3 LITERATURE REVIEW

3.1 African Animal Trypanosomosis

3.1.1 Definition and aetiology

African animal trypanosomosis (AAT) is a disease complex caused by tsetse-fly-transmitted *Trypanosoma brucei brucei, T. congolense* or *T. vivax*. African animal trypanosomosis is most important in cattle but can cause serious losses in pigs, camels, goats, and sheep. Infection of cattle by one or more of the three African animal trypanosomes results in subacute, acute, or chronic disease characterized by intermittent fever, anaemia, occasional diarrhea, rapid loss of condition and often terminates in death (Mulligan, 1970).

T. b. brucei resides in the subgenus *Trypanozoon.* Horses, dogs, cats, camels and pigs are very susceptible to *T. b. brucei* infection. Infection of cattle, sheep, goats and sometimes pigs results in mild or chronic infection (Mulligan, 1970). Although this last observation is widely accepted, Moulton and Sollod (1976) indicated evidences that this organism is widespread in East and West Africa and that it can cause serious disease and high mortality in cattle, sheep, and goats.

In East Africa, *T. congolense* is considered to be the single most important cause of AAT. This trypanosome is also a major cause of the disease in cattle in West Africa. Sheep, goats, horses, and pigs may also be seriously affected. In domestic dogs, chronic infection often results in a carrier state (Mulligan, 1970).

Although *T. vivax* is considered to be less pathogenic for cattle than *T. congolense*, it is nevertheless the most important cause of AAT in West African cattle (Hoare, 1972; Gardiner and Wilson, 1987). This trypanosome readily persists in areas free of tsetse flies (for example, in Central and South America and in the Caribbean), where it is transmitted mechanically by biting flies or contaminated needles, syringes, and surgical instruments (Mulligan, 1970).

3.1.2 Morphology

A sound knowledge of the basic features of the various trypanosomes enables the identification of each species and so the exact cause of the disease. Trypanosomes are classified in the phylum Sarcomastigophora, the order Kinetoplastida and the family Trypanosomatidae (WHO, 1998). The trypanosome consists of a single cell varying in size from 8 to over 50 μm. There are distinct differences in appearance, shape and size between the various species of trypanosomes, allowing specific identification (Uilenberg, 1998). The salivaria group of trypanosomes may or may not have a free flagellum, the kinetoplast is terminal or sub-terminal, and the posterior end of the body is usually blunt. They develop as trypomastigotes within the mammalian host and are usually pathogenic (Mulligan, 1970).

3.1.2.1 The subgenus Nannomonas

This is the smallest of the pathogenic trypanosomes, with a length of 8-24 μm. The blood forms are monomorphic, in that they lack a free flagellum. Generally two variants are to be seen, a shorter form (9-18 μ), the typical *congolense* type and a longer form (up to 25 μ), with individuals intermediate in length between the two (Hoare, 1972). There is evidence which indicates that strains with the most long forms, the so-called 'dimorphic' strains, cause a more severe form of trypanosomosis (Uilenberg, 1998). In stained specimens of *T. congolense* the cytoplasm stains a diffuse, even, pinkish colour and is seldom granular (Uilenberg, 1998). The nucleus is centrally placed. The Kinetoplast is of medium size and is usually situated at the margin of the body, just in front of the posterior extremity (marginal and subterminal). The undulating membrane is poorly developed and inconspicuous (Hoare, 1972).

Recent studies have resulted in a subdivision of the *T. congolense* species in several 'types', which can be distinguished by isoenzymatic differences and molecular techniques. These are designated as *T. congolense* savannah type, *T. congolense* Tsavo type, *T. congolense* forest type, *T. congolense* Kilifi type (Majiwa et al., 1985; Majiwa et al., 1993).

T. simiae species are *polymorphic*, with a length of 15-19 μm. In typical cases some individuals are with and others without a free flagellum. The *Kinetoplast* is of medium size, marginal and subterminal, as in *T. congolense* (Hoare, 1972).

T. gogfreyi has been separated recently from *T. congolense* in the Gambia, on the basis of isoenzymatic and DNA differences, but also because the disease it causes is different. It is pathogenic for pigs, but the disease is more chronic than the one caused by *T. simiae*. Morphologically it is similar to *T. congolense*, with a length of 9-22 μm, but the undulating membrane is described as being usually conspicuous (Hoare, 1972).

3.1.2.2 The subgenus Duttonella

T. vivax was named because of the vigour of its activity under the microscope when examined in fresh preparations. The parasite moves rapidly across the field of view. *T. vivax* as seen in the blood of mammals is also essentially monomorphic, with a free flagellum. Its length, including the free flagellum, varies from 18 to 31 μm. The kinetoplast is large and terminal or almost so. It is much larger than in any of the other pathogenic species, and this is a distinguishing feature. The nucleus is centrally placed. The posterior extremity is swollen and blunt. The undulating membrane is inconspicuous. *T. uniforme* are small trypanosomes (from 12 to 20 μm), otherwise similar to *T. vivax* (Molyneux and Ashford, 1983).

3.1.2.3 The subgenus Trypanozoon

The subgenus Trypanozoon is the most homogeneous group of Salivarian trypanosomes, represented conventionally by species which are morphologically indistinguishable but differ in biological features. This group comprises five members: *T b. brucei, T. b. gambiense, T. b. rhodesiense, T. evansi* and *T. equiperdum*. *T. brucei* is polymorphic, with three main forms, all of which have a small kinetoplast and a conspicuous undulating membrane: (a) Long slender forms (23-30 μm in length) with a free flagellum, which may be up to one half of the length of the organism. The posterior end is pointed and the nucleus is central. The kinetoplast is placed up to 4 μm in front of the posterior extremity. (b) Short stumpy forms (17-22 μm in length) normally without a free flagellum, but in which there may occasionally be individuals with a short free flagellum. The kinetoplast is usually subterminal. The position of the nucleus varies greatly and it is in some cases in the posterior part of the cell, sometimes so far posterior that the kinetoplast is anterior to it (so-called postero-nuclear forms). (c) Intermediate forms, varying in length between the two previously mentioned types. A free flagellum, of varying length, is always present. The nucleus is centrally placed. The posterior

end is somewhat variable in shape, but usually bluntly pointed. The kinetoplast is close to the posterior extremity (Hoare, 1972).

During the course of the infection, there is a change in the trypanosome population from the long thin forms, through the intermediate, to the short stumpy forms, and this altered appearance is accompanied by a change in the type of respiration, as the trypanosome prepares for its period within the tsetse fly. The short stumpy forms are adapted to living and developing in the tsetse, while long thin forms are the true mature blood forms which die in the gut of the insect (Hoare, 1972).

T. evansi is typically represented almost exclusively by thin trypomastigotes comprising slender and intermediate forms corresponding to those in *T. brucei*. The slender forms have a long free flagellum and a narrow posterior extremity, which may be rounded or truncated, with the kinetoplast situated at some distance from the tip. The intermediate forms have a shorter free flagellum and a short, frequently pointed, posterior extremity, with the kinetoplast lying near this end (Hoare, 1972).

T. equiperdum is morphologically indistinguishable from *T. evansi*. Like the latter species, it is typically monomorphic, being represented by thin (slender and intermediate) trypomastigotes possessing a free flagellum. The size of *T. equiperdum* is likewise within the range of that of *T. evansi*. Furthermore, as in the case of *T. evansi*, it is liable to become pleomorphic, exhibiting typical and postero-nuclear stumpy forms (Hoare, 1972).

3.1.3 Host range

Cattle, sheep, goats, pigs, horses, camels, dogs, cats, and monkeys are susceptible to AAT and may suffer syndromes ranging from subclinical mild or chronic infection to acute fatal disease. Rats, mice, guinea pigs, and rabbits are susceptible and useful laboratory species (Molyneux and Ashford, 1983). More than 30 species of wild animals can be infected with pathogenic trypanosomes, and many of these remain carriers of the organisms. Ruminants are widely known to be active reservoirs of the trypanosomes. Wild equidae, lions, leopards, and wild pigs are all susceptible and can also serve as carriers of trypanosomes (Molyneux and Ashford, 1983).

3.1.4 Transmission and distribution

In Africa, the primary vector for *T. congolense*, *T. vivax*, and *T. brucei* is the tsetse fly. These trypanosomes replicate in the tsetse fly and are transmitted through tsetse fly saliva when the fly feeds on an animal. The three main species of tsetse flies for transmission of trypanosomes are *Glossina morsitans*, which favors the open woodland of the savanna; *G. palpalis*, which prefers the shaded habitat immediately adjacent to rivers and lakes; and *G. fusca*, which favors the high, dense forest areas. The distribution of the tsetse-transmitted African trypanosomes is governed by that of their tsetse vectors, which infest an area of sub-Saharan Africa that extends from the southern edge of the Sahara desert (lat. 15° N.) to Angola, Zimbabwe, and Mozambique (lat. 20° S.). Trypanosomosis is also mechanically transmitted by tsetse and other biting flies through the transfer of blood from one animal to another. The most important mechanical vectors are flies of the genus *Tabanus*, but *Haematopota, Liperosia, Stomoxys*, and *Chrysops* flies have also been implicated. In Africa, both *T. vivax* and *T. b. brucei* have spread beyond the "tsetse fly belts" (Roder et al., 1984), where transmission is principally by tabanid and hippoboscid flies. Of the three African animal trypanosomes, only *T. vivax* occurs in the Western Hemisphere in at least 10 countries in the Caribbean and South and Central America (Hoare, 1972).

3.1.5 Pathogenesis and clinical signs

Initial replication of trypanosomes happens at the site of inoculation in the skin; this causes a swelling and a sore (chancre). Trypanosomes then spread to the lymph nodes and blood and continue to replicate. *T. congolense* localizes in small blood vessels and capillaries. *T. brucei brucei* and *T. vivax* localize also in tissue. Antibodies developed to the glycoprotein coat of the trypanosome lyse the trypanosome and result in the development of immune complexes. Antibody, however, does not clear the infection, for the trypanosome has genes that can code for many different surface-coat glycoproteins and change its surface glycoprotein to evade the antibody. Thus, there is a persistent infection that results in a continuing cycle of trypanosome replication, antibody production, immune complex development, and changing surface-coat glycoproteins (Blood et al., 1989).

Immunologic lesions are significant in trypanosomosis, and it has been suggested that many of the lesions (e.g., anaemia and glomerulonephritis) in these diseases may be the result of the deposition of immune complexes that interfere with, or prevent, normal organ function. The most significant and complicating factor in the pathogenesis of trypanosomosis is the profound immunosuppression that occurs following infection by these parasites. This marked immunosuppression lowers the host's resistance to other infections and thus results in secondary disease, which greatly complicate both the clinical and pathological features of trypanosomosis (Blood et al., 1989).

Because simultaneous infections with more than one trypanosome species are very common (Nyeko et al., 1990), and simultaneous infection with trypanosomes and other hemoparasites (*Babesia* spp., *Theileria* spp., *Anaplasma* spp., and *Ehrlichia* spp.) frequently occurs, it is difficult to conclude which clinical signs are attributable to a given parasite. Few adequately controlled studies have been made, and thus a "typical" clinical response to each trypanosome is difficult to reconstruct.

The cardinal clinical sign observed in AAT is anaemia. Within a week of infection with the haematic trypanosomes (*T. congolense* and *T. vivax*) there is usually a pronounced decrease in packed cell volume (PCV), haemoglobin and red blood cells, and within 2 – 3 months the PCVs may drop to below 30 percent of their preinfection values. Also, invariably, present are intermittent fever, oedema and loss of condition. Abortion may be seen, and infertility of males and females may be a sequel. The severity of the clinical response is dependent on the species and the breed of affected animals and the dose and virulence of the infecting trypanosome. Stress, such as poor nutrition or concurrent disease, plays a prominent role in the disease process (Taylor and Authie, 2004).

Haemorrhagic *T. vivax* stocks have been isolated from East Africa. These stocks cause a hyperacute disease, characterised by high parasitaemia, severe anaemia and haemorrhages, which have been related to intravascular disseminated coagulation (DIC). Cattle may die within 2 weeks or, under favourable conditions, rapidly self-cure after 2 months (Taylor and Authie, 2004).

3.1.6 Epidemiology

The epidemiology of African Animal Trypanosomosis depends on three factors, which are the distribution of the vectors, the virulence of the parasite and the response of the host. When we are dealing with the tsetse-transmitted trypanosomosis, much depends on the distribution and the vectorial capacity of the *Glossina* species responsible for transmission. Of the three groups of *Glossina*, the savannah and riverine are the most important since they inhabit areas suitable for grazing and watering. Although the infection rate of *Glossina* with trypanosomes is usually low, ranging from 1 – 20% of the flies, each is infected for life, and their presence in any number makes the rearing of cattle, pigs and horses extremely difficult (Urquhart et al., 1987). Where savannah tsetse are the vectors, the risk of contracting the disease is widespread. When the riverine species are the culprits (in many parts of West and Central Africa), transmission occurs particularly along rivers with dense vegetation along the banks. The proportion of a tsetse population found infected with pathogenic trypanosomes depends not only on its vector capability, but also on the host on which it mainly feeds. For example, repitiles do not carry pathogenic trypanosomes, and there are also major differences between suids and bovids, as the former will infect the flies particularly with *T. simiae* and *T. godfreyi*, while bovids are mainly the source of *T. vivax* and *T. congolense* (Uilenberg, 1998). Biting flies may act as mechanical vectors, but their significance in Africa is still undefined. However, in Central and South America, *T. vivax* is thought to be transmitted readily by such flies (Urquhart et al., 1987).

The parasite virulence, immunogenecity and response to chemotherapeutics are also important factors in the epidemiology of trypanosomosis as the trypanosome species occur in a remarkable variety of genotypes. Since parasitaemic animals commonly survive for prolonged periods, there are ample opportunities for fly transmission, especially of *T. brucei* and *T. congolense*. In contrast, some strains of *T. vivax* in cattle and *T. simiae* in domestic pigs kill their hosts within 1 – 2 weeks, so that the chances of fly infection are more limited (Urquhart et al., 1987).

Species and breed susceptibility are also important in the epidemiology of trypanosomosis as animal hosts differ in their response to trypanosome infection, depending on the species and breed of the individual animals. The level of animal husbandry practices, nutritional status, workload and stress exacerbate the severity of the disease (Urquhart et al., 1987; Uilenberg,

1998). The fact that the parasite affects not only cattle but also wild animals which constitute the reservoirs of the disease, makes the epidemiology of animal trypanosomosis extremely complicated (Hoare, 1972; Uilenberg, 1998).

3.2 Molecular biology of trypanosomes

Trypanosomes exhibit considerable intra-species genetic diversity and variation which can be defined at the level of both the genome and of individual genes (Myler, 1993). The nuclear genome shows considerable inter- and intra-species plasticity in terms of chromosome number and size whereas, the mitochondria (kinetoplast DNA) genome varies considerably in terms of minicircle size and organisation, and within the variable region (VR) of maxicircles.

3.2.1 Nucleus

Trypanosomes have a genome size estimated to be 3-4 x 10^7 bp (Borst et al., 1980), and the DNA is organized into multiple, linear chromosomes, ranging in size from 25 kbp to several Mbp (Van der Ploeg et al., 1984b). The DNA content has been shown to vary among species, subspecies and strains (Dero et al., 1987; Dvorak et al., 1982). The use of PFGE (Schwartz and Cantor, 1984) has enabled understanding of protozoan genome organisation, which was hampered because of the absence of chromosome condensation during metaphase. Also it has shown that trypanosome chromosomes are small in comparison to higher eukaryotes.

All trypanosome species possess a set of large chromosomes (0.7 to 6 Mbp) that contain alleles of the diploid housekeeping genes, which are somehow difficult to resolve by PFGE. In *T. brucei*, the large chromosomes occur as 8-10 homologous pairs (Gottesdiener et al., 1990). A similar number of large chromosomes appear to be present in other trypanosomes (Aymerich and Goldenberg, 1989; Van der Ploeg et al., 1984b). It was revealed that the same gene may be present on chromosomes of widely varying sizes in different stocks (Engman et al., 1987), and that genes linked on the same chromosome in one stock may be not linked in others (Gibson and Garside, 1991).

The genomes of African trypanosomes contain variable numbers of minichromosomes (25-150 kbp), which vary widely between species and even within species (Van der Ploeg et al.,

1984b). *T. congolense*, *T. brucei* and *T.b. rhodesiense* contain about 100-120, *T.b. gambiense* contains about 10, *T. equiperdum* contains one and *T. vivax* and *T. cruzi* contain none (Gibson and Borst, 1986; Van der Ploeg et al., 1984b; Majiwa et al., 1985). The absence of minichromosomes suggests that they may represent an adaptation to the molecular mechanisms used for antigenic variations in African trypanosomes (Gibson and Miles, 1986; Engman et al., 1987; Gibson and Garside, 1991). Physical mapping of these chromosomes and analysis of the majority of their DNA nucleotide sequence showed that they are linear molecules with telomere repeats at their ends and that they lack abnormal structures such as loops, knots or cruciforms. They have most likely replicated from a single bi-directional origin of replication located in the centre of the minichromosome (Myler, 1993).

In addition to minichromosomes and the large chromosomes, which contain housekeeping genes, most trypanosome species also contain intermediate-sized chromosomes that vary in number and size (150-700 kb) among stocks of the same parasite species (Jasmer et al., 1987).

3.2.2 Kinetoplast DNA

All trypanosomes are characterized by the presence of a kDNA network within mitochondrion, which represents the mitochondrial genome that accounts for 10-20% of the total cell DNA (Stuart, 1983; Hajduk et al., 1992). Kinetoplast DNA is organized in a complex network of maxicircles and minicircles.

3.2.2.1 Maxicircles

Maxicircles of all trypanomatids contain a number of genes, which code for components and enzymes that are involved in the electron transport system, which generates energy. The size of maxicircle varies between species of trypanomastids due to differences in the genomic sequence for the edited genes (Read et al., 1993). Basically, maxicircles consist of two distinct regions: the gene-coding region and the variable region (VR). However, the gene-coding regions of maxicircles from different sub-species, species and genera, are generally well conserved. The substantial difference occurs in the VR that varies in length and gene sequence content between species and isolates (Borst et al., 1981; Myler et al., 1993).

3.2.2.2 Minicircles

The sizes of minicircles vary among species while they are constant within species, despite the heterogeneous sequence they display even in the same organism (Stuart, 1983). Minicircles consist of two regions: a small conserved region, which is maintained in all minicircles of each species, and a large non-conserved region, which varies between minicircles. The function of minicircles is to encode guide (g) RNAs needed for RNA editing (Pollard and Hajduk, 1991; Stuart et al., 1992). The high copy number of the minicircle DNA facilitates restriction fragment length polymorphism (RFLP) analysis of the different sequence classes and has lead to the development of the term schizodeme[3] (Morel and Simpson, 1980; Morel et al., 1980).

3.3 Diagnosis

The primary reason for diagnosis of animal trypanosomoses is for the appropriate application of therapeutic and prophylactic measures. Other reasons for diagnosis include the need to target and monitor tsetse control or eradication operations, investigations into the efficacy of chemotherapy and particularly trypanocidal drug resistance, and pathophysiological, epidemiological and socio-economic studies.

The type of diagnostic test used in the detection of infections caused by the animal trypanosomoses will vary according to the epidemiological characteristics of the disease and the strategy for control. Where tsetse-transmitted trypanosomoses occur and where disease prevalence is high, even tests of low diagnostic sensitivity will suffice if chemotherapy or chemoprophylaxis is administered on a herd basis. However, in many situations where mechanically transmitted trypanosomosis is found, drugs are often administered therapeutically to individual infected animals and it is essential that more sensitive diagnostic tests be used in order to detect active infections.

Similar considerations also apply after control campaigns. As the disease prevalence declines, the need for individual treatment as opposed to block treatment becomes an important issue. When chemotherapy has been applied in areas where drug resistance is known to exist, it is also necessary to detect rapidly any failure in treatment.

[3] All trypanosome populations having similar DNA restriction patterns (WHO, 1998).

3.3.1 Clinical diagnosis

Clinical signs of acute bovine trypanosomosis include anaemia, weight loss, roughness of the hair coat, enlargement of peripheral lymph nodes, pyrexia, abortion, reduced milk yield and, in the absence of treatment, death. Hence, trypanosomosis should be suspected when an animal in an endemic area is febrile, anaemic and in poor condition (Blood et al., 1989). The clinical picture depends to some extent on the species of infecting trypanosomes and the susceptibility of the bovine host. Diagnosis of the disease based on clinical manifestations is complicated due to the fact that the disease may have acute, chronic or sub-clinical forms. Confirmation depends on the demonstration of the organism in blood or lymph node smears using the parasitological methods available.

3.3.2 Parasitological diagnosis

The conventional techniques of microscopic examination for the presence of trypanosomes are still widely used, but newer and far more sensitive methods are beginning to supplant them.

3.3.2.1 Blood films

In the early phases of infection, especially with *T. vivax* and *T. congolense*, the parasite can readily be observed by microscopic examination of a wet-mount of blood slides. This method is relatively less sensitive, with a detection limit of about 8.3 x 10^3 trypanosomes per ml of blood (Paris et al., 1982). A drop of fresh blood is taken from the ear vein of an animal with a microscope slide and covered with a glass slip. This preparation is examined microscopically with light or phase contrast microscope at 400x magnification. From the relative size and movement pattern of the parasite, it is possible to guess on the species of the parasites involved, to be confirmed by stained preparation.

Thin and thick blood smears fixed in methanol or acetone and stained with Giemsa may be used in the laboratory to detect blood parasites and determine the trypanosome species involved, respectively. Stained lymph node smears are a very good method for diagnosis,

especially for *T. vivax* and *T. b. brucei*. In chronic *T. congolense* infection, the parasites localize in the microcirculation of the lymph nodes and in other capillary beds, allowing diagnosis by examination of lymph node smears or smears made with blood collected from the ear. Early in infection, blood smears are optimal for the demonstration of *T. congolense*. These techniques are not sensitive enough to detect low parasite levels, characteristic of the disease in large animals at the chronic stage (Woo, 1970; Murray et al., 1977), and as a result several techniques for the concentration of blood trypanosomes have been developed, which increase the chance of trypanosome detection.

3.3.2.2 Concentration techniques

Woo (1970) described a practical method using centrifugation of microhaematocrit capillary tubes containing the blood sample and examination of the buffy coat/plasma junction under the microscope. This method, known as the haematocrit centrifugation technique (HCT), was subsequently improved in the buffy coat technique (BCT) by cutting the capillary tube, expressing the buffy coat/plasma interface on a microscope slide and using dark-ground or phase contrast illumination (Murray et al., 1977). The advantages of these two methods are that diagnostic sensitivity is increased due to a concentration of parasites following centrifugation, and that at the same time the packed red cell volume (PCV) can be determined as a measure of anaemia. Paris et al. (1982) compared the effectiveness of some of these newer diagnostic methods with the traditional blood films and found the BCT to be the most sensitive technique, followed in order of decreasing sensitivity by the HCT, Giemsa-stained thick film, Giemsa-stained thin film and wet blood film. The analytic sensitivity of the BCT depended on the species of trypanosome, with the smallest numbers detectable per millilitre of blood being 2.5×10^2, 5×10^2 and 5×10^3, for *T. congolense*, *T. vivax* and *T. brucei*, respectively. On the other hand, the HCT proved to be the most sensitive microscopic technique to detect *T. brucei* in bovine blood.

Another method available that is not in common use as a field diagnostic technique is the miniature-anion exchange centrifugation technique (m-AECT) described by Lumsden et al. (1979). This method uses miniature anion-exchange columns for the separation of trypanosomes from erythrocytes prior to concentration by centrifugation.

3.3.3 DNA-based techniques

The diagnosis of trypanosomes has been improved, since the 1980s, by DNA-based techniques. DNA diagnosis is either based on hydridization profiles of parasite DNA with DNA probes or polymerase chain reaction (PCR) technology.

A DNA-probe is a known DNA sequence which can be obtained by cloning, or by PCR with labelled nucleotides (enzymes or isotopes). DNA probing entails exposing a denatured DNA sample fixed on nitro-cellulose to a labelled DNA-probe under specific salt and temperature conditions. If the complementary DNA sequence is present in the sample, the probes will bind to it and remain on the nitro-cellulose where they can be visualised (Desquesnes and Dàvila, 2002). Probes have been developed for the main pathogenic trypanosomes (Masiga and Gibson, 1990), but the sensitivity of this technique is limited to 100 parasites (Masiga et al., 1992), which is not sufficient for trypanosome detection in mouthparts of the vectors or in host blood when the parasitaemia is low.

The polymerase chain reaction (PCR) is an *in vitro* technique which allows the amplification of a specific deoxyribonucleic acid (DNA) region that lies between two regions of a known DNA sequence. The technique exploits a thermostable enzyme, *Taq* polymerase, which synthesises a new strand of DNA by copying an original DNA template (Mullis et al., 1986). PCR was originally developed to amplify sequences of interest and to increase the sensitivity of detection using DNA-probes (Saiki et al., 1986). However, since the DNA-probe technique is laborious and time consuming (Desquesnes and Dàvila, 2002), in recent years the development of PCR alone has been mainly devoted to diagnosis without the need of probes.

PCR amplification of DNA is achieved by using oligonucleotide primers. These are s
hort, single-stranded DNA molecules which are complementary to the ends of a defined sequence of DNA template. The primers are extended on a single-stranded denatured DNA template by a DNA polymerase, in the presence of deoxynucleoside triphosphates (dNTPs) under suitable reaction conditions. This results in the synthesis of a new strand complementary to the template strands. Following 30 – 40 cycles, the DNA will have been amplified several million times, and the resulting PCR product can be visualised on an agarose or polyacrylamide gel, after staining with ethidium bromide and exposing it under

ultraviolate light. The specific size of the PCR product is evaluated by simultaneous migration of molecular size markers and a positive control. A negative control is run together to evidence any DNA contamination. The actual sequence amplified can be analysed by sequencing. Generally, once the specificity of the primers has been established, the size of the PCR product is sufficiently characteristic for diagnostic purposes (Newton and Graham, 1997; Desquesnes and Dàvila, 2002). The first developments of PCR for trypanosome identification were published in Moser et al. (1989a,b).

3.3.3.1 Dignostic targets of PCR

In most cases, PCR diagnosis aims to identify the parasite at the species level, which can be done using various targets. The preferred targets are those which are present in a high copy number in the genome of trypanosomatids; the more copies of the target, the greater the chances of amplifying it by PCR. Single copy genes are more difficult to amplify (MacLeod et al., 1997) and are rarely targeted since low parasitaemia is a characteristic of trypanosome infection and the sensitivity would be too low (Desquesnes and Dàvila, 2002).

Mini-chromosomes of the nuclear DNA contain satellite DNA which has been the most favoured target in the development of species-specific primers able to detect very small amounts of parasite DNA. Such primers were developed for the main pathogenic trypanosomes: *Trypanozoon* (Moser et al., 1989a; Masiga et al., 1992), *T. vivax* (Masiga et al., 1992 and *T. evansi* (Artama et al., 1992). Other repetitive sequences have also been investigated, for example, in the detection of *T. vivax* DNA (Masake et al., 1997; Clausen et al., 1998, Morlais et al., 2001).

Because of the diversity of *Trypanosoma* species potentially present in a single host, PCR diagnosis carried out on host material requires several PCR reactions; for example, in cattle, up to five reactions per sample may be required. Research is now focusing on a diagnosis based on the amplification of the internal transcribed spacer-1 (ITS-1) of ribosomal DNA which presents the advantages of being a multi-copy locus (100–200), having a small size (300–800 bp), which varies from one taxon to another but is conserved in size in a given taxon. This may lead to the development of a multi-species-specific diagnostic protocol using a single PCR (Desquesnes and Dàvila, 2002). The limitation of this technique is that the sensitivity of the primers is lower than that of satellite DNA primers, due to partial homology

of the sequences and their limited repetitiveness. ITS1 sequences are only repeated 100-200 times in comparison with 10, 000-20,000 times for satellite DNA. Consequently, the design of new primers is required to reach 100% homology with all T*rypanosoma* species of veterinary interest. A recent study by Geysen et al. (2003) demonstrated that a single polymerase chain reaction (PCR)- restriction fragment length polymorphism (RFLP) assay can be used to characterise all important bovine trypanosome species. This was the first report of a sensitive pan-trypanosome PCR assay amplifying all species including *T. vivax* to a comparable extent, using a single primer pair. Restriction enzyme analysis using *Msp*1 and *Eco*571 gave a clear destinction between *T. congolense*, *T. brucei*, *T. vivax* and *T. theileri*. Several subgroups within the *T. congolense* group could be distinguished using this method, although no difference could be detected between the species belonging to the subgenus *Trypanozoon*. Delespaux et al. (2003) further evaluated this method using mixed infections with *T. b. brucei, T. theileri*, *T. congolense* and *T. vivax*. The results showed that all mixed infections gave clear profiles that could be easily differentiated except in the case of *T. theileri* and *T. congolense* mixed infections where the *T. theileri* band was concealed by the *T. congolense* profile. By reducing the cost of the PCR diagnosis, this technique would allow a greater number of field samples to be tested in epidemiological studies and/or would increase the variety of *Trypanosoma* species that could be detected. Further investigations are required to develop and optimise multi-species-specific diagnostic tools for trypanosomes, which could also serve as a model for such tools in other pathogens.

3.3.3.2 Specificity and sensitivity of PCR diagnosis and typing

Several levels of specificity have been achieved from sub-genus to species, sub-species and even types. Random priming of trypanosome DNA has even allowed "isolate specific" identification. Other work based on microsatellite sequences has provided markers for population genetic studies. Similarly, PCR has allowed an increase in the specificity of diagnosis in vectors such as tsetse flies. Direct determination of the trypanosome species-type in the vector or host is likely to provide a more reliable estimate of parasite prevalence since no selection is exerted on the ability of the parasite to grow either in culture or in animals (Majiwa et al., 1993).

Many natural trypanosome infections of either tsetse or livestock are due to more than one trypanosome species (Nyeko et al., 1990). Precise evaluation of such infections by PCR

requires that the oligonucleotide primers for PCR retain their specificities under standard assay conditions. Species-specific DNA probes have been shown to detect simultaneous infection of cattle with *T. vivax*, *T. b. brucei*, and *T. congolense* when conventional methods revealed only single infections (Nyeko et al., 1990). Clausen et al. (1999) indicated that PCR coupled with DNA probe hybridisation could prove to be a highly sensitive tool for the diagnosis and assessment of the therapeutic efficacy and disease progress especially in chronic trypanosomosis.

Individual hosts often harbour more than one parasite clone. It was previously thought that certain *Nannomonas* species were limited to particular ecological niches but it has been proved that this is not the case, as revealed when these probes are used in epidemiological studies (McNamara et al., 1995). *T. (N.) congolense* comprises morphologically identical but genotypically heterogeneous trypanosomes that express different phenotypes in terms of vector and host specificities, and disease symptoms (Godfrey, 1982; Young and Godfrey, 1983; Majiwa et al., 1986). Four genotypic groups of *T. (N.) congolense* have been recognised. They are designated Savannah-type *T. (N.) congolense*, West African forest/riverine-type *T. (N.) congolense*, Kilifi-type *T. (N.) congolense* and the Tsavo-type *T. (N.) congolense* (Young and Godfrey, 1983; Majiwa et al., 1985; Majiwa et al., 1993). Repetitive, tandomly arranged DNA sequences and oligonucleotide primers, specific for each of these types, for use in polymerase chain reaction (PCR) amplification, have been described (Majiwa et al., 1985; Kimmel et al., 1987; Gibson, et al., 1988; Dickin and Gibson, 1989; Majiwa et al., 1993). The availability of such reagents for rapid, specific and highly sensitive detection and identification of the different types of trypanosomes will ultimately enable a comprehensive evaluation of their role in the epidemiology of the disease they cause (Petterson and Hyypia, 1985; Viscindi and Yolken, 1987; Hide and Tait, 1991).

Applied to the routine detection of *Trypanosoma* infections in animals, PCR provides a highly specific diagnosis. So far, the most used primer sets for trypanosomes have never failed in terms of their specificity (Desquesnes and Dàvila, 2002). However, species-specific primers have not yet been described for some trypanosome species such as *T. equiperdum*. Moreover, although it is possible to distinguish *T. b. rhodesiense* from *T. b. brucei* using three mini-satelite markers (McLeod et al., 2000) or using serum-resistance-associated gene (Welburn et al., 2001), couples of sub-species-specific primers are not yet available (Desquesnes and Dàvila, 2002).

With regards to the sensitivity, PCR allows the detection of a single specific sequence of DNA; consequently, a single parasite (0.1 pg of DNA) or even fewer can be detected when using satellite DNA (Desquesnes and Dàvila, 2002). This very sensitivity also brings a very high risk of false positive results since a very small amount of other biological materials can contaminate the sample. The sensitivity threshold of the trypanosome detection by PCR generally ranges from 1 to 20 parasite/ml of blood, depending on the technique used. Below this level of parasitaemia, PCR cannot detect the infection. It is not a problem of sensitivity; it is only due to the fact that there is no DNA in the sample investigated when the parasitaemia is so low (Desquesnes and Dàvila, 2002). To increase the sensitivity, it should then be necessary to increase the volume of the samples processed and the concentration through the parasitological techniques described.

In all cases, false negative results (in samples where substantial amount the specific DNA is present) can occur due to the presence of inhibiting factors in a sample (haemoglobin, EDTA, etc.), or DNAases introduced into the sample, which provoke destruction of the DNA, and then the specific sequence under investigation (Desquesnes and Dàvila, 2002). There is still a need to design primers based on repetitive sequences for a sensitive detection and specific identification of *T. b. brucei*, *T. b. rhodesiense* and *T. b. gambiense* for epidemiological studies in vectors, domestic and wild reservoirs of human sleeping sickness.

3.4 Control of African Animal Trypanosomosis

Prevention and control of tsetse-transmitted trypanosomosis depends on methods directed to the vectors, the host and the parasites. Each of theses approaches is useful but has important limitations, such as expense, environmental pollution and drug resistance.

3.4.1 Vector control

Several approaches to fly control have been used with varying degrees of success. Many methods widely used for tsetse control in the past have ceased to be used in the last 10-20 years, either because they were ineffective, or because they have become environmentally unacceptable (Uilenberg, 1998). Discriminative bush clearing, extensively used in early tsetse fly eradication campaigns, has been locally useful because it eliminates the breeding places of the tsetse. But, to be completely effective, bush clearing requires destruction of vast areas of

bush and forest, which is ecologically unacceptable. It is still a useful procedure when used locally in conjunction with other control methods (Uilenberg, 1998). Game elimination, and thus elimination of the main source of bloodmeals for the tsetse, was used in early eradication campaigns. This was an ineffective and wasteful procedure. Today, the method has been abandoned, to a large extent on environmental and ethical grounds (Uilenberg, 1998).

Ground and aerial spraying with insecticides and the use of synthetic pyrethroids on cattle have lowered fly densities in some areas, but widespread use would require considerable international cooperation and expense. Widespread application of insecticides has the tremendous disadvantage of also eradicating many other arthropods, several of which are desirable (Uilenberg, 1998). Although few long-term environmental effects of aerial spraying of insecticides have been shown (Douthwaite, 1986; Lambert, 1993), alternatives to the wide-scale application of insecticides are preferred, and the technique is no longer commonly used. Aerial spraying was formerly used to apply non-residual, contact insecticides, particularly endosulfan, by sequential aerial spraying using Ultra-Low-Volume (ULV) techniques (Leak, 1996).

There has been a substantial amount of research for more environmentally acceptable techniques because the widespread use of persistent insecticides or drastic habitat modification is no longer acceptable (Linear, 1985). These include the use of insecticide-impregnated traps or targets (Vale et al., 1988a) and non-impregnated traps (Brightwell et al., 1991) and, more recently, the use of 'pour-on' insecticides applied to cattle. Whilst still depending upon insecticides, the quantities used are much smaller, and the types used are predominantly synthetic pyrethroids with high toxicity for *Glossina* spp. (Zerba, 1988) but low mammalian toxicity. Furthermore, they are not widely distributed in the environment but are much more closely directed at the specific target organism.

Application of the sterile male technique (as used in screwworm eradication in the United States) received considerable attention in the 1980's. Large numbers of male flies, usually sterilised by irradiation, are released and compete with wild males to mate with female flies (Knipling, 1959). Early problems with breeding of the male flies have been overcome, and field trials have been done in both East and West Africa to determine the effectiveness of this approach in vector control. In limited trials, this procedure has reduced fly populations. It has been used successfully in Burkina Faso, Tanzania, Nigeria and, most recently, in Zanzibar

where it eradicated *Glossina austeni* from the 1600 km^2 Unguja Island (Feldmann and Hendrichs, 1998). This method of control is costly, technically demanding and only suitable for relatively small, isolated areas of tsetse infestation (Leak, 1996).

3.4.2 Trypanotolerant cattle breeds

It has long been recognized that certain breeds of West African cattle are considerably more resistant to African trypanosomiasis than others. This is especially true of the West African short-horned cattle (Muturu, Baoule, Laguna, Samba, and Dahomey) and the long-horned cattle (N'Dama). These cattle have existed in the region for over 5,000 years. Susceptibility studies have shown the N'Dama to be the most resistant breed followed by the smaller west African short-horned cattle, but the large and more recently introduced Zebu is the most susceptible (Murray et al., 1979). The mechanisms of trypanotolerance have been extensively studied, and it is now well established that trypanotolerance has a genetic basis (Moulton and Sollod, 1976; Murray et al., 1984). Trypanotolerance in sheep and goats has also been described, but the mechanisms of the tolerance phenomenon have not been defined. It is also known at present that there are differences in susceptibility to trypanosomosis between various East African breeds of cattle and small ruminants. One of the limitations to the use of West African trypanotolerant cattle breeds is their small size; still, genetic studies might lead in the future to the transfer of the trypanotolerance trait to more productive breeds (Uilenberg, 1998).

3.4.3 Vaccination

Enormous advances have been made in the area of immunology in the recent years, but in the case of protozoa in general, some of which ´inhabit the very arsenals of the immune response´, and trypanosomes in particular, many problems remain unsolved. The almost unlimited antigenic variation during infection by one single strain of trypanosome and the antigenic strain diversity within each of the several trypanosome species and types are the main obstacles preventing vaccine development. Some attempts are still being made at vaccine development using internal non-variable antigens or at immunizing against proteins causing pathogenic effects, instead of against the parasite itself. Targeting internal non-variable antigen has the problem that such antigens are out of reach of the host antibodies as long as the trypanosomes are alive and intact (Uilenberg, 1998). Therefore, only little

progress has been made in the development of a vaccine against trypanosomes and no effective vaccine is likely to be marketed in the near future (Geerts and Holmes, 1998).

3.4.4 Chemotherapy and chemoprophylaxis

The application of antitrypanosomal drugs has been the most widely practised means of controlling trypanosomosis in domestic livestock since the early 1950s, either as curative or prophylactic drugs. A programme to eradicate tsetse flies from some 9 million km^2 of Africa is highly ambitious. It will be complex, take many years and possibly cost some US$ 20 billion (Budd, 1999). Thus, control of trypanosomosis will depend in the forseable future on the use of the existing trypanocidal drugs. The challenge, therefore, remains to make optimal use of the three relatively old compounds until new methods of treatment emerge, possibly through unanticipated cross-reactivity with new broad-spectrum anti-protozoal compounds such as those currently being developed for the treatment of malaria and cryptosporidiosis (Holmes et al., 2004)

Despite the fact that chemotherapy is the major means of disease control, development of new antitrypanosomal drugs has been more or less static over the last three decades, due to lack of interest by the pharmaceutical industry to invest into research and development of antitrypanosomal drugs (Gutteridge, 1985). Consequently, this has been a major stimulus for intensive research into the few existing drugs; and in the recent past, considerable body of knowledge has emerged on a number of important aspects, such as drug disposition, mechanisms of action, resistance and toxicity.

The three antitrypanosomal compounds upon which treatment and prophylaxis of cattle trypanosomosis currently depends are isometamidium chloride, homidium chloride or bromide and diminazene aceturate. Whereas, quinapyramine, suramine and melarsomine are primarily used as therapeutic drugs for infections caused by *T. evansi* in equidae, camels and buffaloes, although quinapyramine is also used for prophylactic purpose (Williamson, 1970).

3.4.4.1 Diminazene aceturate

3.4.4.1.1 Chemistry

Diminazene is an aromatic diamidine derived from Surfen (Jensch, 1958). The molecule is marketed as the diaceturate salt and consists of two amidinophenyl moieties linked by a triazene bridge: p,p-diamidinodiazoaminobenzene diaceturate tetrahydrate; N-1,3-diamidinophenyltriazene diaceturate tetrahydrate. In aqueous solution the compound is stable for 2-3 days (Fairclough, 1962). Thus, because of this short duration of stability, diminazene is marketed in combination with the stabilizer phenyldimethyl pyrazolone (antipyrine). Solutions of the preparations can be used without loss of activity for up to 10-15 days when stored at room temperature (Fairclough, 1962).

3.4.4.1.2 Antitrypanosomal activity

The compound was introduced onto the market as a trypanocide and babesiacide for domestic livestock in 1955 (Jensch, 1955). Following its use in the field, it was concluded that intramuscular (i.m.) treatment with diminazene aceturate at a dose of 3.5-mg/kg body weight (b.w.) eliminated *T. congolense* and *T. vivax* infections in cattle. However, infections with *T. brucei* required a dose of 5-mg/kg b.w. (Fussgänger and Bauer, 1958).

Diminazene aceturate is currently marketed under the trade names Azidine®, Berenil®, Ganaseg®, Ganasegur® and Veriben® as both a trypanocide and babesiacide for domestic livestock. Diminazene aceturate is recommended only for use as a therapeutic agent since it is rapidly excreted and therefore thought to have little prophylactic activity (Fussgänger and Bauer, 1958). For all animals the general i.m. dose is 3.5 mg/kg bw. However, twice this amount is recommended for *T. brucei* infections (Hoechst Veterinär, Germany). Standard therapeutic doses of diminazene aceturate (3.5 mg/kg bw) rarely results in signs of toxicity in domestic animals. In camels, however, a single dose of 7.0 mg/kg bw can be highly toxic (Leach, 1961). Diminazene is also relatively toxic in dogs (Losos and Crockett, 1969).

3.4.4.1.3 Mechanism of action

Diminazene binds to trypanosomal kinetoplast DNA (Newton, 1972; MacAdam and Williamson, 1972). This binding does not occur by intercalation (Newton, 1972) but via specific interaction with sites rich in adenine-thymine (A-T) base pairs (Newton, 1972; Brack and Delain, 1975). Non-intercalative binding of diminazene to DNA, with strong affinity for A-T base-pair regions, has similarly been demonstrated *in vitro*, using DNA obtained from various sources (Lane et al., 1991). Such studies have shown that the molecule binds with higher affinity to 5'-AATT-3' than to 5'-TTAA-3' regions of DNA (Hu et al., 1992). Through this specific interaction in trypanosomes, diminazene inhibits synthesis of RNA primers, resulting in accumulation of replicating intermediates, thereby inhibiting kDNA replication (Newton, 1972; Brack and Delain, 1975). In other work, Shapiro and Englund (1990) have shown that diminazene specifically inhibits mitochondrial type II topoisomerase in viable trypanosomes. Thus, inhibition of DNA replication may also occur via this intercalation. Although diminazene probably exerts its action at the level of the DNA, this has not been proven *in vivo* and other mechanisms of action cannot be excluded (Peregrine and Mamman, 1993).

3.4.4.2 Isometamidium and homidium

3.4.4.2.1 Chemistry

Isometamidium chloride (Samorin®), marketed since 1961 as a prophylactic and therapeutic drug (Berg, 1961), and homidium (chloride salt; Novidium®; bromide salt or ethidium bromide: Ethidium ®), marketed since 1952 (Watkins and Woolfe, 1952), are phenanthridinium compounds, whose antitrypanosomal activity was demonstrated more than 60 years ago (Browning et al., 1938).

Isometamidium differs from homidium by an additional moiety of *m*-amidinophenyl-*azo*-amine (Wragg et al., 1958), which in fact is part of the diminazene molecule. Isometamidium can thus be seen as a 'hybrid molecule' which exhibits some of the properties of homidium and diminazene. Moreover, the drug product of isometamidium as marketed contains 70% isometamidium, and the remaining fraction (30%) is a mixture of its two isomers, a small proportion of a bis-compound and homidium (RMB Animal Health).

3.4.4.2.2 Antitrypanosomal activity

Both isometamidium and homidium are active against *T. congolense* and *T. vivax*. Additionally, isometamidium is also of value against infections caused by *T. brucei* and *T. evansi* infections in donkeys, horses and camels. Some studies on *T. evansi* and *T. equiperdum* have shown that the minimum effective dose of isometamidium which killed trypanosome population by 100% within 24 h of drug exposure (MED_{100}) was 1-4 µg/ml (Zhang et al., 1991), and with 96 h drug exposure, 1-300 ng/ml (Brun and Lun, 1994). In the dose range recommended for prophylactic purposes (0.5-1.0 mg kg^{-1} bw), the compound has been used successfully to maintain the productivity of Zebu cattle exposed to tsetse challenge in both village and ranch management systems in East Africa (Trail et al., 1985; Moloo et al, 1987). However, considerable variation in prophylactic activity has been observed in that a dose of 1.0 mg kg^{-1}bw has been shown to confer prophylaxis to cattle for 2-22 weeks (Kirkby, 1964; Pinder and Authié, 1984; Whitelaw et al., 1986; Peregrine et al., 1991). Such variation in prophylactic activity appears to be independent of both the level of trypanosome challenge and the presence or absence of infection at the time of treatment (Peregrine et al., 1988). Variation in drug susceptibility between different trypanosome populations appears to be the major factor determining the duration of prophylaxis.

Homidium was extensively used in the 1960s and 1970s but its usefulness has been greatly reduced due to widespread resistance (Scott and Pegram, 1974). Over the years, it has remained essentially a curative drug in the field, despite claims that the drug has some prophylactic activity, varying from 2 to 19 weeks against field challenge (Leach et al., 1955; Dolan et al., 1990). De Deken et al. (1989) have reported success in protecting rabbits for more than 300 days against seven challenges of *T. congolense* using a slow release device implanted subcutaneously. This finding suggests that the commonly used drug products, Novidium® or Ethidium,® lack prolonged prophylactic activity because the active principle, homidium is rapidly eliminated from the body. Should this slow release device technology prove cost-effective in the field, it will undoubtedly be widely accepted in many areas with a yearlong high incidence of trypanosomosis.

3.4.4.2.3 Mechanism of action

The primary mode of action currently considered to account for the molecular mechanisms of antitrypanosomal activity of phenanthridinium drugs is blockade of nucleic acid synthesis through intercalation between DNA base pairs (Wagner, 1971), inhibition of RNA polymerase (Richardson, 1973), DNA polymerase (Marcus et al., 1982) and incorporation of nucleic acid precursors into DNA and RNA (Lantz and Van Dyke, 1972). Other biochemical reactions that may account partly to their effects include modulation of glycoprotein biosynthesis (Casero et al., 1982), lipid metabolism (Dixon et al., 1971), membrane transport (Girgis-Takla and James, 1974) and selective cleavage of kinetoplast DNA minicircles (Shapiro and Englung, 1990). The mechanism that is considered primary, that is, blockade of nucleic acid synthesis, does not explain the basis of their selective toxicity. However, there are a number of biochemical peculiarities that have been demonstrated in trypanosomes that appear to be candidate targets for drug modulation and that might explain the basis of selective toxicity (Opperdoes, 1985).

3.5 Drug resistance in trypanosomes

Drug resistance, also called drug fastness, may be defined as a loss of sensitivity by a strain of an organism to a compound to which it had previously been susceptible. It implies failure of treatment and prevention, and if no other active drugs are available the animal has to rely on its immune defences alone to combat the disease (Uilenberg, 1998).

Until recently, diminazene aceturate and isometamidium chloride were considered the best therapeutic and prophylactic trypanocides, respectively. The former was reputed as the only to which trypanosomes do not easily develop resistance because of its rapid elimination from the system when compared with the most persistent prophylactic drugs such as isometamidium (Aliu et al., 1984; Rushigajiki et al., 1986). Unfortunately, this view is no longer accepted, as trypanocide resistance has been demonstrated conclusively under laboratory conditions by inoculation of trypanosome stocks into bovines and treating with correct drug dosage regimens, or by administering prophylactic drug dosages and then challenging with tsetse, infected with well-characterized trypanosome populations at regular intervals (Peregrine et al., 1991; Peregrine, 1994). Field and laboratory stocks of diminazene resistant trypanosomes have been reported, some field isolates requiring up to 45 mg/kg diminazene aceturate as the

minimum required dose to achieve cure (Chitamo and Arakawa, 1992; Peregrine and Mamman, 1993). Similarly, isometamidium treatment failures and shortened prophylactic intervals have been attributed to infections with drug-resistant trypanosome species (Sutherland et al., 1991; Peregrine et al, 1991). Widespread development of resistance by trypanosomes to homidium, another trypanocidal that was extensively used as a prophylactic drug, has been reported from East and West Africa (Clausen et al., 1992; Codjia et al., 1993; Mulugeta et al., 1997).

Drugs used in animal disease are generally subject to lower standards of quality control than those used in human disease. Multiple generic variants of the drugs, including isometamidium and diminazene, are available and the variability in quality of these products is striking. The release of preparations that contain low quantities of active drug provokes ideal conditions for the selection of drug resistance as well as leading directly to therapeutic failure (Barrett et al., 2004). This problem of drug resistance in trypanosomes appears to be spreading geographically to many regions in which trypanosomosis occurs. So far, resistance to one or more of the three trypanocidal drugs used in cattle has been reported in at least 13 countries in sub-Saharan Africa (Geerts and Holmes, 1998; Table 1). More worrying, however, are the reported incidences of field stocks that have developed multiple resistances to these trypanocidal drugs (Leach and Roberts, 1981; Moloo and Kutuza, 1990; Ainanshe et al., 1992; Clausen et al., 1992; Mohamed-Ahmed et al., 1992; Codjia et al., 1993; Mulugeta et al., 1997; Afewerk et al., 2000). For instance, Codjia et al. (1993) isolated 11 stocks in 1989 and 10 stocks in 1993 from cattle in Ghibe valley, southwest Ethiopia, which were shown to be resistant to diminazene, isometamidium and homidium. Afewerk et al. (2000) also showed that clones of *T. congolense*, which were derived from primary isolates collected from relapsed cattle in the field after treatment with 1 mg/ kg bw of isometamidium, were resistant to both diminazene and isometamidium when tested in mice; this indicated the appearance of a multiple drug resistant *T. congolense* population in northwestern Ethiopia. Recently, Tewelde et al. (2004) demonstrated the presence of isometamidium resistance in trypanosomes in cattle in western Ethiopia. A recent study in the eastern province of Zambia indicated the presence of trypanosomes resistant both to isometamidium and diminazene (Sinyangwe et al., 2004).

There are a number of evidences to the increase in the prevalence and incidence of drug resistance in trypanosomes in sub-Saharan Africa. The first resistant trypanosome population

identified in Burkina Faso was a *T. congolense* stock derived from a cattle primary isolate in Samorogouan, which showed resistance to isometamidium (Pinder and Authié, 1984). Subsequently, Clausen et al. (1992) identified *T. congolense* isolates from the same area that were resistant to isometamidium and diminazene and homidium, such that trypanosomosis in cattle in Samorogouan was often not cured by some trypanocidal drugs administered at maximum dose rates. In a corresponding chemotherapeutic trial by these authors in previously unexposed Zebu bulls and Sahelian goats infected with one primary *T. congolense* isolate from Samorogouan, the parasite demonstrated a high level of resistance to all three drugs in addition to quinapyramine sulphate at 5 mg/kg body weight in goats. McDermott et al. (2003) have recently reported a widespread resistance to both isometamidium and diminazene by trypanosomes in the Samorogouan region (Kénédougou Province) of Burkina Faso.

Before quinapyramine ceased to be manufactured (Holmes and Scott, 1982), it was widely used in livestock as a therapeutic and prophylactic drug (Fiennes, 1953; Ndoutamia et al., 1993). It was withdrawn not only because resistance to it in trypanosomes appears to develop quite easily and rapidly (Wilson, 1949; Fiennes, 1953; Unsworth, 1954; Newton, 1964; Leach and Roberts, 1981) but also because resistance is always associated with high levels of multiple resistance to diminazene, homidium and isometamidium (Mwambu and Mayende, 1971; Whiteside, 1960). This has been confirmed experimentally by repeated treatment of infected mice with subcurative doses of quinapyramine sulphate (Ndoutamia et al., 1993). Similarly, resistance to isometamidium was induced and increased to 94-fold in *T. congolense* by repeated subcurative treatment of infected mice with isometamidium, which was associated with different levels of cross-resistance to diminazene, homidium and quinapyramine (Peregrine et al., 1997). Nevertheless, it is known that cross-resistance between dininazene and isometamidium rarely occurs in trypanosomes in the field, thus they are used as a "sanative" combination to curtail the development of resistance to either drug (Whiteside, 1960; Moloo et al., 1987). However, strains with cross-resistance to diminazene and isometamidium have been demonstrated in the field and experimentally (Moloo and Kutuza, 1990; Chitamo and Arakawa, 1991, 1992; Ainanshe et al., 1992), although it is possible that cross-resistance in these studies occurred because the stock may have consisted of two phenotypically distinct populations, since heterogeneity of drug resistance to diminazene and isometamidium has been demonstrated (Peregrine et al., 1991). The origin of multiple resistances to these trypanocides by trypanosomes in the field is unclear, but it has been suggested that it might be associated with cross-resistance between the different

compounds as a result of their closely related molecular structures (Whiteside, 1960; Williamson, 1970).

Thus, drug resistance in trypanosomes poses a serious problem to livestock productivity in countries where it has been reported, unless checked and brought under control. The development and spread of drug resistance to the point where drugs become ineffective over large areas of Africa is probably the greatest risk to the future use of the existing three trypanocides. It is also possible that the market will shrink and manufacturers will become unprofitable because of the risk of drug resistance. Addtionally, the spread of generic products, some of which are of doubtful quality, may undermine farmers´confidence in trypanocides (Holmes et al., 2004).

Table 1: Reports of resistance to standard recommended doses of diminazene, homidium, isometamidium and quinapyramine in cattle (modified from Peregrine, 1994).

Country	**Trypanosome**	**Resistance to**	**Reference**
Nigeria	*T. congolense*	H, I	Jones-Davies and Folkers, 1966
Nigeria	*T. vivax*	D	Jones-Davies, 1967
Nigeria	*T. congolense*	D, H, I	Na'Isa, 1967
Nigeria	*T. congolense*	D	MacLennan and Jones-Davies, 1967; Jones-Davies, 1968
Chad	*T.vivax*	D	Graber, 1968
Nigeria	*T. congolense*	D, H, I, Q	Gray and Roberts, 1971
	T. vivax	D, H, Q	Gray and Roberts, 1971
Uganda	*T. vivax*	D	Mwambu and Mayende, 1971
Zimbabwe	*T. congolense*	I	Lewis and Thomson, 1974
Kenya	*T. congolense*	D, H, I, Q	Gitatha, 1979
Nigeria	*T. vivax*	D, H, I	Ilemobade, 1979
Sudan	*T. congolense*	H	Abdel Gadir et al., 1981
	T brucei/*T. vivax*	H	Abdel Gadir et al., 1981
Tanzania	*T. congolense*	D	Mbwambo et al., 1988
Burkina Faso	*T. vivax*	I	Pinder and Authié, 1984
Kenya	*T. vivax*	D, H, I, Q	Röttcher and Schillinger, 1985
Kenya	*T. vivax*	H, I, Q	Schönefeld et al., 1987
Somalia	*T. congolense*	H, I, Q	Schönefeld et al., 1987
Zambia	*T. b. brucei*	I	Chitambo and Arakawa, 1991
Somalia	*T. congolense*	D, I	Ainanshe et al., 1992
Burkina Faso	*T. congolense*	D, H, I, Q	Clausen et al., 1992
Sudan	*T. brucei*	D, H, I	Mohamed-Ahmed et al., 1992
	T. vivax	D, H, I	Mohamed-Ahmed et al., 1992
	T. congolense	H	Mohamed-Ahmed et al., 1992
Ethiopia	*T. congolense*	D, H, I	Codjia et al., 1993
Nigeria	*T. brucei*	D, I	Kalu, 1995
Uganda	*T. b. rhodesiense*	DFMO	Iten et al., 1995
Uganda	*T. brucei*	D, I	Matovu et al., 1997
Ethiopia	*T. congolense*	D, I	Afewerk et al., 2000
Ethiopia	*T. congolense*	I	Tewelde et al., 2004
Zambia	*T. congolense*	D, I	Sinyangwe et al., 2004

D, diminazene ; H, homidium ; I, isometamidium ; Q, quinapyramine; M, melarsopol; DFMO, difluoromethylornithine

3.6 Mechanism and genetics of resistance to trypanocides

In order to identify the potential and novel drug targets and to provide directions to how new chemotherapeutic strategies can be used to reduce development of resistance, an understanding of the mechanisms of drug resistance by trypanosomes is important. Trypanocidal drug resistance could be innate, such as in resistant individuals without previous exposure to the particular drug, or acquired (induced) as a result of drug exposure/pressure, cross-resistance or sometimes by mutagenesis (ILRAD, 1990). Drug resistance in trypanosomes is likely to occur under the same circumstances as for many other parasites, i.e. under large-scale drug use, by using inadequate dosing and by using correct dosing with drugs that are slowly eliminated from the body (Holmes et al., 2004). Reduction in drugs accumulation by the target cell or organism and diminished drug activity in immunosuppressed animals can contribute to the emergency of drug resistance (Frommel and Balber, 1987; Osman et al., 1992). Although contradictory observations have been reported on the genetic stability of isometamidium resistance, recent field observations in Ethiopia, based on cloned populations, showed that the drug-resistant phenotype of *T. congolense* had not altered over a period of four years (Mulugeta et al., 1997).

Hayes and Wolf (1990) distinguished three major types of genetic changes that are responsible for acquired drug resistance: mutations or amplifications of specific genes directly involved in a protective pathway; mutations in genes that regulate stress-response processes and lead to altered expression of large numbers of proteins; and gene transfer. The current possibilities to insert or delete genes will certainly lead to a better insight into the resistance mechanisms (Ten Asbroek et al., 1990; Gaud et al., 1997). Other aspects, such as the stability of drug resistance, its mono- or polygenic nature, dominance or recessiveness, also need to be examined, because of their far-reaching impact on the control of resistance.

3.6.1 Alteration of drug transport

The biochemical basis of trypanosome resistance to trypanocides has not been fully understood. Drug resistance can arise, however, either as a consequence of alteration at the target or changes in the concentration at the target site, or both, because of the fact that anti-microbial agents interact with a drug target. Some experiments have shown that drug-resistant trypanosome clones accumulate lesser drugs than their sensitive counterparts. Frommel and

Balber (1987) described that resistant clones of *T. brucei brucei* and *T. b. rhodesiense* accumulated a lower intracellular quantity of the diamidines, 4′,6-diamidino-2-phenyl-indole (DAPI) and Hoechst 3342, the phenanthridine, ethidium bromide, and the acridine, acriflavine than the sensitive clones. It is likely that the mechanism by which resistance arises to the various trypanocides share common biochemical pathways if they have the same biochemical structure.

In 1990, Shapiro and Englund suggested that the main mode of action of ISMM was the cleavage of kDNA-topoisomerase complexes. The mechanism of resistance to ISMM, however, is less clear. Several authors have reported reduced uptake of isometamidium associated with resistance in *T. congolense*. Sutherland et al. (1991, 1992, 1992a) reported an inverse relationship between drug uptake and resistance to isometamidium in *T. congolense*. Fluorescence microscopic and flow cytometric studies showed a reduction in uptake and accumulation of isometamidium chloride by resistant clones of *T. congolense*. Thus, while incubation for 10 min at 27^0C with 5 mg/ml isometamidium chloride resulted in 79% of susceptible IL 1180 *T. congolense* clone showing increased fluorescence over the controls, only 32% of similarly treated resistant IL 3270 clone were more fluorescent than in the corresponding control sample (Sutherland et al., 1991). Subsequently, Sutherland et al. (1992) showed that the uptake of isometamidium chloride by both sensitive and resistant clones of *T. congolense* was through an energy-dependent, specific, receptor-mediated transport system on the parasite surface. Sutherland and Holmes (1993) proposed that an alteration or replacement of a specific receptor in isometamidium chloride-resistant *T. congolense* results in an increased efflux of the drug and that this increased efflux at least partially mediates the reduction in sensitivity to the compound. Recently, Mulugeta et al. (1997) showed that the maximal uptake rates (V_{max}) of isometamidium in resistant *T. congolense* were significantly lower than in sensitive populations. It remains to be shown whether this is caused by a decreased number of protein transporters of isometamidium in the plasma membrane and/or by changes in the balance between influx and efflux. More recently, Wilkes et al. (1997) have demonstrated changes in mitochondrial electrical potential in isometamidium-resistant *T. congolense*. They indicated that in the presence of drug selection pressure, populations expressing lower mitochondrial electric potential have an advantage, owing to decreased toxicity of the drug.

In addition, it was also found that substances which inhibit drug transport in other organisms like the calcium-channel blocker verapamil (VPL) showed a similar efficacy in an isometamidium-resistant *T. congolense* population (Sutherland et al., 1992a) and in the South-American *Trypansosoma cruzi* species (Neal et al., 1989). In contrast to these findings, studies using *T. brucei* populations did not confirm a resistance reversal effect of VPL (Kaminsky and Zweygrath, 1991). Recently, also in *T. brucei,* a second molecular target potentially involved in the mechanism of resistance, the P-glycoprotein multidrug resistance protein (MRP) family, TbMRPA, was shown to be able to significantly reduce sensitivity to trypanocidal drug when over-expressed experimentally (Shahi et al. 2002). Therefore, membrane pumps like the TbMRPA are of special interest also in other trypanosome species.

3.6.2 Adenosine transport and drug resistance

Trypanosomatids cannot synthesize purines *de novo* and live by scavenging them from their mammalian hosts. *T. b. brucei* were reported to salvage adenosine via the transporter P1, which is specific for adenosine and inosine, and P2, which mediates transport of adenosine and adenine. Cellular uptake of melamine-based arsenicals and diamidines has been shown to occur via the P2 transport activity in *T. brucei* bloodstream forms, and loss of P2 has been implicated in drug resistance (Carter and Fairlamb, 1993; Carter et al., 1995; Ross and Barns, 1996). Recent studies in *T. brucei* have enabled the identification of a P2-type adenosine transporter (TbAT1) gene by functional cloning in *Saccharomyces cervisiae* (Mäser et al., 1999).

Sequencing of the trypanosomal cDNA of TbAT1 gene revealed a 10 nucleotide difference between the wild-type *T. brucei* clone and its drug-resistant derivative; six of the mutations were manifested at amino acid level. Two point mutations within the central fragment of this gene could be used to distinguish between the wild-type TbAT1 gene (TbAT1s) and the melasoprol resistant derivative (TbAT1r). Differences in RFLPs were caused by the mutations involving annulment of a recognition site for *Sfa*N1 restriction enzyme, which is otherwise present in the wild-type *T. brucei* (TbAT1s), and simultaneous introduction of the same site further downstream in the arsenical-resistant derivative clone (TbAT1r). Restriction digestion of a 677 bp central fragment of the wild type transporter yielded fragments 566- and 111- bp, respectively, while that of the corresponding fragment in mutants resulted in 435- and 242-bp bands, respectively. In the same study it was shown that a *T. b. gambiense* isolate from a

melarsoprol relapse in Angola had a *Sfa*N1 restriction pattern similar to that of their strain with laboratory-induced resistance (Mäser et al., 1999). Field *T. b. gambiense*-isolates from northwestern Uganda, an area with high melasoprol treatment failure rates also revealed similar patterns, although some possessed patterns expected in both wild-type and mutated transporters (Matovu et al., 2001a). This mixed pattern may be due to infections by mixed trypanosome populations in the patient, or existence of heterozygous clones (Matovu et al., 2001b). This was supported by a gene deletion study conducted later, which attempted to investigate the role of TbAT1 in drug uptake and drug resistance in *T. brucei* by genetic knockout of TbAT1 (Matovu et al., 2003). This later study demonstrated the total abscence of P2-type transport in TbAT1-null bloodstream form trypanosomes and indicated that loss of TbAT1 reduced the sensitivity of trypanosomes to melaminophenyl arsenicals.

De Koning et al. (2004) have recently investigated the transport of diminazene by *T. b. brucei* and found that this is almost exclusively mediated by TbAT1. They showed that TbAT1 clearly mediated the uptake of both diminazene and pentamidine when expressed in yeast, and [^{3}H] diminazene transport was almost completely absent in TbAT1-null mutant trypanosomes. Thus, loss of this single transport activity is sufficient to explain high levels of resistance observed in laboratory strains and veterinary isolates.

Therefore, in *T. brucei,* sequence polymorphisms in this gene may be correlated with the resistant phenotype. The findings of Mäser et al. (1999) point to the possibility of diagnosis of resistance in the field which, if proved consistent, will directly impact on disease management. To date, there is no marker for resistance, yet such would be an invaluable epidemiological tool, which could be used to assess the situation of drug resistance in trypanosomes in the field. This technique needs to be validated in a large-scale screening of field isolates to elucidate its value in diagnosis of drug resistance. Resistance-related polymorphisms have been used with great success in many cases to establish tests for the molecular differentiation of drug-susceptible and resistant organisms, including bacteria (Bifani et al., 2001), fungi (Bossche, 1997) and helminths (Silvestre and Humbert, 2000; von Samson-Himmelstjerna et al., 2001).

In *T. b. brucei* the P2 transporter may also be responsible for part of the isometamidium uptake, as the drug was found to inhibit P2-mediated adenosine uptake (Mäser et al., 1999). However, apart from the indications that isometamidium is a substrate of the P2-transporter

from a study conducted in the yeast expression system, no further work so far has been conducted using field isolates to investigate the involvement of the gene encoding the P2-transporter (TbAT1) in isometamidium resistance. Therefore, the role of nucleoside transporters in resistance to isometamidium by *T. b. brucei* remains to be examined. Similar sequencing and analysis of the transporter genes in drug sensitive and resistant isolates of *T. b. brucei* may reveal which mutations or combinations do correlate with resistance. If similar results with the ones reported (Mäser et al., 1999; Matovu et al., 2001a) are obtained, then in the future, this can be used as a marker gene for drug resistance in *T. b. brucei*.

Investigations on the alteration in gene transcription patterns, and attempts to identify DNA sequences associated with drug resistance in *T. congolense,* have been carried out (Konde, 1997; De Souza, 2000; Konde and Majiwa, 2000); although some mutations have been detected, linkages to genetic loci affected during the development of drug resistance have not yet been found (Majiwa et al., 2000). Recently, the Amplified Fragment-Length Polymorphism (AFLP) technique (Vos et al., 1995) was used for revealing sequence differences between two isogenic clones of *T. congolense* differing in the level of resistance to isometamidium. The authors reported a putative marker gene that was shown to be present in most of the isometamidium resistant field isolates tested and absent in susceptible isolates (Delespaux et al., 2005). This finding requires further validation using large number of isolates from different geographic regions of sub-Saharan Africa before it can be used as a tool. Therefore, work is still required to investigate the presence of similar transporter genes in *T. congolense*, and their involvement in drug resistance. If target genes for drug resistance could be identified, it would be possible to compare the mRNA or genomic DNA of trypanosomes that differ in sensitivity to the drug and to find differences that may correlate with the phenotype (Konde and Majiwa, 2000).

In conclusion, it is clear that much more work is required in order to elucidate the mechanism of resistance to the currently available trypanocidal drugs. Such studies, as well as being of great value in their own right, may also provide novel methods for the detection of drug-resistant trypanosomes in the future. Recent advances in the manipulation of the gene structure and/or expression, such as gene knockouts, overexpression and RNA interference followed by observation of their effects on the trypanosome phenotype, are useful strategies which would provide us valuable information on the genetic bases of drug resistance in trypanosomes.

3.7 Laboratory induction of drug resistance in trypanosomes

The evolution of trypanocidal resistance is influenced by factors which have been described at four levels, namely the host, the vector, the drug and the parasite (Geerts and Holmes, 1998). Although little is known about the mechanism of development of resistance, it has been proved experimentally that underdosing and immunosuppression of the infected animals lead to the development of drug resistance in trypanosomes. Thus, drug resistant trypanosomes can apparently be derived from populations normally sensitive to curative doses of the drug, by a variety of processes, one of which involves the use of sub-therapeutic doses to treat infected individuals (Osman et al., 1992; Ndoutamia et al., 1993; Mutugi et al., 1994; Fang et al. 1994; Pospichal et al., 1994; Peregrine et al., 1997).

The level of resistance of a clone of *T. congolense* to quinapyramine was successfully increased about 42-fold by repeated subcurative treatment of infected mice over a period of 208 days (Ndoutamia et al., 1993). This resistance was associated with a 6-fold increase in resistance to isometamidium, a 28-fold increase in resistance to homidium, and a 5.5-fold increase in resistance to diminazene in infected mice. In goats infected with the resistant clone, one of five goats treated with 3 mg/kg bw quinapyramine sulphate, none of five goats treated with 0.25 mg/kg bw isometamidium chloride, two of five goats treated with 1 mg/kg bw homidium chloride, and three of five goats treated with 3.5 mg/kg bw diminazene aceturate subsequently relapsed, showing that except for isometamidium, the clone did express multiple drug resistance even in the definitive host. Similarly, it was possible to induce 94-fold resistance to isometamidium in a clone of *T. congolense* by repeated subcurative treatment of infected mice over 11 months from a CD_{50}[4] of 0.018 mg/kg bw for the parental *T. congolense* IL 1180 to a CD_{50} of 1.7 mg/kg bw for the resistant *T. congolense* IL 3343 clone (Peregrine et al., 1997). This clone also exhibited multiple drug resistance with a high level of resistance to homidium chloride (33-fold increase in CD_{50} from 0.37 to 12.1 mg/kg bw) but lower levels of resistance to diminazene aceturate (3.4-fold increase in CD_{50} from 2.3 to 7.8 mg/kg bw) and quinapyramine sulphate (4.2-fold increase in CD_{50} from 0.23 to 0.97 mg/kg bw). Drug resistance in trypanosomes, once induced, appears to be stable and irreversible (Mulugeta et al., 1997).

[4] CD_{50} (median curative dose) is a statistically derived single dose of a substance that can be expected to cure 50 % of the treated animals. The CD_{50} value is expressed in terms of weight of test substances per unit weight of test animal (mg/kg bw). (EPA, 2002).

Osman et al. (1992) demonstrated the importance of the immune system in the development of drug resistance by trypanosomes in mice infected with *T. evansi* through progressive treatment of immunosuppressed mice with subcurative doses of trypanocidals. In this study, it was possible to achieve a high level of resistance to melarsenoxide cysteamine (Mel Cy), diminazene aceturate and isometamidium chloride. In contrast, attempts made by the same authors to develop Mel Cy and diminazene resistance in immunocompetent mice were not successful. They tested the Mel Cy resistant clone for cross-resistance to diminazene and pentamidine and the clone was found to be also highly resistant to these drugs. Mutugi et al. (1994) investigated factors which influence induction of resistance to suramin in two *T. evansi* stocks using cloned and uncloned trypanosomes in immunosuppressed or immunocompetent mice. 3000-fold resistance was achieved in cloned trypanosomes in immunosuppressed mice. Pospichal et al. (1994) produced a population of *T. brucei brucei* with reduced sensitivity to Mel Cy in immunosuppressed mice using subcurative drug treatment, which showed various levels of cross-resistance to melasoprol, diminazene and penthamidine. Thus, in the presence of immunosupression due to, for example, other parasitic diseases in the field, the exposure time of trypanosomes to the drug is increased, which will favour selection for resistance. Other host-related causes may be environmental, e.g., nutritional status and stress, which will render the host unable to play its supportive role in the elimination of infections.

The studies conducted so far showed that a series of trypanosome clones with different degrees of drug resistance can be developed. Such laboratory derived isogenic clones of trypanosomes can be used to study the genetic, biochemical and molecular bases of drug resistance and to investigate the mode of action of the drug. This may provide information on the mechanism of trypanocidal resistance in trypanosomes which might then suggest chemotherapeutic strategies to overcome drug resistance. These laboratory induced drug-resistant trypanosomes also provide useful tools in experimental chemotherapy and have potential applications in the primary screening of candidates of new trypanocides and in calculating cross-resistance between trypanocides.

3.8 Cloning of trypanosomes

An animal can be infected with several different populations of trypanosomes, which may be of the same species but genetically heterogeneous. Thus, trypanosome isolates collected from an infected animal in the field may not be representative of a genetically identical population. Genetically identical populations may be obtained by growing them up from single organisms. Such populations are termed 'clones'. Clones may be grown up either in cultures (convenient for stercorarian trypanosomes) or susceptible animals (convenient for salivarian trypanosomes). Success in establishing infections from single organisms varies widely. Comparison of titration results with simultaneous organismal counts suggests that at some periods of the infection only small proportions of organisms may be infective (Lumsden et al., 1973). Also, differences occur between different antigenic types. McNeillage and Herbert (1968) found that with highly virulent antigenic types one out of two organisms inoculated singly grew in the recipient animals. However, in the case of a less virulent type only one in twenty-three organisms established parasitaemia under identical conditions. The clones can be used to study variations in drug resistance phenotype between different clones of the same population as well as the genetic, biochemical and molecular bases of drug resistance.

3.9 Methods of detection of drug resistance in trypanosomes

Despite reports of trypanosome drug resistance in cattle from at least 13 African countries (Geerts and Holmes, 1998), the reports have usually been of isolated cases, and there has been little attempt to undertake spatial or temporal assessments of the scale of the problem. An important prerequisite to undertake such studies is the availability of reliable and simple tests for drug resistance, as the failure of drug treatment might have causes other than drug resistance, which might include significant under-dosing, treatment with fake drugs, or rapid re-infection after treatment with a short-acting therapeutic drug (Geerts et al., 2001). At present, three types of techniques are commonly used to identify drug resistance: tests in ruminants; tests in mice; and *in vitro* assays. None of these is, however, an ideal test, as they generally require large number of experimental animals, take long periods to perform, and often require adaptation of the parasites to growth in either tissue culture or laboratory rodents. Other tests are still in the phase of development or validation (Holmes et al., 2004). Simpler, more sensitive, fast and reliable methods for typing drug-resistant trypanosomes are

obviously desirable. These would be crucial to help avoiding further increase of resistance levels and waste of resources by continued use of already ineffective drugs.

3.9.1 Tests in ruminants

The optimal animal tests are probably those that are conducted in ruminants, as they provide direct information on drug resistance using recommended doses of trypanocide. The tests commonly consist of infecting a group of cattle or small ruminants with the isolate under investigation and later, when the animals are parasitaemic, treating them with various levels of trypanocide. It is preferable to use at least three animals in each group, because it has been shown that results obtained after inoculation and treatment of one animal are not always reliable (Eisler et al., 2001). The animals are then regularly monitored over a prolonged period (up to 100 days) to determine the effective dose (ED), i.e. the dose that clears the parasites from the circulation, and the curative dose (CD), i.e. the dose that provides a permanent cure (Sones et al., 1988). For these studies, the cattle or small ruminants must be kept in fly-proof accommodation or in a non-tsetse area in order to eliminate the risk of reinfection during the study. A variation of this technique was used by Ainanshe et al. (1992) in Somalia to examine a group of isolates from a district. Blood from a group of infected cattle was inoculated into a single recipient calf, which was monitored, and later, when it was parasitaemic, treated with trypanocide at the recommended dose. A breakthrough infection, indicative that one of the inoculated trypanosome populations was drug-resistant, was inoculated into groups of calves and mice to determine the level of drug resistance. This technique is useful in situations where laboratory facilities are very limited but it only allows a qualitative assessment and does not indicate how many of the isolates inoculated into a single calf were resistant (Holmes et al., 2004).

Eisler et al. (2001) described a standardised test in cattle, to determine the efficacy of recommended curative doses of trypanocidal drugs for the treatment of infection with individual isolates of *T. vivax*, *T. congolense* or *T. brucei*. This test is useful because of the difficulty in extrapolating the curative dose in cattle from the results of tests in mice (Sones et al., 1988). A useful indication of the level of resistance can be obtained from studies in ruminants by recording the length of time between treatment and the detection of breakthrough populations of trypanosomes. The shorter the period, the greater the level of resistance (Ainanshe et al., 1992; Williamson and Stephen, 1960). The main constraints to

this technique are that not all populations might grow equally well and that sensitive isolates might overgrow resistant ones when inoculated together (Sones et al., 1989). However this is not a consistent observation (Burudi et al., 1994). Moreover, the long duration (a follow-up of 100 days is necessary to allow the detection of relapses) and the cost (purchase and maintenance of the animals are expensive) make this method usually impractical to examine a large number of isolates.

3.9.2 Tests in mice

Due to the high costs of tests in the definitive host, tests in laboratory mice have become the preferred method, at least for testing those isolates of *T. congolense* which grow well in mice. After expansion of an isolate in a donor mouse, groups of five or six mice are inoculated with trypanosomes. Twenty-four hours later, or at the first peak of parasitaemia, each group except the control group is treated with a range of drug doses. Thereafter, the mice should be monitored three times a week for 60 days. The ED_{50} or ED_{95} (the effective dose that gives temporary clearance of the parasites in 50 or 95 percent of the animals, respectively) can be calculated, as can the CD_{50} or CD_{95} (the curative dose that gives complete cure in 50 or 95 percent of the animals, respectively). Sones et al. (1988) used groups of five mice, which allowed an easy calculation of ED_{80} and CD_{80} values (one out of five mice was not cleared or cured). These figures should be compared with those obtained using reference-sensitive trypanosome strains. Eisler et al. (2001) described a standardised and simplified method for the investigation of trypanocidal drug resistance using laboratory animals. The first is a single-dose mouse test, which is a simplified approach to the use of mice for investigation of drug resistance on an area-wide basis. It is intended to compare and characterize areas in terms of the extent of drug resistance in *T. congolense* or *T. brucei* by examination of as many isolates as possible, rather than to characterize individual stabilates. The greater the proportion of trypanosome populations in an area that express resistance in naturally infected cattle, the greater the proportion that will show resistance in a test in mice using an appropriate single drug dose. The test uses discriminatory dosages of 1.0 mk/kg isometamidium chloride and 20 mg/kg diminazene aceturate and allows considerable reduction in the number of animals, and also labour needed. This method has shown to be useful in the investigation of trypanocidal drug resistance on an area-wide basis (Geerts et al., 2001). The objective of the standardised multi-dose test (Eisler et al., 2001) is to obtain more detailed information by determining the CD_{50} and CD_{80} values for a given trypanocidal drug.

The test uses dose ranges of 0.01 to 20 mg/kg bw for isometamidium chlorid and for homidium chloride or bromide and 1 to 60 mg/kg bw for diminazene aceturate.

The advantage of the mouse assay is that it is cheaper than the test in cattle. There are several disadvantages, however. Firstly, most *T. vivax* isolates, and also some *T. congolense* isolates, do not grow in mice. Secondly, although there is reasonable correlation between drug sensitivity data in mice and in cattle, higher doses of drug must be used in mice in order to obtain comparable results to those obtained in cattle because of the vast difference in metabolic size (Sones et al., 1988). Thirdly, precise assessment of the degree of resistance needs a large number of mice per isolate, which makes it rather a labour-intensive test. Identification of a discriminatory dose, above which an isolate should be considered as resistant, could drastically reduce the number of mice and the amount of work to be carried out. Finally, it takes as long as 60 days to evaluate the drug sensitivity of an isolate.

3.9.3 *In vitro* assays

Antitrypanosomal activity of drugs can be observed in *vitro* culture conditions (Brun and Kunz, 1989), and techniques have been examined for their usefulness in assessing drug sensitivities (Elrayah and Kaminsky, 1991). For the *in vitro* evaluation of drug sensitivity, procyclic, metacyclic or bloodstream forms of trypanosomes can be used. The advantage of *in vitro* assays is that large numbers of isolates can be examined. However, there are several limitations. The use of procyclic forms is not as reliable as the use of metacyclic and bloodstream forms. Tests with metacyclic trypanosomes correlate well with field observations, but it may take up to 40 to 50 days of *in vitro* incubation to generate metacyclic trypanosomes. *In vitro* assays are expensive to perform and require good laboratory facilities and well-trained staff. In contrast to *T. brucei*, it is very difficult to cultivate *T. congolense* (Holmes et al., 2004). An interesting alternative is the drug incubation *Glossina* infectivity test (DIGIT), in which the trypanosomes are exposed to the drug *in vitro* for a short time and thereafter fed to tsetse flies to check whether or not they develop into metacyclic forms (Clausen et al., 1999). This technique distinguishes resistant from sensitive isolates and does not require experimental animals, but it does require a ready supply of teneral tsetse flies from an artificially reared colony. Further limitation of this technique is that the test is complicated and as a result requires well-equiped laboratory facility and well-trained personel.

3.9.4 Trypanosomal drug ELISAs

Whitelaw et al. (1991) described a competitive ELISA, which allowed the detection of small amounts of isometamidium in the serum of cattle. This technique was further improved by Eisler et al. (1993) and Eisler et al. (1996) and has been validated in cattle under experimental and field conditions (Eisler et al., 1994; 1996; 1997). The test has demonstrated the presence of high drug levels in the blood of cattle harbouring drug-resistant trypanosomes, whereas the same drug concentrations were shown to be prophylactic for drug-sensitive isolates (Eisler et al., 1997). The test is both sensitive, detecting subnanogramme concentrations, and specific. It allows the monitoring of drug levels over extended periods and the evaluation of factors influencing drug disappearance rates from the plasma.

Available data indicate that a considerable individual variation exists after intramuscular injection of isometamidium in cattle (Eisler et al., 1996). One interesting finding has been that the drug disappears more rapidly in animals challenged and becoming infected with drug-resistant trypanosome isolates than in those challenged but protected against infection with sensitive trypanosomes (Eisler et al., 1994). Observations showed that the presence of trypanosomes in animals with an isometamidium concentration of > 0.4 ng/ml suggests resistance; the higher the drug level detected, the greater the degree of resistance that could be inferred (Eisler et al., 1997). Further research is necessary, however, in order to confirm these results in a larger number of animals. Similar drug ELISAs have been developed for the detection of subnanogramme amounts of homidium bromide (Murilla et al., 1999) and a similar test for diminazene is in development (Karanja et al., 2002).

The advantage of the isometamidium ELISA is that large numbers of sera can be tested within a relatively short time. The ELISA may also provide information on drug usage in an area of investigation (Diarra et al., 2001). The disadvantage is that further studies are required to confirm the correlation between protection against tsetse challenge with various trypanosome populations and the isometamidium concentration in the serum. It is not yet possible to draw firm conclusions on the sensitivity or resistance of a trypanosome population at the level of the individual animal. The ELISA should, however, give some indication of the resistance situation at the level of the herd. A further disadvantage is that, while the ELISA may indicate the level of drug that a trypanosome population withstands, it does not provide information about the level required for protection (Geerts and Holmes, 1998).

3.9.5 Longitudinal field studies

Longitudinal parasitological data can be used to detect resistance problems in cattle herds under natural tsetse challenge. Rowlands et al. (1993) showed that the application of a computer model to parasitological data collected over a long period on a monthly basis allowed the incidence of new infections to be distinguished from recurrent infections. An infection was defined as a new infection if it was preceded by two previous months in which monthly collected samples had a PCV ≥ 26% and no trypanosomes were detected. This analysis showed that the prevalence of diminazene-resistant infections in the Ghibe valley, Ethiopia, increased from 6 percent in 1986 to 14 percent in 1989. The advantage of these kinds of data is that they are directly applicable to the field. There are a number of disadvantages, however. Firstly, the true prevalence of drug-resistant infections seems to be underestimated. Secondly, it is retrospective by at least six months. Finally, the technique is quite expensive, if a longitudinal study is not carried out for other purposes.

Another method for the assessment of trypanosomosis risk and the level and prevalence of resistance to isometamidium chloride has been developed using cattle populations under natural challenges in the field (Eisler et al., 2000). This protocol compares new trypanosome infections in a group of cattle treated with isometamidium chloride to an untreated group. The rate at which new infections occur in the two groups is assessed by a comparison of their survival curves over an 8 – 12-week period. This may give a rapid and accurate assessment of isometamidium resistance and the impact of drug use relative to no treatment. This method has been successfully applied to assess isometamidium resistance of trypanosomes in cattle in western Ethiopia (Tewelde et al., 2004). One of the disadvantages of this study is that it cannot be used to assess the resistance situation with regard to diminazene.

The other possibility is a block treatment study, whereby a group of naturally trypanosome-infected animals are treated with prophylactic doses of isometamidium and monitored for relapse of infections. This technique can be used to assess the period of prophylaxis conferred by isometamidium in naturally trypanosome-infected animals under field condition, such that the data has direct relevance to the field, which is not always the case with data obtained in mice (Afewerk et al., 2000). The disadvantage is that the animals are left after treatment to graze in their natural environment, where there is high tsetse-challenge. Consequently, the risk of reinfection after treatment cannot be eliminated, and it is not possible to know whether

the trypanosomes detected in the animals originated from the population present in the animal at the time of treatment or from tsetse feeding on these cattle after treatment. Therefore, further confirmation may be required by isolating the trypanosomes from the animals with relapse infection and testing them in mice or in natural hosts in a fly proof stable (Afewerk et al., 2000). Besides, this test is not suitable for diminazene aceturae because this drug is eliminated from the blood of treated animals in a few days time.

3.9.6 Genetic markers for drug resistance in trypanosomes

Due to the vast limitations of the currently available tests to validate drug resistance in trypanosomes, an alternative approach in the future may be made to identify genetic markers for drug resistance, which might be developed into reagents for the identification of resistant trypanosomes using the polymerase chain reaction (PCR). A PCR-based test could provide a rapid and convenient tool, suitable for large-scale epidemiological surveys of livestock. Developments of such tests require the identification of genetic mutations that may be associated with drug resistance in livestock-infective trypanosomes.

The identification and cloning of the nucleoside transporter gene in *T. brucei* (Mäser et al., 1999), which is putatively a melaminophenyl-arsenical resistance related candidate marker gene, is a recent advance in the molecular diagnosis of drug resistance in *T. brucei*. These putative marker genes have been evaluated in the field using *T. b. rhodesiense* and *T. b. gambiense* isolates collected from patients refractory to melaminophenyl-arsenical treatments. A clear link has been demonstrated between resistance to melaminophenyl-arsenicals and mutation in the TbAT1 gene. However, further studies are required using a large number of field cases before a practically applicable test, which can detect melaminophenyl-arsenical resistance in the field, is developed (Matovu et al., 2001a; Matovu et al., 2003). Apart from the fact that the TbAT1 gene is demonstrated to be involved in the uptake of isometamidium in *T. brucei*, to date no further work has been done to evaluate the involvement of this gene in isometamidium resistance in the livestock-infective *T. b. brucei*. If a similar mechanism is involved in isometamidium resistance in *T. b. brucei* and if there is a consistent link between isometamidium resistance and mutation in this gene, it would be possible to develop a molecular test based on this gene as a marker gene for early detection of isometamidium resistance in *T. b. brucei,* before it becomes a widespread problem. Furthermore, little attempts have been made to detect the presence of a similar transporter gene in *T. congolense*.

Screening for homologues in *T. congolense* to the putatively resistance-related *T. brucei* sequences (TbAT1) would be an important first step in the identification of genetic markers for isometamidium resistance in this species of parasite.

The trypanosomes infective to livestock in Africa have not been as well studied at the molecular level as those that are infective to humans. Consequently, little is known about the genome of *T. congolense*, the trypanosome in which trypanocide resistance has been well documented. Several powerful molecular genetic approaches are now available which can be exploited to detect genetic differences between organisms that differ only in a specific phenotype, but which are otherwise identical. These include: Representational Difference Analysis (RDA) (Lisitsyn et al., 1994), Differential Display (DD) (Liang and Pardee, 1992; Murphy and Pelle, 1994, Rompf and Kahl, 1997) and Suppression subtractive Hybridisation-PCR (SSH-PCR) (Diatchenko et al., 1999). The main advantage of these methods is that prior knowledge of the function or physical location of the gene(s) responsible for the phenotype is not required, yet the methods could lead directly to the gene(s) responsible for the phenotype. These techniques may enable to comparatively scan a genome wide for genomic DNA and/or transcript differences between resistant and sensitive reference trypanosome clones. A number of attempts were made using these methods to compare isogenic clones of *T. congolense* (Peregrine et al, 1997), one of which is sensitive and the other resistant to curative doses of the trypanocidal drug isometamidium chloride (Majiwa et al., 2000; Konde and Majiwa, 2000). Although some mutations have been detected, linkages to genetic loci affected during the development of drug resistance have not yet been found (Majiwa et al., 2000).

Amplified fragment-length polymorphism (AFLP) (Vos et al., 1995) is another technology which requires no sequence information or probes prior to the generation of the fingerprints. This is of particular importance when studying organisms with little DNA sequence information in the existing database as in the case for *T. congolense*. The technique has recently been used for revealing sequence differences between two isogenic clones of *T. congolense* differing in the level of resistance to isometamidium chloride (Delespaux et al., 2005). They were able to identify a gene coding for an 854 amino acid protein which shows polymorphism between the isometamidium sensitive and resistant *T. congolense*. The genetic marker was shown to be present in most of the resistant *T. congolense* field isolates and absent in the susceptible isolates. The gene was found to be homologous to the Adenosine-Tri-Phosphate (ATP)-binding cassette (ABC), and in the isometamidium resistant *T.*

congolense it had a triplet insertion coding for an extra lysine. The presence of the insertion, specifying an extra codon, was found to always be present in the genomes of *T. congolense* clones that were resistant to isometamidium chloride. In addition to DNA sequencing and determination of the localization of these sequences within the genome of *T. congolence*, it is also important to validate such "markers" by applying them to further trypanosome populations with known drug sensitivity phenotypes from different geographical origins. If any of the DNA fragments is linked to the isometamidium resistance phenotype in *T. congolense*, such fragments will form the basis for development of DNA markers for the identification of trypanosomes that are resistant to this trypanocidal drug.

3.9.7 Potential new tests for detection of resistance to isometamidium

Since it has been shown that the rate of isometamidium accumulation in *T. congolense* is a good indicator of the degree of drug resistance and since the mitochondrial electrical potential (MEP) appears to be closely linked with the rate of drug uptake, it might be possible in the near future to develop a quantitative *in vitro* test to evaluate the MEP (Wilkes et al., 1997). If such a test could be carried out using a small number of trypanosomes, it might provide a rapid indication of the level of resistance of a given trypanosome isolate. It is hoped that this test could be conducted on whole blood samples and would not, therefore, suffer from the same limitations as other *in vitro* tests referred to earlier.