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PCR and microsatellite analysis of diminazene aceturate resistance of bovine trypanosomes correlated to knowledge, attitude and practice of livestock keepers in South-Western Ethiopia

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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 the Ghibe valley to evaluate diminazene aceturate (DA) resistance and assess livestock owner's perception of trypanocidal drug use. Four Peasant Associations (PAs) were purposively selected and the cattle randomly sampled in each PAs. At the beginning of the study (t_0), 106 bovines positive for trypanosomes by the haematocrit centrifugation technique (HCT) and 119 negative control animals were recruited for six months follow-up using HCT, 18S-PCR-RFLP, *Dpn*II-PCR-RFLP and microsatellite analysis. Prevalence of trypanosomosis was 18.1% based on the HCT technique and the mean PCV value was $23.6 \pm 5.1\%$ 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 detection showed 38 (59.4%) *Trypanosoma congolense* savannah, 18 (28.1%) *Trypanosoma vivax*, 5 (7.8%) *Trypanosoma 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 *Dpn*II-PCR-RFLP. A positive HCT had a significant effect on PCV ($p < 0.001$) with the mean PCV value equal to $24.4 \pm 0.2\%$ in the absence of trypanosomes and to $20.9 \pm 0.3\%$ in the presence of trypanosomes. PCV increased significantly ($p < 0.001$) with $4.4 \pm 0.5\%$ one month after treatment. All *T. congolense* savannah type were analyzed using microsatellite markers TCM1, TCM3 and TCM4. The main events were new infections (40.0%) and relapses (37.5%) with cures lagging at 22.5%. In 10 purposively selected PAs a semi-structured questionnaire was used. The average herd size was the highest in Abelti PA (6.7 ± 1.8 TLU) and the mean herd size was statistically different ($p = 0.01$) 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 messages should be delivered to promote a rational drug use, improved livestock management and the application of strategic vector control methods.

1. 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 (CFSPH, 2009; Desquesnes and Dia, 2003; FAO, 1983). 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 a 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

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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 odor-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 chemoprophylaxis 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 (Grace, 2003). 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 better gain 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 should be distinguished from a new infection 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 farmers practices to the chemoresistance situation. The specific objectives of this study were (i) to evaluate diminazene aceturate (DA) resistance with the *Dpn*II-PCR-RFLP assay, (ii) following the infection status of *T. congolense* savannah-positive animals using microsatellite loci analysis and (iii) to assess the agro-pastoral knowledge, attitudes and practices (KAP) of livestock owners in the Ghibe valley, Ethiopia.

2. Materials and methods

2.1. 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 range between 29.8 and 44.0 °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).

2.2. Longitudinal study

2.2.1. 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).

2.2.2. Sampling strategy and sample size

This study was conducted from November 2012 to May 2013. 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 (Fig. 1).

During the first two visits, the peasants were requested to gather their cattle at one site where a random sampling was organized by means of a lottery method. The haematocrit centrifugation technique (HCT) was used to detect trypanosome infections in the animals (Woo, 1970).

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 (Fig. 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. 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.

2.2.3. 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.

2.3. 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 min 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).

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