EPIDEMIOLOGICAL INVESTIGATIONS OF BOVINE TRYPANOSOMIASIS IN THE COMMON FLY BELT OF ZAMBIA

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Contents

List of Figures

List of Tables

List of Maps

List of Appendices

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Abstract

The causes of anaemia in cattle are reviewed and it was postulated that trypanosomiasis or malnutrition were the main factors in affecting herd mean packed cell volume (PCV). In 1995/6 ~ 18,000 cattle from 495 herds raised in the common tsetse fly belt of Zambia were examined haematologically, and PCV values were recorded with peripheral blood examined for trypanosomes via the buffy coat smear technique. Giemsa stained thick and thin dried blood smears (T&TS) were also examined. The results were entered into a tailor made Integrated Tsetse and Trypanosomiasis Database and were summarised as mean herd PCV, proportion of herd anaemic and parasitological prevalence. Using a linear regression model, ~ 36% of the variance of the mean herd PCV could be accounted for by parasitological prevalence. A logistic regression of the data gave little improvement. The sensitivities of the buffy coat as well as T&TS examinations were calculated mathematically based on the Poisson distribution and these diagnostic techniques were reckoned to be probably more sensitive than previous work had suggested. The spatial distribution of bovine trypanosomiasis and the herd haematocrit values are displayed using a Geographical Information System (GIS). An indirect enzyme linked immunosorbent assay was developed for the detection of trypanosomal antibodies (Ab-ELISA) in serum using crude somatic antigen from *T. congolense*. The assay was further adapted to carry out tests using circles of dried blood held on filter paper. Inter and intra-assay sources of variation were investigated, as were the effects of sample storage and management. The assay was compared to the indirect fluorescent antibody test, and kinetics of anti-trypanosomal antibody were examined. Twelve thousand blood spot samples were assayed and the data were subject to a rigorous system of quality assurance, with the percentage positivity system of data expression being adopted. The necessary calculations were performed by a computer program. The direct parasitological prevalence and Ab-ELISA sero-prevalence results were compared on a herd basis, and conjectural reasons why there were differences were given. A linear model, with antibody sero prevalence as the independent, only accounted for ~ 50% of the mean herd PCV variance. Protein malnutrition is presumed to account for the remaining 50%. An Antibody ELISA Database was developed for the serological survey results and maps using GIS software are displayed. The costs of direct tests for trypanosomiasis and Ab-ELISA are reviewed and recommendations are given for the development of the assay.

Chapter 1

1. Introduction

The epidemiology of bovine tsetse transmitted trypanosomiasis is a vast and complex subject, which has been reviewed on a multitude of occasions. Qualitative studies into the transmission of the parasite, the vector biology and control, the host and the inter-relation of these factors continue to consume vast resources in Africa and elsewhere. Although the causal agents of African Animal Trypanosomiasis (AAT) have been recognised for almost a century - the diagnosis of the condition, especially under field conditions in Africa where skilled staff and equipment are frequently restricted, is problematic. Not only do staff need to be trained in the correct epidemiological sampling techniques, but also they need to use a quick, reliable diagnostic test. The standard field diagnostic tests used in Zambia[1] are of limited sensitivity, and yet it is important to detect disease, and hence the circumstantial evidence of the presence of the tsetse fly vector, especially when fly challenge is low.

Epidemiology can be defined as "the study of disease in populations and of factors that determine its occurrence" or "the study of the occurrence of disease" (Thrusfield, 1995) or "the study of disease in populations" (Putt *et al.*, 1988). According to Thrusfield, there are five objectives in epidemiology: i) determination of the origin of a disease whose cause is known ii) investigation and control of a disease, whose cause initially is unknown iii) acquisition of information on the ecology and natural history of a disease iv) planning and monitoring of disease control programmes and v)

[1] This refers to tests performed by teams attached the Regional Tsetse and Trypanosomiasis Control Programme (RTTCP)

assessment of the economic effects of a disease and analysis of the costs and economic benefits of alternative control programmes.

Qualitative investigations into the epidemiology of trypanosomiasis have been ongoing since Sir David Bruce discovered the causal agent in KwaZulu/Natal, South Africa at the end of the last century , and since the first half of the twentieth century when the transmission of the parasite was linked to the tsetse fly. Most of the studies of vector biology now centre on the use of odours and attractants with which to attract the fly to some insecticide treated bait. The bait may be of artificial material such as cloth, or may be a moving target using cattle that have been treated with some pyrethroid.

Quantitative investigations into the relationships between tsetse distribution and abundance, and trypanosomiasis distribution and prevalence have intensified in the last decade as personal computers and more "user friendly" software have become available in Africa. The use of modelling, using multivariate analytical techniques to predict the distribution of the vector using remotely sensed data sets is a rapidly developing science, as is the use of Geographical Information Systems to develop some rational decision making process. Quantifying the costs and benefits of AAT disease control however is a relatively new doctrine, which has only recently been addressed by economists (Barratt, 1994; Daniels, 1996).

Planning and monitoring AAT control programmes requires that the distribution and morbidity of the disease first be determined and evaluated. This is necessary to ascertain not only the endemicity of disease and hence to what extent it is a constraint to animal health and thus rural development, but also to gauge the fly species and density in an area - whether some form of vector control is ongoing or not. Knowledge of the severity and distribution of disease or the distribution density, and species of the vector are a pre-requisite to the decision making process of planning where and how to control the disease or vector in a cost effective, strategic manner. The distribution of disease or vector have historically been portrayed by the use of

paper (hard copy) maps. These are invariably at a variety of scales and possibly projections, making interrogation and decision making problematic. Geographical Information Systems are a relatively new science for managing spatial and none spatial data, as well as providing cartographic output and modelling for spatial data sets (Burrough, 1986). In this thesis, survey results are frequently presented in a GIS format as this is a natural progression for ease of presentation of results and for their subsequent manipulation and analyses.

Epidemiological investigations into rationalising trypanosomiasis and tsetse control priorities using a GIS are worthy of a topic for a thesis on their own. In the decision making process for planning tsetse and trypanosomiasis control operations, data sets in addition to tsetse species, density and distribution and trypanosomiasis distribution and level of disease are required in order to apply some prescriptive decision making process (Eastman *et al.*, 1993; Robinson, 1995).

The detection of the tsetse fly vector and trypanosomiases in cattle is problematic. Tsetse of differing species and habits are notoriously difficult to sample when challenge is low and in Southern Africa their detection mostly relies on the use of stationary traps for *G. pallidipes* and *G. brevipalpis* and mobile devices for *G. morsitans*. These methods are usually only able to detect tsetse fly above certain densities and are subject to sampling bias. Improving sampling techniques for tsetse is the topic of much past and future research (RTTCP Regional Office, 1996). In low challenge areas, detection of disease in livestock is a more sensitive indicator for the presence or absence of the vector. A pre-requisite for this however, is that livestock be present in an area in order for the trypanosomiasis disease to be detected. Thus if livestock are not being raised under traditional farming practices this may necessitate the introduction of sentinel herds.

Two differing approaches were investigated to overcome the limitations of the standard RTTCP trypanosomiasis detection methods. An antibody trapping ELISA was developed and cattle haematocrit values were analysed, in order to investigate whether this indirect diagnostic test or herds' haematocrit distributions could be used to predict trypanosomiasis in a herd. These investigations are central to this thesis.

Most epidemiological studies of trypanosomiasis or the use of a developmental diagnostic technique are based on the analyses of historic data sets. Although some historic survey data are presented in this thesis, much of the data is contemporary, and as such this is a unique study of the development of new techniques to detect bovine trypanosomiasis in an African country. The antibody ELISA was actively developed from 1994 - 6 as was the use of eluted blood spots as the source of antibody. The data management systems for survey results and assay quality controls checks for the antibody ELISA were developed as these data were being collected and processed.

Chapter 2

2. A Review of the literature and of the epidemiology of bovine trypanosomiasis with special reference to Zambia

2.1. The epidemiology of African Tsetse transmitted bovine trypanosomiasis of the Common Fly Belt of Zambia

2.1.1. The parasite life cycle

Jordan (1986) has reviewed the life cycle of the parasite whilst its taxonomy, morphology, biology and cyclical transmission have been fully reviewed by Mulligan (1970), Willett (1970), Hoare (1972) and Brown *et al.* (1990). The trypanosome life cycle involves an alternation between two hosts, although mechanical transmission of *T. vivax* has been reported in Africa and is the normal mode of transmission of the parasite in the New World. Classically, the protozoa are maintained in some sylvatic reservoir in wild animals, where they cause little or no disease, and are transmitted to domestic stock after undergoing cyclical transmission in the tsetse fly vector. In areas where game has been largely eliminated and (trypanosome infected) livestock exist above some sufficient threshold, transmission between livestock by tsetse also occurs. This is an important factor in the maintenance of the disease in the Eastern Province of Zambia (Van den Bossche, personal communication) where there are heavy challenges of fly, cattle densities are high, and moderate to high infection rates in the cattle exist.

2.1.2. The parasite

Trypanosomes are haemoflagellate parasite protozoa belonging to the genus *Trypanosoma* Gruby, 1843. The systematic position of trypanosomes is as follows (Levine, 1973).

Phylum	Protozoa
Subphylum	Sarcomastigophora
Superclass	Mastigophorasica
Class	Zoomastigophorasida
Order	Kinetoplastorida
Suborder	Trypanosomatorina
Family	Trypanosomatidae
Genus	Trypanosoma

In Southern Africa, there are three species of salivarian trypanosomes which are cyclically transmitted to cattle by tsetse fly (genus *Glossina*). These three species are *Trypanosoma (Trypanozoon) brucei* Plimmer and Bradford, 1899, *Trypanosoma (Nannomonas) congolense* Broden, 1904 and *Trypanosoma (Dutonella) vivax* Ziemann, 1905 (Mulligan, 1970). The disease these parasites cause in livestock is called *nagana* whilst in man the disease due to *T. brucei* is called human sleeping sickness. Tsetse fly also cyclically transmit *T. simiae* and *T. suis* to pigs in West Africa. Mammalian trypanosomes do not manifest a strict host tropism and can infect a variety of domesticated livestock species, as well as wildlife.

2.1.3. The vector

Of the twenty-three species and sub species of *Glossina* occurring in Africa, only three species of tsetse fly occur in the Common Fly belt of Zambia; *G. pallidipes*, *G. morsitans morsitans* and *G. brevipalpis*. *G. pallidipes* and *G. morsitans* belong to the savannah species of fly, whilst *G. brevipalpis* belongs to the riverine group, especially in the Luangwa valley. *G. morsitans centralis* occurs in the fly belt to the west of the Common Fly Belt which conjoins the fly belts of the Congo drainage

system and West Africa. *G. fuscipes* is confined to the shores of lakes Mweru and Tanganyika and the Luapula River (Everson and Kathuria, 1982) in the north of Zambia.

2.1.4. The host

The disease caused in cattle is of greatest importance in Zambia, as goats have a degree of innate resistance and are of less economic and social importance. In each herd, castrated males (oxen, syn. tollies) and sometimes cows are trained as traction animals. Their health ensures thorough and timely tillage of the land, as well as weeding and transport of the crop at harvest time and fertiliser during the growing season. Although cattle are also cited as important sources of meat and manure, in reality they are rarely culled for income generation nor is their manure used as a fertiliser (personal observation). They are valued as a means of wealth acquisition (including use for *lobola* syn. bride dowry) and little offtake is sustained on a routine basis. This may in part be cultural, or in part because marketing and infrastructure are not well developed in Zambia, which is one of the poorer developing countries in Africa.

2.1.5. The Farming practices in Zambia

One cannot embrace the subject of epidemiology and control of a disease unless farming practices and certain socio-economic factors are considered. Zambians practice a sedentary farming practice based mostly on seasonal maize cultivation, with vegetables being grown perennially. Maize is the favoured crop in Zambia, although sorghum is grown in the drier Zambezi valley. In some areas, primarily the wetter Northern Province where the predominant vegetation type is that of the *brachystygia* broad leafed woodland (syn. miombo) and the soil is leached of minerals, a slash and burn farming regime (syn. *chitimani*) is frequently employed.

In Zambia, there is one rainy season lasting from November to April. The other marked seasons are cold and dry (May to August) or hot and dry (September and October). These seasons affect the tsetse distribution and challenge (and hence the disease risk) as well as cattle grazing and husbandry practices, as the stock seasonally seek water and fodder.

2.2. History of tsetse and trypanosomiasis distribution in Zambia with special regard to the Common Fly Belt

Zambia is a land locked country which covers an area of some 742,400 km^2 in extent and lies between the lines 22° and 34° East of Greenwich and 8° and 18° south of the equator. The greater portion consist of a plateau averaging about 1000 to 1200 metres above sea level. Much of the country is drained by the Zambezi River system, of which the three large tributaries are the Kabompo, Kafue and Luangwa rivers. Most of the Northern and Central Provinces are incorporated in the Congo drainage system. Annual rainfall varies considerably from 50 cm in the south to 150 cm in the north.

There have only been two general surveys in Zambia for tsetse and trypanosomiasis, these having been conducted between 1947 and 1955 under the direction of the Department of Game and Tsetse Control (Steel and Gledhill, 1955; Everson and Kathuria, 1982) and by the European Union funded Regional Tsetse and Trypanosomiasis Control Programme (RTTCP) between 1986 and 1996. Maps of the distribution in intervening years (1968 and 1972) were compiled using data gathered from the Department of Veterinary and Tsetse Control (DVTCS) annual reports. There are six main tsetse fly belts (Map 1) which are as follows (Everson and Kathuria, 1982):

i. The far western belt, connecting with the drainage system of the Kabompo, West Lunga, Musondwezi and Donge rivers

ii. The mid-western fly belt covering the drainage system of the Luswishi, Lunga and Kafue rivers and extending in the south to certain rivers which drain directly into the Zambezi *

iii. the eastern fly belt associated with the Luangwa and Luano valleys but extending for considerable distances on the plateau on its eastern and southern borders

iv. The southern fly belt with a somewhat broken distribution along the valley of the Zambezi valley

v. the mid-northern fly belt in the drainage area of the Chambeshi and Luapula rivers

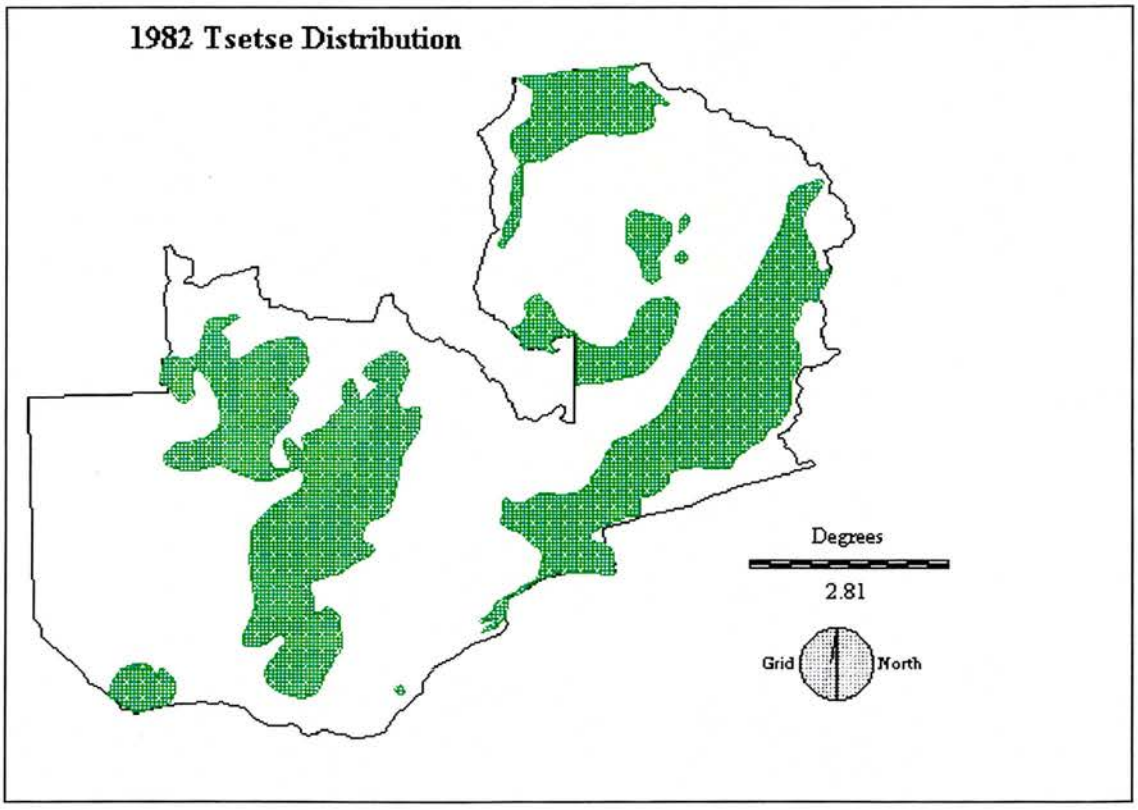
vi. The far-northern fly belt extending between the great lakes of Tanganyika and Mweru.

* recent evidence (Lumamba, personal communication) suggests that belts (i) and (ii) are now contiguous although no survey has confirmed or refuted this.

The eastern fly belt (Map 2) is contiguous with a discrete belt of some 320,000 km^2 which also lies in the Kasungu national park in Malawi, much of Mozambique, and the Zambezi valley of Zimbabwe (Connor, 1989) which is hence referred to as the Common Fly Belt of Southern Africa. The distributions, as reported on the maps dated 1955, 1968, 1972 and 1986 - 93 are given in Appendix 1.

Map 1.1 The 1982 Tsetse distribution map of Zambia of based on the surveys conducted by Steel and Gledhill (1955) as updated by Everson and Kathuria (1982)

Legend Green - areas where tsetse occur



MAP I.2APPROXIMATE DISTRIBUTION OF TSETSE IN THE COUNTRIES OF THE RTTCP (REVISED IN 1994)

Tsetse and trypanosomiasis control have been of major concern to Zambian Government Officials since the turn of the century. To control trypanosomiasis the Veterinary Department relied on the treatment of affected animals. Early tsetse control activities were conducted by the Game and Tsetse Control Department via bush clearing, elimination or control of wild animals hosts of tsetse and the use of fly-sheds. In 1959, Tsetse Control was merged with the Veterinary Services to form the Department of Veterinary and Tsetse Control Services (Connor, 1989), and with an estimated four fifths of the country infected by tsetse, trypanosomiasis was and still is possibly the largest single animal disease problem[2]. Although the range of tsetse control measures that was used increased to include the use of game exclusion fences as well as insecticides, applied both from the ground and aerially, the problem remained serious. It was necessary to administer trypanocides to large numbers of cattle to prevent enormous losses. In 1962, 222,097 doses of trypanocide were used, a figure which rose to 390,740 treatments by 1966 (DVTCS Annual reports), most of which (225,000) were administered to cattle in Eastern Province. In the mid 1970's there was an aerial spraying operation using endosulphan in the area of Eastern Province to the south of the district town of Petauke.

In 1978 almost 750,000 trypanocidal treatments were administered, but with budgetary constraints facing the Department, tsetse and trypanosomiasis control activities could not be sustained. Thus the 594,316 treatments administered in 1984 were only made possible by a donation received from the Food and Agriculture Organisation of the United Nations (FAO). By that time tsetse had now spread into hitherto uninfested areas and the Department sought further external support to contain the deteriorating situation. Emergency assistance was received from the FAO, in the form of large supplies of trypanocides which were delivered in 1985 and 1986 (Connor, 1989).

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[2] Since 1994, a widescale epizoötic of theileriosis may have relegated trypanosomiasis to the disease of secondary national herd health importance.

Meanwhile, Zambia's participation in the RTTCP had been negotiated and a Financing Agreement was signed with the European Commission (EC) in 1985. However, soon afterwards, another Financing Agreement was signed with the EC under the Special Action Programme for the Fight Against Hunger in the World, which was to remove tsetse from an area on the edge of Zambia's Kafue fly belt in the Choma, Kalomo and Namwala districts. This aerial spraying operation was carried out in 1987. A target barrier was erected at the end of spraying operations, but due to insufficient funds within the DVTCS, the barrier was uplifted in 1994.

RTTCP was planned with three phases: a preparatory phase primarily to map the distribution of fly and disease, to conduct research and asses control measures and land use issues; an implementation phase to eradicate fly from the entire Common Fly Belt and evaluation phase to monitor land use and change. Due to innumerable delays in the whole region, RTTCP in Zambia never progressed beyond Phase 1 but did manage to deploy insecticide treated, odour baited targets against tsetse in 600 km^2 of the Lower Gwembe (also called Lusitu) area in the Zambezi Valley of Southern Province, an 850 km^2 ribbon adjoining the Zambezi river to the north of Lusitu, and a 945 km^2 area to the south of the Great East Road in the Petauke District of Eastern Province. In 1994, the operational area in Eastern Province was expanded by 900 km^2 north of the great East Road (Msanzara area) with targets being deployed and maintained by the community (RTTCP Zambia, 1996). In the Senanga West District of Western Province, a target control operation is underway with local community involvement and financial and technical assistance from the Dutch Government.

2.3. Reasons for detecting trypanosomiasis and factors affecting detection of the disease

Generally trypanosomiasis is detected for the following reasons (Hopkins, 1995):

i. for individual and herd health diagnosis and treatment
ii. to detect the presence or absence of disease and hence the presence of the tsetse fly vector
iii. to determine the endemicity of disease and hence to what extent trypanosomiasis is a constraint to animal production and thus the cause of economic loss and hence rural development which in turn leads to the decision making process for control strategies
iv. for research purposes
v. for legislative reasons, if the disease is notifiable.

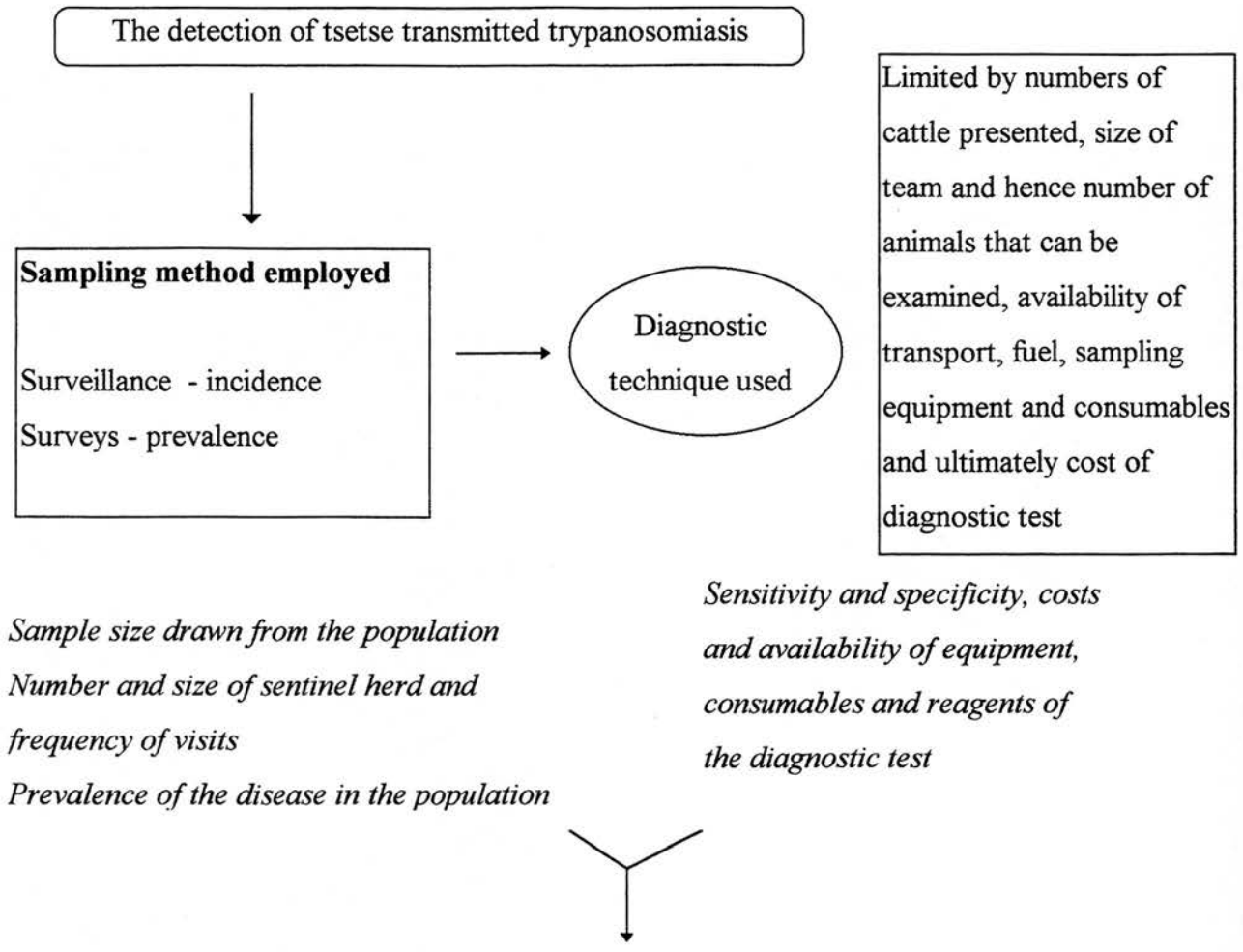
Because the detection of disease is more sensitive than the detection of the vector, it is the preferred method of detecting tsetse challenge in low fly density areas. A pre-requisite however is that cattle (or livestock) are present and the disease picture is not distorted by the overt or covert use of trypanocides (Hopkins, 1995). General surveys may be carried out at the national or provincial level. Before a control programme is implemented their purpose may be to determine baseline data on infection rates and thus whether subsequent annual fluctuations are due to natural changes of season or due to some control measure. During control trypanosomiasis detection can be used to measure the efficacy of the control measure and post control it can be used to detect the possible re-emergence of a tsetse population (Hopkins, 1995).
Some advantages and disadvantages of survey/surveillance for trypanosomiasis vs. sampling for the tsetse fly and why the detection of trypanosomiasis disease is a more sensitive indicator for the presence or absence of the tsetse fly vector are given in Table 2.1.

The major factors affecting the detection of trypanosomiasis (or indeed any disease) are shown diagrammatically in Figure 2.1, these being the (statistical) sampling method(s) used to sample animals from the population and the once the population has been sampled, the diagnostic technique(s) employed. The diagnostic techniques available for AAT are reviewed in Paragraph 2.4, whilst the sample size required to detect or attach confidence limits to disease levels are described in the standard veterinary epidemiology texts (Cannon and Roe, 1982; Putt *et al.* 1988; Thrusfield, 1995).

Table 2.1 A table of the advantages and disadvantages of survey/surveillance for trypanosomiasis vs. sampling for the tsetse fly and postulated reasons why trypanosomiasis detection may be a more sensitive indicator for the presence or absence of tsetse fly

Disadvantages	Advantages
Trypanosomiasis detection requires cattle (or goats) to be present in an area. Goats are somewhat refractory to the disease	Cattle are more attractive than any artificial device
Cost (not known)	Cost (not known)
T. brucei infections are difficult to detect using conventional field diagnostic techniques.	If using certain indirect diagnostic tests, quality control standards can be employed as well precise knowledge of tests' sensitivity and specificity
Relying on the detection of animal trypanosomiasis may require the purchase of sentinel herds.	The single bite from an infected fly may act as *in-vivo* cultivation for the trypanosomes which may be detected by routine diagnostic means.
Ignoring mechanical transmission, the detection of tsetse transmitted trypanosomiasis must, by definition, mean that an animal has been bitten by a tsetse fly	There are however exceptions; trypanocide usage (overt or covert) may mean trypanosomiasis may not be detected by normal direct diagnostic methods (but anti-trypanosome antibodies should still be detectable) also...
	trypanosomiasis may be diagnosed in a population in the absence of tsetse if the cattle have been grazed far away or bought in.
Trypanosomiasis sampling requires some initial knowledge of the cattle population in an area and correct sampling (statistical) methodologies.	Cattle/goats are physically more able to reach inaccessible areas e.g. gullies, dense bush, hills, etc. Antibodies remain in a population.
In areas where *trypanotolerant* cattle are living, there may be no demonstrable parasitaemia, even though cattle are infected and living in a tsetse infested area.	A tsetse team conducting extensive tsetse surveys will only be in an area for a short time whereas livestock will be living there all the year.
It may be difficult to ascertain precisely from where the cattle picked up an infection.	A line of traps manned fly round covers relatively little areas whereas livestock are able to cover a much wider area.
Overt or covert use of trypanocides may result in cattle not being found positive.	In certain circumstances, the edge of a fly belt may advance and retire seasonally. Thus tsetse surveys results will be seasonally dependent. Finding evidence of trypanosomiasis in the area (by direct or indirect methods) will probably be possible in places where tsetse have regressed during unsuitable weather.
Requires reasonably sophisticated equipment e.g., microscopes, microhaematocrit centrifuge, etc..	Vehicle electric nets are probably equally difficult to maintain under general survey conditions

Figure 2.1 Factors involved in the detection of tsetse transmitted trypanosomiasis



Statistical sampling methods for bovine trypanosomiasis (vs. type of sample taken from an individual animal) fall into two categories; surveys during which an estimate of point prevalence is obtained, or surveillance of a group of (ear tagged) animals or sentinel herd, whereby an estimate of the number of new cases hence incidence is determined.

2.4. A Review of diagnostic tests for bovine trypanosomiasis

2.4.1. Introduction

Diagnostic tests for bovine trypanosomiasis have been reviewed extensively. Tests include direct methods (Killick-Kendrick, 1968; Baker, 1970; Robson and Ashkar, 1972; Zwart *et al.*, 1973; Molyneux, 1975; Paris, Murray and Agure, 1980; Paris, Murray and McOdimba, 1982;) or indirect methods (Nantulya, 1990; Luckins, 1992; Connor, 1995; Luckins, 1995; Dwinger, 1997). A list of the available diagnostic tests for bovine tsetse transmitted trypanosomiasis is given in Table 2.2.

Table 2.2 Diagnostic tests for bovine African animal trypanosomiasis

1. Subjective

History, clinical signs and response to treatment

2. Direct (Parasitological)[3]

a. No concentration of trypanosomes
Ear vein wet smear under cover slip or via "hanging drop"
Tail tip capillary wet smear
Giemsa stained thick blood smear examination
Giemsa stained thin blood smear examination
Lymph gland smears

b. Trypanosomes concentrated[4]
Micro-haematocrit centrifuge technique (MHC) - capillary examined intact (Woo)
Buffy coat smear made and examined bright-field, phase contrast or dark ground
Quantitative buffy coat (adaptation of intact capillary MHC)
Capillary concentration techniques
Mini- column anion exchange (DEAE)
Silicone centrifugation technique
Hypotonic shock test and other haemolytic techniques
Double microcentrifugation

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[3] N.B. these can be rendered more sensitive in an individual animal if diminazene is first administered intravenously at 3.5 mg/Kg and blood collected after 5 - 10 minutes. This is the so-called "Berenil flushing" technique (Maxie and Losos, 1977).

[4] The sensitivity of the direct parasitological tests often depends on the species of trypanosome.

3. Sub-inoculation and Xenodiagnosis
Inoculation of suspect blood into rodents and subsequent demonstration of the trypanosomes in the rodents
In vivo culture techniques

4. Indirect (Serological)

a. Demonstration of antibody
- Indirect Fluorescent Antibody Test
- Antibody trapping ELISA
- Immune lysis
- Precipitation on agar gel
- Agglutination on card, latex, charcoal or in tube or flocculation
- Neutralisation of infectivity [5]

b. Demonstration of antigen
- Antigen trapping ELISA

c. Molecular
- Polymerase chain reaction (PCR)
- DNA probes and hybridisation

2.4.2. Subjective methods

In much of Africa where stock are grazed in or near to known tsetse infested areas, subjective diagnosis based on history, clinical signs and response to treatment is probably the only diagnostic technique available for owners and veterinary assistants alike. This technique is expeditious and efficacious (if the disease is due to trypanosomiasis, the correct trypanocide has been given, and there is no drug resistance), and does not rely on the vagaries of Government supplied fuel, transport or drugs. All that is possibly required is the purchase of the trypanocide (if not delivered free of charge by the veterinary services) and the means with which to administer it. Although this diagnostic technique does not elucidate the differential diagnoses of trypanosomiasis, it is the cornerstone of individual or herd disease identification and control in Zambia, if not much of Africa. If the individual or herd

[5] The infectivity of trypanosomes is impaired by exposure to homologous antiserum. Stabilates are incubated with test sera then inoculated into laboratory animals.

responds to therapy the farmer will have overcome the problem, if it does not then more precise disease investigation will be required.

2.4.3. Direct methods

The major advantage of the direct diagnostic methods is that they are 100% specific. Despite the cited sensitivities of the various direct diagnostics tests, when used in the field the reality is that their sensitivities will be heavily influenced by the state of the microscope, its correct configuration, the number of fields examined and magnifying power of the eyepiece and objective, as well as operator skill. The sensitivity of the direct tests can only ever decrease from their most sensitive, as cited by Paris *et al.* (1982). The sensitivity of the standard trypanosome diagnosis techniques, for the three species of trypanosome occurring in cattle are given in Table 2.3.

Table 2.3 The sensitivity of parasitological detection methods in the diagnosis of trypanosomiasis in cattle taken from Luckins (1995) based on data from Robson and Ashkar (1972).

Trypanosome species	No. of animals infected	Proportion of animals positive	
		Blood examination (%)	Mouse inoculation (%)
T. brucei	191	5.2	94.8
T. congolense	409	87.5	43.3
T. vivax.	256	20.2	0 *

* Field strains of *T. vivax* will not grow in rodents

The summary of the work done by Paris *et al.* (1982) to evaluate the parasitological techniques available for the diagnosis of African trypanosomiasis in cattle is given in Table 2.4.

Table 2.4 The threshold sensitivity (in trypanosomes/ml) of different parasitological techniques for the detection of *T. congolense*, *T. vivax* and *T. brucei* (based on Paris *et al.*, 1982)

Techniques	*T. congolense*	*T. vivax*	*T. brucei*
Thin film	2.5×10^4	5×10^4	2.5×10^4
Wet film	1.2×10^4	8.3×10^3	8.3×10^3
Thick film	8.3×10^3	6.25×10^3	5×10^3
HCT Woo	6.25×10^3	1.25×10^3	5×10^2
HCT (dark ground)	2.5×10^2	6×10^2	5×10^2
Mouse inoculation	--	--	1.25×10^2

Little has changed in the development of direct diagnostic techniques in the last fifteen years and as evaluated by Paris *et al.* in 1982, except for the introduction of the technique to examine the tail-tip capillary wet smear. This technique is routinely performed in Zimbabwe (personal observation), and is reported as being as sensitive as the bright field buffy coat technique (Gorrison, personal communication). As such its main advantage is that no generators nor complex micro haematocrit centrifuges are required, and the examination of a blood sample would take a fraction of the time than for a normal buffy coat examination. There is also no need for complex microscopes to be configured for phase contrast or dark ground. There is no requirement for a 12 or 240 volt power supply as an incidental light source (the sun via a mirror) will suffice. However, as the estimation of the degree of anaemia is probably the most reliable indication of the progress of the disease in trypanosome-infected cattle (Hornby, 1952), the single disadvantage with the tail tip capillary technique is that haematocrit data are not gathered.

2.4.4. Indirect methods

Excluding sub-inoculation techniques, indirect methods comprise detection of antibodies raised against the trypanosomes, detection of trypanosomal antigen and molecular manipulations (Connor, 1995).

2.4.4.1. Detection of antibodies

It must be born in mind that antibody detecting methods cannot differentiate between active and recent infections and the duration of antibodies in the blood stream has still not clearly been defined. The breakthrough in immunological diagnoses came with the introduction of primary binding assays for the detection of trypanosomal antibodies. For trypanosomiasis diagnosis in cattle, agglutination tests are now redundant and only the indirect fluorescent antibody test (IFAT) and Antibody ELISA (Ab-ELISA) are being used, albeit on a limited scale. The IFAT technique has not altered much since first described by Bailey, Cunningham and Kimber (1967) for the diagnosis of human sleeping sickness. The test was adapted for use in cattle and evaluated in Eastern Uganda (Wilson, 1969), Kenya (Ashkar and Ochilo, 1972) and Tanzania (Connor and Halliwell, 1987). The technique is still routinely used by the Belgian funded project to give Assistance to the Veterinary Services of Zambia (ASVEZA) in the Eastern Province of Zambia (ASVEZA, 1995). The antigen - antibody bound complex to which a fluorescent dye binds is viewed using an ultra violet light source, and the degree of fluorescence at varying serum (or eluted blood spot) dilutions is scored on some arbitrary scale. The scoring is, however, entirely subjective. IFAT has been shown both sensitive and specific in detecting trypanosomal antibodies in infected cattle, but a stated drawback is that considerable cross trypanosome species reactivity occurs and the test must be performed on the three species of trypanosome to obtain a sensitivity of 94% (Luckins, 1995). Hence, although there is considerable cross reactivity, the results indicate there is a degree of species specificity that requires the use of all three antigens in order to obtain maximum efficiency (Luckins, 1995). The source of antibody for IFAT can be sera,

plasma or eluted blood spot. The development of antibody detection ELISA is reviewed in Chapter 4.

2.4.4.2. Detection of antigen

Using monoclonal antibodies, an antigen trapping ELISA (Ag-ELISA) for the diagnoses of bovine trypanosomiases was first described by Nantulya and Lindqvist (1989). Since that year, work has been ongoing to validate this antigen trapping ELISA (IAEA, 1995; 1997). To date, the assay appears not to have been rationally validated using sera of known provenance collected from African cattle known to be raised under traditional practices in tsetse free areas - nor from sera from trypanosome infected animals with known species and level of parasitaemia. Further validation work is required before this potentially useful diagnostic tool can be relied upon. The assay requires the use of serum (vs. eluted blood spots) from the animal being tested. Latex agglutination tests using whole blood have been described (Nantulya, 1993) but few results are currently available.

2.4.4.3. Molecular methods

Since the early 1990's the techniques of polymerase chain reaction (PCR), relying on isolation and amplification of trypanosome DNA, has become available for trypanosomiasis research. They are certainly highly sensitive and will identify the strain as well as species of trypanosome. As such, they are a very useful research tool but are not yet worthy of consideration of a realistic viable diagnostic technique using blood samples from cattle collected during routine examination.

2.4.5 The objectives of diagnosis

Although the reasons of reasons for detecting trypanosomiasis have been listed in section 2.4, a more focused list of the objectives of trypanosomiasis diagnosis is as follows:

* to study the epidemiology of the disease distribution with a view to developing some strategic approach to a national herd disease problem or the national/international control of the tsetse fly vector
* to investigate individual and herd disease, and thus treatment and control
* to evaluate the efficacy of vector control
* to prove or disprove that eradication has taken place (by long term surveillance or repeated surveys)
* if necessary, to speciate the trypanosomes detected
* to detect drug resistant strains of trypanosomes
* to detect possible reservoirs of *T. brucei* which may act as a zoönotic reservoir
* for epidemiological research, particularly host-parasite inter-relation and effects on animal production
* to identify an individually infected animal and
* for other research purposes.

2.4.6 Why develop diagnostic tests for trypanosomiases?

Bovine trypanosomiasis is characterised in chronic cases by the decline in the number of parasites in the blood stream, often with a concomitant decline in the packed cell volume (PCV). Animals can either succumb to the disease or recover. When recovered they may eliminate the parasite totally from their body system, or it may remain in low numbers in blood or tissue. Under stress, the animal may succumb to infection again. Non patent parasitaemias may also act as a reservoir of infection to biting tsetse fly and thus other animals or possibly man. To complicate matters, *T. brucei* may rarely be detectable in the bloodstream using the standard RTTCP direct diagnostic techniques described in Chapter 3. *T. brucei* infection may not lead to observable sickness nor anaemia thus and may not be diagnosed unless rodent

inoculation is being used. Conventional direct parasitological techniques can only detect the parasite when present in the blood above the densities tabulated above (Paris *et al.*, 1982) and of these techniques, the use of phase contrast is usually favoured. Maintaining microscopes under field conditions is often a daunting task, especially those with complex sub-stage devices and objectives such with the phase contrast system, which soon can loose alignment if not cared for meticulously. The centring of patch stops and condensers to achieve a dark ground configuration also requires considerable operator training. A microscope of robust metallic construction is also required which must be transported in a strong box in the least vibration prone part of a vehicle. Given the need for four wheel drive vehicles and the poor state of most many African roads - then care of field microscopes is fraught with technical problems. The use of micro-haematocrit (MHC) centrifuges requires some generator or inverter[6] to supply 220 volts (again prone to breakdown) although battery operated, hand held and table top MHCs are now available.

The various sensitivities of direct microscopic methods, as cited above, were ascertained under cool, dust free, laboratory conditions. When teams are operating at the inspection site, conditions are very different, especially in the hotter months of the year. This may result in the MHC reaching temperatures that will kill the trypanosomes, precluding their detection and thus compromising the sensitivity of these direct techniques. The presence of ubiquitous African dust on buffy coat smear or on the microscope lenses may result in failure to see trypanosomes, if present. Trypanosomes can be killed for other reasons such as injudicious use of methanol and other chemicals used to clean glass slides. Finally, there is the problem of having adequate trained staff who have the experience, patience and time to screen the smears properly and to recognise trypanosomes on buffy coat or Giemsa stained thick and thin smear. The greater the time taken to examine a buffy coat smear at the examination site will mean fewer animals that can be examined in one morning, and thus a reduced ability to detect disease or attach narrower confidence limits to the

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[6] An inverter is a device for converting 12 volts DC to 220 or 240 volts AC

prevalence of disease, if detected. The **number of animals that can be examined in one morning** is stressed because:

* the ambient temperatures climb towards noon which may cause trypanosomes to be killed due to heat of slides and the MHC rotor
* the farmers will need to send the cattle for grazing and cannot afford to give up a whole day
* the children who often herd the cattle during an examination must report for school
* the skilled staff making the examination cannot be expected to work all day without a break and will want to return to camp for the midday meal and to organise an inspection for the following day, and
* it has been reported that the parasitaemia from the peripheral blood falls around midday (Hornby and Bailey, 1931; Fiennes, 1950 and 1954).

Thus, the time taken to examine blood from one animal is a compromise with how many samples can be examined in one morning. It is for this reason that RTTCP Zambia teams had two trained, experienced microscopists to examine the buffy coat smears.

Although rodent inoculation is part of the cited standard trypanosomiasis diagnostic methods (STDM) as described by Wilson (1969), the practicalities of transporting, identifying individually and by cage, inoculating and screening rodents requires very considerable logistical resources, training and attention to detail. The author would not regard this technique, which is so useful in the laboratory, as a viable routine field diagnostic method during routine trypanosomiasis surveys. One must also bear in mind that *T. vivax* does not grow and only a proportion of *T. congolense* will grow in rodents. There is also the issue of costs of the various diagnostic tests themselves which are discussed in Chapters 3 and 4.

Workers relying on the standard trypanosome detection methods or other routine diagnostic methods identified the poor sensitivity of even the "best" direct diagnostic methods and were perhaps aware of the practical limitations of their routine use in the field. Since the early 1980's, there has been an increase of interest in indirect diagnostic tests - after the IFA test had been developed and assessed in the 70's (Luckins and Mehlitz, 1978). The need for diagnostic tests arose which would overcome the limitations of direct diagnostic tests and yet provide proven and improved sensitivities over direct diagnostic techniques. By using a cadre of well trained and competent staff in central or possibly provincial laboratories, standardised reagents, and specific quality control software for serological tests, it was hoped that the shortfalls of direct diagnostic techniques could be finally overcome. Paris, *et al.* (1982) concluded that for any new diagnostic test to be worthy of consideration, it would have to operate efficiently at levels of less than 10^2 trypanosomes per ml.

Presently, validation of an antigen trapping ELISA is receiving considerable attention (IAEA, 1995; 1997) although other than for identifying animals infected with trypanocide resistant strains of tsetse transmitted trypanosomiasis in cattle - it is difficult to justify why an individual animal diagnostic test is required when the test is performed in some laboratory many weeks later. The development of a "pen-side" test for detecting antigen in cattle remains a laudable goal. There will however be the issue of cost and sustainability of the antigen detecting techniques. Although the antibody trapping ELISA was developed one and a half decades ago (Luckins, 1977a, 1977b) the technique appears to have been "passed over" as a method for conducting widescale epidemiological surveys for trypanosomiasis in Africa. For this reason, this arguably simpler and more sustainable assay for a developing country was investigated. Molecular techniques will remain the domain of the well funded research worker in Africa for the foreseeable future.

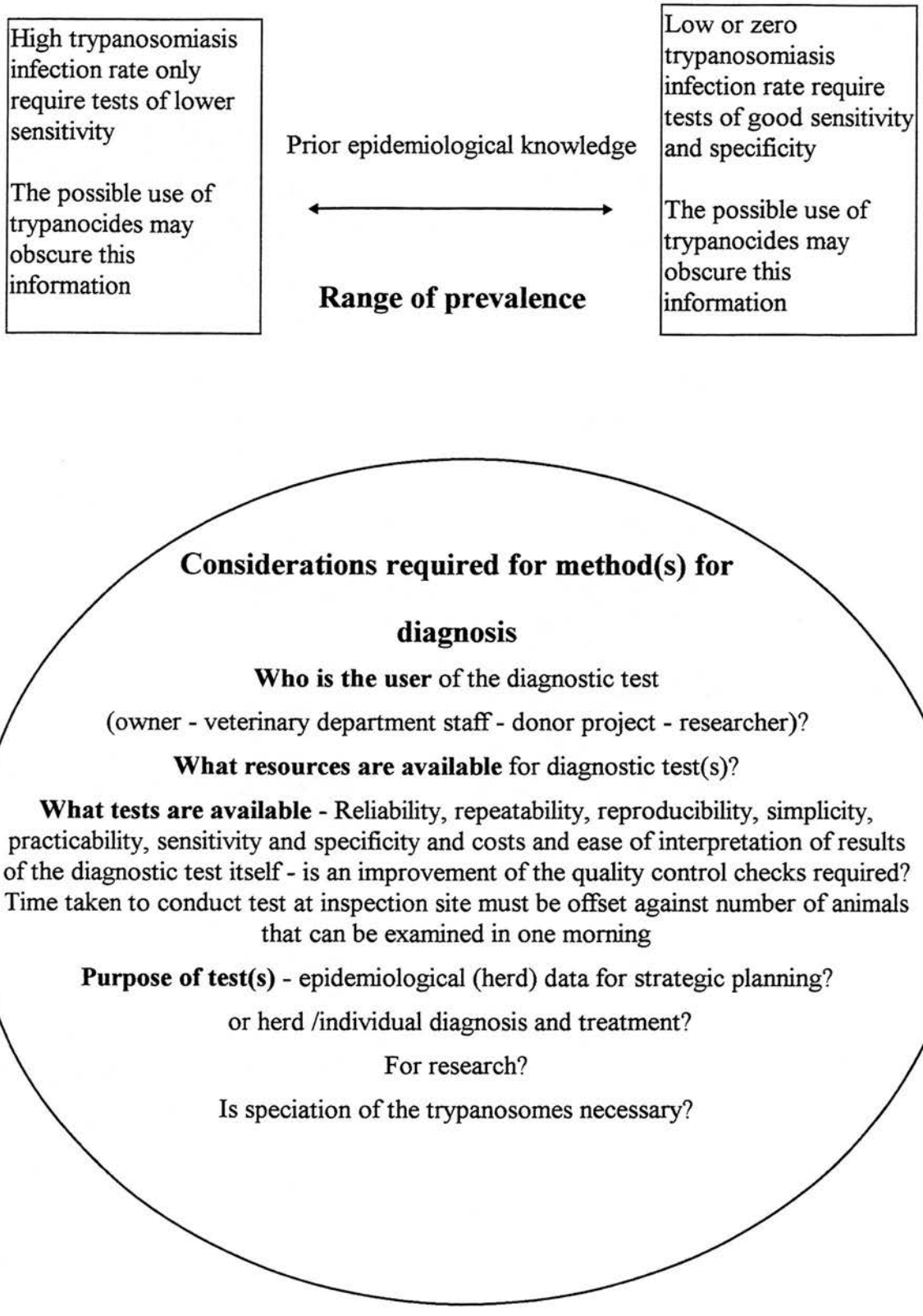
In discussing diagnostic tests for bovine trypanosomiasis one must put in context the objectives of the diagnostic test and thus which factors may dictate its usage. These have been summarised, and the inter-relation of factors that determine the choice of a

diagnostic techniques for tsetse transmitted trypanosomiasis in cattle are portrayed diagramatically in Figure 2.2.

In reality - individual diagnosis and treatment using a diagnostic technique that must be examined in some distant laboratory is probably an unrealistic goal in much of Africa, certainly in Zambia. Veterinary Departments in much of Africa rarely have the resources to visit herds and diagnose and treat affected individuals - yet alone cope with the sampling, screening in a central laboratory, the relocation of the same animals to provide treatment. Treatment based on haematocrit estimation and a good quality buffy coat examination should suffice[7]. Animals in which trypanosomes are identified or with an anaemia are treated at that time. Even this is beyond the capacity of many veterinary departments not receiving donor aid - in Zambia as much as anywhere.

[7] Serial diagnostic testing using buffy coat and PCV estimation at the inspection site to facilitate identification and treatment of infected individuals, followed by some serological laboratory based test is probably the best compromise between responsibility by veterinary departments to treat livestock disease, and obtaining a clear epidemiological picture of the disease and hence vector.

Figure 2.2 The inter-relation of factors that determine the choice of a diagnostic technique(s) for tsetse transmitted trypanosomiasis in cattle



2.5. *The pathogenesis of trypanosomiasis and the causes of low Packed Cell Volume*

2.5.1. The pathogenesis of trypanosomiasis

Trypanosomiasis in domestic stock is characterised by the development of anaemia (Dargie *et al.*, 1979), immunosuppression (Goodwin, 1970, Holmes *et al.*, 1974) and cachexia. There will however be marked differences in clinical signs in cattle which will be due to the species of trypanosome, the virulence of the particular isolate and the host susceptibility (Luckins, 1995). Disease in cattle due to *T. congolense* and *T. vivax* species is much more severe that that caused by *T. brucei*, which is reported to cause little disease and a mild, late, anaemia (Mulligan, 1970; Stephen, 1986).

Anaemia has been considered as the cardinal sign and major disease promoting factor of bovine nagana (Hornby, 1952; Murray, 1979). Immunosuppression has been described as one of the most important pathogenic mechanisms in trypanosomiasis because infected animals generally die, not as a direct result of trypanosome activity but as a result of secondary bacterial and viral infection (Losos and Chouinard, 1979; Silegham *et al.*, 1994). Immunosuppression is obviously difficult to measure during a routine field survey whereas anaemia, via the use of haematocrit estimation, is not.

2.5.2. Bovine trypanosomiasis and anaemia

The development of anaemia is a characteristic occurrence in cattle infected with *T. congolense* (Fiennes, 1950; 1954; Stephen, 1986, Murray and Dexter, 1988) which may be less marked in the case of *T. brucei* and *T. vivax* (Stephen, 1986). The disease has been divided into three stages according to the presence or absence of trypanosomes in the bloodstream and on clinical and pathological findings (Murray, 1979; Dargie *et al.*, 1979). The first stage may last from three to twelve weeks and the onset and severity of anaemia correlates with the level and duration of parasitaemia (Jennings, *et al.*, 1974; Holmes *et al.*, 1974, Mammo and Holmes, 1975). The anaemia during this stage is haemolytic and intravascular in origin

(Fiennes, 1954; Jennings *et al.*, 1974) although extravascular haemolysis has also been described (Naylor, 1971). During stage one anaemia, splenomegaly is a consistent finding (Murray, 1979) and is thought to be due to a combination of lymphoid hyperplasia and erythrophagocytosis (MacKenzie and Cruickshank, 1973; Jennings *et al.*, 1974; Murray *et al.*, 1977). However in some animals a further two stages of anaemia are observed.

Stage two of the anaemia is characterised by intermittent low level anaemia. The haemolysis observed in stage one is continued and associated with sustained hyper activity of the mononuclear phagocyte system (Murray, 1979). Despite this, splenomegaly is no longer prominent. Stage three is characterised by the absence of trypanosomes in the blood and the animal may either remain anaemic or recover. Death can occur during stages two or three of anaemia (Murray, 1979). The effects of nutrition on the degree of anaemia and liveweight change in N'Dama cattle infected with trypanosomes was investigated by Agyemang *et al.* (1990).

2.5.3. The aetiology of African bovine anaemia

Anaemia (syn. olicythaemia, abnormally low packed cell volume) is defined as a deficiency of circulating erythrocytes and may be caused by haemorrhage, by increased destruction or the inefficient production or erythrocytes (Radostits, Blood and Gay, 1994). The packed cell volume is a measure of what percentage of the volume of unclotted blood can be apportioned to the erythrocytes and leukocytes, the normal range cited being 25 - 45% (Merck, 1991).

Trypanosomiasis is associated with an anaemia which will affect the haematocrit values and thus the packed cell volume (PCV) frequency distribution of herds living in zero or low-medium-high tsetse challenge area. The effect of *T. brucei* will be less clear however, because of the species lower pathogenicity compared to *T. congolense* and *T. vivax*. The descriptive statistics for summarising a herd's haematocrit picture are described in Chapter 3. In order to correlate the haematocrit picture from a herd with its trypanosomiasis infection rate, and thus postulate whether the PCV data can be used to predict the trypanosomiasis distribution, other factors which may lead to a change in the PCV distribution in herds managed under traditional African farming systems must be reviewed. Clearly, if trypanosomiasis is one of the few conditions that cause a demonstrable shift to the left of the PCV frequency distribution, then PCV data could be used as a reliable indicator for the disease's presence. When discussing causes of bovine anaemia, a clear distinction must be made between conditions that cause anaemia in individual animals, which will not grossly affect the herd PCV frequency distribution, and those which will affect the herd generally. Thus, if trypanosomiasis is affecting the herd health (cf. nagana tends to be a herd health problem not an individual animal problem) then other reasons that may affect the haematocrit values from the herd must be born in mind and reviewed. Epidemiologically, it is also important to separate causes of anaemia in adult and young stock. The causes of anaemia in cattle living in Africa are summarised in Table 2.5.

Table 2.5 Some causes of bovine anaemia in Africa

(adapted from Radostits *et al.* 1994)

1. Haemorrhagic anaemia

Intestinal parasitism in young animals (also listed under chronic disease)
Parasitism - infestation with *Haemonchus*, *Bunostomum*, or *Fasciola gigantica*
Heavy infestations with ticks or lice
Jugular phlebotomy as practised by pastoralists for food (not in Zambia)
Pyelonephritis with bleeding from a renal lesion
Intra luminal haemorrhage from ulceration or haematomas in the mucosa of the jejunum
Abomasal ulcer sometimes originating from viral leukosis lesion
Enzootic haematuria (bracken poisoning)

2. Haemolytic anaemia

Trypanosomiasis
Theileria annulata infection
Babesiosis and Anaplasmosis
Eperithrozoönosis
Bacillary and post parturient haemoglobinuria
Leptospirosis
Brassica poisoning and Poisoning by certain weeds
Chronic copper poisoning (calves)
Coccidiosis in calves and yearlings
Rare auto-immune and congenital haemolytic anaemias in calves

3. Anaemia due to decreased production of erythrocytes or haemoglobin

a. Nutritional causes

Malnutrition, especially protein malnutrition
Copper and cobalt deficiency
Iron and potassium or pyridoxine deficiency implicated with anaemia in calves but most unlikely in outdoor, traditionally young stock

b. Chronic disease

Chronic suppurative processes
Poisoning with bracken, tri-chloroethylene extracted soya meal or arsenic
Intestinal parasitism e.g. ostertagiasis, trichostrongyliasis

4. Myelophthisic anaemia

These are where the bone marrow cavities are occupied by other, usually neoplastic tissues (very rare)

2.5.4. Differential diagnosis of trypanosomiasis induced anaemia

Although the list of causes of anaemia is extensive, the veterinary premise that "common things occur commonly" holds true and most of the cited aetiological factors occur rarely. The important conditions which will affect individual adult animals are those of anaplasmosis and babesiosis. As these are acute diseases affecting individuals, they would not affect the herd haematocrit picture. Intestinal parasitism, if present, is likely to affect the population of calves and younger animals, but unless these were sampled in a biased manner their haematocrit values would not affect the herd haematocrit picture. The most likely postulated differential diagnosis to bovine trypanosomiasis is therefore infestation with the tropical liver fluke, *Fasciola gigantica* and (protein) malnutrition. Chronic fascioliasis is a persistent, wasting disease and in cattle tends to be self limiting (Hammond and Sewell, 1990). Anaemia is manifest except in lightly infected animals. Animals on a high plane of nutrition are much more resistant to the effects of chronic infections and can tolerate a higher burden of flukes than those on a low plane. Conversely, the disease is exacerbated by inadequate nutrition e.g. during savannah dry season, or by anaemia resulting from intercurrent disease (Hammond and Sewell, 1990).

Nutritional deficiencies (protein malnutrition) have been described as a cause of anaemia (Kerr, 1989). Because haemoglobin is mostly protein, a deficiency of protein may result in anaemia and concomitant hypoalbuminaemia. The anaemia is described as being hypoplastic/aplastic, non regenerative, often microchromic with leptocytes present[8]. The inter-relation between haematological parameters and *T. congolense* infections has been recently investigated by Katunguka Rwakishaya (1996 and 1997).

Although *Theileria* infections are cited as a cause of anaemia, the *T. parva* species giving rise to the East Coast fever and corridor disease complexes occurring in

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[8] Leptocytes are cells with too much membrane area for their size resulting in folds and clumps of haemoglobin (Kerr, 1989).

Zambia are not characterised by anaemia (Brown *et al.*, 1990; Lawrence, de Vos and Irvin, 1994) - this a feature of *T. annulata* which is the causal agent of tropical theileriosis (syn. Mediterranean Coast fever).

2.6. Options for the control of bovine trypanosomiasis

In the absence of suitable immunological control measures for bovine trypanosomiasis, practical control measures include the eradication and control of *Glossina* populations, the exploitation of trypanotolerant cattle and the administration of anti-trypanosomal drugs to cattle (Morrison, Murray and McIntyre, 1981). Because the maintenance of the parasite requires the cyclical transmission via the bite of an infected tsetse fly (excluding mechanical transmission of *T. vivax*), eradication of the parasite through the use of drugs or trypanotolerant stock is unlikely. Tsetse eradication is defined as the complete removal of the vector from some discrete area whereas control is the reduction of the disease or challenge to some acceptable limit. With the advent of techniques to totally eliminate the tsetse fly, eradication of the disease by eradication of the insect vector has become a realistic option if the fly is confined to a discrete pocket. Eradication was thus the goal in certain countries where the fly was confined to a discrete location, for example, the Common Fly Belt of Southern Africa. The fly belt of the *Glossina pallidipes* was successfully eradicated in the KwaZulu - Natal Province of South Africa in the 1960s by DDT ground spraying. It is argued that the finite cost of eradication are less than the infinite costs of control. Given the limited resources available to African Veterinary and Tsetse control services, and the increasing interest in community participation, current policy is now frequently aimed at disease control *versus* vector eradication.

2.6.1. Tsetse control

Tsetse fly have certain characteristics which make their control easier than other arthropod and insect vectors; they are larviporous and are slow to reproduce, the females only mate once in their lifetime, and the genus is particularly susceptible to low doses of insecticides. There are two broad categories for the control of tsetse flies, chemical and non chemical. Chemical control consists of the application of insecticides either from the ground by knapsack or vehicular [9] spraying, from the air either by helicopter or fixed wing aircraft, or onto baits which may be artificial or moving (i.e. cattle). Insecticide application, from the ground using dieldrin or dichlorodiphenyltribroethane (DDT) into the environment may involve their application to specific tsetse resting sites in so-called ground spraying operations. Ground spraying ceased in Zambia in the 1970s and was superseded by the use of aerial spraying. Sequential aerial spraying involves the application of an insecticide, usually either endosulphan or deltamethrin, in cycles timed to kill emerging tsetse fly pupae. The last aerial spraying operation conducted in Zambia was in the Choma Kalomo block of Southern Province 1987.

Synthetic pyrethroids are applied either to black or black - blue cotton targets which have been baited by the use of olfactory stimulants, usually acetone and octenol . Insecticides can also be applied direct to cattle as a dips or pour-on. Bait technology has totally superseded ground and aerial spraying in Zambia as it is seen to be more environmentally friendly and carries none of the inherent risks of low level night flying. Controlling tsetse by the use of some synthetic pyrethroid as dip compound is not a simple control option. The epidemiology of the enzoötic stability of several tick borne diseases (primarily *babesia* and *anaplasma*) or possible presence of reservoirs of *Theileria* species is complex. The epidemiology of these disease and tick biology

[9] Unimog 4 x 4 vehicles were a commonly used type of vehicle in Zambia for ground spraying operations

must also be taken into account when considering dip ingredient as an option for tsetse control[10].

There have been a variety of non-chemical control measures used to reduce *Glossina* populations. The clearance of vegetation and the encouragement of human settlement and agricultural development to create an adverse environment for tsetse were long practised and successful techniques (Ford, 1971; Jordan, 1974). In addition, the destruction of game animals to decrease the flies' food source and therefore size of the natural infection reservoir has been widely practised. Areas where game had been selectively culled were separated from areas where human settlement had taken place or was being actively encouraged were divided by game fences, which prevented stock and wild animal movement.

A sterile insect technique (SIT) has also been developed and is currently in use on the Island of Zanzibar with successful results (RTTCP Regional Office, 1996). This technique involves the rearing of large numbers of male flies, sterilising them by nuclear irradiation, and then releasing them via a container from a helicopter into their natural habitat. They subsequently compete with wild males for mates and thus reduce the reproductive potential of the females. This technique is only viable when tsetse challenge is low, which may require some primary control method to reduce the tsetse numbers.

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[10] In KwaZulu/Natal, South Africa where cattle in close proximity to cape buffalo abound, pour-on pyrethroids applied to every 5th animal after dipping in amitraz has been used as a tsetse control measure.

2.6.2. Trypanotolerance

Trypanotolerance was first described at the beginning of the twentieth century when certain indigenous taurine cattle breeds of West Africa, in particular the N'dama and West African Shorthorn breeds, were observed to survive and be productive in tsetse infected areas (Pierre, 1906). These two breeds survive in large numbers without the aid of trypanocides in areas where significant tsetse challenge occurs (ILCA, 1979). In addition to these two breeds of cattle, many species of *Bovidae* have also been shown to exhibit innate resistance to trypanosomiasis (Murray *et al.*, 1979; Murray, Morrison and Whitelaw, 1982). Not only cattle are trypanotolerant, as sheep and goats in West Africa have also been shown to resist the effects of the disease.

Trypanotolerance is thought to have evolved through natural selection by constant exposure to infection over many generations (ILCA/ILRAD, 1988). However, although resistance to infection probably has a genetic basis, it is not absolute since productivity appears to decrease as the level of challenge increases. Thus N'dama may exhibit wasting and abortion associated with trypanosomiasis (ILCA, 1979). Murray *et al.* (1982) have shown that trypanotolerance is innate. Using animals with no previous exposure to trypanosomiasis, N'dama were significantly more resistant to trypanosomiasis than Zebu and imported exotic breeds in terms of productivity and survival. The prevalence, level and duration of parasitaemia and the severity of anaemia were significantly less in trypanotolerant cattle. Trypanotolerance has also been attributed to differences in immune responsiveness because of the control of parasitaemia in infected animals (Akol *et al.*, 1986; Authié *et al.*, 1993a and 1993b).

Some individuals of trypanotolerant breeds have been reported as being more susceptible to infection than others (Roelants, 1986). However, other reports have suggested N'dama in particular, as well as being trypanotolerant are more resistant to tick-borne diseases such as heartwater, anaplasmosis and babesiosis (Epstein 1971) and streptothricosis (Coleman, 1967). Recent research (Mattioli *et al.*, 1995; Ndao *et al.*, 1995) has shown that N'dama are also more resistant to helminth and tick infestations.

Despite the clear evidence that trypanotolerant breeds survive well under medium tsetse challenge, their use has not been adopted in Zambia. This may in part be due to their small stature and hence limited use for animal draft power (ADP) or because of the prejudice that rural farmers have towards smaller cattle. Such an introduction programme would be reliant on donor funding, as the Government of Zambia has very little financial resources especially for the agricultural sector, and donor agencies have identified the imprudence of attempting to introduce breeds of cattle that are so culturally unacceptable to Southern Africa stock owners.

2.6.3. Chemotherapy

Chemotherapy of bovine tsetse transmitted trypanosomiasis relies on the use of three compounds; diminazene, homidium and isometamidium salts (Mulligan, 1970; Jordan, 1986). The key facts on these drugs are given in Table 2.6:

Table 2.6 Drugs available for bovine trypanosomiasis control in Africa

Generic name	**Compound class**	**Trade name**	**Type**	**Dose (i.m.)**
Diminazene aceturate and diaceturate	Aromatic diamidine	Berenil Dimaphen Dimazan	C	3.5 mg/Kg (*T.* vivax and congolense) 7.0 mg/Kg for T. brucei
Homidium bromide Homidium chloride	Phenanthridine	Ethidium Novidium	C	1.0 mg/Kg
Isometamidium chloride	Phenanthridine aromatic amidine	Samorin Trypanidium	C P	0.25 - 0.5 mg/Kg 0.5 - 1.0 mg/Kg

Type = curative (C) or prophylactic (P)

Despite the above comments on vector control and trypanotolerance, chemotherapy is still the only form of disease control in most parts of Zambia. The restricted number of drugs available in the past four decades, and their usually unrestricted use by owners and lay personal has resulted in problems of drug resistance (Williamson,

1979; Eisler *et al.*, 1994). Drug resistance appears to be acquired by exposure to trypanosomes at sub-therapeutic levels, thereby facilitating selection of a drug resistant sub-population (Whiteside, 1962; Leach and Roberts, 1981). Subtherapeutic drug levels may occur because of incorrect dosage, irregular treatments of prophylactics, or stopping the usage of a prophylactic drug whilst animals are still at risk (Stephen 1986). In the Eastern Province of Zambia, a commonly used cocktail used by farmers is reportedly a mixture of 5 mls of 7% w/v diminazene with 5 mls 1% w/v isometamidium (personal observation). The development of drug resistance has resulted in the use of sanative pairs of trypanocides, which in the Zambian context is effectively an annual dose of diminazene mid way through the dry followed by a prophylactic dose of isometamidium before the rainy season which will impart protection during the rainy season when challenge is highest and the demands for ploughing and weeding, are greatest. The sanitising dose (of diminazene) is meant to clear all trypanosomes from the tissues and blood stream before a chemoprophylactic is administered, and thus eliminating any strains which may have developed isometamidium resistance. With little prospect of the development of new trypanocidal drugs, research is turning to the studies of the distribution and mechanisms of isometamidium resistance (Eisler, personal communication) and the development of novel controlled release device formulations of ethidium and isometamidium (Geerts, personal communication).

Trypanocides are poisonous substances whose use is subject to legislative control and which ought to be administered by trained veterinary staff. In the days of colonial administration, there were staff and an allocated budget for drugs to make this reality. In the past decades the philosophy of free veterinary services has had to change and currently there is increasing interest in cost recovery schemes and privatisation (ASVEZA, 1995) to revitalise the delivery of drugs. Ironically, this has meant drugs are more freely available to farmers which, in the short term has assisted with alleviating the burden of trypanosomiasis, is in the longer term almost certainly leading to increasing numbers of trypanocide-resistant strains of trypanosomes emerging in the population.

2.6.4. The economics of control options

Historically, the costs of tsetse and trypanosomiasis control was borne by colonial and post colonial governments who had the resources to provide free veterinary services and implement widescale tsetse control programmes. In Zambia, the Veterinary and Tsetse Control Department had a relatively large operational budget until the mid 1970s[11]. Human and cattle populations increased, and yet the provision of animal health services was on the decline. Increasing land pressure resulted in a rise in settlement in tsetse infested areas, particularly in the Eastern Province of Zambia. There was thus an increasing need for animal disease control against a scenario of dwindling resources with which to accomplish this goal. As a result, a need has arisen for economic analyses of methods for tsetse and trypanosomiasis control.

Having assessed the most cost-effective technique for tsetse and/or trypanosomiasis control, it remains to be demonstrated that intervention is justifiable (Barrett, 1994). The benefits and costs of control, including those arising outside the boundaries of the project, must be identified, quantified, valued and compared. Standard methods for cost-benefit analysis in developing countries have been described (Gittinger, 1982). Prices for inputs and outputs are adjusted to reflect true value to the economy, rather than prevailing domestic market prices, where these are distorted for example by government subsidy, tax or other form of control. As costs and benefits are likely to arise for many years after the investment, future cash flows are projected. These are translated into equivalent current values by a discounting process in order to arrive at: the *net present value* of the investment; or a *cost-benefit ratio*; or the *internal rate of return* which is a measure of the interest rate earned by investment in the project. These parameters can be used as criteria for deciding whether the investment is justified. The methodological issues in cost-benefit analysis of tsetse and trypanosomiasis control are similar in many respects to those arising for other animal

[11] Much of Zambia's income is derived from the export of copper, much of which was used for munitions. With the cessation of the Vietnam war in the early 1970s, copper prices fell which was reflected by a severe cut in the operational budgets of the various Ministries of the Government of the Republic of Zambia.

health interventions, where modelling approaches have developed considerably in the last decade (e.g. James, 1984).

2.6.5. Strategic tsetse and trypanosomiasis control

Until some new control method is devised, or there is a change in attitude towards aerial and ground spraying techniques, the current choice of tsetse and/or trypanosomiasis control methods in cattle will be dependent on the following factors:

* the cattle and human density
* the trypanosomiasis disease challenge (incidence or prevalence)
* land use potential
* the cost (and availability) of trypanocidal drugs
* the costs of pour-on dips and availability of dips
* the costs of target control operations.

Graphs have been devised (RTTCP Regional Office, 1996) whereby the optimum control technique can be chosen according to the intercept on the graph falling under cattle density, control cost per square kilometre and bovine trypanosomiasis incidence.

Current doctrine within Southern Africa is aimed at socio-economically sustainable tsetse and/or trypanosomiasis control which is part of some integrated rural development strategy. Tsetse or trypanosomiasis control cannot be viewed in isolation from other aspects of rural development, for example provision of health and education services or freely available water. Economic sustainability requires that some marketing infrastructure be in place to absorb the increase in the marketed agricultural products, maize and meat. Given the increase in concern in recent decades over the environmental impact of tsetse control (Jordan, 1986), land use issues must also be born in mind. Although not tangibly and economically quantifiable, the following factors must also be born in mind when selecting some control area and method:

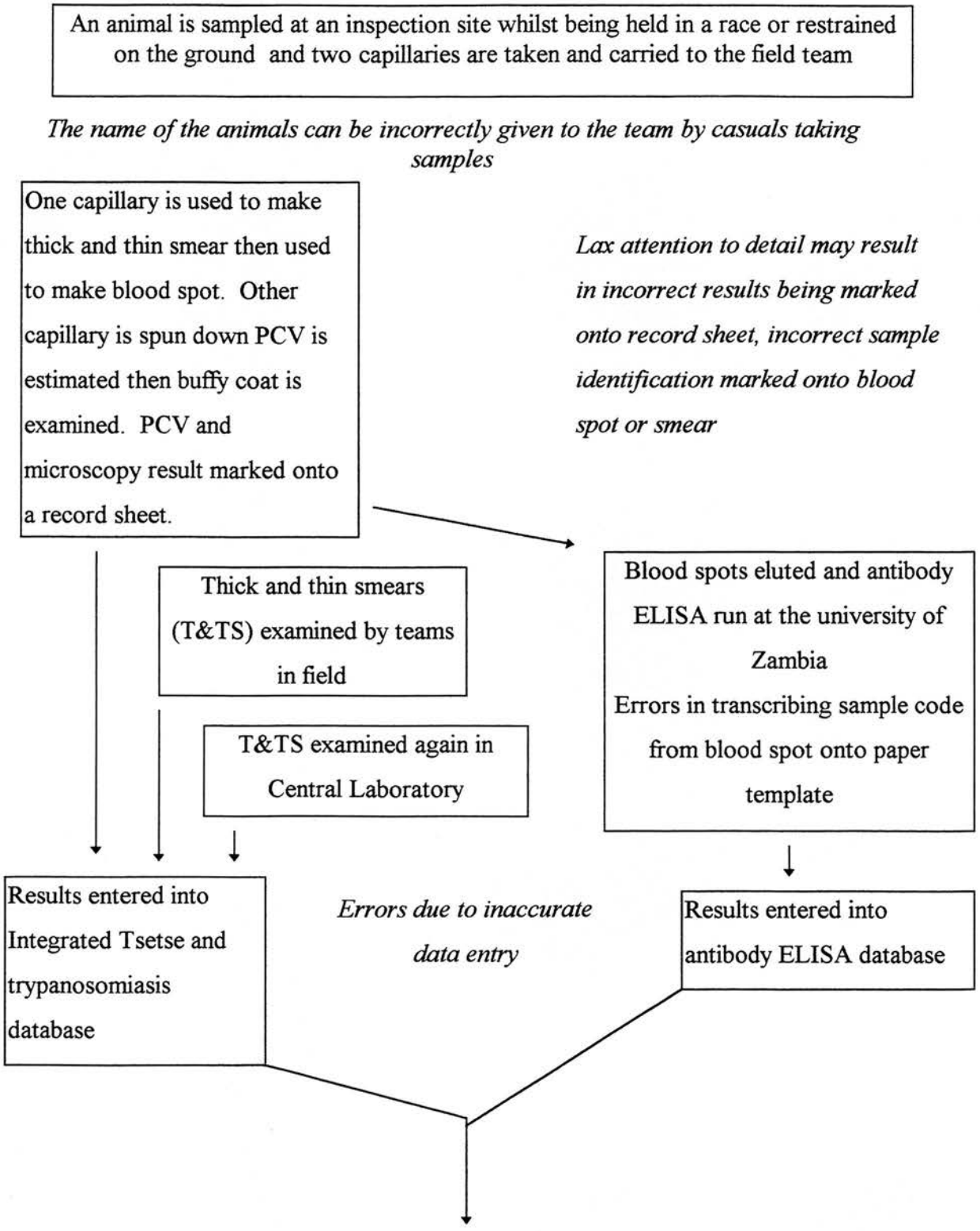
* the available budget and funding considerations
* the community's willingness to participate in control operations
* social and political considerations
* potential land use and environmental considerations
* strategic control considerations.

These considerations are outwith the scope of this thesis and will not be discussed further.

2.7 Sample collection, identification, data management systems and their inter-relationship

Confounding the quantitative investigations into the epidemiology of trypanosomiasis are the limitations dictated by sample and data management. Results from individuals and herds can be managed in some hard copy form (record sheets and manual summaries) and data can be managed digitally and thus accurately and expeditiously to present managers with summaries for their decision making processes. Laboratory and research workers also require an accurate method of cross referencing samples with laboratory results and, in turn, results obtained from both individuals and herds using the various diagnostic tests. Such a system of labelling samples and recording results will lead to error - the bane of any data management system designer. Labelling can be incorrectly marked onto a sample and latterly entered correctly in a database management system (DMS), or there may be straight forward errors in data entry. The occasions when erroneous data can arise are shown in Figure 2.3. This is pointed out because within the results described in this thesis, data occasionally do not correlate as one would intuitively expect they should, this being due to the inherent fallibility of data management (Roos, Sharp and Wajda, 1989). A system of duplicate entry and data verification was devised to help overcome erroneous data entry.

Figure 2.3 Diagram showing where data labelling and data entry errors can occur



When databases are linked, at individual animal or herd summary level, there is a cumulative effect for all the possible occasions above when erroneous sample identification, recording or data entry may have taken place

In a study of the quantitative epidemiology of trypanosomiasis in the Common Fly Belt of Zambia, a natural development was to design several inter-linking DBMS that could manage data separately and yet link results from individual and herd (Robinson and Hopkins, 1995; Hopkins, 1996a; Hopkins 1996b). Herein lies the essence of a relational DMS (Robinson *et al.*, 1997). To this end, an identification system was developed for identifying animals and samples taken during surveys and surveillance. The system needed to be simple yet robust if used by casual workers in the field, many of whom only were educated to the secondary level.

Chapter 3

3. Direct parasitological methods for diagnosing bovine trypanosomiasis and investigations into the relationship with haematocrit values

3.1. Introduction

There have been few developments in the techniques for the field diagnosis of bovine trypanosomiasis (and concurrent packed cell volume estimation) in the past one and a half decades. The way data are managed has however advanced considerably with simpler database management systems and the general usage of personal computers. This led to the design of an Integrated Tsetse And Trypanosomiasis Database (ITTD), which was developed in Zambia by Robinson and Hopkins (1995) and Hopkins (1996a). No comparable database management system exists elsewhere in Africa. The ITTD is able to manage parasitological data from buffy coat and thick and thin examinations, as well as haematocrit values - and summarise the data for each examination site by infection rate, vivax proportion[12], and species of trypanosome identified. The haematocrit data are summarised as mean PCV, mode class, the proportion of animals defined as anaemic (i.e. with a PCV less than or equal to 25%), and as the herds' PCV frequency distribution histogram. The ITTD summarises

[12] The vivax proportion is a refinement of the vivax ratio as described by Jordan (1986). On the premise that *T. vivax* are not being mechanically transmitted, then an increasing proportion of *T. vivax* in an animal population should reflect a younger average age of the *Glossina* vector. This is because *T. vivax* requires less time to undergo cyclical transmission as it undergoes multiplication in the mouthpart of the fly, whereas *T. congolense* and *T. brucei* multiply in the salivary glands and mid gut and salivary glands respectively, which takes longer. Thus, one would hypothesise that an increase in proportion of *T. vivax* in cattle would reflect in a lower mean age of fly, such as would occur at the edge of a fly belt, during a successful control campaign, or during normal seasonal fluctuations.

reports as database files, Geographical Information System (GIS) maps (IDRISI ®[13] vector files), graphs, and for surveillance activities - tables.

3.2. Investigations into the sensitivity of the standard RTTCP Zambia field diagnostic techniques

3.2.1. Introduction

The sensitivities of the direct diagnostic tests have been cited above in Table 2.3 (Paris *et al.*, 1982). These parasitaemias were quantified using a haemocytometer for numbers greater than 10^6 per ml., and results presented using a scoring system based on numbers of trypanosomes per preparation or per field for lower numbers when examined by the dark ground technique. Another system of enumerating parasitaemias is the log equivalent value (LEV), in which the numbers visible per preparation or per field are assigned a simple LEV value ranging from 0.5 to 4.5. The work carried out by Paris *et al.* relied on estimating the parasitaemia then diluting the infected blood with a known volume of trypanosome free blood to arrive at some estimated new, lower parasitaemia. These samples were then examined in order to obtain an estimate of sensitivity or, as the workers' results were expressed, the lowest parasitaemia that could be detected by the various methods.

3.2.2. Materials and methods

In order to determine the sensitivity of the buffy coat technique, a stochastic approach was used based on the Poisson distribution[14]. A more realistic estimate of sensitivity

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[13] IDRISI is not an acronym but a software programme named after an explorer. The programme is developed by: The IDRISI Project, Clark Labs for Cartographic Technology and Geographic Analysis, 950 Main Street, Worcester, MA 01610, USA

[14] The formula used was the probability of observing exactly x trypanosomes on a buffy coat, thick or thin smear taken from a sample with mean m trypanosomes per ml is given by:

$$p = \frac{e^{-m} m^x}{x!}$$

under field conditions was made given the fact that field workers are most unlikely to observe 1 trypanosome per preparation, but are almost certain to observe 1 trypanosome per field. The probabilities of seeing trypanosomes on a buffy coat smear under field conditions are given in Table 3.1:

Table 3.1 The probabilities seeing trypanosomes on a buffy coat smear under field conditions

Numbers of trypanosomes per buffy coat smear	Probability of seeing a trypanosome under normal field conditions
1	0.01
5	0.1
10	0.4
20	0.8
30	0.9
>50	0.99

The calculations assume all trypanosomes remain in and above the buffy coat and not in the erythrocytes, and that the whole buffy coat smear is examined. The volume of blood was based on a standard capillary of 0.1 to 0.12 mm. internal diameter and 75 mm. long, being filled to within 5 mm. of its length and thus allowing for a plug of Cristaseal ® at one end.

Using the formula derived from the Poisson distribution, the probabilities of observing 1, 2, 3, 4 ... or more trypanosomes are totalled to arrive at the probability of one or more trypanosomes occurring. The probability of observing one or more trypanosomes on a smear is the same as the test's sensitivity. In the case of the buffy coat, an additional column gives the probability of observing this number (from the Table 3.1). The probabilities of observing intermediate numbers were interpolated by a logarithmic scale.

Table 3.2 Theoretical sensitivities (lower thresholds of detection) of trypanosomiasis

Buffy coat smear

Volume of blood in a heparinised capillary tube (75 mm long)
60 - 65 mm x 1.1 - 1.2 mm (assumes 5 mm cristaseal and filled to within 5 - 10 mm from top.

Trypanosome density /ml.	*Tryp numbers in capillary*	*Probability of one or more trypanosome*	*Probability of observing one or more trypanosomes*
10	0.6	43.6%	0.6%
20	1.1	68.2%	1.2%
30	1.7	82.1%	2.0%
40	2.3	89.9%	3.0%
50	2.9	94.3%	4.3%
100	5.7	99.7%	14.7%
200	11.5	100.0%	44.4%
300	17.2	100.0%	67.8%
400	22.9	100.0%	81.3%
500	28.6	100.0%	88.4%
1,000	57.3	100.0%	99.7%

Thin blood smear

35 spots made from 45 mm of blood in a capillary tube 1.1 mm internal diameter

Tryp density / ml.	*Tryp numbers in that volume*	*Probability of observing one or more trypanosomes*
50	0.06	5.9%
100	0.12	11.5%
200	0.24	21.7%
300	0.37	30.7%
400	0.49	38.7%
500	0.61	45.7%
1,000	1.22	70.5%
2,000	2.44	91.3%
3,000	3.67	97.4%
4,000	4.89	99.2%
5,000	6.11	99.8%
7,500	9.16	100.0%
50,000	61.09	100.0%

Thick blood smear

24 spots made from 50 mm of blood in a capillary tube 1.1 mm internal diameter

Tryp density /ml.	*Tryp numbers in that volume*	*Probability of observing one or more trypanosomes*
50	0.10	9.4%
100	0.20	18.0%
200	0.40	32.7%
300	0.59	44.8%
400	0.79	54.7%
500	0.99	62.8%
1,000	1.98	86.2%
2,000	3.96	98.1%
3,000	5.94	99.7%
4,000	7.92	100.0%
5,000	9.90	100.0%
7,500	14.85	100.0%
10,000	19.80	100.0%

3.2.3. Results

Table 3.2 gives the calculated sensitivities of the buffy coat, thick and thin smears techniques. An extra probability has been added to the buffy coat examination, in that not only is there the probability of trypanosomes occurring but also whether these will be seen when present in low numbers.

A summary table showing the lowest threshold of detection (i.e. greater than 99% probability of seeing one or more trypanosomes) using the above Poisson distribution and the findings of Paris *et al.* (1982) is given in Table 3.3 below.

Table 3.3 **The summary table showing the lowest threshold of detection as calculated in the thesis and as observed by Paris *et al.* (1982)** (figures in body of table are the lower limits of detection expressed as trypanosomes per ml)

	From Table 3.2	After Paris *et al.* (1982)	
Lower limit of detection shown in body of table	Any species (as calculated by Poisson distribution	Average *T. congolense* and *T. vivax*	Average of all three species of tsetse transmitted trypanosome
Buffy coat smear	100	375	450
Thick smear	~ 3,000	7,280	6,520
Thin smear	4,000	37,500	33,300

3.2.4. Discussion

Using the above approach to determine the sensitivity of the three direct diagnostic techniques, the theoretical sensitivities are clearly much higher than the findings of Paris *et al.* (1982). The observation and hence detection of a parasite in a blood sample is clearly dependant on mathematical probability, as their occurrence in blood follows a classic Poisson distribution. The mathematical approach differs markedly from Paris and workers more empirical approach to the investigation of direct techniques' sensitivities. Paris and his co-workers also observed a difference in the ability to diagnose differing species of trypanosome using the three differing techniques; *T. congolense* being observed at lower parasitaemias than *T. vivax* and *T. brucei* on buffy coat, and *T. brucei* being observed at lower parasitaemias than the other two species on thin and thick smears.

On buffy coat, there is one possible explanation as to why the calculated sensitivities are greater than the empirically derived sensitivities, which could also account for why trypanosome species differences occur. This is due to the differing species propensity to be closely associated with erythrocytes but thus said, this reasoning could not apply to thick and thin smears. Trypanosomes would thus remain mixed with the red blood cells and not separate into the plasma above the buffy coat. *T. congolense* is closely associated with the red cells and yet it is the species observed in the lowest numbers.

Another difference between the calculated sensitivities and those published by Paris *et al.* may be due to the volume of blood used to make smears. The volume of blood used to make a buffy coat smear is a fairly constant 60 - 70 µl, as the dimensions of a glass capillary tube and to what extent it can be "filled" will not alter greatly. To calculate the volume of blood used to make thick and thin smears, a Zambian veterinary assistant was asked to place drops of blood onto a clean slide at the size he would normally use for making thick and thin smears. Making thick and thin smears is as much an art as a science, as too much blood will result in the thin smear being

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greater than one cell thick, thus making examination difficult. The correct methods of making a thick and thin smear are described by Boyt (1980). 45 mm. of blood from a capillary tube made 35 spots of suitable size for thin smears whilst 50 mm. from a capillary tube made 24 spots of suitable size for thick smears. Given the internal diameter of a capillary tube to be 1.2 mm. the volumes of blood required to make thin and thick smears were 1.22 µl and 1.98 µl respectively compared to the 5 µl used by Paris *et al.*. Given these smaller volumes of blood used to the calculations, the dried smears should have had a lower theoretical sensitivity than described by Paris *et al.*. This was not, however, the case.

When calculating the sensitivities of the differing direct methods, especially where very low parasitaemias are being detected, the issue of probability arises. One cannot be categorical in stating that at a certain parasitaemia a trypanosome will be seen and thus trypanosomiasis is diagnosed, whereas below a certain threshold a trypanosome will not be seen therefore the sample will be deemed negative. On buffy coat examination for example, the theoretical probability that one or more trypanosome will occur ranges from 43.6% to 94.3% as the parasitaemia increases from 10 to 50 per millilitre. This does not mean the lower limit of detection is 50 per ml, because at this level of parasitaemia no trypanosome will be present in 1 in 20 occasions. Similarly, solitary trypanosomes will theoretically occur on occasions when the parasitaemia is below 10 per ml. The true sensitivity of the three direct diagnostic tests probably lies between the limits cited by Paris *et al.* (1982) and calculated above.

3.3. Routine RTTCP Zambia examinations

3.3.1. Trypanosomiasis disease sampling methodology

Surveillance of cattle for trypanosomiasis is logistically simpler than for surveys as the cattle can either be purchased and used in an area, or a group of farmers are encouraged to present their cattle on a monthly basis for examination. By uniquely identifying the cattle with ear tags, the true estimate of the number of new cases (i.e. incidence) is determined.

Sentinel herds of a standardised size of 20 cattle were established in Zambia for surveillance purposes. Sixteen to twenty six herds were established in an operational area. This herd size was chosen not only to give an arithmetically "simple" estimate of a incidence but also for practical reasons in order that teams could examine two herds in one morning. The number of sentinel herds per operational area was dictated by the government and project workers' twenty day work period. Herds were examined monthly.

Surveys relied on a systematic sampling technique whereby the sample size was chosen (usually sixty animals), and the sampling frame identified and number of animals presented for examination was ascertained, then every N/n^{th}[15] animal was chosen and sampled. Surveys are not however straightforward in Zambia where the Government dip tanks and inspection systems have failed, and data on cattle census statistics are very difficult to obtain. The sample size chosen from a population of animals presented was based on how many animals the team were able to examine in one morning rather than via the classic epidemiological approach. Teams also had to be given simple instructions on sample size rather than the more complex criteria which require foreknowledge of the disease's likely absence or presence, and if present the likely infection rate. Thus the practical number of sixty animals per inspection was chosen which a well equipped and manned team could examine in one

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[15] where N is the number of animals presented ~ population size and n is the sample size required ~ 60.

morning rather asking the team to refer to tables sample size to detect infected animals at various confidence limits. If and when disease was detected, the teams would continue a systematic sampling approach on the premise that the more animals examined the better, as this would attach narrower confidence limits to the estimate of disease prevalence.

3.3.2. Examination of the blood samples

The materials and methods routinely used during the examination of a sample are given in Appendix 2.

3.3.3. Costs of the RTTCP routine survey method

The costs for a team to examine 1000 head of livestock are given in Tables 3.4 and 3.5. These are broken down into the costs of employing two Departmental attached staff accompanied by 4 casual employees and one driver (Table 3.4) and the costs of consumables and capital equipment (written off over one to five years) in Table 3.5. These figures are based on the composition of a standard RTTCP Zambia trypanosomiasis survey team. Based on a team's ability to examine approximately 1000 animals in one month (16 working days examining 60 animals per day) the total cost per sample is 1.458 ECU of which 21% (0.303 ECU) is spent on the consumables and wear and tear of the equipment whilst 79% (1.155 ECU) is due to the teams salaries, transport and fuel. Of the team's running costs, 43% is due to the depreciation of the vehicle (Landrover) which on average must be replaced every 3 years. As the "overheaded costs" of maintaining a team in the field will not alter, the costs of direct examination are comparatively small compared to the estimated cost of ECU 0.3923 per sample for the Ab-ELISA using eluted blood spots as discussed in Chapter 4.

Table 3.4: Monthly running costs of a team sampling 1000 animals per month

	Monthly cost (ECU)	Capital cost (ECU)	Write off period (Years)	Monthly cost (ECU)	Percentage breakdown
Labour costs					
4 casuals	115.79			115.79	10%
2 attached	56.25			56.25	5%
1 Driver	60.93			60.93	5%
Transport costs					
Running costs	68.25			68.25	6%
Fuel	280			280	24%
Depreciation		18,072	3	502	43%
Tentage and camping equipment		861	1	72	6%
Price per litre of diesel	0.80				
		Total monthly costs for 1000 samples		**1,154.98**	100%
Labour, transport and field equipment costs (ECU) of collecting one sample				**1.155**	79%
Cost of consumables and depreciation of equipment				**0.303**	21%
		Total ECU cost per sample		**1.458**	100%

55

Table 3.5: Costs for examining 1000 animals by direct parasitological methods (not including labour, transport, and camping costs)

	Quantity	Unit cost (ECU)	Capital / Consumable	Write off period (years)	Cost or Depreciation per 1000 samples	Percentage breakdown
Consumables						
Heparinised capillaries	2000	0.03	Consumable	n/a	56.00	18.5%
Slides	1300	0.05	Consumable	n/a	20.72	6.8%
Cover slips	1000	0.02	Consumable	n/a	26.40	8.7%
Lancets	1000	0.03	Consumable	n/a	26.40	8.7%
Methanol	500 ml.	1.11	Consumable	n/a	1.11	0.4%
Giemsa stain	100 ml.	13.57	Consumable	n/a	13.57	4.5%
Staining equipment	500 ml.	2	Consumable	n/a	2.00	0.7%
Miscellaneous inc. diamond pencil	1	4.61	Capital	1	0.38	0.1%
Subtotal consumables				Subtotal	146.58	48.5%
Capital equipment						
Dark ground microscopes	2	3,012.05	Capital	5	100.40	33.2%
Hettich MHC centrifuge	1	500.00	Capital	3	13.89	4.6%
650 watt Generator	1	600.00	Capital	2	25.00	8.3%
250 watt Invertor	1	200.00	Capital	1	16.67	5.5%
Depreciation per month				Subtotal	155.96	51.5%
ECU Cost per 1000 samples (not including labour or transport)					*302.54*	100.0%
				ECU cost per sample	0.303	

Teams examine on average 60 animals per day, 16 days per month = 1000 animals

3.4. The relationship between herd packed cell volume parameters and trypanosomiasis prevalence

With the exception of *T. brucei*, tsetse transmitted trypanosomes have been shown to cause anaemia. In the later stages of infection the presence of trypanosomes may prove difficult to demonstrate in the blood stream although an anaemia may be present. Thus, a critical study of the haematocrit picture in known trypanosome infected and uninfected herds was investigated for use in predicting the presence of the trypanosomiasis. The only other pathological condition which may give rise to a lowering of a herds' PCV being fascioliasis, and even this condition is self limiting or will exacerbate the anaemia caused by trypanosomiasis. In order to examine the strength of the correlation between trypanosomiasis and the PCV distribution, it was first necessary to ascertain that PCV values from infected and uninfected animals were normally distributed, if parametric statistics were to be used. If a strong correlation could be proven, then regression via modelling could be investigated to examine critically whether PCV estimates could be effectively used to predict the presence or absence of trypanosomiasis.

3.4.1. Materials and methods

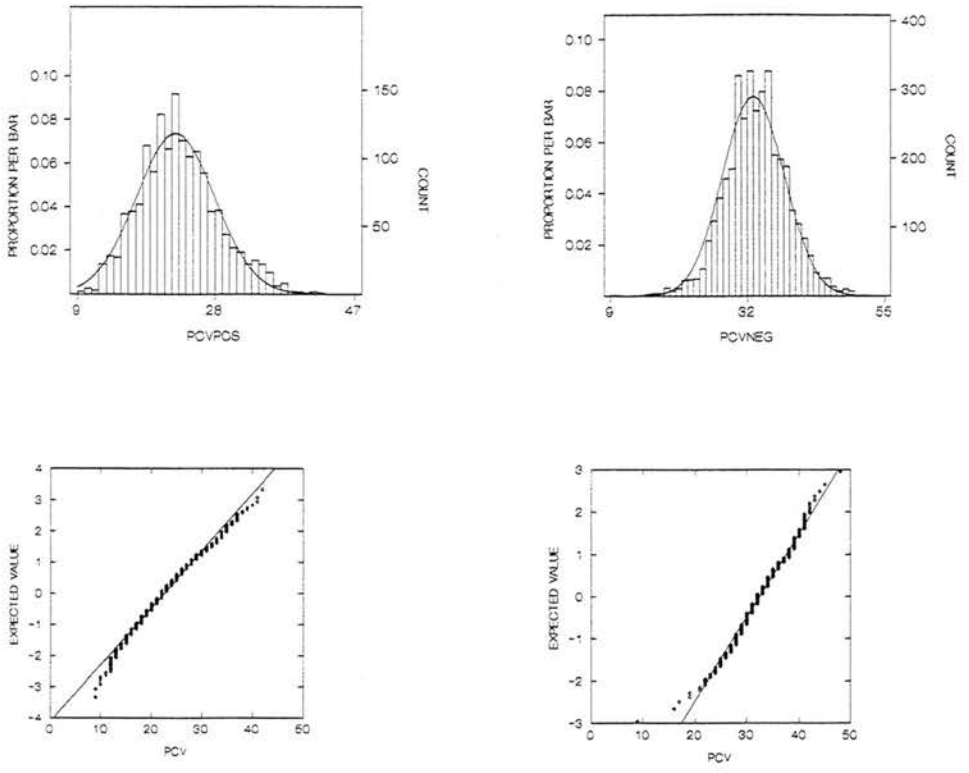
Over 19,300 animals were sampled during the period January 1995 to October 1996 and the parasitological and haematocrit data were entered into the Integrated Tsetse and Trypanosomiasis Database. The PCV distribution from 1599 trypanosome infected animals and 3728 animals from a tsetse locally eradicated area were plotted and visually scrutinised for normality. Of the infected animals 10.9% were diagnosed with *T. vivax*, 85.8% with *T. congolense*, 3.1% had mixed *T. vivax* - *T. congolense* infection whilst only 2 animals were diagnosed with *T. brucei*.

3.4.2. Results

3.4.2.1. The Normality of PCV values from trypanosome infected and non infected animals

Figure 3.1 shows the frequency distributions of PCV values from the infected and uninfected animals were plotted, before and after the data were normalised.

Figure 3.1 The PCV frequency distributions of 1599 trypanosome infected (left) and 3728 trypanosome free animals from a tsetse eradicated area (right), before (top) and after normalisation.

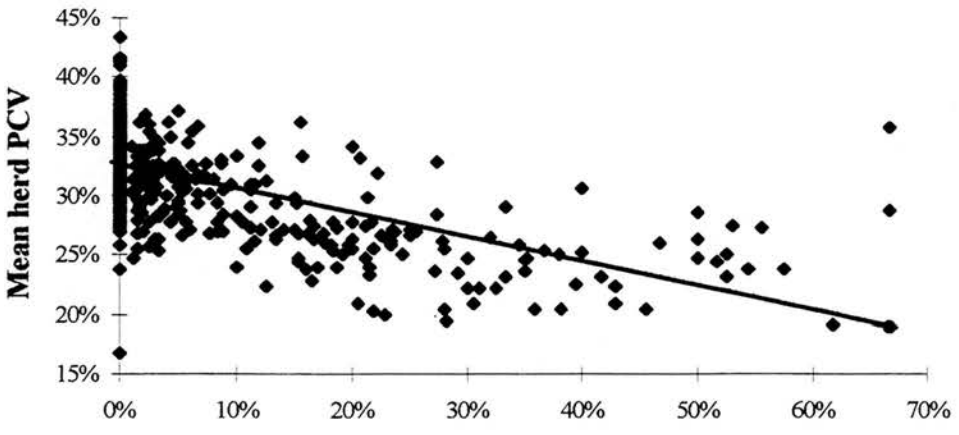


The frequency distributions of haematocrit values from trypanosome infected and free animals are both well approximated by the Gaussian distribution and as such, it was possible to employ usual parametric statistical techniques for herd mean PCV estimates.

3.4.2.2. Herd haematocrit values and prevalence results from routine surveys

The results of this investigation are expressed graphically below, where proportion of animals anaemic in a herd with their logistically transformed values[16], mean herd PCV and herd parasitological prevalence have been plotted. Figure 3.2 shows the relationship between the herd mean PCV and parasitological prevalence with a fitted linear regression line[17] whilst Figure 3.3 shows the relationship between mean herd PCV and parasitological prevalence, both after a logistic transformation.

Figure 3.2 Plot of herd parasitological prevalence and herd mean PCV n = 495



[16] A logistic transformation was used for proportion of herd anaemic and proportion of herd trypanosome infected because both data will be clustered around 0% mark and it is impossible to have values less than 0% or greater than 100%. Hence Logit (P) = Log_e (p/q) where p = number successes (animals infected or animals anaemic) and p + q = n. The data are weighted to account for sample size n.

[17] Linear regression statistics were also deemed acceptable for regression analysis of the mean herd PCV data (with this being the dependant and herd parasitological prevalence being the independent) because mean herd PCV will be normally distributed with mean ~ 30% with extremes <15% or >45% being physiologically impossible.

Figure 3.3 The relationship between mean herd PCV and parasitological prevalence after a logistic transformation n = 495

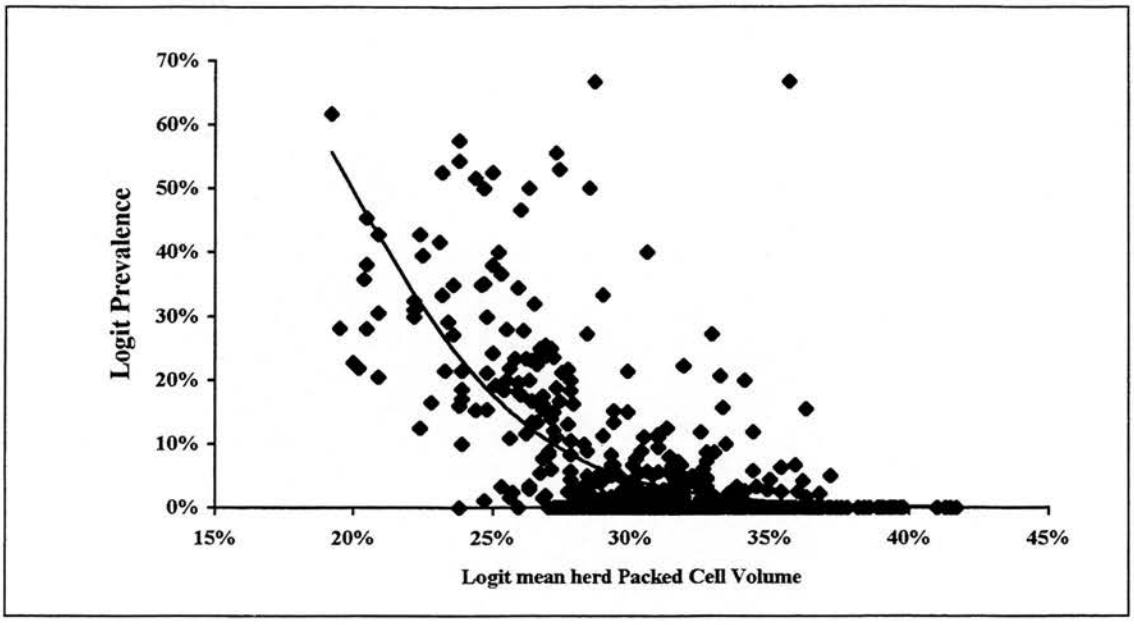


Figure 3.4 shows the relationship between proportion anaemic and herd parasitological prevalence whilst Figure 3.5 shows predicted values of prevalence using PCV as described below

Figure 3.4 **Predicted values of prevalence using PCV data after the logistic regression described below**

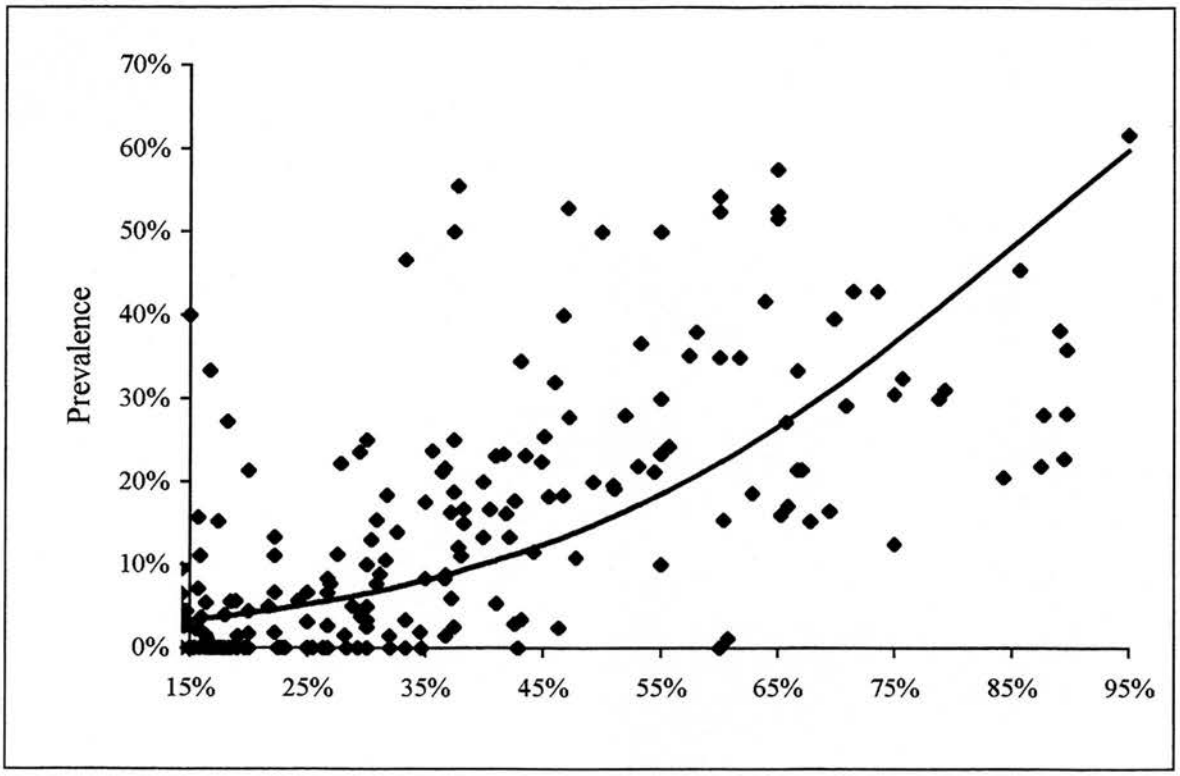
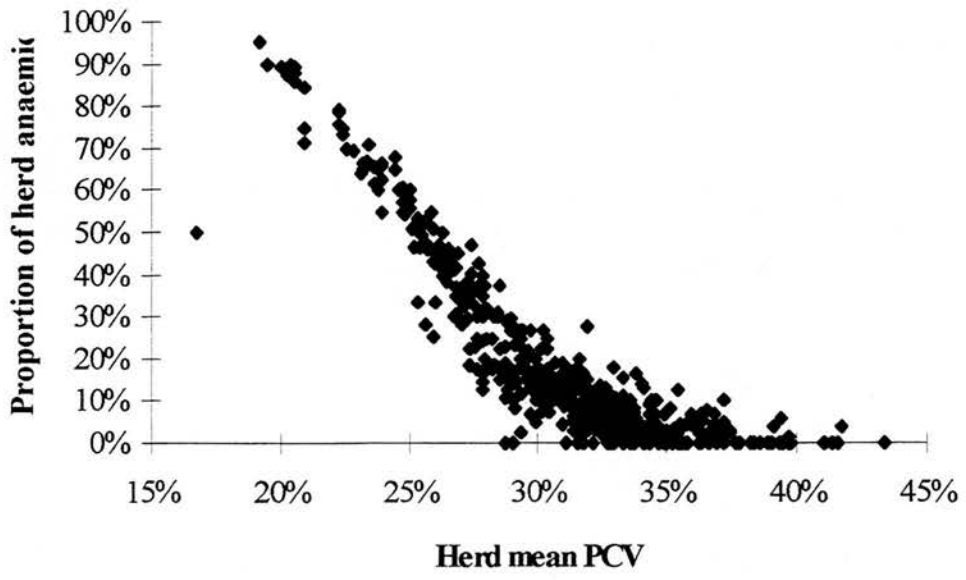


Figure 3.5 Plot of mean herd PCV and proportion of herd anaemic (PCV <= 25%) n = 495



3.4.3. Discussion

Figure 3.5 shows that the mean herd PCV and proportion of animals in a herd that are anaemic are closely correlated with a correlation coefficient of 88%.

When modelling proportion of herd anaemic or herd mean PCV against parasitological prevalence, a logistic transformation was used to fit the data in both cases in an attempt to obtain a closer fit than a linear model. The fit is a maximum likelihood fit using exact binomial errors. The equations for the fits being:

$$Trypanosomiasis\ \text{Prevalence} = \frac{1}{1+e^{a+bx}}$$

where x is either the mean herd PVC or the anaemia and the coefficients a and b (and their correlation coefficients, ρ) are shown in Table 3.6.

Table 3.6: The regression coefficients of PCV and parasitological prevalence after a logistic transformation (top) and with a linear regression (below)

Logistic regression

	Mean herd PCV	Proportion Anaemic
a	−6.08 ± 0.14	+4.071±0.083
b	+30.45 ± 0.94	−4.706±0.057
ρ	−0.99	−0.84

Linear regression

Linear correlation coefficient r	- 0.6009
R Square	0.361
Adjusted R Square	0.359
Standard Error	0.034
Observations	495

ANOVA

	df	*SS*	*MS*	*F*	*Significance F (P)*
Regression	1	0.317	0.317	277.78	8.9785E-50
Residual	493	0.562	0.001		
Total	494	0.879			

a = 0.323 ± 0.0017; b = -0.199 ± 0.0120;

Although regression analyses using logistic transformed data and linear regression on untransformed data are highly significant, the data still differ considerably from the fitted lines. Thus the remaining differences are not simply due to the random errors in the individual data points. Whilst it has been demonstrated both in the literature and examination of results from individuals that trypanosomiasis causes a lowering of the PCV, when using linear regression only ~ 36% of the variance of the mean herd PCV can be accounted for by the model, ~ 64% remains unaccounted for. The same is true for the logistically transformed data, i.e. either the mean herd PCV or the anaemia can be used to get a good estimate of the trend but for individual data sets neither gives a good prediction of the prevalence in an individual herd. Fitting both

simultaneously does not improve the fit because the two are so highly correlated that the improvement in the fit is not significant.

3.5. Results of parasitological and packed cell volume parameters from surveys in the Common Fly Belt of Zambia, 1995 -6

3.5.1. Materials and methods

Individual PCV data were entered into the Integrated Tsetse and Trypanosome Database. PCVs were assigned a single class value in order to deduce the mode PCV class, these are given in Table 3.7.

Table 3.7 Class values used to deduce the mode PCV class for correlation with trypanosomiasis prevalence and producing mode PCV distribution maps

PCV CODE	PCV RANGE
1	less than or including 15%
2	16-20%
3	21-25%
4	26-30%
5	31-35%
6	36-40%
7	41% or over

The mean PCV was calculated for each herd and a lower threshold PCV 25% was chosen to dictate animals with anaemia[18]. Samples for which there was no estimate of PCV were not included in any further calculation which could influence the mean PCV and percentage of animals with low PCVs.

Prevalence was calculated by dividing the number of animals diagnosed positive with trypanosomiasis by the number of animals examined. Because the GIS software package IDRISI can only qualitatively portray 16 differing colours for the output, and a background colour of 0 will be required, then 15 classes are available to represent the trypanosomiasis infection rates. Prevalences above 50% rarely occur (personal

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[18] 25% is the lowest normal value according to Merck (1991) and thus demarcates the pathologically low values from those at the bottom end of the normal range.

observation) because above this level of endemicity cattle are not productive enough to survive, and thus class intervals between 0 and 45% prevalence were assigned and are shown in Table 3.8.

Table 3.8 Class intervals used to portray trypanosomiasis prevalence for GIS output

Crude Parasitological Prevalence %	Crude prevalence class
0%	1
0.001 - 5%	2
5.001 - 10%	3
10.001 - 15%	4
15.001 - 20%	5
20.001 - 25%	6
25.001 - 30%	7
30.001 - 35%	8
35.001 - 40%	9
40.001 - 45%	10
>=45.0001%	11

Over 19,300 animals were sampled during the period January 1995 to October 1996 and the parasitological and haematocrit data were entered into the ITTD. The results were aggregated to the level of inspection site and date, and summarised as database files and exported as ASCII text files in the following format

crushpen code[19] latitude longitude[20] class value[21]

[19] as a text string

[20] latitude and longitude written as decimal degrees to 4 decimal places

[21] an integer

The ASCII text file was then run through a specifically written C programme (TRYP2ID.EXE) to convert it into an IDRISI point vector file of the following format:

class value 1[22]

longitude latitude

00 (at the end of the file)

Because GIS point data is difficult to visualise, the data were rasterised onto a 20 pixels per degree array of Zambia using the IDRISI **Pointras** module.

3.5.2. Results

3.5.2.1. Prevalence map of the Common Fly Belt

The results of the 1995/6 surveys showing prevalence classes per inspection site are given in Map 3.1.

3.5.2.2. Herd mean, mode and percentage of animals with low PCV maps of the Common Fly Belt of Zambia

The results of the 1995/6 surveys showing mean PCV class, mode PCV class and percentage of animals with PCV less than or equal to 25% class per inspection site are given in Maps 3.2, 3.3 and 3.4 respectively.

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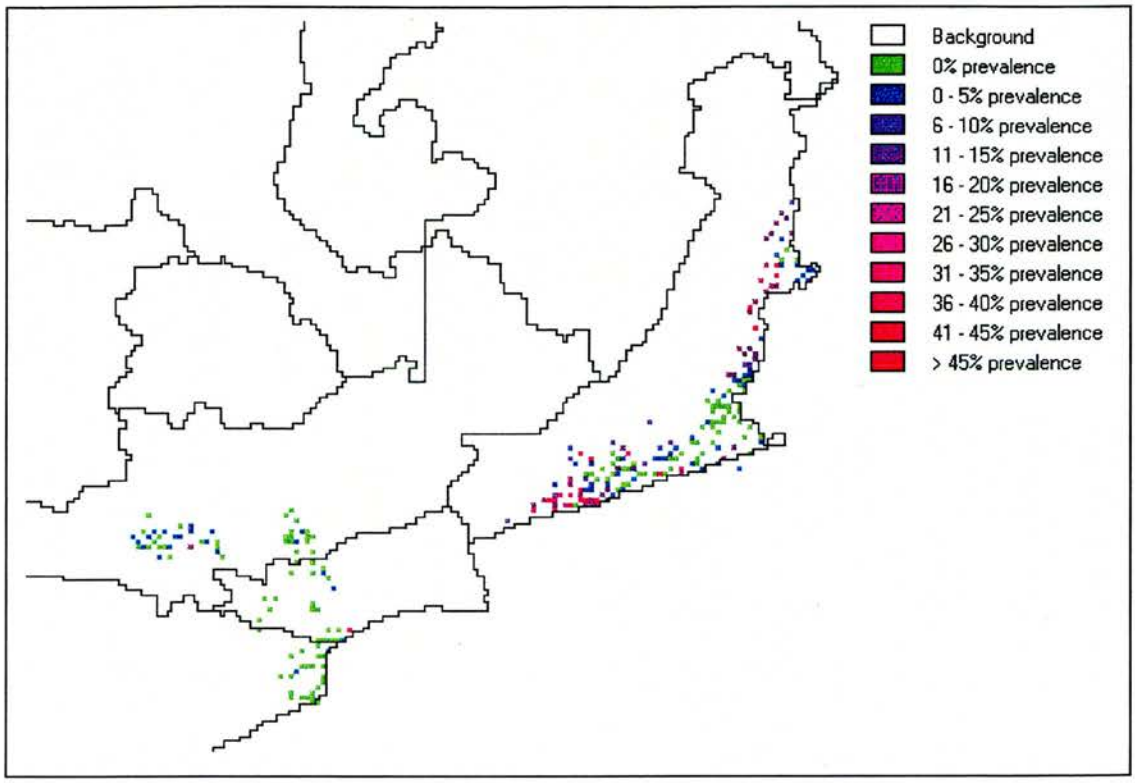
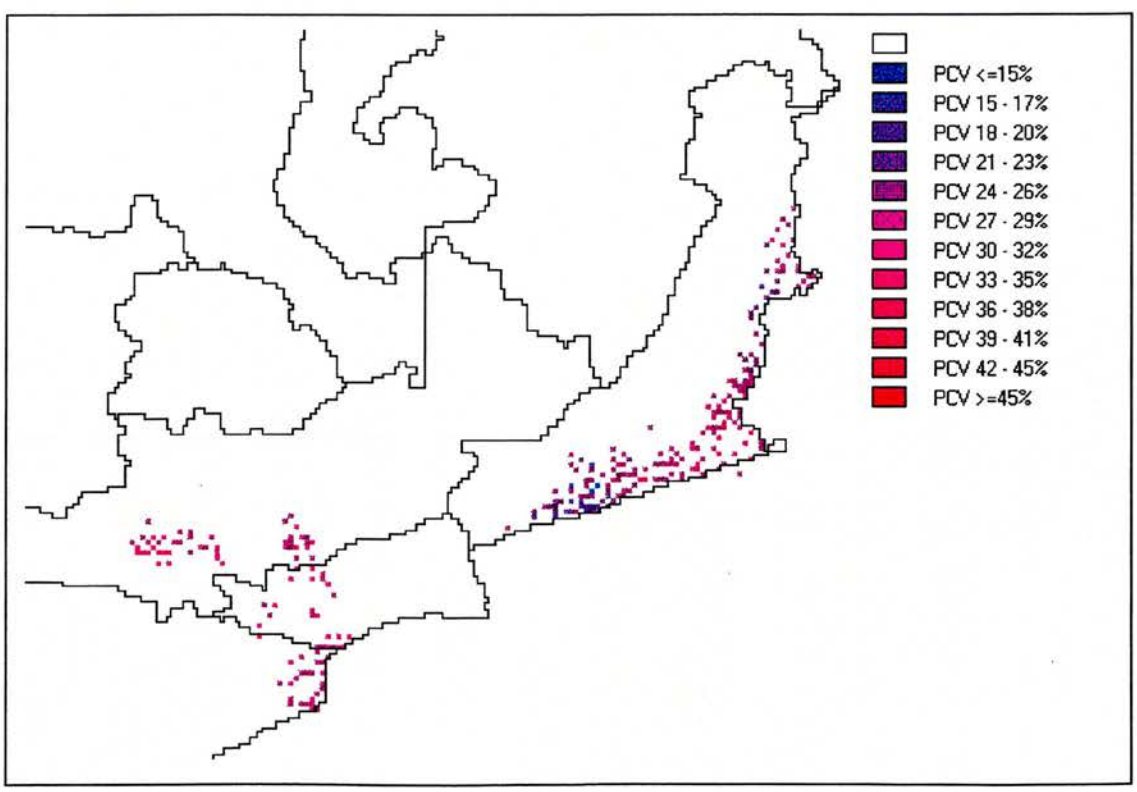
[22] The integer 1 denotes IDRISI vector point data

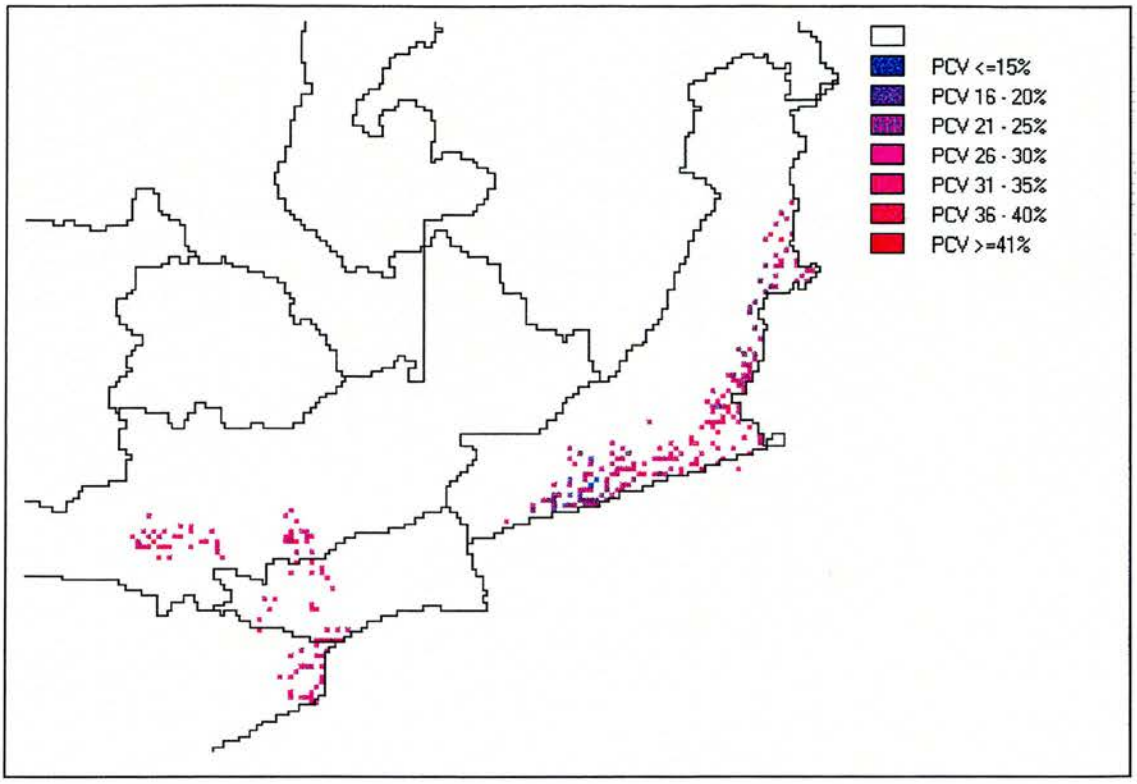
Map 3.1 Map of the Common Fly Belt of Zambia showing trypanosomiasis prevalence rates from 1995/6 survey expressed as classes - see legend



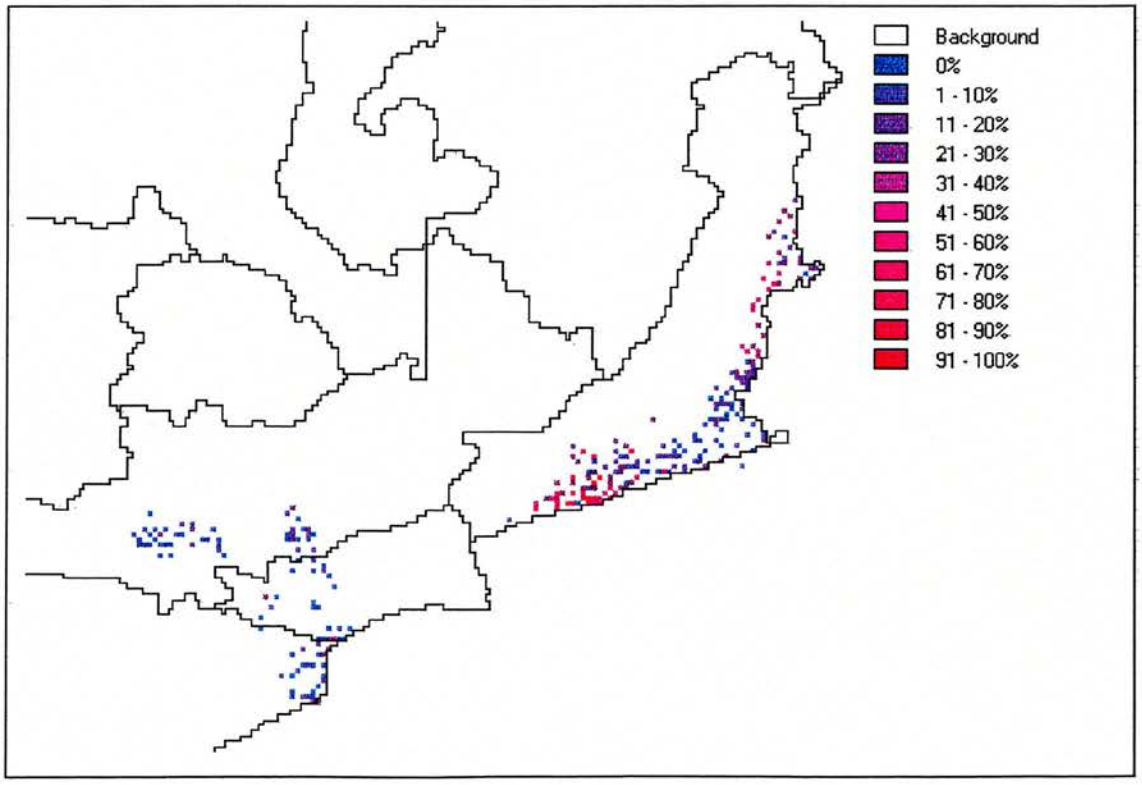
Map 3.2 Map of the Common Fly Belt of Zambia showing herd mean packed cell values expressed as classes - see legend



Map 3.3 **Map of the Common Fly Belt of Zambia showing herd mode classes of packed cell values - see legend**



Map 3.4 **Map of the Common Fly Belt of Zambia showing proportion of animals (expressed as classes - see legend) with PCV values less than or equal to 25%**



3.6. Chapter Discussion

Based on such a large and probably unique data set, it was hoped that by manipulation of the haematocrit data, a strong enough model could have been developed using linear or logistic techniques, to be able to predict (with a reasonable degree of confidence) when trypanosomiasis was present in a herd. Whilst proportion anaemic in a herd and mean herd PCV are closely correlated, as are these two parameters to herd parasitological prevalence, there is a great deal of scatter around either of the fitted lines, and so neither parameter is a useful indicator in predicting trypanosomiasis.

However, when correlating these haematocrit values and parasitological prevalence, it must be borne in mind that the animals diagnosed as positive by these direct parasitological methods will be a sub-set of the animals which were truly parasitaemic (because direct diagnostic tests are only up to ~ 85% sensitive). A more sensitive (indirect) diagnostic test was investigated and this is discussed in Chapter 6.

Maps are clearly the easiest method of portraying survey data, but this is not without problems! Inspection sites where cattle are gathered for examination are represented in the database as a point on the ground. Information derived from such an examination (e.g. disease prevalence by whichever diagnostic test(s) have been employed, or descriptive statistics for haematocrit values) are thus also summarised by this point. GIS data are held as *vectors*, of which there are three types: point, line or polygon, or as a gridded array or *raster image* in which case each cell is called a picture element or pixel (Burrough, 1986). Each format has its strengths and weaknesses and is a large topic of debate. Notwithstanding, spatial data are more easily modelled when in raster format, and thus this is why data for this thesis are presented in this manner. When converting point data to some gridded array, one is required to determine the resolution (defined as pixels per degree [PPD] across one degree of longitude or latitude) of the pixel array. If the resolution is too high (i.e. greater than or equal to 40 PPD), then it is difficult to visualise the pixels, if the resolution is too low, then several points may fall into a pixel and in so doing

information may be lost. Twenty pixels per degree was thus a compromise resolution for the raster array, and was the same resolution at which other important data sets for Zambia were held (e.g. crop use intensity or human and cattle density). Although it is possible to interpolate between pixels using Idrisi prior to modelling, such manipulations are beyond the scope of this thesis.

The trypanosomiasis prevalence maps show the limits of the Common Fly Belt in Zambia, especially areas which are free of the parasite. The sero-prevalence maps as shown in Chapter 5 are probably more informative where areas of low apparent prevalence are more obvious, and do not show up as zero percent parasitological prevalence. The link between PCV and prevalence is apparent in Maps 3.1 and 3.2, but such links are easier to appreciate when plotted on two axes of a graph. The map of mode PCV class was given for thoroughness.

Disease and haematocrit distribution data should probably be displayed at a lower scale, probably at the provincial vs. semi national level. Although a disease of national importance, much of the detail from surveys is lost when trying to display data for half of the country. If being displayed at the provincial level, data from other pertinent data sets (e.g. tsetse density data which are also held as vector point data, or land use and land tenure data which are derived from maps as vector data) would be more easily visualised. As stated above however, if using a GIS for planning strategic control, the software requires that the data be managed in some raster format.

If a vector based GIS were used (e.g. Arc Info, ArcView, Map Info, Atlas GIS for Windows), as well as the political boundary vector files with lakes and rivers, then the survey data would probably be best displayed using a plotter on A0 paper, with locations of diptanks shown as well as prevalence rates (with 95% confidence levels shown) as well as inspection sites visited where disease was not detected but the sample size was inadequate to be 95% confident of having selected at least one positive if disease were present at 5% or above.

Chapter 4

4. The development of an Enzyme-Linked Immunosorbent Assay for detecting anti-trypanosomal antibodies in cattle

4.1. Introduction

The reasons why the antibody - enzyme linked immunosorbent assay (Ab-ELISA) indirect diagnostic test was investigated were discussed in Chapter 1. During the investigations for this thesis, the assay was developed for use as an epidemiological tool to determine sero-prevalence, not for individual diagnoses. The direct diagnostic techniques are reportedly of poor sensitivity which may result in the failure to detect animals with trypanosomiasis, and as a result of which the true prevalence cannot be determined. There are also the problems of the inability to enforce satisfactory quality control criteria on the direct diagnostic methods when teams are working unsupervised in the field. If correct procedures, reagents and quality control software are used in some central or provincial laboratory for an ELISA, and the assay has been properly investigated and established in the first instance and standardised reagents are in use - then correct diagnostic quality control should be insured.

The number of samples that can be examined in one morning via direct diagnostic methods is probably limited to around sixty if a team comprises six members with equipment who can physically fit into one vehicle. If an Ab-ELISA was proven to be a reliable assay, it could be used as a serial test with direct diagnostic techniques for trypanosomiasis, and perhaps supersede the direct tests. If this were possible, many more samples could be collected from an inspection site. This would result in the greater ability to detect disease when occurring at low levels of endemicity, and the ability to attach narrower confidence limits to the infection rate in a population when

the disease was present. These were the main reasons why the Ab-ELISA was investigated.

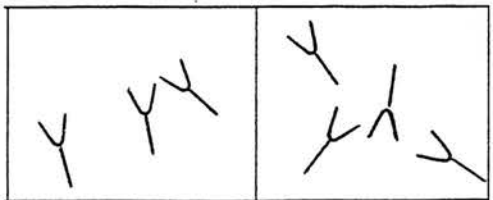
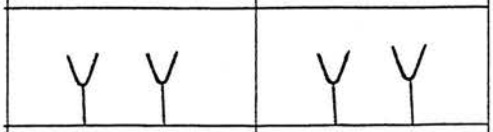
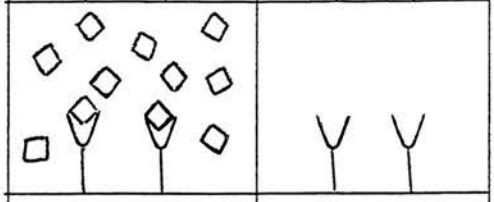
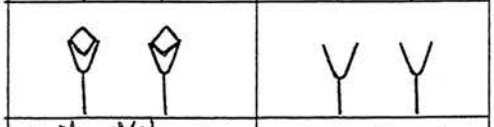
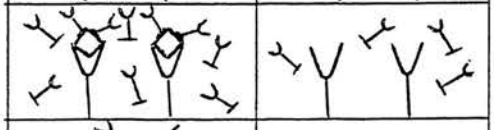
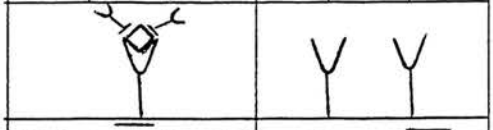
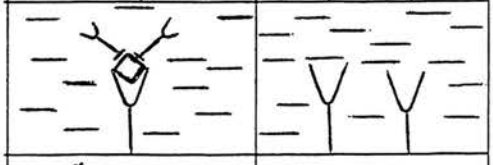
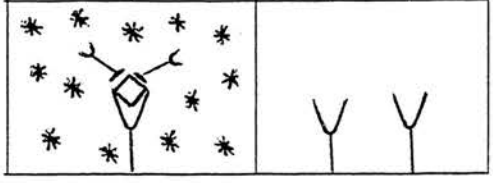
Having developed the assay and determined its sensitivity and specificity, investigations were further required to examine its repeatability and reproducibility (and hence values that could be used in the quality control software), the sources of variation, the effects of sample management and factors that could affect the routine day to day running of the assay. Assay results were compared using antibody derived from sera or eluted blood spot. These were also compared to results from another indirect antibody detecting test, the indirect fluorescent antibody test(IFAT).

4.2. A review of the literature of the tsetse transmitted trypanosomiasis antibody trapping ELISA

4.2.1. A review of the development and usage of the assay

The theory and practice of the ELISA is reviewed by Voller *et al.* (1976). The antibody detecting assay investigated in this thesis is an indirect ELISA which relies on enzyme labelled immunoglobulins. The trypanosome antigen is coupled to a solid phase support and the test sera (or eluted blood spot if used) is incubated in this sensitised carrier. Enzyme labelled antiglobulin (conjugate) is added and this becomes attached to the antigen/antibody (ag-ab) complexes on the carrier surface. The amount of conjugate attached is measured by the amount of substrate it degrades, which is measured quantitatively via a spectrophotometer. Figure 4.1 diagramatically represents the assay. The read-out of the optical density (syn. absorbance) is on a $\log_{10}$ scale.

Figure 4.1 Stages of the antibody trapping ELISA

Assay stage	*Antibodies present*	*Antibodies absent*
The plate is coated overnight with soluble antigen. The soluble trypanosome protein antigen binds to the solid phase support of the plate		
The plate is washed to remove un-bound antigen		
The wells with the sensitised carrier are incubated with test sera. Anti-trypanosome IgG antibodies, if present, bind to the trypanosome antigen		
The plate is washed to remove un-bound antibodies		
Wells are incubated with enzyme labelled anti bovine IgG conjugate		
The plate is washed to remove un-bound conjugate		
A substrate chromagen is added which in the presence of the enzyme degrades to give a quantifiable colour change		
The assay is stopped with acid and the absorbance measured with a spectrophotometer		

Symbols:

Soluble trypanosome antigen

◇ Anti trypanosome IgG antibody

Anti bovine IgG enzyme labelled conjugate

— Substrate chromagen

✳ Colour change

An indirect enzyme linked immunosorbent assay for detecting anti-trypanosome antibodies in cattle was first described by Luckins (1977). The assay has subsequently been shown to have a comparable sensitivity and specificity to IFAT (Luckins, 1995). There is no cross reaction with the non pathogenic stercorarian trypanosome, *T. theileri* nor other haemoprotozoön infections (Luckins, 1977). Although there have been variations in the use of enzyme and substrate systems, the assay has remained little changed since its development. One major advance in interpreting the colour change that the assay causes, is through the adoption of the percentage positivity technique. The use of internationally recognised reference materials and quality control checks has been recommended (Wright *et al.*, 1993). Apart from the work described in this thesis, the only other developmental work with bovine tsetse transmitted trypanosomiasis and antibody ELISA reviewed was carried out in West Africa where work was carried out to study the decline of antibody in artificially infected animals (Bocquenten, Very and Duvallet, 1990) and attempts were made to improve the assay by studying the rate of colour change and volume of reagents per well (Bocquenten and Duvallet, 1990).

Like other indirect ELISAs utilising the chromogenic enzyme/substrate system, the results when read photometrically provide some value of optical density (OD) or absorbance. Several methods have been employed for the expression of antibody activity, none of which have been adopted universally (Wright *et al.*, 1993). With the indirect ELISA there is a positive relationship between the intensity of colour and the amount of antibody bound in the test system. A number of qualitative and quantitative methods have been applied to express these (De Savigny and Voller, 1980). The methods most commonly used include:

* Raw OD values in which the OD is given as a decimal or multiplied by 1000. It is of little use without in-depth knowledge of assay performance, nor are the values useful for intra- or inter- laboratory comparisons.

* Corrected OD values, mostly used with virological systems, in which the subtraction of some control antigen OD compensates for binding of antibody to host cell components.

* End-point titration which is derived from other classical serological assays. The end-point titre is expressed as the reciprocal of the highest serial titration which demonstrates a minimum antibody activity. This technique has no diagnostic advantage over single dilution assays.

* A standard curve in which the OD of each sample tested at a single dilution is read against a standard curve to obtain an estimate of end-point titre. This method assumes a parallel dose/response curve for all samples. The standard curve must be established for every run of the assay and for inter-laboratory comparisons the same set of standards must be available to and used by all laboratories.

* Normalised OD values with the inclusion of a defined positive reference standard in the test and a correction factor is determined on the basis of "expected" vs. "observed" OD values for this reference at a single dilution. All sample OD values are normalised with the correction factor.

* Signal to noise or positive:negative ratio whereby the test sample OD is expressed as a ratio to a negative reference standard. This method assumes that the negative reference standard is truly representative of the normal population of uninfected animals.

* Percent positivity in which the sample tested is expressed as a percentage of a highly positive reference standard. This relative measurement expresses results on a continuous scale from 0% upwards.

Based on the recommendations of Wright *et al.* (1993) the percentage positivity technique was used as the method of expressing the antibody results in this thesis. For

certain experiments conducted on the same plate, results as ODs were analysed. The internal quality control (IQC) checks recommended by Wright *et al.* were also employed.

4.2.2. The nature of trypanosomal antigens

Immunogens are substances which induce an antibody reaction in the host. Antibodies bind to antigens. Tsetse transmitted trypanosomes contain two functional groups of antigens; common (syn. invariant) and variable. Common antigens are components which are the same at all stages of infection whilst trypanosomes are in the bloodstream. They are not exposed on the surface of the trypanosome and it was believed that they elicited only a weak antigenic response by the host (CTVM, 1992). Recent work (Authié *et al.*, 1993) has shown that a 33 kilodalton invariant antigen cysteine protease, "congopain" - which is a member of the papain superfamily, elicits a measurable IgM and IgG response.

Following antibody mediated destruction of each wave of trypanosomes, a wide variety of invariant antigens are released and it has been suggested that these antigens may be involved in inducing the pathology associated with the disease (Mansfield, 1990). The surface coat of trypanosomes alter whilst the parasite is eluding the host's immune system, a technique known as antigenic variation. This surface coat, which is 12 - 15 nm. thick, consists of a monomolecular layer of variable surface glycoprotein (VSG). Only one variable antigen at a time is expressed on an individual trypanosome, which is called the variable antigen type (VAT). When trypanosomes undergo cyclical development in the tsetse fly, the surface coat is shed, and then re-acquired by the metacyclic forms which are infective to mammals.

A cloned trypanosome has the capacity to express several hundred different VATs, but the total number remains unknown. Each cloned infection has a characteristic repertoire of VATs. Cloned parasite populations with the same or very similar repertoires belong to the same serodeme. There are several serodemes within each *Trypanosoma* species.

4.2.3. The Immune response of cattle to trypanosomes

The host's defence system against trypanosome infection comprises two components; non specific protection and antigen specific immunity. Specific immunity is acquired on contact with the antigen and its most important features are its ability to specifically recognise and memorise these antigenic stimuli, so there is an enhanced and accelerated capacity to resist further infection (CTVM, 1992). Two types of immune response exist; humoral (or antibody) and cell mediated. These functions are performed by bursa derived (B cell) and thymus derived (T cell) lymphocytes respectively. Although T cells cannot produce antibody, they are required to initiate and regulate antibody production by B cells.

Cattle infected with trypanosomes display a varied and complex immune response; some antibodies are produced which will react with the VSG of the trypanosome population isolated at that time of infection and thus provide a protective function, whilst other antibodies are produced against the invariant, somatic antigens which remain stable throughout infection. These antibodies show extensive cross-reactions between different populations of the same stock, different stocks of the same trypanosome species, and even different species of trypanosomes (CTVM, 1992) and do not appear to posses any protective activity.

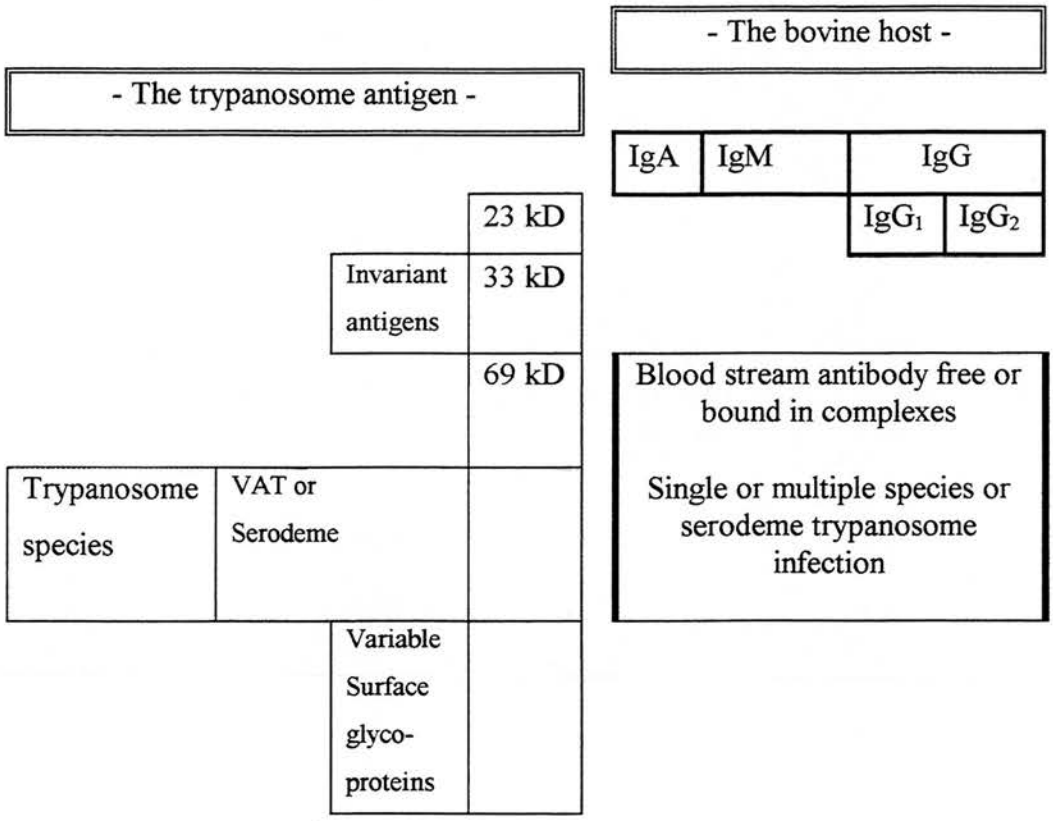
The production of variant specific protective antibodies can be shown to approximate closely to fluctuations in parasitaemia (Wilson and Cunningham, 1972). Fractionation of sera from animals infected with *T. brucei* has shown the protective antibodies are mainly of the IgM class early in infections, but later switch to IgG (Seed, 1972; Nantulya *et al.*, 1979; Musoke, 1981). These findings have also been recorded with *T. congolense* infections (Luckins, 1976; Masake *et al.*, 1983). Concurrent infections with different serodemes or different species of trypanosomes may profoundly alter the ability of the host to respond to challenge (Morrison *et al.*, 1981; Nantulya *et al.*, 1982; Luckins *et al.*, 1983; Luckins, Rae and Gray, 1983; Luckins 1986). The work of Authié and workers (1993a and 1993b) has shown that

the ability of cattle to switch from IgM to IgG in response to congopain is linked with trypanotolerance.

4.2.4. Trypanosome antigen and bovine antibody interaction

Immunologists and diagnosticians alike are interested in the trypanosome antigen and antibody interaction, both in the host and in the laboratory. Elucidating the interaction is complicated by the multitude of antigens and antibodies which can be produced and studying their effects *in vivo* and *in vitro*. Figure 4.2 below portrays the matrix of possible interactions.

Figure 4.2 The antigen - antibody matrix; immune responses in the bovine host to antigens and ability to detect antibody



The ability to detect antibody/antigen binding depends on the type of antigen protein, the nature of the antibody and the method(s) used to visualise the binding. Antibody can be separated into its various moieties by immunoblotting[1] whilst antigens are purified by electroelution from polyacrylamide gels (Authié *et al.*, 1993a). As IFAT relies on antibodies binding to intact trypanosomes fixed to an inert carrier, presumably this test only detects antibodies raised against the VSG coat. With antibody trapping ELISAs, the trypanosomes are lysed and the majority of particulate matter is removed by centrifugation and also, if required, by filtration. How much of the resulting soluble protein is derived from the contents of the trypanosome or from the surface has not been investigated.

The ELISA developed during these studies employed an anti-bovine IgG (whole molecule) peroxidase conjugate. Thus, there is no differentiation between IgG_1 and IgG_2 globulin, and some cross reaction with IgM could be expected[2]. It is also possible that antibodies may not be detected in serum because they are already bound in antigen-antibody complexes (Authié *et al.*, 1993b) and not free to subsequently bind to the antigen adhering to the solid phase of the ELISA plate well. With ELISAs, the colour change will be a function of antibody concentration and avidity, i.e. the overall binding strength of antibodies in the sample.

4.2.5. The choice of cut-off value

With a diagnostic test whose results are expressed on some continuous scale (such as percentage positivity) some cut-off value must be selected in order to dichotomise the results into being positive or negative (Vizard *et al.*, 1990). Given the disease status (presence or absence) of the populations tested, then the cut-off determines which proportion of samples fall into the following classes:

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[1], Identifying the molecular weights of the protein of trypanosome lysate is accomplished by the addition of prestained molecular markers.

[2] Cross reaction between IgG and IgM could be expected due to similarities on the light chain of the bovine immunoglobulins.

Actual health status with Disease

Test result	**Disease present**	**Disease absent**
Positive	a (true positives)	b (false positives)
Negative	c (false negatives)	d (true negatives)
	a+c	b+d

When a cut-off is selected then there will be an inverse relationship between sensitivity and specificity[3]. In selecting a cut-off, the purpose of the diagnostic test must be assessed and the implications of misdiagnoses (false positives and false negatives) must be appraised. As the cut-off value selected dictates the sensitivity and specificity of the test, then in turn it will dictate the predictive value of the test as well as the estimate of true herd prevalence from apparent herd prevalence according to the following equation:

$$\text{True prevalence} = \frac{(\text{apparent prevalence} + \text{specificity}) - 1}{(\text{sensitivity} + \text{specificity}) - 1}$$

where:

$$\text{apparent prevalence} = \frac{\text{Number of animals with PP greater than the cut-off value}}{\text{Number animals sampled}}$$

$$\text{Specificity} = \frac{\text{Number of samples with a PP less than the cut-off value}}{\text{Number of samples from animals without the disease of interest}}$$

$$\text{Sensitivity} = \frac{\text{Number samples with a PP greater than the cut-off value}}{\text{Number of samples from animals with the disease of interest}}$$

A cut-off may be determined by one of several methods (Thrusfield, 1995), for example:

* the mean of the negative population plus two or three standard deviations. This is only pertinent if the unaffected population has some normal distribution. Transformation, such as logarithmic[4] transformation may first be necessary

[3] sensitivity is the ability to diagnose animals with the disease under investigation (true positive) a/a+c whilst specificity is the ability to detect animals without disease (true negatives) d/b+d

[4] other distributions that may be tested are the Weibull and gamma distributions

* fixing the specificity at 95% by choosing the PP representing the 95% percentile of the negative population cumulative frequency. The same method can be employed with the positive population which will result in 95% sensitivity

* by choosing a cut-off value giving the lowest percentage of errors (Se + Sp)/2 (Lessard, 1994) or greatest diagnostic power (Se x Sp) (Eisler and Hopkins, 1996). The cut-off selected by either method is the same.

* choosing the optimum cut-off which minimises the value of *c* which is calculated from the cost of false diagnoses (cost of false negative/cost of false positive), the prevalence of infection, and the density distribution of test values from infected and uninfected animals (Vizard *et al.*, 1990)

The aim of the diagnostic test and the costs of the results (i.e. the costs of the implications of false positives and false negatives results) must be considered in selecting the cut-off. For example, if assessing the sero-prevalence of trypanosomiasis using the Ab-ELISA in a known endemic area, increasing the sensitivity (by lowering the cut-off and at the same time lowering the specificity) would not carry adversely detrimental consequences. Conversely, if wanting to prove trypanosomiasis had been eliminated from an area and hence the tsetse fly had been locally eradicated (and control operations could cease by uplifting of targets) the consequences of false negatives would be very costly. On these occasions one may opt for a cut-off value that is highly sensitive and thus minimise the risk of declaring herds free of the disease when it is still in fact present.

The cut-off value will vary with the batch of antigen, control sera and samples drawn from positive and negative populations. As determining the cut-off reflects a large investment in laboratory effort, then the "test run[5]" which needs to be experimentally established on each occasion should be used for as long as possible when screening test samples.

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[5] screening a batch of test samples using the same batch of antigen and strong positive sera, for which the sensitivity and specificity have been determined.

4.3. Assay development

4.3.1. Materials and methods of the antibody ELISA

The materials necessary, methods and assay protocol employed are given in Appendix 3.

4.3.2. Establishment of optimum assay conditions for antibody ELISA for bovine trypanosomiasis

4.3.2.1. Assay incubation times, temperatures and volumes

In addition to establishing the optimum assay conditions by varying the dilution of antigen, antibody and conjugate, the assay is also affected by the time, volume and temperature that the biologicals are incubated together. Overnight coating of antigen at +4 °C at pH 9.6 is a standard method for ensuring an even and complete binding of the antigen to the surface of the ELISA plate. The period for which antibody is incubated with the antigen coated well should fundamentally influence the performance of the rest of the assay. The shorter the incubation period the less antibody will bind to the antigen and *vice versa*. The serum incubation time can vary between thirty minutes (Rae, 1994), sixty minutes (Eisler, 1996) or longer. Obviously, in order to establish the optimum assay conditions, the maximum ag-ab binding is required with little non-specific binding by bovine antibodies (i.e. proteins other than anti-trypanosome immunoglobulins) to the plate surface. Against this need for maximum ag-ab binding is the practical issue of running plates on a routine basis in the laboratory, as well as managing the samples and data. Thus a compromise serum incubation time of 45 minutes was chosen. The other incubation that will influence the colour change is the time with which enzyme labelled substrate is incubated with the ag-ab complex. If a shorter time has to be chosen out of practical necessity, then this can be compensated for by using a lower conjugate dilution. Thirty minutes conjugate incubation time was chosen. Finally, the substrate incubation time will effect the final colour change. Ten minutes was chosen for the assay. According to Bocquetin and Duvallet (1990) the rate of the colour change after the substrate has

been added gives a better indication of the quantity of antibody, compared to measuring the optical density (OD) at some specified time. The technique to measure **rate** of colour change could not be determined given the equipment and resources available at the University of Zambia. Buffers, reagents and biologicals were used at ambient temperature and standard volumes of 100 µl were used.

4.3.2.2. Principles of assay development

All reagents, apart from soluble trypanosome antigen and control sera, can be purchased from a laboratory supplier. Because the assay is very prone to contamination and hence spurious results, critical attention must be paid to cleanliness of all receptacles used, and the quality of the water used for making up buffers. Fresh double distilled de-ionised water was used for all the Ab-ELISA work carried out. In establishing the assay, the following conditions were established to optimise the test:

* a satisfactory colour change with the strong positive (C++) control sera. OD in the range 0.8 to 2.0 was deemed satisfactory.
* as high as possible differentiation between strong positive and negative control (C-) sera - the so-called "signal to noise" or "binding ratio"
* a low background reading where no sera have been added (conjugate control)
* for future quality control, dilution of a batch of weak positive control sera which falls towards the bottom of the linear part of the curve.

4.3.2.3. Determining the optimum antigen, serum and conjugate dilutions

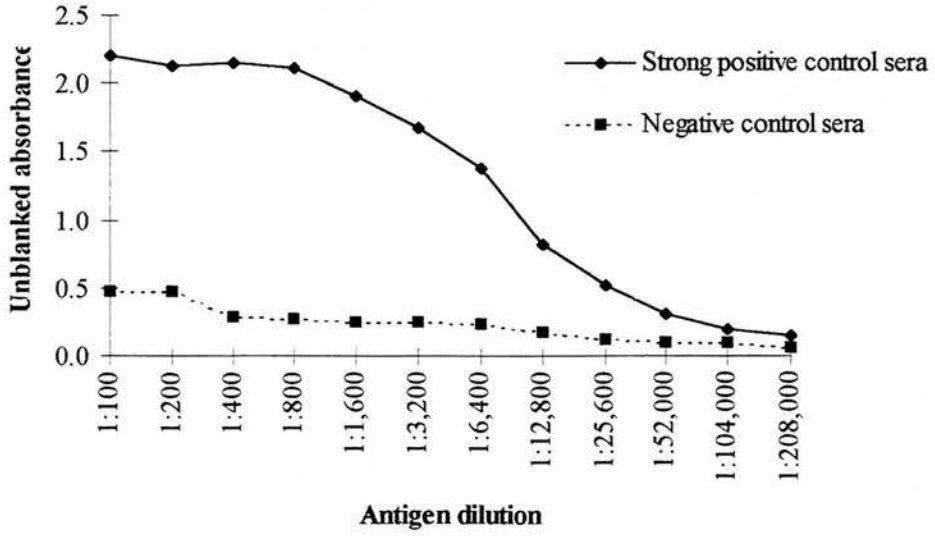
There are two approaches to optimising the dilutions of antigen, serum and conjugate. The method preferred by Rae (1994) relies on selecting an antigen dilution which will provide 1 µg/ml. and coating at 100 µl per well soluble protein (1 mg/ml. stock antigen is diluted 1:1000 coating buffer) and then running a chequerboard titration of C++ serum and conjugate, then selecting the serum and conjugate dilutions which gives the best colour change. An alternative method according to Eisler (1996) follows a two tier approach: i) an antigen titration is performed to asses at what dilution antigen is saturating the binding sites on the well and ii) a quadruple

chequerboard on one plate, whereby for 24 permutations of sera and conjugate dilutions Cc, C++, C+ and C- are assessed and a "binding ratio" is calculated which gives the greatest discrimination between strong positive and negative control sera.

4.3.2.3.1.Materials and methods

When a new batch of antigen is used, its optimum dilution for coating the plate must be ascertained. This is the dilution at which a plateau is reached and little further binding of the antigen protein to the solid phases will occur. Arbitrary dilutions of serum (1:400) and conjugate (1:14,000), normally the same as used for the previous batch of antigen, are used. *T. congolense* antigen with a protein content of 7.2 mg/ml. (the production of which is described below) was serially diluted from 1:100 to its end point of 1:208,000. C++ and C- sera were used. The optical density results are shown in Figure 4.3 below.

Figure 4.3 *T. congolense* antigen titration; Conjugate 1:14,000 Serum 1:400



Having selected an antigen dilution of 1:1000, where the absorption curve clearly levelled off, Cc, C-, C+ and C++ were run on a single plate with sera being diluted 1:50 to 1:1,600 and conjugate was diluted 1:5,000 to 1:40,000.

4.3.2.3.2.Results

The results of this chequerboard titration are given in Table 4.1. The optimum assay conditions were chosen where the binding ratio is highest (serum 1:400, conjugate 1:20,000) and yet the background "noise" where no sera was added had an OD of 0.019.

4.3.2.4. Antigen Production

4.3.2.4.1.Introduction

Crude somatic antigen is produced by lysing trypanosomes which have been harvested from the blood of laboratory animals harbouring a fulminating parasitaemia. Lysis is achieved by ultrasonication or repeated freezing and thawing. Trypanosomes can also be grown by *in vivo* cultivation. Antigen can also be produced by using recombinant DNA techniques.

The batch of soluble antigen used for the Ab-ELISA assay totally dictates all future results from the investigations into the assay. Having committed the considerable resources into establishing the sensitivity and specificity of the assay using standardised times, dilutions, and percentage positivities compared to some reference sera then these should remain constant. Fresh conjugate can be purchased from a supplier, and if required, new strong positive control sera can be produced to give a comparable OD to the previous batch, ideally using a homologous strain of trypanosome infecting similar cattle. Negative control sera can be obtained from any tsetse free area (ideally within Africa) and weak positive control sera can be made by diluting the strong positive in negative sera until the required percentage positivity is achieved. Thus, when investigating the assay, a relatively large volume of

Table 4.1 Chequerboard titration to establish optimum assay conditions

April 1996 Tc antigen 1:1000 45 mins serum incubation and 30 mins conjugate

Serum dilution	50	100	200	400	800	1600	50	100	200	400	800	1600
Conjugate dilution	**Cc**						**C+**					
40K	0.012	0.016	0.015	0.021	0.017	0.015	0.546	0.436	0.276	0.151	0.093	0.061
20K	0.017	0.020	0.017	0.019	0.023	0.019	0.934	0.683	0.490	0.274	0.185	0.124
10K	0.100	0.104	0.113	0.105	0.122	0.119	1.578	1.161	0.873	0.658	0.450	0.308
5K	0.220	0.189	0.185	0.235	0.223	0.234	2.118	1.723	1.331	0.964	0.661	0.501
40K	0.892	0.791	0.660	0.553	0.431	0.330	0.121	0.111	0.080	0.055	0.040	0.032
20K	1.530	1.360	1.161	1.029	0.738	0.584	0.311	0.194	0.143	0.083	0.071	0.054
10K	2.073	2.023	1.818	1.570	1.259	1.027	0.648	0.497	0.368	0.280	0.230	0.191
5K	2.682	2.591	2.379	2.193	1.956	1.539	1.091	0.779	0.676	0.381	0.364	0.290
	C++						**C-**					

Uncorrected optical density shown in body of table

Binding ratios

Serum dilution 1:	50	100	200	400	800	1600
Conjugate dilution 1 40,000	7.4	7.1	8.3	10.1	10.8	10.3
20,000	4.9	7.0	8.1	12.4	10.4	10.8
10,000	3.2	4.1	4.9	5.6	5.5	5.4
5,000	2.5	3.3	3.5	5.8	5.4	5.3

trypanosome antigen is required if the findings of some investigative stage are to be used in the routine running of the assay.

Because *T. congolense* is the most commonly occurring species of trypanosome in Zambia, this species was used for the production of antigen. *T. brucei* antigen had been used for previous investigations into the development of the Ab-ELISA (Rae, 1994) but had been subsequently found to give an inferior binding ratio compared to *T. congolense*. *T. vivax,* especially Southern African strains, are notoriously difficult to rear under laboratory conditions, and will only grow in goats and not laboratory rodents. Given the requirement for a large volume of antigen which was to be used operationally in several laboratories within the Southern Africa Region, rats were used as to grow the *T. congolense*. It was estimated that approximately 15 ml. of antigen whose optimum dilution of 1:1000 was required for the laboratories.

Trypanosomes could be separated from the blood by standard techniques, but could not be lysed by ultrasonication (Luckins, 1977b) as no ultrasonic disintegrator was available within the University of Zambia. Freezing and thawing of the trypanosome pellet gave satisfactory results.

4.3.2.4.2.Materials and methods

Cultivation of trypanosomes in rats

A donor rat was initially inoculated with an isolate of *T. congolense* recovered from cattle in the Eastern Province of Zambia. Eighty rats were inoculated intraperitioneally with 500 µl infected blood (10^5 trypanosomes per millilitre of blood) using the appropriate syringes (2.5 ml) and needles (21G). The parasitaemia of the infected rats was checked daily using microscopic examination of a wet blood smear preparations until a peak parasitaemia is achieved (normally on the fourth day), the determination of which is described by Murray *et al.* (1977). The rats were anaesthetised using diethyl ether then bled by cardiac puncture.

The rats were, in some cases, supplemented with 10% w/v glucose in their drinking water when the parasitaemia attained 10^8/ml. of blood (as estimated using a nuebaur cell counter). The glucose supplemented animals normally survived two days longer than those not supplied with glucose in drinking water (Chitambo, 1996). The use glucose in the drinking water of the rats may have increased the final parasitaemia

Preparation of PBSG for the DE52 column

Stock phosphate buffered saline - glucose, pH 8.0 was prepared using the following reagents;

Na_2HPO_4 (anhydrous)	13.48 gm
$NaH_2PO_4.2H_2O$	0.78 gm
NaCl	4.25 gm
Distilled H_2O	1000 ml

Dilute 6 parts of the stock solution with 4 parts distilled water. Add glucose to give 10% w/v solution.

Preparation of Phyto-Haemagglutinin

Phyto-heamagglutinin is a lectin which causes agglutination of erythrocytes (Leavitt, Felstead and Bachur, 1977). The levels of lectins in Zambian local red kidney beans are sufficient for use in the agglutination of red blood cells. About 200g of local beans were ground very finely using a coffee grinder for 5 minutes. The bean flour was dissolved in 800 ml. of PBSG and stirred for ten minutes to maximise the aqueous extraction of lectins. The mixture was allowed to stand for 15 minutes. The clear supernatant was decanted into a 500 ml. beaker and its pH adjusted to 8.0 (the pH of ion exchange purification of trypanosomes from rat blood). 10 ml. aliquots of the solution were transferred to centrifuge tubes. The solution was centrifuged for 10 minutes at 12,000 rpm in a Beckman centrifuge. The supernatant was either used

fresh or stored at -20°C in a freezer for future use. The use of phyto-haemagglutinin helped to extend the limited supply of costly DEAE cellulose anion exchange media.

Blood preparation prior to loading in the DE52 column and obtaining trypanosome pellets

The infected blood was diluted with an equal volume of PBSG pH 8.0 and then incubated with PHA (1:10 (v/v) PHA: the initial blood volume) for 10 minutes at 37°C in a water bath. It was then centrifuged at 500 rpm for 5 minutes. The red blood cells settled at the bottom leaving the trypanosomes in the supernatant. The trypanosome rich supernatant was loaded onto the column. About 67 grams of DE-52 was used to prepare 1 ml. of antigen. The trypanosomes were separated from blood elements by ion exchange chromatography using DE-52 column as described by Lanham and Godfrey (1970). The trypanosomes were eluted with PBSG adjusted to pH 8.0. and were washed twice with 10 ml. of PBSG, pH 7.4. The trypanosome suspension was concentrated by centrifugation in a Beckman centrifuge at 2500 rpm for 15 minutes at +4 °C. The trypanosome pellet was dispersed in minimal phosphate buffered glucose saline pH 7.4 and stored in a freezer at -79 °C.

Preparation of the trypanosome lysates by freeze thaw technique

The pellets of trypanosomes from above were suspended in 5 ml. buffer (PBS pH 7.4) and lysed by three cycles of 10 minutes of freeze - thaw (from - 70°C to 37°C). In some cases, the pellet was suspended in 5 ml. water and vortexed for 1 minute followed by one cycle of thaw-freeze as above. A wet smear microscope slide was prepared to confirm complete lysis of the trypanosomes. The solution was twice centrifuged in a Beckman centrifuge at 10,000 rpm for 10 minutes at +4°C. A total volume of 15 ml. of pooled concentrated *T. congolense* antigen was thus prepared.

4.3.2.5. Control sera production

When running the Ab-ELISA routinely, three primary reference standards are required (Wright *et al.*, 1993) in addition to running the plate with no sera added to four wells. The controls are run in four wells each, thus occupying a total of sixteen wells. Strong positive, weak positive and negative control sera are run on the plate to i) ensure the assay is performing correctly and not "out of control" and ii) to act as a reference sera with which to compare the test samples' optical densities and thus express the antibody activity as a percentage positivity. The strong positive reference standard should exhibit an antibody activity which lies on the linear portion of the dose response curve, just below the plateau. The weak positive (C+) should exhibit an antibody activity which lies on the linear portion of the dose response curve and should consistently demonstrate antibody activity which is greater than the positive/negative threshold (i.e. cut-off). The negative control sera should consistently demonstrate antibody activity below the positive/negative threshold. Negative control sera (C-) was produced by taking blood from African cattle that had not been in contact with tsetse fly and collecting sera by usual procedures. Strong positive control sera should be selected from animals which exhibit a typical humoral response to the organism in question, this being achieved by experimental infection. It may be derived from a single or pool of sera. Weak positive reference sera may also be made by diluting strong positive sera (C++) in C-. By excluding control sera from four wells (conjugate control), the non specific colour change of the conjugate is assessed.

4.3.2.5.1.Materials and methods

Negative control sera was collected using blood from eight Limousine cows from York Farm, Kafue Road, Lusaka (bled using Vacutainer ® tubes) and four Holstein cows from UNZA dairy herd, Lusaka, Zambia (bled via a 14G needle direct into a 500 ml. conical flask) and pooling the resulting sera.

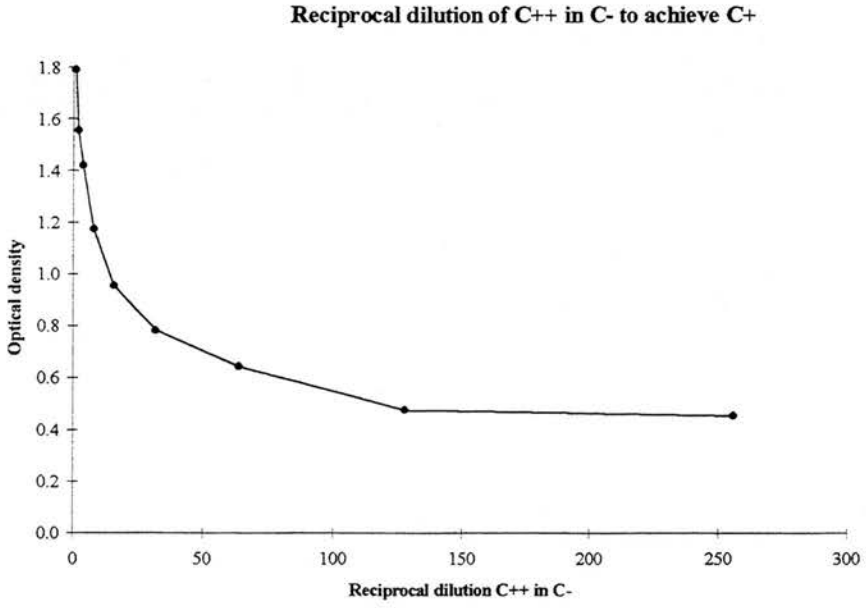
The strong positive control sera was derived in two batches. The first batch was made from a pool of nine sera from the Eastern Province whose sera had exhibited a strong colour change when screened using the CTVM *T. brucei* antigen made in 1994. The second batch was made by artificially infecting two yearling cattle with a field strain of *T. congolense* isolated from the Eastern Province of Zambia. The trial to produce C++, which also yielded data to compare IFAT with Ab-ELISA, is described in Paragraph 4.4 The latter type of production, infection of clean animals with known species of trypanosome, is the preferable method of C++ production as there should be minimal cross reaction between the control sera and antigen. With the pooled sera from field samples, antibodies may have been present against other haemoparasites. Although the Ab-ELISA being investigated is reported to give little or no cross reaction with non tsetse transmitted trypanosomes and haemoparasites, it is preferable to use control sera in which the main antibody present is against tsetse transmitted trypanosomes. This is used to calculate percentage positivities of the test sera rather than control sera where anti *Theileria* or other haemoparasites antibodies may be found.

The batch of C+ was made by diluting C++ in C- and selecting the dilution giving an OD at the bottom of the linear portion of the dose response curve.

4.3.2.5.2.Results

The results of the serial dilution of C++ in C- are shown in Figure 4.4 below.

Figure 4.4 The reciprocal dilution of strong positive in negative control sera to achieve weak positive control sera



In order to make the batch of C+, a dilution of 1 part C++ in 32 parts C- was chosen as this lies at the bottom of the linear portion of the dose response curve.

4.3.3. A standardised quality assurance methodology for the trypanosomosis antibody ELISA and its application in the ongoing assessment of routine use of the assay

4.3.3.1. Use of reference sera and the percentage positivity technique

The optical density values from the antibody trapping ELISA are subject to considerable day to day variation, even when all assay conditions and reagents are identical. This poses two problems: i) to determine when plates are outside the normal limits of colour change and ii) the expression of results from individual samples. These issues have been reviewed by Wright *et al.* (1993) who recommend a

protocol for the standardisation and validation of the ELISA technique following the International Atomic Energy Agency, Food and Agriculture Organisation, World Health Organisation and Office International des Epizooties agreed practices. This paper recommends the use of three control sera occupying four wells on each plate as well as a conjugate control (no sera) and the results from test sera to be expressed relative to the strong positive control sera.

4.3.3.2. Quality assurance and internal - external quality controls

4.3.3.2.1.Introduction

Quality assurance (QA) encompasses all the checks and control activities to ensure that the results of a given ELISA are accurate, precise and reproducible. Quality control (QC) refers to defined activities within the context of QA. Internal QC (IQC) involves those activities which are designed to ensure that the assay performance is precise and reproducible within a given laboratory whilst external QC (EQC) involves those activities which are designed to ensure results are comparable between laboratories. As the Antibody ELISA under investigation was performed in one laboratory, little could be done to investigate EQC.

The IQC checks were performed by several specifically written software programmes[6]. The IQC checks were established for each batch of antigen and differing assay conditions, and were statistical established by repeated testing under routine diagnostic methods. The criteria for plate and test sample acceptance or rejection were based on the recommendations of Wright *et al.* (1993). These are shown in Table 4.2.

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[6] Hopquik.exe written in Borland C language and as one module of the Antibody ELISA database written in Fox Pro for Windows.

Table 4.2 The criteria for plate acceptance or rejection after Wright *et al.* (1993)

Plate IQC	
Four wells on each plate were run with strong positive control sera	The optical densities of three out of four ODs must fall within the range of mean ± 1.96 standard deviations (SD) of repeated testing of the plate.
Four wells on each plate were run with weak positive control sera	The percentage positivities of three out of four wells must fall within the range of mean ± 1.96 SD of repeated testing of the plate
Four wells on each plate were run with negative control sera	The percentage positivities of three out of four wells must fall within the range of mean ± 1.96 SD of repeated testing of the plate
Four wells on each plate were run with no control sera	The percentage of three out of four wells must fall within the range of mean + 1.96 SD of repeated testing of the plate.
Test sample acceptance	
Two replicates of a test sample were run on the sample plate.	The result was rejected if the coefficient of variation was over 12% for samples whose mean OD was over 0.1.

When investigating reproducibility of an assay, there are two forms of bias that can occur. There may be some form of systematic bias which may be correctable, or some form of random variation or bias such as occurs with pipetting errors. Little can be done to correct this latter form of bias.

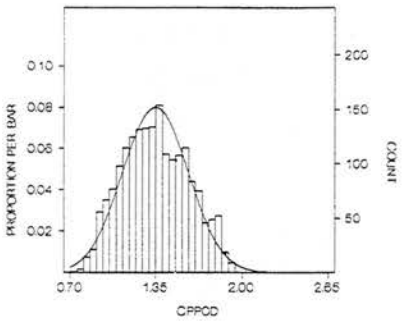
4.3.3.2.2.Materials and methods

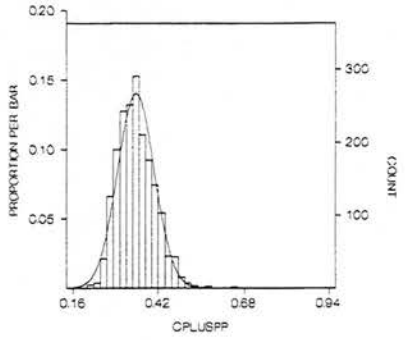
The frequency distribution of ODs and percentage positivities from 475 plates which were run using the same batch of antigen and control sera and assay conditions were plotted and examined for normality. If normally distributed then the mean of the values and their standard deviations could be used to set the upper and lower control limits. These frequency distribution graphs are shown in Figure 4.5. The distributions are normal with the exception of the conjugate control OD and PP which follows a log normal distribution. The criteria for setting the UCL and LCL for the controls was therefore established based on the mean ± 1.96 standard deviations (Table 4.3) which would embrace 95% of the normally distributed control values. The LCL and UCL values used are given in Table 4.4.

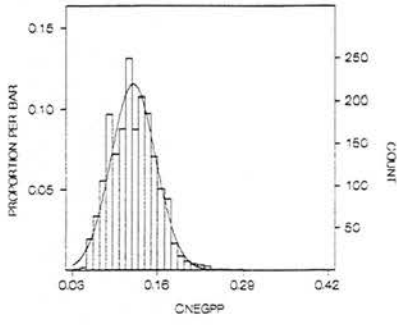
Figure 4.5 The frequency distribution of the optical densities and percentage positivities of the control sera from 475 plates when establishing the assay and testing samples.

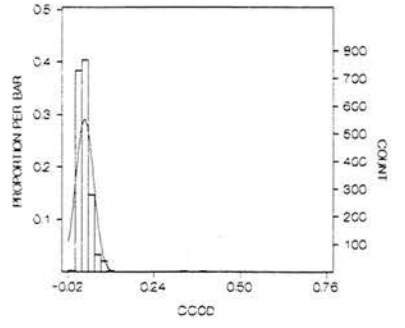
Frequency distribution of the optical densities of the strong positive (above) and conjugate control (below)

Frequency distribution of the percentage positivities of the weak positive (above), negative (middle) and conjugate control (below)









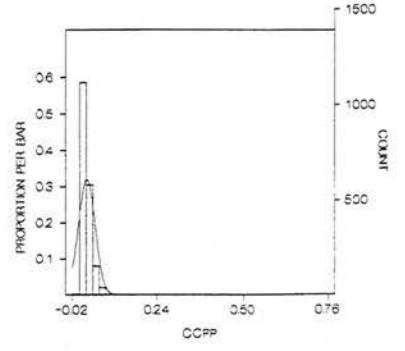


Table 4.3 The means and standard deviations of the optical density (C++) and percentage positivity (C+, C- and Cc) of 475 plates.

	Mean	*SD*
C++ (OD)	1.348	0.249
C+ (PP)	0.350	0.057
C- (PP)	0.122	0.035
Cc (PP)	0.022	0.025
Cc (OD)	0.029	0.027

Table 4.4 The upper and lower control limits used for the IQC checks of the assay when run routinely.

	Lower control limit	*Upper control limit*
C++ (OD)	0.860	1.836
C+ (PP)	23.8%	46.2%
C- (PP)	5.3%	19.1%
Cc (PP)	-2.7%	7.1%

The Cc control limit can be less than zero because OD values are calculated after the subtraction of some blanking value (i.e. the colour change in the absence of the addition of conjugate).

4.3.3.2.3.Discussion

The colour change of the Ab-ELISA clearly follows a normal distribution in its day to day variation. As such, 95% of the observations will fall within the mean ± 1.96 standard deviations. Values outside these limits can be deemed "abnormal" and if observed the plate can be judged as being "out of control". The upper and lower control limits are therefore set by the probability density function of the colour change of the controls (both in absolute and relative terms) when establishing the assay with a batch of antigen and sera.

4.3.4. The effects of water purity, antigen storage and plate coating

4.3.4.1. Effect of water purity when used for plate coating

4.3.4.1.1. Introduction

The water used for making up buffers must be the purest available. Ideally fresh double distilled de-ionised is used. The following experiment was conducted in a laboratory in Zimbabwe which was to perform the antibody ELISA assay but which had repeatedly experienced problems with unsatisfactory binding ratios and an unacceptable conjugate control (background) reading. One possible source of this inability to reproduce assay conditions in neighbouring laboratories may have been due to the poor water quality used to make up the coating buffer.

4.3.4.1.2.Materials and methods

Seven sources of "pure" water were compared to the double distilled de-ionised water which had been used at the University of Zambia (UNZA DDD) to establish the assay. The sources of water compared to UNZA DDD were:

* Millipore water from the Medical Chambers
* water passed though a hand held de-ioniser as used for a *babesia* antibody detecting ELISA (as developed by Australia) in the Zimbabwe Veterinary Laboratory
* Zimbabwe Veterinary Immunology Laboratory double distilled water. The de-ioniser had recently been repaired and recommisionned after being left unused for a period of months
* Zimbabwe Veterinary Toxicology Laboratory double distilled water which was subsequently passed though a hand held de-ioniser
* University of Zimbabwe Clinical Studies laboratory double distilled water
* Water from the Heartwater laboratory - which originated as Millipore water from the Medical Chambers but had been subsequently been handled and stored in a variety of containers, and

* fresh single distilled water from an old fashioned distillation plant in the Tsetse and Trypanosomiasis Control Branch of the Veterinary Department.

4.3.4.1.3.Results

The results are given in Table 4.5. showing the OD and PP values obtained with UNZA double distilled de-ionised water in the left column, and the other sources of water in the centre and right hand columns. The average OD and PP for the four occasions when UNZA DDD was used are shown at the bottom of the table.

4.3.4.1.4.Discussion

The source of the "pure" water has a profound effect on assay results, thus emphasising the importance of this diluent is used ubiquitously throughout the assay. The highest binding ratio was achieved using fresh Millipore water (binding ratio 19.9) which was the highest ever attained using the assay during the period of investigation. This may have been due to a new batch of antigen and a lower working dilution of 1:600 compared to 1:1000. The water from the Heartwater laboratory gave a binding ratio of 7.5 compared to UNZA DDD of 13. The water used in the Heartwater laboratory was in fact the same Millipore pure water from Medical Chambers, the only difference being that it had been held in some container. Presumably the difference in purity is that the container had in some way become contaminated with bacteria or some chemical. The poorest binding ratio observed (3.7) was with tap water that had been passed through a hand held de-ioniser. Although used for a *babesia* antibody detecting ELISA as established by an Australian Project, this water must have contained ionic contaminants which interfered with the binding of the antigen to the solid phase of the well. The next lowest binding ratio (6.9) was that where water from a recently repaired and recommissioned double distillation plant (housed in the Immunology laboratory) had been used to make up the coating buffer. The difference compared to UNZA DDD also demonstrated how susceptible the assay is to less than perfectly "pure" water. The remaining four sources of water all gave comparable binding ratios (11.4 to 12.6) and it is interesting to note that freshly single distilled water from a relatively

inexpensive distillation plant achieved comparable results to the sophisticated plant housed at UNZA.

An analysis of variance demonstrated that there is a significant difference ($P < 0.001$) between the ODs when a plate was coated with UNZA double distilled de-ionised, University of Zimbabwe double distilled and TTCB single distilled water. This was because water from TTCB gave a considerably higher C++ OD and C- OD in absolute terms, but when expressed as percentage positivity the means were very similar.

Table 4.5 The results of the effects of differing water sources for coating ELISA plates

All optical density values have had a substrate blanking value deducted

UNZA Double distilled water			**Heartwater laboratory water**	
	OD	**PP**	**OD**	**PP**
Cc	0.013	1.1%	0.012	1.1%
C++	1.098		1.096	
C+	0.296	27.0%	0.379	34.6%
C-	0.084	7.6%	0.145	13.3%
Binding ratio		*13.1*		*7.54*

UNZA Double distilled water			**Univ. Zimbabwe double dist.**		**Immunology lab. double distilled**	
Cc	0.002	0.2%	0.017	1.3%	0.006	0.5%
C++	1.039		1.253		1.117	
C+	0.329	31.6%	0.403	32.2%	0.388	34.7%
C-	0.075	7.2%	0.092	7.4%	0.163	14.6%
Binding ratio		*13.9*	*13.6*			*6.9*

UNZA Double distilled water			**Toxicology double dist.**		**Single distilled TTCB lab**	
Cc	0.009	1.0%	0.001	0.1%	0.004	0.3%
C++	0.877		0.930		1.389	
C+	0.293	33.4%	0.322	34.7%	0.489	35.2%
C-	0.065	7.4%	0.074	7.9%	0.122	8.8%
Binding ratio		*13.5*	*12.6*			*11.4*

UNZA Double distilled water			**Tap de-ionised water**		**Med Labs Micro Q H20**	
Cc	0.001	0.1%	0.024	2.3%	0.024	2.0%
C++	0.979		1.015		1.177	
C+	0.342	34.9%	0.422	41.6%	0.338	28.7%
C-	0.063	6.4%	0.272	26.8%	0.059	5.0%
Binding ratio		*15.7*	*3.7*			*19.9*

Average for UNZA double distilled de-ionised water		
Cc	0.006	0.6%
C++	0.998	
C+	0.315	31.7%
C-	0.072	7.2%
Binding ratio		*14.0*

4.3.4.2. Antigen storage

4.3.4.2.1.Introduction

One problem sometimes encountered with indirect ELISAs is that over a period of time, there may be an progressive loss of colour change (Clemence, personal communication, personal observation). Reasons for this may include loss of avidity of the antigen or a loss of potency of the conjugate. Loss of conjugate potency is overcome by the relatively easy step of purchasing more from the manufacturer. With antigen this is not an option and so a greater effort must be made to preserve the batch made initially. This is also required to avoid recalibrating (i.e. determining the sensitivity and specificity) the assay when a new batch of antigen is used.

Denaturation of the protein within the soluble invariant antigen may be one of the causes of its loss of avidity. The denaturation of the protein may be as a result of the actions of bacterial proteolytic enzymes or (repeated) freezing and thawing. Thus, the "keeping quality" of antigen may be enhanced by:

* storing the soluble invariant antigen at - 70 °C
* employing semi-sterile conditions when handling rodent blood and separating the trypanosomes
* the removal of bacteria via filtering through a 0.22 μm filter (Authié *et al.*, 1993a)
* the inclusion of protease inhibitors with the antigen (Authié *et al.*, 1993a)
* avoidance of repeatedly freezing and thawing the antigen.

This latter technique can be employed either by coating the plates then storing them frozen as investigated by Sharpe (1979) or by mixing the antigen with an equal volume of glycerol and storing in aliquots at - 70 °C. When an aliquot is subsequently used, it is moved from - 70 °C to - 20 °C where it is no longer frozen solid, and can be pipetted out. If no glycerol has been added to the antigen, it has either to be repeatedly frozen and thawed, or must be removed from the freezer, placed at + 4 °C

and discarded after one to two weeks. These procedures may result in loss of avidity or wastefulness.

4.3.4.2.2.Antigen storage by mixing with an equal volume of glycerol

Materials and methods

The rationale for mixing antigen with an equal volume of glycerol is described above. The addition of such a chemical may, however, have interfered with the assay so as to cause an increase in background colour (conjugate control), a decrease in the optical density of the C++, or adversely affected the PP of the C+ of C-. One half of an ELISA plate was therefore coated normally with a batch of *T. congolense* antigen at 1:600 whilst the other half was coated with the same antigen which had been diluted in an equal volume of glycerol and used at a dilution of 1:300 (working antigen dilution 1:600) to coat the other part of the plate. Two rows each of Cc, C++, C+ and C- at 1:400 were assessed under normal assay conditions.

Results

An analysis of variance was performed after optical density values were converted to percentage positivities for both sides of the plate. The ANOVA table for no glycerol (Treatment 1) and 50:50 with glycerol (treatment 2) is given in Table 4.6.:

Table 4.6 The analysis of variance of the percentage positivities for a plate where half the plate has been coated with antigen mixed with an equal volume of glycerol

```
CONT$
  C+          C++          C-          Cc

 TREAT$
  1           2

DEP VAR:      PP      N:      96  MULTIPLE R: 0.994  SQUARED MULTIPLE R: 0.987

                      ANALYSIS OF VARIANCE

 SOURCE       SUM-OF-SQUARES   DF  MEAN-SQUARE     F-RATIO       P

 CONT$           131905.858    3    43968.619    2298.164       0.000
 TREAT$               0.304    1        0.304       0.016       0.900
 CONT$*TREAT$        24.322    3        8.107       0.424       0.736

 ERROR             1683.622   88       19.132
```

The analyses of variance using percentage positivity shows there to be no significant difference ($P < 0.05$) between the treatments. When the values of the optical densities on the plate are examined however, there is a very large difference between the two treatments. This is shown in Table 4.7

Table 4.7 The results, as optical density and percentage positivity when half the plate has been coated with antigen mixed with an equal volume of glycerol

	Neat antigen		50:50 glycerol : antigen	
	OD	PP	OD	PP
Cc	0.094	7.0%	0.102	6.1%
C++	1.333		1.675	
C+	0.475	35.6%	0.622	37.1%
C-	0.169	12.7%	0.198	11.8%

An analysis of variance of the optical densities (Table 4.8) shows there to be a highly significant ($P<0.001$) difference between the two antigen preparations.

Table 4.8 The analysis of variance of the optical densities for a plate where half the plate has been coated with antigen mixed with an equal volume of glycerol

ANALYSIS OF VARIANCE

SOURCE	SUM-OF-SQUARES	DF	MEAN-SQUARE	F-RATIO	P
CONTROL$	29.517	3	9.839	2245.252	0.000
REP$	0.011	5	0.002	0.486	0.785
TREAT$	0.403	1	0.403	91.903	0.000
CONTROL$ *REP$	0.059	15	0.004	0.895	0.574
CONTROL$ *TREAT$	0.414	3	0.138	31.509	0.000
REP$*TREAT$	0.060	5	0.012	2.745	0.029
CONTROL$ *REP$*TREAT$	0.078	15	0.005	1.191	0.311
ERROR	0.210	48	0.004		

The results demonstrate that whilst inter and intra plate optical densities may differ greatly, when expressed as percentage positivities - there is no significant difference. The conclusion from this trial was that antigen can be mixed with an equal part of glycerol and used for the long term storage of the antigen at - 30 °C or below, which can be moved to the - 20 °C freezer as required and pipetted direct from this aliquot for plate coating.

4.3.4.3. Effect of coating times

4.3.4.3.1. Introduction

The established method of coating ELISA plates is to hold the plate, with suitably diluted antigen and coating buffer, overnight in a +4 °C refrigerator. Whilst running an assay routinely, this means samples cannot be examined on a Monday (or the day after whichever religious day is taken off) unless a technician is prepared to coat the plates on the Sunday evening. Two possible ways of overcoming this practical

problem were examined; leaving the plates coated or washed for three nights or by coating then freezing the plates. If this latter method were viable, then plates could be coated in batches then used as and when required as utilised by the isometamidium competitive ELISA (Eisler *et al.*, 1996).

4.3.4.3.2.Materials and methods

Two plates were coated normally on the night of Day 1 and on day 2 one was washed normally and left in a refrigerator whilst the other was left coated. On the afternoon of Day 3 another plate was coated normally and left in the refrigerator. On Day 4, all plates were washed normally and two rows each of Cc, C++, C+ and C- at 1:400 were assessed under normal assay conditions on the three plates.

4.3.4.3.3.Results

A summary of the results is given in Table 4.9.

Table 4.9 The summary of results when three plates were coated and left at + 4 °C for up to three nights.

	Coated normally overnight (R_x 1)		**Coated, washed, left 3 nights (R_x 2)**		**Left coated for 3 nights (R_x 3)**	
	OD	PP	OD	PP	OD	PP
Cc	0.021	2.3%	0.0221	1.8%	0.020	1.7%
C++	0.885		1.2039		1.188	
C+	0.325	36.8%	0.3644	30.3%	0.401	33.7%
C-	0.175	19.7%	0.1081	9.0%	0.142	11.9%
Binding ratio		5.1		11.1		8.4

The analysis of variance of the three plate treatments is given in Table 4.10.

Table 4.10 The analysis of variance when three plates were coated and left at + 4 °C for up to three nights.

```
CONTROL$
  C+            C++           C-            Cc
 RX$
  1             2             3

DEP VAR:       PP       N:      288  MULTIPLE R: 0.997  SQUARED MULTIPLE R: 0.994

                        ANALYSIS OF VARIANCE

 SOURCE        SUM-OF-SQUARES    DF  MEAN-SQUARE      F-RATIO        P

 CONTROL$          419868.683     3   139956.228    16389.384        0.000
 RX$                   45.630     2       22.815        2.672        0.071
 CONTROL$*RX$        3255.971     6      542.662       63.548        0.000

 ERROR               2356.887   276        8.539
```

4.3.4.3.4.Discussion

The trial showed that it is possible to leave the plates coated for three nights or left washed for three nights before incubating with test sera. The analysis of variance showed there to be no difference between treatments ($P < 0.05$). This is almost certainly due to the performance of the plate coated normally overnight which had a poor signal to noise ratio (C- PP) which was near to the limits of acceptance. When using the normal quality control checks for the two plates left in the refrigerator for three nights, these plates are well within the acceptance limits. The conclusion from this trial is that plates may be coated on a day 1 and run on day 4.

4.3.4.4. The effects of coating then freezing plates

Previous workers (Sharpe, 1979) have examined the possibility of coating plates then freezing them. The purpose of this was to be able to take coated plates, as and when needed from the freezer in order to run the assay. A trial was conducted to coat then freeze a batch of plates (sealed with film) and compare these plates with controls run on a plate coated normally overnight. The percentage positivities of Cc, C+ and C- on the two plates were compared after 8, 15, 22 and 29 days using a Students t test.

The results showed that for all controls, once the plate had been frozen, there was a highly significant ($P < 0.001$) difference between the PPs depending on whether the plate had been coated overnight or stored frozen. The conclusion is that for the bovine trypanosomiasis Antibody detecting ELISA, plates cannot be frozen prior to use.

4.3.5. The use of eluted blood (held dried on filter paper) vs. sera

4.3.5.1. The advantages and disadvantages of eluted blood spots versus sera

The source of antibody for the Ab-ELISA can be serum or plasma, the difference being that the latter contains proteins necessary for blood to clot and some anticoagulant. Antibody can also be recovered by eluting dried blood which has been held on filter paper. The collection of sera in the field requires that c. 4 - 7 mls. of whole blood be drawn from the jugular or tail vein of the animal usually via a Vacutainer®[7] tube. The blood is subsequently allowed to clot, and the sera, which may require "cleaning" up via centrifugation, is then transferred to a stoppered cryogenic vial, labelled clearly, then kept refrigerated or frozen until examined in the laboratory. Preservatives, such as sodium azide, may be added to reduce bacterial contamination, the by-products of which may interfere with the assay. Attention to the temperature of the blood and serum samples is critical. If the blood samples overheat, haemolysis may occur and haemoglobin will discolour the sera. If the blood is kept cool, then although the blood will clot, the clot may not contract making subsequent decanting of the serum problematic. Depending on the sterility of the sample, bacterial growth in the serum may rapidly occur unless the sample is refrigerated then frozen. Finally, transport of frozen serum samples is logistically very difficult under African conditions unless an armoury of cold chain equipment is to hand and functional.

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[7] Becton Dickinson Vacutainer Systems Europe, B. P. 37-38241, Meylan Cedex-France

Plasma is probably no easier to collect under field conditions. The tube into which the blood is drawn must contain some anticoagulant, usually heparin or EDTA, and then samples should be kept cool until centrifuged and the supernatant plasma pipetted into some cryogenic vial. The plasma then must be kept cool or frozen until examined in the laboratory. Correct labelling of the cryogenic vials is not easy, and the tubes themselves are costly. The great advantage of serum samples however is that many tests and assays are designed to use serum as the source of antibody or antigen, and so if collecting sera - a national serum bank could be created and many diseases screened.

To overcome the practical difficulties of serum collection and transport, eluted samples of dried blood held on filter paper disks (blood spots) were tested to determine whether these could act as a suitable source of antibody. The use of eluted blood, dried onto filter paper disks, is a well established source of antibody for IFAT (Anderson, *et al.*, 1961; Bailey, Cunningham and Kimber, 1967; Evengard, Linder and Lundbergh, 1988; BADCP, 1992;) although the technique had not been adapted for use with the Ab-ELISA. The efficacy of the use of eluted blood samples was determined by Rae (1994) and this source of antibody was found to be satisfactory. Blood spots were thus chosen for the collection of antibody samples during epidemiological trypanosomiasis surveys in Zambia for the following reasons:

* they are inexpensive, as blood from four cattle can easily be placed onto a 7 cm. Whatmans filter paper disk
* blood spots can be made from the second (spare) heparinised capillary tube for almost no extra effort - thus saving the costs in time and materials (Vacutainer® tubes and cryogenic vials) necessary for serum samples
* there is no need for additional work to decant the serum from the clotted blood, nor the need for a centrifuge nor power supply to run it

* once dried and stored with a desiccant in a self sealing plastic bag - the blood spots must be kept cool (refrigerated) until moved to a freezer. They are not prone to bacterial contamination and can be safely transported at ambient temperature, provided they are not allowed to exceed 40 °C nor be exposed to direct sunlight.
* they do not suffer the problems of blood being stored too cool once clotted (no clot contraction)
* they are quicker and easier to label than cryogenic vials

4.3.5.2. Materials and methods

The materials necessary for collecting blood spots are:

Whatmans No. 4 filter paper disks, 7 cm. diameter

Self indicating silica gel desiccant

Self sealing freezer bags 15 x 20 cm.

Field absorption refrigerator (gas or kerosene powered) and cool box

Either blood is collected direct onto the filter paper from the ear vein, or it is collected from a heparinised capillary which is moved in a spiralling outwards manner. In the latter method, four samples can be stored on one 7 - 10 cm. filter paper disk.
The filter paper is air dried out of direct sunlight and then placed in the freezer bag with a "handful" of self indicating silica gel, and kept cool until placed in the refrigerator and thence a freezer. Several hundred filter paper disks can be stored in one freezer bag. If the silica gel turns from blue to pink (depending on the colour system) it is replaced and the used silica gel dried out again by gentle warming.

4.3.5.3. Variation of the dried blood results due to the filter paper technique

Three sources of variation may arise because blood has been stored dried on filter paper: the effect of the packed cell volume on plasma dilution; the effect of the filter paper itself and changes in the blood components themselves when it dries; and the distribution of the blood components within the blood spot as it dried and is stored. Whatman No. 4 filter paper was selected for the study as it tends to be an "industry standard" type of filter paper, was relatively freely available in Zambia, and had been successfully used by the Provincial laboratory in the Eastern Province of Zambia (BADCP, 1992) for use with IFAT screening. A standardised "treasury" hole punch of 6.2 mm. diameter, also assessed by Evengard *et al.* (1988), was used to punch out the disks of dried blood.

One possible source of variation in the antibody activity from a hole punched disk from filter paper would be due to the effect of packed cell volume of the blood containing the antibody. Whole blood comprises of red and white blood cells, and plasma. Within the plasma are dissolved proteins, minerals and other blood components. Given that the antibodies will lie extracellularly, then the higher the PCV, the less volume will be available for the antibodies. When whole blood is dried onto filter paper, the erythrocytes are lysed and the haemoglobin mixes freely with the components of the plasma. 70 µl from the heparinised capillary tube is used to make the spot which on average is 20 mm. diameter. As the area of the blood spot on the filter paper will remain the same, no matter what the PCV, and because a hole punch of fixed size is eluted in a fixed volume of buffer, then the PCV will affect the effective dilution of the antibody. A spreadsheet showing the effect of PCV on plasma dilution is shown in Table 4.11. which is graphically portrayed in Figure 4.6.

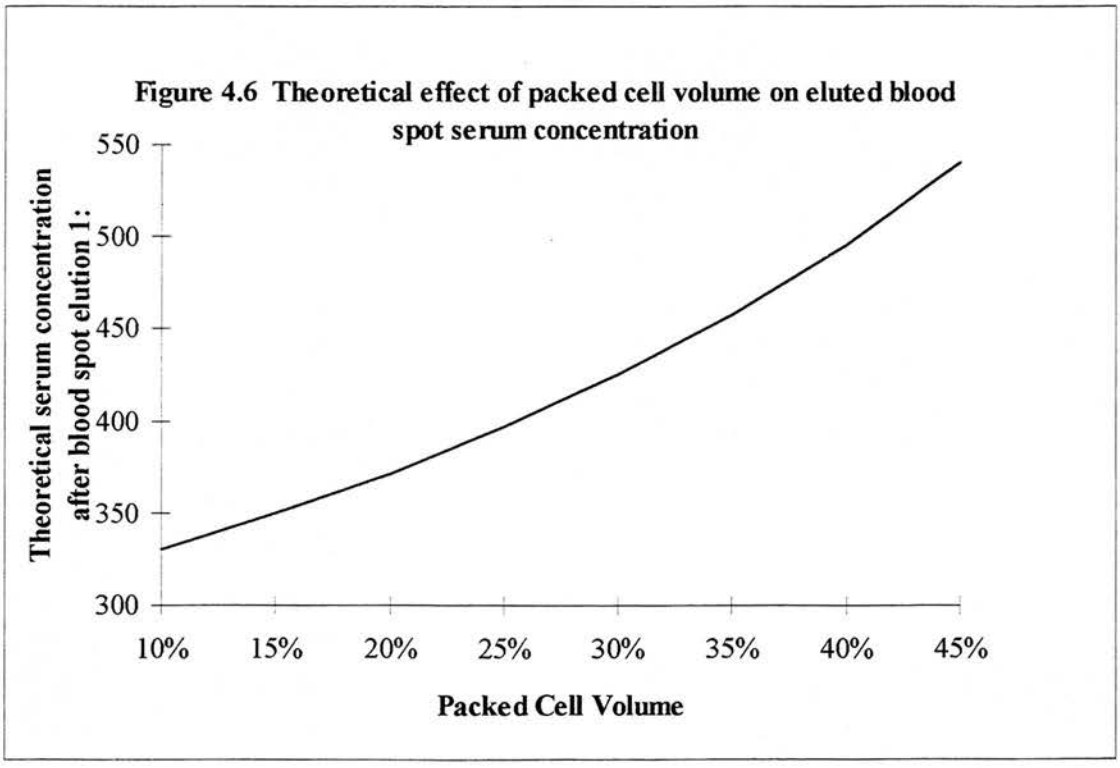
4.3.5.4. Discussion

The use of blood samples dried on to filter paper has many clear advantages over the use of serum or plasma. A full assessment of this technique and its standardisation was made by Evengard *et al.* (1988). An additional advantage is the technique's reported antibody stability when samples were stored at varying temperatures (Van den Bossche, personal communication), and this subject was investigated more thoroughly and is described in paragraph 4.3.7. The site where the hole punch was taken and the inherent variability of the distribution of antibodies within the blood spot was also investigated (paragraph 4.3.5.5). One source of variation that cannot be overcome is the effect of PCV on effective plasma dilution, which in the spreadsheet exercise varied from 1:350 to 1:496 as the PCV ranged from 15% to 40%. This effect on antibody dilution would invariably affect the sample percentage positivity and if some single cut-off value were being used, may dictate whether an animal was deemed positive or negative.

Table 4.11 Spreadsheet to calculate effective serum dilution of 6.2 mm hole punch blood spot in 2 ml PBST buffer

	Value	*Unit*
Volume of blood in capillary tube	70	microlitres
Packed Cell Volume	10%	
Volume of plasma at PCV given	63	microlitres
Radius of blood spot	10	mm.
Area of punch which has diameter 6.2 mm	30.19	sq. mm
Area of blood, spot assuming radius 10 mm	314.16	sq. mm
Fraction of the blood spot in a treasury punch circle	0.10	
Volume of plasma in a hole punch	6.05	microlitres
Effective plasma dilution in 2 ml PBST is 1:	330	

Figure 4.6 Theoretical effect of packed cell volume on eluted blood spot serum concentration



4.3.6. Investigations into the sensitivity and specificity of the trypanosomiasis antibody ELISA using bovine samples of known provenance with regard to infection status

4.3.6.1. Materials and methods

209 sera from cattle raised in closed herds in tsetse free areas and 370 known parasitologically positive animals were assayed with *T. brucei* and *T. congolense* antigen. 464 blood spots from cattle raised in closed herds in tsetse free areas (of which 260 were from traditionally reared cattle and 204 were from commercially managed stock) and 293 known parasitologically positive animals were assayed with the same *T. congolense* antigen. After the subtraction of a substrate blanking value, the results were expressed as a percentage positivity. The strong positive reference sera, against which the results were expressed was pooled sera whose OD had given the strongest colour change during a previous run of the assay (when *T. brucei* was used as antigen).

The sample taken from the negative population was in fact drawn from two sub-populations, pedigree cattle reared commercially and local Zebu breeds reared traditionally. The latter group were sampled in order to achieve a greater comparison between positive and negative sub sets (the positive cattle were all local Zebu breeds reared traditionally). Not only were the cattle sampled from different management systems, but they were sampled in different years. The 204 commercial cattle were sampled June - December 1993 whilst the 260 local cattle were sampled June 1996.

4.3.6.2. Results

4.3.6.2.1.Negative and positive sera and eluted blood spot percentage positivity distributions with *T. brucei* and *T. congolense* antigens

The frequency distribution histograms obtained with sera and *T. brucei* and *T. congolense* antigen, and eluted blood spots with the same *T. congolense* antigen are given in Figure 4.7. 4.8 and 4.9.

Figure 4.7 Histogram of negative and positive sera with T. brucei antigen

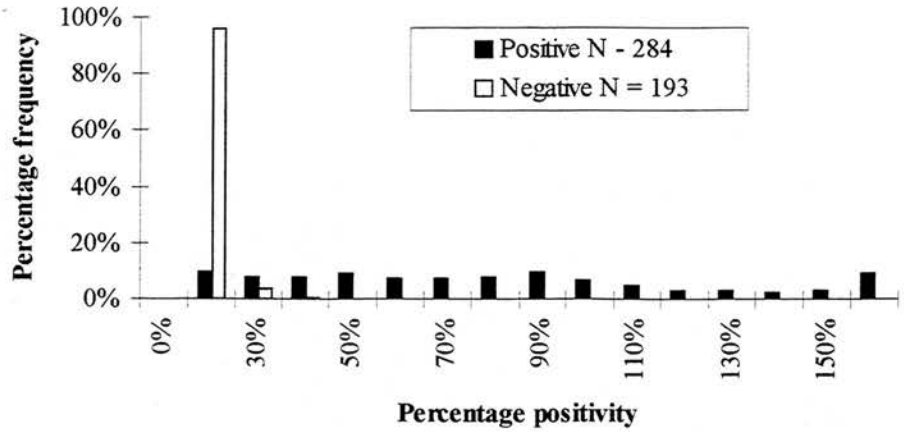


Figure 4.8 Histogram of negative and positive sera with T. congolense antigen

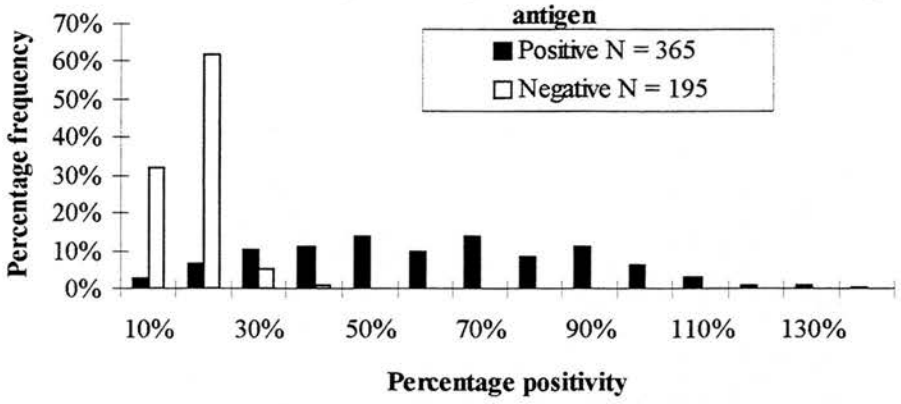
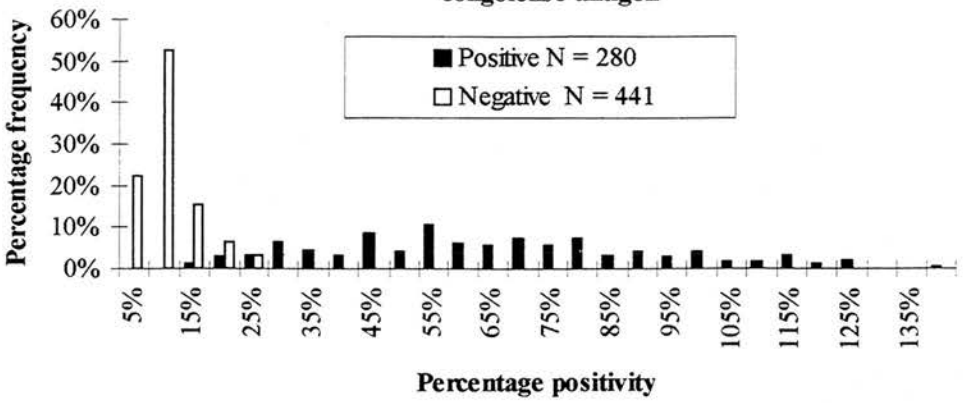


Figure 4.9 Histogram of positive and negative blood spots using T. congolense antigen

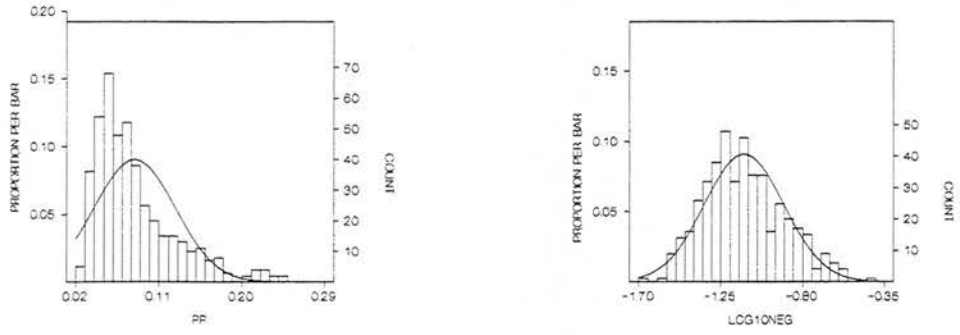


4.3.6.2.2.Negative blood spot data using *T. congolense* antigen from two sub populations

The mean of the PP from the five herds of commercially reared cattle was 0.119 (variance 0.002) whilst from the four traditionally reared herds was 0.058 (variance 0.0003). After log $_{10}$ transformation these values were -0.9563 (variance 0.0295) and -1.2549 (variance 0.0181) respectively. There was a highly significant ($P < 0.001$) difference in the two sub population variances. A two sample students t test assuming unequal variance showed there to be highly significant ($P < 0.001$) difference in the percentage positivities of the two groups.

Histograms of the combined negative population showing raw and $\log_{10}$ transformed data with the best fitted line are given in Figure 4.10 below.

Figure 4.10 The frequency distribution of 293 negative blood spot percentage positivities, before (left) and after (right) a log transformation



4.3.6.3. Discussion

4.3.6.3.1.Negative distribution

The distribution of the combined negative population is clearly skewed to the right. This is not unusual as the negative population PPs can only include positive values (i.e. negative values are not possible) and as such it is common for such data to be skewed to the right (Berk, 1994). The data assume a normal distribution after a logarithmic transformation, as demonstrated in Figure 4.11.

The negative sample was thus clearly drawn from two sub populations; the differences being in breed, management and length of time stored at -20 °C. There was a difference of three years between the two sets of samples being assayed. Other experiments have shown that the PP of samples increases as the proteins degrade (c.f. trial to study effect of heat, UV and sunlight on samples). The author would thus argue that the difference in the two sub-populations is due to time between samples being collected and antibody being measured. It is therefore permissible to pool both sub-populations in order to calculate the specificity, this being because samples used for the assay may be up to three years old.

4.3.6.3.2.Positive distribution

There is no simple "gold standard" diagnostic test for African animal trypanosomiasis. The population used as the positive controls were selected based on them being parasitologically positive, and in three cases through the xenodiagnosis of *T. brucei* in mice. This represents a sub-set of all true positive population as many animals deemed negative by direct parasitological methods would have in fact been true positives. A better estimate of the population of true positives would have been possible by using the best, proven diagnostic tests available. This could have been achieved through the more widespread use of rats and mice for xenodiagnosis, PCR detection methods, Diminazene flushing of suspect positive animals with low PCVs and the experimental infection of animals.

4.3.6.4. The choice of cut-off value

4.3.6.4.1.Introduction

The factors affecting and choice of cut-off value have been reviewed in Paragraph 4.2.5 above. Having optimised the assay conditions and assayed large sample sizes of known provenance with regard to infection status, the various cut-off values with their concomitant sensitivities and specificity's were investigated.

4.3.6.4.2.Materials and methods

As the Ab-ELISA was investigated for sero epidemiological surveys using eluted blood spots and *T. congolense* antigen, these assay conditions are described. The effect of cut-off on the sensitivity and specificity was calculated from the 442 animals from the negative population and 281 animals parasitologically positive.

4.3.6.4.3.Results

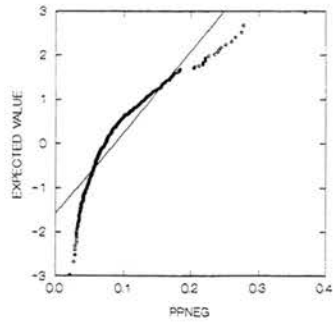
Because the PPs represent two samples drawn from two populations (ie negative and positive), then ideally the type(s) of underlying distribution(s) with its parameters must first be defined. Neither the negative nor positive population were normally distributed, but were well approximated by the log normal and Weibull[8] distributions respectively. This is portrayed in Figures 4.11 These findings are the same as those of Vizard *et al.* (1990) in their studies of the optimum choice of cut-off. In their studies of the "best fitting" type of distribution, Vizard *et al.* employed the Chi Squared test.

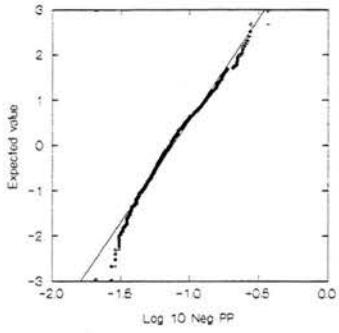
The cut-off point as chosen by one of the methods stated above and respective sensitivity, specificity and diagnostic power are given in Table 4.12.

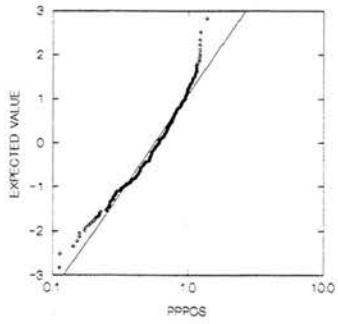
[8] The Weibull transformation is defined by Vizard *et al.* (1990)

Figure 4.11

The probability density functions of the negative population (top) before (left) [mean: 0.086 standard deviation: 0.05] and after (right) a log 10 transformation [mean: - 1.124 standard deviation: 0.22] and the probability density function of the positive population (below) before (left) and after (right) multiplying by 100 and transforming to a Weibull distribution. Untransformed mean: 0.625 (62.5%) standard deviation: 0.272 (27.2%)







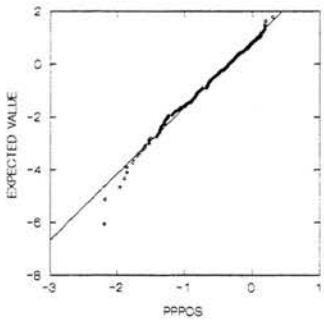
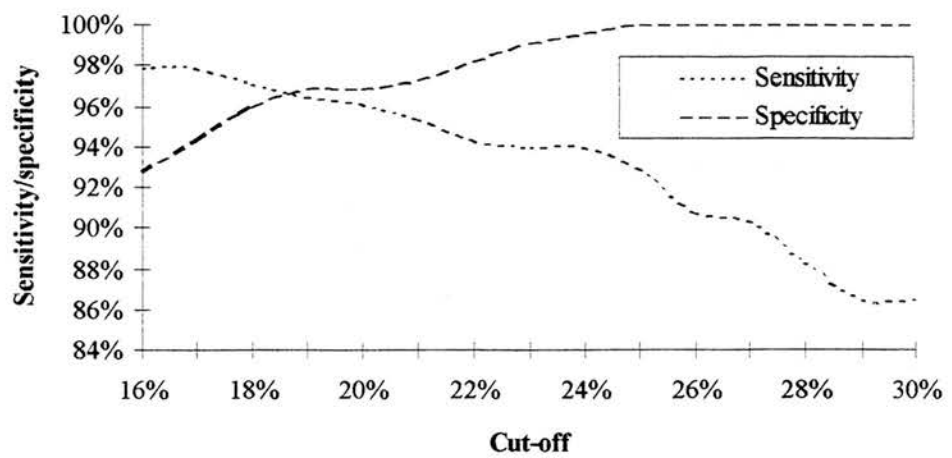


Table 4.12 The effect of cut-off on sensitivity and specificity using eluted blood spots and *T. congolense* antigen by varying methods based on the actual (vs. the fitted log normal and Weibull probability distributions) data

Method	Cut-off	Sensitivity	Specificity	Power (Se* Sp)
Mean of negative population + 2 SD	17.2%	97.5%	94.8%	92.4%
Mean of negative population + 3 SD	21.6%	95.0%	97.5%	92.7%
Antilogged -Log 10 mean + 2 SD of negative population	19.5%	96.4%	96.8%	93.4%
95% percentile (negative population)	17.2%	97.5%	94.8%	92.4%
5% percentile (positive population)	21.7%	94.3%	97.5%	92.0%
Max. (Se + Sp)/2 or Max. Se x Sp	24.0%	94.0%	99.5%	93.5%

The inter-relation between cut-off, sensitivity and specificity are plotted in Figure 4.12.

Figure 4.12 The Inter relation between cut-off, sensitivity and specificity



4.3.6.4.4.Discussion

The choice of cut-off is complex. Not only does the cut-off dictate the apparent prevalence of a sample drawn from some population, but in determining the sensitivity and specificity of the test it will influence the value of the true prevalence. These true prevalence rates should assume a value not greater than unity nor less than zero and is discussed in Chapter 4. If a sample's replicate PPs fall on either side of the cut-off, the sample should be re-tested (Wright *et al.*, 1993). Superimposed on the issue of selection of some finite cut-off percentage positivity are the problems of the day-to-day variation and the repeatability of the assay. One is aiming to avoid samples assayed on one day falling into the positive population and yet on another day being deemed negative, and *vice versa*. Investigations into the repeatability of the assay are thus as important as investigations into the choice of cut-off.

Depending on which of the above methods is chosen to select the cut-off, then the value lies in the range 17.2% to 24% positivity. No consideration about the costs of misdiagnoses has been taken into account. For the sero-epidemiological studies discussed later in this thesis, a cut-off of 24% was chosen as this has high specificity (without being 100%) and has the "lowest percentage of error" viz. the maximum value for (sensitivity + specificity)/2.

4.3.7. Investigations into the error structure of the antibody ELISA for bovine trypanosomosis and the implications thereof

4.3.7.1. Inter-assay variation

4.3.7.1.1.Introduction

The Ab-ELISA showed a day to day variation in the absorbance values of the strong positive controls and negative control percentage positivity, from which the binding ratio is calculated. In order to investigate this inter assay variation, values for the absorbance, expressed both as an optical density and percentage positivity were written to a database every time a plate was assayed.

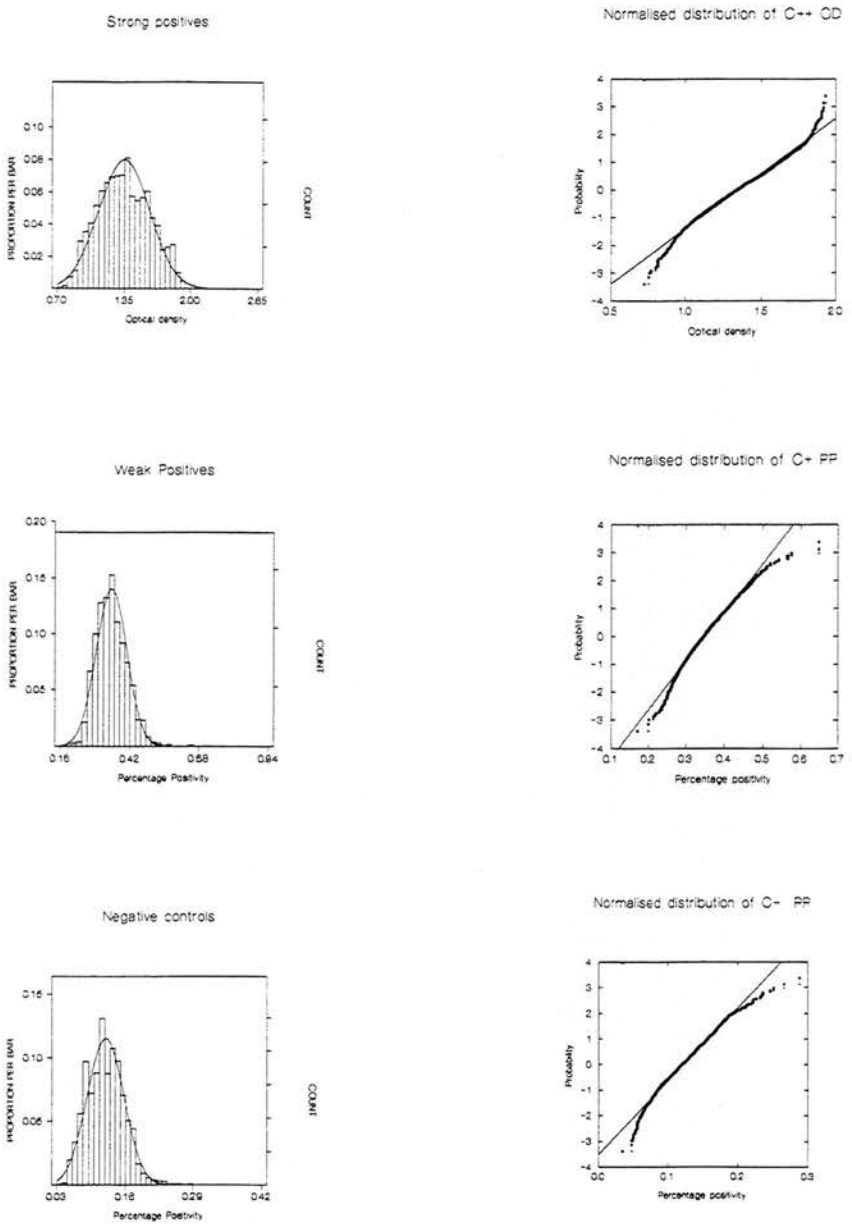
4.3.7.1.2.Results

The intensity of the colour change (syn. absorbance at 450 nm.) of the strong positive reference sera and of the weak positive and negative sera (both expressed as percentage of the strong positive) are graphically presented in Figure 4.13.

4.3.7.1.3. Discussion

These data follow a Gaussian (syn. normal) distribution sufficiently well for parametric statistics to be used. The distributions have relatively few observations in the tails and thus it can be argued to be platykurtotic. The frequency distribution of the optical density of the strong positives has a broad "hump" and short tails. All three distributions have a kurtosis value less than 0.8. When compared to the Chi squared, Exponential, Weibull and Uniform probability distributions, the Gaussian probability density function is the best fitting, and a plot of the observed vs. normalised distributions is as shown in Figure 4.13 The departure from the Gaussian distribution towards the tails is again apparent.

Figure 4.13 The frequency distributions with a fitted "normal distribution" line of the optical density of the strong positive and the percentage positivity of the weak positive and negative control sera (left) and of the same data after they have been normalised (right).



The summary statistics of the three control sera are tabulated in Table 4.13. The coefficient of variation of the negative sera was highest even though the range was smallest. This was because of the low value of the denominator (i.e. the mean of the negative control sera).

Table 4.13 The descriptive statistics from 475 plates (four wells per plate) of the strong positive sera (C++) expressed as an optical density and weak positive and negative sera expressed as a percentage relative to the strong positive

	Control sera		
	CP++ OD	*C+ PP*	*C- PP*
Descriptive statistics			
Mean	1.3478	0.3502	0.1222
Standard Error	0.0057	0.0013	0.0008
Median	1.338	0.346	0.121
Mode	1.279	0.351	0.139
Standard Deviation	0.24921	0.05686	0.03461
Sample Variance	0.06211	0.00323	0.00120
Coefficient of variation	18.5%	16.2%	28.3%
Kurtosis	-0.71643	0.76655	0.19751
Skewness	0.10990	0.55684	0.37183
Range	1.205	0.478	0.254
Minimum	0.725	0.171	0.034
Maximum	1.93	0.649	0.288
Count	1900	1900	1900

The plate reader measures absorbance as an optical density on a logarithmic scale (Rogers, personal communication). There is thus a ten fold difference in colour change between an optical density of 0.1 and 1 and again between 1 and 2.

The range of absorbance of the strong positives was considerable, especially when the absorbance mode on a logarithmic scale is taken into account. This is also referred to as the "day to day" variation of results. Possible reasons for this large day to day variation were investigated, these being the effect of pipetting (pipetting times, pipette tips and the inherent variability of the assay) and any effect of laboratory ambient temperature. Other sources of assay variation are investigated later in this section, these being the variation within a blood spot and a comparison between results from sera and blood spot. Finally, one aspect of data expression is examined when results are expressed as a percentage positivity compared to the strong positive or as a signal to noise ratio when compared to the negative sera.

4.3.7.2. Intra assay variation

4.3.7.2.1.The Coefficient of Variation of a normally run plate

Before analyses could be made into the effects of pipetting times or pipette tips, the intrinsic variation of a plate was examined.

Materials and methods

One plate was incubated normally with one dilution of weak positive sera (1:400) with 100 µl placed in each of 80 wells using the same pipette tip. The plate was run with the normal controls under normal assay conditions to ensure it was within limits.

Results

The results as absorbance (450 nm.) giving the analyses of variance by row and column is given in Table 4.14 whilst details of the absorbance and the source of variance is given in Appendix 5 Tables A5.1 (row variance) and Table A5.2 (column variance).

Discussion

The results show there to be a large variance in the columns ($P < 0.001$) but not in the rows ($P < 0.25$). Given that any effect due to the individual pipetting of the 100 µl diluted sera into the 80 wells was insignificant, one possible explanation for this observation is due to the effect of the use of 8 channel multi-channel pipettes being used to add reagents across the plate. The stages of the assay where single and multi-channel pipettes are used is shown diagrammatically in Figure 4.14.

The effect of washing is likely to be minimal as the wells were washed on 4 or 5 occasions between each step of the assay and if 200 µl were not dispensed into a well, this would be very apparent to the technician. The possible effects of the addition of acid were not considered to play an important part in the between column variation. The effect of coating with antigen or the addition of conjugate and substrate could easily have had a profound effect however. When using an 8 channel multi pipette and adding 100 µl per well - a variation of ± 5 µl between dispensing volumes may have been sufficient to cause such a significant (between) column effect. The difference in volume dispensed by each pipette along each row (the row effect) was not significantly different which one expect, as the multi-channel pipettes are built to a high standard. The column effect could thus have been because the correct amount of 100 µl was not being drawn up then dispensed when reagents were dispensed into the ten columns.

Although pipette tips are manufactured to a high standard and the tolerances (means and standard deviations) measured - pipette tips were recycled in Zambia in order to keep the costs (and the dependence on the need for foreign exchange) to a minimum. Washing comprised soaking in a mild detergent followed by a thorough rinsing.

Table 4.14 The ANOVAs by column and row

Anova: Single Factor by columns

SUMMARY

Groups	*Count*	*Sum*	*Average*	*Variance*
Column 1	8	3.209	0.401125	0.000222
Column 2	8	3.2	0.4	0.000689
Column 3	8	3.125	0.390625	0.000326
Column 4	8	3.033	0.379125	0.000718
Column 5	8	2.855	0.356875	0.000857
Column 6	8	3.225	0.403125	0.00024
Column 7	8	3.131	0.391375	0.000483
Column 8	8	3.148	0.3935	0.000851
Column 9	8	2.808	0.351	0.000969
Column 10	8	2.95	0.36875	0.000399

ANOVA

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	0.025461	9	0.002829	4.916498	4.1E-05	2.016598
Within Groups	0.040279	70	0.000575			
Total	0.06574	79				

Anova: Single Factor by rows

SUMMARY

Groups	*Count*	*Sum*	*Average*	*Variance*
Row 1	10	3.714	0.3714	0.000668
Row 2	10	3.735	0.3735	0.000835
Row 3	10	3.903	0.3903	0.001079
Row 4	10	3.996	0.3996	0.000381
Row 5	10	3.85	0.385	0.001208
Row 6	10	3.754	0.3754	0.000657
Row 7	10	3.941	0.3941	0.001168
Row 8	10	3.791	0.3791	0.000474

ANOVA

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	0.007514	7	0.001073	1.327404	0.250036	2.139657
Within Groups	0.058226	72	0.000809			
Total	0.06574	79				

Figure 4.14 Pipette types used during the various stages of the antibody ELISA

Procedure	**Type of pipette used**
Coating of plate with antigen	← Multi-channel pipette
Washing	Semi-automatic plate washer
Addition of test sera	¬ **Single channel pipette**
Washing	Semi-automatic plate washer
Addition of conjugate	← Multi-channel pipette
Washing	Semi-automatic plate washer
Addition of substrate	← Multi-channel pipette
Quench with acid to stop reaction and give yellow colour change	← Multi-channel pipette

This trial should be repeated on more than one plate to investigate whether there was a consistent column effect. New pipette tips should be used, and a 12 channel multi pipette should have been used down the plate to see whether the column effect became a row effect. The results make interpretation of analyses of variance difficult for the other trials conducted cf. investigation in the effect of site of hole punch within a blood spot. The overall coefficient of variation for this plate (7.5 %) is "respectable" and this value probably reflects the true inherent variability (following a normal distribution) of the response error relationship of the assay.

Introduction

One possible source of variation in the assay is the time test sera (or eluted blood spots) are in contact with antigen bound to the solid phase of the well. Although these test sera were incubated for 45 minutes at 37 °C, there was an inevitable delay between the addition of the first test sera and final test sera before placing the plate in the shaker incubator. A trial was conducted to investigate any such differences.

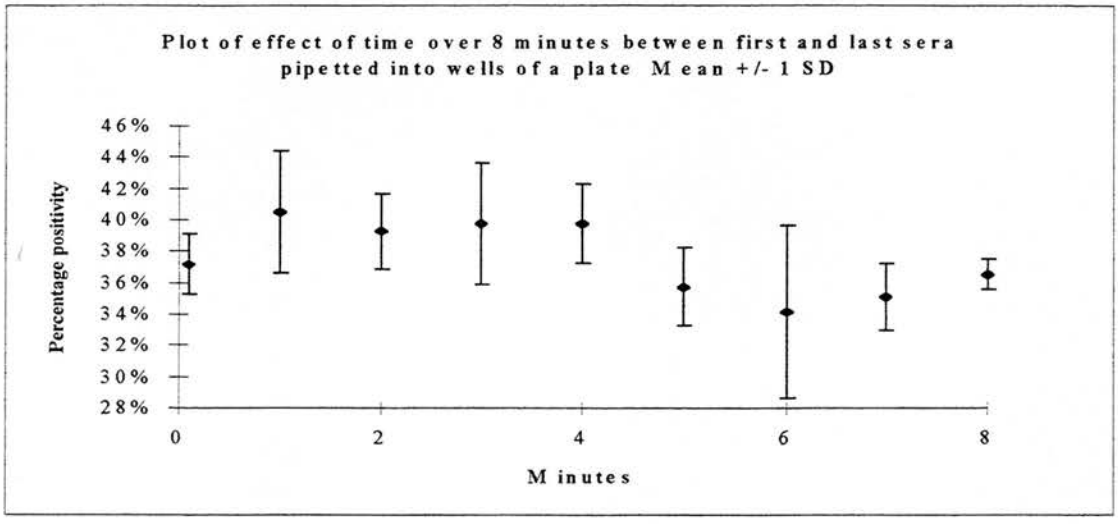
Materials and methods

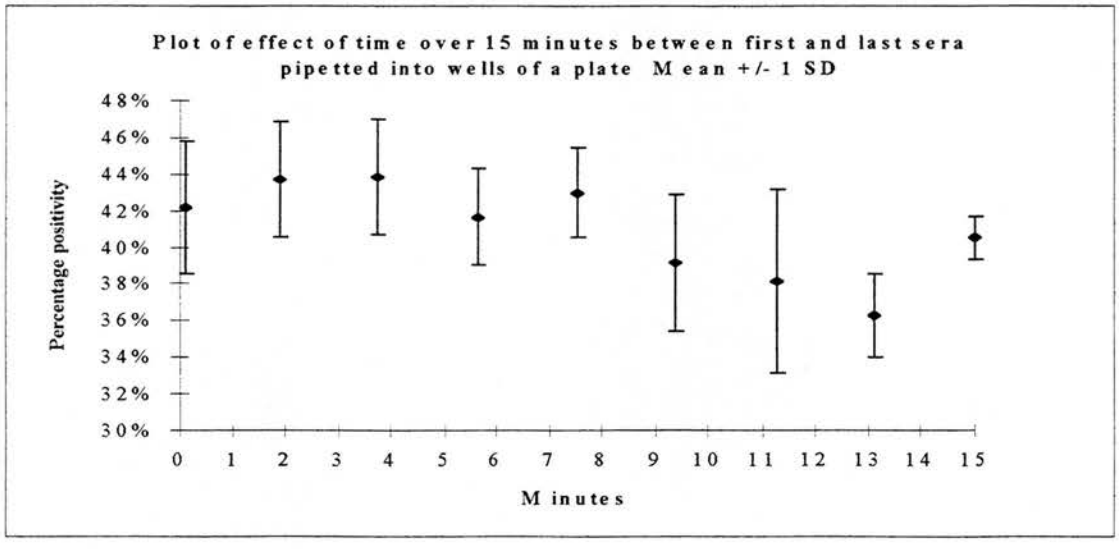
Weak positive test sera were pipetted into the 80 wells on two plates over an eight and fifteen minute period. All other assay conditions were normal.

Results

The results of this trial are presented as graphs of mean percentage positivity ± 1 standard deviation for each minute when test sera were added, and as a scattergram of all the results with a linear regression line fitted. These are shown in Figures 4.15 and 4.16.

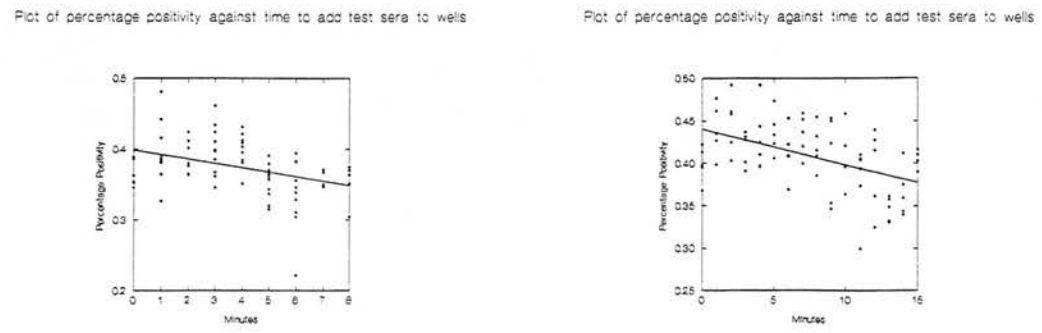
Figure 4.15 The effect of the difference of eight (top) and fifteen (bottom) minutes between the addition of the first and last test sera into the wells of an ELISA plate - prior to the plate being placed in a 37 °C shaker incubator. The mean of the PPs for every minute ± 1 standard deviation are shown.





The same data with individual well values plotted against minutes as scattergram graphs with a fitted linear regression line is given in Figure 4.16.

Figure 4.16 The effect of the difference of eight (left) and fifteen (right) minutes between the addition of the first and last test sera into the wells of an ELISA plate as individual well values with a linear regression line superimposed



Discussion

A regression analysis of variance to predict the percentage positivity as a function of time gave a R^2 of -0.155 and -0.231 respectively when test sera were added to the wells over an eight and fifteen minute period. Both these regressions were significantly different from zero ($P < 0.05$). The steeper fitted line when 15 minutes were taken to add the test sera to the plate shows there is a correlation between the time taken pipette sera from the serum dilution onto the plate and the final optical density. This is not unexpected if antibody has an additional 15 minutes in contact with antigen before a 45 minutes incubation period. The correlation, although not strong, is however present. The conclusion of this trial is that a technician should not delay between the pipetting of the first and last sera dilution/blood spot eluate onto an ELISA plate.

4.3.7.2.3.The effect of pipette tips

Introduction

If the conclusions from the preceding section are correct (c.f. a possible column effect when a plate is run normally) then pipette tips may in part be responsible for the variation in results. The actual pipette itself and pipetting technique used may also have had an influence on the variation of results, but these were not investigated. Two trials were conducted to asses how much variation could be apportioned to the effect of pipette tips.

Materials and methods

In the first trial a new pipette tip was used to make 10 dilutions of sera in PBST and a new pipette tip was used to place 100 µl from each dilution into ten columns. In the second trial one dilution of 1:400 sera was made and ten pipette tips were used to place this single dilution into the ten columns on one plate. If there was no other variance on the plate (which has subsequently been to be not the case), variance due to the effect of pipette tips could have been investigated.

Results

The results of these trials are presented as analyses of variance in Tables 4.15, and 4.16 with details of the absorbance and sources of variance in Appendix 5 Tables A5.3 and A5.4. The results are expressed graphically as the ELISA plate column means ± 1 standard deviation in Figures 4.17 and Figure 4.18.

Table 4.15 The analyses of variance of the first pipetting trial where 10 pipette tips were made to make 10 dilutions and a different pipette tip was used to place 100 microlitres diluted sera into 10 columns

Classic ANOVA: Single Factor

SUMMARY

Groups	*Count*	*Sum*	*Average*	*Variance*
Column 1	8	6.702	0.83775	0.002774
Column 2	8	6.677	0.83462	0.003165
Column 3	8	6.273	0.78412	0.001557
Column 4	8	6.62	0.8275	0.002667
Column 5	8	6.275	0.78437	0.004548
Column 6	8	6.56	0.82	0.003272
Column 7	8	6.527	0.81587	0.0041
Column 8	8	6.196	0.7745	0.004468
Column 9	8	5.684	0.7105	0.002554
Column 10	8	5.136	0.642	0.003387

ANOVA

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	0.282442	9	0.03138	9.658548	1.99E-09	2.016598
Within Groups	0.227443	70	0.00324			
Total	0.509885	79				

Figure 4.17

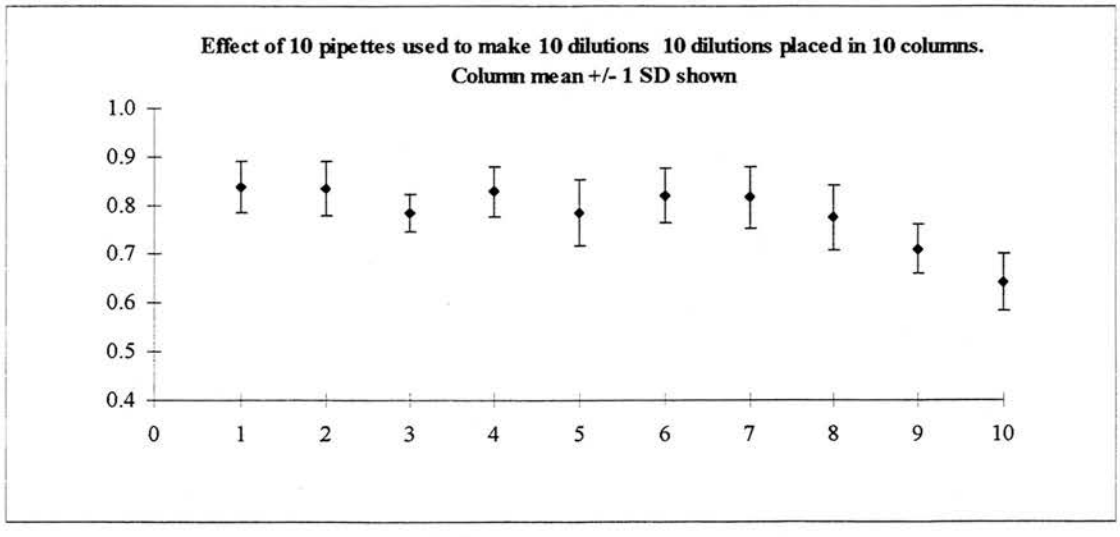


Table 4.16 The analyses of variance of the second pipette trial where one serum dilution was made and 10 pipette tips were used to place 100 microlitres of the single dilution into the 10 columns

Classic ANOVA: Single Factor

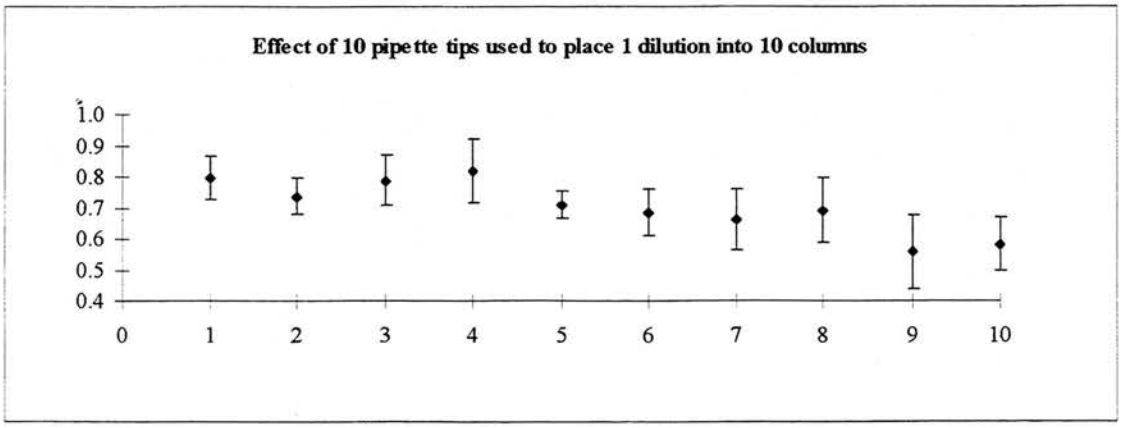
SUMMARY

Groups	*Count*	*Sum*	*Average*	*Variance*
Column 1	8	6.361	0.79512	0.004706
Column 2	8	5.891	0.73637	0.003214
Column 3	8	6.297	0.78712	0.006767
Column 4	8	6.555	0.81937	0.010437
Column 5	8	5.67	0.70875	0.002044
Column 6	8	5.476	0.6845	0.005535
Column 7	8	5.275	0.65937	0.009724
Column 8	8	5.525	0.69062	0.011028
Column 9	8	4.452	0.5565	0.014457
Column 10	8	4.661	0.58262	0.007382

ANOVA

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	0.54877	9	0.06097	8.098055	4.03E-08	2.01659
Within Groups	0.52706	70	0.00752			
Total	1.07583	79				

Figure 4.18



Discussion

Both the analyses of variance demonstrated that there was a significant ($P < 0.001$) difference between the columns and hence between the effect of pipette tip when used to make the dilutions, and when the dilution(s) were added to the wells of the plate. The variance within columns of trial 2 (i.e. the effect of pipetting and of the assay) was considerable, with a mean coefficient of variance of 12.4%. With such a large within column variance it is not possible to estimate the magnitude of the between column (i.e. between pipette tip) variance. Similarly, the trial 1 demonstrates a within column variance CV of 7.3%. This in itself is a respectable value, but makes the analyses of variance difficult to interpret in an attempt to apportion variance to the "between" column (i.e. between pipette tip) effect.

The preceding section shows that the assay has an inherent variability which is due to the biological variation of the performance of the assay and the effect of pipetting (technicians pipetting technique, make of pipette, variability of the pipette tip, time taken to place dilutions into the wells). No doubt the plate reader measuring the absorbance will have variability as well. Whilst nothing can be done about the inherent biological variation of the assay and of the plate reader the variance of results can be minimised by using new pipette tips of a known standard, an improved quality (such as positive pressure dispensing) pipettes, assiduous pipetting technique, and effort to minimise the time between the addition of the first and last serum dilution to a plate - before the plate is placed in the shaker - incubator.

4.3.7.3. The repeatability of the Antibody ELISA

4.3.7.3.1.Introduction

Reproducibility is the ability to duplicate a diagnostic test in other laboratories, and repeatability is the ability to duplicate results from the same samples in the same laboratory using the same test. The repeatability of a test reflects its reliability (Thrusfield, 1995). Whilst most of the reagents for the Ab-ELISA can be procured from some laboratory supplier, the biological reagents fundamental to the performance of the assay (namely antigen and control sera) have to be made specially. Because these biologicals were in such limited supply then none could be spared for use in any other laboratory, other than to establish the assay. As such, very little data were available for the reproducibility of the Ab-ELISA. This could only be investigated in future if some standardised (possible recombinant) antigen and control sera where produced by some reference laboratory. The results from the trial to compare pure water for coating buffer show the assay to have a high level of reproducibility when based on the results of ten plates run in Harare (as described in Paragraph 4.3.4.1).

The repeatability of the assay was investigated in two ways. Firstly, the colour change of the control sera, both in terms of optical density and percentage positivity, were monitored every time a plate was run. These results are discussed in paragraph 4.3.5.1 above and showed the assay to follow a day to day variation that was well approximated by the normal distribution. In a second study, the same serum samples were run repeatedly on up to six occasions in order to study the variation of results and hence the assay's repeatability.

4.3.7.3.2.Materials and methods

Over 200 sera from tsetse free areas and areas where trypanosomiasis was known to occur were repeatedly assayed and results were expressed as percentage positivity. The same assay conditions (batch and dilution of antigen, dilution and batch of C++)

were used throughout. The samples were re-assayed according to the following time-scale and serum reference suffix naming convention given in Table 4.17.

Table 4.17 The suffix naming convention used to investigate the repeatability of the assay and referred to in the test.

Suffix	*Month*	*Samples on plates*
None	1	One pair of replicates from one sample were duplicated on one plate. These data were used to establish the sensitivity and specificity of the assay for sera.
Suffix A and B	4	Samples run in replicate on two plates either on the same morning or same afternoon. The same serum dilution were used for both plates.
Suffix C and D	4	Samples run in replicate on two plates once in the morning and again on the same day's afternoon. The same serum dilution were used for both plates.
Suffix E	5	Samples run in replicate on one plate

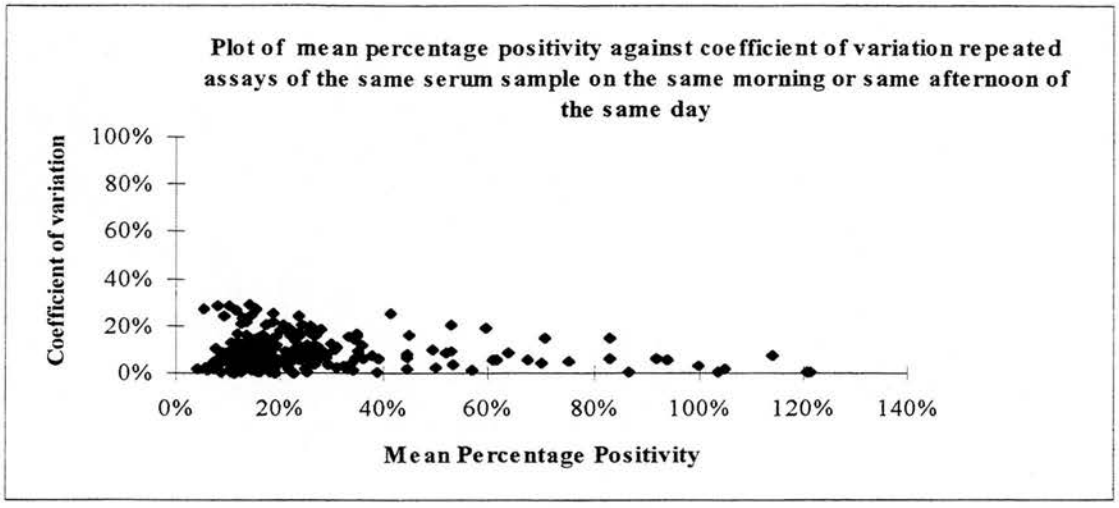
For each study of repeatability, the following statistics were calculated: the arithmetic mean, the standard deviation and the coefficient of variation (CV)[9].

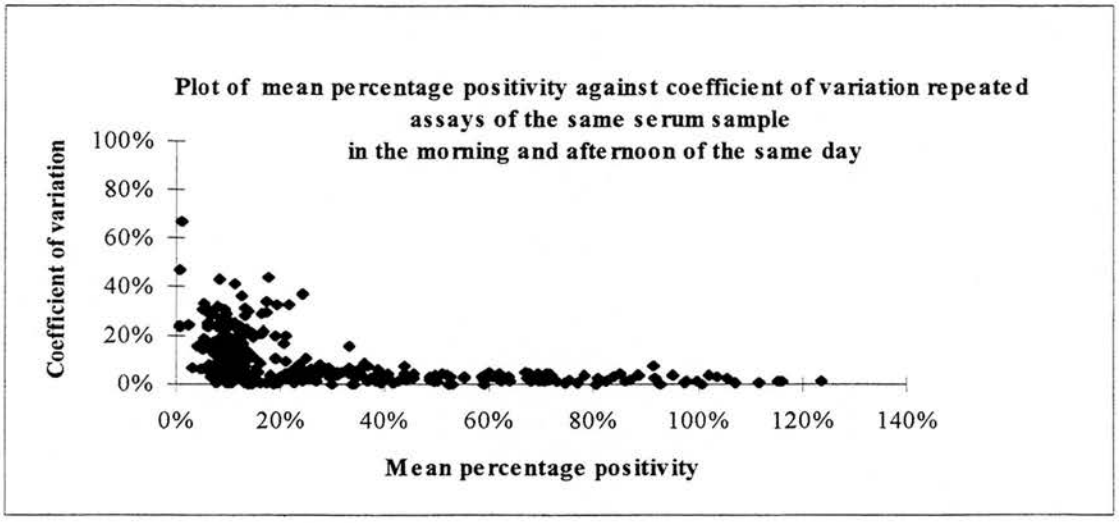
4.3.7.3.3.Results

The results are expressed graphically in Figure 4.19 where the coefficient of variation has been plotted against the mean percentage positivity when serum samples were repeatedly assayed on the same morning or afternoon, on the morning and afternoon of the same day, or on up to six occasions over a five month period. A summary of these results is also given in Table 4.18.

[9] CV = standard deviation/mean x 100 and expressed as a percentage.

Figure 4.19 The repeatability of the antibody ELISA when the same serum sample is assayed on two plates on two plates on the same morning or afternoon (top), in the morning and afternoon of the same day (middle) or on four or five occasions over a five month period (bottom).





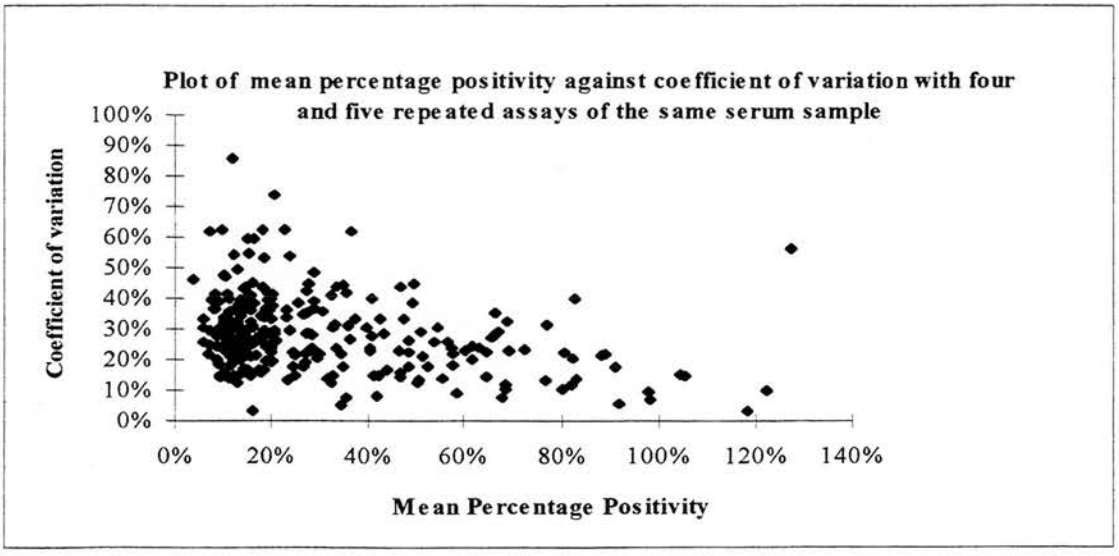


Table 4.18 The summary statistics of the trial to determine the repeatability of the antibody ELISA.

	No. of observations	Average of the CVs	Standard deviation of the CVs
A and B	235	9.1%	6.6%
C and D	310	9.4%	10.2%
A to E and no suffix	278	29.7%	16.2%

4.3.7.3.4.Discussion

Another measure of the repeatability of a diagnostic test is Cochran's Q test or McNemar's change test (Thrusfield, 1995). These statistics were not used as they assume the test being assessed to have a result which discriminates positives from negatives. As the cut-off point for the antibody ELISA being investigated results were not expressed in qualitative terms (i.e. positive or negative) rather on some continuous scale.

The data reflect a similar error response curve as discussed above. As the response increases (expressed as the mean percentage positivity) then so does the standard deviation. As the denominator used to calculate the CV increases, the actual value for the CV falls correspondingly. The converse also holds true and so sera with low PP values have a larger CV.

The preceding graphs and table shows there to be a very large range in the coefficient of variation and hence repeatability of the assay. The range of CV increases as samples were repeated on the same morning/afternoon (mean CV 9.1%) > differing morning/afternoon (mean CV 9.4%) > differing months (mean CV ~ 30%). The range of CVs varies considerably as well, with a standard deviations varying from 6.6 to 16.2%, and in the case of the same samples being run in the morning and again in the afternoon the standard deviation of the CVs was greater than the mean - thus demonstrating the large underlying variability (i.e. poor repeatability) of the assay.

4.3.7.4. The correlation between strong positive control sera absorbance and laboratory ambient temperature

4.3.7.4.1.Introduction

One possible cause of the day to day variation of the absorbance of the strong positive control sera was investigated. Notwithstanding the fact that the intensity of colour change of the strong positive and the relative colour change of the weak positive sera followed a normal distribution and obeyed the central limit theorem, there may have been a correlation between these parameters and laboratory ambient temperature.

4.3.7.4.2.Materials and methods

Ambient room temperature was investigated as the reagents and biologicals (which were brought up to ambient temperature having been stored at + 4 °C) and technicians were all identical during the daily running of the assay over a six month period. The room temperature was regarded as the independent variable whilst the colour change of the C++ was deemed the dependent variable. The room temperature of the laboratory was therefore recorded each time the assay was run.

4.3.7.4.3.Results

The results are presented graphically to show seasonal change of ambient laboratory in Figure 4.20. A scattergram graph of room temperature and strong positive sera absorbance is shown in Figure 4.21 whilst the regression analysis of absorbance against room temperature is given in Table 4.19.

Figure 4.20 A plot of the ambient room temperature in the laboratory against date n = 426

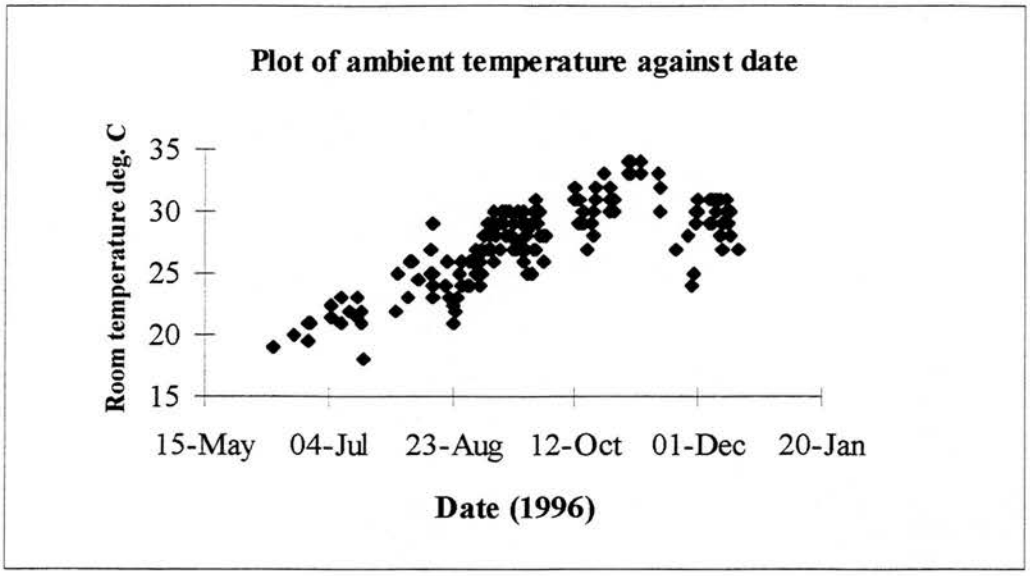


Figure 4.21 A plot of the mean optical density of the strong positives (C++) against laboratory ambient room temperature n = 426

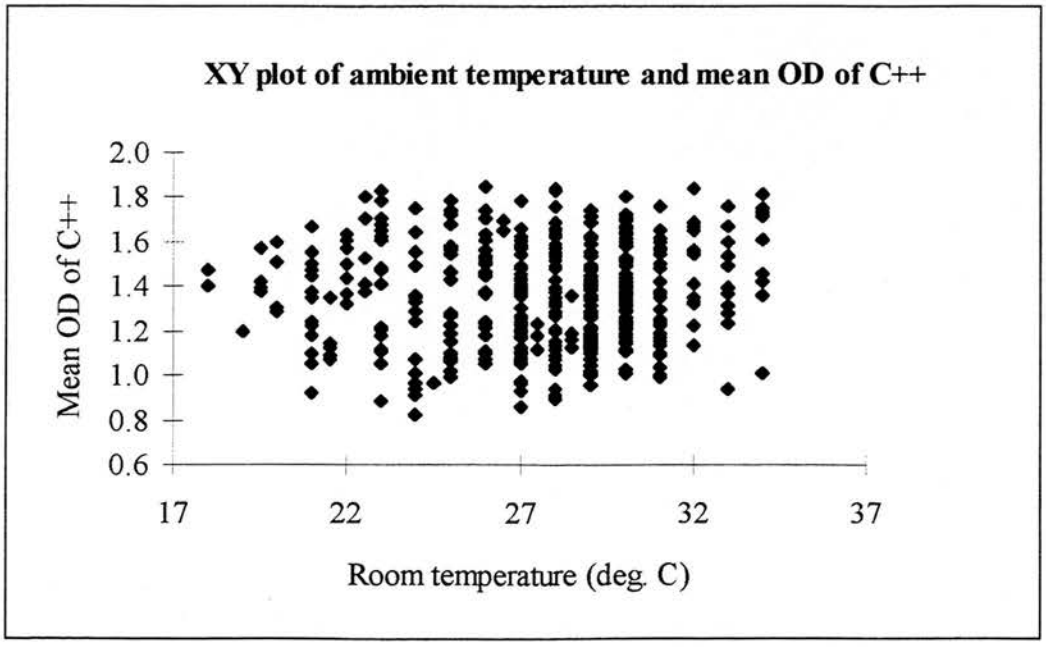


Table 4.19 The regression analysis of the mean absorbance (450 nm.) of the strong positive sera against laboratory ambient room temperature

Regression Statistics	
Linear correlation coefficient R	0.03461
R Square	0.00120
Adjusted R Square	-0.00116
Standard Error	3.30631
Observations	426

ANOVA

	df	*SS*	*MS*	*F*	*Significance F (P)*
Regression	1	5.5592	5.5592	0.5085	0.4762
Residual	424	4635.0371	10.9317		
Total	425	4640.5962			

Discussion

The seasonal temperature change is clearly evident, as the temperature rose from June to mid October (from the cold dry season to the beginning of the rains) and then fell.

The plot of room temperature against mean C++ absorbance shows there is likely to be little or no statistical correlation between the two variables, and this is enforced by the regression analysis which gives a regression coefficient of 3.5%. This is not significantly different from zero ($P > 0.45$) and thus there is no correlation between room temperature and the absorbance of the strong positive control sera.

A conclusion from this trial is that ideally the assay should be performed in a laboratory with some fairly uniform temperature, but if this cannot be avoided, the effect of room temperature will not have an adverse effect on the normal day to day variation of the intensity of colour change of the assay.

4.3.7.5. Intra blood spot variation

4.3.7.5.1.Introduction

A trial was conducted to determine the magnitude in the difference, if any, in antibody activity within the dried blood held on filter paper. Blood, with antibody, may have dried in a uniform manner or there may have been variation in the antibody distribution. When the blood was drying, liquid blood components may have moved to the centre of the spot as the periphery dried or *vice versa*; the thickness of the blood may have affected the elution process; the more rapid drying on the edge of the blood spot may have had an effect on subsequent antibody avidity, or gravity may have influenced the distribution of blood. A large variation in the distribution of antibody may have affected the results of the Ab-ELISA if eluted blood spots were used as a source of antibody .

4.3.7.5.2.Materials and methods

The trial was conducted twice: in the first instance (trial 1), ten punches each were taken from one blood spot and run on one plate (one punch per column) whilst in the second instance (trial 2) ten punches were taken from one filter paper and run on two plates, one spot per column, where columns on both plates had been randomised. On both occasions, each punch was eluted overnight at + 4 °C and the assay was repeated in eight wells occupying one column. Although the site of the hole punches was recorded, they were not clearly categorised into having been taken from the centre or periphery of the blood spot. The assay conditions were not the same[10].

4.3.7.5.3.Results

Results from the individual plate in the first trial were expressed as un-blanked (i.e. without the subtraction of some blanking value) optical densities whilst in the second trial were expressed as a percentage positivities. The analysis of variance of the trials

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[10] In the first trial, antigen was *T. brucei*, conjugate was used at 1:20,000 and the C++ was from the pooled field samples. In the second trial, T. *congolense* was used as the antigen, conjugate was used at 1:14,000 and the control sera was the used from the batch made from artificially *T. congolense* infected cattle.

are given in Tables 4.20 and 4.21, whilst details of the results are given in Appendix 5 Tables A5.5 and A5.6 and Figures 4.22. to 4.25.

Figure 4.22 The variation (as mean ± 1 standard deviation) from the ten punches from a single blood spot run on the same plate Y axis 0.2 - 0.32

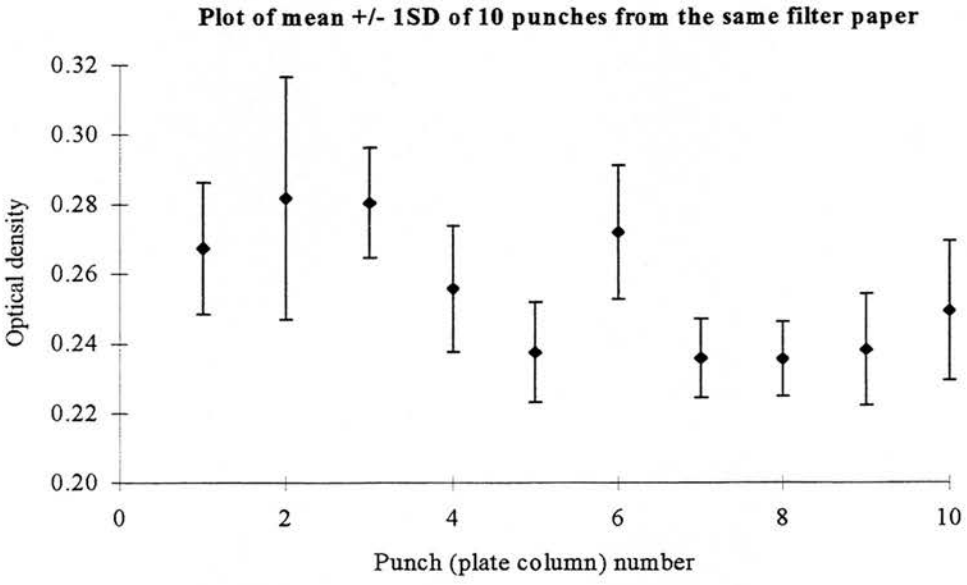

Figure 4.23 The variation (as mean ± 1 standard deviation) from the ten punches from a single blood spot run on the same plate. Y axis 0 - 1

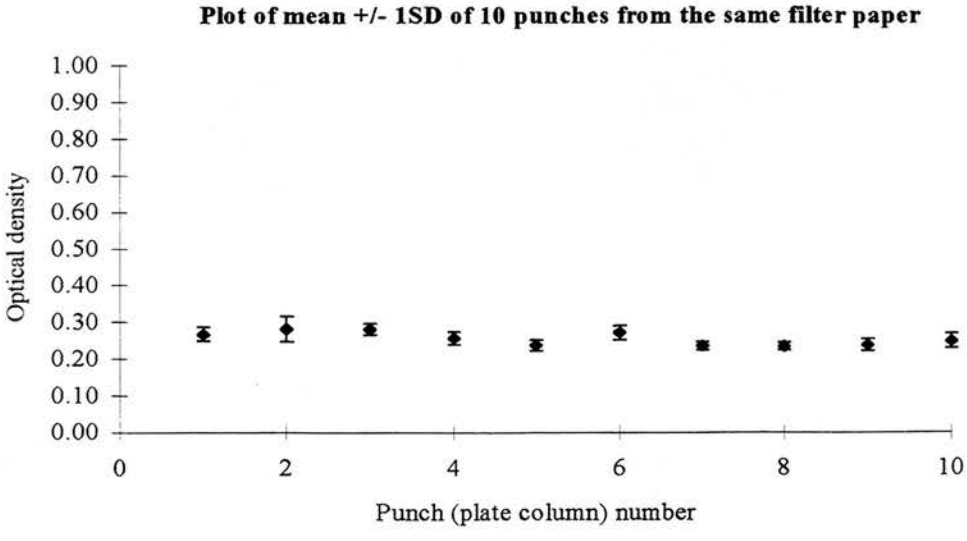

Figure 4.24 The variation (as mean ± 1 standard deviation) from the ten punches from a single blood spot run on two plates with randomised columns Y axis 30 - 65%

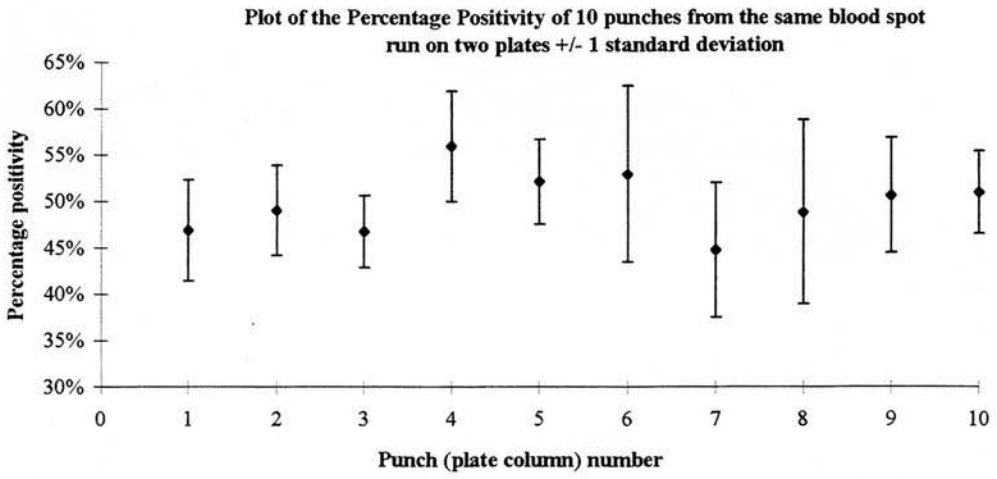

Figure 4.25 The variation (as mean ± 1 standard deviation) from the ten punches from a single blood spot run on two plates with randomised columns. Y axis 0 - 100%

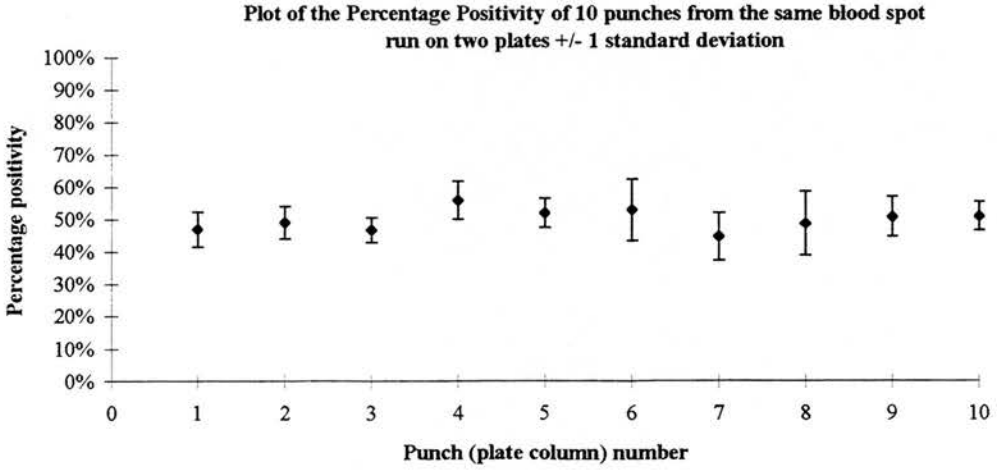

Table 4.20 The analysis of variance of 10 punches from one filter paper run on one plate

Anova: Single Factor

SUMMARY

Groups	*Count*	*Sum*	*Average*	*Variance*
Column 1	8	2.14	0.2675	0.00036
Column 2	8	2.256	0.282	0.00121
Column 3	8	2.245	0.28063	0.00025
Column 4	8	2.047	0.25588	0.00033
Column 5	8	1.901	0.23763	0.00021
Column 6	8	2.177	0.27213	0.00037
Column 7	8	1.887	0.23588	0.00013
Column 8	8	1.885	0.23563	0.00011
Column 9	8	1.907	0.23838	0.00026
Column 10	8	1.996	0.2495	0.0004

ANOVA

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	0.02546	9	0.00283	7.78565	7.6E-08	2.0166
Within Groups	0.02544	70	0.00036			
Total	0.0509	79				

Table 4.21 The analysis of variance of 10 punches from one filter paper run on two plates

Anova: Single Factor

SUMMARY

Groups	*Count*	*Sum*	*Average*	*Variance*
Column 1	16	7.50808	0.46926	0.00299
Column 2	16	7.84935	0.49058	0.00237
Column 3	16	7.486	0.46787	0.0015
Column 4	16	8.95748	0.55984	0.00357
Column 5	16	8.34304	0.52144	0.00208
Column 6	16	8.47749	0.52984	0.009
Column 7	16	7.16583	0.44786	0.00535
Column 8	16	7.82228	0.48889	0.00988
Column 9	16	8.11836	0.5074	0.00381
Column 10	16	8.16124	0.51008	0.00196

ANOVA

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	0.15984	9	0.01776	4.17899	7.8E-05	1.94279
Within Groups	0.63749	150	0.00425			
Total	0.79734	159				

Discussion

Both trials demonstrated a statistical difference in the optical density or percentage positivity between the differing hole punch sites, however the magnitude of this difference varied greatly. In trial 1 when a classic ANOVA was performed, there was a significant difference ($P < 0.05$) between the optical densities from the ten punches (from differing sites). There was also a difference ($P < 0.05$) within replicates from each punch. When the variances were converted to a coefficient of variation (CV) [square root variance/mean] , these same differences are proportionally much less with a CV of 10.1% being calculated for the various regions of the blood spot. A coefficient of variation of less than 12% is usually deemed as acceptable for an ELISA plate (Eisler, personal communication). The magnitude of these differences can be appreciated when the punch means and standard deviations are plotted at two scales on the Y axis; Figure 4.22 (Y axis scale 0.2 - 0.32) shows a large variation of the data from the ten sites whilst Figure 4.23 (Y axis scale 0 - 1) shows the relative magnitudes of the error over which the outcome (i.e. positives and negatives) of the sample would be judged.

The second trial demonstrated a far greater difference between areas from where hole punches were made. Again an ANOVA demonstrated a difference within replicates and between spots. The coefficient of variation within punches was greater than in the first trial (13.1% compared to 7.5%) which would have contributed to the coefficient variation of 36.6% between punches from different areas. The results show there to be a large effect due to the site from where the punch was taken. The two graphs with differing scales on the Y axis, show the greater variation of percentage positivities within and between areas. Even at a PP scale of 0 - 100% the variation is high.

As well as an area effect from where the punch was taken, other causes for the difference between the sites may have included i) a difference in the size of the treasury hole punch ii) pipette tip effect used to fill the wells of each column iii) plate effect (of which it was demonstrated that when one pipette tip is used to fill the 80 wells on a plate there is a demonstrable column effect without there being a row effect) as investigated above or iv) an elution effect i.e. the blood from a filter paper circle may not elute in a repeatable fashion.

This trial needs repeating, with a simpler categorical differentiation between site of hole punch (e.g. centre or periphery) and greater attention (i.e.. more uniform pipetting) to intra spot (i.e. intra plate column) variation. Until this is performed, it is difficult to draw firm conclusions about the effect of hole punch site or intra blood spot variation. Further work is necessary to study the intrinsic variability of the assay, plate and pipette tips, despite preliminary investigations as outlined above.

4.3.7.6. Comparison of results from the same animals using eluted blood spots and sera

Some of the advantages of holding blood for the antibody ELISA dried on filter paper have been discussed above, whilst some of the disadvantages of sera in terms of costs are discussed below. Data were therefore examined to determine the degree of correlation between results (expressed as percentage positivities) when the ELISA was used to determine levels of antibody using either sera or blood spots as a source of antibody. As both blood spots and sera drawn from both positive and negative populations have a Weibull and log normal distribution, then one would expect the two together to approximate a bivariate normal distribution. A plot of the percentage positivities of antibody as obtained from the two sources is shown in Figure 4.26 below. The regression statistics are shown in Table 4.22.

Figure 4.26 The plot of the percentage positivities of antibody as obtained from sera and eluted blood spots.

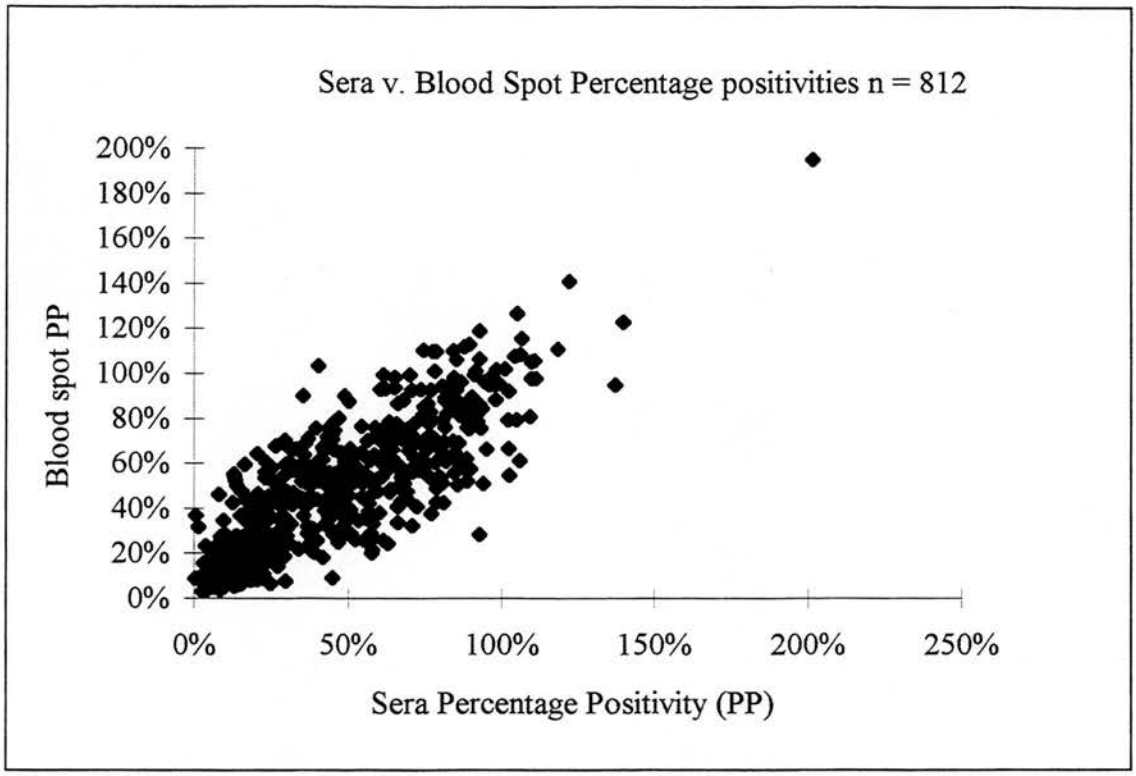

Table 4.22 The regression statistics of eluted blood spot antibody activity (dependant variable) against diluted serum antibody activity.

Regression Statistics	
Linear correlation coefficient R	0.8773
R Square	0.7697
Adjusted R Square	0.7694
Standard Error	0.1440
Observations	812

ANOVA

	df	*SS*	*MS*	*F*	*Significance F (P)*
Regression	1	56.1470	56.1470	2707.0253	0.0000
Residual	810	16.8004	0.0207		
Total	811	72.9474			

The analyses were performed to asses the degree of correlation between the two sources of antibody, not to perform some regression in order to predict the PP of the antibody from blood spot based on the result of the PP from serum. Notwithstanding, because the amount of antibody in a blood spot may be influenced by the PCV (as discussed in 4.2.3 above) then this was plotted as the dependant variable. The linear correlation coefficient R of the two PPs when using the two sources of antibody was 0.877 which demonstrates a highly significant ($P < 0.0001$) degree of correlation.

Serological tests usually use serum as the source of antibody. Whilst eluted blood spots have been used as a source of antibody in the past, frequently for IFAT estimation, little work has been done to compare the results from the two sources. This trial showed that the degree of correlation was very strong, and the implication thereof is the results from samples having used eluted blood spot should have equal credence to results obtained from sera.

4.3.8. A comparison between data from the antibody ELISA when absorbance is expressed as percentage positivity or signal to noise ratio

4.3.8.1. Introduction

Wright *et al.* (1993) cite seven methods of data expression for the intensity of colour change of the indirect (antibody) ELISA[11]. Two methods of expression of the absorbance are: i) compared to the negative reference standard (syn. signal to noise ratio) or ii) expression of the intensity of colour change relative to the strong positive control sera (syn. percentage positivity). During the investigation of the assay, four wells each of strong, weak and negative control sera were included on every plate. These data permitted a comparison of the results obtained for the weak positive control sera to be correlated by the percentage positivity method and the signal noise ratio.

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[11] These have been listed in Section 4.1

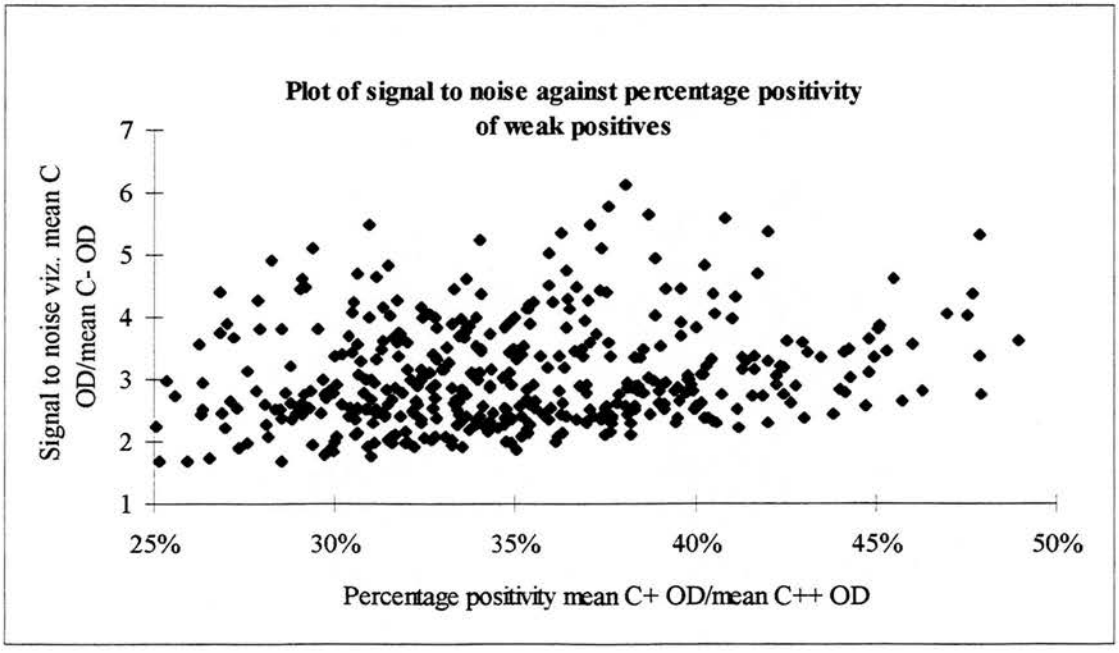
4.3.8.2. Materials and methods

The average of the weak positive control sera were plotted for 426 plates by the two methods of data expression, and a correlation analysis was performed to examine how closely the results were related. Because the signal to noise method of data expression uses a denominator (C- PP) that could theoretically reach zero, and hence the result may reach infinity, the results expressed by this method were also transformed by an *arc-tan* transformation.

4.3.8.3. Results

The results are shown in Figure 4.27 and Table 4.23.

Figure 4.27 The results from 425 plates whereby the absorbance of the weak positives were expressed as percentage positivity and as a signal to noise ratio before (above) and after (below) an arc-tan transformation.



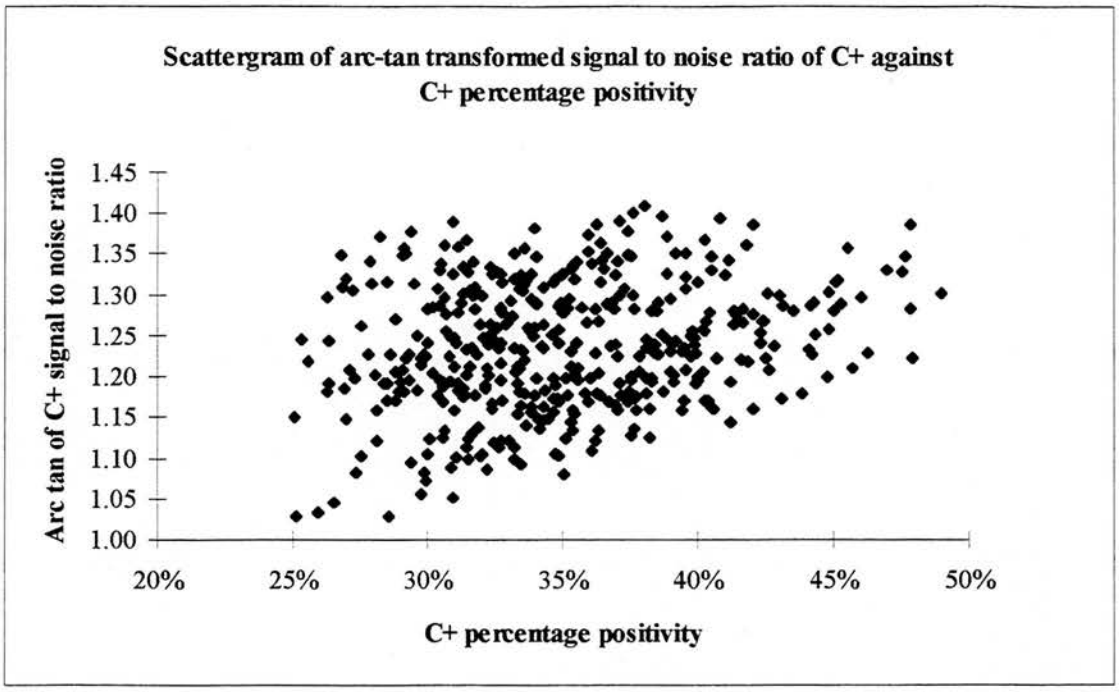


Table 4.23 The regression statistics when data are expressed as percentage positivity and signal to noise ratio before or after an arc-tan transformation.

Regression Statistics - Percentage positivity against signal to noise	
Linear correlation coefficient R	0.1916
R Square	0.0367
Adjusted R Square	0.0344
Standard Error	0.8201
Observations	426

ANOVA

	df	*SS*	*MS*	*F*	*Significance F (P)*
Regression	1	10.863	10.863	16.151	6.91975E-05
Residual	424	285.179	0.673		
Total	425	296.042			

Regression Statistics - Percentage positivity against arc tan transformed signal to noise	
Multiple R	0.2328
R Square	0.0542
Adjusted R Square	0.0520
Standard Error	0.0471
Observations	426

ANOVA

	df	*SS*	*MS*	*F*	*Significance F (P)*
Regression	1	0.054	0.054	24.297	1.18666E-06
Residual	424	0.940	0.002		
Total	425	0.994			

4.3.8.4. Discussion

The results show that even though percentage positivity and signal to noise ratio are both valid methods of data expression, and there is a statistical correlation between both methods ($P < 0.0001$), the correlation is poor. It is not remarkable that the results are correlated, but the lack of strength is perhaps noteworthy. The correlation coefficient of the arc-tan transformed data is only 4% higher than the untransformed data which demonstrates that even though theoretically desirable, this manipulation did not transform the data into values which carried a much improved linear correlation coefficient. The results demonstrate that although absorbance may be expressed as a function of the strong positive or negative control sera (Wright *et al.*, 1993) there is a very great difference in the way these values are expressed when either method is used.

What is also of note is the way the absorbance of the weak positive sera, when expressed as a signal to noise ratio, follow a log normal distribution. This is demonstrated in Figure 4.28 below.

Figure 4.28 The frequency distribution of the intensity of colour change of the means of the weak positives from 426 plates when expressed as a signal to noise ratio (above) and after standardisation (below) both before (left) and after (right) a $\log_{10}$ transformation.

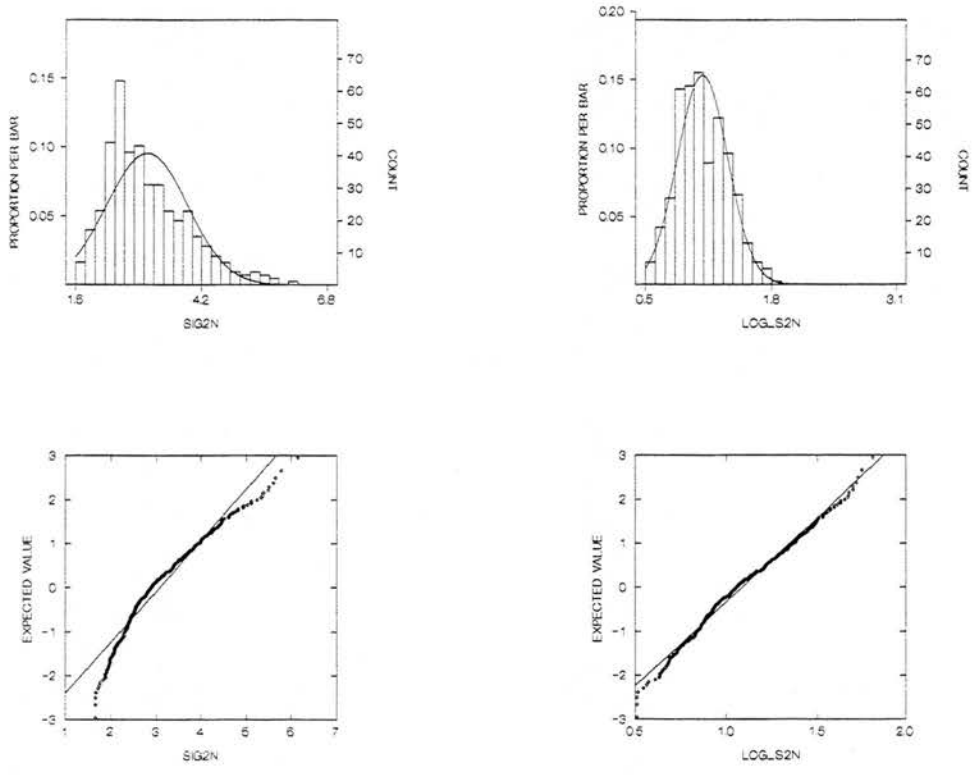
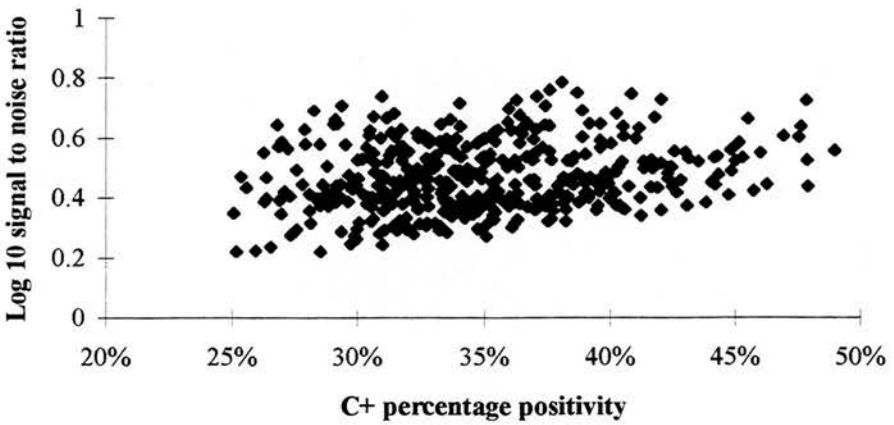


Figure 4.6 above shows that the percentage positivity data for the weak positive control sera follow a normal distribution without the need for a logarithmic transformation.

This is another reason why data expression as the percentage positivity compared to a strong positive reference sera is preferential to comparison to a pool of serum drawn from some negative population. The former follows a Gaussian distribution whilst the latter has a log normal distribution. Figure 4.29 shows that a plot of weak positive data expressed as percentage positivity and plotted against the $\log_{10}$ of the signal to noise ratio. This has a similar correlation than the un-log transformed data. The linear correlation coefficient R for the fitted line is 21.5% which again is significant but poorly correlated.

Figure 4.29 A scattergram plot of the weak positive control sera data expressed as percentage positivity and plotted against the $\log_{10}$ of the signal to noise ratio



One possible reason that the signal to noise data for the weak positives follow a log normal distribution is because of the way the plate reader expresses absorbance on a logarithmic scale.

4.3.9. Investigations into the effect of sample management on the antibody ELISA for trypanosomosis

4.3.9.1. Introduction

The effect of sample management was investigated as well as the antibody ELISA itself. If the activity of antibody in sera or held dried on filter paper varied as a function of storage time and temperature, or whether the antibody activity was effected by (upper) extremes of heat, ultra-violet rays or a combination of both (viz. direct tropical sunlight), then such possible changes would affect the individual sample's antibody ELISA result which in turn would affect the interpretation of the sero-epidemiological findings. A series of experiments were conducted to asses the effects of sample management on antibody activity when measured by the ELISA: i) the effects of heat above 45 °C on serum, ii) the effects of heat above 50°C , ultra-violet light and sunlight on sera dried on filter paper and iii) the effects of long term storage of blood dried on filter paper at four different temperatures.

4.3.9.2. Effect of heat and light on blood spot and sera

4.3.9.2.1.Materials and methods

Strong positive, weak positive and negative control sera were pipetted on to Whatmans No. 4 filter paper in a uniform manner, air dried, then held at - 20 °C before the experiments. Thereafter the filter papers were placed in incubators and hot air ovens at 45°C , 50°C , 55°C 60°C and 65°C. The filter paper for comparison was placed in the +4 °C refrigerator. Treasury hole punches were taken from each of the filter papers every thirty minutes over a four and a half hour period and placed in 2.0 mls PBST. After a minimum of two hours elution time with frequent agitation, the eluted serum paper punches were run on the same plate in the afternoon of the same day. The first two columns were run with the normal controls (C++, C+, C- and Cc) to ensure the plate was within limits. The eluted serum samples were placed in two wells each in the ten remaining columns. All results are expressed as percentage positivities.

To study the effects of ultra-violet (UV) rays, the same protocol was used, with the filter papers being placed in biohazard laminar flow cabinet with a 40 watt UV source (used for managing pathogenic samples) or were held in the self sealing plastic bags with desiccant outside in the sunlight.

The same protocol was used for the study of heat on whole serum, and the sera were held in 2 ml. tubes at the five temperatures, with control sera held at +4°C.

4.3.9.2.2.The effect of heat on antibody activity as whole serum

Results

Because the sera clotted at 65°C, no serum dilutions could be made after 90 minutes. The antibody activity, expressed as percentage positivity against time is shown in Figures 4.30 and 4.31 for 55 °C and 60°C , whilst the effect of 45°C, 50°C and 65°C are all shown in Figure 4.32.

Figure 4.30 The effect of 55 °C on antibody activity in serum

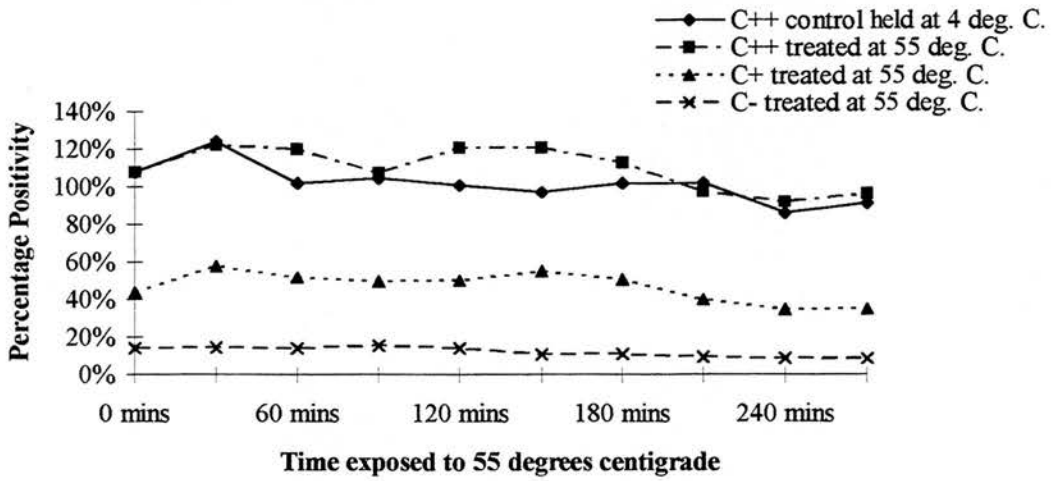


Figure 4.31 The effect of 60 °C on antibody activity in serum

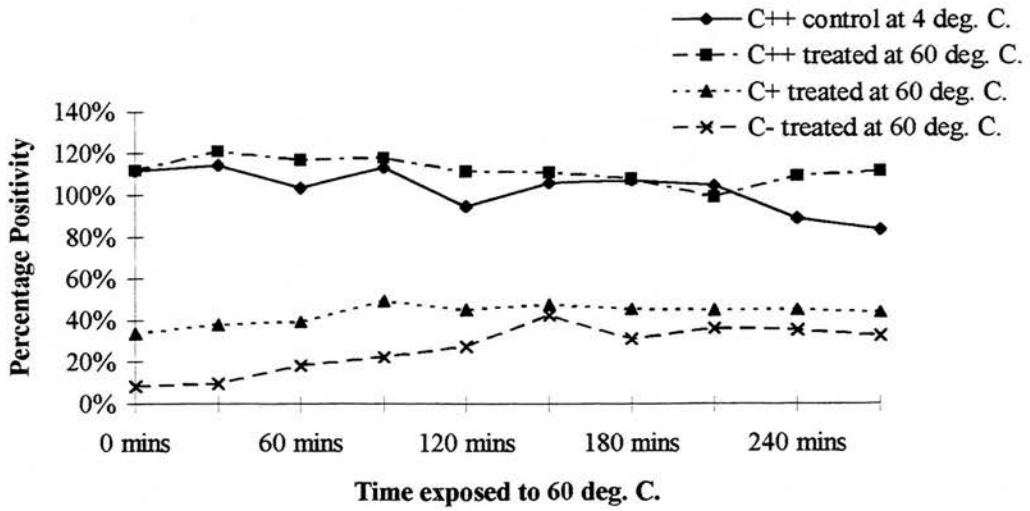
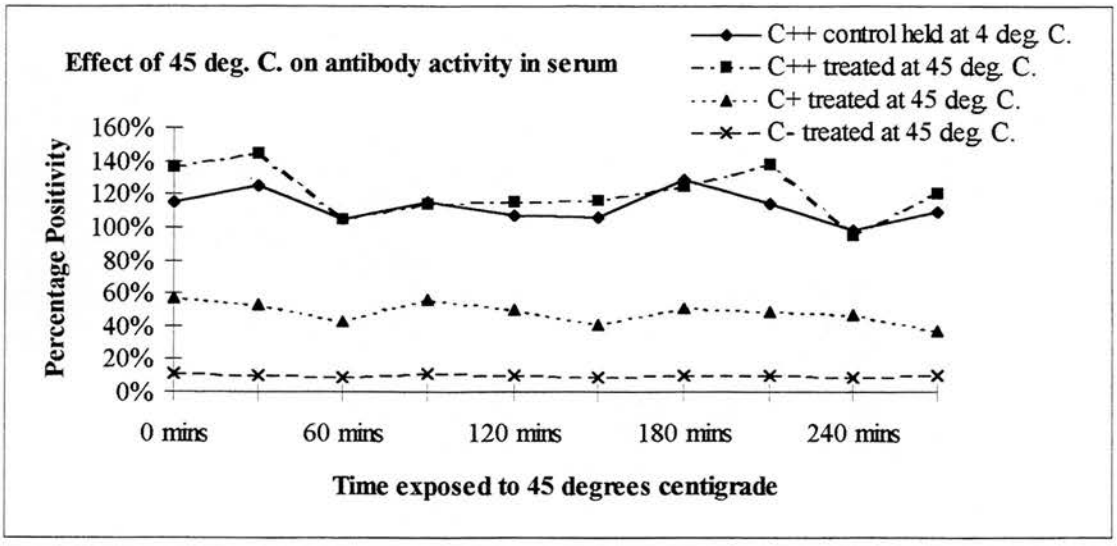
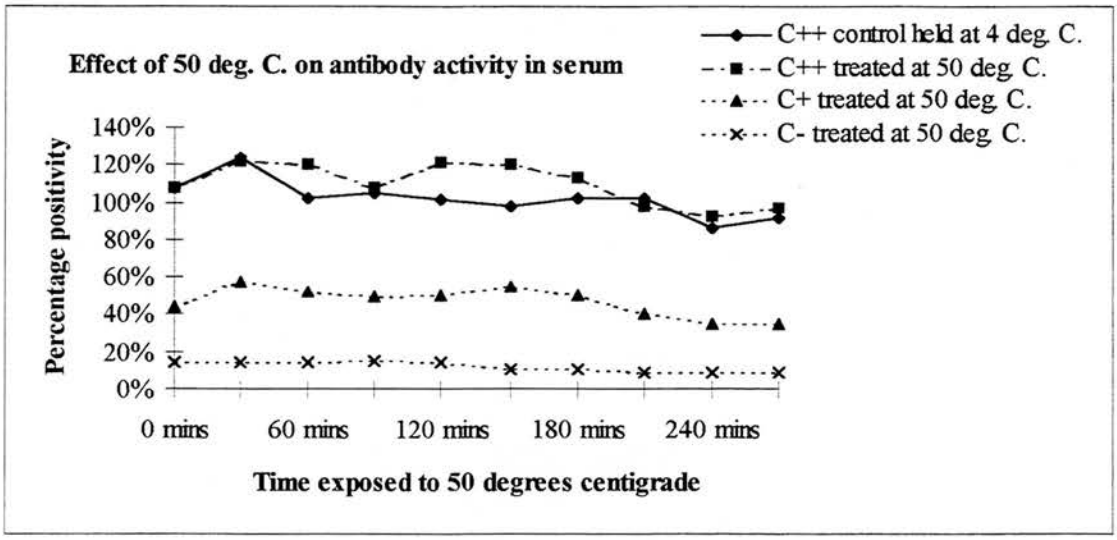
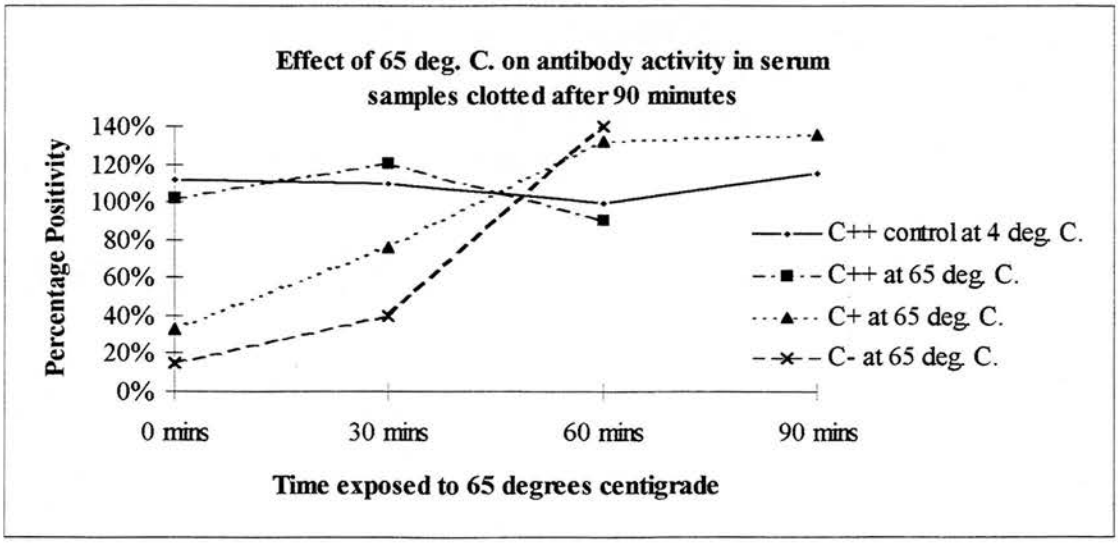


Figure 4.32 The graphs showing effects of heat on antibody activity in whole sera







Discussion

In keeping with the other trials investigating the effect of heat, etc. on antibody activity, there are two clear observations and hence interpretations from the graph. Except for the descriptive statistics of the control held at +4 °C on the 60 °C plate, statistical analyses were not performed on the results as the changes would have been significant. Firstly, the absorbance of the untreated serum (i.e. that held at + 4 °C) is seen to fluctuate when in fact these values should have remained constant. The 20 wells used to measure the control sera absorbance had a mean of 148% PP a standard deviation of 13%, a minimum value of 116% and a maximum of 166% giving a coefficient of variation (CV) of 9% and a range of 50%. Although the CV is not too great, the range of results is remarkable! As sera were diluted at 1:400 in buffer, there can be no elution time effect. There is also no trend, other than at 55°C the observation that PPs of the C++ control and heat treated as well as C+ were all high when the punches were taken at 30 minutes. This means there is a column effect on the plate, which can only be attributable to one of the stages using an 8 channel multi-pipette.

The other observation is the marked difference in the negative control sera between 55 and 60°C. At the lower temperature the absorbance remains constant, whilst at 60°C there is a progressive increase in absorbance for the first 150 minutes, after which some plateau is reached. The conclusion (as also discussed below) is that heat causes the gamma globulins in the negative sera to bind non-specifically to the antigen bound to the solid phase. Conversely, the antibody in the strong positive sera appears not to denature and thus there is no change in avidity to bind to the antigen and initiate the colour change.

There are thus several conclusions from this trial: whilst strong positive sera could be heat treated to kill viruses etc. when inter laboratory reference sera standards were being used, negative control sera would need to be treated with great caution. The trial would need to be repeated on numerous occasions whereby sera with a range of antibody activity would be heat treated at 56°C for 30 minutes (the temperature at

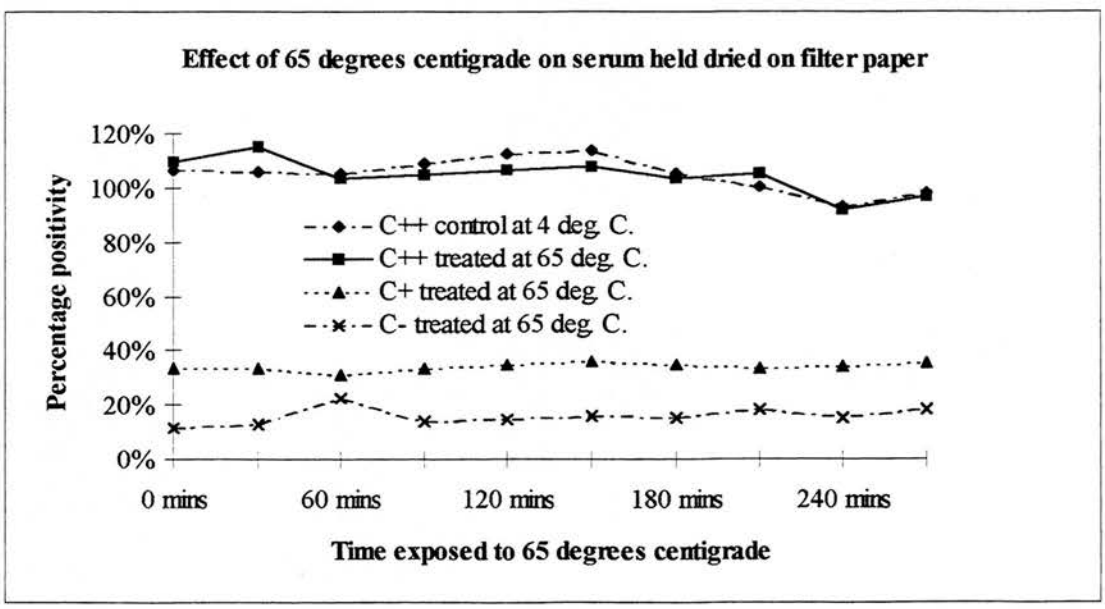
which serum samples must be treated prior to import into UK) , and compared using students T test to un-heat treated sera on the same plate. The incubator used to heat treat the sera would need to be well calibrated for 56 °C (glassware drying ovens were used for the trial which are probably only accurate to ± 2 °C) and sera heat treated for *circa* 45 minutes to ensure the centre of the sera had reached the required 56 °C.

Only after such trials have been performed using more precisely controlled temperatures would it be possible to state whether heat treatment of sera was possibly before importing sera into a country where anti-Foot and Mouth Disease (FMD) serum treatment measures are enforced (for example in South Africa) where sera may be assayed in some regional laboratory.

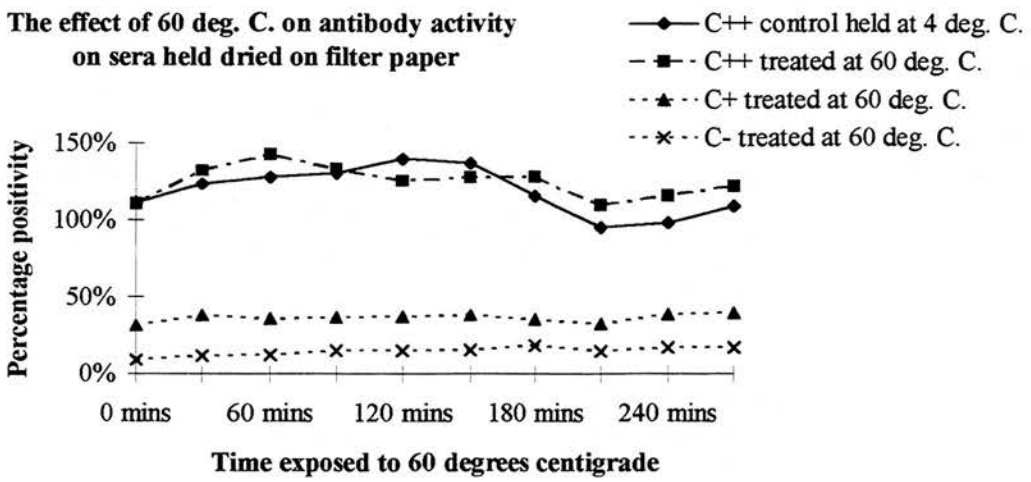
4.3.9.2.3.The effect of heat on antibody activity in sera held dried on filter paper

Results

The results are shown as a graph in Figure 4.33 below.



The graph of the effect of 60°C on serum dried on filter paper is shown in Figure 4.34 below.



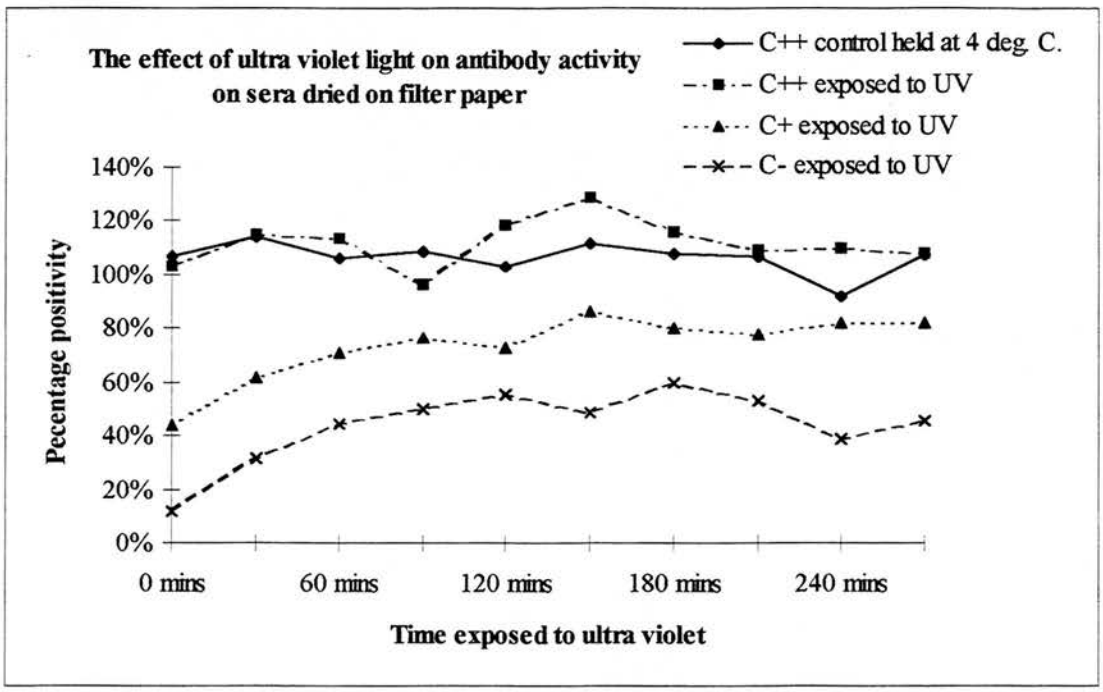
Discussion

Again, statistical analyses were not performed as significant differences over time would have been demonstrated by an analysis of variance. What is apparent however is the stability of the antibody activity in all the sera over the 270 minutes. The absorbance of the C- does not climb, and neither does the absorbance of the heat treated strong positive fall compared to the filter paper held at +4 °C. The conclusion is that sera dried on filter paper could be heat treated prior to importation into a country. A further trial is required however to investigate whether antibody activity in blood dried on filter paper behaves differently to sera dried on filter paper.

4.3.9.2.4.The effect of ultra-violet light on antibody activity in sera held dried on filter paper

Results

The results are given in Figure 4.35 below.



Discussion

The graph demonstrates the continued inherent variability in the repeatability of the absorbance of the strong positive eluted sera. This can only be attributed to a considerable variability of the amount of antibody distributed on the filter paper[12], or to the variability of hole punch size and elution process, or the inherent variability of the assay due to pipetting or other effects.

What is remarkable is the dramatic increase in the absorbance of the negative and weak positive control sera when dried on filter paper. The effect of time exposed to

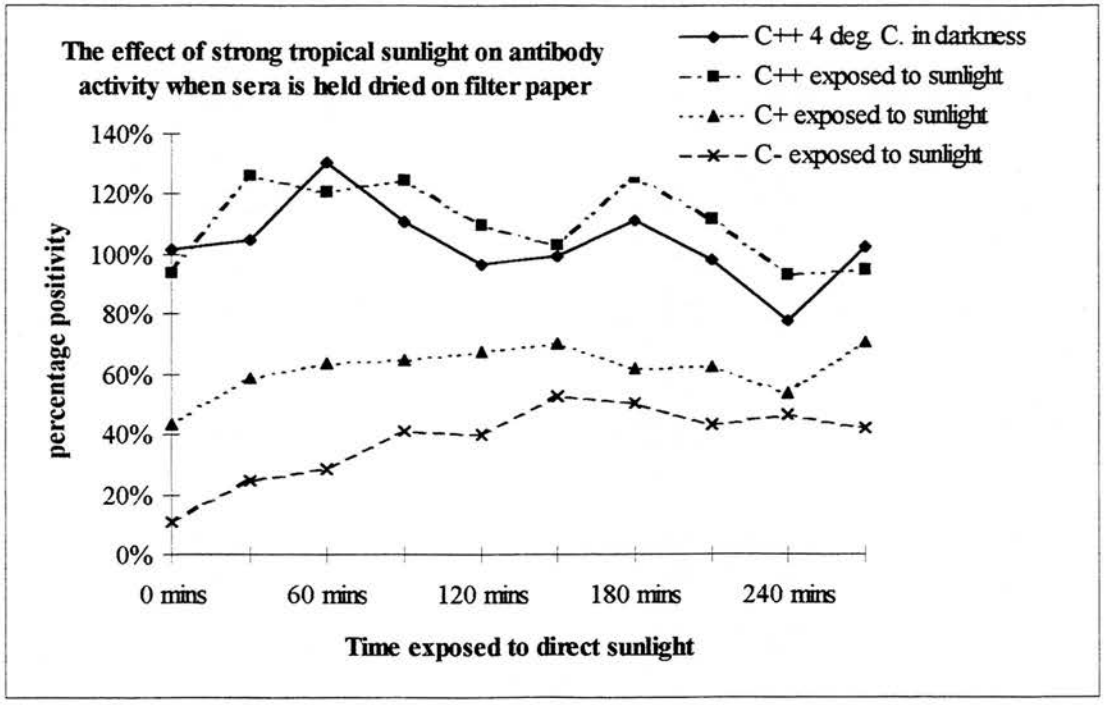
[12] The variability of antibody distribution in dried blood spots was investigated and is described above, the variability of serum dried on filter paper was not however investigated.

UV is immediate. Within the first 60 minutes the percentage positivity of the negative serum has quadrupled (from 10 to 40% PP) whilst the weak positive has increased from ~ 40% to ~ 65% PP. The mechanism for the non specific binding by the globulins to the antigen (or plate wall) must be the same as described above. Although sera dried on filter paper are not naturally going to be exposed to high levels of UV, this was an important trial in the investigation of the effect of strong tropical sunlight (described below) on antibody activity when blood is dried on filter paper.

4.3.9.2.5.The effect of tropical sunlight on antibody activity in sera held dried on filter paper

Results

The results are given in Figure 4.36 below.



Discussion

The graph demonstrates a clear increase in absorbance of the negative and weak positive control sera. This is not unexpected after the investigations into the effects of heat and ultra violet *per se*.

One very clear conclusion that can be drawn by the foregoing investigations into the effects of heat and light on filter paper samples, is that the dried blood spot samples, once air dried and placed in a bag with desiccant, must be kept cool and out of direct sunlight. The study of the effects of storage temperature (out of sunlight) are investigated in the next section.

4.3.9.3. The Effect of temperature and long term storage on antibody activity in dried blood spots on filter paper

4.3.9.3.1.Introduction

A trial was conducted to investigate the effect of storage temperature on antibody activity when blood spots were stored in the dark but at ambient room temperature, in a domestic refrigerator (+4 °C) in the ice compartment of a domestic freezer (-20 °C) or in an ultra low temperature laboratory chest freezer (- 56 °C). The hypothesis was that whilst the antibody activity of dried blood spots at - 20°C and -56°C would remain the same, over a period of months the antibody activity of the blood spots at + 4 °C and room temperature would probably change.

4.3.9.3.2.Materials and methods

At least six large blood spots made from heparinised blood taken from five animals raised in a heavy tsetse challenge area and were placed on Whatman's filter paper and air dried out of the sun. They were then labelled and placed in a self sealing bag with self indicating silica gel desiccant, and were kept cool until reaching the laboratory. In the laboratory, blood spots from each of the five animals were placed together in bags with desiccant (as above) and placed in a drawer, on the bottom shelf of a domestic (+ 4 °C) refrigerator, in a domestic (-20 °C) chest freezer and in a -56°C laboratory chest freezer. Approximately every three weeks a treasury punch was made from each blood spot and after elution in PBST the samples were all run on the same plate (with control sera to ensure the plate was within limits) and the absorbance was expressed as a percentage positivity. Identical assay conditions and reagent were used throughout the trial. 100 µl of each elution was placed in four wells on the ELISA plate and so each sample at each temperature occupied two columns whilst each storage temperature ran across two rows.

4.3.9.3.3.Results

The results are given in Table 4.24. The range of percentage positivities for the control sera and samples under investigation are also presented at the bottom of this table. Because interpretation of this table is difficult, and one is looking for **trends** in the results, the average of all five samples at the four temperatures, as well the weak positive and negative control sera percentage positives have been plotted against time. These results are show in Figure 4.37.

4.3.9.3.4.Discussion

Examination of the tabulated and graphical results does not show any clear trend, other than a slight overall increase in the average of the five samples' percentage positivities at the four storage temperatures. What is very apparent however is the enormous week to week variation of the results, whether samples were stored at -56°C or room temperature. The absolute absorbance of the strong positive control sera and the percentage positivities of the weak positive and negative control sera and conjugate control are all within limits and thus are within the limits of a normal distribution.

The absorbance of the test samples however (expressed as a percentage of the strong positive) demonstrate an enormous fluctuation on a week to week basis. Over the six month period, some samples have a range of 40 - 50%. Percentage positivities "across the board" are sometimes very low and yet the C+ PP and C- PP are performing normally. One conclusion has to be that the antibody activity from eluted blood spots is behaving differently to antibody activity from frozen sera. The author cannot offer a rational explanation.

The trial does demonstrate that at least over a 6 month period, there is no loss of antibody activity when samples are stored at room temperature (in the dark) compared to refrigerators and freezers.

Table 4.24 The results of the blood spot temperature storage trial

						Percentage Positivities					
Day	*PP Control*	*Average of samples*	Week		*Ave OD*	*Sample 1*	*Sample 2*	*Sample 3*	*Sample 4*	*Sample 5*	*Storage temp.*
1	0.5%	50.8%	1	Cc	0.007	35.9%	64.7%	54.3%	51.3%	47.8%	*Room temp*
1	100.0%	52.4%	1	C++	1.487	34.4%	75.6%	56.2%	47.6%	48.1%	*+ 4 deg. C*
1	30.6%	52.7%	1	C+	0.455	38.9%	74.9%	56.2%	52.2%	41.4%	*- 20 deg. C*
1	10.2%	53.1%	1	C-	0.152	40.0%	74.5%	58.3%	44.2%	48.4%	*- 56 deg. C*
22	4.1%	62.5%	4	Cc	0.042	31.5%	86.1%	61.9%	72.6%	60.1%	*Room temp*
22	100.0%	59.3%	4	C++	1.025	19.2%	86.4%	59.1%	69.4%	62.2%	*+ 4 deg. C*
22	26.4%	58.2%	4	C+	0.271	31.9%	87.5%	60.7%	62.6%	48.5%	*- 20 deg. C*
22	11.6%	58.6%	4	C-	0.119	27.3%	90.8%	62.2%	65.2%	47.7%	*- 56 deg. C*
57	1.1%	48.8%	9	Cc	0.019	32.4%	67.4%	42.5%	56.8%	44.9%	*Room temp*
57	100.0%	50.2%	9	C++	1.739	29.5%	66.8%	47.4%	54.7%	52.4%	*+ 4 deg. C*
57	32.8%	47.3%	9	C+	0.570	26.7%	67.0%	44.8%	59.0%	39.1%	*- 20 deg. C*
57	13.2%	51.5%	9	C-	0.230	25.3%	75.6%	50.1%	58.0%	48.7%	*- 56 deg. C*
78	3.5%	40.4%	12	Cc	0.042	25.7%	49.5%	46.2%	39.1%	41.6%	*Room temp*
78	100.0%	44.0%	12	C++	1.182	22.4%	71.7%	41.8%	43.1%	40.8%	*+ 4 deg. C*
78	33.5%	42.3%	12	C+	0.397	24.9%	59.1%	39.6%	46.8%	41.0%	*- 20 deg. C*
78	14.6%	40.5%	12	C-	0.172	25.6%	56.7%	42.7%	37.1%	40.3%	*- 56 deg. C*
99	2.1%	70.0%	15	Cc	0.027	40.0%	98.1%	59.0%	74.5%	78.5%	*Room temp*
99	100.0%	74.1%	15	C++	1.283	39.1%	109.7%	62.8%	75.1%	84.0%	*+ 4 deg. C*
99	34.9%	74.9%	15	C+	0.447	42.3%	107.4%	69.0%	69.9%	85.6%	*- 20 deg. C*
99	16.0%	67.2%	15	C-	0.205	43.4%	100.4%	60.0%	59.5%	73.0%	*- 56 deg. C*
121	1.1%	65.3%	18	Cc	0.012	38.9%	82.9%	60.1%	78.1%	66.6%	*Room temp*
121	100.0%	63.6%	18	C++	1.141	41.4%	80.2%	55.8%	76.0%	64.6%	*+ 4 deg. C*
121	37.0%	65.5%	18	C+	0.422	45.2%	85.0%	59.1%	67.5%	70.6%	*- 20 deg. C*
121	19.3%	59.1%	18	C-	0.220	33.8%	77.4%	57.0%	66.7%	60.7%	*- 56 deg. C*
149	2.4%	71.4%	22	Cc	0.031	42.2%	95.4%	73.8%	76.8%	68.8%	*Room temp*
149	100.0%	71.8%	22	C++	1.315	53.7%	97.9%	64.7%	73.8%	69.1%	*+ 4 deg. C*
149	36.2%	73.1%	22	C+	0.476	44.3%	92.9%	77.8%	77.1%	73.6%	*- 20 deg. C*
149	13.2%	65.9%	22	C-	0.174	44.6%	89.7%	68.1%	67.5%	59.8%	*- 56 deg. C*
169	2.1%	71.4%	25	Cc	0.034	39.0%	99.1%	69.3%	75.7%	73.8%	*Room temp*
169	100.0%	71.5%	25	C++	1.642	50.1%	99.6%	66.5%	68.2%	73.1%	*+ 4 deg. C*
169	44.4%	70.9%	25	C+	0.728	41.3%	99.8%	70.7%	65.5%	76.9%	*- 20 deg. C*
169	16.7%	68.5%	25	C-	0.275	39.9%	93.1%	67.7%	66.2%	75.7%	*- 56 deg. C*
191	1.1%	80.6%	28	Cc	0.013	41.0%	111.0%	72.2%	91.6%	87.2%	*Room temp*
191	100.0%	77.7%	28	C++	1.243	43.0%	105.6%	70.0%	82.6%	87.2%	*+ 4 deg. C*
191	29.6%	77.0%	28	C+	0.369	45.1%	108.0%	66.5%	79.6%	85.6%	*- 10 deg. C*
191	10.4%	74.5%	28	C-	0.129	43.0%	105.4%	65.0%	80.5%	78.5%	*- 30 deg. C*

Range: maximum value - minimum value		*Sample 1*	*Sample 2*	*Sample 3*	*Sample 4*	*Sample 5*	*Storage temp.*
3.7%	Cc	16.5%	61.5%	31.3%	52.4%	45.5%	*Room temp*
0.0%	C++	34.6%	42.9%	28.2%	39.6%	46.3%	*+ 4 deg. C*
17.9%	C+	20.3%	49.0%	38.2%	32.9%	46.5%	*- 20 deg. C*
9.1%	C-	19.2%	48.7%	25.3%	43.5%	38.2%	*- 56 deg. C*

Blood spots always eluted in the morning, tubes held at ambient temperature and plates run in the afternoon

Figure 4.37 Average PPs of samples from 5 animals stored room temp, +4 deg. C., -20 deg. C. and -56 deg. C.

If one ignores the values of the blood spots taken on day 78 (when all the PPs were very low), the trend appears that antibody activity (or more probably non specific binding to the plate wall) increases, at no matter which storage temperature. It is difficult to speculate why this occurs equally at room temperature and - 56 °C but is in keeping with other observations (see above) that inclement conditions cause an increase in absorbance. The increase in non specific binding cannot be too pronounced however, as the samples used for the negative controls to establish the assay had been kept in a - 20 °C freezer for over three years, and yet gave PPs of less than 20%. An increase of PP as a function of time (not temperature) may also account for the differences in blood spot absorbance when samples were less than two months old and over three years.

The important conclusions from the above trials are:

- serum samples should not be exposed to temperatures above 55 °C at which point antibodies are affected
- antibody activity on blood spots is not affected by 55 °C using results extrapolated from sera dried onto filter paper
- antibody activity appears not to be affected by storage temperature, at least in the first six months, provided they are kept in the dark with desiccant
- dried blood spots should never be exposed to direct sunlight
- blood spots should be assayed as soon as possible after they have been taken
- when samples are exposed to inclement conditions, this causes an increase in the non specific binding and hence colour change of the negative and weak positives, rather than a decline in antibody activity of the strong positives.

4.4. Antibody Activity as assessed by IFAT and ELISA

4.4.1. The correlation between IFAT and Antibody ELISA results

4.4.1.1. Introduction

The level of anti-trypanosomal antibody in cattle can be measured by the use of the antibody trapping ELISA or the indirect fluorescent antibody test (IFAT). Both tests may use serum, plasma or eluted blood spots as a source of antibody. The possible main advantages of the antibody ELISA (and hence by contrast the disadvantage of the IFAT) are the rigid quality control checks based on computer software and control sera and an objective (vs. subjective) assessment of antibody activity. The IFAT has been successfully used in Africa for sero-prevalence studies for AAT (Ashkar and Ochilo, 1972; Connor and Halliwell, 1987) and was routinely in use in the Eastern Province of Zambia (BADCP, 1992). Data on the two diagnostic tests were studied when the strong positive control sera was produced by the repeated experimental infection of two oxen.

4.4.1.2. Materials and Methods

Two oxen were housed in fly free accommodation in a tsetse free area of Zambia. They had been infected with a field strain of *T. congolense* a year previously and had been treated by an intra-muscular injection of diminazene aceturate at a dose rate of 7.0 mg./kg.[13]. The animals were inoculated with trypanosomes by subcutaneous injection of infected mouse blood then screened twice weekly when the haematocrit value was measured and the buffy coat was examined for parasitaemia by dark ground microscopy. Sera and plasma as well as blood dried on filter paper were taken and examined by IFAT at the Provincial Veterinary Laboratory in Chipata, Eastern Province, Zambia and the antibody ELISA at the University of Zambia in Lusaka. When a parasitaemia was detected, treatment was by an intra-muscular injection of

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[13] A "double" dose was used to ensure complete sanitising of trypanosomes, especially *T. brucei* which are reported to have a higher CD_{50} than *T. congolense* and *T. vivax*.

diminazene diaceturate at a dose rate of 7.0 mg./kg. A field strain of *T. congolense* which had isolated in the Eastern Province of Zambia and had been cryo-preserved then subsequently grown in mice was used for the infections.

The antibody ELISA was performed using the protocol given in Appendix 3. The strong positive C++ sera produced by the pool of sera from this same trial was used as the control sera by which percentage positivity was calculated. *T. congolense* antigen diluted 1:1000 was used to coat the plates.

The IFAT was performed by a routine method which is described in Appendix 6. *T. congolense* antigen had been produced by the Tropical Institute in Antwerp. IFAT scoring was performed after serial dilution. When viewed under an ultra violet light source, the fluorescence of the trypanosomes was assessed as fluorescing (fluorescence +ve), partially fluorescing (fluorescence +/-) or not fluorescing (fluorescence -ve). and thus samples were scored according to the system given in Table 4.25. A score of 2 or above for a sample is considered positive.

Table 4. 25 The IFAT result scoring system

Dilution	*Fluorescence*	*Score*
1:80	-ve	0
1:80	+/-	1
1:80	+ve	2
1:160	+/-	3
1:160	+ve	4
1:320	+/-	5
1:320	+ve	6
1:640	+/-	7
1:640	+ve	8
1:1,280	+/-	9
1:1,280	+ve	10
1:2,560	+/-	11
1:2,560	+ve	12
1:5,120	+/-	13
1:5,120	+ve	14

4.4.1.3. Results

The results from both animals were combined, and antibody ELISA percentage positivity was plotted against the IFAT score when antibody was obtained from serum, plasma and eluted blood spot are shown in Figure 4.38. The linear correlation coefficient R for the three sources of antibody were 0.833, 0.800 and 0.794 respectively.

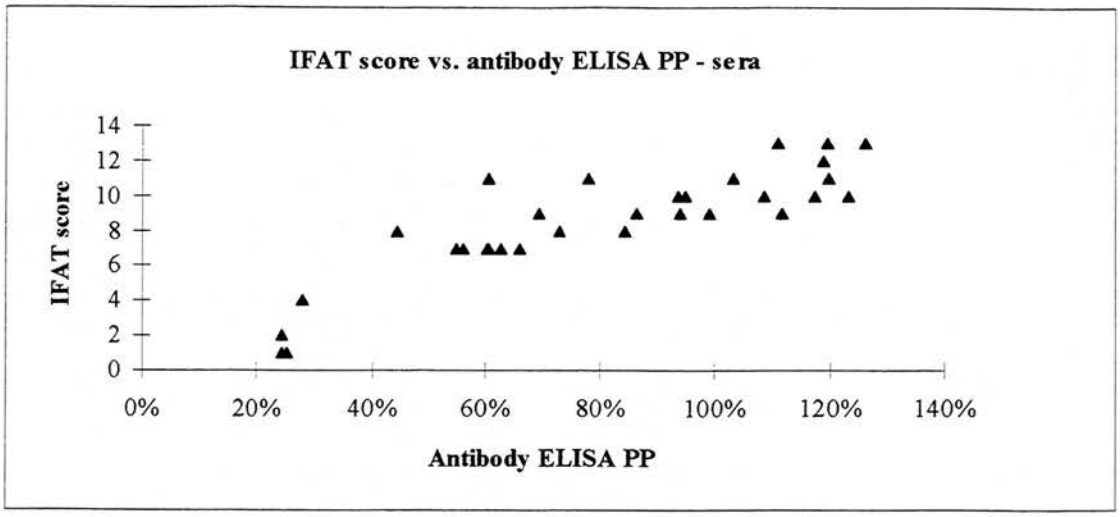
4.4.1.4. Discussion

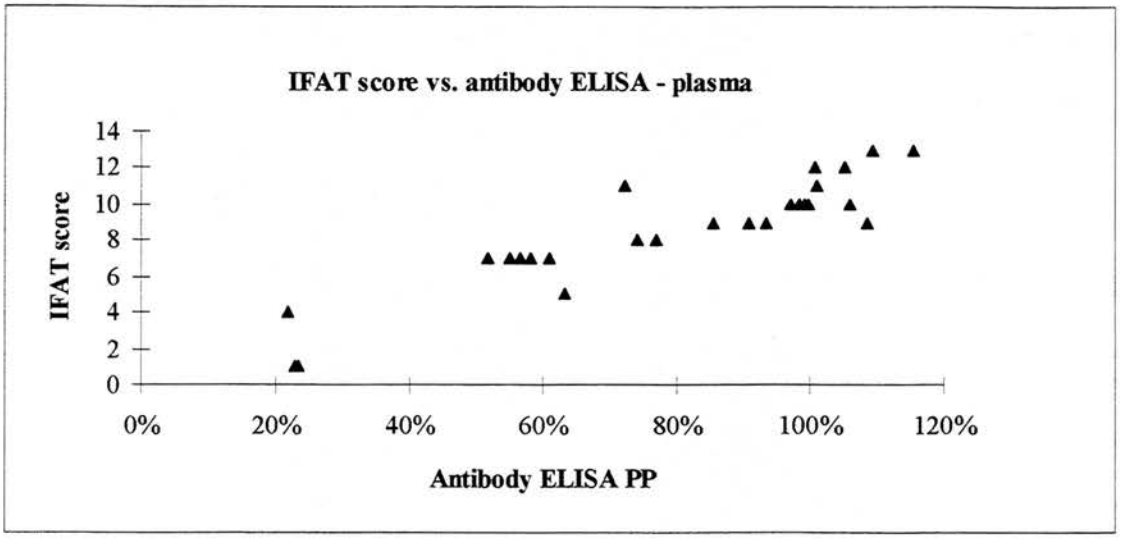
The results show a very good correlation between IFAT score and antibody ELISA, and are the same findings as Jongejan *et al.* (1988). This is a credit to the technician in the Provincial Laboratory who performed the IFAT on the samples. He had no foreknowledge of the ELISA results and has considerable experience in performing the IFAT for trypanosomiasis.

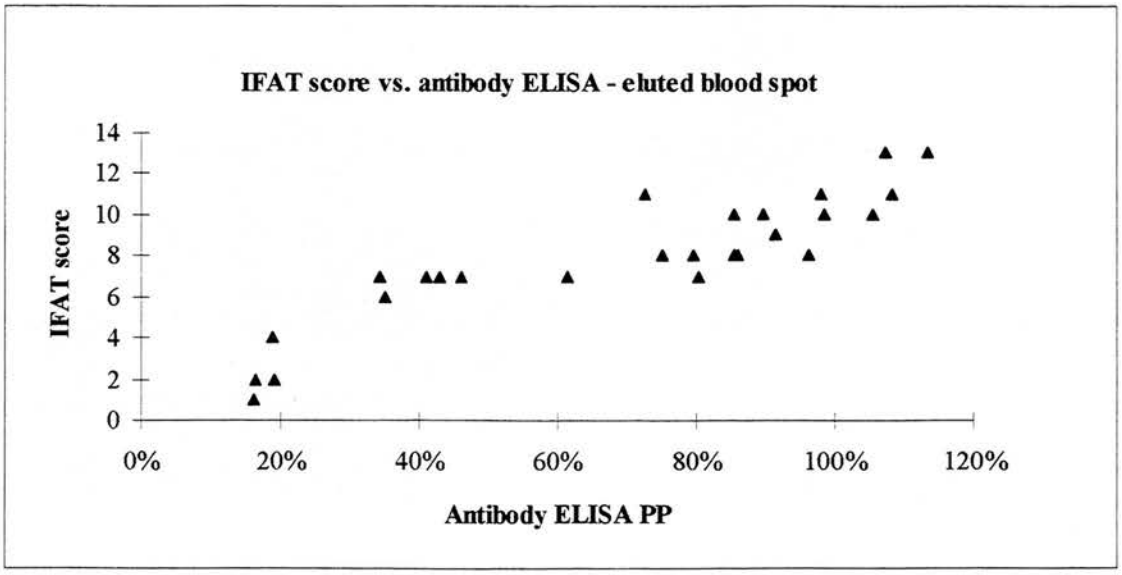
Despite the fact that the two diagnostic techniques may be trapping and hence cause the colour change/fluorescence of antibodies raised against either VSGs (in the case of IFAT) or invariant somatic antigens (in the case of ELISA), there was no reason to expect a difference in the amount of antibody detected by either technique. The presence of intact trypanosomes would stimulate production of antibodies against the VSGs whilst after the host has raised an immune response against the trypanosomes and the fragmented parasites are present in the bloodstream (during which invariant antigens would occur in the blood) antibodies would be raised against these invariant somatic antigens. Hence, any host which has had a trypanosome infection and mounted a successful immune response would have antibodies against both antigen moieties.

The sensitivity and specificity of IFAT was not investigated and so whilst investigating results from individual samples, it was not possible to convert the result into predicative values. Likewise, because the two tests were assessed for individual samples (vs. herds) they could not be compared by McNemar's change test (Thrusfield, 1995). Ideally IFAT and Ab-ELISA results would be compared on a herd basis and sero-prevalence plotted as well as performing a McNemar's change test.

Figure 4.38 The correlation between antibody ELISA percentage positivity and IFAT score using antibody from sera, plasma and eluted blood spots







The lowest R correlation was from the eluted blood spots when used as the source of antibody. This may have been due to the inherent variability of the quantity of antibody (see above) when using this technique to recover antibodies. Notwithstanding, the R value of > 79% showed a very respectable correlation.

One could discuss at length the results when the IFAT scores were 0 - 3 and the ELISA PP results were ~ 20%. Whilst IFAT gives a simple categorical result from a sample (i.e. a score of 0 or 1 is deemed negative and 2 or greater is deemed positive) with antibody ELISA this dichotomy is dependant on the cut-off PP value used to discriminate between positive and negative. The author maintains in this thesis that antibody results are not used to diagnose infection in individual animals rather to asses a herd's trypanosomiasis sero-prevalence. The choice of cut-off is partly dependant on the costs of false positives and negatives and so cannot be used for individual samples. One can see from the graphs that there are the three or four points close the threshold of positivity (for both IFAT and ELISA) which lie close to the approximate cut-off value of 20%.

4.4.2. Observations of the kinetics of antibody responses in trypanosome- infected cattle

4.4.2.1. Introduction

In order to perform the quality control on the Ab-ELISA, control sera are required as discussed above. Strong positive sera may be diluted in negative sera to obtain the weak positive. Strong positive sera (C++) can be obtained via two methods; pooling sera using field samples that have given high absorbance values, or by the experimental infection with trypanosomes of cattle kept under controlled, fly free conditions. Of the two methods, the latter is preferable because, assuming the experimental animals are free from other haemoparasites, then the only difference between control sera and test sera will be the presence or absence of anti-trypanosomal antibodies. If pooled sera from animals of unknown provenance with

regard to other haemo-parasites is used, there could potentially be cross reaction with the antigen.

A trial was therefore conducted, whose primary aim was to produce C++ serum for the Ab-ELISA to be run as a routine sero-epidemiological assay. The control sera was also to have been distributed to other laboratories in order to facilitate the use of the assay. The trial provided results that could be used to compare IFAT with Ab-ELISA (see above) as well as the kinetics of antibody levels. Not only would it be possible to study the rise in antibody, but of greater practical importance would be the rate of decline. Unfortunately, whilst the rise in antibody could be studied (described below) no data were forthcoming from Zambia after this trial to examine the rate of decline.

4.4.2.2. Materials and methods

Two oxen were housed in fly proof quarters in the Eastern Province of Zambia and were infected with a cryo-preserved field strain of *T. congolense* on four occasions. The animals were treated with an intramuscular injection of 7% w/v diminazene at a dose rate of 7.0 mg/kg after a demonstrable parasitaemia of seven days using the buffy coat smear technique or after four weeks after infection. Both animals had been previously infected with a field strain of *T. congolense* approximately seven and a half months previously[14] (Mataa, personal communication).

The animals were examined twice weekly and haematocrit results were recorded as well as buffy coat findings. Parasitaemias were scored according to the system given in Table 4.26.

[14] The animals had previously been treated on 28th July 1995 when parasitaemic. Day 1 of the trial was 4th March 1996.

Table 4.26 The buffy coat dark ground scoring system (x 250) used for the study of the rise in antibody - Taken from "An evaluation of the sensitivity of current trypanosome parasitological diagnostic techniques" by Paris J., Murray M., Agure R. (1980).

Score	Trypanosomes per field (x250)	Trypanosome concentration
6+	Swarming > 100 per field	$> 5 \times 10^6$ / ml
5+	> 10 per field	$> 5 \times 10^5$/ ml
4+	1 - 10 per field	$10^4 - 5 \times 10^5$/ ml
3+	1 per 2 - 10 fields	10^4/ ml approx.
2+	1 - 10 per buffy coat smear	$10^3 - 10^4$/ ml.
1+	1 per buffy coat smear	$10^2 - 10^3$/ ml.

Sera, plasma and blood dried on filter paper were collected weekly and examined by IFAT in the Chipata Provincial Veterinary Laboratory and by Ab-ELISA at UNZA, Lusaka. On several occasions at the end of the trial when the IFAT score was highest, blood was collected from both animals and pooled to make a 500 ml. batch of C++. This same sera was used to express the Ab-ELISA percentage positivity results.

4.4.2.3. Results

The results for animals 808 and 807 are given in Tables 4.27 and 4.28 respectively. The results are also given graphically in Figures 4.39 and 4.40. An average of the IFAT scores was taken and divided by ten in order to portray the IFAT, Ab-ELISA percentage positivity and PCV results on the same axis. The occasions when animals were infected and treated are also shown, as is the presence of parasitaemia on buffy coat smear.

Table 4.27 Results from animal Tag Number : 808

0 in infection or treatment column or 1in Pos BC columns reflects animal infected, treated or positive on buffy coat. Shaded area, samples not taken

				Serum						IFAT Titre/score					
Date	*Trial*	*Ave. IFAT*		*Ab-ELISA*					*Buffy-Coat*	**Serum**		**Plasma**		**Filter paper**	
	day	*Score*	*Ave IFAT/10*	*PP*	*Inf.*	*Rx.*	*PCV*	*Pos BC*	*Score*	*Titre*		*Titre*		*Titre*	
4/03/96	1	1.5	0.2	0.245			0.35		0	+/-80	1			80	2
7/03/96	4	1.3	0.1	0.252			0.34		0	+/-80		1 +/-80		1 80	2
11/03/96	8			0.244											
14/03/96	11	1.3	0.1		0		0.38		0	80	2	+/-80	1	+/-80	1
18/03/96	15						0.40		0						
21/03/96	18	4.0	0.4	0.281			0.39	1	1	160	4	160	4	160	4
25/03/96	22						0.35	1	5						
28/03/96	25	9.0	0.9	1.116		0	0.38	1	4	+/-1280	9	+/-1280	9	+/-1280	9
1/04/96	29						0.32		0						
4/04/96	32	10.0	1.0	1.232			0.35		0	1280	10	1280	10	1280	10
8/04/96	36						0.37		0						
11/04/96	39	13.0	1.3	1.120	0		0.35		0	+/-5120	13	+/-5120	13	+/-5120	13
15/04/96	43						0.35		0						
18/04/96	46	13.0	1.3	1.261			0.35		0	+/-5120	13	+/-5120	13	+/-5120	13
22/04/96	50						0.28		0						
25/04/96	53	11.3	1.1	1.120			0.35		0	+/-2560	11	1280	12	+/-2560	11
29/04/96	57					0	0.31		0						
2/05/96	60	10.0	1.0	1.117			0.32		0	1280	10	1280	10	1280	10
6/05/96	64						0.31		0						
9/05/96	67	11.0	1.1	1.032			0.30		0	+/-2560	11	1280	11	+/-2560	11
13/05/96	71				0		0.32		0						
17/05/96	75	10.0	1.0	1.087			0.35		0	1280	10	1280	10	1280	10
20/05/96	78						0.35		0						
24/05/96	82	9.0	0.9				0.34	1	3	+/-1280	9	+/-1280	9	+/-1280	9
27/05/96	85						0.32	1	3						
30/05/96	88	8.7	0.9				0.30	1	4	+/-1280	9	+/-1280	9	640	8
3/06/96	92					0	0.25	1	4						
6/06/96	95	7.7	0.8				0.26		0	+/-2560	11	+/-2560	1	+/-2560	11
4/07/96	123	12.0	1.2	1.119	0		0.35		0	1280	12		12		
8/07/96	127						0.36		0						
15/07/96	134						0.37	1	3						
18/07/96	137	11.0	1.1				0.35	1	3	+/-2560	11				
22/07/96	141						0.35	1	4						
25/07/96	144						0.34	1	4						
29/07/96	148					0	0.34	1	4						
31/07/96	150	13.0	1.3	1.112			0.34		0	+/-5120	13				
10/09/96	191	13.0	1.3				0.35		0	+/-5120	13				

179

Figure 4.39 Plot of average IFAT score/10, Ab-ELISA PP and PCV over time for animal 808 showing experimental infection and treatment diminazene 7.0 mg/kg and detectable parasitaemia on buffy coat

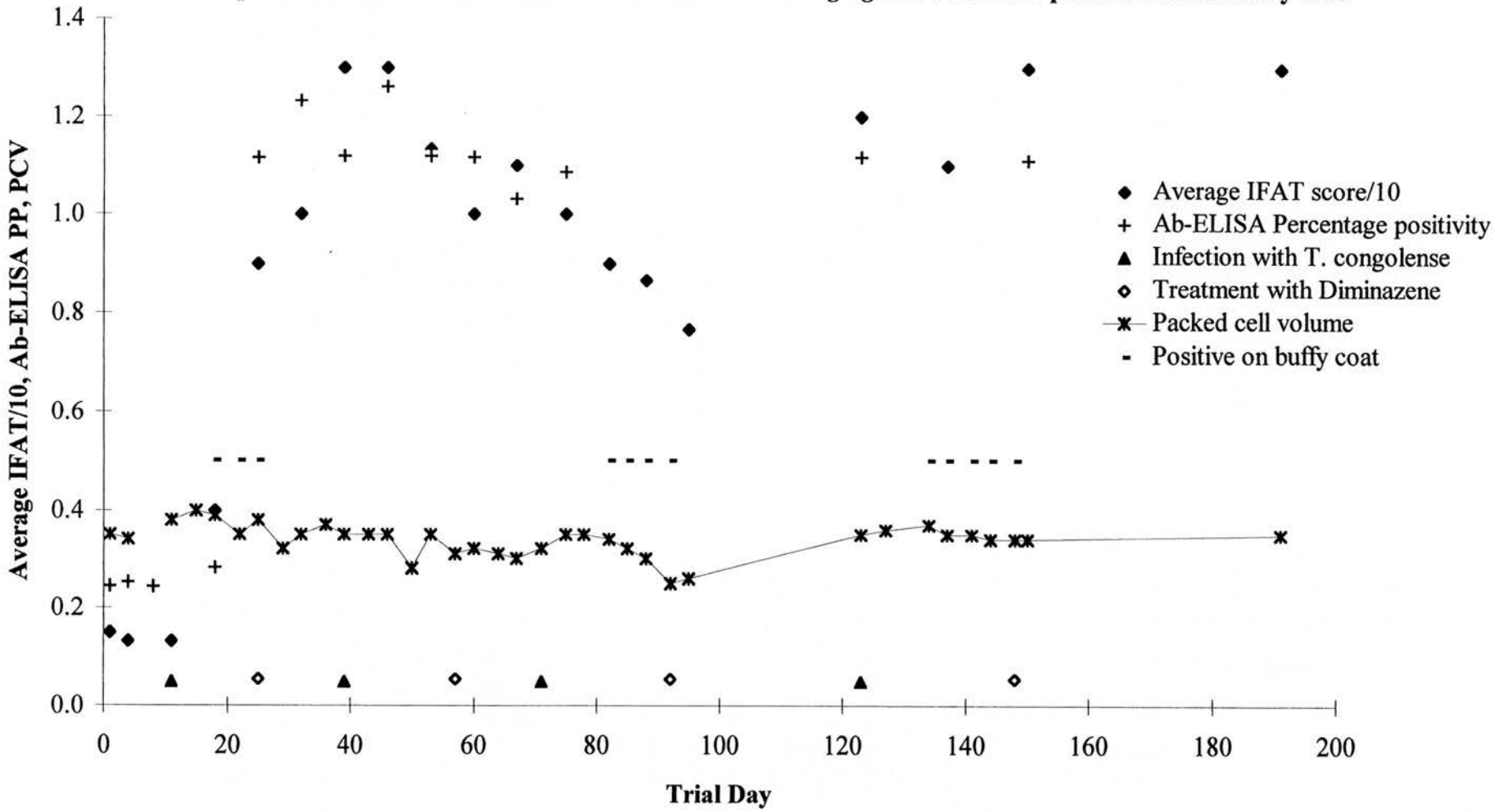
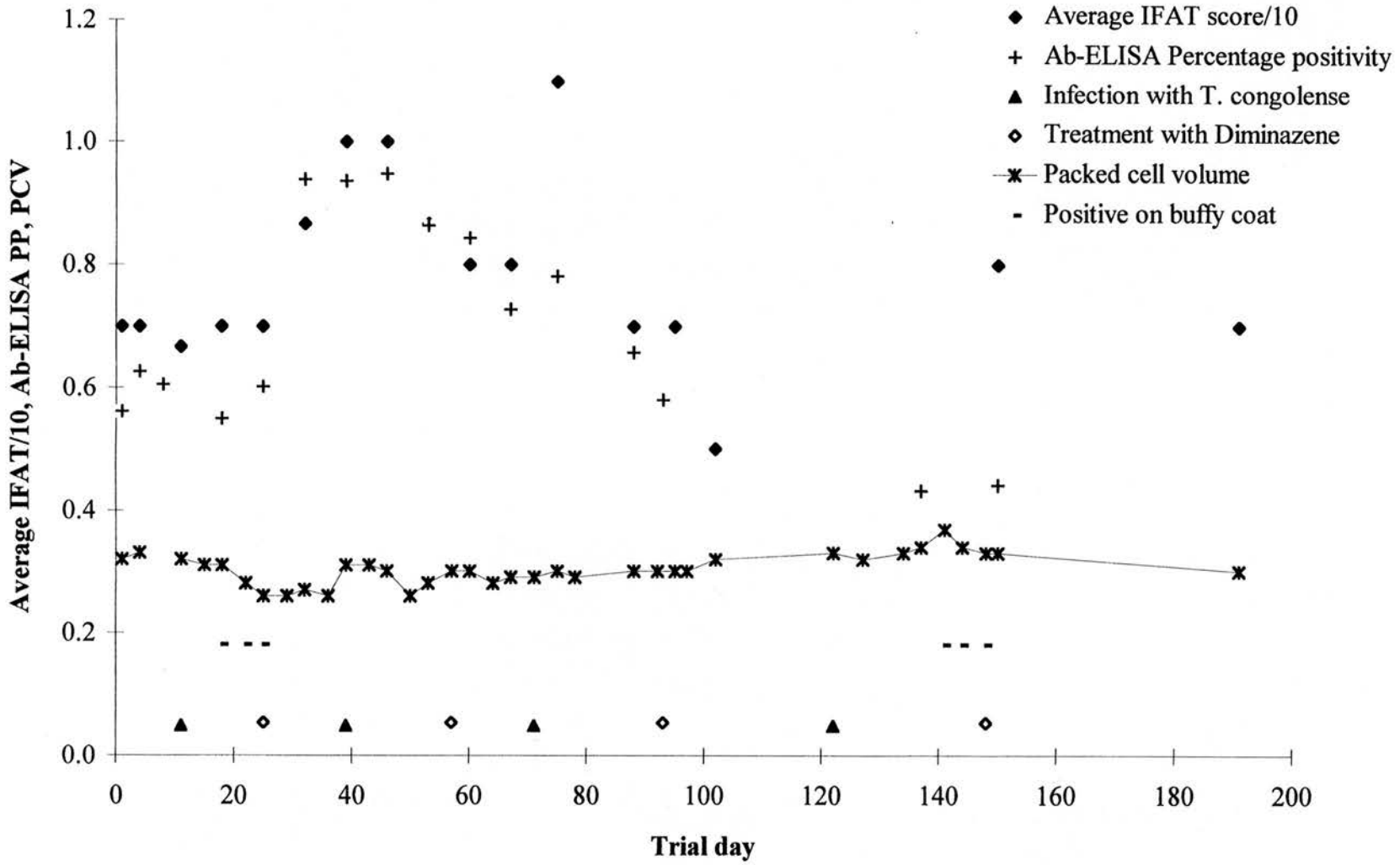


Table 4.28 Results from animal Tag Number : 807

0 in infection, treatment or Pos BC columns reflects animal infected, treated or positive on buffy coat. Shaded area, samples not take**IFAT Titre/score**

Date	*Trial day*	*Ave. IFAT Score*	*Ave IFAT/10*	*Serum Ab-ELISA PP*	*Inf.*	*Rx.*	*PCV*	*Pos BC*	*Buffy-Coat Score*	**Serum** *Titre*	*Score*	**Plasma** *Titre*	*Score*	**Filter paper** *Titre*	*Score*
4/03/96	1	7.0	0.7	0.561			0.32		0	+/-640	7			+/-640	7
7/03/96	4	7.0	0.7	0.627			0.33		0	+/-640	7	+/-640	7	+/-640	7
11/03/96	8			0.605											
14/03/96	11	6.7	0.7		0		0.32		0	+/-640	7	+/-640	7	320	6
18/03/96	15						0.31		0						
21/03/96	18	7.0	0.7	0.549			0.31	0	1	+/-640	7	+/-640	7	+/-640	7
25/03/96	22						0.28	0	4						
28/03/96	25	7.0	0.7	0.602		0	0.26	0	4	+/-640	7	+/-640	7	+/-640	7
1/04/96	29						0.26		0						
4/04/96	32	8.7	0.9	0.939			0.27		0	+/-1280	9	+/-1280	9	640	8
8/04/96	36						0.26		0						
11/04/96	39	10.0	1.0	0.937	0		0.31		0	+/-2560	10	+/-2560	10	+/-2560	10
15/04/96	43						0.31		0						
18/04/96	46	10.0	1.0	0.948			0.3		0	+/-2560	10	+/-2560	10	+/-2560	10
22/04/96	50						0.26		0						
25/04/96	53	8.7	0.9	0.864			0.28		0	+/-1280	9	+/-1280	9	640	8
29/04/96	57					0	0.3		0						
2/05/96	60	8.0	0.8	0.844			0.3		0	640	8	640	8	640	8
6/05/96	64						0.28		0						
9/05/96	67	8.0	0.8	0.728			0.29		0	640	8	640	8	640	8
13/05/96	71				0		0.29		0						
17/05/96	75	11.0	1.1	0.781			0.3		0	+/-1280	11	+/-1280	11	+/-1280	11
20/05/96	78						0.29		0						
30/05/96	88	7.0	0.7	0.658			0.3		0	+/-640	7	+/-640	7	+/-640	7
3/06/96	92						0.3		0						
6/06/96	95	7.0	0.7				0.3		0	+/-640	7	+/-640	7	+/-640	7
4/06/96	93			0.580		0									
8/06/96	97						0.3		0						
13/06/96	102	5.0	0.5				0.32		0	+/-320	5	+/-320	5	+/-320	5
3/07/96	122				0		0.33		0	+/-640					
8/07/96	127						0.32		0						
15/07/96	134						0.33		0						
18/07/96	137			0.433			0.34		0	640	8				
22/07/96	141						0.37	0	1						
25/07/96	144						0.34	0	1						
29/07/96	148					0	0.33	0	4						
31/07/96	150	8.0	0.8	0.442			0.33		0	640	8				
10/09/96	191	7.0	0.7				0.3		0	+/-640	7				

Figure 4.40 Plot of average IFAT score/10, Ab-ELISA PP and PCV over time for animal 807 showing experimental infection and treatment diminazene 7.0 mg/kg and detectable parasitaemia on buffy coat



4.4.2.4. Discussion

The most striking observation is the difference in the humoral activity and response of the two oxen, when measured by IFAT or Ab-ELISA, at the beginning and during the trial.

On Trial day 1, animal 808 had a PP ~ 25% and was negative by IFAT whilst animal 807 had a PP of ~ 60% and was positive on IFAT. After the initial infection, 808's antibody increases by five fold within 20 days whilst 807's less than doubles. Thereafter 808's antibody decline rapidly over the next 50 days and peak again after the third treatment. It is likely that the second inoculation with *T. congolense* did not develop. Antibody levels in 808 remain very high from day 120 to 200. The levels of antibody in 807 never achieved those of 808. Although parasitaemia was only detected over two periods, even on the second infection and treatment the level of antibody activity is only ~ 70% of that of 808 and declines rapidly when measured by the Ab-ELISA. In both animals the PCV falls after the parasitaemia.

The trial demonstrates some interesting results, which deserve further investigation. Antibody levels from 808 are comparable by IFAT and Ab-ELISA yet in 807 they shadow each other from trial day 1 to 100 but thereafter differ considerably. The rate of decline of antibody in both animals may be different, but because the trial ended on day 200, this observation is tentative. The difference in levels of antibody at the beginning of the trial are also not easy to explain. One hypothesis is that animal 807 was harbouring some cryptic infection (possibly *T. brucei* which is likely not have been detected using normal parasitological techniques) which accounted for the high level of antibody and yet poor humoral response because of the trypanosomiasis induced immuno-suppression. Another hypothesis is that individual cattle have markedly differing humoral responses to trypanosomiasis, both in the rate and amount of antibody they produce, and in the rate of decline of antibody.

In order to investigate these observations more thoroughly, a larger, controlled trial needs to be performed. The trial should be conducted using a large group of previously uninfected and infected animals which are free of trypanosomes. Not only should the cattle be infected and treated on one or more occasions, but chronic infections of the three species of trypanosomes should be allowed to develop in order to monitor levels of antibody. Arguably, in a chronically infected animal antibody may remain at some peak level, or decline as immuno-suppression develops. A final part of this crucial trial would be to study the rate of decline of antibody in a group of animals. These studies would be pivotal to being able to differentiate between active and recent infections.

4.5. The correlation between antibody ELISA results and haematocrit values for individual samples

4.5.1. Introduction

The correlation between PCV and Ab-ELISA on a herd basis is described in Chapter 6 below. Due to the relative insensitivity of direct parasitological diagnostic techniques for trypanosomiasis, the correlation between parasitological, serological and haematological results on an individual basis was investigated. Although mode PCV class for each herd had been recorded, this descriptive statistic had previously been demonstrated to be poorly correlated with parasitological results, and so was not investigated further.

4.5.2. Materials and methods

Parasitological and haematocrit results from ~ 18,000 cattle were entered into the Integrated Tsetse and Trypanosomiasis Database whilst the results from ~ 12,000 of this sample drawn from the population were analysed by the Ab-ELISA using antibody obtained from eluted blood spots. Results from individuals (by inspection site code, sample number and sampling date) were related via a relational database,

and the linked results for individual samples were exported for spreadsheet and statistical analyses.

4.5.3. Results

The correlation between haematocrit value and Ab-ELISA percentage positivity before and after a logit[15] transformation are shown in Table 4.29 and Figures 4.41 and 4.42.

Table 4.29 The Pearson correlation matrix between individuals PCV and Ab-ELISA percentage positivity values, before and after a logit transformation

	Packed cell volume (PCV)	Percentage positivity (PP)	Logit PCV	Logit PP
PCV	1.000			
PP	-0.419	1.000		
Logit PCV	0.995	-0.422	1.000	
Logit PP	-0.416	0.983	-0.418	1.000

Number of observations: 12,097

Logit (p) $= \text{Log}_e (p/1-p)$

4.5.4. Discussion

Both the Pearson correlation matrix shown above, and the graphs demonstrate that although there is a negative correlation between PCV and percentage positivity, the association is not strong. The logit transformation only fractionally improves the correlation. Thus, the conclusion is that the antibody ELISA cannot realistically be used for making individual diagnoses, based on the haematocrit value and percentage positivity of that individual.

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[15] Because the percentage positivity and haematocrit values are both proportions, a logit transformation of the date was investigated.

Figure 4.41

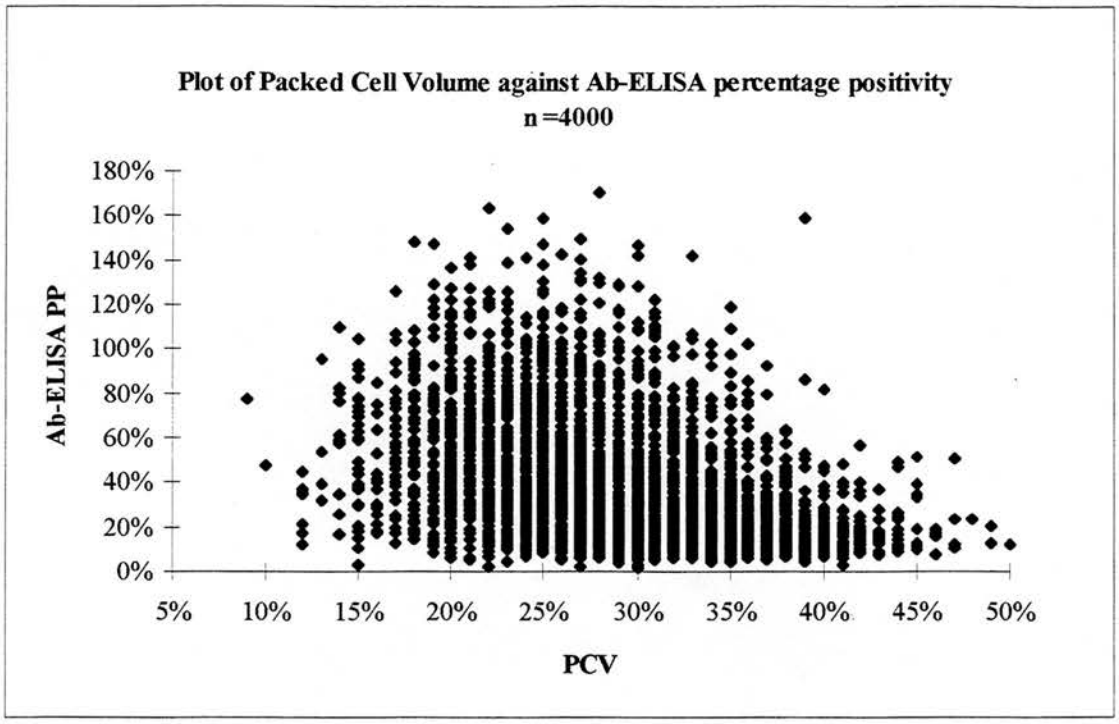
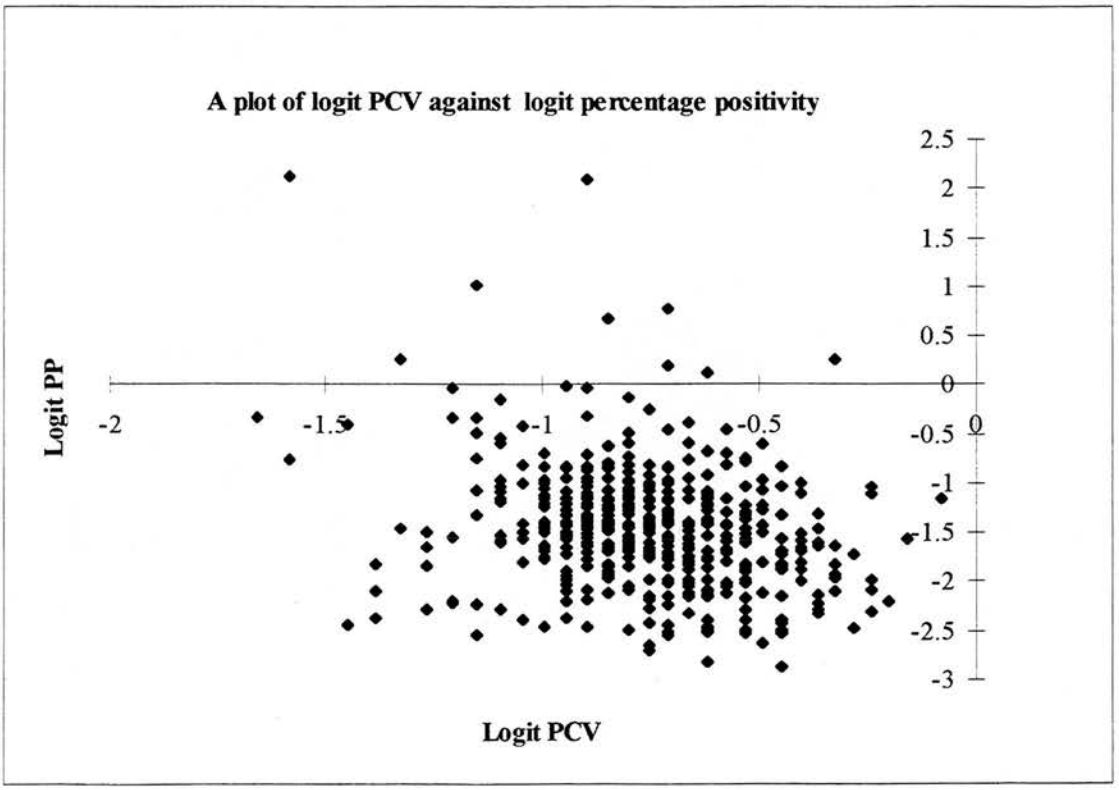


Figure 4.42



4.6. The costs of the Antibody ELISA for trypanosomiasis

4.6.1. Introduction

The ELISA assay is often quoted as being cheap and lending itself to the processing of many samples, and even of mechanisation. This latter method of dealing with many samples was deemed not be a viable, sustainable option in a developing country such as Zambia, where technical back-stopping is very difficult, and spare parts requiring foreign exchange are very difficult to acquire. As Veterinary Departments must operate within a predetermined budget, and the costs of the direct diagnostic techniques have been investigated (see Paragraph 3.4.2.3 of this thesis), the current objective was to investigate some of the costs of running the Ab-ELISA. These costs include sample collection in the field (using blood spots or sera) and running the samples in a laboratory. The transport and labour charges of a field team have not been included in this costing exercise as these are already being spent in order to acquire parasitological data. The exercise was an attempt to quantify, at least roughly, the costs of this laboratory technique under investigation. The overheaded costs of running a laboratory and the provision of services have not been included as these would exist in some Central Laboratory already. The cost of an inexpensive double distillation plant has been included however, as an ample source of suitable water is a pre-requisite for the assay to work. The analysis treats laboratory technician labour as a separate entity as this person may already be paid for by Government (in which case labour costs are not included) or may have to be employed specifically to run the assay and manage the data. The write off period of capital equipment is highly subjective. This equipment can also be used for other assays and laboratory procedures. Although freezers and refrigerators will probably already exist in a laboratory, our experiences showed that the ELISA procedure works best if dedicated equipment, including a personal computer, are made available for the routine running of the assay.

4.6.2. Materials and methods

Spreadsheets where prepared for the costs of processing 1000 samples using the Ab-ELISA, as well as the collection of 1000 serum samples or blood spots. All prices were converted to the European Unit of Currency (ECU) at rates prevailing in October 1996.[16] The costs of buffers and consumables are taken from a procurement contract to the RTTCP dated May 1996. 1994 prices were used for the plate washer, plate reader and refrigerators. The price of antigen was taken from a contract prepared in September 1996 between UNZA and RTTCP Regional Headquarters for 15 mls. of *T. congolense* soluble antigen. The value of the contract was 2000 ECU and the optimum working dilution of the antigen for coating was assessed to be 1:1000. The calculations were based on 25 plates being run each week - which is the normal number that a proficient technician can run. No allowance has been made for rejected plates.

4.6.3. Results

The costs of serum and blood spot sample collection; of consumables, buffers, minor laboratory equipment, capital equipment depreciation and labour; and the costs of antigen and conjugate are given in Appendix 4. The costs per sample with/without labour, sample collection and capital equipment depreciation are summarised below in Table 4.30.

[16] 1 ECU = £0.83p

Table 4.30 Summary costs of antibody ELISA

Costs in body of table are the ECU cost per sample run (based on running 1000 samples per week)

	Serum	Blood spot
Sample collection	0.600	0.026
	Technician labour charges	
	Included	**Not included**
Consumables only - no capital depreciation		
Laboratory costs (sample collection not included)	0.341	0.191
Serum as antibody source	0.941	0.791
Eluted blood spot as antibody source	0.367	0.217
All up cost including consumables and capital depreciation		
Laboratory costs (sample collection not included)	0.413	0.263
Serum as antibody source	1.014	0.864
Eluted blood spot as antibody source	0.440	0.290

The breakdown of the costs shown above, including labour costs is given in Table 4.31 below.

Table 4.31 The breakdown of laboratory costs for trypanosomiasis diagnosis by Ab-ELISA including technician fees

Figures in body of table are ECU

Source of antibody	**Serum**		**Blood spot**	
Item	*Cost*	*%*	*Cost*	*%*
Costs of sample collection - consumables + equipment depreciation	0.600	59%	0.026	6%
ELISA consumables and buffers	0.191	19%	0.191	43%
Laboratory equipment capital depreciation	0.072	7%	0.072	16%
Technician labour costs	0.150	15%	0.150	34%
Total	1.01	100%	0.44	100%

4.6.4. Discussion

The cost of the assay ranges from 0.263 to 1.014 ECU per sample depending upon whether antibody from serum or eluted blood spot is used, or whether labour fees are included in the calculations. Vacutainer® tubes and needles as well as cryogenic vials are expensive consumables, and these costs alone accounted for over 0.5 ECU per sample collected and 59% of the running costs. The model assumes there to be no wastage of these consumables, a rare event under field conditions. The cost of collecting blood dried onto filter paper was less that 0.03 ECU per sample (6% of the total cost), clearly much less expensive than collecting serum. The other major cost of running the assay is the salary paid to the technician running the assay and the helper who will help make elutions, wash glassware and perhaps assist with managing data. The salary of the ELISA technician is difficult to quantify, as it would depend on the opportunity costs of that person, whether they were already receiving a salary from Government, and the "going rate" for persons suitably qualified to run the assay. If the running of an antibody ELISA was being conducted by a Government funded Central or Provincial laboratory, then this cost need not be included. If however a serological survey was being conducted as some specific exercise being sponsored by some aid agency (such as RTTCP funded surveys in Zambia 1997) then this fee must be charged.

4.7. Chapter Discussion

The antibody ELISA for detecting anti-trypanosomal IgG globulins using sera and eluted bloods which had been held dried on filter paper, and using crude soluble *T. congolense* and *T. brucei* antigen was fully investigated. These investigations included development of the assay to maximise discrimination between known positives and negative bovine populations, the effects of water purity for making the coating buffer, inter and intra assay error structures and the quality assurance methodologies for use in the routine use of the assay. The repeatability of the assay as well as choice of cut-off to discriminate between positive and negative samples were also investigated, as were the effects of sample management. The assay was compared to another antibody detection test (IFAT) and observations were made on the kinetics of the antibody response. Finally, some estimate was made of the costs of the assay when being run routinely.

The assay demonstrated the qualities of having potential for use as a routine sero-epidemiological diagnostic technique for determining sero-prevalence, from which can be deduced presence or absence of the tsetse fly vector and if the sero-prevalence is greater than zero - the endemicity of the disease. Knowledge of the endemicity is a pre-requisite for developing some control strategy which may be by the use of vector control or trypanocidal drugs (based on the assumption that trypanotolerant cattle would not be acceptable in Southern Africa). Knowing the poor sensitivity of the direct diagnostic techniques, and of the inability to provide some form of quality control assurance for these direct diagnostic techniques (whether they be performed at the site of the inspection site or in some laboratory equipped with micro-haematocrit centrifuges and microscopes configured for x 20 darkfield examination of the buffy coat) also means the Ab-ELISA is potentially a very useful sero-diagnostic tool. As has been maintained throughout this thesis, for strategic planning purposes, individual

diagnosis and knowledge of species of trypanosome is not necessary[17]. The use of computerised software quality control assurance checks, the ability for one technician to assay ~ 1000 samples a week, and the inherent way the software manages the results in some geo-referenced data management system also demonstrated the usefulness of this sero-epidemiological technique.

At the chosen cut-off, the sensitivity and specificity of the assay are superior to many other diagnostic techniques for AAT. A binding ratio of ~ 19 when using new batches of *T. congolense* soluble invariant antigen and the new control sera proves the assay has the potential to discriminate better between positive and negative populations (for the investigations into the development of the assay, another batch of *T. congolense* antigen was used and the strong positive sera was a pool of sera from the field). If the assay had been investigated further using these batches of antigen and control sera, and the purest source of water (Milli-Q) the results may have been improved upon. Likewise, if positive pressure pipettes had been available for taking the 10 µl sera and placing in the 4 mls. buffer, and if new pipette tips had been used with the multi channel pipettes, then is probable that many of the causes of variation may have been eliminated.

The assay has presently one serious flaw, and this is its poor repeatability when used to measure antibody activity in test sera. This observation was borne out in the trial to investigate the repeatability of the assay, and more so from the trial which assessed the effect of blood spot storage conditions. The test samples appeared to behave differently from the control sera, which the latter having demonstrated a near perfect Gaussian distribution, which one would expect when measuring some biological activity on some continuous scale. One cannot argue that eluted blood spot activity had poorer repeatability than diluted serum activity, as both sources of antibody were

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[17] The only occasions when knowledge of the species of trypanosome may be of use would be when a high proportion of *T. vivax* was occurring and mechanical transmission was a suspect source of the aetiology of the disease, or if there was a high prevalence of *T. brucei* near a human sleeping sickness focus and cattle were a suspect reservoir of the parasite.

used for the two trials. As such, the assay requires considerable further investigations into sample repeatability, as the percentage positivity obviously affects whether the result from the test sample is deemed positive or negative.

A practical problem with the assay is the availability of the biological reagents required to run the test. Whilst nearly all buffers and reagents can be purchased from several laboratory suppliers, antigen and control sera have to be made to order. Having established the assay and determined the cut-off (using samples from animals of known provenance) the batch of positive control sera and antigen could not be altered. Having invested the resources to make the strong positive sera, several litres of serum could be harvested - which would be sufficient for use as an international reference standard throughout Africa for the next decade. The production of soluble invariant antigen is far from simple and requires considerable investment of time, money and laboratory rodents. As such, it was too valuable to be sent to other laboratories for their use.

Whilst *T. congolense* was compared to *T. brucei* as a source of antigen and found to give better discrimination between positives and negatives, it was not possible to produce identical antigen. Mixtures of *T. congolense*/*T. brucei* antigen were not assessed, and *T. vivax* antigen was never produced, owing to the inability to grow Southern African field strains in laboratory animals, and the practical difficulties of artificially growing this species of trypanosome in small ruminants. In order to investigate the assay further, ideally antigen made using recombinant techniques should be assessed, for single and multiple trypanosome species. The need for further investigations into the humoral response and rate of decline of antibody in a large, controlled, group of cattle has been discussed.

Several other trials were designed, but lack of time meant they were not conducted. One trial was to assess the effect of repeated freezing and thawing on antibody activity. Another trial was to study the effect of degradation of antibody activity when serum was left at (African) ambient temperatures. Ten sera were to have been

left at room temperature, then on each day one would be placed in the freezer. On day 11, all sera would have been run on the same plate to study the effect of storage temperature (when microbial growth would presumably occurred).

The quality control software and integral spatial data management system used to develop the assay is probably superior to other data management systems currently in use, which have no spatial component, nor are designed as a relational database in order to link the results from individual samples and herds to results using other diagnostic techniques or even diagnostic techniques for other diseases. The Antibody ELISA database written specifically for the task of developing the assay (and assessing its usefulness as a routine sero-epidemiological technique) could probably be refined and re-written in the Microsoft Visual Basic® language vs. the Microsoft Fox Pro for Windows® application.

If carrying out a purposive serological survey, then the sera could be used for other disease diagnoses, such as the tick borne diseases due to *Anaplasma* and *Babesia* species. This epidemiological knowledge may be necessary in order to ascertain the degree of enzoötic stability or instability of these other diseases, because this knowledge would affect dipping strategy, as the use of pyrethroid dips is one method for controlling the tsetse fly vector.

One final way in which the results from the assay could be improved upon is the issue of choice of cut-off. If the assay had a very high degree of repeatability, then using some finite cut-off to determine whether an animal was positive or negative would be acceptable, and thus could be used to determine a herd's sero-prevalence and calculated true prevalence. This approach used in this chapter was driven pragmatically because the assay was known to have poor repeatability, and the large scale trial to investigate the humoral responses of cattle with single or multiple parasitaemias, and chronically infected animals could not be investigated due to lack of time and money. With an assay giving improved repeatability and results from the trial to study humoral responses, one could possibly use several, more refined techniques for investigating the assay's usefulness as a sero-epidemiological tool.

Instead of using a finite cut-off percentage positivity, two "cut-off" points could be used: a lower cut-off below which one would know with a pre-determined degree of confidence that the sample was from a negative animal, and a higher cut-off above which one would know with a pre-determined degree of confidence that the sample was from a parasitologically positive animal. Even though an animal may not still have a parasitaemia, one would know that it had been infected within a certain period of time. In between these two cut-offs, one would regard the result as falling into a "grey area", and thus rather than re-testing the sample (given that if the assay was highly repeatable the second PP would be the same as the first) one would disregard these values from these animals in determining sero-prevalence.

Another method that could be used to investigate the assay would be to fit the percentage frequency distribution[18] of the herd under examination to some hypothetical distribution. The results given in this chapter have clearly demonstrated that the PPs of a negative population (0% prevalence) have a log normal distribution, whilst those of the parasitological positives (100% prevalence) have a Weibull distribution. Thus, for any hypothetical sample (which would need to have some

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[18] A herd's cumulative frequency distribution could also be used.

minimum size with knowledge of the population size from which it was drawn) ranging from 0 - 100% sero-prevalence, some fitted frequency distribution could be calculated once the mean and standard deviation for the negative and positive populations had been determined. Having run the assay and obtained results from the sample taken from the herd, then the sample frequency distribution would be compared to the range of hypothetical frequency distributions, and by means of a Chi^2 test or similar technique, the best approximate of hypothetical sero-prevalence could be fitted. The usefulness of this technique would then need further investigation.

Chapter 5

5. THE USE OF THE ENZYME-LINKED IMMUNOSORBENT ASSAY FOR THE DETECTION OF TRYPANOSOMAL ANTIBODIES IN EPIDEMIOLOGICAL STUDIES IN CATTLE

5.1. Investigations in areas of known medium and high tsetse challenge, and an area recently subjected to tsetse control activities (Lusitu)

5.1.1. Introduction

Whilst the assay was in its early stages of development, investigations were conducted using serum and blood spot samples from areas of known high (Eastern Province) and medium tsetse challenge (Chongwe-Chinyunyu 100 km to the east of Lusaka) as well as a tsetse eradicated area in Lusitu[1]. Approximately sixty animals per herd were sampled from seven herds, but the size of the population could not be determined and thus no confidence limits to the sero-prevalence could be attached. Both whole blood (for serum) and blood dried on filter paper were collected, and haematocrit values were measured as well as buffy coat smears examined.

[1] Lusitu control area lies some 100 Km south of Lusaka, and is an area bounded by the Zambezi river and the Zambezi escarpment. The area was considered tsetse free as trypanosomiasis had last been diagnosed some two years previously. On the opposite side of the Zambezi lies the Rifa triangle, one of the first areas in Zimbabwe where odour baited targets had been successfully employed to locally eradicate tsetse.

5.1.2. Materials and methods

Serum and blood spot samples were assayed for antibody at the University of Zambia and sera were sent for antigen ELISA[2] testing at the Central Veterinary Research Institute, Lusaka. Blood spots were sent for IFA testing[3] by the Provincial Laboratory in the Eastern Province of Zambia. Regrettably, other than the Ab-ELISA data there were no other indirect diagnostic test results for comparison[4]. For experimental purposes only, the sensitivity and specificity of the Ab-ELISA had been determined using sera as a source of antibody and *T. congolense* as the source of soluble crude somatic antigen[5]. The effect of cut-off on sensitivity and specificity of the assay as well as the power of the tests when using sera are presented in table 5.1 below.

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[2] Using kits distributed by IAEA

[3] Using the IFAT protocol described in Appendix 6

[4] This was part of a collaborative trial to compare the results from antigen ELISA, antibody ELISA, IFAT and parasitology.

[5] The strong positive control sera used was the same pooled sera as described in Chapter 4.

Table 5.1 The effect of cut-off value on sensitivity and specificity using serum as the source of antibody (number negative control = 196, number of parasitological positive = 365)

Cut off	Sensitivity	Specificity	Se x Sp	(Se + Sp)/2
16%	94.2%	77.6%	73.1%	85.9%
17%	92.9%	83.2%	77.2%	88.0%
18%	92.9%	84.7%	78.7%	88.8%
19%	91.8%	89.8%	82.4%	90.8%
20%	91.0%	92.3%	84.0%	91.7%
21%	89.6%	93.4%	83.6%	91.5%
22%	89.0%	94.9%	84.5%	92.0%
23%	87.9%	95.9%	84.4%	91.9%
24%	87.4%	95.9%	83.8%	91.7%
25%	85.8%	96.9%	83.1%	91.3%
26%	84.4%	96.9%	81.8%	90.7%
27%	83.0%	97.4%	80.9%	90.2%
28%	82.2%	98.0%	80.5%	90.1%
29%	81.4%	98.0%	79.7%	89.7%
30%	80.5%	98.5%	79.3%	89.5%

A cut-off value of 22% positivity compared to the strong positive control sera (highlighted row) was selected for the investigations as this has the highest power. Samples were collected in early 1994 and the assay was performed in mid 1996.

5.1.3. Results

The results of the seven herds under investigation as well as those of the negative control population are presented in Table 5.2. For both sources of antibody (with their concomitant sensitivity and specificity), results for each herd for each class of tsetse challenge are expressed as number sampled, number positive (i.e. number with PP > cut-off) and hence apparent prevalence from which the true prevalence is calculated [6].

The frequency distribution of the percentage positivity from eluted blood spots and sera of the sample drawn from the negative population and three herds living in Lusitu (tsetse eradicated area) are shown in Figures 5.1 to 5.3.

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[6] True prevalence = $\frac{\text{Apparent prevalence + specificity -1}}{\text{Se + Sp -1}}$

Table 5.2 The results from seven herds of known challenge and the negative controls using sera or blood spots as source of antibody

Cut-off	*Serum*	*Eluted blood spot*	
22.0%	89.0%	94.0%	*Sensitivity*
24.0%	94.9%	99.5%	*Specificity*

		Serum as source of antibody				***Eluted blood spot as source of antibody***			
Herd Code	**Tsetse Challenge**	**No. sampled**	**No. pos.**	**App. prev**	**True Prev.**	**No. sampled**	**No. pos.**	**App. prev**	**True Prev.**
GLU_XX	2 years after local eradication	69	0	0%	-6%	61	0	0.0%	-0.5%
GLU_F	2 years after local eradication	58	2	3%	-2%	61	1	1.6%	1.2%
GLU_M	2 years after local eradication	58	0	0%	-6%	60	3	5.0%	4.8%
LE_MGO	Medium	54	35	65%	71%	59	41	69.5%	73.8%
EP_ES	High	63	62	98%	111%	56	55	98.2%	104.5%
EP_EW	High	64	57	89%	100%	63	58	92.1%	97.9%
EP_EP	High	65	61	94%	106%	65	61	93.8%	99.8%
Negative controls	None	195	9	5%	-1%	442	2	0.5%	-0.1%

201

Figure 5.1 The frequency distributions of percentage positivity from eluted blood spots from three herds living in a tsetse eradicated area and from all the negative controls

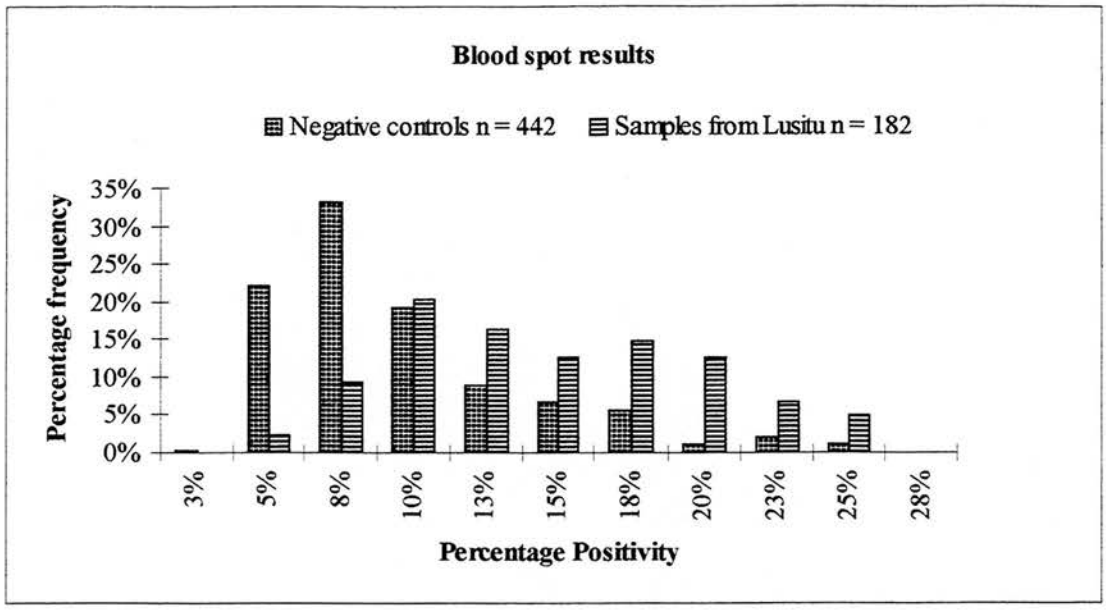


Figure 5.2 The frequency distributions of percentage positivity from eluted blood spots from three herds living in a tsetse eradicated area and from the contemporary negative controls

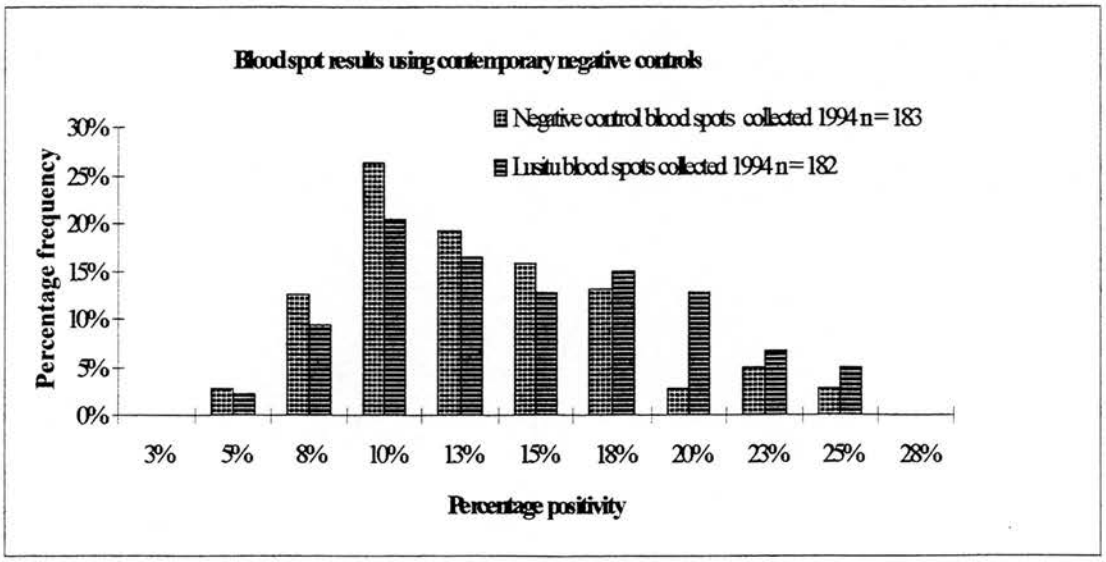
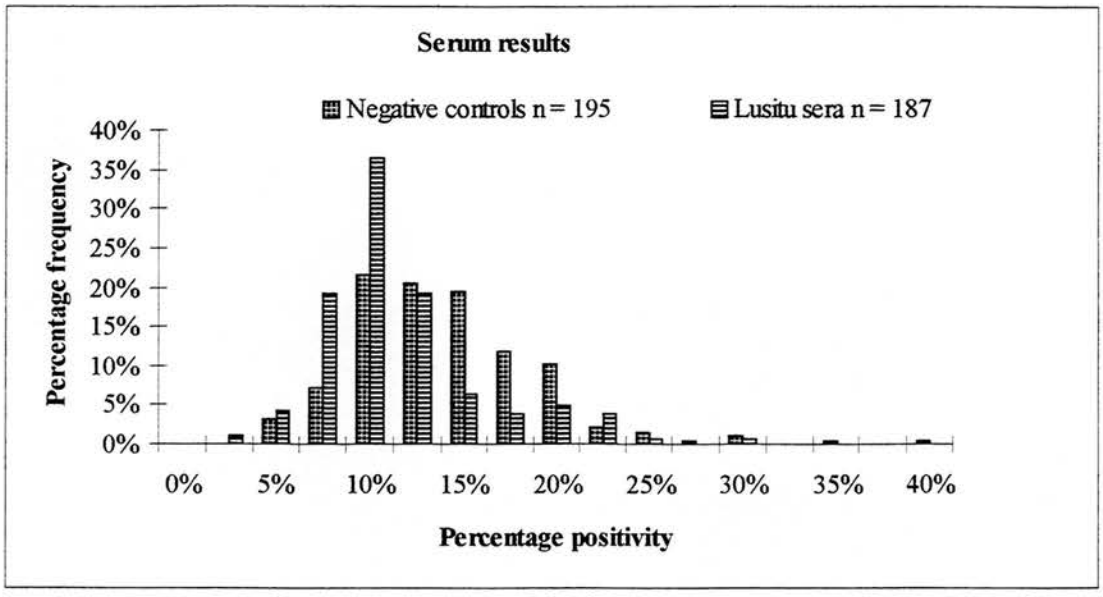


Figure 5.3 The frequency distributions of percentage positivity from sera from three herds living in a tsetse eradicated area and from the negative controls



Students T tests assuming unequal sample variances were performed to examine the difference in the results from sera and eluted blood spots from the all the negative controls and three herds from Lusitu. An F test was conducted on blood spot results from the 182 Lusitu animals as well as 183 negative control blood spots collected at about the same time. These results are given in Appendix 5 Tables A5.7 and A5.8.

5.1.4. Discussion

The results show that for many of the herds the calculated true prevalence is less than zero or greater than 100%. (antibody from serum true prevalence range - 6% to 111% and from eluted blood spots -0.5% to 104.5%). A calculated true prevalence greater than unity or less than zero may result if the incorrect sensitivity or specificity have been deduced. This may arise if i) the point estimate of these parameters was imprecise/poor which may occur if too small a sample size were taken or ii) if the sensitivity and specificity as calculated were based on a test population different to the population being sampled or iii) if the test (Ab-ELISA) is giving spurious percentage positivity results. In the case of blood spots, it has been

demonstrated in the preceding chapter that storage time and conditions may cause an increase in percentage positivity.

The sample sizes used for the positive and negative controls were considered respectable (200 - 450) so hypothesis (i) is less likely. Likewise there was no reason to suspect that the test population (used to establish the sensitivity and specificity of the assay) were any different from the population being sampled. The negative controls were collected from commercially reared cattle managed under tropical conditions and so genotype and some aspects of management could have been argued as the possible cause of difference. The very low percentage positivity results from the additional 260 locally bred cattle sampled in 1996 vs. 1994 may have accounted for an imprecise estimate of specificity.

As has been argued above, when determining the sensitivity of the assay, the parasitologically positive animals were a sub-set of the truly trypanosomiasis infected cattle (because they were only those with a demonstrable parasitaemia by buffy coat). The sensitivity of the assay should possibly been assessed on some better "gold standard" test which could include artificially infected cattle, those proven positive with PCR or antigen ELISA (once these tests in turn had been properly validated), rodent inoculation for possible *T. brucei* infected cattle, or so-called Berenil flushing.

The difference in the percentage positivities between the Lusitu control area and the negative controls is not easy to explain. For the Ab-ELISA results using sera as the source of antibody, there is significantly less ($P < 0.001$) antibody present in the Lusitu samples than in the negative controls. At a cut-off of 22% only two animals were positive (PP values of 23.8% and 28.2% from herd GLU_F). Using antibody from eluted blood spots, there was significantly more ($P < 0.001$) antibody activity in the Lusitu samples than from all the negative controls. This may have been as a result of all the negative controls comprising blood spots made and assayed a month apart ($n = 260$) and yet the Lusitu blood spots were ~ 26 months old. The large

number of "fresh" negative controls would obviously have affected the distribution of the negatives, as well as the mean of the negative blood spots and also the specificity of the assay using eluted blood spots as a source of antibody. A second students T test assuming samples of equal variance (after an F test had been performed) was performed to compare Lusitu eluted blood spots with negative controls collected at the same time. This T test showed there was significant difference in antibody between the contemporary negative and Lusitu results ($P < 0.001$), with there being higher antibody in the Lusitu sample. This is at variance with results using sera as the source of antibody! It could thus be argued that marginally higher levels of antibody may persist in a population were trypanosomiasis has occurred for several years. This could be further investigated by repeated serological and parasitological surveys (or surveillance) in an area where a successful campaign of tsetse eradication had been achieved[7].

[7] As of July 1997, the Island of Zanzibar would be an ideal case study area where *G. austeni* has probably been eradicated by the Sterile Insect Technique (Feldman, personal communication).

5.2. *Mapping of sero-prevalence survey results*

5.2.1. Materials and methods

During the course of the trypanosomiasis general survey in Zambia (Jan. 1995 to December 1996) blood spots were collected from each some 12,000 cattle by emptying the contents of a heparinised capillary tube onto Whatman's No. 4 filter paper. The spots were air dried out of the sun, placed in a bag with self indicating silica gel desiccant, and kept cool until placed in a - 20 °C freezer. The blood spots were eluted in PBST by the usual manner and antibody activity was assessed as a function of percentage positivity compared to some pooled strong positive control sera collected from the field. The ELISA plates were coated with soluble crude somatic *T. congolense* antigen diluted 1:1000.

After running the assay and deduction of the substrate blanking value, plates underwent strict quality control checks using a specially designed database (Antibody ELISA Database - AED) programme written for the occasion in Fox Pro for Windows ®[8]. Results were identified by inspection site code, sampling date and sample number and were expressed as a percentage positivity. If the difference in absorbance between the two wells (expressed as a CV) was too great, the sample was rejected and eliminated from further data management. By specifying the cut-off, sensitivity and specificity the results were summarised by inspection site and date; with numbers sampled, apparent prevalence and calculated true prevalence being output as a database file. The mean herd percentage positivity was also calculated. This could be linked via a relational database to the Integrated Tsetse and Trypanosomiasis Database which managed the parasitological and haematocrit data. As the AED was a spatial database, results were also output as IDRISI point vector files as described in Chapter 3. Classes for apparent prevalence class, calculated true prevalence class and mean percentage positive class are shown below in Table 5.3.

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[8] The Antibody ELISA Database - specifications written by author and coding written by Z. Chitalu of Mt. Makulu, Zambia.

Table 5.3 The class values used for the GIS output.

Apparent or true Prevalence (%)	Apparent, true Prevalence Class or average of percentage positive class
Background	0
<= 0	1
>0 - 0.100	2
0.101 - 0.200	3
0.201 - 0.300	4
0.301 - 0.400	5
0.401 - 0.500	6
0.501 - 0.600	7
0.601 - 0.700	8
0.701 - 0.800	9
0.801 - 0.900	10
0.901 - 1.000	11

5.2.2. Results

The spatial results (i.e. map) of the sero-epidemiological survey as apparent prevalence is given in Map 5.1. whilst Map 5.2 shows the calculated true prevalence. The none spatial results, linked to the parasitological data are presented in Chapter 6.

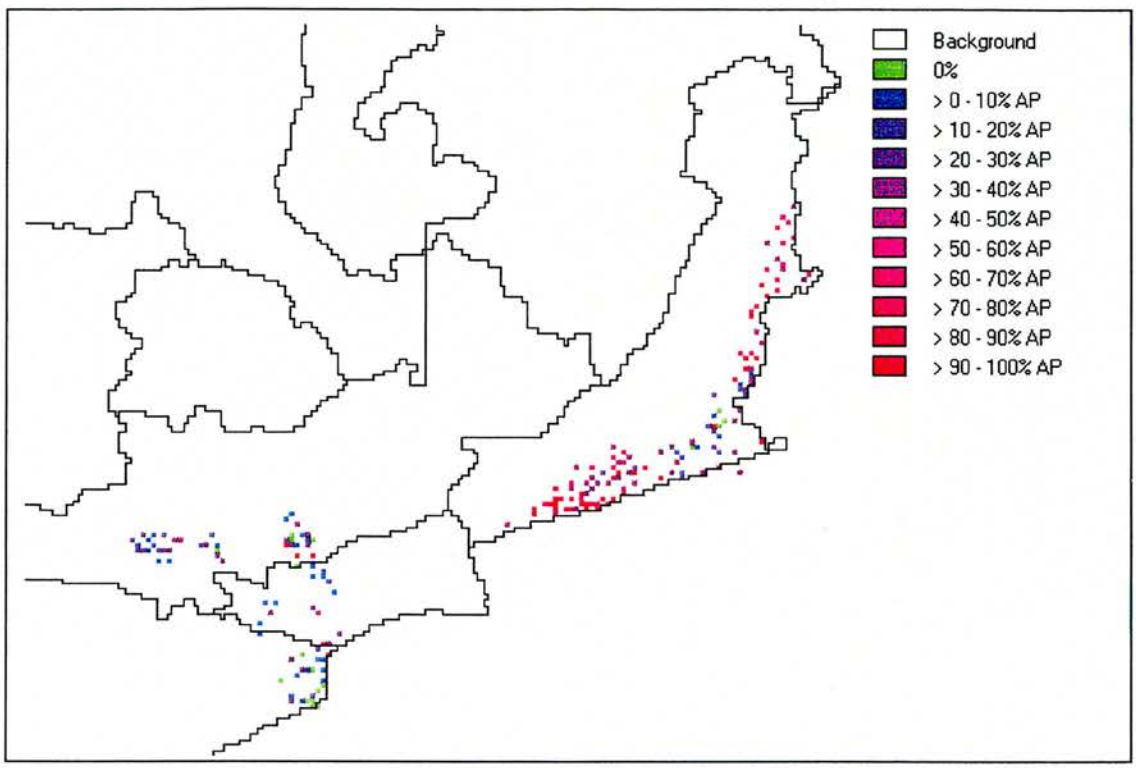
5.2.3. Discussion

The maps clearly show how trypanosomiasis is widespread in the Eastern Province of Zambia, with some pixels near the provincial capital of Chipata (town not shown) being free of disease. These results are in keeping with the parasitological findings described in Chapter 3.5. The disease is also seen to be more widespread over the Common Fly Belt of Zambia, than portrayed by the maps showing the prevalence by direct parasitological means (Map 3.1).

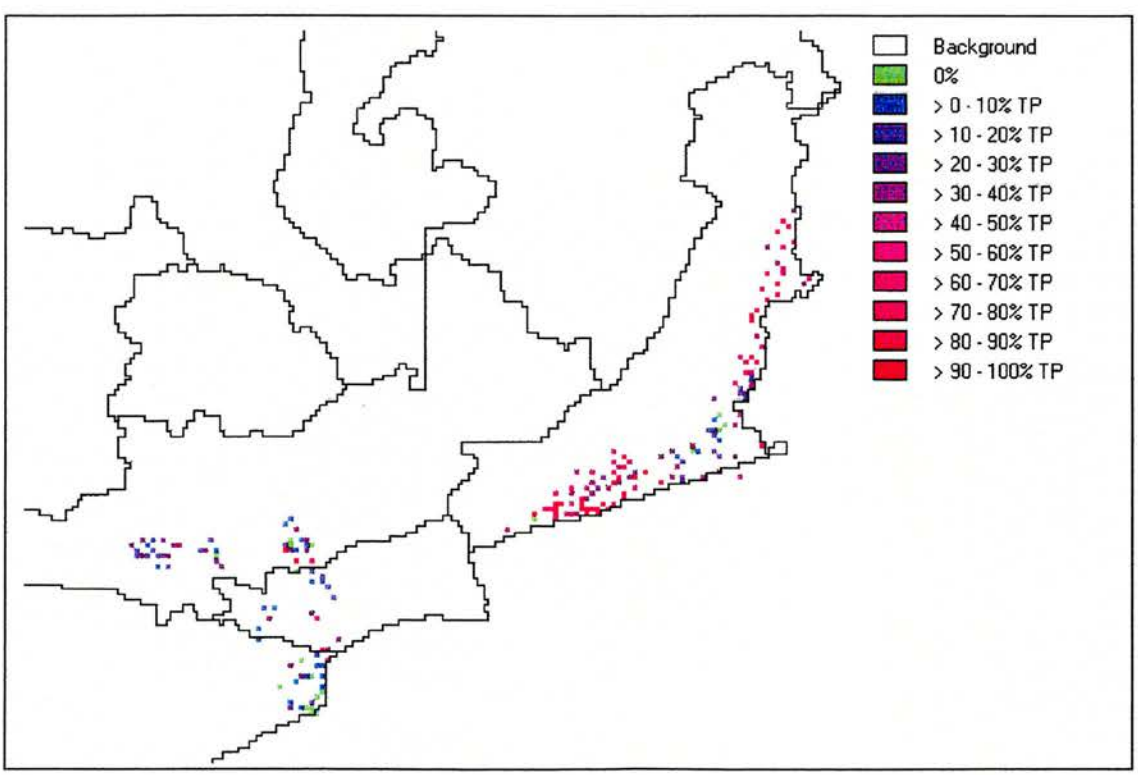
Because of the Ab-ELISAs high degree of sensitivity and specificity, there is little difference between the apparent and calculated true prevalence maps. The same discussions apply to sero-prevalence maps distribution maps as applied to parasitological prevalence maps, and why the need to portray the data at a provincial level may be more meaningful than for the Common Fly Belt of Zambia. The sero-prevalence GIS data set would be more meaningful than the parasitological survey results for determining the distribution of the disease, knowing the diagnostic technique's greater sensitivity.

If one was dealing with trypanosomiasis disease distribution data at the provincial level, and was interpolating between inspection sites (both these techniques are beyond the scope of this thesis), then using sero-prevalence as the data set with which to define some strategic control policy would clearly be advantageous compared to prevalence as determined by normal parasitological means.

Map 5.1 **The apparent sero prevalence of trypanosomiasis using eluted blood spots as the source of antibody, and *T. congolense* as the crude somatic antigen, cut-off 24% (n > 12,000)**



Map 5.2 **The calculated true sero prevalence of trypanosomiasis using eluted blood spots as the source of antibody, and *T. congolense* as the crude somatic antigen, cut-off 24%, sensitivity 94%, specificity 99.5% (n > 12,000)**



Chapter 6

6. THE CORRELATION BETWEEN RESULTS ACHIEVED PARASITOLOGICALLY AND HAEMATOLOGICALLY WITH THOSE FROM THE ANTIBODY TRAPPING ELISA FOR BOVINE TRYPANOSOMIASIS

6.1. Introduction

The correlation between PCV and Ab-ELISA on an individual basis is described in Chapter 4.5 above. Due to the relative insensitivity of direct parasitological diagnostic techniques for trypanosomiasis, the correlations between parasitological, serological and haematological results on a herd basis were investigated. Although mode PCV class for each herd had been recorded, this descriptive statistic had previously been demonstrated to be poorly correlated with parasitological results, and so was not investigated further.

If a strong correlation between serological and haematological results could be demonstrated, and if the antibody ELISA could be demonstrated to be a sensitive and specific, reproducible, etc. assay then the indirect measurements could be used in some regression to define the true prevalence of the disease.

6.2. Materials and methods

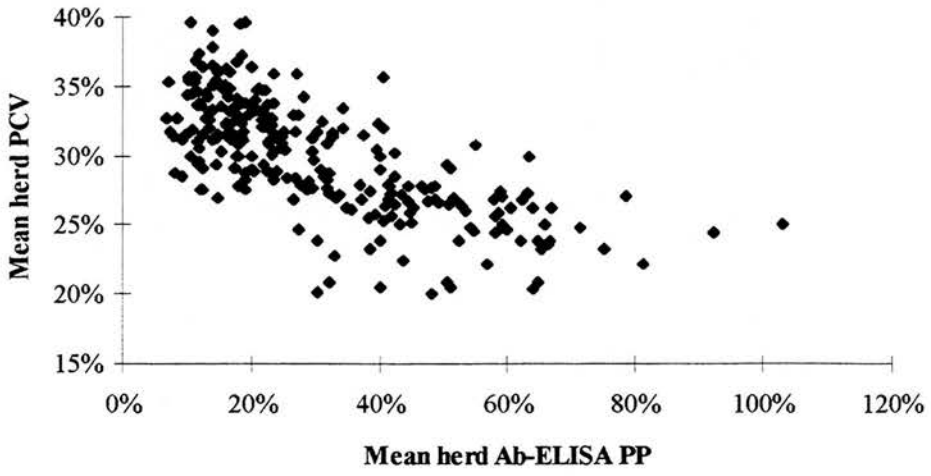
Parasitological and haematocrit results from ~ 18,000 cattle were entered into the Integrated Tsetse and Trypanosomiasis Database whilst the results from ~ 12,000 of this sample drawn from the population were analysed by the Ab-ELISA using antibody obtained from eluted blood spots. Results were summarised by examination site and date using both data management systems. Via a relational database, results were linked for individual samples and by herd summaries. The pertinent summary information on a herd basis for parasitological and haematological data was herd parasitological prevalence and herd mean PCV as well

as proportion of animals found to be anaemic[9]. Using values for cut off, sensitivity and specificity described in Chapter 4.3.6[10], sero-prevalence was summarised as apparent prevalence, calculated true prevalence and mean herd percentage positivity. Summary results from 258 herds were linked, with the average sample size from each herd being 53 samples.

6.3. Results

The results are expressed in Table 6.1 as a Pearson correlation matrix and in Figures 6.1, 6.2 and 6.3. These graphs portray the degree of correlation between mean herd packed cell volume and mean herd percentage positivity; apparent sero-prevalence by the Ab-ELISA and the trypanosomiasis parasitological prevalence and the association between mean herd PCV and herd Ab-ELISA apparent prevalence. Because the Ab-ELISA had undergone quality control checks and was of known sensitivity and specificity, this is plotted as the independent whilst parasitological prevalence is plotted as the dependant.

Figure 6.1 Plot of mean herd percentage positivity and mean herd Packed Cell Volume



[9] Anaemia was defined as any haematocrit value less than or equal to 25%.

[10] Cut-off 24%; sensitivity 94.0%; specificity 99.5%

Table 6.1 The Pearson correlation matrix for antibody ELISA mean herd percentage positivity, apparent and calculated true prevalence against herd trypanosomiasis parasitological prevalence, mean packed cell volume and proportion animals anaemic.

	Ab- ELISA apparent prevalence	Ab- ELISA calculated true prevalence	Mean herd Ab-ELISA percentage positivity	Trypanosomiasis parasitological prevalence	Herd mean PCV
Antibody ELISA apparent prevalence	1.000				
Antibody ELISA true prevalence	1.000	1.000			
Mean herd Ab-ELISA percentage positivity	0.899	0.899	1.000		
Trypanosomiasis parasitological prevalence	0.641	0.641	0.700	1.000	
Herd mean PCV	-0.723	-0.722	-0.709	-0.625	1.000
Proportion of animals anaemic in herd	0.721	0.721	0.737	0.719	-0.909

N.B. This is a smaller data set than used in Chapter 3 as only herds for which there were serological and parasitological data could be used Correlation coefficients between the same variables as given above and in Table 3.6 may be different.

Figure 6.2 Plot of parasitological prevalence and Ab-ELISA apparent sero prevalence (cut-off value 24%) n = 258

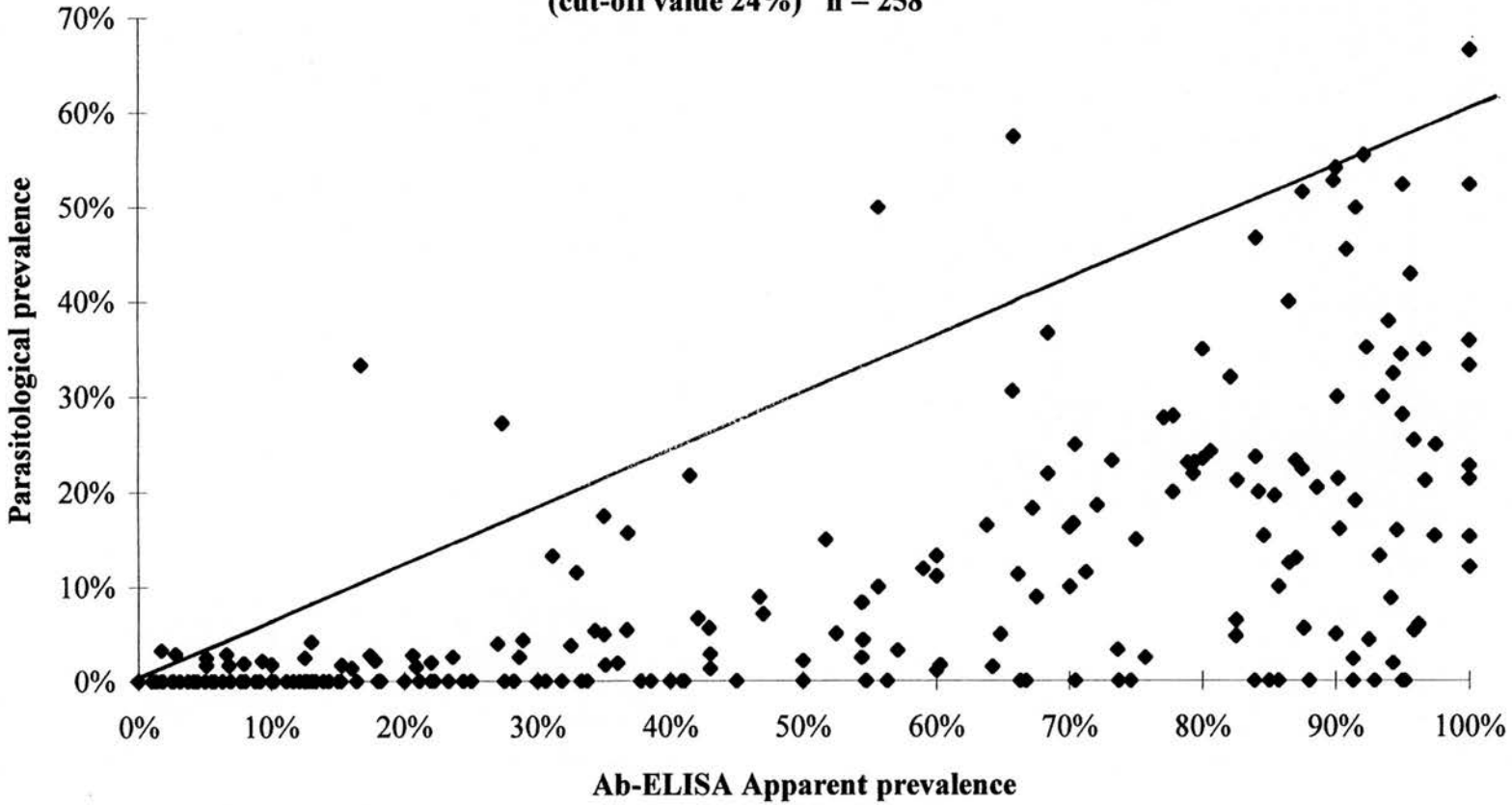
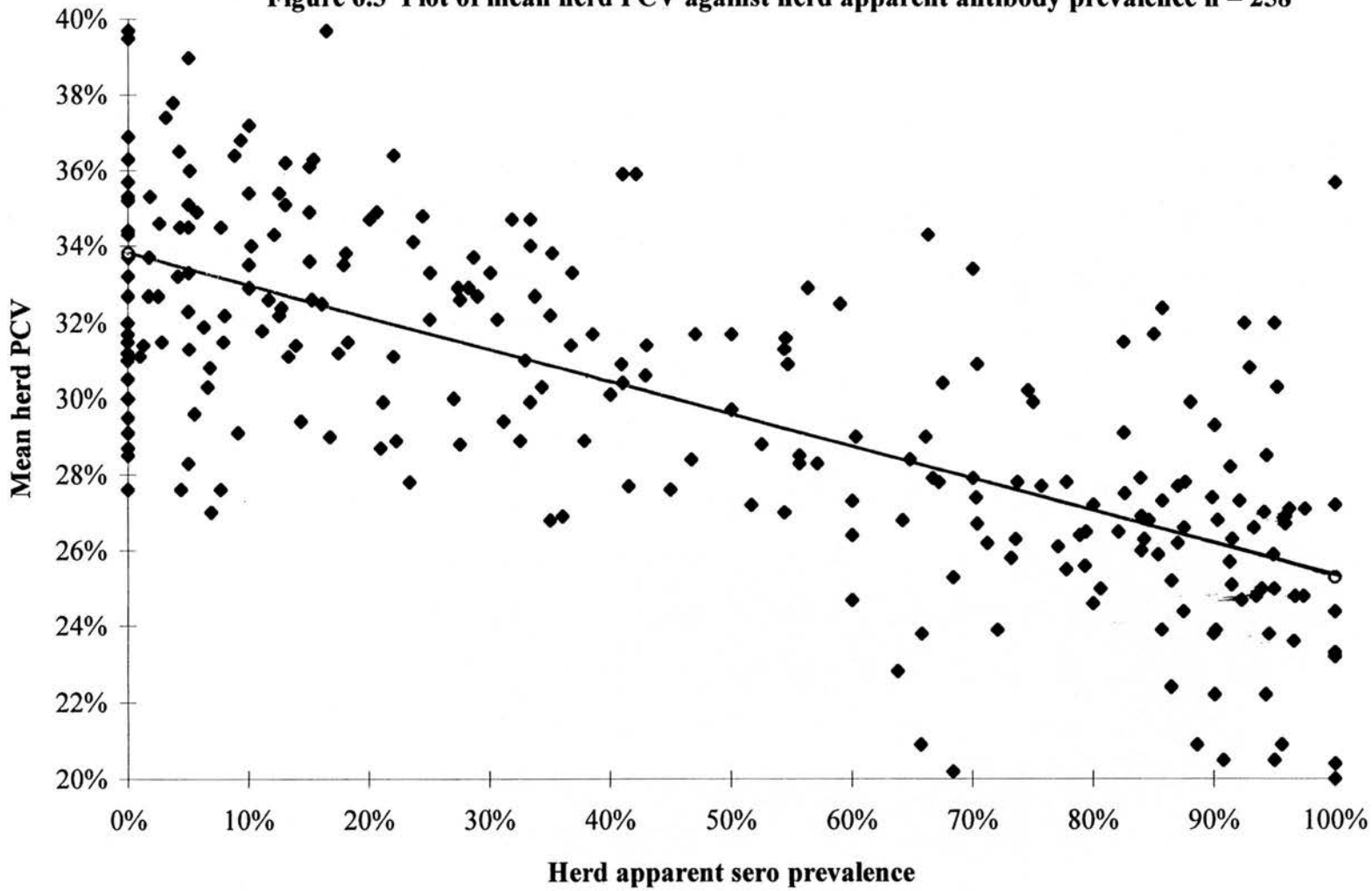


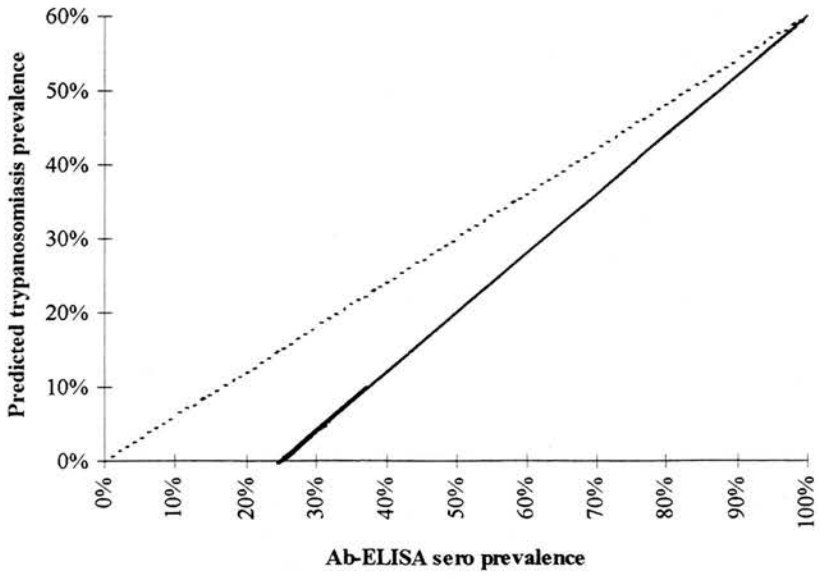
Figure 6.3 Plot of mean herd PCV against herd apparent antibody prevalence n = 258



6.4. Discussion

The Pearson correlation matrix demonstrates the close association between these variables, all of which have an r value of at least 0.62. The very close negative correlation (r ~ -0.9) between herd mean PCV and proportion of herd anaemic is not unexpected as this reflect the greater the number of animals that are anaemic is linked to a reduction in mean herd PCV. Likewise the high positive degree of correlation (r~ 0.9) between mean herd percentage positivity and herd apparent sero-prevalence is expected. Although the correlation between parasitological and apparent sero-prevalence was only 0.64 this is not at variance with the hypothesised relationship between sero-prevalence and parasitological prevalence which is plotted in Figure 6.4.

Figure 6.4 Hypothesized correlation between Ab-ELISA and parasitological prevalence



This hypothetical plot shows two lines where one may expect the two variables (sero-prevalence and parasitological prevalence) to be correlated. The dotted line, intercepting the X and Y axes at zero is so placed because axiomatically, where there is no trypanosomiasis, the sero prevalence should be zero and *vice versa.*

Conversely, one would hypothesis that for any sero-prevalence where the parasitological prevalence is greater than zero, any herd would have a higher sero-prevalence than parasitological prevalence. This would be due to i) the known relative insensitivity of the direct diagnostic techniques and ii) for any sample of cattle that have been in contact with the tsetse fly vector, many more animals will have some detectable level of antibody than detectable parasite. For example, if the demonstrable parasitaemic prevalence in a herd was ~ 15% then one would expect the sero-prevalence to be 50 ± 10% because in order for the herd to have this parasitological prevalence, many more animals would have disease that could be detected using direct parasitological tests (*q.v.* sensitivity of direct diagnostic tests) and more than 15% of the herd would have been recently exposed to the disease and as such would be sero-positive.

The author was not hypothesising a linear relationship between parasitological and sero-prevalence and this is supported by Figure 6.2. Points are expected to fall below the hypothetical line relating sero-prevalence to parasitological prevalence when i) parasitological false negatives are recorded during examination of a herd, ii) trypanocides are in use or iii) an animal has self cured. Whilst the frequency of reason (iii) may not be known under field conditions, what is known is that the sensitivity of direct diagnostic techniques is relatively poor (and hence why indirect diagnostic methods were being investigated) and that trypanocides are widely available in Zambia. Points may move to the left (of the hypothetical fitted line) if the cut-off has been set too high (and as such sero-prevalence is artificially low) or points may move to the right if the cut-off has been set too low.

The field results uphold this hypothesis, with very few points falling above the hypothetical line. Another satisfactory observation of the results is that no herds were parasitologically positive whilst being serologically negative.

The plot of herd apparent prevalence and mean herd PCV shows the two variables have a clear negative correlation ($r \sim 0.72$) which is expected in that both variables are indicating the presence of trypanosomiasis; apparent prevalence specifically and mean herd PCV circumstantially[11]. Using a linear model, the regression coefficient between the two variables is 0.52 (with mean herd PCV as the dependant and Ab-ELISA as the independent) and so again (c.f. Chapter 3 where the regression coefficient between parasitological prevalence and mean herd PCV was ~ 0.34) the data suggest there are other factors in addition to trypanosomiasis which are affecting the PCV on a herd basis.

[11] The word "circumstantially" has been chosen carefully because data given in Chapter 3 do not prove that trypanosomiasis is the primary factor in determining mean herd PCV.

Chapter 7

7. THESIS DISCUSSION

The work described in this thesis has focused on several methods which were investigated to improve the knowledge of the epidemiology of tsetse transmitted bovine trypanosomiasis. In gathering the data, the most sensitive direct field diagnostic tests were used routinely to detect trypanosomiasis, using adequate sample sizes to detect disease[12]. These diagnostic results, as well as the haematocrit values from some 18,000 samples were managed by a tailor made data management system which was able to summarise data as database tables as well as Geographical Information System maps and images. The Antibody ELISA, with a concomitant quality control and geo-referenced data management software was developed to a stage whereby some 12,000 samples were assayed and results could be linked to the parasitological and haematocrit values. A trial of such a scale was probably unique.

The use of a GIS to map disease survey data, and in addition to vector distribution and other data sets (e.g. land use, land tenure, crop use intensity, human and cattle density) to develop strategic planning for trypanosomiasis control, is still at a nascent stage. Whilst reporting disease at a national level and displaying the results, the low resolution of 20 pixels per degree (PPD) was employed out of necessity. In developing this technique in the future, it would probably be better to display and model the data at a provincial level, where a resolution of 80 PPD or greater is possible for the modelling, and vector data can be displayed as points (without being

[12]I.e. was able to detect disease with 95% confidence if greater than 5% prevalence

rasterised) to make scrutiny of the results easier to interpret. Techniques for interpolating between point data also need development.

Whilst there has been a plethora of research work investigating and comparing the sensitivities of direct diagnostic tests, a search of the literature did not reveal the mathematical approach described in Chapter 3. What we do not know is, after centrifugation in a micro-haematocrit capillary tube, what proportion of trypanosomes remain in the erythrocyte fraction? This could possibly be investigated with the emerging polymerase chain reaction (PCR) techniques. With this knowledge, perhaps greater or less credence could be given to the mathematical approach used to evaluate the sensitivity of three direct diagnostic techniques as described in Chapter 3. PCR could possibly also be used to repeat the work of Paris *et al.* in 1982 to compare the sensitivities of the direct parasitological techniques.

There has also been much work to evaluate and compare indirect tests for trypanosomiasis both in cattle and humans. Jongejan *et al.* (1988) compared IFAT and parasitological prevalence in Zambia as well as comparing results from IFAT and Ab-ELISA. These workers also noted the strong correlation between results obtained on herd basis from IFAT and Ab-ELISA. Zwart *et al.* (1973) also compared IFAT with the standard direct diagnostic techniques for trypanosomiasis and had similar findings to those described in this thesis. General surveys for antibody trypanosomiasis in bovines have been conducted in Uganda (Wilson, 1969), the Lambwe Valley of Kenya (Ashkar and Ochilo, 1972), Liberia (Luckins and Mehlitz, 1978), and Tanzania (Connor and Halliwell, 1987) using the IFAT to measure antibody and serum as the source of the antibody.

Whilst the use of blood samples that have been held dried on filter paper as a source of bovine antibody is in routine use by the Provincial laboratory of Eastern Province in Zambia (work unpublished) and has been developed for the diagnosis of human

trypanosomiasis in 1967 by Bailey *et al.* both techniques employed the IFAT, and not the antibody ELISA. Thus the work described in this thesis is also probably unique in that it describes the use of an antibody detecting ELISA using eluted blood samples as the source of antibody. Despite the antibody ELISA's high degree of sensitivity and specificity little work has been done to develop the assay using bovine sera since the technique was first described by Luckins in 1977. In the 1980s workers in Burkina Faso recognised the value of the antibody ELISA for epidemiological studies in cattle and reported on techniques to improve its reproducibility (Bocquentin and Duvallet, 1990). When developing the assay in Zambia, logistical and technical reasons prevented some of these recommended techniques (non activated rabbit serum as a blocking agent, running each tests sample in four wells per plate, reading the optical density as a function of progress vs. time) from being investigated. These same workers (Bocquentin, Very and Duvallet, 1990) also investigated the decline of antibody of naturally or experimentally infected animals after treatment and failed to detect antibody after three and six months using Ab-ELISA and IFAT respectively. The observation in this thesis (as described in Chapter 4) whereby one of the two oxen that was experimentally infected, still had a ~ 60% percentage positivity some twelve months after treatment with a trypanocidal drug and was strongly positive by IFAT is at variance with the observations of the Burkina Faso workers. As stated in Chapter 4, the kinetics of bovine antibody during and after trypanosomiasis infection require considerable further investigation.

Other considerations pertinent to the future use and development of the assay are discussed in Chapter 4.7. Some of the recommendations, e.g. the routine use of Milli-Q water, new pipette tips and positive pressure pipettes, are technically easy to implement but thus said, are costly for a developing African country. The necessary quality control software to manage the Ab-ELISA data has already been written and is satisfactory. This software must continue to be able to link directly with any data management system employed to manage the parasitological and haematocrit

results[13]. The use of the coefficient of variation calculation as the criterion for a replicate's acceptance or rejection when optical density values are low (less than 0.1) probably needs re-thinking, with the absolute difference in OD being used vs. the relative (to the mean of the two values) difference being used.

One real constraint to the future development of the assay could be overcome if some laboratory (e.g. the International Atomic Energy Agency) were to produce standardised antigen and control sera in sufficient quantity for laboratories to reproduce and validate further some of the work described in this thesis, as well as work that has been recommended. Such antigen would ideally be made from a mixture of the three species of trypanosomes[14] and would be free from the practical problems of protein denaturation when in storage, which may be by the addition of some protease inhibitor, by avoidance of bacterial contamination or by mixing with glycerol.

What was perhaps most noteworthy in this thesis was the poor correlation between herd mean packed cell volume and parasitological or antibody sero prevalence. The degree of scatter below the line when plotting sero-prevalence and parasitological prevalence was also somewhat remarkable, but there were sound biological reasons to account for these observations. If mean herd PCV and antibody sero-prevalence are both good indirect parameters for the presence or absence of trypanosomiasis, then one would have expected a good correlation between the two. Chapter 6

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[13] From 1997 the Integrated Tsetse and Trypanosomiasis Database will be superseded by the Disease and Vector Integrated Database (DAVID), developed for the RTTCP and the Overseas Development Administration. This database is much more generic in dealing with vector and disease data, and also manages livestock census data. There are plans for RTTCP to fund the development of DAVID to manage the antibody ELISA results, which will need to manage the indirect ELISA plate layout described in this thesis. The criteria for quality control criteria for the plates will need to be those recommended by Wright *et al.* (1993).

[14] possibly made using recombinant techniques

shows that this is not the case, and knowing that the antibody ELISA demonstrates the presence and absence of the trypanosomiasis disease with a high degree of sensitivity and specificity, leads to the conclusion that there are other factors other than trypanosomiasis that are having a profound effect on PCV on a herd basis. Having reviewed the literature and perused unpublished work it was remarkable that only 30 - 50% of the variance of mean herd PCV could be attributed to trypanosomiasis when measured directly or by Ab-ELISA.

In the absence of proof that other *diseases* profoundly affect PCV, the most likely postulate is that protein malnutrition is the major determinant of haematocrit values in the absence of trypanosome infections. As protein deficiency is a listed cause of mild to moderate anaemia (Kerr, 1989) and having observed the husbandry conditions under which many cattle were raised, this aetiology is the most likely. In the dry season, the grazing (i.e. standing hay syn. foggage) would contain very little crude protein. Cattle owners do not supplement their stock with any protein supplement (personal observation). Knowing that a protein deficiency will induce a mild to moderate anaemia on a herd or individual basis, then this would be accompanied by a hypoalbuminaemia. One possible way to prove or disprove the hypothesis that protein malnutrition was accounting for ~ 50% of the mean herd PCV would be to study the serum or plasma levels in cattle which were anaemic yet had minimal antibodies against trypanosomiasis. By fitting a model whereby mean herd (or individual animal) PCV was a function of trypanosome antibody activity and/or blood albumen levels would probably be able to account for the presently unexplained variance of the herds haematocrit values. Whether blood albumen could be accurately determined from serum, plasma or eluted blood spot would require a trial of its own.

Given the savannah type climate of Zambia, the effect of nutritional dietary protein intake could also be investigated by some time series observations of the PCV data, in the known presence or absence of the disease. Until such work and analyses are

carried out, and until the correlation between blood albumen and PCV are further investigated, one cannot use PCV data with much confidence to predict the presence of disease.

The costs of detecting trypanosomiasis that must be incurred by Government Departments responsible for monitoring animal disease and possibly implementing control strategies were investigated, as it is currently believed by many Governments and donor agencies that the control of animal disease should be cost effective if the control is to be sustained. In the 1980s, it was believed that international co-operation in the approach to a control strategy of a vector such as tsetse was going to be cost effective in Southern Africa (i.e. eradication of tsetse from the Common Fly Belt). This philosophy has now been radically reappraised, and measures are presently aimed at disease control (vs. vector eradication) which will almost certainly employ the more widespread use of trypanocides[15]. Whether employing direct parasitological diagnostic tests or an Ab-ELISA, the detection of disease is an expensive exercise! The costs of transport and a dedicated, well trained team make up ~ 80% of the costs of a direct parasitological examination. Despite the limitations of the sensitivity of the direct diagnostic techniques, good quality dark ground examination of a well made buffy coat smear is probably a very satisfactory trypanosomiasis survey technique. The "quality control" of these buffy examinations is only possibly through frequent field visits by experienced veterinarians, whilst given the insensitivity of thick and thin smears, the subsequent examinations of these smears is a last resort back-stop to ensure positive cases have not been missed in the field.

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[15] Hence the ongoing interest in new formulations of existing trypanocides, the development of drug ELISAs and investigations into the distribution of isometamidium resistance

Hopefully the development of the Ab-ELISA as a reliable diagnostic test could have led to a more sensitive serial diagnostic test when used on a herd basis and eventually possibly even a replacement test. The problems of the repeatability of the assay demonstrated by work described in this thesis, the still unknown antibody kinetics when an animal has been cured of the disease and the practical problems of the supply of the biologicals (viz. antigen and control sera) mean that these issues must be resolved before the assay can be used as a serious contender to good quality direct diagnostic tests. When these issues have been resolved, the assay should act as an excellent tool for monitoring the distribution of disease in space and time (later used during and after control). The use of eluted blood spots as the source of antibody is less than half the costs of collecting serum samples, and hopefully the forthcoming work to assess the indirect ELISA being distributed by IAEA will examine the use of eluted blood spots as well as sera and laboratories will develop the use of using eluted blood spots for screening the enzoötic stability/instability of the tick borne diseases of anaplasmosis and babesiosis.

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APPENDICES

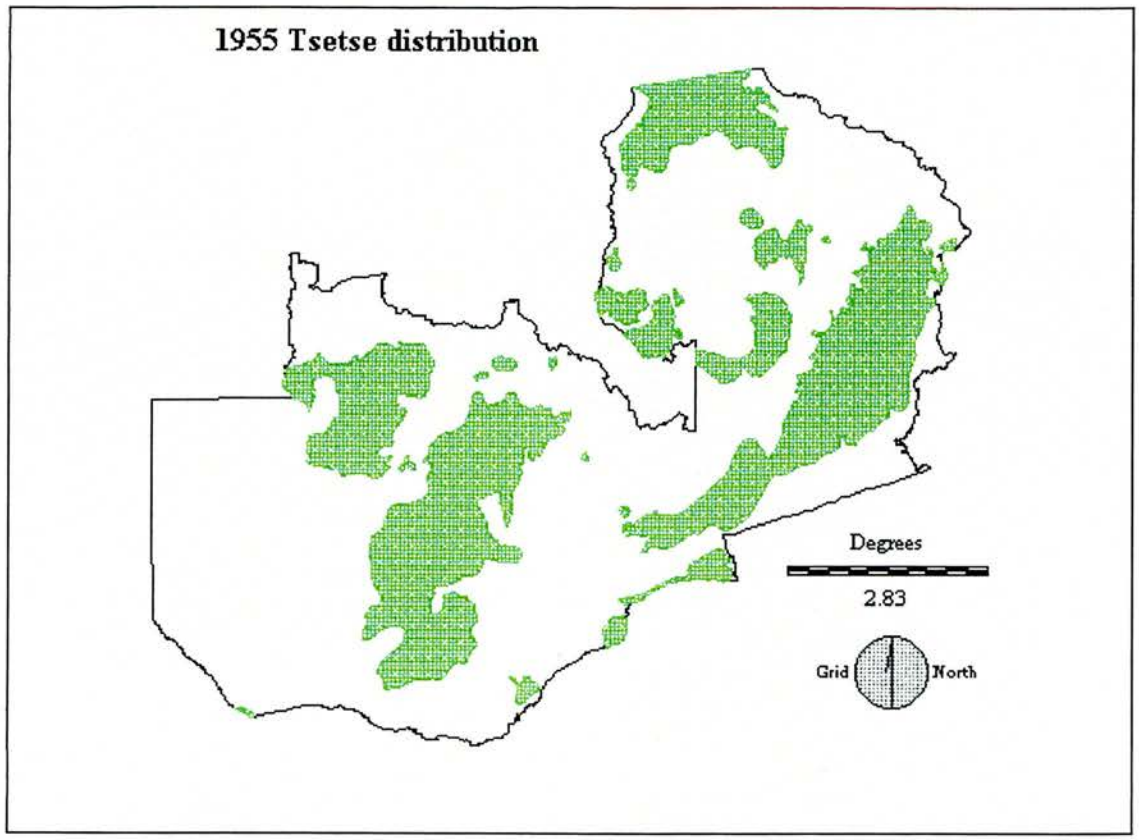
Appendix 2. Materials and methods of the RTTCP direct haematological examination

Materials required

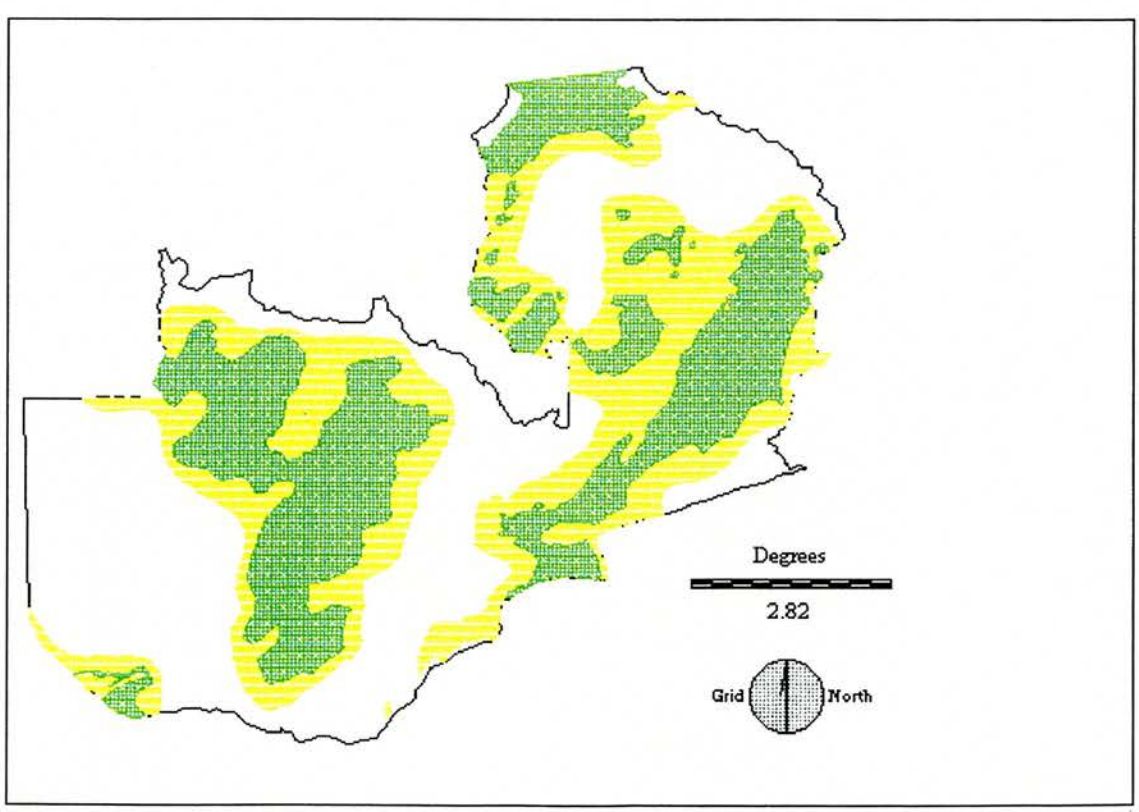
The following equipment was required for a routine RTTCP Zambia examination of blood samples.

Compound microscopes x 2 with condenser and patch stop for dark ground configuration, blue filter x 20, x 40, x 50 (oil) and x 100 (oil) objectives and x 10 eyepieces.
Microhaematocrit centrifuge
Haematocrit reader
Power supply: generator or invertor
Heparinised capillary tubes
Capillary block, clearly numbered 1-30
Trays of Cristaseal®
Lancets
Microscope slides
Slide boxes to hold 100 slides (2)
Coverslips
Diamond pencil
Weighband
Muslin meat cover
Diminazene (Berenil ® or Dimasan ® 10.5g and 1.05g sachets)
10 ml, 20 ml & 30 ml measuring cylinder
150 ml measuring beaker
2.5 litre distilled water
Automatic syringe
Hypodermic needles 18g x 1.5"
Methanol 500 ml (for degreasing slides)

Appendix 1.1 The Distribution of Tsetse based on the surveys conducted by Steel and Gledhill 1945 - 1955 Legend: Green - areas where tsetse occur

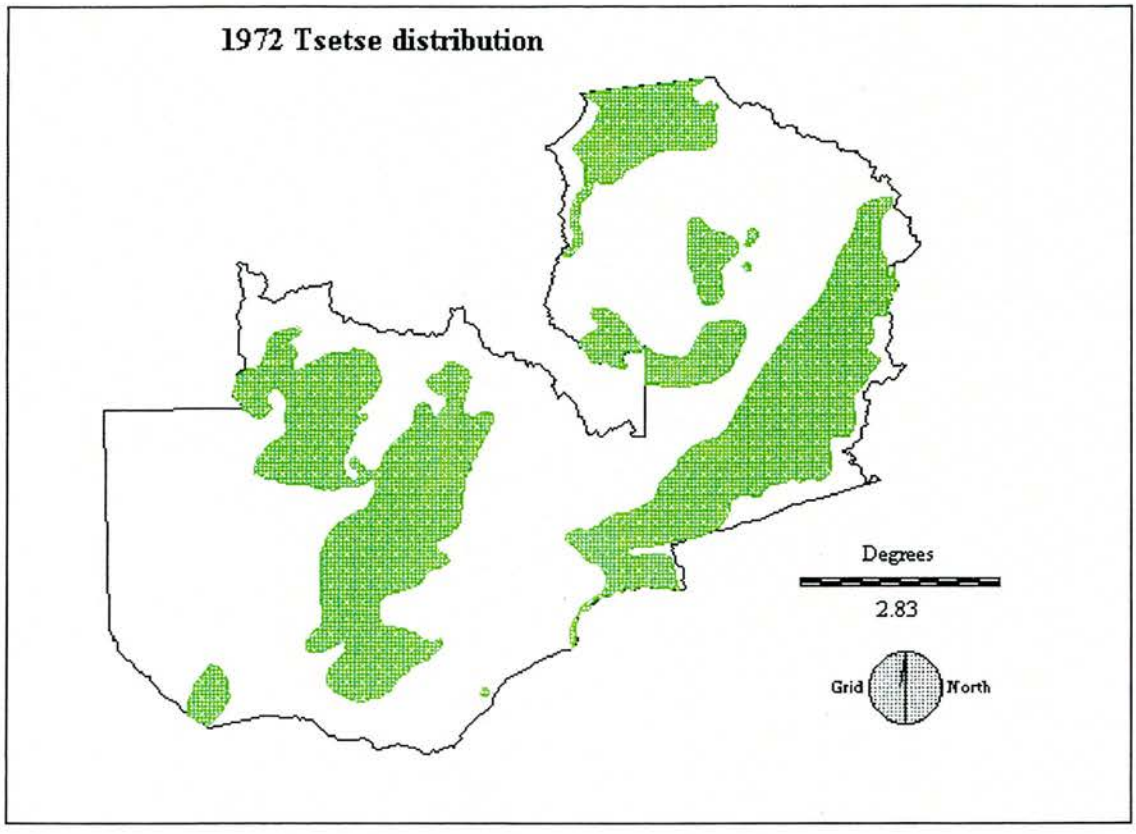


Appendix 1.2 The Distribution of Tsetse and Trypanosomiasis in 1968 based on maps made by the Department of Veterinary and Tsetse Control Services
Legend::Green - areas where tsetse occur; Yellow - areas where trypanosomiasis occurs

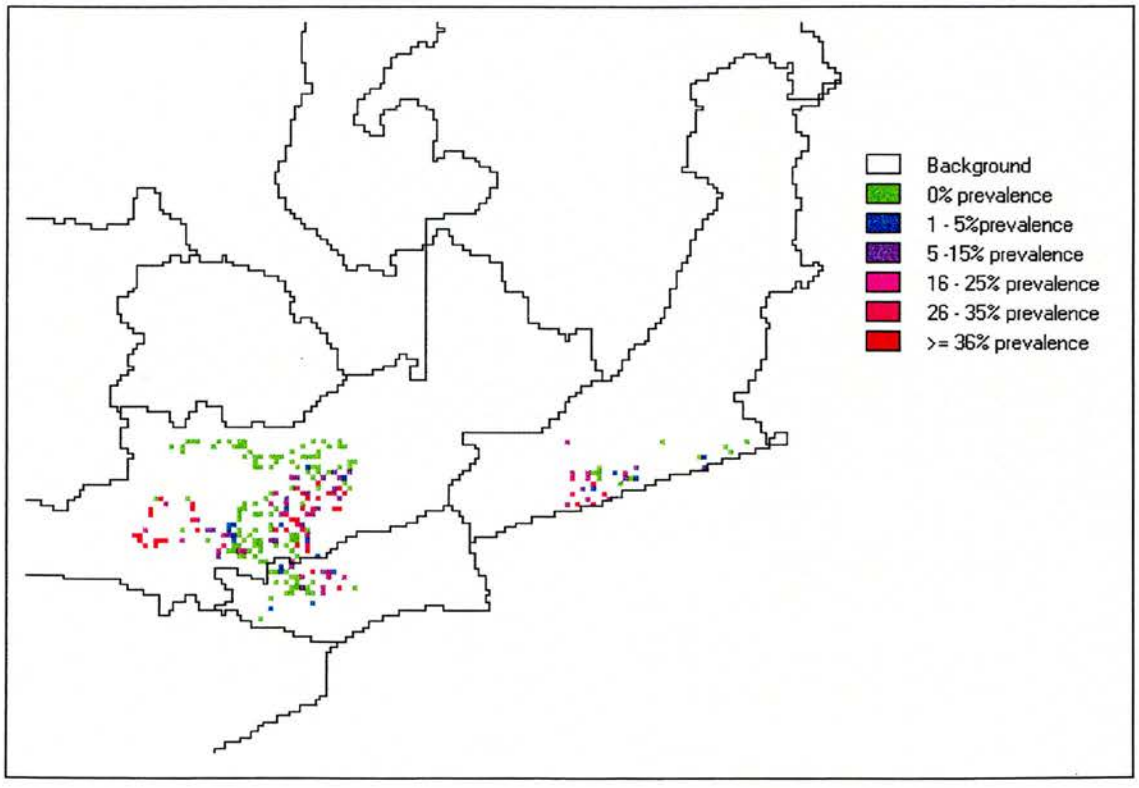


Appendix 1.3 The Distribution of Tsetse and Trypanosomiasis in 1972 based on maps made by the Department of Veterinary and Tsetse Control Services

Legend Green - areas where tsetse occur



Appendix 1.4 Results of the trypanosomiasis surveys conducted by RTTCP in Zambia 1986 to 1993



Methods

Two capillaries taken from a superficial ear vein were filled with blood and one capillary is used to make a thick and thin blood smear then was sealed and placed in numbered tray, whilst the other was spun for 5 minutes in a micro haematocrit centrifuge.

The packed cell volume was measured and the result recorded.

The capillary was cut 1 mm. below the buffy coat and then the buffy coat and some plasma was tapped onto a clean slide and examined at x 20 dark field under a 22 x 22 mm. coverslip.

The unused capillary was broken and expressed in a spiralling fashion onto Whatman's No. 4 filter paper for subsequent Ab-ELISA determination.

Animals with a PCV of 25% or less or where tsetse transmitted trypanosomiasis were diagnosed were treated with diminazene. The dose rate used during surveys was 3.5 mg/kg. During the dose rate 7.0 mg/kg in order to eliminate and *T. brucei* infections.

The thick and thin smears are subsequently stained with 10% Giemsa stain for 30 minutes and then screened using a x 50 objective with trypanosomes being speciated at x 100.

Appendix 3. Antibody ELISA materials, methods and protocol

ELISA reagents, general

Purified water

Double distilled de-ionised distilled water or Millipore Super-Q Plus[1] water

Coating buffer

Carbonate-bicarbonate buffer, 50 mM, pH 9.6. Dissolve one capsule (Sigma[2] C3041) in 100 ml. purified water.

Serum diluent/bloodspot eluent buffer

Phosphate buffered saline (PBST), 10 mM, pH 7.4, containing 0.05% Tween 20 (PBST). Dissolve five tablets (Sigma P1379) in 1 litre purified water. Add 0.5 ml. Tween 20 and mix thoroughly.

Washing buffer

Phosphate buffered saline, 2 mM, pH 7.4, containing 0.05% Tween 20. This solution may be made by diluting PBST 1/5 in purified water, and adding additional Tween 20 at a rate of 0.4 ml. per litre.

Substrate-chromogen buffer

Citrate-phosphate buffer, 50 mM, pH 5.0, containing 0.03% sodium perborate. Dissolve one capsule (Sigma P4922) in 100 ml. purified water.

Substrate-chromogen

3,3',5,5' Tetramethylbenzidine dihydrochloride tablets (Sigma, T3405) added to substrate chromogen buffer at a rate of one 1 mg. tablet per 10 ml.

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[1] Millipore Super-Q Plus water purification system, Millipore (UK) Ltd., The Boulevard, Blackmore Lane, Watford, Hertfordshire, England WD1 8YW

[2] Sigma Chemical Co. Ltd., Fancy Road, Poole, Dorset, England BH17 7NH

Stopping reagent

Sulphuric acid, 2M

ELISA reagents, immunological

Antigen

Crude somatic soluble trypanosome antigen, is used, the production of which is described in paragraph 3.4.1.4.

Test and control bovine sera

Negative control sera must ideally be collected from cattle reared under traditional practices in an area where tsetse are known not to occur and where cattle (from a possible tsetse infested area) have not been bought in. Positive control sera production is described in Paragraph 3.4.1.5.

Anti-immunoglobulin Horseradish Peroxidase (HRP) conjugate

Rabbit anti-bovine IgG (whole molecule) peroxidase conjugate, affinity purified, Sigma A-7414 is purchased and stored frozen. It may be pre-diluted and aliquoted in glycerol.

Equipment required

- ELISA plate reader 450 nm.
- Multi-channel and single channel pipettes and appropriate tips (single pipette range of 5-40 µl and 40-200 µl; multi pipette range of 40 - 200 µl.
- Glassware/10 ml. test tubes and racks (measuring cylinders and beakers (50-1000 ml)
- Automatic dispenser 1-5 ml
- Refrigerator +4 °C
- 37 °C Incubator / orbital shaker
- Reagent reservoirs

- IBM PS/2 Personal computer 80286 or faster, serial printer cable and Procomm software

Consumable materials

- Ninety-six well microtitre ELISA plates, Dynatech Immulon 1 (M129A)[3].
- Cryogenic vials, 2 ml
- Microcentrifuge tubes, 2 ml
- Pipette tips, 200µl

Assay conditions

Well volumes

One hundred microlitre well volumes are used throughout.

Coating conditions

Plates are coated and stored overnight at +4°C.

Serum, blood spot eluate, and conjugate incubation conditions

Sera/eluted blood spots are incubated for forty five minutes at 37°C with orbital shaking. Conjugate is incubated for thirty minutes at 37°C with orbital shaking.

Sera may be diluted the day before the ELISA is run and held overnight +4°C. Blood spots may be eluted the previous day and held overnight +4°C or made up at least three hours before the assay is run.

Washing conditions

ELISA plate contents are ejected manually. Wells are filled completely with washing buffer, using an automatic plate washer. The process is repeated a total of four times after the coating and serum incubations, and five times after the conjugate incubation.

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[3] Dynatech Laboratories, Inc., 14340 Sullyfield Circle, Chantilly, Virginia 22021, USA

Substrate chromogen incubation conditions

Ten minutes at 37°C with orbital shaking.

Test protocol

1. Coat ELISA plates overnight at +4°C using *T. congolense* antigen at an optimal dilution in 50 mM carbonate bicarbonate buffer, 100 µl per well. This optimal antigen dilution has to be determined with each batch of new antigen.
2. Dilute control sera (C++, C+, C-), and test sera at the optimum dilution (again established with each batch of antigen) in PBST; for 1:400 add 10µl control or test sera to 4 ml. PBST in a clean test tube and vortex mix. These dilutions may be stored at +4°C overnight if necessary. For eluted blood spots excise 6.2 mm. disks from dried blood spots on Whatman filter paper using a "treasury punch", and elute for at least 2½ hours in 2.0 ml. PBST in a clean test tube at room temperature with occasional agitation.
3. Wash plates **four** times by filling the wells with washing buffer (PBST diluted 1/5 in purified water, with the addition of a further 0.4 ml. Tween 20 per litre). Blot the plates after the last wash using a "Wettex" sponge reserved exclusively for this purpose. The sponge should be wet first using purified water, and then wrung out thoroughly before use. Avoid undue delays between blotting and addition of diluted sera or blood spot eluates.
4. Add 100µl per well of 1/400 dilutions of test or reference sera, or blood spot eluates to duplicate ELISA plate wells, in accordance with the paper template and incubate for forty five minutes at 37°C with orbital shaking.
5. Wash the plates **four** times, and blot as described above. Avoid undue delays between blotting and addition of conjugate.
6. Add 100µl per well of rabbit anti-bovine IgG (whole molecule) peroxidase conjugate at a final dilution of determined by a chequerboard titration to all the ELISA plate wells, and incubate for thirty minutes at 37°C with orbital shaking.

7. Prior to the end of the conjugate incubation, prepare the substrate-chromogen solution. Dissolve 1 TMB tablet per 10 ml. substrate-chromogen buffer. For 2 plates, 30 ml. will be required, and for 4 plates 50 ml. will be required. Do not store left over substrate-chromogen solution.

8. Wash the plates **five** times, and blot as described above. Avoid **any** delay between blotting and addition of substrate chromogen.

9. Add 50 µl per well of the substrate chromogen solution to all the ELISA plate wells, and to a single column of wells in a blanking plate (i.e. a plate previously unused except for blanking). Incubate for 10 minutes at 37°C with orbital shaking.

10. Add 100µl per well of stopping solution to all the ELISA plate wells, and to the single column of wells in the blanking plate. Stop plates in the same sequence as was used for addition of substrate chromogen solution.

11. Read plates at 450 nm using Procomm software or similar. Blank the reader with the appropriate column of the blanking plate, using the "column mean blank" and "retained blank" settings.

Table A4.1 Costs of collecting 1000 serum samples (excluding labour, transport, and camping costs)

Consumables and capital depreciation only

	Quantity	Unit cost (ECU)	Capital Consumable	Write off period (years)	Cost or Depreciation per 1000 samples (i.e. per month)	Percentage of costs
Consumables						
Plain Vacutainers	1000	0.20	Consumable	n/a	197.84	
Vacutainers needles	1000	0.12	Consumable	n/a	120.58	
Needle holder	1	1.56	Consumable	n/a	1.56	
Cryogenic vials	1000	0.19	Consumable	n/a	193.88	
Pipettes	1000	0.04	Consumable	n/a	37.18	
				Subtotal	*551.03*	*91.8%*
Test tube racks	2	31.18	Capital	1	5.20	
Cryogenic vial racks	2	3.6	Capital	2	0.30	
Capital equipment depreciation (per month)						
18 tube centrifuge	1	350	Capital	3	9.72	
Gas chest freezer	1	850	Capital	3	23.61	
Portable fridge	1	300	Capital	3	8.33	
Cool box	1	60	Capital	3	1.67	
Ice packs	5	5	Capital	5	0.42	
				Subtotal	*49.25*	*8.2%*
				Cost of collecting 1000 serum samples into Cryogenic vials	**600.28**	**100%**
				ECU cost per serum sample	**0.6003**	

Table A4.2 Costs of collecting 1000 blood spot samples (excluding salaries, transport and camping equipment

Consumables						
7.5 cm. filter paper disks		3	Consumable	3.97	11.90	
Self sealing Plastic bags		1 x 25	Consumable	0.46	0.46	
Silica gel desiccant	500 g	0.5	Consumable	8.90	4.45	
				Subtotal	*16.81*	*64.0%*
Capital equipment						
Kerosene ice pack freezer	1	400	Capital	5	6.67	
Kerosene	5	0.47	Consumable	n/a	2.34	
Cool box	1	50	Capital	10	0.42	
Ice packs	1	1	Capital	2	0.04	
				Subtotal	*9.47*	36.0%
			Cost of collection of 1000 blood spots		**26.27**	100%

Table A4.3
Costs of antibody ELISA routine running based on 4 days, 6 plates/day, 4 a.m. 2 p.m. + 1 plate = 1000 samples/week

Item	Sub Unit	Number per week	Unit cost (ECU)	Capital cost	Write off period (years)	Depreciation per 1000 samples (i.e. week)	Total costs (ECU) (per. week) incl. labour	Total costs (ECU) (per. week) excl. labour	Percentage breakdown of costs incl. labour	Percentage breakdown of costs excl. labour
Consumables										
Antigen at ECU 2,000 per 15 ml.			1.60				40.00			
Plates		25	1.23				30.73			
Plate sealer		25	0.29				7.28			
Pipette tips - Coating	10									
Pipette tips - Dispensing eluates/sera	50									
Pipette tips - Substrate	10									
Pipette tips - Stopping	10									
Pipette tips - subtotal	90 per plate	2,250	32.39	Per 1000						
Assume 70% recycled		675	21.86	(per 25 plates plate)			21.86			
Buffers										
Coating buffer P3041 tablets		4	3.80				3.80			
Substrate buffer P4922 tablets		8	8.33				8.33			
PBST 5 litre P4417 tablets		25	11.34				11.34			
Tween (ml)		3	0.27				0.27			
Washing buffer 30 litre										
PBST 5 litre P4417 tablets		30	13.61				13.61			
Tween (mls)		24	2.60				2.60			
TMB tablets		25	30.66				30.66			
Conjugate 25 plates			6.51				6.51			
Sulphuric acid 1 litre 1:10 per week	ml	25	2.93				2.93			
Paper for plate reader - 1 per 100 plates		0.25	5.00				1.25			
Laboratory detergent	5 litres	1	10.00				10.00			
						Subtotal	*191.18*	*191.18*	*46.2%*	*72.6%*
Minor Laboratory equipment										

Measuring cylinders	2	3.85	20.00				3.85			
Aspirator bottles 10 litre	3		20.00				2.00			
Tubes and racks	10		2				2.00			
Vortex mixer	1			75.00	5	0.29	0.29			
Orbital shaker	1			75.00	5	0.29	0.29			
Interval timers	2			20.00	5	0.15	0.15			
Multi channel pippetes	2			414.63	5	3.19	3.19			
P1000	1			159.76	5	0.61	0.61			
P200	2			159.76	5	1.23	1.23			
P20	1			159.76	5	0.61	0.61			
						Subtotal	*14.22*	*14.22*	*3.4%*	*5.4%*
Major Items Capital equipment required										
Plate reader Titertech Multiscan Plus Mk 2	1			6,219.51	5	23.92	23.92			
Computer	1			1,200.00	5	11.82	11.82			
Incubator / shaker	1			1,510.98	5	5.81	5.81			
Denley Well wash 2	1			3,073.17	5	11.82	11.82			
Fridge freezer	1			500.00	5	1.92	1.92			
-20 degrees C chest freezer	1			500.00	5	1.92	1.92			
Double distillation plant + deioniser	1			200.00	5	0.77	0.77			
						Subtotal	*57.99*	*57.99*	14.0%	22.0%
Labour										
Technician		n/a	100.00				100.00	0.00		
Washer - data manager		n/a	50.00				50.00	0.00		
						Subtotal	*150.00*	*0.00*	36.3%	0%
						Total laboratory costs per 1000 samples	**413.39**	**263.39**	100.0%	100%
						Total cost per sample	**0.4134**	**0.2634**		

Table A4.4 Costs of antigen and conjugate (in ECU) to run 25 plates

Antigen	*Volume*	*Unit*	*ECU Cost*
Production of antigen	15	mls.	2,000
1ml costs	1	ml.	133.33
1 plate uses number microlitres at di	12	microlitres	
Costs per plate			1.60
Costs per 25 plates			**40.00**

Antigen dilution 1,000

Volume to coat1 plate in microlitres = 12,000

Conjugate			
Conjugate per 0.5 ml.	0.5	ml.	54.26
To make 2ml of stock 1:20	200	microlitres	21.70
1ml 1:20 stock conjugate costs			10.85
1 plate uses number microlitres at diln.			
Costs per plate			0.26
Costs per 25 plates			**6.51**

Diln. of stock 1:20 = 1: 1,000

Final volume = 12,000

1:20 Conjugate volume = 12

Table A5.1 The analysis of variance by columns on a plate with 80 replicated aliquots of a single serum dilution

Column number	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**
Row **A**	0.401	0.411	0.356	0.389	0.361	0.381	0.368	0.371	0.321	0.355
B	0.393	0.391	0.404	0.403	0.317	0.395	0.372	0.351	0.346	0.363
C	0.403	0.445	0.412	0.4	0.318	0.402	0.386	0.383	0.387	0.367
D	0.405	0.415	0.387	0.373	0.374	0.421	0.429	0.411	0.387	0.394
E	0.431	0.388	0.387	0.412	0.351	0.402	0.376	0.426	0.323	0.354
F	0.406	0.396	0.409	0.342	0.352	0.386	0.385	0.382	0.346	0.35
G	0.389	0.401	0.382	0.372	0.401	0.421	0.418	0.438	0.314	0.405
H	0.381	0.353	0.388	0.342	0.381	0.417	0.397	0.386	0.384	0.362

	1	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**
Mean	0.401	0.400	0.391	0.379	0.357	0.403	0.391	0.394	0.351	0.369
Variance	0.0002	0.0007	0.0003	0.0007	0.0009	0.0002	0.0005	0.0009	0.0010	0.0004
Std. dev.	0.0149	0.0262	0.0180	0.0268	0.0293	0.0155	0.0220	0.0292	0.0311	0.0200
Coefficient of variati	3.7%	6.6%	4.6%	7.1%	8.2%	3.8%	5.6%	7.4%	8.9%	5.4%

Grand mean **0.384**

Variance of the columns

Variance of 10 means	0.00035
Standard deviation	0.0188
Coefficient of variatior	4.9%

Within column variance

Mean of 10 variances	0.0006
Standard deviation	0.0240
Coefficient of variation	6.3%

Variance between columns

Var (means) = Var (between) + Var (within)/8

Variance b	0.0003
Standard d	0.016784
Coefficient	4.4%

Variance between columns

Var (means) = Var (between) + Var (within)/1

Var between areas	0.0006
Standard deviation	0.0252
Coefficient of variation	6.6%

Table A5.2 The analysis of variance by row on a plate with 80 replicated aliquots of a single serum dilution

Column number	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**Mean**	**Var.**	**Std. dev.**	**CV**
Row **A**	0.401	0.411	0.356	0.389	0.361	0.381	0.368	0.371	0.321	0.355	0.371	0.00067	0.0258	7.0%
B	0.393	0.391	0.404	0.403	0.317	0.395	0.372	0.351	0.346	0.363	0.374	0.00084	0.0289	7.7%
C	0.403	0.445	0.412	0.4	0.318	0.402	0.386	0.383	0.387	0.367	0.390	0.00108	0.0328	8.4%
D	0.405	0.415	0.387	0.373	0.374	0.421	0.429	0.411	0.387	0.394	0.400	0.00038	0.0195	4.9%
E	0.431	0.388	0.387	0.412	0.351	0.402	0.376	0.426	0.323	0.354	0.385	0.00121	0.0348	9.0%
F	0.406	0.396	0.409	0.342	0.352	0.386	0.385	0.382	0.346	0.35	0.375	0.00066	0.0256	6.8%
G	0.389	0.401	0.382	0.372	0.401	0.421	0.418	0.438	0.314	0.405	0.394	0.00117	0.0342	8.7%
H	0.381	0.353	0.388	0.342	0.381	0.417	0.397	0.386	0.384	0.362	0.379	0.00047	0.0218	5.7%

Grand mean **0.384**

Variance of the rows

Variance of 10 means	0.00011
Standard deviation	0.0104
Coefficient of variatio	2.7%

Within row variance

Mean of 10 variances	0.0008
Standard deviation	0.0284
Coefficient of variation	7.4%

Variance between rows

Var (means) = Var (between) + Var (within)/8

Variance b	0.000006
Standard d	0.002502
Coefficient	0.7%

Variance between rows

Var (means) = Var (between) + Var (within)/1

Var between areas	0.0001
Standard deviation	0.0107
Coefficient of variation	2.8%

Table A5.3 The results of the first pipetting trial where 10 pipette tips were made to make 10 dilutions and a different pipette tip was used to place 100 microlitres diluted sera into 10 columns

	1	2	3	4	5	6	7	8	9
A	0.815	0.859	0.835	0.826	0.776	0.804	0.846	0.795	0.72
B	0.936	0.82	0.798	0.825	0.733	0.779	0.822	0.726	0.688
C	0.825	0.807	0.761	0.828	0.706	0.752	0.783	0.813	0.696
D	0.894	0.92	0.828	0.874	0.839	0.888	0.909	0.83	0.756
E	0.82	0.794	0.765	0.841	0.82	0.852	0.808	0.741	0.688
F	0.775	0.751	0.712	0.71	0.692	0.745	0.686	0.642	0.619
G	0.84	0.902	0.781	0.876	0.841	0.876	0.847	0.836	0.731
H	0.797	0.824	0.793	0.84	0.868	0.864	0.826	0.813	0.786

	1	2	3	4	5	6	7	8	9
Mean	0.838	0.835	0.784	0.828	0.784	0.820	0.816	0.775	0.711
Variance	0.0028	0.0032	0.0016	0.0027	0.0045	0.0033	0.0041	0.0045	0.0026
Std. dev.	0.0527	0.0563	0.0395	0.0516	0.0674	0.0572	0.0640	0.0668	0.0505
Coefficient of variati	6.3%	6.7%	5.0%	6.2%	8.6%	7.0%	7.8%	8.6%	7.1%

Grand mean **0.799**

Variance of the columns

Variance of 10 means	0.00165
Standard deviation	0.0406
Coefficient of variatio	5.1%

Within column variance

Mean of 10 variances	0.0032
Standard deviation	0.0569
Coefficient of variation	7.1%

Variance between columns

Var. (means) = Var. (between) + Var. (within)/8

Variance b	0.0012
Standard d	0.035253
Coefficient	4.4%

Variance between columns

Var. (means) = Var. (between) + Var. (within)/1

Var. between areas	0.0029
Standard deviation	0.0538
Coefficient of variation	6.7%

Table A5.4 The results of the second pipette trial where one serum dilution was made and 10 pipette tips were used to place 100 microlitres of the single dilution into the 10 columns

	1	2	3	4	5	6	7	8	9	10
A	0.839	0.813	0.837	0.857	0.767	0.687	0.681	0.723	0.58	0.557
B	0.872	0.746	0.898	0.859	0.665	0.647	0.633	0.663	0.583	0.605
C	0.746	0.717	0.811	0.86	0.723	0.646	0.678	0.716	0.577	0.621
D	0.869	0.778	0.834	0.908	0.78	0.812	0.817	0.78	0.621	0.656
E	0.784	0.687	0.774	0.887	0.699	0.653	0.635	0.63	0.317	0.499
F	0.668	0.632	0.627	0.593	0.654	0.568	0.46	0.472	0.444	0.433
G	0.813	0.753	0.724	0.838	0.691	0.749	0.684	0.806	0.635	0.702
H	0.77	0.765	0.792	0.753	0.691	0.714	0.687	0.735	0.695	0.588

	1	2	3	4	5	6	7	8	9	10
Mean	0.795	0.736	0.787	0.819	0.709	0.685	0.659	0.691	0.557	0.583
Variance	0.0047	0.0032	0.0068	0.0104	0.0020	0.0055	0.0097	0.0110	0.0145	0.0074
Std. dev.	0.0686	0.0567	0.0823	0.1022	0.0452	0.0744	0.0986	0.1050	0.1202	0.0859
Coefficient of variati	8.6%	7.7%	10.5%	12.5%	6.4%	10.9%	15.0%	15.2%	21.6%	14.7%

Grand mean **0.702**

Variance of the columns

Variance of 10 means	0.00762
Standard deviation	0.0873
Coefficient of variatio	12.4%

Within column variance

Mean of 10 variances	0.0075
Standard deviation	0.0868
Coefficient of variation	12.4%

Variance between columns

Var. (means) = Var. (between) + Var. (within)/8

Variance b	0.0067
Standard d	0.081735
Coefficient	11.6%

Variance between columns

Var. (means) = Var. (between) + Var. (within)/1

Var. between areas	0.0143
Standard deviation	0.1196
Coefficient of variation	17.0%

Table A5.5 The results from the ten punches from a single blood spot run on the same plate.

Punch number		**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**
Replicate	**1**	0.273	0.364	0.279	0.274	0.260	0.294	0.251	0.245	0.257	0.274
	2	0.277	0.287	0.298	0.269	0.243	0.284	0.245	0.235	0.231	0.254
	3	0.251	0.263	0.263	0.232	0.214	0.242	0.218	0.236	0.230	0.240
	4	0.251	0.278	0.268	0.237	0.242	0.275	0.236	0.229	0.228	0.212
	5	0.252	0.252	0.269	0.252	0.234	0.266	0.224	0.237	0.235	0.268
	6	0.269	0.269	0.269	0.243	0.236	0.275	0.231	0.227	0.219	0.237
	7	0.260	0.265	0.302	0.258	0.223	0.248	0.236	0.221	0.240	0.248
	8	0.307	0.278	0.297	0.282	0.249	0.293	0.246	0.255	0.267	0.263
		1	2	3	4	5	6	7	8	9	10
Mean		0.268	0.282	0.281	0.256	0.238	0.272	0.236	0.236	0.238	0.250
Variance		0.0004	0.0012	0.0003	0.0003	0.0002	0.0004	0.0001	0.0001	0.0003	0.0004
Std. dev.		0.0190	0.0348	0.0159	0.0181	0.0145	0.0193	0.0113	0.0107	0.0160	0.0200
Coefficient of variati		7.1%	12.4%	5.7%	7.1%	6.1%	7.1%	4.8%	4.5%	6.7%	8.0%

Grand mean **0.256**

Variance of punch means

Variance of 10 means	0.00035
Standard deviation	0.0188
Coefficient of variatic	7.4%

Within punch variance

Mean of 10 variances	0.0004
Standard deviation	0.0191
Coefficient of variation	7.5%

Variance between punches

Var (means) = Var (between) + Var (within)/reps

Variance b	0.0003
Standard d	0.017557
Coefficien	6.9%

Variance between punches from different areas

Var (means) = Var (between) + Var (within)/1

Var between areas	0.00066
Standard deviation	0.025728
Coefficient of variation	10.1%

Table A5.6 The results from the ten punches from a single blood spot run on two plates with randomised columns.

Punch number		**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**
Replicate	**1**	0.488	0.474	0.489	0.609	0.565	0.588	0.493	0.562	0.519	0.503
Plate 1	**2**	0.468	0.487	0.471	0.588	0.563	0.601	0.487	0.609	0.574	0.528
	3	0.493	0.478	0.468	0.570	0.526	0.644	0.511	0.581	0.595	0.535
	4	0.555	0.508	0.465	0.601	0.570	0.653	0.539	0.633	0.566	0.580
	5	0.455	0.474	0.435	0.568	0.514	0.580	0.526	0.556	0.516	0.544
	6	0.530	0.508	0.432	0.637	0.554	0.655	0.552	0.580	0.576	0.547
	7	0.509	0.491	0.461	0.606	0.546	0.611	0.477	0.573	0.524	0.539
	8	0.482	0.470	0.423	0.591	0.556	0.581	0.447	0.526	0.543	0.495
Replicate	**1**	0.445	0.483	0.471	0.523	0.508	0.445	0.367	0.406	0.464	0.518
Plate 2	**2**	0.375	0.498	0.445	0.495	0.461	0.385	0.375	0.372	0.410	0.470
	3	0.407	0.489	0.469	0.509	0.487	0.445	0.348	0.369	0.439	0.512
	4	0.454	0.445	0.463	0.504	0.498	0.442	0.438	0.406	0.448	0.445
	5	0.398	0.456	0.457	0.516	0.479	0.411	0.360	0.379	0.426	0.457
	6	0.570	0.661	0.598	0.677	0.601	0.540	0.511	0.504	0.583	0.580
	7	0.449	0.465	0.466	0.486	0.476	0.460	0.363	0.407	0.473	0.457
	8	0.428	0.462	0.474	0.476	0.440	0.436	0.373	0.358	0.464	0.451

Spot number/column	1	2	3	4	5	6	7	8	9	10
Mean	0.469	0.491	0.468	0.560	0.521	0.530	0.448	0.489	0.507	0.510
Variance	0.003	0.002	0.001	0.004	0.002	0.009	0.005	0.010	0.004	0.002
Std. dev.	0.055	0.049	0.039	0.060	0.046	0.095	0.073	0.099	0.062	0.044
Coefficient of variati	11.7%	9.9%	8.3%	10.7%	8.8%	17.9%	16.3%	20.3%	12.2%	8.7%

Grand mean **0.499**

Variance of punch means

Variance of punch me	0.00111
Standard deviation	0.03332
Coefficient of variatio	6.7%

Within punch variance

Mean of 10 variances	0.00425
Standard deviation	0.06519
Coefficient of variation	13.1%

Variance between punches

Var. (means) = Var. (between) + Var. (within)/reps

Variance between are	0.00084
Standard deviation	0.0291
Coefficient of variatio	5.8%

Variance between punches from different areas

Var. (means) = Var. (between) + Var. (within)/1

Var. between areas	0.0333
Standard deviation	0.1825
Coefficient of variation	36.6%

Table A5.7 F test and T test comparing percentage positivities from Lusitu and negative controls

Sera results

F-Test Two-Sample for Variances

	PP_neg_sera	*PP Sera Lusitu*
Mean	0.129205128	0.102374332
Variance	0.002709432	0.001923956
Observations	195	187
df	194	186
F	1.408260936	
P(F<=f) one-tail	0.009478538	
F Critical one-tail	1.270819006	

t-Test: Two-Sample Assuming Unequal Variances

	PP_neg_sera	*PP Sera Lusitu*
Mean	0.129205128	0.102374332
Variance	0.002709432	0.001923956
Observations	195	187
Hypothesized Mean Difference	0	
df	374	
t Stat	5.456045315	
P(T<=t) one-tail	4.43755E-08	
t Critical one-tail	1.648936632	
P(T<=t) two-tail	8.8751E-08	
t Critical two-tail	1.966327545	

Eluted blood spot results

F-Test Two-Sample for Variances

	PP_Neg_BS	*PP_Lusitu_BS*
Mean	0.083475113	0.13510989
Variance	0.001948254	0.002539071
Observations	442	182
df	441	181
F	1.303254146	
P(F<=f) one-tail	0.01484195	
F Critical one-tail	0.809852629	

t-Test: Two-Sample Assuming Unequal Variances

	PP_Neg_BS	*PP_Lusitu_BS*
Mean	0.083475113	0.13510989
Variance	0.001948254	0.002539071
Observations	442	182
Hypothesized Mean Difference	0	
df	301	
t Stat	-12.05093402	
P(T<=t) one-tail	7.38745E-28	
t Critical one-tail	1.649932528	
P(T<=t) two-tail	1.47749E-27	
t Critical two-tail	1.967878234	

Table 5.8 The T test to compare eluted blood spot results from Lusitu and contemporary negative blood spot control samples

F-Test Two-Sample for Variances

	PP neg Blood spot (no BN)	*PP Lusitu Blood spot*
Mean	0.119153005	0.13510989
Variance	0.002084768	0.002539071
Observations	183	182
df	182	181
F	1.217915439	
P(F<=f) one-tail	0.092533791	
F Critical one-tail	0.782802712	

t-Test: Two-Sample Assuming Equal Variances

	PP neg Blood spot (no BN)	*PP Lusitu Blood spot*
Mean	0.119153005	0.13510989
Variance	0.002084768	0.002539071
Observations	183	182
Pooled Variance	0.002311293	
Hypothesized Mean Difference	0	
df	363	
t Stat	-3.170555567	
P(T<=t) one-tail	0.000825436	
t Critical one-tail	1.649061687	
P(T<=t) two-tail	0.001650873	
t Critical two-tail	1.966518539	

Appendix 6 The protocol for the Indirect Fluorescent Antibody Test (IFAT)

IFAT MATERIALS

1. Biologicals

Whole *T. congolense* antigen

2. Reagents

2.1. Stock solutions

PBS (5x)-stock (0.05 M)

NaCl	36 g
Na_2HPO_4.anh.	7.39 g
$Na_2HPO_4.H_2O$	8.33 g
$Na_2HPO_4.2H_2O$	9.27 g
KH_2PO_4.anh.	2.15 g
NaN_3	1 g

Add distilled water to 1000 ml, filter and store at 4 °C

Before use: Dilute stock solution 5x in distilled water i.e. take 200 ml PBS-stock solution and add 800 ml distilled water, this constitutes PBS-working solution. (0.01M pH 7.2.

2.2. Working solutions

2.2.1. Evans Blue

Evans Blue powder	10 mg
PBS-working solution (0.01 M)	10 ml

Renew the solution after 2 weeks.

2.2.2. PBS-BSA (Buffer for plasma dilutions and antigen-coating)

PBS-working solution (0.01 M)	5 ml
Bovine Serum Albumin	10 mg

2.2.3. Mounting medium

PBS-working solution (0.01 M) 5 ml

Bring carefully to pH 8.0 with NaOH 10%, while stirring the solution. Add equal volume of glycerol (Analar, commercial concentration), mix well and store at 4 °C.

Renew the solution every 2 weeks.

IFAT PROCEDURE

2.3. Prepare PBS-working solution 0.01 M.

Prepare PBS-BSA. Effective cleaning of the Teflon-coated slides is achieved by soaking the slides in ethanol (denaturated in ether) for 1 hour. Leave to dry in upright position. Confettis of dried blood (treasury punch hole punches) with a diameter of 6.2 mm are punched out, placed in a microtitre-plate and soaked by adding 100 µl of PBS-BSA solution (giving more or less a 1:50 dilution). Soaking takes 60 minutes, during this period the tray is shaken on the orbital shaker. Put another microtitre-plate on top of the first one during the shaking, to avoid evaporation of the PBS. The positive and negative reference samples are treated the same way.

2.3.1. Preparation of the antigen

Take the bottle of antigen with the trypanosomes in suspension (**stored at -20 °C**). Shake the bottles for 1 minute. Take 200 µl out of the bottle using a syringe with 21 1/2 G needle or automatic pipette and transfer this to test-tubes. Add 40 µl PBS-BSA to the 400 µl antigen suspension (enough for 15 slides) and Vortex. Pass this mixture through a 25G needle to break up all the clumps.

NOTE : According to the instructions *T. congolense* can be mixed with *T. vivax*, in which case 200 µl of *T. congolense* and 200 µl of *T. vivax* antigen is brought together in the test-tube, to this 400 µl of antigen we add 80 µl of PBS-BSA (on principle, 1 µl PBS-BSA is added to each 5 µl antigen).

2.3.2. Coating of the antigen

Take 50 µl of the antigen suspension in an automatic pipette (P100), place a bubble on each spot of the Welcome-Teflon® slide, suck it up again and do the same for each spot. Vortex the antigen each time after coating 2 slides. Use separate slides for each antigen, or use a mixture of antigens. Leave the slides to dry at 37 °C.

2.3.3. Rehydration of the antigen

Rehydrate the antigen-preparations for 15 minutes by dropping (Pasteur pipette) 25 to 50 µl of PBS onto each reaction area. After this period shake off the buffer by tapping the edge of the slide against a sheet of absorbent paper.

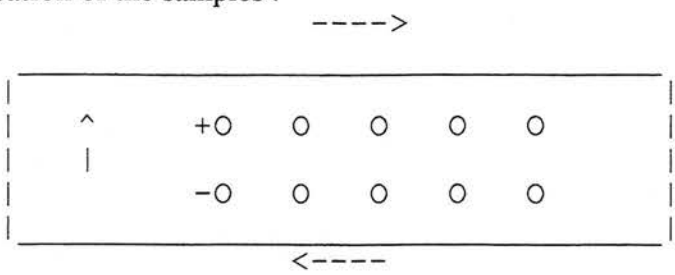
Take the test-sera plus a negative and positive reference serum, dilute the sera 1:40 (975 µl PBS-BSA plus 25 µl serum). Vortex mix.

2.4. Application of the samples.

Take 25 µl of each plasma or confetti dilution and transfer onto the Teflon slide.

Reference sera: Positive and negative controls at 1:40 are placed respectively on upper left spot and spot below to allow detection of any seepage under Teflon-layer. Eventually add lymphocyte lysate-control as well.

Application of the samples :



Leave slides for 30 min in humid chamber at room temperature (not more than 30 minutes). Hereon until mounting, slides should never be allowed to dry!

2.4.1. Washing procedure:

Buffer: plain and properly diluted PBS (kept at 4 °C)

Procedure: tip off the sera, transfer the slides to a Coplin Jar filled with buffer beforehand. Once all the slides are rinsed in this way transfer them to another Coplin Jar, filled with buffer beforehand. Leave the slides for 15 minutes. Transfer the slides to another Coplin Jar, filled with buffer beforehand, and leave for at least another 15 minutes. Never pour buffer onto the slides, but fill the jars beforehand. total washing time should not be less than incubation time. If necessary slides can be left in washing buffer for 1 hour or more.

2.4.2. Conjugate

Conjugate is stored in aliquots of 100 µl (or less) at -20 °C, in the deep freezer. Take 400 µl of the Evans Blue stock solution in an aliquot-tube. Centrifuge both the conjugate- and the Evans Blue-aliquot tubes at 4000 RPM for 5 min. Fill test-tube with 3.55 ml PBS. Clip off the tops of both aliquot tubes and extract 400 µl Evans Blue and 50 µl conjugate. Add both to the 3.55 ml PBS and vortex.

This brings the final concentration of conjugate to 1:80 and Evans Blue to 1:10,000

Take the slides out of the washing solution, tip off washing fluid but do not wipe off.

Put 25 µl of the conjugate solution on each spot using a 25 G needle. Incubate slides for 30 minutes in humid chamber in the dark at room-temperature.

2.4.3. Washing procedure.

Using plain and properly diluted PBS Buffer(kept at 4_C), tip off the sera, transfer the

slides to a Coplin Jar filled with buffer beforehand. Once all the slides are rinsed in this way transfer them to another Coplin Jar, filled with buffer beforehand. Leave the slides for 15 minutes.

Transfer the slides to another Coplin Jar, filled with buffer beforehand, and leave for 15 at least another minutes.

2.4.4. Mounting

Take slides out of jar and tip off washing fluid. Put 3-4 drops mounting fluid at the level of the left row of the spots. Take a coverslip, bring left side in contact with the mounting fluid and put the coverslip slowly down, while inclining the slide slightly downwards to the right. Remove air-bubbles by sliding the coverslip sideways. Remove all excess of the mounting fluid by blotting the edges so that the coverslip does not float any more.

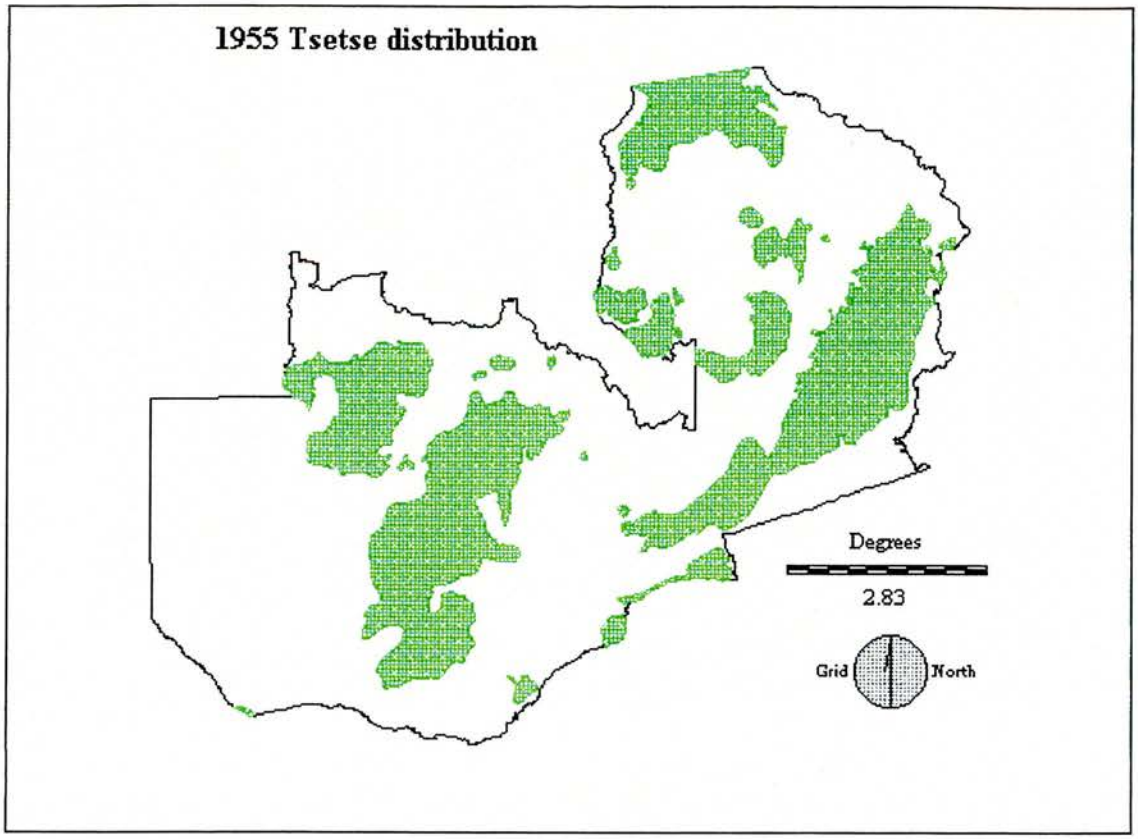
Slides are now ready for microscopy, but can be stored overnight in the dark at 4 °C, if necessary.

2.4.5. Microscopy

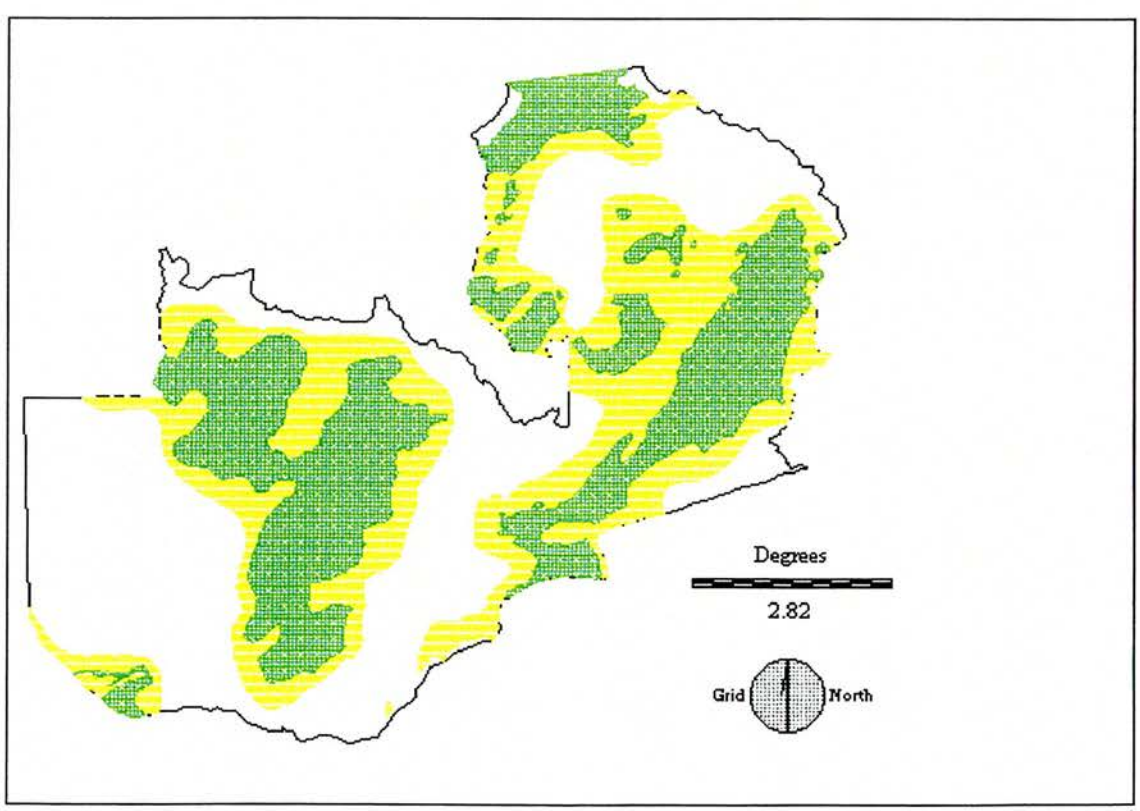
Switch on the microscope at least 15 minutes before usage. The best combination of optics is 6.3 x 40 Phaco oil (but 6.3 x 63 with Phaco oil may be used if necessary).

Use immersion oil of good quality. According to the lenses available, water immersion gives an equally clear image (500x). Check orange-coloured zone between spots for seepage under Teflon. Score fluorescence.

Appendix 1.1 The Distribution of Tsetse based on the surveys conducted by Steel and Gledhill 1945 - 1955 Legend: Green - areas where tsetse occur

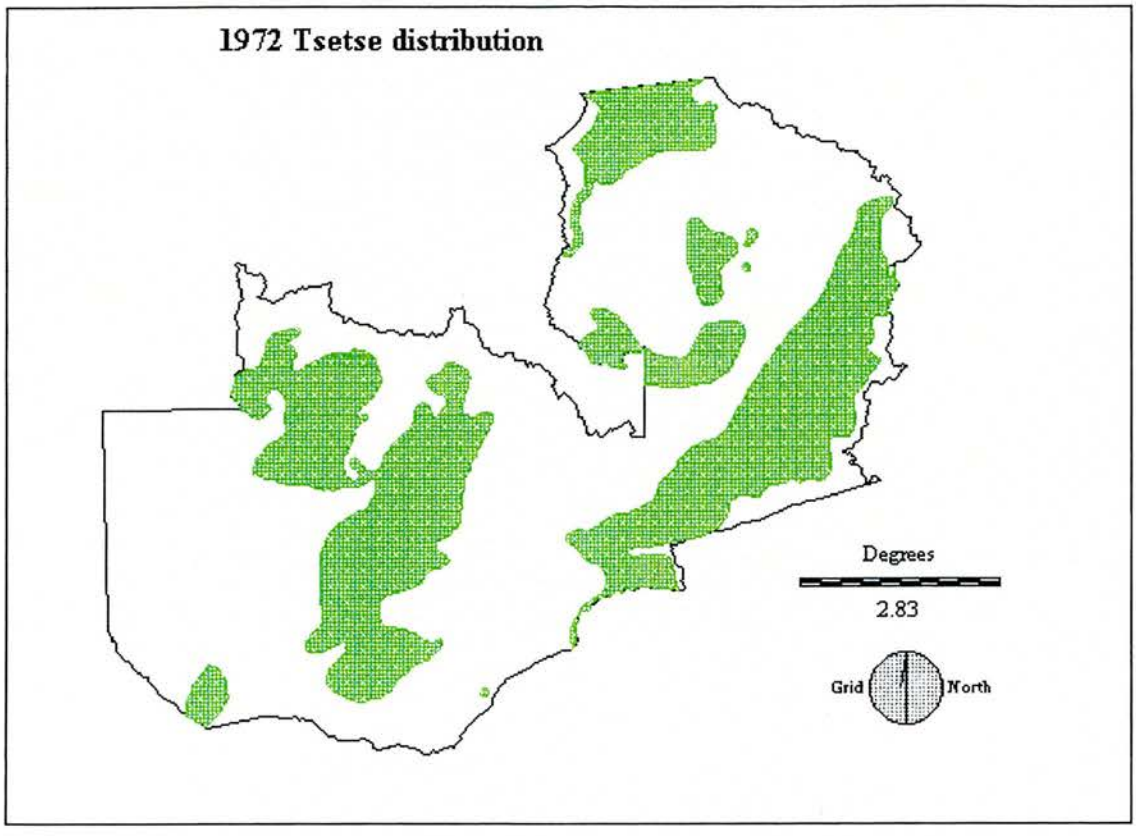


Appendix 1.2 The Distribution of Tsetse and Trypanosomiasis in 1968 based on maps made by the Department of Veterinary and Tsetse Control Services
Legend::Green - areas where tsetse occur; Yellow - areas where trypanosomiasis occurs

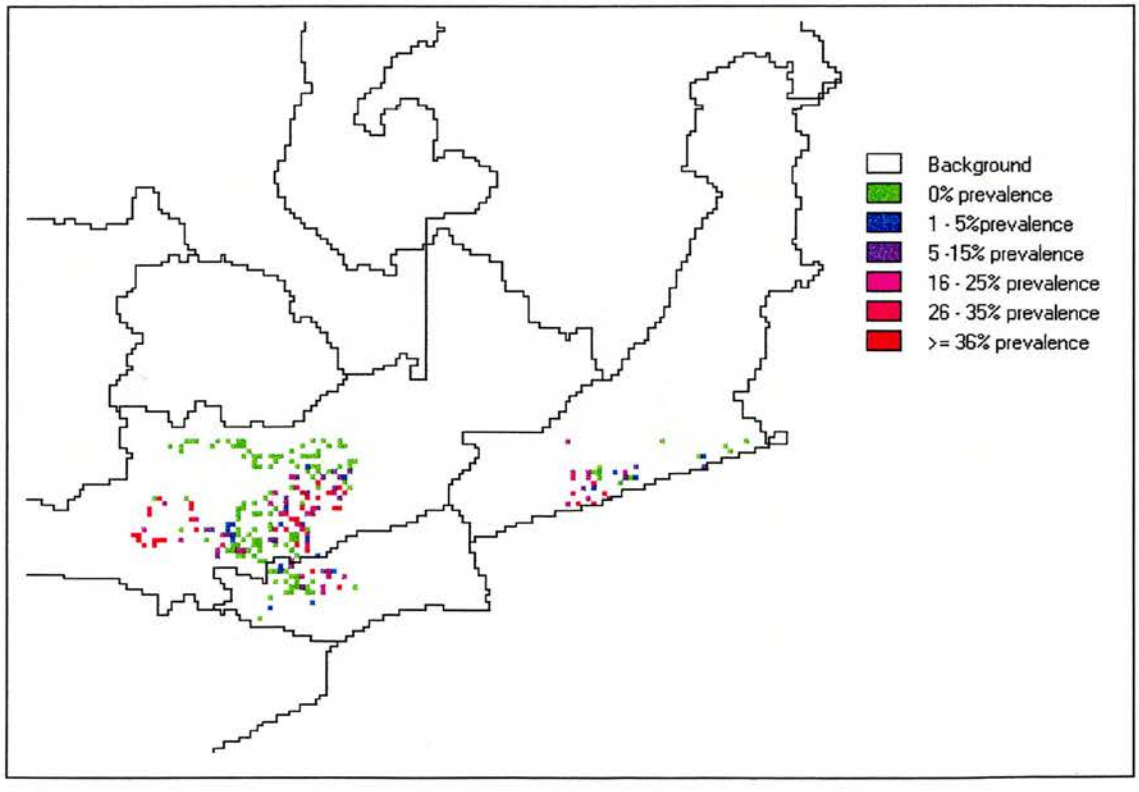


Appendix 1.3 The Distribution of Tsetse and Trypanosomiasis in 1972 based on maps made by the Department of Veterinary and Tsetse Control Services

Legend Green - areas where tsetse occur



Appendix 1.4 Results of the trypanosomiasis surveys conducted by RTTCP in Zambia 1986 to 1993



Appendix 2. Materials and methods of the RTTCP direct haematological examination

Materials required

The following equipment was required for a routine RTTCP Zambia examination of blood samples.

Compound microscopes x 2 with condenser and patch stop for dark ground configuration, blue filter x 20, x 40, x 50 (oil) and x 100 (oil) objectives and x 10 eyepieces.
Microhaematocrit centrifuge
Haematocrit reader
Power supply: generator or invertor
Heparinised capillary tubes
Capillary block, clearly numbered 1-30
Trays of Cristaseal®
Lancets
Microscope slides
Slide boxes to hold 100 slides (2)
Coverslips
Diamond pencil
Weighband
Muslin meat cover
Diminazene (Berenil ® or Dimasan ® 10.5g and 1.05g sachets)
10 ml, 20 ml & 30 ml measuring cylinder
150 ml measuring beaker
2.5 litre distilled water
Automatic syringe
Hypodermic needles 18g x 1.5"
Methanol 500 ml (for degreasing slides)

Methods

Two capillaries taken from a superficial ear vein were filled with blood and one capillary is used to make a thick and thin blood smear then was sealed and placed in numbered tray, whilst the other was spun for 5 minutes in a micro haematocrit centrifuge.

The packed cell volume was measured and the result recorded.

The capillary was cut 1 mm. below the buffy coat and then the buffy coat and some plasma was tapped onto a clean slide and examined at x 20 dark field under a 22 x 22 mm. coverslip.

The unused capillary was broken and expressed in a spiralling fashion onto Whatman's No. 4 filter paper for subsequent Ab-ELISA determination.

Animals with a PCV of 25% or less or where tsetse transmitted trypanosomiasis were diagnosed were treated with diminazene. The dose rate used during surveys was 3.5 mg/kg. During the dose rate 7.0 mg/kg in order to eliminate and *T. brucei* infections.

The thick and thin smears are subsequently stained with 10% Giemsa stain for 30 minutes and then screened using a x 50 objective with trypanosomes being speciated at x 100.

Appendix 3. Antibody ELISA materials, methods and protocol

ELISA reagents, general

Purified water

Double distilled de-ionised distilled water or Millipore Super-Q Plus[1] water

Coating buffer

Carbonate-bicarbonate buffer, 50 mM, pH 9.6. Dissolve one capsule (Sigma[2] C3041) in 100 ml. purified water.

Serum diluent/bloodspot eluent buffer

Phosphate buffered saline (PBST), 10 mM, pH 7.4, containing 0.05% Tween 20 (PBST). Dissolve five tablets (Sigma P1379) in 1 litre purified water. Add 0.5 ml. Tween 20 and mix thoroughly.

Washing buffer

Phosphate buffered saline, 2 mM, pH 7.4, containing 0.05% Tween 20. This solution may be made by diluting PBST 1/5 in purified water, and adding additional Tween 20 at a rate of 0.4 ml. per litre.

Substrate-chromogen buffer

Citrate-phosphate buffer, 50 mM, pH 5.0, containing 0.03% sodium perborate. Dissolve one capsule (Sigma P4922) in 100 ml. purified water.

Substrate-chromogen

3,3',5,5' Tetramethylbenzidine dihydrochloride tablets (Sigma, T3405) added to substrate chromogen buffer at a rate of one 1 mg. tablet per 10 ml.

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[1] Millipore Super-Q Plus water purification system, Millipore (UK) Ltd., The Boulevard, Blackmore Lane, Watford, Hertfordshire, England WD1 8YW

[2] Sigma Chemical Co. Ltd., Fancy Road, Poole, Dorset, England BH17 7NH

Stopping reagent

Sulphuric acid, 2M

ELISA reagents, immunological

Antigen

Crude somatic soluble trypanosome antigen, is used, the production of which is described in paragraph 3.4.1.4.

Test and control bovine sera

Negative control sera must ideally be collected from cattle reared under traditional practices in an area where tsetse are known not to occur and where cattle (from a possible tsetse infested area) have not been bought in. Positive control sera production is described in Paragraph 3.4.1.5.

Anti-immunoglobulin Horseradish Peroxidase (HRP) conjugate

Rabbit anti-bovine IgG (whole molecule) peroxidase conjugate, affinity purified, Sigma A-7414 is purchased and stored frozen. It may be pre-diluted and aliquoted in glycerol.

Equipment required

- ELISA plate reader 450 nm.
- Multi-channel and single channel pipettes and appropriate tips (single pipette range of 5-40 µl and 40-200 µl; multi pipette range of 40 - 200 µl.
- Glassware/10 ml. test tubes and racks (measuring cylinders and beakers (50-1000 ml)
- Automatic dispenser 1-5 ml
- Refrigerator +4 °C
- 37 °C Incubator / orbital shaker
- Reagent reservoirs

- IBM PS/2 Personal computer 80286 or faster, serial printer cable and Procomm software

Consumable materials

- Ninety-six well microtitre ELISA plates, Dynatech Immulon 1 (M129A)[3].
- Cryogenic vials, 2 ml
- Microcentrifuge tubes, 2 ml
- Pipette tips, 200µl

Assay conditions

Well volumes

One hundred microlitre well volumes are used throughout.

Coating conditions

Plates are coated and stored overnight at +4°C.

Serum, blood spot eluate, and conjugate incubation conditions

Sera/eluted blood spots are incubated for forty five minutes at 37°C with orbital shaking. Conjugate is incubated for thirty minutes at 37°C with orbital shaking.

Sera may be diluted the day before the ELISA is run and held overnight +4°C. Blood spots may be eluted the previous day and held overnight +4°C or made up at least three hours before the assay is run.

Washing conditions

ELISA plate contents are ejected manually. Wells are filled completely with washing buffer, using an automatic plate washer. The process is repeated a total of four times after the coating and serum incubations, and five times after the conjugate incubation.

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[3] Dynatech Laboratories, Inc., 14340 Sullyfield Circle, Chantilly, Virginia 22021, USA

Substrate chromogen incubation conditions

Ten minutes at 37°C with orbital shaking.

Test protocol

1. Coat ELISA plates overnight at +4°C using *T. congolense* antigen at an optimal dilution in 50 mM carbonate bicarbonate buffer, 100 µl per well. This optimal antigen dilution has to be determined with each batch of new antigen.
2. Dilute control sera (C++, C+, C-), and test sera at the optimum dilution (again established with each batch of antigen) in PBST; for 1:400 add 10µl control or test sera to 4 ml. PBST in a clean test tube and vortex mix. These dilutions may be stored at +4°C overnight if necessary. For eluted blood spots excise 6.2 mm. disks from dried blood spots on Whatman filter paper using a "treasury punch", and elute for at least 2½ hours in 2.0 ml. PBST in a clean test tube at room temperature with occasional agitation.
3. Wash plates **four** times by filling the wells with washing buffer (PBST diluted 1/5 in purified water, with the addition of a further 0.4 ml. Tween 20 per litre). Blot the plates after the last wash using a "Wettex" sponge reserved exclusively for this purpose. The sponge should be wet first using purified water, and then wrung out thoroughly before use. Avoid undue delays between blotting and addition of diluted sera or blood spot eluates.
4. Add 100µl per well of 1/400 dilutions of test or reference sera, or blood spot eluates to duplicate ELISA plate wells, in accordance with the paper template and incubate for forty five minutes at 37°C with orbital shaking.
5. Wash the plates **four** times, and blot as described above. Avoid undue delays between blotting and addition of conjugate.
6. Add 100µl per well of rabbit anti-bovine IgG (whole molecule) peroxidase conjugate at a final dilution of determined by a chequerboard titration to all the ELISA plate wells, and incubate for thirty minutes at 37°C with orbital shaking.

7. Prior to the end of the conjugate incubation, prepare the substrate-chromogen solution. Dissolve 1 TMB tablet per 10 ml. substrate-chromogen buffer. For 2 plates, 30 ml. will be required, and for 4 plates 50 ml. will be required. Do not store left over substrate-chromogen solution.

8. Wash the plates **five** times, and blot as described above. Avoid **any** delay between blotting and addition of substrate chromogen.

9. Add 50 µl per well of the substrate chromogen solution to all the ELISA plate wells, and to a single column of wells in a blanking plate (i.e. a plate previously unused except for blanking). Incubate for 10 minutes at 37°C with orbital shaking.

10. Add 100µl per well of stopping solution to all the ELISA plate wells, and to the single column of wells in the blanking plate. Stop plates in the same sequence as was used for addition of substrate chromogen solution.

11. Read plates at 450 nm using Procomm software or similar. Blank the reader with the appropriate column of the blanking plate, using the "column mean blank" and "retained blank" settings.

Table A4.1 Costs of collecting 1000 serum samples (excluding labour, transport, and camping costs)

Consumables and capital depreciation only

	Quantity	Unit cost (ECU)	Capital Consumable	Write off period (years)	Cost or Depreciation per 1000 samples (i.e. per month)	Percentage of costs
Consumables						
Plain Vacutainers	1000	0.20	Consumable	n/a	197.84	
Vacutainers needles	1000	0.12	Consumable	n/a	120.58	
Needle holder	1	1.56	Consumable	n/a	1.56	
Cryogenic vials	1000	0.19	Consumable	n/a	193.88	
Pipettes	1000	0.04	Consumable	n/a	37.18	
				Subtotal	*551.03*	*91.8%*
Test tube racks	2	31.18	Capital	1	5.20	
Cryogenic vial racks	2	3.6	Capital	2	0.30	
Capital equipment depreciation (per month)						
18 tube centrifuge	1	350	Capital	3	9.72	
Gas chest freezer	1	850	Capital	3	23.61	
Portable fridge	1	300	Capital	3	8.33	
Cool box	1	60	Capital	3	1.67	
Ice packs	5	5	Capital	5	0.42	
				Subtotal	*49.25*	*8.2%*
			Cost of collecting 1000 serum samples into Cryogenic vials		**600.28**	**100%**
			ECU cost per serum sample		**0.6003**	

Table A4.2 Costs of collecting 1000 blood spot samples (excluding salaries, transport and camping equipment

Consumables						
7.5 cm. filter paper disks		3	Consumable	3.97	11.90	
Self sealing Plastic bags		1 x 25	Consumable	0.46	0.46	
Silica gel desiccant	500 g	0.5	Consumable	8.90	4.45	
				Subtotal	*16.81*	*64.0%*
Capital equipment						
Kerosene ice pack freezer	1	400	Capital	5	6.67	
Kerosene	5	0.47	Consumable	n/a	2.34	
Cool box	1	50	Capital	10	0.42	
Ice packs	1	1	Capital	2	0.04	
				Subtotal	*9.47*	36.0%
			Cost of collection of 1000 blood spots		**26.27**	100%

Table A4.3
Costs of antibody ELISA routine running based on 4 days, 6 plates/day, 4 a.m. 2 p.m. + 1 plate = 1000 samples/week

Item	Sub Unit	Number per week	Unit cost (ECU)	Capital cost	Write off period (years)	Depreciation per 1000 samples (i.e. week)	Total costs (ECU) (per. week) incl. labour	Total costs (ECU) (per. week) excl. labour	Percentage breakdown of costs incl. labour	Percentage breakdown of costs excl. labour
Consumables										
Antigen at ECU 2,000 per 15 ml.			1.60				40.00			
Plates		25	1.23				30.73			
Plate sealer		25	0.29				7.28			
Pipette tips - Coating	10									
Pipette tips - Dispensing eluates/sera	50									
Pipette tips - Substrate	10									
Pipette tips - Stopping	10									
Pipette tips - subtotal	90 per plate	2,250	32.39	Per 1000						
Assume 70% recycled		675	21.86	(per 25 plates plate)			21.86			
Buffers										
Coating buffer P3041 tablets		4	3.80				3.80			
Substrate buffer P4922 tablets		8	8.33				8.33			
PBST 5 litre P4417 tablets		25	11.34				11.34			
Tween (ml)		3	0.27				0.27			
Washing buffer 30 litre										
PBST 5 litre P4417 tablets		30	13.61				13.61			
Tween (mls)		24	2.60				2.60			
TMB tablets		25	30.66				30.66			
Conjugate 25 plates			6.51				6.51			
Sulphuric acid 1 litre 1:10 per week	ml	25	2.93				2.93			
Paper for plate reader - 1 per 100 plates		0.25	5.00				1.25			
Laboratory detergent	5 litres	1	10.00				10.00			
						Subtotal	*191.18*	*191.18*	*46.2%*	*72.6%*
Minor Laboratory equipment										

Measuring cylinders	2	3.85	20.00				3.85			
Aspirator bottles 10 litre	3		20.00				2.00			
Tubes and racks	10		2				2.00			
Vortex mixer	1			75.00	5	0.29	0.29			
Orbital shaker	1			75.00	5	0.29	0.29			
Interval timers	2			20.00	5	0.15	0.15			
Multi channel pippetes	2			414.63	5	3.19	3.19			
P1000	1			159.76	5	0.61	0.61			
P200	2			159.76	5	1.23	1.23			
P20	1			159.76	5	0.61	0.61			
						Subtotal	*14.22*	*14.22*	*3.4%*	*5.4%*
Major Items Capital equipment required										
Plate reader Titertech Multiscan Plus Mk 2	1			6,219.51	5	23.92	23.92			
Computer	1			1,200.00	5	11.82	11.82			
Incubator / shaker	1			1,510.98	5	5.81	5.81			
Denley Well wash 2	1			3,073.17	5	11.82	11.82			
Fridge freezer	1			500.00	5	1.92	1.92			
-20 degrees C chest freezer	1			500.00	5	1.92	1.92			
Double distillation plant + deioniser	1			200.00	5	0.77	0.77			
						Subtotal	*57.99*	*57.99*	14.0%	22.0%
Labour										
Technician		n/a	100.00				100.00	0.00		
Washer - data manager		n/a	50.00				50.00	0.00		
						Subtotal	*150.00*	*0.00*	36.3%	0%
				Total laboratory costs per 1000 samples			**413.39**	**263.39**	100.0%	100%
				Total cost per sample			**0.4134**	**0.2634**		

Table A4.4 Costs of antigen and conjugate (in ECU) to run 25 plates

Antigen	*Volume*	*Unit*	*ECU Cost*
Production of antigen	15	mls.	2,000
1ml costs	1	ml.	133.33
1 plate uses number microlitres at di	12	microlitres	
Costs per plate			1.60
Costs per 25 plates			**40.00**

Antigen dilution 1,000

Volume to coat1 plate in microlitres = 12,000

Conjugate			
Conjugate per 0.5 ml.	0.5	ml.	54.26
To make 2ml of stock 1:20	200	microlitres	21.70
1ml 1:20 stock conjugate costs			10.85
1 plate uses number microlitres at diln.			
Costs per plate			0.26
Costs per 25 plates			**6.51**

Diln. of stock 1:20 = 1: 1,000

Final volume = 12,000

1:20 Conjugate volume = 12

Table A5.1 The analysis of variance by columns on a plate with 80 replicated aliquots of a single serum dilution

Column number	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**
Row **A**	0.401	0.411	0.356	0.389	0.361	0.381	0.368	0.371	0.321	0.355
B	0.393	0.391	0.404	0.403	0.317	0.395	0.372	0.351	0.346	0.363
C	0.403	0.445	0.412	0.4	0.318	0.402	0.386	0.383	0.387	0.367
D	0.405	0.415	0.387	0.373	0.374	0.421	0.429	0.411	0.387	0.394
E	0.431	0.388	0.387	0.412	0.351	0.402	0.376	0.426	0.323	0.354
F	0.406	0.396	0.409	0.342	0.352	0.386	0.385	0.382	0.346	0.35
G	0.389	0.401	0.382	0.372	0.401	0.421	0.418	0.438	0.314	0.405
H	0.381	0.353	0.388	0.342	0.381	0.417	0.397	0.386	0.384	0.362

	1	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**
Mean	0.401	0.400	0.391	0.379	0.357	0.403	0.391	0.394	0.351	0.369
Variance	0.0002	0.0007	0.0003	0.0007	0.0009	0.0002	0.0005	0.0009	0.0010	0.0004
Std. dev.	0.0149	0.0262	0.0180	0.0268	0.0293	0.0155	0.0220	0.0292	0.0311	0.0200
Coefficient of variati	3.7%	6.6%	4.6%	7.1%	8.2%	3.8%	5.6%	7.4%	8.9%	5.4%

Grand mean **0.384**

Variance of the columns

Variance of 10 means	0.00035
Standard deviation	0.0188
Coefficient of variatior	4.9%

Within column variance

Mean of 10 variances	0.0006
Standard deviation	0.0240
Coefficient of variation	6.3%

Variance between columns

Var (means) = Var (between) + Var (within)/8

Variance b	0.0003
Standard d	0.016784
Coefficient	4.4%

Variance between columns

Var (means) = Var (between) + Var (within)/1

Var between areas	0.0006
Standard deviation	0.0252
Coefficient of variation	6.6%

Table A5.2 The analysis of variance by row on a plate with 80 replicated aliquots of a single serum dilution

Column number	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**Mean**	**Var.**	**Std. dev.**	**CV**
Row **A**	0.401	0.411	0.356	0.389	0.361	0.381	0.368	0.371	0.321	0.355	0.371	0.00067	0.0258	7.0%
B	0.393	0.391	0.404	0.403	0.317	0.395	0.372	0.351	0.346	0.363	0.374	0.00084	0.0289	7.7%
C	0.403	0.445	0.412	0.4	0.318	0.402	0.386	0.383	0.387	0.367	0.390	0.00108	0.0328	8.4%
D	0.405	0.415	0.387	0.373	0.374	0.421	0.429	0.411	0.387	0.394	0.400	0.00038	0.0195	4.9%
E	0.431	0.388	0.387	0.412	0.351	0.402	0.376	0.426	0.323	0.354	0.385	0.00121	0.0348	9.0%
F	0.406	0.396	0.409	0.342	0.352	0.386	0.385	0.382	0.346	0.35	0.375	0.00066	0.0256	6.8%
G	0.389	0.401	0.382	0.372	0.401	0.421	0.418	0.438	0.314	0.405	0.394	0.00117	0.0342	8.7%
H	0.381	0.353	0.388	0.342	0.381	0.417	0.397	0.386	0.384	0.362	0.379	0.00047	0.0218	5.7%

Grand mean **0.384**

Variance of the rows

Variance of 10 means	0.00011
Standard deviation	0.0104
Coefficient of variatio	2.7%

Within row variance

Mean of 10 variances	0.0008
Standard deviation	0.0284
Coefficient of variation	7.4%

Variance between rows

Var (means) = Var (between) + Var (within)/8

Variance b	0.000006
Standard d	0.002502
Coefficient	0.7%

Variance between rows

Var (means) = Var (between) + Var (within)/1

Var between areas	0.0001
Standard deviation	0.0107
Coefficient of variation	2.8%

Table A5.3 The results of the first pipetting trial where 10 pipette tips were made to make 10 dilutions and a different pipette tip was used to place 100 microlitres diluted sera into 10 columns

	1	2	3	4	5	6	7	8	9
A	0.815	0.859	0.835	0.826	0.776	0.804	0.846	0.795	0.72
B	0.936	0.82	0.798	0.825	0.733	0.779	0.822	0.726	0.688
C	0.825	0.807	0.761	0.828	0.706	0.752	0.783	0.813	0.696
D	0.894	0.92	0.828	0.874	0.839	0.888	0.909	0.83	0.756
E	0.82	0.794	0.765	0.841	0.82	0.852	0.808	0.741	0.688
F	0.775	0.751	0.712	0.71	0.692	0.745	0.686	0.642	0.619
G	0.84	0.902	0.781	0.876	0.841	0.876	0.847	0.836	0.731
H	0.797	0.824	0.793	0.84	0.868	0.864	0.826	0.813	0.786

	1	2	3	4	5	6	7	8	9
Mean	0.838	0.835	0.784	0.828	0.784	0.820	0.816	0.775	0.711
Variance	0.0028	0.0032	0.0016	0.0027	0.0045	0.0033	0.0041	0.0045	0.0026
Std. dev.	0.0527	0.0563	0.0395	0.0516	0.0674	0.0572	0.0640	0.0668	0.0505
Coefficient of variati	6.3%	6.7%	5.0%	6.2%	8.6%	7.0%	7.8%	8.6%	7.1%

Grand mean **0.799**

Variance of the columns

Variance of 10 means	0.00165
Standard deviation	0.0406
Coefficient of variatio	5.1%

Within column variance

Mean of 10 variances	0.0032
Standard deviation	0.0569
Coefficient of variation	7.1%

Variance between columns

Var. (means) = Var. (between) + Var. (within)/8

Variance b	0.0012
Standard d	0.035253
Coefficient	4.4%

Variance between columns

Var. (means) = Var. (between) + Var. (within)/1

Var. between areas	0.0029
Standard deviation	0.0538
Coefficient of variation	6.7%

Table A5.4 The results of the second pipette trial where one serum dilution was made and 10 pipette tips were used to place 100 microlitres of the single dilution into the 10 columns

	1	2	3	4	5	6	7	8	9	10
A	0.839	0.813	0.837	0.857	0.767	0.687	0.681	0.723	0.58	0.557
B	0.872	0.746	0.898	0.859	0.665	0.647	0.633	0.663	0.583	0.605
C	0.746	0.717	0.811	0.86	0.723	0.646	0.678	0.716	0.577	0.621
D	0.869	0.778	0.834	0.908	0.78	0.812	0.817	0.78	0.621	0.656
E	0.784	0.687	0.774	0.887	0.699	0.653	0.635	0.63	0.317	0.499
F	0.668	0.632	0.627	0.593	0.654	0.568	0.46	0.472	0.444	0.433
G	0.813	0.753	0.724	0.838	0.691	0.749	0.684	0.806	0.635	0.702
H	0.77	0.765	0.792	0.753	0.691	0.714	0.687	0.735	0.695	0.588

	1	2	3	4	5	6	7	8	9	10
Mean	0.795	0.736	0.787	0.819	0.709	0.685	0.659	0.691	0.557	0.583
Variance	0.0047	0.0032	0.0068	0.0104	0.0020	0.0055	0.0097	0.0110	0.0145	0.0074
Std. dev.	0.0686	0.0567	0.0823	0.1022	0.0452	0.0744	0.0986	0.1050	0.1202	0.0859
Coefficient of variati	8.6%	7.7%	10.5%	12.5%	6.4%	10.9%	15.0%	15.2%	21.6%	14.7%

Grand mean **0.702**

Variance of the columns

Variance of 10 means	0.00762
Standard deviation	0.0873
Coefficient of variatio	12.4%

Within column variance

Mean of 10 variances	0.0075
Standard deviation	0.0868
Coefficient of variation	12.4%

Variance between columns

Var. (means) = Var. (between) + Var. (within)/8

Variance b	0.0067
Standard d	0.081735
Coefficient	11.6%

Variance between columns

Var. (means) = Var. (between) + Var. (within)/1

Var. between areas	0.0143
Standard deviation	0.1196
Coefficient of variation	17.0%

Table A5.5 The results from the ten punches from a single blood spot run on the same plate.

Punch number		**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**
Replicate	**1**	0.273	0.364	0.279	0.274	0.260	0.294	0.251	0.245	0.257	0.274
	2	0.277	0.287	0.298	0.269	0.243	0.284	0.245	0.235	0.231	0.254
	3	0.251	0.263	0.263	0.232	0.214	0.242	0.218	0.236	0.230	0.240
	4	0.251	0.278	0.268	0.237	0.242	0.275	0.236	0.229	0.228	0.212
	5	0.252	0.252	0.269	0.252	0.234	0.266	0.224	0.237	0.235	0.268
	6	0.269	0.269	0.269	0.243	0.236	0.275	0.231	0.227	0.219	0.237
	7	0.260	0.265	0.302	0.258	0.223	0.248	0.236	0.221	0.240	0.248
	8	0.307	0.278	0.297	0.282	0.249	0.293	0.246	0.255	0.267	0.263
		1	2	3	4	5	6	7	8	9	10
Mean		0.268	0.282	0.281	0.256	0.238	0.272	0.236	0.236	0.238	0.250
Variance		0.0004	0.0012	0.0003	0.0003	0.0002	0.0004	0.0001	0.0001	0.0003	0.0004
Std. dev.		0.0190	0.0348	0.0159	0.0181	0.0145	0.0193	0.0113	0.0107	0.0160	0.0200
Coefficient of variati		7.1%	12.4%	5.7%	7.1%	6.1%	7.1%	4.8%	4.5%	6.7%	8.0%

Grand mean **0.256**

Variance of punch means

Variance of 10 means	0.00035
Standard deviation	0.0188
Coefficient of variatic	7.4%

Within punch variance

Mean of 10 variances	0.0004
Standard deviation	0.0191
Coefficient of variation	7.5%

Variance between punches

Var (means) = Var (between) + Var (within)/reps

Variance b	0.0003
Standard d	0.017557
Coefficien	6.9%

Variance between punches from different areas

Var (means) = Var (between) + Var (within)/1

Var between areas	0.00066
Standard deviation	0.025728
Coefficient of variation	10.1%

Table A5.6 The results from the ten punches from a single blood spot run on two plates with randomised columns.

Punch number		1	2	3	4	5	6	7	8	9	10
Replicate	1	0.488	0.474	0.489	0.609	0.565	0.588	0.493	0.562	0.519	0.503
Plate 1	2	0.468	0.487	0.471	0.588	0.563	0.601	0.487	0.609	0.574	0.528
	3	0.493	0.478	0.468	0.570	0.526	0.644	0.511	0.581	0.595	0.535
	4	0.555	0.508	0.465	0.601	0.570	0.653	0.539	0.633	0.566	0.580
	5	0.455	0.474	0.435	0.568	0.514	0.580	0.526	0.556	0.516	0.544
	6	0.530	0.508	0.432	0.637	0.554	0.655	0.552	0.580	0.576	0.547
	7	0.509	0.491	0.461	0.606	0.546	0.611	0.477	0.573	0.524	0.539
	8	0.482	0.470	0.423	0.591	0.556	0.581	0.447	0.526	0.543	0.495
Replicate	1	0.445	0.483	0.471	0.523	0.508	0.445	0.367	0.406	0.464	0.518
Plate 2	2	0.375	0.498	0.445	0.495	0.461	0.385	0.375	0.372	0.410	0.470
	3	0.407	0.489	0.469	0.509	0.487	0.445	0.348	0.369	0.439	0.512
	4	0.454	0.445	0.463	0.504	0.498	0.442	0.438	0.406	0.448	0.445
	5	0.398	0.456	0.457	0.516	0.479	0.411	0.360	0.379	0.426	0.457
	6	0.570	0.661	0.598	0.677	0.601	0.540	0.511	0.504	0.583	0.580
	7	0.449	0.465	0.466	0.486	0.476	0.460	0.363	0.407	0.473	0.457
	8	0.428	0.462	0.474	0.476	0.440	0.436	0.373	0.358	0.464	0.451

Spot number/column	1	2	3	4	5	6	7	8	9	10
Mean	0.469	0.491	0.468	0.560	0.521	0.530	0.448	0.489	0.507	0.510
Variance	0.003	0.002	0.001	0.004	0.002	0.009	0.005	0.010	0.004	0.002
Std. dev.	0.055	0.049	0.039	0.060	0.046	0.095	0.073	0.099	0.062	0.044
Coefficient of variati	11.7%	9.9%	8.3%	10.7%	8.8%	17.9%	16.3%	20.3%	12.2%	8.7%

Grand mean **0.499**

Variance of punch means

Variance of punch me	0.00111
Standard deviation	0.03332
Coefficient of variatio	6.7%

Within punch variance

Mean of 10 variances	0.00425
Standard deviation	0.06519
Coefficient of variation	13.1%

Variance between punches

Var. (means) = Var. (between) + Var. (within)/reps

Variance between are	0.00084
Standard deviation	0.0291
Coefficient of variatio	5.8%

Variance between punches from different areas

Var. (means) = Var. (between) + Var. (within)/1

Var. between areas	0.0333
Standard deviation	0.1825
Coefficient of variation	36.6%

Table A5.7 F test and T test comparing percentage positivities from Lusitu and negative controls

Sera results

F-Test Two-Sample for Variances

	PP_neg_sera	*PP Sera Lusitu*
Mean	0.129205128	0.102374332
Variance	0.002709432	0.001923956
Observations	195	187
df	194	186
F	1.408260936	
P(F<=f) one-tail	0.009478538	
F Critical one-tail	1.270819006	

t-Test: Two-Sample Assuming Unequal Variances

	PP_neg_sera	*PP Sera Lusitu*
Mean	0.129205128	0.102374332
Variance	0.002709432	0.001923956
Observations	195	187
Hypothesized Mean Difference	0	
df	374	
t Stat	5.456045315	
P(T<=t) one-tail	4.43755E-08	
t Critical one-tail	1.648936632	
P(T<=t) two-tail	8.8751E-08	
t Critical two-tail	1.966327545	

Eluted blood spot results

F-Test Two-Sample for Variances

	PP_Neg_BS	*PP_Lusitu_BS*
Mean	0.083475113	0.13510989
Variance	0.001948254	0.002539071
Observations	442	182
df	441	181
F	1.303254146	
P(F<=f) one-tail	0.01484195	
F Critical one-tail	0.809852629	

t-Test: Two-Sample Assuming Unequal Variances

	PP_Neg_BS	*PP_Lusitu_BS*
Mean	0.083475113	0.13510989
Variance	0.001948254	0.002539071
Observations	442	182
Hypothesized Mean Difference	0	
df	301	
t Stat	-12.05093402	
P(T<=t) one-tail	7.38745E-28	
t Critical one-tail	1.649932528	
P(T<=t) two-tail	1.47749E-27	
t Critical two-tail	1.967878234	

Table 5.8 The T test to compare eluted blood spot results from Lusitu and contemporary negative blood spot control samples

F-Test Two-Sample for Variances

	PP neg Blood spot (no BN)	*PP Lusitu Blood spot*
Mean	0.119153005	0.13510989
Variance	0.002084768	0.002539071
Observations	183	182
df	182	181
F	1.217915439	
P(F<=f) one-tail	0.092533791	
F Critical one-tail	0.782802712	

t-Test: Two-Sample Assuming Equal Variances

	PP neg Blood spot (no BN)	*PP Lusitu Blood spot*
Mean	0.119153005	0.13510989
Variance	0.002084768	0.002539071
Observations	183	182
Pooled Variance	0.002311293	
Hypothesized Mean Difference	0	
df	363	
t Stat	-3.170555567	
P(T<=t) one-tail	0.000825436	
t Critical one-tail	1.649061687	
P(T<=t) two-tail	0.001650873	
t Critical two-tail	1.966518539	

Appendix 6 The protocol for the Indirect Fluorescent Antibody Test (IFAT)

IFAT MATERIALS

1. Biologicals

Whole *T. congolense* antigen

2. Reagents

2.1. Stock solutions

PBS (5x)-stock (0.05 M)

NaCl	36 g
Na_2HPO_4.anh.	7.39 g
$Na_2HPO_4.H_2O$	8.33 g
$Na_2HPO_4.2H_2O$	9.27 g
KH_2PO_4.anh.	2.15 g
NaN_3	1 g

Add distilled water to 1000 ml, filter and store at 4 °C

Before use: Dilute stock solution 5x in distilled water i.e. take 200 ml PBS-stock solution and add 800 ml distilled water, this constitutes PBS-working solution. (0.01M pH 7.2.

2.2. Working solutions

2.2.1. Evans Blue

Evans Blue powder	10 mg
PBS-working solution (0.01 M)	10 ml

Renew the solution after 2 weeks.

2.2.2. PBS-BSA (Buffer for plasma dilutions and antigen-coating)

PBS-working solution (0.01 M)	5 ml
Bovine Serum Albumin	10 mg

2.2.3. Mounting medium

PBS-working solution (0.01 M) 5 ml

Bring carefully to pH 8.0 with NaOH 10%, while stirring the solution. Add equal volume of glycerol (Analar, commercial concentration), mix well and store at 4 °C.

Renew the solution every 2 weeks.

IFAT PROCEDURE

2.3. Prepare PBS-working solution 0.01 M.

Prepare PBS-BSA. Effective cleaning of the Teflon-coated slides is achieved by soaking the slides in ethanol (denaturated in ether) for 1 hour. Leave to dry in upright position. Confettis of dried blood (treasury punch hole punches) with a diameter of 6.2 mm are punched out, placed in a microtitre-plate and soaked by adding 100 µl of PBS-BSA solution (giving more or less a 1:50 dilution). Soaking takes 60 minutes, during this period the tray is shaken on the orbital shaker. Put another microtitre-plate on top of the first one during the shaking, to avoid evaporation of the PBS. The positive and negative reference samples are treated the same way.

2.3.1. Preparation of the antigen

Take the bottle of antigen with the trypanosomes in suspension (**stored at -20 °C**). Shake the bottles for 1 minute. Take 200 µl out of the bottle using a syringe with 21 1/2 G needle or automatic pipette and transfer this to test-tubes. Add 40 µl PBS-BSA to the 400 µl antigen suspension (enough for 15 slides) and Vortex. Pass this mixture through a 25G needle to break up all the clumps.

NOTE : According to the instructions *T. congolense* can be mixed with *T. vivax*, in which case 200 µl of *T. congolense* and 200 µl of *T. vivax* antigen is brought together in the test-tube, to this 400 µl of antigen we add 80 µl of PBS-BSA (on principle, 1 µl PBS-BSA is added to each 5 µl antigen).

2.3.2. Coating of the antigen

Take 50 µl of the antigen suspension in an automatic pipette (P100), place a bubble on each spot of the Welcome-Teflon® slide, suck it up again and do the same for each spot. Vortex the antigen each time after coating 2 slides. Use separate slides for each antigen, or use a mixture of antigens. Leave the slides to dry at 37 °C.

2.3.3. Rehydration of the antigen

Rehydrate the antigen-preparations for 15 minutes by dropping (Pasteur pipette) 25 to 50 µl of PBS onto each reaction area. After this period shake off the buffer by tapping the edge of the slide against a sheet of absorbent paper.

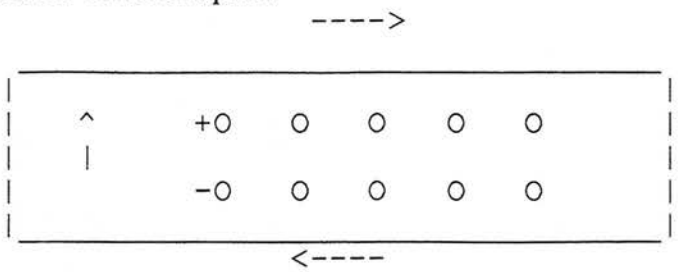
Take the test-sera plus a negative and positive reference serum, dilute the sera 1:40 (975 µl PBS-BSA plus 25 µl serum). Vortex mix.

2.4. Application of the samples.

Take 25 µl of each plasma or confetti dilution and transfer onto the Teflon slide.

Reference sera: Positive and negative controls at 1:40 are placed respectively on upper left spot and spot below to allow detection of any seepage under Teflon-layer. Eventually add lymphocyte lysate-control as well.

Application of the samples :



Leave slides for 30 min in humid chamber at room temperature (not more than 30 minutes). Hereon until mounting, slides should never be allowed to dry!

2.4.1. Washing procedure:

Buffer: plain and properly diluted PBS (kept at 4 °C)

Procedure: tip off the sera, transfer the slides to a Coplin Jar filled with buffer beforehand. Once all the slides are rinsed in this way transfer them to another Coplin Jar, filled with buffer beforehand. Leave the slides for 15 minutes. Transfer the slides to another Coplin Jar, filled with buffer beforehand, and leave for at least another 15 minutes. Never pour buffer onto the slides, but fill the jars beforehand. total washing time should not be less than incubation time. If necessary slides can be left in washing buffer for 1 hour or more.

2.4.2. Conjugate

Conjugate is stored in aliquots of 100 µl (or less) at -20 °C, in the deep freezer. Take 400 µl of the Evans Blue stock solution in an aliquot-tube. Centrifuge both the conjugate- and the Evans Blue-aliquot tubes at 4000 RPM for 5 min. Fill test-tube with 3.55 ml PBS. Clip off the tops of both aliquot tubes and extract 400 µl Evans Blue and 50 µl conjugate. Add both to the 3.55 ml PBS and vortex.

This brings the final concentration of conjugate to 1:80 and Evans Blue to 1:10,000

Take the slides out of the washing solution, tip off washing fluid but do not wipe off.

Put 25 µl of the conjugate solution on each spot using a 25 G needle. Incubate slides for 30 minutes in humid chamber in the dark at room-temperature.

2.4.3. Washing procedure.

Using plain and properly diluted PBS Buffer(kept at 4_C), tip off the sera, transfer the

slides to a Coplin Jar filled with buffer beforehand. Once all the slides are rinsed in this way transfer them to another Coplin Jar, filled with buffer beforehand. Leave the slides for 15 minutes.

Transfer the slides to another Coplin Jar, filled with buffer beforehand, and leave for 15 at least another minutes.

2.4.4. Mounting

Take slides out of jar and tip off washing fluid. Put 3-4 drops mounting fluid at the level of the left row of the spots. Take a coverslip, bring left side in contact with the mounting fluid and put the coverslip slowly down, while inclining the slide slightly downwards to the right. Remove air-bubbles by sliding the coverslip sideways. Remove all excess of the mounting fluid by blotting the edges so that the coverslip does not float any more.

Slides are now ready for microscopy, but can be stored overnight in the dark at 4 °C, if necessary.

2.4.5. Microscopy

Switch on the microscope at least 15 minutes before usage. The best combination of optics is 6.3 x 40 Phaco oil (but 6.3 x 63 with Phaco oil may be used if necessary).

Use immersion oil of good quality. According to the lenses available, water immersion gives an equally clear image (500x). Check orange-coloured zone between spots for seepage under Teflon. Score fluorescence.