *T. congolense*; T.v. as *T. vivax*; T. th. As *T. theileri;* N as no amplification; U as unidentified and R as resistant

The probability of getting a 50µl sample with no parasite is thus not negligible. Furthermore, the buffy coats that are examined with the microscope or that are submitted to PCR-RFLP diagnosis are two different individual samples. It is thus expected to find some discrepancies between the two methods. An option to avoid this problem could be to replace the sampling on

filter paper by whole blood (500µl) mixed to a protective buffer such as AS1 (Qiagen®) or a 6M guanidine hydrochloride / 0.2 M EDTA buffer (Avila *et al*., 1990). This alternative presents a double advantage: (i) the sampling volume is larger, increasing the probability of getting at least one trypanosome in the collected blood and (ii) it decreases the risk of contamination between samples. This is usually not a big issue as all samples are stored individually and as the parasitaemia is normally very low but it can constitute a hinder in cases of high parasitaemias as it is observed in some spots (Moti et al., unpublished data). The 18S-PCR-RFLP was as expected, more sensitive than BCE with three times more positive samples. Our results (17.8%) with the 18S-PCR-RFLP for the prevalence of trypanosomosis are in concordance with previous studies (13.3%) (Rowlands et al., 2001).

The results of the species determination with *T. congolense* savannah type as predominant species are also comparable with previous studies (Mulugeta et al., 1997).The prevalence of *T. congolense* infections using BCE fluctuated around 5 to 6% and were not significantly different across the 8 kebeles. Our results indicate that the monthly systematic administration of DA (Veriben®) and deltamethrin pour-on treatment in the priority zones manage to reduce the burden of the disease to a level comparable to the non priority zones where no systematic and organized interventions were found necessary. There, the control relies on the sole administration of trypanocidal drugs based on clinical signs or breeder assumptions. The fact that farmers continue administrating trypanocides even when the presence of drug resistance is confirmed in the region, might be not surprising as it was demonstrated that even for infections with drug resistant trypanosomes the treatment with trypanocides remained beneficial. In an experimental model where cattle was inoculated with ISM-resistant *T. congolense* and treated at the first peak of parasitaemia, the impact of the infection on the PCV was not very pronounced with an average PCV reduction 8 to 14 weeks after treatment of only 5.9% (95% CI: 4.5-7.3) (Delespaux et al., 2010). Limiting the treatment to anaemic animals would certainly decrease the volume of drugs used as well as the costs for the farmers as recommended by Van den Bossche and Delespaux (2011).

Cases of drug resistance in the Ghibe valley has been described earlier. Rowlands *et al.* (1991) documented 12/12 isolates resistant to DA and 11/12 isolates to ISM in South-Western Ethiopia. Codjia *et al*. (1993) collected 12 isolates from cattle bred in the Ghibe valley and reported resistance to the maximum recommended dose of DA (7.0 mg/kg B.W.). Later, in the same region, concerns raised about the threat of drug resistance for the efficiency of animal production (Mulugeta et al., 1997; Chaka and Abebe, 2003). Tewelde et al. (2004) also reported ISM resistance in cattle sampled from upper Didessa valley of Western Ethiopia by the use of block treatments (Eisler et al., 2000). Our study shows that molecular tools greatly facilitate the diagnosis of DA resistance allowing for more large scale surveys but still has to be complemented by tests in mice or block treatments for the diagnosis of ISM resistance as no trustable molecular tool has been developed for this drug.

The molecular technique for the diagnosis of DA resistance in *T. congolense* proved to be sensitive, fast and reliable even if only half of the samples positive with the 18S-PCR-RFLP gave amplification products with the *Dpn*II-PCR-RFLP. This can be explained by the fact that the target gene of the 18S-PCR-RFLP is multicopy as opposite to the target gene of the *Dpn*II-PCR-RFLP which is a single copy gene. The correlation between the *Dpn*II-PCR-RFLP and the gold standard (test in mice) was 100% as all 12 isolates tested in mice for DA-resistance were also diagnosed as such by the molecular tool allowing for a reliable extrapolation to all the *T. congolense* isolates.

The test in mice complements the *Dpn*II-PCR-RFLP by providing information on ISM resistance. All isolates were resistant to ISM too jeopardizing thus the use of the sanative pair to overcome drug resistant trypanosomes as proposed by Whiteside (1962). Briefly, the concept of the sanative pair recommends the use of two trypanocides (e.g. DA and ISM) which are chemically unrelated and, therefore, are unlikely to induce cross-resistance. The first of the pair is used until resistant strains of trypanosomes appear and then the second is substituted and used until the resistant strains have vanished from cattle and tsetse (Whiteside, 1962).

The relative control of the disease in the area, despite the high level of drug resistance, is a relief as a recent resettlement program launched in the region by the government for farmers from draught struck areas is placing the Ghibe Valley under high pressure for food production (Getachew, 2005; Lemecha et al., 2006).

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Chapter 4

Detection of African animal trypanosomes: The haematocrit centrifugation technique compared to PCR with samples stored on filter paper or in DNA protecting buffer

Adapted from:

Moti, Y., Fikru, R., Büscher, P., Van Den Abbeele, J., Duchateau, L., Delespaux, V. (2014). Detection of African animal trypanosomes: the haematocrit centrifugation technique compared to PCR with samples stored on filter paper or in DNA protecting buffer. Veterinary Parasitology, 203, 253–8.

4.1 Abstract

The present study aimed to compare the trypanosome specific 18S-PCR-RFLP using samples stored either on Whatman filter papers (PCR-RFLP-fp) or in a commercial cell lysis and DNA protecting buffer (PCR-RFLP-pb) with the haematocrit centrifugation technique (HCT), a method widely used for the diagnosis of African Animal Trypanosomosis. Out of 411 head of cattle, 49 (11.92%) (CI = 8.95-15.45) scored positive for the presence of trypanosomes by HCT whereas 75 (18.25%) (CI = 14.63-22.33) and 124 (30.17%) (CI = 25.77-34.86) scored positive using PCR-RFLP-fp and PCR-RFLP-pb, respectively. Out of the 49 positives by HCT, 14 (28.57%) (CI = 16.58-43.26) and 28 (57.14%) (CI = 42.21-71.18) were concordant by PCR-RFLP-fp and PCR-RFLP-pb, respectively. None of the PCR techniques detected parasites from the *Trypanozoon* group. Although HCT detected more cases of *T. vivax* (33), species identification using PCR-RFLP-fp and PCR-RFLP-pb were significantly different ($p < 0.001$) from the HCT technique. The use of DNA protective buffer is thus recommended as the output of the PCR-RFLP-pb is improved and the risk of contamination between samples is reduced.

Keywords: Trypanosoma, Buffer, Ethiopia, Cattle, *Trypanosoma vivax*, *Trypanosoma congolense*

4.2 Introduction

African Animal Trypanosomosis (AAT) is a parasitic disease that causes serious economic losses in the livestock production system. Diverse clinical signs are observed from anaemia, loss of body condition and emaciation to death when untreated. In sub-Saharan Africa, the disease is caused by *Trypanosoma congolense*, *Trypanosoma vivax* and *Trypanosoma brucei.* Its distribution overlaps with the presence of the tsetse fly except for *T. vivax* and *Trypanosoma evansi* that can be transmitted mechanically by other hematophagous biting flies (CFSPH, 2009). AAT restricts agricultural production, limits the availability of food and contributes to poverty across rural sub-Saharan Africa (Grady et al., 2011).

Accurate diagnosis of trypanosome infections is required for a proper understanding of the epidemiology of the disease, which can then result in the implementation of adequate control strategies (FAO, 1992; FAO, 1998; Getachew, 2005). The diagnosis mainly involves examination of clinical signs and laboratory methods. A presumptive field diagnosis is often based on finding an anaemic animal with a poor body condition in an epidemic or endemic area. Even then, because of the various clinical manifestations, diagnosis of trypanosomosis cannot be based on these clinical signs alone (Nantulya, 1990) as no pathognomonic clinical sign can confirm the disease. The presence of the parasite must thus be confirmed to ensure a proper diagnosis. Different techniques are therefore more or less routinely used by veterinarians: direct microscopy, concentration techniques, laboratory animal inoculation, detection of anti-trypanosomal antibodies and molecular assays (Moser et al., 1989; Murray et al., 1977; Nantulya, 1990). Each of these techniques presents some strong and weak points.

Serodiagnosis is a sensitive method but does not differentiate active infections from cured ones, nor does it distinguishes between different species of trypanosomes due to a poor specificity of the method (Desquesnes, 1997). However, Bossard et al. (2010) recently developed an inhibition ELISA based on the Heat Shock Protein 70 that is closely related to the mammalian Immunoglobulin Binding Protein. The specificity of this method has been reported to be much better than previous tests.

Parasitological techniques such as the haematocrit centrifugation technique (HCT), Buffy Coat examination (BCE) and stained blood smear examination have been reported to be of low sensitivity but good specificity (Gardiner, 1989). These techniques are of limited significance when parasitaemias are low as often observed in endemic areas (Desquesnes, 1997).

The polymerase chain reaction (PCR) has been reported to be the most sensitive and specific technique to detect trypanosomal DNA in either the vector or the host (Delespaux et al., 2006; Geysen et al., 2003; Lefrancois et al., 1998; Majiwa et al., 1993; Reifenberg et al., 1997; Solano et al., 1999). There have been several advances in the development of PCR using different

primer sets for the identification of trypanosomes at species and sub-species level. For instance, Masiga et al. (1992) designed oligonucleotide primers targeting the satellite DNA monomer to accurately identify *T. simiae*, *T. congolense*, *T. brucei* and *T. vivax*. Geysen et al. (2003) developed a semi-nested PCR-Restriction Fragment Length Polymorphism (RFLP) based on the 18S ribosomal small subunit for the detection of all trypanosome species including the three subtypes of *T. congolense* using three primers. Desquesnes et al. (2001) identified the three *T. congolense* subtypes, the *Trypanozoon* group, *T. vivax*, *T. simiae* and *Trypanosoma theileri* using a single PCR based on the Internal Transcribed Spacer 1 (ITS-1) of rDNA. However, PCR has been scarcely applied to assess the prevalence of trypanosomosis on field samples, due to its time-consuming and costly aspects as well as the requirement for technical expertise (Solano et al., 1999). For our study, the semi-nested PCR-RFLP developed by Geysen et al. (2003) was preferred to other molecular methods because of its high sensitivity (multicopy gene) and its ability of differentiating the three subtypes of *T. congolense*. This PCR cannot differentiate species within the trypanozoon group but being not or weakly pathogenic for cattle, this issue was not considered as a constraint.

Separating the sampling from the laboratory PCR activities involves the storage of the biological material for later processing. This might constitute a critical point as contamination between samples can easily occur in field conditions. Replacing the conventional filter papers for storage by blood aliquots preserved in a DNA protecting buffer decreases the time for the preparation of buffy-coats spotted on filter papers (e.g. impregnation, drying and storage), decreases also the potential contact between samples and thus the risk of contamination. Furthermore, the volume of blood that is collected is larger and subsequently allows the extraction of more target DNA. This should allow a higher number of detected cases by the PCR-RFLP. The objective of this study was to compare the detection of parasites by HCT, a technique routinely used in the field, with PCR-RFLP on samples that were stored either on Whatman® filter papers (PCR-RFLP-fp) or in a DNA protecting buffer (PCR-RFLP-pb). In the absence of gold standard, specificities and sensitivities were not calculated.

4.3 Materials and Methods

4.3.1 Study animals and site

A heterogeneous group of 411 cattle of different ages and sexes were selected from different villages (kebeles) in and around the Ghibe valley, South-Western Ethiopia (Jimma and Gurage zones). Details on the epidemiology of trypanosomosis and trypanocidal drug resistance in the area have been mentioned elsewhere in Fikru et al. (2012) and Moti et al. (2012) respectively.

4.3.2 Protocol

4.3.2.1 Blood sample collection

From each animal, whole blood was collected by puncture of the jugular vein into 5 ml heparinized Venosafe® tubes (Terumo Europe) for further analysis using haematocrit centrifugation technique and molecular assay.

4.3.2.2 Haematocrit Centrifugation Technique (HCT)

The blood samples were then examined using the HCT as described by Woo (1970). Briefly, blood was collected in a microhaematocrit capillary tube and sealed on one end. The capillary tube was centrifuged at 15,000 rpm for 5 minutes to concentrate the trypanosomes in the buffy coat layer. Then, the capillary tube was placed in a Woo viewing chamber and a cover slip of 24x24 mm was placed on the capillary tube. The space between the tubes and the cover slip was filled with a drop of water to reduce light diffraction. The buffy-coat plasma junction was examined for the presence of trypanosomes and species was identified through the type of their motility. The Packed Cell Volume (PCV) was also measured and a value below 24 % was considered as anaemia (Marcotty et al., 2008).

4.3.2.3 Molecular assay

4.3.2.3.1 Preparation of the buffy coats spotted on filter paper and DNA extraction

The buffy coat was prepared from about 50µl of heparinized blood in plain capillary tubes and was extracted from each capillary tube after the HCT was completed by cutting the tube approximately 1 mm below the red cell/plasma interface. The buffy coat from each tube was spotted on filter papers (Whatman® n° 4, GE Healthcare Bio-Sciences AB, Sweden) and dried protected from direct sun light and flies. The filter papers were kept in individual envelopes and then in a plastic bag containing silica gel for storage at 2-8°C (refrigerator) until further processing.

DNA extractions were done using the saponin-PBS method described by de Almeida et al. (1998). Briefly, four confetti's of 5 mm in diameter i.e. approximately 2/3 of the total surface of the dried buffy coat spot, were punched out for DNA extraction using 10% Chelex® (BIORAD, Belgium). To avoid contamination, two stainless steel punching tools were alternated after they were flame-sterilized and cooled.

4.3.2.3.2 Whole blood in protecting buffer and DNA extraction

Aliquots of whole blood (800µl) were kept in an equal volume of AS1 buffer® (Qiagen, USA) for cell lysis and DNA preservation at ambient temperature for a period of less than 3 months. DNA extraction was done from 200µl of blood/AS1 buffer® combination using QIAamp mini blood kit® according to the manufacturer's instructions (Qiagen, USA). Extracted DNA was stored at -20°C till further processing.

4.3.2.3.3 Trypanosome species identification

For species identification, DNA amplifications were done using three primers targeting the gene coding for the small ribosomal subunit 18S (semi-nested PCR) and followed by digestion of the amplicons using *Msp*1 enzyme (New England BioLabs, USA) as described by Geysen et al.

(2003). PCR conducted on DNA extracted from buffy coat spotted on filter paper was abbreviated PCR-RFLP-fp. When the DNA extraction was performed from whole blood in protection buffer PCR-RFLP-pb was used.

4.3.3 Data analysis

The percentage of positivity for each trypanosome species detected by the different methods together with their 95% exact (Clopper-Pearson) confidence intervals was calculated. Techniques (HCT and PCR-RFLP's) were compared pairwise with respect to estimated prevalences by the Fisher exact test at the 5% significance level. The distribution of the positive cases of *T. vivax* and *T congolense* was also compared pairwise between the methods by the Fisher exact test at the 5% significance level using SAS Version 9.3 (SAS Institute Inc., Cary, NC, USA).

4.4 Results

4.4.1 Percentage positivity using different sampling methods

Compared to HCT, the percentage of positivity for the presence of trypanosomes was increased by more than 50% and 250% when using PCR-RFLP-fp and PCR-RFLP-pb respectively (see Table 4.1). The percentages of positivity for the three tests were statistically different from each other when compared two by two ($p < 0.001$). The non-pathogenic trypanosome species *T. theileri* was excluded from the analysis to avoid exaggerating the differences between the tests during comparison.

4.4.2 Comparison of HCT and PCR-RFLP-fp

Out of 49 HCT positive samples, 14 (28.57%) (CI = 16.58-43.26) also scored positive when using PCR-RFLP-fp. In contrast, PCR-RFLP-fp detected more positive cases compared to HCT, but the difference was not statistically significant (Table 4.2).

Table 4.1 The number and percentage of positivity of trypanosome infections detected by HCT, PCR-RFLP-fp and PCR-RFLP-pb in cattle blood samples from Ethiopia

Species	**HCT positive**	**PCR-RFLP-fp positive**	**PCR-RFLP-pb positive**
	N (% - LCL/UCL)	**N (% - LCL/UCL)**	**N (% - LCL/UCL)**
Tc	16 (3.89-2.24/6.25)	73 (17.76-14.19/21.81)	100 (24.33-20.26/28.78)
Tv	23 (5.60-3.58/8.28)	2 (0.49-0.06/1.18)	23 (5.56-3.58/8.28)
Tc + Tv	8 (1.95-0.84/3.80)	0 (0.00/0.089)	1 (0.2-0.01/1.35)
T + Tv	2 (0.49-0.06/1.18)	0 (0.00/0.089)	0 (0.00/0.089)
Total	**49 (11.92–8.95/15.45)**	**75 (18.25–14.63/22.33)**	**124 (30.17–25.77/34.86)**

With HCT: haematocrit centrifuge technique, PCR-RFLP-fp: 18S-PCR-RFLP using samples stored on Whatman filter papers, PCR-RFLP-pb: 18S-PCR-RFLP using samples stored in a commercial cell lysis and DNA protecting buffer, N: number, LCL: lower confidence level, UCL: upper confidence level, Tv:*T. vivax;* Tc: *T. congolense* savannah*;* T: T*rypanozoon.*

Table 4.2 Comparison of the detection of trypanosome infection using HCT, PCR-RFLP-fp and PCR-RFLP-pb on cattle blood samples from Ethiopia

		PCR-RFLP-pb Negative	**PCR-RFLP-pb Positive**	**Total**
HCT Negative	PCR-RFLP-fp Negative	243	58	301
	PCR-RFLP-fp Positive	23	38	61
	Total	266	96	362
HCT Positive	PCR-RFLP-fp Negative	11	24	35
	PCR-RFLP-fp Positive	10	4	14
	Total	21	28	49

Table 4.1. shows the results of species identification. Out of 411 samples, 24 (5.8%) were detected as *T. congolense* savannah by HCT (16 single and 8 mixed) whereas PCR-RFLP-fp detected 73 (17.8%) positives (all single); 33 (8.0%) (CI = 5.59-11.09) were detected as *T. vivax* by HCT (23 single and 10 mixed) whereas PCR-RFLP-fp detected 2 (0.48%) (CI = 0.06-1.75) (all single). None were detected as *Trypanozoon* by PCR-RFLP-fp whereas HCT detected 2 (0.5%) (2 mixed). The difference in species identification using PCR-RFLP-fp compared to HCT was statistically significant ($p < 0.001$).

4.4.3 Comparison of HCT versus PCR-RFLP-pb

Out of 49 HCT positive samples, 28 (57.1%) also scored positive when using PCR-RFLP-pb. On the other hand, PCR-RFLP-pb detected more positives compared to HCT (Table 4.2) and the difference was statistically significant ($p < 0.001$).

For species identification, out of 411 samples, 24 (5.84%) (CI = 3.78-8.56) were detected as *T. congolense* savannah by HCT (16 single and 8 mixed) whereas PCR-RFLP-pb detected 101 (24.57%) (CI = 20.49-29.03) positives (100 single and 1 mixed); 33 (8.02%) (CI = 5.59-11.09) were detected as *T. vivax* by HCT (23 single and 10 mixed) whereas PCR-RFLP-pb detected 24 (5.8%) (CI = 3.78-8.56) (23 single and 1 mixed); 2 (0.48%) (CI = 0.06-1.75) were detected as *Trypanozoon* by HCT (2 mixed) whereas PCR-RFLP-pb detected none. Difference in species identification using PCR-RFLP-pb compared to HCT was statistically significant ($p < 0.001$).

4.4.4 Comparison of PCR-RFLP-pb and PCR-RFLP-fp

Out of 124 PCR-RFLP-pb positive samples, 42 (33.9%) (CI = 26.37-43.25) also scored positive when using PCR-RFLP-fp. On the other hand, PCR-RFLP-fp detected 33 positives that were not detected by PCR-RFLP-pb. The difference in detection of infection was statistically significant ($p < 0.001$).

For species identification, out of 411 samples, 101 (24.57%) (CI = 20.49-29.03) were detected as *T. congolense* savannah by PCR-RFLP-pb (100 single and 1 mixed) whereas PCR-RFLP-fp detected 73 (17.76%) (CI = 14.19-21.81) cases (all single); 24 (5.84%) (CI = 3.78-8.56) were detected as *T. vivax* by PCR-RFLP-pb (23 single and 1 mixed) whereas PCR-RFLP-fp detected 2 (4.87%) (CI = 0.6-1.75) (all single); no *Trypanozoon* was detected by none of the PCR. Considering *T. congolense* and *T. vivax* detection, the percentage positivity was higher with PCR-RFLP-pb ($p < 0.001$).

4.4.5 Relationship of PCV value with HCT and PCR-RFLP-fp or -pb

The mean PCV was 26.2±5.0 and anaemic condition of the animal (PCV < 24) was found matching with diagnostic test positivity for trypanosomosis in 29 (59.2%) (CI = 44.23-73.01), 36 (48%) (CI = 36.35-60.07) and 53 (42.7%) (CI = 34.21-52.30) cases for HCT, PCR-RFLP-fp and PCR-RFLP-pb, respectively (Table 4.3).

Table 4.3 Relationship between PCV category and presence of trypanosomes infection with the three different tests used on cattle blood from Ethiopia

PCV category	HCT +N (%, LCL/UCL)	PCR-RFLP-fp +N (%, LCL/UCL)	PCR-RFLP-pb +N (%, LCL/UCL)
PCV < 24 (n = 109)	29 (59.2-44.23/73.01)	36 (48-36.35/60.07)	53 (42.7-34.21/52.30)
PCV ≥ 24 (n = 302)	20 (40.82-28.06/54.94)	39 (52.00-40.78/63.02)	71 (57.26-48.42/65.66)
Total (n = 411)	49	75	124

With N: number; LCL: lower confidence level; +: positive; UCL: upper confidence level

4.5 Discussion

The aim of this study was to compare the HCT with the PCR-RFLP on samples from the same animal but stored under two different conditions, i.e. either buffy coat spots on filter paper or whole blood in lysis/stabilization buffer. Filter papers are commonly used to store blood or buffy coat samples for epidemiological surveys. This method for field sample storage presents multiple advantages as a low cost, a low weight allowing easy transport and long term preservation. However, two important drawbacks were observed from a long time practice in the field i.e. a potential contamination between samples by contact between the filters or unclean hands of the technician and the low quantity of blood that is effectively used for DNA extraction. To overcome these drawbacks, an alternative storage method is available using a commercial buffer (QIAGEN AS1® - QIAGEN, USA) that is lysing the cells and protecting the DNA present in the solution for several months at ambient temperature.

The main outcome of this study is clearly better trypanosome detection with PCR performed on DNA extracted from a larger volume of whole blood (± three times the volume). The larger the volume of the blood sample used for DNA extraction, the higher the probability of detecting trypanosomes at low concentration. PCR-RFLP-fp method is performed routinely with 30 µl aliquots and PCR-RFLP-pb with 100 µl blood samples. The number of positives would have certainly increased if QIAGEN Midi-kit® (uses up to 2ml blood) was used rather than the Mini-kit (uses up to 200 µl blood) for the DNA extraction. However, the price has to be considered with 0.44€ and 7€ for the mini and midi kits, respectively. In our study, the detection efficiency of the PCR was higher (124 against 75) when using PCR-RFLP-pb compared to PCR-RFLP-fp. The 65% gain in detection might however be less than expected when tripling the volume of the sample used for DNA extraction. An explanation could be a lower efficiency of the QIAGEN kit compared to the chelex-based extraction. However Hsiang et al. (2010) reported that the two methods were equally efficient.

Beside the considerations made here above, some results deserve more accurate observation such as (i) negative PCR's that coincide with a positive HCT, (ii) two samples that were detected as *T. vivax* by PCR-RFLP-fp and as *T. theileri* by PCR-RFLP-pb.

Due to the sampling procedure i.e. a simultaneous processing of the microscopic examination and preparation of the buffy coats on filter paper or blood in the protective buffer, detection of trypanosome was done for each animal on three independent samples (obtained from the bleeding of the same animal, but being three distinct aliquots from the same 5 ml Vacutainer® tube). Parasitaemia observed in the field are commonly oscillating between the detection limits of the HCT and of the 18S-PCR being around 250-500 parasites/ml and 25 parasites/ml, respectively (Geysen et al., 2003; Paris et al., 1982). These low parasitaemia are likely to explain some differences between the aliquots that are tested with the different methods. Some capillary tubes filled from the same 5 ml Vacutainer® tube might contain no trypanosome or a cluster of trypanosomes or even different species of trypanosomes. As stated above, the detection limit of the 18S-PCR is situated around 25 trypanosomes/ml meaning about 1 trypanosome/capillary tube. At this low level of parasitaemia, some of the capillaries may contain 1, 2 or more trypanosomes and some may be empty. This situation was previously reported by Junqueira et al. (1996) with negative PCR's correlated to positive xenodiagnosis. This is likely the explanation of the positive HCT versus negative PCR and the two samples that were detected as *T. vivax* by PCR-RFLP-fp and as *T. theileri* by PCR-RFLP-pb.

The number of *T. vivax* detected by HCT (33) was significantly higher than by PCR-RFLP-fp and PCR-RFLP-pb that detected 2 and 24, respectively. A lower sensitivity for *T. vivax* detection using an 18S based PCR had already been reported elsewhere (Njiru et al., 2005). This might be explained by the limited sensitivity of the PCR for this particular species linked to a high genetic diversity of the 18S locus. Additionally, the microscopical observation of *T. vivax* is strongly facilitated by the high mobility of this species. On the other hand, the PCR-RFLP-fp and PCR-RFLP-pb detected more *T. congolense* infections than the ITS1 PCR on the same samples (Fikru

et al. 2012). The discrepancy in this case can be explained by the fact that the 18S-PCR is a semi-nested PCR with, in principle, a lower detection limit.

Finally, the question should be raised to know if the infection or the disease has to be diagnosed. This was already explored for trypanosomosis in goats in Burkina Faso where sick animals were always microscopically positive (Vitouley et al., 2012). Diagnosing the disease with a less sensitive method like the HCT keeps its full sense in the strategies aiming at reducing the economic impact of the disease at the herd level. In our study, the proportion of anaemic animals was higher in the HCT positive animals (59.2%) compared to 48% and 42.7% for PCR-RFLP-fp and PCR-RFLP-pb, respectively. HCT remains a robust field method as it will be more efficient than PCR to detect clinical cases that have to be treated. Indeed, in routine veterinary practice diagnosing the disease remains the priority compared to detecting parasites. Alternatively, the PCR constitutes a valuable tool for epidemiological surveys and for drug efficacy testing where an evidence of cure is necessary. The parasitological technique combined with the clinical examination is ideal when the focus is on the disease. The molecular techniques are useful when the focus is on the presence of the parasite (presence/absence, geographic distribution, herd prevalences). The multi-species PCR present the advantage of decreasing the work load and the final cost of the operation. Only one PCR has to be done on each sample and the restriction step for species diagnosis applies only for the positives.

4.6 Conclusion

Major differences were observed during this study between the detection of trypanosome by the three methods. Therefore, it is of a high importance to consider carefully the protocol of any trypanosome prevalence survey before comparing any results. Prevalences might vary by a factor 2 to 3 depending on the method that is used. Haematocrit values should also be systematically correlated with infection status as this is providing useful information on the impact of the disease in a specific area. Highly virulent parasites will strongly affect the PCV values of infected animals as opposed to more endemic situations where a better host/parasite

balance is observed. This distinction is essential for the establishment of a coherent control strategy.

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Chapter 5

Close-up on diminazene aceturate resistance by PCR and microsatellite analysis correlated to knowledge, attitude and practice of livestock keepers: the case of Ghibe river basin in South-western Ethiopia

Based on:

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5.1 Abstract

African Animal Trypanosomosis is threatening the agricultural production and cattle breeding more severely than any other livestock disease in the continent, even more since the advent of drug resistance. A longitudinal study was conducted from November 2012 to May 2013 in Ghibe valley to evaluate diminazene aceturate (DA) resistance, follow infection dynamics of *T. congolense* after treatment and assess the livestock owner's perception of trypanocidal drug use. For blood sampling, four Peasant Associations (PAs) were purposively selected. At baseline, 106 infected and 119 negative control animals were recruited for six months follow-up using HCT, 18S-PCR-RFLP and microsatellite analysis. In another 10 purposively selected PAs, a semi-structured questionnaire was used to interview farmers about drug use. Prevalence of trypanosomosis was 18.1% based on the HCT technique and the mean PCV value was 23.6±5.1% for the 587 sampled cattle. Out of the 106 HCT positive, 64 (60.4%) were positive for the presence of trypanosomes using the *18S*-PCR-RFLP. Species detected by this method were 38 (59.4%) *T. congolense* savannah, 18 (28.1%) *T. vivax*, 5 (7.8%) *T. theileri* and 3 (4.7%) *T. congolense* Kilifi. Among the *T. congolense* savannah samples, 31 (81.6%) showed a DA resistant RFLP profile, 2 (5.3%) a mixed profile and 5 did not amplify using the *DpnII*-PCR-RFLP. A positive HCT had a significant effect on PCV ($P < 0.001$) with the mean PCV value equal to 24.4±0.2% in the absence of trypanosomes and to 20.9± 0.3% in the presence of trypanosomes. PCV increased significantly ($P < 0.001$) with 4.4 ± 0.5% one month after treatment. All *T. congolense* savannah type were analysed using the microsatellite markers TCM1, TCM3 and TCM4 to follow the evolution of infection after treatment. The main events were new infections (40.0%) and relapses (37.5%) with cures lagging at 22.5%. From the questionnaire survey, the average herd size in Tropical Livestock Unit (TLU) was highest in Abelti PA (6.7±1.8) and a statistically significant difference ($p=0.01$) in the mean herd size in the 10 PAs. Trypanosomosis was designated as the main disease affecting cattle by 97% of the respondents. DA was used by 95.5% of the farmers though more than half of them (51.9%) were not familiar with isometamidium (ISM). There was a trend to overdose young small animals and to underdose large ones. Oxen were treated very frequently (nearly 20 times/year) and calves almost never.

To improve the situation in the Ghibe valley, extension packages should be delivered to promote a rational drug use, improved livestock management and the application of strategic vector control methods.

Keywords: Trypanosoma congolense, 18S-PCR-RFLP, microsatellite, trypanosomes, PCV, Diminazene, cattle, Ghibe

5.2 Introduction

Livestock contributes significantly to the economy of developing countries. However, animal diseases including trypanosomosis are still major constraints to the livestock-sector productivity (Forman et al., 2012). African Animal Trypanosomosis (AAT) is a disease transmitted biologically by tsetse and mechanically by various hematophagous biting flies like stomoxes or tabanids. The disease causes morbidity and mortality in wide geographic areas estimated to 9 million km^2 across Africa (Jahnke et al., 1988). This insect-borne disease is caused by kinetoplastid protozoa and affects most species of domestic livestock and wild animals. (Getachew, 2005; Osório et al., 2008). AAT has been listed among the neglected tropical diseases affecting marginalized populations (FAO, 2002). Moreover, AAT afflicts the poorest and remotest populations with limited or no access to veterinary health services. It is threatening the agricultural production and cattle breeding more severely than any other livestock disease in the continent (Getachew, 2005; Pagabeleguem et al., 2012; WHO, 2012).

With a population now exceeding 80 million, Ethiopia is the second most populated country in Africa after Nigeria. Most Ethiopians live in highland areas, with 85% of the population being rural and relying on low productivity agriculture. The ever increasing population pressure in those highland areas has led to an expansion of agriculture to more marginal zones (Awulachew et al., 2007) which are potentially productive but tsetse infested (Getachew, 2005; Reid et al., 2000). To help people to survive in these marginal areas, trypanosomosis control activities have been successfully implemented: (i) in the Ghibe valley using 'pour-on' insecticides (Leak et al.,

1995; Rowlands et al., 2000), (ii) in Didessa valley using community based odour-baited, insecticide impregnated target/trap technology as part of the Eastern African Regional Programme and, finally (iii) in the southern Rift valley, the eradication of the flies using the sterile insect technique (Getachew, 2005). However, due to the lack of natural barriers and to the non-sustained control efforts, flies reinvade the tsetse-cleared territories. Thus, farmers still rely on chemotherapy and chemoprohylaxis to maintain their livestock in acceptable health condition. Unfortunately, treatment failures and chemoresistance against the existing trypanocidal drugs have also been reported (Codjia et al., 1993; Moti et al., 2012; Mulugeta et al., 1997; Peregrine et al., 1997; Tewelde et al., 2004).

The way chemotherapy and chemoprophylaxis is performed in the field and its efficacy depends on multiple factors such as farming type, herd size and structure, breed, knowledge of the disease, availability of veterinary services and drugs and legislation about drug administration. Those parameters can fairly be evaluated by standardized questionnaires. In addition, molecular techniques like microsatellite loci length analysis allows population genetics and phylogenetic analysis (Duvallet et al., 1999; Morrison et al., 2009) but also to gain better insight in the infection dynamics of trypanosomosis. Most of the papers on AAT differentiate between infected and uninfected animals (Abebe and Wolde, 2010; Cherenet et al., 2004; Kebede and Animut, 2009; Leak et al., 1993; Mekuria and Gadissa, 2011; Mulugeta et al., 1997; Rowlands et al., 2001, 1993; Sinshaw et al., 2006; Tafese et al., 2012) but do not follow the infection status of a specific animal over time. The evolution of an infection after a treatment can be a cure or a "relapse". However, a relapse can be the result of different events i.e. (i) the parasite was not killed by the treatment, (ii) the parasite was killed but a new infection occurred and (iii) the parasite was not killed and a new infection occurred by one or several strains. When setting up this study, our overall objective was to gain a more precise perspective of the drug resistance by examining it from a dynamic point of view and to link farmer's practices to the chemoresistance situation. The specific objectives of this study were (i) to evaluate diminazene aceturate (DA) resistance with the *DpnII*-PCR-RFLP assay, (ii) to follow the evolution after treatment of *T. congolense* savannah infection using microsatellite loci analysis and (iii) to

assess the agro-pastoral Knowledge, Attitudes and Practices (KAP) of livestock owners in the Ghibe valley, Ethiopia.

5.3 Materials and Methods

5.3.1 Study site description

The present study was conducted in the Ghibe valley area which is located at about 180 km southwest of Addis Ababa. The mean annual rainfall hardly reaches 900 mm. The mean monthly temperature ranges from 29.8 to 44.0^{o}C (Rowlands et al., 2001). Agriculture is mainly based on a small-scale mixed crop-livestock production with little financial input. Cattle are used as draught power and as an alternative banking system. Mechanization of the farming activities concerns only a few larger exploitations. The study site and the peasant associations (PAs) were selected on the ground of a previous cross-sectional study (Moti et al., 2012). The landscape is topographically heterogeneous, consisting of upper plateaus (1400-1800 m elevation) separated by deep gorges of the Gilgel Ghibe and Ghibe Rivers (Reid et al., 2000). The intense agricultural expansion is thinning out the forest and natural vegetation coverage. These changes in land use and cover is a direct consequence of trypanosomosis control and prevention (Reid et al., 2000).

5.3.2 Longitudinal study

5.3.2.1 Study animals

The cattle included in this study were all of zebu breed and classified as calves (< 1 year), young adults (1-4 years), and adults (> 4 years), including cows, bulls and oxen. Three classes of body conditions were considered i.e. lean, medium and fat (Nicholson and Butterworth, 1986).

5.3.2.2 Sampling strategy and sample size

This study was conducted from November 2012 to May 2013. Four PA's were purposively selected (ease of access and proximity of suitable tsetse habitat according to expert opinion): Yatu, Keta Wabe, Keta Bosso and Boke (Figure 5.1).

During the first two visits, the peasants were requested to gather their cattle at one site where they were purposively selected (the closest animal at the moment of blood taking). The haematocrit centrifugation technique (HCT) was used to detect trypanosome infections in the animals.

To recruit a group of 106 positive animals, a total of 587 cattle were bled. The 106 positive and 119 negative control animals were recruited and ear-tagged (Figure 5.2). The remaining 362 negative cattle were not further followed up. The selected animals were visited monthly for a period of six months. During each sampling, blood samples were collected for HCT on the spot and aliquots were preserved for further molecular analysis. In between the monthly visits, farmers were asked to report any acutely sick animal to the local development agent who was responsible for veterinary assistance and had to report any treatment.

5.3.2.3 Ethical consideration

The experimental protocol and sampling were approved by the Ethical Committee of Jimma University and the Zonal Agricultural Bureau. The background of the study was explained to the PA's and consent of the farmers was asked for sampling of their animals.

5.3.2.4 Blood collection

Two ml of blood was collected from the jugular vein in EDTA treated BD Vacutainer® tubes (Becton-Dickinson, USA) for parasitological examination. About 0.75 ml was preserved in an equal volume of saturated 6 M guanidine buffer for further molecular analysis.

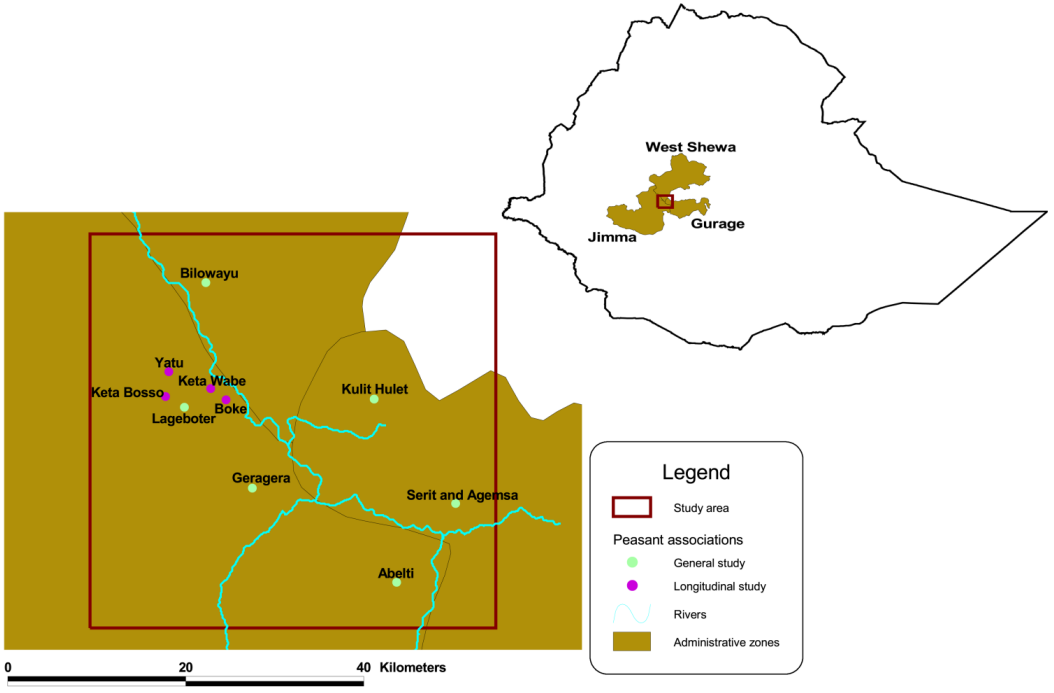

Figure 5.1 Map of Ethiopia depicting sites of questionnaire survey and longitudinal study (left) in the Ghibe.

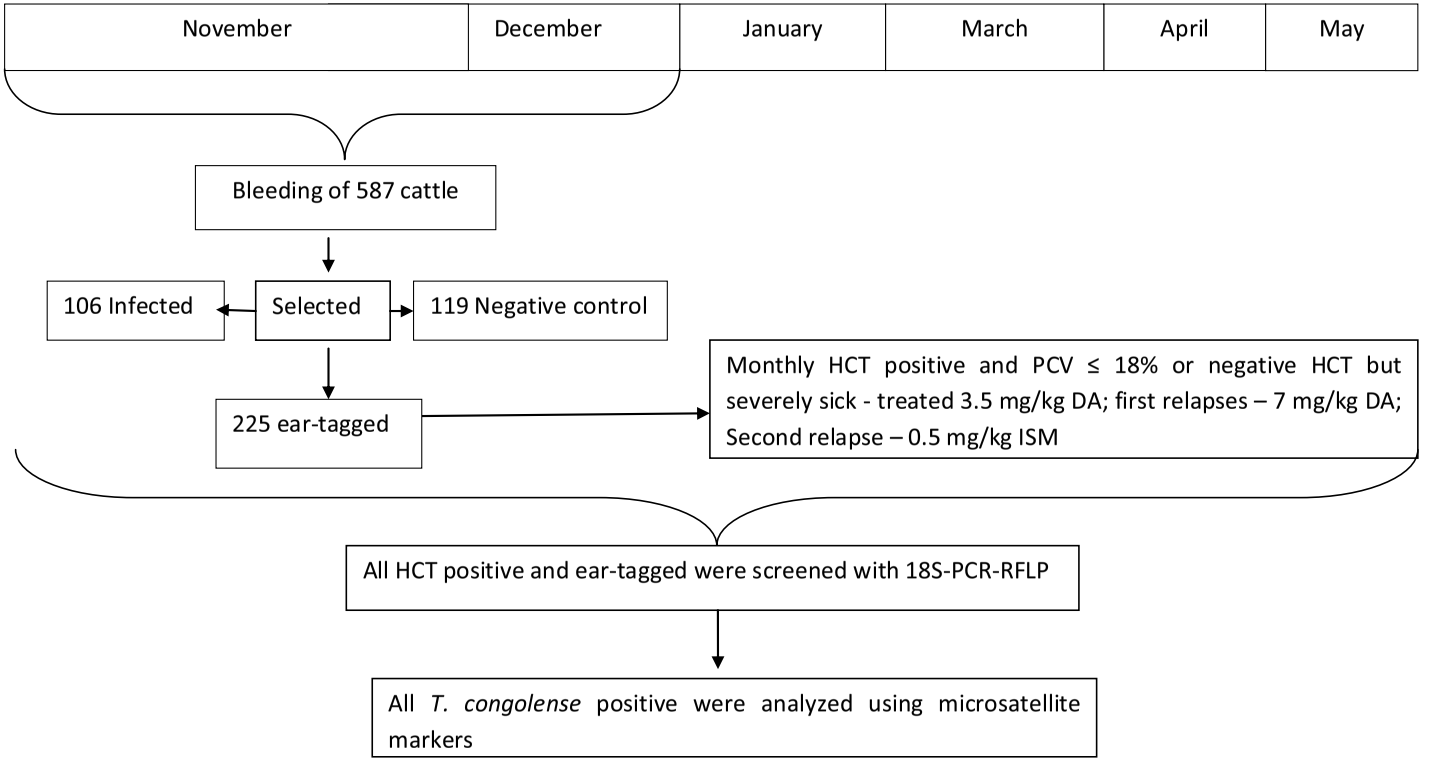

Figure 5.2 Sketch of longitudinal study protocol followed during DA resistance study in the Ghibe valley.

As an incentive, antibiotics, acaricides and anthelmintics were provided for the treatment of sick animals. In addition, minor surgical operations and advice on animal husbandry were provided for free.

5.3.2.5 Haematocrit Centrifugation Technique

Blood from the EDTA treated BD Vacutainer® tubes (Becton-Dickinson, USA) was collected in microhaematocrit capillary tubes and sealed on one end with Cristaseal® (Hawksley). The capillary tubes were centrifuged at 15, 000 rpm for 5 minutes and then placed in a Woo viewing chamber. The leukocyte - plasma interface was examined for the presence of trypanosomes (Woo, 1970). The packed cell volume (PCV) was measured and values below 24% were considered as anaemia (Marcotty et al., 2008; Van den Bossche and Rowlands, 2001).

Cattle found positive for any species of pathogenic trypanosome with a PCV lower or equal to 18% and negative cattle with a PCV lower or equal to 18% were treated with 3.5 mg diminazene aceturate (DA) /kg B.W. (Veriben®, CEVA Santé Animale, France). Any animal relapsing after treatment with 3.5 mg DA/kg B.W. was treated with the double dose. Any animal relapsing after administration of this double dose was treated with 0.5 mg isometamidium chloride (ISM) /kg B.W. (Veridium®, CEVA Santé Animale, France).

5.3.2.6 Molecular Assays

5.3.2.6.1 DNA extraction and 18S-PCR-RFLP screening

For the 106 HCT positive cattle, DNA was extracted from 200µl of the mixture blood/guanidine buffer using the commercial kit QIAamp® DNA Blood Mini kit (Quiagen Inc, Valencia, USA). For trypanosome species confirmation, DNA amplifications were done using three primers targeting the gene coding for the small ribosomal subunit 18S (semi-nested PCR) and followed by digestion of the amplicons using *Msp*1 and *Eco*571 enzymes (New England BioLabs, USA) as described by Geysen et al. (2003).

5.3.2.6.2 Diminazene aceturate DpnII-PCR-RFLP resistance test

All *T. congolense* positive samples (based on 18S-PCR-RFLP) were amplified using two primers targeting the P1-type purine transporter TcoNT10 gene. The accession number of TcoNT10 gene is TcIL3000_9_2500 (http://www.genedb.org). Then, the PCR products were digested by *Dpn*II restriction enzyme for DA resistance determination (Delespaux and de Koning, 2013; Delespaux et al., 2006; Munday et al., 2013; Vitouley et al., 2011).

5.3.2.6.3 Microsatellite loci analysis of T. congolense samples

Five species specific polymorphic microsatellite markers TCM1, TCM2, TCM3, TCM4 and TCM7 (Morrison et al., 2009) were considered in the analysis of the *T. congolense* samples. Briefly, Morrison et al. (2009) identified these markers by screening of *T. congolense* genome sequence with the Tandem Repeat Finder software, which resulted in the identification of 4500 loci containing repeats. Selected 25 candidate microsatellite loci were tested initially against a reference panel of *T. congolense* isolates to determine the level of polymorphism and potential sub-group specificity and against *T. brucei* and *T. vivax* genomic DNA to ensure species-specificity. In our study, the 6-FAM-labeled PCR products using TCM1, TCM3 and TCM4 were sent to GenoScreen (http://www.genoscreen.fr/, Campus de l'Institut Pasteur de Lille, 59000 Lille Cedex, France) for length determination by capillary electrophoresis. Animals were only considered if data were available for at least two observation points for one single microsatellite locus (Annex 1). For estimating possible events, the binomes values (allele 1 and 2 of the available loci) were considered at two observation points T_1 and T_2. The same values at T_1 and T_2 were considered as a relapse, any new value at T_2 as a new infection and any value disappearing at T_2 as a cured infection.

5.3.3 Questionnaire Survey

A semi-structured questionnaire was adapted from Van den Bossche et al. (2000). Interviewers were trained on data collection and the questionnaire was pre-tested with colleagues before

use in 10 purposively selected PA's with the following inclusion criteria (i) ease of access, (ii) similarity in agro-ecological characteristics and (iii) trypanosomosis prevalence. The 10 selected PA's were situated in the Jimma, Gurage and West Shoa Zone (Figure 5.1). The average cattle herd sizes were compared following conversion to tropical livestock units (TLU) at a conversion rate of 1 for bulls, 0.7 for cows, 0.5 for weaned calves and 0.2 for suckling calves (Machila et al., 2003). Among those 10 PA's, four of them were considered for the longitudinal study. From each PA, about 15 farmers were randomly selected for a total of 156 interviewees. The questionnaire included questions on socio-economic parameters and on knowledge, attitude and practice in trypanocidal drug use.

5.3.4 Statistical analysis

The association between disease prevalence and anaemia, age, sex and study site was investigated by the chi square test at the 5% significance level.

The difference in PCV was tested between the group of animals infected at the beginning of the study (t_0) and control animals. First, PCV at t_0 was compared between the two groups using a fixed effects model with group as fixed effects factor. Next, the PCV profile over time was compared between the two groups using a mixed model with time, group and their interaction as categorical fixed effects and animal as random effect.

In order to assess the relationship between the presence of trypanosomes (HCT) and PCV throughout the whole study, a mixed model was fitted with HCT as categorical fixed effect and animal as random effect.

In order to assess the treatment effect, the PCV change after treatment, i.e., the difference in PCV between the month after the treatment and the month when the treatment was given is used as a response variable in a mixed model with treatment as categorical fixed effect and animal as random effect.

Data generated from questionnaire survey were analyzed using descriptive statistics like means, frequencies and percentages for different parameters. All analyses were made using Statistical Package for Social Sciences (SPSS) version 16 software.

5.4 Results

5.4.1 Prevalence and diminazene aceturate resistance

Based on the HCT technique, the prevalence of trypanosomosis at t_0 was 18.1 % (n=106). Details per species are provided in table 5.1.

Table 5.1 Infection rate per species of trypanosomes (HCT for the 587 animals of the study) in the Ghibe valley

Species diagnosed	Positives	Infection rate (%)
T. vivax	13	2.2
T. congolense	54	9.2
T. brucei	3	0.5
T.v + T.c	26	4.4
T.v + T.b	2	0.3
T.c + T.b	8	1.4
Total	106	18.1

T.v:*T. vivax*, T.c: *T. congolense*, T.b: *T. brucei*

The mean PCV value and standard error was 23.6±5.1% with a range between 9 and 36%. Almost half of the animals (47.9%) had a PCV value below 24% (anaemic) and being anaemic was significantly associated ($P < 0.01$) with the presence of trypanosomes. Infection rates varied significantly ($p < 0.05$) between study sites, body conditions and sexes (Table 5.2).

At t_0, out of the 106 trypanosome HCT-positive blood samples, 64 (60.4%) were positive for the presence of trypanosomes using the *18S*-PCR-RFLP. Species detected were 38 (59.4%) *T. congolense savannah*, 18 (28.1%) *T. vivax*, 5 (7.8%) *T. theileri* and 3 (4.7%) *T. congolense* Kilifi.

Table 5.2 Association of trypanosomosis in the Ghibe valley with study sites, age, sex and body condition

Variable	Categories	Total sampled	Number positive (%)	p-value
Study site	Yatu	120	10 (8.3)	P ≤ 0.001
	Keta Bosso	129	15 (11.6)	
	Boke	235	56 (23.8)	
	Keta Wabe	103	25 (24.3)	
Age	Calf	29	10 (34.5)	P = 0.06
	Young	100	16 (16.0)	
	Adult	458	80 (17.5)	
Sex	Male	370	81 (21.9)	P = 0.002
	Female	217	25 (11.5)	
Body condition	Lean	124	32 (25.8)	P = 0.02
	Medium	250	45 (18.0)	
	Fat	213	29 (13.6)	

The 38 *T. congolense* savannah were screened for DA resistance with the *DpnII*-PCR-RFLP: 31 (81.6%) showed a RFLP profile which may be linked to DA resistance, 2 (5.2%) a mixed profile and the remaining 5 (13.2%) did not amplify. The three *T. congolense* Kilifi gave a negative *DpnII*-PCR.

5.4.2 Longitudinal study

The PCV's evolution in time for the two groups of animals (infected or negative at t_0) is shown in Figure 5.3. The mean PCV value of the negative control animals (24.9 ± 0.5%) differed significantly from that of the infected animals (20.9 ± 0.5%) at the start of the study. The interaction between group and time was significant (P < 0.001), due to the fact that the PCV values of the two groups were coming closer together with time.

At t_0, the presence of trypanosomes (HCT) had a significant effect on PCV (P < 0.001) with the mean PCV value equal to 24.4± 0.2% in the absence of trypanosomes and to 20.9± 0.3% in the

presence of trypanosomes. PCV increased significantly ($P < 0.001$) with 4.4 ± 0.5% one month after treatment.

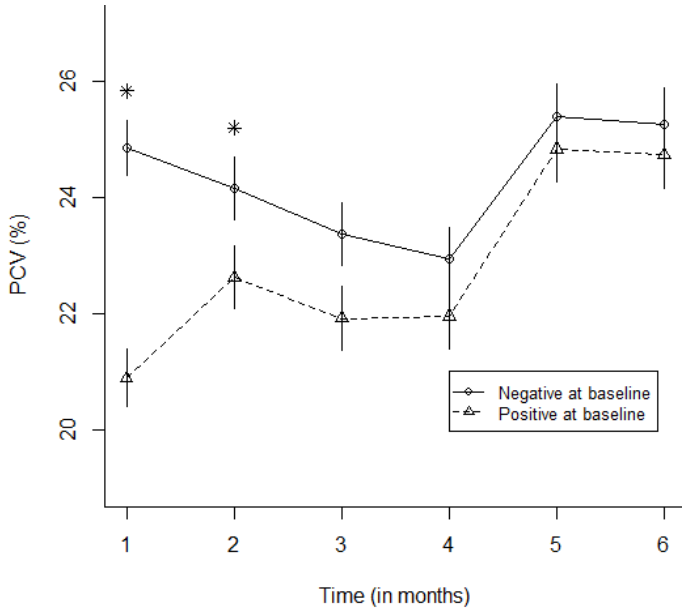

Figure 5.3 PCV evolution for the two groups of animals (HCT negative or positive at t_0) followed by longitudinal study in the Ghibe valley. Asterisk denotes a significant result between the two groups and the bars show the standard error.

5.4.2.1 Microsatellite loci analysis (T.congolense savannah)

Out of the five primer pairs, only three gave satisfactory amplification. TCM2 and TCM7 showed no detectable PCR product after gel electrophoresis. From the 38 samples that were confirmed by 18S-PCR-RFLP as *T. congolense* savannah type, microsatellite data are available for 35 of them (Annex 1).

When an animal is positive after treatment, this can be due to different events: (i) a relapse, (ii) a new infection, (iii) or a combination of both. The distribution of the events is summarized in

table 5.3 and detailed information is available in Annex 2. Most of the events (70 - 80%) took place one or two months after treatment. New infections (40.0%) and relapses (37.5%) are the main events with cures lagging at 22.5%.

Table 5.3 Microsatellite loci analysis results depicting infection events in cattle from the Ghibe valley as a function of the number of months after treatment

Month(s) after treatment	Number of relapses	Number of new infections	Number of cured infections
1	15	11	8
2	13	15	8
3	1	4	2
4	4	6	2

5.4.3 Questionnaire survey

5.4.3.1 Socio-economic status and herd composition profile

The majority of the respondents (98.1%) were male and the mean age of the respondents was 44.6±13.9 years ranging from 18 to 85 years. The family size ranged between 1 and 14 with an average of 5.9±2.3. The mean number of illiterate, grade 1-6, grade 6-12 and higher education per family of the respondents were 2.6±1, 2.8±1.6, 2.0±1.3 and 1.3±0.5, respectively. All interviewed farmers practice mixed farming (crop and livestock production) and for 99.4% of the respondents crop production contributes the most to the family income. The average cattle herd size expressed in TLU was the highest in Abelti PA (6.7±1.8) and all animals were of indigenous zebu breed (Table 5.4). There was a statistically significant difference (p=0.01) in mean cattle herd size between the different PA's considered. Beside cattle, the vast majority of the interviewed farmers have also donkeys and oxen, 70.5 and 98.1%, respectively. Most of them also rely on poultry breeding. For the majority of the respondents (98.7%), trypanosomosis was designated as the main disease affecting their cattle.

Table 5.4 Average herd Tropical Livestock Unit (TLU) profiles of different PAs in the Ghibe valley

Peasant associations	Number interviewed	Average herd TLU ± SD	Minimum	Maximum
Yatu	15	5.16±4.7	1	20
Serit and Agemsa	16	3.80±1.3	2	6
Keta Bosso	15	4.06±1.7	2	7
Legeboter	17	5.44±4.1	2	17
Keta Wabe	15	5.66±1.7	2	8
Boke	16	5.15±2.5	2	10
Bilowayu	15	3.71±1.3	2	6
Gerangera	16	3.92±2.3	2	10
Abelti	15	6.56±1.8	4	10
Kulit Hulet	16	3.62±1.1	2	6

5.4.3.2 DA and ISM use

DA was used by 95.5% of the farmers the year preceding this survey compared to 48.1% for ISM. The majority of the respondents (89.7%) treated their animals because they thought that the animals had trypanosomosis, but for ISM only 6.4% of the respondents used it for prevention and 37.8% for treatment of sick animals. The majority of the farmers (91.3%) used different doses of DA according to the different age categories, for ISM this percentage fell to 46.8% and 51.9% of them didn't use the drug at all. There was a trend to overdose young small animals and to under-dose large ones (Table 5.5).

Oxen are treated frequently (nearly 20 times/year), calve almost never and cows/bulls are situated in between (Table 5.6). In 66% of the cases, farmers are treating their animals by themselves (Table 5.5). The average number of DA sachets used per herd/year was 26.1±16.4 ranging from 3 to 83 sachets. The average number of ISM sachets used per herd/year was 2.1±1.4 ranging from 1 to 6 sachets.

Table 5.5 Different practices of DA and ISM use by farmers in the Ghibe valley

Drug use Practices		DA – % of farmers	ISM – % of farmers
Use of the trypanocide		95.5	48.1
In order to treat trypanosomosis		89.7	37.8
Same dose administered to all age categories		3.2	1.3
Dose calves (0-1 year)	Dosing correctly	0.6	-
	Under dosing	0.6	-
	Over dosing	86.5	47.4
Dose young cattle (1-4 years)	Dosing correctly	5.1	-
	Under dosing	-	0.6
	Over dosing	87.2	48.1
Dose adult cattle (>4 years)	Dosing correctly	93.6	7.7
	Under dosing	-	40.4
	Over dosing	-	0.6
Dilute and keep left over of ISM		-	0.6
Treatment season	Wet	7.9	19.9
	Dry	8.5	31.2
	Both	83.6	48.9
Drug dilution medium	Boiled water	13.6	
	Well water	67.3	
Drug administration	Farmer	66.7	
	Veterinary assistant	30.8	

5.5 Discussion

5.5.1 Prevalence and resistance

Trypanosomosis control and prevention by the Ethiopian government and non-governmental organizations in the Ghibe valley dates back to the second half of the 20th century (Rowlands et al., 1999). This control was supposed to significantly decrease the prevalence of the disease even though eradication was not achieved. However, our study revealed an important prevalence of 18.1% in the Ghibe river basin. This is higher than a prevalence of 12.1% reported

previously in the Metekel zone (Tesfaye et al., 2012) and of 10.1% in the Awi zone (Kebede and Animut, 2009) but it must be recalled that our study sites were purposively selected for their high tsetse challenge.

Table 5.6 Reported DA and ISM treatments by farmers in the Ghibe valley

Type of animal	**Treated using DA (%)**	**Average number of DA treatments/animal/year**	**Treated using ISM (%)**	**Average number of ISM treatments/animal/year**
Calves (0-1yr)	-	-	1 (0.6)	2
Young (1-4yr)	33 (21.2)	4.8±3.4	4 (2.6)	1.8±0.5
Cows treated	96 (61.5)	7±5.6	47 (30.1)	4.2±3.2
Bulls treated	51 (32.7)	6.9±4.7	21 (13.5)	3.3±2.2
Oxen treated	145 (93)	19.4±11.8	69 (44.2)	8.1±6.4

Despite the intensive agricultural expansion in the locality leading to fragmentation of suitable tsetse habitat and the intensive trypanocidal drug use, trypanosomosis prevalence remains high in the Ghibe valley. Two factors are likely to contribute to this situation. Firstly, drug resistance in the region that was already reported by other groups (Codjia et al., 1993; Moti et al., 2012; Rowlands et al., 1993) and confirmed by our *DpnII*-PCR-RFLP and microsatellite analysis (numerous relapses after treatment). Secondly, even though the tsetse habitat is becoming highly fragmented, cattle gets easily in contact with the vector at watering points, especially in the dry season when flies are clustering around unspoiled river banks.

Based upon a strong statistical correlation that was demonstrated between the *Dpn*II-PCR-RFLP and the DA-resistant phenotype (Chitanga et al., 2011; Delespaux et al., 2008, 2006; Mamoudou et al., 2008; Moti et al., 2012), this test remains a reliable read-out for DA-resistance although Munday et al. (2013) showed that the target gene in this test codes for a P1-type purine transporter not directly involved in DA transport.

5.5.2 Longitudinal study

Anaemia is one of the classical clinical signs of trypanosomosis (Van den Bossche and Rowlands, 2001) and an inverse correlation between the PCV's value and the presence of trypanosomes is often observed (Rowlands et al., 2001). In our study, the average PCV value of infected animals was, as expected, significantly lower than those of the HCT negative controls for the first two months of follow up.

No significant difference in PCV was observed during the last four months of observation between t_0 negative and positive group. Indeed, the treatment of any positive animal in both groups is explaining why PCV values converge. Even if DA resistance is present, the effect of treatment on the PCV is still observable. This phenomenon was previously observed in cattle and goats inoculated with drug resistant trypanosomes and treated with trypanocidal drugs (Delespaux et al., 2010; Vitouley et al., 2012).

In the publication of Morrison et al. (2009), seven microsatellite loci were used. However, we were not able to reproduce their results with our samples from the Ghibe valley. The nested PCR was creating some strong artefacts (results not shown). Therefore, we worked with a single run and lost sensitivity. Our data was scantier than expected, with only three loci that amplified properly. However, some conclusions can be drawn. Most of the events (relapse, new infection or cure) were observed during the first two months of follow up. In other words, the probability for an animal to remain free of parasites for more than two months was low. Relapses and new infections constituting, in similar proportion, about 80% of the events indicates that the tsetse challenge is high and drug resistance is present to a large extent.

5.5.3 Questionnaire survey

Most of the farmers had donkeys and oxen showing the importance of the animal traction in the area. Besides crop production, most of the farmers also relied on poultry (short cycle

species for meat production) for quick cash money availability allowing a.o. the purchase of trypanocidal treatments.

DA was more popular among the farmers compared to ISM. Livestock keepers are focusing on sick animals and do not think in terms of prophylaxis. The multi-dose packaging of ISM is a limiting factor. With a mean herd size of around 6, the unused diluted ISM is more a constraint than an advantage. Treatments were definitely focused on the most valuable animals i.e. those used for transport or ploughing. This is in agreement with the observations of Van den Bossche et al. (2000) in Eastern Zambia. The high frequency of treatment of those productive animals is certainly one of the factors explaining the chemoresistance in the area.

The majority of the farmers used water straight from a well for dilution and administered the treatment themselves. The use of unsterilized water might cause of abscesses after injection. Those abscesses are sequestering the drug and lead to lower drug serum concentration and might lead to poor drug efficacy, therapy failure and possible drug resistance. This result is in agreement with the reports of other research groups (Tesfaye et al., 2012; Van den Bossche et al., 2000).

5.6 Conclusions and implications

The situation in the Ghibe valley could be summarized as follows: (i) the tsetse challenge remains high despite habitat fragmentation, (ii) the trypanosomosis prevalence remains high, (iii) drug resistance is present in the area, (iv) veterinary services are scanty, (v) knowledge of the farmers about drug use is low and (vi) many trypanocidal drugs brands from all continents are available from local shops and markets.

Solutions to improve the situation include the delivery of extension packages to promote a rational drug use (appropriate drug quality, correct dilution, right dose, and focus on clinical cases) and to improve the livestock management (appropriate feeding, strategic deworming).

As drug resistance is a reality in the area, strategic vector control should be promoted such as insecticidal spraying of the herds with permethrin or derivates. Years of national efforts for controlling the tsetse fly in Ethiopia have brought some temporary relief to the farmers but flies rapidly reinvade cleared areas. If the budget of the government allows it, then this effort should be continued and extended to areas that are not benefiting from it. The alternative for the farmers is to become self-sustainable but this requires knowledge and efforts. Awaiting for a potential eradication of the tsetse fly, if ever achieved, the EU funded project Trypanosomosis Rational Chemotherapy (TRYRAC) (http://www.trypanocide.eu/) is meanwhile promoting a self-sustainable alternative which is a solution when national programmes for vector control are not implemented.

The last recommendation would be to test the quality of the drugs that are circulating in the area. This is now possible since two African laboratories were equipped for this purpose as part of a FAO - Galvmed initiative namely the Laboratoire de Contrôle des Médicaments Vétérinaires (LACOMEV) in Dakar and the Tanzania Food and Drug Authority (TFDA) Quality Control Laboratory in Dar es Salaam. Those two laboratories are performing standardised tests upon internationally agreed quality standards.

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ANNEX 1. Summary of longitudinal follow up of the microsatellite markers used for analysis of *T. congolense* samples from the Ghibe valley

	November			**December**			**January**			**March**			**April**			**May**		
Animal ID	TCM 1	TCM 3	TCM 4	TCM 1	TCM 3	TCM 4	TCM 1	TCM 3	TCM 4	TCM 1	TCM 3	TCM 4	TCM 1	TCM 3	TCM 4	TCM 1	TCM 3	TCM 4
77		+	+								+	+						
236		+			+													
239								+	+		+							
241								+	+		+							
247								+	+		+	+					+	
252	+	+						+	+		+							
267	+	+	+					+	+									
268		+	+					+	+		+							
269	+	+	+		+													
270	+	+								+	+	+						
271				+	+			+		+	+	+						
274							+	+	+	+	+	+						
283	+	+								+	+	+	+	+				
285	+	+									+	+				+	+	
289				+	+	+	+	+			+							
298	+	+									+							
310													+	+		+		
311		+		+	+	+				+	+	+	+	+				
318		+											+	+				
331								+			+							
332	+	+	+		+						+							
333				+	+	+	+	+	+	+	+							

339								+	+	+	+	+	+	+						
365				+	+			+												
373							+	+	+	+	+	+								
381											+		+				+	+		
429	+	+								+	+	+								
542				+	+					+	+	+								
543				+	+	+		+	+				+	+			+	+		
544											+						+	+		
554				+	+	+	+				+									
561					+			+												
570													+	+			+	+		
574										+	+	+	+	+	+	+				
586					+			+	+											

TCM=*T. congolense* microsatellite marker
With + as microsatellite data available for the animal at an observation moment

ANNEX 2: Details of the *T. congolense* samples from the Ghibe valley analysed using three microsatellite markers and size of the alleles at each microsatellite locus

Month and animal ID	Locus	Allele size 1	Allele size 2	Allele size 3	Allele size 4	Allele size 5	Events	Months post treatment
November 77	TCM3	182	184				NI	4
November 77	TCM4	157	160					
March 77	TCM3	190	193					
March 77	TCM4	157	160					
November 236	TCM3	190	190				R+NI	1
December 236	TCM3	182	190	193				
January 239	TCM3	184	193				2 NI	2
March 239	TCM3	172	184	190				
January 241	TCM3	190	193				R	2
March 241	TCM3	190	193					
January 247	TCM3	182	190				R	2
March 247	TCM3	182	190				TR+R+NI	2
May 247	TCM3	190	193					
November 252	TCM3	182	182				NI	2
January 252	TCM3	184	193				R+NI	2
March 252	TCM3	184	190	193			-	-
November 267	TCM3	182	190				R+NI	2
November 267	TCM4	157	157					
January 267	TCM3	182	182					
January 267	TCM4	157	160					
November 268	TCM3	190	193				NI	2
November 268	TCM4	157	157					
January 268	TCM3	172	184					
January 268	TCM4	157	160				TR+R+NI	2
March 268	TCM3	182	184	190				
November 270	TCM1	171	181				TR+R+NI	4
November 270	TCM3	182	184	190	193			
March 270	TCM1	171	179					
March 270	TCM3	190	193					
December 271	TCM3	190	190					
December 271	TCM1	171	171				R+NI	1
January 271	TCM3	184	190				TR+R+NI	2
March 271	TCM1	171	179					
March 271	TCM3	184	193					
January 274	TCM1	177	177				TR+R+NI	2
January 274	TCM3	172	184					

January 274	TCM4	157	160					
March 274	TCM1	171	177					
March 274	TCM3	172	182	184	190	193		
March 274	TCM4	157	160					
November 283	TCM1	171	171				R+NI	4
November 283	TCM3	182	190					
March 283	TCM1	171	177					
March 283	TCM3	172	182	184	190	193		
April 283	TCM1	171	177				TR+R	1
April 283	TCM3	172	184					
November 285	TCM3	182	190				TR+R+NI	4
March 285	TCM3	172	184	190			TR+R	2
May 285	TCM3	190	190					
December 289	TCM1	171	171				TR+R+NI	1
December 289	TCM3	182	190					
January 289	TCM1	157	161	171	177			
January 289	TCM3	190	190					
November 298	TCM3	190	193				NI	4
March 298	TCM3	182	184					
November 311	TCM3	190	193				R	1
December 311	TCM1	179	183					
December 311	TCM3	190	193					
December 311	TCM4	157	160				TR+NI	3
March 311	TCM1	171	171				TR+NI	1
March 311	TCM3	184	190					
March 311	TCM4	157	157					
April 311	TCM1	179	183					
April 311	TCM3	190	193					
November 318	TCM3	184	193				R	4
April 318	TCM3	184	193					
November 332	TCM3	172	184				NI	1
December 332	TCM3	190	190				R+NI	3
March 332	TCM3	172	182	184	190	193		
December 333	TCM1	171	171				R	1
December 333	TCM3	190	190					
December 333	TCM4	205	207					
January 333	TCM1	171	171					
January 333	TCM3	190	190					
January 333	TCM4	157	240				R+NI	2
March 333	TCM1	171	171					
March 333	TCM3	182	190					

January 331	TCM3	190	193	TR+R+NI	2
March 331	TCM3	182	190		
January 339	TCM3	182	184	NI	2
January 339	TCM4	157	160		
March 339	TCM1	171	183	R+NI	1
March 339	TCM3	190	193		
March 339	TCM4	233	236		
April 339	TCM1	171	171		
April 339	TCM3	182	190		
December 365	TCM1	171	171	R	1
December 365	TCM3	190	190		
January 365	TCM3	190	190		
January 373	TCM1	171	171	R	2
January 373	TCM3	182	190		
January 373	TCM4	157	157		
March 373	TCM1	171	171		
March 373	TCM3	182	190		
March 373	TCM4	157	157		
March 381	TCM3	184	190	TR+R+NI	2
April 381	TCM1	171	183	R	1
May 381	TCM1	171	183		
May 381	TCM3	184	193		
November 429	TCM1	171	171	NI	4
November 429	TCM3	182	182		
March 429	TCM1	171	171		
March 429	TCM3	190	190		
December 542	TCM1	171	181	TR+NI	3
December 542	TCM3	182	190		
March 542	TCM1	171	171		
March 542	TCM3	184	184		
December 543	TCM1	171	179	R	1
December 543	TCM3	190	193		
January 543	TCM3	190	193	NI	3
April 543	TCM1	177	177	TR+NI	1
April 543	TCM3	172	184		
May 543	TCM1	171	179		
May 543	TCM3	190	193		
March 544	TCM1	190	190	TR+NI	2
March 554	TCM3	172	184		
May 544	TCM1	171	181		
May 544	TCM3	182	190		

December 554	TCM1	171	177		R	1
January 554	TCM1	171	177			
December 561	TCM3	182	182		TR+NI	1
January 561	TCM3	172	184			
April 570	TCM1	177	177		R+NI	1
April 570	TCM3	172	184			
May 570	TCM1	177	179			
May 570	TCM3	172	184			
March 574	TCM1	171	179	183	TR+R+NI	1
March 574	TCM3	190	193			
March 574	TCM4	157	160			
April 574	TCM1	179	183			
April 574	TCM3	190	193			
April 574	TCM4	157	160			
December 586	TCM3	182	184		TR+NI	1
January 586	TCM3	172	184			
November 269	TCM3	182	190	193	TR+R	1
December 269	TCM3	190	190			
April 310	TCM1	171	177		R	1
May 310	TCM1	171	177			

Legend: R=relapse, NI=new infection, TR=effectively treated

Chapter 6

General discussion and future perspectives

6.1 General Discussion

The trypanosomosis burden in the Ghibe valley, Southwestern Ethiopia, remains high, despite the long and continuous efforts that have been undertaken in the region to control the disease. The research presented in this dissertation addresses the trypanosomosis problem sequentially as follows.

i. What is the prevalence/incidence of trypanosomosis in the Ghibe valley, Southwestern Ethiopia, and which trypanosome species are contributing most to the burden?
ii. What is the magnitude of DA and ISM resistance in the area?
iii. What is the perception/knowledge of the farmers on trypanocidal drugs, and how does it impact on the trypanosomosis burden generally and on the drug resistance problem specifically?
iv. Based on the insight gained on the trypanosomosis burden in the Ghibe valley, what strategy could be recommended for control.

6.1.1 High prevalence of *T. congolense* savannah infections in the Ghibe river basin

The majority of *T. congolense* strains identified using the 18S-PCR-RFLP belonged to the savannah subgroup. Three (2.8%) strains belonging to the Kilifi subgroup were also identified during the longitudinal study. Three different subgroups of *T. congolense* have been described so far, i.e., the savannah, Kilifi and forest (OIE, 2013; Rodrigues et al., 2014). This has an implication in the disease epidemiology since the pathogenicity among those different *T. congolense* subgroups and even within the savannah subgroup greatly varies (Van den Bossche et al., 2011). Moreover, besides pathogenicity, Rodrigues et al. (2014) showed that they also differ in resistance to drugs, vector species and geographical distribution. Bengaly et al. (2002) reported more severe symptoms and consistently higher levels of parasitaemia, lower haematocrit and lower leukocyte counts in the savannah-type infection compared to the other two types.

Different diagnostic tests exist for the detection of trypanosomes, and for the classification in the different subgroups. The specificity and sensitivity of such diagnostic tests are crucial in epidemiological studies in order to draw a correct conclusion. Therefore, part of the work was devoted to the evaluation of different sampling strategies and tests. In brief, samples were preserved in two different ways and the conventional microscopy test; HCT and PCR-RFLP were compared for the identification of trypanosomes. PCR using larger volume of blood in a protection buffer for DNA extraction, detected more positive cases than buffy coat samples on filter paper. The percentage of samples positive for trypanosomes was increased by more than 50% and 250% when using PCR-RFLP-fp and PCR-RFLP-pb, respectively as compared to HCT.

For the trypanosome species identification, PCR-RFLP-pb was found to be more efficient for *T. vivax* whereas PCR-RFLP-fp detected more *T. congolense*. The discrepancies in detection of trypanosome by the three techniques (HCT, PCR-RFLP-fp, and PCR-RFLP-pb) may be explained by uneven distribution of the parasite in blood samples because of the low parasitaemia in field conditions. This assumption is very much in agreement with Cox et al. (2010) who tested the hypothesis that parasite DNA contained in a blood sample may be unevenly distributed or clustered when using FTA (Fast Technology for the Analysis of nucleic acids) cards, with the result that taking a single punch as a template for a PCR-based diagnostic test may result in a false negative result simply because that punch did not contain any parasite DNA. They further explained that the likelihood of any single punch giving a false-negative is inversely related to the parasitaemia (i.e. parasite density) in the host animal blood. Therefore, in order to decrease the probability of false negative results, rather than using a single punch, the examination of extra punches would give a more accurate estimation of the disease prevalence (Ahmed et al., 2011; Cox et al., 2010). Likewise our study demonstrated that increasing the volume of whole blood sample in protection buffer improved the sensitivity of the PCR-RFLP assay compared to buffy coat spot sampling on filter paper.

Given the ongoing control and prevention using chemotherapy and chemoprophylaxis, the need for reliable diagnostic tools is unquestionable to evaluate the disease burden. The

collection of appropriate samples in a suitable medium is also as vital as choosing the particular diagnostic tool. Sampling on filter paper seems to be the easiest method to collect blood samples for molecular diagnosis in field situation. Though the detection rate when using the buffer solution was found higher, it demands more logistics for sampling and special medium for storage of samples. Unfortunately, the molecular analysis of trypanosomosis still remains costly and time consuming and thus, farmers continue to use clinical diagnosis for immediate treatment of sick animals.

The high prevalence of *T. congolense* savannah in the cattle of the Ghibe might seem astonishing at first sight. Indeed, as Van den Bossche et al. (2010) explained, the anthropogenic environmental changes have important repercussions on the level of interaction between tsetse and cattle and, hence on the distribution, epidemiology and socio-economic impact of bovine trypanosomosis. In the Ghibe, a re-settlement program was launched for people starving in the North of the country (Getachew, 2005; Lemecha et al., 2006). This demands huge surfaces of land for agriculture to produce crops. It resulted in a high degradation and fragmentation of the tsetse habitat which logically should have reduced the fly pressure. However, flies are persisting along the remaining unspoiled river banks and can feed easily on cattle, especially at the end of the dry season when cattle are gathered at strategic watering points. Furthermore, as reported by Akoda et al. (2009), nutritional stress is increasing the susceptibility of the vector to the trypanosomes. The lower host availability might be compensated by a higher susceptibility of the vector. A second explanation of this high trypanosomosis prevalence, is the development of drug resistance. Indeed irrational drug use for both chemotherapy and chemoprophylaxis is the rule in the Ghibe valley. In those trypanosomosis endemic areas, farmers treat their cattle intensively to benefit of the draft power for agriculture. Since their treatment is not based on scientific knowledge and sound clinical evaluation, over-usage and under-dosage are key factors leading to trypanocidal drug resistance (Grace, 2003).

6.1.2 The magnitude of DA and ISM resistance in the Ghibe valley, South-western Ethiopia

The chemo-resistance of the isolated trypanosomes was confirmed using in vivo tests in mice, PCR-RFLP and microsatellite analysis. Briefly, all isolates were found resistant to DA and ISM at all doses in the mice model. The same isolates turned-out to be resistant to DA using *Dpn*II-PCR-RFLP. Field samples from the same area (without isolation in mice) confirmed the high level of resistance in the area with 94.6% of resistant profiles, 2.7% of sensitive profiles and 2.7% of mixed profiles. In addition, at the baseline survey of the longitudinal study *Dpn*II-PCR-RFLP revealed 81.6% of resistant profiles and 5.3% of mixed profiles. No sensitive profile was observed.

Our resistance report is also supported by the microsatellite analysis allowing differentiation between clearance of infection, relapse or new infection after administration of the treatment. Although more new infections were found, the substantial number of relapses documented confirms the presence of trypanocidal drug resistance. However, some areas of concern during our microsatellite analysis include (i) field blood samples were used for DNA extraction whereas other researchers used *T. congolense* isolated in mice which increased their DNA quality and (ii) four out of seven microsatellite primers that we used didn't result in the desired amplicons for the majority of our samples. The results obtained by Morrison et al. (2009) were not reproducible in our hands.

Though different researchers reported the occurrence of DA and ISM resistance (Chaka and Abebe, 2003; Mulugeta et al., 1997; Tewelde et al., 2004) in Ethiopia , this is, to the best of our knowledge, the first confirmation of *T. congolense* DA resistance in the Ghibe valley of Ethiopia based on molecular technique. There are different in vivo and in vitro methods of detecting resistant trypanosomes (Eisler et al., 2001; Peregrine and Mamman, 1993) but the *Dpn*II-PCR-RFLP method employed here was found to be an easy and fast method of DA resistance diagnosis compared to the mice model. Thus, it can be applied in a wider region for mapping of DA resistance in endemic areas like the Ghibe valley in Ethiopia.

To avoid the use of laboratory animals, a molecular assay for ISM resistance diagnosis is needed. At present, no genetic marker for ISM resistance is available. Moreover, some studies infer that trypanosome stocks that are sensitive to recommended therapeutic doses in mouse would be equally sensitive to therapeutic doses in cattle. However, isolates that were found to be resistant in mouse test models might gave varying results in cattle (Joshua et al., 1995). For instance, *T. congolense* that was resistant to 14 mg/kg diminazene aceturate in cattle was successfully treated with 56 mg/kg in mice (Mbwambo et al., 1988). From this perspective, when ISM resistance is reported in the mice model, interpretation and dose extrapolation for cattle needs precaution.

Evidently, the development of resistance to both DA and ISM has serious implications in the strategies to be followed to control trypanosomosis using chemotherapeutic and chemo-prophylactic measures. Regular resistance reports play a critical role in making decisions on the existing control and prevention strategies. The sustainable use of trypanocidal drugs in the future has been said to face two threats, next to the resistance of the trypanosome. First, availability might not be guaranteed. Pharmaceutical companies have been reluctant to develop new drugs against trypanosomosis since the market is perceived to be unprofitable in view of the poverty of African farmers. Furthermore, companies are reluctant to maintain the production of existing drugs if this requires significant investment. Secondly, fake drugs are a growing international threat to both human and animal health (Geerts et al., 2001; Holmes, 1997; Pecoul et al., 1999). Thus, integrated tsetse and trypanosomosis control measures are an absolute necessity to decrease the impact of this disease.

6.1.3 Farmer's perception/knowledge about trypanocidal drugs

Sustainable use of currently available drugs is only possible through rational drug use practices. The existing situation of drug use is not regulated or coordinated at any level. To safeguard animal dependent agriculture of the poor community, trypanocidal drugs market chain and use need to be well regulated by responsible private and government stakeholders.

Livestock owners in Ghibe over- and misuse both DA and ISM to fight the disease. As trypanosomosis has been shown to be the major animal health problem in Ghibe, researchers in other regions also reported the same and revealed similar unwise use of trypanocidal drugs (Gechere et al., 2012; Machila et al., 2003; Van den Bossche et al., 2000). Obviously, these improper practices might be the reason for the occurrence of drug resistance (Eisler et al., 1997; Geerts et al., 2001).

Though limited by issues of resistance development, chemotherapy has been and is being fundamental to the control of African trypanosomosis (Alsford et al., 2013). The widespread occurrence of resistance necessitates regular monitoring and evaluation of any ongoing control and prevention methods. It has been suggested that privatization of veterinary services in Africa including Ethiopia has led to a situation where drug administration is in the hands of farmers or extension workers, who are unskilled in differential diagnosis and lack knowledge about appropriate drug use (Van den Bossche et al., 2000). Thus, judicious and/or strategic use of currently available trypanocidal drugs in trypanosomosis endemic areas like Ghibe valley should be possible to properly utilize draft animal power for agricultural practice and livestock farming. In addition, control of trypanosomosis is more effective when using an integrated approach than solely relying on chemotherapy. Community education interventions to improve the knowledge, attitude and practice of trypanocidal drug use by livestock owners through different media (radio, booklets and leaflets) need to be in place. This type of community education interventions has been implemented with smallholder farms in Kenya and reported to be successful (Machila et al., 2007).

6.1.4 Recommendations for trypanosomosis control in the Ghibe valley, South-western Ethiopia

In this study, despite the high level of drug resistance, PCV levels of animals improved due to treatment administered after proper diagnosis. It was clear that PCV of animals negative at baseline decreased during the course of follow-up months mainly due to new trypanosome

infections. In contrast, for animals positive at baseline, PCV improvement of about 4.4% was observed one month after treatment. This suggests that a better balance between the host and the parasite is created by chemotherapy, in favor of the host. In the absence of other diseases causing anaemia, the PCV value is a good indicator of trypanosome infection in animals (Biryomumaisho et al., 2013; Marcotty et al., 2008). This has been witnessed by a study conducted in Zambia where the average herd PCV decreased with increasing prevalence of trypanosomal infections (Van den Bossche and Rowlands, 2001). Marcotty et al. (2008) also reported that the presence of a trypanosomal infection significantly reduced the PCV, independently of the age and sex of the infected animal. Therefore, decisions to intervene and control trypanosomosis cannot be based solely on the presence of trypanosome infected animals but should be supported by an assessment of the impact of the infection on animal condition and, hence, animal production (Van den Bossche and Rowlands, 2001). Thus, should farmers continue treatment despite the prevailing drug resistance? The answer is yes, but not in the way they currently do.

Given the high number of drug treatments in some groups of animals in the Ghibe valley and the fact that high a treatment frequency is one of the most important reasons for the development of drug resistance, it could be wise to reduce the treatment frequency. This could be achieved by more strategic and focused treatments. i.e. only animals with PCV lower than 24% and/or with clinical signs of trypanosomosis.

It was clear from our field observations that chemotherapy should not be the only method for trypanosomosis control and prevention. It has to be supplemented by other vector control strategies such as applying insecticide (deltamethrin) to cattle (pour-on insecticide application) (Shereni, 1990), impregnated targets and odour-baited traps (Vale, 1993; Vale et al., 1988). As an alternative, 'restricted application of insecticide' using strategic deltamethrin spraying (legs, belly, anal and genital region, bottom part of the neck and chest) in combination with other methods could be used as more efficient and cost-effective control of tsetse (Torr et al., 2007). In addition, farmers need to be educated about strategic use of trypanocidal drugs, and the art

of drug marketing should be shaped in such a way to promote rational drug use (Grace, 2003; Grace et al., 2006). Furthermore, it is worth mentioning that the choice of vector or parasite control options is influenced by multiple factors (Allsopp, 2001; Reid et al., 2000; Schofield and Kabayo, 2008) which needs to be considered before action. Moreover, the paper of Scoones (2014) examined 'the ongoing and long-running debates about vector and parasite control methods, tracing the histories and associated politics of each'.

Finally, regular large-scale epidemiological studies on drug resistance and trypanosomosis are crucial in making the correct conclusions on existing control and prevention strategy. Based on these studies, mapping drug resistance hot spot areas shall help in application of focused control measure. Besides, information from this large scale epidemiological study can be used to provide advice to the government on re-settlement program for food security. Because this re-settlement program is one among the factors that catalyses the dynamics of host, parasite and environment interaction (Cottontail et al., 2009; Mattioli et al., 2004; Patz et al., 2004; Samdi et al., 2012; Van den Bossche et al., 2010).

6.1.5 Conclusion

DA resistant strains of *T. congolense* savannah circulate in cattle in and around Ghibe valley at high prevalence. Resistance of *T. congolense* savannah to DA using PCR-RFLP and microsatellite analysis was confirmed for the first time in Ethiopia. ISM resistance was also demonstrated using the mice model and thus, confirming the widespread occurrence of resistance in Ghibe. Farmers use available trypanocidal drugs without any scientific knowledge about control and prevention which worsens the drug resistance problem. It is time to provide farmers with adequate education on rational drug use. It is also important to continue active surveillance in animals to understand the dynamics of drug resistance in endemic areas like Ghibe valley. Furthermore, conducting large scale disease and socio-economic surveys helps to monitor ongoing control and prevention strategy and adapt them to the dynamic environment.

6.2 Future Perspectives

1. As DA and ISM will continue to be used for chemotherapy and chemoprophylaxis, conducting epidemiological studies of trypanocidal drug resistance in different tsetse belts of Ethiopia is an immediate necessity. With a better understanding of the epidemiology and magnitude of drug resistance at the national level, investigation of factors contributing to development of resistance should follow. This would help to revise the ongoing control and prevention strategy for use in trypanosomosis endemic areas in Ethiopia.

2. A strategy for the distribution and control of trypanocidal drugs from the top to the bottom-line users should be developed in the near future. In general, veterinary drugs do not seem to be properly managed when considering the pharmaceutical guidelines in the country. For example, any business oriented person can dispense trypanocidal drugs to the needy people in the Ghibe valley region and in Ethiopia in general. Such issues can be addressed by advocating standard operating procedures, besides active community education and putting laws/directives in place. A farmer's guide (extension material) on efficient and effective use of trypanocidal drugs needs to be prepared and disseminated.

3. Traditional trypanocidal drugs used by farmers should be scientifically validated. Although there are new drugs in the pipeline, investigation of indigenous treatment knowledge among livestock owners in trypanosomosis endemic areas like Ghibe valley might generate novel drugs and help solving the problem.

4. During this study *T. congolense* from Ghibe was not satisfactorily characterized using microsatellite markers developed elsewhere for the same species. This brought the assumption that *T. congolense* originating from Ghibe might possess different sequences in the regions bordering the microsatellite loci. Thus, more focused research that involves designing oligonucleotide sequences for amplification of selected microsatellites for *T. congolense* deserves an immediate attention for further genotyping.

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Summary

Summary

Trypanosomosis can affect various species of mammals but, from an economic point of view, tsetse-transmitted trypanosomosis is particularly important in cattle. The disease is a serious constraint to livestock production in areas of the north and southwest Ethiopia below 2000 meters above sea level. Similarly to other trypanosomosis endemic areas, trypanocidal drugs remain popular among farmers of the Ghibe Valley because they allow treatment of individual animals and they are relatively cheap and widely available. Unfortunately due to this widespread use of chemotherapy for control of trypanosomosis, drug resistance has been reported from different parts of Ethiopia raising several questions. Therefore the objectives of this thesis were i) to review literature on trypanocidal drug resistance in East Africa ii) to assess the magnitude of diminazene aceturate (DA) and isometamidium (ISM) resistance in the Ghibe Valley iii) to compare the haematocrit centrifugation technique (HCT) with PCR with different sample storage methods for the identification of trypanosome infection and type and iv) to assess the knowledge, attitude and practices of livestock owners towards trypanocidal drug use.

The first chapter of this thesis is focused on describing the parasite, the vector and their interaction and the impact of the disease on the host. Control options are reviewed.

The second chapter consists of a concise literature review on trypanocidal drug resistance and related problems in East African countries.

The third chapter contains a description of the results of a cross-sectional study that was carried out to assess the prevalence of trypanosomosis in tsetse controlled and non-controlled areas and to evaluate DA resistance in *T. congolense* savannah type. Therefore, the *Dpn*II-PCR-RFLP was used in parallel with the gold standard, i.e., the mice test. Twelve isolates were successfully characterized by both methods. All isolates were resistant to both drugs at all doses. The effects of tsetse control operations in the area are discussed.

In the fourth chapter, a comparison is made between the trypanosome specific 18S-PCR-RFLP using samples stored either on Whatman filter papers (PCR-RFLP-fp) or in a commercial cell lysis

and DNA protecting buffer (PCR-RFLP-pb) with the haematocrit centrifugation technique. The PCR-RFLP-pb detected more cases than the PCR-RFLP-fp and HCT. None of the PCR techniques detected parasites from the *Trypanozoon* group. Although HCT detected more cases of *T. vivax*, species identification using PCR-RFLP-fp and PCR-RFLP-pb were significantly better than the HCT technique.

The fifth chapter is presenting a six months longitudinal study conducted to evaluate dynamically DA resistance using microsatellite analysis to differentiate relapses from new infections. In addition, knowledge, attitudes and practices of livestock owners about trypanocidal drugs was assessed using a standardized questionnaire. The major findings of the study were (i) a high prevalence of the disease in the study area, (ii) a high tsetse challenge and (iii) a high degree of drug resistance. The questionnaire survey highlighted trypanosomosis as the main disease affecting cattle and DA as the main drug used in the area. Both DA and ISM were not properly used by farmers. Finally, trypanosomosis control options in the Ghibe valley are discussed.

In the sixth chapter, the overall findings of each research objective are discussed and integrated in a broader context of strategic disease control. Future prospects and research are proposed.

Samenvatting

Trypanosomose treedt op bij verschillende zoogdieren, maar is economisch vooral van belang bij de veestapel, waar het op de eerste plaats wordt verspreid door tseetseevliegen. De ziekte belemmert in grote mate de dierlijke productie in noord en zuidwest Ethiopië in gebieden lager dan 2000 meter boven de zeespiegel. Net zoals in andere gebieden waar trypanosomose endemisch is, zijn trypanociden (medicijnen die trypanosomen doden) populair bij de veehouders in de Ghibe Vallei omdat ze kunnen ingezet worden bij individuele dieren en goedkoop en algemeen beschikbaar zijn. Het overtollige gebruik van deze trypanociden voor de controle van trypanosomose heeft evenwel resistentie van de trypanosomen tegen de trypanociden in de hand gewerkt, zoals gerapporteerd werd in verschillende gebieden in Ethiopië. Resistentie tegen trypanociden is het hoofdthema van deze thesis, en de objectieven van de thesis zijn als volgt: i) de literatuur omtrent trypanociden resistentie in Oost Afrika doornemen en samenvatten, ii) het inschatten van het resistentieprobleem in de Ghibe Vallei voor de twee meest gebruikte trypanociden, diminazene aceturate (DA) en isometamidium (ISM), iii) het vergelijken van de haematocriet centrifugatie techniek (HCT) met PCR voor de identificatie van de aanwezigheid en het type van trypanosoma waarbij tevens gebruik gemaakt wordt van verschillende methoden om bloedstalen te verzamelen en te bewaren en iv) het evalueren van de kennis, de attitude en de werkwijze van veehouders met betrekking tot gebruik van trypanociden.

Het eerste hoofdstuk bevat een beschrijving van de parasiet, de vector en hun interactie en verder wordt beschreven welke impact de ziekte op de gastheer heeft. Ook verschillende controlemodaliteiten worden aangehaald.

Het tweede hoofdstuk bestaat uit een overzicht van de literatuur met betrekking tot trypanocide resistentie in Oost Afrika en daaraan gerelateerde problemen.

In **het derde hoofdstuk** worden de resultaten van een cross-sectionele studie besproken die tot doel had het trypanosomose probleem in de Ghibe Vallei in kaart te brengen. De prevalentie van trypanosomose werd vergeleken tussen gebieden in de Ghibe Vallei met en zonder controle van tseetseevliegen, en de DA en ISM resistentie werd geëvalueerd in de *T. congolense*

savannah type populatie. Daartoe werd de *Dpn*II-PCR-RFLP tests gebruikt samen met de gouden standaard, i.e., de muis infectie test. Twaalf isolaten werden gekarakteriseerd aan de hand van de twee technieken waarbij alle isolaten resistent bleken te zijn voor beide trypanociden bij elke dosis. Tenslotte werd ook nog het effect van tseetseesvliegen controle in het gebied besproken.

In het vierde hoofdstuk wordt een vergelijking gemaakt tussen de trypanosoom specifieke 18S-PCR-RFLP test gebaseerd op bloedstalen die ofwel bewaard worden op Whatman filter papier (PCR-RFLP-fp) of in een commercieel cell lyse en DNA beschermende buffer (PCR-RFLP-pb) enerzijds en de standaard haematocriet centrifugatie techniek. Met de PCR-RFLP-pb tst werden meer positieve stalen gevonden dan met de PCR-RFLP-fp en HCT techniek. Met de PCR gebaseerde technieken werden geen parasieten van de *Trypanozoon* groep gedetecteerd. Met de HCT techniek werden meer positieve *T. vivax* stalen gevonden maar de species identificatie met PCR-RFLP-fp en PCR-RFLP-pb was meer performant dan de HCT techniek.

Het vijfde hoofdstuk houdt een bespreking in van een longitudinale studie (6 maanden opvolging) die werd opgezet om DA resistentie te evalueren op basis van microsatelliet analyse; op basis van het longitudinale karakter van de gegevens kan een onderscheid gemaakt worden tussen een nieuwe en een voortdurende infectie. Ook de kennis, attitude en werkwijze van eigenaars van vee met betrekking tot gebruik van trypanociden werd in kaart gebracht aan de hand van een gestandardiseerde vragenlijst. De belangrijkste bevindingen van de studie waren als volgt: (i) er is een hoge prevalentie van trypanosomose in het studiegebied, (ii) en ook een groot aantal tseetseevliegen en (iii) een hoge graad van trypanocide resistentie. Op basis van het bevragen van de veehouders kan geconcludeerd worden dat trypanosomose gezien wordt als de belangrijkste bedreiging voor de veestapel en dat DA algemeen gebruikt wordt als trypanocide in het gebied. Noch DA noch ISM wordt op een correcte manier gebruikt door de veehouders. Tenslotte worden nog de verschillende opties om trypanosomose te controleren in de Ghibe vallei besproken.

In het zesde en laatste hoofdstuk worden de specifieke bevindingen per onderzoeksobjectief besproken en geïntegreerd in de meer algemene context van strategische ziektebeheersing. Tenslotte worden nog een aantal nieuwe en belangrijke onderzoekstopics opgelijst

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Curriculum vitae

Curriculum vitae

Moti Yohannes was born on November 17, 1979, in Nekemte town of Ethiopia. He studied primary and secondary educations in Nekemte. In 1999, he joined Addis Ababa University, Faculty of Veterinary Medicine as a student and obtained degree of Doctor of Veterinary Medicine (DVM) in 2005. After graduation in 2005, he worked in Jimma University College of Agriculture and Veterinary Medicine as a lecturer in the Department of Microbiology and Veterinary Medicine. Then, in 2007 he joined University of GADVASU, School of Public Health and Zoonoses to pursue his Masters in Veterinary Public Health (MVPH). After completing his study in 2009, he started working in Jimma University at different levels of responsibilities from handling different courses to conducting multidisciplinary research in collaboration with other professionals. Since January 2013, he has been promoted to the rank of Associate Professor in the Department of Microbiology and Veterinary Public Health. Besides, he is working as a Focal Person of One Health Central and Eastern Africa (OHCEA) since 2010. He has published 18 research outputs and his research interest areas among others drug resistance dynamics, epidemiological investigation of zoonotic diseases, food safety and utilization/proper disposal of animal wastes.

In January 2010, he started a PhD research in the area of *Trypanosoma congolense* drug resistance in collaboration with the Institute of Tropical Medicine (ITM) and Ghent University in the framework of JU-IUC program. His doctoral research was funded by VLIR-UOS in the framework of JU-IUC program under the guidance of his Promoters: Prof. Luc Duchateau and Dr. Vincent Delespaux. He conducted intensive field works in the Ghibe valley of Ethiopia and undertaken advanced laboratory tests on *T. congolense* trypanocidal drug resistance detection and characterization which resulted in scientific publications in peer reviewed international journals.

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