4. MATERIALS AND METHODS

4.1 Study area

The study was carried out in Adamaoua plateau situated in the northern part of Cameroon; it covers more than 72,000 km^2 and is one of the ten provinces of the republic of Cameroon. There are five subdivisions in the Adamaoua province (Djerem; Faro and Déo; Mayo Banyo; Mbéré and Vina) (Fig 4.1). Suchel (1972) describes two types of climate in the region: one covering the northern part of the Adamaoua plateau, the other covering the meridional reserve.

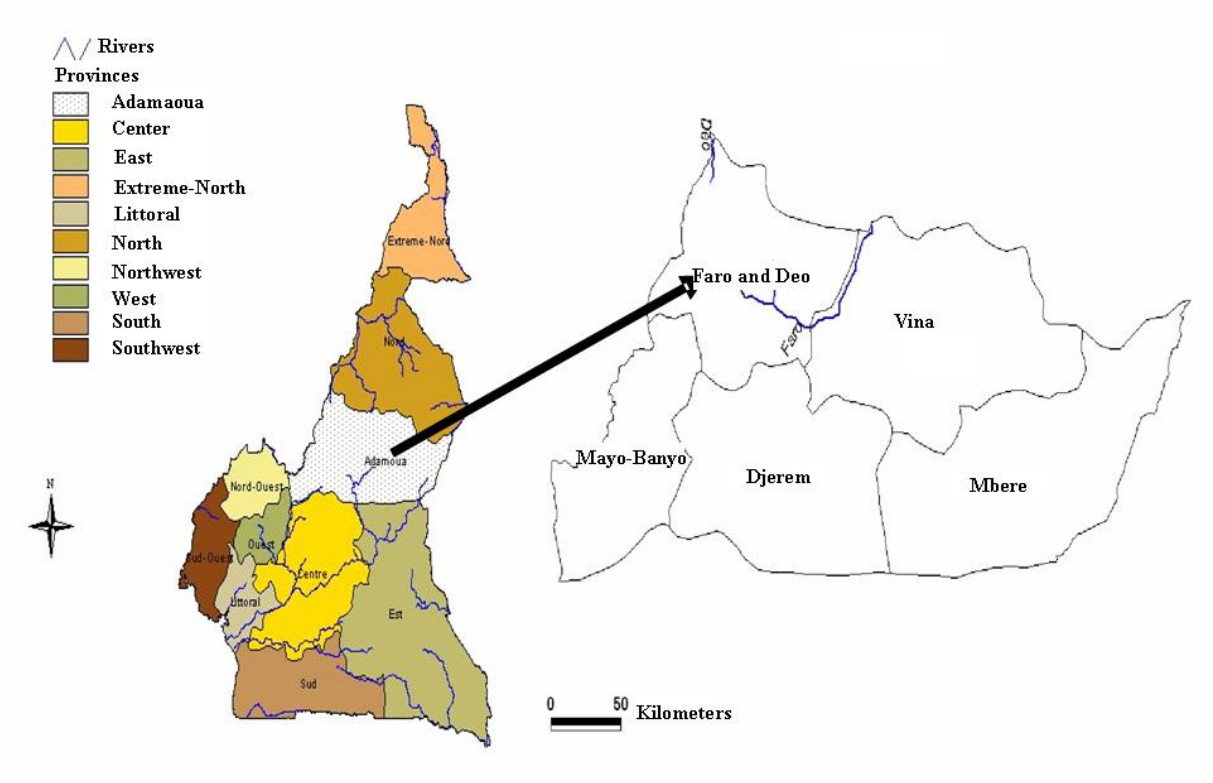

Figure 4.1 Map of the Adamaoua province, Cameroon

The Faro and Deo Division, our study area, is located in the northern part and has a Soudano-Sahelian climate. It lies at an altitude of 1000-1100m above sea level with an average rainfall of 1800 mm. The rainy season lasts from March/April to October and most of the rainfall occurs between June and September. The Adamaoua plateau is covered with savannah consisting for more than 90% of *Daniellia olivert* and *Lophira lanceolata* (Letouzey, 1969).

The most common tree species are *Isoberlina* spp and *Sporobolus africanus* (De-Wispelaere, 1994). The environment is very suitable for intensive cattle rearing. The White Fulani or Akou and the Red Fulani or Djafoun are indigenous East African zebu breeds which arrived with transhumance and immigration of people (Boutrais, 1978). The other breed known by its local name Goudali or Peuhl of Adamaoua is an original breed of the province, more precisely from the Vina Division. The cattle are kept under traditional husbandry systems with communal herding. The husbandry practice is purely extensive. Occasionally, salt is offered to the animals. In the dry season many herds move from the plateau to the tsetse infested valley (transhumance).

The Faro and Deo Division, which covers 11,000 km^2, has an approximately 74,559 head of cattle (PACE, 2005) and 67,413 inhabitants (PNVRA, 2001). The government policy in this division has been to establish a zone of high livestock productivity by the creating big ranches, such as the Faro ranch. At the end of the tsetse eradication campaigns in 1994, the territory was divided in the following three zones situated from South to North (Fig 4. 2):

1. The plateau: most of the tsetse flies (*G. m. submorsitans*), with the exception of some small pockets, were cleared from this zone in 1994. Large ranches with big cattle herds are present on the plateau. There is only limited transhumance to the tsetse-infested valley in the dry season, often involving only part of the herd. Cattle are regularly dipped with insecticides during the rainy season.

2. Buffer zone: The MSEG has designated this zone as a barrier to tsetse invasion from the valley to the Adamaoua plateau (Boutrais and Cuisance, 1995). Therefore, it was advised that all stationary herds should be treated regularly with insecticides (pyrethroids). According to the MSEG and our observations, insecticides are used quite frequently although the treatment frequency varies according to the breeder's financial resources. Some treat all year round and others only during the dry or the rainy season. The most commonly used products are pyrethroids (Butox®, Cypermil®, Didétéki®, Ectopor® and Eradick®).

3. The valley: The plain of Koutine is an agricultural zone where the cattle keepers of the plateau and some herds from neighboring Nigeria pass the dry season (transhumance). The biggest tsetse threat originates from the forest area where the flies have never been controlled. This forest area borders the tsetse-infested game reserves of Faro (Boutrais and Cuisance, 1995). In the West, the valley is bordered by the tsetse-infested Gashaga reserve forest in Nigeria (Fig 4.2). In 1994, *Glossina morsitans submorsitans, G. fuscipes fuscipes*

and *G. tachinoides* were present in the valley. *Glossina morsitans submorsitans* was present in the buffer zone and on the plateau (Cuisance and Boutrais, 1995). Livestock owners consider trypanosomosis as the most important disease constraint (Caphavet, 2003).

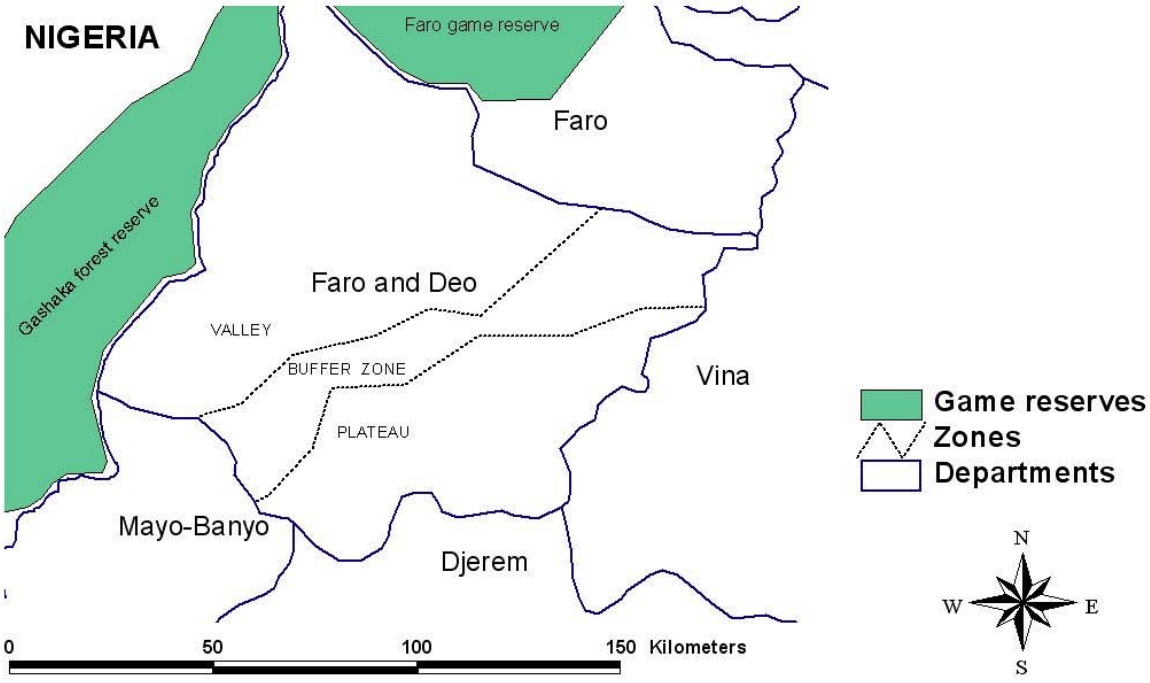

Figure 4.2 Map of the study area, indicating the three zones of the Faro and Deo Division

4.2 Sentinel herds

In order to assess the trypanosomosis risk, nine sentinel herds, three in each zone (buffer, valley and plateau), were established and monitored between March 2004 and February 2005. All selected herds were geo- referenced and are shown in Fig 4.3. The average number of cattle per herd was 35. In each of the herds, 20 adults animals were randomly chosen and ear tagged. The owner, weight, age, breed and sex of each animal were recorded.

At the start of the study period, all the sentinel animals received a curative intramuscular treatment of diminazene aceturate (Berenil®, Hoechst) of 7mg/kg body weight dose. Blood was collected monthly from each sentinel animal and examined using parasitological

diagnostic methods. Animals which were infected with trypanosomes received a curative treatment with diminazene aceturate. The monthly trypanosomosis incidence was calculated as the number of new positive cases expressed as a percentage of the total number sampled, without taking into account the prophylactic effect of the drug. The sentinel herds were kept under traditional village management. To assess the daily movement of the sentinel animals and their grazing limits, the sentinel herds were followed for one day at the beginning of the rainy season, and their position noted every half hour by GPS (Global Positioning System, Garmin 75). The sentinel herds did not leave their respective zones during the observation period.

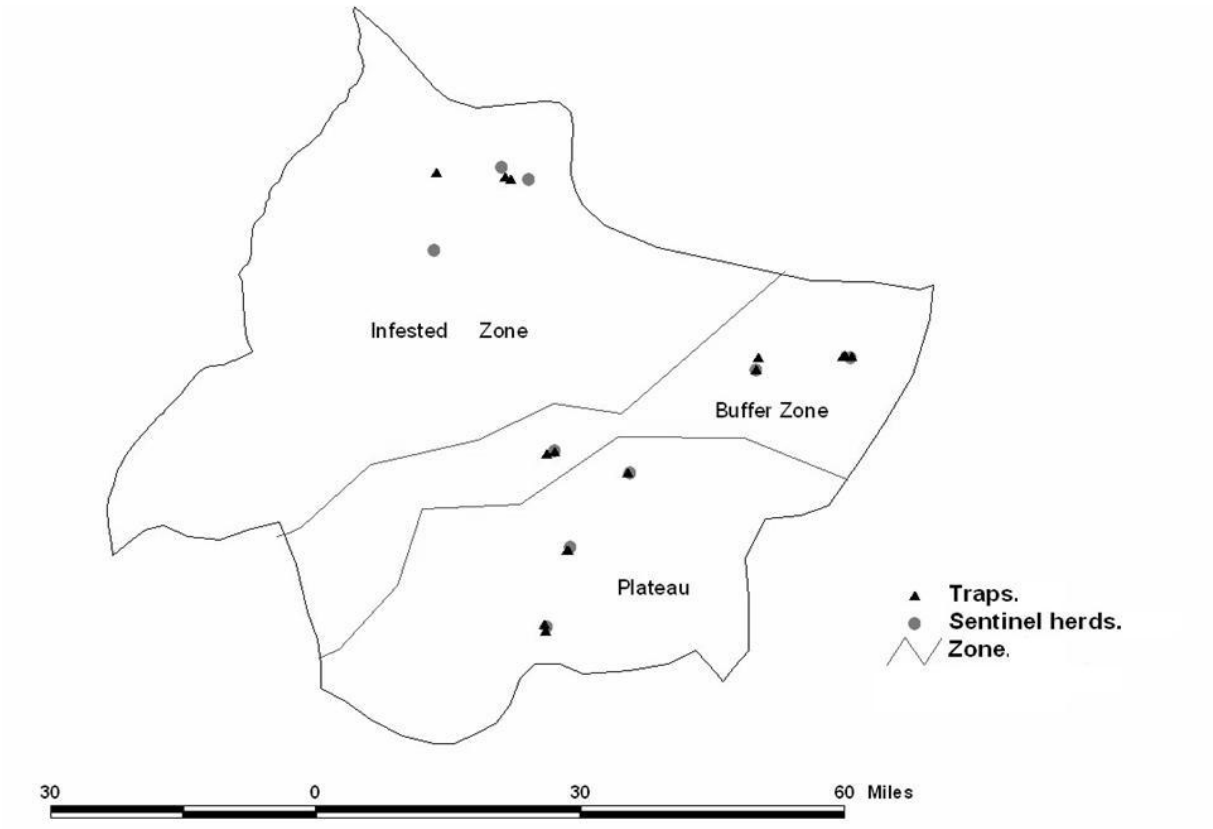

Figure 4. 3 Map showing the study area, the Faro and Deo Division within the Adamaoua Province of Cameroon and the location of the sentinel herds and tsetse traps

4.3 Tsetse monitoring around sentinel cattle

Between April 2004 and March 2005, a total of 15 geo-referenced biconical traps (Challier *et al.,* 1977) (Fig 4.4) were used to monitor the tsetse flies in the study area. Four traps were deployed on the plateau, 7 in the buffer zone and 4 in the valley. From April 2005 until

September 2005 the number of traps was increased to 7 on the plateau, 10 in the buffer zone and 5 in the valley. Traps were checked twice a month. Records were kept of the number, species and sex of the tsetse captured in each trap. A monthly mean index of abundance (IA) of tsetse was calculated as the mean number of flies (males and females) captured per trap per day.

Figure 4. 4 An example of a Biconical trap used in the study

4.4 Parasitological techniques

Thin blood films were prepared from a drop of blood obtained by puncture of visible ear veins using a lancet. These films were fixed on site with methanol and stained with 10% Giemsa solution at the laboratory. Whole blood was collected at the same time from the ear veins using heparinized capillary tubes. These tubes were sealed with "Cristaseal" (Hawksley, Lancing, UK) and centrifuged immediately in a micro-haematocrit centrifuge for 5 minutes. at 7,500 g. A portable diesel generator of 6.5 kW was used as a power source in the field. After centrifugation, the packed cell volume (PCV) of each sample was estimated

using a Hawksley micro-haematocrit reader (Hawksley and Sons, Lancing, UK). The haematocrit tubes were cut a few millimeters below the junction of the buffy coat levels. The erythrocytes, buffy coat and the uppermost layer of red blood cells of each specimen were extruded onto a microscope slide and examined with a phase-contrast microscope at x 40 objective lens for the presence of motile trypanosomes (Murray *et al.,* 1977). The trypanosome species were identified by their movement, morphology and their sizes as described by Hoare (1972).

4.5 ELISA for detecting antitrypanosomal antibodies

To determine the level of challenge of cattle during transhumance in the valley, the sero-conversion of calves originating from the plateau was monitored after their first transhumance into the valley. For this purpose, hundred seronegative calves (age 5 to 11 months of) belonging to 6 different herds were ear-tagged and went on transhumance between November 2004 and March 2005. Their blood was checked for the presence of anti-trypanosomal antibodies after transhumance. Blood of each selected calf was collected from an ear vein into two heparinized microhaematocrit centrifuge capillary tubes. The first tube was used for the buffy coat technique (BCT) and the haematocrit centrifugation technique (HCT). The second tube was extruded onto a filter paper (Whatman no. 4) for serological analysis. All filter papers were labelled, indicating the date of collection, place of collection and the sample number. Samples were air dried on direct sunlight and placed in plastic bags with silica gel. The bags were sealed and kept cool.

Eluted blood spots were screened for the presence of trypanosomal antibodies using an indirect Enzyme-Linked Immunosorbent Assay (ELISA) with *Trypanosoma congolense* CP 81 antigen (Rebeski *et al.,* 2000). Negative control samples were obtained from non-infected cattle in the tsetse-free zone of Kousseri in Cameroon. Positive control sera were obtained from cattle experimentally infected with *T. congolense* (Van den Bossche *et al.,* 2004).
500 µl High Performance ELISA (HPE) buffer (CLB, Amsterdam, The Netherlands) were added to the filter paper (6 mm diameter) containing the blood spots and incubated overnight at 4°C . Microwells of immunoassay plates (Immulon 1B®, Dynex, USA) were coated with 100 µl of antigen (10 µg protein/ml) of *T. congolense* CP 81 antigen diluted in carbonate buffer (pH 9,6). Plates were sealed and incubated overnight at 4°C. After incubation, the wells were emptied and completely filled with wash buffer (PBS-T), which was prepared using 0,01M PBS buffer and 0,05% of Tween 20 (Sigma, USA). After that the wells were

filled with 100µl of eluted blood spots, the plates were sealed and incubated at 37°C for 1 hour with continuous shaking. Following a four-wash cycle with PBS-T, each well was filled with 100µl of peroxidase-conjugate (rabbit anti whole bovine IgG molecule; Sigma, USA). The plates were incubated and washed as described above. Therefore, each well was filled with 100µl of chromogen/substrate (Ortho-Phenylene Diamine, Dakocytomation, Denmark). The were then incubated at 37°C for 30 minutes and 50 µl of H_2S0_4 added to each well to stop the reaction. The absorbance was measured at 490 nm wavelength using a multichannel spectrophotometer (Multiskan RC version 6.0, Finland). Samples giving percentage positivity (PP) exceeded the mean of negative samples plus three standard deviations were considered positive (Desquesnes *et al.,* 2001).

4.6 Traps-transects and tsetse monitoring in the valley

Between January 2005 to December 2005, the tsetse population was monitored along four traps-transects traversing the three zones (plateau, buffer zone and valley) of the study area. A total of 32 geo-referenced biconal traps (Challier and Laveissiere, 1973) were deployed, with 8 traps along a transect in the valley (Alme Parc), 3 traps along the second transect (Lib Mbak) in the Buffer zone and 3 traps along the third transect (Tig Wog) on the plateau. A fourth transect (Border Plateau) involving 18 traps started on the plateau, went through the Buffer zone and ended in the valley. Depending on the location of the traps, these transects were divided into a "transhumance section" and a "game area section" (Nigeria direction) (Fig 4. 5). Traps 5 to 11 belonged to "transhumance section" and traps 12 to 18 belonged to "game area section" (Nigeria direction). The trap-transects traversed the various vegetation types and human settlements. An additional four traps on the plateau and seven traps in the buffer zone were deployed along a river and in a cattle grazing area. Traps were checked at 24 hours every week for one year. The number of tsetse caught was recorded and the species and sex identified. A monthly average Index of Apparent Abundance (IAA) was calculated as the average number of flies (males and females) captured per trap per month.

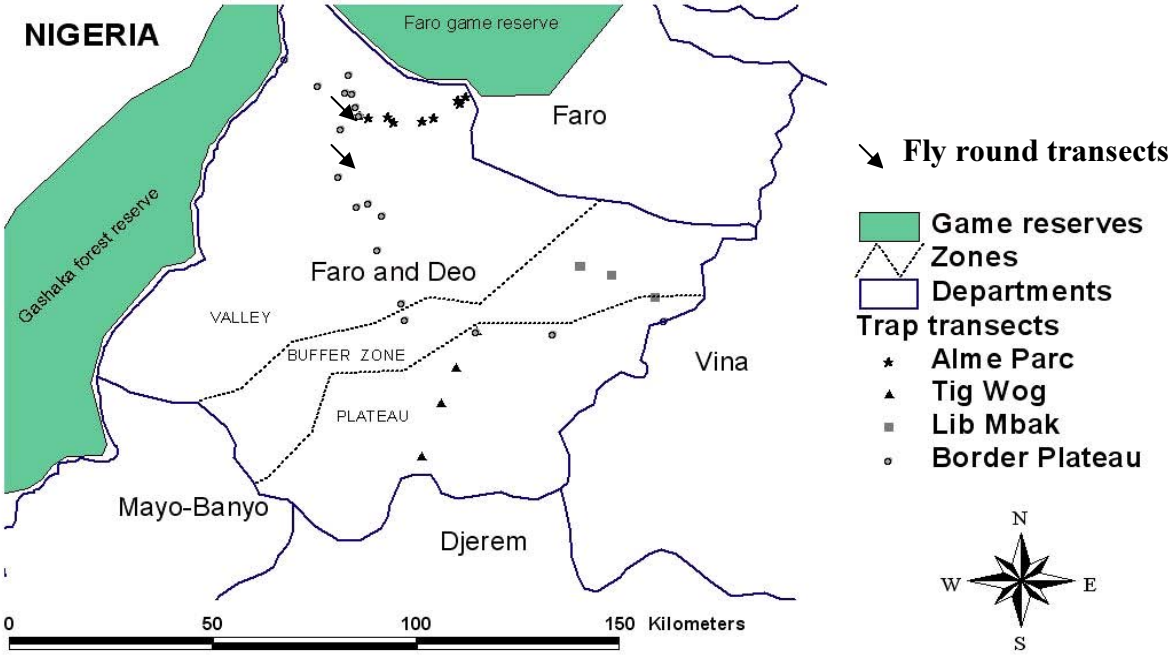

Figure 4. 5 Location of trap and fly round transects on the plateau, in the buffer zone and in the valley of the Faro and Deo Division, Adamaoua Province, Cameroon (January 2005– December 2005)

4.7 Fly rounds along transects in the valley

Between January 2005 and December 2005, the tsetse population was monitored along two transects in the valley using the fly rounds. The first transect was located in a transhumance area and the second in a game area. Each transect was about 6 km long and had 30 geo-referenced stops at approximately 200 m intervals. Fly rounds were conducted along these transects as described by Potts (1930) and Ford *et al.* (1959). Teams of two men traversed them (Fig 4.6) . The teams used a black screen (1.5 x 1 m). The screen hung from a bamboo pole and was kept hanging vertically with a second bamboo pole at the bottom. The fly round team remained at each stop for 2 minutes and, using hand-nets captured tsetse flies that landed on the screen. All captured flies were killed immediately using a pair of forceps and put in a tube. Each stop had a separate tube.

Figure 4. 6 Fly round team in the infested zone of the Adamaoua Plateau, Cameroon

Transects were traversed once a month alternately in opposite directions. The fly rounds started early in the morning, between 07.00 and 08.00 hours or 15.00 and 16.00 hours, in the rainy and hot seasons when it was not possible to make rounds early because of rain and heat.

Records were kept of the number and sex of the captured tsetse at each stop. Monthly mean IAA of tsetse was calculated as the monthly number of flies (males and females) captured per stop per fly-round.

4.8 Isometamidium-block treatment study at Kontcha village

Cross-sectional study in the valley

In order to determine the prevalence of trypanosome infections in the local cattle population a cross-sectional study was conducted from October to December 2004. Four different villages (Mayo Baleo, Mayo Zaoule, Almé and Kontcha) in the valley were selected because of their large cattle populations. A total of 493 animals in 19 herds from the 4 villages were sampled. Age, sex, breed and previous history of treatment were recorded. Blood samples were collected from the ear vein of each animal into two heparinized capillary tubes.

Samples were then examined for trypanosomes using the phase-contrast buffy-coat technique (Murray *et al.,* 1977). In parasitaemic animals, the trypanosome species were identified using Giemsa-stained thin smears.

Questionnaire Survey

During the first 2 weeks of October 2004, a questionnaire (Annex 1) survey was carried out with a major objective of getting some basic information about usage of trypanocidal drugs and insecticides, cattle breeders' knowledge about trypanosomosis and the vectors. A total of 64 breeders were interviewed from the four villages mentioned above.

Longitudinal study

This study design was carried out in the village of Kontcha because out of the 4 villages it had the highest prevalence of trypanosome infections. Prior to the start of the investigation, farmers were informed about the objective and the protocol of the study. In addition, they were requested not to treat their animals.

This longitudinal study was conducted using the method described by Eisler *et al. (*2000). Two herds were included in the study. In each herd, 40 animals were ear-tagged and randomly assigned into two groups, 20 controls and 20 for isometamidium treatment. The proportion of animals infected with *Trypanosoma congolense, T. vivax and T. brucei* was determined before treatment. Prior to treatment, the body weight of each animal was estimated by measuring the thorax with Rondo®. This was done in order to calculate the required dose of each animal. At day 0, the 20 cattle in the treatment group in each herd were treated intramuscularly with isometamidium chloride (Trypamidium®, Lot Z412971B, Merial, France) at 1mg/kg body weight. The 20 cattle in the control group in each herd were treated intramuscularly with diminazene aceturate (Berenil®, Lot BN 01A037, Hoechst, Germany) at 7mg/kg body weight. After treatment, cattle were left to graze in their natural environment. Both groups of cattle were examined for trypanosome parasites using BCT every 14 days from day 0 to day 56. When trypanosomes were detected, the trypanosome species present were identified and blood samples from positive cattle for *Trypanosoma congolense* and *T. brucei* were inoculated intraperitoneally into mice. Irrespective of the group, animals found infected with trypanosomes during the follow-up period were treated with diminazene aceturate at 7 mg/kg body weight.

4.9 Drug sensitivity studies in mice using trypanosome isolates from Kontcha village

Six stabilates of *T. congolense* isolated within the 14 and 42 days-period after treatment were tested for isometamidium and diminazene sensitivities using the single dose mouse test described below in 4. 10.

4.10 Drug sensitivity studies in mice using trypanosome isolates from the valley and buffer zone

Collection of trypanosome stabilates

Two-hundred twenty one cattle from 17 herds were sampled in both the buffer zone and the infested zones (valley) of the Adamaoua Plateau between May to November 2005. Blood samples were collected from 10 to 15 randomly selected animals per herd. At the time of sampling of cattle, background epidemiological data that included sex, age, weight, disease records and drug usage were obtained.

Blood samples of 0,5 ml from each cattle found positive for *T. congolense* or *T. brucei* using the buffy coat technique (Murray *et al.,* 1977) were inoculated intraperitoneally into mice aged between 5 to 8 weeks and weighing on average 30g . The mice were purchased from the National Veterinary Laboratory (LANAVET, Garoua). They were not immuno-suppressed prior to trypanosome infection. Infected mice were then transported to the laboratory IMT/UDs of the University of Dschang (Cameroon) for follow up. They were kept on a standard pellet ration and watered ad libidum. At high parasitaemia, from 7.1 onwards on the Herbert and Lumsden scale (Herbert and Lumsden, 1976), mice were euthanized and stabilates were prepared in liquid nitrogen using DMSO/PSG (20%) as a cryopreservative. The cryotubes were kept for 24h in the vapour phase of the liquid nitrogen before placing them directly in contact with pure liquid nitrogen. Twenty trypanosomes stabilates were prepared (Annex 2).

Single-dose test in mice

The single-dose test in mice was carried out according to the protocol described by Eisler *et al.* (2001). Twenty trypanosomes isolates were tested at a single discriminatory dosage at 1mg/kg for isometamidium and 20 mg/kg for diminazene. The tests were performed partly in Cameroon (LANAVET, Garoua) and partly in Belgium (ITM, Antwerp).

Experimental animals

White mice of the OF1 breed, 8-10 weeks old, weighing 25-30g, were obtained from the National Veterinary Laboratory (LANAVET in Cameroon) or from IFFA Credo® (France) for the tests at the Institute of Tropical Medicine (ITM, Belgium). They were maintained on a commercial pelleted ration and water ad libitum. They were housed in a fly-proof stable at the Department of Parasitology and Department of Animal Health in LANAVET and ITM, respectively.

Infection

Stabilates of trypanosomes were inoculated in two mice and they were followed up until a parasitaemia developed. The mice in which drug-sensitivity studies were carried out were infected i.p. with 1 x 10^5 trypanosomes with blood from infected mice taken at the first parasitaemic peak.

Experimental design

Each trypanosome isolate was inoculated into 3 groups of 6 mice each. About 24 hours after inoculation, the mice were treated. The drugs were dissolved in an appropriate quantity of sterile distilled water. Mice were weighed on a flat pan balance for the purpose of calculating the correct drug dosages. The first group of 6 mice was treated with 1mg/kg bw isometamidium chloride (Trypamidium-Samorin®, Merial France, A445971), the second group with 20 mg/kg bw diminazene aceturate (Berenil®, Hoechst AG, Germany, 01W005) and the third group was left untreated as control. The control group received the same amount of distilled water without drug i.p. afterwards; the mice were monitored twice a week for the presence of trypanosomes through the examination of wet smears of tail blood. Treated groups were followed until relapse occurred or for 60 days post-treatment (Eisler *et al*., 2001). In the control group, the mice were euthanized when parasitaemia was observed.

4.11 Statistical analysis

Sentinel Herds

Chi-square ($\chi 2$) from the Logistic regression analysis (STATA Corporation, 2003) was used to find out if differences existed between incidence rates of cattle trypanosomosis in both the buffer zone and valley. Analysis of variance was used to compare the PCV of the cattle on the plateau, valley and the buffer zone.

Trap and fly round transects

Data were managed using MS Exel. Graphs were drawn using MS Exel and significance testing performed with MINITAB Statistical Software (MINITAB Inc 2000). Prior to significance testing, fly counts were normalized using $\log_{10}$ (X+1) transformation. Comparisons of the sex ratios and seasonal fly catches were performed.

Isometamidium-block treatment

Kaplan-Meier survival curves were plotted from 0 to 8 weeks for both the diminazene and the isometamidium treated groups using STATA (STATA Corporation, 2003). The duration of the period during which the animals remained free of trypanosomes was used to assess resistance to isometamidium. The Cox proportional risk model was used to compare both groups. When cattle were lost during the study, they were excluded from the analysis even if they rejoined the study in the next sampling. The following parameters were used to assess drug resistance (adapted from Eisler *et al.,* 2000): 1. Resistance to isometamidium was suspected if more than 25% of the isometamidium treated cattle became parasitaemic within 8 weeks after treatment; 2. Resistance to diminazene was suspected when trypanosomes were present two weeks after treatment.