# DEVELOPMENT OF MONOCLONAL ANTIBODY-BASED ASSAYS FOR DETECTION AND DIFFERENTIATION OF TRYPANOSOME SPECIES IN THE TSETSE FLY (*GLOSSINA* SPP.)

**A Thesis Presented to**
**The Board of Graduate Studies,**
**University of Ghana, Legon.**
**Ghana.**

**In fulfilment of the**
**Requirements for the Degree**
**of Doctor of Philosophy (Ph.D.)**
**(Animal Science).**

**By**

**KWABENA MANTE BOSOMPEM**
**BSc. (Hons.)**

**Department of Animal Science,**
**Faculty of Agriculture,**
**University of Ghana,**
**Legon, Accra. Ghana.**

**March, 1993.**

## DECLARATION

I do hereby declare that except for references to other people's investigations which have been duly acknowledged, this exercise is the result of my own original research, and that this thesis, either in whole, or in part, has not been presented for another degree elsewhere.

KWABENA MANTE BOSOMPEM
(Student)

PROF. R. K. G. ASSOKU
(Supervisor)

DR. V. M. NANTULYA
(External supervisor)

PROF. R. K. G. ASSOKU
(HEAD, Department of Animal Science,)
University of Ghana, Legon,
Ghana.

# DEDICATION

To Dr. J. E. Fletcher for the help in determining my career, and to my wife Ernestina and my children for their understanding of the circumstances.

## ACKNOWLEDGEMENT

It is my first duty to acknowledge with pleasure, my indebtedness to all the individuals, organisations and institutions that have contributed in various ways to the formulation, execution and submission of the work described in this thesis.

I will begin by expressing my sincere thanks to the Animal Science Department, Faculty of Agriculture, University of Ghana, for making me the beneficiary of the Research Fellowship awarded by the International Laboratory for Research on Animal Diseases (ILRAD), and to my employers, Noguchi Memorial Institute for Medical Research (NMIMR), University of Ghana Legon, for granting me a study leave to undertake this study. I am also grateful to the ILRAD Administration and the Training Department for providing such excellent facilities and atmosphere under which this work was carried out.

I am particularly indebted to Prof. Reginald K. G. Assoku and Dr. Vinand Nantulya, my supervisors, not only for the guidance, encouragement and stimulating discussions, but also for their unfailing courtesy and cooperation. I also wish to express my deep gratitude to Dr. Rachael Masake, for acting as my ILRAD supervisor during the absence of Dr. Nantulya. I am particularly appreciative of the way in which Dr. Nantulya continued to find time for this project, even after his departure from active service in ILRAD. I am thankful to him.

I am extremely thankful to Dr. S. K. Moloo, head of the ILRAD tsetse laboratory, for the help and encouragement he offered, and all his technicians, particularly Messrs Joseph Muia, John Kabata and Clement Sabwa for their unfailing cooperation and assistance in all the experiments involving the use of laboratory-bred tsetse flies.

I gratefully acknowledge the support granted to me by the Kenya Trypanosomiasis Research Institute (KETRI) in making it possible to conduct part of this work on the Galana Ranch, and the assistance received from that institute from Dr. Elizabeth Opiyo. I am thankful to Dr. Chris Green for his assistance in trapping tsetse flies, and Mr. T. Wanyama for helping me in both trapping and dissection of tsetse at the Ranch.

I owe a special debt of gratitude to Dr. Ian Gumm and Dr. Ron Kaminsky for supplying culture-derived trypanosomes for this project whilst they were in ILRAD, and Mr. Francis Chuma of ILRAD for similar assistance. Also, I remember Dr. Joe McNamara of the Tsetse Research Laboratory, Bristol, England for supplying *Trypanosoma grayi* antigens for this study and Dr. Andrew Peregrine of ILRAD for making this arrangement possible.

The helpful advice and encouragement that was freely given by Dr. Phelix Majiwa, Dr. M. Touré and Mr. Stephen Minja all of ILRAD, is greatly appreciated.

I wish to thank Dr. M. K. Shaw for technical help in immunolocalization studies; Henry Gathuo for introducing me to tissue culture and immunofluorescence techniques; Messrs John Ngatti, James Thuo, Jackson Makau, Benson Gichuki and Stephen Ngava for helping me to master the various techniques described in this work; David Elsworth and J. Mwaura for advice on preparation and presentation of illustrations, and Francis Shikhubari for his expert assistance in photography.

Finally, I wish to thank Mr. Maxwell Appawu of the NMIMR, for the immeasurable help he offered during my absence from Ghana.

  

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# ABBREVIATIONS

| | |
|---|---|
| ABTS | Azino-bis(3-ethylbenzthiazoline-6-sulfonic acid) |
| APS | Ammonium persulphate |
| $^{o}C$ | Degrees centigrade |
| cm | Centimetre(s) |
| DAB | Diaminobenzidine tetrahydrochloride |
| DE-52 | Diethylaminoethyl cellulose |
| DMSO | Dimethylsulfoxide |
| DNA | Deoxyribonucleic acid |
| EA | East African |
| EATRO | East African Trypanosomiasis Research Organisation |
| EDTA | Ethylenediaminetetracetate |
| ELISA | Enzyme-linked immunosorbent assay |
| Fab | Fraction antibody binding portion of the immunoglobulin molecule |
| FBS | Foetal bovine serum |
| FITC | Fluorescein isothiocynate |
| gm | Gramme(s) |
| HAT-medium | Hypoxanthine, aminopterin and thymidine medium |
| HEPES | N-2-hydroxyethyl-piperazine-N-2-ethane sulfonic acid |
| hr | Hour |
| HRPO | Horseradish peroxidase |
| HT-medium | Hypoxanthine and thymidine medium |
| IFAT | Indirect immunofluorescent antibody test |
| Ig | Immunoglobulin |
| IgG | Immunoglobulin G |
| IgM | Immunoglobulin M |
| ILRAD | International Laboratory for Research on Animal Diseases |
| IU | International units |
| kDa | Kilodalton |
| l | Litre(s) |
| 2-ME | 2-mercaptoethanol |
| MoAb | Monoclonal antibody |
| ml | Millilitre(s) |
| min | Minutes |
| mm | Millimetre(s) |
| MW | Molecular weight |
| $\mu$ | Microns |
| $\mu$g | Micrograms |
| $\mu$l | Microlitre(s) |
| $\mu$m | Micrometre(s) |
| nm | wavelength in nanometres |
| NC | Nitrocellulose |
| NMIMR | Noguchi Memorial Institute for Medical Research |
| NP-40 | Nonidet P-40 |
| PBS | Phosphate buffered saline |
| PEG | Polyethylene glycol |
| pH | Negative logarithm base ten of hydrogen ion concentration |
| PSG | Phosphate saline glucose |
| PTM | Peritrophic membrane |
| RPMI(1640) | Rosewell Parke Memorial Institute Medium 1640 |
| SDS | Sodium dodecyl sulphate |
| SDS-PAGE | Sodium dodecyl sulphate polyacrylamide gel electrophoresis |
| TBS | Tris buffered saline |
| TEMED | N,N,N'-,N'-tetramethylethylenediamine |
| VAT | Variable antigen types |

| | | |
|---|---|---|
| W | | Watt(s) |
| WA | | West African |
| WHO | | World Health Organisation |
| *X*g | | Times gravitational force |
| 4CIN | - | 4-Chloro-1-Naphthol |

## SUMMARY

Diagnosis of trypanosome infections in tsetse (*Glossina spp.*) is currently based on dissection and microscopic examination of different organs of the vector for the presence of infecting trypanosomes. This procedure is slow and labour intensive, and it is not accurate.

The work reported in this thesis was conducted with the objective of developing a field applicable, monoclonal antibody (MoAb)-based assay for the detection of, and differentiation between trypanosome species in infected *Glossina* species. The development of a more accurate alternative technique to the dissection method would provide a useful tool for estimation of the trypanosomiasis risk in various localities.

MoAbs had been previously generated against invariant antigens of various trypanosome subgenera, including the *Trypanozoon*, *Nannomonas* and *Duttonella*, and the *T. congolense* species. Some of these MoAbs were selected and used in the studies described in this thesis. It was, however, necessary to produce additional trypanosome species-specific MoAbs for several reasons: Firstly, there was no *T. simiae* specific MoAb. Secondly, the selected *T. vivax* specific MoAbs were less sensitive compared with some of the *T. congolense* and *Nannomonas* species-specific MoAbs, in terms of the minimum number of procyclics or epimastigotes that they could detect. Thirdly, some of the selected MoAbs could not be used in the Western immunoblot assay to identify the antigens involved.

The additional trypanosome species-specific MoAbs produced included three *Trypanozoon* subgenus-specific MoAbs (KT39a/18.17, KT43/33.32 and KT43/27.32), two *Duttonella* subgenus-specific MoAbs (KD32/48.17 and KD37/19.3), two *Nannomonas* subgenus-specific MoAbs (KN4/13.9 and KN5/6.15) and one *T. simiae* specific MoAb KNS7/14.X. Characterization of the specific antigens, using the indirect immunofluorescent antibody test

(IFAT), Western immunoblot analysis, proteinase-K digestion and periodate oxidation, showed that some of the new MoAbs detected trypanosome species or subgenus-specific antigens that previously had not been identified. These new antigens included a *T. brucei* species-specific antigen which localized in the cytoplasm of *T. brucei* procyclics and differed from previously identified antigens which were located at the cell membrane.

Employing the nitrocellulose membrane (NC)-based dot-enzyme-linked immunosorbent assay (dot-ELISA), it was found that the selected MoAbs could identify and differentiate between *in vitro* propagated trypanosome species. The dot-ELISA detected *in vitro* propagated *T. brucei*, *T. congolense* and *T. simiae* procyclics, and *T. vivax* epimastigotes, in both single and artificially mixed preparations. The assay had a specificity more than 99.9% and a sensitivity as good as 10 trypanosomes per dot for some *T. brucei*, *T. congolense* and *Nannomonas* specific MoAbs. The integrity of trypanosome antigens applied onto NC membrane remained unaffected for up to 60 days of storage at 4°C under desiccated conditions or under similar conditions at room temperature (17-26°C). Each of the derived MoAbs was able to detect isolates of the respective trypanosome species obtained from different geographical areas, and none cross-reacted with *T. grayi*. Furthermore, each of the *T. congolense* specific MoAbs reacted with all three types of this parasite species (savannah, Kilifi and riverine-forest types) that were tested.

The dot-ELISA developed, however, could not be applied directly to the diagnosis of trypanosome infections in tsetse because of non-specific staining of NC membrane by samples prepared from tsetse gut, due to the presence of pigments and undigested blood meals. This non-specific staining was eliminated by prior decoloration of tsetse gut samples on NC membrane, using 5% hydrogen peroxide ($H_2O_2$). This finding enabled successful detection and differentiation of *T. brucei*, *T. congolense* and *T. simiae* parasites in the gut of experimentally infected *Glossina*, using a modified dot-

ELISA. The sensitivity of the assays was; 90.5% in detecting *T. brucei* infections, 85.4% in detecting *T. congolense* infections and 94.4% in detecting *T. simiae* infections. The sample from the gut of each tsetse fly could be replicated in 15 dots onto NC membrane for testing. When stored on NC membrane at room temperature (19-26$^{o}$C) under desiccated conditions, the samples did not show any loss in activity over a period of 90 days.

The dot-ELISA technique was also used successfully to detect *T. brucei* parasites in the salivary glands of experimentally infected *Glossina*. This application of the technique was, however, achieved following the substitution of $Na_2EDTA$ buffer for PBS or PSG which were used in detecting *T. brucei* in infected tsetse gut. The assay was more than 99.9% specific and 90% sensitive in detecting *T. brucei* in infected tsetse salivary glands.

*Trypanosoma congolense* and *T. vivax* infections were successfully detected in the mouthparts of experimentally infected tsetse flies using dot-ELISA. However, it was not possible to replicate tsetse proboscide samples for testing with different MoAbs due to the very low parasite numbers in this organ. The recommended strategy, therefore, was to randomly sort all tsetse flies suspected to be infected with *T. congolense, T. simiae* or *T. vivax* into three separate groups and test them with different MoAbs.

In a limited field evaluation of the dot-ELISA, 2 out of 104 *G. pallidipes* flies identified by dissection and microscopy to carry midgut infections, were also positive by dot-ELISA. Using the dissection technique, such infections would have been diagnosed as immature *T. brucei* and/or *T. congolense* and/or *T. simiae*. However, by the dot-ELISA, it was possible to determine with certainty that the two flies were infected with *T. congolense* parasites. Furthermore, the dot-ELISA detected *T. congolense* antigens in the midguts of an additional 6(5.8%) of the 104 *G. pallidipes* and 17(4.4%) of 390 *G. longipennis* that were negative by the dissection method, suggesting that the dot-ELISA may be more sensitive than the dissection method. This

ability of the dot-ELISA to detect more infections than the dissection method, suggests that the MoAb-based dot-ELISA is not only a more precise tool for identification and differentiation of trypanosome species in the vector, but that it may also be more sensitive than the dissection method.

# CHAPTER 1

# INTRODUCTION

## 1.1 General introduction

Trypanosomiasis is the name given to a disease complex caused by several species of microscopic flagellate haemoprotozoan parasites called trypanosomes. These organisms are members of the group previously belonging to the phylum *Protozoa*, but recently re-assigned the kingdom *Protista*, subkingdom *Protozoa*, and phylum *Sarcomastigophora* (Margulis, 1974; Molyneux and Ashford, 1983; Cortiss, 1984; Corlis, 1986). The trypanosomes are further classified as belonging to the class *Zoomastigophorea*, order *Kinetoplastida*, suborder *Trypanosomatina*, family *Trypanosomatidae* and the genus *Trypanosoma* (Hoare, 1972). The trypanosomes that cause the African trypanosomiases are grouped under four subgenera, three of which are of medical and veterinary importance, namely, the *Trypanozoon*, which comprises the *brucei*-type of organisms, *Duttonella* which includes *T.* (*D.*) *vivax* and *T.* (*D.*) *uniforme*, and *Nannomonas* which consists of *T.* (*N.*) *congolense* and *T.* (*N.*) *simiae*. Tables 1a and 1b show the systematic classification and detailed speciation of members of the genus *Trypanosoma*.

At the end of the 19th century, the British scientist David Bruce, demonstrated that the trypanosome which caused Nagana in livestock of Zululand was transmitted by tsetse flies (Bruce, 1895; Hoare, 1972). This trypanosome (*T. brucei brucei*) was later named after him by Plimmer and Bradford (1899). From then onwards tsetse flies were known to transmit trypanosomiasis, but the mode of transmission remained unclear. It was actually believed that trypanosomes underwent no multiplication or development in the vector. This hypothesis of "mechanical" (non-cyclical) transmission remained unchallenged until in 1909 when Kleine, a German investigator working in German East Africa (Tanzania), discovered that

Table 1a: Classification of kinetoplastida (after Molyneux and Ashford, 1983)

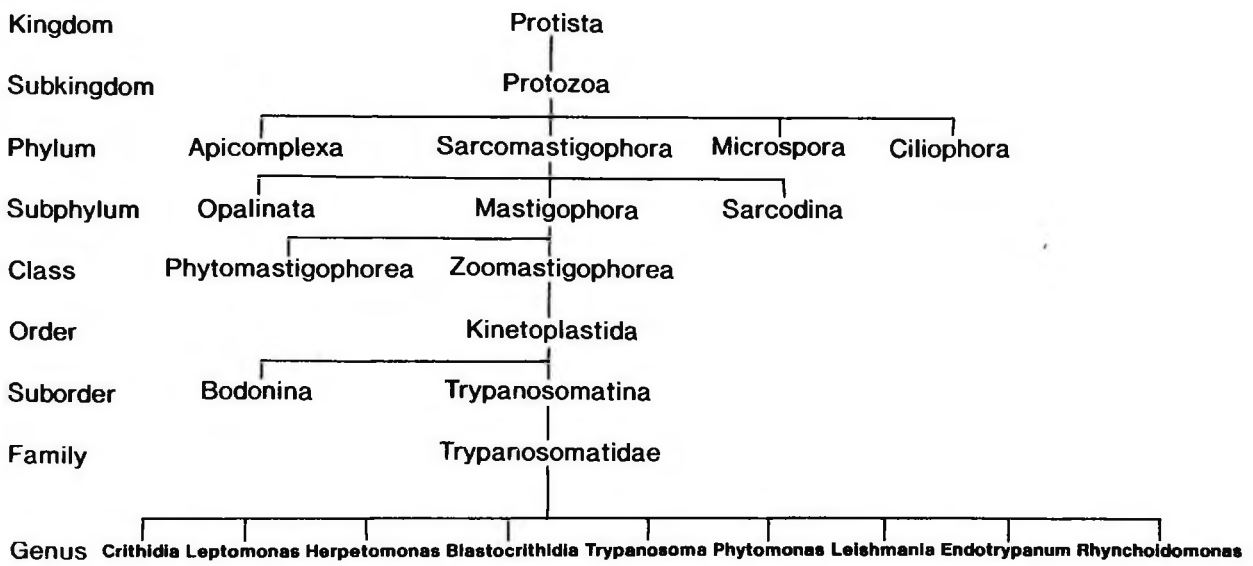
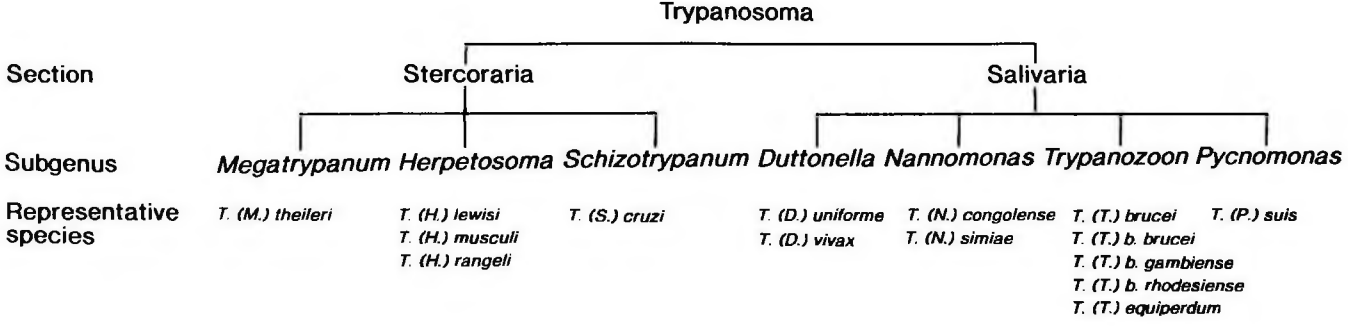


Table 1b: Classification of the genus Trypanosoma (after Molyneux and Ashford, 1983)



*Glossina palpalis* could transmit *T. brucei* 50 days after the fly had taken an infective feed (Bruce, Hamerton, Bateman and Mackie, 1910; Hoare, 1972). His results were confirmed by Bruce and co-workers in 1910, and by Robertson in 1913 for the human parasite, *T. gambiense*. It has since been established that trypanosomes undergo cyclical development in the tsetse fly (Vickerman, Tetley, Hendry and Turner, 1988; Moloo and Gray, 1989).

In Africa trypanosomiasis is an important, frequently fatal, disease of man and livestock, transmitted by tsetse flies (*Glossina spp.*). Indeed, all mammalian trypanosomes occurring in Africa, with the exception of *T. evansi* and *T. equiperdum*, are tsetse-borne, their distribution thus being influenced by the distribution of these flies. There are 30 species and subspecies of the genus *Glossina*, all of which are capable of transmitting trypanosomiasis (Glasgow, 1970; Hoare, 1970). The result, therefore, is trypanosomiasis being endemic throughout much of tsetse (*Glossina*)-infested tropical Africa.

The *Glossina* infest a variety of habitats, ranging from wooded savannah to forest, and the vegetation along banks of rivers and lakes, in 37 countries (FAO/WHO/OIE, 1982). This area of tsetse fly belt, covering about 9-10 million $Km^2$ of land or 30% of the African continent (Molyneux and Ashford, 1983), lies between latitude $15^oN$ and $30^oS$ (Figure 1), and occupies potentially fertile land which, if cleared, could support an additional 120 million head of cattle (Leach and Roberts, 1981; FAO/WHO/OIE, 1983; Jordan, 1986; Hornigberg, 1986). It has further been estimated that, as a result of the extensive distribution of this disease on the continent, Africa produces 70 times less protein per hectare than Europe (Allsopp, Hall and Jones, 1985).

**Figure 1**

The distribution of cattle and tsetse flies in Africa.

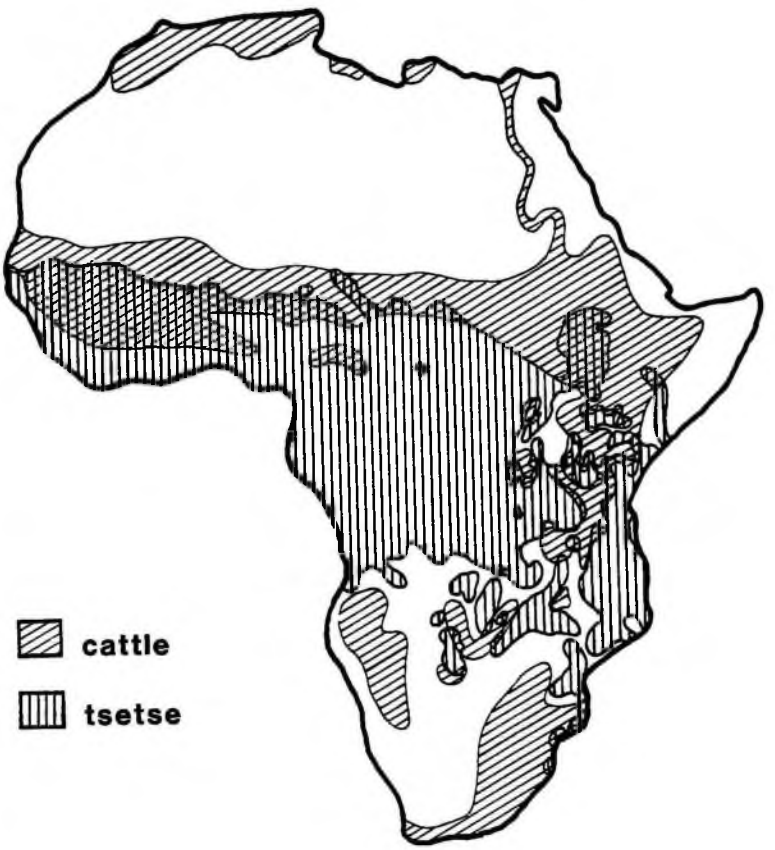



**Figure 1**

The principal tsetse-borne trypanosomes pathogenic to livestock, are *Trypanosoma congolense*, *T. vivax*, *T. brucei brucei* and *T. simiae*. The first three trypanosome species are pathogenic to cattle, sheep and goats, while *T. simiae* affects pigs principally. The disease in man (sleeping sickness) is caused by *T. b. gambiense* and *T. b. rhodesiense*. Trypanosomiasis also occurs outside the tsetse fly belt where, it is transmitted mechanically. The most important trypanosome species in these areas are *T. vivax* and *T. evansi*, transmitted by other haematophagous *Diptera* such as *Tabanidae*, and *T. equiperdum* which is sexually transmitted. *T. evansi* is a major disease pathogen of camels, pigs, water-buffaloes and cattle, whilst *T. equiperdum* affects horses and donkeys.

In animals, African trypanosomiasis is characterized by intermittent fever, fluctuating parasitaemia, profound anaemia, severe loss of appetite, and progressive loss of bodily condition. Growth of young animals is stunted, while adults show decreased fertility, and an increased rate of abortion. Typically, the disease is chronic, extending over several months, often terminating in death if untreated, although cases of spontaneous self-cure have been reported (Nantulya, Musoke, Rurangirwa and Moloo, 1984; Nantulya, Musoke and Moloo, 1986).

In man, the Rhodesian form of the disease, caused by *T. b. rhodesiense*, is usually severe and acute with myocarditis as the main cause of death, whereas the Gambian sleeping sickness, caused by *T. b. gambiense*, is usually mild and chronic. The progress of human trypanosomiasis is characterized by increasing anaemia, leucopoenia and wasting of limb, abdominal and thoracic muscles. The invasion of the meninges and brain by trypanosomes ushers in the sleeping sickness syndrome as evidenced by drowsiness in day time and insomnia at night. In most cases, mental deterioration becomes apparent over a period of months or years.

Since the establishment of the fact that tsetse flies are vectors of human and animal trypanosomiasis, the *Glossina* species have been the target of numerous attempts to control the disease (reviewed by Molyneux, 1982). These measures include: elimination of the tsetse shelter and breeding sites by clearance of bushes (ii) reduction of tsetse food sources by elimination of game animals (iii) hand catching of tsetse flies (iv) the use of tsetse traps aimed at reducing or possibly eliminating fly populations (v) the use of insecticide impregnated screens baited with odour attractants, designed to kill flies on contact (vi) aerial and ground application of insecticides designed to kill tsetse flies in their habitats (vii) genetic control of tsetse populations achieved by the release of sterile males into the natural fly habitat, and (viii) biological control of the flies, using organisms that are predatory or pathogenic to the *Glossina* species. These diverse efforts aimed at controlling trypanosomiasis through the elimination of the vector, have so far achieved limited success. Consequently, the tsetse fly continues to play an important role in the epidemiology of both human and animal trypanosomiasis. The need to identify the proportion of tsetse populations carrying pathogenic trypanosomes as well as the determination of the identity of the infecting trypanosomes can, therefore, not be over emphasized. Such information is needed, for example, to determine trypanosomiasis challenge to man, and livestock.

The standard method for identification of trypanosome infections in the tsetse fly, is by dissection. The infections are then diagnosed at the subgeneric level according to the site of development of the trypanosomes in the vector. Thus, if trypanosomes are found in the gut and salivary glands, the infections are deemed to be due to the *Trypanozoon*. If however, they are found in the gut and proboscis, then they are assumed to belong to the *Nannomonas* subgenus. Again, infections confined to only the midgut are classified as immature *Nannomonas* or *Trypanozoon* (Lloyd and Johnson, 1924), while

infections confined to the proboscis alone would be ascribed to the *Dutonella* subgenus (Figure 2). It is, however, not possible by these criteria to differentiate between the recognized species of trypanosomes within the various subgenera, since all species in a subgenus have identical cycles of development in the vector. The differential diagnosis is further complicated when mixed infections occur. Moreover, attributing tsetse midgut infections to the species of the *Trypanozoon* or *Nannomonas* subgenus could be misleading. This is because the crocodile parasite, *T. grayi*, is also known to infect the midgut of riverine tsetse (Hoare, 1929; McNamara and Snow, 1991).

It has been reported that *T. congolense* and *T. simiae*, in an infected tsetse proboscis, can be differentiated by the arrangement of the parasites in that organ (Janssen and Wijers, 1974). McNamara and Snow (1991) tried this method in The Gambia, and reported that, they could not differentiate *T. simiae* from *T. congolense* by the criterion of arrangement of the trypanosomes in the tsetse proboscis. They attributed this failure to two possible causes: The first was their use of a different fly species (*G. morsitans submorsitans*), Janssen and Wijers (1974) having used *G. pallidipes*, *G. austeni and G. brevipalpis*. The second explanation offered was that it could have been due to mixed infections, an explanation which they, however, thought was unlikely.

Another method used to identify infecting trypanosomes in the vector at the species level is by microscopic examination of stained smears, and identifying the respective organisms by their morphological characteristics. It is, however, known that this technique has many limitations; for example, it is not possible to differentiate between the insect forms of *T. congolense* and those of *T. simiae* by morphological criteria. In the introduction to his monograph, Hoare (1972) indeed drew attention to the limitations of morphological differentiation of trypanosomes, in these words:

**Figure 2**

Venn diagram illustrating the localization of trypanosomes of the different subgenera in the salivary glands, midgut and proboscis of infected tsetse flies:

X = mixed infections involving the *Trypanozoon* and *Duttonella*;

Y = *Trypanozoon* infections with trypanosomes located in the salivary glands and midgut, and with trypanosomes present in the proboscis, especially just after feeding;

Z = trypanosome infections that may also be due to *T. grayi*.

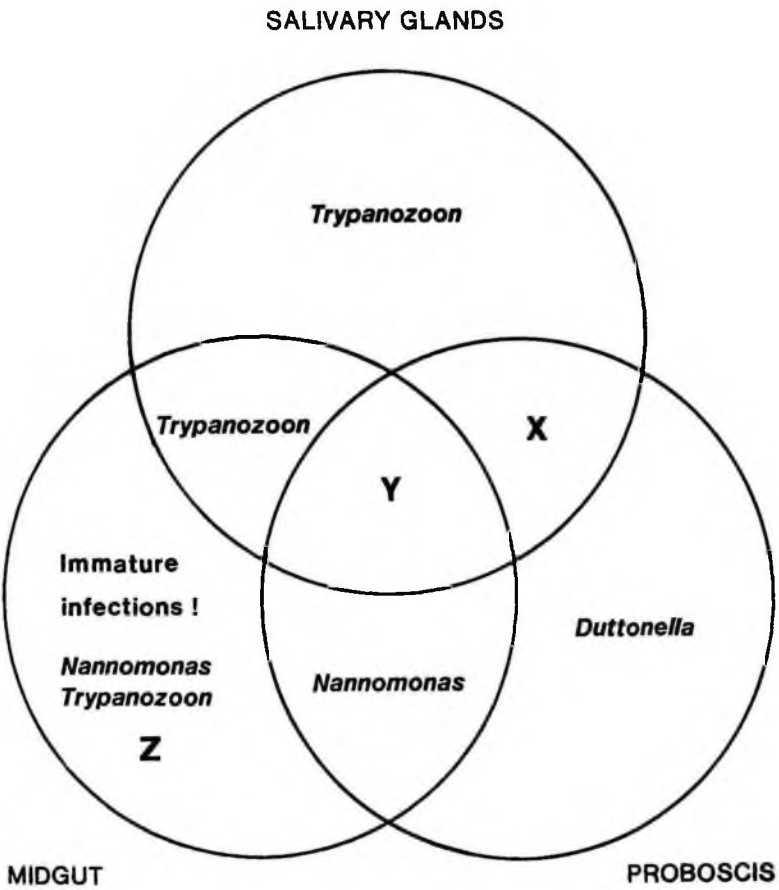



Figure 2

"While the systematics of these trypanosomes serves its purpose adequately in the case of morphologically distinct species, it does not provide objective criteria for the recognition and classification of intraspecific biological variants".

Trypanosome species in the tsetse fly can also be identified by transmission to various animal hosts to determine the host range. By this method, infected tsetse flies are fed on susceptible hosts either by fly bite or by syringe inoculation of trypanosomes isolated from such flies (Moloo and Kutuza, 1974). This procedure has been used to determine the identity of quite a number of trypanosome populations. The procedure also allows the identification of some trypanosome species by examination of bloodstream forms in stained infected blood smears. Several disadvantages, however, limit the regular practice of the method. Firstly, some trypanosome species, and sometimes even strains, exhibit host specificity; such organisms may even fail to grow in the experimental hosts used. Secondly, selective pressure exerted by the *in vivo* environment in the experimental host could lead to suppression of some trypanosome sub-populations. In this way, mixed infections could easily be misidentified. Thirdly, the procedure could be expensive where larger animals, such as cattle, are required, and certainly it would raise moral questions in situations where human volunteers are needed (Corson, 1935; Heish, McMahon and Manson-Barh, 1958).

Another approach used in the identification of trypanosome species is by iso-enzyme analysis of the populations isolated from the vector. By this method it is possible to determine the species identity of some stocks. The approach, however, requires parasites to be isolated and grown in large numbers before characterization could be undertaken (WHO, 1979; Gashumba, Gibson and Opiyo, 1986). This latter requirement is a significant disadvantage

to the routine use of this method for the identification of trypanosome infections. Moreover, the results obtained with this method are not very accurate.

The latest approach to identification of trypanosome species in the vector is the use of recombinant DNA probes (Gibson, Dukes and Gashumba, 1988; Kukla, Majiwa, Young, Moloo and Ole-MoiYoi, 1987; Majiwa and Webster, 1987; Majiwa, 1989). This is a more accurate and more sensitive technique which does not require trypanosomes to be first isolated and grown before identification. To date, these probes have shown that there are five types of *T. congolense* (Kilifi type, West African riverine/forest type, Savannah type, "Godfrey" Type and Tsavo type), recognized by five different probes. It is quite likely, however, that other *T. congolense* types exist which do not react with the five probes, so that a negative result would not necessarily show that a given *Nannomonas* trypanosome population does not belong to the *T. congolense* species. The probes for *T. vivax* have also been found to hybridize with some *T. vivax* stocks (Gardiner, 1989). There is no probe yet that can hybridize with all stocks. Probes have also been derived which can distinguish between *T. simiae* and *T. congolense* in the vector (Majiwa and Webster, 1987), but those for *T. brucei* cannot distinguish between the subspecies.

Despite its high specificity, the use of recombinant DNA probes also has several disadvantages. Firstly, the probes are so specific that they fail to detect some trypanosome populations within the species. Secondly, the technique currently employs radioactive reagents which represent a potential biohazard. Thirdly, the present technique is expensive and not readily applicable in the field. It has thus been suggested that non-radioactive reagents for labelling DNA probes would diminish cost and minimize the biohazard (Langer, Waldrop and Ward, 1981; Leary, Brigati and Ward, 1983; Forster,

McInnes, Skingle and Symons, 1985; Kukla *et al.,* 1987). In this connection, recent advances in the search for non-radioactive probes have indicated that a plant-derived non-radioactive molecule, "digoxigenin", can be used in place of radioactive labels (Holtke, Sagner, Kessler and Schmitz, 1992). However, any DNA test that utilizes this non-radioactive reporter molecule will require field validation.

The need for accurate data on the epidemiology of tsetse-transmitted trypanosomiasis continues to call for the development of simple, accurate and field-applicable assays for identifying trypanosomes in infected *Glossina* species. An attractive possibility would be to develop monoclonal antibody-based assays to directly identify the various species. That this may be a feasible approach is evidenced by the work of Zavala and colleagues (1984) and Petros, Procell, Campbell and Collins (1989) in which malaria sporozoites were detected in mosquitoes by monoclonal antibody-based enzyme immunoassays. Furthermore, it is evident in the literature that nitrocellulose membrane-based enzyme immunoassays could be so designed as to be rapid, easy to perform and interpret, reagent conservative, cost effective and field portable (Pappas, 1988a). Moreover, it has been demonstrated by monoclonal antibody analysis that some of the invariant antigens of trypanosomes are indeed species-specific (Nantulya, Musoke, Rurangirwa, Saigar and Minja, 1987) and that some are expressed by the procyclic (vector) stages of the parasites.

Based on these observations, an investigative study was to be conducted, utilizing monoclonal antibodies as specific reagents, to develop a reliable, field-applicable technique for the detection and identification of trypanosome species in infected *Glossina* species.

## 1.2 Objectives of the study

**1.2.1** To produce and characterize trypanosome species-and subgenus-specific monoclonal antibodies against vector stages of *T. brucei*, *T. vivax*, *T. congolense* and *T. simiae*.

**1.2.2** To determine the usefulness of the monoclonal antibodies so produced as diagnostic reagents by investigating their reactivity with known trypanosome species from different geographical areas.

**1.2.3** To utilize selected monoclonal antibodies in the development of field-applicable assays for identification of *in vitro* propagated insect stages, namely, procyclics and epimastigotes of the tsetse-transmitted trypanosomes.

**1.2.4** To apply the assays developed in detecting and differentiating between the trypanosome species in experimentally-infected tsetse flies, and to standardize the assays.

**1.2.5** To conduct a limited field evaluation of the assays developed in naturally infected tsetse flies.

## 1.3 Justification of study

The development of a simple, sensitive and specific-field applicable test for the diagnosis of trypanosome infection and for the differentiation between trypanosome species in *Glossina*, would provide an important tool for accurate epidemiological studies on *Glossina*-transmitted trypanosomiasis. Such a development or facility would also greatly facilitate the easy estimation of the disease risk in any one location, and thus generally provide for the proper management of trypanosomiasis.

# CHAPTER 2

# LITERATURE REVIEW

## 2.1 The Tsetse Fly and Trypanosomiases

Tsetse is a word, meaning "fly destructive to cattle". As early as 1857, David Livingstone, (cited by Hoare, 1972) suspected that trypanosomiasis of domestic animals (Nagana) was acquired from tsetse flies that fed on game animals. He noted particularly the prevalence of *Glossina morsitans* in the Zambezi basin and wrote: "...the bite of this poisonous insect is certain death to ox, horse and dog..." but "harmless to man and wild animals". However, it was not until 40 years later that the first scientific evidence exposing the involvement of tsetse and wild game in the epidemiology of Nagana was produced (Bruce, 1895). Earlier in 1843, Gruby, a Hungarian scientist (cited by Stephen, 1986) had provided the generic name *Trypanosoma* to haemoflagellate protozoan parasites observed by him and others in amphibians. Bruce (1895) correctly assigned the flagellate parasites he saw in the blood of cattle suffering from Nagana to the genus *Trypanosoma*, and went further to incriminate tsetse flies as the vectors of the disease. Trypanosomes were then thought to be transmitted without multiplication or development in tsetse flies, a hypothesis of "mechanical" (non-cyclical) transmission which survived long after cyclical transmission in the tsetse was demonstrated (Kleine, 1909). Although the term "tsetse" was first applied to *G. morsitans*, it now refers to the entire *Glossina*-species (Austen, 1911).

## 2.2 Systematics, Identification and Distribution of Tsetse Flies

The tsetse flies are two winged insects belonging to the class *Insecta*, order *Diptera*, family *Glossinidae* and genus *Glossina*. Twenty-two species and eight subspecies of tsetse flies (Table 2) belonging to the genus *Glossina* and classified under the three subgeneric groups- *morsitans, palpalis* and *fusca*, are recorded in the literature (Austen, 1903; Ford and Katondo, 1975,1977; Katondo, 1984; Leak and Jeannin, 1984; and Moloo, 1985).

**Table 2:** **Species and subspecies of *Glossina***

| Riverine or *Palpalis* group | Savannah or *Morsitans* group | Forest or *Fusca* group |
|---|---|---|
| *G. palpalis palpalis*[s1] | *G. morsitans morsitans*[s4] | *G. fusca fusca*[s5] |
| *G. palpalis gambiensis*[s1] | *G. morsitans submorsitans*[s4] | *G. fusca congolensis*[s5] |
| *G. tachinoides* | *G. morsitans centralis*[s4] | *G. longipennis* |
| *G. fuscipes fuscipes*[s2] | *G. pallidipes* | *G. tabaniformis* |
| *G. fuscipes quanzensis*[s2] | *G. austeni* | *G. nigrofusca nigrofusca*[s6] |
| *G. fuscipes martinii*[s2] | *G. longipalpis* | *G. nigrofusca hopkinsi*[s6] |
| *G. caligenea* | *G. swynnertoni* | *G. fuscipleuris* |
| *G. pallicera pallicera*[s3] | | *G. brevipalpis* |
| *G. pallicera newsteadi*[s3] | | *G. medicorum* |
| | | *G. haningtoni* |
| | | *G. vanhoofi* |
| | | *G. nashi* |
| | | *G. schwetzi* |
| | | *G. severini* |

[s] subspecies.

Today, tsetse flies are found only in Africa, including inshore islands such as Zanzibar and Fernando Po, but excluding Madagascar and Pemba (Glasgow, 1970). There are two reported cases of tsetse flies outside the African continent. Fossil records of four extinct species of the genus *Glossina*, were discovered in the Florissant beds of Colorado in the United States of America (Buxton, 1955), and estimated to have lived some 35 million years ago (Glasgow, 1970; Gerster, 1986). It is, however, believed that the genus is now extinct in the America's (Glasgow, 1970). Also, Carter (1906) reported the presence of *Glossina tachinoides* in Saudi Arabia. This report, however, has since not been confirmed.

The members of the genus *Glossina* are yellowish or greyish brown, dark brown to almost blackish brown flies (Glasgow, 1970). They vary in length from 7.5 millimetres (measured from the face to the end of the abdomen excluding the proboscis and wings) in the smallest species, such as *G. tachinoides* to as much as 14 millimetres in the largest species such as *G. longipennis* (Austen, 1911; Glasgow, 1970). It is quite easy to identify a tsetse fly when it is in the resting position (Austen, 1911). This identification is possible because when at rest the wings of tsetse lie closed flat over the back, one on top of the other like the blades of a pair of scissors (Figure 3), while the ensheathed proboscis projects horizontally in front of the head. The most certain way to identify the *Glossina*-species is by demonstration of the secondary branching of the hairs of the antenal arista (Figure 4a) and the presence of a hachet-shaped discal cell in each wing (Glasgow, 1960) (See Figure 4b).

The three subgeneric groups (*morsitans*, *palpalis* and *fusca*) into which the tsetse flies are divided are determined by the construction of the male genitalia (Newstead, Evans and Potts, 1924), though differences are known to exist between the groups in other anatomical aspects (Glasgow, 1970). These groups can also be generally defined by the ecological habitats which

**Figure 3**

Diagram showing the position of the wings in a resting tsetse fly, dorsal view. Note the folding of the wings one over the other, like the blades of a pair of scissors.

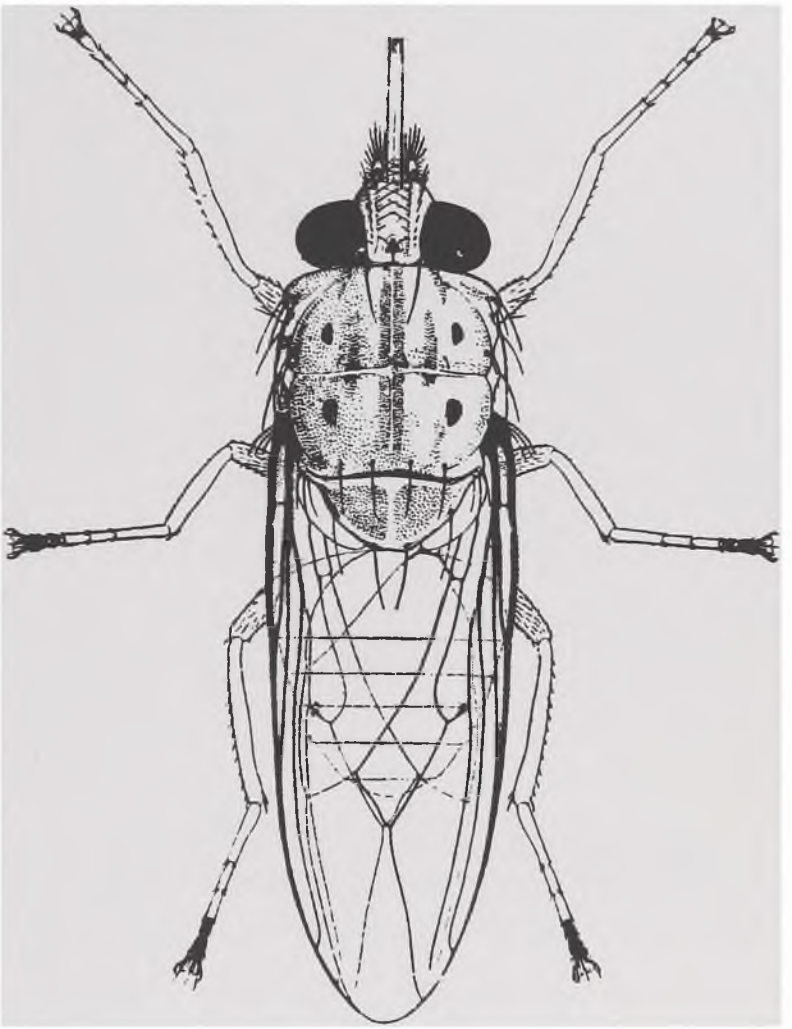


**Figure 3**

**Figure 4a**

Left antenna of a tsetse fly showing the branched arista (After Austen, 1911).

**Figure 4b**

Diagram showing the wing venation of *Glossina*. 1, 2, 3, 4 represent the first, second, third and fourth wing veins, respectively. Note the hatchet shaped cell.

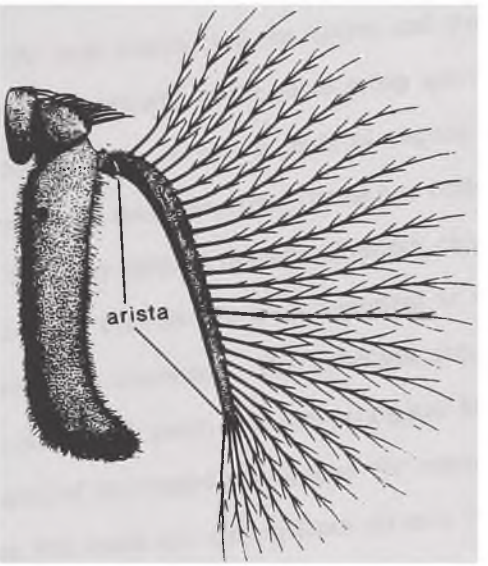



Figure 4a

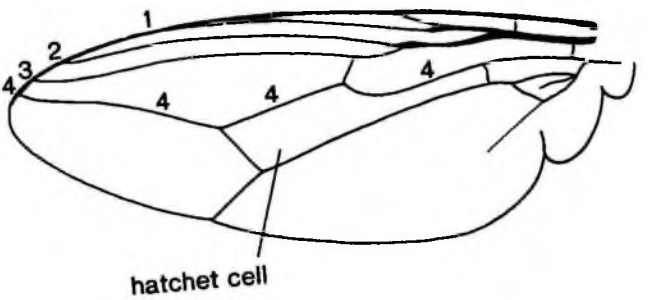



Figure 4b

they inhabit. Thus, species of the *morsitans* group are present throughout much of the savannah (grassy woodlands) of Africa. Their distribution is mainly limited by the cold winter conditions of the south and the hot and dry conditions of the north of West and Central Africa. The *palpalis* group on the other hand is mainly limited to the humid areas of Africa, such as the mangrove swamps, the rain forests, and the shores and the gallery forests along rivers or lakes. The majority of the *fusca* group species are limited to the more thickly forested areas of Africa, such as tropical forests or forest outliers in the savannah. As such, they rarely come into contact with domestic animals and hence feed very little on domestic livestock (Jordan, 1988). The *morsitans* group of tsetse flies are the most important as far as the African animal trypanosomiases are concerned. This is because these species will feed readily on cattle, sheep and goats, and the ecological habitat they occupy coincides with much of the rangelands utilized for animal rearing (Jordan, 1988). The tsetse flies under this circumstance act as a vital vectoral link in the transmission of trypanosomiasis between a wide range of reservoir hosts (mainly wild game) and domestic livestock (Hoare, 1970). Of the six tsetse species that are vectors of sleeping sickness, three (*G. palpalis*, *G. tachinoides* and *G. fusca*) belong to the *palpalis* group and are mainly vectors of the *gambiense* variant, whereas the other three (*G. morsitans, G. swynnertoni* and *G. pallidipes*) of the *morsitans* group are the usual vectors of the *rhodesiense* variant (Buxton, 1955; Rennison, 1956; Nash, 1969; Apted, Ormerod, Smyly, Stronach and Szlamp, 1963; Ashcroft, 1963).

## 2.3 Sources of Tsetse Blood meal

Tsetse flies are obligate haematophagous insects. Host seeking behaviour in tsetse flies has been investigated extensively (Vale, 1974a, 1974b, 1974c; Vale and Hargrove, 1975; Vale, Hargrove, Jordan, Langley and Mews, 1976). This is because the disposition of the vector to feed on the

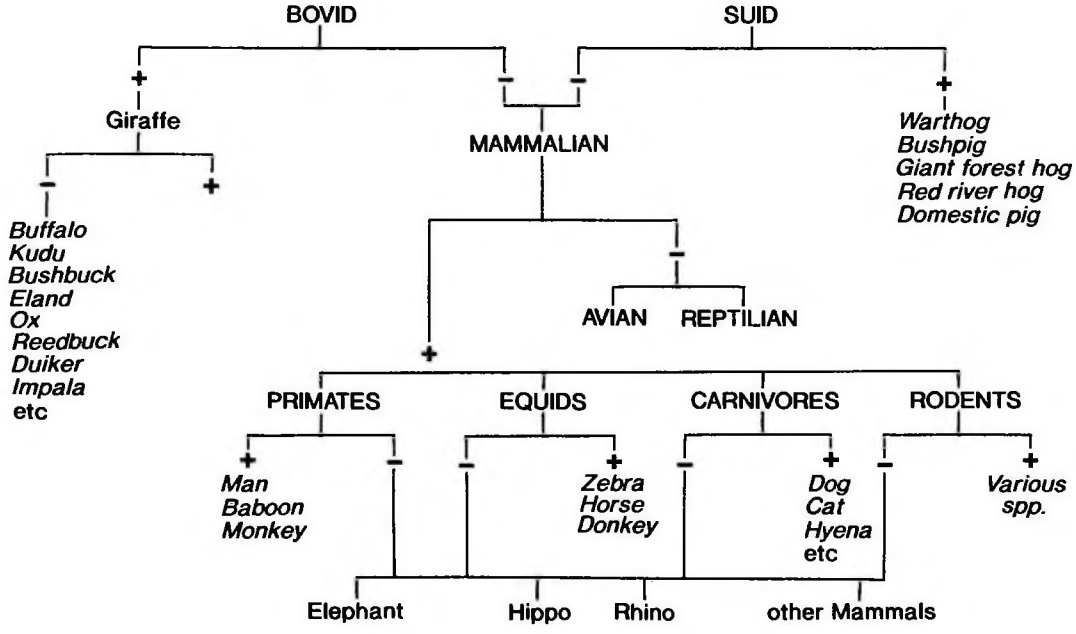
vertebrate host has been considered as one of the most important factors in the epidemiology of trypanosomiasis. It has been reported that the life-cycle of tsetse flies permits a high level of independence in the selection of hosts (Stephen, 1986). Thus, one fly species will feed on a particular type of host animal in one area, but yet on another type of animal in another location (Ashcroft, 1959; Moloo, Kutuza and Boreham, 1980; Madubunyi, 1987). The technique of tsetse blood meal identification, first introduced by Lloyd and Johnson (1924), has permitted researchers to make very important findings in terms of tsetse host preferences (reviewed by Weitz, 1963). It was, for example, for a long time thought that sheep and goats possess natural resistance to trypanosomiasis (Leach and Roberts, 1981). However, through blood meal identification it is now known that these animals are rarely used as sources of food despite their presence in tsetse infested areas (Jordan, Lee-Jones and Weitz, 1962; Leach, 1973). Also, it has been determined by such methods that wild pigs (suids) are a preferred source of blood for several species of *Glossina* (Jackson, 1940; Glasgow, Istherwood, Lee-Jones and Weitz, 1958; Jordan, Lee-Jones and Weitz, 1961). Today, tsetse flies are known to feed on a wide range of domestic and wild animals as determined by tsetse blood meal analysis Table 3.

## 2.4 Feeding and Transmission of Trypanosomes by *Glossina*

A newly emerged tsetse fly is referred to as "teneral" which is, by definition, a tsetse fly that has not yet taken its first blood meal. This means that a teneral tsetse fly will certainly not be infected with trypanosomes, since the parasites are not transmitted in utero (Mulligan, 1970).

The tsetse proboscis is ensheathed in a maxillary palp and projects horizontally in front of the head of the fly when it is not feeding (Austen, 1911; Stephen, 1986).

Table 3: Procedure for identification of tsetse fly blood meals (after Mulligan, 1970)




Antisera used to identify groups by the precipitin test are shown in capitals.
Antisera used to identify species by the precipitin test are shown in roman type.
Antisera used to identify species by the Haemagglutination-Inhibition test are *italicized*.

The proboscis is made up of three parts, the labium, labrum and hypopharynx. The labium, the thickest of the three structures has a large number of small teeth (labellar teeth) at the tip, which are moved by muscles contained in the enlarged base of the proboscis (thecal bulb). The labrum on the other hand forms a tube (food canal) through which blood is sucked during feeding, and the hypopharynx, an extremely narrow tube, is used for pumping saliva into host tissues during feeding.

When a tsetse alights on a suitable host, it disengages the haustellum (labium, labrum and hypopharynx) from the palpi and directs it downwards at a right angle to the skin, and penetration commences. Temperature has been identified as the most important factor required to elicit this feeding reaction (Dethier, 1954). With temperatures substantially above ambient, flies have been successfully induced to probe quite unsuitable surfaces such as glass and paper. The flexible haustellum explores the deeper epidermal layers of the skin, an action associated with copious but intermittent outpouring of saliva through the hypopharynx (Gordon, Crewe and Willett, 1956). This outpouring of saliva continues throughout the probing of the tissues and even as extravasated blood is sucked into the food canal by the action of the cibarial pump, and the fly starts to engorge (Gordon and Crewe, 1948; Stephen, 1986). It is during this issue of saliva that salivarian trypanosomes are introduced into host tissues by an infected tsetse fly (Hoare, 1972; Stephen, 1986). The salivary secretion of tsetse flies has been shown to contain a highly efficient anticoagulant (Lester and Lloyd, 1928) which was later identified as an antithrombin (Hawkins, 1966; Parker and Mant, 1979), and isolated and characterized from *G. morsitans centralis* by Klickstein, Moloo and ole-MoiYoi (1985). This anticoagulant facilitates the passage of blood through the fine tubes of the foregut of the fly (Bursell, 1970), and together with five fibrinolytic proteases, they prevent clotting of the blood during

storage in the crop (Hawkins, 1966; Parker and Mant, 1979; Endege, Lonsdale-Eccles, Olembo, Moloo and ole-MoiYoi, 1989).

## 2.5 Transformation and Cyclical Development of Trypanosomes in Tsetse Flies

### 2.5.1 The Trypanosomes

The genus *Trypanosoma* is subdivided into two sections (introduced by Hoare, 1964): (a) salivaria and (b) stercoraria. These divisions are based on the site of development of the organism in the vector and on the mode of transmission to the vertebrate host. The salivarian African trypanosomes (Table 4) complete their development in the mouthparts of the insect vector and transmission is inoculative (Hoare, 1972; Leach and Roberts, 1981), whereas, the stercorarian group of trypanosomes complete their development in the faecal medium of the hind gut of the vector and transmission is contaminative.

The mammalian trypanosomes that develop in tsetse are: the *Trypanozoon* (namely, *T.* (*T.*) *b. gambiense, T.* (*T.*) *b. rhodesiense* and *T.* (*T.*) *b. brucei*); the *Pycnomonas* (*T.* (*P.*) suis); the *Duttonella* (namely, *T.* (*D.*) *vivax* and *T.* (*D.*) *uniforme*); and the *Nannomonas* (namely, *T.* (*N.*) *congolense* and *T.* (*N.*) *simiae*) (See Table 1b). The crocodile parasite, *T. grayi*, is also tsetse-borne; its developmental stages in the fly must, therefore, be recognised in order to avoid confusing it with other trypanosomes sharing the same vector.

### 2.5.2 Transformation of Trypomastigotes into Procyclics in *Glossina*

An uninfected tsetse fly feeding on an infected host, ingests trypanosomes contained in the blood meal. These trypanosomes undergo a series of divisions and differentiation termed "cyclical development" in the vector (Figure 5). In the case of the *Trypanozoon* and the *Nannomonas*,

**Table 4: The salivarian and stercorarian African trypanosomes (After Mulligan, 1970)**

| Subgenera | Species | Localization in fly | | | |
|---|---|---|---|---|---|
| | | Proboscis | Salivary glands | Midgut | Hindgut |
| *Duttonella* | *T. vivax*<br>*T. uniforme* | Trypomastigotes, epimastigotes and meta-trypanosomes | No development | No development | No development |
| *Nannomonas* | *T. congolense*<br>*T. simiae* | Trypomastigotes epimastigotes and meta-trypanosomes | No development | Trypomastigotes | No development |
| *Trypanozoon* | *T. brucei*<br>*T. rhodesiense*<br>*T. gambiense* | No development | Trypomastigotes epimastigotes and meta-trypanosomes | Trypomastigotes | No development |
| *Pycnomonas* | *T. suis* | No development | Trypomastigotes epimastigotes and meta-trypanosomes | Trypomastigotes | No development |
| Crocodile parasite | *T. grayi** | No development | No development | Epimastigotes (including filamental forms) and trypo-mastigotes | Epimastigotes and meta-trypanosomes |

* stercorarian trypanosome

## Figure 5

Diagram illustrating the life cycles of the three major tsetse-transmitted African trypanosomes, in the mammalian host and the tsetse fly vector.

Heavy outlines indicate mammalian infective parasite forms. Light outlines indicate uncoated vector stages which do not infect mammals. *Trypanosoma b. brucei* develops in the tsetse midgut, proventriculus and salivary glands, where infective metacyclic forms are produced. Parasite development in the fly takes 3 to 5 weeks. *Trypanosoma congolense* develops over 2 to 4 weeks in the tsetse midgut, proventriculus and mouthparts, where infective metacyclic forms are produced. *Trypanosoma vivax* develops entirely in tsetse mouthparts. The developmental cycle may be as short as 10 days.

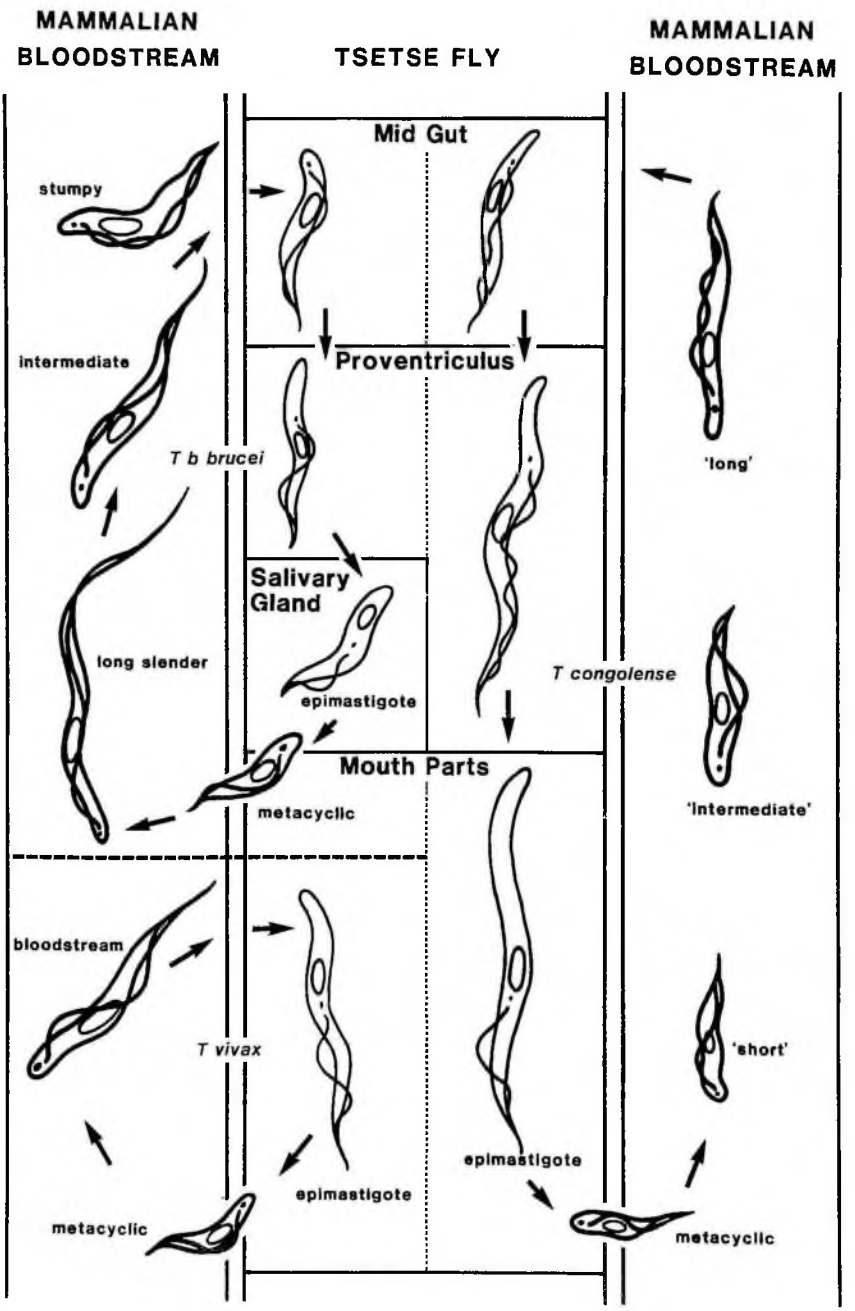



Table 5

transformation of trypomastigotes to procyclic midgut forms in the fly commences immediately upon ingestion of an infective blood meal (Vickerman, 1965; Vickerman and Preston, 1976; Englund, Hadjuk and Marini, 1982). The transformation processes involve a series of morphological and physiological changes that include: a loss of the surface coat; cessation of the synthesis of the variable surface glycoprotein; reticulation of the mitochondria, and activation of mitochondrial enzymes and the cytochrome electron-transport system; induction of procyclin synthesis; and displacement of the positions of the nucleus and kinetoplast (Vickerman and Preston, 1976; Barry and Vickerman, 1979; Ghiotto, Brun, Jenni and Hecker, 1979; Frevert, Herzberg, Reinwald and Risse, 1986; Roditi and Pearson, 1990). These changes enable the trypanosome to adapt to the new environment in the fly midgut.

### 2.5.3 Cyclical Development of the *T. brucei* subgroup

The human pathogens, *T. b. gambiense* and *T. b. rhodesiense*, and the animal pathogen, *T. b. brucei* (one of the three species that cause the disease "Nagana" in cattle), have a similar course of development in the vector. When a tsetse fly feeds on an animal infected with the pleomorphic *brucei*-type trypanosomes, it ingests trypomastigotes contained in the blood meal. The ingested organisms may consist of some long forms, intermediate forms and short stumpy forms (Hoare, 1956; Stephen, 1986). It has been determined that only the short stumpy forms, and possibly some intermediate forms with well developed mitochondria containing many cristae, are capable of establishing infections in the fly (Stephen, 1986). These bloodstream forms of the parasites undergo active transformation and division in the midgut as large procyclic trypomastigotes, which further penetrate the peritrophic membrane (PTM) of the gut to reach the ectoperitrophic space where they migrate forward to the proventriculus and cease dividing to become elongate mesocyclic

trypomastigotes. In this form, the parasites traverse the PTM and migrate via the oesophagus, proboscis lumen, and hypopharynx to the vector's salivary glands. Here, the parasites assume the epimastigote form and undergo multiplication during which they are anchored by their flagella to the vector's salivary gland epithelium by a process involving the production of flagellopodia over and around salivary gland microvilli (Molyneux, 1977; 1983; Tetley and Vickerman, 1985; Molyneux and Jefferies, 1986). The epimastigotes then differentiate into the free, non-dividing metacyclic trypomastigotes (Vickerman *et. al.,* 1988) which alone among the fly forms, can infect a mammal. The entire developmental cycle takes 3-5 weeks (Robertson, 1913; Lloyd and Johnson, 1924; Vickerman *et al.,* 1988). Infections with *brucei*-type trypanosomes, therefore, localize in the midgut and salivary glands of infected tsetse flies. However, the discovery by Mshelbwala (1972) that *brucei*-type trypanosomes infect the haemolymph of *Glossina* (confirmed by Otieno, 1973; Otieno and Darji, 1977) and the ability of these trypanosomes to penetrate the midgut wall and into the haemolymph (Evans and Ellis, 1975) have clearly indicated that cyclical development of the *brucei*-group of trypanosomes is not yet fully elucidated. Thus, as questioned by WHO (1979), it is possible that *brucei*-type trypanosomes infecting the haemolymph invade the salivary glands by other routes.

### 2.5.4 Cyclical Development of *T. suis*

*Trypanosoma (P.) suis*, a pathogen of pigs, is the only known species in the subgenus *Pycnomonas*. It is reported that this organism has stages of development in the vector that are similar to that of the *brucei*-group of trypanosomes (Peel and Chardome, 1954; Hoare, 1972). Peel and Chardome (1954) had shown that procyclics of *T. (P.) suis* develop in the intestine and proventriculus of *G. brevipalpis*. Later, some proventricular forms differentiate and assume the epimastigote form which then pass forward to the

salivary glands. The labial cavity is later invaded by these organisms from the salivary glands, and the cycle completed by the appearance of metatrypanosomes in the hypopharynx. This trypanosome (*T. suis*) has, however, been found on only four separate occasions (Ochmann, 1905; Geisler, 1912; Peel and Chardome, 1954; and Janssen and Wijers, as reported by Stephen, 1986). As a result, a detailed description of its life-cycle in the tsetse fly has not been reported in the literature.

### 2.5.5 Cyclical Development of *T. congolense* and *T. simiae*

The two species of the *Nannomonas* subgenus (*T. congolense* and *T. simiae*) have an identical cycle of development in the tsetse fly. Their cyclical development is similar to that of the *brucei* group of organisms, except that the elongate mesocyclic trypomastigotes do not proceed to the salivary glands. Instead, the epimastigotes multiply, attached to the chitinous wall of the food canal (labrum) and the premetacyclic trypomastigotes swim to the hypopharynx where they mature into metacyclics. Kaddu and Mutinga (1980) reported the penetration of the midgut cells of *G. pallidipes* by *T. congolense* organisms. A similar phenomenon in *T. brucei* infections is now known to lead to *brucei*-type trypanosome infections in the fly haemolymph. So far, however, no such occurrence has been reported in *T. congolense* infections in the tsetse fly. The development of *T. congolense* in *Glossina* from the date of the first infected blood meal to the first infective metacyclics is about 15 days (Harley and Wilson, 1968; Nantulya, Doyle and Jenni, 1978).

### 2.5.6 Cyclical Development of *T. vivax*

*T. vivax*, the other major tsetse-borne African trypanosome, omits the fly midgut phase altogether. Vickerman and others (1988) suggested that the vector procyclic phase of *T. vivax* possibly occurred deep in the foregut (cibarium) and quickly transformed into epimastigotes which invade the

proboscis by attaching to the inner wall of the labrum. The dividing epimastigotes then generate metacyclics in the same manner as the species of the *Nannomonas* subgenus (Vickerman, *et al.*, 1988; Jefferies, Helfrich and Molyneux, 1987). In a later study, Moloo and Gray (1989) confirmed the earlier hypothesis that cyclical development of *T. vivax* was initiated at sites other than the proboscis. They observed *T. vivax* trypomastigotes, pre-epimastigotes and epimastigotes in the cibarium/oesophageal region of the tsetse fly, between 1 and 48 hrs after an infective feed, and concluded that the parasites migrated from that region to the labrum where they established an active infection that matured with infective metatrypanosomes in the hypopharynx. The time lapse between ingestion of bloodstream trypanosomes and extrusion of metacyclics in this species may be as short as 10 days.

### 2.5.7 Cyclical Development of *T. grayi*

*Trypanosoma grayi*, the crocodile parasite, is a stercorarian trypanosome. Upon ingestion, the bloodstream forms of this parasite differentiate and transform to procyclic stages in the midgut of the fly. These trypanosomes are confined to the midgut lumen by the PTM for two to three days after an infective meal. They then escape from the peritrophic membrane by migrating to the hind gut where they take up permanent residence between the PTM and the gut wall by six to eight days after the infective meal, and mature with the appearance of metatrypanosomes in the hindgut (Hoare, 1931).

## 2.6 Differentiation between Vector Stage Trypanosomes by Morphological and Mensural Criteria

While the trypanosomes in the mammalian host and some of the infective metatrypanosomes in the insect can be identified to the species level by morphological and mensural criteria (Table 5 and Figure 6), the procyclic

**Table 5: Some morphological differences of African trypanosomes**

| Subgenera | Species | Size | Morphological Characteristics | | |
|---|---|---|---|---|---|
| | | | Kinetoplast | Free flagellum | Undulating membrane |
| *Duttonella* | *T. vivax*<br>*T. uniforme* | 18-31$\mu$ | kinetoplast large and terminally placed | Present in all stages | Inconspicuous |
| *Nannomonas* | *T. congolense*<br>*T. simiae* | 8-24$\mu$ | Kinetoplast medium and marginal and subterminal | Absent in all stages | Inconspicuous |
| *Trypanozoon* | *T. b. brucei*<br>*T. b. rhodesiense*<br>*T. b. gambiense* | 17-27$\mu$ | Kinetoplast small, subterminal | Present in all stages except infective forms | Conspicuous |
| *Pycnomonas* | *T. suis* | 9-19$\mu$ | Kinetoplast small, sub-terminal and marginal | Present in all stages | Inconspicuous |

## Figure 6

Diagram showing trypanosome mensural data (After Stephen, 1986):

L = total parasite length including the free flagellum,

P-K = distance from the posterior end of the parasite to the kinetoplast,

K-N/2 = distance from the kinetoplast to the mid- point of the nucleus,

P-N/2 = distance from the posterior end of the parasite to the mid-point of the nucleus,

N/2-A = distance from the mid-point of the nucleus to the anterior end of the body,

F = length of the flagellum.

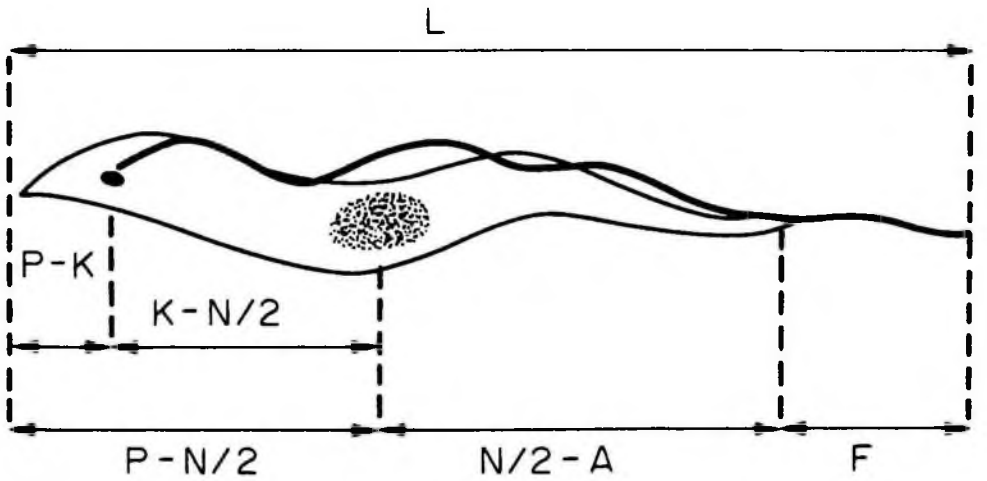



Figure 6

insect forms in the vector are practically indistinguishable by these methods even when there are marked differences between the bloodstream forms (Hoare, 1970; Godfrey, 1977; Stephen, 1986). Thus, though morphology is correctly identified as an intrinsic character (Lumsden, 1974), its successful application in the differential diagnosis of the procyclic vector stages of trypanosomes is still awaited. For this reason, the method currently used for detecting trypanosomes in tsetse in routine epidemiological surveys, relies mainly on the location of the trypanosomes in the vector for differential diagnosis at the subgeneric level (Hoare, 1970; McNamara and Snow, 1991). The disposition of *T. vivax (Duttonella), T. congolense (Nannomonas)* and *T. brucei (Trypanozoon)* in *Glossina*, is shown in Figure 7.

## 2.7 Digestion in *Glossina*

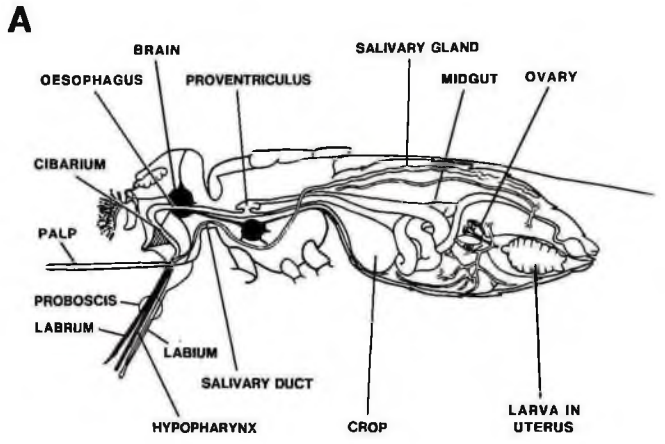
In the *Glossina*-species, the blood meal is first directed to the anterior portion of the midgut. When this portion is filled to capacity, the intake is then diverted to the oesophageal diverticulum (crop), which distends enormously to accommodate a blood meal that may weigh considerably more than the insect itself (Gerster, 1986). The blood in the crop does not clot and no absorption takes place there. The discharge of material from the anus of tsetse flies during and just after feeding has been observed (Lester and Lloyd, 1928). It is known that the crocodile parasite (*T. grayi*) is transmitted by tsetse flies through faecal contamination by this process (Hoare, 1972).

The digestive tract of tsetse flies is made up of: the oesophagus; proventriculus; crop; anterior, middle and posterior midguts; the hindgut; and rectum (Figure 8). The blood in the midgut becomes localised in three sections which were identified by Wigglesworth (1929). In the anterior region, the blood is concentrated by the removal of water without digestion of the blood components. By 2 hr 30 min after feeding most of the blood in the

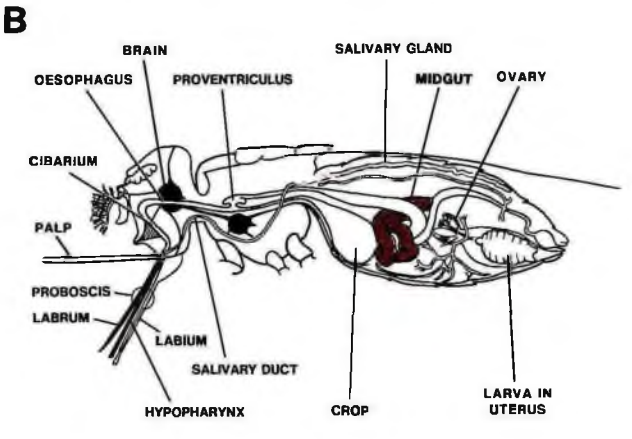
## Figure 7 (A, B and C)

Diagram illustrating the disposition of the three major salivarian African trypanosomes in infected *Glossina*.

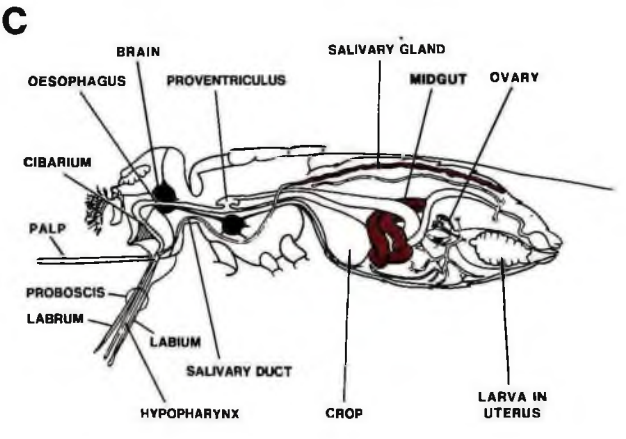
The red colour indicates the presence of trypanosomes. Localization of *T. vivax* in the mouthparts (A); Localization of *T. congolense* in the mouthparts and midgut (B); Localization of *T. brucei* in the salivary glands and midgut (C).




*Trypanosoma vivax* in *Glossina*




*Trypanosoma congolense* in *Glossina*




*Trypanosoma brucei* in *Glossina*

**Figure 8**

Diagram showing the different sections of the digestive tract of *Glossina*.

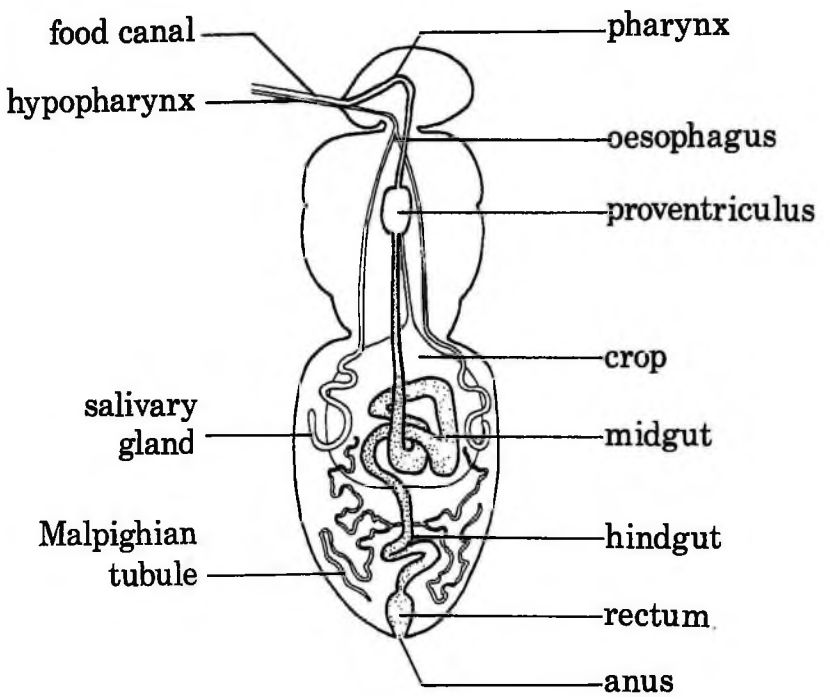



**Figure 8**

crop is passed into the midgut. Blood in the second section of the midgut turns dark and becomes amorphous, whilst the unclotted blood at the anterior section remains bright red. By 24 hours, the crop is empty and the blood meal in the midgut is again divided into regions by folds in the gut. The posterior region has regular, columnar epithelial cells which become vacuolated late in the digestive process and are believed to play a role in absorption (Gooding, 1972). By 48 hours the blood in the gut greatly decreases and the dark mass progressively contracts posteriorly (Lester and Lloyd, 1928).

It was reported by Bursell (1970) that haematin is split off from the haemoglobin molecule during the early stages of digestion in tsetse flies, but it is not absorbed from the gut. The wastes in the faeces of tsetse flies, therefore, include haematin, and other substances, such as, pigment, uric acid, histidine and arginine (Bursell, 1965).

Investigations into the rate of digestion indicate that wild tsetse flies tend to digest their blood meals faster than laboratory colonized flies. Weitz and Buxton (1953) determined the rate of digestion of blood meals of various haematophagous arthropods using the precipitin test. They found that laboratory reared *G. morsitans* fed on man, ox, sheep or goat were all positive for precipitin reactions after 2 days and on the third day gave 100%, 100%, 75% and 90% positive reactions, respectively. In contrast, wild caught *G. swynnertoni* estimated to have fed 3 days earlier, gave only 28% positives and those estimated as having fed four days earlier gave only 7% positives. Also, Langley (1967a) reported that field-caught male and female *G. morsitans* feeding on oxen, digest their blood meals more rapidly than laboratory reared males or females feeding on bovine or guinea-pig blood. Langley (1967b) reported that *G. morsitans* feeding on ox blood in the field, excrete their blood meals more rapidly than field-caught males feeding upon guinea-pigs in the laboratory. Furthermore, Langley (1967b) published the results of an

experiment in which field-caught male tsetse flies, fed several times on guinea-pigs in the laboratory, were shown to digest each meal more slowly than the preceding one, until by the third meal, the rate of digestion was just slightly greater than that of laboratory-reared flies. These differences in the rate of digestion led Langley (1966) to propose that the feeding behaviour of the flies affected the subsequent rate of digestion. This proposition was, however, questioned by Gooding (1972) when he pointed out that non-teneral field-caught male tsetse feeding upon guinea-pigs digest this meal at the same rate when held in continuous light as opposed to total darkness, even though the flies were less active in total darkness.

## 2.8 Mechanical Transmission of the African Trypanosomiases

It has been necessary to determine the role played by tsetse flies in comparison to other biting flies in the transmission of the African trypanosomiases. This is because control of the disease centres around the use of chemotherapy and vector control by tsetse population reduction or elimination (reviewed by Molyneux, 1982). Mechanical transmission of the salivarian trypanosomes is believed to involve a few genera of the haematophagous *Diptera*, namely; *Stomoxys*, *Haematopota*, *Chrysops* and *Lyperosa*. Among these, Mahmoud and Gray (1980) considered the *Tabanus* (family *Tabanidae*, genus *Haematopota*) as most important since their mouthparts are suited for protecting bloodfilms, and since they inflict very painful bites which increase the frequency at which their feeding is interrupted, thereby facilitating the quick transfer of trypanosomes from one host to another as they seek to complete their meals (reviewed by Luckins, 1988). Jordan (1986), however, argued that although mechanical transmission of the African animal trypanosomiasis may be important in some localities, there is no evidence that the disease can persist in the absence of the tsetse fly. Data collected from extensive areas of northern Nigeria have provided support for

the conclusion that tsetse-transmitted trypanosomiasis cannot be maintained in the absence of *Glossina*-species. Jordan (1986) reported that in these areas tsetse flies have been eradicated by the use of persistent insecticide spraying, backed by consolidation of reclaimed land through intensive human use. As a result, trypanosomiasis is no longer a significant problem (Putt, Shaw, Mathewman, Bourn, Underwood, James, Hallam and Ellis, 1980). Difficulties in detecting very low density tsetse populations was given by Wells (1972) as one of two reasons contributing to the erroneous conclusion that trypanosomiasis exists in some localities where tsetse are apparently absent. The other reason being the movement of animals from tsetse infested to tsetse free areas, coupled with the recrudescence of parasitaemia in chronically infected animals (Bennett, 1933; Jordan, 1986).

## 2.9 Factors that Govern the Establishment of Trypanosomes in *Glossina*

For a long time it was assumed that the relationship between tsetse flies and the trypanosomes they transmit was largely determined by factors beyond the control of the fly. Trypanosome infection rates in the fly were then thought to be governed mainly by the availability of infected hosts, which was reported by Jordan (1965) as the most important environmental factor influencing particularly *T. vivax* infections in the vector. Though an infective feed is an essential prerequisite for flies to be infected, high levels of host infection has not been observed to lead to high infection rates in wild tsetse flies. Harley (1966) and Harley and Wilson (1968) published the results of an experiment in which pupae of *G. pallidipes* and *G. fuscipes* were collected from the field in Uganda, and the emergent flies fed on animals infected with *T. congolense*. He observed that the trypanosome infection rates obtained in these flies, all of which had taken an infective feed, were not considerably greater than those of wild flies of the same two species caught from the same

location. These results suggested that some tsetse flies (some members of the same species) possess an intrinsic refractoriness to trypanosome infections. The importance of these intrinsic fly factors in determining their susceptibility to trypanosome infections was further demonstrated by Maudlin and Dukes (1985), when they successfully selected two lines of *G. morsitans morsitans*, one of which was susceptible and the other refractory to infection with a single procyclic stock of *T. congolense*.

Varying degrees of *Glossina*-species susceptibility to trypanosome infections have been reported by many workers (Robertson, 1912; Taylor, 1932; Duke, 1933; Godfrey, 1961; Roberts and Gray, 1972; Moloo *et al.* 1980; Ibrahim, Ingram and Molyneux, 1984; Vickerman, 1985; Madubunyi, 1987; Maudlin and Welburn, 1987; Moloo and Kutuza, 1988; Maudlin, 1991). As a result of these investigations, several factors that determine whether a pathogenic trypanosome will complete it's cycle of development from trypomastigotes in a blood meal to metatrypanosomes in the saliva of an infected tsetse fly have been identified (Stephen, 1986). Of these, intrinsic factors that relate to the inate constitution of the fly and the trypanosome, and extrinsic factors, that are mainly environmental, such as mean ambient temperature (Ford and Leggate, 1961; Moloo, Kutuza and Boreham, 1980), principal food source of tsetse species (Jordan, 1965) and variation in infectibility of different tsetse species (Roberts and Gray, 1972), have been reported to be of importance. The need to identify and understand the intrinsic factors associated with the fly, led to research into the genetic and physiological basis of the susceptibility of tsetse to trypanosome infections (Maudlin, 1972; Maudlin, 1985; Maudlin and Dukes, 1985). Initial studies by Maudlin (1972) on selection for susceptibility in *Glossina* indicated that *T.* (*N.*) *congolense* and *T.* (*T.*) *brucei* receptivity in the fly is controlled by a stable trait which was maternally inherited (Maudlin and Dukes, 1985; Maudlin,

Dukes, Luckins and Hudson, 1986). Maudlin and Ellis (1985) and Jennings and Urquhart (1985) associated this inherited character with the presence of rickettsia-like-organisms (RLOs) in the egg cytoplasm of the fly.

The RLOs are typical rod-shaped bacteria which occur within the cytoplasm of midgut cells of some tsetse flies, including *G. morsitans morsitans, G. morsitans centralis, G. pallidipes, G. fuscipes fuscipes, G. brevipalpis, G. palpalis palpalis* and *G. nigrofusca* (Reinhardt, Steiger and Hecker, 1972; Huebuer and Davey, 1974; Pinnock and Hess, 1974; Roberts and Pell, 1976; Hecker and Moloo, 1981; Moloo and Shaw, 1989; Maudlin, Welburn and Mehlitz, 1990). These RLOs appear to cause no obvious pathology in the fly (Shaw and Moloo, 1991).

Studies by Croft, East and Molyneux (1982) and East, Molyneux, Maudlin and Dukes (1983) have also revealed that *Glossina* haemolymph has anti-trypanosomal properties specific to salivarian pathogenic trypanosomes and not to other trypanosomes or flagellates. From another study, Maudlin and Ellis (1985) found that of 40 *T. congolense* "susceptible" lines of *G. morsitans morsitans*, 36 had RLOs within their midgut epithelium, compared with only 7 out of 40 "refractory" lines of the same fly species. Based on this observation, Maudlin (1985) suggested that the midgut RLOs might inhibit in some way the anti-trypanosome factors in the midgut and haemolymph, possibly lectins, leading to the establishment of *T. congolense* infections in the tsetse midgut. Welburn and Maudlin (1989) and Welburn and Maudlin (1990) subsequently demonstrated that killing of trypanosomes by lectins is proportional to the number of RLOs in the tsetse midgut. Also, Welburn and Maudlin (1991) have recently reported a quantitative relationship between RLOs and susceptibility in which teneral flies with heavier RLO infections were found to be more susceptible to trypanosome infections. In addition, Ingram and Molyneux (1988) have identified the specificities of various

*Glossina* lectin-like molecules, and partially characterized a haemolymph lectin from *G. fuscipes*. It has also been discovered that *Glossina* haemolymph lectin is involved in signalling the maturation process of trypanosomes infecting the fly midgut (Maudlin and Welburn, 1988; Welburn and Maudlin, 1989; Welburn and Maudlin, 1990). RLOs influence trypanosome establishment in the fly midgut by producing a chitinase that generates glucosamine which, in turn, inhibits the trypanocidal activity of the lectins (Hawking, 1977). Excess uninhibited midgut or haemolymph lectin may, however, stimulate the trypanosomes that establish in the fly midgut to mature (Matthyssens *et al.*, 1987). However, upon comparing the presence of midgut RLOs with sucseptibility to *T. congolense* and *T. brucei* infections in *G. morsitans centralis*, Moloo and Shaw (1989) and Shaw and Moloo (1991) were unable to positively correlate RLO occurrence with susceptibility to infection of tsetse with those two trypanosome species, indicating that the RLO story is not yet complete.

Evidence has been provided by Maudlin (1991) that infections of *Glossina* salivary glands with *Trypanozoon* organisms is controlled by a sex-linked recessive character, promoted by a gene on the X chromosome. He further suggested that the gene products may be the haemolymph lectin which Welburn and Maudlin (1990) reported to control trypanosome maturation. So far, no genetic mechanism has been identified to regulate the number of midgut RLOs in tsetse flies. It is, therefore, not known whether the number is determined only by random variations in the number of RLOs transmitted from a female to her offspring; or by environmental factors such as temperature during puparial life; or availability of blood meals (Brun and Jenni, 1987; Gooding, 1972).

Apart from RLOs and lectins, other tsetse midgut factors have been identified to operate against the establishment and transformation of trypanosomes in the fly. These include trypsin or a trypsin-like enzyme which

is essential for transformation of *T. b. brucei* trypomastigotes into procyclic (midgut) forms *in vitro* (Yabu and Takayanagi, 1988). Others include midgut trypanolysin and trypanoagglutinins that act against the initial establishment of the ingested parasites (Molyneux and Stiles, 1991). Also, viral particles that may occur in the tsetse gut have been reported to have an influence on trypanosome infections in the fly, and their presence seems to induce resistance to infections (Clair, 1988). Furthermore, *Glossina* species are known to be susceptible to infection by bacteria, fungi, helminths and even other protozoa (reviewed by Nash, 1970). In the presence of certain bacteria for example there is a tendency for the fly gut flagellates to disappear (Peel and Chardome, 1954).

Both cellular and humoral immunity have been reported in insects (reviewed by Lackie, 1988). The implications of invertebrate immunity for vector-borne parasites was clearly communicated by this author when he wrote: "Vector-borne parasites must cope with the immune defences both of their vertebrate and invertebrate hosts; their survival in insects is just as remarkable and may require just such elegant evasion mechanisms as are necessary in the definitive host". Lackie (1988) divided the mechanisms of insect immunity into three categories: (1) melanization and haemocytic encapsulation of small biotic particles such as bacteria and protozoa through phagocytism and/or entrapment within haemocytic aggregates (2) haemocytic killing mechanisms such as may be exerted by lysosomal "marker" enzymes, and (3) immunorecognition as demonstrated by haemocytic response towards cuticular grafts, organ implants, or transfused haemolymph (Lackie, 1986), and by the discovery of an insect immune protein (Sun, Lindstrom, Boman, Faye and Schmidt, 1990).

Kaaya and Darji (1988) reported that the humoral defence system of tsetse flies differs in response due to age, sex and antigen types. In their

studies, these authors demonstrated higher antibacterial immune response in female than in male *G. morsitans morsitans*. They further showed that inoculation of live *T. b. brucei* or *T. congolense* into tsetse did not induce production of lysozyme or antibacterial activity. However, tsetse so inoculated or naturally infected with *T. b. brucei* or *T. congolense* did not show any evidence of immunosupression when challenged with live *Escherichia coli*. These results may suggest that tsetse humoral immune defence mechanisms against trypanosomes are not as elaborate as those against bacteria.

Other fly factors that influence the suitability of tsetse as vectors of trypanosomes are: the age of the fly at which it ingests its first infective blood meal and the principal food source of the tsetse species. It is generally acknowledged that feeding tsetse on non-infected blood prior to an infective meal considerably reduces the chances of establishing mature infections in the vector (Robertson, 1912; 1913; Van Hoof, Henrard and Peel, 1937; Wijers, 1958; Ward, 1968; Clarke, 1969). This is in part attributed to the physiological status of the peritrophic membrane (reviewed by Langley, 1977). Wigglesworth (1929) had long ago observed that in newly-emerged *G. morsitans, G. tachinoides* and *G. palpalis*, the peritrophic membrane was ragged and discontinuous. It became well formed immediately after the first blood meal and contained the entire meal, suggesting that if trypanosomes were present, they could more readily traverse the endoperitrophic space to the ectoperitrophic space. Moreover, surveys of the feeding habits of tsetse strongly indicate that the choice of blood tends to be species-characteristic (Jordan, 1965; Stephen, 1986), so that if a preferred host is refractory to infection by any group of trypanosomes, the tsetse species that feeds on that animal may not be exposed to those trypanosomes.

Despite the diverse mechanisms that may operate against the establishment or maturation of trypanosomes in *Glossina*, it has been reported

that a single trypanosome is sufficient to infect a tsetse fly (Maudlin and Welburn, 1989). However, not all tsetse flies that ingest trypanosomes from infected animals develop mature infections, since many of such infections are aborted (Bruce, Hamerton, Watson and Bruce, 1915; Lynhurst, 1933; Vickerman *et al.,* 1988). Nevertheless, laboratory-based tsetse infection experiments normally give very high infection rates. Thus, as high as 60 to 100 percent infection rates may be obtained for *T. vivax* in *G. palpalis*, *G. tachinoides* and *G. morsitans* (Stephen, 1963; Clarkson and McCabe, 1970; Roberts and Gray, 1972; Moloo and Kutuza, 1988), with some exceptions as exemplified by the work of Desowitz (1963). Similarly, it is known that given access to suitable sources of trypanosomes, one can obtain up to 100 percent *T. congolense* infection rates in *G. palpalis, G. tachinoides* and *G. morsitans* (Stephen, 1986), albeit also with some exceptions (Godfrey, 1961).

On the other hand, trypanosome infection rates in wild-caught tsetse flies are usually low, with some less than 10% as reported by Jordan (1974). This situation is clearly shown in the summary of infection rates for tsetse species in southern Nigeria and southern Camerroon compiled by Jordan (1961) (See Table 6). The literature is replete with the information that trypanosome infection rates in any species of fly is highest for *T. vivax*, followed by *T. congolense* and then *T. brucei* (reviewed by Buxton, 1955; Jordan, 1974). This trend is believed to be attributable to the varying complexity of the life-cycles of the different species of the salivarian trypanosomes. Thus, *T. vivax* with the simplest cycle of development involving only the mouthparts of the fly, is most likely to be successful, whereas *T. congolense* infections that require invasion of the midgut will be less frequent. *T. brucei*, with the most complex cycle involving the midgut and the salivary glands, is expected to be even less (Hoare, 1972). It is also established that the trypanosome infection rates in some fly species may be considerably higher than that in others, as shown in Table 6.

**Table 6**

**Trypanosome infection rates for tsetse species of Southern Nigeria and Camerroon (After Jordan, 1961)**

| Species of fly | Number dissected | % positive | Type of infection | | |
|---|---|---|---|---|---|
| | | | vivax % | congolense % | brucei % |
| *G. caliginea* | 230 | 36.5 | 84.5 | 14.3 | 1.2 |
| *G. nigrofusca* | 182 | 24.2 | 88.6 | 11.4 | 0 |
| *G. longipennis* | 4,360 | 21.5 | 82 | 18.1 | 0.1 |
| *G. fusca* | 1,300 | 15.8 | 88.8 | 10.7 | 0.5 |
| *G. medicorum* | 225 | 15.5 | 87.2 | 12.8 | 0 |
| *G. haningtoni* | 59 | 8.5 | 5 | 0 | 0 |
| *G. tabaniformis* | 3,389 | 3.2 | 60.9 | 38.2 | 0.9 |
| *G. pallicera* | 119 | 2.5 | 3 | 0 | 0 |
| *G. palpalis* | 2,497 | 1.8 | 75.6 | 24.4 | 0 |

Based on this, very important conclusions have been made on the susceptibility of the various *Glossina* species to trypanosome infections and their ability to transmit the parasites. The *palpalis* group of tsetse flies (Table 2), for example, are known to be poor vectors of *T. congolense*. On the other hand, species of the *fusca* group (Table 2) can be heavily infected with trypanosomes (Jordan, 1988), but the forest ecological zone that this group inhabits makes them less important as vectors of trypanosomiasis in domestic animals. The *morsitans* group of tsetse flies (Table 2), which occupies the savannah zone, is considered the most important vector of trypanosomiasis in livestock. It is also known that *G. fuscipes* and *G. palpalis* are hardly able to transmit trypanosomes of the subgenus *Nannomonas*, whereas *G. morsitans* and *G. pallidipes* would readily transmit these trypanosomes (Godfrey, 1961; Harley and Wilson, 1968). Again, *G. palpalis*, *G. tachinoides* and *G. morsitans submorsitans* are known to be efficient vectors of *T. vivax* (Stephen, 1963; Clarkson and McCabe, 1970; Roberts, and Gray, 1972).

## 2.10 Effect of Trypanosome Infections on the Vector

The effect of trypanosome infections on the tsetse fly has been reviewed by Molyneux and Stiles (1991), while trypanosome-mechanoreceptor association in the labrum of tsetse has been demonstrated by Jenni, Molyneux, Liversey and Galun (1980) and Liversey, Molyneux and Jenni (1980). This association, which is believed to enhance probing activity by the fly, was confirmed by Liversey *et al.* (1980) by transmission electron microscopy. Jenni *et al.* (1980) had demonstrated that *G. morsitans morsitans* infected with *T. brucei* probed more frequently than uninfected flies, and Roberts (1981) subsequently observed a similar activity during *T. congolense* infection. In related studies, Moloo (1983a, 1983b) was unable to confirm these findings. It is important, though, to mention that a similar effect of *Leishmania* infection on the behaviour of the sandfly has been established (Beach, Kiilu and

Leeuwenberg, 1985). The possibility that parasites infecting the foregut of the vector may impair the flow of blood at feeding and thereby change the insect's probing behaviour has indeed been published (Jefferies, Liversey and Molyneux, 1986). Again, although Tarimo, Snow, Butler and Dransfield (1985), Ryan (1984) and more recently, Nitcheman (1990), have demonstrated reduced longevity in trypanosome infected tsetse flies, this effect of trypanosomes on the vector is still not conclusive (Molyneux and Stiles, 1991).

One of the most important effects of trypanosome infection on the vector is the enhancement of vector susceptibility to insecticides. Golder and co-workers (1982, 1984) for instance, have published the results of studies which showed that *T. brucei* infections increased tsetse susceptibility to endosulfan and a pyrethrum extract, chemicals that are used to control uninfected *Glossina*. Using deltamethrin, Nitcheman (1990) has confirmed these findings when he found that pregnant female *Glossina* infected with *T. congolense*, were significantly more susceptible to that chemical than uninfected females of the same physiological status.

## 2.11 Trypanosomiasis Challenge

The distribution of tsetse flies in the infested areas of Africa is not uniform. There are extremes ranging from abundant fly presence, presenting what may be subjectively called "heavy" trypanosomiasis challenge to livestock, to the complete absence of the fly (tsetse free localities) in which disease challenge is non-existent (Jahnke, Tacher, Keil and Rojat, 1988). Studies on trypanosomiasis risk (challenge) are centred on evaluation of this risk using three components: (1) tsetse relative density determined from catches in biconical traps (Challier and Laveisiere, 1973; Leak, Awoume, Colardelle, Duffera, Feron, Mahamat, Mawuena, Minengu, Mulungo, Mankodaba, Orduer, Pelo, Sheria, Tikubet, Toure and Yangari, 1988) and expressed as the mean number of tsetse caught per trap per day. Biconical

traps are chosen for this purpose because more is known about the biases of that trap compared to other traps with equivalent efficiency; (2) trypanosome infection rates in tsetse which are determined by dissecting the proboscides, salivary glands and midguts of tsetse and identifying the trypanosome types using the method of Lloyd and Johnson (1924); and (3) the proportion of blood meals taken from the target host as determined by various techniques (Wietz, 1956; Rurangirwa, Minja, Musoke, Nantulya, Grootenhuis and Moloo, 1986; Pant, Houba, and Engers, 1987). The product of these three factors gives an index of tsetse challenge which is compared with trypanosome prevalence in cattle (Clair, 1988). It is important to describe the relationship between tsetse challenge and trypanosome prevalence (in both tsetse and livestock) since such information will provide the basis for realistic comparisons of the productivity of trypanotolerant and susceptible breeds of livestock under various levels of tsetse challenge. Also, this could assist livestock producers in making decisions such as those involving chemoprophylactic and therapeutic drug strategies.

The relationship between tsetse challenge and trypanosome prevalence in livestock is, however, a complex one involving at least thirteen factors (Whiteside, 1958; Molyneux, 1977; Jordan, 1986). One of these factors is the trypanosome infection rates in the population of tsetse flies (ILCA/ILRAD, 1986). At present, information on the infection rates in flies (as analysed by dissecting the flies) is not sufficiently accurate due to the difficulties in differentiating infections by the various trypanosome species.

## 2.12 Methods for Diagnosis of Trypanosome Infections in the Tsetse Fly

### 2.12.1 The Dissection Method

Lloyd and Johnson (1924) introduced a procedure for the diagnosis of trypanosome infections in tsetse flies. This method involves dissection and microscopic examination of tsetse fly organs for the presence of infecting

trypanosomes. Mature trypanosome infections in *Glossina* are further differentiated, based on the morphological characteristics of infective forms in the vector through the examination of stained preparations. Also, inoculation of mammalian infective metatrypanosomes into susceptible laboratory animals allows the determination of the range of animals that can be infected and permit subsequent characterization based on the morphological characteristics of the bloodstream forms of the parasites. As mentioned earlier (Chapter 1), the dissection method of diagnosis is possible because tsetse-transmissible trypanosomes are found to localize in specific organs in the vector (See Figure 2). Even though the method permits differentiation of trypanosome infections in the vector up to the subgeneric level only, it has been used extensively (Hoare, 1972; Stephen, 1986), and it is the method of choice for epidemiological surveys (WHO, 1979). The method, however, is not accurate enough to identify mixed or immature infections (Hoare, 1970) since the immature stages of the parasites in the vector do not have any species-specific morphological features.

It has been reported that a single tsetse fly can become infected with double or even triple trypanosome infections and transmit such mixed infections to susceptible hosts (Moloo, Faiqa Dar and Kamunya, 1982; Gibson and Ferris, 1992). Stephen (1986) pointed out that there is enough evidence to indicate that a single tsetse fly could be infected with both *T. vivax* and *T. congolense* and lose the flagellates in the gut but yet retain an infection with both species in the mouthparts. Such an infection would be assigned to *T. vivax*, yet the fly would continue to transmit both organisms. *T. grayi*, as reported by Hoare (1929), is a stercorarian type of trypanosome that shares the same vector with the salivarian trypanosomes. Though this organism infects both the midgut and the hindgut of the vector, the hindgut is normally not dissected during routine field dissections, because *T. grayi* is not of medical or veterinary importance (McNamara and Snow, 1991). As a result, mixed

infections involving *T. grayi* and *T. vivax* will appear to be of the *Nannomonas* type (Stephen, 1986). As mentioned earlier, immature trypanosome infections in the vector are those confined to the midgut. Such infections are said to be either *Nannomonas* or *Trypanozoon*, but it is now certain that *T. grayi* could be mistaken for one of those. The attribution of salivary gland infections to the *brucei*-group of organisms has also been questioned. Stephen (1986) noted that *T. (P.) suis*, which also infects the salivary gland, is still circulating, and had asserted that Janssen and Wijers (1974) were the last to find this organism which he later confirmed. The discovery of *brucei*-type infections in the haemolymph of infected tsetse flies, coupled with the very low salivary gland infection rates in the vector in areas where animals are known to be heavily infected with *T. brucei* species, led to the suggestion that the site of development of *T. brucei* infective forms in the fly is doubtful (WHO, 1979). It has, moreover, been pointed out (WHO, 1979) that salivary gland infections may remain undetected either because the optical conditions used for microscopy are of insufficient standard or because the infection in the gland may be localized.

### 2.12.2 Xenodiagnosis and use of Host Restriction

Inoculation of mammalian infective metatrypanosomes into susceptible experimental animals allows the identification of some species through the determination of host range. Also, the morphological characteristics of the bloodstream forms help to confirm the identity of the organisms. This technique can be used to differentiate some trypanosome species. For instance, it has been argued that, whilst *T. simiae* causes a fatal disease in pigs, *T. congolense* does not. On the other hand, *T. simiae* does not grow in rodents, whilst *T. congolense* does (Hoare, 1970). Blood from inoculated animals can also be sub-inoculated into other susceptible hosts. Wenyon (1926) pointed out that sub-inoculation of blood from animals with inapparent

trypanosome infections into laboratory animals has been very useful in revealing infections of that nature. Bloodstream forms of trypanosomes isolated from these animals can also be used in other laboratory studies, such as isoenzyme analysis. The good sensitivity of animal sub-inoculation in revealing mature trypanosome infections in the vector was clearly shown by Ward and Bell (1971) when they reported that feeding *T. brucei* infected tsetse flies individually on mice gave transmission rates that were five times higher than those revealed by salivary gland dissections. Another noteworthy utilization of the animal inoculation method was provided by Moloo and Kutuza (1974). They investigated sleeping sickness in an area in Tanzania where all attempts to find the vector of the disease had failed. In that study, they triturated batches of 50 wild-caught *G. swynnertoni* by grinding them with glass powder in a mortar to which 2ml of a borate diluent was added and injected 0.5ml of the supernatant solution intraperitoneally into each of five mice, and successfully isolated nine (9) strains of *brucei*-subgroup trypanosomes.

Differentiation of trypanosome species by host restriction, however, is also limited in application. It is recognized that whereas some *T. congolense* strains will grow in rodents, others may not (Godfrey, 1961; Mackenzie and Boyt, 1969; Young and Godfrey, 1983; Masake, Nantulya, Musoke, Moloo and Nguli, 1987). Also, Chardome and Peel (1967) described two variants of *T. congolense* restricted to pigs, and a strain of *T. simiae* that caused a chronic disease in pigs. Thus, strains of *T. congolense* may be mistaken for *T. simiae* and *vise versa*. Yet, Mackenzie and Boyt (1969) and Roberts (1971) considered that the ability of *T. simiae* to infect and cause a fatal disease in pigs was the most reliable characteristic distinguishing it from *T. congolense*. Moreover, later studies by Janssen and Wijers (1974) and Agu (1984) revealed that the species of the tsetse fly transmitting *T. simiae* can influence the virulence of the parasites. This means that host restriction alone cannot be

used reliably to differentiate *T. congolense* species from *T. simiae*. It is also known that *vivax*-type infections, especially the East African stocks, can easily go undetected because the experimental hosts used (laboratory rodents such as mice, guinea-pigs or rabbits) are usually refractory to that organism (Stephen, 1986). Moreover, the delay in diagnosis of some of these cases by the long prepatent periods and the cost of maintaining the experimental animals, makes this method unsuitable for routine diagnosis of infections in the vector, especially in the field.

### 2.12.3 Molecular Diagnosis

The realization that molecular patterns underlie the diverse expressions of life led to the development and use of biochemical methods in the study of the relationship between life forms (Taxonomy). Two important methods used for the characterization of parasites are isoenzyme typing and DNA hybridization analysis. Both methods have been extensively applied in the characterization of trypanosomes (ole-MoiYoi, 1987; Gashumba, Baker and Godfrey, 1988).

#### 2.12.3.1 Isoenzyme typing

The term "isoenzyme typing" was first introduced by Markett and Moller (1959) when they described techniques capable of revealing the different molecular forms in which proteins with the same enzymatic specificity (isoenzymes) may exist. To analyse trypanosome enzymes, soluble proteins in parasite lysates (prepared from parasites grown *in vitro* or *in vivo*) are separated by electrophoresis on thin layer starch gels. By this procedure, electrophoretic variants of the enzymes are resolved into separate bands as a result of differences in their molecular sizes and charges (Holmes and Scopes, 1974). Masters and Holmes (1975) explained that these differences were the result of genetically functional adaptations of the parasite to different

environments. Analysis of the patterns (zymodemes) identified on the basis of isoenzyme profiles, has clearly demonstrated enzyme polymorphism amongst trypanosome stocks and species. For example, Young and Godfrey (1983) reported marked genetic differences between stocks of *T. congolense* originating from the humid regions of West Africa compared with stocks from the arid savannah areas of Africa. The major differences were in the frequencies of the electrophoretic variants of phosphoglucomutase and peptidase. Also, Gashumba, Gibson and Opiyo (1986) found, by isoenzyme electrophoresis, distinct differences between two stocks of *T. congolense* isolated from a sheep and a goat and 112 other stocks of the same species. Moreover, by isoenzyme analysis, intra-species variations have been shown in morphologically indistinguishable trypanosomes that, however, have different behavioural characteristics (Godfrey, 1979; Miles, Lanham, De Souza and Pavoa, 1980; Gibson, Marshall and Godfrey, 1980). Gibson and others (1988) had also found the frequencies of electrophoretic variants of the enzymes, phosphoglucomutase and isocitrate dehydrogenase, in *T. brucei* to be different between stocks from East and West Africa. Also, Godfrey and Kilgour (1976) having examined 33 isolates of *brucei*-type trypanosomes, found differences between isolates collected from patients suffering from chronic *gambiense*, acute *rhodesiense* and animal but non-human infective *T. b. brucei*, by the isoenzyme profiles of two aminotransferases. However, in a later study, Gibson, Wilson and Moloo (1983) failed to differentiate between a *T. evansi* stock obtained from Kenya and a West African *T. b. brucei* strain, despite the fact that some East African *brucei*-type trypanosomes have been successfully differentiated from *T. evansi*. Thus, the wide occurrence of intra-species differences makes it difficult to identify with certainty all the different species of trypanosomes by isoenzyme analysis. Besides, isoenzyme characterization requires large numbers of parasites that must be grown *in vitro* or *in vivo* prior to analysis (WHO, 1979; Gashumba, Gibson and Opiyo,

1986). This requirement, together with other recorded limitations, confirms that this technique is neither consistent nor suitable for direct identification of trypanosomes in *Glossina*.

### 2.12.3.2 DNA hybridization techniques

Research into the possibility of using genomic differences in differentiating between trypanosome species was started as early as the 1940's (Vanderplank, 1944). Progress in this promising new area of diagnosis was, however, slowed down by the fact that the chromosomes of microbes belonging to the order *Kinetoplastida* do not condense at metaphase, thus preventing their study by microscopy (Vickerman and Preston, 1970). However, following the development of new techniques several methods have been devised and used to study the trypanosome genome.

Recently, recombinant DNA probes have been developed and applied to the identification of trypanosomes in tsetse flies (Gibson, Dukes, and Gashumba, 1988; Kukla, Majiwa, Young, Moloo and ole-MoiYoi, 1987; ole-MoiYoi, 1987; Majiwa, 1989; Majiwa and Otieno, 1990). The development of DNA probes has been based on the finding that certain nucleotide sequences of parasite genomic DNA are species-specific. These sequences are of two types, namely, sequences existing in a single copy and those existing in multiple copies. The genomic DNA repeat sequences (multiple copies) are known as "satellite DNA" because they band separately from the bulk of the nuclear DNA in density centrifugation due to a high AT content, and they have no coding function.

The principle of DNA hybridization is that denaturation of duplex DNA by heat or by decreasing hydrogen ion concentration (increasing pH) leads to a reversible separation of the two complementary strands. Under appropriate conditions, however, the two separated strands can gradually reunite. In DNA hybridization tests, species-specific parasite DNA fragments are identified,

isolated and produced in large quantities in a plasmid or phage vector (Vogelstein and Gillespie, 1979; Maniatis, Fritsch and Sambrook, 1982; Sloof, Bos, Konings, Menke, Borst, Gutteridge and Leon, 1983; Gibson, White, Laird and Borst, 1987; Gibson, Dukes and Gashumba, 1988).

The isolated fragments are preferably purified and labelled with a tracer (often a radioisotope) such as $^{32}P$ so that its hybridization can be detected. The labelled reagent can then be used to probe either purified parasite DNA or lysed whole organisms. Prior to application of the probe, the test DNA is treated with denaturing agents and split into single strands, thereby making it possible for complementary DNA sequences in the probe and the test DNA to hybridize. This hybridization reaction is very specific since two polynucleotide chain fragments will only associate if they are highly complementary, tolerating only a small degree of mismatch. Imprecisely matched duplexes are less stable when subjected to heat in buffers of low ionic-strength, thus providing the basis for discrimination between precisely and imprecisely matched duplexes. The repetitive nature of satellite DNA makes it possible for more labelled probe to hybridize, hence, giving strong reactions when targeted. Bound label is revealed by autoradiography if the probe was labelled with a radioisotope.

The percentage of satellite DNA in parasite genome can be significantly high, for instance, in *T. brucei* and *T. cruzi*, it is 12% and 9% respectively (Borst, Fase-Fowler, Frasch, Hoeijmakers and Weijers, 1980; Castro, Craig and Castaneda, 1981; Sloof, Bos, Konnings, Borst, Gutteridge and Leon, 1983; Keohavong and Thilly, 1989). However, where the number of sequence repeats in the target are few, the sensitivity of the test is low. In such cases the incorporation of an amplification step based on DNA polymerase chain reaction greatly enhances the sensitivity (White, Arnheim and Erlich, 1989).

The DNA probes, however, also have some major disadvantages. Firstly they appear to be so specific that most of them detect intra-species

differences. Gibson and coworkers (1988) obtained 5 species-specific DNA probes for trypanosomes of the *Trypanozoon* and *Nannomonas* subgenera. Each probe consisted of one repeat unit of the major repetitive DNA of a trypanosome species or intra-species group. Though one probe hybridized with all members of subgenus *Trypanozoon* (except *T. equiperdum* which was not tested), and another probe hybridized with *T. simiae*, three probes were needed to identify the limited number of stocks of *T. congolense* tested. Moreover, in 1990, a new *Nannomonas* trypanosome was isolated in Tsavo, Kenya, to which none of the existing DNA probes for detecting *T. congolense* was able to hybridize (Majiwa, Maina, Waitumbi, Mihok and Zweygarth, 1993). Consequently, another probe was made for this new, Tsavo-type *T. congolense* (Majiwa *et al.,* 1993). A similar situation led to the discovery of another *T. congolense* genotype, designated as "*T. congolense* 'Godfrey' type" (McNamara, Gibson, Mohammed, Snow and Godfrey, 1991). This trend of development suggests that some more *T. congolense* types that require new probes may still be out in the field. For example *T. (N.) vanhoofi* has been encountered on very few occasions since its discovery and it is not known whether any of the existing DNA probes will hybridize to it. Again, none of the probes so far derived for *T. vivax* identification can hybridize with all *T. vivax* isolates (Kukla *et al.*, 1987; Dickin and Gibson, 1989; Gardiner, 1989). The second drawback is that the probes are radioactively labelled with $^{32}P$. The biohazard associated with the use of radioactive reagents is an important limitation. There are, however, prospects for overcoming this limitation through the introduction of non-radioactive labels (Shroyer, Morichi, Koji, and Nakone, 1987; Saiki, Chang, Levenson, Warren, Boehm, Kazazian and Erlich, 1988; Keller, Cumming, Huang, Monak and Ting, 1988; Majiwa, 1989). Recently, Holtke, Sagner, Kessler and Schmitz (1992) published a protocol in which nucleic acids were labelled with a plant derived substance, "digoxigenin", to make non-radioactive probes. These probes were then

detected with anti-digoxigenin antibody *Fab* fragments, coupled to alkaline phosphatase and 3-(4-methoxyspiro [1,2-dioxetane -3,2'-tricyclo [3.3.1.1] decan]-4-yl) phenyl phosphate as substrate. According to this report, the protocol is fast and simple, and the sensitivity is comparable or better than that of radioactive probes. The option to omit the use of X-ray films may also cut down on costs. Currently, work is in progress on the use of this non-radioactive probe for the detection and differentiation between trypanosomes (P. A. O. Majiwa, personal communication). If successfully developed for the detection of trypanosomes in infected tsetse flies, this non-radioactive probe will still need critical evaluation with regard to sensitivity and specificity under field conditions.

## 2.13 Monoclonal Antibodies as Diagnostic Reagents

The discovery and development of the monoclonal antibody (MoAb) technology by Köhler and Milstein (1975) have greatly improved the performance of immunological assays as a result of replacement of polyclonal antibodies with these new immunodiagnostic reagents (Sikora and Smedley, 1984). The underlying principle involves the immortalization of a single B-lymphocyte by fusing it with a non-secretor tumour lymphocyte *in vitro*, thereby enabling endless production of a single antibody molecule with specific reactivity to a single antigenic epitope against which it was derived. The extraordinary specificity of epitope detection provided by MoAbs has made it possible to develop standardizable immunodiagnostic reagents of high specificity. As a result, MoAbs are now being used in immunoparasitology: (1) as probes for localization of antigen, and analysis of its organization and availability; (2) for studies of antigenic heterogeneity (variability) in parasite populations; (3) for detection of cloned DNA in various vectors; and (4) for parasite typing and parasite detection (Goding, 1980; Mitchell, 1984; Sikora and Smedley, 1984).

Recent studies in malaria have revealed the potential use of MoAbs for the detection of parasites in their vectors. Zavala and others (1984) for instance, developed a two-site immunoradiometric assay (IRMA) for identification of malaria sporozoites in the mosquito vector. This test can detect sporozoite densities of less than 100. A field trial of this assay in The Gambia confirmed its sensitivity and reliability. To circumvent the limitations of radiometric tests, Burkot, Zavala, Gwadz, Collins, Nussenzweig and Roberts (1984) developed an enzyme-linked immunosorbent assay (ELISA) for identification of malaria parasites in mosquitoes. Using an extract of dried infected mosquitoes as antigen, they performed an ELISA which proved sensitive enough to detect one infected mosquito in a pool of 20, and could detect as few as 100 sporozoites. Furthermore, Beier, Perkins, Wirtz, Whitmire, Mugambi and Hockmeyer (1987) have published data on the field evaluation of an ELISA for *Plasmodium falciparum* sporozoite detection in *Anopheline* mosquitoes. Using a MoAb that bound a repetitive epitope on the circumsporozoite protein of *P. falciparum*, they confirmed by ELISA, that 88% of 44 sporozoite-positive gland dissections were *P. falciparum*, and thus demonstrated the sensitivity and specificity of the test. Also important is the work by Petros and others (1989), in which a nitrocellulose membrane (NC)-based dot-ELISA was successfully applied in the detection of sporozoites of *P. inui* in *Anopheles dirus*. This appreciable specificity and sensitivity of the NC membrane-based dot-ELISA, explains the increasing use of the technique in field diagnosis of parasitic diseases in recent years (Pappas, 1988a).

## 2.14 The Nitrocellulose Membrane-Based Dot-ELISA

NC membrane-based dot enzyme-linked immunosorbent assay (dot-ELISA) is a highly versatile solid-phase immunoassay that requires small amounts of reagents for antibody or antigen detection (Pappas, 1988a). Dot-ELISA has also been used extensively in the diagnosis of human and veterinary

protozoan and metazoan parasitic diseases, and they have been shown to be also field applicable (Pappas, Hajkowski and Hockmayer, 1983; Pappas, Hajkowski, Cannon and Hockmayer, 1984; Pappas, Hajkowski and Hockmayer, 1984; Kumar, Band, Samantaray, Dang and Talwar, 1985; Pappas, Schantz, Cannon and Wahlquist, 1986; Zheng, Zhao and Feng, 1986; Guimaraes, Celeste and Franco, 1986; Walton, Pappas, Sierra, Hajkowski, Jackson and Custodio, 1986; Boctor, Stek, Peter and Kamal, 1987; Londner, Rosen, Sintov and Spira, 1987; Pappas, 1988b; Lussier, Brodeur and Winston, 1989; Cardosa and Tio, 1991; Corral, Orn and Grinstein, 1992).

## 2.15 Significance of Antigenic Variation in the Use of MoAbs for Differentiating between Trypanosome Species

In studies on trypanosomiasis, efforts have been made to develop species-specific assays for diagnosis of the disease in the vertebrate host. The potential usefulness of MoAbs in this area of diagnosis was, however, not realised immediately due to the ability of bloodstream forms of trypanosomes to undergo "antigenic variation".

Each bloodstream form trypanosome is covered by a replaceable surface coat composed of a variable surface glycoprotein (VSG). This electron opaque coat that covers the pellicular surface of trypanosomes was first described by Vickerman (1969). The VSG coat was later shown by Cross (1973; 1975) to be made up of a matrix of identical glycoprotein molecules that differed in amino acid sequences. Due to these sequence differences, each VSG specifies a variable antigen type (VAT) which is unique in antigenic specificity. The fact that individual trypanosomes within a population possess different VATs meant that various MoAbs of different specificities would be needed to identify any given trypanosome population in situations where the bloodstream form trypanosome surface antigens were to be utilized. The magnitude of this problem was further revealed by the discovery that a single

trypanosome possesses at least 1000 genes that code for VSGs (Van der Ploeg, Valerio, De Lange, Bernads, Borst and Grosveld, 1982) and is capable of generating $10^4$ to $10^5$ VATs (Capbern, Giround, Baltz and Meltern, 1977; Van der Ploeg *et al.*, 1982). Following serological studies, the total repertoire of VATs that an individual trypanosome can generate was assigned the term "serodeme" (WHO, 1978).

In trypanosome infections, some VATs tend to predominate. In an immunocompetent host, such VATs are quickly eliminated as potent antibodies are produced against them. However, in a large population of dividing organisms, the gene encoding the expression of a particular VAT may be switched off in a trypanosome and a different gene controlling the expression of a different VAT may become active (Miller, Allan and Turner, 1984; Boothroyd, 1985). A new trypanosome population expressing a different VSG may then emerge, multiply, and produce a parasitaemic peak. As new antibodies are produced against the VSGs of the infecting organisms, those expressing the predominant VATs are rapidly eliminated leading to drastic reduction in the infecting parasite population. Once again, a few organisms that successfully acquire new surface coats as a result of gene switching, multiply as antibodies are produced against the new antigens that they express. This phenomenon of antigenic variation leads to successive parasitaemic peaks during the course of a trypanosome infection.

Apart from their unsuitability as targets for immunodiagnosis, the VSGs expressed by trypanosomes are also known to be too antigenically diverse to be useful as a basis for a vaccine against the disease (Shapiro and Pearson, 1986). Antigenic variation has been reviewed by many workers (Vickerman, 1969; Gray and Luckins, 1976; Doyle, 1977; Cross, 1978; Vickerman, 1978; Cross, Holder, Allen and Boothroyd, 1980; Donelson and Turner, 1985). It is in general a mechanism exploited by parasitic protozoa to

evade the immune response of their vertebrate hosts (Brown and Vickerman, 1986).

## 2.16 Suitability of MoAbs as Specific Reagents for Differentiating Between Trypanosome Species

Rapid advances in the use of MoAbs as specific reagents for the diagnosis of trypanosomiasis were achieved following the realization that trypanosomes possess species-specific antigens that are expressed in the non-variable components of the cell body (Parish, Morrison and Pearson, 1985; Richardson, Jenni, Beecroft and Pearson, 1986; Nantulya, Musoke, Rurangirwa, Saiga and Minja, 1987). As a result, simple, MoAb-based invariant antigen capture ELISA tests for the detection of species-specific trypanosome circulating antigens in the blood of infected animals and humans, have been developed and are currently being evaluated in the field (Nantulya, 1989; Nantulya and Lindqvist, 1989; Nantulya, Bayjana Songa and Hamers, 1989a; Nantulya, Lindqvist, Diall and Olaho-Mukani, 1989b). Moreover, data published by Nantulya *et al.* (1987) clearly shows that some of the trypanosome species-specific invariant antigens are expressed by the procyclic vector stages of the parasites.

These facts indicate that a MoAb-based ELISA system for identification of trypanosomes in tsetse flies may be a viable possibility. If such a test is applicable in the field, it will facilitate the collection of accurate epidemiological data needed for the management of trypanosomiasis. It was in the light of this that the studies described in this thesis were conducted with the aim of developing field applicable assays for the detection of trypanosomes in the vector.

# CHAPTER 3

# GENERAL MATERIALS AND METHODS

## 3.1 Trypanosomes

### 3.1.1 *Trypanosoma brucei* stocks and clones

*T. b. brucei* stocks IL375, IL2616 and IL3579, were derived from an East African stock STIB 247, which was isolated in 1971 from a naturally infected Kongoni in Serengeti, Tanzania (Geigy and Kauffmann, 1973). The *T. b. brucei* clone, MiTat 1.2 (previously designated clone 221) was derived from stock LUMP 427 which was isolated from a naturally infected tsetse in 1960 from Tororo, Uganda (Described by Cross in 1977). The *T. b. brucei* stock CP 2137 was isolated in 1986 from a dog in Nairobi, Kenya, whilst stock CP547 was isolated in 1985 from a cow in Jilib, Somalia (Zweygarth and Kaminsky, 1989). *T. b. gambiense* TH-17/78E(020) is a West African stock from Cote d'Ivoire, and has been described by Mehlitz and co-workers (1981); *T. b. gambiense* TREU 1442 is also a stock isolated in West Africa (Nigeria). The *T. b. rhodesiense* stocks IL1984 and IL1478 were isolated from Lugala, Uganda and Lambwe Valley, Kenya, respectively.

### 3.1.2 *Trypanosoma vivax* stocks and clones

West African (WA) *T. vivax* stock IL1392 was derived from a stabilate IL560 following tsetse transmission from one goat to another. The stabilate WA IL560 was derived, as described by Moloo (1981), from Zaria Y486 which was originally isolated in 1973 from a Zebu cow in northern-Nigeria (Leeflang, Buys and Blotkamp, 1976). WA stocks IL2160 and IL3096 were both derived from IL1392; IL2160 was obtained by passage through a mouse, whilst IL3096 was obtained from a goat following tsetse transmission. East African (EA) stock IL2337 was a derivative of IL1480 originally isolated in 1978 from a naturally infected cow at Galana Ranch, Tana River District, Kenya (Gardiner, Assoku, Whitelaw and Murray, 1989). EA stock IL2005 was a rodent-adapted derivative of stock ILV21 which was isolated from a

naturally infected cow in 1972 from Teso, North Eastern Uganda (Gathuo, Nantulya and Gardiner, 1987). EA stock IL3895 was isolated from a naturally infected cow from the Kipini area of Kenya. WA *T. vivax* clone ILDat (ILRAD *Duttonella* antigenic type) 1.9, was obtained from the primary isolate Y486 (Barry and Gathuo, 1984). The identity of *T. vivax* stocks IL1392 and IL2337 had been confirmed using isoenzyme and DNA analysis (Allsopp and Newton, 1985; Gibson, *et al.*, 1980; Kukla, *et al.*, 1987). Clone IL3841 is a South American *T. vivax*, obtained from the Atlantic coast of Lorica, Colombia (Dirie, Otte, Thatthi and Gardiner, 1993).

### 3.1.3 *Trypanosoma congolense* stocks and clones

*T. congolense* stock IL3779 had been obtained from the infected mouthparts of a female *G. pallidipes* fly from Nguruman, Kenya, and passaged through sub-lethally irradiated BALB/c and Swiss mice. The stock ILC49 had been isolated from a naturally infected cow in 1966, from Transmara, Southern-Kenya. CP81 was a stock derived from the primary isolate, Transmara 1 Strain, maintained by the Deutsche Gesellschaft für Technische Zusammenarbeit (GTZ)-supported Project, "Chemotherapy of Trypanosomiasis Research", Veterinary Research Laboratory, Kabete, Kenya (Hirumi and Hirumi, 1991). The Kilifi type *T. congolense* clone K/82/IL/60/1 was a derivative of stock K60, which was isolated in 1982 from a naturally infected cow at the Kilifi plantations, in Kenya (Masake, *et al.*, 1987). Savannah type *T. congolense* IL1180 and IL2079 were cloned from the primary isolates STIB 212 and STIB 249A, respectively. These two isolates were obtained from different naturally infected lions in the Serengeti National Park, Tanzania in 1971 (Geigy and Kauffmann, 1973). Clone IL13-E3 had been derived from EATRO 209, which was isolated by Van Hoeve in 1962 from a naturally infected cow at Ikulwe Government Farm Institute, Busoga, Uganda. Kilifi type *T. congolense* clone K/83/IL/97/2 was from stock K97,

which had been isolated from a naturally infected cow at the Kilifi plantations, Kenya in 1983, Savannah type clone IL 3274 had been derived from stock Banankeledaga/83/CTRA/67, also isolated from a naturally infected cow in 1983 from Bobo-Dioulasso, Burkina Faso (Peregrine, Knowles, Ibitayo, Scott, Moloo and Murphy, 1991). *T. congolense*, riverine-forest type IL3900, had also been obtained from Bobo-Dioulasso, Burkina Faso, whilst *T. congolense* savannah type MBOI/NG/60/1-148, riverine-forest type MSUS/LR/77/TSW103, Kilifi type MOVS/KE/81/WG84, and *T. grayi* GPAG/GM/88/BAN1 were supplied by Dr. J. McNamara of the Tsetse Research Laboratory, Bristol, England.

### 3.1.4 *Trypanosoma simiae* stocks and clones

The *T. simiae* stock KETRI 2431 (IL3631) was initially derived from the EATRO 1875 stock, which had been isolated in 1970 from a tsetse fly (*G. austeni*) at Ukunda, Kenya. This stock (KETRI 2431) had been examined by DNA hybridization techniques and identified as a *T. simiae* species by Majiwa and Webster in 1987. Both *T. simiae* stocks, IL3706 and CP11 (IL3879) had been derived from KETRI 2431 by passage through pigs, and both found to cause acute infections in swine. *T. simiae* stock IL3815, which also causes acute infections in pigs, was derived from CP813 which was isolated in 1981 from naturally infected *G. pallidipes* from the Muhaka forest, Ukunda, Kenya. *T. simiae*, TS1 and TS4 were both clones derived from KETRI 2431.

## 3.2 Resuscitation of cryopreserved trypanosomes

### 3.2.1 Procyclics

A vial containing 1 x $10^7$ procyclic trypanosomes in 1ml of stabilating medium, was retrieved from liquid nitrogen at -196°C and immediately transferred to a waterbath at 37°C. The thawed stabilate was

diluted to 10ml with semidefined maintenance (SM) medium (Cunningham, 1977), modified by the addition of L-glutamine and gentamicin (GIBCO-BIOCULT, Schiphol, The Netherlands; and Schering, Kenilworth, NJ, USA) to a final concentration of 2.4mM and 0.59$\mu$g/ml, respectively. This medium was supplemented with 10%(v/v) heat-inactivated (56°C/30min) foetal bovine serum (FBS) purchased from Hyclone Laboratories Inc., USA and filtered through a 0.2 to 0.45$\mu$m membrane bottle filter (Costar, Cambridge, MA, USA). (This medium will henceforth be referred to as "complete-(SM) medium"). The suspended trypanosomes were washed twice with complete-(SM) medium by centrifugation at 400 *Xg* for 10 min. After the second wash the pelleted trypanosomes were resuspended into 5ml of complete-(SM) medium and pipetted into sterile tissue culture flasks (surface area, 25cm$^2$) (Costar, USA; or Falcon, Becton Dickinson Labware, Oxnard, California). The flasks were then gassed with 5% $CO_2$ in air for 10 to 20 seconds, closed tightly and kept at 27°C in an Incubator (Heraeus-Christ B5060, Hanau, Germany). The culture was subsequently maintained by subculture from one flask to another.

### 3.2.2 Bloodstream forms

Bloodstream form trypanosomes stabilated in blood diluted to 10% glycerol in capillary tubes, were retrieved from liquid nitrogen and thawed rapidly in a waterbath at 37°C. The contents of the capillary tubes were removed by syringe and diluted in phosphate-saline-glucose (PSG) buffer, pH 8.0. This preparation was then used to infect mice ($1x10^5$ trypanosomes/mouse) and rats ($1x10^6$ trypanosomes/rat).

## 3.3 *In vitro* cultivation of bloodstream forms

Bloodstream form trypanosomes, cultivated *in vitro*, were supplied by Dr. R. Kaminsky and Mr. F. Chuma, both of the International Laboratory

for Research on Animal Diseases (ILRAD), Nairobi. The *T. brucei* and *T. vivax* trypomastigotes were propagated in culture, using the procedures described previously (Kaminsky, Chuma and Zweygarth, 1989; Kaminsky and Zweygarth, 1989; Zweygarth, Kaminsky and Gray, 1991; Zweygarth, Gray and Kaminsky, 1991), whilst *T. congolense* trypomastigotes were grown *in vitro* using the method previously described by Hirumi and Hirumi (1991).

### 3.4 Infection of mice and rats

Outbred 3-4 month old BALB/c x Swiss mice and 3-month old Sprague Dawley rats, were infected with different stocks and clones of *T. brucei, T. vivax* or *T. congolense*. Rats and mice were sublethally irradiated (650 rad) using a $^{137}$cesium radiation source at least 2 hr prior to inoculation with trypanosomes. Each mouse was injected intraperitoneally with $1x10^6$ trypanosomes retrieved from storage in liquid nitrogen. Wet-film preparations of tail blood from each mouse were examined daily and the level of parasitaemia assessed by the matching method of Herbert and Lumsden (1976). Highly parasitaemic mice were anaesthetised by confinement in a chamber saturated with vaporized diethyl-ether and subsequently bled by cardiac puncture using a gauge 22 needle, and sodium citrate as anticoagulant in a (0.1M $Na_3C_6H50_7.2H_20$, 0.01M $C_6H_{12}0_6$) buffer. Some infected blood was diluted with PBS, pH 7.4, to about $2x10^7$ trypanosomes/ml. This preparation was then used to infect more mice (0.5ml or $1x10^7$ trypanosomes/mouse) and rats (1ml or $2x10^7$ trypanosomes/rat) by the intraperitoneal route. Inoculated mice and rats were bled from the tail veins daily and the blood examined for the presence of trypanosomes by microscopy, as stated above.

### 3.5 Isolation of trypanosomes from mouse, rat, or pig blood

Bloodstream form trypomastigotes were isolated from infected mouse, rat or pig blood by anion exchange column chromatography on

diethylaminoethyl (DEAE) cellulose (DE-52) (Whatman, Kent, England) equilibrated in phosphate-buffered saline-glucose (PSG) according to Lanham and Godfrey (1970). PSG buffers, with different ionic strengths (I), were used for separating trypanosomes from blood elements of each of the above listed mammals. Thus, for mice and rats, PSG consisted of 38mM $Na_2HPO_4$, 2mM $NaH_2PO_4.2H_2O$, 29mM NaCl and 1%(w/v)D-glucose, with (I) of 0.145 and pH 8.0, and 57mM $Na_2HPO_4$, 3mM $NaH_2PO_4.2H_2O$, 44mM NaCl and 1%(w/v)D-glucose, with (I) of 0.217 and pH 8.0, respectively, were used to separate *T. brucei, T. vivax* or *T. congolense* from infected blood. The PSG used to isolate *T. simiae* from infected pig blood, consisting of 28mM $Na_2HPO_4$, 1.5mM $NaH_2PO_4.2H_2O$, 22mM NaCl and 1.5%(w/v)D-glucose, with (I) of 0.109 and pH 8.0. Trypanosomes were eluted into at least 100ml of the appropriate elution buffer at 4°C and washed twice at the same temperature by centrifugation at 450 *Xg* for 10 min each. The supernatants were discarded after the second wash, and the pelleted trypanosomes stored at -20 or -80°C until used.

## 3.6 Cryopreservation of cultivated trypanosomes

### 3.6.1 Procyclics

Procyclic trypanosome cultures containing about $1x10^6$ trypanosomes/ml were centrifuged at 700 *Xg* for 10 min. The trypanosome pellets were resuspended in complete-(SM) medium containing 7.5%(v/v) dimethyl sulphoxide (DMSO) (E. Merk, Darmstadt Germany) to give $1x10^6$ to $1x10^7$ procyclics/ml (Hirumi, Doyle and Hirumi, 1977). One millilitre aliquots of the suspensions were pipetted into 2ml cryopreservation vials (Greiner, Labortechnick, Germany) and immediately placed in plasticine-insulated large screw cap plastic bottles (freezing jackets), which were then suspended in the vapour phase of liquid nitrogen in a stabilate storage tank (LR-30A Cryogenic equipment, Union Carbide) for 2 hr. The frozen vials

were removed and immediately transferred onto labelled stabilation canes and immersed in liquid nitrogen. After one week, one vial was recovered and thawed rapidly at 27°C in a waterbath and the parasites assessed for viability on the basis of their motility as observed by microscopy at x400 magnification.

### 3.6.2 Epimastigotes

Epimastigote colonies from three-month old axenic cultures of EA *T. vivax* stocks IL2337 and IL3895 were scraped from culture flasks. The medium containing trypanosomes was removed and centrifuged at 700 *Xg* for 10 min. The epimastigote pellets were then resuspended in complete-(M-DMEM) medium, containing 7.5%(v/v) DMSO to give $1x10^6$ to $1x10^7$ epimastigotes/ml. The rest of the stabilation procedure was the same as described above (section 3.6.1).

### 3.6.3 Bloodstream forms

Heparinised blood from a trypanosome infected animal was diluted slowly by dropwise addition of 20% glycerol in PSG to give a final concentration of 10% glycerol. The diluted blood was mixed thoroughly and left for 15 min to equilibrate on ice and then transferred into capillary tubes (approximately 50$\mu$l/tube). Both ends of the capillary tubes were sealed with plasticine and transferred into 75 x 12mm plastic tubes (Greiner, Labortechnick, Germany) together with a narrow hard paper label. Both ends of each tube were punctured to allow ingress of liquid nitrogen, and placed in a freezing jacket which was then suspended in the vapour phase of liquid nitrogen for 2 hr. The remaining stabilation procedure was the same as described under section (3.6.1) except that thawing of stabilates was done at 37°C.

### 3.7 Myeloma cell lines and their maintenance

Several drug (8-azoguanine) resistant parental myeloma cell lines of BALB/c origin were used. X63-AG8.653 (X63 for short) was introduced by Kearney, Radbruch, Liesegang and Rajewsky in 1979, whilst Sp2/OAG14 (Sp2 for short) was developed by Shulman, Wilde and Köhler in 1978. Both X63 and Sp2 are non-secretor myeloma cell lines. Also used was NSI/1Ag4.1, a kappa (k) chain secretor myeloma introduced by Köhler, Howe and Milstein (1976). All three myeloma cell lines were adapted for *in vitro* propagation in growth-medium. This medium was composed of Rosewell Parke Memorial Institute Medium 1640 (RPMI-1640), modified by addition of 2.4mM L-glutamine, 0.59 $\mu$g/ml gentamicin, and supplemented with 10%(v/v) heat-inactivated (56°C/30min) FBS. The myeloma cells were cultivated in 25 or 75cm$^2$ sterile plastic flasks (Costar, Cambridge, MA, USA or Falcon, Oxnard, California) in 5 and 20ml medium, respectively, gassed with a mixture of 5% $CO_2$ in air and incubated at 37°C (Heraeus-Christ B5060 EC/$CO_2$, Hanau, Germany). The cells were allowed to grow to a concentration of 1x10$^7$/ml and maintained in culture by subculturing a third of the cell suspension with two-thirds volume of fresh growth-medium into new flasks. To ensure that healthy cells were used for fusion, the cells were kept in logarithmic growth phase (about 5x10$^5$ cells/ml) for at least 4 days prior to cell fusion (Pearson, Pinder, Roelants, Kar, Lumsdin, Mayor-Withey and Hewet, 1980).

### 3.8 Propagation and storage of hybridomas

Doubly cloned hybridoma cells secreting MoAbs of desired specificity were transferred from 100$\mu$l of growth-medium in 96 well plates to 500$\mu$l of medium in 24 well plates, and the hybrids allowed to multiply before increasing the volume to 1ml. The hybridoma cells were grown in the 24 well plates, with medium changes every three days until 1x10$^6$ cells could be

transferred to 25cm$^2$ tissue culture flasks in 3ml of growth-medium. The cells were then grown until the medium turned acid before increasing the volume of medium in the flasks. 1x10$^5$ hybrid cells/ml in a total of 20-25 ml of growth medium were transferred to 75cm$^2$ tissue culture flasks. The hybrids were then maintained by subculturing into other flasks when the medium turned acidic. Acidic culture fluids, containing MoAbs were harvested after centrifugation at 450 *Xg* for 10 min to remove the cells, and the latter stored frozen at -20°C. Some of the hybridoma cell cultures were maintained in the logarithmic phase of growth, at least 4 days before preservation in liquid nitrogen, by subculturing as soon as the cell concentration reached approximately 5x10$^5$/ml. This was to ensure that healthy cells were preserved. Suspensions of cells in the logarithmic growth phase were centrifuged at 400 *Xg* for 10 min at 4°C and the supernatant removed. The pelleted cells were resuspended in growth-medium containing 7.5%(v/v) DMSO to give 1-2x10$^7$ cells/ml. 1ml aliquots of these cell suspensions were placed in 2ml cryopreservation vials and frozen in liquid nitrogen, following the method described under section 3.6.1.

### 3.9 Production of ascites

To produce MoAbs in large quantities, hybridoma cells were passaged *in vivo* to obtain antibody from ascitic fluid. By this method, antibody concentrations as high as 10 to 20 $\mu$g/ml of ascites (Clark and Waldmann, 1986) could be harvested. BALB/c mice were primed 2-3 weeks, before the injection of cells, with pristane (tetramethylpentadecane; Sigma, USA) administered intraperitoneally at a dose of 0.5ml/mouse. The mice were then inoculated intraperitoneally with between 5x10$^6$ and 1x10$^7$ monoclonal antibody producing hybridoma cells in logarithmic growth phase. These cells produced ascitic fluid, 2 to 8ml per mouse, in approximately 2 weeks. The mice were sacrificed by terminal anaesthesia with diethyl-ether and the ascitic

fluid drained from the peritoneum and centrifuged at 2,000 *Xg* for 30 min at room temperature. Fatty material floating at the top of the centrifuged fluid was aspirated off and the ascitic fluid pipetted into another tube. Second generation hybrid cells in the pellet were resuspended in PBS, pH 7.4, and passaged in mice for the production of more ascitic fluid. Harvested fluid was stored frozen at -20°C.

## 3.10 Purification of monoclonal antibodies

### 3.10.1 Salt fractionation of ascitic fluid and hybridoma culture supernatants

Ascitic fluid or hybridoma culture supernatants, frozen at -20°C, were thawed at 37°C in a waterbath. Immunoglobulins in these fluids were precipitated with saturated ammonium sulphate, $[(NH_4)_2SO_4]$, added slowly whilst stirring to 50%(v/v) final concentration. The precipitates formed were pelleted by centrifugation at 3,000 *Xg* for 40 min, and dissolved in distilled water. The precipitation was repeated once more and the precipitates resuspended as above. The antibody solutions were pipetted into dialysis membrane bags, molecular weight cut-off 3,500, and dialysed against Tris-buffered saline (0.05M Tris, 0.01M NaCl, 0.02% $NaN_3$,pH 8.6) (Dalchau and Fabre, 1986) at 4°C over a 24 hr period with one buffer change. Monoclonal antibody fluids purified by this process, were used in the dot-immunobinding assay or were subjected to further purification as described under sections 3.10.2 and 3.10.3.

### 3.10.2 Ion exchange chromatography

Ascites or culture fluids containing IgG MoAbs, were concentrated by the ammonium sulphate precipitation method and dialysed against Tris-buffered-saline, pH 8.6, as described above. Dialysed fractions of the same monoclonal antibody were pooled and passed through a column of DE-52

(Whatman, Kent, England) equilibrated in the same buffer. Approximately 1.5ml of gel was used for every 1.0ml of unprecipitated ascitic fluid or 200ml of unprecipitated culture fluid. The column was washed with one column volume of the buffer and eluted with a linear gradient of 10 to 300mM NaCl (500ml total buffer for a 50ml column) (Dalchau and Fabre, 1986). 10ml fractions were collected in tubes and each fraction assayed for antibody activity using antibody-detection micro-plate ELISA. Fractions with specific antibody activity were pooled and concentrated by filtration through an X-100 membrane fitted to an Amicon concentration chamber (Amicon Corporation, Ireland). Samples were filtered under 2 bar pressure of nitrogen gas.

### 3.10.3 Gel filtration

IgM monoclonal antibodies, precipitated and dialysed as before, were purified by gel filtration through a Sephadex G-200 (Pharmacia, Uppsala, Sweden) column, using the dialysis buffer (Tris-buffered-saline pH 8.6). Sephadex G-200 was prepared as recommended by the manufacturer. Samples were applied in volumes of up to 5% of the column volume. IgM eluted ahead of any IgG's, and fractions were collected in 10ml volumes and assayed for antibody activity using antibody-detection micro-plate ELISA (section, 4.3.13.1). Fractions with specific antibody activity were pooled and concentrated by filtration, using the amicon concentration chamber.

## 3.11 Preparation of enzyme and fluorescein antibody conjugates

### 3.11.1 Preparation of horseradish peroxidase antibody conjugates

Purified trypanosome species-specific monoclonal antibodies as well as goat anti-mouse immunoglobulins, were conjugated to horseradish peroxidase (HRPO) using the periodate method described by Wilson and Nakane (1978). The protein concentration of antibody fractions was estimated, using the Coomassie Blue G-250 based reagent (Pierce, Rockford,

Illinois, USA). The desired amount of antibody IgG (MW 160,000) or IgM (MW 900,000) in terms of protein to be conjugated, was pipetted into a tube. HRPO enzyme (Sigma, USA) was conjugated to antibody at a ratio of 1:1 (enzyme : antibody) by molecular weight. This molecular weight ratio was essentially 1:4 (enzyme : antibody) for IgG's and 1:22.5 for IgM. Assuming an 80% coupling efficiency, the calculated value for enzyme was multiplied by 100, then divided by 80, to give the final weight of the enzyme required. For IgM, the value for enzyme was multiplied by 5 to account for the pentameric structure of the immunoglobulin. Thus, to conjugate 32mg of IgG, the amount of HRPO required was calculated as follows: $32x1 \div 4x100 \div 80 =$ 10mg. Hence, in conjugating 32mg of IgG 10mg of HRPO was weighed out and dissolved in 2ml of 50mM sodium-acetate buffer, pH 4.0. HRPO was oxidized using sodium metaperiodate ($NaIO_4$) at a ratio of 1:1 by weight. 10mg of $NaIO_4$ was weighed out and dissolved in 1ml of sodium-acetate buffer, and the $NaIO_4$ solution then added dropwise to the HRPO solution and the mixture kept in the dark for 15 to 20 min. A 300$\mu$l volume of ethyleneglycol ($C_2H_6O_2$) was added to the mixture and stirred. The mixture was fractionated through a Sephadex G-25 column (1.0cm diameter), allowing 30ml column volume for every 5ml of enzyme solution. It was eluted with sodium-acetate buffer. The pH of the eluted enzyme was adjusted to pH 9.6 by adding 1 to 2ml of carbonate-bicarbonate buffer, pH 9.6, followed by dropwise addition of saturated $Na_2CO_3$. The pH of the antibody solution was likewise adjusted to pH 9.6. The antibody and enzyme solutions were added together, covered with aluminium foil and stirred slowly for 1 hr at room temperature. Three hundred milligrams of glycine ($NH_2.CH_2.COOH$) was then added and mixed and the pH adjusted to 8.0, using 1M HCl.

The conjugate was kept at 4°C overnight, after which it was precipitated with 33%(v/v) saturated ammonium sulphate solution for IgG or 50% for IgM, and then centrifuged for 2 min at 9,900 *Xg* and the pellet

dissolved and made up to the original volume with glycine/$Na_2$EDTA buffer (0.4M glycine, 0.3M NaCl and 20mM $Na_2$EDTA, pH 8.0). Twenty milligrams of ovalbumin was added and mixed to dissolve, and the solution centrifuged once more to remove particulate matter. The conjugate solution was then passed through a 0.45$\mu$m, followed by a 0.22$\mu$m membrane filter (Millipore Products Division, Bedford, MA, USA) to remove polymerized conjugate. Finally, an equal volume of glycerol was added, mixed and stored at -20°C.

### 3.11.2 Preparation of goat anti-mouse glucose oxidase conjugate

An IgG fraction of goat anti-mouse immunoglobulins was conjugated to glucose oxidase, using the periodate method. The protein concentration of the antibody to be conjugated was estimated as described earlier (section 3.11.1) and 20mg of it pipetted into a tube. The enzyme was conjugated to IgG antibody at a ratio of 1:1 (enzyme : antibody) by molar mass. Since both the enzyme and IgG had identical molecular weights (160,000), the ratio of 1:1 remained unchanged in terms of actual weights. Assuming 80% coupling efficiency, however, an increased amount of enzyme was required. Thus, for 20mg of IgG protein, 25mg of enzyme was required. The enzyme was weighed out and dissolved in 1ml of sodium-acetate buffer, pH 4.0. Also 8mg of $NaIO_4$ was weighed and dissolved in 1ml of sodium-acetate buffer. Oxidation of the enzyme was initiated by dropwise addition of the $NaIO_4$ solution, followed by 20 min incubation at room temperature without stirring. Fifty microlitres of ethyleneglycol was added to the mixture and incubated for a further 15 min to stop the oxidation. The enzyme solution was then centrifuged at 9,900 *Xg* to remove slight turbidity. The pH of the goat anti-mouse immunoglobulin, as well as that of the oxidized enzyme, were adjusted to pH 9.6 as described under section 3.11.1. The oxidized enzyme was then added dropwise to the antibody solution and the mixture incubated for 2 hr at

room temperature under slow stirring. One hundred milligrams of glycine were added and mixed to dissolve, followed by 1ml of glycine/$Na_2$EDTA buffer and the pH adjusted to 7.5, with 1M HCl.

The conjugate was then incubated at 4°C overnight, after which it was precipitated with 33%(v/v) saturated ammonium sulphate solution and centrifuged for 2 min at 9,900 *Xg*. The precipitate was immediately dissolved in glycine/$Na_2$EDTA buffer and centrifuged once more to remove any particulate matter. The rest of the procedure was the same as described previously for conjugation with HRPO (see section 3.11.1).

### 3.11.3 Preparation of goat anti-mouse FITC conjugate

An IgG fraction of goat anti-mouse immunoglobulins were conjugated to fluorescein isothiocynate (FITC) isomer 1 (Sigma, USA) as described by Clark and Shepard (1963) and modified by Katende, Musoke, Nantulya and Goddeeris (1987). Briefly, 5ml of the antibody fraction containing 50mg total protein was dialysed against freshly prepared carbonate-bicarbonate buffer (0.036M $NaHCO_3$, 0.014M $NaCO_3$, pH 9.6) overnight at 4°C. The antibody was further dialysed against 50ml of the same buffer, containing 1mg/ml FITC and 0.025% sodium azide, under continuous stirring in the dark at 4°C for 24 hr. It was then passed through a Sephadex G-50 column to separate unconjugated FITC from conjugated immunoglobulins. The conjugate was further fractionated by ion-exchange on diethylaminoethyl cellulose (DE-52) column. A 0.1 to 0.5M NaCl gradient in phosphate buffer (9.3mM $Na_2HPO_4$, 0.7mM $NaH_2PO_4$, pH 7.8) was used to elute optimally conjugated, overconjugated and underconjugated antibody in different peaks. The optimally conjugated antibody fraction was eluted with 0.25 to 0.3M NaCl.

# CHAPTER 4

# PRODUCTION AND CHARACTERIZATION OF MONOCLONAL ANTIBODIES

## 4.1 Summary

Mouse monoclonal antibodies (MoAbs) were produced against invariant antigens of the vector forms of *T. brucei, T. vivax, T. congolense* and *T. simiae*. To generate B-cell hybridomas, NSI/Ag4.1, Sp2/OAG14 or X63/AG8.653 myeloma cells were fused with splenic lymphocytes from BALB/c mice that had been immunized intraperitoneally or intravenously with various preparations of *T. brucei, T. congolense* or *T. simiae* procyclic or *T. vivax* epimastigote antigens. Hybridoma culture supernatants were screened for MoAb by indirect immunofluorescent antibody test (IFAT), micro-plate ELISA or by dot-enzyme immunoassay. Three *T. brucei* specific MoAbs (KT39a/18.17, KT43/33.32 and KT43/27.32), two *T. vivax* specific MoAbs (KD32/18.17 and KD37/19.3), two *Nannomonas* species-specific MoAbs (KN4/13.9 and KN5/6.15) and one *T. simiae* specific MoAb (KNS7/14.X) were derived. These MoAbs were added to a selection of trypanosome species-specific MoAbs derived by Nantulya *et al.* (1987) and (A. Jaye, unpublished), and characterized. IFAT studies showed that some of the *T. brucei* specific antigenic epitopes localize at the surface membrane and others in the cytoplasm of *T. brucei* procyclics. All the *Nannomonas* specific MoAbs stained the surface membrane, whilst all the *T. congolense* and the *T. simiae* MoAb stained internal antigens. Western immunoblot analysis, using procyclic lysates, revealed that the *T. brucei* MoAb that stained cytoplasmic antigens could not bind any antigens in that assay, whilst two of those that stained the membrane bound antigens of MW between 21 and 47 kDa. Three *T. congolense* MoAbs bound antigens of MW between 21 and 40 kDa, whilst one bound antigens between 51 and 85 kDa. Only one of the *Nannomonas* MoAbs (TC16/5.12.3) could detect antigens in this assay: It bound 18 and 89 kDa antigens in *T. congolense* procyclics and a 31 kDa antigen in *T. simiae* procyclics. Proteinase-K digestion and periodate oxidation studies showed

that, three *T. brucei* MoAbs bound protein antigenic determinants, and one bound a glycoprotein or lipoprotein antigen. Two of the *T. vivax* MoAbs also bound protein antigenic determinants, whilst one detected a carbohydrate or a lipid antigen. All the *T. congolense* MoAbs bound protein determinants, whilst all the *Nannomonas* and the *T. simiae* MoAbs detected carbohydrate determinants in glycoprotein or glycolipid antigens.

Reactivity studies revealed that all the *T. congolense* MoAbs react with the three types of *T. congolense* (the savannah, Kilifi and the riverine-forest types) that were tested. Also, all the trypanosome species-specific MoAbs were able to detect different trypanosome stocks isolated from different geographical areas, and none cross-reacted with *T. grayi*.

These findings indicate that the MoAbs generated in this study may facilitate the development of MoAb-based assays for detecting and differentiating the vector forms of the tsetse-borne trypanosomes.

## 4.2 Introduction

Köhler and Milstein (1975) discovered and first described the method for *in vitro* production of MoAbs by cell-fusion techniques. Since then, these reagents (MoAbs) have been shown to be highly specific probes for the analysis of complex antigenic mixtures (Pearson and Anderson, 1980).

Parish, Morrison and Pearson (1985) produced MoAbs that bound specifically to *Trypanosoma congolense*. Also, MoAbs capable of distinguishing between three of the most important species of the tsetse-transmitted African trypanosomes (*T. congolense, T. vivax* and *T. brucei*) were produced by Nantulya *et al.* (1987). These MoAbs were produced against invariant surface antigens of the insect forms (procyclics/epimastigotes) of the afore-mentioned trypanosome species, and some of these were utilized in the development of a sandwich micro-plate-based ELISA for the diagnosis of trypanosomiasis caused by *T. congolense, T. vivax* and *T. brucei* in the mammalian host (Nantulya, 1989; Nantulya and Lindqvist, 1989; Nantulya *et al.,* 1989b). In those studies, evaluation of the usefulness of the MoAbs centred around their ability to trap trypanosome species-specific antigens in the sera of infected mammals.

In order to select some of those MoAbs for ultimate application in the diagnosis of trypanosome infections in the vector, the specificity, as well as the sensitivity in terms of the minimum number of trypanosomes that could be detected by each of the extant MoAbs in the NC membrane-based dot-ELISA were determined (see Chapter 5). Based on that work, and on the ability of those MoAbs to bind antigens in the Western immunoblot technique, some of the existing MoAbs were selected for use in this study. The selected extant MoAbs included TR7/47.37.16 (*T. brucei* specific), TV8/8.33.32 (*T. vivax* specific), TC39/30.25.95, TC40/30.15.40 (*T. congolense* specific), and TC16/5.12.33, TC6/25.25.4 (*Nannomonas* specific) all derived by Nantulya *et*

*al.* (1987). Also included were TC6/42.6.3 (*T. congolense* specific) derived by Parish, Morrison and Pearson (1985), and C2, which was derived by A. Jaye (unpublished) against electroeluted antigen bands bound by TC39/30.38.16.

It was, however, decided to produce additional trypanosome species-specific MoAbs for three reasons:

1. The *T. brucei* specific MoAb selected (TR7/47.37.16) was an IgM. Earlier studies had shown that this antibody could not work in the Western immunoblot analysis, indicating that extensive characterization of the antigen detected by this antibody would be difficult.

2. The selected *T. vivax* specific MoAb (TV8/8.33.42) could detect only a minimum number of about $1x10^3$ East or West African *T. vivax* epimastigotes by dot-ELISA. This, compared with the *T. brucei* and *Nannomonas* species-specific MoAbs, which could detect 10 organisms per dot. Since it was known that in *T. vivax* infections, few trypanosomes can be found in the mouthparts, additional *T. vivax* specific MoAbs with higher sensitivity were needed.

3. The striking absence of a *T. simiae* specific MoAb (Table 7) despite previous attempts to produce one of that specificity was noted, and efforts were, therefore, initiated to derive new MoAbs with specificity to this species.

The derivation of additional *T. brucei, T. vivax* and *Nannomonas* species-specific MoAbs, as well as fresh attempts to derive a *T. simiae* specific MoAb, and the characterization of all the selected MoAbs, are reported in this Chapter. Experiments performed to investigate the usefulness of the trypanosome species-specific MoAbs as diagnostic reagents, in terms of their

ability to react with trypanosome stocks from different geographical areas, are also reported.

## 4.3 Materials and methods

### 4.3.1 Experimental animals

#### 4.3.1.1 Mice and Rats

Inbred male and female BALB/c mice, aged between 12 and 16 weeks, and nursling BALB/c mice, aged 14 days, were used for the production of MoAbs. Male outbred BALB/c x Swiss, aged between 12 and 16 weeks, and 12-week old Sprague Dawley rats, were used for the *in vivo* propagation of bloodstream form trypanosomes. All the mice and rats used in this study were obtained from the ILRAD colonies.

### 4.3.2 Preparation of trypanosome antigens

#### 4.3.2.1 Fixation of procyclics or epimastigotes for IFAT and Immunization

Procyclic or epimastigote trypanosomes were harvested from axenic cultures and washed twice in 0.01M phosphate-buffered saline (PBS), pH 7.4, by centrifugation at 400 *Xg* for 10 min at 4°C. After the last wash, a suspension of $4x10^6$ trypanosomes/ml in PBS was added to an equal volume of 2% formaldehyde (May and Baker Ltd., Dagenham, England) and incubated overnight at 4°C to fix and stabilize the membrane antigens, as described for surface coat antigens of bloodstream trypanosomes (Nantulya and Doyle, 1977). The fixed procyclics were washed again, as described above, and resuspended in PBS to a final concentration of $2x10^6$ trypanosomes/ml and used in indirect fluorescent antibody test (IFAT). Also, mice were immunized with $1x10^7$ fixed trypanosomes/mouse in order to induce humoral immune response, with antibodies directed against membrane bound antigens. Spleen localized B-lymphocytes from such mice were later used for the production of MoAbs.

**4.3.2.2** Preparation of procyclics and epimastigotes for immunolocalization by IFAT

Several fixatives were tested in order to determine the sensitivity of the trypanosome species-specific antigenic epitopes to aldehyde fixation. This was useful in determining whether it would be possible to localize the antigens by electron microscopy and IFAT.

**4.3.2.3** Fixation with paraformaldehyde or glutaraldehyde

Procyclic or epimastigote trypanosomes were harvested from axenic cultures and washed twice in PBS, pH 7.4, as described earlier (section 3.5.1 and 3.5.2). After the second wash, $1x10^8$ trypanosomes were suspended in 10ml of 8% paraformaldehyde or 0.5% glutaraldehyde diluted in PBS and pipetted into 15ml sterilin tubes (Sarstedt, Germany). The trypanosomes were then incubated at room temperature (18-23°C) for one hr to fix and stabilize the membrane antigens (Nantulya and Doyle, 1977). Thereafter, the parasites were pelleted by centrifugation at 400 *Xg* for 10 min at room temperature and the supernatant discarded. The trypanosome pellets were resuspended and washed twice in PBS. After the final wash, the parasites were resuspended in 2ml of PBS and 1ml of each sample pipetted into a different tube. One tube of each sample was kept at 4°C. The fixed trypanosomes were used to assess membrane surface localization of the antigens by IFAT, whilst the other half of the sample was processed further to assess internal localization of the antigens (section 4.3.2.5).

**4.3.2.4** Fixation with a mixture of paraformaldehyde and glutaraldehyde

*In vitro* propagated procyclic or epimastigote trypanosomes were fixed with a mixture of 4% paraformaldehyde and 0.5% glutaraldehyde diluted in PBS containing 0.2% picric acid and 0.5mM $CaCl_2$. The fixation procedure was the same as described above (section 4.3.2.3).

**4.3.2.5** Permeabilization of fixed procyclic or epimastigote cell membranes

Procyclic or epimastigote trypanosomes, fixed with either paraformaldehyde or glutaraldelyde or a mixture of both and washed in PBS, pH 7.4, were processed further in order to permeabilize the cell membrane. Fixed trypanosomes were incubated overnight in 0.1% Triton X-100 in PBS at 4°C. By this treatment, fixed trypanosome membranes were permeated by Triton X-100 making it possible to immunolocalize internal trypanosome antigens. After this treatment, the trypanosome suspensions were spun down at 400 *Xg* for 10 min at 4°C. The pelleted parasites were resuspended in PBS and washed twice by centrifugation, and resuspended in PBS to a final concentration of $2x10^6$ trypanosomes/ml and stored at 4°C, ready for immunofluorescence studies.

**4.3.2.6** Extraction of crude trypanosome antigens

The medium used to extract trypanosome antigens (extraction medium) consisted of a mixture of phenylmethyl-sulfonylfluoride (PMSF), N-$\alpha$-P-Tosyl-L-lysine chloromethyl Ketone (TLCK) and N-Tosyl-L-phenylalanine chloromethyl Ketone (TPCK) all from Sigma, Chemical Company, USA. They were reconstituted in absolute ethanol and diluted in PBS pH 7.4 to a final concentration of 1mM PMSF, 0.2mM TLCK, and 0.05mM TPCK. Protease inhibitors, Leupeptin and E-64, were then added each to a final concentration of 10$\mu$g/ml.

Trypanosome pellets, frozen at -20°C in Eppendorf tubes (Eppendorf-Netheler-Hinz GmbH, Humburg), were retrieved and extraction medium added before thawing. The trypanosomes were resuspended in the medium by pipetting up and down, vortexed for 1 min and frozen rapidly by immersion in liquid nitrogen (-196°C) in an insulated bucket. The tubes were then transferred to a waterbath at 37°C and the frozen suspension rapidly thawed. This freeze-thaw process was repeated two more times vortexing after

each thaw (Nantulya *et al.*, 1987). The sample was then spun at 9,900 *Xg* in a microcentrifuge (Eppendorf-Netheler-Hinz GmbH, Humburg) for 5 min and the supernatant removed and labelled "first extract". Fresh extraction medium was added to the pellet, mixed and the extraction process repeated to obtain the second, third and fourth extracts. The "first extract", which contained a large amount of trypanosome DNA, was discarded, whilst the "second", "third" and sometimes the "fourth" extract (depending on the concentration of the antigens), were pooled and used.

#### 4.3.2.7 Purification of trypanosome crude antigen extracts for immunization

Trypanosome crude antigen extracts, prepared as described above (section 4.3.2.6) were purified by column chromatography to obtain protein-free fractions which retained antigenic specificity to the homologous trypanosome species (Ijagbone, Staak and Reinhard, 1989). The crude trypanosome antigen extracts were dialysed against the elution buffer (O.1M Tris/HCl+1M NaCl pH 8.0) at 4°C overnight. Sephadex G-200 (Pharmacia Fine Chemicals, Uppsala, Sweden) was treated as recommended by the manufacturer. G-200 gel filtration was conducted in a cold room at 4°C in glass columns (100 x 2.5cm) using a peristaltic pump adjusted to a flow rate of 12ml/hr. A fraction collector and UV-monitor (LKB, Broma, Sweden) were used to collect fractions and monitor protein concentration at 280nm. All individual fractions were tested for antigenicity by antigen detection micro-plate ELISA. The results indicated two distinct peaks of antigenic activity. Pooled fractions corresponding to the two peaks were tested for protein by Lowry's method (Lowry, Rosebrough, Farr and Randall, 1951). These peaks were pooled and dialysed against 0.15M Tris/HCl buffered saline, pH 8.3, and fractionated by DEAE cellulose (Whatman, DE-52 pre-swollen) ion-exchange chromatography on a column measuring 40 x 2.5cm. Two peaks were eluted

again, of which the first peak contained antigenic activity while the second was antigenically inactive. The antigenically active peak was found to be protein-free and specific to the homologous trypanosome species by the Sandwich-ELISA.

### 4.3.3 Immunization of mice for MoAb production

Inbred male or female BALB/c mice were immunized with trypanosome antigens prepared by the different methods described below, in order to produce trypanosome species-specific MoAbs.

#### 4.3.3.1 Immunization with formaldehyde fixed trypanosomes

Male and female inbred BALB/c mice, aged between 12 and 16 weeks, were injected intraperitoneally with $1x10^7$ formaldehyde-fixed *T. brucei* or *T. simiae* procyclics or epimastigotes of *T. vivax*. After an interval of 3-4 weeks, the mice were given another intraperitoneal injection of $1x10^6$ fixed trypanosomes. This booster immunization was repeated at 6, 10, 14, and 16 weeks. Seven to ten days after the fifth booster, the mice were bled from the tail veins and tested for antibody response to homologous trypanosomal antigens by the micro-plate ELISA method. The best responder mice, with an antibody titre of 1:10,000 or more, were rested for 8 weeks and given a final booster with either $1x10^6$ trypanosomes intraperitoneally, or $1x10^5$ trypanosomes intravenously. Three to four days after this booster, the mice were bled from the retro-orbital venous plexus. The sera were separated and stored at -20°C as positive control prefusion sera. Immediately after bleeding, the mice were killed by terminal anaesthesia using diethyl-ether, and their spleens aseptically removed for cell fusion.

#### 4.3.3.2 Immunization with crude or purified trypanosome antigen extracts

BALB/c mice were immunized with either crude or purified trypanosome antigen extracts, both prepared as described earlier (sections 4.3.2.6, 4.3.2.7) as well as with electro-eluted proteins from polyacrylamide gels (4.3.10.6). Mice were injected intraperitoneally with 0.5ml total inoculum, consisting of 50-100$\mu$g of trypanosome antigens emulsified in Freund's complete adjuvant (Difco, Michigan, USA) supplemented with $10^9$IU of *Bordetella pertusis*. The first and second intraperitoneal booster immunizations were given respectively, 2 and 6 weeks later, using similar concentrations of trypanosome antigens as in the primary immunization, but this time in Freund's Incomplete adjuvant. The immunized mice were bled from the tail veins 7 to 10 days after the second booster, and screened for antibody response to the immunizing antigens using antibody detection micro-plate ELISA. Mice with high antibody titre (1:10,000 or more) were each given a final intraperitoneal booster of 50$\mu$g or intravenous booster of 20$\mu$g of the antigen preparation diluted in PBS, pH 7.4. Pre-fusion serum was prepared from blood obtained by orbital bleeding 3 to 4 days after the final booster. The mice were subsequently killed as described above and their spleens aseptically dissected out for cell fusion.

### 4.3.4 Screening of immunized mice for antibody response

The tip of the tail of each mouse was cut with a pair of scissors and the tail gently squeezed from the base towards the tip. Drops of blood from the severed tail veins were aspirated using a 10$\mu$l Eppendorf pipette and then transferred each into an Eppendorf tube containing 490$\mu$l of PBS, pH 7.4 and mixed thoroughly. The tubes were then spun at 9,900 *Xg* for 1 min and the supernatants pipetted into different tubes, and the sedimented blood cells discarded. Each supernatant was tested for antibody activity by titration in threefold dilutions on a polystyrene microtitre-plate previously coated with

trypanosome crude antigen extracts. The antibody activity was assayed as described under section 4.3.13.1. Mice with antibody titre of 1:10,000 or more were selected for cell fusions.

### 4.3.5 Cell fusion and selection for hybridomas

Three to four days after the final booster, the best immune responder mice were killed by terminal anaesthesia, with diethyl-ether and their spleens dissected out aseptically. Cell fusion was done following the method described by Pearson and others (1980) with some modifications. All the stages in this procedure were carried out under aseptic conditions in a flow-hood (Flow Laboratories).

The spleen from each mouse was separately minced with fine scissors and the splenocytes suspended in growth-medium in a 10ml conical sterilin tube (Sterilin, Teddington, Middlesex, UK). Clumps and membrane fragments were allowed 3 min to settle and the resulting cell suspension pipetted into another tube and washed once with serum-free RPMI-1640 by centrifugation at 400 *Xg* for 10 min. The cells were adjusted to $1\text{-}2\text{x}10^7$/ml and pipetted into a 50ml conical Falcon tube (Falcon, Becton Dickinson Labware, Oxnard California). The myeloma cells were washed similarly and adjusted to $1\text{-}2\text{x}10^6$/ml in serum-free RPMI-1640 and 10ml of the suspension mixed with the spleen cells at a ratio of 1:10 myeloma to spleen cells. The mixed cell suspension was centrifuged at 400 *Xg* for 10 min and the supernatant removed completely. 0.5ml of polyethylene glycol (PEG) Solution 1 [5g (PEG) 4000 (Serva, Heidelberg, Germany) liquefied by autoclaving and cooled to 60°C, and 7.0ml of serum-free RPMI-1640, and 1.5ml of dimethylsulfoxide (DMS0)], was added dropwise to the cell pellet. The cells were gently resuspended by stirring with the same pipette for 1 min, allowing mixing and agglutination of cells. With a new pipette, 0.5ml of PEG Solution 2 [5g of PEG autoclaved as above, in 15ml of serum-free RPMI-1640], was

added dropwise and mixed using the pipette for 2-3 min. This was followed by slow addition of 4 ml of fusion-medium [growth-medium further modified by addition of 10mM N-2-hydroxyethyl-piperazine-N-2-ethane sulfonic acid (HEPES) and 0.05mM 2-mercaptoethanol (2-ME)]. Forty millilitres more of fusion-medium were added and the cells mixed and then spun at 400 *Xg* for 10 min. The supernatant was discarded and the cell pellet resuspended in 48ml of fusion-medium. 1ml aliquots of this suspension were dispensed into two 24 well culture plates (24 well Tissue Culture Cluster, Costar) and 0.1ml ($2x10^6$/ml) spleen cells (which form a feeder layer) added to each well.

Plates were incubated at 37°C in a humidified atmosphere of 5% $CO_2$ in air mixture. The next day, 0.8ml of fusion medium was removed from each well and replaced with 1ml of HAT-medium consisting of 0.1mM hypoxanthine, $4x10^{-4}$mM aminopterin and 0.016mM thymidine dissolved in fusion medium. HAT-medium was replaced every 3 days until day 10 and changed to HT-medium consisting of 0.1mM hypoxanthine and 0.016mM thymidine dissolved in fusion medium. Usually between day 8 and 14, wells with large colonies of hybrid cells, as determined by observation under an Inverted microscope (Nikon, 46212, Japan), were marked and the media allowed to turn acid (yellow) and then tested for antibody activity (4.3.13.1). Hybrid cells from wells showing positive antibody activity were immediately cloned, and some stabilated as soon as possible (4.3.6). More supernatants were tested for antibody as the hybrids grew. When selected hybrids were growing well, the HT-medium was replaced with normal growth-medium.

### 4.3.6 Screening, cloning and stabilation of hybridomas

Culture fluids from wells with hybridoma cell colonies obtained after cell fusion were screened for antibody activity, using the antibody detection micro-plate ELISA or nitrocellulose membrane based dot-immunobinding assay (dot-ELISA). Hybrid cells from fusion wells that had

antibody activity were selected for cloning on the basis of optical densities or intensity of staining. Some cells from these wells were transferred to 25cm$^2$ tissue culture flasks, and grown until 2x10$^7$ cells could be frozen in liquid nitrogen (Pearson *et al.,* 1980). Cells were stabilated in growth medium, containing 7.5%(v/v) of DMSO. The remaining hybridomas were resuspended uniformly in the wells by gentle pipetting and live cells counted in an improved Neubauer Counting Chamber. Nigrosine was used as vital stain to distinguish live cells from dead cells. The hybrids were then cloned by limiting dilution in growth medium. A cell suspension was made to give 1 cell/60$\mu$l of medium and 50$\mu$l amounts pipetted into each well in 96 well tissue culture plates previously incubated (37$^o$C overnight) with 50$\mu$l/well (1x10$^6$/ml) splenocytes or thymocytes from 2-week old BALB/c mice. The 96 well plates were then incubated at 37$^o$C in a humidified atmosphere containing 5% $Co_2$ in air. The plates were left undisturbed for 10 days and then examined for visible cell colonies by viewing the bottom of the wells against light. Wells with single visible cell colonies were marked and the cells transferred into 500$\mu$l of growth-medium in 24-well culture plates. 1ml fresh growth-medium was added to the wells when the cell numbers increased and the culture fluids allowed to become acidic before testing for antibody. Antibody positive culture fluids were concentrated 10-15 times, and used for determination of immunoglobulin class by double immunodifusion, whilst the cells secreting the antibody were recloned.

### 4.3.7 IFAT procedure

Trypanosome procyclic or epimastigote suspensions containing 2x10$^6$ trypanosomes/ml, were prepared as described (sections 4.3.2.3, 4.3.2.4 and 4.3.2.5). Indirect immunofluorescence was performed as described by van Meirvenne, Janssens and Magnus (1975) with modifications. Fifty microlitres of the parasite suspension were delivered using a 50$\mu$l pipette into a

well on the Teflon-coated multitest microscope slide (Cooper Wellcome, Fremhodegen, Belgium) and the suspension immediately withdrawn leaving behind a thin film of fluid containing trypanosomes. The withdrawn fluid was transferred to the next well and the procedure repeated until all the 10 wells on a slide had received the parasite suspension. The slides were then dried for 15 min at 37°C and immersed in a bath of PBS, pH 7.4 for 5 min to wash off loosely bound trypanosomes. The slides were removed and 15$\mu$l of serial two-fold diluted trypanosome species-specific MoAbs added to the wells leaving out a few wells for assessing non-specific conjugate binding. The slides were incubated at room temperature for 15 min in a moist chamber. Excess MoAb was removed with absorbent tissue placed at the edges of the reaction wells. The slides were then dipped a few times in a bath of PBS and washed twice by immersion in fresh buffer for 2x10 min with one buffer change. FITC-labelled goat anti-mouse immunoglobulin prepared as described by Clark and Shepard (1963) and as modified by Katende *et al.* (1987) was added to the wells and incubated as above. The slides were washed as before and mounted in 50% glycerol in Tris-buffered saline, pH 9.0, for microscopy. The parasites were examined for fluorescence using the Leitz Ortholux fluorescence microscope (Leitz Wetzlar, Germany), fitted with x63/1.30 oil phase contrast objective and x6.3 periplan eyepieces under epiploem illumination with a 200W ultra-high pressure mercury vapour lamp.

### 4.3.8 Live IFAT procedure

The trypanosome species-specific MoAbs were also tested by IFAT, for reactivity with live procyclics and epimastigotes. This assay was performed as described by Barbet and McGuire (1978). Trypanosomes were incubated with MoAbs for 10 min on ice, then resuspended in 1% formaldehyde to prevent endocytosis. They were then washed twice in PBS by centrifugation at 400 *Xg* for 10 min. FITC-labelled goat anti-mouse

immunoglobulin was added to the parasite suspension to achieve a working dilution of 1:100. 1% nigrosine was added as counter stain and the suspension incubated for 10 min. The trypanosomes were washed as before, and coated on microscope slides by cytospin (Shandom, Southern, UK) at 2,200 *Xg*. The slides were mounted in 50% glycerol and the parasites examined for fluorescence as described under section 4.3.7.

### 4.3.9 Dot-ELISA procedure

#### 4.3.9.1 Detection of *in vitro* propagated trypanosomes by dot-ELISA

Whole trypanosomes harvested from *in vitro* cultures were suspended in buffer and applied onto NC membrane filters in dots and air dried as described earlier. All incubations and washings in this assay were performed on a gentle rocker at room temperature. The antigen "dotted" membranes were cut into strips and placed in the wells of a slot tray and incubated for 1 hr with "blocking solution" containing 5%(w/v) skimmed milk in Tris-buffered saline (TBS) (50mM Tris and 150mM NaCl, pH 8.0). The blocking solution was discarded and the strips further incubated for 3 hr with trypanosome species-specific MoAbs diluted in blocking solution. The specific antibody solution was poured out and the strips washed twice at 10 min intervals in TBS, pH 8.0. This was followed by a 1 hr incubation with peroxidase-conjugated goat anti-mouse immunoglobulin diluted 1:500 in blocking solution. After three washes the strips were incubated for 3 min in substrate solution containing 0.15%(v/v) hydrogen peroxide ($H_2O_2$) and 0.05%(w/v) chromogen (3,3'-diaminobenzidine) in phosphate-$Na_2$EDTA buffer (10mM $NaH_2PO_4$, 10mM $Na_2HPO_4$ and 10mM $Na_2$EDTA). The strips were rinsed twice with deionised water, after which the substrate reaction was stopped by immersing them for 5 min in deionised water acidified by the addition of a few drops of concentrated hydrochloric acid (HCl). The results

were read visually. Positive readings were observed as brown dots, whereas negative results remained colourless.

### 4.3.10 Characterization of MoAbs

#### 4.3.10.1 Determination of immunoglobulin class and subclass

The class and subclass specificity of the murine immunoglobulins (Igs) were determined by double immunodiffusion, a technique described by Ouchterlony (1967). Commercially prepared antisera (sheep anti-mouse Ig against murine Ig isotypes: $IgG_1$, $IgG_{2a}$, $IgG_{2b}$, $IgG_3$ and IgM) purchased from Sigma, were used as recommended by the manufacturer. A 1%(w/v) agarose (Bethesda Research Laboratories, USA) solution was prepared by melting solid agarose in phosphate buffered saline (15mM NaCl, 1.1mM $Na_2HPO_4$ and 0.1mM $KH_2PO_4$, pH 7.4). The molten agarose was poured onto microscope slides and allowed to solidify. Wells were then cut into the solid gel and each was filled with approximately 10$\mu$l of reagent. Antisera were placed in a central well and test samples (culture suparnatants concentrated ten-fold by ammonium sulphate precipitation) were placed in the surrounding wells. A precipitin line formed in-between a sample well and the homologous antiserum in a central well. The precipitin reaction was allowed to develop in up to 48 hr at room temperature in a wet chamber and observed by viewing the gels against light. For preservation, agarose gels were thoroughly washed with PBS, pH 7.4, followed by distilled water to remove unprecipitated proteins, dried, and stained using Coomassie Brilliant blue (Williams, 1971).

**4.3.10.2** Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE)

Electrophoresis of trypanosome proteins was performed, using the Bio Rad Protean II cell apparatus (Bio Rad, Italy) and following the SDS-Tris-glycine discontinuous buffer system (Laemmli, 1970).

**4.3.10.3** Assembly of slab gel apparatus, and preparation of resolution and stacking gels

The gel casting apparatus consisted of four transparent glass plates (16cm x 20cm), four 1.5mm thick plastic spacers, and four plastic clamps. These were assembled such that the two glass plates wiped clean, using 70% ethanol, were separated by the plastic spacers along the 16cm edges and clamped together. The assembled glass plates were then secured vertically on a gel casting platform so that the lower gap between each pair of plates was sealed by a rubber gasket.

Resolution acrylamide gradient gels (7.5-15%) were prepared as follows:

| Solution A, 7.5% resolution gel (one gel) | |
|---|---|
| Deionised water | 7.34ml |
| 30% (w/v) acrylamide, | |
| 0.8%(w/v) N'-methylene bis-acrylamide | 3.75ml |
| 1.5M,pH 8.8,tris-(hydroxymethyl) | |
| -aminomethane (Tris) | 3.75ml |
| 10% (w/v) sodium dodecyl sulphate (SDS) | 0.3ml |
| N,N,N'-,N'-tetramethylethylenediamine (TEMED) | 0.01ml |
| 10%(w/v) ammonium persulphate (APS) | 0.04ml |

Solution B. 15% resolution gel (one gel)

| | |
|---|---|
| Deionised water | 3.84ml |
| 30%(w/v)acrylamide; 0.8%(w/v) bis-acrylamide | 7.5ml |
| 1.5M Tris, pH 8.8 | 3.75ml |
| 10%(w/v) SDS | 0.3ml |
| TEMED | 0.01ml |
| 10% APS | 0.04ml |

The acrylamide/bis-acrylamide solution and TEMED were stored in brown bottles whilst 10% APS was prepared fresh and added to Solutions A and B just before gel casting. All solutions were kept at 4°C, except 10% SDS which was kept at room temperature.

A 7.5-15% resolution acrylamide gradient gel was prepared by slowly mixing solution A and B using Bio-Rad Model 385 gradient former. Each solution was swirled to mix and poured into one of the two separate chambers of the gradient mixer. Solution B, with the highest acrylamide concentration was placed in the chamber next to the outlet. The valve between the two chambers was opened and a magnetic stirrer placed in solution B started. A peristaltic pump (LKB, Sweden) set at a flow rate of 3ml/min was used to deliver the gel mixture via rubber tubing into the space between the two glass plates. The gel former apparatus was then immediately rinsed with distilled water. The poured gel was overlaid with 500-1000$\mu$l of water-saturated butanol using a micropipette or pasteaur pipette and left for approximately 1 hr to polymerize. After the resolution acrylamide gel (separating gel) had set, the gel overlay was removed and the top of the gel rinsed with distilled water. A stacking gel (Solution C) prepared as below was then poured on top of the separating gel and a comb carefully inserted to cast the desired wells for sample application.

Solution C. 3% stacking gel (one gel)

| | |
|---|---|
| Deionised water | 7.5ml |
| 30%(w/v) acrylamide; 0.8%(w/v) bis-acrylamide | 1.5ml |
| 0.5M Tris, pH 6.8 | 3.0ml |
| 10%(w/v) SDS | 0.125ml |
| TEMED | 0.01ml |
| 10% APS | 0.05ml |

The stacking gel was allowed 30 min to polymerize and the comb removed. The cast gel units were then assembled in a Bio-Rad Protean II cell electrophoresis apparatus. The upper electrophoretic chamber, at the cathode, was filled with 0.4 litres of running-buffer (24.8mM Tris, 191.8mM Glycine and 3.47mM SDS) and the lower chamber, at the anode, filled with 1.6 litres of running-buffer diluted with 0.5 litres of deionised water.

**4.3.10.4** Preparation of samples and electrophoresis run

The trypanosome crude extracts were adjusted for protein and diluted with sample buffer [150mM Tris, pH 6.8, 104mM SDS, 3%(v/v) mercaptoethanol, 30%(v/v) glycerol and 4%(v/v) bromophenol blue] at 2:1 sample to buffer ratio, to give a final protein concentration of approximately 1mg/ml. Standard high molecular weight markers (Rainbow Markers, MW 14,300 to 200,000; Amersham International plc, Amersham, UK) were diluted 1:1 with sample buffer without bromophenol blue. All the samples and standard markers were boiled for 5 and 1 min, respectively, at 100°C in a waterbath and centrifuged at 9,900 *Xg* for 5 min to remove particulate matter. About 200$\mu$g of the boiled trypanosome extracts or 5 to 10$\mu$l of standard molecular weight markers were loaded per lane of about 0.5cm width. Electrophoresis was performed using the Bio-Rad protean II cell apparatus, cooled to 10°C with a Lauda RC20 cooler (Bremen, Germany). A constant voltage using electrophoresis power supply (EPS 500/400, Pharmacia Fine

Chemicals) either at 50-70V/gel overnight, or at 300V/gel for 3-4 hr was applied until the bromophenol blue tracer dye migrated almost to the end of the separating gel. At the completion of the run, the assembled gel units were removed from the electrophoresis chamber and dismantled.

**4.3.10.5** Staining, destaining and western immunoblot

A vertical strip of the gel was cut using a surgical blade, and transferred into a plastic tray containing staining solution [0.5%(w/v) coomassie blue, 10%(v/v) acetic acid and 30%(v/v) isopropyl] for 15 min. The stained gel was then transferred to a destaining solution containing 10%(v/v) methanol and 7%(v/v) acetic acid on a gentle rotor (Red-Rotor Model PR70, Hoeffer) with several changes of the solution until stained protein bands were clearly visible in the gel (Weber and Osborn, 1969). Separated trypanosome proteins were transferred electrophoretically from unstained gels to nitrocellulose sheets, as described by Towbin, Staechelin and Gordon (1979) and Burnette (1981). Briefly, 3mm Whatman chromatography paper (Whatman, Maidstone, England) was soaked with transfer buffer [25mM Tris, 192mM glycine, 0.1%(w/v) SDS and 20%(v/v) methanol] and placed on top of a scouring pad (Scotch-Brite, Hoefer Scientific Instruments, USA) wetted in the same buffer and supported by a stiff plastic grid. The gel was then placed on top of the chromatography paper. A sheet of nitrocellulose filter (0.45$\mu$m pore size, Schleicher and Schuell, Inc., Keen, NH, USA) trimmed to fit the gel, was briefly wetted with transfer buffer and carefully placed on top of the gel without trapping air bubbles. A second chromatography paper and scouring pad, both soaked in the same buffer, and a plastic grid were added in that order and clipped. The sandwiched gel was then fitted in a Transphor Electrophoresis Unit, Model TE50 (Hoeffer Scientific Instruments, San Francisco, USA) filled with transfer buffer. Electrophoretic transfer was run at a constant voltage (either at 10V overnight

or at 70V for 3 hr) with the nitrocellulose sheet facing the cathode. The nitrocellulose was removed, cut into strips and immuno-assayed using the method described for the detection of *in vitro* propagated trypanosomes by dot-ELISA (section 4.3.9.1).

**4.3.10.6** Electro-elution of proteins from polyacrylamide gels

Individual coomassie-stained protein bands of interest were cut out of polyacrylamide gels using a scalpel blade, and chopped into small pieces. The two screw cups of the electrophoresis concentration chambers of the Ecu-040 electrophoresis elution apparatus (CBS Scientific Company, Inc., USA), were fitted with Spectra/Por dialysis membrane, molecular weight cut-off 3,500 (Spectrum Medical Industries, Inc., USA) and the cut gel placed in the large wells. All the chambers of the apparatus were filled with elution buffer (50mM ammonium bicarbonate, 0.97mM 1,4'-Dithiothreitol and 0.1%(w/v) SDS) to volumes recommended by the manufacturer. Protein elution was performed at a constant current of 12mA/cell for 17-24 hr at room temperature. The elution buffer was then carefully replaced with dialysis buffer (10mM $NH_3HCO_3$ and 0.02%(w/v) SDS) without disturbing the eluted proteins concentrated in the small well of the concentration chamber. Dialysis was performed using the same current for 2 hr. At the end of the run, the dialysis buffer was carefully pipetted out of the concentration chambers and the eluted proteins resuspended in a small volume of PBS, pH 7.4. These samples were immediately used to immunize mice for the production of MoAbs, or stored frozen at -20°C before use.

**4.3.11 Determination of the biochemical nature of antigenic epitopes**

**4.3.11.1** Detection of MoAbs specific for carbohydrate epitopes

The micro-plate ELISA based periodate oxidation at acid pH described by Woodward, Young and Bloodgood (1985) was used in

determining whether the antigenic epitopes detected by the specific MoAbs were carbohydrate in nature. Crude trypanosome extracts were diluted in coating buffer (34.5mM $NaHCO_3$ and 15.1mM $Na_2CO_3$) at dilutions previously determined by titration, and dispensed (100$\mu$l/well) into a 96-well micro-plate (Immulon, Dynatech Laboratories, Chantilly, Virginia, USA) and blocked overnight at 4$^{o}$C. The plates were rinsed once with washing buffer consisting of 0.05%(v/v) Tween 20 in PBS, pH 7.4, followed by a second rinse using 50mM sodium acetate buffer, pH 4.5. Sets of wells were then incubated with varying concentrations of periodate (0, 10, and 20mM) in sodium acetate buffer (100$\mu$l/well) for 1 hr at room temperature in the dark. The plates were rinsed once with sodium acetate buffer, and incubated with 1% glycine, 100$\mu$l/well, for 30 min at room temperature, after which they were rinsed five more times with washing buffer. The wells were incubated with MoAbs of murine origin diluted appropriately, in washing buffer, 100$\mu$l/well, for 1 hr at room temperature, and the plates washed five times with washing buffer to get rid of excess unbound antibody. To each well was then added 100$\mu$l of HRPO-conjugated goat anti-mouse antibodies diluted at 1:1000 in washing buffer. The plates were washed five times with washing buffer and incubated with substrate solution [40mM 2,2'-azino bis-(3-ethylbenz-thiazoline sulfonic acid) diammonium salt (ABTS) and 0.01%(v/v) hydrogen peroxide in 50mM citric acid buffer, pH 4.0]. The reaction was allowed to proceed for 30 min and the plates read at a wavelength of 414nm using a Titertek Multiskan micro-plate ELISA reader (MCC/340, Labsystems and Flow Laboratories, Finland).

#### 4.3.11.2 Detection of MoAbs specific for protein epitopes

Monoclonal antibodies (MoAbs) with specificity for protein antigenic epitopes were detected using enzymatic digestion with proteinase-K according to the methods described by Martin, Larose, Hamel, Lagac`e and

Brodeur (1988) and Lussier *et al.* (1989) with some modifications. Fifty micrograms of proteinase-K (Bethesda Research Laboratories, USA) diluted in PBS, pH 7.4, was added to 100$\mu$g of fresh trypanosome crude antigens extracted in PBS by the freeze and thaw method. One hundred microgram amounts of each antigen extract were pipetted into two different eppendorf tubes. Fifty micrograms of proteinase-K in 50$\mu$l PBS was added to one sample, whilst the other sample was diluted with 50$\mu$l of plain PBS. Both tubes were incubated at 37$^{o}$C in a waterbath for 1 hr and 3$\mu$l samples pipetted onto nitrocellulose strips in dots. The strips were assayed as described under section 4.3.9.1.

### 4.3.12 Micro-plate ELISA

Non-competitive ELISA techniques were used for the detection of antibody, employing the double antibody sandwich method (Cheng, L.Y., 1987; Beards and Bryden, 1981) and for the detection of antigen, using the indirect-system (Sandwich-ELISA) as described by Nantulya *et al.* (1987) and Nantulya (1989).

#### 4.3.12.1 Coating microtitre plates with antigen or antibody

Trypanosome extracts or purified MoAbs were diluted in carbonate-bicarbonate buffer consisting of 34.5mM $NaHCO_3$ and 15.1mM $Na_2CO_3$, pH 9.6 (coating buffer). Flat-bottomed 96-well microtitre plates were coated with 100$\mu$l/well of 10-15$\mu$g of crude antigen or 2.5-5.0 $\mu$g/ml of purified MoAb. The coating concentrations were determined by chequerboard titrations as described by Voller, Bidwell and Bartlett (1980). The plates were covered and the adsorption of antigen or antibody onto the polystyrene wells achieved by overnight incubation at 4$^{o}$C.

### 4.3.13 Micro-plate ELISA procedure

#### 4.3.13.1 Antibody detection ELISA

Antibody detection ELISA was used for screening hybridoma culture fluids for the selection of trypanosome species-specific MoAbs. This method was also used for screening sera from immunized mice. In this assay, micro-ELISA plates were coated with antigens of a particular trypanosome species, and then rinsed once with washing buffer to remove excess unbound antigen. Culture fluids and positive controls (prefusion sera taken from immunized mice diluted, 1:500) and negative controls (normal mouse serum diluted 1:500) or titrated sera from immunized mice, were transferred to a micro-ELISA plate (100$\mu$l/well) and incubated for 15 min at 37$^{o}$C. The micro-ELISA plates were rinsed once with washing buffer to remove excess unbound antibody, and all the wells incubated with 100$\mu$l/well goat anti-mouse HRPO conjugate diluted at 1:1000 for 15 min at 37$^{o}$C. The plates were then washed 3 times, each by 10 min incubation with washing buffer to remove excess unbound conjugate. The presence of bound conjugate was revealed by the addition of substrate solution consisting of 40mM 2,2'- azino-bis (3-ethylbenzthiazoline-6-sulfonic acid) (ABTS) and 0.01%(v/v) hydrogen peroxide in 50mM citric acid buffer, pH 4.0. The substrate was incubated for 30 min at room temperature. The colourless substrate solution changed to green in wells with bound enzyme conjugates. The optical densities were read at 414nm wavelength using a Titertek Multiskan micro-ELISA reader.

#### 4.3.13.2 Antigen detection sandwich-ELISA

A simplified sandwich-ELISA using MoAb-coated polystyrene micro-ELISA plates was used for antigen detection. The plates were coated with 100$\mu$/well of 2.5-5.0 $\mu$g/ml of purified MoAbs and rinsed once with washing buffer to remove uncoated antibody. The plates were then incubated for 15 min at 37$^{o}$C with 100$\mu$l/well of trypanosome antigen extracts titrated

serially (using washing buffer as diluent) to give protein concentrations of 10-0.714 $\mu$g/ml. Antigen molecules were captured specifically by the coating MoAb. The plates were rinsed twice with washing buffer to remove uncaptured antigen. This was followed by incubation with 100$\mu$l/well horseradish peroxidase-labelled MoAb, diluted 1:500 in washing buffer at 37$^{o}$C for 15 min. During this step, the conjugated MoAb reacted with the antigen that had previously been captured by the coating MoAb. The plates were washed, the substrate solution added, and the results read as described for antibody detection-ELISA (section 4.3.13.1).

**4.3.13.3** Inhibition ELISA

Inhibition ELISA was used to study the relationship between trypanosome species-specific MoAbs. By this method it was possible to determine whether the binding of one antibody inhibited binding by another. Micro-ELISA plates were coated with antigens of a particular trypanosome species and rinsed once with washing buffer. Sets of wells were then incubated for 15 min at 37$^{o}$C with 100$\mu$l/well serial dilutions of different MoAbs specific for the coating trypanosome species. MoAbs with specificity to different trypanosome species (non-related MoAbs) were titrated as above and used as controls. A second control consisted of a set of wells that were not incubated with any antibody. The plates were rinsed twice to remove excess unbound antibody and then incubated as above with a uniform concentration of MoAb-HRPO conjugate of one of the specific MoAbs used earlier. The plates were washed three times (10 min/wash) to remove excess antibody-conjugate. Substrate solution was added and the optical densities read as described previously (section 4.3.13.1). The effect of the non-related MoAbs on conjugate binding, was interpreted as protein to protein interactions that were not due to specific inhibition. The conjugate activity in the control wells that were not incubated with antibody, gave the level of conjugate

binding without interference. The results of these inhibition experiments were interpreted by two-way analysis of variance (Snedecor and Cochram, 1980).

## 4.4 Results

### 4.4.1 Selection of MoAbs from those produced previously

Experiments were carried out using the nitrocellulose membrane-based dot-ELISA, the indirect immunofluorescent antibody test (IFAT) and the micro-plate ELISA assays, to re-examine the specificities of the MoAbs produced previously by Nantulya *et al.* (1987). Also, the sensitivity, in terms of the minimum number of procyclics or epimastigotes that could be detected by the extant MoAbs in the dot-ELISA, was studied. Following those experiments, the MoAbs listed in Table 7 were selected for further studies.

The selected MoAbs reacted specifically with various trypanosome species or subspecies as shown in Table 7. The minimum number of trypanosomes that each of these MoAbs could detect, are however reported in Chapter 5.

### 4.4.2 Immunizing Antigens and Antibody Responses in Immunized Mice

The BALB/c mice responded well to the various trypanosome antigen preparations that were used in the immunizations. In general, high serum antibody responses, with titres far beyond 1:10,000, were obtained against homologous trypanosome antigens, as determined by the double-antibody sandwich micro-plate ELISA. However, differences were found in the ability of the three different trypanosome antigen preparations (namely, formaldehyde-fixed whole trypanosomes, trypanosome crude-antigen extracts or purified trypanosome antigens) to induce antibody responses that were essentially species-specific.

Figure 9, illustrates the mean antibody responses of mice following immunization with *T. brucei* procyclic crude antigen extract (BPCAE). The individual curves in this figure show that the mice produced antibodies that

**Table 7:** **Reactivity of selected extant MoAbs with procyclics or epimastigotes of different trypanosome species/subspecies as determined by dot-ELISA, IFAT and micro-plate ELISA**

| Monoclonal Antibody | Isotype | *T. brucei* * | *T. vivax* ** | *T. congolense* * | *T. simiae* * |
|---|---|---|---|---|---|
| TR7/47.37.16 | IgM | + | - | - | - |
| TV8/8.33.42 | $IgG_3$ | - | + | - | - |
| C2 | $IgG_1$ | - | - | + | - |
| TC6/42.6.3 | $IgG_1$ | - | - | + | - |
| TC40/30.15.40 | IgM | - | - | + | - |
| TC39/30.25.95 | IgM | - | - | + | - |
| TC16/5.12.33 | $IgG_1$ | - | - | + | + |
| TC6/25.25.4 | $IgG_3$ | - | - | + | + |

* procyclics.
** epimastigotes.
+ =antibody reacts with trypanosomes.
- =antibody does not react with trypanosomes.

**Figure 9**

Serum antibody response of BALB/c mice against antigens of different trypanosome species following immunization with *T. brucei* procyclic crude antigen extract. Each point represents the mean of three test readings obtained for three different mice ± the standard error.

O.D. = Optical density.

T.b.Ag = Curve showing serum antibody response against *T. brucei* antigen.

T.c.Ag = Curve showing serum antibody response against *T. congolense* antigen.

T.v.Ag = Curve showing serum antibody response against *T. vivax* antigen.

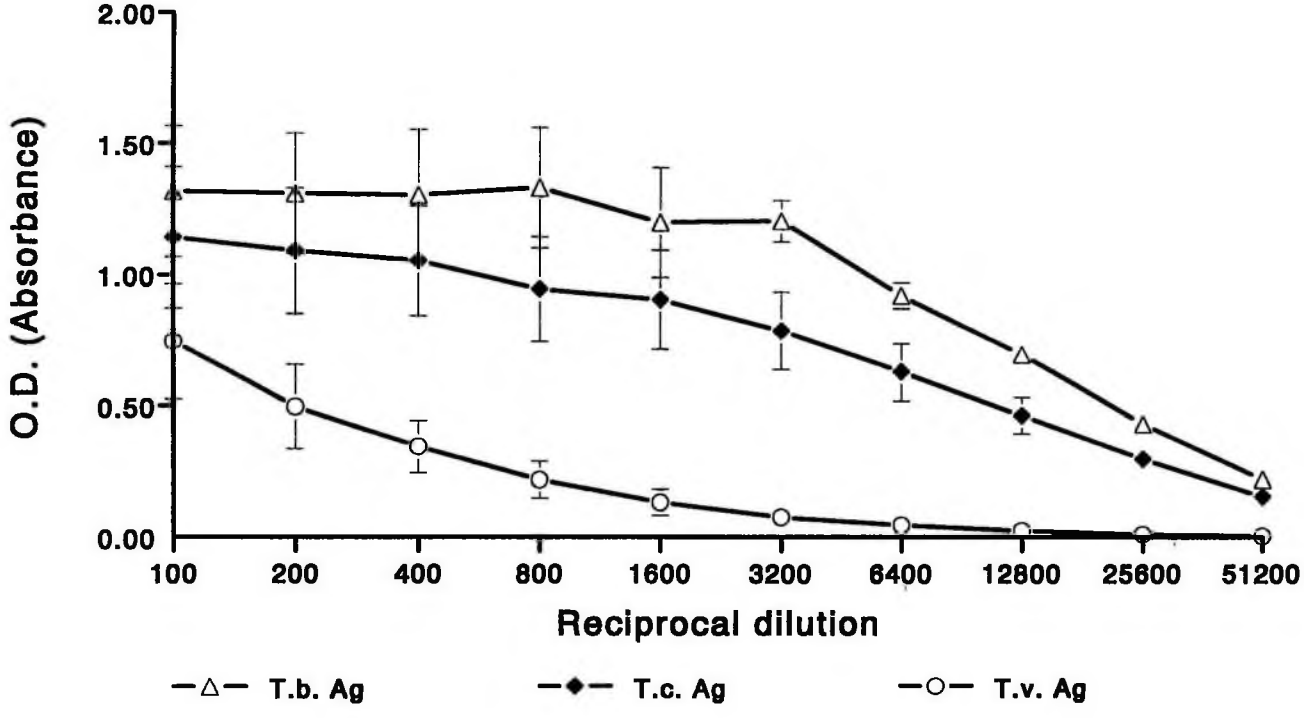



Figure 9

cross-reacted with equal concentrations of antigens derived from three different trypanosome species (*T. brucei, T. congolense* and *T. vivax*). Even though the mean antibody titre against immunizing *T. brucei* antigens was the highest, the titres recorded against *T. congolense* or *T. vivax* were substantially high, with both maintaining optical densities $\geq 0.5$ for serum dilutions of up to 1:200. On the other hand, the mean antibody response against *T. brucei* antigens was maintained at optical densities $\geq 1.0$ for serum dilutions of up to 1:6,400.

The mean antibody responses of other mice which were immunized with purified *T. brucei* procyclic antigens (PBPA), are shown in Figure 10. At a serum dilution of 1:100, the optical density of the mean response against *T. brucei* antigens was $> 1.0$ whereas, that against *T. congolense* or *T. vivax* was $< 0.25$. At a serum dilution of 1:400, the mean antibody response against *T. brucei* antigens still gave an optical density $> 1.0$, whereas, that against *T. congolense* and *T. vivax* were reduced to zero. At a serum dilution of 1:12,800, the optical density of the reactivity against *T. brucei* antigens, remained higher than that against *T. congolense* or *T. vivax* at 1:100 serum dilution. This immunization with purified *T. brucei* procyclic antigens, thus demonstrates antibody responses that were essentially species-specific.

The ability of purified trypanosome antigens to induce antibody responses that were essentially species-specific, was also demonstrated for *T. vivax* and *T. simiae*.

In the case of *T. vivax*, BALB/c mice were immunized with purified *T. vivax* epimastigote antigens (PVEA), and screened for serum antibody response against equal concentrations of *T. vivax*, *T. brucei*, *T. congolense* and *T. simiae* antigens. The individual curves shown in Figure 11, illustrate the mean antibody responses against the different antigens. At a serum dilution of 1:400, the mean optical density of serum antibody reactivity against *T. vivax* antigens was $> 1.5$, whilst that against the other trypanosome antigens, was $< 0.5$. The mean serum reactivity against *T. vivax* antigens maintained

**Figure 10**

Serum antibody response of BALB/c mice against antigens of different trypanosome species following immunization with purified *T. brucei* procyclic antigens. Each point represents the mean of three test readings obtained for three different mice ± the standard error.

O.D. = Optical density.

T.b.Ag = Curve showing serum antibody response against *T. brucei* antigen.

T.c.Ag = Curve showing serum antibody response against *T. congolense* antigen.

T.v.Ag = Curve showing serum antibody response against *T. vivax* antigen.

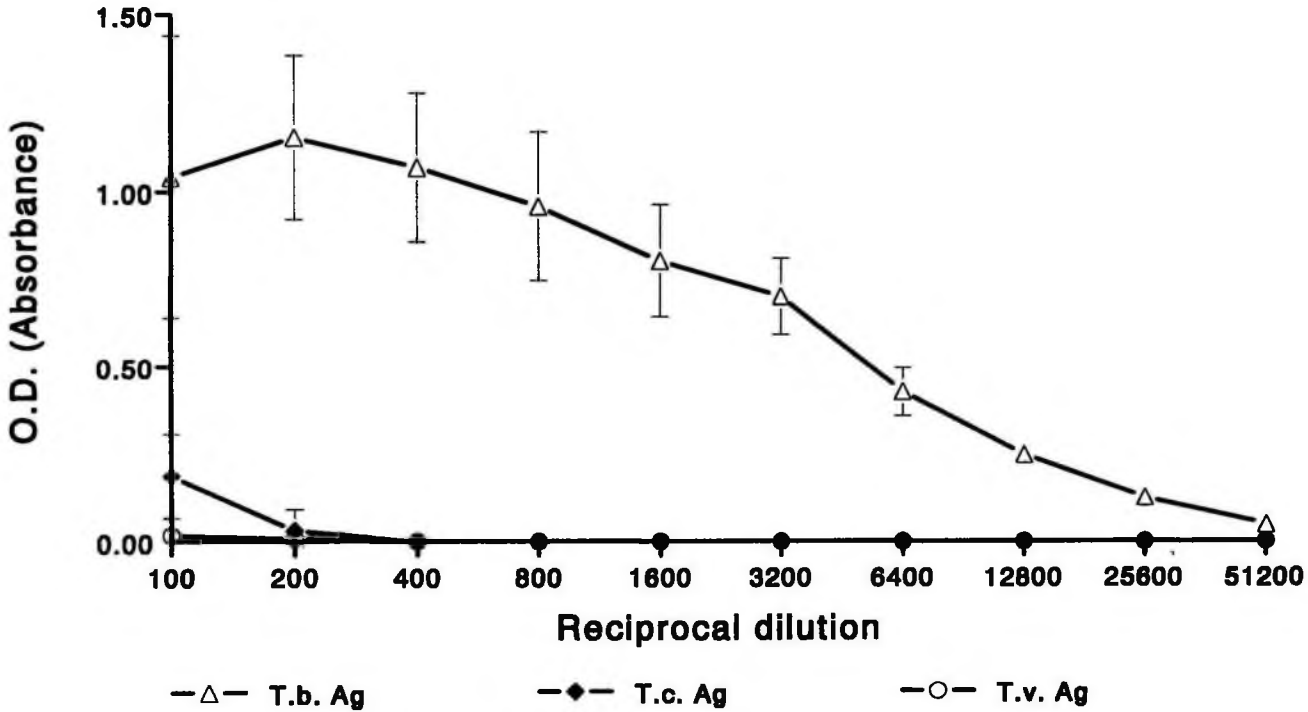



Figure 10

**Figure 11**

Serum antibody response of BALB/c mice against antigens of different trypanosome species following immunization with purified *T. vivax* epimastigote antigens. Each point represents the mean of three test readings obtained for three different mice ± the standard error.

O.D. =Optical density.

T.v.Ag =Curve showing serum antibody response against *T. vivax* antigens.

T.b.Ag =Curve showing serum antibody response against *T. brucei* antigens.

T.c.Ag =Curve showing serum antibody response against *T. congolense* antigens.

T.s.Ag =Curve showing serum antibody response against *T. simiae* antigens.

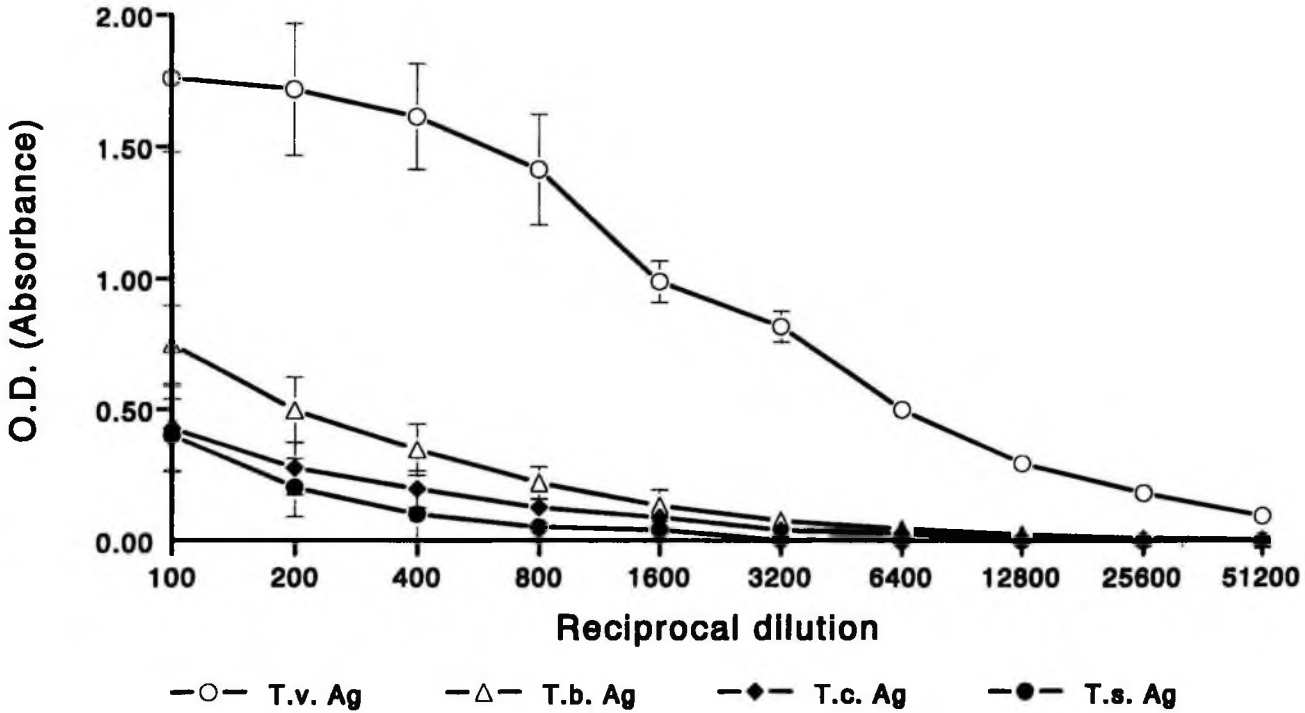



Figure 11

an optical density of about 0.5 at a serum titre of 1:6,400, when reactivity against antigens of the other trypanosome species were almost nonexistent (Figure 11).

Serum antibody responses of mice immunized with purified *T. simiae* procyclic antigens (PSPA) are shown in Figure 12. The individual curves show the mean serum antibody responses against equal amounts of *T. simiae*, *T. brucei* and *T. vivax* antigens. An elevated antibody response is clearly shown against *T. simiae* antigens as compared with the responses against antigens of the other trypanosome species. At a serum titre of 1:100, the mean optical density of the reactivity against *T. simiae* antigens was $>1.25$, whereas, that against antigens of *T. brucei, T. congolense* and *T. vivax* was each below 0.5. Furthermore, for serum dilutions $\geq$1:3,200, the reactivity on *T. simiae* antigens gave a mean optical density of about 0.75, whereas there were no reactions at all against antigens of the other trypanosome species, thus demonstrating specific reactivity with *T. simiae*.

Screening for serum antibody responses in immunized mice, was also performed using the IFAT technique. Table 8 summarizes the results obtained for serum antibody responses of one of the mice immunized with formaldehyde fixed *T. vivax* epimastigotes (FFVE). To enable the selection of the best responder mice for cell fusion, using this method of screening, the fluorescence on test trypanosomes were graded from; negative (-); weak positive (+); to strong positive (++++). Antibodies produced in this mouse reacted strongly with epimastigote antigens of East African *T. vivax* (EATV) as well as those of West African *T. vivax* (WATV), with antibody titres of up to 1:400 and 1:800 respectively (Table 8). Also the antibodies cross-reacted weakly $\leq$(++) with procyclic antigens of *T. brucei* (TB), *T. congolense* (TCK) and *T. simiae* (TS), with antibody titres ranging from 1:50 to 1:100.

**Figure 12**

Serum antibody response of BALB/c mice against antigens of different trypanosome species following immunization with purified *T. simiae* procyclic antigens. Each point represents the mean of three test readings obtained for three different mice ± the standard error.

O.D. =Optical density.

T.s.Ag =Curve showing serum antibody response against *T. simiae* antigens.

T.c.Ag =Curve showing serum antibody response against *T. congolense* antigens.

T.b.Ag =Curve showing serum antibody response against *T. brucei* antigens.

T.v.Ag =Curve showing serum antibody response against *T. vivax* antigens.

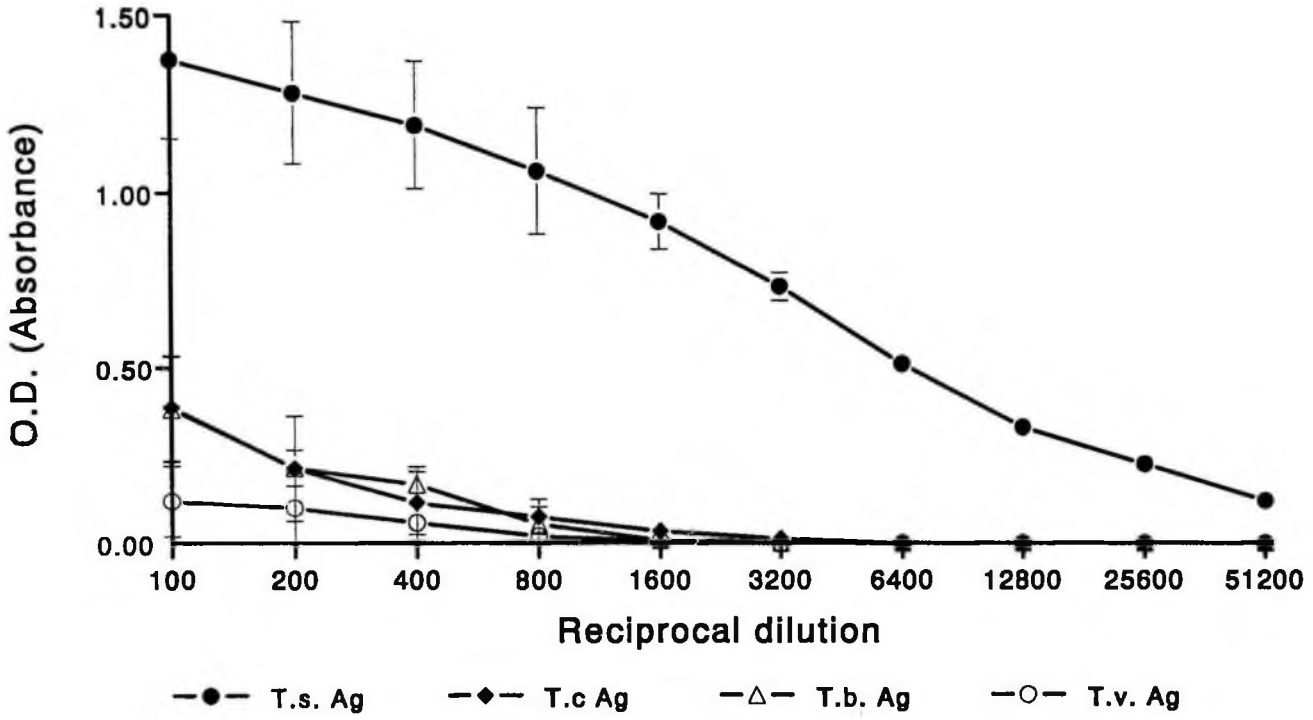



Figure 12

**Table 8**

**Antibody response of a BALB/c mouse following immunization with formaldehyde fixed *Trypanosoma vivax* epimastigotes**

| Serum dilution | Reactivity of serum with different trypanosome species by IFAT | | | | |
|---|---|---|---|---|---|
| | EATV | WATV | TB | TCK | TS |
| 1/50 | ++++ | ++++ | ++ | + | + |
| 1/100 | +++ | +++ | + | + | + |
| 1/200 | ++ | +++ | | - | |
| 1/400 | ++ | + | - | - | |
| 1/800 | - | + | | - | - |

EATV = East African *T. vivax* epimastigotes.
WATV = West African *T. vivax* epimastigotes.
TB = *T. brucei* procyclics.
TCK = *T. congolense* Kilifi type procyclics.
TS = *T. simiae* procyclics.

Other mice immunized with FFVE produced serum antibodies that reacted similarly as the one described above.

### 4.4.3 Cell Fusions and Selection for Hybridomas

Twenty eight independent cell fusions were made for the production of trypanosome species-specific MoAbs. Of those fusions, 10, 8 and 10 were made for the production of *T. brucei*, *T. vivax* and *T. simiae* specific MoAbs, respectively. Antibody secreting hybridomas were cloned at least twice by limiting dilution, and the class and subclass of the secreted MoAbs determined by the Ouchterlony double immunodiffusion method (section 4.3.10.1).

#### 4.4.3.1 Anti-*T. brucei* MoAb secreting hybridomas

The results of ten independent cell fusions made towards the production of *T. brucei* specific MoAbs are summarized in Table 9. Four of these fusions (TB39a, TB40a, TB40b and TB42) were carried out using spleen cells from mice immunized with formaldehyde fixed *T. brucei* procyclics (FFBP). From these fusions, only one hybridoma from TB39a had the desired specificity for *T. brucei*. Another hybridoma obtained from TB40a secreted a MoAb that reacted specifically with *T. brucei* antigens in the double antibody sandwich ELISA. However that MoAb cross-reacted with antigens of *T. congolense* and *T. simiae* when tested using the Western immunoblot technique (Table 9). Cell fusions (TB40c, TB40d and TB41) were undertaken using spleen cells from mice immunized with crude antigen extracts of *T. brucei* procyclics (BPCAE) (Table 9). None of the hybridoma cells derived from those three fusions secreted any MoAb that was *T. brucei* specific, despite a high number of fusion wells with antibody activity (Table 9). The highest success in deriving hybridomas that secreted *T. brucei* specific MoAbs was achieved from fusions TB43 and TB44, both of which were carried out using spleen cells

**Table 9: Results of cell fusions made towards the production of *T. brucei* specific MoAbs**

| Fusion | Origin of spleen cells | Immunogen | Myeloma parent | Spleen cell count for fusion | Numbers of wells showing growth of hybrids | Number (%) of positive wells [a] | Number of wells with specific reactivity[b] | Number of hybrids cross-reacting by other tests | Number of stable hybrids secreting specific antibody[s] |
|---|---|---|---|---|---|---|---|---|---|
| TB39a | BALB/c | FFBP | NSI/1Ag4.1 | $3.6x10^6$ | 16/48 | 10(62%) | 1 | 0 | 1 |
| TB40a | " | | | $4.1x10^6$ | 48/48 | 41(85%) | 1 | 1[w] | 0 |
| TB40b | ' | " | ' | $3.2x10^8$ | 42/48 | 31(74%) | 0 | n/a | n/a |
| TB40c | " | BPCAE | | $2.1x10^6$ | 45/48 | 36(80%) | 0 | n/a | n/a |
| TB40d | " | " | ' | $3.2x10^5$ | 48/48 | 47(98%) | 0 | n/a | n/a |
| TB41 | " | " | " | $5.2x10^7$ | 20/48 | 10(50%) | 0 | n/a | n/a |
| TB42 | " | FFBP | " | $8x10^6$ | 12/48 | 3(25%) | 0 | n/a | n/a |
| TB43 | " | PBPA | X63/AG8.653 | $6.9x10^7$ | 42/48 | 16(38%) | 4 | 0 | 3 |
| TB44 | " | ' | | $9.4x10^6$ | 40/48 | 6(15%) | 4 | 0 | 2 |
| TB45 | " | " | ' | $7.9x10^7$ | 48/48 | 48(100%) | 3 | 3[w] | 0 |

[a] Supernatants were tested in double antibody sandwich ELISA using *T. brucei* antigen coated micro-ELISA plates.
[b] Tested in double antibody sandwich ELISA using 4 different antigens (*T. brucei, T. vivax, T. congolense* and *T. simiae*).
[s] Hybridoma cells which secreted specific antibody into culture supernatants after two months of continuous culture *in vitro*.
[w] Number of hybridomas that secreted cross-reacting MoAbs when tested using the Western immunoblot technique.
n/a not applicable.
FFBP = Formaldehyde fixed *T. brucei* procyclics.
BPCAE = *T. brucei* procyclic crude antigen extract.
PBPA = Purified *T. brucei* procyclic antigens.

from mice immunized with a purified *T. brucei* procyclic antigen preparation (PBPA). These two fusions yielded 5 *T. brucei* specific antibody-producing hybridomas (Table 9). One other fusion which was undertaken using spleen cells from a mouse that was immunized with PBPA produced three hybridomas that secreted MoAbs that reacted specifically with *T. brucei* in the micro-plate ELISA. However, those MoAbs were found to cross-react with *T. congolense*, *T. simiae* and *T. vivax* in the Western immunoblot assay (Figure 13). Cell clones that were isolated from *T. brucei* specific antibody-secreting hybridomas were found to be stable as determined by sustained MoAb secretion in continuous *in vitro* culture for two months.

Figure 14 shows an example of the micro-plate ELISA results when used for screening 45 out of 48 wells from the *T. brucei* cell fusion TB43. The reactions in wells A(5&6) and F(9&10) occur on all three microtitre plates, indicating that the hybridoma cells from the corresponding tissue culture well secreted antibodies that cross-reacted with antigens from all the three species of trypanosomes. On the other hand, the reactions in wells B(1&2), C(5&6), D(5&6), E(5&6) and F(5&6) are only seen on the *T. brucei* coated plate, demonstrating that the hybridoma cells from the corresponding tissue culture wells were secreting antibodies that react specifically with only *T. brucei* antigens.

#### 4.4.3.2 Anti-*T. vivax* MoAb secreting hybridomas

Table 10 gives a summary of all the cell fusions performed with the aim of producing *T. vivax* specific MoAbs. Five of these fusions (TV30, TV31, TV32, TV33 and TV34) were made using spleen cells from mice immunized with formaldehyde fixed *T. vivax* epimastigotes. Of these one fusion (TV34) produced no wells with antibody activity even though two wells had hybridoma cell colonies (Table 10), and two (TV30 and TV33) produced

**Figure 13**

Western immunoblot cross-reactivity of a MoAb secreted by a hybridoma which was produced by fusing a myeloma cell with a spleen lymphocyte from a BALB/c mouse immunized with purified *T. brucei* procyclic antigens. Strip (A) shows the reactivity with electrophoresed *T. brucei* IL2616 antigens; (B) with *T. congolense* K/83/IL/97/2 antigens; (C) with *T. simiae* KETRI 2431 antigens, and (D) with *T. vivax* IL1392 antigens. Notice the weak cross-reactivity of the MoAb with a 58 kDa antigen.


Figure 13

**Figure 14**

Screening for *T. brucei* specific MoAbs in culture supernatants from cell fusion TB43 using micro-plate ELISA. The spleen cell donor BALB/c mouse was immunized with purified *T. brucei* procyclic antigens. Plate I was coated with *T. brucei* procyclic antigen; Plate II with *T. congolense* procyclic antigen, and Plate III with *T. vivax* epimastigote antigen. Culture supernatants from fusion wells were tested in duplicates in identical wells on all three plates. Wells G(11&12) were incubated with 1:100 dilution of normal mouse serum as negative controls, and H(11&12) were incubated with 1:100 dilution of pre-fusion mouse serum as positive controls. Also, wells A(1&2) was used to assess non-specific conjugate binding by omitting the addition of samples.

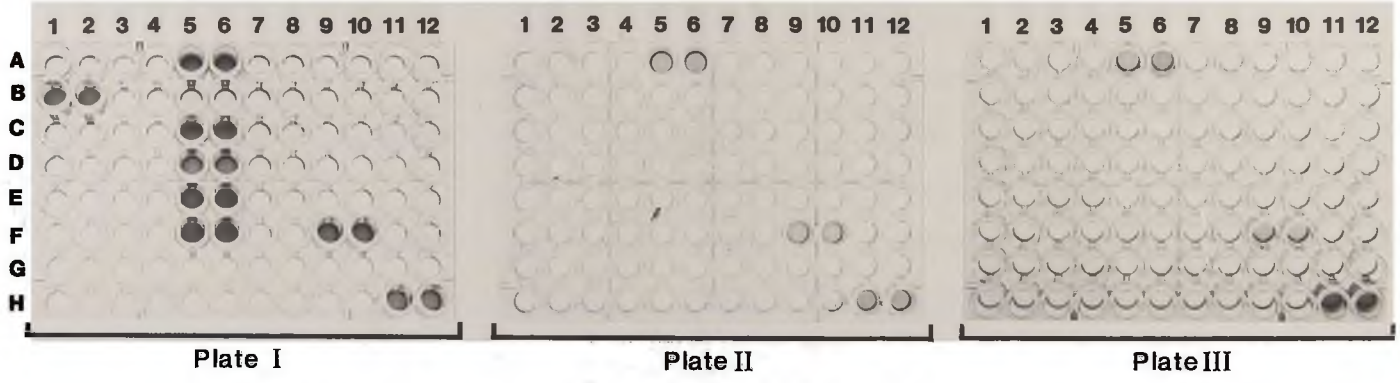



**Figure 14**

**Table 10: Results of cell fusions made towards the production of *T. vivax* specific MoAbs**

| Fusion | Origin of spleen cells | Immunogen | Myeloma parent | Spleen cell count for fusion | Numbers of wells showing growth of hybrids | Number (%) of positive wells[a] | Number of wells with specific reactivity[b] | Number of hybrids cross-reacting by other tests[w] | Number of stable hybrid secreting specific antibody[s] |
|---|---|---|---|---|---|---|---|---|---|
| TV30 | BALB/c | FFVE | NSI/1Ag4.1 | $1.25x10^7$ | 5/48 | 2(40%) | 0 | n/a | n/a |
| TV31 | " | ' | ' | $1.65x10^7$ | 17/48 | 7(41%) | $1^I$ | 0 | 1 |
| TV32 | " | " | ' | $5.8x10^7$ | 38/48 | 19(50%) | $3^I$ | 0 | 3 |
| TV33 | | ' | | $8.8x10^6$ | 26/48 | 4(15%) | 0 | n/a | n/a |
| TV34 | " | " | Sp2/OAG14 | $6.4x10^5$ | 2/48 | 0(0%) | 0 | n/a | n/a |
| TV35 | " | CVEAL | | $5x10^6$ | 15/48 | 5(33%) | 0 | n/a | n/a |
| TV36 | | " | X63/AG8.653 | $2.4x10^6$ | 45/48 | 30(67%) | 0 | n/a | n/a |
| TV37 | ' | PVEA | | $3.2x10^7$ | 48/48 | 48(100%) | $9^D$ | 0 | 9 |

[a] Supernatants were tested in double antibody sandwich ELISA using *T. vivax* antigen coated micro-ELISA plates.
[b] Tested in double antibody sandwich ELISA using 4 different antigens (*T. brucei, T. vivax, T. congolense* and *T. simiae*).
[s] Hybridoma cells which secreted specific antibody into culture supernatants after one month of continuous culture *in vitro*.
[I] Supernatants were tested in Indirect Fluorescent Antibody Test (IFAT) using 4 different antigens (*T. vivax, T. brucei, T. congolense* and *T. simiae*).
[D] Tested in dot enzyme immunoassay (dot-ELISA) using 4 different antigens (*T. vivax, T. brucei, T. congolense* and *T. simiae*).
[w] Tested for cross-reactivity using the Western immunoblot technique.
n/a not applicable.
FFVE = Formaldehyde fixed *T. vivax* epimastigotes.
CVEAL = Crude *T. vivax* epimastigote antigen lysate.
PVEA = Purified *T. vivax* epimastigote antigens.

wells with antibody activity, but none of them was specific for *T. vivax*. The remaining two fusions (TV31 and TV32) produced four hybridomas of desired specificity for *T. vivax* as determined by IFAT. All the positive clones derived from those wells were stable (Table 10).

None of two fusions (TV35 and TV36) made with spleen cells from mice immunized with crude *T. vivax* epimastigote antigen lysate (CVEAL) produced wells with specific activity against *T. vivax*, although both fusions had wells with antibody activity against trypanosome antigens. Cell fusion (TV37) was made using spleen cells from mouse MTV32 immunized with a purified *T. vivax* epimastigote antigen (see Figure 11). The products from this cell fusion were screened by dot-ELISA (Figure 15). All the 48 fusion wells showed antibody activity against the *T. vivax* epimastigote antigen (Figure 15). Of these, wells (1, 4, 19, 32, 33, 34, 35, 42 and 43) contained antibodies that reacted specifically with the *T. vivax* antigen. The cells from wells 19, 33 and 34 were selected for cloning based on the stronger reactivity of the secreted antibodies and the relatively fewer cells that were present in those wells.

The hybridoma (KD37/19.3) derived from well 19 was selected for further studies.

#### 4.4.3.3 Anti-*T. simiae* MoAb secreting hybridomas

Of ten cell fusions carried out with the aim of producing *T. simiae* specific MoAbs, four were made using spleen cells from mice previously immunized with formaldehyde fixed *T. simiae* procyclics (FFSP). All these four fusions (TS1, TS2, TS3 and TS4) produced hybridomas which secreted antibodies to trypanosome antigens (Table 11). However, only two of those fusions (TS3 and TS4) yielded hybridomas with antibodies specific for either *T. simiae* or the *Nannomonas* subgenus. TS3 had one well with hybrids that secreted *T. simiae* specific antibodies. These hybrids were, however, not

**Figure 15**

Screening for *T. vivax* specific MoAbs in culture supernatants from wells of cell fusion TV37 using dot-ELISA. The spleen cell donor BALB/c mouse was immunized with purified *T. vivax* epimastigote antigens. Culture supernatant from each of the 48 fusion wells was incubated with one strip of NC membrane which was previously "dotted" with $1x10^5$ *T. congolense* K/83/IL/97/2 (TCK), *T. brucei* IL2616 (TB) and *T. simiae* KETRI 2431 (TS) cultured procyclics, and $1x10^5$ *T. vivax* IL1392 (TV) cultured epimastigotes. (▲) represents culture supernatants that contained antibodies that reacted specifically with *T. vivax* epimastigotes; (■) represents culture supernatants that contained antibodies that cross-reacted with all the four trypanosome species that were tested.

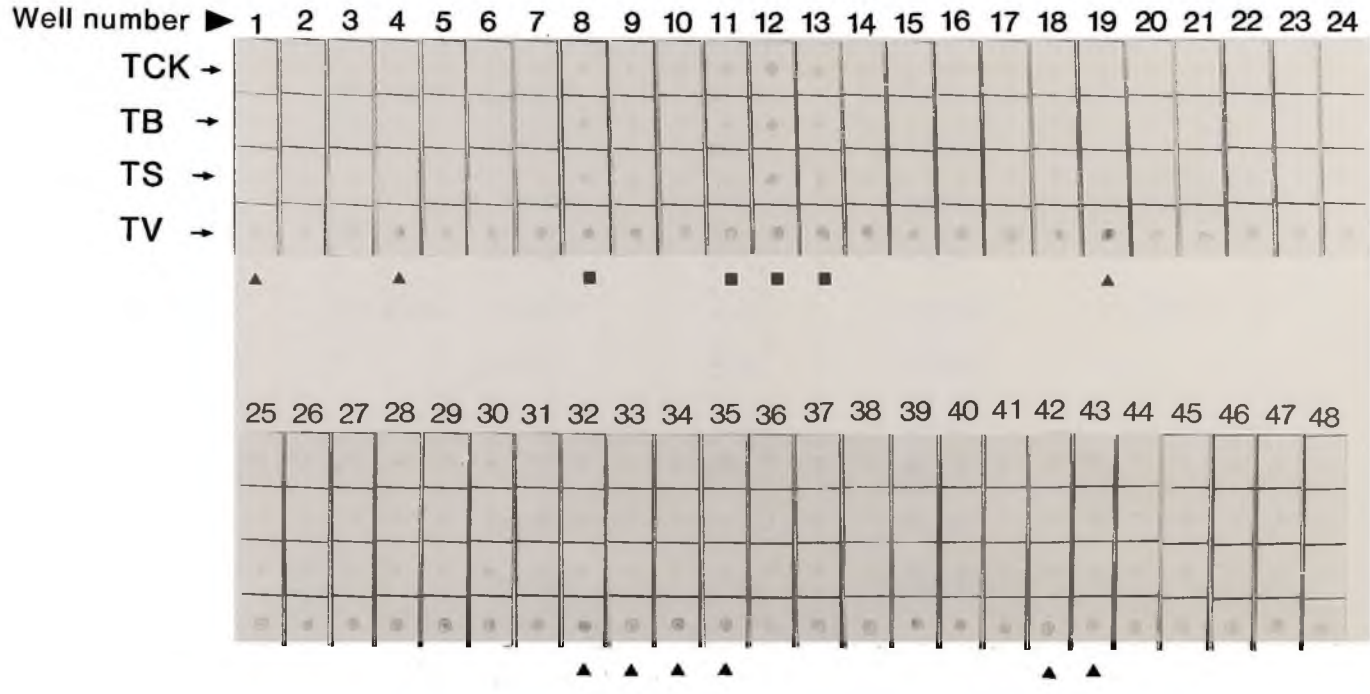



**Figure 15**

**Table 11: Results of cell fusions made towards the production of *T. simiae* specific MoAbs**

| Fusion | Origin of spleen cells | Immunogen | Myeloma parent | Spleen cell count for fusion | Numbers of wells showing growth of hybrids | Number (%) of positive wells[a] | Number of wells with specific reactivity | Number of hybrids cross-reacting by other tests[W] | Number of stable hybrids secreting specific antibody[S] |
|---|---|---|---|---|---|---|---|---|---|
| TS1 | BALB/c | FFSP | NSI/1Ag401 | $1.2x10^7$ | 11/48 | 6(54%) | 0 | n/a | n/a |
| TS2 | " | " | | $6.8x10^6$ | 9/48 | 7(78%) | 0 | n/a | n/a |
| TS3 | " | " | | $2.3x10^5$ | 8/48 | 4(50%) | $1^I$ | 0 | 0 |
| TS4 | | | | $7.1x10^6$ | 10/48 | 8(80%) | $1^{*I}$ | 0 | 1 |
| TS5 | " | CSPAL | Sp20AG14 | $3.8x10^6$ | 16/48 | 12(75%) | $2^D,11^{*D}$ | 0 | 1 |
| TS6 | ' | | " | $4.4x10^7$ | 48/48 | 47(98%) | 0 | n/a | n/a |
| TS7 | " | PSPA | X63/AG8.653 | $7.2x10^7$ | 48/48 | 48(100%) | $1^D$ | 0 | 0 |
| TS8 | " | (41.7-43.6)kDa** | | $6.9x10^7$ | 8/48 | 2(25%) | 0 | n/a | n/a |
| TS9 | ' | 75 kDa** | | $8.9x10^7$ | 13/48 | 6(46%) | 0 | n/a | n/a |
| TS10 | " | 107 kDa** | " | $1.1x10^7$ | 18/48 | 10(56%) | 0 | n/a | n/a |

[a] Supernatants were tested in double antibody sandwich ELISA using *T. simiae* antigen coated micro-ELISA plates.
[S] Hybridoma cells which secreted specific antibody into culture supernatants after one month of continuous culture *in vitro*.
[I] Supernatants were tested in Indirect Fluorescent Antibody Test (IFAT) using 4 different antigens (*T. simiae, T. congolense, T. brucei* and *T. vivax*).
[D] Tested in dot enzyme immunoassay (dot-ELISA) using 4 different antigens (*T. simiae, T. congolense, T. brucei* and *T. vivax*).
[W] Tested for cross-reactivity by the Western blot technique.
n/a Not applicable.
FFSP = Formaldehyde fixed *T. simiae* procyclics.
CSPAL = Crude *T. simiae* procyclic antigen lysate.
PSPA = Purified *T. simiae* procyclic antigens.
* Hybridoma secreting *Nannomonas* species-specific monoclonal antibody.
** Antigen band electro-eluted from polyacrylamide gels.

stable as they stopped antibody secretion when maintained in continuous culture *in vitro*. As a result, the hybrid was lost during cloning. The fusion (TS4) had one well with specificity for the *Nannomonas* subgenus. This hybrid was cloned successfully.

Two fusions (TS5 and TS6) were made using spleen cells from mice immunized with crude *T. simiae* procyclic antigen lysate (CSPAL). One of these fusions (TS5) produced one hybridoma with specific antibodies to the *Nannomonas* subgenus (Table 11). However, none of the hybridomas from TS6 secreted antibodies that were specific for either *T. simiae* or the *Nannomonas* subgenus. The fusions (TS8, TS9 and TS10) were made using spleen cells from mice immunized with *T. simiae* antigen bands of varying molecular weights that had been electro-eluted from polyacrylamide gels (Table 11, Figure 16). These bands were selected based on the following rationale. *T. simiae* and *T. congolense* procyclic extracts were electrophoresed side by side on polyacrylamide gels and the resolved bands studied for differences in molecular weight (MW). All *T. simiae* antigen bands that occur at MW where there were no corresponding *T. congolense* bands, were pin-pointed. Reasoning that some of the *T. simiae* unique bands may contain antigens that define *T. simiae* specificity, the bands were separately electro-eluted and used for immunizing BALB/c mice. Though each of the three fusions made with spleen cells from mice immunized with electro-eluted antigen bands produced hybridomas with antibody activity, none of them was specific for *T. simiae* or the *Nannomonas* subgenus.

The fusion (TS7) which was made using spleen cells from the mouse MTS7 immunized with a purified *T. simiae* procyclic antigen produced the most promising results (Figure 17). Figure 17 shows the result of this cell fusion as screened by dot-ELISA. All the wells from this fusion had colonies of hybrid cells that were secreting antibodies to trypanosome antigens. Of these, 11 wells had antibodies specific to the *Nannomonas* subgenus, whereas 2

**Figure 16**

Comparison of coomassie stained polyacrylamide gel electrophoresed *T. simiae* (KETRI 2431) and *T. congolense* (K/83/IL/97/2) antigens. Lane 'C' shows the staining pattern of *T. simiae* antigens; Lane 'B' the staining pattern of *T. congolense* antigens; and lane 'A', the molecular weight markers. *T. simiae* antigen bands without corresponding *T. congolense* bands of identical molecular weights were numbered 1 to 8.

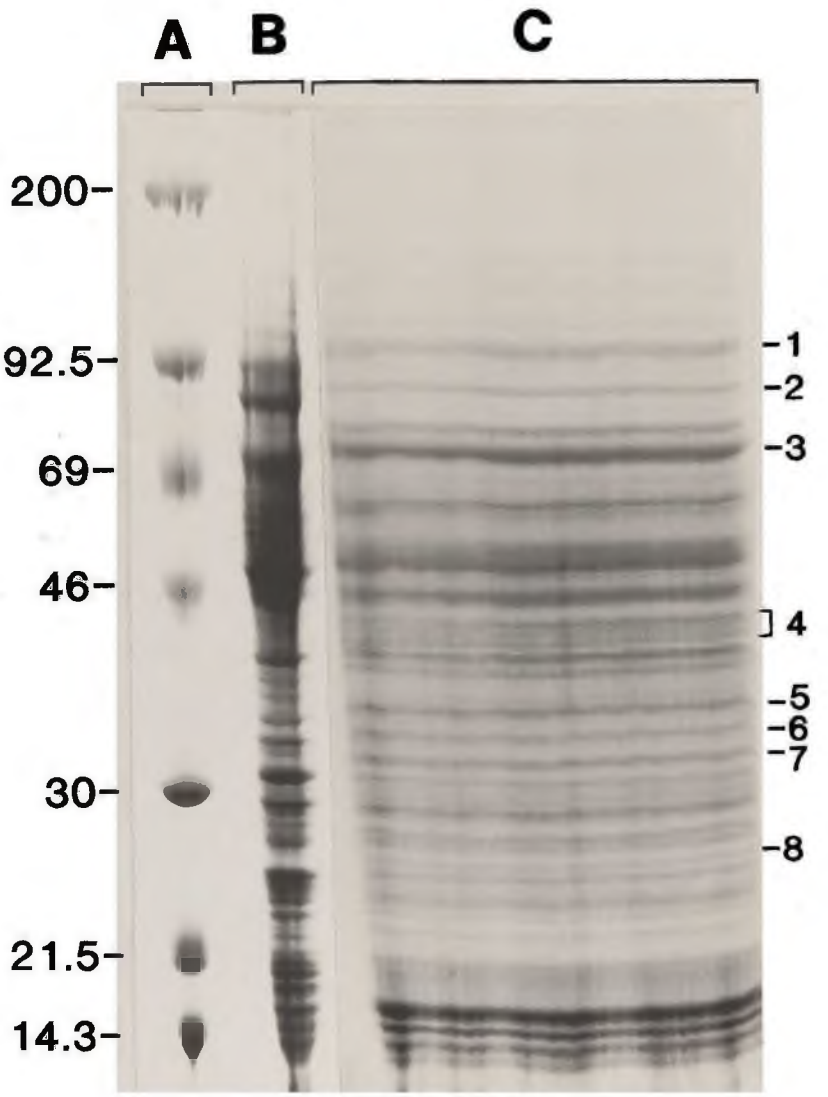



Figure 16

**Figure 17**

Screening for *T. simiae* specific MoAbs in culture supernatants from wells of cell fusion TS7 using dot-ELISA. The spleen cell donor BALB/c mouse was immunized with purified *T. simiae* procyclic antigens. Culture supernatant from each of the 48 fusion wells was incubated with one strip of NC membrane which was previously "dotted" with $1x10^5$ *T. congolense* K/83/IL/97/2 (TCK), *T. brucei* IL2616 (TB) and *T. simiae* KETRI 2431 (TS) cultured procyclics, and $1x10^5$ *T. vivax* IL1392 (TV) cultured epimastigotes. (▲) represents culture supernatants that contained antibodies that reacted mainly with *T. simiae* procyclics; ( □ ) represents culture supernatants that contained antibodies that reacted specifically with trypanosomes of the *Nannomonas* subgenus; ( ○ ) represents culture supernatants that contained antibodies that cross-reacted with all the four trypanosome species that were tested.

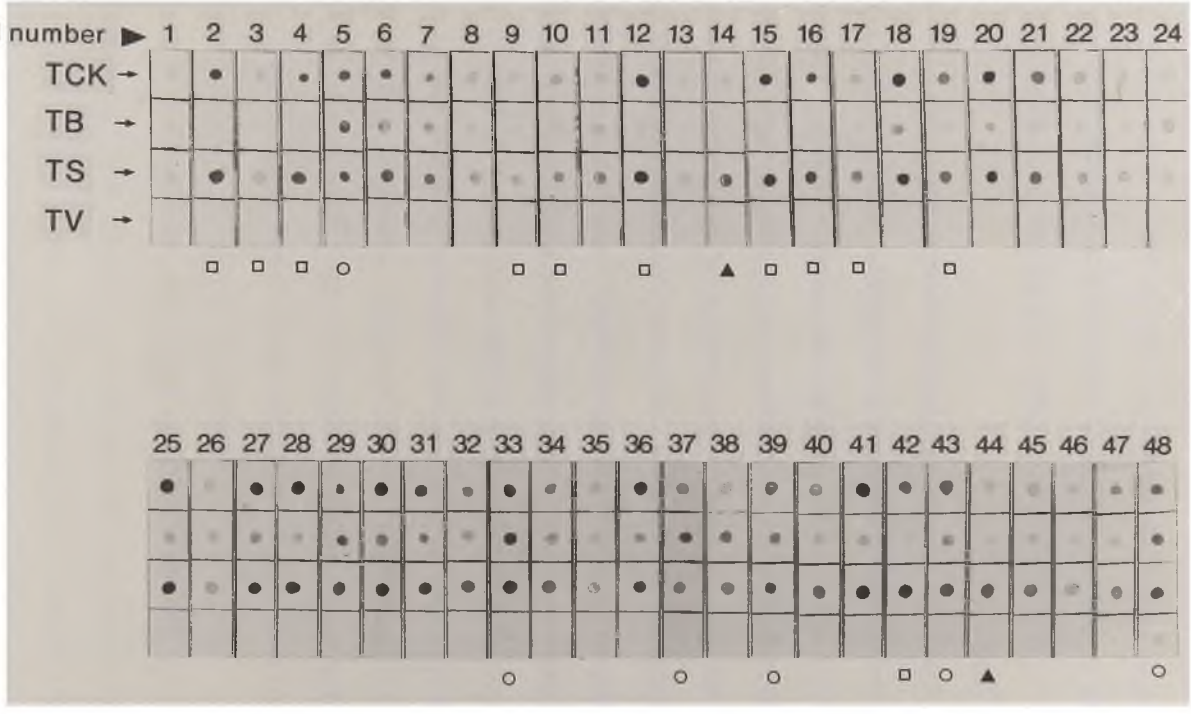



**Figure 17**

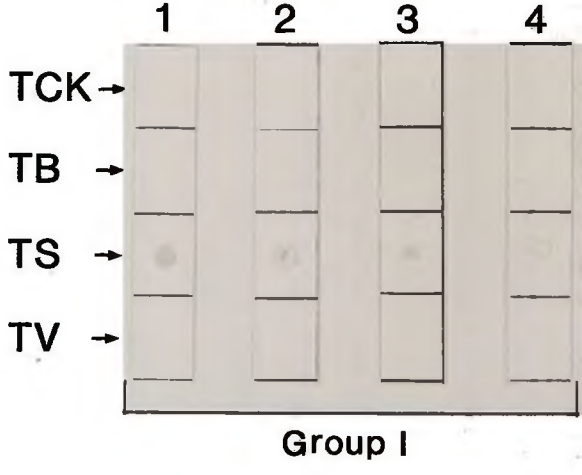
wells (14 and 44) had activity confined mainly to the *T. simiae* antigen. The antibodies from well 14 reacted strongly with the *T. simiae* antigen dot and very weakly with the *T. congolense* antigen dot, but not at all with the dots representing *T. brucei* or *T. vivax*. Likewise, the antibodies from well 44 reacted strongly with the *T. simiae* antigen dot, but showed weak reactivity with both the *T. congolense* and the *T. brucei* antigen dots. It also did not react with the dot representing the *T. vivax* antigen. Based on this reactivity, the cells from those two wells were extensively cloned.

Of the first clones originating out of well 14, only 4 out of 200 tested positive and all were specific to *T. simiae* when screened by dot-ELISA (Figure 18). The clones marked 1, 2 and 3 were selected for further cloning on the basis of their stronger reactivity. Screening of the re-clones revealed that only about 1% of the cells continued to secrete the *T. simiae* specific MoAb, suggesting that the hybridoma was unstable. It was, therefore, decided to explore the possibility of isolating some stable hybrids by re-cloning positive clones several times over. Unfortunately, after several re-cloning attempts, the trend remained unchanged. As a result, continuous culturing of these cells was not possible. However, culture supernatants obtained from the earlier cultures were concentrated by ammonium sulphate precipitation and dialysed. This fraction was tested by dot-ELISA and found to be active, and used for further characterization of the antibody and antigen. Some cells from the original well 14 as well as cells from the first and second positive clones were cryopreserved in liquid nitrogen.

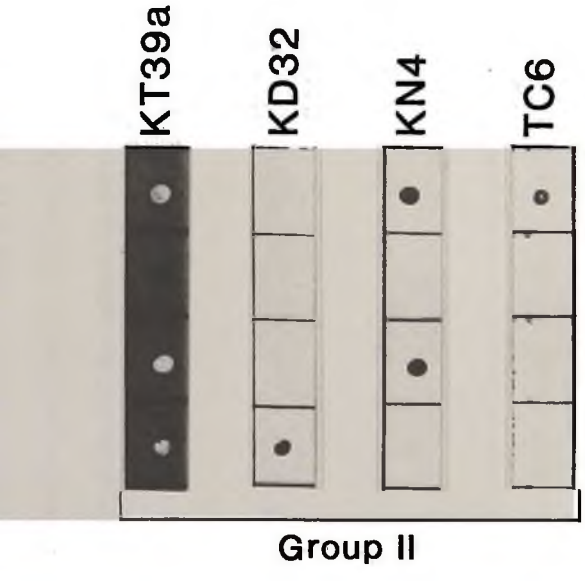
Two hundred first clones were derived from TS7 fusion well 44, but none of these tested positive when screened by dot-ELISA. Hence, only two hybrids (one from fusion TS3 and the other from fusion TS7) secreting antibodies with specific reactivity to *T. simiae* were ever produced, and none of these hybrids was stable.

**Figure 18**

Dot-ELISA reactivity of MoAbs secreted by four hybridoma cell clones obtained from well 14 of cell fusion TS7. Culture supernatant from each clone was tested for antibody reactivity by incubating with a strip of NC membrane that was previously "dotted" with $1x10^5$ *T. congolense* K/83/IL/97/2 (TCK), *T. brucei* IL2616 (TB) and *T. simiae* KETRI 2431 (TS) cultured procyclics, and $1x10^5$ *T. vivax* IL1392 (TV) cultured epimastigotes. Strips 1, 2, 3 and 4 (Group I) shows the specific reactivity of the MoAbs secreted by the four hybrid clones, with *T. simiae* parasites. The strips shown in Group II illustrate specific reactivity of control MoAbs in the same test: *T. brucei* specific MoAb (KT39a); *T. vivax* specific MoAb (KD32); *Nannomonas* specific MoAb (KN4); and *T. congolense* specific MoAb (TC6).




Figure



### 4.4.4 Reactivity of the New MoAbs Produced

From the 28 fusions that were made, 8 additional hybridomas each of which secreted a trypanosome species or subspecies specific MoAb were derived. The specificity of each of the MoAbs was confirmed by testing for cross-reactivity using the more sensitive Enzyme-linked Immunoelectrotransfer Blot Technique (Western immunoblot) analysis. The isotypes and reactivity patterns of the 8 additional MoAbs are listed in Table 12. Three of these MoAbs [KT39a/18.17 (IgM), KT43/33.32 ($IgG_1$) and KT43/27.32 ($IgG_{2a}$)] were *T. brucei* specific; two [KD32/48.17 ($IgG_1$) and KD37/19.3 ($IgG_1$)] were *T. vivax* specific; one [KNS7/14.X($IgG_1$)] was *T. simiae* specific; and two [KN4/13.9($IgG_3$) and KN5/6.15($IgG_1$)] were *Nannomonas* subgenus-specific.

### 4.4.5 Characterization of MoAbs and the Antigens that they Detect

The remaining sections of this Chapter record the results of the characterization studies of the MoAbs listed in (Tables 7 and 12), and the specific antigens that they detected. For purposes of convenience, abbreviated names of those MoAbs will henceforth be used in the text. Table 13 lists the full names of the MoAbs and their abbreviated forms.

### 4.4.6 Immunolocalization of the Species-specific Antigens bound by the MoAbs

Immunolocalization studies made by IFAT, revealed that some trypanosome species-specific antigens bound by the MoAbs were located on the surface membrane of procyclics or epimastigotes (Figures 19a, 19b and 19c) whilst others were intracytoplasmic (Figure 19d). Table 14 summarizes the results on the localization of the specific antigens bound by all the MoAbs as determined by IFAT. Three out of four *T. brucei* specific antigens localize to the surface membrane of *T. brucei* procyclics, whereas one is intracytoplasmic. Likewise, two out of three *T. vivax* specific antigens

**Table 12**

**Reactivity of the new MoAbs against procyclics or epimastigotes of different trypanosome species**

| Monoclonal Antibody | Isotype | *T. brucei* * | *T. vivax* ** | *T. congolense* * | *T. simiae* * |
|---|---|---|---|---|---|
| KT39a/18.17 | IgM | + | - | - | - |
| KT43/33.32 | $IgG_1$ | + | | - | |
| KT43/27.32 | $IgG_{2a}$ | + | | - | |
| KD32/48.17 | $IgG_1$ | - | + | - | - |
| KD37/19.3 | $IgG_1$ | - | + | | |
| KNS7/14.X | $IgG_1$ | - | - | | + |
| KN4/13.9 | $IgG_3$ | - | - | + | + |
| KN5/6.15 | $IgG_1$ | - | - | + | + |

* procyclics.
** epimastigotes.
+ = antibody reacts with trypanosomes.
= antibody does not react with trypanosomes.

**Table 13**

**Abbreviated forms of the names of selected MoAbs**

| Monoclonal Antibody (FULL NAME) | Abbreviated name | Isotype | Specificity |
|---|---|---|---|
| TR7/47.37.16 | TR7 | IgM | *T. brucei* |
| KT39a/18.17 | KT39a | IgM | *T. brucei* |
| KT43/33.32 | KT43/33 | $IgG_1$ | *T. brucei* |
| KT43/27.32 | KT43/27 | $IgG_{2a}$ | *T. brucei* |
| TV8/8.33.42 | TV8 | $IgG_3$ | *T. vivax* |
| KD32/48.17 | KD32 | $IgG_1$ | *T. vivax* |
| KD37/19.3 | KD37 | $IgG_1$ | *T. vivax* |
| C2 | C2 | $IgG_1$ | *T. congolense* |
| TC6/42.6.3 | TC6 | $IgG_1$ | *T. congolense* |
| TC40/30.15.40 | TC40 | IgM | *T. congolense* |
| TC39/30.25.95 | TC39 | IgM | *T. congolense* |
| KNS7/14.X | KNS7 | $IgG_1$ | *T. simiae* |
| TC16/5.12.33 | TC16 | $IgG_1$ | *Nannomonas* |
| TC6/25.25.4 | TC6/25 | $IgG_3$ | *Nannomonas* |
| KN4/13.9 | KN4 | $IgG_3$ | *Nannomonas* |
| KN5/6.15 | KN5 | $IgG_1$ | *Nannomonas* |

**Figure 19a**

Light micrograph of *Trypanosoma simiae* (KETRI 2431) procyclics showing surface membrane fluorescence following incubation with KN4 and anti-mouse-FITC. Photographed at x100 magnification.

f = flagella.

m = membrane.

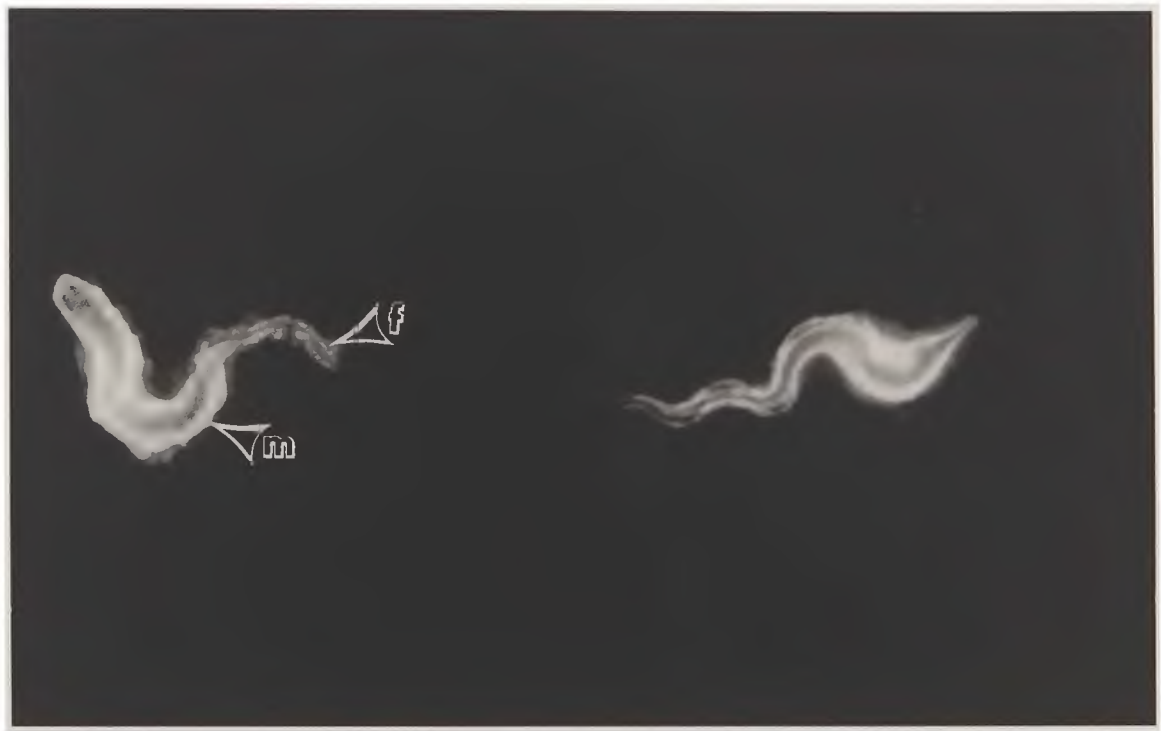



**Figure 19a**

**Figure 19b**

Light micrograph of *Trypanosoma vivax* (IL1392) epimastigotes showing surface membrane fluorescence following incubation with KD32 and anti-mouse-FITC.
Photographed at x100 magnification.

f = flagella.
m = membrane.

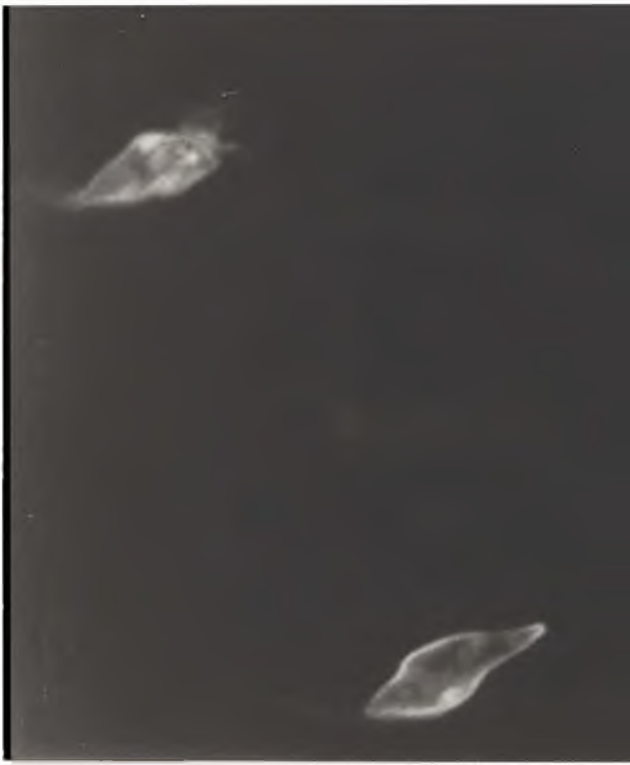


**Figure 19b**

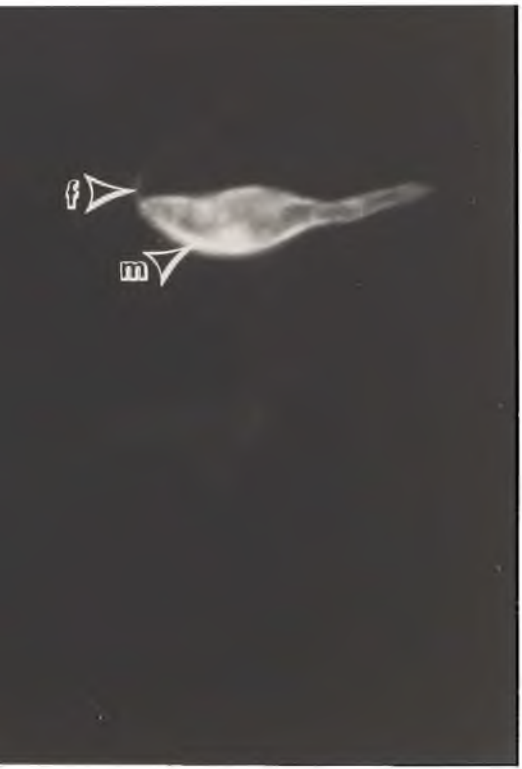
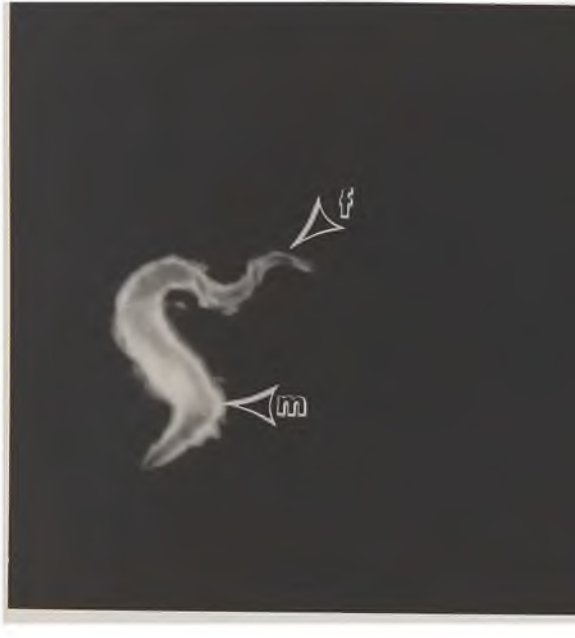


**Figure 19c**

Light micrograph of *Trypanosoma brucei* (IL2616) procyclics showing surface membrane fluorescence following incubation with KT39a and anti-mouse-FITC.
Photographed at x100 magnification.

f = flagella.
m = membrane.




Figure

19c

**Figure 19d**

Light micrograph of *Trypanosoma brucei* procyclics showing cytoplasmic staining following incubated with KT43/27 and anti-mouse-FITC. Photographed at x100 (A) without and (B) with fluorescence.

m = membrane.

n = nucleus.

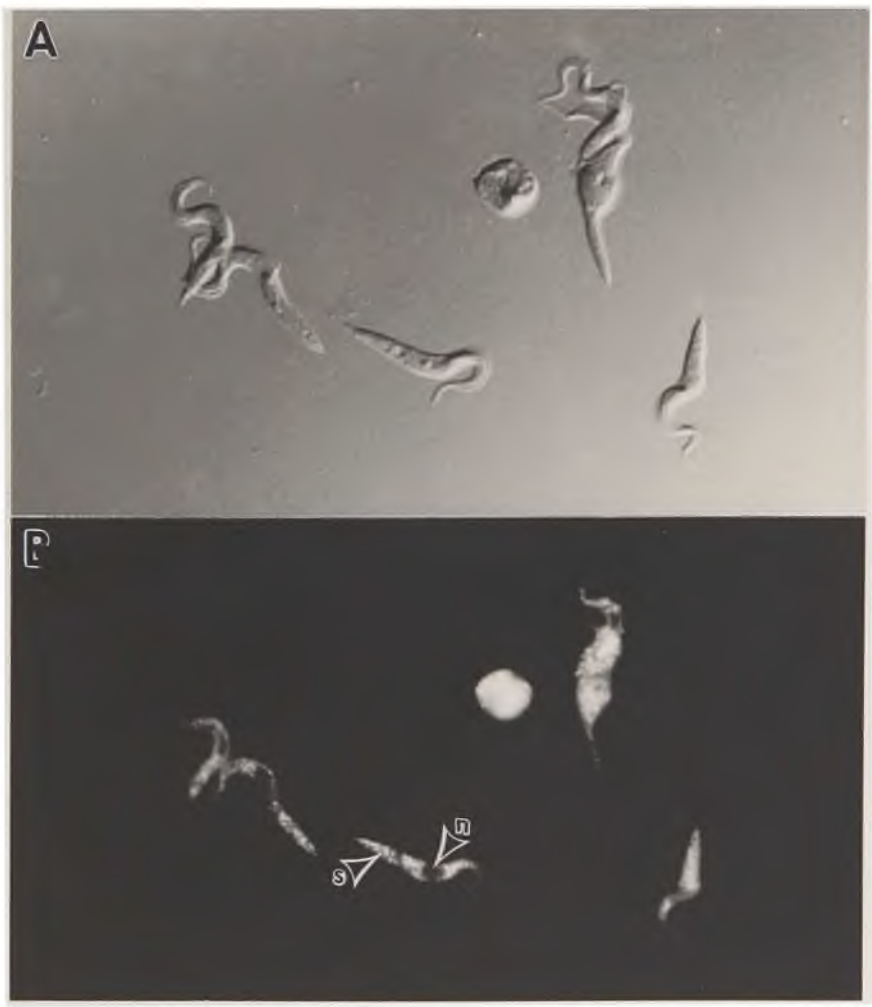



**Figure 19d**

**Table 14**

**Immunolocalization of the trypanosome species-specific antigens by indirect fluorescent antibody test (IFAT)**

| Monoclonal Antibody | Isotype | Specificity | Membrane staining | Cytoplasmic staining |
|---|---|---|---|---|
| TR7 | IgM | *T. brucei* | + | |
| KT39a | IgM | *T. brucei* | + | |
| KT43/33 | $IgG_1$ | *T. brucei* | + | |
| KT43/27 | $IgG_{2a}$ | *T. brucei* | | + |
| TV8 | $IgG_3$ | *T. vivax* | + | |
| KD32 | $IgG_1$ | *T. vivax* | + | - |
| KD37 | $IgG_1$ | *T. vivax* | | + |
| C2 | $IgG_1$ | *T. congolense* | | + |
| TC6 | $IgG_1$ | *T. congolense* | | + |
| TC40 | IgM | *T. congolense* | | + |
| TC39 | IgM | *T. congolense* | | + |
| KNS7 | $IgG_1$ | *T. simiae* | - | + |
| TC16 | $IgG_1$ | *Nannomonas* | + | |
| TC6/25 | $IgG_3$ | *Nannomonas* | + | |
| KN4 | $IgG_3$ | *Nannomonas* | + | - |
| KN5 | $IgG_1$ | *Nannomonas* | + | - |

+ = stained.
= not stained.

are surface membrane antigens of epimastigotes, and one is intracytoplasmic. All the *T. congolense* specific antigens are located within the cytoplasm of procyclic organisms. The *T. simiae* specific antigen is also located in the cytoplasm of procyclics. Unlike the *T. congolense* specific MoAbs, all the *Nannomonas* subgenus-specific antigens are located on the surface membranes of procyclics.

IFAT analysis, using live trypanosomes, showed that three *T. brucei* specific MoAbs (TR7, KT39a and KT43/33); four *Nannomonas* specific MoAbs (TC16, TC6/25, KN4 and KN5), and one *T. vivax* specific MoAb (KD32) bound antigens on the surface of living trypanosomes.

Characterization of the specific antigens by the Western immunoblot technique revealed that some of the MoAbs could not work in this assay (Table 15). Each trypanosome species-specific MoAb was assayed on SDS-PAGE separated antigens of four different trypanosome species (*T. brucei, T. vivax, T. congolense* and *T. simiae*). Of the four *T. brucei* specific MoAbs, TR7 (an IgM antibody) bound three protein bands of MW between 21 and 27 kDa whilst KT43/33 (an $IgG_1$ antibody) bound multiple bands ranging between 21 and 47 kDa (Figure 20). The third *T. brucei* antibody, KT39a, an IgM isotype, bound a 90 kDa protein band whilst the fourth, KT43/27, an $IgG_{2a}$, did not work in this assay.

The *T. vivax* specific MoAbs TV8 ($IgG_3$), KD32 ($IgG_1$) and KD37 ($IgG_1$) did not bind any bands in electrophoresed *T. vivax* bloodstream form or epimastigote lysates.

C2 and TC6, both *T. congolense* specific and of $IgG_1$ isotype, bound protein bands within the same molecular weight range (30-40) kDa. C2 bound a doublet of protein bands at 30 and 31 kDa and another doublet at 38 and 40 kDa, whilst TC6 bound only one band at 30 kDa. The *T. simiae* specific MoAb, KNS7 ($IgG_1$), did not work in the Western immunoblot assay. Also,

**Table 15**

**The molecular weights of the antigens detected by the trypanosome species-specific MoAbs as determined by Western immunoblot analysis**

| Monoclonal Antibody | Isotype | Specificity | Molecular weight (kDa) |
|---|---|---|---|
| TR7 | IgM | *T. brucei* | (21, 24, 27) |
| KT39a | IgM | *T. brucei* | (90) |
| KT43 | $IgG_1$ | *T. brucei* | (21 - 47) |
| KT43/27 | $IgG_{2a}$ | *T. brucei* | — |
| TV8 | $IgG_3$ | *T. vivax* | — |
| KD32 | $IgG_1$ | *T. vivax* | — |
| KD37 | $IgG_1$ | *T. vivax* | — |
| C2 | $IgG_1$ | *T. congolense* | (30 - 40) |
| TC6 | $IgG_1$ | *T. congolense* | (30) |
| TC40 | IgM | *T. congolense* | (51, 60, 85) |
| TC39 | IgM | *T. congolense* | (21 - 30) |
| KNS7 | $IgG_1$ | *T. simiae* | — |
| TC16 | $IgG_1$ | *Nannomonas* | $(18, 89)^{TCK}$ $(31)^{TS}$ |
| TC6/25 | $IgG_3$ | *Nannomonas* | — |
| KN4 | $IgG_3$ | *Nannomonas* | — |
| KN5 | $IgG_1$ | *Nannomonas* | — |

— = antibody does not bind any antigens.
TCK = *T. congolense* Kilifi type procyclic lysate.
TS = *T. simiae* procyclic lysate.

**Figure 20**

Specific reactivity of the MoAb KT43/33 in the Western immunoblot assay and the molecular weights of the antigens that it bound. Strip 'A' shows the reactivity of KT43/33 with antigens in electrophoresed *T. brucei* IL2616 procyclic lysate. Strips B, C and D contained respectively, electrophoresed *T. congolense*, *T. simiae* and *T. vivax* antigens. KT43/33 reacted specifically with *T. brucei* antigen peptides of molecular weights ranging between 21 and 47 kDa.

three of the four *Nannomonas* specific MoAbs did not work. However, TC16, also *Nannomonas* specific, bound antigens in electrophoresed *T. congolense* and *T. simiae* procyclic extracts at 18, 89 kDa and 31 kDa, respectively (Table 15).

### 4.4.7 The Biochemical Nature of the Trypanosome Species-specific Antigenic Determinants

Periodate oxidation of carbohydrate residues and proteinase-K modification of polypeptide residues were used to study the biochemical nature of the specific antigenic determinants bound by the MoAbs.

Binding by three of the four *T. brucei* specific MoAbs was totally abrogated by treatment with proteinase-K (Table 16). This result suggests that the antigenic determinants involved are protein in nature. The fourth *T. brucei* specific MoAb (KT39a) was only partially affected by proteinase-K digestion, but not by sodium periodate oxidation, indicating that the antigenic determinant involved is partly protein and partly either a carbohydrate or a lipid (Table 16).

Two of the three *T. vivax* specific MoAbs bound a proteinase-K sensitive antigenic determinant, whilst the remaining one (TV8) was insensitive to both periodate oxidation and proteinase-K digestion.

All the four *Nannomonas* as well as the *T. simiae* specific MoAbs detected periodate sensitive antigenic determinants in glycoprotein or glycolipid antigens. In contrast, all the *T. congolense* specific MoAbs detected protein antigenic epitopes, as evidenced by their sensitivity to proteinase-K and insensitivity to periodate oxidation (Table 16).

### 4.4.8 Distribution of the Specific Antigenic Determinants (Epitopes) on Antigens

The antigen detection sandwich-ELISA was used to study the

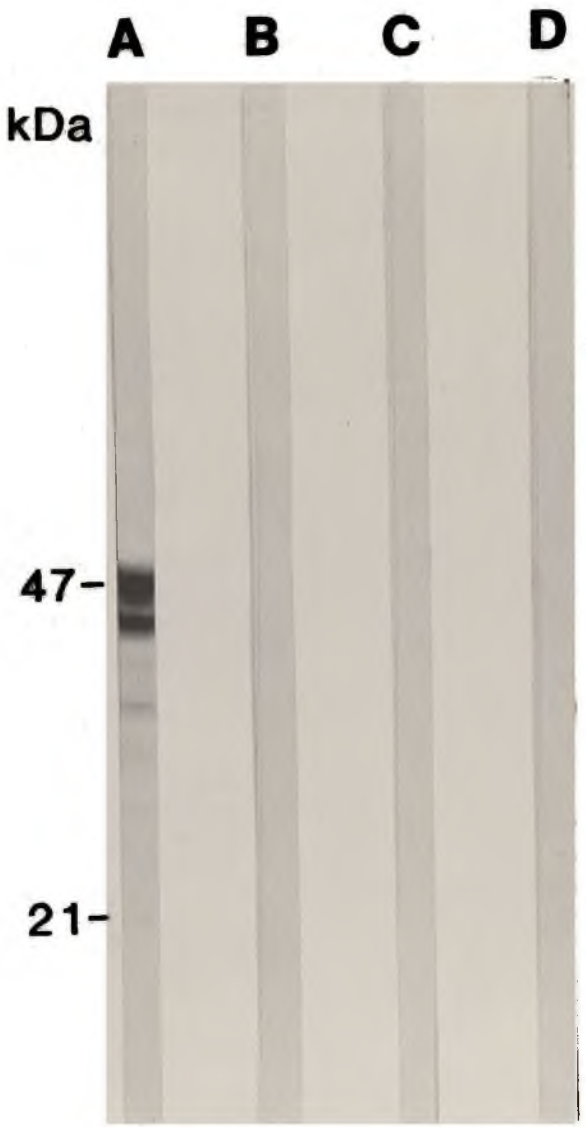



Figure 20

**Table 16**

**The nature of the antigens detected by the trypanosome species-specific MoAbs as determined by periodate and proteinase-K digestion**

| Monoclonal Antibody | Isotype | Specificity | Sensitivity to | | Nature of antigenic epitope |
|---|---|---|---|---|---|
| | | | Periodate | Proteinase-K | |
| TR7 | IgM | *T. brucei* | - | + | P |
| KT39a | IgM | *T. brucei* | - | +/- | P, C?, L? |
| KT43 | $IgG_1$ | *T. brucei* | | + | P |
| KT43/27 | $IgG_{2a}$ | *T. brucei* | | + | P |
| TV8 | $IgG_3$ | *T. vivax* | - | - | C?, L? |
| KD32 | $IgG_1$ | *T. vivax* | - | + | P |
| KD37 | $IgG_1$ | *T. vivax* | - | + | P |
| C2 | $IgG_1$ | *T. congolense* | - | + | P |
| TC6 | $IgG_1$ | *T. congolense* | | + | P |
| TC40 | IgM | *T. congolense* | | + | P |
| TC39 | IgM | *T. congolense* | | + | P |
| KNS7 | $IgG_1$ | *T. simiae* | + | - | C |
| TC16 | $IgG_1$ | *Nannomonas* | + | - | C |
| TC6/25 | $IgG_3$ | *Nannomonas* | + | - | C |
| KN4 | $IgG_3$ | *Nannomonas* | + | | C |
| KN5 | $IgG_1$ | *Nannomonas* | + | | C |

+ = sensitivity to periodate or proteinase-K.
- = insensitivity to periodate or proteinase-K.
± = partial sensitivity to proteinase-K.
P = protein antigenic determinant.
C = carbohydrate antigenic determinant.
L = lipid antigenic determinant.
? = not certain.

distribution of trypanosome species-specific antigenic epitopes on antigens. In these experiments, MoAbs specific to each trypanosome species or subgenus were placed into separate groups. Each antibody in a group was used to trap the antigen(s) on which its specific epitope is expressed. The trapped antigen(s) was then revealed by HRPO conjugate of each antibody in the group in separate experiments.

The results obtained for the *T. brucei* specific MoAbs are shown in Table 17a. When the MoAb TR7 was used to capture the antigen(s) on which its specific epitope is expressed, it was possible to reveal the captured antigen(s) using the conjugates of all the *T. brucei* specific MoAbs, including the homologous conjugate of TR7 (Table 17a). This meant that the TR7 specific antigenic epitope is repeated on the captured antigen(s), so that when the antigen(s) was trapped by TR7 the same antigen(s) could be revealed by that MoAb's conjugate. Also, the result suggested that the antigen(s) captured by TR7 expressed all the antigenic epitopes bound by the other *T. brucei* specific MoAbs. Similar results were recorded when KT39a was used as capture antibody (Table 17a). The results also showed that the IgM MoAbs were better capture antibodies compared with the IgG's. This was clearly shown by the strong reactivity of the conjugate of KT43/33($IgG_1$) with the antigen(s) captured by KT39a(IgM), and yet the absence of reactivity when the conjugate of KT39a was used to reveal the antigen(s) captured by KT43/33 (Table 17a). Furthermore, it was evident from the pattern of reactivity that KT43/33 was the best antibody for revealing captured antigen (Table 17a).

Each *T. congolense* MoAb captured antigen(s) that could be revealed by conjugates of all the others including that of the capture MoAb (Table 17b). This result suggested that the antigen(s) captured by these MoAbs is likely to be the same. Also, the ability of the MoAbs to capture their

**Table 17a**

**Relationship between the *T. brucei* specific MoAbs as revealed by sandwich-ELISA**

| Capture antibody | Reveal antibody conjugate | | | |
|---|---|---|---|---|
| | TR7 | KT39a | KT43/33 | KT43/27 |
| TR7(IgM) | ++ | + | ++++ | ++ |
| KT39a(IgM) | + | + | ++++ | ++ |
| KT43/33($IgG_1$) | + | - | ++ | |
| KT43/27($IgG_{2a}$) | | | + | |

+ = ability of MoAb conjugate to reveal captured antigen.
- = MoAb conjugate unable to reveal captured antigen.

**Table 17b**

**Relationship between the *T. congolense* specific MoAbs as revealed by sandwich-ELISA**

| Capture antibody | Reveal antibody conjugate | | | |
|---|---|---|---|---|
| | C2 | TC6 | TC40 | TC39 |
| C2 | + | ++ | + | + |
| TC6 | + | + | ++ | + |
| TC40 | ++ | + | + | ++ |
| TC39 | ++ | + | ++ | +++ |

+ = ability of MoAb conjugate to reveal captured antigen.

respective antigens and reveal them by their own conjugates, indicated that the antigenic determinants are repeated on the antigen(s).

As shown in Table 17c, the *Nannomonas* subgenus-specific MoAb TC6/25 captured antigen(s) that could be detected by conjugates of itself, KN4 and KN5 but not TC16. Likewise, the antigen(s) captured by TC16 could not be revealed by any of the conjugates other than that of TC16 itself. These results indicate that the antigenic epitope bound by TC16 is on a different antigen. It is also seen from Table 17c that both KN4 and KN5 captured antigen(s) that could not be revealed by their own conjugates. This observation suggests that the epitopes bound by these MoAbs (KN4 and KN5) are not significantly repetitive on the respective antigens.

The *T. vivax* MoAb, TV8, captured antigen(s) that could be revealed by conjugates of each of the three *T. vivax* MoAbs (Table 17d), suggesting that the antigenic determinants bound by all these MoAbs are distributed on the same antigen(s). KD32 captured antigen(s) that could be revealed by its own conjugate, but weakly by conjugates of TV8 or KD37, yet the same KD32 conjugate could reveal very well the antigen(s) captured by TV8. This suggests that the weak reactivity of the TV8 conjugate may be due to altered epitope accessibility due to conformational changes in the antigen, brought about by the binding to KD32.

### 4.4.9 Inhibition ELISA

To further elucidate the relationship between the specific epitopes bound by the different MoAbs (section 4.4.8), experiments were conducted to examine the effect of the binding of one MoAb on binding by another, using micro-plate-based inhibition ELISA.

The results obtained showed that the *T. brucei* specific MoAbs, KT43/33 and KT43/27 could not inhibit each other, showing that the two epitopes were different. However, two other *T. brucei* specific MoAbs

**Table 17c**

**Relationship between the *Nannomonas* specific MoAbs as revealed by sandwich-ELISA**

| Capture antibody | Reveal antibody conjugate | | | |
|---|---|---|---|---|
| | TC6/25 | TC16 | KN4 | KN5 |
| TC6/25 | + | - | + | + |
| TC16 | - | + | - | |
| KN4 | + | - | - | + |
| KN5 | + | | + | |

+ = ability of MoAb conjugate to reveal captured antigen.
- = MoAb conjugate unable to reveal captured antigen.

**Table 17d**

**Relationship between the *T. vivax* specific MoAbs as revealed by sandwich-ELISA**

| Capture antibody | Reveal antibody conjugate | | |
|---|---|---|---|
| | TV8 | KD32 | KD37 |
| TV8 | ++ | ++ | + |
| KD32 | ± | + | ± |
| KD37 | + | | + |

+ = ability of MoAb conjugate to reveal captured antigen.
= MoAb conjugate unable to reveal captured antigen.
± = very weak reactivity.

(KT39a and TR7) inhibited each others binding, suggesting that the two MoAbs are directed at the same epitope.

Each of the four *T. congolense* specific MoAbs (C2, TC6, TC39 and TC40) was able to inhibit the binding of the others.

**4.4.10** Reactivity of the Various MoAbs with Different Trypanosome Stocks and Clones

The aim of the experiments described here was to determine the suitability of the trypanosome species-specific MoAbs as diagnostic reagents. This assessment was based on their ability to react with trypanosomes from different geographical areas.

All the trypanosome stocks and clones used in the present study, are listed, together with their places of origin, in Table 18.

With the exception of KT43/27, the *T. brucei* specific MoAbs detected all the different developmental stages of *T. brucei* organisms that were tested (Table 19). The reactivity patterns indicate that KT43/27 was able to detect all the *in vitro* propagated *T. brucei* procyclic organisms. However, the same MoAb could not detect *T. brucei* bloodstream forms or insect forms from tsetse gut or salivary glands.

Studies with the *T. vivax* specific MoAbs showed that TV8 and KD37 could detect all the epimastigotes and blood stream forms tested (Table 20). Thus, in addition to East and West African *T. vivax*, these two MoAbs detected IL3841 which originated from Colombia, South America. These MoAbs also detected *T. vivax* insect forms of IL3096 from tsetse mouthparts. In contrast, KD32 which was derived against epimastigotes of West African *T. vivax* (IL1392), was unable to detect bloodstream forms of IL1392, IL2160 and IL3841, even though the same MoAb detected bloodstream forms of

**Table 18: Trypanosome stocks and clones from different geographical areas used in determining the range of reactivity of the trypanosome species-specific MoAbs**

| Species | Trypanosome stock/clone | Origin |
|---|---|---|
| *T. congolense* | *K/83/IL/97/2 | Kilifi, Kenya (K) |
| | K/82/IL/60/1 | Kilifi, Kenya (K) |
| | IL3779 | Nguruman, Kenya (S) |
| | CP81 | Taita, Kenya (S) |
| | *ILC49 | Transmara, Kenya (S) |
| | *IL13-E3 | Busoga, Uganda (S) |
| | *IL2079 | Serengeti, Tanzania (S) |
| | *IL1180 | Serengeti, Tanzania (S) |
| | IL3900 | Bobodioulasso, Burkina Faso (R) |
| | *IL3274 | Banankeledaga, Burkina Faso (R) |
| | MSUS/LR/77/TSW103 | Duoplay, Liberia (K) |
| | MOVS/KE/81/WG84 | Matuga, Kenya (R) |
| | MBOI/NG/60/1-148 | Donga Valley, Nigeria (S) |
| *T. simiae* | KETRI 2431 | Ukunda, Kenya |
| | *TS1 | Ukunda, Kenya |
| | *TS4 | Ukunda, Kenya |
| | IL3815 | Ukunda, Kenya |
| *T. vivax* | IL3895 | Kipini, Kenya |
| | IL2005 | Teso, Uganda |
| | IL1392 | Zaria, Nigeria |
| | IL2160 | Zaria, Nigeria |
| | IL3096 | Zaria, Nigeria |
| | *ILDat 1.9 | Zaria, Nigeria |
| | *IL3841 | Lorica, Colombia |
| *T. b. brucei* | CP 2137 | Nairobi, Kenya |
| | MiTat 1.2 | Lugala, Uganda |
| | CP 547/R | Jilib, Somalia |
| | IL2616 | Serengeti, Tanzania |
| | IL375 | Serengeti, Tanzania |
| | IL3579 | Serengeti, Tanzania |
| *T. b. gambiense* | TREU 1442 | Nigeria |
| | Th-17/78 E(020) | Cote d'Ivoire |
| *T. b. rhodesiense* | IL1984 | Lugala, Uganda |
| | IL1478 | Lambwe Valley, Kenya |
| *T. grayi* | GPAG/GM/88/BAN1 | Bansang, The Gambia |

* = clone.
K = Kilifi type.
S = savannah type.
R = riverine-forest type.

**Table 19:** **Reactivity of the *T. brucei* specific MoAbs with different stocks and clones of *T. brucei* as defined by dot-ELISA**

| Monoclonal antibody | IL2616 (Proc) | Th-17/87 (Proc) | TREU-1442 (Proc) | MiTat1.2 (Proc) | IL1984 (Proc) | IL1478 (Proc) | CP2137 (b/d) | CP547/R (b/d) | IL375 (i/f)G | IL375 (i/f)SG | IL3579 (i/f)G | IL3579 (i/f)SG |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| TR7 | + | + | + | + | + | + | + | + | + | + | + | + |
| KT39a | + | + | + | + | + | + | + | + | + | + | + | + |
| KT43/33 | + | + | + | + | + | + | + | + | + | + | + | + |
| KT43/27 | + | + | + | + | + | - | - | - | - | - | - | - |

\+ = antibody reacts with trypanosomes.
\- = antibody does not react with trypanosomes.
(Proc) = procyclic forms propagated *in vitro*.
(b/d) = bloodstream forms propagated *in vivo*.
(i/f)G = insect forms from the gut.
(i/f)SG = insect forms from the salivary glands.

**Table 20:** **Reactivity of the *T. vivax* specific MoAbs with different stocks and clones of *T. vivax* as defined by dot-ELISA**

| Monoclonal antibody | IL1392 (Epis) | IL1392 (b/d) | IL3895 (Epis) | ILDat1.9 (Epis) | IL2160 (b/d) | IL2005 (b/d) | IL3841 (b/d) | IL3096 (i/f)MP |
|---|---|---|---|---|---|---|---|---|
| TV8 | + | + | + | + | + | + | + | + |
| KD32 | + | - | + | + | - | + | - | + |
| KD37 | + | + | + | + | + | + | + | n.t. |

\+ =antibody reacts with trypanosomes.
\- =antibody does not react with trypanosomes.
(Epis) =epimastigote forms propagated *in vitro*.
(b/d) =bloodstream forms propagated *in vivo*.
(i/f)MP =insect forms from the mouthparts.
n.t =not tested.

IL2005 (Table 20), indicating a differential expression of the epitope involved in insect stages of the parasite, and in bloodstream forms of some stocks (KD37 was not tested against the insect forms of IL3096 because the MoAb was derived late in the course of these studies).

Each of the four *T. congolense* specific MoAbs detected all the different stocks and clones of *T. congolense* that were tested, regardless of the developmental stages of the organism (Table 21).

The reactivity of the *Nannomonas* specific MoAbs is given in Table 22. The four specific MoAbs detected all the epimastigote and tsetse gut forms of *T. congolense*, as well as the procyclics of *T. congolense* and *T. simiae*. The reactivity of these MoAbs with bloodstream forms, however, presented a different picture. Whilst KN5 detected all the *T. congolense* bloodstream forms that were tested, TC6/25, TC16 and KN4 were unable to detect the bloodstream forms of IL2079, and CP81. Yet, the same MoAbs (TC6/25, TC16 and KN4) could detect bloodstream forms of IL1180, IL3779 and IL3900, suggesting that the antigenic epitopes detected by these MoAbs were not expressed in the bloodstream forms of all the different stocks of *T. congolense* organisms.

All the specific MoAbs were screened against *T. grayi* procyclics. The object of this exercise was to determine whether any of the MoAbs would cross-react with *T. grayi*, since it also infects tsetse flies. None of the MoAbs reacted with the *T. grayi* parasites when tested with the dot-ELISA (Figure 21). The results of the screening of TC6/25, KN4, TC6/42, KT39a and KD32 on *T. grayi*, and *T. congolense* (savannah type, riverine/forest type and Kilifi type) are summarized in Figure 21. As it is indicated, the *T. congolense* MoAb (TC6) and *Nannomonas* MoAbs (TC6/25 and KN4) detected all the different types of *T. congolense* that were tested.

**Table 21:** **Reactivity of the *T. congolense* specific monoclonal antibodies with different stocks and clones of *T. congolense* as defined by dot-ELISA**

| Monoclonal antibody | IL/60/1 (Proc) | IL/97/2 (Proc) | IL2079 (Epis) | IL3900 (b/d) | IL2079 (b/d) | ILC49 (b/d) | CP81 (Epis) | CP81 (b/d)* | CP81 (i/f)G | IL1180 (b/d) | IL1180 (i/f)G | IL13-E3 (i/f)G | IL3274 (i/f)G | IL3779 (i/f)G | MOVS (Proc) | MBOI (Proc) | MSUS (Proc) |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| C2 | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + |
| TC39 | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + |
| TC40 | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + |
| TC6 | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + |

\+ = antibody reacts with trypanosomes.
IL/60/1 = K/82/IL/60/1.
IL/97/2 = K/83/IL/97/2.
(Proc) = procyclic forms propagated *in vitro*.
(Epis) = epimastigote forms propagated *in vitro*.
(b/d)* = bloodstream forms propagated *in vitro*.
(b/d) = bloodstream forms propagated *in vivo*.
(i/f)G = insect forms from the gut.
MOVS = MOVS/KE/81/WG84.
MBOI = MBOI/NG/60/1-148.
MSUS = MSUS/LR/77/TSW103.

**Table 22:** **Reactivity of the *Nannomonas* specific MoAbs with different stocks and clones of *T. congolense* and *T. simiae* as defined by dot-ELISA**

| Monoclonal antibody | IL/97/2 (Proc) | IL/60/2 (Proc) | TSc1 (Proc) | TSc4 (Proc) | IL2079 (Epis) | IL2079 (b/d)* | CP81 (Epis) | CP81 (b/d)* | CP81 (i/f)G | IL1180 (b/d) | IL1180 (i/f)G | IL3779 (b/d) | IL3779 (i/f)G | IL3900 (b/d) | CP813 (i/f)G | IL3274 (i/f)G | MOVS (Proc) | MBOI (Proc) | MSUS (Proc) |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| TC6/25 | + | + | + | + | + | - | + | - | + | + | + | + | + | + | + | + | + | + | + |
| TC16 | + | + | + | + | + | - | + | - | + | + | + | + | + | + | + | + | + | + | + |
| KN4 | + | + | + | + | + | - | + | - | + | + | + | + | + | + | + | + | + | + | + |
| KN5 | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + |

\+ = antibody reacts with trypanosomes.
\- = antibody does not react with trypanosomes.
IL/60/1 = K/82/IL/60/1.
IL/97/2 = K/83/IL/97/2.
(Proc) = procyclic forms propagated *in vitro*.
(Epis) = epimastigote forms propagated *in vitro*.
(b/d)* = bloodstream forms propagated *in vitro*.
(b/d) = bloodstream forms propagated *in vivo*.
(i/f)G = insect forms from the gut.
(i/f)SG = insect forms from the salivary glands.
MOVS = MOVS/KE/81/WG84.
MBOI = MBOI/NG/60/1-148.
MSUS = MSUS/LR/77/TSW103.

**Figure 21**

Reactivity of some trypanosome species-specific MoAbs against $1x10^4$ parasites/dot of *Trypanosoma grayi* (TG), and *T. congolense* savannah type ($TC^{ST}$), Kilifi type ($TC^{KT}$) and $3x10^3$ forest type ($TC^{FT}$) in the dot-ELISA. The control antigens consisted of $1x10^5$ parasites per dot of *T. congolense* K/83/IL/97/2 (TCK), *T. brucei* IL2616 (TB) and *T. simiae* KETRI 2431 (TS) cultured procyclics, and $1x10^5$ *T. vivax* IL1392 (TV) cultured epimastigotes. Strip 'C' was a conjugate control; not incubated with specific MoAb. KD32 and KT39a were respectively, *T. vivax* and *T. brucei* specific MoAbs. TC6/25 and KN4 were *Nannomonas* subgenus-specific, and TC6 was a *T. congolense* specific MoAb. Note that none of the MoAbs reacted with *T. grayi*.

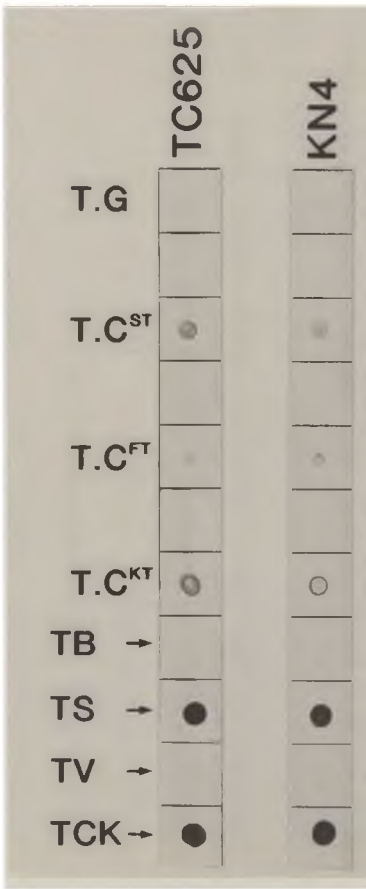



**Figure 21**

## 4.5 Discussion

The main aim of this study was to obtain trypanosome species-specific MoAbs that could be useful in developing a field applicable assay for detecting and differentiating trypanosome species in the vector (*Glossina spp.*).

It had been previously shown that *T. brucei, T. vivax, T. congolense* and the *Nannomonas* subgenus, possess species-specific and/or subgenus specific antigens (Parish, Morrison and Pearson 1985, Richardson *et al.*, 1986; Nantulya *et al.*, 1987).

The initial step, therefore, was to re-examine the specificity and sensitivity of the extant trypanosome species-specific MoAbs that were available at ILRAD, to select some for further study and then to produce additional MoAbs, if necessary, for eventual detection and differentiation of vector-borne trypanosome species. These studies were made using IFAT, micro-plate ELISA, dot-ELISA and Western immunoblot analysis, and the selection criteria were as follows: (1) specificity as determined in all the assay systems, (2) sensitivity in terms of the minimum number of insect form trypanosomes that can be detected in the dot-ELISA and (3) the ability of a MoAb to bind antigens in the Western immunoblot assay. A series of MoAbs were also produced against insect forms of *T. brucei*, *T. vivax, T. simiae* and the *Nannomonas* subgenus as indicated, and each of these MoAbs was screened using IFAT, micro-plate ELISA, dot-ELISA and Western immunoblot assays, and their specific reactivity with the trypanosome species against which they were derived established.

Data presented in this Chapter shows that immunization of BALB/c mice with trypanosome antigens purified according to the method described by (Ijagbone *et al.*, 1989) increased the fusion success rate as well as the chances of obtaining hybridomas that secreted trypanosome species-specific MoAbs.

Although this method of antigen purification was originally described for the purpose of improving serological diagnosis of trypanosomiasis, it has successfully been used in this study to produce *T. brucei* specific MoAbs (KT43/33, KT43/27), *T. vivax* specific MoAb (KD37) and *T. simiae* specific MoAb (KNS7). It has also been demonstrated in this study that the dot-ELISA procedure is suitable for screening the products of cell fusions, especially where it is desired to screen MoAb against a large number of different antigens simultaneously. This suggestion is in agreement with the findings of Hawkes, Niday and Gordon (1982), that the NC membrane-based dot-ELISA was well suited for screening of MoAbs.

Two different hybridomas that secreted *T. simiae* specific MoAb were derived. However, both hybridomas were not stable since the ability to secrete the specific MoAb was lost when grown in continuous culture. This phenomenon has indeed been reported by Pearson, Pinder, Raelants, Kar, Lundin, Mayor-Withey and Hewett (1980), the underlying reason having been attributed to the loss of chromosome chains (Goding, 1980). Hybrid cells are unstable and tend to lose chromosomes especially during the early cell divisions following fusion (Goding, 1980). It is, therefore, possible for hybrid cells to lose one or more of the chromosomes that encode the genes for the expression of immunoglobulins. The stability of the cells increases with chromosome loss, and eventually the cells become relatively stable. Consequently, it has been established that recloning hybrid cells should produce higher cloning efficiency and increase numbers of active cells. This was, however, not the case with the *T. simiae* hybridomas reported here. This observation may be explained by the fact that the chances of a hybrid cell losing immunoglobulin genes never really ceases. Conditions necessary to minimise the occurrence of unstable hybrid cells include; a suitable immunization schedule; the state of health of the cells used in cell fusion; rapid identification of MoAb secreting cells; early cell cloning and the use of feeder

cells; and the choice of FBS used in the preparation of medium (reviewed by Goding, 1980). Though these conditions were closely followed, the two *T. simiae* specific MoAb secreting hybridomas were not stable. This occurrence is, however, not the first, since it has been reported that some hybrids are inherently more stable than others. Hence even with intensive care and repeated cloning, some hybrid cells will lose production (Goding, 1980). The results, however, show that *T. simiae* specific antigens exist at least in the procyclic stage of that species, and that these antigens are sufficiently immunogenic and can be utilized in traditional cell fusion methods for the production of *T. simiae* specific MoAbs. This is very useful information since no *T. simiae* specific MoAb has been reported in the literature. The low frequency in occurrence of MoAbs that are specific for *T. simiae* compared with *T. congolense* may be attributed to fewer or less immunogenic *T. simiae* specific antigens.

IFAT studies carried out to immunolocalize the antigens detected by the MoAbs, revealed that antigens that define *T. brucei* specificity were not restricted to the cell membrane. Thus, the MoAb KT43/27 was also shown to bind antigens located in the cytoplasm of *T. brucei* procyclics, and membrane bound *T. brucei* specific antigens have been reported earlier (Nantulya *et al.*, 1987). Despite the obvious differences in location, it was not possible to determine whether the antigenic molecule bound by KT43/27 was different from that bound by any of the other *T. brucei* specific MoAbs.

Parish, Morrison and Pearson (1985) reported the identification of an antigen specific to *T. congolense* using MoAbs. This MoAb, TC6/42.6.4, was shown to bind a membrane antigen that was made accessible to the antibody following treatment of bloodstream trypanosomes with acetone. In this study, it was shown using IFAT, that the four *T. congolense* specific MoAbs tested did not bind to live procyclic trypanosomes, unlike the *Nannomonas* subgenus-specific MoAbs which all bound to live procyclic trypanosome membranes. It

has also been shown using IFAT that the *T. simiae* specific MoAb did not bind to live *T. simiae* procyclics.

All the MoAbs produced reacted specifically with the trypanosome species against which they were derived when tested in the IFAT, micro-plate ELISA and the dot-ELISA. However, not all of them bound their respective antigens with the Western immunoblot analysis, whether as MoAb in culture supernatants or as purified fractions. This, perhaps, was not surprising, since in SDS-PAGE analysis, antigen samples were heated to 100°C in SDS in the presence of a reducing agent, a process that could lead to the denaturation of antigens (Su and Prestwood, 1990). Also, the conformation of the antigens after they are transferred to NC membrane is not known. Moreover, it had been reported that the reactivity of immune serum made against native antigens is usually much weaker when tested against denatured antigens (Arnon, 1973). Chaicumpa, Ruangkunaporn, Kalambaheti, Limavongpranee, Kitikoon, Khusmith, Pungpak, Chongsa-Nguan and Sornmani (1991) had also pointed out that antisera against native proteins normally contain some clonal products which recognize the denatured antigens, allowing the Western immunoblot to function. In contrast, MoAbs against native antigens may or may not bind the denatured products. It is therefore expected that many clones of MoAb may fail to bind denatured antigens (Goding, 1983), which could explain why the *T. simiae* KNS7 failed to bind any antigens with the Western immunoblot assay. This inability of KNS7 to bind the specific antigens, did not allow purification of that antigen by electro-elution. As a result, it would have been necessary to resort to immunoaffinity or immuno-precipitation techniques for isolation of the antigen. However, time did not permit such a study to be conducted.

The *T. brucei* MoAbs TR7 and KT43/33; *T. congolense* MoAbs (C2, TC39 and TC40); and *Nannomonas* MoAb TC16, reacted with multiple bands in the Western immunoblot assay. This observation is not new, since, it has

been reported that certain MoAbs often show variable staining intensities or multiple band staining (Braun, Pereira, Norrid and Roizman, 1983; Mandrell and Zollinger, 1984; Steinemann, Fenner, Binz and Parish, 1984; Turner, 1983). The case of TR7 and KT39a may be explained by the findings obtained from micro-plate based antigen capture studies, which indicated that the two *T. brucei* specific MoAbs bound repeated determinants which possibly were located in multiple peptide bands. It has indeed been reported that the usual treatment of antigens with a reducing agent (sodium dodecyl sulphate) and heat in the Western immunoblot assay, leads to the breakdown of antigens into several peptides (Chung, 1987). Moreover, autodegradation of the antigens could produce a similar effect. Both of these processes could lead to the distribution of the antigenic epitopes detected by MoAbs on several peptides of varying molecular weights, and thus lead to multiple band staining (Chaicumpa *et al.*, 1991). It has also been argued that MoAbs that reveal multiple bands in the Western immunoblot assay may be binding common sequences or repeated determinants (Bers and Garfin, 1985; Chaicumpa, Thin-inta, Khusmith, Tapchaisri, Echeverria, Kalamba-heti and Chongsa-Nguan, 1988) in different polypeptide chains produced by denaturing conditions.

The studies described in this Chapter also showed that each of the four *T. congolense* specific MoAbs captured antigen molecules that could be revealed by conjugates of all the others. These results thus suggest that the antigenic epitopes for those MoAbs were located on the same antigen molecule(s). Furthermore, inhibition ELISA studies which showed that each of those *T. congolense* specific MoAbs could inhibit binding by any of the others, suggested two possibilities: (1) that the antigenic epitope(s) recognised by all those four MoAbs was the same, or (2) that the antigenic epitopes detected by these MoAbs were not necessarily the same, instead they might be located so close to one another in a way that binding by one MoAb led to interference in the binding of another MoAb.

Proteinase-K digestion of peptide residues and periodate oxidation of carbohydrate residues have been described and used by several workers in the characterization of the antigens detected by MoAbs (Bright, Chen, Flebbe, Lei and Morrison, 1990; Woodward *et al.*, 1985). Investigations of the biochemical nature of the epitope specificities of the MoAbs produced had revealed that three of the *T. brucei* MoAbs bound protein specific antigenic determinants, since their binding was completely abrogated by proteolysis. Binding by the fourth *T. brucei* MoAb KT39a was, however, only partially affected by proteolysis, suggesting that the antigenic determinant was at least partly protein in nature. Binding by that same antibody was not affected by periodate oxidation of carbohydrate residues. It was, however, difficult to rule out any part played by carbohydrate. This is because according to Woodward *et al.* (1985), antigenic determinants affected by periodate oxidation are carbohydrates in glycoprotein or glycolipids, yet some carbohydrate residues are insensitive to periodate oxidation. It is, therefore, likely that KT39a bound a glycoprotein or a lipoprotein. The studies also showed that the four *T. congolense* MoAbs bound protein antigenic determinants, whilst the four *Nannomonas* subgenus-specific MoAbs were directed at carbohydrate antigenic determinants.

The only *Nannomonas* specific MoAb (TC16) that bound antigens with the Western immunoblot assay, was also shown to bind an antigenic determinant which was different from the common determinant bound by the other three *Nannomonas* MoAbs. This meant that there were at least two different antigenic determinants that defined *Nannomonas* species specificity, both of which were of carbohydrate nature.

Although none of the *T. vivax* specific MoAbs could bind antigens in the Western immunoblot assay, the results obtained using micro-plate ELISA revealed that the antigenic determinant bound by TV8 was different from that bound by KD32. This observation is supported by the finding that TV8

reacted with the bloodstream forms of the South American *T. vivax* IL3841, whilst KD32 could not. Also, the determinant bound by TV8 was insensitive to proteinase-K digestion whilst that bound by KD32 was sensitive to that treatment. It may be further argued that the antigenic determinants bound by TV8 was different from that bound by KD37. This is because unlike TV8, the KD37 epitope was sensitive to proteolysis, and yet both MoAbs detected the bloodstream forms of IL3841. These findings could mean that there were at least three different antigenic determinants that express *T. vivax* species specificity, two of which were proteins and the other of carbohydrate or lipid nature.

Studies on the reactivity of the trypanosome species-specific MoAbs with trypanosome stocks isolated from different geographical areas, have clearly shown that the reactivity of some of the MoAbs was indeed broad. The studies showed that two of the *T. vivax* specific MoAbs, TV8 and KD37, were capable of detecting *T. vivax* originating from East and West Africa, as well as from South America. All the *T. congolense* species-specific and *Nannomonas* subgenus-specific MoAbs were also shown to be capable of detecting the three different types of *T. congolense* (savannah, riverine-forest and Kilifi types) tested. In the application of DNA probes for the differentiation of trypanosome species, the absence of a probe that could hybridize with all the different types of *T. congolense* has been a major limitation (Kukla *et al.*, 1987). It is important to mention here, though, that the recently identified Tsavo type *T. congolense* was not tested in this study because of failure to obtain suitable samples.

Another important observation was that some MoAbs showed a stage specificity in their reactivity. Of the *Nannomonas* specific MoAbs, for instance, only KN5 detected all the different *T. congolense* bloodstream forms tested. The others could not react with the bloodstream stages of two *T. congolense* stocks even though they reacted with the insect stages of the same

stocks. It was also shown that unlike TV8 and KD37, the *T. vivax* specific MoAb KD32 reacted with the vector stages but not the bloodstream forms of the parasite. These observations were not unusual since MoAbs that were specific to the procyclic stages of trypanosomes had been reported earlier (Richardson *et al.*, 1986). A second point was that one of the *T. brucei* specific MoAbs, KT43/27, failed to react with some *in vitro* propagated *T. brucei* procyclics and insect forms from the gut and the salivary glands of tsetse infected with some *T. brucei* stocks. Moreover, KT43/27 could not react with bloodstream forms isolated from laboratory rodents (mice and rats) infected with two different *T. brucei* stocks. This observation suggests that the antigenic epitope bound by KT43/27 might not be expressed in the procyclic as well as other stages of some *T. brucei* stocks. However, it should be noted that of the four *T. brucei* MoAbs, only KT43/27 could bind cytoplasmic antigens; and this was the only *T. brucei* specific MoAb that did not bind any antigens with the Western immunoblot assay. The internal localization of the antigen detected by KT43/27 suggested that, of all the *T. brucei* specific MoAbs, it was the one that would most likely be affected by degradative substances such as proteases and lysozymes from the parasites during sample preparation. It is also likely that the dot-ELISA technique was not suitable for detecting the antigen targeted by KT43/27, possibly because when applied to NC membrane, the antigen could bind in such a way that the epitope detected by KT43/27 was concealed.

These findings indicated that, apart from KT43/27, the MoAbs included in this study were likely to be useful in the development of MoAb-based assays for the detection and differentiation of procyclic forms of African trypanosomes, propagated *in vitro* as well as those in the vector (*Glossina spp.*). In addition, some of the newly derived MoAbs could be useful in studies aimed at diagnosing trypanosomiasis in the vertebrate host. The usefulness of the MoAbs as diagnostic reagents in detecting and differentiating

between culture derived vector stage trypanosome species, would be the subject of investigation in the next Chapter, as a prelude to the diagnosis of infections in the vector.

# CHAPTER 5

## DIFFERENTIATION BETWEEN *IN VITRO* PROPAGATED INSECT-STAGE TRYPANOSOME SPECIES USING DOT-ELISA

## 5.1 Summary

A sensitive and specific nitrocellulose (NC) membrane-based dot-ELISA, utilizing a panel of monoclonal antibodies (MoAbs), was developed for differentiation between *in vitro* derived procyclic forms of *Trypanosoma brucei, T. congolense* and *T. simiae*, and epimastigotes of *T. vivax*. Trypanosomes were applied onto NC membrane in dots and probed with unlabelled trypanosome species-specific MoAb. Bound MoAb was revealed by enzyme labelled anti-mouse IgG and precipitable chromogenic substrate. The assay detected the afore-mentioned trypanosome species in both single and artificially mixed preparations. Six *T. brucei*, four *T. vivax*, seven *T. congolense* and three *T. simiae* procyclic stocks and clones from different geographical areas were tested and identified using the specific MoAbs in the dot-ELISA which had a specificity greater than 99.9%. Some of the *T. brucei*, *T. congolense* and *Nannomonas* specific MoAbs could detect as low as 10 trypanosomes per dot, whilst one *T. vivax* MoAb was able to detect a minimum of 100 trypanosomes per dot in mono-species preparations. A concentration of $1x10^4$ trypanosomes/$\mu$l/dot was eventually determined as ideal for testing in the dot-ELISA. Antigen dots made from the different trypanosome species, and stored at 4°C under desiccated conditions did not show any loss in activity in up to 90 days. However, when stored under similar conditions at room temperature (17-26°C), the *T. congolense* specific antigen remained unaffected up to 60 days, and then showed decreased activity when tested on day 90. The ability of the dot-ELISA to distinguish between the various stocks and clones of trypanosomes that were used, and the ability to identify the constituent species in mixed trypanosome preparations, indicated that this test might prove useful as a laboratory tool for the determination of the identity of *in vitro* derived procyclic trypanosomes. Also, the dot-ELISA

developed could be a useful first step in the development of a field applicable MoAb-based assay for diagnosis of trypanosome infections in the tsetse fly.

## 5.2 Introduction

In the studies described in this Chapter, the trypanosome species and subgenus-specific MoAbs derived against *in vitro* propagated procyclics of *T. brucei*, *T. congolense* and *T. simiae*, and epimastigotes of *T. vivax*, were employed in the development and standardization of a simple, sensitive and specific NC membrane-based dot-ELISA for the differentiation of readily available *in vitro* cultivated forms of those trypanosome species.

It was intended to ultimately apply the assay to the diagnosis of trypanosome infections in infected tsetse flies. The feasibility of this approach stems from the finding that procyclic tsetse midgut forms and culture forms of the African trypanosomes express similar antigens (Richardson *et al.*, 1986; Pearson, Moloo and Jenni, 1987). The NC membrane-based dot-ELISA was selected for this application for two reasons. Firstly, it offered the best opportunity for the development of a diagnostic test that is simple and easy to perform, in addition to being both sensitive and specific. Secondly, such an assay could be easily modified for field diagnosis of trypanosome infections in the tsetse fly (*Glossina spp.*).

Furthermore, the introduction of a simple, specific and sensitive assay capable of detecting and differentiating between *in vitro* propagated trypanosome species, would facilitate other studies. For example, the search for a solution to the trypanosomiasis problem has necessitated extensive studies into the biology, biochemistry, response to chemotherapy, antigenic constitution, as well as characterization of the causative organisms. To facilitate these studies, techniques have been developed for *in vitro* culture of various developmental stages of the parasite. Thus the procyclic, epimastigote, metacyclic and the bloodstream forms of trypanosomes can be propagated in large numbers in artificial cultures *in vitro* (Hirumi, Doyle and Hirumi, 1977; Ross, Gray, Taylor and Luckins, 1985; Zweygarth, Gumm,

Gray, Cheruiyot, Webster and Kaminsky, 1989; Hirumi, Nelson and Hirumi, 1983; Baltz, Baltz, Giroud and Crocket, 1985; Brun and Schönenberger, 1979). This has made it possible to conduct trypanosome drug sensitivity analysis *in vitro* (Kaminsky and Zweygarth, 1989; Kaminsky, Chuma and Zweygarth, 1989; Ross and Taylor, 1990). Moreover, *in vitro* culturing of trypanosomes has made possible, the isolation of the parasites from various organs of infected tsetse flies into artificial cultures (Cunningham, 1977; Trager, 1959; Gumm, 1991; Gray, Cunningham, Gardiner, Taylor and Luckins, 1981).

This unlimited opportunity to grow freshly isolated trypanosomes in culture, and the ability to cryopreserve samples of such organisms, calls for the availability of simple reliable techniques for ascertaining the species of trypanosomes present in *in vitro* cultures. Two diagnostic techniques, DNA hybridization analysis and isoenzyme characterization, are currently used for this purpose (Gashumba, Gibson and Opiyo, 1986; Kukla *et al.*, 1987). These methods are, however, not simple enough and cannot be performed in most laboratories. The ability of a simple MoAb-based dot-ELISA to detect and differentiate between culture derived procyclic trypanosomes, is reported.

## 5.3 Materials and methods

### 5.3.1 *In vitro* cultivation of trypanosomes

#### 5.3.1.1 Cultivation of procyclic forms

Procyclic trypanosomes were cultivated in culture using complete-(SM) medium filtered through 0.2 to 0.45$\mu$m membrane bottle filter (Costar). Cryopreserved *T. brucei, T. congolense* and *T. simiae* procyclic trypanosomes were resuscitated into culture medium and the cultures initiated as described earlier (section 3.2.1). The trypanosomes were allowed to multiply until the trypanosome density reached approximately $1x10^7$/ml. The *T. brucei* IL2616 procyclics were well adapted to culture. Maintenance of this trypanosome stock was achieved by removal of all but 1ml of the $1x10^7$/ml trypanosome suspension from a culture flask and replacement with fresh medium in quantities of up to 20 times the residual volume. However, the *T. congolense* clone K/83/IL/97/2 and *T. simiae* clones TS1 and TS4 procyclics were poorly adapted to culture. These were maintained by removal of half of the $1x10^7$/ml procyclic suspension and replacement with an equal volume of new medium usually thrice weekly. The trypanosomes were grown in 15-20ml volumes in $75cm^2$ flasks (Costar; Falcon). The cultures were gassed whenever the flasks were opened. Flasks were closed tightly soon after gassing and incubated at 27°C. Old flasks that had been used for maintaining over six passages were replaced with new ones.

#### 5.3.1.2 Cultivation of epimastigote forms

*In vitro* propagation of *T. vivax* epimastigotes, East African (EA) stock IL2337 and West African (WA) stock IL1392 were achieved by transformation of freshly isolated bloodstream forms into epimastigotes at 27°C in Iscove's modified Dulbecco's minimum essential medium (M-DMEM,; Flow Laboratories, Irvine, Scotland, UK) that had transferrin,

soybean lecithin, and bovine serum albumin incorporated in it (Iscove and Melchers, 1978). Foetal bovine serum (FBS), purchased from Hyclone Laboratories Inc., was heat inactivated at 56°C for 30 min, and used at 20%(v/v) final concentration. This medium was modified again by the addition of 0.3% (w/v) sodium bicarbonate and adjusting the pH to 7.0 (It will henceforth be referred to as "complete-(M-DMEM) medium").

Mice were infected by intraperitoneal injection of $1x10^5$ trypanosomes in 0.5ml of PSG, pH 7.4. Blood taken from the tail veins of infected mice was examined microscopically, at x400 magnification, for trypanosomes as described by Herbert and Lumsden (1976). Mice with peak parasitaemia of more than $1x10^9$ trypanosomes/ml were killed by terminal anaesthesia with diethyl-ether and immediately sterilized by immersion in 70% ethanol. Infected blood was drawn aseptically by cardiac puncture, using a 22 gauge hypodermic needle, into heparinized syringes containing 5IU heparin/ml. Five microlitre volumes of infected blood were slowly deposited by pipette at the bottom, close to the edges of three of the wells in a six well plate (Costar, USA) containing 1ml of complete-(M-DMEM) medium each. The culture plate was then incubated at 27°C for 90 min. During this incubation, bloodstream form trypanosomes migrated from the infected blood into the medium. Five hundred microlitres of medium, containing the trypanosomes, were pipetted away from the deposited blood from each of the three wells and transferred to the three remaining wells containing 1ml of complete-(M-DMEM) medium each. The three new wells were incubated for another 90 min, after which 1ml volumes of medium, containing trypanosomes, were pipetted from each well away from the site of deposition and pooled (3ml) into a $25cm^2$ culture flask. The flask was capped tightly and incubated at 27°C. No attempt was made to change or add medium to the culture for at least five days, and even then only if an increase in trypanosome numbers and a decrease in pH were observed. Thereafter, up to 50% of the

medium was changed over 2 to 3 days. Subculture of the EA IL2337 and WA IL1392 were made when colonies of epimastigote forms covered at least 75% of the plastic surface (Gumm, 1991). Epimastigote colonies were scraped off, using disposable cell scrapers (Costar). Half of the medium containing epimastigotes was transferred from one flask to a new one, and an equal volume of fresh medium added.

**5.3.1.3** Transformation of bloodstream form trypanosomes into procyclics

Transformation of *T. brucei* and *T. congolense* bloodstream trypomastigotes into procyclics was initiated at 27°C in complete-(SM) medium. Infected parasitaemic mice were killed by terminal anaesthesia as usual and immediately dipped into 70% ethanol. Infected blood was drawn aseptically into a heparinised syringe by cardiac puncture as previously described. The blood was washed two times in complete-(SM) medium by centrifugation at 400 *Xg* for 10 min. The pelleted blood cells and trypanosomes were resuspended in complete-(SM) medium to a final concentration of $1x10^6$-$1x10^7$ red bloodcells/ml and 4ml volumes pipetted into $25cm^2$ sterile culture flasks (Costar). The flasks were gassed with 5% $CO_2$ in air for 10-20 seconds, tightly closed and incubated at 27°C. Each flask was examined daily, using an inverted microscope (Nikon, 46212, Japan) under x200 magnification. When the trypanosome density reached approximately $1x10^7$/ml, half of the medium was removed and replaced by an equal volume of fresh medium. This process was repeated until all blood cells were eliminated from the culture. The cultures were then expanded by two fold volume increases and transferred to larger ($75cm^2$) culture flasks (Costar).

### 5.3.2 Sample Preparation for Dot-ELISA

#### 5.3.2.1 Preparation of trypanosomes for dot-ELISA

Procyclic forms of *T. brucei* and *T. congolense,* as well as epimastigote forms of *T. vivax* were propagated in culture as described under sections 5.3.1.1 and 5.3.1.2. Trypanosomes were harvested from *in vitro* cultures and washed 2 times in PBS, pH 7.4, PSG, pH 8.0 or normal saline, by centrifugation at 1000 *Xg* for 5 min each. The trypanosome pellets were resuspended in small volumes of the appropriate buffer and counted, using an Improved Neubauer counting chamber.

To allow for the proper estimation of the minimum number of trypanosomes detected, the trypanosome suspensions were adjusted to $1x10^8$ trypanosomes/ml and ten-fold serial dilutions made down to $1x10^3$ trypanosomes/ml. 1$\mu$l samples were then pipetted from those tubes and placed in dots onto strips of NC membrane, pore size 0.45$\mu$m. Trypanosome numbers ranging between $1x10^5$ trypanosomes/dot and 0 trypanosomes/dot were thus obtained. Each sample was dotted onto several strips so that strips with the same samples could be tested against different trypanosome species-specific MoAbs. Also, procyclic *T. congolense* (savannah, riverine/forest and Kilifi types) and *T. grayi*, each suspended in normal saline and dotted onto NC filters ($1x10^4$ or $7x10^3$) per dot, were prepared and donated by Dr. J. McNamara of the Tsetse Research Laboratory, Bristol, England.

The ability of the MoAbs to identify mixed trypanosome populations was also studied. Trypanosome mixtures, each consisting of equal numbers of two different species from the group (*T. brucei, T. congolense, T. simiae* and *T. vivax*), were made in all possible combinations. Each mixed sample was titrated in PBS (pH 7.4) to give $1x10^5$, $1x10^4$, $1x10^3$, $1x10^2$, $1x10^1$ and 0 trypanosomes per microlitre, and 1$\mu$l volumes dotted onto NC membrane strips. Trypanosomes were also lysed by suspension in distilled water, and the lysed suspensions titrated to determine the effect of lysis on

specificity and sensitivity of the MoAbs. The antigen "dotted" NC membrane strips were left to dry at room temperature (17-26°C) for 15 min before they were assayed.

The suitability of some other membrane supports, namely, 0.45μm pore size hybridization transfer membranes, Hybond-C (Cat. RPN.203C, Lot.18425), Hybond-N (Cat. RPN.303N, Lot.20849) both from Amersham, and 0.45μm pore size immuno-affinity membrane (Pall Immunodyne Lot.141743; Pall Bio Support Division, East Hills, NY, USA), was also investigated.

### 5.3.3 Dot-ELISA Procedure

#### 5.3.3.1 Detection of *in vitro* propagated trypanosomes by dot-ELISA

*In vitro* derived trypanosomes were suspended in buffer and applied in dots onto NC membrane filters, and tested as described previously (Chapter 4, section 4.3.9).

#### 5.3.3.2 Titration of specific MoAbs and enzyme conjugated antibody

Trypanosome species-specific MoAbs were tested in the dot-ELISA to determine the optimal working dilution and time of incubation. MoAb in culture supernatants were tested in four-fold dilutions, starting with a dilution of 1:2. Ammonium sulphate concentrated MoAb fractions were tested in two-fold dilutions (starting with 1:5 dilution), and affinity purified MoAb fractions were tested in two-fold dilutions, starting from 1:250. All MoAb dilutions were also tested for optimum time of incubation, namely 30 min, 1hr, and 3hr. Enzyme-conjugated antibody was also tested in two-fold dilutions from 1:250 to 1:2,000. The dilution and time of incubation at which an antibody reacted specifically and gave the highest intensity of colour development were selected and tested against various conjugate incubation times (15 min, 30 min and 1hr) for optimisation.

## 5.4 Results

### 5.4.1 Optimal working dilution and incubation period for MoAbs and enzyme-conjugate

Investigations into the influence of period of incubation on the reactivity of the specific MoAbs, indicated that a 3hr incubation period was suitable for all the MoAbs. In contrast, different MoAb dilutions were determined to be ideal, depending on the source of MoAb and the purification method used (Table 23). Figure 22 shows the results obtained when purified fractions of KT43/33 and KN4 were tested to determine the optimal working dilutions. Both MoAbs reacted specifically at all the dilutions tested. The intensity of the positive colour reactions remained virtually unchanged from 1:250 to 1:1,000 and decreased at 1:2,000. A working dilution of 1:1,000 was selected for both MoAbs.

One hour incubation of enzyme-conjugate was found to give better reactions when compared to 30 min or 15 min incubations (Figure 23).

### 5.4.2 Specificity of the dot-ELISA in identifying trypanosomes in mono-species preparations

The ability of a panel of trypanosome species-specific MoAbs to differentiate between *in vitro* derived procyclics of *T. brucei, T. congolense* and *T. simiae* and epimastigotes of *T. vivax*, was investigated with the dot-ELISA. Figure 24 summarizes the reactivity of two of the *T. brucei* specific MoAbs (TR7 and KT39a) in this assay. It was observed that both TR7 and KT39a reacted with the dot containing *T. brucei* antigen, and neither of them reacted with the antigen dots representing *T. congolense, T. simiae* or *T. vivax*. At 1:5 dilution, the culture supernatant containing KT39a MoAb, clearly left negative impressions on the *T. congolense* and *T. simiae* dots, thereby

**Table 23**

**Working dilution of the specific MoAbs used in the dot-ELISA**

| Monoclonal Antibody | Isotype | Specificity | Source of antibody | Dot-ELISA titre |
|---|---|---|---|---|
| TR7/47.37.16 | IgM | *T. brucei* | A.S. ppt | 1:20 |
| KT39a/18.17 | IgM | *T. brucei* | C.S. | 1:50 |
| KT43/33.32 | $IgG_1$ | *T. brucei* | Purified | 1:1,000 |
| KT43/27.32 | $IgG_{2a}$ | *T. brucei* | Purified | 1:1,000 |
| TV8/8.33.42 | $IgG_3$ | *T. vivax* | Purified | 1:1,000 |
| KD32/48.17 | $IgG_1$ | *T. vivax* | Purified | 1:1,000 |
| KD37/11.1 | $IgG_1$ | *T. vivax* | A.S. ppt | 1:50 |
| C2 | $IgG_1$ | *T. congolense* | Purified | 1:500 |
| TC6/42.6.3 | $IgG_1$ | *T. congolense* | A.S. ppt | 1:20 |
| TC40/30.15.40 | IgM | *T. congolense* | Purified | 1:2,000 |
| TC39/30.25.95 | IgM | *T. congolense* | Purified | 1:2,000 |
| KNS7/14.X | $IgG_1$ | *T. simiae* | A.S. ppt | 1:20 |
| TC16/5.12.33 | $IgG_1$ | *Nannomonas* | Purified | 1:1,000 |
| TC6/25.25.4 | $IgG_3$ | *Nannomonas* | Purified | 1:1,000 |
| KN4/13.9 | $IgG_3$ | *Nannomonas* | Purified | 1:1,000 |
| KN5/6.15 | $IgG_1$ | *Nannomonas* | A.S. ppt | 1:20 |

A.S. ppt = x20 concentration of culture supernatant by ammonium sulphate precipitation.
C.S. = culture supernatant.
Purified = purified MoAb fraction.

**Figure 22**

Titration of purified fractions of KT43/33 (*T. brucei* species-specific) and KN4 (*Nannomonas* subgenus-specific) MoAbs to determine the optimal dilution of antibody for use in the dot-ELISA. Each strip of NC membrane was applied with varying concentrations of trypanosomes per dot (Tryps/dot) of *T. congolense* K/83/IL/97/2 (TCK), *T. brucei* IL2616 (TB) and *T. simiae* KETRI 2431 (TS) cultured procyclics. MoAbs were tested at several dilutions as shown.

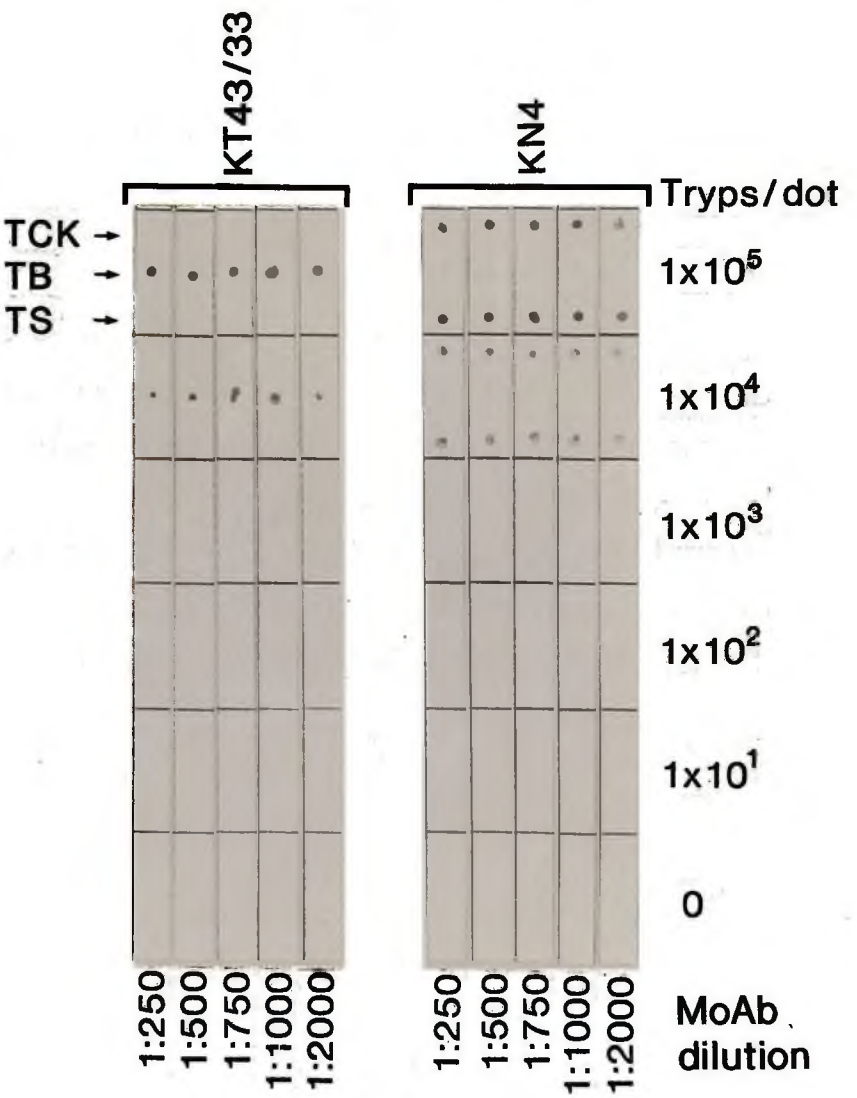



Figure 22

**Figure 23**

Titration of goat anti-mouse horseradish peroxidase conjugate to determine optimal incubation time for reactions in the dot-ELISA. Each strip of NC membrane was applied with varying concentrations of trypanosomes per dot (Tryps/dot) of *T. congolense* K/83/IL/97/2 (TCK), *T. brucei* IL2616 (TB) and *T. simiae* KETRI 2431 (TS) cultured procyclics. Strip 'C' represents a conjugate control, that was not incubated with specific MoAb.

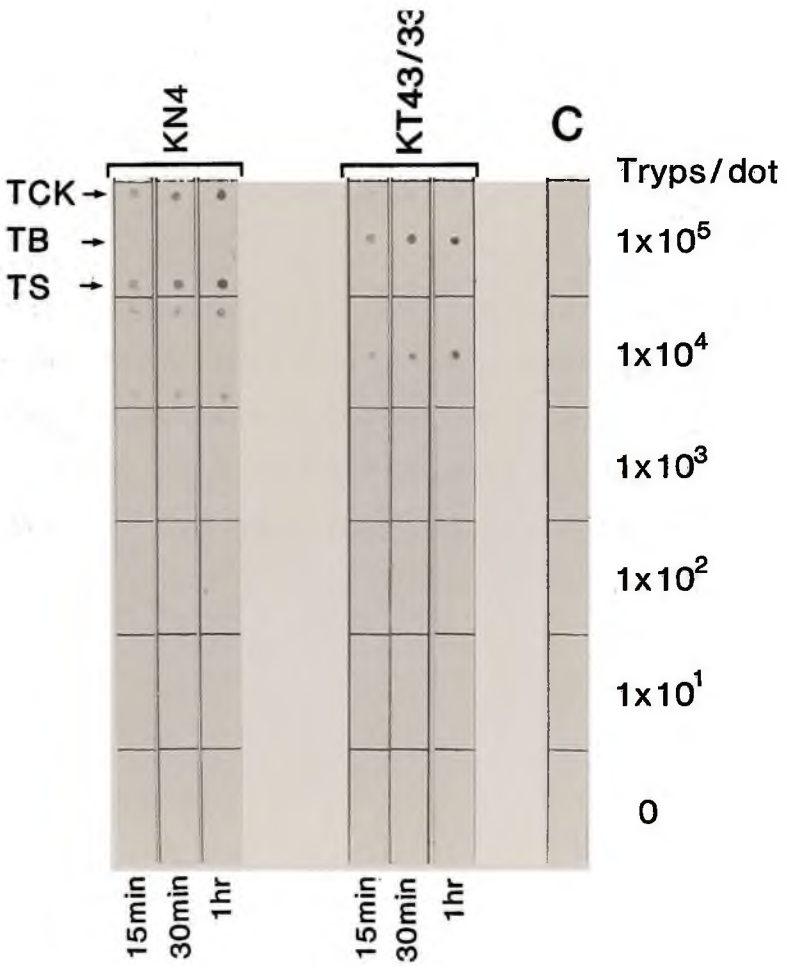



Figure 23

**Figure 24**

An illustration of the specific reactivity of two *Trypanosoma brucei* specific MoAbs (TR7 and KT39a) in the dot-ELISA. Each strip shown was applied with various concentrations of trypanosomes per dot (Tryps/dot) of either *T. vivax* IL1392 cultured epimastigotes (TV) or procyclics of *T. congolense* K/83/IL/97/2 (TCK), *T. brucei* IL2616 (TB) and *T. simiae* KETRI 2331 (TS).

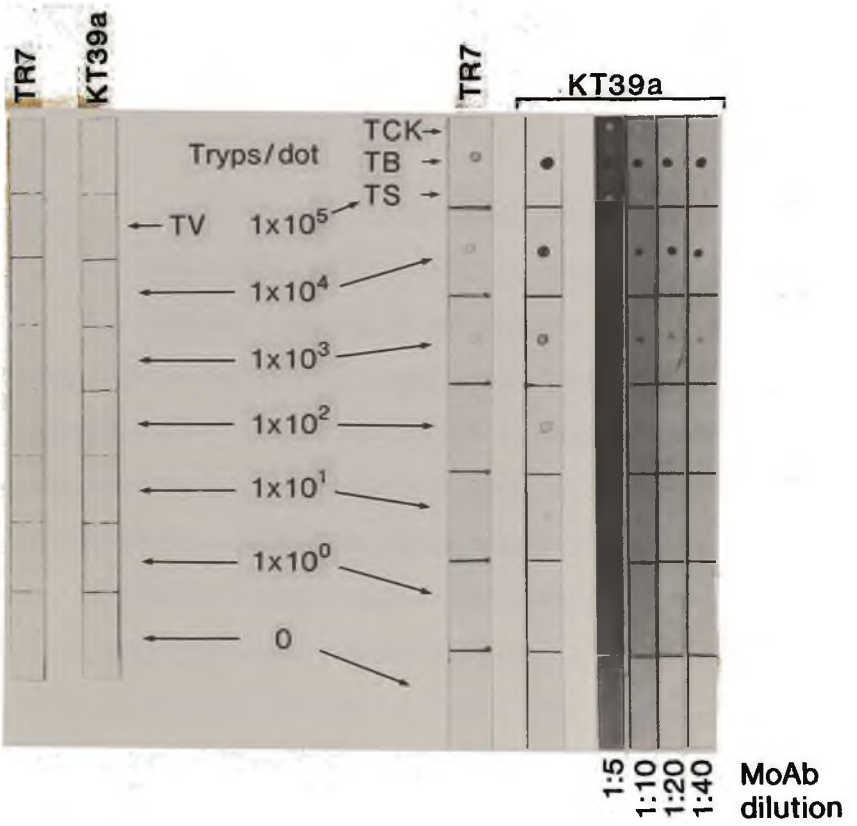



Figure 24

illustrating clear *T. brucei* specificity (Figure 24). Figures 25, 26 and 27 illustrate respectively, the specificity of two *T. vivax* specific MoAbs (TV8 and KD32), four *T. congolense* specific MoAbs (C2, TC6, TC40 and TC39), and four *Nannomonas* subgenus-specific MoAbs (TC16, TC6/25, KN4 and KN5). These specific reactions remained unaltered even at trypanosome concentrations of $1x10^5$ trypanosomes/dot in 1 $\mu$l volumes. The reactivity pattern of the entire panel of MoAbs, as determined by dot-ELISA, is shown in (Table 24).

### 5.4.3 Specificity of the dot-ELISA in identification of the constituent trypanosome species in artificially mixed preparations

Mixtures consisting of two different trypanosome species each, were made in all possible combinations, using *T. brucei, T. vivax, T. congolense* and *T. simiae* organisms. The mixed trypanosome suspensions were doted onto NC membrane and tested to determine the ability of the various MoAbs to differentiate the constituent species. Figure 28 illustrates the ability of the *T. brucei* specific MoAb (KT39a) to detect *T. brucei* in *brucei/congolense* and *brucei/simiae* mixtures. In the same experiment, KT39a did not react with the *congolense/simiae* mixture. Also, Figure 28 shows the specific detection of *T. congolense* by C2, TC6, TC40 and TC39 in *congolense/brucei* and *congolense/simiae* mixtures. The absence of a reaction on the *brucei/simiae* dot, clearly showed that those MoAbs were indeed *T. congolense* specific. TV8 and KD32, both *T. vivax* specific, had been shown not to react with antigen mixtures of *brucei/congolense*, *brucei/simiae* and *congolense/simiae* (Figure 29). Yet, the *Nannomonas* specific MoAbs (TC16, TC6/25, KN4 and KN5) are shown to detect their target species (*T. congolense* and *T. simiae*) in those mixtures.

**Figure 25**

Reactivity of *Trypanosoma vivax* specific MoAbs (TV8 and KD32) in the dot-ELISA. Each strip shown was applied with various concentrations of trypanosomes per dot (Tryps/dot) of either *T. vivax* IL1392 cultured epimastigotes (TV) or procyclics of *T. congolense* K/83/IL/97/2 (TCK), *T. brucei* IL2616 (TB) and *T. simiae* KETRI 2431 (TS).

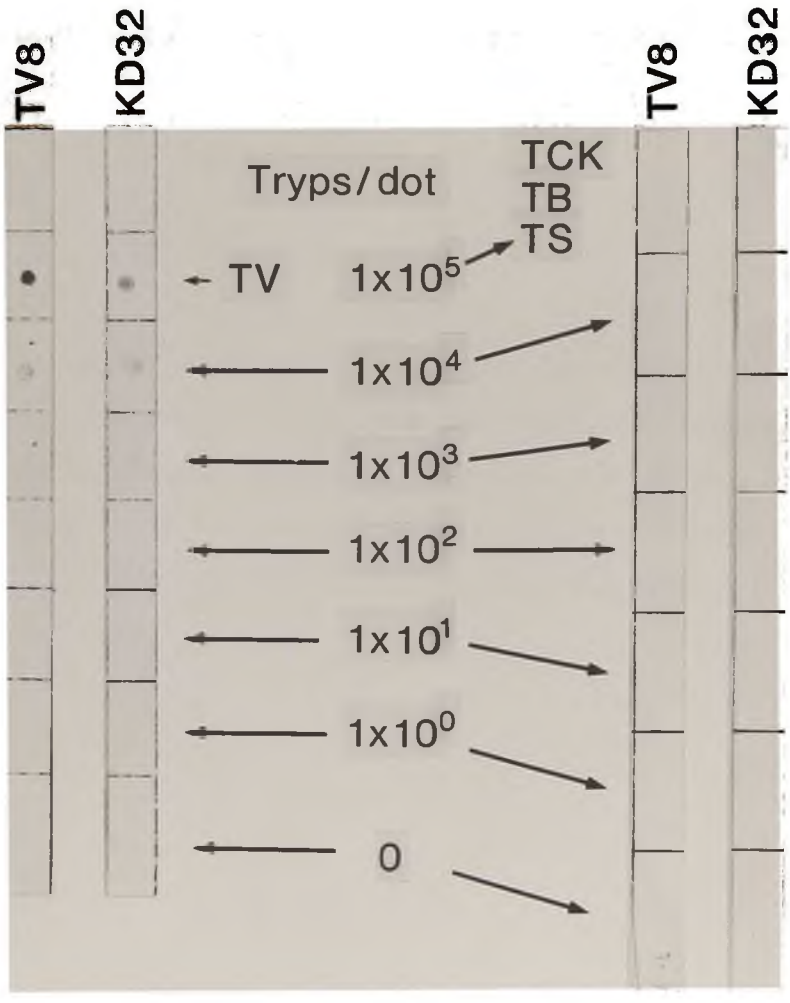



**Figure 25**

**Figure 26**

Reactivity of *Trypanosoma congolense* specific MoAbs (C2, TC6, TC40 and TC39) in the dot-ELISA. Each strip shown was applied with various concentrations of trypanosomes per dot (Tryps/dot) of either *T. vivax* IL1392 cultured epimastigotes (TV) or procyclics of *T. congolense* K/83/IL/97/2 (TCK), *T. brucei* IL2616 (TB) and *T. simiae* KETRI 2431 (TS).

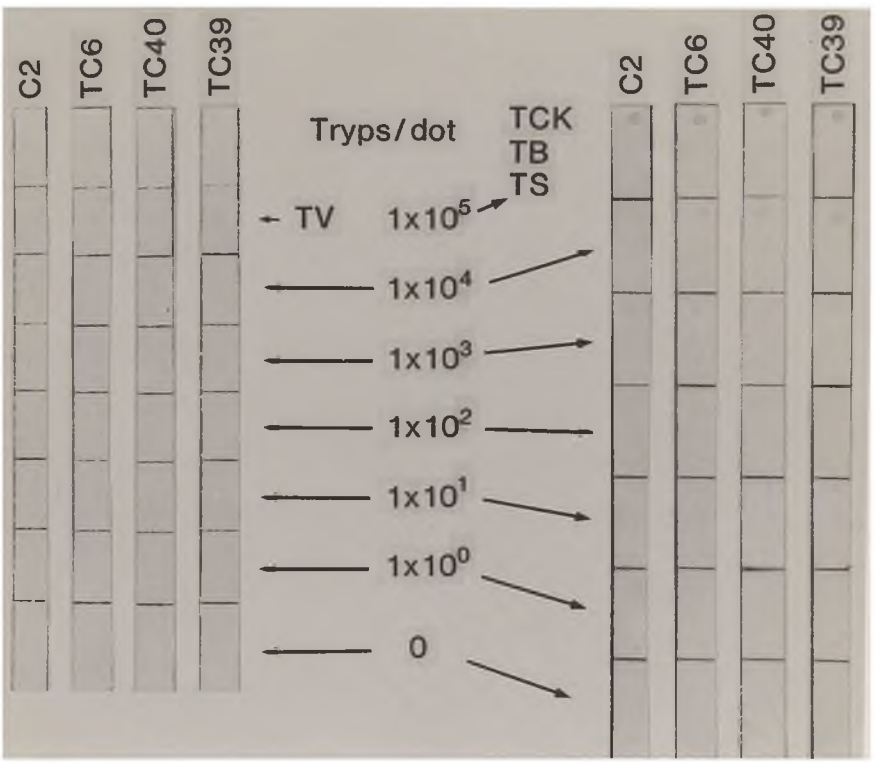



**Figure 26**

**Figure 27**

Reactivity of *Nannomonas* subgenus-specific MoAbs (TC16, TC6/25, KN4 and KN5) in the dot-ELISA. Each strip shown was applied with various concentrations of trypanosomes per dot (Tryps/dot) of either *T. vivax* IL1392 cultured epimastigotes (TV) or procyclics of *T. congolense* K/83/IL/97/2 (TCK), *T. brucei* IL2616 (TB) and *T. simiae* KETRI 2431 (TS).

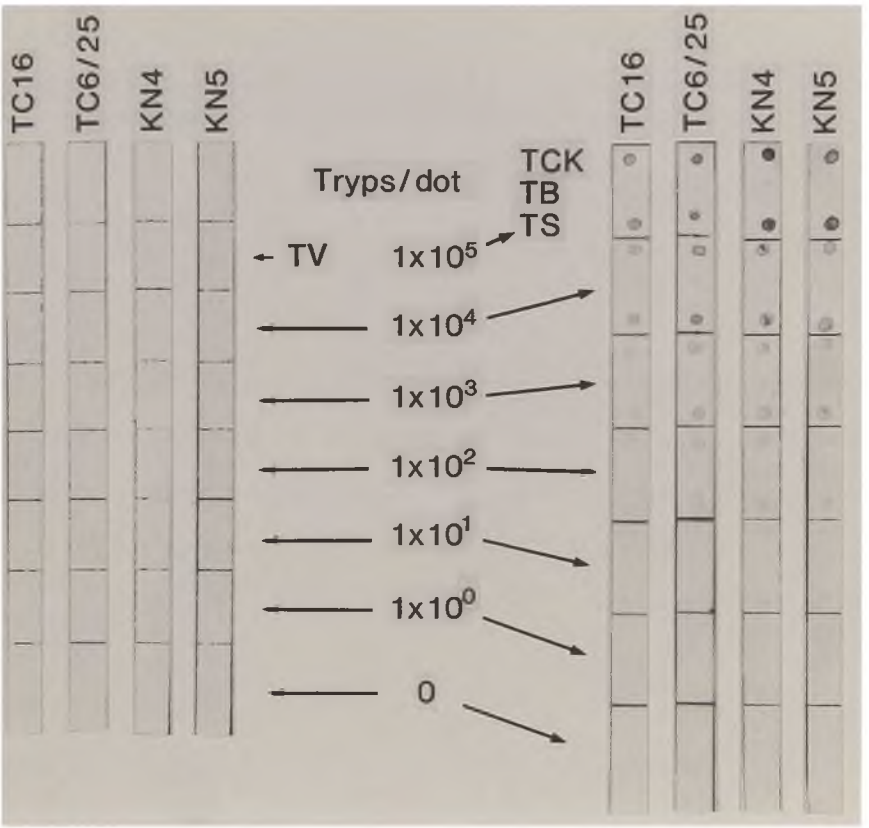



**Figure 27**

**Table 24**

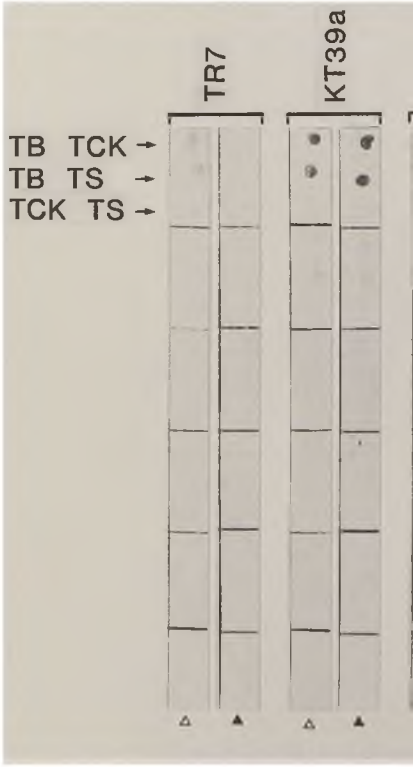
**The specificity of the MoAbs as determined by their reactivity with procyclics and epimastigotes of different trypanosome species in the dot-ELISA**

| Monoclonal Antibody | Isotype | *T. brucei* * | *T. vivax* ** | *T. congolense* * | *T. simiae* * |
|---|---|---|---|---|---|
| TR7/47.37.16 | IgM | + | | | |
| KT39a/18.17 | IgM | + | | | |
| KT43/33.32 | $IgG_1$ | + | - | - | |
| KT43/27.32 | $IgG_{2a}$ | + | - | - | |
| TV8/8.33.42 | $IgG_3$ | - | + | - | - |
| KD32/48.17 | $IgG_1$ | | + | | - |
| KD37/11.1 | $IgG_1$ | - | + | | - |
| C2 | $IgG_1$ | - | | + | |
| TC6/42.6.3 | $IgG_1$ | - | - | + | |
| TC40/30.15.40 | IgM | - | | + | |
| TC39/30.25.95 | IgM | | | + | |
| KNS7/14.X | $IgG_1$ | - | | - | + |
| TC16/5.12.33 | $IgG_1$ | | | + | + |
| TC6/25.25.4 | $IgG_3$ | | | + | + |
| KN4/13.9 | $IgG_3$ | | - | + | + |
| KN5/6.15 | $IgG_1$ | | - | + | + |

* procyclics.
** epimastigotes.
+ = antibody reacts with trypanosomes.
= antibody does not react with trypanosomes.

**Figure 28**

Reactivity of *T. brucei* species-specific MoAbs (TR7 and KT39a) and *T. congolense* specific MoAbs (C2, TC6, TC40 and TC39) with mixtures of cultured trypanosome procyclics in the dot-ELISA. Each NC membrane strip was applied with varying concentrations of trypanosome mixtures per dot (Tryps/dot/spp.). The trypanosome species used were: *T. brucei* IL2616 (TB), *T. congolense* K/83/IL/97/2 (TCK) and *T. simiae* KETRI 2431 cultured procyclics. (▲) indicates NC membrane strips which were "dotted" with trypanosome antigens prepared by suspending whole organisms in deionised water. (△) indicates NC membrane strips which were "dotted" with whole trypanosomes suspended in phosphate buffered saline (PBS) pH 7.4.



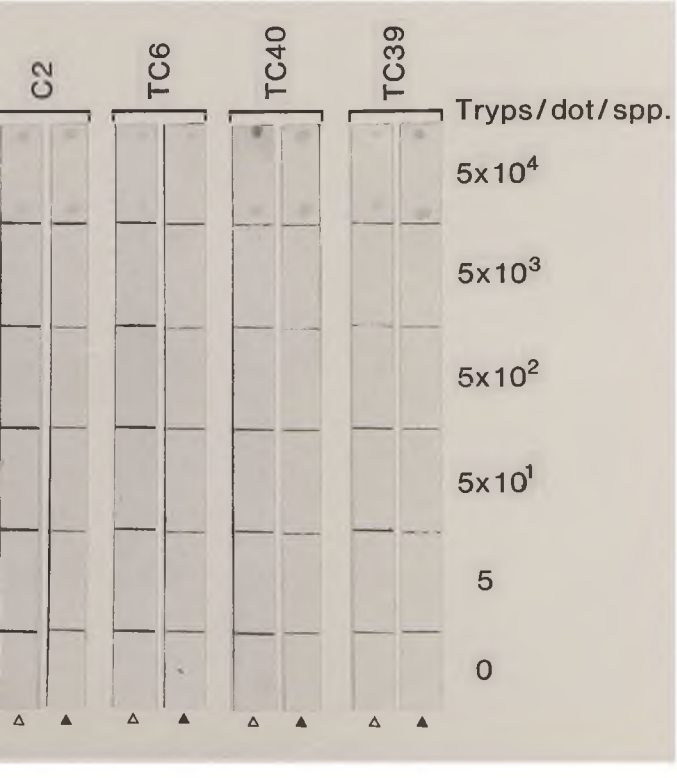



**Figure 28**

**Figure 29**

Reactivity of *T. vivax* specific MoAbs (TV8 and KD32) and *Nannomonas* subgenus-specific MoAbs (TC16, TC6/25, KN4 and KN5) with mixtures of cultured trypanosome procyclics in the dot-ELISA. Each NC membrane strip was applied with varying concentrations of trypanosome mixtures per dot (Tryps/dot/spp.). The trypanosome species used were: *T. brucei* IL2616 (TB), *T. congolense* K/83/IL/97/2 (TCK) and *T. simiae* KETRI 2431 cultured procyclics. (▲) indicates NC membrane strips which were "dotted" with trypanosome antigens prepared by suspending whole organisms in deionised water. (△) indicates NC membrane strips which were "dotted" with whole trypanosomes suspended in phosphate buffered saline (PBS) pH 7.4.

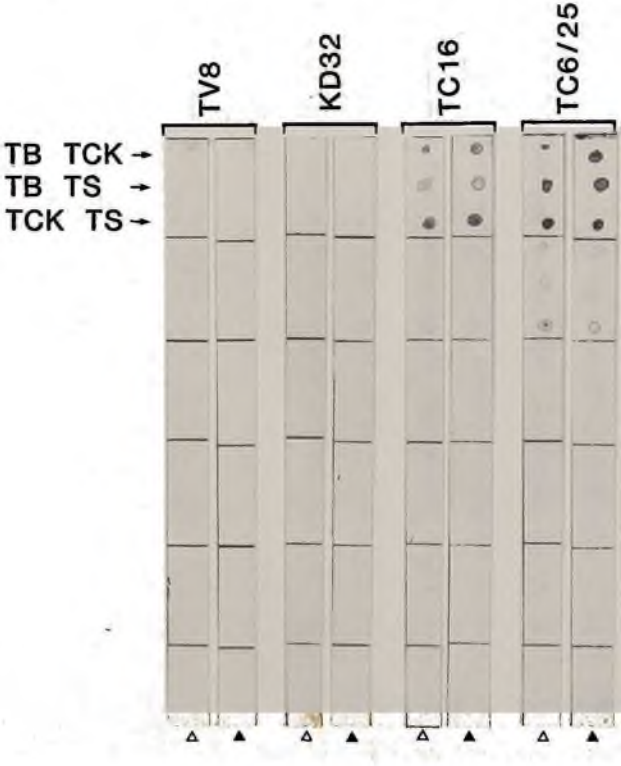
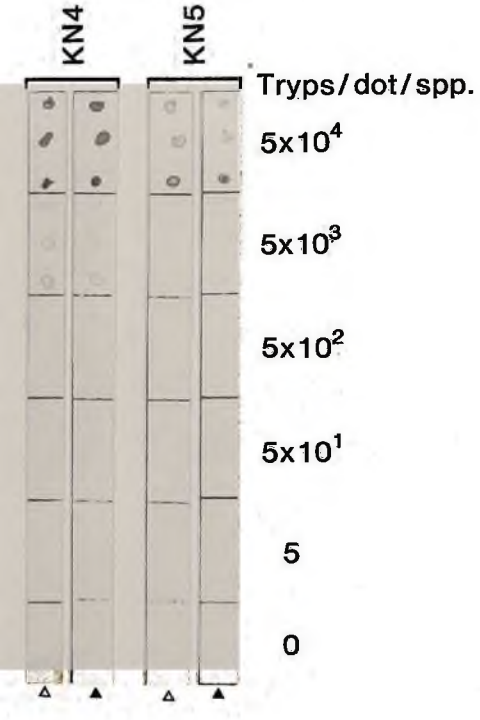



Figure 29



Suspending mixed trypanosomes in PBS and dotting them as whole organisms or lysing the mixed organisms in deionised water before applying the samples in dots onto NC membrane, gave similar results (Figure 28, 29). On the whole, it was found that each of the MoAbs could detect it's target trypanosome species, irrespective of which other species were present (Table 25). Also, the MoAbs were tested against trypanosome stocks or clones from different geographical areas and shown to detect specifically all the different isolates (Table 26).

### 5.4.4 The suitability of ascites as a source of MoAb for the dot-ELISA

The use of ascitic fluid as a source of specific MoAb for the dot-ELISA presented some problems. Most ascites fractions cross-reacted extensively with all the different trypanosome species when used at dilutions lower than 1:100. At higher dilutions, specific reactions were usually obtained, but this was normally at the expense of sensitivity. Figure 30 shows the results obtained from the titration of ascites containing the *T. congolense* specific MoAb TC39. The trend clearly illustrates the decreasing sensitivity as specific reactivity on the *T. congolense* antigen dot (TCK) was being achieved. This loss in sensitivity was unreasonably high when compared to MoAb purified from culture supernatants, and remained unchanged even when ascites was purified. It was, therefore, decided to discontinue the use of ascites as a source of specific MoAb for the dot-ELISA in favour of direct use of culture supernatants or purified fractions thereof.

### 5.4.5 Cross-reactivity in the dot-ELISA

Cross-reactivity due to factors such as the source of MoAb and concentrations of MoAb or conjugate or antigen, was encountered in the dot-ELISA, prior to standardization of the assay. High concentrations of purified MoAb or HRPO-conjugated antibody, increased the non-specific reactivity,

**Table 25:** **Ability of the specific MoAbs to identify trypanosome species in artificial mixtures of cultured insect stages of the parasites**

| Monoclonal Antibody | Isotype | Specificity | Reactivity of MoAbs with trypanosome mixtures tested | | | | | |
|---|---|---|---|---|---|---|---|---|
| | | | *T. brucei* & *T. congolense* | *T. brucei* & *T. simiae* | *T. brucei* & *T. vivax* | *T. congolense* & *T. simiae* | *T. congolense* & *T. vivax* | *T. simiae* & *T. vivax* |
| TR7/47.37.16 | IgM | *T. brucei* | + | + | + | - | - | - |
| KT39a/18.17 | IgM | *T. brucei* | + | + | + | - | - | - |
| KT43/33.32 | $IgG_1$ | *T. brucei* | + | + | + | - | - | - |
| KT43/27.32 | $IgG_{2a}$ | *T. brucei* | + | + | + | - | - | - |
| TV8/8.33.42 | $IgG_3$ | *T. vivax* | - | - | + | - | + | + |
| KD32/48.17 | $IgG_1$ | *T. vivax* | - | - | + | - | + | + |
| KD37/11.1 | $IgG_1$ | *T. vivax* | - | - | + | - | + | + |
| C2 | $IgG_1$ | *T. congolense* | + | - | - | + | + | - |
| TC6/42.6.3 | $IgG_1$ | *T. congolense* | + | - | - | + | + | - |
| TC40/30.15.40 | IgM | *T. congolense* | + | - | - | + | + | - |
| TC39/30.25.95 | IgM | *T. congolense* | + | - | - | + | + | - |
| KNS7/14.X | $IgG_1$ | *T. simiae* | - | + | - | + | - | + |
| TC16/5.12.33 | $IgG_1$ | *Nannomonas* | + | + | - | + | + | + |
| TC6/25.25.4 | $IgG_3$ | *Nannomonas* | + | + | - | + | + | + |
| KN4/13.9 | $IgG_3$ | *Nannomonas* | + | + | - | + | + | + |
| KN5/6.15 | $IgG_1$ | *Nannomonas* | + | + | - | + | + | + |

+ = antibody reacts with trypanosomes.
- = antibody does not react with trypanosomes.

**Table 26:** **Reactivity of the specific MoAbs with different stocks and clones of *T. brucei*, *T. vivax*, *T. congolense* and *T. simiae* as defined by dot-ELISA**

| | Reactivity of the monoclonal antibodies with uncoated procyclic or epimastigote trypanosomes of: | | | | | | | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | *T. brucei* | | | | | | *T. vivax* | | | | *T. congolense* | | | | | | | *T. simiae* | | |
| Monoclonal antibody | IL2616 (Proc) | Th-17/87 (Proc) | TREU-1442 (Proc) | MiTat1.2 (Proc) | IL1984 (Proc) | IL1478 (Proc) | IL1392 (Epis) | IL3895 (Epis) | ILDat1.9 (Epis) | CP2331 (Epis) | IL/60/1 (Proc) | IL/97/2 (Proc) | 1L2079 (Proc) | CP81 (Proc) | MOVS (Proc) | MBOI (Proc) | MSUS (Proc) | TS1 (Proc) | TS4 (Proc) | KETRI 243 (Proc) |
| TR7 | + | + | + | + | + | + | - | - | - | - | - | - | - | - | - | - | - | - | - | - |
| KT43/33 | + | + | + | + | + | + | - | - | - | - | - | - | - | - | - | - | - | - | - | - |
| KT39a | + | + | + | + | + | + | - | - | - | - | - | - | - | - | - | - | - | - | - | - |
| KT43/27 | + | + | + | + | + | + | - | - | - | - | - | - | - | - | - | - | - | - | - | - |
| TV8 | - | - | - | - | - | - | + | + | + | + | - | - | - | - | - | - | - | - | - | - |
| KD32 | - | - | - | - | - | - | + | + | + | + | - | - | - | - | - | - | - | - | - | - |
| KD37 | - | - | - | - | - | - | + | + | + | + | - | - | - | - | - | - | - | - | - | - |
| C2 | - | - | - | - | - | - | - | - | - | - | + | + | + | + | + | + | + | - | - | - |
| TC6 | - | - | - | - | - | - | - | - | - | - | + | + | + | + | + | + | + | - | - | - |
| TC40 | - | - | - | - | - | - | - | - | - | - | + | + | + | + | + | + | + | - | - | - |
| TC39 | - | - | - | - | - | - | - | - | - | - | + | + | + | + | + | + | + | - | - | - |
| KNS7 | - | - | - | - | - | - | - | KNS7 | - | - | - | - | - | - | - | - | - | - | - | - |
| TC16 | - | - | - | - | - | - | - | - | - | - | + | + | + | + | + | + | + | + | + | + |
| TC6/25 | - | - | - | - | - | - | - | - | - | - | + | + | + | + | + | + | + | + | + | + |
| KN4 | - | - | - | - | - | - | - | - | - | - | + | + | + | + | + | + | + | + | + | + |
| KN5 | - | - | - | - | - | - | - | - | - | - | + | + | + | + | + | + | + | + | + | + |

\+ = antibody reacts with trypanosomes.
\- = antibody does not react with trypanosomes.
IL/60/1 = K/82/IL/60/1
IL/97/2 = K/83/IL/97/2
MSUS = MSUS/LR/77/TSW103
(Proc) = procyclic forms propagated *in vitro*
(Epis) = epimastigote forms propagated *in vitro*
MOVS = MOVS/KE/81/WG84
MBOI = MBOI/NG/60/1-148

**Figure 30**

Reactivity of ascitic fluid containing the *T. congolense* species-specific MoAb (TC39) with *T. congolense* K/83/IL/97/2 (TCK), *T. brucei* IL2616 (TB) and *T. simiae* KETRI 2431 cultured procyclics. Trypanosomes were titrated tenfold from $1x10^5$ to 0 trypanosomes per dot (Tryps/dot). Note the decreasing sensitivity as the ascites was diluted further in order to achieve specificity.

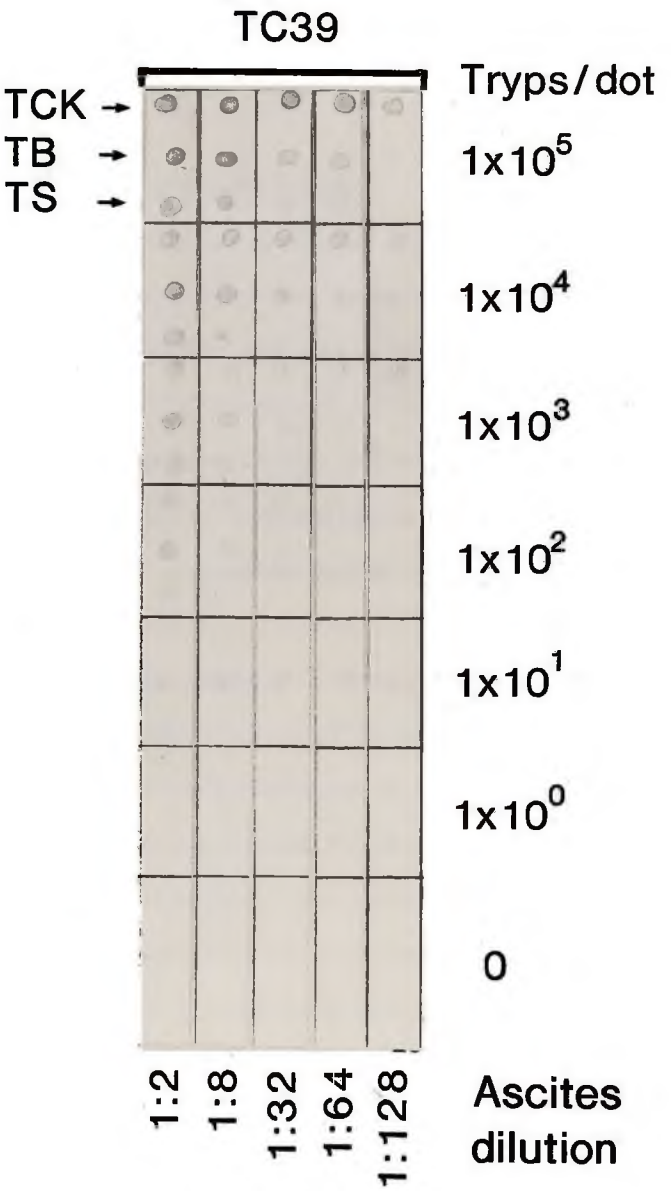



Figure 30

even though culture supernatants, or higher dilutions of the same MoAbs fractions, reacted specifically. Similarly, it was found that excess antigen ($\geq 1x10^8$ trypanosomes/dot/$\mu$l) induced non-specific reactions, although the observed reaction intensities were generally low.

All cross-reactions were, however, abolished following standardization of the assay.

### 5.4.6 Sensitivity of the dot-ELISA

Application of the dot-ELISA in identification of trypanosomes in mono-species cultures revealed that, some MoAb fractions of TR7, KT39a, TC40, TC6/25 and KN4 were capable of detecting a minimum of 10 trypanosomes per dot, but one of the *T. vivax* specific MoAbs (KD37) detected a minimum of 100 organism per dot. Table 27 summarizes the results of the sensitivity in terms of the minimum number of trypanosomes detectable in mono-species culture preparations by the dot-ELISA, using the panel of MoAbs prepared.

The minimum number of trypanosomes that could be detected in the dot-ELISA increased by a factor of 50, when the assay was applied for the specific identification of trypanosomes in artificially mixed preparations. Thus, a MoAb fraction that detected a minimum of 100 trypanosomes per dot in the mono-species preparations, now would detect a minimum of 100 x 50 (5,000) trypanosomes per dot in the mixed preparations. Table 28 summarizes the sensitivity in terms of the minimum number of trypanosomes that could be detected by the various MoAbs in the dot-ELISA when applied for the differentiation of artificially mixed trypanosomes. This drop in sensitivity could be due to competition between antigens of the mixed trypanosome species for the binding sites on the NC membrane.

Analysis of the data on sensitivity in terms of the minimum number of trypanosomes that could be detected in mono-species trypanosome cultures,

**Table 27: Sensitivity in terms of the minimum number of trypanosomes detected in mono-species preparations by the dot-ELISA**

| Parasite number detected | Specific monoclonal antibodies | | | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | *T. brucei* | | | | *T. vivax* | | | *T. congolense* | | | | *T. simiae* | | *Nannomonas* | | |
| | TR7 | KT39a | KT43/33 | KT43/27 | TV8 | KD32 | KD37 | C2 | TC6 | TC40 | TC39 | KNS7 | TC16 | TC6/25 | KN4 | KN5 |
| $10^5$ | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + |
| $10^4$ | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + |
| $10^3$ | + | + | + | - | + | + | - | + | + | + | + | - | + | + | + | + |
| $10^2$ | + | + | - | - | - | + | - | + | + | + | + | - | - | + | + | - |
| $10^1$ | + | + | - | - | - | - | - | - | - | + | - | - | - | + | + | - |
| $10^0$ | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - |
| 0 | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - |

+ = visible reactions observed with that number of trypanosomes in a dot.
- = visible reaction not observed with that number of trypanosomes in a dot.

**Table 28: Sensitivity in terms of the minimum number of trypanosomes detected in mixed trypanosome preparations by the dot-ELISA**

| Parasite number detected | Specific monoclonal antibodies | | | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | *T. brucei* | | | | *T. vivax* | | | *T. congolense* | | | | *T. simiae* | *Nannomonas* | | | |
| | TR7 | KT39a | KT43/33 | KT43/27 | TV8 | KD32 | KD37 | C2 | TC6 | TC40 | TC39 | KNS7 | TC16 | TC6/25 | KN4 | KN5 |
| $5x10^4$ | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + |
| $5x10^3$ | + | + | + | - | + | + | - | - | + | + | + | - | + | + | + | + |
| $5x10^2$ | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - |
| $5x10^1$ | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - |
| 5 | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - |
| 1 | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - |
| 0 | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - |

\+ = visible reactions observed with that number of trypanosomes in a dot.
\- = visible reaction not observed with that number of trypanosomes in a dot.

revealed that this sensitivity varied between different assays as well as between different fractions of the same MoAb. Thus, using the most active fractions, some of the MoAbs could detect as low as 10 trypanosomes per dot. However, the repeatability of the assay was better when higher trypanosome numbers were used. It was found that all the MoAbs could detect $5x10^3$ trypanosomes/spp./dot, and this was 100% reproducible, compared with 60% reproducibility obtained with 10 organisms/dot.

Based on these results, and the intensity of the positive colour reactions obtained, $1x10^4$ trypanosomes/dot was considered to be the most suitable parasite number for use in identifying trypanosome cultures with the dot-ELISA.

### 5.4.7 Stability of dotted antigens

NC membrane strips with trypanosomes deposited as dots were stored under desiccated conditions at 4°C, and at room temperature (17-26°C), and tested for antigenic reactivity at intervals of 30 days using the panel of MoAbs. No significant loss in assay reactivity was observed for periods of up to 60 days, regardless of whether the strips were blocked with 5% skimmed-milk diluted in TBS before storage or not. However, after 90 days of storage at room temperature, the *T. congolense* specific MoAbs showed reduced reactivity, with a loss in sensitivity from $1x10^2$ to $1x10^4$ trypanosomes/dot, irrespective of whether the strips were pre-blocked or not. Again, no change in reactivity was observed in strips stored at 4°C for up to 90 days.

### 5.4.8 Use of different immuno-affinity membranes

The effects of different brands of immuno-affinity membranes (NC membrane, Hybond-C, Hybond-N and Pall Immunodyne) on the dot-ELISA reactions, were also investigated. No differences in either specificity or sensitivity were observed with regard to the NC membrane, Hybond-C and

Hybond-N. The Pall Immunodyne membrane, however, gave less satisfactory results when compared with the other three membranes. There was loss of sensitivity of the order of x10 magnitude, from $1x10^3$ to $1x10^4$ trypanosomes/dot. This drop in sensitivity was attributed to a tendency for samples to diffuse into the Pall Immunodyne membrane, leading to lack of clearly defined positive staining reaction zones.

## 5.5 Discussion

In order to develop a MoAb-based assay for the differentiation of *in vitro* propagated insect stage trypanosome species, emphasis was placed on the utilization of an existing technique that offered the best opportunity to develop an assay which would be simple and easy to perform, in addition to being both sensitive and specific. It was anticipated that such an assay could easily be modified for use in the field in the diagnosis of trypanosome infections in infected tsetse flies (*Glossina spp.*).

Of the existing techniques namely, dot-ELISA, micro-plate ELISA and radio-immunoassay, the assay that could best be developed for purposes of this study, was the NC membrane-based dot-ELISA. The dot-ELISA is a highly versatile, solid-phase immunoassay useful for both antibody or antigen detection. The technique is rapid, easy to perform and interpret, reagent conservative, cost effective and field portable (Pappas, 1988a).

The dot-ELISA described in this Chapter was successfully applied to the identification of *in vitro* derived vector stages of *T. brucei, T. congolense, T. simiae* and *T. vivax*. The assay could correctly identify the trypanosome species involved in mono-species preparations as well as in artificial mixtures of trypanosome species. This assay was also able to identify the various stocks and clones of the different trypanosome species isolated from different geographical areas. This broad application of the dot-ELISA is a distinct advantage over the DNA hybridization technique which at present employs probes that are intra-species specific (Gardiner, 1989) and, therefore, are known to fail to identify new genotypes (McNamara *et al.*, 1991; Majiwa *et al.*, 1993).

Some of the MoAbs detected as low as 10 trypanosomes/dot with the dot-ELISA. This high sensitivity, in terms of the minimum number of trypanosomes detected, was not surprising, since it had been reported that NC

membrane avidly binds a wide variety of parasite antigen preparations (Pappas *et al.*, 1983; Pappas *et al.*, 1986; Zimmerman, Nelson and Clark, 1985; Boctor *et al.*, 1987; Whelen, Richardson and Wikel, 1986). Furthermore, unlike plate-ELISA in which adsorption of soluble antigens to well surface had been reported to vary significantly (Burt, Carter, and Kricka, 1979; Kricka, Carter, Burt, Kennedy, Holder, Holliday, Telford and Wisdom, 1980), with the dot-ELISA technique the entire antigen applied is immobilized on the NC membrane, which has a large surface area as a result of its porosity, and thus increases the sensitivity of the assay (McFarlane, Tolley, Major, McFarlane and Williams, 1983; Kumar, Band, Samantaray, Dang and Talwar, 1985). However, in this work, it was found that the smaller the number of trypanosomes dotted onto NC membrane (ie., at least 100 or less) the less reproducible was the assay results. This reduced reproducibility is believed to be due to small variations in the test conditions which may affect MoAb binding; some of these conditions were temperature, pH, concentrations of buffers and washing. This finding therefore suggested that the dot-ELISA would not be suitable, especially under field conditions if such low numbers of trypanosomes were to be detected. However, the finding that at least 1,000 or more trypanosomes/dot could produce 100% reproducible results, indicated that at higher antigen concentrations, this dot-ELISA method could be robust and suitable for use in the field.

Using the dot-ELISA method, Pappas and colleagues (1983) had shown that as little as $2.5 \times 10^4$ parasites/dot gave sensitive, specific and reproducible results. In this study, $1 \times 10^4$ trypanosomes/dot, applied in 1 $\mu$l volumes, was established to give satisfactory results, and this was therefore recommended for use in the dot-ELISA for subsequent studies. The application of antigen in small volumes was shown to give better results, since the intensity of the colour that developed was more dependent on the density, than on the amount of antigen in a dot (Towbin and Gordon, 1984). Volumes

as small as 0.1 μl/dot (Hawkes, Niday and Gordon, 1982) and 1 μl/dot (Pappas, Hajkowski and Hockmeyer, 1983) had been used previously.

In this dot-ELISA method, the use of whole ascites or immunoglobulin fractions was found unsuitable, as they cross-reacted extensively. Ascitic fluids contain antibodies secreted by hybridoma cells, as well as some of the animal's own immunoglobulins. Consequently, immunoglobulins, purified from this source are not monospecific (Boeye, 1986). Hence, depending on the type of work, the contaminating antibodies in ascites may be more or less a nuisance.

Pappas, Hajkowski and Hockmeyer (1984) attributed the decreased reactivity of antigen in dotted samples stored on NC membrane to oxidation of adsorbed antigen by nitro groups present in the NC membrane matrix. This phenomenon is likely to be, in part, the cause of the deterioration of the *T. congolense* specific antigen when dotted samples were stored at room temperature (17-26°C) for more than 60 days. Another likely cause of decreased reactivity was epitope sensitivity to temperature variations. It has been reported that other types of membrane, for example cellulose membrane (Londner, Rosen, Sintov and Spira, 1987) and opaque, white plastics (Lin and Halbert, 1986) could be used in dot-ELISA. In this study, it was found that some brands of membrane supports were more suitable than others in the dot-ELISA.

Positive results obtained with the dot-ELISA method were easily observed visually as brown dots on white NC filter paper, and the assay did not require a high level of technical expertise to perform or interpret. The potential of this procedure for diagnosis of trypanosome infections in the vector (*Glossina spp.*) of the African trypanosomes was noted, and this was indeed the subject of investigation in the studies described in the next chapter.

# CHAPTER 6

## DETECTION AND DIFFERENTIATION BETWEEN TRYPANOSOMES IN EXPERIMENTALLY-INFECTED TSETSE FLIES (*GLOSSINA SPP.*) USING DOT-ELISA

## 6.1 Summary

A modification of the NC membrane-based dot-ELISA developed in Chapter 5, was successfully used to detect and differentiate between *T. brucei*, *T. congolense* and *T. simiae* procyclics in the midguts of experimentally infected tsetse flies. The modification of the assay consisted of (a) the lysis of *T. congolense* or *T. simiae* in NC membrane applied sample dots using Triton X-114, and (b) a hydrogen peroxide destaining step, in which stains made on NC membrane strips by applied sample dots were removed. The afore-mentioned trypanosome species were specifically detected and differentiated without any cross-reactivity. In these assays, *T. brucei* and *T. congolense* parasites were detected directly using MoAbs specific to each of them, whereas *T. simiae* parasites were detected by exclusion using a *T. congolense* specific and *Nannomonas* subgenus-specific MoAb. The sensitivity of the assay was 90.5% in detecting *T. brucei* infections, 85.4% in detecting *T. congolense* infections and 94.4% in detecting *T. simiae* infections. The sample preparation from the gut of each tsetse fly could be replicated in 15 different dots, allowing some samples to be stored for testing at a later date. Sample dots stored at room temperature (19-26$^{o}$C) under desiccated conditions did not show any loss in activity in 90 days. However, after seven days of storage, a ring-pattern reaction appeared on most sample dots that were tested with the *T. brucei* specific MoAb, irrespective of whether *T. brucei* antigens were present or not. These ring reactions, however, did not interfere with the correct interpretation of the assay results.

Substitution of the PBS or PSG sample buffers used in the original dot-ELISA described in Chapter 5 for $Na_2EDTA$ buffer, led to the detection of *T. brucei* parasites in the salivary glands of infected tsetse flies using a *T. brucei* specific MoAb. This dot ELISA had a specificity greater than 99.9% and a sensitivity of 90%. Also, a dot-ELISA employing *T. vivax*

and *T. congolense* specific MoAbs and utilizing the biotin-streptavidin reaction amplification technique, was successfully used to detect *T. vivax* and *T. congolense* in the mouthparts of infected tsetse flies. The specificity of the assays were as good as for detecting *T. brucei* in infected tsetse salivary glands, but the sensitivity was lower, 43.8% in detecting *T. vivax* and 55.6% in the case of *T. congolense*.

The successful modification and application of the dot-ELISA in detecting and differentiating between trypanosome species in the midguts, salivary glands and mouthparts of experimentally infected tsetse flies, is the best indication that the assays developed may be capable of specific identification of trypanosome species in naturally infected *Glossina* species.

## 6.2 Introduction

Earlier studies of the life-cycle of trypanosomes in the vector established that each trypanosome subgenus characteristically develops in a particular organ of the tsetse fly (Lloyd and Johnson, 1924). As a result, the standard method for diagnosis of trypanosome infections in tsetse, has been by dissection and microscopy. By this method, infections in the gut and salivary glands have been deemed to be due to the *Trypanozoon* subgenus, whereas, infections in the midgut and proboscis have been assigned to the *Nannomonas* subgenus. Infections confined to the midgut are classified as immature *Nannomonas* or *Trypanozoon*, whilst those confined to the proboscis have been ascribed to the *Duttonella* subgenus. It is, however, not possible by these criteria to differentiate between the recognised species within the various subgenera, since all species within a subgenus have identical cycles of development in the vector (Hoare, 1972). The differential diagnosis is further complicated when mixed infections occur in the vector (Godfrey, 1966). Besides, *Trypanosoma grayi* and *T. suis* are known to reside in the midgut and salivary glands of infected tsetse, respectively (Hoare, 1972), and their presence, therefore, could lead to a misdiagnosis.

Currently, a recombinant DNA-based technique, first applied to trypanosome identification by Kukla *et al.* (1987), provides the best known alternative for the detection of, and differentiation between trypanosome species in the *Glossina* species. This technique has subsequently been used for the identification of *Nannomonas* species (Gibson *et al.*, 1988), differentiation between *T. congolense* and *T. simiae* (Majiwa and Webster, 1987; McNamara, Dukes, Snow and Gibson, 1989; Majiwa and Otieno, 1990; McNamara and Snow, 1991) and identification of *T. brucei* and *T. vivax* (Kukla *et al.*, 1987; Dicken and Gibson, 1989) in infected tsetse flies. However, this method also has some disadvantages. The most important disadvantage is that the existing

DNA probes employed in the technique are intra-species specific (Majiwa *et al.*, 1993). As a result, the probes have shown that there are five types of *T. congolense* (Kilifi type, West African riverine-forest type, Savannah type, Godfrey type and Tsavo type) recognised by five different probes. The most recent discovery, the Tsavo type, was established following failure of the four previously existing *T. congolense* probes to hybridize to a new *Nannomonas* isolate from Tsavo, Kenya. It is also quite likely that other *T. congolense* populations exist which would not react with any of the five probes developed, so that a negative result would not necessarily indicate that a given *Nannomonas* trypanosome population does not belong to the *T. congolense* species. Another, disadvantage is that the technique is not simple enough, and therefore cannot be performed in most laboratories. The development of a test which is simple, rapid, sensitive and specific and applicable under both laboratory and field conditions is, therefore, highly desirable.

In the studies described in the previous chapter, a nitrocellulose (NC) membrane based dot-ELISA that utilizes trypanosome species-specific MoAbs was developed for identification and differentiation between *in vitro* derived trypanosome species. In the study reported here, the dot-ELISA thus developed, was successfully modified and used to detect and differentiate between *T. brucei, T. congolense* and *T. simiae* procyclics in the midgut, *T. brucei* in the salivary glands, and *T. congolense* and *T. vivax* in the mouthparts of experimentally infected *Glossina* species.

## 6.3 Materials and methods

### 6.3.1 Experimental animals

#### 6.3.1.1 Goats

Adult male castrated goats (crossbreeds between East African Masai and Galla), aged 8-10 months and weighing between 20 and 25Kg, were used in this study. They were purchased from farms in the Kumanchu location, Laikipia district of Kenya, an area known to be free from tsetse flies and trypanosomiasis. The goats were quarantined in fly-proof housing for one month, after being dipped and treated with long-acting tetracycline, coccidiostats and anthelmintics on arrival. Prior to being used, they were confirmed to be uninfected with trypanosomes, using the thick, thin and wet blood films as well as by the darkground/phase contrast buffy coat technique described by Murray, Murray and McIntyre (1977).

#### 6.3.1.2 Pigs

Male and female, 6-month old, Large-white pigs were bred at the Veterinary Research Laboratory, Kabete, Kenya, an area known to be free from tsetse and trypanosomiasis. Those which were purchased for this study, came from herds kept in the area.

The animals were kept in fly-proof quarters and screened for trypanosomiasis, using the haematocrit centrifugation technique as well as the thick, thin and wet blood film microscopy method.

### 6.3.2 Tsetse flies

#### 6.3.2.1 Laboratory bred tsetse flies

The tsetse flies used came from the ILRAD laboratory-reared *Glossina morsitans centralis* which had previously been obtained from the East African Trypanosomiasis Research Organisation (EATRO), Tororo, Uganda in

1979 (Moloo, Kutuza, Bakakimpa, Kamunya, Desai and Pereira, 1985). This colony was first initiated in 1969 at EATRO, with adults which had emerged from pupae collected in the field at Singida, mainland Tanzania (Moloo and Kutuza, 1969). The *Glossina pallidipes* flies used had originated from Nguruman and Shimba Hills in Kenya, and were also part of the ILRAD tsetse colony collection.

### 6.3.3 Infection of goats, pigs and tsetse flies

#### 6.3.3.1 Infection of goats

Seven goats were each infected with one of the following 7 trypanosome stocks or clones: *T. brucei* stock IL375; *T. vivax* stocks IL3096 and IL2337; *T. congolense* stock IL3779 and clones, IL1180, IL3274, and IL13-E3. Each goat was infected by the intramuscular route with about $1x10^7$ trypanosomes, diluted in 3ml of phosphate-buffered saline-glucose (PSG), pH 8.0 (Lanham and Godfrey, 1970). To monitor parasitaemia, each goat was bled daily from a marginal ear vein, and the blood examined for the presence of trypanosomes, using the wet blood film phase contrast microscopy at x400 magnification or the microhaematocrit/dark ground technique (Murray *et al.*, 1977).

#### 6.3.3.2 Infection of pigs

Three pigs were each infected subcutaneously in the neck region with either *T. simiae* stock CP11(IL3879) or IL3815. To monitor parasitaemia, peripheral blood was drawn daily from either an ear or tail vein and examined for trypanosomes as described for goats above.

**6.3.3.3** Infection, maintenance and identification of infected tsetse flies

*T. brucei, T. vivax* and *T. congolense* parasites were transmitted to *G. m. centralis* flies from infected goats and *T. simiae* to *G. pallidipes* from infected pigs, by fly feeding.

Five days after infections had become patent in goats, and 1 day afterwards in pigs, teneral tsetse were allowed to feed on the shaven flanks of an infected goat for a period of 30 days in the case of *T. brucei* and 25 days for *T. vivax* and *T. congolense*. Thereafter, the flies were starved for two days and those with mature infections identified by the warm-slide probe method of Burtt (1946). With regard to *T. simiae*, teneral tsetse were fed once only on infected pigs, after which the flies were maintained by feeding on rabbits for 25 days and starved for 2 days prior to probing. All tsetse flies confirmed to be infected by the extrusion of metacyclics were maintained by feeding on rabbits.

**6.3.4 Preparation of samples for dot-ELISA**

**6.3.4.1** Dissection of tsetse and extraction of midgut tissue

Tsetse flies were killed by crushing the thorax with gentle pressure exerted with a finger or by anaesthesia using chloroform, and the wings and legs pulled off. Several ways of dissection and preparation of the midgut samples were examined:

**6.3.4.1.1** The fly was placed on a microscope slide under a dissecting microscope (Wild M5A binocular; Wild, Heerbrugg, Switzerland) at x120 magnification and about 30$\mu$l of PBS (pH 7.4) or PSG (pH 8.0) added. The abdomen of the fly was then torn open at the ventral surface, using a pair of forceps and a dissecting pin. The gut was pulled into the buffer on the slide and the midgut cut out and covered with a coverslip and examined for trypanosomes using a compound microscope (Leitz larlux; Leitz Wetzlar, Germany) at

x320 magnification. The examined tissues were then transferred with forceps into an Eppendorf tube containing 50$\mu$l of the appropriate buffer.

**6.3.4.1.2** Alternatively, the tsetse gut was dissected out onto a slide as above, and cut below the proventriculus and above the rectum. The whole gut, including the foregut, midgut and hindgut, was examined microscopically for the presence of trypanosomes and then transferred into 50$\mu$l of either PBS or PSG.

**6.3.4.1.3** In the last method tried, the distal quarter of the abdomen of tsetse was excised with a pair of dissecting scissors and discarded (Kukla *et al.*, 1987). The whole abdominal contents were then squeezed out by applying firm pressure in a rolling motion, from the anterior portion toward the posterior end of the abdomen. The protruding gut was then torn apart with forceps and transferred into 50$\mu$l of buffer.

**6.3.4.2** Preparation and application of tsetse midgut suspensions onto NC membrane

Tsetse gut tissue dissected out as indicated variously above, and suspended in either PBS or PSG, were treated again in three different ways before applying sample dots onto NC membrane strips for testing: (1) Suspended tsetse gut samples were agitated to release trypanosomes into the buffer, by tapping the base of the tube gently with a finger. The samples were then allowed to stand for up to 1 hr, and agitated once more before pipetting out 3$\mu$l samples onto NC membrane strips in dots. (2) The gut suspensions were mixed gently by pipetting the fluid up and down a few times, using a 50$\mu$l micropipette, and 3$\mu$l samples dotted soon after. (3) Trypanosomes in suspended gut tissues were released by physical

maceration, using the tip of a 50μl micropipette, together with vigorous pipetting up and down about 10 times, before 3μl sample volumes were applied in dots onto NC membrane strips for testing. As controls, $1x10^5$ *T. brucei*, *T. congolense* and *T. simiae* procyclics, and epimastigotes of *T. vivax* obtained from *in vitro* cultures were separately applied in dots onto each NC membrane strip.

**6.3.4.3** Preparation of touch blots

Tsetse abdomen was slit open as above (section 6.3.4.1.3). The abdomen was squeezed several times and the protruding gut touch-blotted by pressing gently onto NC membrane. Alternatively, the protruding gut was cut with a scalpel blade before touch-blotting.

**6.3.4.4** Preparation of dot-blots using lysed trypanosomes

Attempts were made to lyse gut forms of *T. congolense* and *T. simiae* so as to expose internal trypanosome species-specific antigens for uptimum reactivity in the dot-ELISA.

**6.3.4.4.1** Lysis of *T. congolense* or *T. simiae* in infected tsetse gut samples in suspension

Several detergents, namely, Nonidet P-40 (NP-40), Saponin, Sodium dodecyl sulphate (SDS), all from (Sigma, England); and Triton X-114 (Fluka Chemie AG, Switzerland), were tested in experiments intended to lyse *T. congolense* or *T. simiae* procyclic midgut forms in infected tsetse gut samples. Each of the detergents was used to prepare a "lysis-buffer" which consisted of 0.05, 0.1, 0.2, 0.3, 0.4 or 0.5% detergent, and 10μg/ml Leupeptin and E-64 in either PBS or PSG. Fifty microlitre aliquots of each concentration of lysis-buffer were pipetted into Eppendorf tubes, and infected tsetse midguts dissected out and suspended in them. Trypanosomes were

released into the sample lysis buffers by gently pipetting the fluid up and down a few times, using a 50$\mu$l pipette. The samples were allowed to stand for 30 min, and mixed once again before 3$\mu$l volumes were pipetted out and applied onto NC membrane strips in dots.

**6.3.4.4.2** Lysis of *T. congolense* or *T. simiae* gut forms in sample dotted NC membrane strips

NC membrane strips were "dotted" with experimentally-infected *T. congolense* or *T. simiae* tsetse gut samples, as previously described. The sample dotted strips were then incubated in different concentrations of detergent solutions for varying time periods (either 30 min, 1hr or 2hr, at room temperature of 19-25°C) without shaking. The detergents used were either: NP-40, Saponin, SDS or Triton X-114. Each was tested at six different concentrations (0.05, 0.1, 0.2, 0.3, 0.4, or 0.5%), diluted in blocking solution which consisted of 5% (w/v) skimmed milk in Tris-buffered saline (TBS)(50mM Tris and 150mM NaCl, pH 8.0).

**6.3.4.5** Preparation of tsetse salivary glands for dot-ELISA

Tsetse salivary glands were dissected out in one of two ways:

**6.3.4.5.1** Each fly was placed on a microscope slide, ventral surface up, and 30$\mu$l of PSG added onto the head region. The thorax was then pinned down with a dissecting needle close to the base of one wing, and the head grasped with fine forceps and pulled gently, but steadily, away from the thorax in a straight line, under a dissection microscope at x120 magnification. The glands were pulled out whilst immersed in the buffer until they were out of the thorax. When a gland broke, it was seized with forceps and pulled out.

**6.3.4.5.2** A fly was placed on a slide as above, and 30$\mu$l of buffer added to the abdominal area. The abdomen was then torn open at the anterior

end close to the thorax and the elastic tissue pulled backwards towards the posterior end. The exposed gut was pulled back and a drop of PSG added into the cavity. The tissues at the anterior most part of the exposed abdominal cavity were grasped with a pair of fine forceps and pulled into the buffer on the slide. The two salivary glands were easily located by this method.

The isolated salivary glands were transferred, using forceps, into 10$\mu$l of 5mM $Na_2EDTA$ buffer. The suspended glands were pipetted up and down several times, using a 10$\mu$l micro-pipette, before 3$\mu$l volumes were pipetted onto NC membrane strips in dots.

**6.3.4.6** Preparation of tsetse proboscides for dot-ELISA

Several ways of dissection and preparation of tsetse mouthparts were explored:

**6.3.4.6.1** The proboscis of a tsetse fly was cut a third of the way down from the thecal bulb, with a pair of scissors. The thorax was then squeezed gently to expel a drop of fluid through the cut proboscis and gently touch-blotted onto a demarcated spot on NC membrane.

**6.3.4.6.2** The head of a tsetse fly was placed on a microscope slide, ventral surface up. The posterior base of the thecal bulb, close to the head, was pressed gently but firmly with a dissecting needle placed almost parallel to the slide surface, and pulled away from the head. The labrum and hypopharynx were then examined under a light microscope (Leitz labrlux; Leitz Wetzlar, Germany) at x320 magnification, for the presence of trypanosomes. The separated proboscis was then transferred into about 10$\mu$l of distilled water, under a dissection microscope at x180 magnification, and the labrum, labium and hypopharynx separated, using two dissecting

needles, before they were transferred into about 10$\mu$l of distilled water in an Eppendorf tube. The sample was left to stand for about 1hr before pipetting up and down about ten times using a 5$\mu$l micropipette, and five microlitres transferred onto a spot on NC membrane. The sample was air dried at room temperature and the remaining 5$\mu$l pipetted onto the same spot.

**6.3.4.6.3** Tsetse proboscis was separated from the head and dissected as described above, in about 7$\mu$l of distilled water, PBS or PSG or 5mM $Na_2EDTA$ buffer, in the wells of a teflon coated multitest immunofluorescence slide. The dissected labrum and hypopharynx were then cut into pieces in the wells with a small knife, and the suspension transferred from the well directly onto NC membrane in a dot.

**6.3.4.6.4** The sample preparation, as previously described (section 6.3.4.6.3) was repeated using silicon coated multitest slides.

**6.3.4.6.5** The proboscis was separated from the head of tsetse and cut at the base of the thecal bulb. The mouth parts were then transferred into an Eppendorf tube containing about 7$\mu$l of distilled water, PBS, PSG, or 5mM $Na_2EDTA$ buffer. The sample was left to stand for at least 1 hr, after which the proboscis was broken up into small pieces with the tip of a micropipette. The sample was pipetted up and down about 5 times and the whole volume transferred onto a NC membrane in a dot.

### 6.3.5 Estimation of trypanosome numbers in midgut suspensions

Tsetse midgut suspensions were prepared and mixed as described earlier, and large particulate tissue matter removed and discarded. A small volume was then pipetted out of each sample preparation and dropped onto a microscope slide, and covered with coverslip.

The number of trypanosomes per millilitre in each sample was then estimated using the "rapid matching method" of Herbert and Lumsden (1976).

### 6.3.6 Monoclonal antibodies

The panel of trypanosome species, and subgenus-specific MoAbs used in this study were produced and selected as described in Chapter 4.

### 6.3.7 Dot-ELISA procedure

#### 6.3.7.1 Detection of *T. brucei* in midguts of infected tsetse flies by a modified dot-ELISA I

All incubations and washings in this assay were performed at room temperature on a gentle rocker. Each tsetse sample was applied in dots onto several strips so that every sample could be assayed for reactivity with various trypanosome species-specific MoAbs. The strips were first destained by incubating for 1 hr in a "destaining solution" containing 5%(v/v) $H_2O_2$ in blocking solution. They were then washed three times, 10 min each, with TBS pH 8.0, and then incubated for 3 hr with specific MoAbs, diluted in a blocking solution. This was followed by two washes (10 min/wash) with the same buffer. The strips were then incubated for 1 hr with goat anti-mouse immunoglobulins labelled with horseradish peroxidase (HRPO) (Sigma, USA) and diluted in a blocking solution. They were washed two times, after which they were immersed for three minutes in a substrate solution containing 0.15%(v/v) hydrogen peroxide ($H_2O_2$) and 0.05%(w/v) 3,3'-diaminobenzidine (DAB) in phosphate-$Na_2$EDTA buffer (10mM $NaH_2PO_4$, 10mM $Na_2HPO_4$ and 10mM $Na_2$EDTA). The strips were then rinsed two times in deionised water and the substrate reaction stopped as described earlier (Chapter 4, section 4.3.8.1). The results were read visually. Positive reactions appeared as brown dots, whereas negative results remained colourless. A control

section on each strip was applied with $1x10^5$ trypanosomes/dot of *T. brucei*, *T. congolense* and *T. simiae*. These were used to assess the performance of the specific MoAbs. The conjugate control strips dotted with all the test samples were assayed omitting the step in which specific MoAb was added. Any reactions on these trips therefore gave a measure of non-specific background reactivity.

**6.3.7.2** Detection of *T. congolense* and *T. simiae* in the midguts of infected tsetse flies by a modified dot-ELISA II

NC membrane strips applied with sample dots were first incubated for 1 hr with 0.1%(v/v) Triton X-114 dissolved in blocking solution, and washed three times (10 min/wash) with Tris-buffered saline pH 8.0. The Triton X-114 was used to lyse whole trypanosomes in the dotted samples so as to expose internal trypanosome antigens. The rest of the procedure was the same as described previously for the detection of *T. brucei* in infected tsetse midgut.

**6.3.7.3** Detection of *T. brucei* in the salivary glands of infected tsetse flies by dot-ELISA

Salivary gland samples were prepared as described earlier and dotted onto NC membrane strips in triplicates. All the steps in this assay were performed at room temperature. Also, all incubations and washings were done on a gentle rocker. The strips were first blocked by incubation in blocking solution for 1 hr. The blocking solution was discarded and the strips further incubated for 3 hr with trypanosome species-specific MoAbs diluted in blocking solution. They were then washed three times (10 min/wash) with TBS and incubated for 1 hr with HRPO-conjugated goat anti-mouse immunoglobulins diluted in blocking solution. They were washed as above

and then incubated with substrate solution for 3 min. The substrate reaction was stopped and the results read as before.

**6.3.7.4** Detection of *T. vivax* and *T. congolense* in the mouthparts of infected tsetse flies by dot-ELISA

Each tsetse proboscide sample was placed onto a NC membrane strip in a single dot. The sample dotted strips were assayed either following the procedure described earlier for the detection of *T. brucei* in the salivary glands or following the modified procedure briefly described here under: Briefly, after washing off excess monoclonal antibody, the strips were incubated for 1 hr with biotinylated sheep anti-mouse immunoglobulins, diluted in Tris-buffered saline pH 8.0 and containing 1%(w/v) bovine serum albumin. The strips were washed three times as usual to remove excess unbound biotinylated antibody and then incubated for 1 hr with streptavidin-conjugated horseradish peroxidase (diluted in blocking solution). This was followed by washing as described above, before incubating with the substrate. As usual, a control section was incorporated into the system, consisting of four dots containing, respectively, $1x10^5$ trypanosomes/dot of *T. vivax, T. congolense, T. brucei* and *T. simiae*. (There were no controls with the conjugate since tsetse proboscide samples were not replicated).

## 6.4 Results

### 6.4.1 Detection of trypanosomes in the midgut of experimentally infected tsetse flies, using the standardized dot-ELISA developed for differentiating between *in vitro* derived insect stage trypanosomes

Gut samples prepared from laboratory-reared tsetse flies and applied in dots onto white NC membrane, were found to stain the membrane with varying coloration and intensity. The stains were predominantly either, reddish, reddish-brown, brown or blackish-brown to black, and occasionally greenish to almost colourless, depending on the stage of digestion in the fly. The strip labelled "P" in Figure 31 shows the stains made on NC membrane by "dotted" tsetse gut samples from five *T. congolense* infected flies, five uninfected flies, and *in vitro* derived procyclic *T. congolense*, *T. brucei* and *T. simiae*. Two important observations were evident. Firstly, midgut samples from both infected and uninfected tsetse flies stained the membrane. Secondly, sample dots consisting of *in vitro* derived trypanosomes did not stain the membrane.

Figure 31 shows an example of the results of direct application of the dot-ELISA developed and standardized in Chapter 5 when applied for the detection of *T. congolense* in the midgut of experimentally infected *G. m. centralis*. The *Nannomonas* subgenus-specific MoAb (TC6/25) and the *T. congolense* specific MoAb (TC6) reacted with samples from the *T. congolense* infected flies as expected (Figure 31). However, these MoAbs also reacted with samples from all the uninfected flies. The reactions with the control trypanosome dots on the strips incubated with these two MoAbs, showed cross-reactivity with other trypanosome species to which they were known not to react (Chapter 5). Furthermore, both the *T. brucei* (KT39a) and *T. vivax* (KD32) specific MoAbs, reacted with samples from all the *T. congolense*

infected as well as all the uninfected flies. These false reactions were also seen on samples that were tested as conjugate controls (without incubation with specific MoAbs), suggesting that the reactions were not the result of non specific binding of the MoAbs. Comparison of the staining on the preserved NC membrane strip **P** with those on the tested strips, revealed that the staining intensity of the sample from uninfected fly UF5 was amplified by the dot-ELISA (Figure 31).

### 6.4.1.1 Removal of high background activity from the dot-ELISA

The high background encountered was assumed to be due to two causes: (1) persistent staining of the NC membrane due to the physical coloration of the test samples, and (2) non-specific reactivity due to interference by haem. To remove the background, investigations were conducted into several alternatives. These were: (1) testing only flies with very low amount of undigested blood meal (2) changing the handling of samples prior to dotting, such as using the touch blot technique (3) changing the enzyme substrate chromogen system so that positive reactions are distinguishable from the background (4) reducing the amount of biological debris in the tsetse gut samples, such as by partial isolation of trypanosomes (5) removing the background without destroying the diagnostic antigens such as by a destaining process, and (6) developing an alternative assay in which background originating from tsetse gut samples present no problems. The findings on each of these investigations are presented serially here under:

#### 6.4.1.1.1 Testing flies with very low amounts of undigested blood meal

The suitability of the option to test only flies with minimal amounts of residual blood meal clearly depended on the proportion of flies that could be tested using that selection criterion. To determine this proportion in

**Figure 31**

Dot-ELISA of midgut samples from five *T. congolense* (IL1180) infected tsetse flies (IF1 to IF5) and five uninfected control flies (UF1 to UF5). The sample from each fly was dotted across in a row, one replicate dot on each strip. A control sector at the lower section of each strip was applied with $1x10^5$ trypanosomes per dot of *in vitro* propagated *T. congolense* K/83/IL/97/2 (TCK), *T. brucei* IL2616 (TB) and *T. simiae* KETRI 2431 (TS). The antigen "dotted" strips were tested using *Nannomonas* subgenus-specific MoAb (TC6/25), a *T. brucei* species-specific MoAb (KT39a), a *T. congolense* specific MoAb (TC6) and a *T. vivax* specific MoAb (KD32). Strip 'C' was a conjugate control, and strip 'P' was preserved without processing through the dot-ELISA. Note the sample stains shown on strip 'P'.

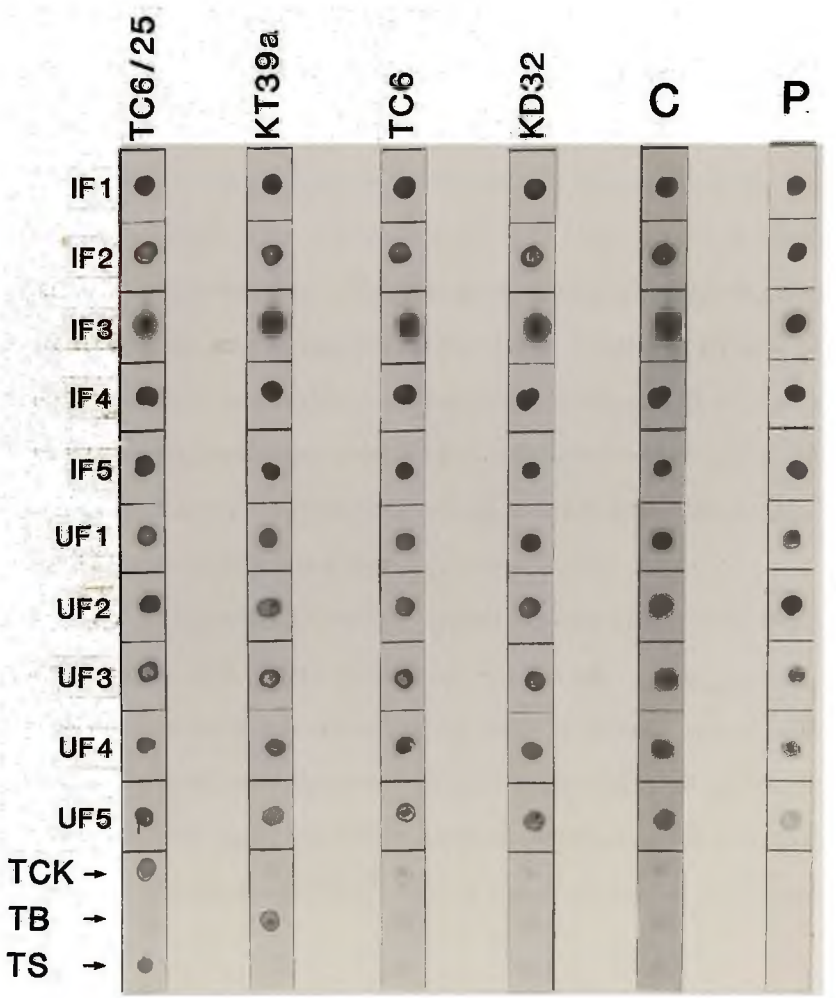



Figure 31

the case of laboratory reared tsetse flies, an experiment was first conducted using newly emerged and 8-week old *G. m. centralis*.

Figure 32a shows the staining pattern obtained from midgut samples prepared from newly emerged male and female *G. m. centralis* from 1 hr after feeding (day 0) through day 1, 2, 3 to 4 after feeding. Non-specific staining was observed with samples obtained up to 4 days post-feeding. Samples prepared and dotted on day 0, 3 and 4 after feeding presented less staining problems, compared with the samples prepared and dotted on days 1 and 2 after feeding. Analysis of the results obtained from the experiments conducted showed that $<10\%$ of male, and $<5\%$ of female flies could be tested 1 hr after feeding. The flies that were most suitable for testing were those that did not fully engorge. None of the flies from either sex could be tested on day 1 and 2 after feeding, though the female flies presented more intense staining problems. By day 3 and 4 after feeding, about 10% of the flies from either sex could be tested, and there appeared to be no differences in the intensity of staining with reference to sex.

The pattern of staining from dotted gut samples of the eight week old *G. m. centralis* flies is shown in Figure 32b. It was evident that gut samples from male flies stained NC membrane less intensely when compared with samples from female flies. Whilst about 10% of male flies could be tested 1 hr after feeding and 35% could be tested on day 3 or 4 after feeding, less than 2% of female flies could be tested on day 3 and 4 post-feeding combined.

**6.4.1.1.2** Use of different sample application techniques

Efforts were made to reduce non-specific staining by reducing the amount of undigested material that was applied onto NC membrane for testing. Investigations were conducted into the suitability of use of the tsetse abdominal touch blot technique previously described by Kukla *et al.* (1987).

**Figure 32a, b**

The staining pattern obtained on NC membrane following application of midgut samples prepared from male (♂) and female (♀) G. morsitans centralis from 1 hr after feeding (day 0) through days 1 to 4 after feeding. (a) staining pattern from young flies that have fed only once; (b) staining pattern from 8-week old adult flies. Midgut samples were prepared from four randomly selected flies from each of the two sex groups on days 0, 1, 2, 3 and 4 after feeding, and applied in dots onto NC membrane. The strips were tested in the dot-ELISA without incubating with specific MoAbs, but with conjugate and substrate, so as to determine non-specific background.

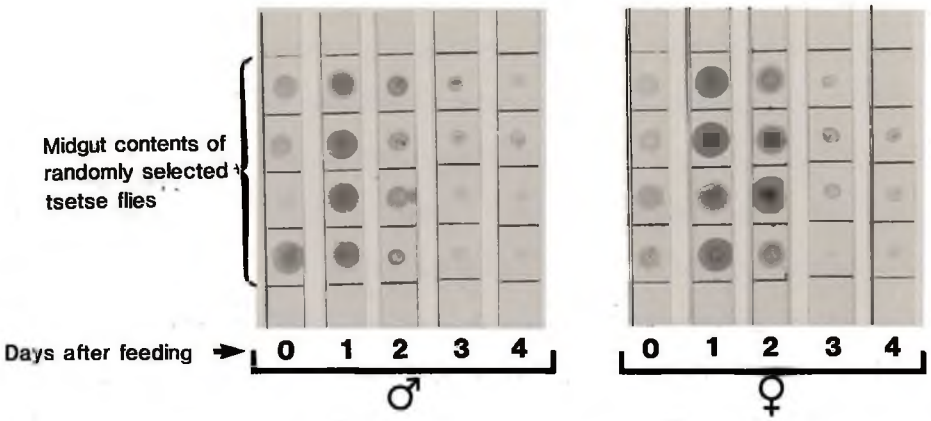



Figure 32a

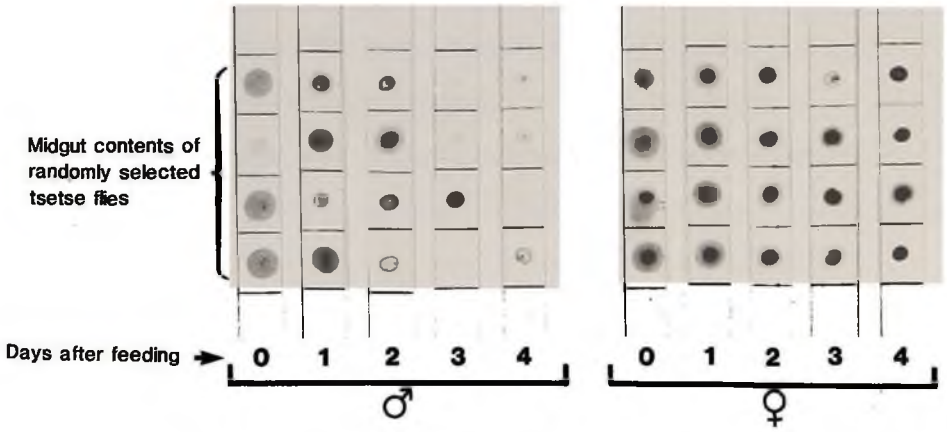



Figure 32b

The results showed that touch blotted samples left very little or no stains on NC membrane. However, the sensitivity of the dot-ELISA was so much lowered that less than 5% of *T. brucei* midgut infections could be detected.

**6.4.1.1.3** Use of different enzymes and chromogenic substrates

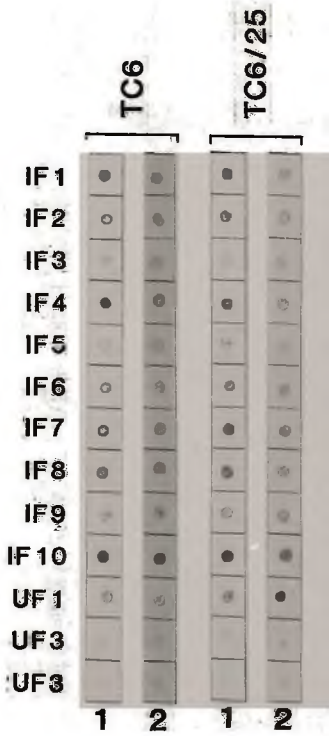
It was thought likely that the false reactions could be the result of oxidation of the chromogenic substrate [3,3'-diaminobenzidine (DAB)] by $H_2O_2$ through catalysis by haem which acted as a peroxidase (Saunders, Holmes-Siedle and Staak, 1964). To abolish those non-specific reactions, the HRPO enzyme used in the dot-ELISA was replaced with glucose oxidase which generates $H_2O_2$ by its action on glucose (Decker, 1977), thereby permitting the omission of $H_2O_2$ in the substrate solution. The presence of haem without glucose oxidase, therefore, would result in no false reactions since $H_2O_2$ would be absent. Figure 33 summarizes the results of a comparison of HRPO-conjugate and glucose oxidase-conjugated antibodies in the detection of *T. congolense* (IL1180) in the midguts of experimentally infected *Glossina*. The results showed that the glucose oxidase-conjugate was not able to eliminate the background. It was also found that the glucose oxidase-conjugate gave a higher assay background compared to HRPO. The chromogenic substrate 4-Chloro-1-Naphthol (4C1N) which normally gives a blue reaction, was compared with the brown reaction given by DAB so as to determine whether it was possible to distinguish positive reactions from the background by colour. The results obtained showed that neither the blue reaction of 4C1N nor the brown reaction of DAB could allow clear distinction of positive reactions from the background in the dot-ELISA.

**6.4.1.1.4** Removal of background activity by destaining

A destaining step involving the use of hydrogen peroxide ($H_2O_2$) was introduced into the dot-ELISA and investigated to establish

**Figure 33**

Comparison of HRPO-conjugated and glucose oxidase-conjugated antibodies in the detection of *T. congolense* (IL1180) in the midguts of experimentally-infected *G. morsitans centralis* using dot-ELISA. Each strip was "dotted" with samples from ten infected flies (IF1 to IF10) and three uninfected control flies (UF1 to UF3). The strips marked '1' were tested using HRPO-conjugate, and those marked '2' with glucose oxidase-conjugate. The MoAbs used were: TC6, *T. congolense* specific; TC6/25, *Nannomonas* subgenus-specific; TR7 and KT39a, *T. brucei* species-specific; and KD32, *T. vivax* specific. Strip 'P' was preserved without processing through the dot-ELISA.



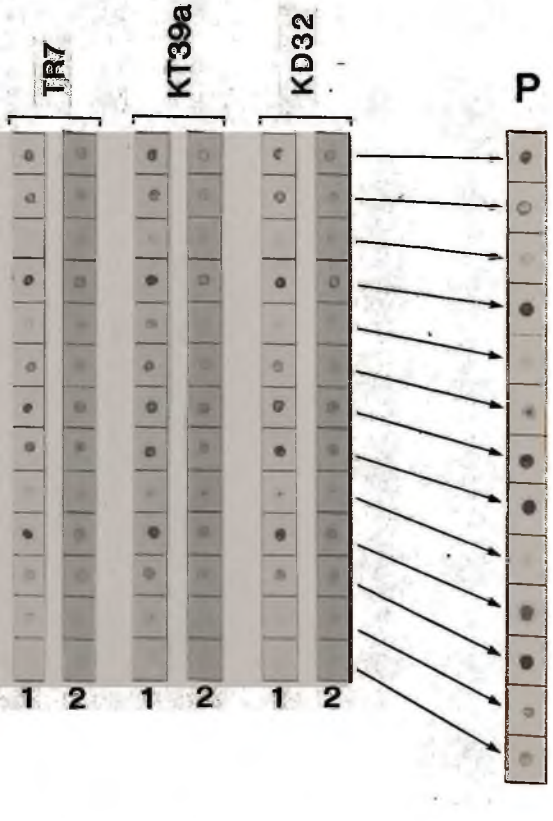



Figure 33

whether it could remove the non-specific stains. Figure 34 shows the results of an experiment in which the stains made by gut samples from *T. brucei* infected flies as well as uninfected flies were removed by the destaining method. Strip **P** which was not subjected to any testing, shows the stains made by the original dotted samples. Strip **C** was run through the already established dot-ELISA as conjugate control (without incubation with specific MoAbs). This strip showed the non-specific reactions and stains described earlier. The third strip $\mathbf{C^m}$ was run through a modified dot-ELISA that included an $H_2O_2$ destaining step, also as a conjugate control. The removal of all the non-specific stains in the sample dots on strip $\mathbf{C^m}$ are clearly shown. It is important to mention, however, that the $H_2O_2$ treatment at this dilution appeared to be harsh, considering the manner in which the sample dots foamed, and pieces of debris broke loose and floated. Two important questions, therefore, arose from this experiment. These were: (1) the effect of $H_2O_2$ on the diagnostic antigens detected by the MoAbs, and (2) the stability of those antigens on the NC membrane during treatment with $H_2O_2$. These questions were subsequently addressed in the following experiments.

**6.4.1.2** The effect of hydrogen peroxide on the trypanosome species-specific antigens detected by the MoAbs

Figure 35 shows the effect of a 1 hr incubation of varying concentrations of $H_2O_2$ on the *T. brucei* specific antigenic epitope detected by KT43/27. The reactions on the control strip (incubated with 0% $H_2O_2$) and those on strips incubated with 0.5-30% $H_2O_2$ showed that both the specificity and sensitivity of the MoAb were unaffected by this treatment. In a similar experiment illustrated in Figure 36, the *Nannomonas* subgenus-specific MoAb KN4 was shown to react specifically with $H_2O_2$ treated *T. congolense* and *T. simiae* parasites without any noticeable changes in sensitivity.

**Figure 34**

Removal of non-specific stains from NC membrane strips "dotted" with *G. morsitans centralis* midgut samples from five *T. brucei* infected flies (IF1 to IF5) and five uninfected control flies (UF1 to UF5), using $H_2O_2$ in the dot-ELISA. The sample from each fly was dotted across in a row, one replicate dot on each strip. 'P' was a preserved strip that was not subjected to any testing. It shows the original stains made on NC membrane by the applied gut samples. 'C' was a strip processed through the dot-ELISA as conjugate control, without incubation with specific MoAb, and strip '$C^m$' was processed through a modified dot-ELISA that included an $H_2O_2$ destaining step.

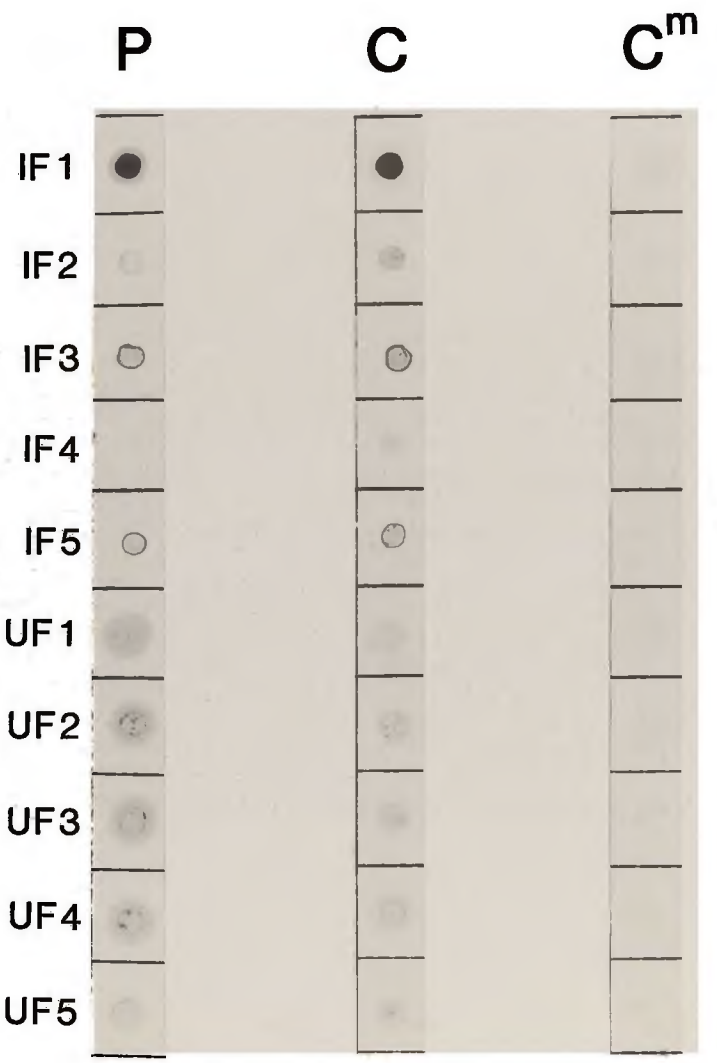



Figure 34

**Figure 35**

The effect of $H_2O_2$ on the *T. brucei* species-specific antigenic epitope detected by KT43/27. *In vitro* derived *T. congolense* K/83/IL/97/2 (TCK), *T. brucei* IL2616 (TB) and *T. simiae* KETRI 2431 (TS) were applied onto NC membrane strips starting from $1x10^5$ trypanosomes per dot to 1 trypanosome per dot for each species.

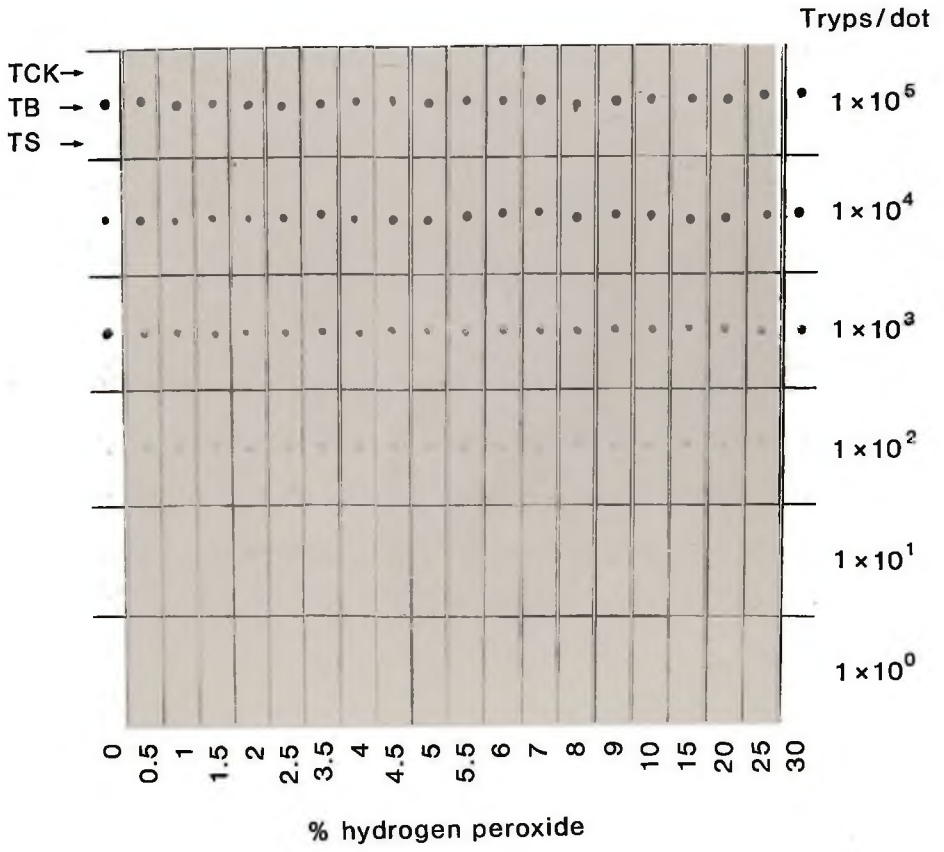



**Figure 35**

**Figure 36**

The effect of $H_2O_2$ on the *Nannomonas* subgenus-specific antigenic epitope detected by KN4. *In vitro* derived *T. congolense* K/83/IL/97/2 (TCK), *T. brucei* IL2616 (TB) and *T. simiae* KETRI 2431 (TS) were applied onto NC membrane strips starting from $1x10^5$ trypanosomes per dot to 1 trypanosome per dot for each species.

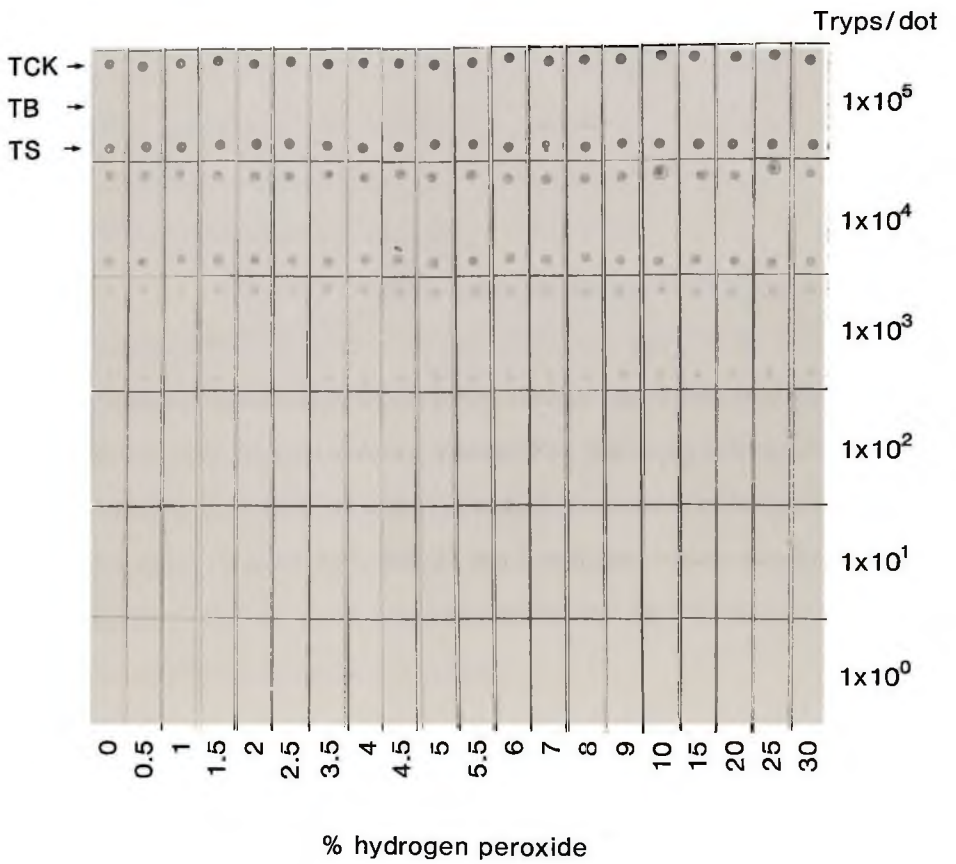



**Figure 36**

Further, experiments were conducted to determine the effect of varying concentrations of $H_2O_2$ over longer periods of incubation (up to 12hr) on the non-specific staining of NC membrane by tsetse gut samples, as well as the specificity and sensitivity of the dot-ELISA, when used in detecting *in vitro* derived trypanosomes. The results are summarized in Figure 37. The non-specific stains were substantially removed by as low as 1% $H_2O_2$. Between 3% and 7% $H_2O_2$ concentration, the background remained unchanged. Further reduction of the background occurred between 7% and 12% $H_2O_2$ concentration. The sensitivity of the assay remained unaffected with up to 12% $H_2O_2$, after which it dropped.

From these results, it was concluded that at high concentrations and long periods of incubation, $H_2O_2$ has an effect on the integrity of the trypanosome species-specific antigens. It was also found that when used for destaining NC membrane bound sample dots, 5% $H_2O_2$ incubated for 1 to 2hr reduced the background substantially to levels where it no longer interfered in the interpretation of the results of the dot-ELISA. Subsequently, destaining with 5% $H_2O_2$ was incorporated into the dot-ELISA. This will henceforth be referred to as the "modified dot-ELISA".

### 6.4.1.3 The use of other oxidizing agents in destaining antigen dotted strips

Following the discovery that $H_2O_2$ could be used in destaining NC membrane strips dotted with tsetse gut samples, investigations were conducted into the probable use of some other oxidizing agents. The chemicals used in these experiments were: potassium dichromate and potassium permanganate (Sigma, England), and calcium hypochlorite (bleaching powder)(Laboratory Chemicals, Kobian, Kenya). None of these chemicals was able to destain the pigmented sample dots on the strips.

**Figure 37**

The effect of varying concentrations of hydrogen peroxide incubated for 12 hr, on the non-specific staining of NC membrane by tsetse gut samples, and on the sensitivity in terms of the minimum number of culture derived trypanosomes detectable with the modified dot-ELISA. (H) represents the highest background or highest sensitivity. (L) indicates no background staining or no reactivity. (C) represents a critical point above which sensitivity of the assay is reasonably high and below which the background is acceptably low. The arrow indicate the $H_2O_2$ concentration 13% at which a drop in the sensitivity of the assay was first recorded.

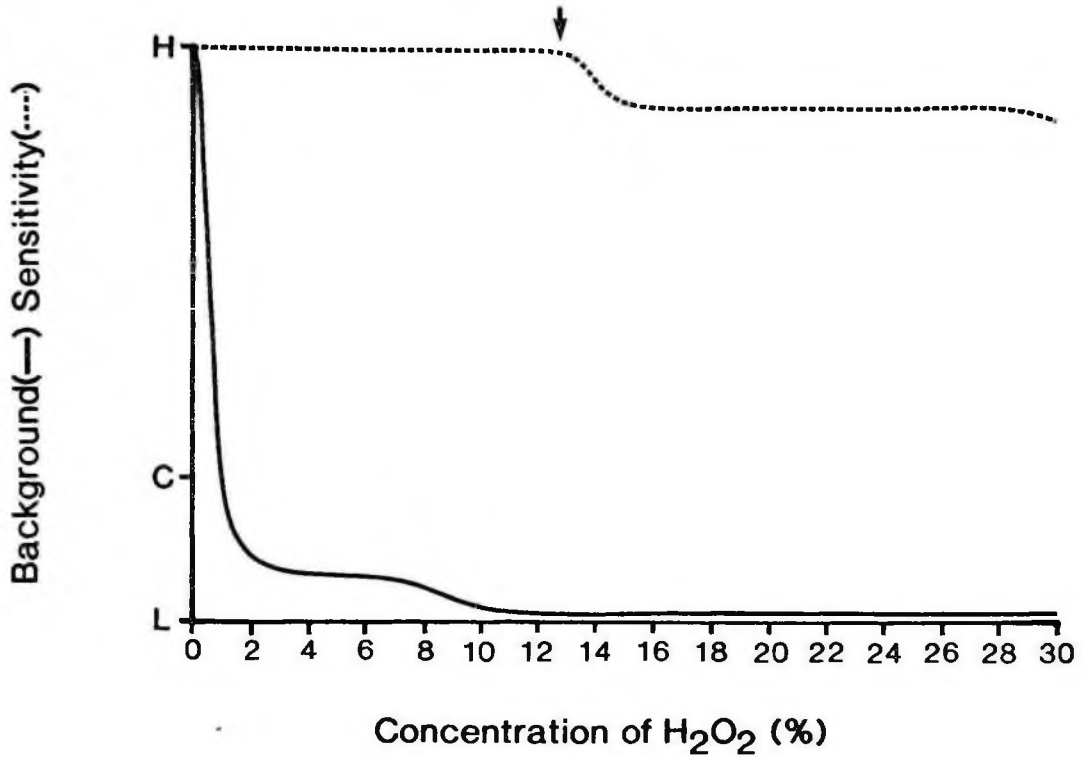



**Figure 37**

### 6.4.2 Detection of *T. brucei* in the midguts of experimentally infected *Glossina* species using the "modified dot-ELISA"

Figure 38 shows the results of an experiment in which *T. brucei* infections in tsetse midguts were specifically detected. In the strip incubated with the *T. brucei* specific MoAb (KT39a), four out of five infected flies gave positive reactions, while the uninfected control flies were negative. Also, the *T. brucei* specific MoAb (KT39a) reacted specifically in the test, as shown by its reactivity with the control *T. brucei* dot. None of the control MoAbs specific for *Nannomonas* (KN4) or *T. congolense* (TC6) gave positive reactions with any of the flies tested, whereas they reacted with their appropriate controls, thus underscoring the specificity of the test. The elimination of non-specific reactions and stains previously encountered in the dot-ELISA was further confirmed by the absence of reactions on the sample dots tested on the conjugate control strip **C**, as well as the strip tested with the *T. vivax* specific MoAb (KD32).

This successful detection of *T. brucei* in the midguts of both male and female *G. m. centralis* showed that: (1) $H_2O_2$ destaining does not release the trypanosome species-specific antigens bound to NC membrane, and (2) the modified dot-ELISA is suitable for detecting *T. brucei* in infected tsetse midguts.

#### 6.4.2.1 Determination of the best stage to incorporate hydrogen peroxide destaining into the modified dot-ELISA

Following the successful detection of *T. brucei* in the midguts of experimentally infected tsetse flies using the modified dot-ELISA, experiments were conducted to determine the effect of: (a) blocking and destaining the NC membrane strips at the same time (TEST I), and (b) destaining the strips before blocking (TEST II). The results obtained are shown in Figure 39. The original stains made on the NC membrane strips by the applied gut samples are

**Figure 38**

Detection of *T. brucei* (IL375) in the midguts of experimentally infected *G. morsitans centralis* using a modified dot-ELISA. Midgut samples from five IL375 infected flies (IF1 to IF5) and five uninfected control flies (UF1 to UF5), as well as *in vitro* propagated control trypanosomes consisting of $1x10^5$ parasites per dot of *T. congolense* K/83/IL/97/2 (TCK), *T. brucei* IL2616 (TB) and *T. simiae* KETRI 2431 (TS) were applied in dots onto each of the strips. The MoAbs used were: *Nannomonas* subgenus-specific (KN4); *T. congolense* specific (TC6); *T. brucei* species-specific (KT39a): and *T. vivax* specific (KD32). Strip 'C' was assayed as a conjugate control.

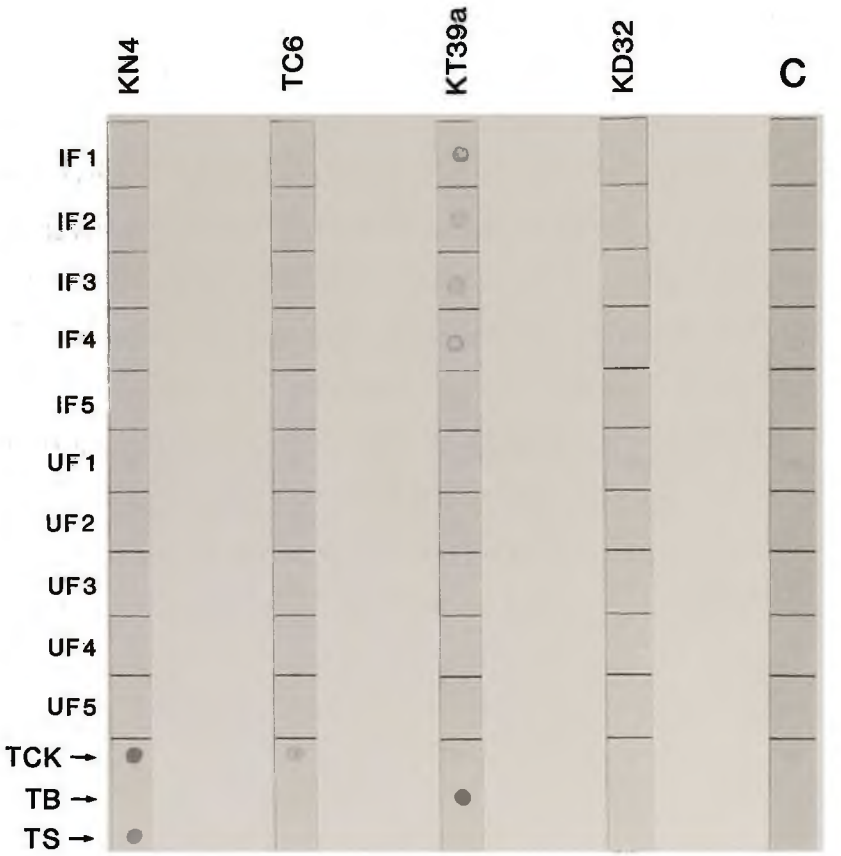



Figure 38

**Figure 39**

Comparison of the effect of destaining and blocking sample "dotted" NC membrane strips simultaneously (TEST I), with destaining the strips before blocking (TEST II), in the modified dot-ELISA. Each of the strips was applied with midgut samples from, five *T. brucei* IL375 infected *G. morsitans centralis* (IF1 to IF5) and five uninfected control flies (UF1 to UF5). A control sector at the lower section of each strip was applied with $1x10^5$ trypanosomes per dot of *in vitro* propagated *T. congolense* K/83/IL/97/2 (TCK), *T. brucei* IL2616 (TB) and *T. simiae* KETRI 2431 (TS). The MoAbs used were: *T. brucei* species-specific (KT39a), *T. congolense* specific (TC6); and *Nannomonas* subgenus-specific (TC6/25). The strips marked 'C' were conjugate controls and 'P' was the preserved strip showing the original staining of NC membrane by the applied samples.

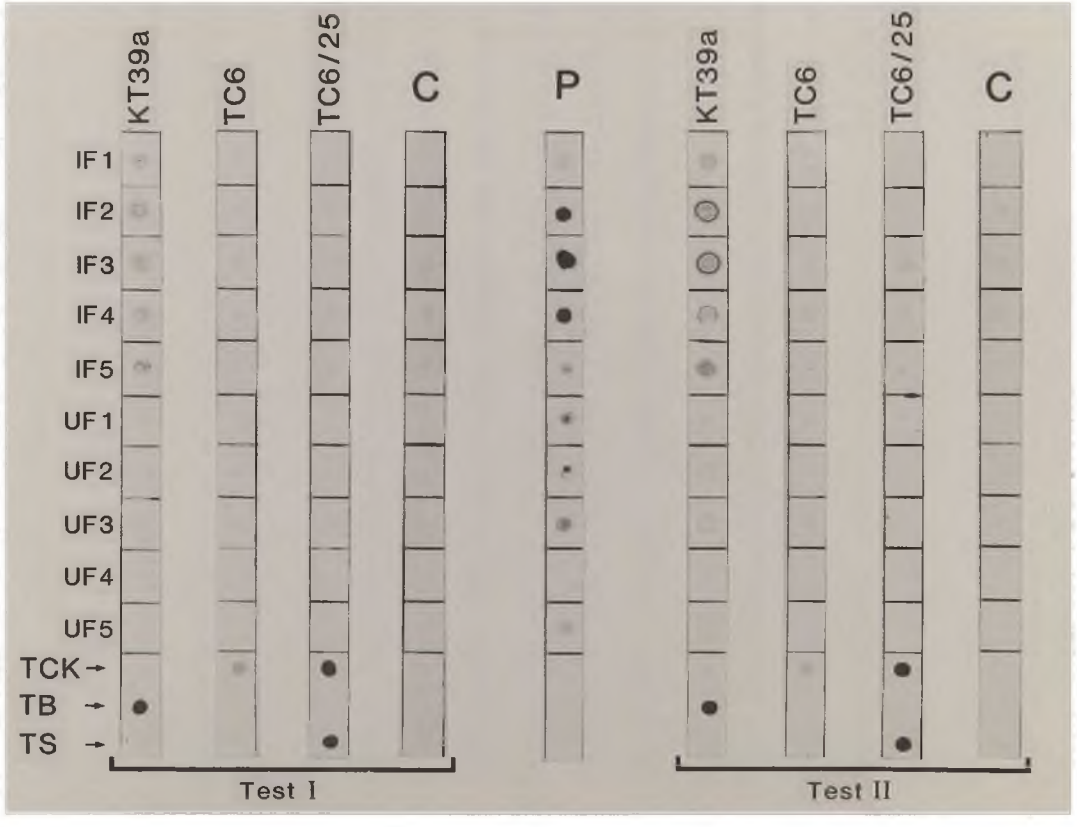



**Figure 39**

shown on the preserved (untested) strip **P**. The conjugate control strips which were not incubated with specific MoAb, showed no reactions in both tests, indicating that the non-specific stains were removed by the destaining procedures used in both Test I and Test II. Both the *T. congolense* specific MoAb (TC6) and the *Nannomonas* specific MoAb (TC6/25) were shown to react specifically with the TCK and TS control trypanosome dots, and there were no differences between the two destaining procedures in terms of the removal of non-specific stains. The strips incubated with the *T. brucei* specific MoAb KT39a showed that the MoAb reacted specifically with the *T. brucei* control antigen and detected all the five infected flies in both Test I and II. However, whilst all the five uninfected flies tested negative on the KT39a strip in Test I, the results obtained for four out of the same five flies in Test II showed the presence of faint brown rings around the areas where sample dots were applied. These ring reactions occurred also on uninfected samples. However, as shown in Figure 39, the ring-pattern on negative results could be differentiated from positive results by the absence of the central stained cores characteristic of positive reactions when the ring phenomenon occurred on them.

Further experiments showed that: (1) this ring-pattern reaction occurred only when the *T. brucei* specific MoAb KT39a was used in the modified dot-ELISA, (2) in most cases, the ring-pattern did not occur on all the infected or uninfected samples in the same test, and (3) the occurrence of the ring-pattern was more frequent when NC membrane strips were destained before blocking. Based on these findings, $H_2O_2$ destaining was combined with blocking of the NC membrane strips in all subsequent experiments.

**6.4.2.2** The effect of different substrate systems on the non-specific ring-pattern reaction

Since the occurrence of the ring-pattern was not completely abolished by the combination of blocking and destaining of NC membrane strips, experiments were conducted to determine the effect of different substrate systems on the occurrence of the rings, and in the interpretation of the modified dot-ELISA results. Figure 40 shows the results of an experiment in which three different substrate systems were tested. The absence of reactions on the conjugate control strips ($\mathbf{A}^{o}$, $\mathbf{B}^{o}$, $\mathbf{C}^{o}$ and $\mathbf{D}^{o}$) showed that the samples tested in this experiment gave no background. On the other hand, the specific reactivity of KT39a on the *T. brucei* control parasite dots on all the tested strips were clearly shown. The reactions on the two replicate strips developed with substrate system **A** showed that all the five infected flies were positive. However, a ring-pattern was present on the sample dots from two uninfected flies (UF2 and UF3), as well as four of the infected flies. The absence of these non-specific ring-patterns on the conjugate control strip $\mathbf{A}^{o}$, suggested that the specific MoAb (KT39a) influenced the occurrence of the non-specific ring reactions. However, it was also shown that the ring reactions on the infected samples had centrally stained portions which were absent on the uninfected samples with the ring-patterns. This showed that despite the occurrence of the ring-patterns on both infected and uninfected samples, it was possible to distinguish positive and negative reactions. Strips developed with substrate system **B** showed non-specific ring-reactions on the same samples that the phenomenon was observed using substrate system **A**. However, substrate system **B** detected fewer infected flies (4), compared with the detection of all the five infected flies by system **A**. This loss in sensitivity made substrate system **B** less attractive. The strips developed with substrate system **C**, showed no non-specific rings on the uninfected samples, even though ring-patterns were clearly seen on two of the infected samples. It is,

**Figure 40**

Comparison of different substrate systems and their effects on non-specific ring-pattern reactions obtained with the *T. brucei* species-specific MoAb (KT39a) in the modified dot-ELISA. Each of the strips was "dotted" with gut samples from five *T. brucei* IL375 infected *G. morsitans centralis* (IF1 to IF5), five uninfected control flies (UF1 to UF5), and control *in vitro* propagated trypanosomes consisting of $1x10^5$ parasites per dot of *T. congolense* K/83/IL/97/2 (TCK), *T. brucei* IL2616 (TB) and *T. simiae* KETRI 2431 (TS). The substrate systems were: **A** = 0.05% (w/v) DAB and 0.15% (v/v) $H_2O_2$ in phosphate-$Na_2EDTA$ buffer (10mM $NaH_2PO_4$, 10mM $Na_2HPO_4$ and 10mM $Na_2EDTA$); **B** = 0.05% (w/v) DAB and 0.15% (v/v) $H_2O_2$ in Tris-buffered saline (50mM Tris and 150mM NaCl, pH 8.0); **C** = **A** plus 5mM $NiSO_4.6H_2O$; and **D** = **B** plus 5mM $NiSO_4.6H_2O$. Strips $A^o$, $B^o$, $C^o$ and $D^o$ were run through the assay as conjugate controls for the different substrate systems.

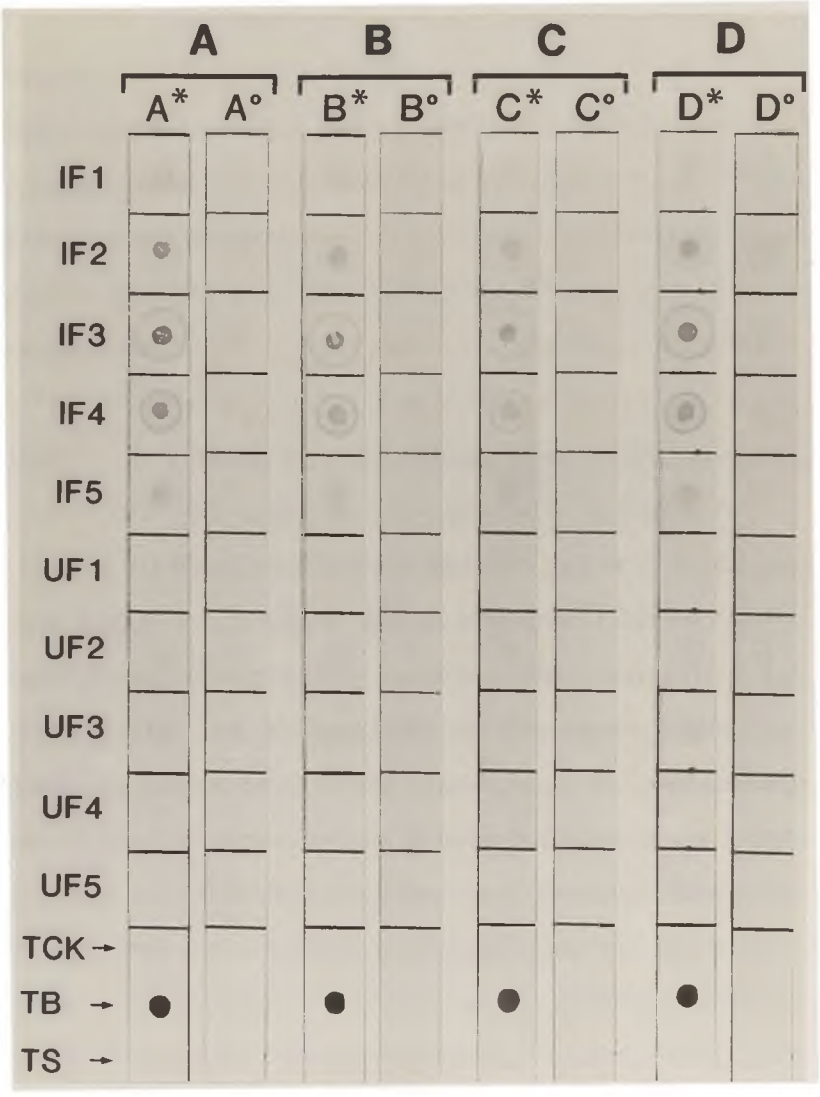



**Figure 40**

however, important to note that only 3 of the infected flies were detected with this substrate system. This lower sensitivity made the substrate system **C**, also less attractive. Substrate system **D** gave completely different results, as the reactions observed were bluish-black instead of brown.

This bluish-black colour was preferred by 5 out of 6 technicians who were made to choose between substrate **A** or **D**, basing their selection on which substrate they would prefer to work with if they had to tell the positive reactions from the negative ones. However, substrate **D** detected four of the five infected flies, compared with substrate **A** which detected all. This superior sensitivity, led to the selection of substrate system **A** for further use in the dot-ELISA.

### 6.4.3 Detection of *T. congolense* in the midguts of experimentally infected *Glossina* using the modified dot-ELISA

The modified dot-ELISA used to identify *T. brucei* infections in tsetse midgut was also applied for the detection of *T. congolense* in the same organ in experimentally infected tsetse flies. The results obtained with one of these experiments are shown in Figure 41. The preserved strip **P** showed the original stains made by the applied samples on the NC membrane strips. The removal of all the stains made by the uninfected tsetse midgut samples on the test strips, using $H_2O_2$ destaining, was clearly illustrated. As expected, the *T. brucei* specific MoAb KT39a did not detect any of the flies tested. Yet, its reactivity with the control *T. brucei* parasite dot confirmed that KT39a reacted specifically in the test. Both the *Nannomonas* subgenus-specific MoAb (KN4) and the *T. congolense* specific MoAb (TC6) reacted specifically on the control parasite dots. The detection of all the five infected flies by KN4, suggested that the infections were due to *T. congolense* or *T. simiae* or both. Since it was known that the flies were infected with *T. congolense*, it was expected that TC6 would also detect the infections. However, as shown in Figure 41, TC6

**Figure 41**

Detection of *T. congolense* IL3274 in the midguts of experimentally infected *G. morsitans centralis* using a modified dot-ELISA. Each strip was applied with midgut samples from five *T. congolense* infected flies (IF1 to IF5) and five uninfected control flies (UF1 to UF5), as well as cultured control trypanosomes consisting of $1x10^5$ parasites per dot of *T. congolense* K/83/IL/97/2 (TCK), *T. brucei* IL2616 (TB) and *T. simiae* KETRI 2431 (TS). The MoAbs used were: *Nannomonas* subgenus-specific (KN4); *T. congolense* specific (TC6); and *T. brucei* species-specific (KT39a). Strip 'P' was not subjected to any testing and it shows the original stains made on NC membrane by the applied samples.

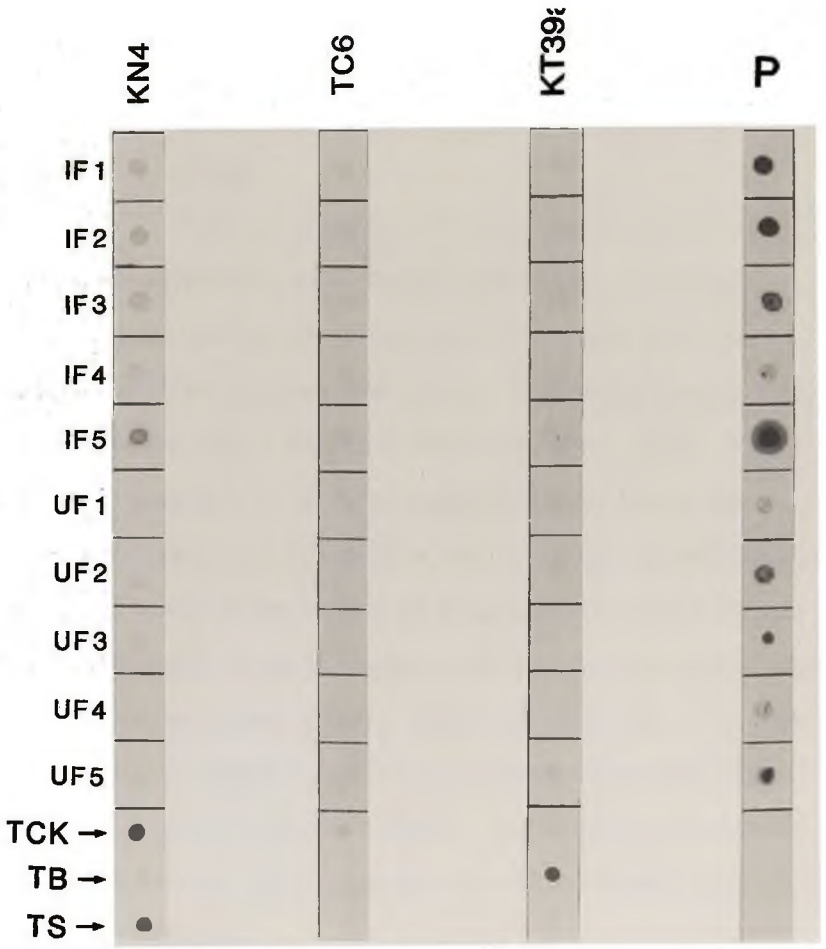



Figure 41

reacted very weakly with the infected samples. This inability to strongly detect clearly, the *T. congolense* IL1180 midgut infections was found to be the case for all the *T. congolense* specific MoAbs. On the contrary, each of the *Nannomonas* subgenus-specific MoAbs was able to detect those infections with strong positive reactions.

**6.4.3.1** Improved detection of *T. congolense* in the midguts of experimentally infected tsetse flies using the modified dot-ELISA

Attempts were made to improve the accessibility of the *T. congolense* species-specific internal antigens (Chapter 4) for reactivity in the dot-ELISA, by lysis of the trypanosomes in gut suspensions before applying samples onto NC membrane for testing. The results, however, showed an abolition of the weak reactivity of the *T. congolense* specific MoAbs, and a decreased reactivity of the *Nannomonas* subgenus-specific MoAbs. It was assumed that the decreased binding of the *Nannomonas* specific MoAbs, could be due to increased competition for binding to NC membrane between the trypanosome species-specific antigens and the mass of debris released by midgut contents. Figure 42 shows a light micrograph of *T. congolense* midgut procyclic forms together with the characteristic mass of biological debris usually present in the tsetse gut samples. To circumvent this problem, it was decided to first dot the gut samples onto NC membrane, before lysing the bound trypanosomes.

Preliminary investigations showed that each of the detergents used (NP-40, Saponin, SDS and Triton X-114), was capable of enhancing the reactivity of the *T. congolense* specific MoAbs. Triton X-114 was, however, selected due to its superior ability to give stronger reactions. Figure 43 shows the results of an experiment in which *T. congolense* (IL1180) in the midguts of

**Figure 42**

Light micrograph of a trypanosome infected tsetse midgut sample showing biological debris and *T. congolense* parasites (t).

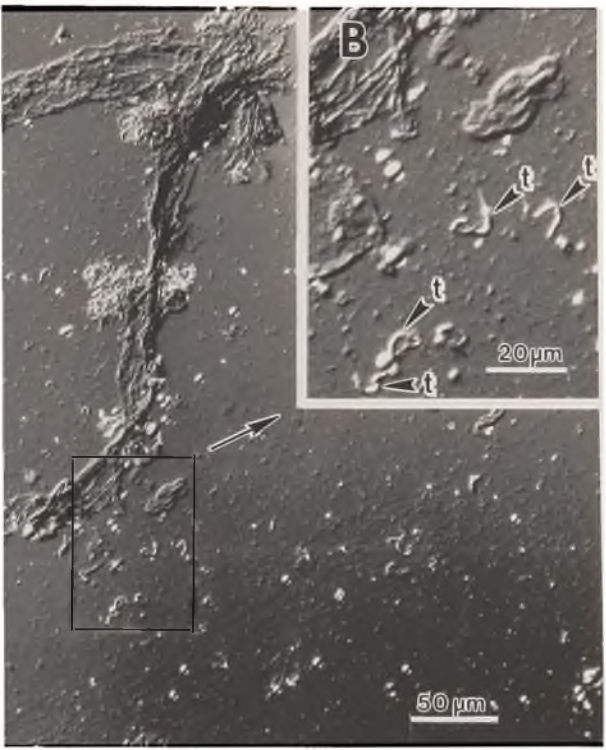



**Figure 42**

**Figure 43**

Detection of *T. congolense* IL1180 in the midguts of experimentally infected *G. morsitans centralis* using a modified dot-ELISA. Each of the NC membrane strips was "dotted" with midgut sample preparations from, five infected flies (IF1 to IF5), and five uninfected control flies (UF1 to UF5). A control sector at the lower section of each strip was applied with $1x10^5$ trypanosomes per dot of *in vitro* propagated *T. congolense* K/83/IL/97/2 (TCK), *T. brucei* IL2616 (TB) and *T. simiae* KETRI 2431 (TS). The antigen "dotted" strips were tested using *Nannomonas* subgenus-specific MoAb (KN4), *T. congolense* specific MoAb (TC6) and *T. brucei* species-specific MoAb (KT39a). Strip 'C' was tested as a conjugate control.

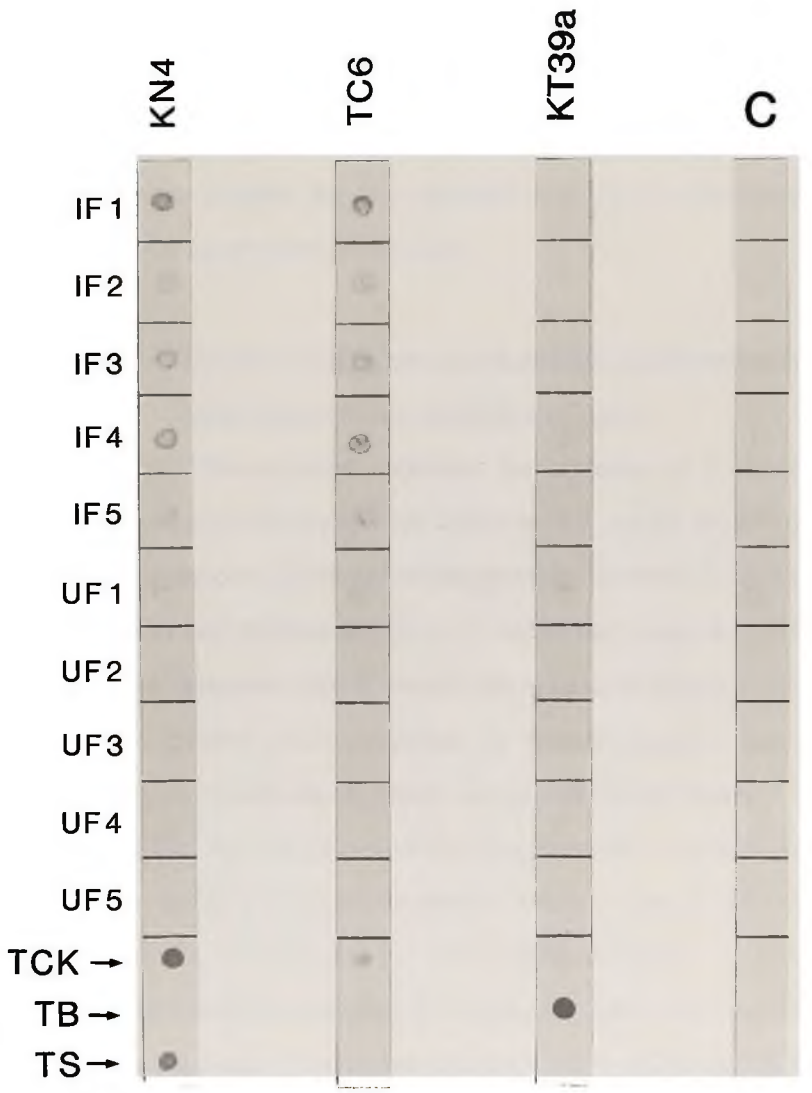



**Figure 43**

experimentally infected *G. m. centralis* were detected following lysis of trypanosomes in sample bound NC membrane strips using 0.1% Triton X-114. Both the *Nannomonas* subgenus-specific MoAb (KN4) and the *T. congolense* specific MoAb (TC6) elicited stronger reactions and detected clearly, 4 out of the five infected flies. Samples from all the uninfected flies tested negative, and the reactions on the control trypanosome dots showed that both MoAbs reacted specifically. The *T. brucei* specific MoAb KT39a reacted with the *T. brucei* control parasite dot and detected none of the flies tested, thus, demonstrating the specificity of the test.

### 6.4.4 Detection of *T. simiae* in the midguts of experimentally infected tsetse flies using the modified dot-ELISA

The dot-ELISA described for detection of *T. congolense* in infected tsetse guts was used for the detection of *T. simiae* in the same organ. Figure 44 summarizes the results of an experiment in which *T. simiae* parasites were detected and differentiated from *T. congolense* using the modified dot-ELISA. The preserved strip **P**, which was not run through the assay, shows the original staining of NC membrane by the gut samples. The conjugate control strip **C**, which was not tested with specific MoAb shows no reactions, confirming that the non-specific stains were removed. As seen on the strip tested with the *T. brucei* specific MoAb (KT39a), none of the tested tsetse samples reacted with this MoAb even though the specific reactivity of the antibody was clearly illustrated by its reaction with the *T. brucei* control. Both the *Nannomonas* and *T. congolense* specific MoAbs (KN4 and TC6) reacted specifically in the assay as shown by their reactivity with the control *T. congolense* and *T. simiae* parasite dots. Yet it was clearly shown that, whereas KN4 detected all the five infected flies, TC6 did not, thus indicating that the infections were due to *T. simiae*. *T. simiae* infections were successfully

**Figure 44**

Detection of *T. simiae* CP11 in the midguts of experimentally infected *G. morsitans centralis* using a modified dot-ELISA. Each of the NC membrane strips was "dotted" with midgut sample preparations from, five infected flies (IF1 to IF5), and five uninfected control flies (UF1 to UF5). A control sector at the lower section of each strip was applied with $1x10^5$ trypanosomes per dot of *in vitro* propagated *T. congolense* K/83/IL/97/2 (TCK), *T. brucei* IL2616 (TB) and *T. simiae* KETRI 2431 (TS). The antigen applied strips were tested using *Nannomonas* subgenus-specific MoAb (KN4), *T. congolense* specific MoAb (TC6) and *T. brucei* species-specific MoAb (KT39a). Strip 'C' was tested as a conjugate control, and strip 'P' was preserved without processing through the dot-ELISA. Note the successful removal of sample stains shown on strip 'P'.

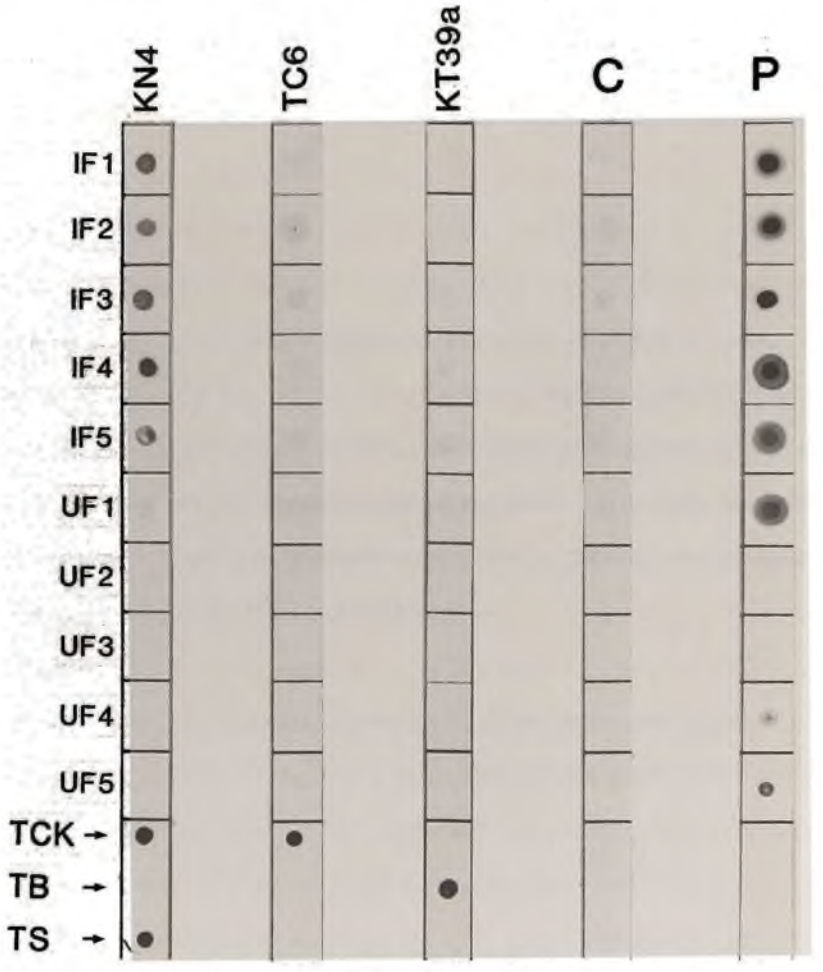



**Figure 44**

detected in males and females of both experimentally infected *G. m. centralis* and *G. pallidipes*.

### 6.4.5 Selected method of dissecting out tsetse gut tissue, choice of sample buffer, and method of gut sample preparation

Dot-ELISA assays performed using gut tissue dissected out as described in the materials and methods, indicated that, infecting trypanosome species in tsetse gut could be detected using any of the gut sections.

In the preparation of tsetse gut for testing, no significant differences were found between the use of PBS or PSG as sample buffers.

The release of trypanosomes from suspended tsetse gut tissue by mixing through gentle pipetting and application of samples onto NC membrane was found to be the best method. Using this method, positive reactions were strong and easily seen. The release of trypanosomes by complete disruption of gut tissue gave the strongest positive reactions. However, the method also gave high background on negative sample dots, thereby making interpretation of the results of the dot-ELISA difficult.

### 6.4.6 MoAbs selected for use in the modified dot-ELISA

Of the four *T. brucei* specific MoAbs tested in the modified dot-ELISA, KT43/27 ($IgG_{2a}$) was found to be unsuitable, since it could not detect *T. brucei* infections in the midguts of experimentally infected *Glossina*. The other three MoAbs [TR7 (IgM), KT43/33 ($IgG_1$) and KT39a (IgM)] were able to detect those infections. KT39a was selected for routine use in the detection of *T. brucei* in infected tsetse guts on the basis of its ability to strongly react with this parasite species in that organ

Four *T. congolense* specific MoAbs [TC6 ($IgG_1$), C2 ($IgG_1$), TC39 (IgM) and TC40 (IgM)] were tested in the modified dot-ELISA for the detection of *T. congolense* in the midguts of infected tsetse flies. TC6, TC39

and TC40 reacted more strongly with infected samples compared to C2. Though TC6, TC39 and TC40 did equally well, TC6 was selected for use in the modified dot-ELISA.

From the four *Nannomonas* subgenus-specific MoAbs tested, the two $IgG_3$ MoAbs (KN4 and TC6/25) reacted more strongly with positive samples compared to the two $IgG_1$ (TC16 and KN5). Both KN4 and TC6/25 were found to be suitable for the identification of *T. congolense* and *T. simiae* infections in the midguts of infected *Glossina*.

### 6.4.7 Specificity of the modified dot-ELISA

As reported under section 6.4.2.1 and 6.4.2.2, a ring-pattern reaction occurred round some uninfected samples when tested in the modified dot-ELISA using the *T. brucei* specific MoAb KT39a. Since the ring reactions could be differentiated from specific reactions, they did not lead to difficulty in the interpretation of the results. Hence, none of the 315 uninfected tsetse flies that were tested using the *T. brucei* specific MoAb KT39a gave false positive reactions in the modified dot-ELISA.

Of one hundred and ten (110) uninfected flies tested using the *Nannomonas* subgenus-specific MoAb (KN4) and the *T. congolense* specific MoAb (TC6), none reacted positively in the dot-ELISA.

### 6.4.8 Sensitivity of the modified dot-ELISA

The ability of the modified dot-ELISA to detect trypanosomes in the midguts of experimentally infected tsetse flies was 91.6% (Table 29). Further breakdown of this sensitivity into the ability to detect *T. brucei*, *T. congolense* or *T. simiae* are also provided in the same table. Of 95 *T. brucei*, 130 *T. congolense* and 90 *T. simiae* infected flies tested, 86, 111 and 85 were detected, giving sensitivities of 90.5, 85.5 and 94.4% respectively.

**Table 29**

**Detection of trypanosomes in the midguts of experimentally infected *Glossina* by a modified dot-ELISA**

| Trypanosome species | Number of infected flies tested | Number of flies positive | % positive (sensitivity) |
|---|---|---|---|
| *T. brucei* | 95 | 86 | 90.5 |
| *T. congolense* | 130 | 111* | 85.4 |
| *T. simiae* | 90 | 85 | 94.4 |
| Total | 315 | 282 | 91.6 |

* The number of flies detected by both *T. congolense* and *Nannomonas* specific MoAbs used in the assays.

In the case of the detection of *T. congolense*, two different MoAbs (one *Nannomonas* and one *T. congolense* specific) were used. Confirmed *T. congolense* infections were those that were detected by both the *Nannomonas* and the *T. congolense* specific MoAbs. As shown in Table 29, 111 out of 130 *T. congolense* infected flies were detected by both the *Nannomonas* MoAb and the *T. congolense* specific MoAbs, giving a sensitivity of 85.4%. However, as shown in Table 30, more of the known *T. congolense* infections were detected by the *Nannomonas* MoAb (117) compared to those detected by the *T. congolense* specific MoAb (111). Thus, 6 out of 130 known *T. congolense* infected flies were detected by the *Nannomonas* but not the *T. congolense* specific MoAb. Also, as shown in Table 30, one out of the 111 *T. congolense* infections detected by the *T. congolense* specific MoAb, was not detected by the *Nannomonas* specific MoAb.

Investigations into the sensitivity of the modified dot-ELISA in terms of the number of trypanosomes required in test samples before they could be detected positive, revealed that, as low as $5x10^5$ trypanosomes/ml or $1.5x10^3$ trypanosomes/dot could be detected in gut samples.

### 6.4.9 Cross-reactivity in the modified dot-ELISA

Some results did raise questions about cross-reactivity. Figure 45 illustrates one such case. The three MoAbs, TC6/25 (*Nannomonas* specific), TC6 (*T. congolense* specific) and KT39a (*T. brucei* specific) used in this experiment were shown to react specifically with the control trypanosome dots.

As shown on the strip tested with the *Nannomonas* specific antibody (TC6/25), four of the five infected flies were clearly positive, suggesting that those flies were infected with *T. congolense* or *T. simiae*. Three of these four flies (IF2, IF3 and IF4) were negative on the strips tested with the *T. congolense* specific MoAb (TC6) as well as the *T. brucei* specific

**Table 30**

**Detection of *T. congolense* in the midguts of experimentally infected *Glossina* by a modified dot-ELISA**

| Trypanosome species | Number of infected flies | Number of flies positive when tested using: | | Percentage of flies detected as | |
|---|---|---|---|---|---|
| | | *Nannomonas* species-specific MoAbs | *T. congolense* specific MoAbs | *T. congolense* | *T. simiae* |
| *T. congolense* | 130 | 117 | 111* | 85.4 | 4.6** |

* One of the flies was detected by the *T. congolense*, but not the *Nannomonas* specific MoAb.

** *T. congolense* infections that could be mistakenly attributed to *T. simiae*.

**Figure 45**

Detection of *T. simiae* CP813 in the midguts of experimentally infected *G. morsitans centralis* using a modified dot-ELISA. Each of the NC membrane strips was "dotted" with midgut samples from five *T. simiae* infected flies (IF1 to IF5) and five uninfected control flies (UF1 to UF5), as well as cultured control trypanosomes consisting of $1x10^5$ parasites per dot of *T. congolense* K/83/IL/97/2 (TCK), *T. brucei* IL2616 (TB) and *T. simiae* KETRI 2431 (TS). The MoAbs used were: *Nannomonas* subgenus-specific (TC6/25); *T. congolense* specific (TC6); and *T. brucei* species-specific (KT39a). Strip 'C' was processed through the modified dot-ELISA as a conjugate control.

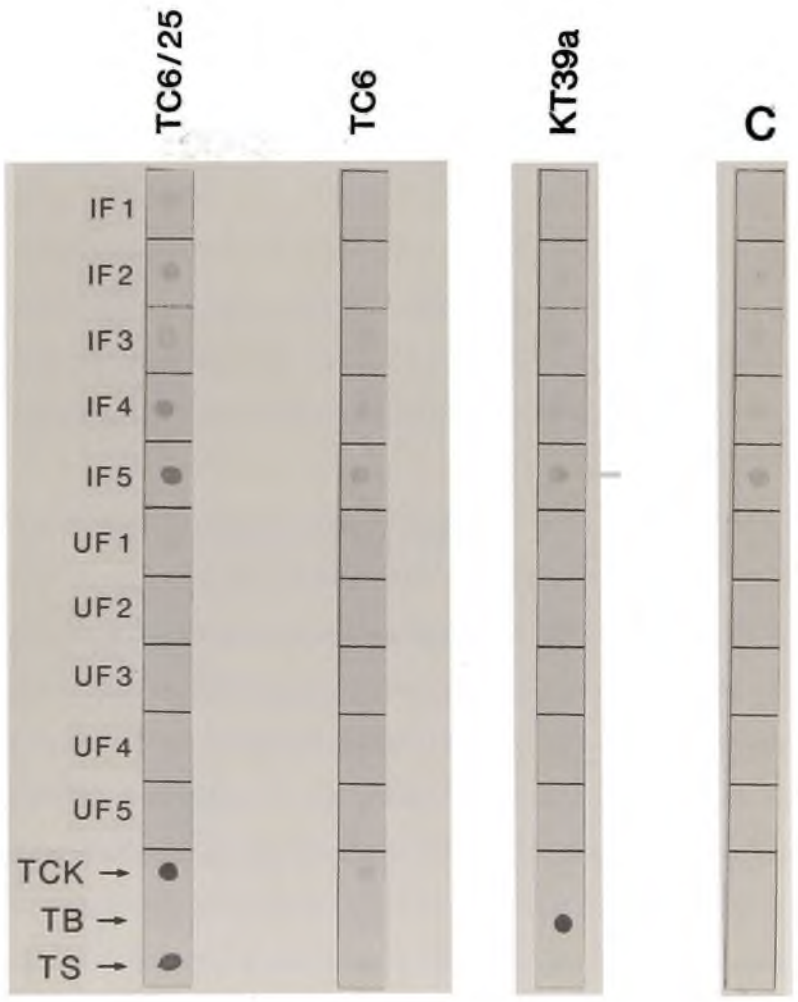



Figure 45

MoAb (KT39a), when compared with the control strip **C**, indicating by exclusion that those flies were infected with *T. simiae*. However, the sample from one fly IF5, appeared to be positive when tested with TC6 as well as KT39a, thus suggesting cross-reactivity. The results also showed that the sample from fly IF5 gave a reaction when tested on the conjugate control strip **C** which was not incubated with any specific MoAb, suggesting that the reactions seen on the sample dots from IF5 were non-specific. Further analysis of the results revealed that, whilst the intensity of the reactions on the sample dots from IF5 were uniform on the conjugate control, and *T. congolense* and *T. brucei* incubated strips, it was far more intense on the strip tested with the *Nannomonas* subgenus-specific MoAb. This indicated that despite a general background given by that sample, the *Nannomonas* subgenus-specific MoAb was the only one that reacted with it.

### 6.4.10 Stability of dotted samples

Sample "dotted" NC membrane strips were stored under desiccated conditions at room temperature (19-26°C). Some of the strips were retrieved and tested for antigen potency at various time intervals with selected trypanosome species-specific MoAbs (KT39a, TC6, KN4 and TC6/25), in the modified dot-ELISA. No significant loss in assay reactivity was observed in up to 90 days of storage for any of the tested MoAbs. However, after 7 days of storage, the frequency of occurrence, as well as the intensity of the non-specific ring reaction increased on strips tested with the *T. brucei* specific MoAb (KT39a).

### 6.4.11 Detection of *T. brucei* in the salivary glands of experimentally infected tsetse flies

Attempts to use the dot-ELISA developed and standardized for the differentiation of *in vitro* derived trypanosomes, in detecting *T. brucei* in

the salivary glands of infected tsetse flies were not successful. None of the four *T. brucei* specific MoAbs (KT39a, TR7, KT43/33 and KT43/27) was able to detect *T. brucei* (IL375) in microscopically confirmed *G. m. centralis* infected salivary glands, even though each of the MoAbs reacted specifically with *T. brucei* control parasite dots. The most likely causes of this failure to detect *T. brucei* in infected salivary glands, were thought to be: (1) interference due to factors such as enzymes, and (2) inability of the sample buffers (PBS and PSG) to release infecting trypanosomes from the salivary glands and promote their binding onto NC membrane in a way that they can be detected.

Addition of protease inhibitors (Leupeptin and E-64) to PBS, PSG or plain distiled water, did not solve the problem. Further experimentation revealed that 5mM $Na_2EDTA$ was a suitable sample buffer for the assay. Figure 46 shows the results of one experiment in which using 5mM $Na_2EDTA$ as sample buffer, the dot-ELISA was used to detect *T. brucei* in the salivary glands of infected tsetse flies. The two *T. brucei* specific MoAbs (KT39a and KT43/33) reacted specifically with the *T. brucei* control parasite dots, and the conjugate control strip **C** showed no background. The *T. brucei* specific MoAb (KT39a) which was selected for diagnosing *T. brucei* in infected tsetse gut was able to detect 3 of the 5 infected salivary glands. In comparison, KT43/33 clearly detected all the five *T. brucei* infected salivary glands. As shown in Figure 46, the reactivity of KT39a on the control *T. brucei* parasite dot was always far stronger than that of KT43/33. The superior ability of KT43/33 to detect *T. brucei* salivary gland infections was confirmed in all other experiments.

**Figure 46**

Detection of *T. brucei* IL375 in the salivary glands of experimentally infected *G. morsitans centralis* using dot-ELISA. Each of the NC membrane strips was "dotted" with salivary gland samples from five infected flies (IF1 to IF5) and five uninfected control flies (UF1 to UF5). A control sector at the lower section of each strip was applied with $1x10^5$ trypanosomes per dot of *in vitro* propagated *T. congolense* K/83/IL/97/2 (TCK), *T. brucei* IL2616 (TB) and *T. simiae* KETRI 2431 (TS). The MoAbs used were: KT43/33 and KT39a, both *T. brucei* species-specific. Strip 'C' was processed as a conjugate control.

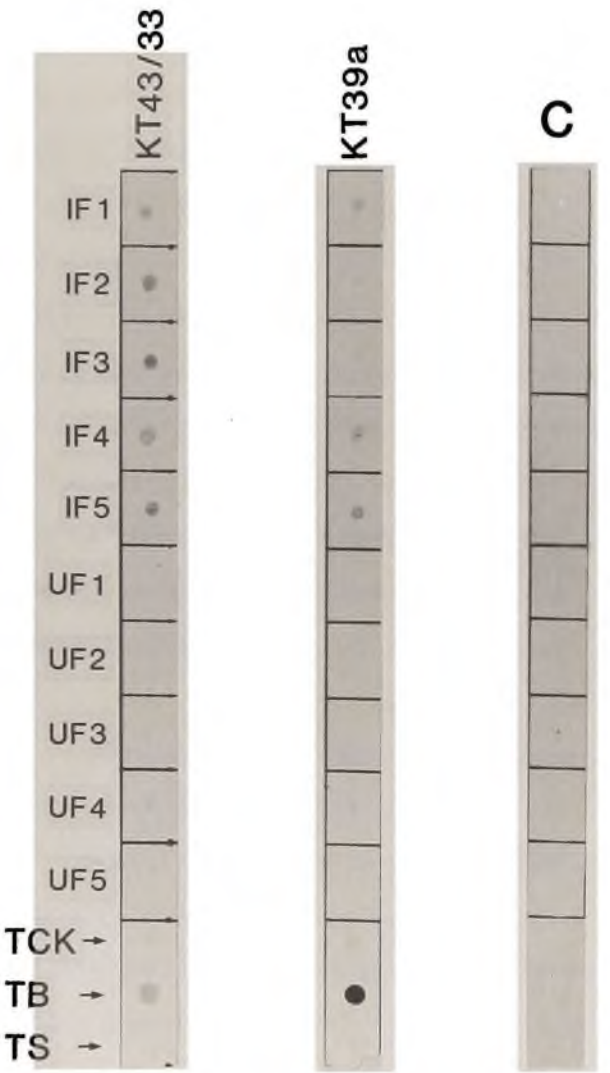



**Figure 46**

**6.4.12** Specificity and sensitivity of the dot-ELISA used in detecting *T. brucei* in experimentally infected tsetse salivary glands

During the development and standardization of the assay, sample dots made from some uninfected salivary glands reacted positively in the dot-ELISA. Whenever they occurred, such false positive reactions were found on all dots made from the same sample, irrespective of whether they were incubated with specific MoAb or not. The presence of such reactions on strips tested as conjugate control (without reaction with specific MoAb), indicated that the reactions were indeed not specific (background). Investigations into the cause of this background revealed that it was due to contamination of salivary gland samples with pigments originating from the gut, through spillage of gut contents during dissection. Following this finding, dissection of the salivary glands were always completed before dissection of the gut in the same fly. As a result, none of 70 uninfected salivary glands tested using the standardized dot-ELISA were positive. Of 70 *T. brucei* infected salivary glands that were tested, 63 reacted positively (Table 31), giving a sensitivity of 90%.

**6.4.13** Detection of *T. congolense* and *T. vivax* in the proboscides of experimentally infected tsetse flies

Several methods of tsetse proboscide sample preparations were tested using dot-ELISA. The most simplified method investigated was the excision of the proboscis about a third way from the tip using a pair of scissors and subsequent dotting of fluid expelled by squeezing the thorax of the fly. Samples obtained from *T. congolense* or *T. vivax* infected tsetse proboscides using this method were, however, not successfully diagnosed using the dot-ELISA. In an attempt to reveal very weak, inconspicuous reactions that may be present, the biotin/streptavidin reaction amplification steps were incorporated into the dot-ELISA procedure. Nevertheless, none of the

**Table 31**

**Detection of trypanosomes in the salivary glands of experimentally infected *Glossina* by dot-ELISA**

| Trypanosome species | Number of infected flies | Number of flies positive by test | % positive (sensitivity) |
|---|---|---|---|
| *T. brucei* | 70 | 63 | 90 |

microscopically confirmed *T. congolense* or *T. vivax* infected tsetse proboscides tested, using the method, was positive. Another procedure involving the suspension of dissected tsetse mouthparts in distiled water which was expected to lyse infecting trypanosomes and release their constituent antigens, was tried. However, this method of tsetse proboscide sample preparation also failed to give positive results using known infected mouthparts.

In order to ensure the release of trypanosomes from infected proboscides, tsetse mouthparts were dissected in distiled water, PBS, PSG or 5mM $Na_2EDTA$ in the wells of multitest immunofluorescence slides, and the labrum and hypopharynx cut into pieces using a small knife, and the sample fluids transferred from the wells directly onto NC membrane strips in dots. This method of sample preparation, permitted the detection of about 20% of *T. congolense* infected tsetse proboscides using the biotin/streptavidin amplified dot-ELISA. The use of distiled water did not give any positive results even though microscopically confirmed infected proboscides were tested. Microscopic examination of the sample remnants in multitest slide wells in which mouthparts were dissected revealed the presence of some trypanosomes, showing that not all the infecting organisms were transferred onto NC membrane for testing. Following that finding, further experiments were conducted using silicon coated multitest slides. The results were, however, not better than those obtained earlier using the uncoated slides.

In order to maximise the number of trypanosomes transferred onto NC membrane for testing, each tsetse proboscis including the thecal bulb was separated from the insects head and tested. In this experiment, all the infected as well as the uninfected proboscides tested positive, irrespective of which sample buffer was used (data not shown). The loss of specificity was in part attributable to higher concentrations of biological debris originating from the massive thecal bulb. To reduce the amount of biological debris in the test

samples without drastically reducing the number of trypanosomes available for testing, the thecal bulb was cut at a position leaving about a third of it still attached to the mouthparts, and the two thirds discarded. The mouthparts together with the third of the thecal bulb were transferred into Eppendorf tubes and disrupted with the tip of a pipette. The samples were then transferred onto NC membrane in dots and tested using the amplified dot-ELISA. The results of this experiment revealed that PBS, PSG or 5mM $Na_2EDTA$ could be used to detect *T. congolense* (IL1180) infections in the mouthparts of infected *G. m. centralis*.

Using this method of sample preparation, *T. vivax* (IL3096) was successfully detected in the mouthparts of experimentally infected *G. m. centralis* (Figure 47a). Both strips were tested using the *T. vivax* specific MoAb KD32. The reactivity of the MoAb with only the *T. vivax* (TV) control antigen showed that it reacted specifically in the test. Whilst none of the uninfected proboscides was positive, the infected fly proboscides were all positive on each of the two strips.

Figure 47b shows the results obtained for another experiment in which two *T. congolense* specific MoAbs (TC6 and TC39) were used to detect that parasite in tsetse mouthparts. Both MoAbs were shown to react specifically with the *T. congolense* control antigen dots (Figure 47b). Furthermore, each of the MoAbs detected clearly, four out of five infected proboscides, and none of the uninfected samples.

### 6.4.14 Specificity and sensitivity of the dot-ELISA used in detecting *T. congolense* and *T. vivax* in experimentally infected tsetse proboscides

Using the standardized dot-ELISA, a total of 45 and 64 uninfected tsetse proboscides were tested using the *T. congolense* specific MoAb (TC6) and the *T. vivax* specific MoAb (KD32), respectively. None of

**Figure 47a, b**

Detection of *T. vivax* (A) and *T. congolense* (B) in the mouthparts of experimentally infected *G. morsitans centralis* using dot-ELISA. Each infected or uninfected tsetse mouthpart was processed and placed onto NC membrane in a single dot for testing. KD32 was a *T. vivax* specific MoAb, whilst TC6 and TC39 were both *T. congolense* specific.

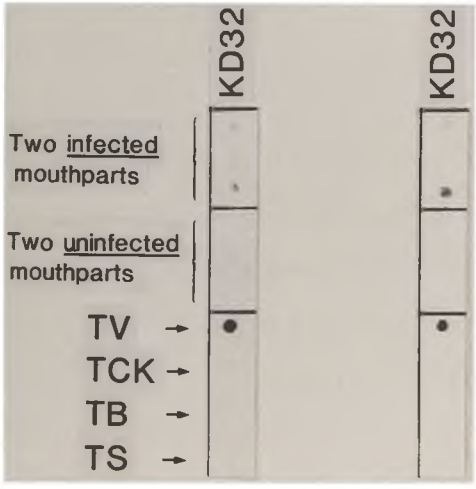



Figure 47a

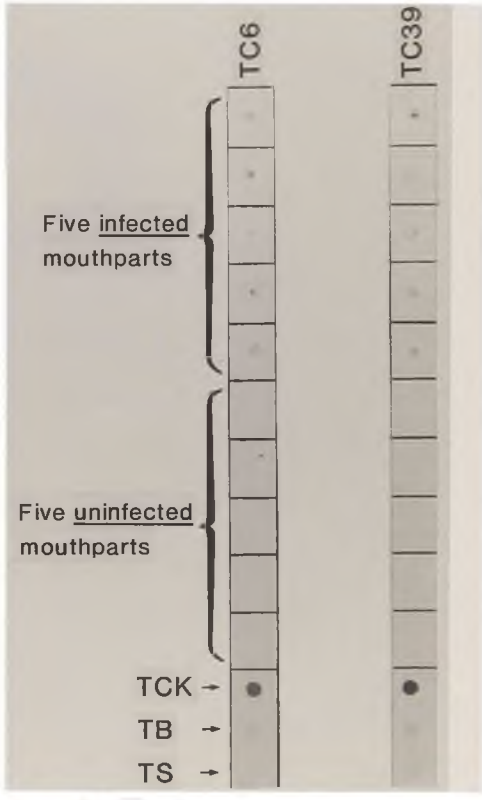



Figure 47b

these uninfected proboscides tested positive in the dot-ELISA, giving a specificity of 100%. The overall sensitivity of the assay was, however, lower than 50% (Table 32). Thus, of 45 *T. congolense* infected tsetse proboscides that were tested, 25 reacted positively, giving a sensitivity of 55.6%, whilst 28(43.8%) of the 64 *T. vivax* infected proboscides were detected.

**Table 32**

**Detection of trypanosomes in the mouthparts of experimentally infected *Glossina* by dot-ELISA**

| Trypanosome species | Number of infected flies | Number of flies positive by test | % positive (sensitivity) |
|---|---|---|---|
| *T. congolense* | 45 | 25 | 55.6 |
| *T. vivax* | 64 | 28 | 43.8 |

## 6.5 Discussion

The primary aim of this particular part of the study, as indicated before, was to apply the dot-ELISA developed and standardized for the differentiation of *in vitro* derived insect stage trypanosomes, to the detection of *T. brucei*, *T. congolense* and *T. simiae* in the gut, *T. brucei* in salivary glands, and *T. congolense* and *T. vivax* in the proboscides of experimentally infected tsetse flies.

Unlike trypanosome suspensions from *in vitro* cultures, which did not stain NC membrane upon dotting, tsetse gut suspensions were found to stain the membrane when applied for testing. The staining colours ranged from red through brown to black, with occasional greenish or colourless shades, depending on the state of digestion of the blood meal in the fly. These stains affected the specificity of the dot-ELISA when used for the detection and differentiation of trypanosome species in infected tsetse guts. Also, false positive reactions were encountered even in uninfected control sample dots processed with substrate and chromogen alone, ie., without incubation with MoAb or anti-MoAb HRPO-conjugate. This observation suggested that there were peroxidase-like substances in the sample dots. Such substances could utilise $H_2O_2$ and cause the oxidation of the chromogenic substrate (DAB), and thereby elicit positive reactions. This indeed appeared to be the case.

According to Bursell (1970), haematin is split off from haemoglobin in the gut of tsetse during the early stages of digestion, but it is not absorbed from the gut. It means therefore that haematin is likely to be present in much of the digestive residue found in the tsetse gut sections that were dissected out and processed for testing. Moreover, Saunders and co-workers (1964) reported that some haem-proteins could utilize either oxygen or hydrogen peroxide ($H_2O_2$) as a substrate and carry out oxidation, hydroxylation or oxygenation. Williams (1974) called these haem-proteins,

peroxidases, in accordance with earlier practice, but also indicated that they could as well be oxidases or oxygenases. Thus, the high background activity encountered with the dot-ELISA, was attributable to: (1) staining of NC membrane by pigmented gut samples, and (2) false reactivity resulting from oxidation of DAB by haem.

It was reasoned, therefore, that one method of eliminating the non-specific reactions was to change the enzyme/substrate system to one that did not require the addition of free $H_2O_2$ to the solution. To achieve this, the HRPO enzyme was substituted for glucose oxidase, which made it possible to use a substrate solution in which $H_2O_2$ was substituted for glucose. This change of enzyme, however, did not substantially reduce the non-specific staining. Moreover, the use of glucose oxidase-conjugated antibodies, introduced an additional problem of higher "assay background" which made it more difficult to detect weak positive reactions. A similar contribution to high assay background has been reported for alkaline phosphotase-conjugated antibodies (Pappas, 1988b). Consequently, the use of glucose oxidase-conjugated antibodies in the dot-ELISA was abandoned.

Another possibility was to degrade the haem-proteins in the test samples through a chemical process, such as oxidation, prior to analysis of the samples with the dot-ELISA. The most suitable of the oxidizing agents tried was $H_2O_2$.

$H_2O_2$ is a strong oxidising agent. Earlier research by Lemberg (cited by Jackson, 1974) had shown that the first step in haemoglobin catabolism is an oxidative degradation of the molecule, which leads to separation of the haem from globin and the loss of iron. Treatment of haematin with excess $H_2O_2$ might lead to a similar degradation of the haem-protein, with accompanying loss of colour. This discovery that $H_2O_2$ could be used to decolorize pigmented samples obtained from the guts of laboratory reared tsetse flies, without affecting the integrity of trypanosome species-

specific diagnostic antigens, suggests that: (1) the modified dot-ELISA that utilized this innovation, may do even better when it came to detecting and differentiating between trypanosome species in naturally infected *Glossina*; since such flies are known to digest their blood meals faster (Langley, 1967b) and will, therefore, present less staining problems; (2) $H_2O_2$ destaining may be potentially useful in the development of colorimetric assays for the detection of parasites in pigmented samples.

Tellez-Giron, Ramos, Dufour, Alvarez and Montante (1987) reported the occurrence of a non-specific ring-pattern reaction in a dot-ELISA that they used for detecting *Cysticercus cellulosae* antigens in cerebrospinal fluid. There was, however, no explanation offered for the occurrence of the so-called "ring phenomenon". In this study, a similar non-specific "ring-pattern" reaction occurred on some sample dots, despite the use of $H_2O_2$. These ring reactions did not occur on samples tested as conjugate controls, thus showing that specific MoAb played a role in their occurrence. Of the four *T. brucei* specific MoAbs tested with the dot-ELISA, only one (KT39a) induced the ring reactions, although not all samples tested with that particular MoAb showed the phenomenon. Despite the need to elucidate the mechanisms responsible for the ring formation, it has been shown in this work that the presence of the rings did not hamper interpretation of the results of the modified dot-ELISA.

The inability to enhance the reactivity of the *T. congolense* specific MoAbs by lysing the infecting organisms in suspension, was attributable to two causes. Firstly, it is known that some detergents such as NP-40 could remove protein from NC membranes (Lin and Kasamatsu, 1983), suggesting that their presence in the sample suspensions could reduce the binding efficiency of the antigens. Secondly, lysing the trypanosomes, together with the mass of biological debris present in the gut suspensions, could enormously increase the competition for binding between released

trypanosome antigens and other substances. The method of applying *T. congolense* infected gut suspensions onto NC membrane before lysing the parasites in the bound sample, circumvented the above listed problems and led to successful identification of these infections with the modified dot-ELISA. The success of this procedure could be explained by the earlier finding that the *T. congolense* specific MoAbs detected antigens that were not exposed on the surface of intact trypanosomes (Chapter 4).

Parasite detection and identification methods intended for field use need to be simple, in addition to being specific and sensitive. Methods that reduce time spent on sample preparation are, therefore, desirable. In this study, tsetse gut samples collected by simply cutting away the distal end of the insect's abdomen and squeezing out its contents for testing in the dot-ELISA, gave results that were comparable, in terms of detection and identification of parasites, to those following the more time consuming methods of tsetse midgut dissection. The suitability of this simplified method of tsetse gut tissue extraction for the dot-ELISA, was indicated by the finding that none of the trypanosome species-specific MoAbs cross-reacted with *T. grayi*, which is known to reside mainly in the hindgut of tsetse flies infected with the species (Hoare, 1972).

The tsetse gut sample preparation methods, employed in this dot-ELISA, afford the opportunity of analysing multiple dots of the same sample, using different species and/or subgenus-specific MoAbs. This has made it possible, for instance, to use *T. congolense* specific MoAbs and *Nannomonas* subgenus-specific MoAbs to specifically detect *T. congolense* infections in tsetse gut, or detect *T. simiae* infections in this organ in the same fly, by exclusion of *T. congolense*. Also, with the present dot-ELISA, it should be possible to tell whether a tsetse fly was infected with *T. grayi* in the gut, by excluding the presence of *T. congolense, T. simiae*, and *T. brucei*.

Theoretically, it would be expected that all *T. congolense* infections should be detected by *T. congolense* specific MoAbs and by the *Nannomonas* subgenus-specific MoAbs. However, it was shown in this study that *T. congolense* infections could be detected by *Nannomonas* specific MoAbs without being detected by *T. congolense* specific MoAbs. It is, however, important to note that the frequency of occurrence of this discordant reaction was low (<5%) as only 6 out of 117 *T. congolense* infected flies were detected by the *Nannomonas* but not by the *T. congolense* specific MoAb. This inability of the *T. congolense* specific MoAb to detect all the *T. congolense* tsetse gut infections revealed by the *Nannomonas* MoAb, could be attributed to differences in sensitivity between the two groups of MoAbs.

Another observation made in this study was that one *T. congolense* tsetse gut infection out of 110 (<1%) was detected by the *T. congolense* specific MoAbs without being detected by *Nannomonas* specific MoAbs in the same experiment. Two possible explanations could be offered for this unusual reactivity: Firstly the occurrence of 1 out of 110 is statistically insignificant and could be simply due to variations in test conditions, such as the amount of residual Triton X-114 or $H_2O_2$ that remained on a test strip following washings. Secondly, it might be the result of the destruction of the *Nannomonas* subgenus-specific antigen by factors such as enzymes in tsetse gut which nevertheless did not affect the *T. congolense* species-specific antigen. The likelihood of the second possibility was supported by earlier findings, that the *T. congolense* specific antigenic epitope(s) were of protein nature, whilst the *Nannomonas* specific antigenic epitope(s) were of carbohydrate nature (Chapter 4).

The opportunity to test each tsetse gut sample at least 15 times using the dot-ELISA, made the technique even more suitable for the purpose of detecting and differentiating between trypanosome species in infected tsetse gut. This is because, five main species of trypanosomes (*T. brucei*, *T.*

*congolense*, *T. simiae*, *T. suis* and *T. grayi*) are known to infect the gut of the *Glossina* species. This ability to replicate tsetse gut originated test samples, therefore, offered two additional advantages. The first was the possibility of detecting mixed infections in tsetse by employing the different trypanosome species and subgenus-specific MoAbs. The second advantage was the opportunity to utilize undigested tsetse blood meal in unused gut samples, in the identification of tsetse host, using methods such as described with the micro-plate ELISA developed by Rurangirwa, Minja, Musoke, Nantulya, Grootenhuis and Moloo (1986).

In this study, a total of 315 tsetse flies experimentally infected with *T. brucei*, *T. congolense* or *T simiae* in the guts, were tested using the modified dot-ELISA. The sensitivity of the assays was high, as 90.5% of the tsetse infected with *T brucei*, 85.4% of those infected with *T. congolense* and 94.4% of those infected with *T. simiae* were correctly identified. This gave an overall sensitivity of 91.6% for detecting and differentiating between trypanosome species in the guts of experimentally infected tsetse flies. The specificity of these assays was greater than 99.9%.

Decreased reactivity of the *T. congolense* specific antigen was recorded when culture-derived vector stage trypanosomes were applied and stored on NC membrane at room temperature (17-26$^{o}$C) for more than 60 days (Chapter 5). However, no significant loss in reactivity was observed in up to 90 days of storing trypanosome infected tsetse gut samples under similar conditions. This stability of the antigens, introduces a degree of flexibility in this test, since the collected samples need not be analysed at once.

The *T. brucei* specific MoAb (KT39a) which was used for detecting this parasite in infected tsetse gut, was not used for the detection of the parasite in the salivary glands of the vector. This was because, another *T. brucei* specific MoAb (KT43/33) performed better and was, therefore, selected. This difference in the performance of the two MoAbs was explained

as follows. It was found in Chapter 4 of this thesis that, KT39a bound to an antigenic epitope which was only partially sensitive to proteinase-K and which was located on a 90 kDa antigen peptide. On the other hand, KT43/33 bound to an antigenic epitope which was completely susceptible to proteinase-K and which was located on an antigen which localized in a series of peptide bands ranging between 21 and 47 kDa. These findings suggested that the *T. brucei* species-specific antigens detected by the two MoAbs (KT39a and KT43/33), were indeed different. It is, therefore, possible that the *T. brucei* specific antigens detected by the two MoAbs are expressed in different quantities in the different life cycle stages of the parasite. If this were the case, then the antigen detected by KT39a was better expressed in the procyclic stages of the parasites which predominate in the vector's midgut, whilst the antigen detected by KT43/33 was better expressed in the epimastigote and metacyclic stages in the vector's salivary glands. Another possible cause of the differences in the performance of the two MoAbs, was differential susceptibility of the specific antigens to degradative enzymes that may be present in the tsetse salivary glands.

As shown in the case of the identification of trypanosomes in the guts of infected tsetse flies, the sensitivity of the dot-ELISA for the detection of *T. brucei* in infected salivary glands was high (90%). The specificity of the assay was greater than 99.9%. Furthermore, the opportunity to test each pair of tsetse salivary glands at least three times, indicated that if the need arose, and MoAbs were made against *T. suis* which can also infect the vector's salivary glands, then this parasite could also be tested for alongside *T. brucei* in the dot-ELISA.

It has also been shown in this work that both *T. congolense* and *T. vivax* could be detected in the proboscides of infected tsetse flies. However, the sensitivity of the dot-ELISA in detecting these two trypanosome species in the target organ was low (55.6% for *T. congolense* and 43.8% for

*T. vivax*). This low detection rate of trypanosomes in the proboscis of *Glossina*, might be related to three factors: (1) the relative numbers of *T. congolense* or *T. vivax* parasites present in the proboscis at a certain point in their life cycles in the vector; (2) difficulties in releasing trypanosomes from the proboscis as a result of anatomical peculiarities of that organ, and (3) differences in sensitivity of the trypanosome species-specific MoAbs employed in the study. The inability to test each tsetse proboscis more than once, is clearly a drawback. One way to circumvent this limitation is to randomly place suspected tsetse proboscides into two groups, and to test one group with *T. vivax* specific MoAb and the other with *T. congolense* specific MoAb.

From this study, it could be concluded that the dot-ELISA is potentially a practical method for the diagnosis of trypanosome infections in tsetse flies. However, in order to determine the full potential of this technique, there was the need to investigate its applicability in the field. This was done, and the results obtained were recorded in the next chapter.

# CHAPTER 7

## FIELD EVALUATION OF A DOT-ELISA DEVELOPED FOR THE DETECTION AND DIFFERENTIATION OF TRYPANOSOME SPECIES IN INFECTED TSETSE FLIES (*GLOSSINA SPP.*)

## 7.1 Summary

A rapid, visually read, dot-ELISA developed for the detection and differentiation of trypanosome species in tsetse flies (*Glossina spp.*), was field tested alongside the standard fly dissection method on a ranch in south eastern Kenya.

Of a total of 104 *G. pallidipes* dissected, two were found to be infected with trypanosomes in their midguts. By the dissection method the infecting trypanosome species could not be identified, as both flies were free from salivary gland infections. However, using the dot-ELISA, the two flies were shown to be infected with *T. congolense* in their midguts. The midguts of an additional 6(5.8%) of the 104 *G. pallidipes* tested positive for *T. congolense* in the dot-ELISA, even though no trypanosomes were seen on dissection. The infection rate for this fly species as determined using the dot-ELISA, therefore, was 7.7% for *T. congolense* in midgut infections compared to 1.9% identified by fly dissection. The salivary glands and mouthparts of the 6 additional tsetse flies identified by dot-ELISA, were all negative as determined by the two techniques.

None of 390 *G. longipennis* flies dissected and examined for trypanosomes in the midgut, salivary glands and mouthparts was shown, by this method, to be infected. Using the dot-ELISA, however, 17(4.4%) of the flies tested positive for *T. congolense* in the midguts, whilst the salivary glands and mouthparts of the same flies were negative.

Thus, the dot-ELISA appears to be more sensitive than the fly dissection method under field conditions. Moreover, the dot-ELISA was performed in the field without electricity. It was simple to perform, and was not affected by high ambient temperatures (22-32$^{o}$C), or by contamination of reactants with dust.

## 7.2 Introduction

The tsetse dissection method, first introduced by Lloyd and Johnson (1924), is the method used in routine epidemiological surveys to determine trypanosome infection rates in *Glossina*. This is still the case even though a recombinant DNA technique was introduced by Kukla and colleagues (1987), for the diagnosis of trypanosome infections in the tsetse fly. The reason for this is partly because, the present DNA probes cannot recognise all the intra-species variants of targeted trypanosome species. Secondly, the DNA technique is not simple enough to be performed in most laboratories.

In the studies recorded in this Chapter, the dot-ELISA developed for differentiating between *in vitro* propagated trypanosome species (Chapter 5), and which was successfully modified for detecting and differentiating between infecting trypanosome species in the mouthparts, salivary glands or midguts of experimentally-infected *Glossina* species (Chapter 6), was evaluated in the field for the diagnosis of natural trypanosome infections in the vector.

It is shown here, that the dot-ELISA developed hereto, is capable of detecting and identifying infecting trypanosome species in naturally infected tsetse flies when the assay was performed under field conditions.

## 7.3 Materials and methods

### 7.3.1 The dot-ELISA kit

#### 7.3.1.1 Nitrocellulose (NC) membrane template

Lines were drawn on NC membrane sheets to form a grid consisting of square and rectangular shaped areas as shown in Figure 48. The columns representing the outlines of demarcated strips were numbered, and *in vitro* propagated procyclic *T. congolense* (TCK), *T. brucei* (TB) and *T. simiae* (TS), and epimastigotes of *T. vivax* (TV) were applied, $1x10^5$ trypanosomes/dot onto each of the demarcated NC membrane strips as shown in Figure 48, to provide the controls. The NC membrane sheets were sealed in polythene bags and transported to the field for use.

#### 7.3.1.2 Materials

1. Two Bio-Rad slot incubation trays
2. One 500ml plastic measuring cylinder
3. Two 500ml plastic beakers
4. One rubber pipette aid
5. 10ml plastic pipettes
6. 0.5 to 10$\mu$l adjustable pipette and pipette tips
7. 1.5ml Eppendorf tubes
8. Multitest IFA slides.

#### 7.3.1.3 Chemicals, reagents and buffers

Chemicals, reagents and buffers, enough for screening at least 1,000 tsetse flies, were transported to the field. These were:

**Figure 48**

A nitrocellulose membrane grid showing demarcated spaces for sample application.

TCK = $1x10^5$ trypanosomes/dot of *T. congolense* Kilifi type culture procyclics.

TB = $1x10^5$ trypanosomes/dot of *T. b. brucei* culture procyclics.

TS = $1x10^5$ trypanosomes/dot of *T. simiae* culture procyclics.

TV = $1x10^5$ trypanosomes/dot of *T. vivax* culture epimastigotes.

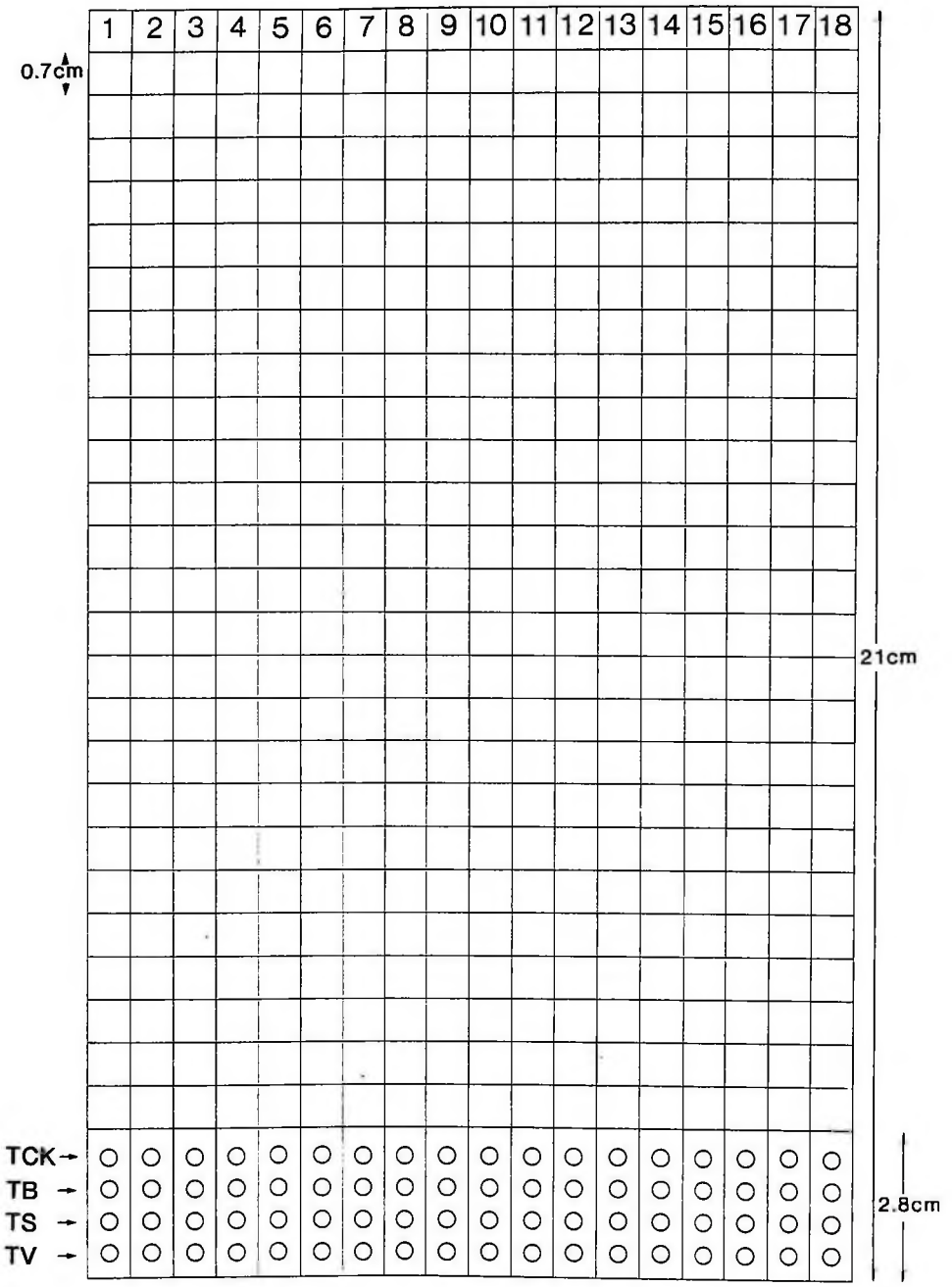



**Figure 48**

1. 50mg weights of 3-3'diaminobenzidine (DAB) in 1.5ml Eppendorf tubes, each enough for assaying samples from 100 tsetse flies
2. 5g weights of skimmed milk in sealed polythene bags, each enough for assaying samples from 50 tsetse flies
3. 1ml of concentrated hydrochloric acid
4. 50ml of 30% hydrogen peroxide ($H_2O_2$)
5. Aliquots of trypanosome species or subgenus-specific MoAbs and goat anti-mouse horseradish peroxidase (HRPO)-conjugated antibodies kept on ice in thermos flasks.
6. 100ml of phosphate buffered saline, pH 7.4
7. 100 ml 5mM $Na_2$EDTA buffer
8. 1.5 litres of x10 concentrated Tris buffered saline, pH 8.
9. 2ml of Triton X-114
10. 10 litres of deionised water
11. 200ml of Phosphate/$Na_2$EDTA buffer

### 7.3.2 The dissecting kit

#### 7.3.2.1 Equipment

1. Dissection microscope
2. Compound microscope

#### 7.3.2.2 Other materials

1. Microscope slides and cover slips
2. Kit of dissecting instruments
3. Phosphate buffered saline, pH 7.4, listed under 7.3.1.3

### 7.3.3 Study area and trapping of tsetse flies

#### 7.3.3.1 Study area

Field evaluation of the dot-ELISA was conducted at the Galana Ranch which covers an area of approximately 6000 $km^2$ in the coastal hinterland of Kenya. The mean altitude is 270 m, with an average annual rainfall of 550 mm (Wilson, Gatuta, Njogu, Mgutu and Alushula, 1986). The vegetation consists of riverine thickets along the Galana river, and thick coastal bush in the east which gives way to grasslands and scattered thickets in the west. Four species of tsetse have been identified on this ranch, inhabiting different ecological zones that make up to 35% of the area of the Ranch. The tsetse species were: *Glossina longipennis* in the drier savannah areas, away from the river; *G. austeni*, restricted to the river-bed; *G. brevipalpis*, in forested areas in the east; and *G. pallidipes* in thickets along the river and in the east.

#### 7.3.3.2 Trapping of wild tsetse flies

Male and female *G. longipennis* and *G. pallidipes* flies were trapped from their natural habitats using the F4 and biconical traps, respectively. The F4 traps (Figure 49) were set up in the late afternoon at about 4.00 pm in the grassland areas infested by *G. longipennis* and emptied the following day at about 9 am. Biconical traps (Figure 50) were also set up around 4.00 pm, in the *G. pallidipes* infested areas, in the east of the Ranch, and emptied the following day at about 4.00 pm.

### 7.3.4 Experimental design

Tsetse flies were killed by anaesthesia using chloroform, and sorted into groups according to species. The flies were also separated into tenerals and non-tenerals. The teneral flies were discarded.

**Figure 49.** F4 trap used for catching *G. longipennis in* the savannah areas at the Galana Ranch.

**Figure 50.** Biconical trap used for catching *G. pallidipes* in the thickets in the west of the Galana Ranch.

As shown in Figure 51 for *G. pallidipes*, each species of tsetse was first separated on the basis of sex. The flies in either sex (male and female) were subsequently sorted randomly, each into two groups; M1 and M2 for the males and; F1 and F2 for the females (Figure 51). The males in group M1 were then added to the females in group F1 to form batch 1 tsetse flies whose mouthparts were dissected and examined microscopically, and tested with a *T. vivax* specific MoAb (KD32/48.17) using the dot-ELISA described in Chapter 6. The mouthparts of the second batch of flies consisting of flies from M2 and F2, were also dissected, but tested with a *T. congolense* specific MoAb (TC6/42.6.3) in the dot-ELISA.

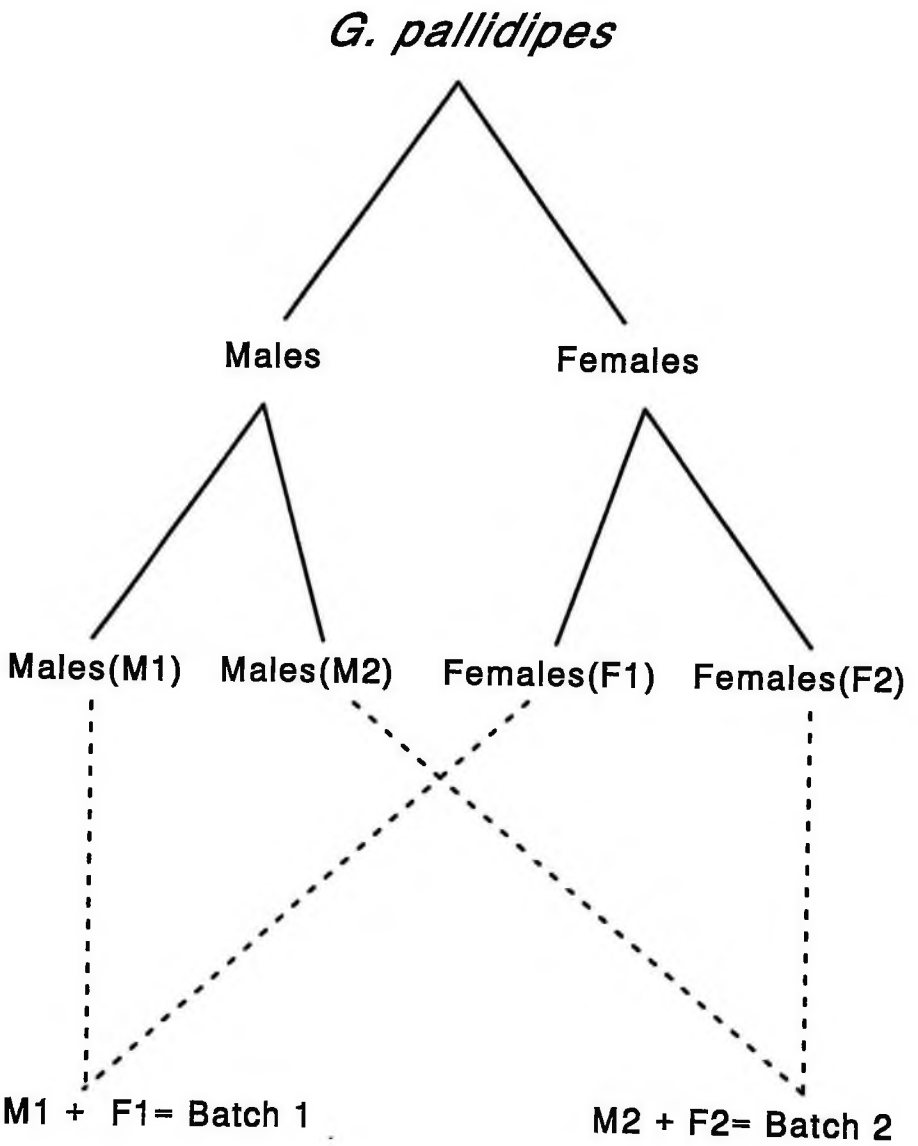
The midgut and salivary glands of all the flies were also dissected and examined microscopically for trypanosomes and also tested using the dot-ELISA as described in Chapter 6.

### 7.3.5 Dot-ELISA procedure

Tsetse proboscide, midgut and salivary gland samples were prepared and applied onto the NC membrane template (Figure 48) following the procedure described in Chapter 6, and the NC membrane cut out into strips. The dot-ELISA procedures were also as previously described (Chapter 6, section 6.3.7) except that the shaking of the slot incubation trays was accomplished manually.

**Figure 51**

Sorting *G pallidipes* into two batches, for testing for infecting trypanosomes in the mouthparts. Flies in 'Batch 1' were tested with a *T. congolense* specific MoAb, whereas flies in 'Batch 2' were tested with a *T. vivax* specific MoAb.



## 7.4 Results

### 7.4.1 Detection of trypanosome infections in tsetse flies using the dissection method

The mouthparts, salivary glands and midguts of 104 *G. pallidipes* (44 males and 60 females), and 390 *G. longipennis* (154 males and 236 females) were dissected and examined for trypanosomes. Two of the *G. pallidipes* flies (1.9%), both of them females, were found to be infected with trypanosomes in the midguts (Table 33), and no trypanosomes were seen in the mouthparts or salivary glands of the same flies. Trypanosomes were not detected in any of the *G. longipennis*.

### 7.4.2 Detection of trypanosome species in tsetse flies using the dot-ELISA

The mouthparts, salivary glands and midguts of all the dissected tsetse flies were also tested using the dot-ELISA.

Of the 104 *G. pallidipes* that were tested, 8(7.7%) were positive for *T. congolense* in the midgut (Table 33). These included the two flies which were determined by the dissection method to be infected with trypanosomes in their midguts. Gut samples from all the 8 flies reacted positively with the *T. congolense* specific MoAb as well as with the *Nannomonas* subgenus-specific MoAb (Table 34). No *T. vivax* or *T. brucei* antigens were detected in any of the organs that were tested.

Out of the 390 *G. longipennis* tested using the dot-ELISA, 17(4.3%) were positive for trypanosome infections in the midguts (Table 33). Nine of those 17 midguts gave positive reactions with *T. congolense* and the *Nannomonas* specific MoAbs (Table 35), whilst the remaining 8 midguts tested positive with only the *T. congolense* specific MoAb (Table 35).

**Table 33**

**Detection of trypanosome infections in the midguts of *G. pallidipes* and *G. longipennis* using the dissection and dot-ELISA techniques**

| Tsetse species | Number tested | Dissection | Dot-ELISA |
|---|---|---|---|
| | | Number (% positive) | Number (% positive) |
| *G. pallidipes* | 104 | 2(1.9%)* | 8(7.7%) |
| *G. longipennis* | 390 | 0 | 17(4.3%) |
| Total | 494 | 2(0.4%) | 25(5.1%) |

* Infections detected only in the midgut by the fly dissection method.

**Table 34**

**Detection and differentiation of trypanosome species in the midguts of *G. pallidipes* using the dot-ELISA**

| Tsetse flies detected by assay | Reactivity of tsetse midguts with different trypanosome species-specific MoAbs | | | |
|---|---|---|---|---|
| | *T. congolense* | *T. brucei* | *Nannomonas* | *T. vivax* |
| 1 | + | | + | - |
| 2 | + | | + | - |
| 3* | + | | + | - |
| 4 | + | | + | |
| 5* | + | - | + | |
| 6 | + | | + | |
| 7 | + | | + | |
| 8 | + | | + | |

* Tsetse flies with infected midguts as determined by the dissection method.

**Table 35**

**Detection and differentiation of trypanosome species in the midguts of *G. longipennis* using the dot-ELISA**

| Tsetse flies detected by assay | Reactivity of tsetse midguts with different trypanosome species-specific MoAbs | | | |
|---|---|---|---|---|
| | *T. congolense* | *T. brucei* | *Nannomonas* | *T. vivax* |
| 1 | + | | + | |
| 2 | + | - | + | - |
| 3 | + | | + | - |
| 4 | + | | + | - |
| 5 | + | | + | - |
| 6 | + | | + | - |
| 7 | + | | + | - |
| 8 | + | | + | - |
| 9 | + | - | + | - |
| 10 | + | | | - |
| 11 | + | - | - | - |
| 12 | + | | | - |
| 13 | + | | | - |
| 14 | + | | | - |
| 15 | + | | | - |
| 16 | + | | | - |
| 17 | + | | | - |

### 7.4.3 Problems encountered, and special observations made during tl performance of the dot-ELISA under field conditions

In the laboratory, all incubations in the dot-ELISA were made slot trays under continuous shaking on an electrical powered gentle rocke Under field conditions, the unavailability of electrical power necessitate improvisation. Consequently, shaking of the slot trays during the variou incubation steps in the first dot-ELISA performed in the field wa accomplished manually by tilting the trays in a rocking motion continuousl for 1-2 min, and then allowing the trays to stand for 15 min before repeatin the shaking. The reactivity of the specific MoAbs on the control trypanosom dots included in the test were, however, weaker than those obtained in th laboratory using the electric rocker. In order to eliminate the effect c interrupted shaking on the reaction intensities, an improvised technique wa tested. The improvisation consisted of suspending the incubation trays on rope that was tied to the branch of a tree, and swinging to emulate the simpl pendulum motion (Figure 52). In addition, the trays were rocked manually a before. This procedure increased the intensity of the reactions in the dot ELISA.

**Figure 52.** Field improvisation of a rocker during incubations. Note the suspended tray.

## 7.5 Discussion

The aim of the studies conducted in this Chapter was to evaluate the applicability of the dot-ELISA in terms of its ability to detect and identify trypanosome species involved in natural infections in the vector, when the assay was performed under field conditions. The field conditions under which the dot-ELISA was evaluated, constituted a realistic situation likely to be encountered in other tsetse infested areas.

The NC membrane strips, applied with control trypanosomes, and the reagents and buffers proved to be stable over the period of one week that they were used in the field. The presence of strong winds, coupled with the abundance of sandy dust, brought about contamination of reagent solutions with dust and plant debris during the performance of the assays. However, this contamination, together with the high ambient temperatures (22 to 31$^{o}$C) prevailing during the assays, did not adversely affect the results. This indicated that the dot-ELISA was robust as far as conditions such as temperature and dust were concerned.

In this study, only 2 midgut infections were detected in a total of 494 tsetse flies that were screened using the dissection method, on the Galana Ranch. However, in an earlier study conducted on the same ranch by Wilson, Gatula, Njogu, Mgutu and Alushula (1986), *T. vivax*, *T. congolense* and *T. brucei* infection types were all detected in tsetse flies, using the dissection method, with *T. vivax* infections being the most common. The most likely explanation for these differences in observations made in the present study and that of Wilson *et al.* (1986), is that, the tsetse and trypanosomiasis control programme being implemented on the ranch (Opiyo, Njogu and Omuse, 1990) may have altered the status of trypanosome infections and transmission.

This field study revealed that the trypanosome infection rate in *G. pallidipes* was higher than that in *G. longipennis*, regardless of the technique

used. This finding was in agreement with an earlier report which showed that *G. pallidipes* was the major vector of trypanosomiasis on the Galana Ranch (Opiyo, Dolan, Njogu, Sayer and Mgutu, 1987).

The identity of the trypanosome species involved in the two midgut infections which were detected in *G. pallidipes* could not be determined using the dissection method, since there were no accompanying infections in the mouthparts or salivary glands of the two flies. Using the dot-ELISA, however, it was possible to identify *T. congolense* as the parasite species involved, since the parasites reacted with both the *Nannomonas* and the *T. congolense* specific MoAbs. Furthermore, it was possible to ascertain the absence of *T. brucei* in those infections. Unfortunately, at the time that this study was conducted, the *T. simiae* specific MoAb reported in Chapter 4 had not been derived, and was not, therefore, included in the evaluation.

It was also found that the dot-ELISA detected *T. congolense* antigens in the midguts of some parasitologically negative flies. This was a new observation that could be attributed to three possible causes, namely: (1) correct identification of infected tsetse by the dot-ELISA as a result of differences in sensitivity between the two tests, (2) false detection of uninfected tsetse due to reactivity of MoAbs with circulating *T. congolense* antigens present in the blood meal ingested from infected hosts, and (3) false detection of uninfected tsetse due to reactivity of MoAbs with trypanosome antigens originating from ingested whole bloodstream form trypanosomes that were unable to establish an active infection. However, this observation would appear to indicate that the dot-ELISA had a higher sensitivity compared with the tsetse dissection method. A statistical comparison of the dissection technique with the dot-ELISA using Chi-square analysis, indicated that the two tests were significantly different in their ability to detect trypanosomes in tsetse midgut, at $P<0.05$. This possibility of diagnosing trypanosome infections in the midguts of tsetse flies, even though no infecting parasites are seen using

the dissection method, has not been indicated or discounted in the use of the recombinant DNA technique. This is simply because, in all the studies that the recombinant DNA technique was tried in the field, only flies shown by the dissection method to be infected with trypanosomes, were tested.

It was also interesting to note that gut samples from some of the parasitologically negative tsetse flies could react with the *T. congolense* specific MoAb without reacting with the *Nannomonas* subgenus-specific MoAb in the dot-ELISA. Three likely explanations could be offered for this unusual reactivity. Firstly, it could be due to differences in the levels of biochemical substances such as enzymes which are able to affect the integrity of the epitope on the *Nannomonas* subgenus-specific antigen, without affecting the *T. congolense* specific antigen. If this were true, it would mean that the species of tsetse may be an important factor, since the observation was associated with *G. longipennis*, but not *G. pallidipes*. Secondly, it could be that the *G. longipennis* were infected with different *T. congolense* variants, some of which may not be expressing the *Nannomonas* subgenus-specific antigen detected by the MoAb. This possibility is however unlikely, since the *Nannomonas* specific MoAb was able to react with *T. congolense* organisms isolated from different geographical areas (Chapter 4). Thirdly, the *T. congolense* specific MoAb could be cross-reacting with unidentified antigens present in the tsetse gut. However, this possibility was also thought to be unlikely, since in extensive studies with experimental tsetse flies (Chapter 6), no cross-reactivity was observed.

The results obtained from this limited evaluation of the dot-ELISA clearly showed that the technique was a practical alternative to the dissection method which is currently employed in the diagnosis of trypanosome infections in *Glossina* species. This is especially so, considering the fact that each of the MoAbs used had been shown to react with various trypanosome isolates from different geographical areas. Besides, the materials required can easily be

transported to field locations, and the assay could be performed without the need for electricity. The test is rapid, simple to perform, and will be inexpensive in screening large numbers of tsetse flies at a time. Moreover, it is specific and detects more trypanosome infections in field caught tsetse flies in comparison with the dissection method. These are strong indications that the dot-ELISA could contribute greatly to studies aimed at further elucidating the role played by the tsetse fly in the epidemiology of the African trypanosomiases.

# CHAPTER 8

# GENERAL DISCUSSION AND CONCLUSIONS

Since the discovery that tsetse flies were the main vectors of the African trypanosomiases, attempts have been made to reduce the disease prevalence by vector control and by the use of trypanocidal drugs. These methods have, however, been expensive and relatively unsuccessful. As a result, vast areas of land are still infested with tsetse flies, while areas previously cleared of the fly are prone to reinfestation (MacLennan, 1981). This, together with the emergence of drug resistant strains of trypanosomes (Kupper and Wolters, 1983; Pinder and Authie, 1984), reveals the true extent of the threat posed by trypanosomiasis.

Estimation of the trypanosomiasis risk or challenge to domestic animals or humans, requires the determination of several factors, including tsetse relative density, the proportion of blood meals taken from target hosts, and trypanosome infection rates in tsetse (Lloyd and Johnson, 1924; Challier and Laveisiere, 1973). Assessment of these parameters is currently important, as sites are investigated for trypanosomiasis risk in relation to productivity of trypanotolerant breeds (Leak *et al.*, 1988).

The diagnosis of trypanosome infections in the tsetse fly, at present, is by dissection. This method, however, can only be used to identify the parasites up to the subgeneric level (Hoare, 1972; Stephen, 1986; McNamara and Snow, 1991). It is, therefore, essential to develop a more accurate method that is capable of detecting and differentiating between trypanosome species in infected tsetse flies. In this thesis, the suitability of a MoAb-based approach was investigated.

Several species of parasitic protozoa (including *Plasmodia spp.*, *Leishmania spp.*, *T. cruzi* and *Trypanosoma spp.*) possess species and/or subgenus-specific antigens (Santoro, Cochrane, Nussenzweig, Nardin, Nussensweig, Gwardz and Ferreira, 1983; Flint, Schechter, Chapman and Miles, 1984; McMahon-Pratt, Bennet and David, 1982; Parish, Morrison and

Pearson, 1986; Nantulya *et al.,* 1987). In the case of the *Trypanosoma,* this had been shown by the generation of MoAbs that are specific to the *T. congolense* species, and the *Nannomonas, Trypanozoon* and *Duttonella* subgenera. However, no MoAbs specific to *T. simiae* or any of the subspecies that constitute the *Trypanozoon* subgenus had been produced. In studies described in Chapter 4, a MoAb specific to *T. simiae* (KNS7/14.X) was produced using spleen cells from a BALB/c mouse that had been immunized with purified *T. simiae* antigens obtained using the purification procedure described by Ijagbone *et al.,* (1989). The production of a *T. simiae* specific MoAb has shown for the first time that both trypanosome species within the *Nannomonas* subgenus (*T. congolense* and *T. simiae*), possess immunogenic species-specific antigens that could be used to differentiate between them.

The potential usefulness of the trypanosome species-specific MoAbs as diagnostic reagents was demonstrated through their reactivity with vector stages of the parasites that had been isolated from different geographical areas (Chapter 4). In those experiments, it was recorded that the majority of the MoAbs could also detect the bloodstream forms of the target parasite species, indicating that some of the MoAbs may also be useful in the diagnosis of trypanosomiasis in the mammalian host. A similar observation was made by Nantulya and co-workers (1987) for MoAbs that they produced against membrane antigens of procyclic trypanosomes, and which were later utilized in developing diagnostic assays for detecting circulating bloodstream trypanosome antigens in both infected humans and animals (Nantulya and Lindqvist, 1989; Nantulya, 1989; Nantulya, Doua and Molisho, 1992).

Since it had previously been established that procyclic tsetse midgut forms and culture forms of the African trypanosomes express similar antigens (Richardson *et al.,* 1986; Pearson, Moloo and Jenni, 1987), efforts were first initiated to establish a simple, sensitive and specific MoAb-based assay that could detect and differentiate between culture derived *T. brucei, T. congolense*

and *T. simiae* procyclics, and *T. vivax* epimastigotes. The NC membrane-based dot-ELISA was the preferred choice. The reason for this was that, this technique had been shown to be simple, sensitive, specific and field portable (Pappas, 1988a). Ensuing experiments led to the development and standardization of a simple, sensitive and specific NC membrane-based dot-ELISA (Chapter 5) that utilized trypanosome species-specific MoAbs in the detection and differentiation of *in vitro* propagated forms of the aforementioned trypanosome species. In these experiments, the dot-ELISA correctly identified the trypanosome species in both single and artificially mixed trypanosome populations. This finding showed that the trypanosome species present in artificial cultures could be ascertained using the dot-ELISA. This was considered important since culture derived trypanosomes were becoming increasingly utilized in trypanosome research (Ross and Taylor, 1990). Hence, there was the need for simple reliable techniques that could be used in identification and confirmation of the propagated trypanosome species. The finding also indicated that the assay might be suitable for detecting and differentiating between trypanosome species in infected tsetse flies.

Genotypic diversity had been recorded among members of the *Nannomonas* subgenus, especially the *T. congolense* species (Majiwa, Masake, Nantulya, Hamers and Matthyssens, 1985; Majiwa, Hamers, van Meirvenne and Matthyssens, 1986). As a result of this diversity, efforts aimed at producing a DNA probe that could hybridize to all the intra-species variants of *T. congolense*, have been unsuccessful.

As reported in Chapter 4, the *T. congolense* specific and *Nannomonas* subgenus-specific MoAbs reacted with all the *T. congolense* isolates including the Savannah, Kilifi and riverine-forest types that were tested. This result showed that despite the reported genotypic differences, these *T congolense* genotypes were closely related antigenically, thus

suggesting that the MoAb approach to diagnosis of trypanosome infections in *Glossina* may have an advantage over the recombinant DNA technique.

The gut of an infected tsetse fly contains trypanosomes as well as digestive residue originating from ingested blood meal. The digestive residue is normally pigmented, mostly by haematin which is split off the haemoglobin molecule early in the digestion of tsetse blood meal, but which is not absorbed (Bursell, 1970). Efforts to test tsetse gut samples for the presence of infecting trypanosomes using the NC membrane-based dot-ELISA (Chapter 5) encountered non-specific background. This background was attributable to two likely causes (Chapter 6); namely, (1) staining of NC membrane by pigmented gut samples, and (2) false reactivity resulting from the oxidation of the chromogenic substrate (DAB) by haem. The non-specific stains were removed using hydrogen peroxide ($H_2O_2$) as a destaining agent in a modified dot-ELISA, without any significant effect on the integrity of the trypanosome species-specific diagnostic antigens (Chapter 6). This ability of $H_2O_2$ to decolorize NC membrane applied pigmented gut samples was attributed to its ($H_2O_2$'s) oxidative degradation of haem (Jackson, 1974), and of haematin (Chapter 6). It should be of interest to further investigate the ability of $H_2O_2$ to decolorize NC membrane applied infected gut samples as well as faecal material obtained from other haematophagous arthropod vectors, such as the *Triatomine bugs* that transmit *T. cruzi*. This could lead to further development of this $H_2O_2$ innovation and allow maximum utilization of colorimetric assays, which are generally simple and can be read visually (without the aid of sophisticated equipment).

The modified dot-ELISA was shown to be capable of identifying *T. brucei*, *T. congolense* and *T. simiae* organisms in the guts of experimentally infected tsetse flies. The assay had an overall sensitivity greater than 90 percent and a specificity greater than 99.9 percent. This successful detection of trypanosome species in the guts of laboratory-colonized tsetse flies,

suggested that the modified dot-ELISA would be even more readily applied in the detection of trypanosome species in wild tsetse flies which are known to digest their blood meals faster (Langley, 1967a).

Two main trypanosome species (*T. brucei* and *T. suis*) are known to infect the salivary glands of the *Glossina* species (Hoare, 1972; Mulligan, 1970). In this study, a *T. brucei* species-specific MoAb was employed in the dot-ELISA for the identification of *T. brucei* in experimentally infected tsetse salivary glands (Chapter 6). However, the assay could not detect the parasites in the target organ when PBS or PSG were used as sample buffer, even though these buffers were the preferred choice for the preparation of tsetse gut samples for testing with the dot-ELISA (Chapter 6). This observation was explained as below: Tsetse saliva is known to contain several enzymes and inhibitors including an antithrombin (Parker and Mant, 1979) and fibrinolytic proteases (Endege *et al.*, 1989). These enzymes and inhibitors were believed to affect trypanosome antigens in salivary glands during sample preparation, leading to interference with the dot-ELISA. Investigations conducted into the use of alternative sample buffers showed that 5mM $Na_2EDTA$ was suitable for treating tsetse salivary glands prior to testing with the dot-ELISA (Chapter 6). The successful use of $Na_2EDTA$ for this purpose, was explained as follows. $Na_2EDTA$ is an enzyme inhibitor. Its use in the sample buffer may, therefore, have been necessary to inactivate salivary gland enzymes that affected the *T. brucei* diagnostic antigen or interfered with the reaction between specific MoAb and the target antigens.

*Trypanosoma suis* had been found on only three separate occasions since its discovery (Stephen, 1986). As a result, suitable antigens of this parasite could not be obtained for immunization and production of specific MoAbs to this species. However, the opportunity to replicate test samples from tsetse salivary glands for testing with the dot-ELISA, showed that should the need arise and specific MoAbs made against *T. suis*, this parasite could

also be tested alongside *T. brucei* using the dot-ELISA. The sensitivity of the dot-ELISA in detecting *T. brucei* parasites in the salivary glands of tsetse was high (90%), and the specificity was greater that 99.9%, with no cross-reactivity recorded.

The overall sensitivity of the dot-ELISA in detecting trypanosomes in the mouthparts of experimentally infected *Glossina* was low, less than 50% (Chapter 6). This low detection rate was believed to be due to the relatively low number of trypanosomes present in infected proboscides as well as to difficulties in releasing trypanosomes from the proboscis as a result of anatomical peculiarities of this organ. The specificity of the assay was, however, high (greater than 99.9%).

Natural trypanosome infection rates in *Glossina* determined by dissection are usually low, less than 10% as reported by Jordan (1974). Evaluation of the dot-ELISA in the field, revealed similarly low trypanosome infection rates (Chapter 7). In this field study, two tsetse flies that were identified by dissection as infected with trypanosomes in the midguts, were also positive by the dot-ELISA (Chapter 7). Using the dissection technique, such infections were attributable to immature *T. brucei* or *T. congolense* or *T. simiae* (Lloyd and Johnson, 1924). However, using the dot-ELISA, it was possible to tell that those two flies were infected with *T. congolense* (Chapter 7).

The sensitivity of the dissection method had been shown to be below 100% by many investigators. For example, Ward and Bell (1971) performed experiments with the animal sub-inoculation method of revealing mature trypanosome infections in the vector, and reported that feeding *T brucei* infected tsetse flies individually on mice gave transmission rates that were about five times higher than those revealed by salivary gland dissections. Also, Moloo and Kutuza, (1974), used the animal sub-inoculation method to incriminate *G swynnertoni* as the vector of sleeping sickness in an area in

Tanzania, where attempts to find the vector of the disease using the dissection method had failed. In a field evaluation (Chapter 7), the dot-ELISA detected *T congolense* antigens in the guts of tsetse flies that had been assumed to be uninfected as determined using the dissection technique. This high detection rate by the dot-ELISA, could be attributed to superior sensitivity of the technique as compared with the dissection method, thus suggesting that the dot-ELISA could be a better choice for revealing the true extent of the vectoral capacity of the *Glossina* species.

In conclusion, this study provides useful information on the suitability of MoAbs as diagnostic reagents for detecting and differentiating between the vector stages of the African trypanosomes. It also provides evidence that a MoAb-based dot-ELISA could be employed as a practical alternative to the dissection technique which is currently used for diagnosis of trypanosome infections in *Glossina* species.

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