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Assessment of Trypanocidal Drug Resistance on *Trypanosoma congolense* in Amaya District of South West Shewa, Ethiopia

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Abstract

The study was conducted in January to April of 2013 to assess occurrence of diminazene diaceturate and isomethamidum chloride hydrochloride resistant Trypanosoma congolense isolates in Ameya district, Ethiopia. Initial survey was conducted on 51 cattle owners with the help of questionnaires to assess potential risk factors for trypanocidal drug resistance and trypanosomosis prevalence in the study area while the experimental study was carried out at Ambo on 10 calves purchase from Ambo market, tested and placed in Ambo University farm for two months prior to the artificial infection. The animals were divided into two groups {Diminazene diaceturate(Group-I) and Isometamidium chloride(Group-II)} of five calves. The survey result on trypanocidal drug administration revealed that 9 (17.6%) of the respondents self administer the drugs to their animals, 22 (43.1%) by veterinary professionals and the remaining 20(39.2) by other farmers, community health workers (CHWs) or drug smugglers. It also showed that 80.4% of the respondents treated only sick cattle, where as 19.6% treated sick and healthy cattle as well. The interviewees replied that trypanocidal drugs was used by 49% for the last 15-20 years, and 31.4% for the last 25-30 years ,and 13.7 % for the last 5-10 years. In addition drug sell by non professionals, prolonged use of trypanocidal drug (up to 40 years) and use of trypanocidal drugs by farmers might have resulted in under-dosing with any of the three trypanocidal drugs (diminazene diaceturate, isometamidium chloride and homidium bromide). Drug sensitivity revealed that T. congolense developed resistance to both doses of diminazene diaceturate(7% solution) at 3.5 and 7 mg/Kg body weight in Group I ,and to isometamidum chloride hydrochloride(1% solution) at therapeutic doses of 0.5 mg/Kg body weight and double dose of 1.0 mg/Kg body in Group II. There was a 100% relapse following treatment with therapeutic dose of both drugs where as a 60% and 100% relapses recorded following double doses treatment with diminazene diaceturate and isometamidum chloride hydrochloride, respectively. During the 100 days of trial period there was statistically significant difference ($P<0.05$) in mean packed cell volume (PCV) of the two groups at therapeutic dose of treatment while no significant difference ($P>0.05$) was observed at double doses. In conclusion, Trypanosoma congolense infection was found to have developed high level of resistance against the tested drugs in the area. Therefore, strict regulation and supervision of trypanocidal drugs usage together with integrated vector control methods are suggested in order to alleviate trypanocidal drug resistance. Furthermore, invention and introduction of new drugs and detailed experimental works are also recommended.

Keywords: Ameya, Calves, Diminazene diaceturate, drug resistance, isomethamidum chloride, *Trypanosoma congolense*

Introduction

Trypanosomosis is a complex, debilitating, zoonotic hemoprotozoan disease of human and animals (WHO, 1998). African trypanosomosis is responsible for 3 million livestock death and 55,000 people death annually in agriculture and mixed farming (Abenga *et al*., 2003). In this region at least 46 million cattle are exposed to the risk of contracting tsetse-borne trypanosomosis, as are millions of sheep, goats, donkeys, camels and horses (Reid *et al., 1998).* A study conducted by Kristjanson *et al*.(1999) estimated the direct annual cost of trypanosomosis to be about 1.34 billion US$. African livestock producers are administering an estimated 35 million curative and prophylactic treatments annually which costs the producers and the government at least 35 million US$ (Geerts and Holmes, 1998). The direct losses from trypanosomosis in livestock include mortality, morbidity, impaired fertility and the cost of implementing and maintaining tsetse fly and trypanosomosis control operations. Indirect losses stem from farmers responses to the perceived risk of the disease, including the reduction and in some cases, the exclusion of livestock from tsetse-infested grazing lands and reduced crop production due to insufficient animal draught power (ILRAD, 1993), exerting adverse effect on the whole pattern of agricultural activities in the continent. It is transmitted by 22 species and 33 sub-species of tsetse flies in Africa (WHO, 1998; Kristjason *et al*., 1999). In Ethiopia also, it has been described as a major impediment to the livestock development and agricultural production; contributing negatively to the overall development in general and to food self-reliance efforts of the country in particular (Zewdu *et al.,* 2003). The most important trypanosomes in terms of economic loss in domestic livestock in Ethiopia are the tsetse transmitted species: *T. congolense, T. vivax* and *T. brucei* (Abebe, 2005). It has been shown that in Ethiopia, at least 10 million heads of cattle are already exposed to the disease and over 14 million heads are at risk of contracting the disease at any one time such that total direct and potential annual losses from the disease exceed US$ 236 million (OAU, 2001). Furthermore, livestock cannot be introduced into some 155,000-220,000 Km^2 of the most fertile land in the southwest and western regions (ILRI, 2002).

As part of the Gibe river system, tsetse-transmitted animal trypano-somosis has been incriminated as the primary disease highly curtailing the production potential of livestock sub-sector particularly in the fertile lowland and midlands of Amaya district of South West Shewa zone. There are two principal approaches to control trypanosomosis in Ethiopia. These are control or elimination of tsetse and biting flies and prevention or treatment of animals using

trypanocidal drugs. Among the trypanocidal drugs available for the treatment of animal trypanosomosis, diminazene diaceturate(Berenil®) and Isomethamidium Chloride (Semidium)® have been used the most because of their availability and relatively low toxicity to cattle. However, there have been reports of increasing development of resistance in the parasite to the commonly used trypanocidal drugs. Furthermore, the unlikelihood of new trypanocides appearing in the foreseeable future together with the low adoption of other alternatives to the use of trypanocidal drugs create additional dilemma in the management of African animal trypanosomosis (Bett *et al*., 2004; Chaka and Abebe, 2003). There are at least 18 African countries (e.g. Burkina Faso, Chad, Côte d'Ivoire, Ethiopia, Kenya, Nigeria, Somalia, the Sudan, the United Republic of Tanzania, Uganda, Zimbabwe, the Central African Republic, Zambia, Cameroun, Mozambique, Benin, Ghana and Togo) in which trypanocidal drug resistance has been reported (Chitanga *et al*., 2011; Delespaux *et al*.,2008). This is probably an underestimation of the true situation, because in several countries surveys for resistance have not yet been carried out or cases of resistance have not been published. Case surveys conducted in some Sub-Saharan countries, including Ethiopia, showed that almost all of the commercially available trypanocidal drugs are gradually losing their efficacy due to the development of multiple drug resistance to both diminazene diaceturate and isometamidium chloride and this is threatening the last stand to overcome drug resistance through the use of the sanative pair (McDermott *et al*., 2003; Mulugeta *et al*., 1997; Tewelde *et al*., 2004).

Studies conducted in different tsetse-infested zones of Ethiopia had revealed the occurrence of various degrees of trypanosomes resistance to both diminazene diaceturate and isometamidium chloride. Multiple drug resistance had been reported in the Abay/Didessa tsetse belt in Metekel district (Afewerk, 1998), Didessa valley in Bedelle district (Tewelde, 2001), Birbir valley of Baro Akobo tsetse belt, in Gawo Dalle district of Kellem Wollega (Eteya, 2008) and in Ghibe/Omo tsetse belt adjacent to the upper Diddessa river valley, in Kindo Koysha wereda, Southern Ethiopia (Mesfine Ademe and Getachew Abebe, 2000). In North Omo Zone, southern Ethiopia, Asefa and Abebe (2001) had also reported multiple drug resistant of *T. congolense* against diminazene diaceturateand isometamidium chloride in naturally infected donkeys. Most of the studies conducted in Ethiopia to assess the therapeutic and prophylactic efficacy of trypanocidal drugs involved experimentally infected mice where it was possible to demonstrate the general status of resistance to the drugs used in cattle after natural challenge. However, an assessment on occurrence of trypanocidal drugs resistant local isolates of trypanosomes has never been

conducted in the study area. Therefore, the objective of the study was to assess the trypanocidal efficacies of diminazine diaceturate (YZ-Dimnazene) and Isometha-midium chloride in calves artificially infected with isolates of *T. congolense* from naturally infected cattle and to assess potential risk factors for trypanocidal drug resistance in cattle of Amaya district.

Methodology

Description of the study area

The study was carried out at Amaya district from where the prevalence study was conducted and field isolates were obtained and Ambo where the experimental work on drug resistance was conducted. Amaya district is about 180 Km southwest from Addis Ababa and located in South-West Shewa zone of Oromia regional state, Central Ethiopia with Gindo as its administrative town. The district has 39 kebeles (peasant associations (PAs)), of which 19 (47%), 10 (36%) and 7 (17%) PAs are lowland, midland and highland, respectively. The topography of these areas is mainly plain and thoroughly cultivated. Mixed livestock and crop farming is the dominant form of production where rain-fed agriculture is the common production system. The vegetations are confined on the riversides, and the types of vegetations are dominated by thick and scattered thickets with short grasses on the upper reaches of the rivers. The district has a total of 15,715 local and 37 cross breed cattle. In addition, there are 30,881 sheep, 34,815 goats, 7,803 horses, 3,150 mules, 13,046 donkeys and 85,823 poultry socioeconomic data (2012).

Ambo University where the in vivo drug sensitivity test was conducted is situated at an altitude of 2060 – 2090 m.a.s.l, 08° 58.954'- 08° 59.102' N latitude and 037° 50.490'- 037° 50.839'E longitude, 114 km west of Addis Ababa, Central Oromia, Ethiopia. The temperature ranges from 15°C - 29°C with a mean average of 22°C and a mean annual rain fall ranging from 800-1000 mm with an average of 900 mm. The highest rainfall concentration occurs from June to September and the mean monthly relative humidity varies from 64.6% in August to 35.8% in December. Ambo has a livestock population of 272,168 (137,448 cattle; 40,985 sheep; 20,968 goats; 11,901 donkeys; 8,679 horses; 337 mules and 51,850 poultry). The total human population of Ambo is estimated to be 112,129 with a total of 55,491 (50.08 %) female and 55, 305 (49.92 %) male (AWRADO, 2009).

Prevalence questionnaire survey

Five peasant associations (Pas) namely Gambela Ashute Talgo (Ashute site), Gombore Aliyi (Gombere, Eteya and Gombore Baticha sites), Mari Kereyu Sekela (Mari-Magari and Mari sites), Moko Ujuba, Kota (Kota site) and Cha'a Kase (Atnafo site) were randomly selected for the

trypanosomes prevalence study. A structured questionnaire format was developed and administered to randomly selected 51 volunteer cattle owners in Amaya, who were interviewed individually. The main questions included in the questionnaire format were about herd composition, management, importance of trypanosomosis in the area, extent and type of trypanocidal drugs utilization, source of drugs, common treatment places and trypanosomosis control methods being practiced.

Drug sensitivity study

Experimental animals

Study animals were calves of Horro breed cattle of around 1-1.5 year old (6 males and 4 females) and weighing mean body weight of 110.32 $\pm$ 11.76 Kg at the beginning of the experiment. The calves were purchased from Ambo market (particularly from high land origins of Ambo area)two months prior to the commencement of the experiment, ear tagged and treated with long acting oxytetracycline 20% (YZ.OXY20%, HEBEI YUANZHENG-PARMACEUTICAL Co.,LTD)was injected intramuscularly at dose of 1ml/20kg body weight; Ivermectin (TECMECTIN,HEBEI YUANZHENG-PARMACEUTICAL Co.,LTD) at 200µg/Kg subcutaneously and 1.05g diminazene diaceturate (YZ-Diminazin, HEBEI YUANZHENG-PARMACEUTICAL Co.,LTD) were administered intramuscularly at7% solution at dose of 3.5 mg/Kg body weight .Their blood was examined by buffy coat technique and found to be free from trypanosomes, other hemoparasites and blood sucking gastrointestinal tract parasites prior to challenge. The calves grazed during the day and confined in shed at night separately in the main campus of Ambo University, Ethiopia, which is located in tsetse flies free area. Initial packed cell volume (PCV) values of each animal was recorded and considered as normal PCV value (27.4%). As approved by Ethical Committee of Ambo University (RKTT/21/11), blood samples were collected from cattle of study area and from the experimental animals (calves) after properly securing and aseptically preparing around the veins.

Blood sample collection, examination and inoculation into experimental animals

Cross sectional parasitological study was carried out in Amaya district to estimate the prevalence of trypanosomosis infection at different sites and to isolate the strains that were used to infect the cattle to conduct experimental trial on trypanocidal drug sensitivity. The study was carried out by simple random sampling technique based on the selection of house-hold heads by lottery system. Blood samples were collected after properly securing the animal and aseptically preparing around the marginal ear veins.

The dark ground or phase contrast buffy coat technique had been used for parasitological examination. This technique is recommended for diagnosing low parasitaemia, helps to identify trypanosome species and its quantification. Paired blood samples were collected from each animal using two heparinized haematocrit capillary tubes and filled to a level of ¾ of their height from marginal ear vein after pricking the vein with the tip of a lancet and sealed with wax sealant at one end, centrifuged at 12000 rpm for 5 minutes and then measured by Haematocrit reader to know PCV values for determination of anemia. The capillary tube was cut by diamond pencil 1mm below the buffy coat red blood cell junction to include the top layer cells up to 1cm plasma as the trypanosomes concentrate at this site after centrifugation. The content of the capillary tube was expressed onto a clean microscope slide, covered with a 22x22 mm cover slip. Then the slide was examined for trypanosomes based on the type of movement in the microscope field. The use of 10x eyepiece in combination with a 25x objective gives optimal viewing, by allowing large visual fields and sufficient magnification for ready identification of trypanosomes. This technique is the most sensitive of the parasitological tests for the detection of *T. congolense* and *T. vivax* detecting trypanosomes to an estimated level of just over 10^2 parasites per ml (Murray *et al*., 1977**).** Confirmations of trypanosome species by morphological characteristics were done after staining the blood smear with giemsa and examination with oil immersion microscopy with 100 x powers of magnifications according to procedures of Murray *et al*. (1977) and Paris *et al.* (1982). Then all the relevant data (species of trypanosome, number and identification of the animals by location, peasant association, village, owner, and cattle's name given by owner, parasitaemic and non parasitaemic animals, PCV values, sex) were recorded for analysis.

Then about 10ml blood was collected into vacutainer tube coated with ethylene diamino-tetraacetic acid (EDTA) from jugular vein of highly parasitaemic cattle of Amaya district and were transported under ice pack in cold chain to the Ambo University. The blood were incubated to about 38^0C for half an hour and trypanosomes viability was tested then 2ml of the blood samples with high parasitemia (about $1x10^5$ parasite) were inoculated into jugular vein of the experimental animal using 19 guage syringe.

Trypanocidal drugs: Among the commonly used trypanocidal drugs in the study area, Diminazene diaceturate (YZ-Diminazin, HEBEI YUANZHENG-PARMACEUTICAL Co.,LTD) and Isomethamidium chloride hydrochloride 125mg (Semidium, SeQuent Scientific Ltd., A-68, MIDC Indl. Area, Additional Ambernath, India) were selected for this study because of their availability. Diminazene diaceturate was injected as a 7% solution at initial dose of 3.5

mg/Kg body weight and double dose 7 mg/Kg body weight while Isomethamidium chloride hydrochloride was injected as 1% solution at curative/initial dose of 0.5 mg/Kg body weight and double dose of 1.0 mg/Kg body weight. Both drugs were accurately administered deep intramuscularly to animals on the basis of their body weight measurement taken before treatment by Cattle and pig weighing tape or band (DALTON SUPPLIES LTD. NETTLEBED.HENLEY-ON. THAMES. OXON. ENGLAND) according to Arora *et al*.(1981).

Experimental drug sensitivity test in calves

The drug resistance testing in calves was carried out as per the standard protocol for drug resistance testing in cattle according to Eisler *et al*. (2001).The experimental animals were grouped into two treatment groups of five animals each with equal number of the males and females randomly assigned to either of the treatment groups in complete block design as shown below:

Group I (n= 5; 3 males and 2 females): Treated with diminazene diaceturateat therapeutic dose (3.5 mg/kg body weight) and double dose (7mg/kg body weight), intramuscular route (IM). Group II (n=5; 3 males and 2 females): treated with Isomethamidium chloride hydrochloride (Semidium) at curative dose (0. 5 mg/kg body weight) and double dose (1mg/kg body weight), administered IM.

Ten *T.congolense* isolates from Ameya district (Gombore and Mari Magari peasant associations) were inoculated into the experimental cattle's. On the first days of high parasitaemia post infection the calves in the treatment groups were individually treated with 7% diminazene diaceturate, 1% isomethamidium chloride hydrochloride at a dose of 3.5, and 0.5 mg/kg body weight respectively. The second treatment (double dose of the initial treatment) was given to all the animals immediately after showing relapse with the therapeutic dose and peak parasitaemia (score: 3), when PCV decreased below 20 percent or when clinical signs of trypanosomosis was observed. Animals were regarded as cured if no parasitaemia was detected following treatment while the isolate were considered sensitive only if all the five calves (5/5) were cured otherwise considered resistant.

Haematology and parasitological examination

Blood was taken from ear vein and examined by dark ground or phase contrast buffy coat technique. Presence or absence of Trypanosomosis and the level of parasitemia was then estimated according the standard method of scoring by Paris, *et al.,* (1982). Recording of the PCV and buffy coat examination for parasite were carried out three times per week and the animals were monitored for duration of 100days. Date of treatment with therapeutic dose, double dose, and date of relapses after the respective treatments was recorded for each

animal. Level of parasitemia was scored based on parasite count at high power microscope field (40x) as (0: no parasite/field (aparasitemia), 1: $\leq$ 5 parasite/field, 2: parasite count of greater than 6 but less than10 per field, 3: $\geq$ 10 parasites/field was taken as high parasitemia).

Statistical analysis

The collected data were subjected to analysis using the SPSS statistical software programme for windows version 15. Student t-test (for normal distribution) and Mann-Whitney test (for distributions significantly different from normal) were used to compare the mean PCV values at different times of aparacitemic and paracitemic conditions of the experimental animals at $\alpha = 0.05$, while descriptive statistics was used to summarize questionnaire survey data.

Results

Survey

Herd composition and management

All respondents in the study area keep their cattle with small ruminants and equines regardless of age, sex and physiological status, but cattle constitute the major part. Only extensive management was practiced in the study area, where all animals share communal grazing land and common watering point. All the interviewees also replied that bovine trypanosomosis is the major problem in their areas. Provision of supplementary feed to cattle was not commonly practiced except for the draft oxen when they were sick.

Treatment and control of trypanosomosis

Of the total 51 respondents, 11 (21.6%) gets their sick animals treated commonly at veterinary clinics (governmental or private) whereas 40 (78.4%) treat their sick animals at home as well as at veterinary clinics. The respondents reported that they get their animals treated at home and veterinary clinics and used to purchase trypanocidal drugs from veterinary clinics as well as other sources. As a control method of the disease, the respondents used a combination of trypanocidal drugs, deltamethrin poured on cattle and targets impregnated with deltamethrin for control of trypanosomosis (Table 1).

Table 1. Common treatment places, sources of drugs and trypanosomosis control methods practiced in the study area

Variables	Responses	No. of respondent(%)
Common treatment place	Vet. clinic (public and private)	11(21.6)
	At Vet. clinic and at home	40(78.4)
Source of veterinary drugs	Vet. clinics (private, Government)	25(49)
	Vet. clinic and other sources	26(51)
Trypanosomosis control methods being practiced in the study area	Use of trypanocidal drug	10(19.6)
	Give supplement and feed separateyly and trypanocidal drug and tsetse trap	2(3.9)
	Use trypanocidal, tsetse trap and pour on application	15(29.4)
	Use of trypanocidal drug and pour on application	24(47.1)

Characteristics of Trypanocidal drugs usage in the study area: In the study area, 24 (47.1%) of the respondents had been using and assume that isomethamidium chloride was the most effective trypanocidal drug, while 13 (25.5%) and 6 (11.8%) considered diminazene and homidium as the most effective one, respectively. In addition typanocidal drugs were administered to cattle by different personnel. Accordingly, 9 (17.6%) of the respondents administered the drugs by themselves, and 22 (43.1%) by veterinary professionals and 20(39.2) by other farmers, community animal health workers (CAHWs) and drug smugglers.. In the study area, trypanocidal drugs had been used for the last 15-30 years. Regarding treatment approach, most of the respondents (80.4%) treated only the sick animals and 84.3% of them replied that their sick animals treated with the trypanocidal drugs were cured. (Table 2).

Table 2. Types of trypanocidal drugs used, personnels administering the drug and extent of used in the study area

Variables	Responses	No. of respondent (%)
Trypanocidal drug commonly used and considered effective	Isomethamidium	24(47.1)
	Diminazine	13(25.5)
	Homidium	6(11.8)
	dont know	8(15.7)
Personnels administering trypanocidal drugs	Farmers themselves	9(17.6)
	Vet professionals	22(43.1)
	Farmers,CAHWs, and smugglers	20(39.2)
Time since a given trypanocidal drugs used	For the last 5-10 years	7(13.7)
	For the last 15-20 years	25(49)
	For the last 25-30 years	16(31.4)
	For the last 35-40 years	2(3.9)
	Don't know the time since when	1(2)
Status after treatment	Cure	43(84.3)
	Not cure	8(15.7)
Treatment approach	Treat all cattle (sick and healthy)	10(19.6)
	Treat only sick ones	41(80.4)

Hematological Examination

Based on Buffy coat examination of the blood samples, infection of *T. congolense* observed in the samples of naturally infected cattle was also detected in the experimental animals. Comparison of mean PCV values recorded at different aparasitemic and parasitemic status of animals in the two treatment groups showed no statistical significant difference ($p > 0.05$) (Table 3). However, all the mean PCV values of animals in both treatment groups remained significantly lower ($p < 0.05$) than the normal PCV (i.e. Pi) throughout the observation period (Table 4).

Table 3. Comparison of treatment groups mean PCV values at different aparacitemic and paracitemic status of animals

PCV Status	Treatment group	N	Mean	Std. Deviation	p-value
P	Diminazene	5	21.00	3.674	0.354
	Isometamedium	15	20.53	1.846	
P1	Diminazine	9	18.00	5.148	0.864
	Isometamedium	16	19.00	2.129	
P2	Diminazene	30	21.17	3.524	0.278
	Isometamedium	12	20.50	2.541	
P3	Diminazene	27	20.56	3.238	0.058
	Isomethamedium	18	19.06	2.796	
P4	Diminazine	17	19.82	2.481	0.225
	Isomethamedium	19	20.47	2.606	
P5	Diminazine	5	17.60	4.393	0.194
	Isometamedium	16	19.88	2.125	

P: PCV of aparacitemic after inoculation, P1: PCV of paracitemic before treatment, P2: PCV of aparacitemic after treatment, P3: PCV of relapse with normal dose, P4: PCV of aparacitemic after double dose, P5: PCV of relapse with double dose. N=number of readings

Table 4. Comparison of mean PCV values recorded at different aparacitemic and paracitemic status of animals

	Test value (Pi= 27.4)			
Test variable	**mean**	**df**	**Std.Deriation**	**p- value**
P	20.65	19	2.32	0.00
P1	18.64	24	3.45	0.00
P2	20.98	41	3.26	0.00
P3	19.96	44	3.13	0.00
P4	20.17	35	2.53	0.00
P5	19.33	20	2.87	0.00

Pi: initial PCV (normal value), P: PCV of aparacitemic after inoculation, P1:PCV of paracitemic before treatment, P2: PCV of aparacitemic after treatment, P3:PCVof relapse with normal dose, P4: PCV of aparacitemic after double dose, P5: PCV of relapse with double dose.

At normal dose, diminazene has significantly improved ($P<0.05$) the mean PCV value than isomethamidium. However, no statistical significant difference ($P> 0.05$) was observed in both treatment groups when the doses were doubled (Table 5).

Table 5. Comparison of mean PCV of the two treatment groups at normal and double dose

Dose of drug used	Mean PCV values		P-value
	Diminazene aceturate	Isometamidium chloride	
Therapeutic dose	20.88 (n=57)	19.63(n=22)	0.043
Double dose	19.32(n=30)	20.23(n=30)	0.170

Drug sensitivity test in calves

In both treatment groups, the mean days for which the animals remained free of parasite in the peripheral blood post treatment varied. In group I, following treatment with diminazene diaceturate at therapeutic dose (3.5mg/kg body weight), trypanosomes reappeared in all the five calves after 17.60 days. However, treatment of same five calves by doubling the dose (7mg/Kg body weight) of diminazene diaceturate led to the recovery of two calves from the infection and relapse was observed in 3 of the calves after 36.67 days.

Subsequent to the treatment of group II animals with isomethamedium chloride hydrochloride at a therapeutic dose (0.5 mg/kg body weight) and double dose (1.0 mg/kg body weight), relapse was seen in all animals after mean of 6.40 days and 18.80 days, respectively. Therefore, from the current study it was found that isolates of *T. congolense* were found to be resistant to the therapeutic as well as double doses of both drugs. There was a 100% relapse following treatment with normal dose of both drugs where as a 60% and 100% relapses were recorded following double dose treatment with diminazene and isomethamedium, respectively (Table 6).

Table 6. Resistance of *T.congolense* field isolates in calves against the effects of diminazene diaceturate and isometamedium chloride hydrochloride

Treatment group	Dose of drug used (mg/kg)	Number of calves with relapse/total (%)	Mean relapse days ± SD
Diminazene	3.5	5/5 (100%)	17.60±5.94
	7.0	3/5 (60%)	36.67 ± 32.33
	7.0	2/5 (40%)	Cured
Isomethamidium	0.5	5/5 (100%)	6.40± 5.64
	1.0	5/5 (100%)	18.80±15.81
	1.0	0/5	No Cured

Discussion

Prevalence survey

Results of the questionnaire survey indicated all (100 %) of the respondents rank trypanosomiasis as first and serious health problem of cattle in their district and isometamidium chloride hydrochloride or diminazene diaceturate groups are used to treat the disease. Similar finding was obtained in a surveys conducted in west Ethiopia, at Bedelle by Tewelde (2001) and at Gawo Dalle by Eteya (2008) who identified trypano-somosis as the most important livestock disease in the study areas (95.5% and 100% of respondents in the area, respectively). In the present study 56.9% of the respondents administer the drugs by non-professionals, 9 (17.6%) by themselves, 43.1% by veterinary professionals and 20(39.2) by other farmers, community animal health workers (CAHWs) and drug smugglers. Eteya (2008) had also reported 52.65% of farmers treat their animals at home by themselves while Afework (1998) and Tewelde (2001) had reported that 43% and 57% drug application was by farmers and uncertified individuals respectively. This is in agreement with the present finding.

Questionnaire survey results regarding practice of current veterinary drug trading showed that apart from veterinary pharmacy, veterinary drugs were being purchased from different illegal sources including farmers' home, human pharmacy, and drug smugglers on open local market, at homes or in shops intended for other trading activities. Due to these, instead of getting their cattle treated by veterinary professionals, farmers were treating their animals at home by themselves, by other farmer whom they think is experienced and by drug smugglers. The typanocidal drugs were not only used for treatment purpose, but alone or in combination with other trypanosomosis control methods as a prophylactic measure for long time. In addition, there was a trend of treating all animals including healthy ones once the trypanosomosis is detected in a herd. The majority 41(80.40%) of the respondents have been using one type of trypanocidal drug consider as effective for at least 15 years. In the present study isometamidium chloride was the most commonly used as well as considered most effective drug by 24 (47.10%) of respondents followed by diminazene diaceturate and homidium which were considered effective by 13(25.5%) and 6(11.8%) of respondents respectively. However, of the total respondents, 8(15.7%) indicated that there is no cure after using the drug they thought most effective. This is not in line with reports of Eteya (2008) in which 80% of respondents interviewed in GawoDalle reported that animals were not cured after treatment.

Drug resistance is known to occur under certain circumstances such as under large scale drug use,

inadequate dosing and due to use of trypanocidal drugs that are well known to be mutagenic (Geerts *et al*., 2001). Many of the practices of the farmers particularly treating animals at home by non-professionals, purchasing drug from illegal sources, indiscriminate use of the same type of trypanocidal drug for extended period of years might have contributed for the drug resistance documented in current study in many ways and hence can be considered as risk factors majorly, such practice might have resulted in under dosing which is considered as one of the major causes of resistance development because farmers or unskilled persons have a tendency to underestimate the weight of their animals when they treat them or over dilute the drugs in powder form when they prepare it themselves. In the present finding 35% of the respondents reported that the time of first trypanosomosis out breaks and use of trypanocidal drug was about 25 - 40 years and that time they used to treat their animals by one of homidium salts ("Red tablet"). This finding is almost in agreement, with the report of Eteya (2008) in Gawo-Dalle where 28% of the respondents replied that they used trypanocidal drugs for the last 45 years. The respondents have also replied that the trypanocidal drug has been in use for over forty years , which can also lead to trypanocidal drug resistance and this is in agreement with the report of Waller, (1994), where it has been reported that repeated use of chemicals as pesticides or chemotherapeutic agents inevitably leads to the development of resistance in the target organisms and observed that resistance systematically occurs within approximately ten years of their introduction as antimicrobials, insecticides, trypanocides and anthelmintics to the market. This has occurred with the trypanocidal drugs such as isometamidium chloride (ISMM), the homidium salts and diminazene aceturate, which were introduced during the 1950s to Africa. Therefore, the use of trypanocidal drugs in this area for several decades, the irregular and inappropriate mass treatments, and widespread use of trypanocidal drugs altogether might have induced a high level of drug resistance to the currently available drugs in the *T. congolense* populations in the area. In this study 16% of the respondents complained that the treated animals did not recover. This was not in line with the report of Eteya (2008) in which 80% of the interviewed respondents reported that animals were not cured after treatment.

Hematological examination and Drug sensitivity test in experimental calves

Comparison of mean PCV values recorded at different aparacitemic and paracitemic status of animals in between the two treatment groups showed no significant difference ($p>0.05$). Moreover, all the mean PCV values of animals in both treatment groups remained significantly below ($p < 0.05$) the normal PCV (i.e. Pi) throughout the observation period.

However, treatment by diminazene at therapeutic dose of 3.5 mg/Kg has resulted in significant improvement in mean PCV value as compared to that of isomethamidium at therapeutic dose. On the other hand, assessment of trypanocidal activity of the two drugs showed that the isolates of *T. congolense* from study sites were found to be resistant to the curative and double doses of both drugs.

In the present work study with 10 trypanosome isolates collected from cattle in Amaya district, the outcome of the trypanocidal drug resistance tests in cattle clearly showed the presence of trypanosome strains that have developed resistance to both currently available trypanocides. The results showed that isometamidium chloride at both dose of 0.5 and 1.0 mg/kg body weight failed to kill the parasite and it was found that five out of five animals were resistant with the isolates of *T. congolense*. Similarly the isolates of *T. congolense* were resistant to diminazene diaceturate at a dose of 3.5 mg/kg body weight (five out of five; 100% were relapsed) and to 7 mg/kg body weight (three out of five) (60%) failed to clear the infection. The resistance of *T. congolense* against these two drugs in this study is thus in agreement with the earlier findings in different tsetse infested parts of Ethiopia (Codja *et al*., 1993; Mulugeta *et al*., 1997; Rowlands *et al*., 1993 and Tewelde *et al*., 2004; Afewerk *et al*., 2000 ; Chaka and Abebe, 2003). In a trial made to compare the therapeutic efficiency of diminazene diaceturateat doses of 3.5 and 7.0 mg/kg body weight in two of the herds at Ghibe, Rowlands *et al*. (1993) indicated that the proportion of animals that relapsed by day 20 following treatment although decreased in the higher dosage (25% vs 55%), it was not able to cure all the infections. This work was again more in accordance with experimental work that was further substantiated by Codjia *et al.* (1993) in which they inoculated blood samples from 12 trypanosome isolates collected from cattle in the Ghibe valley in 1989 into Boran (*Bos indicus*) calves. Twelve isolates produced infections, which were shown to be *T. congolense* and resistant to treatment with diminazene diaceturate at dose of 7.0 mg/kg boy weight. Eleven of the infections were also resistant to isometamidium chloride at a dose of 0.5 mg/kg body weight, where, except for one isolate sensitive to 0.5 mg/kg body weight isometamidium chloride, all were found 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. This multiple-resistant phenotype was even expressed at the clonal level. Eteya (2008) documented resistance of the *T. congolense* and *T. brucei* to diminazene and isomethamidium and homidium in the experimental work in which 15 isolates of *T. congolence* and *T. brucei* were inoculated of GawoDalle district of Birbir valley (BaroAkobo river system) showed a similar high level of drug resistance to treatment with 3.5 and 7.0 mg/kg body weight diminazene and 0.5 and 1.0 mg/kg isometamidium and, 1 and 2 mg/Kg

body weight homidium. Mulugeta et al. (1997) indicated a long-term occurrence of *T. congolense* resistance to diminazene, isometamidium and homidium in cattle of Ghibe, Ethiopia. The study carried out by Afewerk *et al.* (2000) showed the presence of multiple-drug-resistant *T. congolense* in the village cattle of Metekel district, northwest Ethiopia. *T. congolense* isolates from Ghibe, Bedelle, Sodo and Arbaminch showed a similar level of drug resistance to treatment with 3.5 mg/kg body weight diminazene and 0.5 and 1.0 mg/kg isometamidium (Chaka and Abebe, 2003). The magnitude of drug resistant trypanosomes across Ethiopia is not well documented. However, the present study on a few isolates of *T. congolense* indicated the potential risk for the future in the greater part of tsetse infested areas, where the proportional infection rate of cattle by *T. congolense* is increasing and where dependence on regular drug treatment for trypanosomosis control, which is a common practice now in Ethiopia, may lead to the risk of major drug resistance development.

When compare the relapse time between the two drugs in therapeutic dose about three times more relapse interval of diminazene diaceturate3.5 mg/kg than 0.5 mg/Kg isometha-midium chloride hydrochloride and about two times more relapse interval of 7 mg/kg diminazene and 1 mg/kg isomethamidium chloride hydrochloride in second dose was observed. In general when we compare the relapse time of diminazene second dose is more by about two times than its first dose, whereas in the case of isometamidium chloride second dose relapse time is more by about three times than its first dose. Ainanshe *et al*., 1992, reported that a useful indication of the level of resistance obtained from studies in ruminants by recording the length of time between treatment and the detection of breakthrough populations of trypanosomes which implies that the shorter the period, the greater the level of resistance. Accordingly, in the present study finding due to its short period of relapse with normal dose (mean: 6.40 days) and relapse after double dose (mean: 18.80 days), greater level of resistance was detected after treatment with isomethamidium chloride hydrochloride than diminazene diaceturate which have a relatively longer period of relapse with normal dose (mean: 17.60 days) and relapse after double dose (mean: 36.67 days). Therefore, as opposed to the assumption of farmers of Amaya district, the results of the experimental study showed isomethamidium found to be more resistant than diminazene. This could be due the fact that farmers had been using isomethamidium indiscriminately for long time and is in agreement with the finding that changing drugs or alternative use of drugs in different time reduced the chance of drug resistance (Uilenberg, 1998).

Conclusion and Recommendation

The study result showed that there was a widespread (high level) occurrence of diminazene and isomethamidium resistant populations of *T. congolense* due to indiscriminate and frequent use of the two drugs in Amaya district of Ethiopia . This is a serious threat to cattle production in this area , however, relatively greater level of resistance was recorded for isomethamidium than diminazene. A number of risk factors like (inexpertise use of drugs) treating animals at home by non-professionals which led to under dose, purchasing drug from illegal sources, and indiscriminate use of the same type of trypanocidal drugs for extended period of years and irregular mass treatment (sick and healthy ones)by trypanocidal drugs might have contributed to the development of drug resistance. This study has in addition proved that the two common chemotherapeutic and chemoprophylactic agents (diminazene aceturate and isomethamidium chloride) currently in use would not effectively control trypanosomiasis in the study area. Based on the conclusions, the following recommendations are forwarded;

- More emphasis should be given to the vector control in integrated disease control strategy in the area
- Treatment should only be given to clinically sick animals in order to reduce the frequency of drug usage and occurrence of resistance, hence the need for strict supervision of trypanocidal drugs handling and distribution
- Invention and introduction of new drugs and detailed experimental works are also recommended.
- There is an urgent need for detailed experimental work in the field to monitor the development of drug resistance in tsetse-infested areas of Ethiopia.

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