J. Protozool. Res. 10, 191-201 (2000)

Determination of serum isometamidium levels in sheep and goats under field conditions using isometamidium-ELISA

J. O. WESONGAH*[1], G. A. MURILLA[1], J. K. KIBUGU[1] and T. W. JONES[2]

[1] Kenya Trypanosomiasis Research Institute, P.O. Box 362, Kikuyu, Kenya.

[2] The University of Edinburgh, Center for Tropical Veterinary Medicine, Edinburgh, EH25 9R Scotland, UK.

* Corresponding author: J. O. WESONGAH Tel: +254-154-32960-4; Fax: 254-154-32397: e-mail: ketri@net2000ke.com

Key words: Isometamidium chloride; ELISA; Sheep; Goats; Trypanosomosis

ABSTRACT

Twenty eight sheep and 28 goats were treated with isometamidium chloride (Samorin®, hone Merieux, Lyon, France) at a dose of 1 mg/kg body weight (bw) by intramuscular injection. All the animals were grazed in a tsetse-infested area. They were monitored for anaemia, body weight, anti-trypanosome antibodies and serum isometamidium concentration using the isometamidium enzyme-linked immunosorbent assay (ELISA) for more than 80 days after treatment. Serum isometamidium levels were higher in goats than in sheep throughout the experimental period. Isometamidium was still detectable in sheep and goats for up to 77 and 98 days, respectively, alter treatment (detection limit=0.1 ng/ml). The isometamidium elimination half-lives in sheep and goats were approximately 13.8 and 17.4 days, respectively. No trypanosomes were detected in either the isometamidium-treated or untreated control animals. The present study demonstrated that the isometamidium-ELISA, originally developed for use in cattle, may be equally useful in monitoring the drug in sheep and goats. The elimination half-lives and serum isometamidium levels were markedly higher in goats than in sheep. This could have important implications for chemoprophylaxis in small ruminants under field conditions.

INTRODUCTION

Animal trypanosomosis is a major constraint to livestock production in large areas of sub-Saharan Africa (Spath, 2000). Only the trypanotolerant breeds can survive, reproduce and remain productive in tsetse infested areas with minimum requirement of trypanocidal drug treatment (Murray et al., 1982), Isometamidium chloride is the only drug that is used for prophylaxis against trypanosome infections in livestock in Africa.

A number of studies have been carried out using parasitological methods to investigate the use of isometamidium chloride in sheep and goats under natural tsetse challenge (Griffin and Allonby 1979a; Okech et al., 1997). The studies showed that sheep and goats could be protected against trypanosomosis by isometamidium chloride for periods ranging from 6 to 16 weeks. In the present study three methods were used, isometamidium-enzyme linked immunosorbent assay (Isometamidium-ELISA), parasitological methods and antibody ELISA in an attempt to correlate serum isometamidium concentrations to the presence of trypanosome infections.

Previously, Braide and Eghianruwa (1980) attempted to evaluate isometamidium residues in goat tissues using the spectrophotometric methods. They were however unable to follow the drug for long periods due to limitations of those methods.

MATERIALS AND METIIODS

Study area

The study was carried out at the National Range Research Station of the Kenya Agricultural Research Institute (KARI) in Makueni district, Eastern Province of Kenya (Fig. 1). The study area comprises of open grass savannah and Acacia-Commiphora bushland, typical of ecological Zone V, with low erratic annual rainfall. The tsetse population at Kiboko area consists principally of *Glossina pallidipes*, *G. longipennis* and *G. brevipalpis*. *G. pallidipes* is the most common, followed by *G. brevipalpis* (Griffin and Allonby 1979b).

The ranch management system has instituted a program in which sheep and goats are protected against trypanosomosis by prophylactic treatment with isometamidium chloride at a dose of 1 mg/kg body weight every 3 months.

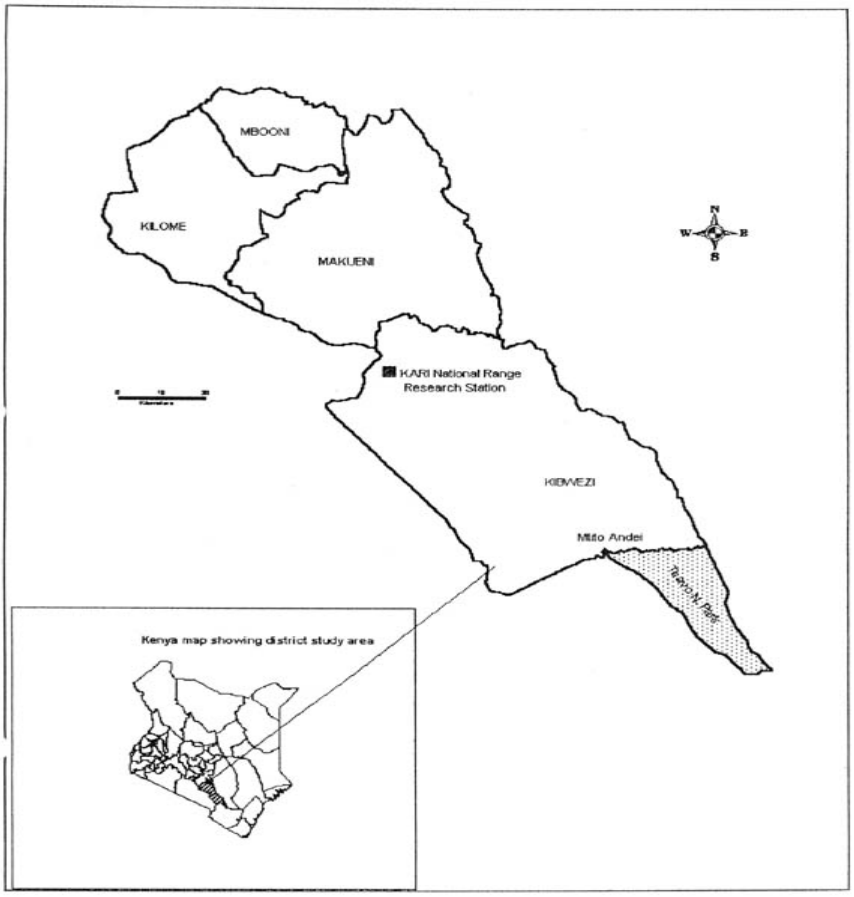

Fig. 1 map showing Kiboko study area in Makueni District, Kenya

Experimental animals

Experimental animals consisting of two different breeds of sheep (Red Maasai and Black Head Somali) and goats (Small East Africa and Galla) were used (Table 1). Twenty-eight sheep were randomly selected from a population of 38 sheep on Kiboko ranch. However, all the 28 goats available on the ranch were used. Fourteen sheep and 16 goats were purchased from farmers in the vicinity of the ranch and used as untreated controls. All the experimental animals were identified by eartags and left to graze under natural conditions. The animals were deformed with 1.5% levamisole hydrochloride and 3% w/v oxyclozanide (Levafas, Norbrook Laboratories

Ltd) at l m1/ 2kg body weight once every 3 months and dipped monthly using Stelladone® (Ciba-Geigy, Switzerland) to control ticks and tick-borne diseases. All the animals on the ranch including those that were not used for the experiment were grazed together to ensure they were exposed to similar tsetse challenge.

Prior to the start of the experiments all the animals were screened for

Table 1. Experimental groups of sheep and goats and the type of treatment adminisrrated

Breed	Group 1	Group 2	Group 3	Group 4
	Control sheep	Control goats	Ismm tx sheep**	Ismm tx goats**
Red Maasai	7*	-	14	-
Black Head Somali	7	-	14	-
Small East Africa	-	11	-	16
Galla	-	5	-	12
Total	14	16	28	28

* No. of animals
**Ismm tx:Isometamidium treated

anti-trypanosome antibodies against *Trypanosoma congolense*, *T. brucei* and *T. vivax* using an antibody trapping ELISA described by Masake et a1. (1995). As *T. congolense* antigen is known to cross-react with antibodies produced against *T. vivax*, and *T. brucei* (Luckins, 1977) this could be used to screen for any previous exposure of animals to any of the 3 trypanosome species indicated. All the animals were weighed monthly using a mechanical weighing balance. Six biconical traps were also set up monthly within the grazing area and collections of tsetse catches made every 48 hrs to determine the fly species and density. The experiment covered a period of 6 months consisting of both wet (September to December) and dry (June to August) seasons.

Treatment

Isometamidium chloride (Samorin®, Rhone Merieux, Lyon, France) was administered at 3 monthly intervals. The drug was prepared immediately before use as a 2 % w/v solution in distilled water and was given as a single bolus at 1 mg/kg body weight by intramuscular injection into the neck muscles of each of the animals except the controls.

Experimental design

The animals were divided into 4 treatment groups as shown in Table 1.

Serum collection

Blood samples were collected two days before isometamidium treatment to determine anti-trypanosome antibody levels and for use in the preparation of isometamidium standards as

described by Eisler et al. (1996). Following isometamidium chloride treatment, blood samples for serum preparation were collected weekly to determine drug concentrations as described by (Eisler et a1., 1996) and for determination of the presence of anti-trypanosome antibodies using the Antibody-ELISA as described by Masake et al. (1995). Blood samples were also collected weekly from the ear vein for the determination of packed cell volume (PCV) as described by Woo (1970) and for monitoring parasitaemia by examination of buffy coats (Murray et al., 1977).

Drug analysis

Isometamidium-enzyme-linked immunosorbent assay (EIJISA)

Sera collected from sheep and goats following isometamidium treatment were tested using the isometamidium enzyme-linked immunosorbent assay (ELISA) described by (Eisler et a1., 1996). All the plates used for the isometamidium-enzyme linked immunosorbent were Immulon 4®, from Dynatech laboratories, Chantilly, USA. Briefly, 96-well microtitre plates were coated by overnight incubation of 100 µm of 1/10,000 dilution of hyperimmune rabbit anti-isometamidium serum in carbonate/bicarbonate buffer, 0.1 M pH 9.2. Plates were washed 5 times using 1/5 dilution of phosphate buffered saline (PBS), 0.2M pH 7.4, containing 0.05% Tween 20 (PBST, Sigma, St Louis, USA), and blotted dry. Test sera, isometamidium standards and quality control standards were pre-diluted (ten fold) in PBST and vortexed. Each pre-dilution was added to duplicate wells on microtitre plates (100 µl per well), which were shaken for 10 min and then Stored at 4^{o}C overnight. Plates were then washed as described above, and 100 µl isometamidium horseradish peroxidase-conjugate diluted 1/128,000 in PSST was added to every well, followed by incubation at 37^{o}C with shaking for 15 min. Plates were again washed 5 times as described above, and 100 µl of a mixture containing substrate and chromogen solutions (Hydrogen peroxide and 3,3,5,5 tetramethylbenzidine-TMB) (Sigma) added to each well. Ten minutes later, the reaction was stopped using 1M orthophosphoric acid, turning the blue colored complex to yellow.

Detection of anti-trypanosome antibodies

The antibody detection ELISA technique described by Masake et al. (1995) was used to assess the presence of anti-trypanosome antibodies in all the sheep and goats over the experimental period of over 80 days. Briefly, 96-well microtitre plates pre-coated with 10 µl of *T.*

congolense antigen, prepared from a trypanosome density of l x 10^9 parasites/ml, were obtained from the International Livestock Research Institute (ILRI), Nairobi, Kenya. Plates were treated with blocking buffer (0.2% casein in phosphate-buffered saline containing 0.25% Tween 20) for 20 min, followed by washing 5 times with washing buffer (PBST). Thereafter, 150 pl of samples diluted 1/200 were added to each well, followed by incubation at 37 ^oC for 40 min with shaking. Plates were then washed 5 times to remove the unbound antibody. One hundred and fifty microtitres of rabbit anti-goat IgG labelled with horseradish peroxidase diluted 1/20,000 in PBST plus 1% skimmed milk were added to each well. Plates were further incubated at 37^oC with shaking for 30 min. Plates were then washed 5 times with soaking periods of 10 minutes in washing buffer. One hundred microtitres of a mixture of (1: 1) hydrogen peroxide and chromogen solution (H_2O_2/TMB; Sigma) was added to each well followed by incubation for 40 min with shaking. The reaction was stopped with 0.1% of sodium dodecyl-sulphate (SDS) and the absorbance of each well measured photometrically at 620 nm using the ELISA reader interfaced to a desktop computer.

A checkerboard titration was used to determine the optimum conjugate dilutions using known negative and positive control serum samples. A cut-off point of OD values was established to be 0.35 and 0.75 for sheep and goats respectively using frequency distribution curves.

Data analysis

Data collected included PCV, Serum isometamidium concentrations, antibody levels and live weight. Mean±SD values were calculated for all data sets. The values were compared within and between various treatment groups (at 95% C.I) using the Welch's corrected t test (Instat biostatistics software).

Data handling

A multi-channel microtitre reader fitted with a 450 nm filter (Bio-Tek EL-311 SX, Bio-Tek Instruments Inc. Vermont, USA) and interfaced to a desktop computer (Compaq Pressario series 5000, USA), via the RS232C serial connection was used to read and record the optical densities of the samples. Samples were analyzed in duplicate, intra plate coefficient variances (CVs) above 12% and inter plate duplicates with CVs $\geq$ 2 25% were excluded from the

data. An estimate of the rate of decline of isometamidium over this period (7-105 days) was obtained by performing linear regression (log mean concentrations versus time after treatment) for both sheep and goats.

RESULTS

No flies belonging to the Genus *Glossina* were caught in any of the traps during the study period. Both the isometamidium-treated and control sheep remained free of detectable trypanosomes throughout the study period. All the control sheep and goats were negative for anti-trypanosome antibodies while 3 and 4 of the isometamidium treated goats and sheep respectively were positive prior to the start of the experiments.

Serum isometamidium concentration

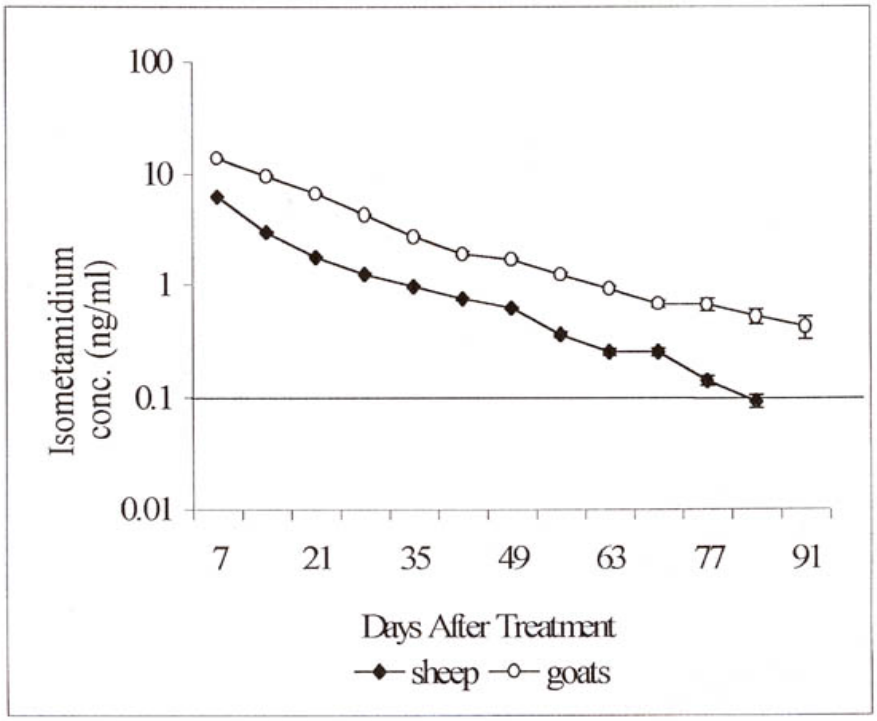

Fig. 2 Mean serum isometamidium (±SE)concentration in sheep and goats after i.m. administration of the drug at 1 mg/kg b.w.
There was low individual variation in serum isometamidium concentration, hence error bars are not visible.

Sheep

Mean serum isometamidium concentration versus time plot obtained following

intramuscular administration of the drug to sheep is shown in Fig. 2. Mean serum isometamidium concentration of 6.2±0.06 ng/ml was detected at 7 days after treatment. This serum concentration then declined to 3.0±l.5 ng/ml and 1.2±0.03 ng/ml at 14 and 29 days respectively after treatment. Mean serum isometamidium concentration of 0.14±0.19 ng/ml were still detectable in 50% of the isometamidium-treated sheep up to 77 days after treatment. The drug was undetectable in all the sheep at 105 days after treatment given that the detection limit of the assay was 0.1 ng/m1. An apparent drug elimination half-life of approximately 13.8 days (R^2=0.99) was estimated in sheep using linear regression (Fig. 2).

Goats

Mean serum isometamidium concentration versus time plot obtained following intramuscular administration of the drug to goats is shown in Fig. 2. Mean serum isometamidium concentration of 13.7±0.07 ng/ml was detected 7 days after treatment. This serum drug concentration then declined to 9.5i: 3.8 ng/ml and 4.3±0.03 ng/ml at 14 and 29 days respectively after treatment. Mean serum isometamidium concentrations of 0.4±0.06 ng/ml were still detectable in approximately 14% of the isometamidium-treated goats up to 98 days after treatment. However, the drug was undetectable in all the goats at 105 days after treatment. An apparent drug elimination half-life of approximately 17.4 days (R^2=0.97) was estimated in goats (Fig. 2).

In comparison, the mean serum isometamidium concentrations were significantly higher ($P<0.05$) in goats than in sheep (Fig. 2) although a similar profile of isometamidium decline was observed in both species. The elimination half-life estimated in goats was markedly longer than that of sheep (Fig 2).

DISCUSSION

The current study has established that serum isometamidium levels of 0.14±0.2 ng/ml and 0.4±0.06 ng/ml could be detected in sheep and goats for periods up to 77 and 98 days respectively, after prophylactic treatment. The drug concentration was persistently higher in goats than in sheep. Previously, serum isometamidium concentration in goats had not been described beyond 48 hours (Kinabo and McKellar, 1990; Braide and Eghianurwa, 1980) and there has not been any previous information on isometamidium persistence in sheep. The availability of the

enzyme-linked immunosorbent assay for isometamidium has, therefore, made it possible to establish the drug profiles in sheep and goats.

The results from studies carried out by Eisler et al. (1994) showed that isometamidium was detectable in serum of treated cattle up to 3 months after treatment using the isometamidium-ELISA. The results also suggested that serum isometamidium concentration of 0.4 ng/ml could afford reasonable protection against *T. congolense* infection. In the present study, serum isometamidium concentration in both sheep and goats were 0.6±0.02 ng/ml and 0.5±0.08 ng/ml at 49 and 85 days respectively. These levels exceed the protective levels reported in cattle after prophylactic treatment (Eisler et al., 1994). However, isometamidium concentration obtained in the present study could not be related to the trypanosome infections as none of the animals became infected with trypanosomes throughout the period of study, as determined by the buffy coat method (Murray et al., 1977) and antibody ELISA.

The serum isometamidium concentration versus time profiles established in the present study differed markedly from those reported previously. For instance, Braide and Eghianruwa (1980), reported mean serum isometamidium concentration of 2.17 μg/ml at 24 hours after intramuscular administration of isometamidium chloride in goats at a dose rate of 0.5 mg/kg body weight. Subsequent serum samples taken at seven days, two weeks and three weeks after single dose injection did not contain any detectable amounts of the drug, presumably because of the low sensitivity of the analytical method used. In the present study serum isometamidium levels were higher in goats than in sheep throughout the experimental period. This is due to the shorter, elimination half-life of the drug in sheep (13.8 days) as compared to goats (17.4 days) suggesting that isometamidium persisted longer in goats than in sheep under conditions of the study. This implies that goats, under isometamidium prophylactic treatment would be protected against trypanosomosis under field conditions for longer periods than sheep. Although no trypanosome infections were detected in the present study, these findings agree with observations made by Okech et a1. (1997) of longer periods of protections in goats than sheep given isometamidium prophylactic treatment under medium to high tsetse challenge in the field.

In conclusion the use of isometamidium-ELISA made it possible to detect and monitor isometamidium in sheep and goats treated with the drug at a dose of 1 mg/kg body weight for

periods in excess of 70 days. Generally, the serum drug concentrations and elimination half-lives were markedly higher in goats than sheep. This difference in isometamidium metabolism between the two species could have important implication for chemoprophylaxis under field conditions.

ACKNOWLEDGEMENTS

We would like to thank the Department for International Development (DFID) and the Kenya Government through KETRI for funding this project, the Residue Analysis staff for their assistance with this work and Dr. Karimi (KARI) for his professional assistance. We would also like to thank Dr. Mark Eisler for the ELISA software, isometamidium conjugate and antibody. We are grateful to Drs J. M. Ndungu, D. K. Masiga, and S. Nyamwaro for constructive criticism of the paper. This paper is published with the kind permission of the Director, KETRI.

REFERENCES

Braide, V. B. and Eghianruwa, K. I. 1980. Isometamidium residues in goat tissues after parenteral administration. *Res. Vet. Sci.* 29:111-113.

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

Eisler, M. a, Elliott, C. T. and Holmes, P. H. 1996. A simple competitive enzyme-linked immunoassay for the detection of the trypanocidal drug isometamidium. *Therap. Drug Mon*. 18:73-79.

Griffin, L. and Allonby, E. W. 1979a. The economic effects of Trypanosomiasis in sheep and goats at a range station in Kenya. *Trop. Anim. Hlth. Prod*. 11:127-132.

Griffin, L. and Allonby, E. W. 1979b. Studies on the epidemiology of trypanosomiasis in sheep and goats in Kenya. *Trop. Anim. Hlth. Prod*. 11:133-142.

Kinabo, L. D. B., and McKellar, Q. A. 1990. Isometamidium in goats: Disposition kinetics mammary excretion and tissue residues. *Br. Vet. J*. 146:405-412.

Luckins, A. G., 1977. Detection of antibodies in trypanosome-infected cattle by means of

microplate enzyme -linked immunosorbent assay (ELISA). *Trop. Anim. Hlth. Prod.*, 9:53-62.

Masake, R. A. Moloo, S. K., Nantulya, V. M. Minja, S. H., Makau, J..M. Njuguna, J. T. 1995. Comparative sensitivity of antigen-detection enzyme immunosorbent assay and microhaematocrit centrifugation technique in the diagnosis of *Trypanosoma brucei* infections in cattle. *Vet. Parasitol*. 56:37-46.

Murray, M., Murray, P. K. and Mclntyre, W. I. M. 1977. Improved parasitological technique for diagnosis of African Trypanosomiasis. *Trans. Roy. Soc. Trop. Med. Hyg*., 71: 325-326.

Murray, M., Morrison, W. I. and Whitelaw, D. D. 1982. Host susceptibility to African Trypanosomosis: trypanotolerance. *Adv. Parasitol*., 21:1-68.

Okech, G., Masinde, A., Stevenson, P. and Ndungu, J. M. 1997. The role of isometamidium chloride in chemoprophylaxis against trypanosomiasis of small ruminants in Kenya. Trypanosomiasis Research and Control (ISCTRC), Maputo, Mozambique, pp 390-397.

Spath, J. 2000. Feeding patterns of the three sympatric tsetse species (*Glossina* spp) (Diptera, Glossinidae) in the preforest zone of Cote d'Ivoire. *Acta Trop*., 75:109-118.

Woo, P. T. K. 1970. The haematocrit centrifuge technique for the diagnosis of African trypanosomiasis. *Acta Trop*., 4:385-386.