# EVALUATION OF DIAGNOSTIC TESTS FOR *TRYPANOSOMA EVANSI* AND THEIR APPLICATION IN EPIDEMIOLOGICAL STUDIES IN INDONESIA

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## DECLARATION

I declare that this thesis has been composed by myself and that I have fully acknowledged contributions by other persons to the work conducted.

## ABSTRACT

The diagnosis of *Trypanosoma evansi* infections is problematic because low, fluctuating parasitaemias are typical of sub-acute and chronic infections. Antigen-detection ELISAs (Ag-ELISAs) that have been developed to detect trypanosomal antigens in serum are reported to be better indicators of current *T. evansi* infections than either parasitological or antibody-detection tests. Two *T. evansi* Ag-ELISAs based on different monoclonal antibodies (2G6 Ag-ELISA and Tr7 Ag-ELISA) were evaluated using buffaloes in Southeast Asia, where *T. evansi* is endemic and livestock are important for draught power, meat and investment. The two Ag-ELISAs were standardised in the UK, following international guidelines on data expression and quality assurance. Diagnostic sensitivities were estimated using buffaloes either experimentally infected (n=35) or naturally infected (n=139) with *T. evansi* and compared with estimates obtained for the microhaematocrit test (MHCT), mouse inoculation (MI), three antibody-detection tests (IgM ELISA, IgG ELISA and card agglutination test {CATT}). Diagnostic specificities were estimated with non-exposed British cattle (n=249) and Australian buffaloes (n=263), and positive and negative predictive values were calculated. Field studies were conducted in Central Java to estimate prevalence and true incidence rates of *T. evansi* infections in buffaloes. No previous studies have compared two *T. evansi* Ag-ELISAs, estimated the prevalence of *T. evansi* infections in multiple villages within a district or true incidence rates.

The repeatability and robustness of the two Ag-ELISAs were shown to be high. Profiles of antigenaemia varied between individual buffaloes and between the two Ag-ELISAs. Antigen and antibody responses were first detected 7 to 42 days after infection, but in some buffaloes responses fluctuated below cut-off values during infection, whilst in other buffaloes antigen and antibody responses persisted after trypanocidal drug treatment. With the naturally-infected buffaloes, the diagnostic sensitivity estimate of the Tr7 Ag-ELISA (81%) was significantly higher than that of the 2G6 Ag-ELISA (71%), and the IgG ELISA sensitivity (89%) was significantly higher than either the IgM ELISA or CATT sensitivities (78%). The diagnostic specificity estimates obtained with the British cattle were 83% for the 2G6 Ag-ELISA and 78% for the Tr7 Ag-ELISA, and with the Australian buffaloes were 75% for the 2G6 Ag-ELISA, 78% for the Tr7 Ag-ELISA, 100% for the CATT, 89% for the IgM ELISA and 92% for the IgG ELISA. Only slight agreement was found between the two Ag-ELISAs (*kappa* = 0.20), but moderate agreement between the IgG ELISA and CATT (*kappa* = 0.58). Positive and negative predictive values ranged from 24% to 99% for prevalence values from 10% to 90%, and true prevalence was underestimated at higher test prevalence values and overestimated at lower test prevalence values.

In Central Java, 2387 buffaloes were blood sampled in 59 villages, and estimates of test prevalence were 4% with the MHCT, 9% with MI, 58% with the 2G6 Ag-ELISA and 70% with the Tr7 Ag-ELISA, but prevalence values differed between districts and between villages. True incidence rates per animal-year at risk were 0.44 with the Tr7 Ag-ELISA and 0.22 with the 2G6 Ag-ELISA. Of 239 market buffaloes sampled, 10% were parasitaemic, 39% antigenaemic, 56% positive by IgG ELISA and 47% positive by CATT, representing an important source of *T. evansi*.

The *T. evansi* Ag-ELISAs and antibody-detection tests used in this study have many advantages as screening tests over commonly used parasitological tests, in terms of their diagnostic sensitivity and ability to rapidly test large numbers of samples. The two *T. evansi* Ag-ELISAs could be applied in high prevalence areas, whilst antibody-detection tests (in particular, the IgG ELISA or CATT) would be more appropriate to test buffaloes in low prevalence areas or to confirm the negative-status of buffaloes prior to movement within Indonesia or export. Future work should aim to improve the specificities of the Ag-ELISAs, which were low in this study in contrast to previous reports. The CATT had a high positive predictive value even with low prevalence and could be adapted more readily to test individual buffaloes in the field. The selection of diagnostic tests for *T. evansi* depends not only on test validity parameters, but also on the prevalence of *T. evansi* in the test population, the principal testing objectives and practical considerations.

To my mother, Lilas Davison,

and to my late father, Dr William Davison.

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| | |
|---|---|
| Ab-ELISA | Antibody-detection enzyme-linked immunosorbent assay |
| Ag-ELISA | Antigen-detection enzyme-linked immunosorbent assay |
| APC | Antigen presenting cell |
| BCT | Buffy coat test |
| BS | Body score |
| BW | Body weight |
| CATT | Card agglutination test |
| CBPP | Contagious bovine pleuropneumonia |
| CFT | Complement fixation test |
| CI | Confidence interval |
| CTVM | Centre for Tropical Veterinary Medicine |
| CV | Coefficient of variation |
| DEAE | Diethylaminoethyl cellulose |
| DNA | Deoxyribonucleic acid |
| EDTA | Ethylene diaminetetraacetic acid |
| ELISA | Enzyme-linked immunosorbent assay |
| FAO | Food and Agriculture Organisation of the United Nations |
| HRPO | Horseradish peroxidase |
| $I$ | True incidence rate |
| IAEA | International Atomic Energy Agency |
| IFAT | Immunofluorescent antibody test |
| Ig | Immunoglobulin |
| IHA | Indirect haemagglutination test |
| ILRI | International Livestock Research Institute |
| INI ANSREDEF | Indonesia International Animal Science Research and Development Foundation |
| $k$ | *Kappa* value |
| kDa | Kilodalton |
| kDNA | Kinetoplast DNA |
| LCL | Lower control limit |
| mAb | Monoclonal antibody |
| MHCT | Microhaematocrit test |
| MI | Mouse inoculation test |
| NPV | Negative predictive value |
| OD | Optical density |
| OIE | Office International des Epizooties |
| $P$ | Probability |
| P | True prevalence |
| $P^T$ | Test prevalence |
| PBS | Phosphate buffered saline |
| PSG | Phosphate buffered saline glucose |
| PCR | Polymerase chain reaction |
| PCV | Packed-cell volume |
| pers. comm. | personal communication |
| PP | Percent positivity |
| PPV | Positive predictive value |

| | |
|---|---|
| *R* | Intraclass correlation coefficient |
| RAPD | Random amplification of polymorphic DNA |
| RIA | Radioimmunoassay |
| SD | Standard deviation |
| SE | Standard error |
| TMB | Tetramethylbenzidine dihydrochloride |
| UCL | Upper control limit |
| UK | United Kingdom |
| VAT | Variable antigen type |
| VSG | Variable surface glycoprotein |
| WBF | Wet blood film |
| WHO | World Health Organization |

# CHAPTER 1

## INTRODUCTION

During the last 100 years, much research has been undertaken on human and animal trypanosomoses, which are a group of diseases caused by infection of susceptible hosts by protozoan parasites called trypanosomes. Transmission of these parasites between hosts is principally by haematophagous flies, of which tsetse flies are important in sub-Saharan Africa. Trypanosomoses are commonly divided into tsetse-transmitted trypanosomoses and non-tsetse-transmitted trypanosomoses and the former have received substantial research funding because of the devastating effect of this group of diseases on people (e.g., human sleeping sickness) and livestock (e.g., nagana) in Africa. In South America, a non-tsetse-transmitted trypanosomosis called Chagas' Disease, caused by *Trypanosoma cruzi,* is a common problem in people living in poor quality housing which provides a habitat for the reduvid bug vector. The importance of non-tsetse-transmitted animal trypanosomoses (NTTAT), which include surra caused by *Trypanosoma evansi*, has been acknowledged recently by the formation of an *ad hoc* NTTAT International Working Group of the Office International des Epizooties (OIE), Paris.

*Trypanosoma evansi* is a protozoan parasite that can infect a range of host species including buffaloes, cattle, horses, camels, sheep, pigs and goats, causing a disease commonly known as surra. By contrast with the tsetse-transmitted trypanosomes found in Africa, *T. evansi* can be transmitted by a variety of biting flies, in particular those of the family Tabanidae, and is widely distributed in Africa, the Middle East, Asia, and Central and South America. Changing disease patterns of surra, particularly in terms of morbidity and case-fatality rates, have been observed in many countries over the last century. Earlier this century, epidemic outbreaks of surra with high case-fatality rates were first reported in horses and camels in India and then in buffaloes, cattle and horses in Indonesia and other countries of South-east Asia. Today, surra is endemic in many regions, but epidemics of acute trypanosomosis with high case-fatality rates continue to be reported in some countries, for example China

and Vietnam. Surra is considered to be an important disease of livestock by Indonesian and other national veterinary services, but its economic impact on the livestock industries of Southeast Asia has yet to be fully evaluated.

A variety of laboratory and field tests for trypanosomosis have been used for routine diagnosis and in epidemiological studies. Early diagnostic tests were based on the detection of trypanosomes in blood, but many chronically-infected animals have low, fluctuating parasitaemias which are not detected by these tests. With advances in immunological techniques, the detection of infected animals was improved by the development of antibody-detection tests to detect immune responses of the host. The diagnostic sensitivities of these tests, which include the complement fixation test (CFT), immunofluorescent antibody test (IFAT) and enzyme-linked immunosorbent assay (ELISA), are higher than the sensitivities of standard parasitological tests. However, serum antibodies are not detectable in early infections and antibody responses remain high after chemotherapy, and differentiation between trypanosome species is limited by cross-reactivity of heterologous antigens and antibodies.

More recently, trypanosomosis antigen-detection ELISAs (Ag-ELISAs), which have the potential to be better indicators of current infection status than antibody-detection tests, have been developed. The Ag-ELISAs have typical advantages of ELISA systems including the ability to rapidly and economically test large numbers of samples. The use of defined monoclonal antibodies in these Ag-ELISAs to capture serum trypanosomal antigens released during infection enables improved standardisation between test runs and between laboratories compared with the crude antigen preparations usually employed for antibody-detection tests.

The epidemiology of *T. evansi* in many countries is not well understood and attempts to investigate it further have been restricted by the lack of suitable diagnostic tests with a high sensitivity and specificity. Previous studies have relied on parasitological tests or antibody-detection tests that have the limitations described above and that frequently have poorly defined diagnostic sensitivity and specificity. Trypanosomosis Ag-ELISAs have been reported to detect earlier stages of infection and to give

negative results more rapidly after effective chemotherapy than antibody-detection tests. However, these findings were based on studies to evaluate *T. evansi* Ag-ELISAs that used small numbers of experimentally-infected animals or animals of unconfirmed infection status living in endemic areas.

Improved diagnostic tests of known diagnostic sensitivity and specificity, therefore, need to be developed to optimise the outputs of future epidemiological studies on *T. evansi* and for assessment of the economic impact of the disease. The aim of this project was to evaluate two Ag-ELISAs for the diagnosis of *T. evansi* infections of buffaloes (and cattle) and to apply these tests to estimate the prevalence and incidence of *T. evansi* infections in an area of Central Java with a high density of buffaloes. Buffaloes and cattle represent a valuable investment of farmers and provide draught power, meat, and milk in this region of Indonesia and elsewhere in Southeast Asia.

Different monoclonal antibodies were employed in the two *T. evansi* Ag-ELISAs which were established in Indonesia after their initial standardisation and optimisation in the United Kingdom. The diagnostic sensitivity and specificity of the *T. evansi* Ag-ELISAs were estimated using buffaloes experimentally infected or naturally infected with *T. evansi* and buffaloes from Australia, where *T. evansi* is not found. Commonly-used parasitological and antibody-detection tests were also evaluated to compare their test performance with the *T. evansi* Ag-ELISAs, and diagnostic parameters including diagnostic sensitivity and specificity, *kappa*, and positive and negative predictive values were estimated.

# CHAPTER 2

# LITERATURE REVIEW

## 2.1 Introduction

Of all pathogenic trypanosome species, *T. evansi* has the largest geographical distribution and can infect a wide range of mammals (Luckins, 1988). Several reviews have been published that have comprehensively outlined previous trypanosomosis research (Mulligan and Potts, 1970; Hoare, 1972; Mahmoud and Gray, 1980; Luckins, 1988; Sileghem *et al.,* 1994). In this review, the diagnosis and epidemiology of animal trypanosomoses, and in particular of *T. evansi* in South-east Asia, are focused on and information is given on the morphology and molecular characteristics of trypanosomes, the host's immune response and pathological changes that occur following infection. The potential economic impact of the disease (surra) caused by *T. evansi* and different control strategies are discussed, and background information is given on farming systems used in the area of Central Java where the project work was undertaken.

In 1880, Evans (1881, 1882) was the first to attribute disease, a wasting condition of horses and camels he observed in India, to trypanosomal infection, although it was not until 1888 that this organism was classified within the genus *Trypanosoma* by Balbiani (cited by Hoare, 1972) and later was called *T. evansi*. *Trypanosoma evansi* has been assigned many different synonyms based on different hosts and geographical locations; for example, *T. equinum*, *T. hippicum* and *T. venezuelense* (Wells, 1984). It was hypothesized that *T. evansi* evolved from *T. brucei* through the adaptation of *T. brucei* strains carried away from tsetse fly habitats, possibly by infected camels and cattle on the trade routes between Africa and Asia (Hoare, 1972). This theory has been supported by the similarity found between isoenzyme electrophoretic patterns of *T. brucei* and *T. evansi* (Gibson *et al.,* 1983). *Trypanosoma evansi* and other salivarian trypanosomes are thought to have been introduced into South America with

imported domesticated animals such as zebu cattle and horses from Africa and water buffaloes from India (Wells, 1984).

Several names have been used to describe the trypanosomoses of domesticated and wild animals. Nagana is the disease caused by tsetse-transmitted trypanosomes (*T. brucei*, *T. congolense*, *T. vivax*), dourine in horses is caused by *T. equiperdum*, and surra by *T. evansi*. The name surra is derived from a local term in India "sur gia" meaning 'gone rotten' (Evans, 1881,1882), and in Africa, *T. evansi* infections are known by various Arabic names, including mbori, salaf, debab, and tahaga (Hoare, 1972).

## 2.2 Classification

Trypanosomes of veterinary importance can be divided into two groups, namely Salivaria and Stercoraria, on the basis of their mode of development in the insect vector and host species (Hoare, 1964). Salivarian trypanosomes (e.g., *T. brucei, T. congolense* and *T. vivax*) complete their development in the anterior part of the vector and the infective metacyclic stage of the parasite is transmitted in saliva of the fly during feeding. The infective stages of stercorarian trypanosomes (e.g., *T. theileri*) are present in the faeces of the vector and transmission is by faecal contamination. *Trypanosoma evansi* and *T. equiperdum* are classified as salivarian trypanosomes within the sub-genus *Trypanozoon* with *T. brucei,* but unlike the latter trypanosome they are not capable of cyclical development within insect vectors.

Table 2.1 lists the main characteristics of principal trypanosomes of veterinary importance. In addition to the distribution indicated in Table 2.1, *T. evansi* has been also found in Turkey and the Transvolga and Caucasus regions of Russia and *T. equiperdum* has been reported in Poland, Italy and Russia. *Trypanosoma theileri* infection of livestock is common in Indonesia (Partoutomo, 1993) and many other countries including the United Kingdom, but usually is not pathogenic. However, there have been reports of clinical trypanosomosis in a calf in Ireland (Doherty *et al.,* 1993) and in a cow in Iran (Seifi, 1995) infected with *T. theileri.*

Trypanosome infection of a susceptible animal may cause acute, subclinical or chronic disease, or may be inapparent with a latent infection. The term trypanosomosis has recently been introduced to replace trypanosomiasis in the standardisation of terminology for parasitic diseases (Kassai *et al.*, 1988) and therefore will be used in this thesis. Trypanosomiasis has been used commonly in the past to describe the infection status of animals, but without distinguishing between trypanosome infection with disease and trypanosome infection without clinical signs.

**Table 2.1** Trypanosomes of veterinary importance

| Group | Species | Host animals | Mode of transmission | Distribution |
|---|---|---|---|---|
| Salivaria | *T. brucei, T. vivax* *T. congolense* *T. simiae, T. suis* | Wide range of domesticated animals | Cyclically by tsetse flies | Sub-Saharan Africa |
| Salivaria | *T. vivax* | Domesticated ruminants | Mechanically by haematophagous flies | Africa, South America, Mauritius |
| Salivaria | *T. evansi* | Wide range of domesticated animals | Mechanically, principally by haematophagous flies | Africa, Asia, the Middle East, South and Central America |
| Salivaria | *T. equiperdum* | Horses | Venereally | Tropics and sub-tropics |
| Stercoraria | *T. theileri* | Domesticated and wild bovines | Via faeces of infected vector | Temperate and tropical areas |
| Stercoraria | *T. cruzi* | Man, dogs, cats, pigs | Via faeces of infected reduvid bugs | Central and South America |

Adapted from Hunter and Luckins (1990)

## 2.3 Morphology and molecular characterisation

*Trypanosoma evansi* has the typical morphology of other *Trypanozoon* trypanosomes. In blood smears stained with standard Romanovsky methods, the parasite is seen as long, thin, curving trypomastigotes. Typically *T. evansi* is monomorphic although stumpy forms have been reported (Hoare, 1972). The flagellum, kinetoplast and nucleus are used to differentiate *T. evansi* from other trypanosome species, with the

exception of slender forms of *T. brucei* which are morphologically similar. *Trypanosoma evansi* has an elongated nucleus, small subterminal kinetoplast, and a flagellum that extends along the length of the body to beyond the anterior end where it forms a 'free flagellum' (Stephen, 1986). Electron microscopic studies have shown details of the plasma membrane, outer surface coat of glycoproteins, basal body of the flagellum, nucleus, kinetoplast and Golgi apparatus (Vickerman and Tetley, 1977).

Strains of *T. evansi* from diverse geographical areas are morphologically indistinguishable but phenotypic differences have been identified by characteristic banding patterns of soluble isoenzymes (Gibson *et al.*, 1983; Boid and Mleche, 1985; Boid, 1988; Lun *et al.*, 1992). The homology of isoenzyme patterns found in different populations of *T. evansi* is consistent with the theory that *T. brucei* is a progenitor of *T. evansi* (Gibson *et al.*, 1980).

The kinetoplast DNA (kDNA) consists of unusual mitochondrial DNA that is organised into a complex network of circular molecules called maxicircles and minicircles (Simpson, 1986) However, it has been shown that *T. evansi* differs from other trypanosomes by its lack of maxicircle kDNA (Lun and Desser, 1995). The minicircle sequences found in different isolates of *T. evansi* are similar (Borst *et al.*, 1987) which further supports the theory of *T. evansi* originating from *T. brucei*, possibly from a mutant lacking maxicircle kDNA (Lun *et al.*, 1992; Lun and Desser, 1995).

Molecular characterisation that detects differences in chromosome DNA by pulsed-field gradient gel electrophoresis is more capable of distinguishing between *T. evansi* isolates than isoenzyme patterns and analysis of kinetoplast DNA (Lun *et al.*, 1992; Waitumbi and Young, 1994). Characterisation of trypanosome isolates has been further refined by the development of specific hybridization probes. Trypanosomes have been typed using polymerase chain reaction (PCR) techniques involving arbitrary priming of DNA or AP-PCR (Waitumbi and Murphy, 1993) or random amplification of polymorphic DNA or RAPD (Majiwa *et al.*, 1993).

## 2.4 Antigenic variation

Trypanosomes have antigens that are classified as nonvariable or common antigens (e.g., some structural proteins) and variable antigens (e.g., surface coat glycoproteins). The variant surface glycoprotein (VSG) of bloodstream-form trypanosomes and infective metacyclic trypanosomes of cyclically transmitted trypanosomes is important in the immune response of the host and has been investigated intensively (Cross, 1975; Gray and Luckins, 1976; Doyle, 1977). The VSG consists of about 500-600 amino acids and is anchored at the carboxy terminus to the plasma membrane of both the trypanosome body and flagellum (Vickerman and Luckins, 1969; Maizels, 1990; Cross, 1990). Variant-specific antigenic epitopes are found at the N-terminus, however, cleavage of the molecules attaching VSGs can expose cryptic cross-reactive determinants (Shak *et al.*, 1988).

Following infection of the host, different variable antigenic types (VATs) of trypanosomes appear (Vickerman, 1969, 1978; Cross, 1975), a phenomenon known as 'antigenic variation', by which the parasites are able to evade the immune responses of the host. The VATs develop by sequential expression of different VSGs by insertion of their genes at specific expression sites of the chromosome (Borst and Cross, 1982). A succession of parasitaemic peaks occurs in the blood of the host which is characteristic of trypanosome infections (Gray and Luckins, 1976). Therefore, VSGs are considered important in the regulation of parasitaemia, and more than 1000 VSG genes with an almost unlimited number of possible variations have been detected using hybridisation studies (Van der Ploeg *et al.*, 1982). Strains of trypanosomes, known as serodemes, can be characterised immunologically by agglutination tests and express a different 'repertoire' of VATs (Lumsden *et al.*, 1973).

## 2.5 Immunology

### 2.5.1 Immune responses to trypanosome infections

The immune response of the host can both modulate current trypanosome infections and provide protective immunity to subsequent trypanosome infections. *Trypanosoma evansi* and the tsetse-transmitted trypanosomes live primarily in the blood of the host,

do not have intracellular parasitic stages, and therefore are targeted by antibodies that control the level of parasitaemia in the host. However, the parasite is able to evade the immune response by antigenic variation, described above. Both T cell-dependent and T cell-independent B cell responses directed at specific VSG epitopes are likely to be involved in this process (Reinitz and Mansfield, 1990). In T cell-dependent B cell responses, helper T cells are stimulated to produce cytokines (e.g., interleukins and gamma interferon) which regulate B cell and macrophage cell functions (Mansfield, 1994).

Antigens released during the breakdown of trypanosomes interact with B cells and T cells by different pathways. B cells can recognise and bind to free antigen by their surface immunoglobulin molecules. In contrast, T cells have surface receptors that only recognise antigen associated with host proteins of the Class II major histocompatibility molecules (MHC II) which are present on the surface of antigen-presenting cells (APCs; Grencis, 1990). Uptake and presentation of antigen can occur by a variety of APCs widely distributed throughout the host tissues, including macrophages, endothelial cells, Langerhans cells, B lymphocytes, T cells, gut epithelial cells, fibroblasts and dendritic cells.

Trypanosomes and their breakdown products are removed from host tissues and the peripheral circulation by mononuclear phagocytic cells. In mice, the clearance of radiolabelled *T. brucei* was shown to be primarily by antibody-mediated macrophage uptake in the liver, and uptake was reduced in irradiated mice with lower circulating antibody levels (MacAskill *et al.*, 1980). Dempsey and Mansfield (1983a) also showed that hepatic clearance of *T. rhodesiense* infections in mice was mediated by both IgM and IgG antibodies, and that normal levels of complement were not required.

Some B cell responses and specific antibody production are suppressed during trypanosome infections. However, other humoral components of the immune system are activated, and involve: 1) an increase in the cellularity of the spleen (B cells and macrophages) and lymph nodes; 2) an increase in the number of some B cells; 3) an increase in immunoglobulins, especially IgM; 4) the appearance of antibodies

(heterophilic antibodies) that react with non-trypanosomal antigens; and 5) the presence of autoantibodies, rheumatoid factors and immune complexes (Sileghem *et al.*, 1994). There is a rapid expansion of B cells producing polyreactive low-affinity IgM antibodies while the generation of B cells producing VAT-specific, monoreactive, high-affinity IgM and IgG antibodies is limited (Casali *et al.*, 1987; Casali and Notkins, 1989). Much of the current knowledge about the immune response of the host to trypanosome infections has been obtained using either mouse models or naturally infected humans; only relatively recently have findings from trypanosome-infected cattle been reported.

In trypanosome-infected cattle, total IgM antibodies usually increase more rapidly after infection, and attain a higher level than IgG antibodies (Morrison *et al.*, 1985). Luckins and Mehlitz (1976) found that in cattle experimentally infected with either *T. brucei*, *T. congolense*, *T. vivax* or a mixture of these species, IgM levels increased within 14 days of infection compared with IgG levels which increased by 25-60 days after infection. In some animals, high levels of both immunoglobulin isotypes were maintained for more than one year. The pattern of antibody responses varied between individual cattle, and in some cattle an anamnestic response occurred after superinfection with a second or third species of trypanosome. In a later study, naive Hereford and Boran steers were inoculated with a clone of *T. brucei,* and responded with increases in both IgM and IgG antibodies detected by IFAT that peaked at 14 days post-infection (Morrison *et al.*, 1982). Similar antibody responses were found in other cattle inoculated with irradiated trypanosomes of the same clone.

Monitoring changes in lymphocyte sub-populations during trypanosome infections has been made possible by the recent development of monoclonal antibodies (mAb) specific for lineage-restricted cell surface antigens in cattle. Both Boran cattle infected with a *T. congolense* clone as a primary challenge, and those re-challenged with an homologous clone, showed a reduction in their $CD4^+$ T cell and $\gamma\delta$ T cell populations and the proportion of $CD5^+$ B cells increased (Williams *et al.*, 1991). The proliferation of $CD5^+$ B cells has been correlated with an increase in serum IgM levels, in cattle experimentally infected with a *T. congolense* clone (Naessens and Williams, 1992).

Morrison and colleagues (1982) showed that naive steers inoculated with $10^7$ irradiated trypanosomes of a *T. brucei* clone were resistant to an homologous re-challenge 14 days later, but only partial immunity was given by a lower inoculating dose of $10^6$ trypanosomes. The antibody responses in these immune cattle were primarily directed against exposed VSG antigenic determinants. VAT-specific antibodies have been considered to be the principal components of the host's response that modulate infections, either by limiting parasite replication or by increased removal of parasites from the circulation, and may confer immunity for subsequent homologous infections. (Akol and Murray, 1983).

Most trypanosome infections do not produce a long-lasting immunity, however, because of the ability of the parasites to change their surface VSG molecules which are the main target of the host's immune response. After natural infection of the host, acute trypanosomosis may develop with rapid death, or the host may develop chronic trypanosomosis and later die or be prematurely slaughtered, if not treated. The ability of the host to eliminate an infection ('self-cure') has been reported in experimentally infected animals (Morrison *et al.,* 1985; Williams *et al.,* 1991; Onah, 1992). Self-cure has only occasionally been reported in field infections (Randall and Schwartz, 1936) and the likelihood of it occurring under repeated field challenge is questionable (Luckins, 1988). Furthermore, the confirmation of self-cure is dependent on the sensitivity of the diagnostic test that is used and inapparent infections may go undetected for long periods; animals imported into British zoological collections have been found to be infected many years after their arrival (Hoare, 1972).

Acquired resistance or non-sterile immunity can develop when susceptible cattle become resistant to the effects of trypanosomal infection after repeated natural challenge, although parasites may be found in their blood (Urquhart and Holmes, 1987). Studies in Uganda (Wilson *et al.,* 1975), Kenya (Wilson *et al.,* 1976) and Ethiopia (Bourn and Scott, 1978; Rowlands *et al.,* 1994a) have shown that cattle can develop adequate levels of non-sterile immunity with strategic chemotherapy. Within breeds, the trypanotolerance of individual cattle varies between areas of different trypanosomosis risk and management systems.

Trypanotolerance has been attributed to a variety of host responses including antibody responses (Murray *et al.*, 1982; Pinder *et al.*, 1988), anaemia (Murray and Dexter, 1988) and activity of blood phagocytes (Nantulya, 1986). Trypanotolerant cattle are able to control the parasitaemia and reduce trypanosome-associated pathology, especially anaemia (Murray *et al.*, 1982), and the PCV is suggested as a good indicator of tolerance (Paling *et al.*, 1991).

Genetic characteristics of the host and extrinsic factors can influence the course of trypanosome infections (Murray *et al.*, 1982). It is known that different breeds of cattle vary in their resistance to trypanosome infections (Dolan, 1987) and that there can be marked differences between individual animals of the same breed (Roelants *et al.*, 1983). N'Dama and West African shorthorn cattle breeds are more trypanotolerant than other breeds (e.g., Boran cattle) and research has been conducted into the genetic basis of this resistance, which may be related to the efficacy of the host immune response (Murray *et al.*, 1982). Following experimental re-challenge with an homologous clone of *T. congolense*, N'Dama cattle were able to control the parasitaemia and anaemia more effectively than Boran cattle. These breed differences may have been associated with the decreases in $CD4^+$ T cells, $\gamma\delta$ T cells and neutrophils that occurred in infected Boran cattle, but were not found in the N'Dama cattle (Williams *et al.*, 1991). Furthermore, isotypic responses of N'Dama and Boran cattle to a range of invariant antigens have been investigated and it has been suggested that cattle resistant to trypanosomosis may produce protective antibodies against some invariant antigens (Authie *et al.*, 1993b). N'Dama cattle were shown to produce $IgG_1$ antibodies against a 33 kDa cysteine protease of *T. congolense* which were not detected in susceptible Boran cattle and which may protect against pathological changes associated with this protein (Authie *et al.*, 1993a).

Some workers have questioned the theory that resistant cattle have enhanced VAT-specific antibody responses, and thereby are able to control trypanosome infections. Boran and N'Dama cattle experimentally infected with a *T. congolense* clone had similar antibody titres and isotypes against surface VSG epitopes

(Williams *et al.*, 1996). However in this study, the N'Dama cattle did have higher $IgG_1$ responses to cryptic VSG epitopes exposed by formaldehyde fixation and reduced non-specific IgM responses compared with Boran cattle.

### 2.5.2 Immunosuppression

Immunosuppression has been reported in *T. evansi*-infected livestock and is a characteristic feature of tsetse-transmitted trypanosomoses. However, the underlying immunological mechanisms of immunosuppression are not clearly defined, although a number of hypotheses have been put forward (Sztein and Kierszenbaum, 1993; Sileghem *et al.*, 1994). Both modulation of the primary trypanosome infection and the host's response to other pathogens can be affected. In trypanosome-infected cattle, opportunistic pathogens can cause death rather than trypanosomosis *per se*.

Early studies showed that mice infected with *T. brucei* did not develop a strong antibody response to injected sheep red blood cells (Goodwin, 1970). The production of VAT-specific antibodies was suppressed in mice re-challenged with a homologous clone of *T. rhodesiense,* although the degree of suppression depended on the stage of the primary infection at which the secondary infection was given (Dempsey and Mansfield, 1983b). *Trypanosoma rhodesiense*-infected mice, however, were able to remove trypanosomes of a homologous challenge from circulation, suggesting that the mononuclear phagocytic system was still functional (Dempsey and Mansfield, 1983a). Much of the experimental work on immunosuppression has been conducted using mouse models, and extrapolation of these results to bovine trypanosomosis without further studies in cattle has been questioned (Sileghem *et al.*, 1994).

There are a few reports of immunosuppression in livestock associated with natural or experimental *T. evansi* infections. Onah (1992) showed that antibody and cell-mediated responses were reduced in sheep experimentally infected with *T. evansi* following *Pasteurella multocida* vaccination. In Thailand, an increased prevalence of brucellosis was observed in buffaloes with natural *T. evansi* infections (Bajyana Songa *et al.*, 1987a), and in Indonesia *T. evansi*-infected buffalo calves were reported to be predisposed to sarcoptic mange (Partoutomo, 1993).

*Trypanosoma congolense* infection in cattle was shown to suppress antibody responses to *Brucella abortus* (S-19) vaccine (Rurangirwa *et al.*, 1983) and both experimental and natural *T. congolense* infections slightly reduced antibody responses to clostridial vaccines (Holmes *et al.*, 1974). In another study, cattle infected with either *T. congolense* and/or *T. vivax* isolates had slightly depressed antibody responses after contagious bovine pleuropneumonia (CBPP) booster vaccination, but up to 50% of the trypanosome-infected cattle succumbed to CBPP after exposure to infection whereas none of the vaccinated control cattle were affected (Ilemobade *et al.*, 1982).

Two hypotheses have been proposed to explain the reduction in antibody titres found in trypanosome-infected cattle: 1) increased catabolization of serum immunoglobulin (Nielsen *et al.*, 1978) and 2) antigenic competition (Nantulya *et al.*, 1982). However, it is now clear that T cell-dependent mechanisms are also involved. Impaired cytokine (interleukin 2 and gamma interferon) production may be important in the immunology of bovine trypanosomosis. Macrophages appear to have a central role and may produce an undefined factor that affects T cell function leading to a reduction in interleukin 2 secretion and expression of interleukin 2 receptors, and altered B cell responses (Sztein and Kierszenbaum, 1993; Sileghem *et al.*, 1994).

## 2.6 Clinical signs and pathology

When trypanosome-infected haematophagous flies bite susceptible animals, a cutaneous oedematous swelling called a chancre develops at the bite site. Since very few trypanosomes are transmitted by individual flies, multiplication in the skin increases the numbers of trypanosomes to invade the host's tissues and circulation, and may increase the antigenic heterogeneity by the appearance of additional VATs (Luckins *et al.*, 1992). The antigenic stimulation by multiplying trypanosomes and the resultant cellular infiltration may promote the induction of immunity (Luckins *et al.*, 1992). Cyclically transmitted trypanosomes transform from metacyclic trypomastigotes into bloodstream trypomastigotes prior to entering the bloodstream via the lymphatic system.

Trypanosome infections can cause three distinct disease syndromes in ruminants: 1) anaemia and weight loss; 2) haemorrhagic disease; and 3) meningoencephalitis. Fluctuating parasitaemias are a feature of trypanosome infections and the degree of anaemia is associated with the level and duration of parasitaemia which depend on the trypanosome species and serodeme and host species (Morrison *et al.*, 1985). The PCV of the host can fall to a critical level followed by death of the host or if the parasitaemia declines, the PCV may recover to normal levels with survival of the host.

In Indonesia, *T. evansi* infections in cattle and buffaloes are typically chronic with associated weight loss and anaemia (Dieleman, 1983; Partoutomo, 1993). However, a wide range of other clinical signs have been attributed to *T. evansi* infections in cattle and buffaloes, including fever, salivation, diarrhoea, oedema, jaundice, conjunctivitis, lacrimation, mucopurulent nasal discharge, dyspnoea, alopecia, urticaria, swelling of superficial lymph nodes, abortion and infertility, weakness, incoordination and paralysis (Damayanti, 1991). The effect of *T. evansi* infection on fertility is not fully understood, although there have been reports of cessation of oestrous activity possibly due to body weight loss (Payne *et al.*, 1993) and abortion (Paikne and Dhake, 1972; Lohr *et al.*, 1986).

Although less common, acute disease and high mortality have been reported in Indonesia following movement of livestock between areas with different endemic strains of *T. evansi* (Payne *et al.*, 1990) and after importation of naive stock (Payne *et al.*, 1991d). Factors such as the stress of movement, adverse weather, nutritional deficiencies, physiological changes and concurrent disease may result in clinical trypanosomosis in previously latently infected animals (Lohr *et al.*, 1985; Luckins, 1988). Furthermore, *T. evansi* has itself been suggested as a predisposing factor for disease outbreaks caused by *Clostridium perfringens* in camels (Wernery *et al.*, 1991).

In a study of natural *T. evansi* infections in local and imported buffaloes in Indonesia, non-specific gross pathological findings included carcass emaciation, pale mucous membranes and myocardium and haemorrhagic or congested lungs

(Damayanti, 1993). Histopathological lesions of non-suppurative interstitial myocarditis, multifocal necrosis of spleen and liver, and interstitial pneumonia were considered more specific to *T. evansi* infections.

Some serodemes of *T. brucei* and *T. evansi* can experimentally produce a meningoencephalitis associated with trypanosomes in the CSF (A.G. Luckins pers. comm.; Losos and Ikede, 1972). Inflammatory reactions in the central nervous system (Morrison *et al.,* 1983), myocardium and skeletal muscle (Damayanti, 1991) may be caused by antigen-antibody complexes formed during the host response to *T. evansi* infection.

The degree of anaemia is related to the susceptibility of different breeds of cattle (Murray *et al.,* 1982), and in acute trypanosomosis anaemia is commonly the cause of the host's death (Murray and Dexter, 1988). The development of anaemia has been attributed to several factors including erythrophagocytosis, dyserythropoiesis, macrophage activation, opsonisation of erythrocytes, adsorption of parasite antigens on erythrocytes, changes in the fragility of the erythrocyte membrane and tumour necrosis factor-α (Murray and Dexter, 1988; Sileghem *et al.,* 1994). Specific antibodies may bind antigens on the surface of erythrocytes resulting in their sequestration in the reticuloendothelial system (Kobayashi *et al.,* 1976). Macrophages were observed phagocytosing erythrocytes (Fiennes, 1954) and their activity is thought to be important in extravascular anaemia (Murry and Dexter, 1988).

## 2.7 Diagnosis

A variety of tests have been used for the diagnosis of trypanosome infections in different hosts but an ideal test, in terms of diagnostic sensitivity and specificity and practical application, has yet to be developed (Killick-Kendrick, 1968; Luckins, 1988; Nantulya, 1990). The typical clinical signs of bovine trypanosomosis described above, are not pathognomonic and many trypanosome-infected animals have only mild clinical signs or inapparent infections. In this thesis, diagnostic sensitivity is defined as the proportion of trypanosome-infected animals detected by a test, as distinct from analytical sensitivity (Stites and Rogers, 1991), and diagnostic specificity as the

proportion of uninfected animals which are negative by a test. In areas with a high prevalence of trypanosomosis (e.g., in areas of China with endemic *T. evansi*), the detection of only a proportion of infected animals within a group by a test of relatively lower sensitivity may be adequate, if treatment is given on a herd basis. In low prevalence areas or in the final stages of an eradication programme, the identification of individual infected animals is important.

The requirements of trypanosomosis diagnostic tests depend on many factors including the trypanosome species, host species, geographical location, control strategies employed and the relative cost of mis-diagnosis. Important test characteristics include high diagnostic sensitivity and specificity, high reproducibility and repeatability, user-friendly test protocols and interpretation of test results, and potential for use in laboratories with limited infrastructure and financial resources. Reproducibility describes the ability of a test to produce consistent results when performed in different laboratories (inter-laboratory variation) compared with repeatability which describes the ability of a test to produce consistent results when run two or more times under the same conditions (intra-laboratory variation) (Thrusfield, 1995). The term robustness (Kemeny, 1991) is used in this thesis to describe the repeatability of a test under different conditions including sample processing and storage.

Some diagnostic tests have been developed with the aim of detecting trypanosome-infected animals and differentiating between trypanosome species. Diagnostic tests for trypanosomosis can be divided into: 1) parasitological tests (detection of intact trypanosomes); 2) serological tests (detection of host antibody responses); and 3) tests that detect antigenic or molecular parasite components.

Traditional diagnostic tests relied on the detection of trypanosomes in wet blood films (Evans, 1881, 1882) or blood smears (Hoare, 1972). By these methods trypanosomes can be distinguished from other organisms if experienced workers examine well-prepared smears, but infections are frequently missed because of the low level and fluctuating nature of parasitaemias, particularly in chronic stages of disease

(Killick-Kendrick, 1968; Mahmoud and Gray, 1980). Parasitological tests allow differentiation between some trypanosome species by their characteristic size, flagellum, kinetoplast and motility. For example, *T. theileri* is distinguishable from *T. evansi* by its larger size and slower motility, but *T. evansi* cannot be distinguished morphologically from *T. brucei* or *T. equiperdum*.

To improve diagnostic sensitivity, methods that concentrate trypanosomes in blood samples were developed and continue to be used. Trypanosomes can be directly observed in microhaematocrit capillary tubes after centrifugation (MHCT) (Woo, 1969) or using a modification of this test with darkground phase-contrast microscopy, called the buffy coat technique (BCT; Murray, 1977). A more elaborate concentration method involves separation of trypanosomes from blood using miniature anion-exchange columns (Lumsden *et al.*, 1977).

Some trypanosome species (*T. brucei*, *T. congolense*, *T. evansi*) will proliferate if parasitaemic blood is inoculated into laboratory rodents, and this has been used to increase the detection rate of these parasites. Mouse inoculation (MI) detected 95% of all *T. brucei* infections found in cattle (n = 178), sheep (n = 5) and goats (n = 8) in a survey in Kenya, whereas only a small number were detected by wet blood film examination (Robson and Ashkar, 1972). In Argentina, MI detected 88% and the MHCT detected 71% of *T. evansi* infections in horses (n = 52) (Monzon *et al.*, 1990), but the MHCT had the advantage that it required only limited resources and could be conducted in the field and PCVs were recorded at the same time. An unusual parasitological detection method is xenodiagnosis which has been used experimentally to detect latent trypanosome infections by feeding uninfected vectors on infected hosts, followed by transfer of the vectors to naive hosts or laboratory mice (Masake *et al.*, 1995a).

In general, parasitological tests have a lower sensitivity, are time-consuming and often impractical for testing large numbers of samples. The disadvantages of parasitological tests, together with advances in immunology, stimulated the development of a range of diagnostic tests that detect components of the host's immune response to

trypanosome infections. Both non-specific and specific serological tests have been used for trypanosomoses. The gelatinisation of serum immunoglobulins from trypanosome-infected animals by formalin and mercuric chloride has been used (Bennett and Kenny, 1928), but neither of these tests were specific for trypanosomal infections since immunoglobulins can be precipitated in both trypanosome-infected animals as well as animals infected with other pathogens. More specific serological tests have been developed, including primary binding tests: immunofluoresent antibody test (IFAT) (Wilson, 1969) and antibody-detection ELISAs (Ab-ELISAs) (Luckins and Mehlitz, 1978) and secondary binding tests: complement fixation test (CFT) (Watson, 1920) and indirect haemagglutination test (IHA) (Jatkar and Singh, 1971).

The CFT has been used for the diagnosis of dourine in horses for many years (Watson, 1920) and remains an international reference test for *T. equiperdum* (Luckins, 1992). Problems in antigen preparation and test standardisation have limited its use for other trypanosome species, although it has been used for detection of *T. evansi* infections in buffaloes in the Philippines (Randall and Schwartz, 1936) and in horses, cattle, buffaloes and camels in China (Lun *et al.*, 1993). Caporale and colleagues (1981) compared the CFT with other serological tests for dourine and concluded that the antibody-detection ELISA gave comparable results and used lower quantities of a more stable antigen.

The IFAT was developed using acetone-fixed trypanosomes in blood smears to detect species-specific antibodies in infected cattle (Luckins and Mehlitz, 1978), although some heterologous cross-reactions were found by other workers (Ashkar and Ochilo, 1972). In camels experimentally infected with *T. evansi*, the IFAT was positive from 10-15 days post-infection, and had a higher sensitivity than the formol gel and mercuric chloride tests in both experimentally and naturally-infected camels (Luckins *et al.*, 1979).

Enzyme-linked immunosorbent assays (ELISAs) have several advantages over other diagnostic tests including higher sensitivity, the capacity to test large numbers of

samples and produce quantitative results which are read by a spectrophotometer (Voller *et al.*, 1976). *Trypanosoma evansi* antibody-detection ELISAs gave similar results compared with an IFAT when used to test sera from experimentally-infected rabbits (Luckins *et al.*, 1978), naturally-infected cattle (Luckins and Mehlitz, 1978) and experimentally and naturally-infected camels (Luckins *et al.*, 1979). These antibody-detection ELISAs used crude antigen extracts from sonicated trypanosomes and cross-reactions with heterologous antibodies were found (Luckins, 1977). To overcome this problem, workers have attempted to purify antigenic components by selection of specific internal cytoskeleton proteins by Western Blot analysis (Imboden *et al.*, 1995) or by fractionation of non-protein antigens by column chromatography (Ijagbone *et al.*, 1989). In the latter work, the composition of the trypanosome non-protein antigenic component was not determined but, interestingly, it was more antigenic and specific than the protein components tested. In the future, the use of recombinant antigens could improve specificity and allow better standardisation of antibody tests (Masake *et al.*, 1995b).

In contrast to antibody-detection ELISAs and IFAT which involve reactions between host antibodies and species-specific or common antigens, the card agglutination test (CATT) uses variable surface antigens (e.g., LiTat 1.3) present in salivarian trypanosomes (Bajyana Songa *et al.*, 1987b). The CATT has been used for the diagnosis of *T. evansi* infections in camels (Zweygarth *et al.*, 1984) and in buffaloes, cattle, pigs and sheep (Bajyana Songa *et al.*, 1987a). For testing buffalo sera, rabbit anti-IgG immunoglobulin was added and higher serum dilutions were used to reduce non-specific agglutination. Most infected animals were positive by two weeks after experimental infection, but the authors pointed out that the test only detects antibodies against LiTat 1.3 VAT, and further VATs might need to be included to increase the sensitivity of the test for animals infected with different trypanosome populations (Bajyana Songa *et al.*, 1987a).

The main disadvantages of the serological tests described above are that a positive result does not indicate the current infection status, but only previous exposure of the host to trypanosomes. Moreover, after chemotherapy, antibody levels can remain

elevated for long periods (Luckins *et al.*, 1979; Nantulya *et al.*, 1989). Furthermore, cross-reactions with antibodies against other trypanosome species is a major problem in countries where multiple trypanosome species occur, for example with tsetse-transmitted trypanosomes in Africa (Luckins, 1992). Despite these limitations, serological tests have been widely applied to obtain useful data for epidemiological studies on trypanosomosis, although often without prior test standardisation or validation (Van Meirvenne and Le Ray, 1985).

More recently, antigen-detection ELISAs (Ag-ELISAs) have been developed in which antigen released from trypanosomes is captured using either polyclonal or monoclonal antibodies. Studies describing the use of trypanosomosis Ag-ELISAs are given in Table 2.2. The major potential advantages of these tests compared with antibody-detection tests is their reported ability to identify an active trypanosome infection, as indicated by antigenaemia, sooner after infection, and to monitor clearance of trypanosomes as indicated by the disappearance of antigenaemia, more quickly after chemotherapy (Nantulya *et al.*, 1987). The use of monoclonal antibodies enables these tests to be better standardised compared with antibody-detection tests (Nantulya, 1990).

Rae and Luckins (1984) detected serum antigens in rabbits and goats experimentally infected with *T. congolense* or *T. evansi* by Ag-ELISAs using polyclonal antibodies. A number of monoclonal antibodies against pathogenic trypanosomes have been produced which form the basis of diagnostic tests for detecting infections with African trypanosomes (Nantulya *et al.*, 1987). Nantulya and colleagues (1987) reported that these monoclonal antibodies did not cross-react with heterologous trypanosome species in IFAT or ELISA, although there were reactions with trypanosomes of the same sub-genus. Thus, *T. congolense* monoclonal antibodies reacted with *T. simiae* in the IFAT and *T. brucei* monoclonal antibodies reacted with *T. rhodesiense* and *T. gambiense*. Previous workers also had produced monoclonal antibodies against procyclic *T. congolense,* but these did not recognise antigens of bloodstream forms or live trypomastigotes (Parish *et al.*, 1985).

In recent years, these monoclonal antibodies have been used in several trypanosomosis Ag-ELISAs. Nantulya and Lindqvist (1989) used Ag-ELISAs to detect cattle experimentally infected with *T. brucei*, *T. vivax* or *T. congolense* by tsetse transmission. Antigenaemia was detected by 10-12 days (*T. vivax* and *T. congolense*) and by 8-14 days (*T. brucei*) post-infection, and coincided with the first detectable parasitaemia. It was reported that the Ag-ELISAs captured free serum antigens and may have given false-negative results, if antigen was unavailable because it was bound to antibody in immune complexes.

Antigen-detection tests using monoclonal antibodies which have been modified for field use include a latex agglutination test (Suratex; Nantulya, 1994), antigen-capture tube ELISA (Waithanji *et al*, 1993) and a dot-ELISA (Bosompem *et al.*, 1996). The latex agglutination test was reported by Nantulya (1994) to detect higher numbers of naturally-infected camels (30/32 in one herd, and 60/60 in another herd) than the BCT (5/32 in the first herd) or MI (28/60 in the other herd). In camels experimentally infected with *T. evansi*, antigenaemia was first detectable two to three weeks post-infection by the latex agglutination test, and disappeared three to four weeks after Cymelarsan treatment (Olaho-Mukani *et al.*, 1996). A dot-ELISA using nitrocellulose membranes detected *T. congolense* in 7.7% (8/104) of *Glossina pallidipes* and 4.4% (17/390) of *G. longipennis* (Bosompem *et al.*, 1996). However, the presence of trypanosomes was confirmed by standard dissection techniques in two tsetse flies only.

Recently, the antigen recognised by the *T. vivax* monoclonal antibody (Tv27) developed by Nantulya and colleagues (1987) has been characterised and recombinant antigen produced by expression in *Esherishia coli* and baculovirus systems (Masake *et al.*, 1995b). This *T. vivax* antigen had a molecular weight of 10 kDa when prepared from parasite lysates and was shown to be distributed throughout the cytosol and nucleus of metacyclic bloodstream forms and procyclic trypomastigotes of *T. vivax* but its function is unknown.

The specificity of Ag-ELISAs used to distinguish tsetse-transmitted trypanosomes has been questioned since there have been discrepancies in the proportions of trypanosome species detected by Ag-ELISAs compared with parasitological tests and furthermore, positive Ag-ELISA results have been reported in areas where there was no other evidence of trypanosome infections in the animals tested. For example, in Burkina Faso Ag-ELISA results suggested trypanosome infections in 66% of 1633 cattle, with the majority of infections being *T. congolense* followed by *T. brucei.* However, BCT detected infections in 9% of the cattle which were predominantly *T. vivax* followed by *T. congolense* (Bengaly *et al.,* 1995). Therefore, it is unclear how the proportion of Ag-ELISA positive cattle related to the true proportion of trypanosome-infected cattle and to the true proportions of different trypanosome species.

None of the diagnostic tests currently available for trypanosomoses has a diagnostic sensitivity and specificity of 100%. Without reliable estimates of these parameters, test results cannot be interpreted accurately and can be misleading. The gold standard can be defined as the means by which the presence or absence of a pathogen or disease can be determined (Smith, 1995). However, in the validation of new tests which have a higher sensitivity than the best test available or accepted gold standard, the differentiation of true positives from false-positives in a population of naturally-infected animals is problematic. For diseases for which there is no accepted gold standard, diagnostic tests are evaluated using experimentally-infected animals, and therefore they are assumed to be positive. Potential drawbacks of this approach, include the economic constraints on the number of animals and length of study, and the repeated sampling of individual animals giving measures which are not independent. Furthermore, single clones or isolates of a pathogen are often used, which may have been passaged several times and may not be representative of natural infections, to infect animals which may themselves not be representative of the target population. For example, a screening test should be validated using infected and non-infected animals from the target population, whereas a clinical diagnostic test for the differential diagnosis of diseased animals should be validated using animals with similar clinical signs (Thrusfield, 1995). For trypansomosis, many studies have

compared multiple diagnostic tests because of the lack of a suitable gold standard, and examples of these studies are given in Table 2.3.

Molecular techniques have been applied to the diagnosis of many diseases including trypanosomosis, and in particular for the differentiation of closely related parasites (Nantulya, 1991). The polymerase chain reaction (PCR) has been used to detect *T. evansi*-infected hosts and vectors (Panyim *et al.,* 1992), and has been used to identify *T. godfreyi*, a previously undescribed trypanosome of the *Nannomonas* subgenus (Masiga *et al.,* 1996). The PCR has the potential to detect both inter-species and intra-species differences of trypanosome isolates, but it requires the synthesis of specific pairs of oligonucleotides primers for each system. More recently, modified PCR techniques (AP-PCR and RAPD) have been developed which can detect smaller differences in parasite DNA and do not require information of the target DNA sequence (Waitumbi and Murphy, 1993).

An important advantage of molecular techniques is their ability to differentiate trypanosome species. Transmission studies, which need to detect very small numbers of trypanosomes in insect vectors, have used DNA probes to identify trypanosomes in tsetse flies (Kukla *et al.,* 1987). However, standard tsetse dissection methods are still used because these molecular techniques are not widely available and the DNA probes cannot recognise all intraspecies variants (Bosompem *et al.,* 1996). Although molecular techniques are useful research tools, their use in the routine diagnosis of trypansomosis is unlikely in the near future because of the high cost of equipment and consumables that are required.

## 2.8 Transmission

*Trypanosoma evansi* is mechanically transmitted by biting flies (Luckins, 1988) and lacks genes for mitochondrion formation, which are prerequisites for cyclical development of trypanosomes in the tsetse fly (Borst *et al.,* 1987). In South America, the vampire bat, *Desmodus rotundus,* can act as a vector and carnivores can be infected by ingestion of meat from parasitaemic animals (Hunter and Luckins, 1990). Although transplacental transmission of trypanosomes has been reported, it is not

**Table 2.2** Studies which have used antigen-detection ELISAs for the diagnosis of trypanosome infections

i) Using experimental trypanosome infections

| Trypanosome species | Source of samples | Type of infection | Antibody type | Days PI when first positive | Sensitivity | Specificity | Reference |
|---|---|---|---|---|---|---|---|
| *T. congolense* | Mouse blood (serial dilutions) | | mAb | | | | Parish *et al.*, 1985 |
| *T. congolense* | Goats, cattle | E | mAb | | 94.3%<br>82.5% | 100%<br>(20/20) | }Masake and Nantulya, 1991<br>} |
| *T. equiperdum* | Rabbits, horses | E | mAb | 14d | | | }Bishop *et al.*, 1995 |
| *T. evansi* | Rabbits | E | mAb | 7-14 | | | Frame *et al.*, 1990 |
| *T. brucei* | Rabbits, goats | E | poly | 3 | 1.5 µg/ml | | }Rae and Luckins 1984 |
| *T. congolense* | | E | poly | 3-14 | 1.5 µg/ml | | } |
| *T. evansi* | | E | poly | 3-8 | 1.5 µg/ml | | } |
| *T. vivax* | | E | poly | 3 | 1.5 µg/ml | | } |
| *T. brucei* | *In vitro* cultures | | mAb | | | | }Nantulya *et al.*, 1987 |
| *T. congolense* | | | | | | | } |
| *T. vivax* | | | | | | | } |
| *T. brucei* | Cattle | E | mAb | 8-14 | | | }Nantulya and Lindqvist, 1989 |
| *T. congolense* | | | | | | | } |
| *T. vivax* | | | | | | | } |
| *T. rhodesiense* | Vervet monkeys | E | poly | 7 | | | Liu *et al.*, 1988 |
| *T. rhodesiense* | Vervet monkeys | E | mAb | 14-70 | | | Gichuki *et al.*, 1994 |

E: Experimental infection; mAb: Monoclonal antibody; poly: Polyclonal antibody; PI: Post-infection

**Table 2.2** continued

ii) Using both experimental and natural trypanosome infections or only natural trypanosome infections alone

| *Trypanosoma species* | Source of samples | Type of infection | Location of field infections | Antibody type | Days PI when first positive | Sensitivity | Specificity | Reference |
|---|---|---|---|---|---|---|---|---|
| *T. evansi* | Rabbits, dogs, cattle, goats, pigs, | E | | mAb* | 5-10 (rabbits) | | | }Nantulya *et al.*, 1989a<br>}<br>} |
| *T. evansi* | buffaloes | N | Thailand | mAb* | | | | } |
| *T. evansi* | Horses, mules | E | | mAb* | | | | }Monzon *et al.*, 1995 |
| | | N | Argentina | mAb* | | 74% (58/78) | 100% (60/60) | }<br>} |
| *T. evansi*<br>*T. equiperdum* | Rabbits, horses | E, N | Bolivia | mAb | 7-14 | | | }Bishop, 1992<br>} |
| *T. evansi* | Camels | N | Mali | mAb* | | 94% (16/17) | | }Nantulya *et al.*, 1989b |
| | | | Kenya | | | 90% (18/20) | 100% (30/30) | } |
| *T. evansi* | Camels | N | Kenya | poly | | 60.9% | 97.8% | }Diall *et al.*, 1992 |
| | | | | mAb* | | 83.3% | 100% | } |
| *T. evansi* | Camels | N | Kenya | mAb* | | | | Waithanji *et al.*, 1993 |
| *T. evansi* | Buffaloes, | N | India | poly | | 76% (47/62) | | }Singh *et al.*, 1995 |
| | horses | N | | | | 74% (14/19) | | } |
| *T. brucei*,<br>*T. congolense*<br>*T. vivax* | Cattle | N | Burkina Faso | mAb | | 75% | 98% | }Bengaly *et al.*, 1995<br>}<br>} |
| *T. brucei*<br>*T. congolense*,<br>*T. simiae*<br>*T. vivax* | Tsetse flies | N | Kenya | mAb | | | 100% (6/6) | }Bosompem *et al.*, 1996<br>}<br>}<br>} |
| *T. rhodesiense* | Humans | N | Kenya | mAb | | 90.1% | 100% | Nantulya, 1989 |

*: Group-specific anti-*T. rhodesiense* mAb used for *T. brucei* and *T. evansi* Ag-ELISAs

E: Experimental infection; mAb: Monoclonal antibody; N: Natural infection; poly: Polyclonal antibody; PI: Post-infection

**Table 2.3** Studies using multiple diagnostic tests for trypanosome infections

| Diagnostic tests | Trypanosome species | Sample source | Location | Reference |
|---|---|---|---|---|
| Ag-ELISA, BCT | *T. brucei* | Cattle | | Masake *et al.*, 1995a |
| Ab-ELISA, IFAT, non-specific Ig tests | *T. evansi* | Camels | Sudan | Luckins *et al.*, 1979 |
| CATT, CFT, IHA | *T. evansi* | Buffaloes, cattle, pigs, sheep | Thailand | Bajyana Songa *et al.*, 1987a |
| Ag-ELISA, Ab-ELISA | *T. evansi* | Camels | Sudan | Rae *et al.*, 1989 |
| Parasitological tests | *T. evansi* | Horses | Argentina | Monzon *et al.*, 1990 |
| Ab-ELISA, CFT, IFAT, RIA | *T. equiperdum* | Horses | Italy | Caporale *et al.*, 1981 |
| Parasitological tests | *T. brucei*, *T. congolense, T. vivax* | Cattle, goats, sheep | Kenya | Robson and Ashkar, 1972 |
| Ab-ELISA, IFAT | *T. brucei*, *T. congolense, T. vivax* | Cattle | Liberia | Luckins and Mehlitz, 1978 |
| Parasitological tests | *T. brucei*, *T. congolense, T. vivax* | Cattle, mouse blood (serial dilutions) | | Paris *et al.*, 1982 |
| Ag-ELISA, BCT | *T. brucei*, *T. congolense, T. vivax* | Cattle | Gabon | Trail *et al.*, 1992 |

Ab-ELISA: Antibody-detection ELISA; Ag-ELISA: Antigen-detection ELISA; BCT: Buffy coat technique; CATT: Card agglutination test; CFT: Complement fixation test; IFAT: Immunofluoresence antibody test; Ig: Immunoglobulin; IHA: Indirect haemagglutination assay; RIA: Radioimmunoassay

considered an important transmission route of natural infections (Ogwu and Nuru, 1981) and there is no direct evidence that this mode of transmission has occurred in *T. evansi* infections.

In India, Rogers (1901) showed that *T. evansi* could be transmitted by tabanids fed on susceptible hosts shortly after an interrupted feed on an infected host. Later, observations of camels in India supported the theory that *T. evansi* could be mechanically transmitted and implicated the role of *Lyperosia* and *Stomoxys* species in areas where no significant numbers of *Tabanus* species were found (Leese, 1912). In Indonesia, much of the research on the transmission of *T. evansi* was carried out more than fifty years ago by Nieschulz and his colleagues (Nieschulz, 1926, 1927 and 1928; Nieschulz and Ponto, 1927). This pioneering early work showed that more than 25 *Tabanus* species could experimentally transmit *T. evansi*. More recently, African *Stomoxys* species have been shown to transmit *T. evansi* to Balb/c mice after feeding on parasitaemic blood (Mihok *et al.*, 1995). Flies of other genera of the Tabanidae family, for example *Chrysops* and *Haematopota*, are capable of transmitting *T. evansi* but together with *Stomoxys*, are considered less important vectors of this parasite than *Tabanus* species (Foil, 1989).

Despite this earlier work, data on the transmission of trypanosomes by flies are lacking, and much of the current knowledge is based on studies carried out many years ago (Foil, 1989). Furthermore, changes in habitat as a result of the introduction of intensive agricultural systems such as rice cultivation, will undoubtedly have affected fly populations. The probability of transmission occurring depends on a variety of factors including the species, age and density of the host, the level of host parasitaemia, the trypanosome species, and the species and feeding behaviour of the vector (Foil, 1989). The feeding of flies on *T. evansi*-infected hosts needs to be interrupted and feeding continued on a susceptible host within hours; the shorter the interval, the higher the probability of effective transmission.

In Indonesia, surveys have identified 18 *Tabanus* species, eight *Chrysops* species and seven *Haematopota* species and *Stomoxys* species and *Lyperosia* species

(Sigit *et al.*, 1983). Buffalo flies (*Haematobia exigua*) are numerous, but have not been shown to transmit *T. evansi*. Many areas have suitable habitats for tabanid breeding sites including wet pasture, pools, streams, beaches and irrigated fields and in a tropical climate, two to three fly generations can be produced each year (Chainey, 1993). In West Java, *T. evansi* was found in 25% of *Tabanus rubidus* examined, with the infection rates of trapped flies varying between villages from 0 to 63% (Hartini and Aziz, 1991). Since the early work of Nieschulz, little information has been collected on the distribution of fly populations throughout Indonesia and on their role in the dynamics of *T. evansi* transmission.

## 2.9 Aspects of epidemiology

The epidemiology of the pathogenic animal trypanosomoses involves complex interactions between insects and other vectors, trypanosomes and domesticated and wild animal hosts. In addition, environmental conditions can influence trypanosome infection rates of vectors and hosts, and therefore the rate of trypanosome transmission between them (Molyneux and Ashford, 1983). Aspects of the epidemiology of animal trypanosomoses, in particular the transmission of *T. evansi* and distribution and patterns of the occurrence of *T. evansi* infections in Indonesia, are described below.

The relationship between the likelihood of disease (i.e., surra) and the abundance of biting flies was known by keepers of horses and camels in India (Evans, 1881, 1882), before the transmission route had been proven. Geographical and seasonal variations in the prevalence of *T. evansi* infections in cattle, horses and camels in India were described by Basu (1945).

Several workers have described the natural history of *T. evansi* in Asia (Wilson, 1983; Lohr *et al.* 1985; Luckins, 1988). Wilson (1983) listed 12 parameters that had been used to study the epidemiology of trypanosomoses of cattle, sheep, goats and camels in Kenya and which he suggested could be applied to epidemiological studies on *T. evansi* in Indonesia for the future development of control strategies and estimation of economic losses. The parameters included parasite determinants (parasite attack

rate, mortality rate, weight loss and concurrent diseases), host determinants (breed, age, sex, population at risk, herd structure and stocking density and previous exposure) and other determinants (e.g., management systems). The incidence and pathogenicity of *T. evansi* infections varies with the host species, parasite strain and geographical location (Luckins, 1988). The application of molecular karyotyping techniques could enable the dynamics of different trypanosome populations to be investigated more fully (Waitumbi and Young, 1994).

In Indonesia, although epidemic outbreaks of surra were common in the years following the first report of *T. evansi* in 1897 by Penning (1900), the disease is now endemic. Occasional outbreaks of clinical trypanosomosis do still occur, however, related to animal movement between islands or importation of naive animals or other factors that may promote relapse parasitaemias in apparently healthy animals (Adiwinata and Dachlan, 1969; Payne *et al.* 1990, 1991d).

It is not known why the incidence of acute trypanosomosis has declined so dramatically in Indonesia. Selection of both parasite and host populations may have occurred such that endemic strains now have a lower pathogenicity, and the intensification of agricultural systems may have affected vector habitats (Luckins, 1988). Furthermore, serological studies have shown that the antigenic diversity of *T. evansi* stocks from Indonesia is more limited than those found in African tsetse-transmitted trypanosomes (Jones and McKinnell, 1985), and it has been suggested that this lack of diversity may explain the high morbidity and low mortality now associated with *T. evansi* infections of livestock in Indonesia (Payne *et al.*, 1991c). By comparison, recent outbreaks of trypanosomosis in the Philippines, Vietnam and China have been associated with high case-fatality rates and high morbidity (Luckins, 1988; Lun *et al.*, 1993).

In Indonesia, there is a high prevalence of *T. evansi* infections in buffaloes and cattle, but these species are considered to be less susceptible to acute trypanosomosis than horses (Wilson, 1983; Payne, 1989; Partoutomo, 1993). At field sites on various islands of Indonesia, few horses were found to have trypanosomal antibodies and it

was concluded that those infected had had a low survival rate (Payne *et al.*, 1991c). However, local horses on Sulawesi were reported to survive in endemic trypanosomosis areas, where imported Australian horses required regular trypanocidal treatment (B. Copeman pers. comm.).

More recently, quantitative methods have been applied to the epidemiology of trypanosomoses. For example, using multiple logistic regression a strong association was found between *T. vivax* infections of cattle in Colombia and low-lying swampy areas and tabanid activity (Otte *et al.*, 1994), and in cattle in the Ghibe Valley, Ethiopia, the incidence of new *T. congolense* infections, prevalence of recurrent infections and relapse to treatment were shown to be associated with season, herd, age and sex (Rowlands *et al.*, 1993).

Epidemiological studies, such as those described above, are dependent on the validity of the diagnostic tests. The lack of diagnostic tests with high sensitivity and specificity for trypanosomoses has limited the value of epidemiological studies because of the problem of differentiating trypanosome-infected from non-infected animals and vectors. Many studies have used parasitological tests alone (e.g., Otte *et al.*, 1994), but these tests do not detect animals with a low parasitaemia, and in one study, a low PCV was taken as an indicator of trypanosome infection in cattle with no detectable parasitaemia (Rowlands *et al.*, 1993). In Asia, surveys have been conducted to estimate the prevalence of *T. evansi*, as shown in Table 2.4, and the results are dependent on the diagnostic tests, with higher prevalence estimates obtained by serological tests. Sample sizes are included in Table 2.4 because of their importance for extrapolation of data for regional or national prevalence estimation, but in most studies random sampling was not used and sampling protocols were not given. The precision of prevalence estimates was rarely included in survey results, an exception is the study of *T. evansi* infections in cattle in Cambodia which reported a prevalence of 27% with an associated 95% confidence interval of 22% to 33% (O'Sullivan, 1995).

**Table 2.4** Surveys of *Trypanosoma evansi* infections of cattle and buffaloes in Asia

| Location | Sample size | | Prevalence (%)* | | Diagnostic tests | References |
|---|---|---|---|---|---|---|
| | Cattle | Buffaloes | Cattle | Buffaloes | | |
| Philippines | 425 | 61 | 12 | 25 | CFT | Randall and Schwartz, 1936 |
| Philippines | 2,141 | 2,642 | 3 | 19 | CFT | Topacio and Acevado, 1938 |
| India | 1004 | | 5-32 | | Not given (blood smear?) | Basu, 1945 |
| Philippines | 1,640 | | 1 | | Thick smear | Venus and Dumag, 1967 |
| Indonesia | 179 | 194 | (1) 34<br>(2) 37 | (1) 53<br>(2) 52 | (1) IFAT<br>(2) Ab-ELISA | Luckins, 1983 |
| Thailand | | 1,145 | (1) 17-24<br>(2) 4-15 | | (1) CFT,<br>(2) Thick smear, MI | Lohr *et al.*, 1985 |
| China | | | | (1) 96<br>(2) 78<br>(3) 82 | (1) Ab-ELISA<br>(2) IHA<br>(3) CFT | Shen *et al.*, 1985 |
| Indonesia | 628 | 98 | (1) 6<br>(2) 1 | (1) 7<br>(2) 6 | (1) MHCT<br>(2) MI | Suhardono *et al.*, 1985 |
| Indonesia | 1522 | 276 | (1) 1<br>(2) 44 | (1) 6<br>(2) 48 | (1) MHCT<br>(2) Ab-ELISA | Payne *et al.*, 1991c |
| India | Total of 200 | | (1) 23-46<br>(2) 4-6 | (1) 24<br>(2) 8 | (1) IFAT<br>(2) MI | Ray *et al.*, 1992 |
| China | More than 7 million buffaloes and cattle | | 3-16 | 3-16 | CFT, IHA | Lun *et al.*, 1993<br>(Review of studies 1950-1989) |
| Cambodia | 341 | | 27 | | Ag-ELISA | O'Sullivan 1995 |

*:If more than one test was used, the results for each test are given by a number corresponding to the test;
Ab-ELISA: Antibody-detection ELISA; Ag-ELISA: Antigen-detection ELISA;
CFT: Complement fixation test; IHA: Indirect haemagglutination test;
MHCT: Microhaematocrit test; MI: Mouse inoculation test

The variety of host species which are susceptible to *T. evansi* infections has contributed to the widespread distribution of this parasite. In Indonesia, several wild animals have been experimentally infected with *T. evansi* and the Grey Javan monkey, *Macaca irus mordax*, and several deer species became chronically infected (Kraneveld and Mansjoer, 1952). However in Indonesia, domesticated animals are considered to be the principal reservoirs of *T. evansi*, particularly in areas of intensive agriculture, for example rice cultivation, where there is minimal contact between wild animals and livestock (Luckins, 1988). Dogs can be infected with *T. evansi* (Singh *et al.*, 1993), but usually have a high mortality and in Indonesia feral dogs are not common in most areas.

## 2.10 Control

In the past, trypanosomosis control programmes, particularly for tsetse-transmitted trypanosomoses, have targeted both trypanosome and vector populations. Several drugs have been used for chemotherapy and chemoprophylaxis, but a limited number of trypanocidal drugs are now available, and strains of drug-resistant trypanosomes have developed (Hunter and Luckins, 1990). There have been few national control programmes for *T. evansi* in Asia, but in Africa large-scale programmes have been implemented involving the widespread use of insecticides, killing of wild animals, re-location of farming communities and destruction of fly-breeding sites (Nantulya, 1986).

Studies have shown that with strategic trypanocidal drug treatment, cattle can have acceptable growth rates (Wilson *et al.*, 1975; Rowlands *et al.*, 1994a), work output (Bourn and Scott, 1978) and reproductive performance (Rowlands *et al.*, 1994b) in areas of endemic trypanosomosis. In general, however, there has been limited success in the control of trypanosomoses, and therefore alternative immunologically-based strategies have been investigated, including the use of genetically resistant livestock breeds (Murray *et al.*, 1982) and identification of immunomodulatory factors (Sileghem *et al.*, 1994) which aim to reduce the effects of disease without necessarily altering infection rates. Vaccination against variable surface antigens has not been effective because of the ability of trypanosomes to rapidly change their surface antigen

and suitable non-variable antigenic determinants have not been identified (Nantulya, 1986).

In Indonesia, strict official regulations existed in 1912 for the control of *T. evansi*. These stipulated isolation of all animals for three months, slaughter of clinical cases and no movement of animals between affected areas and livestock markets (Dieleman, 1983). These regulations were modified when an effective treatment (suramin, Naganol) became available. Today, livestock movement between islands is under government control, and a proportion of animals are screened by blood smear examination, but this test would not detect all *T. evansi*-infected animals. Furthermore, re-location of people and livestock between islands as part of governmental transmigration programmes could potentially increase the risk of trypanosomosis outbreaks (Luckins, 1988).

Currently in Asia, the control of *T. evansi* relies on the use of chemotherapy and chemoprophylaxis (Wilson, 1983; Lun *et al.*, 1993), and in China researchers are testing new compounds for trypanocidal activity (Lun *et al.*, 1993). In Indonesia, the trypanocides diminazene aceturate (Berenil), isometamidium chloride (Trypamidium) and suramin (Naganol) have been widely used, but drug-resistant trypanosome strains have been reported (Dieleman, 1983) and suramin is no longer readily available. Cymelarsan has been shown to be effective when given at a high dose (0.75 mg/kg) in experimental buffaloes (Payne *et al.*, unpublished data) and cattle (Payne *et al.*, 1994a), but it is expensive and, as yet, marketed for use in camels only. The control of biting flies, for example by the use of pesticides on breeding sites and trapping adult flies, is difficult (Foil, 1989). However, new insecticide-impregnated eartags have been shown to control tabanids in Australia (R. Tozer pers. comm.) which may be potentially useful. In the future, integrated control strategies for trypanosomosis, using immunotolerant livestock breeds and strategic chemotherapy and chemoprophylaxis of livestock populations at risk, may be more effective. However, for this approach to be successful, improved diagnostic tests and a better understanding of the epidemiology of *T. evansi* are required.

## 2.11 Economic impact

The economic importance of a disease on livestock production can be considered at different levels: the individual farmer and farmer groups, the consumers and the national economy (Dijkuizen *et al.*, 1994). In Indonesia, there is a lack of data on the impact of trypanosomosis on livestock production (Wilson, 1983), without which the development of control strategies and the rationalisation of limited resources by governmental animal health departments is difficult (Hutabarat and Holden, 1991). National annual losses of US $22.4 million (Ronohardjo *et al.*, 1986), unit losses of US $45/draught animal/year due to reduced work output (Rukmana, 1979), and a benefit return of US $9.14/beef animal from increased growth after trypanocidal treatment (Payne *et al.*, 1994b), have been estimated.

In Indonesia, the long-term impact of chronic trypanosomosis on production is likely to be more economically important in contrast with other countries, for example China and Vietnam, where high mortality and other losses associated with epidemics of trypanosomosis occur (Luckins, 1988; Lun *et al.*, 1993). Experimental studies showed reduced body weight gain in Friesian Holstein calves (Payne *et al.*, 1992) and anoestrus in a Friesian Holstein heifer after 16% loss in body weight (Payne *et al.*, 1993). Abortion has been reported from Thailand (Lohr *et al.*, 1986) and India (Paikne and Dhake, 1972). Other financial losses, for example the premature sale of sick animals, have not been fully quantified. Data on economic losses associated with trypanosomosis in Indonesia and elsewhere are difficult to obtain, partly because of the problem of diagnosis and the complexity of interactions with other diseases, which may explain the lack of such information.

## 2.12 Farming systems in Indonesia, with special reference to project areas in Central Java

The livestock industry is of major importance to the national economy of Indonesia contributing 12% of the agricultural Gross Domestic Product, and the government plans to increase livestock production by 6.4% during the current five-year national development plan (Soehadji, 1994). A priority of the government is the improvement in the income and welfare of smallholder and landless farmers associated with

increased livestock production. In 1989, there were approximately 10 million cattle and 3.2 million buffaloes in Indonesia (Anon, 1989). With a human population approaching 190 million, Indonesia imports livestock for internal markets, and ambitious government livestock development programmes aim to import large numbers of cattle and buffaloes for breeding and slaughter from Australia. Low productivity of both local and imported cattle and buffaloes in Indonesia is reported, with mortality rates up to 19% in local buffaloes and 18% in imported cattle (Hardjosubroto, 1985).

The majority of buffaloes in Indonesia are swamp buffaloes with small numbers of dairy Murrah buffaloes in northern Sumatra and other regions. On Java the climate is tropical with high rainfall in many months. Figure 2.1 shows the location of the five districts in Central Java where the project field work was undertaken and Figure 2.2 gives the monthly rainfall in these areas. Both smallholder and landless farmers own buffaloes, and each farmer usually owns one to four animals (Anon, 1986). In this region, pairs of female buffaloes are preferred for draught power which are either worked by their owners or hired to other farmers. Typical work includes preparation of rice fields before transplantation of seedlings, as shown in Figure 2.3. Buffaloes which are used only by their owner usually work for one to three months per year (i.e., one month per rice crop), but hired buffaloes may work for much longer periods. The main buffalo work periods are April to May and November to December, but vary in areas with irrigation or crop rotation. In some areas, for example Tegal district, adult male buffaloes are hired by sugar-cane companies for approximately six months per year for the sugar-cane harvest, and in a few locations buffaloes are kept primarily for fattening. Typically, buffaloes are communally grazed on rice fields after harvest or alternatively, a cut-and-carry system utilising grasses cut from non-cultivated areas is practiced. In some areas, buffaloes are grazed in forests or teak plantations, where periods of high biting-fly activity were reported by farmers.

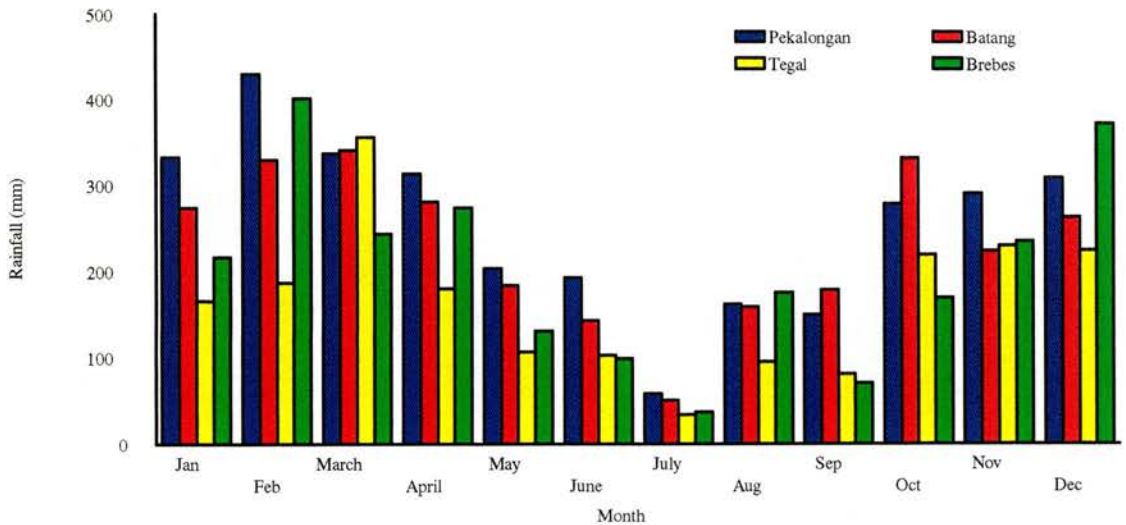
In Central Java, two types of animal housing are commonly used: 1) traditional stalls for one to five buffaloes adjacent to the farmer's house; and 2) communal animal housing (*kandangs*) for 10 to 100 buffaloes which are located away from the central

village area. In recent years, the use of *kandangs* has been promoted by the Government to improve village sanitation and farmers have benefited from the shared responsibility of guarding their buffaloes. Buffaloes represent a significant investment of farmers whose annual income may be as low as Rupiah 500, 000 (£140) with adult buffaloes costing more than the equivalent of £450 each.

**Figure 2.1** Map of Java, Indonesia, showing the five districts visited during the project


A : Batang
B : Pekalongan
C : Pemalang
D : Tegal
E : Brebes

**Figure 2.2** Monthly rainfall (millimetres) in four districts of Central Java in 1992




(Source: District government veterinary services visited during project; data from Pemalang district were not available)

**Figure 2.3** A pair of buffaloes working in rice fields in Central Java

# CHAPTER 3

# STANDARDISATION AND OPTIMISATION OF ANTIGEN-ELISAS FOR THE DIAGNOSIS OF *TRYPANOSOMA EVANSI* IN CATTLE AND BUFFALOES

## 3.1 Introduction

*Trypanosoma evansi* Ag-ELISAs which use monoclonal antibodies to detect serum trypanosomal antigens have been developed (Nantulya and Lindqvist, 1989; Nantulya *et al.*, 1989b; Luckins, 1991; Masake and Nantulya, 1991). The use of monoclonal antibodies in these Ag-ELISAs enables better standardisation of test reagents between batches and laboratories (Luckins, 1992) because monoclonal antibodies are homogeneous with identical affinities. This is a major advantage of these tests compared with antibody-detection tests that are based on crude, undefined antigens. In *T. evansi* Ag-ELISAs the same monoclonal antibody is employed in a sandwich ELISA format for both capture and detection. Such a design is possible because the antigen has multiple copies of the target epitope and because binding of antigen with the capture monoclonal antibody does not alter the accessibility of the epitopes for the detection monoclonal antibody (McCullough, 1993).

Since their development several years ago, however, *T. evansi* Ag-ELISAs have not been fully standardised or evaluated in terms of their diagnostic sensitivity and specificity, and their performance under different conditions (Luckins, 1992). The current international application of *T. evansi* Ag-ELISAs for both research and diagnosis (Touratier, 1993; Anon, 1994) underlines the need to standardise test protocols and reagents to facilitate comparison of results from different laboratories. Previous studies have used standard 96-well polystyrene ELISA plates (Nantulya *et al.*, 1989a; Bishop, 1992; Diall *et al.*, 1992; Monzon *et al.*, 1995) or polystyrene tubes (Nantulya *et al.*, 1989b; Waithanji *et al.*, 1993). These *T. evansi* Ag-ELISAs can be readily applied to test a variety of animal species, because, in contrast to antibody-detection ELISAs, no host animal species-specific conjugate is involved. Camels have been tested in the majority of published work of the application

of *T. evansi* Ag-ELISAs (Nantulya *et al.*, 1989b; Waithanji *et al.*, 1993) and their use for buffaloes has rarely been reported.

Guidelines for the international standardisation of antibody-detection ELISA protocols and reagents have been produced which describe data expression, primary reference standards, quality assurance and diagnostic validation (Wright *et al.*, 1993). A similar approach has been adopted here for *T. evansi* Ag-ELISAs, in particular the use of percent positivity (PP) values instead of optical densities (OD) to express results, the production of control sera and quality assurance. The latter term refers to quality control parameters which are used to quantify intra-assay and inter-assay variation, including upper and lower control limits which are established by repeat testing under routine conditions and are used for the acceptance or rejection of each ELISA plate within a laboratory and between laboratories.

The aims of this study were to: 1) standardise working protocols for two *T. evansi* Ag-ELISAs based on different monoclonal antibodies; 2) obtain positive and negative reference sera; 3) establish quality control parameters; 4) determine when cattle become antigenaemic after experimental infection with *T. evansi*; and 5) obtain preliminary estimates of specificity using British cattle sera. Two *T. evansi* Ag-ELISAs (2G6 Ag-ELISA and Tr7 Ag-ELISA) were standardised at the Centre for Tropical Veterinary Medicine (CTVM), Edinburgh, prior to their establishment at the Research Institute for Veterinary Science (Balitvet), Indonesia, for subsequent evaluation using buffaloes experimentally and naturally infected with *T. evansi*. The two *T. evansi* monoclonal antibodies, 2G6 and Tr7, were developed at the CTVM, Edinburgh, and the International Livestock Research Institute (ILRI), Kenya, respectively, and specifically recognise antigenic determinants present only in trypanosomes of the sub-genus *Trypanozoon* (Nantulya *et al.*, 1987; Frame *et al.*, 1990; Luckins, 1991).

## 3.2 Materials and methods

### 3.2.1 Experimental *Trypanosoma evansi* infection of calves

Two Limousin-cross calves (Calf 915 and Calf 917) approximately 3 months old, were housed in an isolation unit at the CTVM. Before the start of the study, sera from both calves were tested by the Scottish Agricultural College Veterinary Services laboratory, Edinburgh, to confirm their negative antibody status for bovine virus diarrhoea and enzootic bovine leucosis. The calves were inoculated intravenously with $2 \times 10^6$ trypanosomes of an Indonesian isolate of *T. evansi*, TREU 1994, derived from a single passage of a field isolate (Simorejo/84/Bakit 233) originally collected from a cow in Java. The trypanosomes were obtained from mice with a high parasitaemia, and separated from the blood by ion exchange chromatography on a DEAE cellulose column (DE52; Whatman Laboratory Sales Ltd.; Lanham and Godfrey, 1970), as described in Appendix I. The separated trypanosomes were washed three times with phosphate-buffered saline, pH 8.0, containing 1% glucose (PSG) and counted with an haemocytometer. The trypanosomes were diluted with PSG to obtain $5 \times 10^5$ trypanosomes per ml and each calf was inoculated with 4 ml of the trypanosome suspension.

Post-infection, blood samples from the calves were collected every two to four days in plain Vacutainers (606430; Becton Dickinson) and Vacutainers containing lithium heparin (368484; Becton Dickinson). The heparinised blood was examined by wet blood film and MHCT (Woo, 1969). In the latter technique, blood was centrifuged in a microhaematocrit tube for five minutes. The PCV was then recorded and the intact microhaematocrit tube was placed on a glass slide holder and examined under a microscope for the presence of trypanosomes moving near the buffy coat. Blood samples without anticoagulant were left to clot overnight at 4°C. The next day, the sera were taken off after centrifugation at 800 g for ten minutes. Sera were stored in sterile 5 ml plastic bijoux at -20°C and tested by the *T. evansi* Ag-ELISAs, and by the IgM ELISA, IgG ELISA and CATT as described in Appendix I.

### 3.2.2 Collection of positive and negative reference sera

Before infection, several blood samples were collected from Calf 915 and Calf 917 to obtain a pool of sera for use as a negative Ag-ELISA control (C-). After infection with *T. evansi* TREU 1994, the antigenaemia was monitored by 2G6 Ag-ELISA and when the optical density (OD) was between 0.50 to 1.0, 100 ml blood were collected from each calf over three days (31-33 days post-infection) for the 2G6 low-positive control (C+). This procedure was repeated at a later stage (53-55 days post-infection) to obtain the 2G6 high-positive control (C++) at which stage the OD was >1.0. With the Tr7 Ag-ELISA, the OD of the first serum pool (31-33 days) was found to be higher than the OD of the second serum pool (53-55 days), and therefore sera collected 31-33 days post-infection were used as the Tr7 high-positive control (C++), and sera collected 53-55 days post-infection were used as the Tr7 low-positive control (C+).

### 3.2.3 *Trypanosoma evansi* Ag-ELISA reagents and protocol

The same basic Ag-ELISA protocol was used for all 2G6 Ag-ELISAs and Tr7 Ag-ELISAs that were conducted.

#### *3.2.3.1 Antigen-detection ELISA reagents*

The reagents were labeled with either a blue marker (2G6 Ag-ELISA) or a red marker (Tr7 Ag-ELISA).

Coating monoclonal antibodies

The 2G6 monoclonal antibody is an $IgG_1$ isotype derived from antibodies against *T. evansi* which recognises a 70 kDa antigen of *T. evansi*. The Tr7 monoclonal antibody is an IgM isotype derived from antibodies against *T. rhodesiense* which recognises a 15 kDa antigen common to *T. brucei* and *T. evansi;*, this was provided by Dr R. Masake, ILRI. The 2G6 and Tr7 monoclonal antibodies were stored at dilutions of 1 mg/ml and 2.5 mg/ml, respectively, in sterile phosphate-buffered saline (PBS), pH 7.2, in 50-100 µl aliquots at -20°C.

Reference control sera

The negative (C-), low-positive (C+) and high-positive (C++) control sera were stored in 25 ml plastic Universal tubes. One of these 25 ml aliquots was further aliquoted into 2 ml plastic vials, and all the sera were stored at -20°C or -40°C; 2 ml working aliquots of each control were kept at 4°C.

Horse-radish peroxidase conjugated monoclonal antibodies

The conjugates were the same monoclonal antibodies used for coating the microtitre plates, but were labeled with horse radish peroxidase (HRPO) and stored in 100-250 µl aliquots at -20°C. After preliminary assays, the 2G6 conjugate was passed through a purification column (FreeZyme Conjugate Purification Kit, Pierce & Warriner (UK) Ltd.) to reduce the quantity of free enzyme. The 2G6 conjugate was stored in sterile PBS diluted 1:2 with glycerol. The Tr7 conjugate was supplied partially diluted in 50% glycerol/glycine/EDTA buffer.

Coating buffer

Carbonate-bicarbonate buffer, pH 9.6, 0.05 M, (C-3041; Sigma) was prepared by dissolving one capsule in 100 ml deionised water and kept at 4°C for up to two weeks.

Washing buffer

Phosphate-buffered saline, pH 7.2, with 0.05% polyoxyethylene sorbitan monolaurate (Tween 20) (PBS/0.05T) was prepared following the protocol given in Appendix I, and kept at room temperature for up to four weeks.

Serum and conjugate diluents

The concentration of Tween 20 was higher (0.50%) in the serum and conjugate diluents than in the washing buffer (0.05%). The diluent buffer (PBS/0.50T) was prepared by dissolving one PBS tablet (P-4417; Sigma) in 200 ml deionised water and adding one ml of Tween 20. The PBS/0.50T, pH 7.4, was kept at room temperature for up to five days only, because fungal contamination was noticed after longer storage periods.

Substrate buffer

The phosphate-citrate substrate buffer, 0.05 M, pH 5.0, containing 0.03% sodium perborate (S-P4922; Sigma) was prepared by dissolving one capsule in 100 ml deionised water, kept in the dark at room temperature and used within 30 minutes.

Substrate chromogen

The chromogen solution was prepared by dissolving one tetramethylbenzidine dihydrochloride tablet (TMB) (T-3405; Sigma) in ten ml of freshly prepared substrate buffer per plate, and used within ten minutes.

*3.2.3.2 Basic antigen-ELISA protocol*

1) Coating monoclonal antibody was added, 100 µl per well, to a 96-well microtitre ELISA plate that was then shaken on an incubator/shaker (Dynatech Laboratories Ltd.) at ambient temperature for 30 seconds to ensure even distribution of the solution on the bottom of the wells. The plate was covered and then stored overnight at 4°C.

2) The following day, the ELISA plate was washed (three one minute cycles) with washing buffer using an automatic microtitre plate washer (Dynatech Ultrawash Plus). After washing, excess buffer was removed by patting the inverted plate on a paper towel. Test samples and control sera were diluted with PBS/0.50%T and added, 100 µl per well, in duplicate and quadruplicate wells, respectively. Four controls were included on each plate: the conjugate control (Cc) which was serum diluent buffer only, high-positive (C++), low-positive (C+), and negative (C-) controls. The plate was covered and put on the incubator/shaker at 37°C for 30 minutes. The plate layout used for testing serum samples is shown in Figure 3.1 and the plate layouts used for titrations are described in the text below.

3) After serum incubation, the plate was washed as before. The conjugate was diluted with PBS/0.50%T and 100 µl added to each well of the plate. The covered plate was put on the incubator/shaker at 37°C for 30 minutes.

4) After the conjugate incubation, the plate was washed again. The freshly prepared substrate/chromogen solution was added, 100 µl per well, and the plate covered and incubated at 37°C for 10 minutes on the incubator/shaker.

5) The enzymatic reaction was stopped by addition of 50 µl 2 M sulphuric acid per well. The ODs of individual wells were read immediately using a Labsystems Multiskan Plus II ELISA plate reader (Life Sciences International (UK) Ltd.) with a 450nm filter. Before reading the test results, the plate reader was blanked against an empty ELISA plate of the same type used for the test.

### 3.2.4 Standardisation and optimisation of *Trypanosoma evansi* Ag-ELISAs

#### *3.2.4.1 Microtitre ELISA plate type*

High- and low-binding 96-well polystyrene microtitre ELISA plates (Immulon 1, 2, 3 and 4; Dynatech) were tested. Immulon 3 plates are reported by the manufacturers to bind up to 30% more protein than the low-binding, Immulon 1 plates. Immulon 2 and Immulon 4 plates are surface-treated during manufacture to enhance protein binding and Immulon 4 plates are reported to give lower well-to-well, plate-to-plate and batch-to-batch variation and to bind up to 50% more protein than Immulon 2 plates (Dynatech data sheet). Sera from Calf 915 and Calf 917 were not available when this study was conducted, and therefore negative rabbit serum (Scottish Antibody Production Unit) and positive and negative sheep sera obtained from the CTVM were titrated against a range of dilutions of coating monoclonal antibody and conjugate in Assays 1 to 3, as shown in Table 3.1. The Tr7 Ag-ELISA reagents were not available at this stage of the project.

#### *3.2.4.2 Substrate*

Once the plate type (Immulon 2) had been selected and sera were available from the two experimentally-infected calves, two different substrates were compared. The TMB substrate/chromogen prepared with TMB tablets as described above was compared with a two component liquid TMB formulation (Chromogen E8073, Buffer E8071; Cambridge Veterinary Sciences). Duplicate Immulon 2 plates were tested with negative calf serum (C-) in columns 2 to 6, PBS/0.50T in columns 1 and

12, and positive serum (Calf 915, Day 55) in columns 7 to 11. A 1:2 serum dilution and 1:4000 conjugate dilution were used, and tablet TMB was used on one plate and soluble TMB on the other plate.

**Table 3.1** Assays to compare the performance of different types of ELISA plates for *Trypanosoma evansi* antigen-ELISAs.

| Assay | Immulon ELISA plate | Coating monoclonal antibody (μg/ml) | Type of serum* | Serum dilutions | Conjugate dilutions |
|---|---|---|---|---|---|
| 1 | 1, 2, 4 | 10 | Rabbit (-)<br>Sheep (+) | 1:1-1:8 | 1:1000-1:4000 |
| 2 | 1, 2, 3, 4 | 2.5 | Sheep (+)<br>Sheep (-) | 1:20 | 1:1000-1:16,000 |
| 3 | 2, 4 | 2.5, 5 | Sheep (+)<br>Sheep (-) | 1:5-1:40 | 1:1000-1:8000 |

*: (-): Pre-infection serum; (+): Serum from experimental *T. evansi*-infected sheep.

*3.2.4.3 Optimisation of reagent dilutions*

Optimal reagent dilutions were determined for both the 2G6 Ag-ELISA and Tr7 Ag-ELISA using the standardised Ag-ELISA format (i.e., Immulon 2 plates and tablet TMB substrate) and positive (C+ and C++) and negative (C-) control sera from the experimentally-infected calves. Chequerboard titrations were conducted to determine which reagent dilutions gave optimal differentiation between positive and negative serum ODs, as shown by calculation of the ratio of positive to negative control ODs ($OD_{C++}/OD_{C-}$) (Voller *et al.*, 1976). The dilution ranges used were 1.25-10 μg/ml for coating monoclonal antibody, 1:1-1:1:40 for serum and 1:1000-1:64,000 for conjugate. For the Tr7 Ag-ELISA, recommended dilutions of 1:500 monoclonal antibody (0.5 μg/ml), 1:10 serum and 1:1000 conjugate were used.

3.2.5 Quality control parameters and data expression

International guidelines on data expression and quality assurance for ELISAs were adopted (Wright *et al.*, 1993). The standard plate layout used for 40 test samples is shown in Figure 3.1 which includes four replicate wells for each control Cc, C-, C+ and C++).

**Figure 3.1** Standard plate layout for *Trypanosoma evansi* antigen-detection ELISA

| | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| Cc | Cc | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 |
| Cc | Cc | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 |
| C++ | C++ | 11 | 12 | 13 | 14 | 15 | 16 | 17 | 18 | 19 | 20 |
| C++ | C++ | 11 | 12 | 13 | 14 | 15 | 16 | 17 | 18 | 19 | 20 |
| C+ | C+ | 21 | 22 | 23 | 24 | 25 | 26 | 27 | 28 | 29 | 30 |
| C+ | C+ | 21 | 22 | 23 | 24 | 25 | 26 | 27 | 28 | 29 | 30 |
| C- | C- | 31 | 32 | 33 | 34 | 35 | 36 | 37 | 38 | 39 | 40 |
| C- | C- | 31 | 32 | 33 | 34 | 35 | 36 | 37 | 38 | 39 | 40 |

Cc: Conjugate control; C++: High positive control; C+: Low positive control;
C-: Negative control; 1-40: Samples 1 to 40.

*Data expression*

The PP value of the OD of each well was calculated using the equation:

$$PP = \frac{\text{Single well OD}}{\text{Mean OD}_{C++}} \times 100$$

where the mean $OD_{C++}$ equals the mean of the two intermediate high-positive control ODs[1] i.e. the highest and lowest of the four high-positive control ODs were discarded and the mean of the two remaining ODs was calculated.

*Upper and lower control limits*

The OD and PP values of the high-positive control (C++), and the PP values of the low-positive (C+), negative (C-) and conjugate (Cc) controls were used to determine whether each test was within acceptable limits of variability, as defined by the upper (UCL) and lower control limits (LCL). Upper (UCL) and lower (LCL) control limits for each of the Ag-ELISA controls (Cc, C-, C+ and C++) were determined for the standardised 2G6 Ag-ELISA and Tr7 Ag-ELISA. To establish these limits, six plates

with multiple replicate wells for each control were assayed by the same operator on different days by either the 2G6 Ag-ELISA or Tr7 Ag-ELISA, under as near to ideal conditions as possible. New plastic ware was used for each assay and exact incubation times were used. The Ag-ELISAs were performed using the Ag-ELISA protocol given in section 3.2.3.2 and optimal reagent dilutions. The conjugate control (Cc) was added to columns 1-3, the negative control (C-) to columns 4-6, the low-positive control (C+) to columns 7-9, and the high-positive control (C++) to columns 10-12. As a further assessment of non-specific binding of reagents, one plate was tested by each Ag-ELISA with the conjugate control (Cc) in all wells.

For acceptance or rejection of individual test plates, three different criteria were used. At the first level, at least two of the four replicate high-positive control (C++) ODs had to be within the established upper and lower control limits. For the second level, at least three of the four replicate PP values for each control (C++, C+, C- and Cc) had to be within their respective upper and lower control limits. Finally, the difference between ODs of replicate test samples had to be <10%.

### 3.2.6 Data analysis

Variation between the ODs of replicate wells was assessed by calculation of the coefficient of variation (CV) from the mean and standard deviation (SD) using the equation:

$$CV\,(x) = \frac{SD}{mean\,(x)} \times 100\ \%$$

(Armitage and Berry, 1994)

The upper and lower control limits for all the Ag-ELISA controls (C++, C+, C- and Cc) were determined by the 90th and 10th percentiles of the replicate well OD or PP values, respectively. Percentile values were used because the data did not have a Normal distribution, even after log-transformation. The percentiles were obtained by sorting the data in ascending order using Minitab software[1], and excluding data from all edge-wells.

[1] Minitab Inc. (Version 10.1) 3081 Enterprise Drive, State College PA, 16801-3008 USA.

3.2.7 Preliminary estimation of diagnostic specificity using British cattle sera

Batches of sera that had been submitted for routine, biochemical profiles from principally dairy farms in the United Kingdom were obtained from A. Dowell, Department of Veterinary Clinical Studies, University of Edinburgh. A total of 249 sera from 18 herds were tested by both Ag-ELISAs, and herd-specific diagnostic specificities were estimated with associated 95% confidence intervals using the software CIA[2], with the exact binomial method for the herd-specific specificities and the Normal approximation for the overall specificity. The overall diagnostic specificity was calculated as the number of test-negative animals divided by the total number of animals tested, and herd-specific diagnostic specificities were calculated as the number of test-negative animals divided by the number of animals tested in each herd, and were expressed as a percentage. Differences between the specificity estimates of the two Ag-ELISAs were calculated with 95% confidence intervals using CIA, and positive differences between these estimates were considered significant at the 5% level, if the lower limit of the 95% confidence interval was above zero.

## 3.3 Results

3.3.1 Standardisation and optimisation of antigen-detection ELISAs

*Type of microtitre ELISA plate and substrate*

Table 3.2 shows the ratios of positive to negative control ODs obtained using different ELISA plate types and reagent dilutions in Assays 1, 2 and 3. A wider range of reagent dilutions was investigated, but only results for those dilutions that were representative of the working dilutions eventually chosen are shown. Insufficient positive control serum from Assay 1 and Assay 2 was available for Assay 3, therefore only intra-results were compared. In each assay, higher ratios of positive to negative control ODs were obtained with high-binding plates (Immulon 2, 3 and 4), with less diluted serum or higher dilutions of conjugate. In Assay 3, the ranges of OD ratios obtained were 5.1 to 24.6 with Immulon 2 plates and 7.7 to 24.0 with Immulon 4 plates. Immulon 2 plates were chosen for all subsequent Ag-ELISAs because

[2] Confidence Interval Analysis (CIA) computer programme, British Medical Journal, BMA House, Tavistock Square, London, WC1H 9JR.

acceptable positive/negative OD ratios were obtained with this plate type which was less expensive than Immulon 4 plates

When substrate types were compared, the ranges of coefficients of variation of replicate wells in columns were 16-18% (Cc), 3-9% (C-), 10-18% (C++) using tablet TMB and 3-12% (Cc), 10-20% (C-) and 12-18% (C++) using soluble TMB. Although soluble TMB gave less inter-well variation with the conjugate control, it did not reduce inter-well variation for the negative or high-positive controls, and therefore tablet TMB was chosen which was considered to be more convenient for transportation overseas.

*Optimisation of reagent dilutions*

The initial optimal dilutions determined for the 2G6 Ag-ELISA using chequerboard titration and sheep control sera, were 2.5 μg/ml (coating monoclonal antibody), 1:20 (serum) and 1:8000 (conjugate). Although with these dilutions sheep sera gave high positive ODs, when the same dilutions were used for cattle sera the 2G6 Ag-ELISA was unable to detect antigenaemia in the experimentally-infected calves. Therefore, it was necessary to re-titrate the 2G6 Ag-ELISA reagents for use with cattle serum using pre-infection and post-infection sera from the two experimentally-infected calves.

Figure 3.2 shows the results of titrations of the high positive control (C++) and conjugate in the standardised 2G6 and Tr7 Ag-ELISAs, using the reference bovine control sera obtained from Calf 915 and Calf 917. The ODs of the negative (C-) and conjugate (Cc) controls of the 2G6 Ag-ELISA were higher than the ODs of these controls in the Tr7 Ag-ELISA, at the lower conjugate dilutions.

**Table 3.2** Ratios of positive to negative control optical densities ($OD_{C++}/OD_{C-}$) obtained using different types of ELISA plates (Immulon 1, 2, 3 and 4) and reagent dilutions in the *Trypanosoma evansi* 2G6 antigen-detection ELISA.

i) Assay 1: Serum and conjugate titrations, using 10 µg/ml coating monoclonal antibody, negative rabbit control serum and positive sheep control serum.

| Conjugate | Plate type | Serum dilution | | | |
|---|---|---|---|---|---|
| | | 1:1 | 1:2 | 1:4 | 1:8 |
| 1:1000 | 1 | 7.5 | 4.1 | 1.9 | 1.1 |
| | 2 | 6.9 | 6.6 | 5.6 | 3.7 |
| | 4 | 4.9 | 4.6 | 3.7 | 4.0 |
| 1:2000 | 1 | 5.8 | 3.2 | 1.5 | 1.1 |
| | 2 | 10.4 | 9.6 | 7.9 | 5.9 |
| | 4 | 9.5 | 7.4 | 5.3 | 4.3 |
| 1:4000 | 1 | 5.3 | 2.9 | 1.7 | 1.3 |
| | 2 | 15.7 | 12.9 | 9.0 | 6.0 |
| | 4 | 11.8 | 9.3 | 6.8 | 3.8 |

ii) Assay 2: Conjugate titration, using 2.5 µg/ml coating monoclonal antibody, and negative and positive sheep control sera at 1:20 dilution.

| Conjugate | Plate type | | | |
|---|---|---|---|---|
| | 1 | 2 | 3 | 4 |
| 1:1000 | 2.3 | 3.6 | 4.4 | 4.9 |
| 1:2000 | 2.2 | 6.4 | 5.4 | 6.9 |
| 1:4000 | 1.9 | 7.7 | 6.2 | 9.3 |
| 1:8000 | 1.6 | 9.8 | 5.9 | 10.3 |
| 1:16000 | 1.2 | 9.9 | 4.3 | 9.8 |

iii) Assay 3: Conjugate and serum titrations, using 2.5 µg/ml and 5.0 µg/ml coating monoclonal antibody (mAb) and positive and negative sheep control sera.

| Conjugate | Plate type | 2.5 µg/ml coating mAb | | | | 5.0 µg/ml coating mAb | | | |
|---|---|---|---|---|---|---|---|---|---|
| | | Serum dilution | | | | Serum dilution | | | |
| | | 1:5 | 1:10 | 1:20 | 1:40 | 1:5 | 1:10 | 1:20 | 1:40 |
| 1:1000 | 2 | 6.3 | 8.0 | 6.9 | 5.1 | 7.1 | 7.0 | 6.2 | 5.7 |
| | 4 | 9.6 | 10.4 | 10.2 | 7.7 | 9.9 | 9.2 | 10.0 | 8.1 |
| 1:2000 | 2 | 11.5 | 12.5 | 9.6 | 7.8 | 10.7 | 8.0 | 9.1 | 6.4 |
| | 4 | 17.4 | 16.1 | 14.3 | 9.1 | 16.5 | 15.1 | 13.1 | 8.3 |
| 1:4000 | 2 | 16.4 | 15.8 | 11.7 | 7.3 | 16.7 | 14.4 | 10.9 | 7.8 |
| | 4 | 21.6 | 18.4 | 15.1 | 9.3 | 22.0 | 19.4 | 14.9 | 9.1 |
| 1:8000 | 2 | 17.0 | 15.6 | 12.0 | 7.5 | 24.6 | 16.9 | 12.2 | 7.1 |
| | 4 | 24.0 | 18.8 | 15.3 | 7.9 | 23.5 | 20.5 | 14.5 | 8.1 |

The ratios of positive to negative control ODs obtained with the Tr7 Ag-ELISA were also greater than with the 2G6 Ag-ELISA. Figure 3.3 shows the titrations of the reference bovine control sera, using optimal dilutions of coating monoclonal antibody and conjugate given in Table 3.3, and shows that the ratios of positive to negative control ODs decreased more rapidly with increasing serum dilution in the 2G6 Ag-ELISA than in the Tr7 Ag-ELISA.

The reagent dilutions recommended for the Tr7 Ag-ELISA were found to give high ratios of positive to negative control ODs and are shown in Table 3.3 with the optimal reagent dilutions obtained for the 2G6 Ag-ELISA. The ratios of the high-positive to negative ($OD_{C++}/OD_C$) and low-positive to negative ($OD_{C+}/OD_{C-}$) control ODs were 51.9 and 17.8 for the 2G6 Ag-ELISA and 42.8 and 23.1 for the Tr7 Ag-ELISA, respectively, using these optimal dilutions.

### 3.3.2 Establishment of upper and lower control limits

In the repeated quality control plates some ODs of edge wells were higher than the ODs of inner replicate wells, and coefficients of variation of replicate column wells were higher for columns 1 and 12 than columns 2 to 11. Because of this observed 'edge-effect', the ODs of all edge wells were excluded from the calculation of the upper and lower control limits which are given in Table 3.4. For this, a blank value of 0.04 was deducted from all ODs, and PP values were then calculated using mean $OD_{C++}$ values of 0.886 (2G6 Ag-ELISA) and 1.314 (Tr7 Ag-ELISA).

In general, higher coefficients of variation were found more frequently with the 2G6 Ag-ELISA than the Tr7 Ag-ELISA, as shown by the frequency distributions shown in Figure 3.4. With the ELISA plates tested with conjugate controls (Cc) in all wells, the range of well ODs was 0.083-0.163 (10% CV) for the 2G6 Ag-ELISA and 0.078-0.106 (6% CV) for the Tr7 Ag-ELISA.

**Figure 3.2** Titrations of the conjugate and high-positive serum control (C++; 1:2 to 1:16) in the standardised *Trypanosoma evansi* antigen-detection ELISAs, using optimal dilutions of coating monoclonal antibody.

i) 2G6 antigen-detection ELISA using 2.5 µg/ml coating monoclonal antibody.
(C++ {1:2} ____ , C++ {1:4} ---- , C++ {1:8} __ __ , C++ {1:16} _-_-_ , C- ____, Cc ___ )

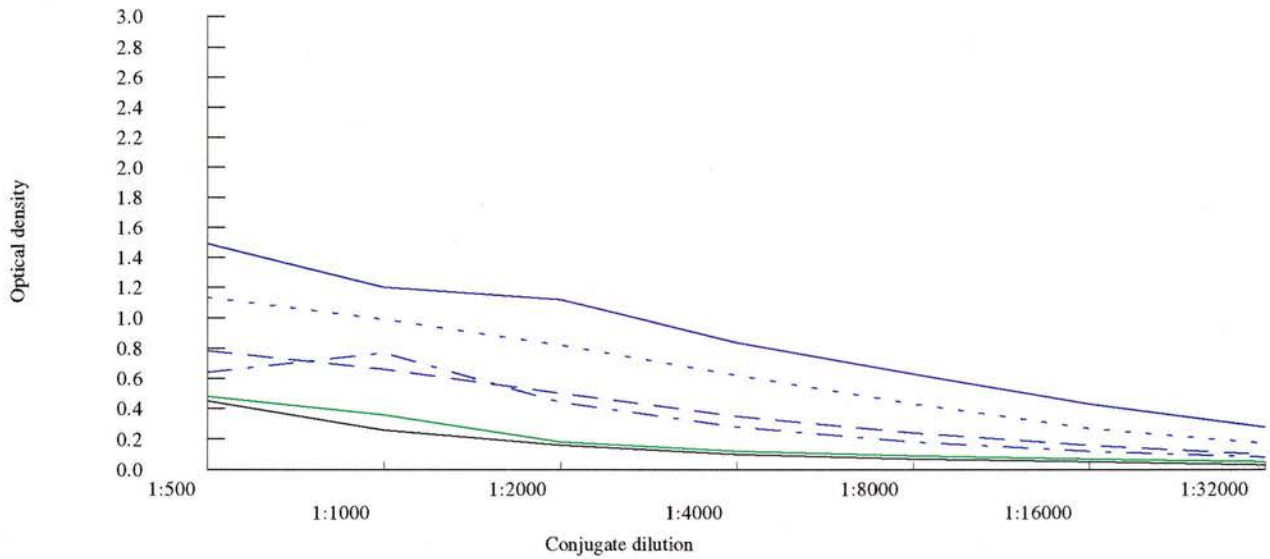


**Figure 3.2** continued

ii) Tr7 antigen-detection ELISA using 0.5 µg/ml coating monoclonal antibody
(C++ {1:2} ____ , C++ {1:4} ---- , C++ {1:8} __ __ , C++ {1:16} _ - _ - _ , C- ____, Cc ____ )

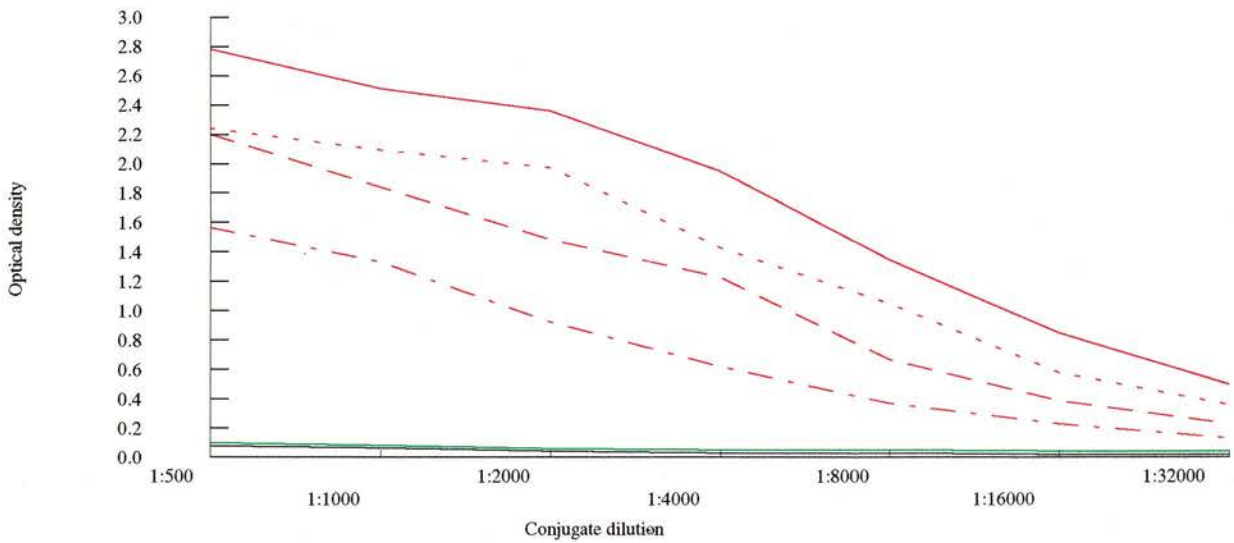


**Figure 3.3** Titrations of standardised *Trypanosoma evansi* antigen-ELISA controls (C++, C+, C-, Cc), using optimal dilutions of coating monoclonal antibody and conjugate.

i) 2G6 antigen-detection ELISA using 2.5 µg/ml coating monoclonal antibody and a 1:6000 conjugate dilution. (C++ ___ , C+ --- , C- ___ , Cc ___ )

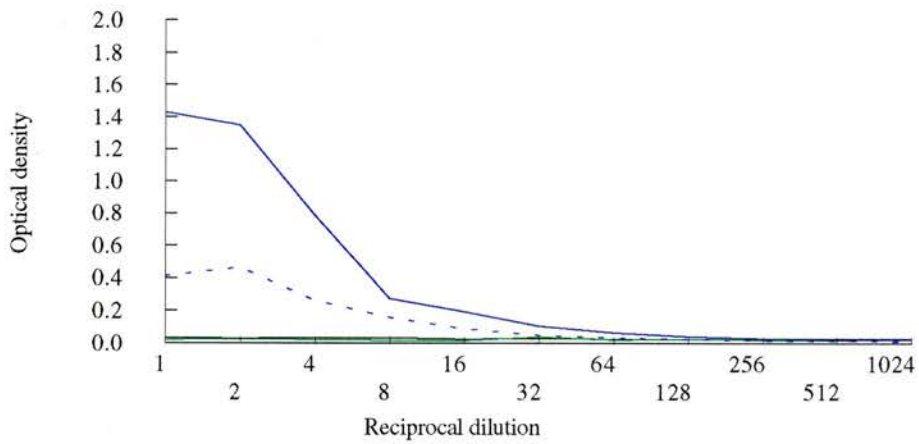


**Figure 3.3** continued

ii) Tr7 Ag-ELISA using 0.5 µg/ml coating monoclonal antibody and a 1:1000 conjugate dilution (C++ ___ , C+ --- , C- ___ , Cc ___ )

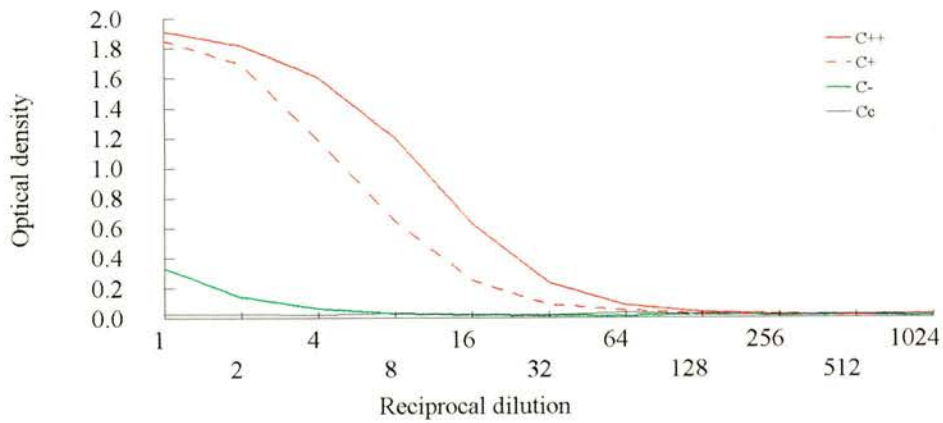


**Table 3.3** Optimal reagent dilutions of standardised *Trypanosoma evansi* antigen-detection ELISAs for testing bovine sera.

| | 2G6 Ag-ELISA | Tbr7 Ag-ELISA* |
|---|---|---|
| Monoclonal antibody | 1:400 (2.5 μg/ml) | 1:500 (0.5 μg/ml) |
| Serum | 1:2 | 1:10 |
| Conjugate | 1:4000 | 1:1000 |

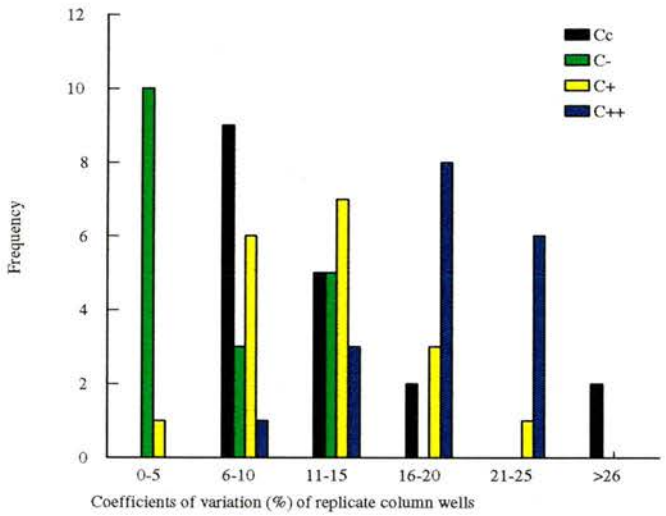
*: Dilutions recommended by supplier

**Table 3.4** Upper (UCL) and lower (LCL) control limits for 2G6 Ag-ELISA and Tr7 Ag-ELISA given as optical densities with percent positivity values in brackets.

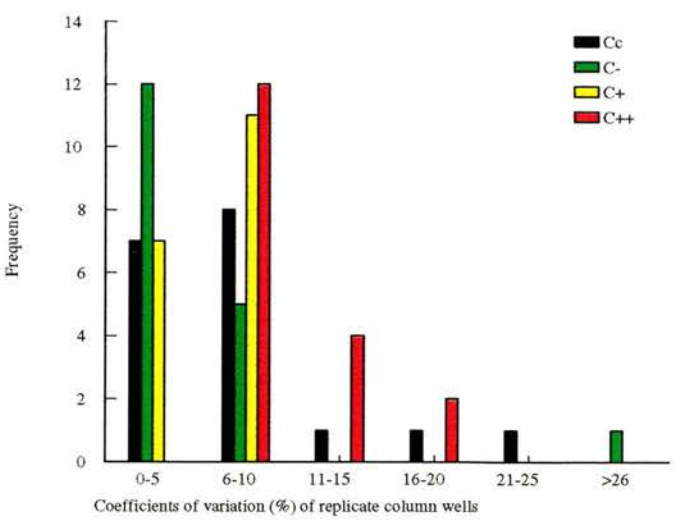
| Control | 2G6 Ag-ELISA | | Tr7 Ag-ELISA | |
|---|---|---|---|---|
| | LCL | UCL | LCL | UCL |
| C++ | 0.672 (76%) | 1.080 (122%) | 1.110 (84%) | 1.508 (115%) |
| C+ | 0.278 (31%) | 0.434 (49%) | 0.485 (37%) | 0.876 (67%) |
| C- | 0.046 (5%) | 0.076 (9%) | 0.070 (5%) | 0.094 (7%) |
| Cc | 0.042 (5%) | 0.086 (10%) | 0.058 (4%) | 0.078 (6%) |

**Figure 3.4** Frequency distributions of coefficients of variation calculated from eight replicate column wells of the controls (C++, C+, C-, Cc) on the repeated quality control plates of the *Trypanosoma evansi* antigen-detection ELISAs.

i) 2G6 antigen-detection ELISA




ii) Tr7 antigen-detection ELISA



### 3.3.3 Experimental *Trypanosoma evansi* infection of calves

The serum antigen responses detected by the two Ag-ELISAs in Calf 915 and Calf 917 after experimental infection with *T. evansi* are shown in Figure 3.5, together with serum antibody levels. By 10 days post-infection, both the 2G6 and Tr7 Ag-ELISAs had detected an increase in serum antigen in Calf 915 above 20 PP; this value was chosen empirically as a cut-off value because it was equal to or greater than twice the pre-infection PP values obtained by the two Ag-ELISAs.

In Calf 917, antigenaemia was detected by 10 days post-infection by the 2G6 Ag-ELISA and by 13 days post-infection by Tr7 Ag-ELISA. The profiles of antigenaemia differed between the two Ag-ELISAs and between the two calves. For example, in Calf 915 there was a more rapid rise in detectable antigen by both Ag-ELISAs than in Calf 917. With the 2G6 Ag-ELISA, detectable antigen responses declined below 20 PP by approximately three weeks post-infection in both calves, and then increased again.

In both calves, IgM and IgG-specific trypanosomal antibody responses were detected ten days post-infection, and in Calf 917 antibody responses increased more rapidly than antigenaemias. In Calf 915 there was a strong CATT response from 13 days post-infection, but in Calf 917 the CATT response was weakly positive by 21 days, strongly positive between 24 to 42 days, and weakly positive from 43 days, post-infection.

### 3.3.4 Preliminary estimation of diagnostic specificity using British cattle sera

The estimates of specificity of the 2G6 Ag-ELISA, with 95% confidence intervals, were 83% (78, 87) and 91% (87, 94) and of the Tr7 Ag-ELISA were 78% (72, 82) and 87% (83, 91), using 20 PP and 30 PP cut-off values, respectively. The differences between the specificity estimates of the two Ag-ELISAs was 5% (-1, 12) with the 20 PP cut-off value and 4% (-2, 9) with the 30 PP cut-off value. A total of 52 samples gave false-positive results using the 30 PP cut-off value, but only four of these samples were positive by both Ag-ELISAs.

The herd-specific estimates of specificity (Table 3.5) ranged from 58% to 100% (2G6 Ag-ELISA) and 53% to 100% (Tr7 Ag-ELISA), using the 20 PP cut-off value, and 76% to 100% (2G6 Ag-ELISA) and 65% to 100% (Tr7 Ag-ELISA), using the 30 PP cut-off value. Significant differences were found between some herd-specific estimates. For example, the differences between the specificities obtained for Herd 2 and Herd 18 were 29% (8, 51) with the 2G6 Ag-ELISA and 41% (15, 67) with the Tr7 Ag-ELISA, using a 20 PP cut-off value.

**Table 3.5** Estimates of herd-specific specificity, with 95% confidence intervals (in brackets), of the 2G6 Ag-ELISA and the Tr7 Ag-ELISA, using 20 PP and 30 PP cut-off values

| Herd | n | 2G6 Ag-ELISA | | Tr7 Ag-ELISA | |
|---|---|---|---|---|---|
| | | 20 PP | 30 PP | 20 PP | 30 PP |
| 1 | 13 | 69 (39, 91) | 77 (46, 95) | 100 (75, 100) | 100 (75, 100) |
| 2 | 17 | 71 (44, 90) | 76 (50, 93) | 53 (28, 77) | 82 (57, 96) |
| 3 | 17 | 82 (57, 96) | 88 (64, 99) | 82 (57, 96) | 94 (71, 100) |
| 4 | 17 | 88 (64, 99) | 100(81,100) | 71 (44, 90) | 71 (44, 90) |
| 5 | 12 | 75 (43, 95) | 92 (62, 100) | 58 (28, 85) | 83 (52, 98) |
| 6 | 16 | 88 (62, 98) | 88 (62, 98) | 81 (54, 96) | 81 (54, 96) |
| 7 | 5 | 60 (15, 95) | 80 (28, 100) | 80 (28, 100) | 100 (48, 100) |
| 8 | 19 | 58 (34, 80) | 84 (60, 97) | 74 (49, 91) | 84 (60, 97) |
| 9 | 19 | 89 (67, 99) | 89 (67, 99) | 84 (60, 97) | 89 (67, 99) |
| 10 | 10 | 70 (35, 93) | 90 (56, 100) | 80 (44, 98) | 90 (56, 100) |
| 11 | 17 | 88 (64, 99) | 94 (71,100) | 65 (38, 86) | 65 (38, 86) |
| 12 | 13 | 92 (64, 100) | 92 (64, 100) | 77 (46, 95) | 92 (64, 100) |
| 13 | 13 | 100 (75, 100) | 100 (75, 100) | 77 (46, 95) | 85 (55, 98) |
| 14 | 5 | 80 (28, 100) | 80 (28, 100) | 80 (28, 100) | 100 (48), 100) |
| 15 | 16 | 94 (70, 100) | 100 (79, 100) | 81(54, 96) | 94 (70, 100) |
| 16 | 17 | 88 (64, 99) | 100 (81,100) | 76 (50, 93) | 82 (57, 96) |
| 17 | 6 | 83 (36, 100) | 100 (54, 100) | 100 (54, 100) | 100 (54, 100) |
| 18 | 17 | 100 (81, 100) | 100 (81, 100) | 94 (71, 100) | 100 (81, 100) |

n: Number of cattle tested; PP: Percent positivity

**Figure 3.5** Trypanosomal antigens and antibodies in sera from Calf 915 and Calf 917 after experimental infection with *Trypanosoma evansi* TREU 1994
(2G6 Ag-ELISA ___ , Tr7 Ag-ELISA ___ ; IgM ELISA ----, IgG ELISA ___ )

i) Calf 915

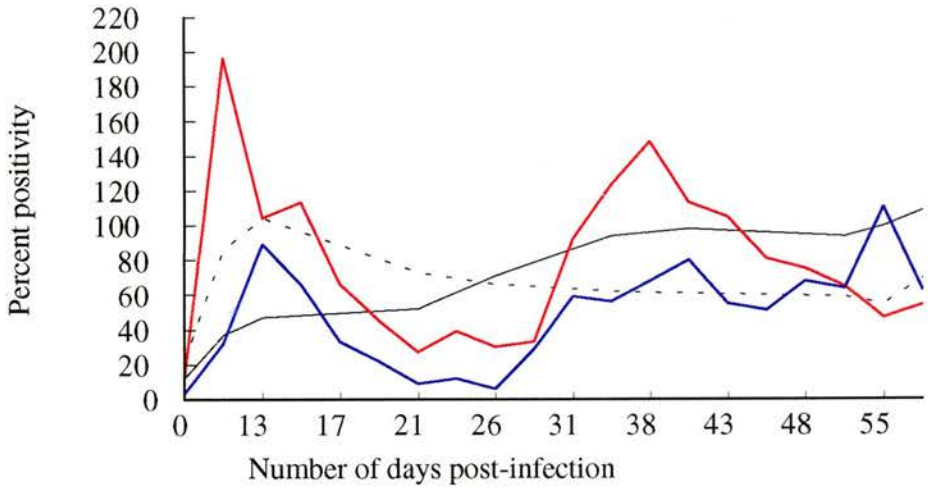
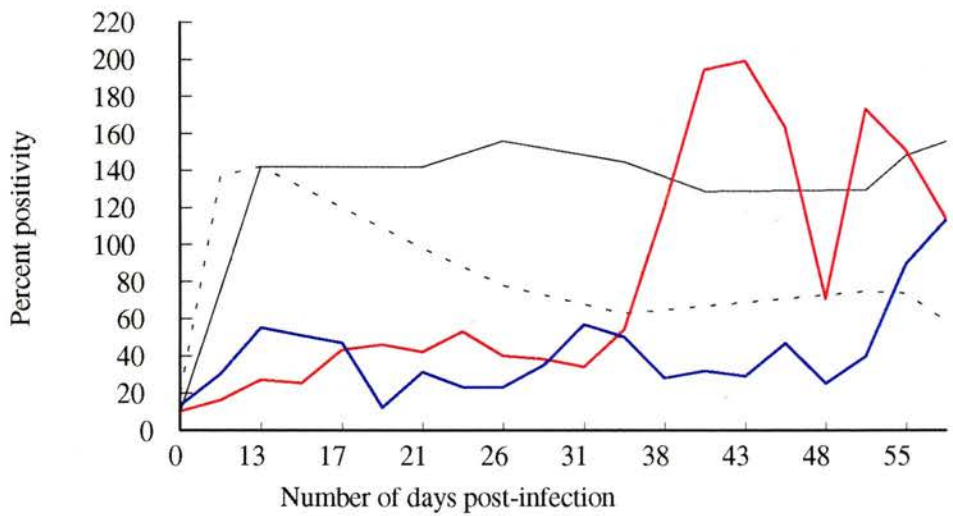


**Figure 3.5** continued

ii) Calf 917



### 3.4 Discussion

The aim of this initial work was to standardise the two *T. evansi* Ag-ELISAs prior to their establishment and evaluation in Balitvet, Indonesia. The same protocol was used for each Ag-ELISA in terms of the plate type, incubation times and substrate, but reagent dilutions were optimised separately for each Ag-ELISA by chequerboard titration. Guidelines on data expression and quality assurance recommended by the FAO/IAEA Central Laboratory for ELISA and Molecular Techniques in the Diagnosis of Animal Diseases, Seibersdorf, Vienna, were adapted for the Ag-ELISAs (Wright *et al.*, 1993).

High-binding microtitre ELISA plates were chosen because higher ratios of positive to negative control ODs were obtained with this plate type which also have the advantage that less reagents are required. Initially, optimal reagent dilutions were determined by chequerboard titrations using a highly positive sheep serum. However, using a serum dilution of 1:20, antigenaemia was not detected in the calves experimentally infected with *T. evansi* and re-titration was necessary once positive and negative sera from these calves were available. These findings suggested that very high antigen responses were detectable in the positive sheep serum control compared to the cattle sera, underlining the importance of using sera of the appropriate target species to determine optimal reagent dilutions.

The assumption was made that cattle serum is similar to buffalo serum in the level and binding capacity of trypanosomal antigens and antibodies. Furthermore, anti-bovine IgG conjugate was employed in the antibody-detection tests used for buffalo sera later in the project. With doubling serum dilutions, the OD of the 2G6 high-positive control (C++) declined more rapidly than the OD of the Tr7 high-positive control (C++), possibly due to a lower level of 2G6-specific serum antigen available for binding. Therefore, the level of serum antigen may be a more important limiting factor in the 2G6 Ag-ELISA, which used a 1:2 serum dilution, than in the Tr7 Ag-ELISA, which used a 1:10 serum dilution.

The serum antigens recognised by these Ag-ELISAs have not yet been fully characterised, but are thought to be released by dying trypanosomes. The Tr7 monoclonal antibody recognises a 15 kDa plasma membrane antigen common to *T. brucei*, *T. rhodesiense* and *T. gambiense* (Nantulya *et al.*, 1987), and the 2G6 monoclonal antibody recognises a 70 kDa undefined structural antigen (A.G. Luckins, pers. comm.).

Sufficient reference control sera (C++, C+ and C-) were obtained from the experimentally-infected calves for the quality control of the two *T. evansi* Ag-ELISAs to be conducted in the project work in Indonesia. Quality control limits were established as 'bench marks' of acceptable plate-to-plate and day-to-day variation within Balitvet (intra-laboratory variation) and between the CTVM and Balitvet (inter-laboratory variation). To obtain upper and lower control limits, 90th and 10th percentiles were considered to be more appropriate because the data from repeated assays did not have a Normal distribution. The variation between replicate control wells was greater in the 2G6 Ag-ELISA than in the Tr7 Ag-ELISA, as indicated by a higher number of coefficients of variation above 10%. These findings may reflect differences in reagent characteristics, in particular the origin and preparation of the monoclonal antibodies. The 2G6 monoclonal antibody was prepared from the IgG fraction and precipitated by salt fractionation using ammonium sulphate (A.G. Luckins, pers. comm.) and the Tr7 monoclonal antibody was prepared from the IgM fraction, separated by filtration through a sepharose column (Nantulya *et al.*, 1987).

Differences were observed between the profiles of antigenaemia detected by the two *T. evansi* Ag-ELISAs in Calf 917 and a high antigenaemic response was not detected until the later stages of infection in this calf. Both calves were antigenaemic by ten days post-infection, but the antigenaemias fluctuated throughout the course of infection and, of importance with respect to diagnostic sensitivity, the antigen detected by the 2G6 monoclonal antibody declined below the 20 PP cut-off value by three weeks post-infection.

The estimates of specificity of the 2G6 Ag-ELISA (83% {78, 87}) and Tr7 Ag-ELISA (78% {72, 82}) were not significantly different. *Trypanosoma theileri* is the only bovine trypanosome found in the UK and is reported not to be recognised by *T. evansi* Ag-ELISAs (Delafosse *et al.,* 1995); therefore all the sera were assumed to be true negative samples. The number of false-positive results was unexpectedly high and suggests that either there were non-immunological reactions or immunological reactions occurring. Different types of reactions may have been involved because the majority of false-positive samples were positive with one Ag-ELISA only. Another explanation is the binding of cross-linking heterophilic antibodies (e.g., anti-murine antibodies) to the Ag-ELISA monoclonal antibodies, and it has been shown that a variety of substances found in sera from a wide range of animal species can multivalently bind mouse IgG present (Boscato and Stuart, 1988).

Although the herd sample sizes were small, the variation in specificity estimates found between some herds demonstrated the potential bias that could occur in the selection of a negative population. Ideally, animals of the same species and living in the area where the test is to be used should be sampled (i.e., representative of the target population). Because of the difficulty of identifying non-exposed buffaloes in Indonesia, the possibility of obtaining sera from buffaloes in Australia, where *T. evansi* is not found, was investigated for further evaluation of specificity. These preliminary estimates of specificity suggested that a higher cut-off value should be considered, but the results from the experimental calves showed that this would reduce sensitivity. Therefore a single cut-off value was not chosen and both the 20 PP and 30 PP cut-off values were used in subsequent project work to evaluate the diagnostic sensitivity and specificity of the two Ag-ELISAs.

# CHAPTER 4

# EXPERIMENTAL INFECTION OF BUFFALOES WITH TWO INDONESIAN ISOLATES OF *TRYPANOSOMA EVANSI*

## 4.1 Introduction

Validation of diagnostic tests for *T. evansi* infections of livestock in Southeast Asia requires testing both *T. evansi*-infected and uninfected livestock from this region to estimate diagnostic sensitivity and specificity, respectively. However, the identification of *T. evansi*-infected animals is problematic because of the low diagnostic sensitivity of currently available tests (Luckins, 1992), and therefore confirmation of the negative-status of animals can be difficult in endemic areas where a large proportion of the population have been exposed to the parasite. Newly developed tests would be expected to be better compared with established tests, but this means that the interpretation of positive results by new tests for animals which are negative by all other tests can be difficult.

For example, in field studies in Gabon 40% of 106 parasitologically-negative N'Dama cattle that had been exposed to natural tsetse-challenge were positive by Ag-ELISA (Trail *et al.*, 1992). In another study, nine out of 20 camels sampled in Kenya and 13 out of 20 camels sampled in Mali were considered to be infected with *T. evansi* on the basis of detectable antigenaemia alone, but were negative by parasitological tests (Nantulya *et al.*, 1989b). In Indonesia, an Ag-ELISA based on a *T. brucei*-specific monoclonal antibody was used to test 37 buffaloes in Lampung, southern Sumatra, and 78% of the buffaloes were found to be antigenaemic compared with 27% positive by MHCT (Anon, 1990).

One approach to test validation is to ensure that animals are truly infected with *T. evansi* by using experimentally-infected animals, and this has been used by several workers (e.g. Nantulya and Lindqvist, 1989; Nantulya *et al.*, 1989a; Masake *et al.*, 1995a; Monzon *et al.*, 1995). However, experimental studies have often used small numbers of animals which were not always the target species for

which the test was intended, and therefore the results obtained may have been biased both by the choice of animals and individual animal variation. Another common limitation is the short period of monitoring in comparison to the length of time which animals may be naturally infected (i.e., for several years). Experimental infections are not truly representative of all the different stages of natural infections, namely acute and chronic stages, and furthermore the presence or absence of associated clinical signs may depend on the strain of parasite chosen. For example, in studies of animals experimentally infected with different *Trypanosoma* species monitoring periods of 60 days (Nantulya *et al.*, 1989a) and 40 days (Nantulya and Lindqvist, 1989) were used. In another study using Boran cattle, although the animals were monitored for 600 days, only four individual cattle were used (Masake *et al.*, 1995a). Higher detectable antigenaemias occur at certain stages of infection, and therefore sampling animals during these stages could bias the estimation of sensitivity towards 100%.

The overall aims of the present study were to: 1) determine the diagnostic sensitivity of two standardised Ag-ELISAs for the detection of *T. evansi* infections in individual Indonesian buffaloes, and 2) determine the ability of the assays to monitor trypanosome clearance after chemotherapy. To achieve this, 35 buffaloes were infected experimentally on different occasions with two isolates of *T. evansi* and treated with a trypanocidal drug to: 1) obtain profiles of antigenaemia of individual buffaloes; 2) determine when antigens are first detectable post-infection; 3) determine the number of buffaloes that are positive by the Ag-ELISAs compared to other diagnostic tests; and 4) monitor changes in antigenaemia after chemotherapy.

Buffaloes were chosen instead of cattle for this study because of their economic importance throughout Southeast Asia, the lack of data on serum antigen responses in buffaloes after infection with *T. evansi* and because buffaloes are an important target population for the application of these Ag-ELISAs. Two Indonesian isolates of *T. evansi* were used for the experimental infections: an isolate of low pathogenicity was used for the primary infection and a more pathogenic isolate for the secondary infection. Trypanocidal chemotherapy was given to eliminate pre-existing natural *T. evansi* infections prior to the primary infection and to eliminate the primary

infection prior to the secondary infection. The two standardised Ag-ELISAs based on the 2G6 and Tr7 monoclonal antibodies (described in Chapter 3) were compared with the MHCT, MI test, IgM ELISA, IgG ELISA and CATT.

This chapter focuses primarily on the serum antigen and antibody responses of individual buffaloes and groups of buffaloes experimentally infected with *T. evansi*. The diagnostic sensitivities of the Ag-ELISAs and the other diagnostic tests were estimated at different stages of infection and these results are presented in Chapters 6 and 7.

## 4.2 Materials and Methods

### 4.2.1 Buffaloes

Thirty-five male swamp buffaloes were purchased in three batches from the local market in Bogor, Java, Indonesia. Buffaloes were identified with ear-tag numbers 500 to 513 (batch 1), 514 to 526 (batch 2), and 527 to 534 (batch 3). The buffaloes were approximately 18-24 months old and none was found to be parasitaemic by the MHCT performed on ear-vein blood at the time of purchase (one to two months prior to the main study). The buffaloes were kept at Balitvet, Bogor, in a fly-proof animal house in pens with metal-bar partitions. The buffaloes were each given a maintenance diet of approximately 12 kg fresh elephant grass (*Pennisetum purpureum*) and 1-2 kg commercial cattle concentrate ration.

### 4.2.2 Procedures prior to the main study

On arrival at Balitvet, the buffaloes were ear-tagged, and weighed using a digital weigh balance (Ruddweigh Pty). Faecal samples were examined for helminth eggs, and all buffaloes were given anthelmintics orally (triclabendazole, Fasinex 10%, 36 mg/kg, Ciba Animal Health; and fenbendazole, Panacur SC 10%, 7.5 mg/kg, Hoechst Roussel Vet Limited).

Jugular blood samples were taken every three days over a minimum of four weeks' settling-in period. Giemsa-stained thin blood smears were examined for the presence of *Anaplasma marginale, Babesia bigemina* and *Babesia bovis*, and were all

negative. Some buffaloes were found to have *T. evansi* infections by MHCT (Buffaloes 503, 506, 509, 511, 514, 517 and 532) or by MI (Buffaloes 521 and 530) or to have *T. theileri* infection (Buffaloes 505, 507 and 513). All buffaloes were treated 17 days prior to the primary infection with the trypanocidal drug Cymelarsan (Rhone-Merieux Ltd) at an intramuscular therapeutic dose of 0.75 mg/kg, previously shown to be effective for *T. evansi* infections of buffaloes in Indonesia and which is rapidly excreted (Payne *et al.*, 1992). After treatment, the negative *T. evansi*-infection status of the buffaloes was confirmed by at least four weekly MI tests before the experimental infection was given.

### 4.2.3 Experimental protocol

The timetable of the main procedures conducted in this study is given in Table 4.1 and the notation used to describe the different buffalo groups is summarised in section 4.2.5.

#### Trypanosoma evansi *isolates*

Two *T. evansi* isolates were used for the experimental infections. *Trypanosoma evansi* Bakit 259, which had been passaged five times after isolation from a buffalo in Tuban, East Java, was chosen for the primary infection and *T. evansi* Bakit 362, which had been passaged three times after isolation from a buffalo in Bangkalan, Madura Island, was chosen for the secondary infection. Mice were inoculated with stabilates of the isolates to expand the numbers of trypanosomes. The mice were bled when a high parasitaemia was found by WBF examination of tail blood. Each buffalo was injected intravenously with $2 \times 10^7$ trypanosomes, as described previously in Chapter 3.

#### *Stage 1: Primary infection with* Trypanosoma evansi *Bakit 259*

All 35 buffaloes were infected with *T. evansi* Bakit 259 and monitored for 105 days, as described below.

*Stage 2: Cymelarsan chemotherapy*

After monitoring the primary infection for 105 days, the buffaloes shown to be parasitaemic at least once during this period by either MHCT or MI (n = 24) were randomly divided into two groups: $T_{CYPOS}$ (treated group) and $T_{CON}$ (untreated control group). All buffaloes in Group $T_{CYPOS}$ (n = 12) were injected intramuscularly with Cymelarsan (0.75 mg/kg) and all buffaloes in Group $T_{CON}$ (n = 12) were left untreated. The remaining 11 buffaloes were not found parasitaemic on any sampling days of the primary infection and were designated Group $T_{CYNEG}$. All buffaloes in Group $T_{CYNEG}$ were given Cymelarsan (0.75 mg/kg) intramuscularly because, although aparasitaemic, several buffaloes in this group had detectable antigen and antibody responses during the primary infection.

The buffaloes of all three groups were then monitored for 67 days, after which the untreated control buffaloes (Group $T_{CON}$) were treated intramuscularly with Cymelarsan (0.75 mg/kg) to eliminate all *T. evansi* Bakit 259 infections prior to the secondary infection.

*Stage 3: Secondary infection with* Trypanosoma evansi *Bakit 362*

The buffaloes (except Buffalo 503 which died on Day 177 from an unknown cause) were injected intravenously with $2 \times 10^7$ trypanosomes of *T. evansi* Bakit 362 at 35 days (Group $T_{CON}$), 67 days (Group $T_{CYNEG}$) or 74 days (Group $T_{CYPOS}$), after Cymelarsan chemotherapy. The 34 buffaloes were then monitored for 45-64 days. Group $T_{CON}$ buffaloes were infected 19 days after those in Groups $T_{CYPOS}$ and $T_{CYNEG}$, to allow a total of 35 days for serum antigen and antibody responses to decline after chemotherapy. At the end of the monitoring period, all buffaloes were injected intramuscularly with Cymelarsan (0.75 mg/kg).

Approximately three months after treatment at the end of the study, 18 buffaloes were still available for sampling and, 12 of these buffaloes were sampled after a further five months. During this eight month period the buffaloes were kept in the fly-proof animal house.

### 4.2.4 Monitoring procedures

Jugular blood samples were collected weekly from each buffalo into 10 ml plain sterile Vacutainers and 7 ml sterile Vacutainers containing 15% EDTA $K_3$ anticoagulant. The blood samples with anticoagulant were examined by WBF and MHCT (Woo, 1969), and the PCVs were recorded. From each sample, 0.5 ml was inoculated intraperitoneally into two laboratory mice which were monitored for 30 days by WBF examination of tail blood every two to three days. For the secondary infection, one mouse per buffalo was used to reduce the total number of mice required. The WBF results were recorded as the total number of trypanosomes seen in 20 microscope fields at x 400 magnification. The MHCT results were scored from 0 to 3 according to the total number of trypanosomes seen in the microhaematocrit tube: negative (score 0), 1-5 trypanosomes (score 1), 6-20 trypanosomes (score 2) and more than 20 trypanosomes (score 3).

**Table 4.1** Timetable of procedures and monitoring periods of the primary and secondary *Trypanosoma evansi* experimental infections and Cymelarsan chemotherapy of the Indonesian buffaloes.

| Procedure/monitoring period* | Day |
|---|---|
| Cymelarsan treatment prior to experiment | -17 |
| All buffaloes infected with *T. evansi* Bakit 259 | 0 |
| End of *T. evansi* Bakit 259 monitoring period | 105 |
| Group $T_{CYPOS}$ treated with Cymelarsan | 107 |
| Group $T_{CYNEG}$ treated with Cymelarsan | 114 |
| End of post-treatment monitoring period | 161 |
| Group $T_{CON}$ treated with Cymelarsan | 165 |
| Groups $T_{CYPOS}$ and $T_{CYNEG}$ infected with *T. evansi* Bakit 362 | 181 |
| Group $T_{CON}$ infected with *T. evansi* Bakit 362 | 200 |
| End of *T. evansi* Bakit 362 monitoring period | 245 |
| All buffaloes treated with Cymelarsan | 245 |
| 18 buffaloes blood sampled post-treatment | (3 months) |
| 12 buffaloes blood sampled post-treatment | (8 months) |

*: Buffalo group notation explained in section 4.2.5

Blood samples without anticoagulant were left to clot overnight at 4°C and the next day the sera were collected by centrifugation at 800 g for ten minutes and stored in

sterile 2 ml plastic tubes. The sera were stored at -20°C and later tested by the 2G6 Ag-ELISA, Tr7 Ag-ELISA, IgM ELISA, IgG ELISA and CATT described in Chapter 3 and Appendix I. All the samples were tested by the Ag-ELISAs and antibody-detection tests, except in the primary infection where samples collected 105 days post-infection were not tested by antibody tests.

The buffaloes were weighed every two weeks. During the experiment, several buffaloes had clinical signs of mange and were given 200 μg/kg ivermectin subcutaneously (Ivomec, MSD AGVET). A PCV below 26% was considered an indicator of anaemia (Rowlands *et al.*, 1993), and blood samples with a PCV less than 26% were examined by Giemsa-stained thin blood smear. Several buffaloes with a low PCV were found to have concurrent *A. marginale* infections and were treated with 4 mg/kg imidocarb diproprionate (Imizol, Mallinckrodt Veterinary Ltd), and the treatment was repeated two weeks later, if no increase in PCV was observed following the first treatment.

### 4.2.5 Data analysis

The results were stored in a database using the computer software Microsoft Access[1]. Serum antigen and antibody profiles were graphed for individual buffaloes. The method of summary measures was used to analyse the data from some of the monitoring periods (Matthews *et al.*, 1990). The summary measures obtained were: 1) the number of days post-infection when a buffalo was first detected positive; and 2) the total number of positive weekly tests of individual buffaloes. To compare the latter summary measures between tests, the Friedman two-way analysis of variance by ranks was used with the null hypothesis that there was no difference between tests, using a 5% significance level and adjustment for rank ties (Siegel and Castellan, 1988). Friedman statistic values were computed using Minitab and the Chi-square distribution was assumed because the number of tests was greater than five. Where there was a significant difference at the 5% level, the method of multiple comparisons between tests was used by calculation of the critical difference

[1] Microsoft Access Relational Database Management System for Windows (version 1.1), Microsoft Corporation, One Microsoft Way, Redmond, WA 98052-6399, USA.

of the rank sums to determine which tests differed significantly (Siegel and Castellan, 1988). The proportions of positive tests obtained by different diagnostic tests were calculated, with 95% confidence intervals, using the software CIA. The exact binomial method was used, or if given, the Normal approximation where nP and n(1-P) were both greater than a threshold level defined by the software.

The Student's *t*-test for paired data was used to test the null hypothesis that there was no decrease in the mean PCV of each buffalo group after infection, and the Student's *t*-test for independent samples was used to test the null hypothesis that there was no difference between the mean PCVs of the buffalo groups either before or after infection (Bailey, 1981). The *t*-values were calculated, with adjustment for small sample size, and results interpreted for a one-tailed test at the 5% significance level.

The naturally-acquired *T. evansi* infections found in some buffaloes upon arrival at Balitvet could have affected the antigen and antibody responses found during the primary infection. For this reason, the buffaloes were divided into groups according to their parasitaemic status prior to the primary infection for the data analysis. Not all buffaloes were confirmed to be parasitaemic with either the primary or secondary infections, and therefore the buffaloes were further divided into groups according to whether or not a parasitaemia was detected on at least one occasion after experimental infection.

*Buffalo groups for data analysis*

1) Primary infection with *Trypanosoma evansi* Bakit 259

The following notation was used:

a) Group $259_{POS}$: Buffaloes shown to be parasitaemic on at least one occasion after infection with *Trypanosoma evansi* Bakit 259;

b) Group $259_{PREPOS}$: Buffaloes shown to be parasitaemic on at least one occasion after infection with *Trypanosoma evansi* Bakit 259, but that were parasitaemic with natural *T. evansi* infection(s) after their arrival in Balitvet;

c) Group $259_{NEG}$: Buffaloes not shown to be parasitaemic after infection with *T. evansi* Bakit 259.

2) Cymelarsan chemotherapy

The following notation was used:

a) Group $T_{CYPOS}$: Parasitaemic buffaloes with the primary infection that were treated with Cymelarsan;

b) Group $T_{CON}$: Parasitaemic buffaloes with the primary infection that were untreated controls;

c) Group $T_{CYNEG}$: Buffaloes not shown to be parasitaemic with the primary infection, but that were treated with Cymelarsan.

3) Secondary infection with *Trypanosoma evansi* Bakit 362

The following notation was used:

a) Group $362_{POS}$: Buffaloes shown to be parasitaemic with the secondary infection;

b) Group $362_{NEG}$: Buffaloes not shown to be parasitaemic with the secondary infection.

*Cut-off values*

Empirical cut-off values of 20 PP (2G6 Ag-ELISA, Tr7 Ag-ELISA and IgG ELISA) and 30 PP (IgM ELISA) were chosen based on the standardisation of these ELISAs and because the best estimates of sensitivity and specificity were obtained with these values when naturally-infected buffaloes and non-exposed buffaloes were tested (described in Chapter 6). The supplier's recommendations were followed for the interpretation of the CATT results (see Appendix I). For analysis of data from the primary infection, buffaloes were considered positive if their ELISA PP value was twice the Day 0 PP value, to account for the serum antigen and antibody responses found in some buffaloes prior to infection. For the secondary infection, the cut-off values 20 PP and 30 PP given above were used because a higher proportion of buffaloes had PP values below these cut-off values on Day 0, after a longer period between chemotherapy and the secondary infection.

## 4.3 Results

### 4.3.1 Primary infection of buffaloes with *Trypanosoma evansi* Bakit 259

*Clinical signs, concurrent infections and packed cell volume*

During the primary infection with *T. evansi* Bakit 259, clinical signs of mange (pruritus, crust formation, skin thickening and alopecia, especially around the perineal region and dorsal part of the neck) were observed in 11 buffaloes (Buffaloes 505, 508, 509, 512, 513, 514, 518, 521, 529, 530, and 532). No other clinical signs were seen. Buffaloes 501, 503, 509, 510, 519 and 525 were found to have *A. marginale* infections and Buffalo 510 was found to have a concurrent *B. bovis* infection. Buffaloes 504, 505 and 516 were found to have infections with *T. theileri* on Day 49, Day 112 and Day 196, respectively.

The PCV of 13 buffaloes declined below 26% during the primary infection and, of these, nine buffaloes (Buffaloes 506, 508, 510, 511, 514, 525, 527, 530 and 532) were parasitaemic on at least one occasion, whilst four buffaloes (Buffaloes 502, 503, 509 and 531) were not found parasitaemic with *T. evansi*. Of buffaloes with a PCV less than 26%, Buffaloes 503, 509, 510 and 525 had *A. marginale* infections. The PCV of Buffalo 510, which had concurrent *T. evansi, A. marginale* and *B. bovis* infections, declined from 29% pre-infection to 13% by 56 days post-infection, but in this buffalo *T. evansi* was found only on Day 28 post-infection, by MI.

During the primary infection, PCVs of individual buffaloes were lower than pre-infection levels from 35 to 49 days post-infection, and in some buffaloes were below 26% up to 77 days post-infection, although in Buffaloes 525 and 532 PCVs were below 26 % up to 105 days post-infection. The mean PCVs of the three buffalo groups (shown in Table 4.2) had declined from pre-infection values by 49 days post-infection, and increased again by 98 days post-infection. By Day 49, there was a significant decrease in the mean PCV of Group $259_{POS}$ ($0.01 < P < 0.02$) and of Group $259_{PREPOS}$ ($0.02 < P < 0.05$). There was no significant difference between the mean PCVs of Groups $259_{POS}$ and $259_{PREPOS}$ ($0.10 < P < 0.20$) or between Groups $259_{PREPOS}$ and $259_{NEG}$ ($0.05 < P < 0.10$).

*Parasitological tests*

Table 4.3 shows the results of weekly MHCT tests and Table 4.4 the results of the weekly MI tests of individual buffaloes during the primary infection. *Trypanosoma evansi* was detected in buffaloes by MI on more occasions than by the MHCT, but some buffaloes were found parasitaemic on only one or two occasions by either parasitological test.

**Table 4.2** Mean packed cell volumes (PCV), with the standard deviation given in brackets, of different buffalo groups during the primary *Trypanosoma evansi* Bakit 259 infection

| Buffalo group | Number of buffaloes | PCV % (SD) | | |
|---|---|---|---|---|
| | | Number of days from primary infection | | |
| | | -1 | 49 | 98 |
| Group $259_{POS}$ | 18 | 36.4 (5.0) | 32.9[1] (8.0) | 37.1 (5.2) |
| Group $259_{PREPOS}$ | 6 | 33.3 (3.0) | 31.0[2] (4.9) | 34.7 (4.8) |
| Group $259_{NEG}$ | 11 | 35.6 (5.0) | 33.7 (6.3) | 36.0 (6.1) |

[1]: Significant decrease from pre-infection level ($0.01 < P < 0.02$)
[2]: Significant decrease from pre-infection level ($0.02 < P < 0.05$)

Buffaloes 515 (Day 98), 522 (Day 91) and 533 (Days 28 and 56) were positive by MHCT but never by MI, and were positive only on the days indicated in brackets. Eight buffaloes were shown to be parasitaemic by MI, but not by MHCT, and one of these, Buffalo 511, was found to be parasitaemic on one occasion only.

The earliest time after infection that buffaloes were first found positive was 14 days by MI (five buffaloes) and 21 days by MHCT (two buffaloes). During the first 12 weeks of the primary infection, 13% (8, 17) of all Group $259_{POS}$ samples were positive by MHCT and 43% (36, 49) were positive by MI. In Group $259_{PREPOS}$, 11% (5, 21) of all samples were positive by MHCT and 50% (38, 62) were positive by MI. For comparison with the secondary infection the proportions of parasitological tests that were positive during the first six weeks of the primary infection were also calculated: 15% (8, 22) of Group $259_{POS}$ samples were positive by MHCT and 37% (28, 46) were positive by MI.

In the mice, parasitaemia was first detected 3 to 21 days post-inoculation. Wet blood film examination only detected a parasitaemia in Buffalo 524 (21 days post-infection) and Buffalo 519 (35 days post-infection), and therefore this test was not used after 12 weeks post-infection.

*Profiles of antigenaemia of individual buffaloes*

A wide range of individual profiles of antigenaemia was found in the buffaloes experimentally infected with *T. evansi* 259. Prior to infection, 18 of the 35 buffaloes had PP values below 20 PP by the 2G6 Ag-ELISA compared with eight buffaloes by the Tr7 Ag-ELISA, and only Buffaloes 515 and 532 had PP values less than 20 PP by both Ag-ELISAs. Trypanosomal antigens were detected 7 to 42 days post-infection both by the 2G6 Ag-ELISA and Tr7 Ag-ELISA.

For the presentation and interpretation of the results, the buffaloes were categorised into three groups according to the 2G6-specific antigenaemia profile found during the primary infection as follows: Group 1) buffaloes that had a PP value lower than 20 PP pre-infection and in which an antigen response was detected post-infection (Figures 4.1 to 4.10); Group 2) buffaloes that had a PP value higher than 20 PP pre-infection and in which an antigen response was detected post-infection (Figures 4.11 to 4.14); and Group 3) buffaloes that did not have a detectable antigen response post-infection, including buffaloes that had a persistent antigenaemia (Figures 4.15 to 4.18). To demonstrate the variation found between individual buffaloes, a total of 18 representative examples of buffaloes in Group 1 (n = 10), Group 2 (n = 4) and Group 3 (n = 4) are given. The serum antigen and antibody profiles of these buffaloes after Cymelarsan treatment and following the secondary infection with *T. evansi* Bakit 362 are also included in these figures.

In Group 1, Buffalo 529 (Figure 4.8) had similar antigenaemia profiles using both the 2G6 Ag-ELISA and Tr7 Ag-ELISA, with peaks of antigenaemia detected seven days post-infection (2G6 Ag-ELISA) and 42 days post-infection (both Ag-ELISAs). However, in this buffalo no antigenaemia was detectable 21-28 days post-infection and after 70 days post-infection. Other buffaloes in Group 1 had similar profiles of

antigenaemia, but with peaks of antigenaemia occurring at 21-28 days (Buffaloes 532 and 534; Figures 4.9 and 4.10), 42 days (Buffalo 515; Figure 4.5) and 49 to 70 days (Buffaloes 514 and 528; Figures 4.4 and 4.7) post-infection. Buffalo 515 had a PP value above 20 only on Day 42 post-infection by either Ag-ELISA and was parasitaemic only on Day 98 post-infection by MHCT and never by MI. Most buffaloes were found to be parasitaemic before antigenaemic, unless they had been naturally infected before the start of the study (e.g. Buffalo 514). In Buffalo 505 (Figure 4.1) a small peak of antigenaemia coincided with the onset of parasitaemia, but no detectable parasitaemia was found during the higher, second antigen peak 63 days post-infection. Buffaloes 512 and 519 (Figures 4.2 and 4.6) had different patterns of antigenaemia detected by the two Ag-ELISAs: in Buffalo 512 the Tr7-specific antigenaemia persisted from a high pre-infection level to the end of the monitoring period, and in Buffalo 519 the Tr7-specific antigen response was earlier and higher than the 2G6-specific antigen response.

All the buffaloes in Group 2 had a high Tr7-specific (and 2G6-specific) antigen response both before and during the primary infection. However, peaks of antigenaemia were detected above the high pre-infection levels by both Ag-ELISAs, and these occurred at the same stage of infection in some buffaloes (e.g., Buffaloes 501 and 527; Figures 4.11 and 4.14), but at different stages in other buffaloes. In Buffaloes 522 and 525 (Figures 4.12 and 4.13) antigens were first detected by the two Ag-ELISAs at the same time, but later in infection higher PP values were obtained with the 2G6 Ag-ELISA than the Tr7 Ag-ELISA.

All buffaloes in Group 3 had a persistent Tr7-specific antigenaemia, but no detectable 2G6-specific antigen response. Of these, Buffaloes 502 and 504 (Figures 4.15 and 4.16) were not shown to be parasitaemic, whereas Buffaloes 518 and 526 (Figures 4.17 and 4.18) were found to be parasitaemic, but on one occasion only.

**Table 4.3** Detection of the primary infection with *Trypanosoma evansi* Bakit 259 in experimental buffaloes by weekly microhaematocrit tests (MHCT)

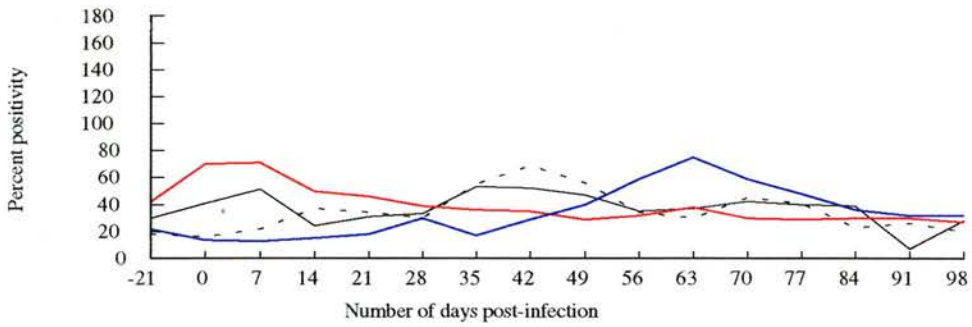
| Buffalo | Number of days post-infection | | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | 7 | 14 | 21 | 28 | 35 | 42 | 49 | 56 | 63 | 70 | 77 | 84 | 91 | 98 | 105 |
| 500 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| 501 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| 502 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| 503 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| 504 | 0 | 0 | 0 | 0 | 0 | 0 | **Th** | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| 505 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| 506 | 0 | 0 | 0 | 0 | **1** | **3** | **1** | 0 | 0 | 0 | **2** | 0 | **1** | 0 | 0 |
| 507 | 0 | 0 | 0 | 0 | 0 | **1** | **2** | **1** | **2** | **1** | 0 | 0 | **1** | 0 | **2** |
| 508 | 0 | 0 | 0 | **2** | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 1 | 0 | 0 | 0 |
| 509 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| 510 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| 511 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | **2** | 0 |
| 512 | 0 | 0 | 0 | **2** | 0 | 0 | 1 | 0 | 0 | **2** | 0 | 0 | **1** | 0 | 0 |
| 513 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| 514 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| 515 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | **2** | 0 |
| 516 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| 517 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| 518 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| 519 | 0 | 0 | 0 | 0 | **1** | 0 | 0 | 0 | 0 | 0 | **1** | 0 | 0 | **1** | 0 |
| 520 | 0 | 0 | 0 | 0 | 0 | **1** | 0 | **1** | 0 | 0 | 0 | 0 | **1** | **2** | 0 |
| 521 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| 522 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | **1** | 0 | 0 |
| 523 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| 524 | 0 | 0 | **3** | **1** | 0 | 0 | 0 | 0 | **1** | 0 | 0 | 0 | **1** | 0 | 0 |
| 525 | 0 | 0 | **1** | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| 526 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| 527 | 0 | 0 | 0 | **1** | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| 528 | 0 | 0 | 0 | 0 | **1** | **1** | 0 | 0 | **2** | **1** | 0 | 0 | 0 | **1** | 0 |
| 529 | 0 | 0 | 0 | **1** | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | **1** | 0 |
| 530 | 0 | 0 | 0 | 0 | 1 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| 531 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| 532 | 0 | 0 | 0 | 0 | 0 | 1 | 1 | 0 | 0 | 0 | 0 | **2** | 0 | 0 | 0 |
| 533 | 0 | 0 | 0 | **1** | 0 | 0 | 0 | **1** | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| 534 | 0 | 0 | 0 | **1** | 0 | 0 | 0 | **1** | 0 | 0 | 0 | 0 | 0 | 0 | 0 |

MHCT scored as: 0 (negative); 1 (1-5 trypanosomes); 2 (6-20 trypanosomes); or 3 (more than 20 trypanosomes), positive results are emboldened; nd: Not done; Th: *T. theileri*

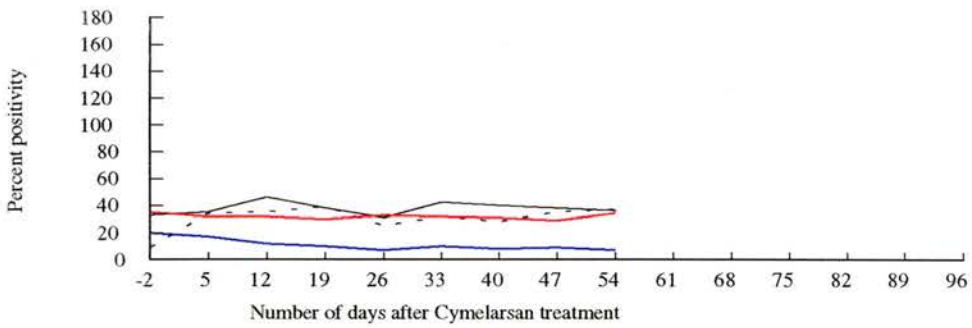
**Table 4.4** Detection of the primary infection with *Trypanosoma evansi* Bakit 259 in experimental buffaloes by weekly mouse inoculation (MI) shown as the number of days when a mouse was first found parasitaemic post-inoculation

| | Number of days post-infection | | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Buffalo | 7 | 14 | 21 | 28 | 35 | 42 | 49 | 56 | 63 | 70 | 77 | 84 | 91 | 98 | 105 |
| 500 | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - |
| 501 | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - |
| 502 | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - |
| 503 | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - |
| 504 | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - |
| 505 | - | - | 6 | 6 | 6 | - | - | - | - | - | - | - | - | - | - |
| 506 | - | 16 | - | 6 | 3 | 6 | 6 | 9 | 16 | 4 | 9 | 10 | 10 | 8 | 11 |
| 507 | - | - | - | - | 3 | 3 | 6 | 3 | 18 | 4 | 10 | 8 | 6 | - | 11 |
| 508 | - | 10 | 6 | 6 | 6 | 12 | 6 | - | - | 6 | 10 | 8 | 10 | 8 | 11 |
| 509 | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - |
| 510 | - | - | - | 15 | - | - | - | - | - | - | - | - | - | - | - |
| 511 | - | - | - | - | - | - | - | - | 20 | - | - | - | - | - | - |
| 512 | - | - | - | 3 | 6 | 6 | 6 | - | 3 | 6 | 10 | 8 | 8 | 11 | 6 |
| 513 | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - |
| 514 | - | - | - | - | - | 9 | 6 | 13 | - | 6 | - | 13 | 17 | - | - |
| 515 | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - |
| 516 | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - |
| 517 | - | - | 6 | - | 9 | 6 | 6 | 6 | 11 | - | 13 | 10 | 10 | 13 | 13 |
| 518 | - | - | - | - | 6 | - | - | - | - | - | - | - | - | - | - |
| 519 | - | - | 6 | 9 | 3 | 6 | 6 | 6 | - | 6 | 10 | 6 | 8 | 6 | 8 |
| 520 | - | - | - | - | 3 | 21 | 6 | 6 | 3 | 6 | 17 | 8 | 8 | 8 | 6 |
| 521 | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - |
| 522 | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - |
| 523 | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - |
| 524 | - | - | 1 | 12 | 3 | 6 | 6 | 6 | - | 6 | 10 | 13 | 10 | 11 | 11 |
| 525 | - | 8 | - | 6 | - | - | 9 | - | - | - | 16 | - | 8 | - | - |
| 526 | - | - | 20 | - | - | - | - | - | - | - | - | - | - | - | - |
| 527 | - | - | - | - | 6 | 3 | 3 | - | - | - | - | 13 | - | - | - |
| 528 | - | - | - | 6 | 3 | 6 | 6 | 3 | 6 | 6 | 10 | 6 | 8 | 11 | 15 |
| 529 | - | - | - | - | 3 | 3 | 6 | 6 | 8 | 6 | 10 | 6 | 8 | 6 | 8 |
| 530 | - | - | 13 | 6 | 9 | 6 | - | - | - | - | - | - | - | - | - |
| 531 | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - |
| 532 | - | 8 | - | 6 | 6 | 3 | 6 | - | - | 8 | 13 | 4 | 14 | - | - |
| 533 | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - |
| 534 | - | 16 | 4 | 6 | 6 | 6 | 6 | - | - | 6 | 10 | 10 | 8 | 11 | - |

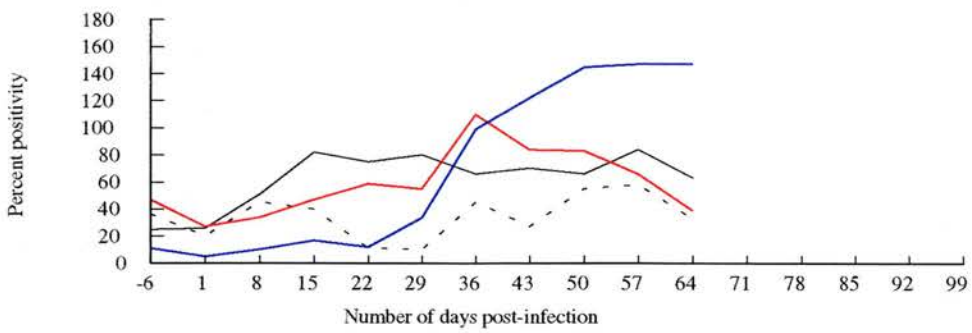
**Figure 4.1** Trypanosomal antigens and antibodies in serum from **Buffalo 505** after primary infection, treatment and challenge with an heterologous stock of *Trypanosoma evansi* (2G6 Ag-ELISA ____ , Tr7 Ag-ELISA ____ , IgM ELISA ----, IgG ELISA ____)




a) Primary infection with *Trypanosoma evansi* Bakit 259

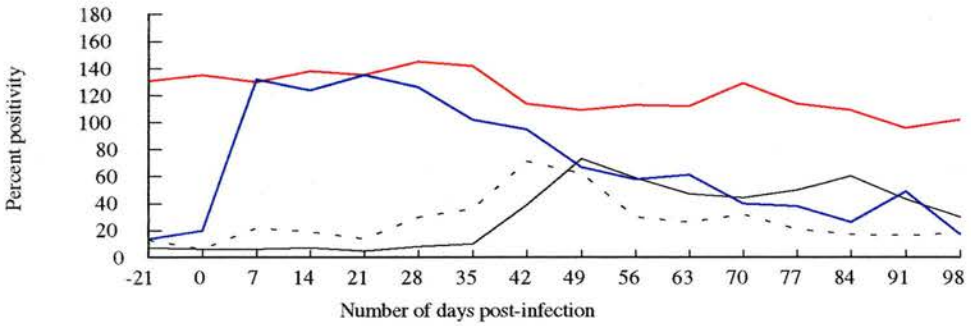



b) Cymelarsan treatment on day 107 of *Trypanosoma evansi* Bakit 259 infection

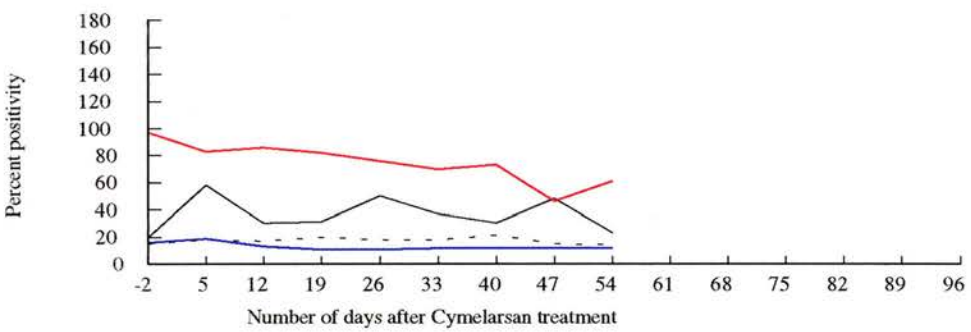



c) Challenge infection with *Trypanosoma evansi* Bakit 362

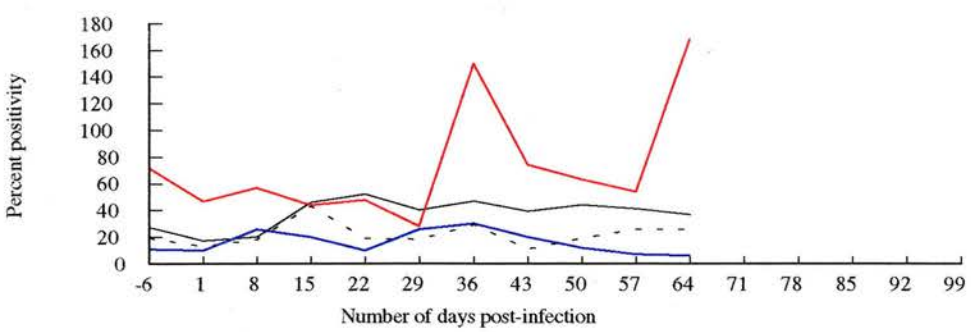
**Figure 4.2** Trypanosomal antigens and antibodies in serum from **Buffalo 512** after primary infection, treatment and challenge with an heterologous stock of *Trypanosoma evansi* (2G6 Ag-ELISA ____ ,Tr7 Ag-ELISA ____ , IgM ELISA ----, IgG ELISA ____)




a) Primary infection with *Trypanosoma evansi* Bakit 259

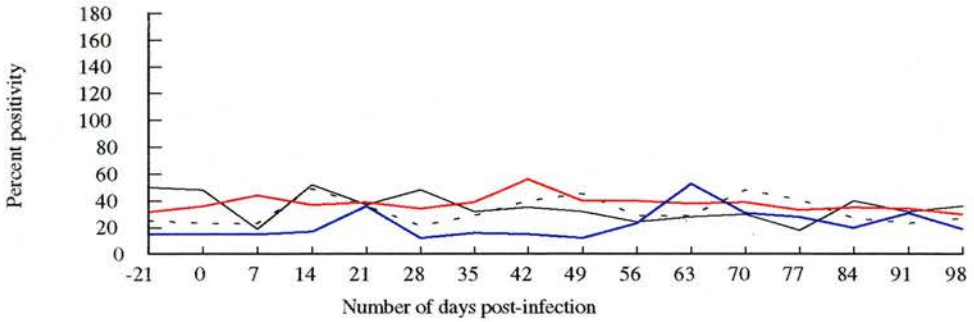



b) Cymelarsan treatment on day 107 of *Trypanosoma evansi* Bakit 259 infection

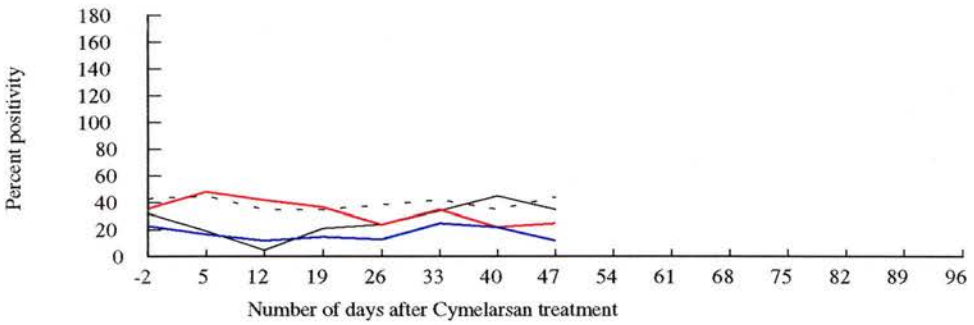



c) Challenge infection with *Trypanosoma evansi* Bakit 362

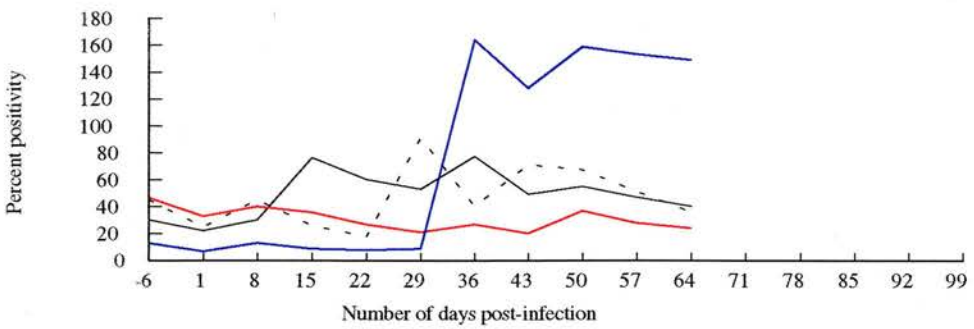
**Figure 4.3** Trypanosomal antigens and antibodies in serum from **Buffalo 513** after primary infection, treatment and challenge with an heterologous stock of *Trypanosoma evansi* (2G6 Ag-ELISA ____ ,Tr7 Ag-ELISA ____ , IgM ELISA ----, IgG ELISA ____)




a) Primary infection with *Trypanosoma evansi* Bakit 259 (not shown parasitaemic)

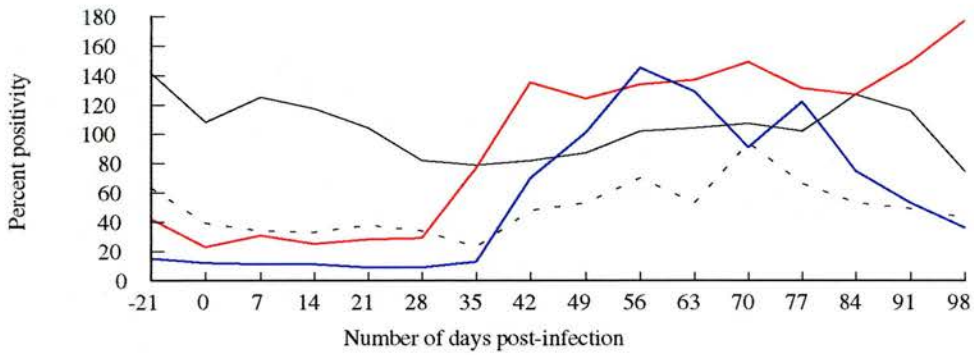



b) Cymelarsan treatment on day 114 of *Trypanosoma evansi* Bakit 259 infection

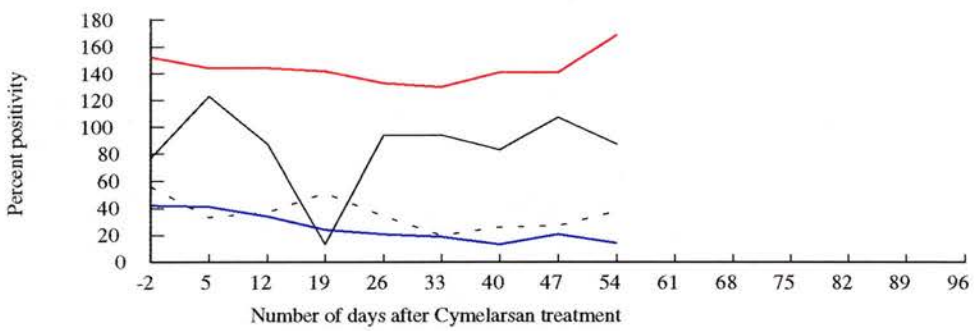



c) Challenge infection with *Trypanosoma evansi* Bakit 362

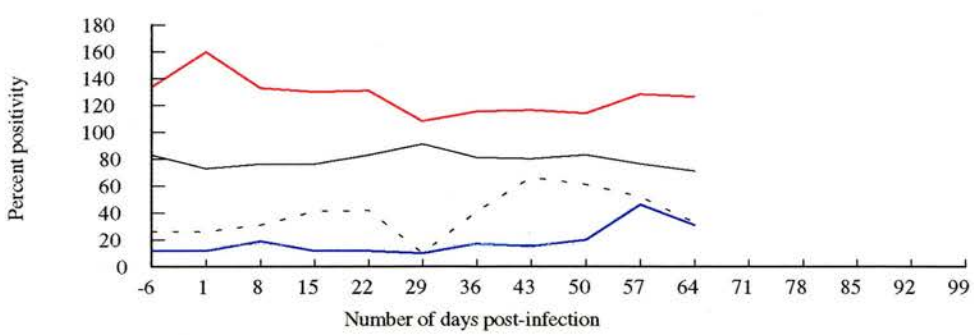
**Figure 4.4** Trypanosomal antigens and antibodies in serum from **Buffalo 514** after primary infection, treatment and challenge with an heterologous stock of *Trypanosoma evansi* (2G6 Ag-ELISA ____ ,Tr7 Ag-ELISA ____ , IgM ELISA ----, IgG ELISA ____)




a) Primary infection with *Trypanosoma evansi* Bakit 259

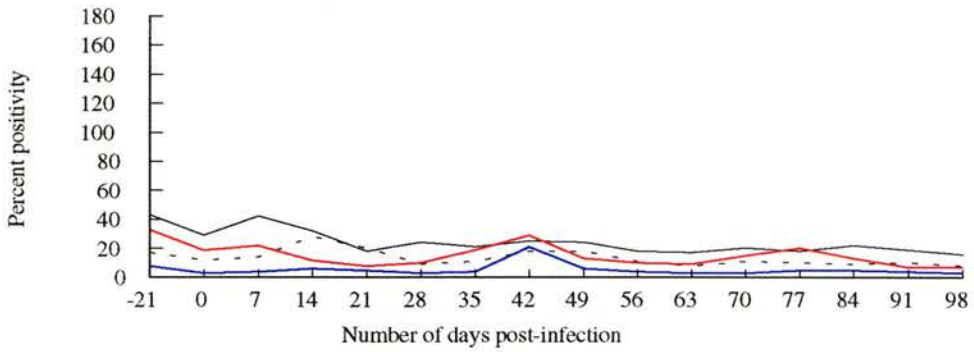



b) Cymelarsan treatment on day 107 of *Trypanosoma evansi* Bakit 259 infection

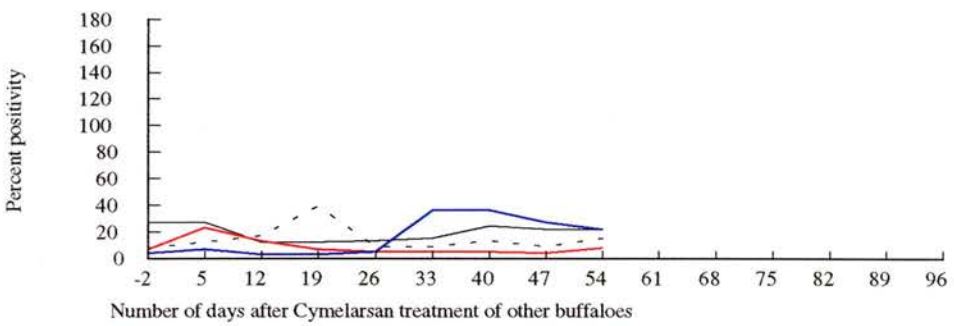



c) Challenge infection with *Trypanosoma evansi* Bakit 362

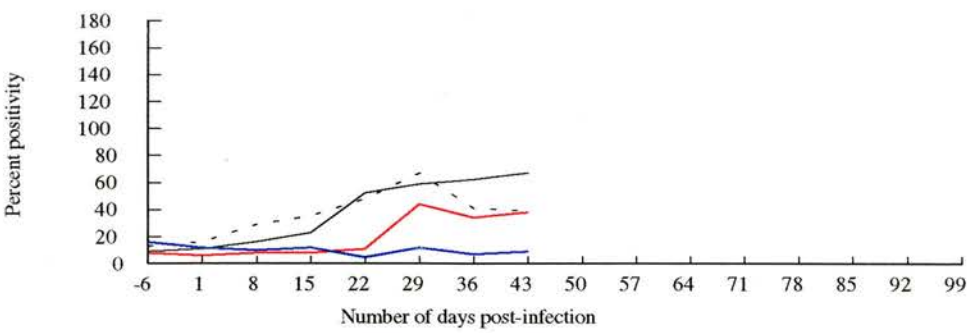
**Figure 4.5** Trypanosomal antigens and antibodies in serum from **Buffalo 515** after primary infection, treatment and challenge with an heterologous stock of *Trypanosoma evansi* (2G6 Ag-ELISA ____ ,Tr7 Ag-ELISA ____ , IgM ELISA ----, IgG ELISA ____)




a) Primary infection with *Trypanosoma evansi* Bakit 259

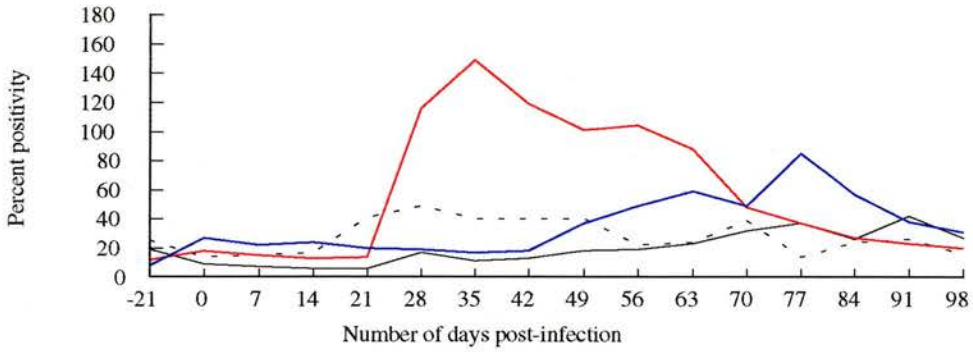



b) Untreated control in treatment stage of *Trypanosoma evansi* Bakit 259 infection

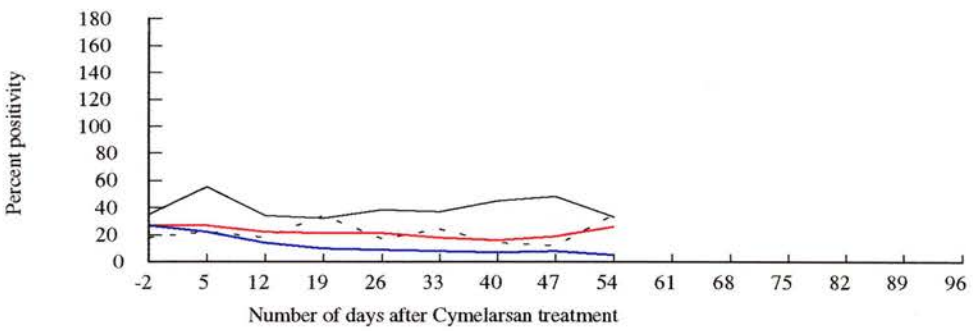



3. Challenge infection with *Trypanosoma evansi* Bakit 362

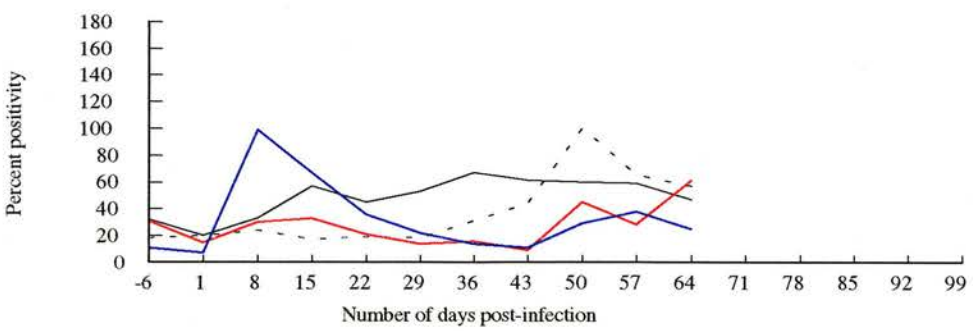
**Figure 4.6** Trypanosomal antigens and antibodies in serum from **Buffalo 519** after primary infection, treatment and challenge with an heterologous stock of *Trypanosoma evansi* (2G6 Ag-ELISA ____ , Tr7 Ag-ELISA ____ , IgM ELISA ----, IgG ELISA ____)




a) Primary infection with *Trypanosoma evansi* Bakit 259

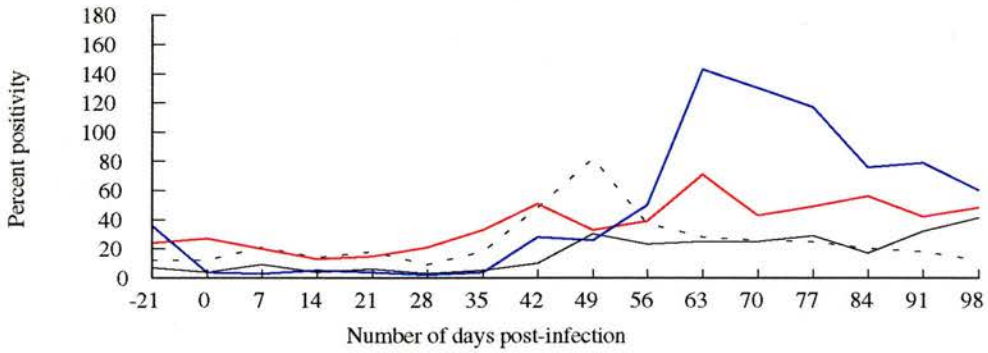



b) Cymelarsan treatment on day 107 of *Trypanosoma evansi* Bakit 259 infection

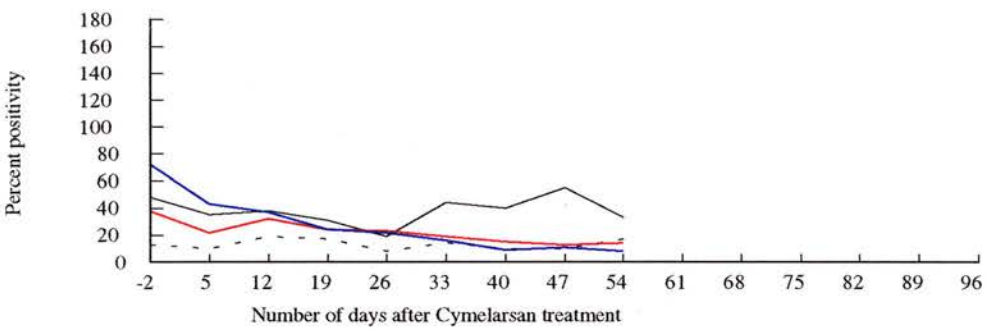



c) Challenge infection with *Trypanosoma evansi* Bakit 362

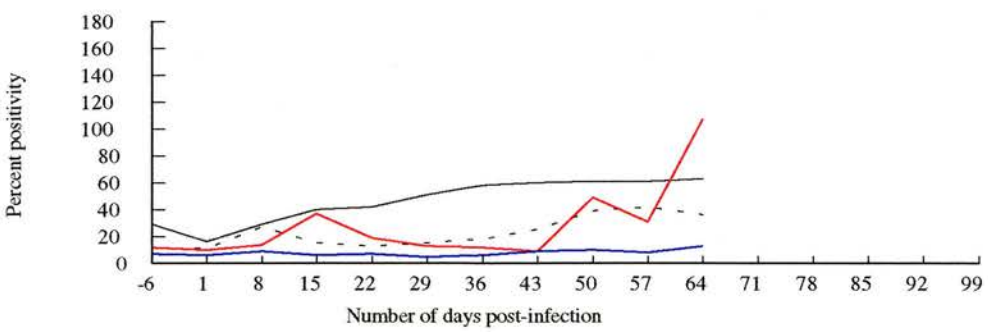
**Figure 4.7** Trypanosomal antigens and antibodies in serum from **Buffalo 528** after primary infection, treatment and challenge with an heterologous stock of *Trypanosoma evansi* (2G6 Ag-ELISA ____ , Tr7 Ag-ELISA ____ , IgM ELISA ----, IgG ELISA ____)




a) Primary infection with *Trypanosoma evansi* Bakit 259

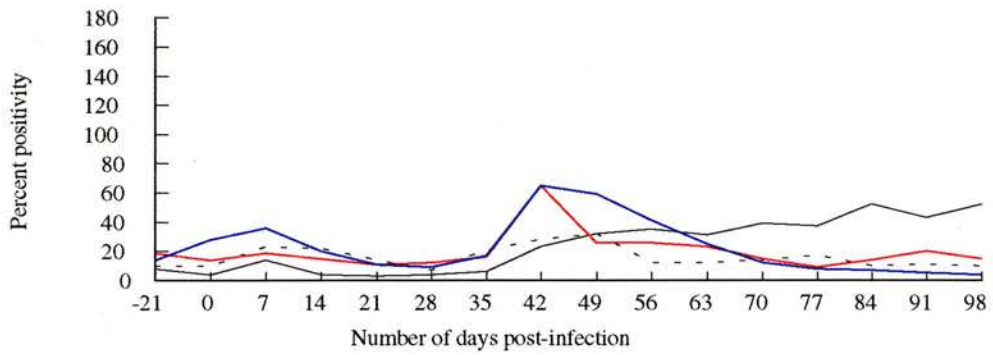



b) Cymelarsan treatment on day 107 of *Trypanosoma evansi* Bakit 259 infection

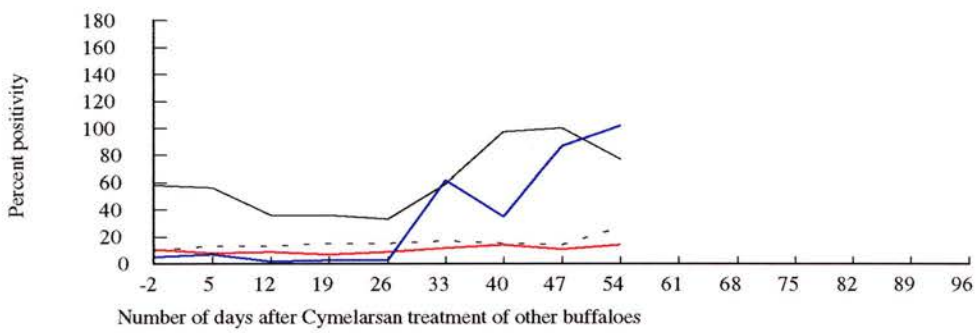



c) Challenge infection with *Trypanosoma evansi* Bakit 362

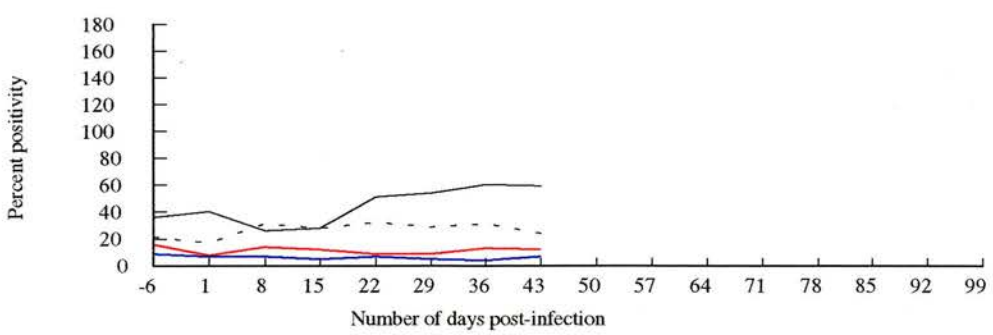
**Figure 4.8** Trypanosomal antigens and antibodies in serum from **Buffalo 529** after primary infection, treatment and challenge with an heterologous stock of *Trypanosoma evansi* (2G6 Ag-ELISA ____ , Tr7 Ag-ELISA ____ , IgM ELISA ----, IgG ELISA ____)




a) Primary infection with *Trypanosoma evansi* Bakit 259

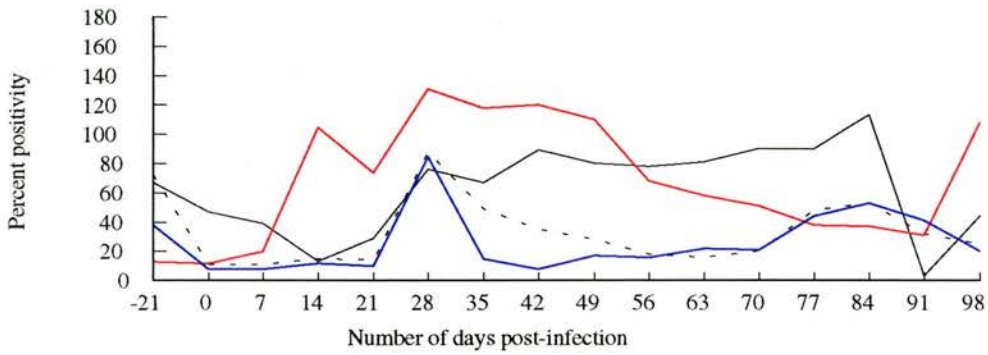



b) Untreated control in treatment stage of *Trypanosoma evansi* Bakit 259 infection

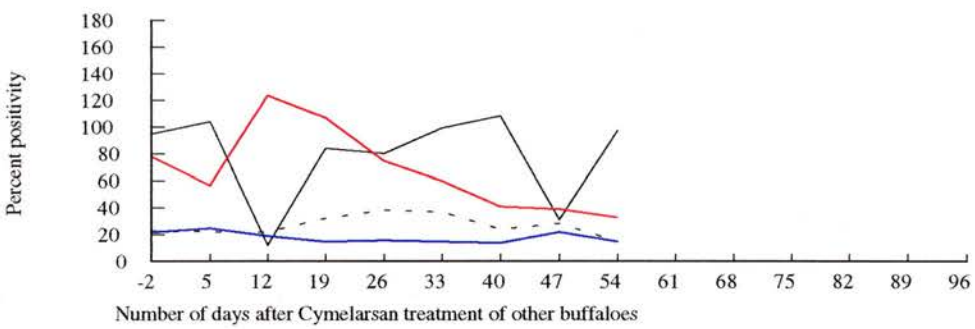



c) Challenge infection with *Trypanosoma evansi* Bakit 362

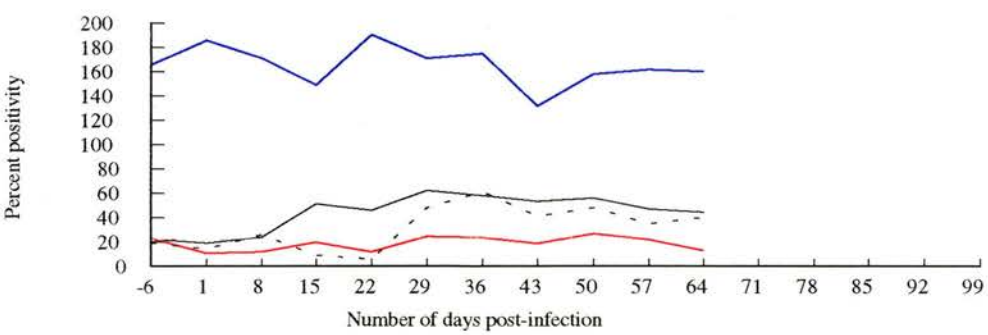
**Figure 4.9** Trypanosomal antigens and antibodies in serum from **Buffalo 532** after primary infection, treatment and challenge with an heterologous stock of *Trypanosoma evansi* (2G6 Ag-ELISA ____ , Tr7 Ag-ELISA ____ , IgM ELISA ----, IgG ELISA ____)




a) Primary infection with *Trypanosoma evansi* Bakit 259

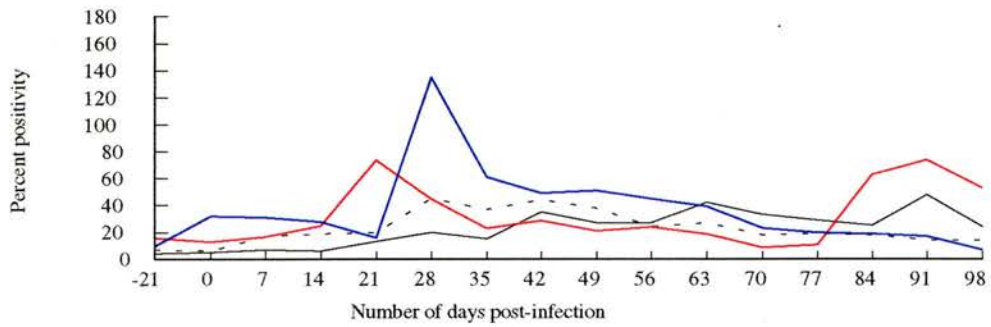



b) Untreated control in treatment stage of *Trypanosoma evansi* Bakit 259 infection

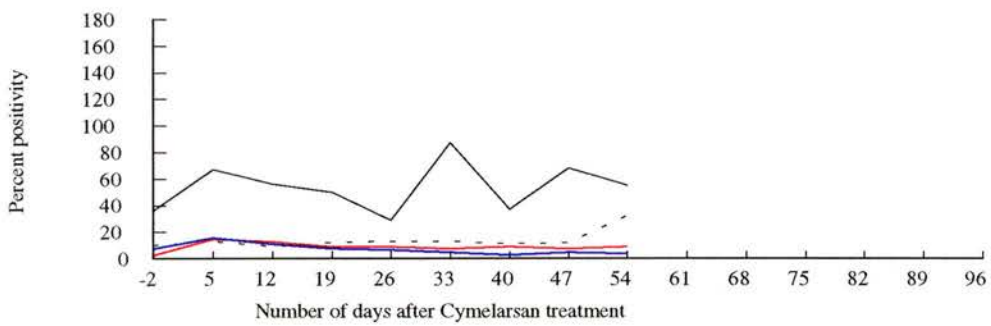



c) Challenge infection with *Trypanosoma evansi* Bakit 362

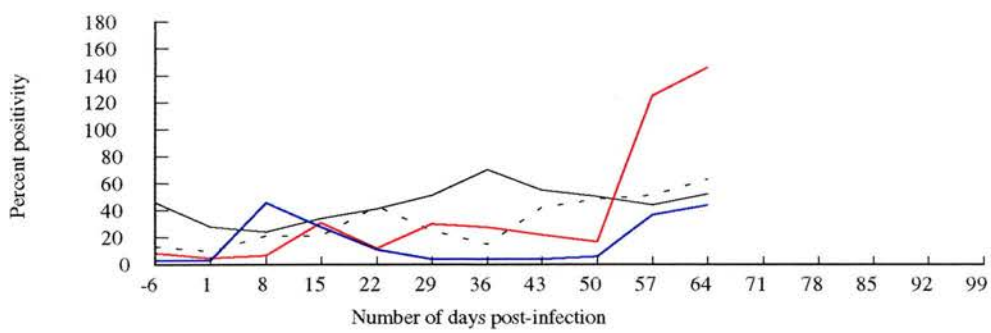
**Figure 4.10** Trypanosomal antigens and antibodies in serum from **Buffalo 534** after primary infection, treatment and challenge with an heterologous stock of *Trypanosoma evansi* (2G6 Ag-ELISA ____ , Tr7 Ag-ELISA ____ , IgM ELISA ----, IgG ELISA ____)




a) Primary infection with *Trypanosoma evansi* Bakit 259

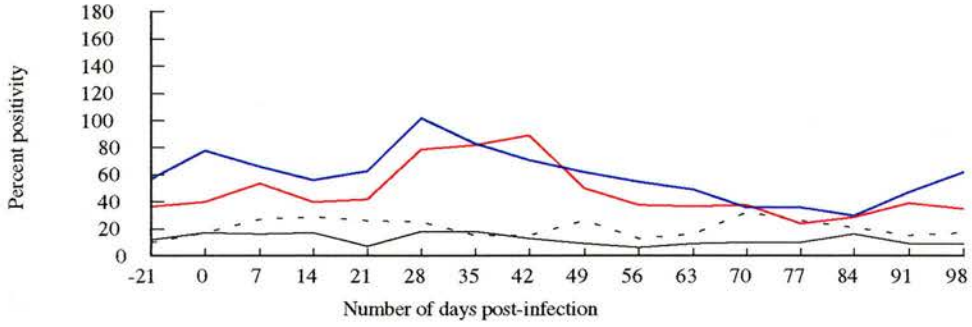



b) Cymelarsan treatment on day 107 of *Trypanosoma evansi* Bakit 259 infection

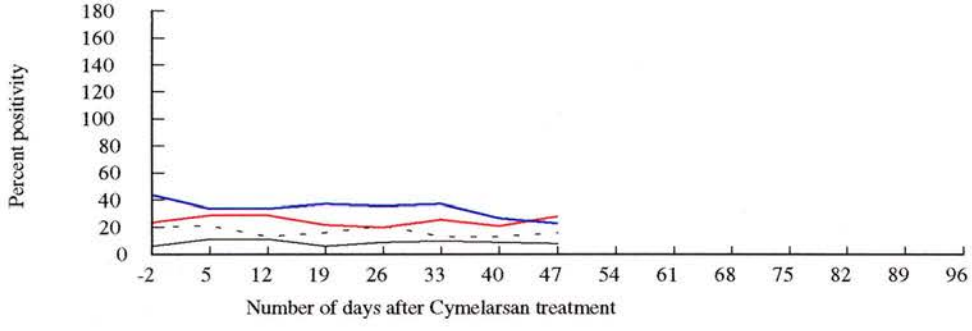



c) Challenge infection with *Trypanosoma evansi* Bakit 362

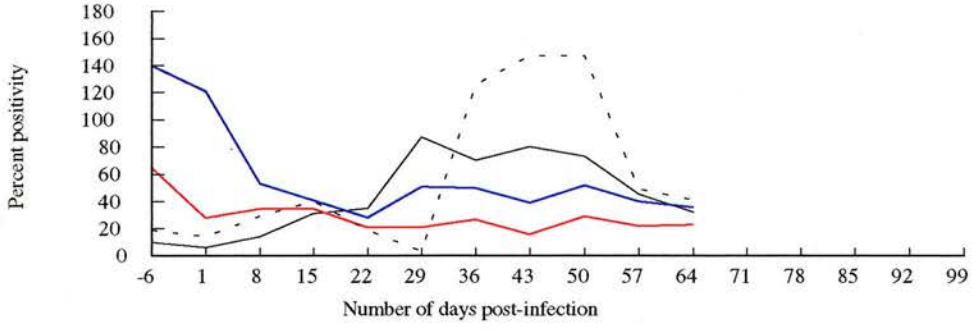
**Figure 4.11** Trypanosomal antigens and antibodies in serum from **Buffalo 501** after primary infection, treatment and challenge with an heterologous stock of *Trypanosoma evansi* (2G6 Ag-ELISA ____ , Tr7 Ag-ELISA ____ , IgM ELISA ----, IgG ELISA ____)




a) Primary infection with *Trypanosoma evansi* Bakit 259 (not shown parasitaemic)

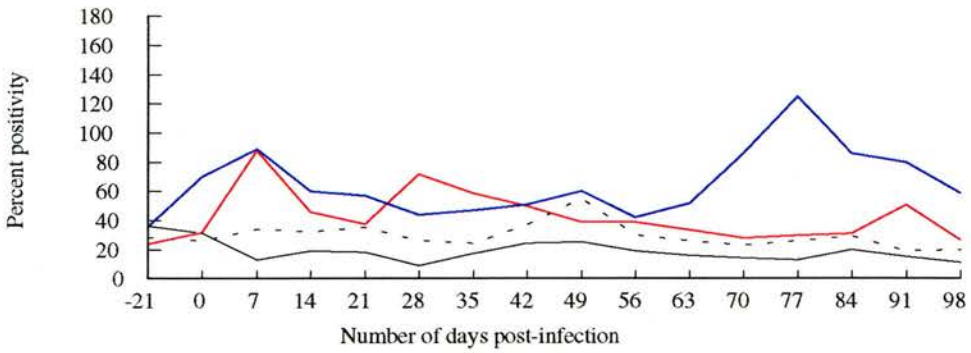



b) Cymelarsan treatment on day 114 of *Trypanosoma evansi* Bakit 259 infection

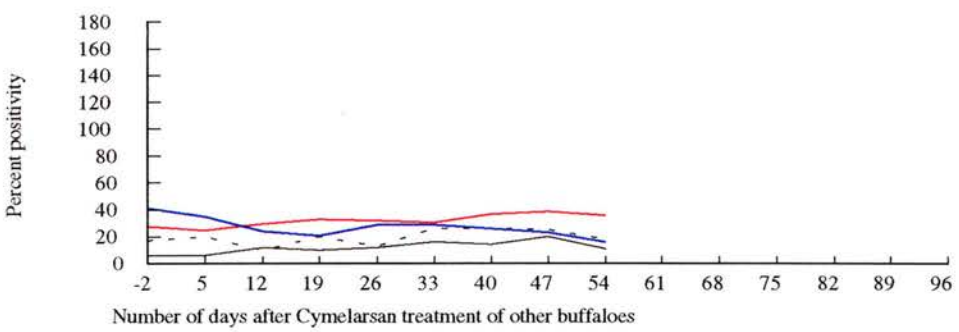



c) Challenge infection with *Trypanosoma evansi* Bakit 362

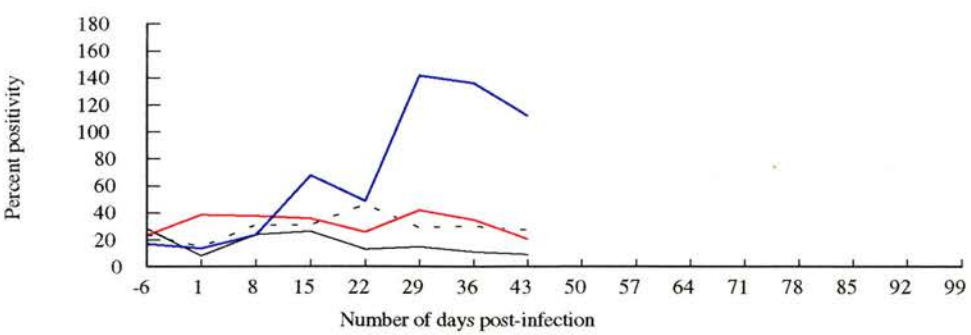
**Figure 4.12** Trypanosomal antigens and antibodies in serum from **Buffalo 522** after primary infection, treatment and challenge with an heterologous stock of *Trypanosoma evansi* (2G6 Ag-ELISA ____ , Tr7 Ag-ELISA ____ , IgM ELISA ----, IgG ELISA ____)




a) Primary infection with *Trypanosoma evansi* Bakit 259

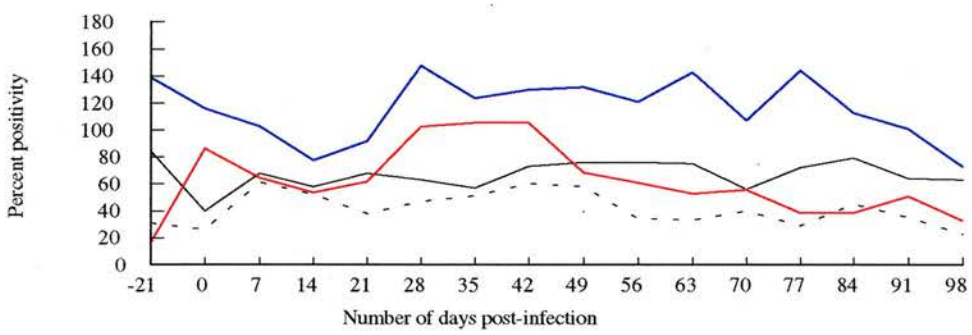



b) Untreated control in treatment stage of *Trypanosoma evansi* Bakit 259 infection

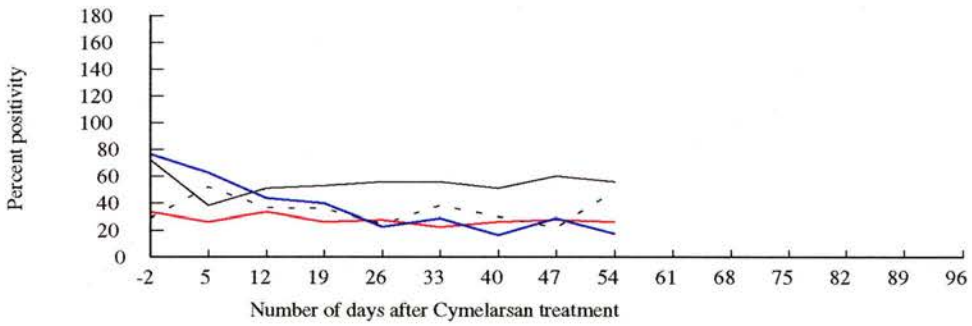



c) Challenge infection with *Trypanosoma evansi* Bakit 362

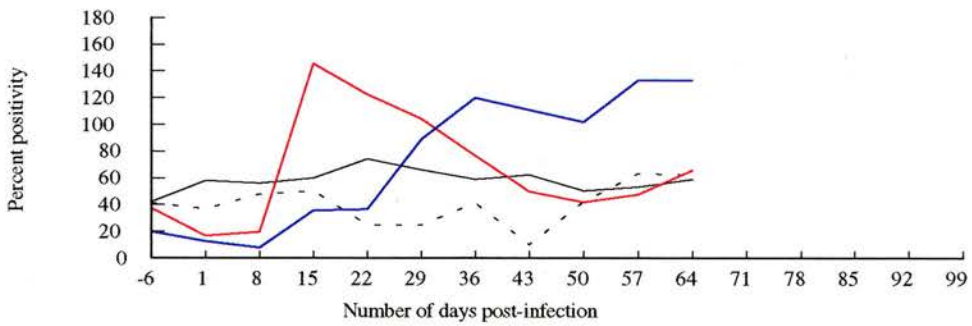
**Figure 4.13** Trypanosomal antigens and antibodies in serum from **Buffalo 525** after primary infection, treatment and challenge with an heterologous stock of *Trypanosoma evansi* (2G6 Ag-ELISA ____ , Tr7 Ag-ELISA ____ , IgM ELISA ----, IgG ELISA ____)




a) Primary infection with *Trypanosoma evansi* Bakit 259

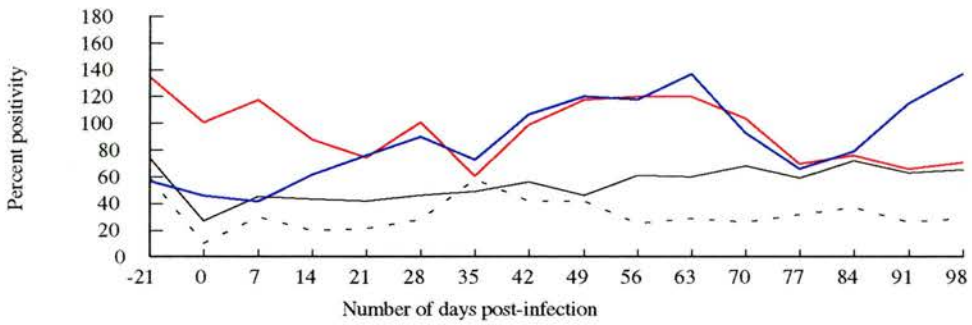



b) Cymelarsan treatment on day 107 of *Trypanosoma evansi* Bakit 259 infection

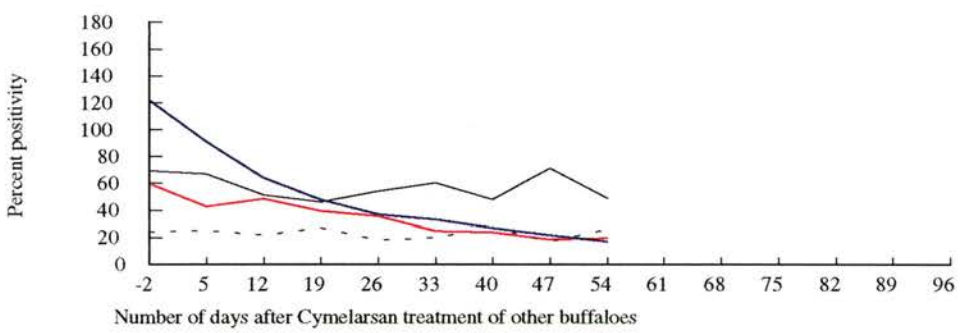



c) Challenge infection with *Trypanosoma evansi* Bakit 362

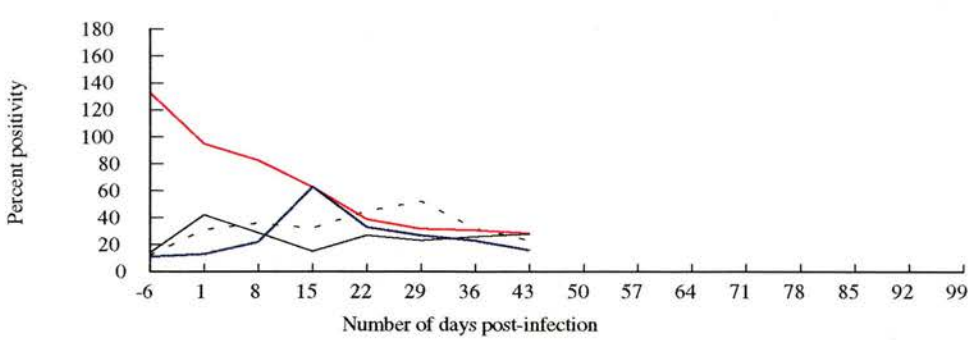
**Figure 4.14** Trypanosomal antigens and antibodies in serum from **Buffalo 527** after primary infection, treatment and challenge with an heterologous stock of *Trypanosoma evansi* (2G6 Ag-ELISA ____ , Tr7 Ag-ELISA ____ , IgM ELISA ----, IgG ELISA ____)




a) Primary infection with *Trypanosoma evansi* Bakit 259

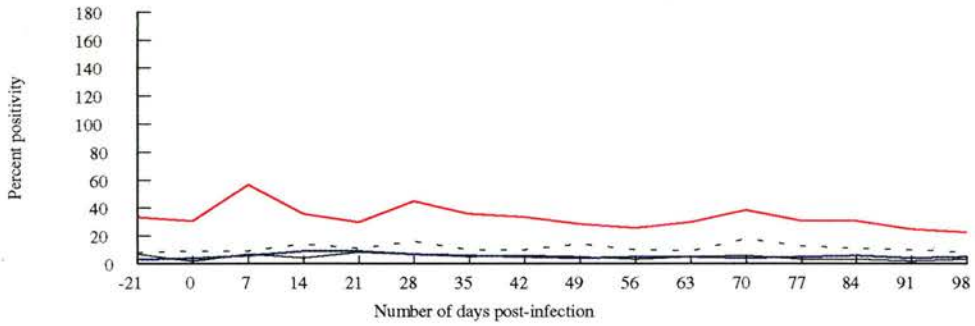



b) Untreated control in treatment stage of *Trypanosoma evansi* Bakit 259 infection

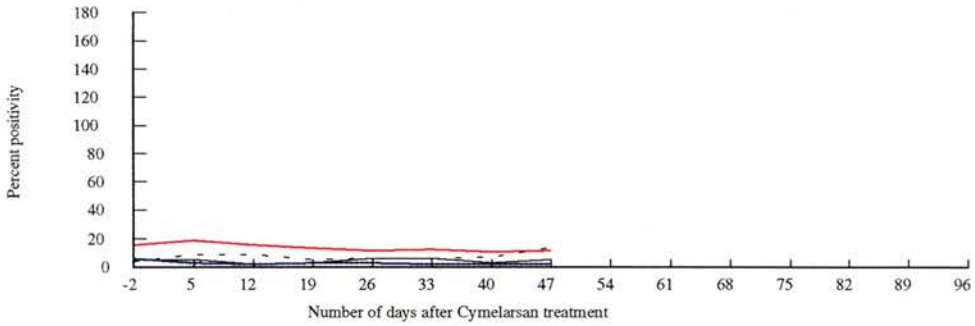



c) Challenge infection with *Trypanosoma evansi* Bakit 362

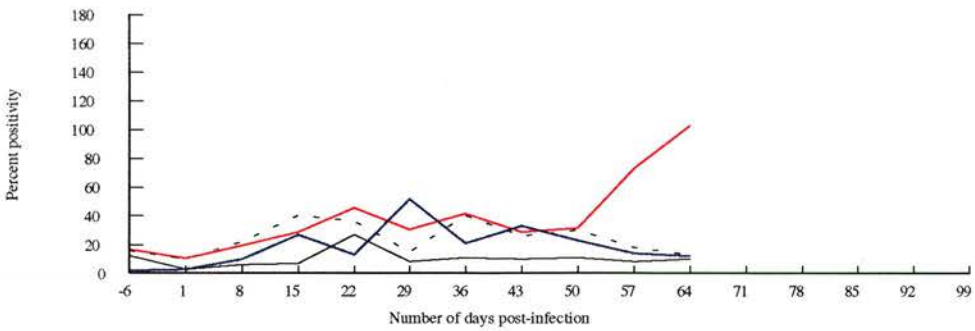
**Figure 4.15** Trypanosomal antigens and antibodies in serum from **Buffalo 502** after primary infection, treatment and challenge with an heterologous stock of *Trypanosoma evansi* (2G6 Ag-ELISA ____ , Tr7 Ag-ELISA ____ , IgM ELISA ----, IgG ELISA ____)




a) Primary infection with *Trypanosoma evansi* Bakit 259 (not shown parasitaemic)

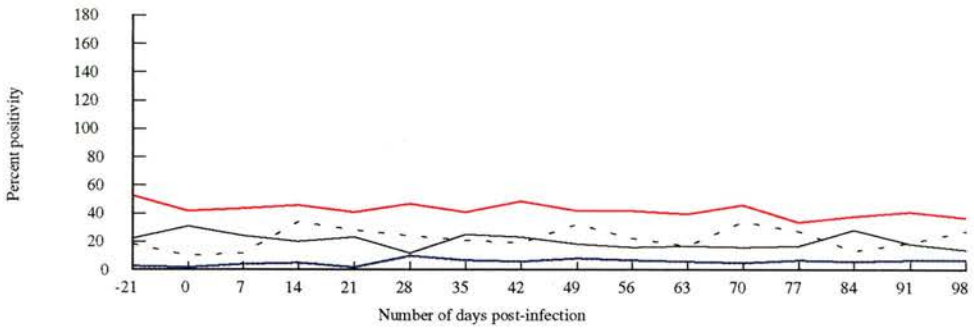



b) Cymelarsan treatment on day 114 of *Trypanosoma evansi* Bakit 259 infection

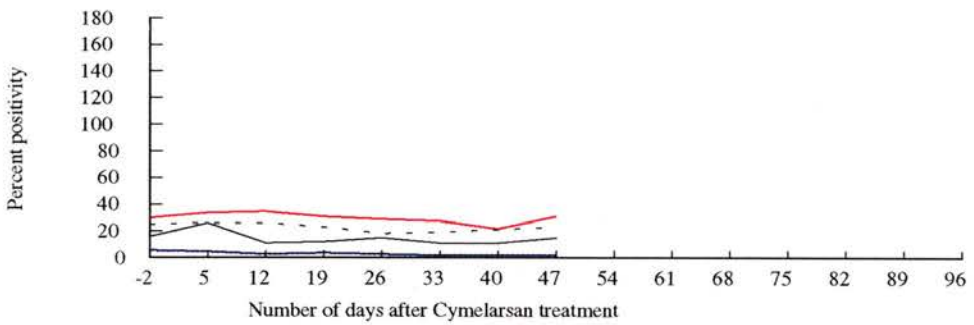



c) Challenge infection with *Trypanosoma evansi* Bakit 362

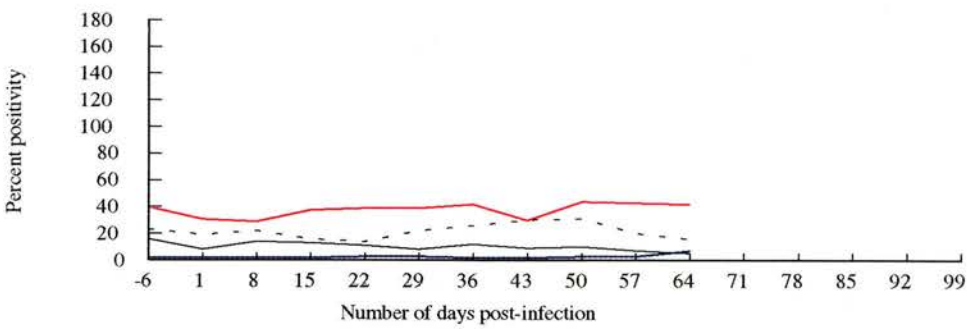
**Figure 4.16** Trypanosomal antigens and antibodies in serum from **Buffalo 504** after primary infection, treatment and challenge with an heterologous stock of *Trypanosoma evansi* (2G6 Ag-ELISA ____ , Tr7 Ag-ELISA ____ , IgM ELISA ----, IgG ELISA ____)




a) Primary infection with *Trypanosoma evansi* Bakit 259 (not shown parasitaemic)

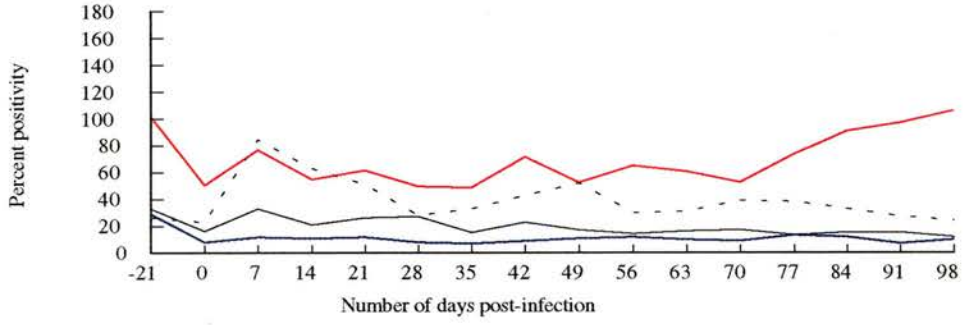



b) Cymelarsan treatment on day 114 of *Trypanosoma evansi* Bakit 259 infection

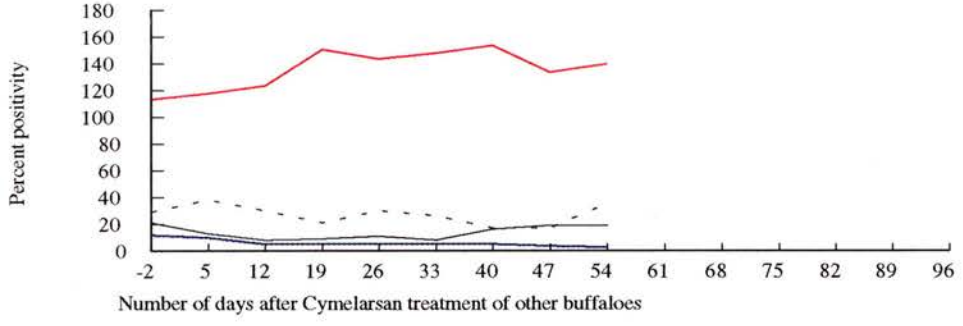



c) Challenge infection with *Trypanosoma evansi* Bakit 362 (not shown parasitaemic)

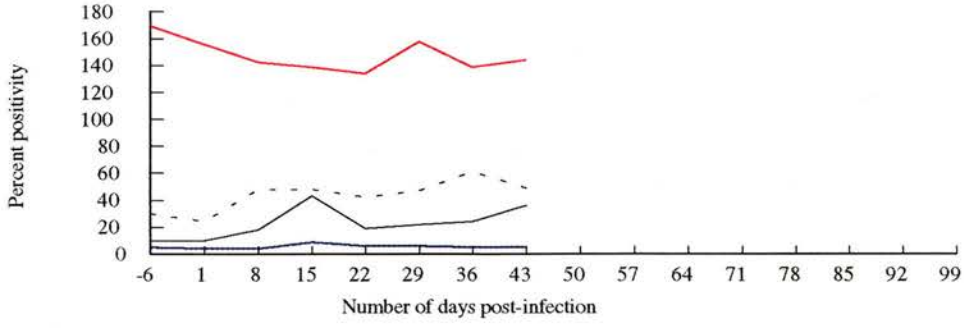
**Figure 4.17** Trypanosomal antigens and antibodies in serum from **Buffalo 518** after primary infection, treatment and challenge with an heterologous stock of T*rypanosoma evansi* (2G6 Ag-ELISA ____ , Tr7 Ag-ELISA ____ , IgM ELISA ----, IgG ELISA ____)




a) Primary infection with *Trypanosoma evansi* Bakit 259

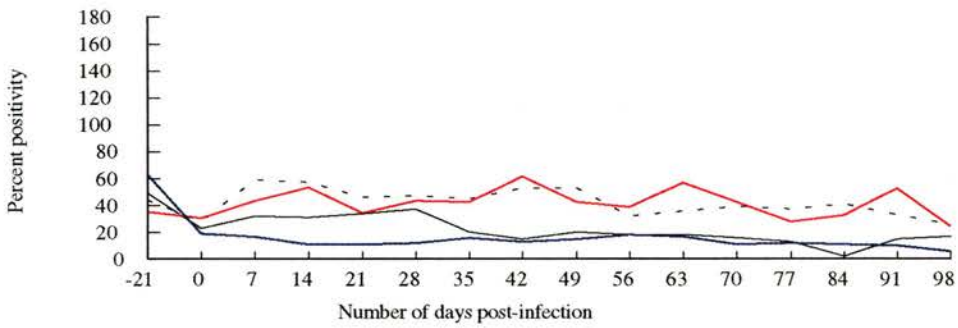



b) Untreated control in treatment stage of *Trypanosoma evansi* Bakit 259 infection

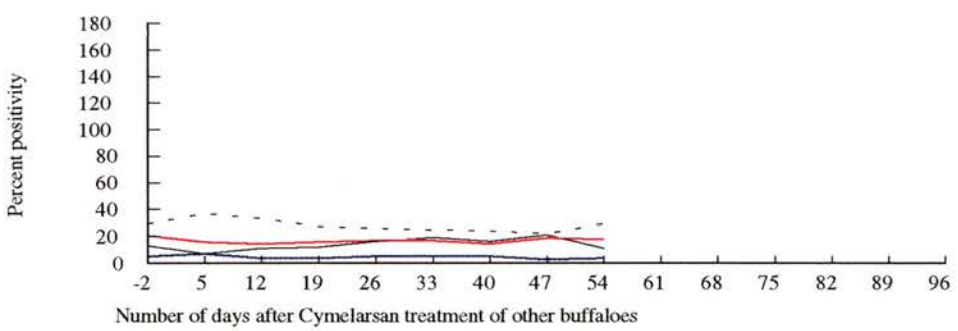



c) Challenge infection with *Trypanosoma evansi* Bakit 362

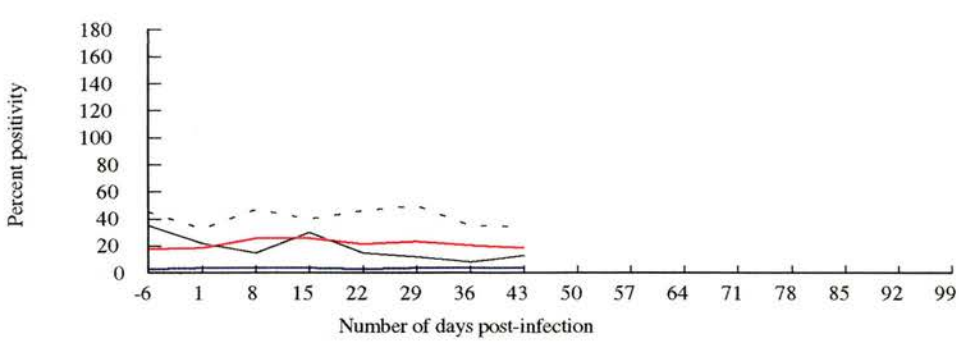
**Figure 4.18** Trypanosomal antigens and antibodies in serum from **Buffalo 526** after primary infection, treatment and challenge with an heterologous stock of *Trypanosoma evansi* (2G6 Ag-ELISA ____ , Tr7 Ag-ELISA ____ , IgM ELISA ----, IgG ELISA ____)




a) Primary infection with *Trypanosoma evansi* Bakit 259




b) Untreated control in treatment stage of *Trypanosoma evansi* Bakit 259 infection




c) Challenge infection with *Trypanosoma evansi* Bakit 362

*Profiles of serum antibody responses of individual buffaloes*

On Day 0, 14 buffaloes had PP values below 20 PP by IgG ELISA and 32 buffaloes had PP values below 30 PP by IgM ELISA, whilst 19 buffaloes were negative by CATT. In general, both IgM and IgG antibodies were first detected 7 to 42 days post-infection and throughout the remainder of the monitoring period, using the cut-off value of twice $PP_{Day\ 0}$.

Using the three groups described above, most buffaloes in Group 1 had low pre-infection antibody-detection ELISA PP values, except Buffaloes 514, 515 and 532 (Figures 4.4, 4.5 and 4.9). Buffalo 514 was naturally infected with *T. evansi* on arrival at Balitvet and had high PP values by IgG ELISA throughout the primary infection. In some buffaloes IgM antibodies were not detected until 49 days post-infection (e.g., Buffalo 529; Figure 4.8) and IgG antibodies were not detected until 49-63 days post-infection (e.g., Buffaloes 519 and 528; Figures 4.6 and 4.7). In buffaloes with late antibody responses, for example Buffaloes 505, 519, 528, and 529, peaks in antigenaemia were also detected later in infection.

In all three groups some buffaloes had low antibody responses, and as observed with patterns of antigenaemia, PP values obtained by both IgM ELISA and IgG ELISA fluctuated below the respective cut-off values. In Group 2, high PP values were obtained by the Ag-ELISAs, whilst low PP values were obtained by the antibody assays; for example, in Buffaloes 501 and 522 (Figures 4.11 and 4.12) IgG ELISA PP values remained close to the cut-off value even though persistently high PP values were found by the Ag-ELISAs. Buffalo 522 was found to be parasitaemic on Day 91 post-infection only, and Buffalo 501 was never found to be parasitaemic. In Group 3, Buffaloes 518 and 526 (Figures 4.17 and 4.18) had high Tr7-specific antigenaemias and high IgM ELISA PP values, but both the 2G6-specific antigenaemias and IgG PP values remained low.

*Number of occasions on which positive tests were obtained in individual buffaloes and in buffalo groups using different diagnostic tests*

The numbers of individual buffaloes which tested positive during the 12-week monitoring period are shown in Tables 4.5, 4.6 and 4.7 for Group $259_{POS}$, Group $259_{PREPOS}$ and Group $259_{NEG}$, respectively. The ranking of the tests in descending order of total number of positive tests was CATT > MI > IgG ELISA > IgM ELISA > 2G6 Ag-ELISA > Tr7 Ag-ELISA > MHCT for Group $259_{POS}$, and CATT > Tr7 Ag-ELISA > MI > IgM ELISA > 2G6 Ag-ELISA > IgG ELISA > MHCT for Group $259_{PREPOS}$. None of the buffaloes was parasitaemic in Group $259_{NEG}$, and low numbers of samples were positive by Ag-ELISA, but higher numbers of positive tests were found by the three antibody tests. Using Friedman's two-way analysis of variance by ranks, a significant difference was found between the number of weekly samples found positive by different tests in Group $259_{POS}$ ($P < 0.001$), Group $259_{PREPOS}$ ($0.010 < P < 0.025$) and Group $259_{NEG}$ ($P < 0.001$). By the method of multiple comparisons, the critical difference in rank sums was 38.9 in Group $259_{POS}$, 22.8 in Group $259_{PREPOS}$ and 30.4 in Group $259_{NEG}$. Significant differences in rank sums in Group $259_{POS}$ existed between the two highest ranked tests given above (CATT and MI) and the two lowest ranking tests (Tr7 Ag-ELISA and MHCT), in Group $259_{PREPOS}$ between the CATT and the three lowest ranked tests (2G6 Ag-ELISA, IgG ELISA and MHCT), and in Group $259_{NEG}$ between the two highest ranked tests (CATT and IgG ELISA) and two lowest ranked tests (MI and MHCT).

In Group $259_{POS}$, 25% (19, 30) of all samples (n = 270) were positive by 2G6 Ag-ELISA compared with 15% (11, 20) by Tr7 Ag-ELISA. In Group$259_{PREPOS}$, 40% (29, 53) of all samples (n = 90) were positive by 2G6 Ag-ELISA and 53% (41, 65) by Tr7 Ag-ELISA, and of the non-parasitaemic buffaloes (Group $259_{NEG}$), 14% (8, 20) of all samples (n = 165) were positive by 2G6 Ag-ELISA and 3% (1, 8) by Tr7 Ag-ELISA. By both IgG ELISA and by IgM ELISA, 34% (28, 41) of 252 Group $259_{POS}$ samples were positive and 55% (48, 61) were positive by CATT. In Group $259_{PREPOS}$, 24% (14, 35) of 84 samples were positive by IgG ELISA, 42% (30, 54) by IgM ELISA and 78% (66, 87) by CATT. Of the non-parasitaemic

buffaloes (Group259$_{NEG}$), 8% (4, 14) 154 samples were positive by IgG ELISA and 13% (7, 19) by IgM ELISA compared with 59% (51, 68) by CATT.

**Table 4.5** Group 259$_{POS}$: Number of positive tests using different diagnostic tests in individual buffaloes sampled weekly for 12 weeks after primary infection with *Trypanosoma evansi* Bakit 259.

| Buffalo | Number of positive tests of 12 weekly tests | | | | | | |
|---|---|---|---|---|---|---|---|
| | MHCT | MI | 2G6 Ag-ELISA‡ | Tr7 Ag-ELISA‡ | IgG ELISA‡ | IgM ELISA‡ | CATT |
| 505 | 0 | 3 | 8 | 0$^+$ | 0$^+$ | 6 | 7 |
| 507 | 5 | 9 | 0$^+$ | 0$^+$ | 0$^+$ | 6 | 5 |
| 508 | 2 | 9 | 10 | 10$^+$ | 12 | 7 | 10 |
| 510 | 0 | 1 | 2 | 1$^+$ | 0$^+$ | 6 | 9$^+$ |
| 512 | 3 | 8 | 10 | 0$^+$ | 7 | 6 | 6 |
| 515 | 0* | 0 | 1 | 0 | 0$^+$ | 0 | 1 |
| 518 | 0 | 1 | 0 | 0$^+$ | 1 | 2 | 9 |
| 519 | 2 | 9 | 3$^+$ | 8 | 5 | 0 | 1 |
| 520 | 2 | 8 | 1$^+$ | 0$^+$ | 5 | 7 | 3 |
| 522 | 0* | 0 | 0$^+$ | 2$^+$ | 0$^+$ | 0 | 10 |
| 524 | 3 | 9 | 3$^+$ | 5$^+$ | 12 | 10 | 8 |
| 525 | 1 | 4 | 0$^+$ | 0$^+$ | 0$^+$ | 0 | 12$^+$ |
| 526 | 0 | 1 | 0 | 0$^+$ | 0$^+$ | 0 | 12$^+$ |
| 527 | 1 | 4 | 5$^+$ | 0$^+$ | 6$^+$ | 0 | 12$^+$ |
| 528 | 4 | 9 | 7 | 2$^+$ | 8 | 6 | 4 |
| 529 | 1 | 8 | 2$^+$ | 1 | 8 | 5 | 2 |
| 533 | 2 | 0 | 0$^+$ | 0 | 0 | 0 | 0$^+$ |
| 534 | 2 | 9 | 1$^+$ | 4 | 10 | 12 | 7 |
| Total | 28 | 92 | 53 | 33 | 74 | 73 | 118 |

*: Detected positive between 13-15 weeks post-infection
‡: Positive if PP greater than twice the PP value on Day 0
$^+$: PP value on Day 0 was greater than 20 PP (Ag-ELISAs and IgG ELISA) or 30 PP (IgM ELISA) cut-off value, or positive by CATT

### 4.3.2 Cymelarsan chemotherapy

*Clinical signs, concurrent infections and packed cell volume*

Buffaloes 503 and 509 (in Group T$_{CYNEG}$: not shown to be parasitaemic pre-treatment), Buffalo 525 (in Group T$_{CYPOS}$: shown to be parasitaemic pre-treatment) and Buffalo 532 (in Group T$_{CON}$) had clinical signs of mange and had *A. marginale* infections. Of all the buffaloes, only Buffalo 525 had a PCV less than 26%; which increased to 29% within two weeks after treatment, and thereafter remained above 26%.

**Table 4.6** Group $259_{PREPOS}$: Number of positive tests using different diagnostic tests in individual buffaloes sampled weekly for 12 weeks after primary infection with *Trypanosoma evansi* Bakit 259

| Buffalo | Number of positive tests of 12 weekly tests | | | | | | |
|---|---|---|---|---|---|---|---|
| | MHCT | MI | 2G6 Ag-ELISA‡ | Tr7 Ag-ELISA‡ | IgG ELISA‡ | IgM ELISA‡ | CATT |
| 506 | 4+ | 10 | 3 | 0+ | 5+ | 11 | 9+ |
| 511 | 0*+ | 1 | 0+ | 7+ | 0+ | 3 | 7+ |
| 514 | 0+ | 5 | 7 | 8+ | 0+ | 1+ | 12+ |
| 517 | 0+ | 8 | 7 | 0+ | 11 | 0 | 12+ |
| 530 | 1 | 4+ | 6+ | 12+ | 0+ | 9 | 6 |
| 532 | 3+ | 8 | 6 | 11 | 1+ | 6 | 10 |
| Total | 8 | 36 | 29 | 38 | 17 | 30 | 56 |

*: Detected positive between 13-15 weeks post-infection

‡: Positive if PP greater than twice the PP value on Day 0

+: PP value on Day 0 greater than 20 PP (Ag-ELISAs and IgG ELISA) or 30 PP (IgM ELISA) cut-off value, or positive by CATT

**Table 4.7** Group $259_{NEG}$: Number of positive tests using different diagnostic tests in individual buffaloes sampled weekly for 12 weeks after primary infection with *Trypanosoma evansi* Bakit 259

| Buffalo | Number of positive tests of 12 weekly tests | | | | | | |
|---|---|---|---|---|---|---|---|
| | MHCT | MI | 2G6 Ag-ELISA‡ | Tr7 Ag-ELISA‡ | IgG ELISA‡ | IgM ELISA‡ | CATT |
| 500 | 0 | 0 | 0+ | 0+ | 11+ | 7 | 12+ |
| 501 | 0 | 0 | 0+ | 2+ | 0 | 1 | 5 |
| 502 | 0 | 0 | 0 | 0+ | 0 | 0 | 0+ |
| 503 | 0+ | 0 | 3+ | 0+ | 2+ | 1+ | 5+ |
| 504 | 0 | 0 | 10 | 0+ | 5+ | 2 | 11+ |
| 509 | 0+ | 0 | 1 | 0+ | 2+ | 0 | 0 |
| 513 | 0 | 0 | 3 | 0+ | 10+ | 6 | 12+ |
| 516 | 0 | 0 | 0+ | 2+ | 12+ | 0 | 2+ |
| 521 | 0 | 0+ | 0+ | 0+ | 9+ | 8+ | 11 |
| 523 | 0 | 0 | 1 | 0+ | 11+ | 12 | 11+ |
| 531 | 0 | 0 | 0+ | 0 | 10 | 2 | 9 |
| Total | 0 | 0 | 18 | 4 | 80 | 39 | 78 |

‡: Positive if greater than twice the PP value on Day 0

+: PP value on Day 0 greater than 20 PP (Ag-ELISAs and IgG ELISA) or 30 PP (IgM ELISA) cut-off value, or positive by CATT

Within all three groups, the observed increase in the mean PCV (shown in Table 4.8) 56 days after treatment was not significant ($P > 0.90$). Before treatment, there was no significant difference ($P > 0.90$) between the mean PCVs of the treated group (Group $T_{CYPOS}$) and control group (Group $T_{CON}$), but there was a significant difference ($0.02 < P < 0.05$) between these groups 56 days post-treatment.

**Table 4.8** Mean packed cell volumes (PCV), with standard deviation given in brackets, for different buffalo groups infected with *Trypanosoma evansi* Bakit 259 before and after Cymelarsan chemotherapy

| Buffalo group | Number of buffaloes | PCV % (SD) | |
|---|---|---|---|
| | | Pre-treatment | 56 days post-treatment |
| Group $T_{CYPOS}$ | 12 | 36.2 (6.0) | 38.6 (5.2) |
| Group $T_{CYNEG}$ | 11 | 37.1 (6.0) | 38.9 (5.3) |
| Group $T_{CON}$ | 12 | 34.7 (3.5) | 35.0 (3.9) |

*Parasitological tests*

With the exception of Buffalo 522 which was positive on Day 31 post-treatment by MI, none of the treated buffaloes was found to be parasitaemic after chemotherapy.

*Clearance of serum antigen and antibody after chemotherapy*

Tables 4.9 and 4.10 show the change in the positive/negative test status of individual buffaloes treated with Cymelarsan in Group $T_{CYPOS}$ and $T_{CYNEG}$. The number of positive buffaloes that became negative to each test for the duration of the post-treatment monitoring period was seven buffaloes by CATT, two buffaloes by 2G6 Ag-ELISA, two buffaloes by Tr7 Ag-ELISA and none by IgG ELISA. The number of days taken to become test-negative post-treatment ranged from 12 to 47 days. The majority of buffaloes continued to fluctuate between positive and negative test status by some tests and remained either negative or positive throughout the monitoring periods by other tests, as shown in Table 4.9. Of the treated buffaloes that were not previously shown to be parasitaemic (Group $T_{CYNEG}$), only Buffaloes 502 and 509 were negative using all tests both before and after treatment, and Buffalo 501 became negative by 26 days post-treatment, by CATT.

*Untreated control group (Group $T_{CON}$)*

Buffaloes of the untreated control group (Group $T_{CON}$) were monitored from 119 to 161 days after the primary infection; whilst none of the 12 buffaloes was positive by MHCT, five buffaloes were MI-positive (Table 4.11). Some of the buffaloes remained positive by both Ag-ELISAs, and on fewer occasions by CATT. Buffaloes 524 and 529 were negative by Tr7 Ag-ELISA, but positive by MI, 2G6 Ag-ELISA and IgG

**Table 4.9** Group $T_{CYPOS}$: Changes in positive/negative test status after chemotherapy using different diagnostic tests in individual buffaloes with the primary infection *Trypanosoma evansi* Bakit 259.

| Buffalo | Test results and number of days taken for decline in analyte* | | | | |
|---|---|---|---|---|---|
| | 2G6 Ag-ELISA§ | Tr7 Ag-ELISA§ | IgG Ab-ELISA§ | IgM Ab-ELISA§ | CATT |
| 505 | - | + | + | +/- | - |
| 506 | - | +/- | + | - | 12 days |
| 507 | + | + | +/- | - | - |
| 512 | - | + | + | - | 33 days |
| 514 | +/- | + | +/- | +/- | +/- |
| 517 | - | 47 days | + | - | 47 days |
| 519 | 12 days | +/- | + | +/- | - |
| 525 | +/- | + | + | +/- | +/- |
| 528 | 33 days | 33 days | +/- | - | 33 days |
| 530 | + | + | + | 19 days | 40 days |
| 533 | +/- | - | - | - | 33 days |
| 534 | - | - | + | - | - |

§: Positive if PP greater than 20 PP (Ag-ELISAs and IgG ELISA) or 30 PP (IgM ELISA)
*: Number of days post-treatment is given when buffaloes that were positive pre-treatment became negative for the remainder of the monitoring period
-: Buffaloes which were negative pre- and post-treatment
+: Buffaloes which were positive pre- and post-treatment
+/-: Buffaloes which fluctuated between positive and negative status post-treatment

ELISA, and Buffalo 518 was found positive only by Tr7 Ag-ELISA and CATT. Buffalo 522 was positive only by the Ag-ELISAs and not by the antibody-detection tests. By Friedman's two-way analysis of variance by ranks, a significant difference was observed between the number of samples found positive by different tests ($P < 0.001$), and a significant difference in rank sums ($\geq 26.9$) existed between the MHCT and the 2G6 Ag-ELISA, Tr7 Ag-ELISA and IgG ELISA.

### 4.3.3 Secondary infection of buffaloes with *Trypanosoma evansi* Bakit 362

*Clinical signs, concurrent infections and packed cell volume*

After the secondary infection with *T. evansi* Bakit 362, Buffaloes 513, 515, 516 and 533 had clinical signs of mange. *Anaplasma marginale* was found in blood smears of Buffaloes 509 and 525.

**Table 4.10** Group $T_{CYNEG}$: Changes in positive/negative test status after chemotherapy using different diagnostic tests in individual buffaloes infected with primary infection *Trypanosoma evansi* Bakit 259, but that were not shown to be parasitaemic.

| Buffalo | Test result and time taken for decline in analyte* | | | | |
|---|---|---|---|---|---|
| | 2G6 Ag-ELISA§ | Tr7 Ag-ELISA§ | IgG Ab-ELISA§ | IgM Ab-ELISA§ | CATT |
| 500 | + | + | + | +/- | +/- |
| 501 | + | +/- | - | - | 26 days |
| 502 | - | - | - | - | - |
| 503 | +/- | +/- | - | - | - |
| 504 | - | + | - | - | +/- |
| 509 | - | - | - | - | - |
| 513 | +/- | + | +/- | + | +/- |
| 516 | - | - | +/- | - | - |
| 521 | + | + | +/- | - | +/- |
| 523 | - | + | +/- | +/- | +/- |
| 531 | + | +/- | +/- | +/- | +/- |

§: Positive if PP greater than 20 PP (Ag-ELISAs and IgG ELISA) or 30 PP (IgM ELISA)
*: Number of days post-treatment is given when buffaloes that were positive pre-treatment became negative for the remainder of the monitoring period
-: Buffaloes which were negative pre- and post-treatment
+: Buffaloes which were positive pre- and post-treatment
+/-: Buffaloes which fluctuated between positive and negative status post-treatment

**Table 4.11** Group $T_{CON}$: Number of positive tests in buffaloes sampled at weekly intervals from 119 to 161 days after primary infection with *Trypanosoma evansi* Bakit 259 using different diagnostic tests

| Buffalo | Number of positive tests of eight weekly samples | | | | | |
|---|---|---|---|---|---|---|
| | MHCT | MI | 2G6 Ag-ELISA§ | Tr7 Ag-ELISA§ | IgG Ab-ELISA§ | CATT |
| 508 | 0 | 7 | 8 | 8 | 8 | 2 |
| 510 | 0 | 0 | 4 | 2 | 3 | 3 |
| 511 | 0 | 0 | 1 | 4 | 3 | 5 |
| 515 | 0 | 0 | 4 | 1 | 4 | 1 |
| 518 | 0 | 0 | 0 | 8 | 0 | 2 |
| 520 | 0 | 5 | 8 | 8 | 8 | 1 |
| 522 | 0 | 0 | 7 | 8 | 0 | 0 |
| 524 | 0 | 6 | 8 | 0 | 8 | 2 |
| 526 | 0 | 0 | 0 | 0 | 1 | 5 |
| 527 | 0 | 0 | 7 | 6 | 0 | 3 |
| 529 | 0 | 7 | 4 | 0 | 8 | 0 |
| 532 | 0 | 1 | 1 | 8 | 7 | 3 |
| Total | 0 | 26 | 52 | 53 | 50 | 27 |

§: Positive if PP greater than 20 PP cut-off value

In contrast with the primary infection, only Buffaloes 509, 525 and 530 had PCVs below 26% during the secondary infection. Table 4.12 shows the mean PCVs of the two buffalo groups pre-infection and at the end of the monitoring period of 45 days (30 buffaloes) and 64 days (20 buffaloes). The mean PCV of buffaloes shown to be parasitaemic (Group $362_{POS}$) was not significantly lower ($P > 0.90$) 64 days post-infection than pre-infection.

**Table 4.12** Mean packed cell volumes (PCV), with standard deviation given in brackets, of different buffalo groups before and after the secondary infection with *Trypanosoma evansi* Bakit 362.

| Buffalo group | Number of buffaloes | PCV % (SD) | | |
|---|---|---|---|---|
| | | Pre-infection | Number of days post-infection | |
| | | | 45 | 64 |
| Group $362_{POS}$ | 30 | 35.3 (5.5) | 34.3 (4.8)* | 33.1 (7.3)** |
| Group $362_{NEG}$ | 4 | 35.6 (2.8) | 33.9 (3.2) | nd |

nd: Not done (only two buffaloes in Group $362_{NEG}$ were monitored up to 64 days)
*: n = 30; **: n = 20.

*Parasitological tests*

Thirty of the 34 buffaloes were shown to be parasitaemic on at least one occasion, by either MHCT and/or MI (data shown in Tables 4.13 and 4.14) and of these, 13 buffaloes were positive by WBF at various sampling days throughout the infection. The number of days when mice were first found to be parasitaemic varied from 2 to 27 days post-inoculation. One day after infection with *T. evansi* Bakit 362, 12 buffaloes were positive by MHCT and 21 buffaloes were positive by MI. All the buffaloes that were positive by MHCT were positive also by MI, whereas eight buffaloes were positive by MI, but negative by the MHCT. In those buffaloes shown to be parasitaemic (Group $362_{POS}$), 32% (26, 38) of 240 weekly tests were positive by MHCT compared with 55% (49, 61) by MI during the first six weeks of infection.

*Profiles of antigenaemia of individual buffaloes*

As observed with the primary infection, there was much variation between the antigenaemia profiles of individual buffaloes found with the secondary infection. Compared with the primary infection, more buffaloes had no detectable antigenaemia prior to the secondary infection using a 20 PP cut-off value; 23 buffaloes were

negative by 2G6 Ag-ELISA, and 17 buffaloes were negative by Tr7 Ag-ELISA, pre-infection.

The range of antigenaemia profiles of individual buffaloes was as varied as that described for the primary infection and, in general the antigenaemia profile found with the primary infection was markedly different from the profile found with the secondary infection in the same buffalo, in both the magnitude of the antigen response and the stage of infection when it was detected. The results are discussed using the same groups given above for the primary infection (i.e., Groups 1, 2 and 3). In Group 1, Buffaloes 512, 514, 528, 529 and 534 (Figures 4.2, 4.4, 4.7, 4.8 and 4.10) had a greater 2G6-specific antigen response in the primary infection than the secondary infection. By contrast, Buffaloes 505 and 513 (Figures 4.1 and 4.3) had a greater 2G6-specific antigen response with the secondary infection, and in Buffalo 519 (Figure 4.6) the response was earlier with the secondary infection than the primary infection.

In Group 1, Buffalo 519 (Figure 4.6) had a 2G6-specific antigenaemia that first peaked eight days post-infection with the secondary infection compared with 77 days post-infection with the primary infection. Buffalo 529 (Figure 4.8), which had no detectable 2G6-specific or Tr7-specific antigen response after the secondary infection, was parasitaemic on each sampling day (but did have a 2G6-specific antigen response after the primary infection). Buffaloes 505 and 519 (Figures 4.1 and 4.6) were shown to have both 2G6- and Tr7-specific antigen responses and were parasitaemic on most sampling days of the secondary infection.

Buffalo 512 (Figure 4.2) had a high Tr7-specific, but low 2G6-specific antigen response whereas Buffalo 513 (Figure 4.3) had a high 2G6-specific, but low Tr7-specific antigen response, and both buffaloes were found to be parasitaemic on several occasions. In Buffalo 514 (Figure 4.4) the high Tr7-specific antigenaemia which had persisted from the primary infection and after chemotherapy, remained high throughout the secondary infection.

**Table 4.13** Detection of the secondary infection with *Trypanosoma evansi* Bakit 362 in experimental buffaloes by weekly microhaematocrit test (MHCT)

| | Number of days post-infection | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| Buffalo | -6 | 1 | 8 | 15 | 22 | 29 | 36 | 43 | 50 | 57 | 64 |
| 500 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| 501 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| 502 | 0 | **1** | **2** | **2** | **1** | **1** | **1** | **2** | 0 | **1** | 0 |
| 504 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| 505 | 0 | 0 | 0 | 0 | 0 | 0 | **1** | **3** | 0 | 0 | **1** |
| 506 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| 507 | 0 | **1** | 0 | **1** | **1** | **1** | 0 | **3** | **1** | 0 | **3** |
| 508* | 0 | **2** | 1 | **1** | **2** | 0 | 0 | **2** | nd | nd | nd |
| 509 | 0 | 0 | 1 | 0 | 0 | 0 | 0 | 0 | **2** | **3** | 0 |
| 510* | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | nd | nd | nd |
| 511* | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | nd | nd | nd |
| 512 | 0 | **1** | **3** | **1** | **1** | **1** | 0 | 0 | 0 | **1** | **2** |
| 513 | 0 | 1 | 0 | 0 | 0 | **2** | 0 | **1** | 0 | 0 | 0 |
| 514 | 0 | 1 | 0 | 0 | 0 | 0 | **3** | 0 | 0 | **3** | 0 |
| 515* | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | nd | nd | nd |
| 516 | 0 | **1** | 0 | 0 | 0 | 0 | 0 | **2** | 0 | **1** | 0 |
| 517 | 0 | 0 | 0 | 0 | 0 | **1** | 0 | **1** | 0 | 0 | 0 |
| 518* | 0 | 0 | 0 | **1** | 0 | 0 | **1** | 0 | nd | nd | nd |
| 519 | 0 | 0 | 0 | 0 | 0 | **1** | **1** | **1** | 0 | 0 | 0 |
| 520* | 0 | **2** | **1** | 0 | **3** | **1** | **1** | **1** | nd | nd | nd |
| 521 | 0 | 0 | **2** | 0 | **3** | 0 | 0 | 0 | 0 | 0 | 0 |
| 522* | 0 | **2** | 0 | 0 | 0 | 0 | 0 | 0 | nd | nd | nd |
| 523 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| 524* | 0 | **1** | **1** | **1** | **1** | 0 | 0 | 0 | nd | nd | nd |
| 525 | 0 | 0 | **2** | 0 | **1** | 0 | 0 | 0 | 0 | 0 | 0 |
| 526* | 0 | **1** | 0 | 0 | 0 | 0 | 0 | 0 | nd | nd | nd |
| 527* | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | nd | nd | nd |
| 528 | 0 | **1** | 0 | 0 | **1** | 0 | 0 | 0 | 0 | **1** | 0 |
| 529* | 0 | 0 | 0 | **1** | **1** | **2** | 0 | **3** | nd | nd | nd |
| 530 | 0 | 0 | 0 | 0 | **1** | 0 | 0 | 0 | 0 | 0 | **1** |
| 531 | 0 | 0 | **2** | 0 | **1** | 0 | 0 | 0 | 0 | 0 | 0 |
| 532* | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | nd | nd | nd |
| 533 | 0 | 0 | 0 | **3** | 0 | **1** | 0 | **1** | 0 | 0 | **1** |
| 534 | 0 | 0 | 0 | **1** | **2** | **2** | 0 | 0 | **2** | 0 | 0 |

*: Group $T_{CON}$ buffaloes were infected nine days after Groups $T_{CYPOS}$ and $T_{CYNEG}$ and, for direct comparison results are presented according to the number of days post-infection for each buffalo. Results were scored as: 0 (negative); 1 (1-5 trypanosomes); 2 (6-20 trypanosomes); or 3 (more than 20 trypanosomes), positive results are emboldened;
nd: Not done.

**Table 4.14** Detection of the secondary infection with *Trypanosoma evansi* Bakit 362 in experimental buffaloes by weekly mouse inoculation (MI) shown as the number of days when a mouse was first found parasitaemic post-inoculation

| | Number of days from infection of buffaloes | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| Buffalo | -6 | 1 | 8 | 15 | 22 | 29 | 36 | 43 | 50 | 57 | 64 |
| 500 | - | - | 4 | - | 15 | - | - | - | - | - | - |
| 501 | - | 6 | 6 | - | 6 | - | - | 6 | - | 13 | - |
| 502 | - | 4 | 4 | 4 | 2 | 6 | 6 | - | 6 | 13 | 6 |
| 504 | - | - | - | - | - | - | - | - | - | - | - |
| 505 | - | 4 | 4 | 6 | 6 | - | - | 3 | 6 | - | 3 |
| 506 | - | - | - | - | - | - | - | - | - | - | - |
| 507 | - | 4 | 4 | 4 | 6 | 6 | 11 | D | 6 | - | 3 |
| 508* | - | 2 | 6 | 6 | 3 | 6 | - | 4 | nd | nd | nd |
| 509 | - | - | 4 | 8 | - | - | 6 | 8 | 6 | 6 | 6 |
| 510* | - | - | 3 | 11 | - | - | - | - | nd | nd | nd |
| 511* | - | - | - | - | - | - | - | - | nd | nd | nd |
| 512 | - | 4 | 4 | 6 | 4 | - | 6 | - | 6 | 6 | 4 |
| 513 | - | 4 | 4 | - | - | - | - | 8 | - | - | - |
| 514 | - | 4 | 6 | - | - | - | 6 | - | - | 3 | 4 |
| 515* | - | - | - | - | 8 | - | - | 4 | nd | nd | nd |
| 516 | - | 4 | 4 | 4 | 13 | 6 | 6 | - | - | 13 | 4 |
| 517 | - | 6 | - | 6 | 6 | 6 | 6 | 6 | 9 | - | - |
| 518* | - | 6 | 8 | - | 8 | - | 6 | - | nd | nd | nd |
| 519 | - | 4 | 6 | 4 | 4 | 6 | 6 | 3 | - | 9 | 6 |
| 520* | - | 2 | - | 6 | 3 | 3 | 3 | 13 | nd | nd | nd |
| 521 | - | 4 | 2 | - | 2 | 8 | - | - | - | - | - |
| 522* | 27 | 6 | 15 | - | - | - | | 8 | nd | nd | nd |
| 523 | - | - | 6 | - | 4 | - | - | - | - | - | - |
| 524* | - | 4 | - | - | - | 6 | - | - | nd | nd | nd |
| 525 | - | - | 2 | - | 9 | - | 9 | - | - | - | - |
| 526* | - | - | - | - | 18 | - | - | - | nd | nd | nd |
| 527* | - | 6 | - | - | - | - | - | - | nd | nd | nd |
| 528 | - | 4 | 4 | 6 | 6 | 6 | 6 | - | 6 | 4 | - |
| 529* | - | 4 | 6 | - | - | 6 | 9 | 4 | nd | nd | nd |
| 530 | - | - | 4 | 6 | 9 | 6 | - | - | - | 6 | 6 |
| 531 | - | - | 4 | - | - | - | - | - | - | - | - |
| 532* | - | - | - | - | - | - | - | - | nd | nd | nd |
| 533 | - | 4 | 4 | 4 | 2 | 6 | 6 | D | D | 6 | 4 |
| 534 | - | 4 | 4 | 4 | 15 | - | - | 6 | 3 | - | - |

*: Group $T_{CON}$ buffaloes were infected nine days after Groups $T_{CYPOS}$ and $T_{CYNEG}$; for direct comparison results are presented according to the number of days post-infection for each buffalo; nd: Not done; D: Mouse died with no result.

In general, in Group 2 buffaloes, Ag-ELISA PP values which had been high prior to the primary infection had declined prior to the secondary infection. However, in Buffaloes 501 and 527 (Figures 4.11 and 4.14) high antigen responses were observed prior to the secondary infection, despite their low antigen responses after chemotherapy. In Buffalo 527, the Tr7-specific antigen response declined, but the 2G6-specific antigen response peaked 15 days post-infection, and this buffalo was found to be parasitaemic one day after infection only.

In Group 3, a persistent Tr7-specific antigenaemia was detectable in Buffalo 504 (Figure 4.16) before and after the primary and secondary infections, but this buffalo was never shown to be parasitaemic and was negative by the 2G6 Ag-ELISA throughout the study. Buffalo 518 (Figure 4.17) also had a persistent Tr7-specific antigenaemia with no 2G6-specific antigen response, but this buffalo was parasitaemic on several occasions. Buffalo 526 (Figure 4.18) was found to be parasitaemic on two sampling days, but had no detectable antigen response and Buffalo 502 (Figure 4.15) was parasitaemic on most sampling days and had both 2G6- and Tr7-specific antigen responses.

*Profiles of serum antibody responses of individual buffaloes*

In Buffaloes 513, 515 and 519 (Figures 4.3, 4.5 and 4.6) in Group 1, the IgM and IgG antibody responses increased more quickly and to a higher PP value with the secondary infection than with the primary infection, typical of an anamnestic response. In Buffalo 505 (Figure 4.1) and Buffalo 513, antibody responses preceded antigen responses. On most sampling days Buffaloes 514 and 532 (Figures 4.4 and 4.9), which had persistent Tr7-specific and 2G6-specific antigenaemias, respectively, were positive by IgM and IgG ELISAs, however Buffalo 532 was never shown to be parasitaemic.

Fluctuations in IgM and IgG antibody levels below their respective cut-off values of 30 PP and 20 PP were observed in buffaloes in all three groups. For example in Group 2, Buffalo 501 (Figure 4.11) had detectable IgM and IgG antibody responses by 15 days post-infection, but both antibody responses were declining by 57 days

post-infection. In Buffalo 525 (Figure 4.13) antigen responses were high, but the IgM antibody response fluctuated markedly.

In Group 3, the IgM response found in parasitaemic buffaloes (Buffaloes 502, 518 and 526; Figures 4.15, 4.17 and 4.18) was higher than the IgG response. In Buffalo 504 (Figure 4.16), which was never found to be parasitaemic, no antibody responses occurred but in Buffalo 502, which was parasitaemic on most sampling days, an increase in IgG antibody was detected on Day 22 only, whilst IgM responses fluctuated around the cut-off value. Buffaloes 502 and 506 had no detectable IgG antibody by the end of the monitoring period (64 days post-infection).

*Number of occasions on which positive tests were obtained in individual buffaloes and in buffalo groups using different diagnostic tests*

The numbers of buffaloes found positive by different tests during the six-week monitoring period are shown in Table 4.15 (Group $362_{POS}$) and Table 4.16 (Group $362_{NEG}$). The ranking of the tests, in descending order of total number of positive tests, was IgG ELISA > CATT > Tr7 Ag-ELISA > MI > 2G6 Ag-ELISA > IgM ELISA > MHCT for Group $362_{POS}$ and IgG ELISA/CATT > Tr7 Ag-ELISA > 2G6 Ag-ELISA > IgM ELISA > MHCT/MI for Group $362_{NEG}$. All buffaloes in Group $362_{NEG}$ were found to be positive by one or more antigen and antibody tests on several occasions. By Friedman's two-way analysis of variance by ranks, there was a significant difference ($P < 0.001$) between the number of samples found positive by different tests in Group $362_{POS}$, but no significant difference ($P = 0.15$) between tests in Group $362_{NEG}$. In Group $362_{POS}$ the critical difference in rank sums was 50.3 and higher differences in rank sums existed between the MHCT and three highest ranking tests (IgG ELISA, CATT and Tr7 Ag-ELISA).

In Group $362_{POS}$ (buffaloes shown to be parasitaemic), 54% (47, 62) of 240 samples were positive by 2G6 Ag-ELISA compared with 76% (69, 82) by Tr7 Ag-ELISA. In Group $362_{NEG}$ (buffaloes not shown to be parasitaemic), 23% (10, 42) of 30 samples were positive by 2G6 Ag-ELISA compared with 47% (28, 66) by Tr7 Ag-ELISA.

*Buffaloes sampled 84 and 251 days after chemotherapy given during the secondary infection*

None of the buffaloes tested 84 days (n = 18) and 251 days (n = 12) after treatment of the secondary infection was shown to be parasitaemic. The numbers of buffaloes that tested positive (shown in Table 4.17) were eight (2G6 Ag-ELISA), eleven (Tr7 Ag-ELISA), seven (IgM ELISA), nine (IgG ELISA) and eight (CATT) at 84 days post-treatment and four (2G6 Ag-ELISA), eight (Tr7 Ag-ELISA), seven (IgM ELISA), nine (IgG ELISA) and one (CATT) at 251 days post-treatment.

**Table 4.15** Group $362_{POS}$: Number of positive tests in buffaloes sampled weekly for six weeks after secondary infection with *Trypanosoma evansi* Bakit 362 using different diagnostic tests

| Buffalo | Number of positive tests of six weekly tests | | | | | | |
|---|---|---|---|---|---|---|---|
| | MHCT | MI | 2G6 Ag-ELISA‡ | Tr7 Ag-ELISA‡ | IgG ELISA‡ | IgM ELISA‡ | CATT |
| 500 | 0 | 2 | 6+ | 6+ | 6+ | 4 | 6 |
| 501 | 0 | 3 | 6+ | 5+ | 5 | 3 | 5 |
| 502 | 6 | 5 | 4 | 5 | 1 | 3 | 4 |
| 505 | 2 | 4 | 3 | 6+ | 6+ | 3+ | 6 |
| 507 | 4 | 5 | 6+ | 6+ | 5 | 0 | 5 |
| 508 | 4 | 5 | 3+ | 3 | 6+ | 4+ | 5 |
| 509 | 1 | 4 | 0 | 5 | 5 | 4 | 4 |
| 510 | 0 | 2 | 1 | 2+ | 0 | 6 | 2 |
| 512 | 4 | 4 | 3 | 6+ | 5 | 1 | 5 |
| 513 | 2 | 2 | 2 | 6+ | 6+ | 4 | 5 |
| 514 | 1 | 2 | 0 | 6+ | 6+ | 4 | 6 |
| 515 | 0 | 2 | 0 | 3 | 5 | 5 | 4 |
| 516 | 1 | 5 | 6 | 5 | 5 | 2 | 5 |
| 517 | 2 | 5 | 1 | 3 | 6+ | 1 | 5 |
| 518 | 2 | 3 | 0 | 6+ | 4 | 6 | 6 |
| 519 | 3 | 6 | 4 | 3 | 6+ | 2 | 3 |
| 520 | 5 | 5 | 6+ | 6+ | 6+ | 5 | 6 |
| 521 | 2 | 3 | 6+ | 6+ | 6 | 2 | 1 |
| 522 | 0 | 2 | 6 | 6+ | 2 | 3 | 4 |
| 523 | 0 | 2 | 0 | 6+ | 5 | 4+ | 6 |
| 524 | 3 | 1 | 6+ | 3 | 6+ | 5 | 5 |
| 525 | 2 | 3 | 5 | 5 | 6+ | 3+ | 6 |
| 526 | 0 | 1 | 0 | 5 | 1 | 6+ | 6 |
| 527 | 0 | 0* | 5 | 6+ | 5 | 5 | 0 |
| 528 | 1 | 5 | 0 | 1 | 6 | 0 | 4 |
| 529 | 4 | 5 | 0 | 0 | 6+ | 3 | 5 |
| 530 | 1 | 4 | 6+ | 5+ | 6+ | 3 | 4 |
| 531 | 2 | 1 | 6+ | 2 | 6 | 3 | 5 |
| 533 | 3 | 5 | 5+ | 5 | 3 | 1 | 4 |
| 534 | 3 | 3 | 2 | 4 | 6+ | 2 | 5 |
| Total | 58 | 99 | 98 | 136 | 147 | 97 | 137 |

‡: Positive if PP greater than 20 PP (Ag-ELISAs and IgG ELISA) or 30 PP (IgM ELISA)

+: PP value on Day 0 was greater than 20 PP (Ag-ELISAs and IgG ELISA) or 30 PP (IgM ELISA) cut-off value, or positive CATT

*: Positive on Day 1 post-infection only

**Table 4.16** Group $362_{NEG}$: Number of positive tests in buffaloes sampled weekly for six weeks after secondary infection with *Trypanosoma evansi* Bakit 362 using different diagnostic tests

| Buffalo | Number of positive tests of six weekly tests | | | | | | |
|---|---|---|---|---|---|---|---|
| | MHCT | MI | 2G6 Ag-ELISA‡ | Tr7 Ag-ELISA‡ | IgG ELISA‡ | IgM ELISA‡ | CATT |
| 504 | 0 | 0 | 0 | $6^{+}$ | 0 | 0 | 5 |
| 506 | 0 | 0 | 5 | $1^{+}$ | $6^{+}$ | 0 | 1 |
| 511 | 0 | 0 | $1^{+}$ | 2 | 0 | 3 | 6 |
| 532 | 0 | 0 | 0 | 2 | $6^{+}$ | 2 | 0 |
| Total | 0 | 0 | 6 | 11 | 12 | 5 | 12 |

‡: Positive if greater than 20 PP (Ag-ELISAs and IgG ELISA) or 30 PP (IgM ELISA)

+: PP value on Day 0 was greater than 20 PP (Ag-ELISAs and IgG ELISA) or 30 PP (IgM ELISA) cut-off value, or positive CATT

**Table 4.17** Ag-ELISA and CATT results 84 days and 251 days after treatment of buffaloes infected with *Trypanosoma evansi* Bakit 362

| Buffalo | 84 days post-treatment | | | | | 251 days post-treatment | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | Ag-ELISA (PP) | | Antibody ELISAs (PP) | | CATT | Ag-ELISA (PP) | | Antibody ELISAs (PP) | | CATT |
| | 2G6 | Tr7 | IgM | IgG | | 2G6 | Tr7 | IgM | IgG | |
| 501 | 22 | 12 | 42 | 31 | + | 27 | 22 | 27 | 14 | - |
| 502 | 3 | 10 | 12 | 6 | - | 3 | 18 | 14 | 4 | - |
| 504 | 2 | 27 | 17 | 5 | - | 4 | 38 | 20 | 3 | - |
| 505 | 18 | 101 | 40 | 44 | + | na | na | na | na | na |
| 506 | 3 | 8 | 11 | 10 | - | 2 | 40 | 15 | 6 | - |
| 507 | 41 | 32 | 18 | 20 | - | 33 | 60 | 17 | 5 | - |
| 513 | 137 | 20 | 31 | 25 | + | 75 | 27 | 28 | 11 | - |
| 515 | 6 | 11 | 14 | 16 | - | 4 | 9 | 17 | 9 | - |
| 516 | 11 | 15 | 15 | 17 | - | na | na | na | na | na |
| 517 | 5 | 27 | 19 | 25 | - | na | na | na | na | na |
| 518 | 6 | 167 | 33 | 13 | + | 13 | 180 | 27 | 5 | - |
| 519 | 28 | 70 | 20 | 24 | + | 14 | 20 | 21 | 7 | - |
| 521 | 96 | 33 | 23 | 22 | - | 154 | 49 | 15 | 10 | - |
| 523 | 8 | 58 | 56 | 28 | + | na | na | na | na | na |
| 525 | 70 | 23 | 38 | 21 | + | na | na | na | na | na |
| 526 | 7 | 10 | 35 | 40 | + | 4 | 17 | 26 | 6 | + |
| 533 | 21 | 22 | 28 | 5 | - | 14 | 35 | 17 | 3 | - |
| 534 | 45 | 101 | 13 | 8 | - | na | na | na | na | na |

na: Not available for sampling

## 4.4 Discussion

To estimate the diagnostic sensitivity of two *T. evansi* Ag-ELISAs, 35 Indonesian buffaloes were experimentally infected on two separate occasions with different isolates of *T. evansi* (Bakit 259 and Bakit 362). The Ag-ELISAs were compared with parasitological and antibody-detection tests in their ability to detect *T. evansi* infections in individual buffaloes and groups of buffaloes, and to monitor trypanosome clearance after chemotherapy. The lack of an established gold standard test for *T. evansi* with a high sensitivity and specificity meant that it was necessary to use experimentally-infected animals for test validation.

With the primary infection, parasitaemic buffaloes (Group $259_{POS}$) were positive by CATT or MI significantly more frequently than by Tr7 Ag-ELISA or MHCT. In this group of buffaloes, 25% (19, 30) of all weekly samples were positive by 2G6 Ag-ELISA and 15% (11, 20) by Tr7 Ag-ELISA, compared with 34% (28, 41) both by IgM ELISA and by IgG ELISA and 55% (48, 61) by CATT. With the secondary infection, parasitaemic buffaloes (Group $362_{POS}$) were positive by CATT, IgG ELISA or Tr7 Ag-ELISA significantly more frequently than by MHCT. In this group of buffaloes, 54% (47, 62) of all weekly samples were positive by 2G6 Ag-ELISA and 76% (69, 82) by Tr7 Ag-ELISA compared with 54% (47, 62) by IgM ELISA, 82% (76, 87) by IgG ELISA and 76% (69, 82) by CATT.

Twenty-one buffaloes during the primary infection and 30 buffaloes during the secondary infection, were found to be parasitaemic on at least one occasion. The MI test detected parasitaemias in buffaloes on more occasions than the MHCT, but several buffaloes were shown to be parasitaemic on one or two occasions only. During the first six weeks of infection in all parasitaemic buffaloes, 15% (8, 22) by MHCT and 37% (28, 46) by MI with the primary infection (Group $259_{POS}$) were positive compared with 32% (26, 38) by MHCT and 55% (49, 61) by MI with the secondary infection (Group $362_{POS}$). The higher rate of detection of the secondary infection (detectable from one day post-infection) was possibly due to a higher replication rate of the trypanosomes, which were from a more pathogenic isolate. With the primary infection, buffaloes were first found to be parasitaemic two to three

weeks post-infection compared with one day post-infection with the secondary infection. Buffaloes 515 and 522 were not detected parasitaemic until 13 to 15 weeks after the primary infection. Transmission of *T. evansi* between buffaloes within the animal house may have occurred because some *Stomoxys* flies were observed inside the building which was not completely fly-proof (no uninfected control buffaloes were present), alternatively these buffaloes may have been mis-classified as parasitaemic due to operator error, although antigen responses were detected in both these buffaloes several weeks before trypanosomes were found in the blood.

Although the MI test was more sensitive than the MHCT, it was more labour-intensive and some buffaloes were only parasite-positive by MHCT. A disadvantage of the MI test was the 30-day mouse monitoring period, but this procedure was necessary because parasitaemias were first detected in mice up to 27 days post-inoculation; this period did not appear to be associated with the stage of infection in the buffalo. The majority of positive MHCT tests were scored one (i.e., one to five trypanosomes seen) suggesting that these infections could be missed with a rapid examination of the microhaematocrit tube or by use of a poor quality microscope, both of which are common under field conditions.

The results of the different tests were considered for each experimental infection separately. Comparisons between the primary and secondary infections were not possible because of the different cut-off values used; cut-off values twice pre-infection levels were used in the primary infection because of the number of buffaloes that had pre-existing serum antigen and antibody responses. It was not possible to test buffaloes prior to purchase except by MHCT, however the buffaloes used may be considered more representative of buffaloes in the field than naive buffaloes. To reduce background levels for the secondary infection, a longer post-treatment period (five to ten weeks) was allowed. Although fewer buffaloes were antigenaemic before the secondary infection, 12 buffaloes by 2G6 Ag-ELISA and 18 buffaloes by Tr7 Ag-ELISA were still antigenaemic. There was no reduction in the number of buffaloes that were positive by the antibody-detection ELISAs, but fewer buffaloes were

positive by the CATT before the secondary infection than before the primary infection.

A wide range of profiles of antigenaemia in individual buffaloes was obtained. Several buffaloes had similar profiles with both the primary and secondary infections, while other buffaloes had strong antigen responses with one infection, but negligible responses with the other infection. Three major types of antigen responses were observed: 1) development of antigenaemia; 2) no detectable antigenaemia; and 3) persistence of a pre-infection antigenaemia. In buffaloes that had an antigen response (1), antigenaemia was first detected 7 to 42 days post-infection. In terms of sensitivity, an important finding in some buffaloes was the fluctuation of antigen (and antibody) levels below cut-off values at various times post-infection. Few previous studies have monitored animals experimentally infected with *T. evansi* for long periods. However, in a study of four cattle experimentally infected with *T. brucei* and monitored for 600 days post-infection, antigen responses also fluctuated in two cattle close to or below the cut-off value on many sampling days (Masake *et al.*, 1995a).

With both the primary and secondary infections, antigenaemia peaked around 42 days post-infection in many buffaloes, and thereafter declined. Although some buffaloes had similar 2G6-specific and Tr7-specific profiles of antigenaemia (e.g., Buffalo 501), in other buffaloes the two antigens appeared at different stages of infection (e.g., Buffalo 519). For example, some buffaloes (e.g., Buffaloes 518 and 504) had a persistent antigenaemia by one Ag-ELISA, but were not antigenaemic by the other Ag-ELISA and sometimes had minimal antibody responses and parasitaemias that were detectable on one or two occasions only. During the later stages of the primary infection (119 to 161 days post-infection), only five of twelve buffaloes were shown to be parasitaemic (by MI); two of these buffaloes were also positive by the 2G6 Ag-ELISA and IgG ELISA, but negative by the Tr7 Ag-ELISA. Throughout this chronic stage of the infection several buffaloes were negative by one or more of the diagnostic tests used.

A wide range of serum antibody profiles was also found in the individual buffaloes. As with antigenaemia, most buffaloes had detectable antibody 7 to 42 days post-infection, but remained antibody-positive throughout the remainder of the monitoring period. Buffaloes with late peaks of antibody responses also often had late peaks of antigenaemia (e.g., Buffaloes 514 and 528). The IgM response did not always precede the IgG response and in some buffaloes one or other response was negligible, and not all antigenaemic buffaloes had associated antibody responses (e.g., Buffaloes 501, 502 and 515). In several buffaloes a more rapid antibody response was observed with the secondary infection than with the primary infection, typical of an anamnestic response. IgM levels fluctuated below the cut-off value in some buffaloes. Variations in IgM responses have been reported by Luckins and Mehlitz (1978) in cattle naturally infected with tsetse-transmitted trypanosomes in West Africa, and the diagnostic value of IgM-detection tests has been questioned because of observed IgM fluctuations (Clarkson *et al.*, 1975).

Clinical signs of mange were seen in several buffaloes, particularly with the primary infection, and blood smear examination showed that some of these buffaloes had concurrent *A. marginale* or *B. bovis* infections. Mange has been reported to be a common finding in buffaloes, particularly buffalo calves, naturally infected with *T. evansi* in Indonesia (Partoutomo, 1993). Several buffaloes had a PCV less than 26%, and not all of these were shown to have a *T. evansi* parasitaemia, but some had *A. marginale* infections. The PCV of Buffalo 510 declined to 13% 56 days post-infection and this buffalo had concurrent *T. evansi*, *A. marginale* and *B. bovis* infections. Cymelarsan treatment has not been shown to be effective against *T. theileri*, and therefore it was not unexpected that a few buffaloes were found to be infected with this trypanosome which is common in Indonesia. It is unlikely that *T. theileri* infections affected the Ag-ELISA results because these tests do not recognise antigens of this trypanosome (Delafosse *et al.*, 1995). The mean PCV of buffaloes infected with the primary infection had declined significantly ($0.01 < P < 0.02$) from pre-infection levels by 49 days post-infection, but increased again by 98 days post-infection. By contrast, there was no significant change ($P > 0.90$) in the mean PCVs by 64 days post-infection of the secondary infection

which was with the more pathogenic of the two *T. evansi* isolates, however fewer buffaloes had concurrent *A. marginale* infections during the secondary infection, possibly due to the Imidocarb treatment given during the primary infection.

After Cymelarsan treatment of the primary infection, none of the buffaloes was shown to be parasitaemic during the 74 day monitoring period, except Buffalo 522 which was parasitaemic 31 days post-treatment, but without an associated antigen or antibody response. There was no significant increase in mean PCV within groups ($P > 0.90$), but the mean PCV of the treated group ($Group_{CYPOS}$) was significantly higher ($0.02 < P < 0.05$) than the mean PCV of the untreated control group ($Group_{CON}$) at 56 days post-treatment.

Previous studies have reported clearance of *T. vivax* and *T. congolense* antigens within two weeks and of *T. brucei* antigens within four weeks, of trypanocidal treatment (Nantulya and Lindqvist, 1989). However in the present study, antigen clearance was much more variable; in some buffaloes antigenaemia persisted post-treatment for up to 74 days (after the primary infection) and up to eight months (after the secondary infection). Another important finding was the absence of a detectable antigenaemia in several buffaloes prior to treatment. Six of the 12 parasite-positive buffaloes ($Group_{CYPOS}$) that were treated became negative by CATT, but fewer buffaloes became negative by the other tests and none of the eight buffaloes positive by IgG ELISA became negative. In another study, antigen responses had already declined, in some of the cattle experimentally infected with *T. brucei, T. congolense* or *T. vivax*, before treatment was given 35-40 days post-infection (Nantulya and Lindqvist, 1989). However, the importance of these observations in relation to false-negative results was not discussed and no untreated controls were monitored to assess antigen fluctuations in the absence of treatment.

The present study showed that estimates of diagnostic sensitivity of the Ag-ELISAs and antibody-detection tests were higher than the MHCT for the detection of experimental *T. evansi* infections in Indonesian buffaloes. Furthermore, these tests could be used more readily than MI to test large numbers of samples. The persistence

of antigenaemia post-treatment in a proportion of buffaloes suggests that these Ag-ELISAs may have limitations for the differentiation of current *T. evansi* infections from previous exposure, which is known to be a major disadvantage of antibody-detection tests (Nantulya, 1990; Luckins, 1992). Antigen responses were found to fluctuate during the experimental *T. evansi* infections, often close to or below the cut-off value, and there was a wide variation in the profiles of antigenaemia of individual buffaloes. Antigenaemia may be directly related to the number of trypanosomes killed by the host's immune system and the rate of clearance of antigenic components from the circulation. Alternatively, antigen may be present that is bound to antibody such that epitopes are not available for binding with Ag-ELISA monoclonal antibodies, thus giving false-negative results (Waitumbi and Nantulya, 1993). The variation in profiles of antigenaemia between buffaloes and between *T. evansi* isolates demonstrates the potential for bias when animals and trypanosome isolates are selected for the estimation of diagnostic sensitivity using limited numbers of experimentally-infected animals.

# CHAPTER 5

# EVALUATION OF *TRYPANOSOMA EVANSI* ANTIGEN-ELISAS: I) ASSESSMENT OF TEST PERFORMANCE UNDER DIFFERENT TEST AND SAMPLE CONDITIONS

## 5.1 Introduction

An ideal diagnostic test should be capable of rapidly testing large numbers of samples at low cost, while giving valid results. Test users should be aware of how a particular test will perform when used to test samples under their laboratory conditions. The usefulness of a diagnostic test is dependent on its ability to accurately detect infection or disease in the long run; this is defined as test validity. Reliability is another important characteristic of diagnostic tests which is indicated by the agreement between results of the same test performed two or more times on the same samples by the same operator (repeatability) and by the agreement between results of the same test performed on the same samples by different laboratories or operators (reproducibility) (Thrusfield, 1995). Another test characteristic that is of particular importance in developing countries is 'robustness'. This term is used to describe the reliability of a test under sub-optimal working conditions which include sample quality and processing and components of the test (Kemeny and Chantler, 1988).

Suppliers of commercial diagnostic tests, particularly for medical diagnostics, commonly provide technical data on test performance and include quality assurance parameters with each test kit. The World Health Organization (WHO) and The International Union of Immunological Societies (IUIS) have responsibility for immunological standards used in some diagnostic tests (Kemeny, 1991). There is an increasing number of commercial veterinary diagnostic kits available based on ELISA formats. Internal quality controls are included to measure day-to-day and plate-to-plate variation, but external quality controls are not always available, and therefore comparison of inter-laboratory and inter-kit results can be problematic. Standard reference material is particularly important for quantitative assays and can be classified as primary, secondary and tertiary (working) standards

(Hamilton and Adkinson, 1988). In this classification, the primary standard is collected, tested and aliquoted under the guidance of an internationally recognised body, the secondary standard is cross-standardised to the primary standard and can be made available in larger quantities by a producer. The working standard is a sample pool that is cross-standardised to the primary or secondary standards in multiple replicate assays and can be used on a routine basis by the test user.

Many governmental and private laboratories have used, and continue to use, tests that have been developed 'in-house' or supplied as separate components by collaborating institutes (e.g. tests for trypanosomosis and tick-borne diseases). Frequently 'in-house tests' have not been properly validated and do not include quality controls with test performance parameters. The use of non-validated or improperly validated tests without appropriate quality controls produces data of limited value which cannot be compared readily with data of other diagnostic institutions or research workers. The lack of international standards for veterinary diagnostic tests has been recognised by the IAEA, Vienna, which has initiated programmes to promote the standardisation of ELISAs for a limited number of animal diseases, including foot and mouth disease, rinderpest, trypanosomosis and babesiosis (Wright *et al.*, 1993).

A wide range of different ELISA systems have been developed successfully for veterinary diagnostics. In the UK, the governmental Veterinary Laboratories Agency routinely uses 13 different ELISAs for the diagnosis of bacterial, viral and parasitic diseases of cattle. An advantage of ELISAs is that these assays can be used to test large numbers of samples because of the automation of procedures (Ruitenberg *et al.*, 1977). However, in any test system variation will exist and it is essential that underlying causes are identified and that attempts are made to minimise or correct them. Sources of variation in ELISAs can be divided into inter-laboratory and intra-laboratory components of systematic and random error. Within one laboratory, variation will exist between operator technique, test run days, different plates and individual wells on the same plate. Random variation originating from these causes can be reduced by operator training, and standardisation and optimisation of the test system. The physical characteristics of samples, the timing and temperature of

different incubation stages of the assay, and the preparation of reagent dilutions (determined by the accuracy of pipetting and mixing) are all important sources of random variation (Kemeny and Chantler, 1988) which affect test validity, reliability and robustness. However, systematic error, or bias, may not necessarily affect these test parameters (e.g. a highly reliable test may not be valid, if the results are consistently biased). It is therefore essential that potential sources of both random and systematic error in tests are properly recognised and minimised.

Prior to the establishment of two *T. evansi* antigen-ELISAs in Indonesia and other countries for their application as diagnostic tests and epidemiological tools, their validity, reliability and robustness were assessed. In this study, the effects of temperature and duration of sample storage and the serum separation method were investigated to assess the suitability of the tests for working conditions in Indonesia. Blood samples with anticoagulant are commonly taken for parasitological examination by Indonesian veterinary officers, and therefore plasma was compared with serum because it would be a convenient sample which would avoid two samples having to be taken from each animal.

The performance of the two standardised *T. evansi* antigen-ELISAs, described in Chapter 3, was investigated under different test conditions. Various blocking agents and serum titrations were also used in an attempt to reduce the proportion of false-positive results observed with British cattle and Australian buffalo sera (see Chapters 3 and 6).

## 5.2 Materials and Methods

The standardised 2G6 Ag-ELISA and the Tr7 Ag-ELISA described in Chapter 3 were used for the experiments described below which were conducted either at the CTVM or Balitvet, Indonesia. The Australian buffalo sera had been heat-treated at 56°C for 30 minutes to comply with UK importation regulations. The use of the 2G6 Ag-ELISA alone is indicated in some studies because insufficient reagents were available for the Tr7 Ag-ELISA to be included.

### 5.2.1 Repeatability of antigen-ELISAs

Blood samples taken 33 and 34 weeks post-infection from the 34 buffaloes experimentally infected with *T. evansi* BAKIT 362, described in Chapter 4, were used. The samples were kept at 4°C overnight and the sera collected the following day after centrifugation at 800 g for 10 minutes and then stored at -20°C. Sera were tested with the 2G6 Ag-ELISA and the Tr7 Ag-ELISA on one day and then re-tested by both Ag-ELISAs by the same operator on a subsequent day, at Balitvet.

### 5.2.2 Effect of the method of serum separation, and temperature and duration of serum storage on antigen-ELISA results

Sera were prepared from blood samples taken from the 34 buffaloes experimentally infected with *T. evansi* BAKIT 362 at 33 weeks post-infection. After overnight storage at 4°C, 300 µl of sera were pipetted off each sample prior to centrifugation, and stored as 100 µl aliquots at -20°C, 4°C and 25°C. The remaining sera were separated from the blood clots by centrifugation at 800 g for 10 minutes and stored at -20°C. The non-centrifuged serum aliquots were tested by 2G6 Ag-ELISA after one day and seven days storage at -20°C, 4°C and 25°C, and after 14 days storage at -20°C and 4°C only. The centrifuged serum samples were tested after one day storage at -20°C. All samples were tested at Balitvet.

### 5.2.3 Comparison of antigen-ELISA results obtained with serum, serum left with blood clot and plasma samples

Blood samples were collected into plain Vacutainers and Vacutainers containing EDTA from 40 buffaloes in Brebes district, as described in Chapter 8. The blood samples were kept on ice overnight in an insulated plastic box and transported the next day to Balitvet for processing. The serum or plasma were removed within 48 hours of sample collection, by centrifugation at 800 g for 10 minutes and stored at -20°C. The 40 pairs of samples (serum and plasma) were tested by the 2G6 Ag-ELISA and Tr7 Ag-ELISA at Balitvet.

Blood samples were also collected into plain Vacutainers and Vacutainers containing EDTA from 40 buffaloes in Batang district, as described in Chapter 8. The samples

were kept on ice overnight. The following day, 2 ml of serum were pipetted off each clotted sample into a 2 ml sterile plastic tube. The sera were kept on ice together with the Vacutainers containing the remainder of the sera on the blood clots and the Vacutainers with anticoagulant. Seven days after sample collection, the sera left with the blood clots and the plasma samples were centrifuged at 800 g for 10 minutes at Balitvet, and all the samples were then stored at -20°C. The three triplicate samples (serum, serum left with blood clot and plasma) from the same buffalo were tested by the 2G6 Ag-ELISA and Tbr7 Ag-ELISA.

5.2.4 Effect of serum titration on percent positivity values of false-positive samples

Twelve sera which gave high ODs (false-positive results) when tested by the standardised 2G6 Ag-ELISA using a 1:2 serum dilution were chosen from the Australian buffalo sera that were used for specificity estimation (described in Chapter 6). The samples were re-tested by 2G6 Ag-ELISA using serum dilutions of 1:1 to 1:32 and the high-positive (C++), low-positive (C+) and negative (C-) 2G6 Ag-ELISA controls were included. Percent positivities (PP) were calculated for OD values obtained at different dilutions using the OD of the high-positive control (C++) at the 1:2 dilution.

5.2.5 Effect of various blocking agents in the antigen-ELISA

The effect of the inclusion of blocking agents in the 2G6 Ag-ELISA and Tr7 Ag-ELISA was investigated at the CTVM. The standardised Ag-ELISAs which included the blocking agent Tween 20 at 0.50% in the conjugate and serum diluents, and at 0.05% in the washing buffer were used (described in Chapter 3). The effect of the addition of 1% bovine serum albumin (fraction V, A-4503; Sigma), 0.5% normal mouse serum (provided by D. Riebeski, IAEA) and 1% ovalbumin (Scottish Antibody Production Unit) added to the serum and conjugate diluents on the PP values of known positive, negative and false-positive sera was investigated. The PP values were calculated using the high-positive control (C++) included on the same plate as the test samples with the same blocking buffers.

*5.2.5.1 Blocker Study 1*

Eighty of the British cattle sera that were used for estimation of specificity and a range of sera from Calf 915 and Calf 917 experimentally-infected with *T. evansi* TREU 1994 (described in Chapter 3) were used in Blocker Study 1. Initially, the 80 British cattle sera were tested either with no additional blockers or with 0.5% normal mouse serum in the serum diluent. Then a smaller number of British cattle sera that were negative (n = 7) or found to be false-positive by either of the two Ag-ELISAs (n = 5) together with sera collected from Calf 915 (28, 43 and 50 days post-infection) and from Calf 917 (0, 19 and 55 days post-infection) were tested by both Ag-ELISAs. In these Ag-ELISAs either no additional blocker was added or one of the following blockers was included in both serum and conjugate diluents: 1% bovine serum albumin; 0.5% normal mouse serum; or 1% ovalbumin.

*5.2.5.2 Blocker Study 2*

A second attempt was made to investigate the potential of different blockers using a different panel of sera because false-positive results continued to be observed. In the second study, a further 16 British cattle sera, 11 of which had previously given a false-positive result by at least one of the Ag-ELISAs, were tested. The sera were tested with the 2G6 Ag-ELISA and Tr7 Ag-ELISA with the following blocker(s) added to both serum and conjugate diluents: 1% bovine serum albumin; 0.5% normal mouse serum; or both 1% bovine serum albumin and 0.5% normal mouse serum.

*5.2.5.3 Blocker Study 3*

Twenty false-positive sera from the Australian buffaloes were selected for a third blocker study, together with sera collected from Calf 915 and Calf 917 on Days 0, 10, 14, 17, 19, 26, 31, 33, 43 and 60 post-infection. The following blocker combinations were used in the 2G6 Ag-ELISA: 1) 0.5% normal mouse serum in the serum diluent; 2) 1% bovine serum albumin in the conjugate diluent; 3) 0.5% normal mouse serum in the serum diluent and 1% bovine serum albumin in the conjugate diluent; 4) both blockers in both serum and conjugate diluents.

### 5.2.6 Data analysis

The data were considered as either continuous data (PP values) or nominal data (positive or negative values) using a 20 PP cut-off value. To assess the repeatability of the Ag-ELISAs, PP values were transformed logarithmically to base 10 to assume Normality, and the transformed values were then used to calculate estimates of the intraclass correlation coefficient ($R$) with an associated lower 95% confidence interval (Fleiss, 1986). Intraclass correlation coefficients ($R$) were also calculated to compare results obtained with different types of samples (e.g., serum and plasma) or with samples stored under different conditions. In the blocker studies, the differences between estimates of specificity obtained with British cattle sera, either with or without normal mouse serum as an Ag-ELISA blocker, were assessed by McNemar's change test with a continuity correction, using the Chi-squared distribution (Armitage and Berry, 1994). The ability of various 2G6 Ag-ELISA blocker combinations to change the proportion of positive and negative results in different test runs was evaluated using Cochran's Q test (Cochran, 1950).

## 5.3 Results

### 5.3.1 Repeatability of the antigen-ELISAs

Table 5.1 and Table 5.2 show the number of buffaloes found positive at 33 and 34 weeks after infection with *T. evansi* Bakit 362 when tested on two occasions by the 2G6 Ag-ELISA and Tr7 Ag-ELISA, respectively. There were fewer changes in the positive/negative status of samples between Run 1 and Run 2 of the Tr7 Ag-ELISA (one change in Week 33 sera and two changes in Week 34 sera) than with the 2G6 Ag- ELISA (four changes in Week 33 sera and three changes in Week 34 sera). Those samples in which the positive/negative status was rated differently in Run 2 compared with Run 1 had PP values near the cut-off value of 20 PP, and were from different buffaloes on the two occasions that the repeatability was assessed. For the Tr7 Ag-ELISA, the intraclass correlation coefficient estimate ($R$), with lower 95% confidence interval was 0.99 ($\geq$0.98) for both the Week 33 and Week 34 sera data. For the 2G6 Ag-ELISA, the intraclass correlation coefficient estimates ($R$) were 0.90 ($\geq$0.83) and 0.95 ($\geq$0.92), respectively.

**Table 5.1** Number of buffaloes found positive at 33 and 34 weeks after infection with *Trypanosoma evansi* Bakit 362 when tested on two occasions by 2G6 Ag-ELISA

| Run 1 | Week 33 | | Week 34 | |
|---|---|---|---|---|
| | Run 2 | | Run 2 | |
| | Positive | Negative | Positive | Negative |
| Positive | 18 | 4 | 17 | 1 |
| Negative | 0 | 12 | 2 | 14 |

**Table 5.2** Number of buffaloes found positive at 33 and 34 weeks after infection with *Trypanosoma evansi* Bakit 362 when tested on two occasions by Tr7 Ag-ELISA

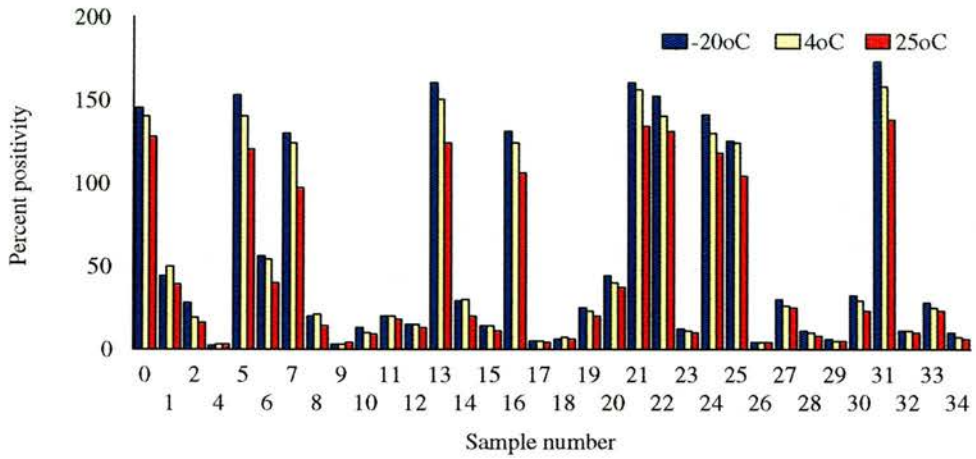
| Run 1 | Week 33 | | Week 34 | |
|---|---|---|---|---|
| | Run 2 | | Run 2 | |
| | Positive | Negative | Positive | Negative |
| Positive | 27 | 1 | 28 | 2 |
| Negative | 0 | 6 | 0 | 4 |

5.3.2 Effect of serum separation method and temperature and duration of serum storage on antigen-ELISA results

When the 2G6 Ag-ELISA results for sera separated with or without centrifugation and stored at -20°C were compared, only serum from Buffalo 514 changed in positive/negative status; the non-centrifuged serum aliquot gave a positive result and the centrifuged serum aliquot gave a negative result. The PP values of this serum sample were close to the 20 PP cut-off value. The intraclass correlation coefficient estimate ($R$) with lower 95% confidence interval calculated from results of sera separated with and without centrifugation and stored at -20°C, was 0.99 ($\geq$0.98).

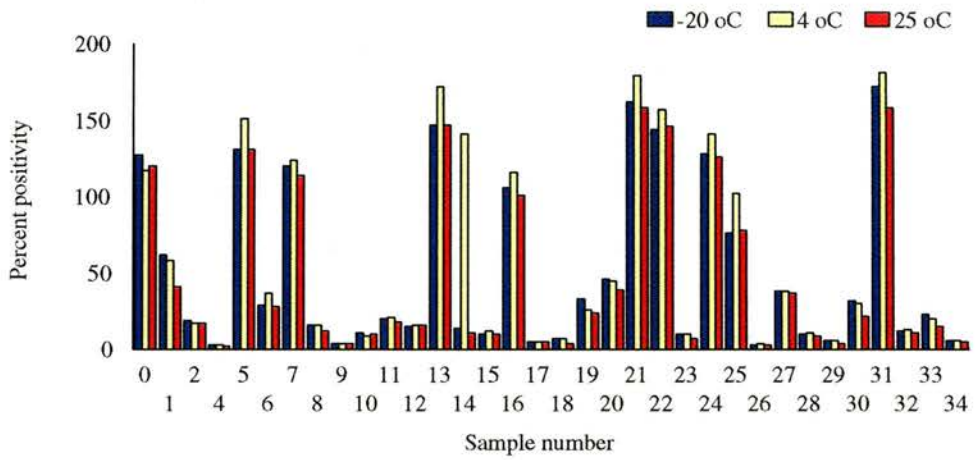
The PP values of the 34 buffalo sera tested by 2G6 Ag-ELISA after storage for one, seven and 14 days at different temperatures are shown in Figures 5.1, 5.2 and 5.3, respectively. In general, PP values were lower after storage at 4°C or 25°C than after storage at -20°C, except that sera stored for seven days had higher values after storage at 4°C. Greater changes in PP values were observed more commonly in samples with a high PP value, while PP values near the 20 PP cut-off value varied less. For example, there was almost no difference between the PP values of Samples

**Figure 5.1** Percent positivity values of buffalo sera tested after storage for one day at -20°C, 4°C and 25°C by 2G6 Ag-ELISA



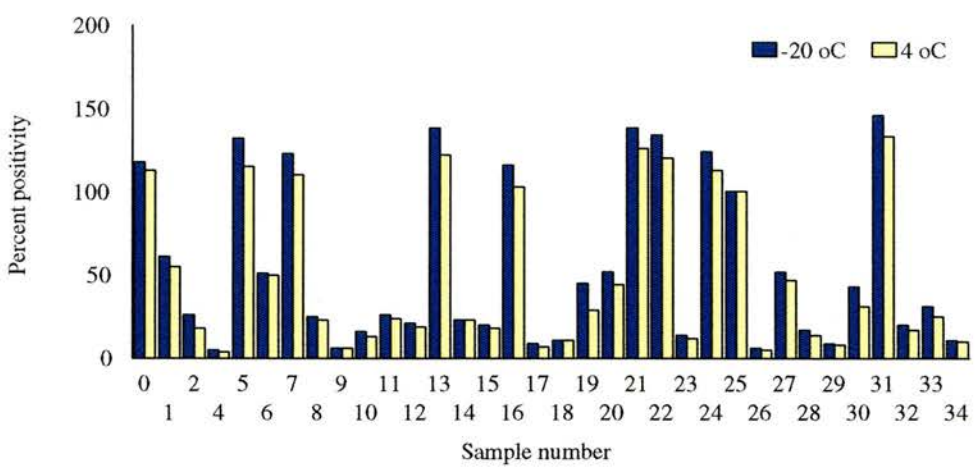

(Samples 0 to 34 are from Buffaloes 500 to 534, respectively)

**Figure 5.2** Percent positivity values of buffalo sera tested after storage for seven days at -20°C, 4°C and 25°C by 2G6 Ag-ELISA




(Samples 0 to 534 are from Buffaloes 500 to 534, respectively)

**Figure 5.3** Percent positivity values of buffalo sera tested after storage for 14 days at -20°C and 4°C by 2G6 Ag-ELISA




(Samples 0 to 34 are from Buffaloes 500 to 534, respectively)

14 and 15 (< 25 PP) that were stored at 4°C and -20°C for 14 days, whereas the PP values of Samples 13 and 16 (> 100 PP) were lower after 14 days storage at 4°C (shown in Figure 5.3). Serum from Buffalo 514 (Sample 14 in Figure 5.2) that was stored for seven days at 4°C had a high value of 141 PP. Overall, the intraclass correlation coefficient estimate ($R$) with lower 95% confidence interval for samples stored at -20°C and 4°C for 14 days was 0.99 (≥0.98).

### 5.3.3 Comparison of antigen-ELISA results obtained from serum, serum left with blood clot, and plasma samples

There were few differences in the positive/negative status of matched samples (serum, serum left with blood clot and plasma) by either Ag-ELISA. In the Visit 4 samples (data in Table 5.3), the serum from Buffalo 12E was positive (23 PP), but the plasma sample was negative (17 PP) by Tr7 Ag-ELISA, and the serum from Buffalo 25E was negative (20 PP), but the plasma sample was positive (22 PP) by 2G6 Ag-ELISA. In the Visit 5 samples (data in Table 5.4), more triplicate samples did not have the same positive/negative status as each other. Of 40 triplicate samples, 11 triplicate samples had different results from serum, serum left with blood clot and plasma samples by 2G6 Ag-ELISA, and three triplicate samples had different results by Tr7 Ag-ELISA. The number of buffaloes that had a different positive/negative result from serum versus serum left with blood clot was seven by 2G6 Ag-ELISA and two by Tr7 Ag-ELISA and three buffaloes had a different positive/negative result from serum versus plasma samples by both Ag-ELISAs. Higher estimates of the intraclass correlation coefficient were obtained for the sera and plasma sample results than the triplicate sample results, particularly for samples tested by 2G6 Ag-ELISA, as shown in Table 5.5.

### 5.3.4 Effect of serum titration on percent positivity values of false-positive samples

Figure 5.4 shows the effect of doubling serum titrations on the ODs obtained with the false-positive Australian buffalo sera with the 2G6 Ag-ELISA. The OD of the high-positive control (C++) declined more rapidly than the OD of the low-positive control (C+). At the 1:8 dilution, the high-positive control (C++) was 23% of its OD at the 1:2 dilution (i.e., 23 PP), and the low-positive control (C+) was 13 PP. At the

1:16 dilution, the PP of the high-positive control (C++) was 14 PP and of the low-positive control (C+) was 9 PP, both of which are below the 20 PP cut-off value. However, at a 1:16 dilution, the PP values of Australian buffalo Sample 1 (145 PP), Sample 2 (21 PP), Sample 3 (32 PP), Sample 4 (37 PP) were above this cut-off value. The effects of doubling dilutions on the ODs of seven additional false-positive Australian buffalo sera are shown in Figure 5.5. The PP values of all the samples, except Sample 8, were reduced to below the 20 PP cut-off value by a 1:8 dilution.

### 5.3.5 Effect of various blocking agents in the antigen-ELISA using sera from calves experimentally infected with *Trypanosoma evansi* TREU 1994, and selected British cattle and Australian buffalo sera

#### *5.3.5.1 Blocker Study 1*

With the addition of 0.5% normal mouse serum to the serum diluent, estimates of specificity using 80 British cattle sera were 73% (61, 82) by 2G6 Ag-ELISA and 83% (72, 90) by Tr7 Ag-ELISA compared with 78% (67, 86) and 71% (60, 81), respectively, previously found without this blocker. Although the specificity of the 2G6 Ag-ELISA was not improved, there was a significant increase in the specificity of the Tr7 Ag-ELISA ($0.01 < P < 0.025$).

The effects of 1% bovine serum albumin, 0.5% normal mouse serum and 1% ovalbumin added separately to both the serum and conjugate diluents of the 2G6 Ag-ELISA and Tr7 Ag-ELISA were compared (data given in Table 5.6). The addition of 0.5% normal mouse serum reduced the PP value of the high-positive control (C++) by 45% of its PP value in the standard 2G6 Ag-ELISA and Tr7 Ag-ELISA. The addition of 1% ovalbumin in both Ag-ELISAs and of 1% BSA in the Tr7 Ag-ELISA, increased the PP value of the high-positive control (C++), whereas the addition of 1% BSA in the 2G6 Ag-ELISA reduced the high-positive control (C++) PP value.

**Table 5.3** Percent positivities (PP) of serum and plasma samples collected from 40 buffaloes in Brebes district during Visit 4 and tested by the 2G6 Ag-ELISA and Tr7 Ag-ELISA.

| Buffalo | 2G6 Ag-ELISA (PP) | | Tr7 Ag-ELISA (PP) | |
|---|---|---|---|---|
| | Serum | Plasma | Serum | Plasma |
| 1E | 10 | 8 | 10 | 9 |
| 2E | 49 | 30 | 69 | 65 |
| 3E | 4 | 4 | 19 | 15 |
| 4E | 3 | 2 | 55 | 48 |
| 5E | 7 | 7 | 15 | 13 |
| 6E | 6 | 4 | 13 | 13 |
| 7E | 6 | 5 | 14 | 16 |
| 8E | 77 | 137 | 113 | 114 |
| 9E | 54 | 39 | 26 | 30 |
| 10E | 8 | 10 | 52 | 59 |
| 11E | 73 | 76 | 32 | 32 |
| 12E | 35 | 46 | 23 | 17 |
| 13E | 14 | 12 | 66 | 40 |
| 14E | 4 | 3 | 4 | 4 |
| 15E | 6 | 3 | 8 | 7 |
| 16E | 30 | 33 | 19 | 15 |
| 17E | 4 | 4 | 16 | 16 |
| 18E | 3 | 3 | 47 | 31 |
| 19E | 116 | 87 | 8 | 8 |
| 20E | 4 | 4 | 38 | 34 |
| 21E | 2 | 2 | 7 | 9 |
| 22E | 7 | 8 | 20 | 18 |
| 23E | 23 | 28 | 8 | 10 |
| 24E | 7 | 11 | 9 | 5 |
| 25E | 20 | 22 | 104 | 87 |
| 26E | 10 | 19 | 6 | 5 |
| 27E | 4 | 4 | 10 | 9 |
| 28E | 120 | 141 | 8 | 6 |
| 29E | 3 | 2 | 16 | 14 |
| 30E | 2 | 2 | 16 | 18 |
| 31E | 28 | 25 | 110 | 96 |
| 32E | 4 | 4 | 44 | 31 |
| 33E | 23 | 37 | 10 | 9 |
| 34E | 2 | 3 | 18 | 13 |
| 35E | 57 | 67 | 40 | 33 |
| 36E | 4 | 5 | 15 | 12 |
| 37E | 10 | 9 | 37 | 53 |
| 38E | 42 | 64 | 15 | 15 |
| 39E | 57 | 80 | 11 | 11 |
| 40E | 2 | 4 | 14 | 8 |

**Table 5.4** Percent positivities (PP) of triplicate samples (serum, serum left with blood clot and plasma) collected from 40 buffaloes in Batang district during Visit 5 and tested by the 2G6 Ag-ELISA and Tr7 Ag-ELISA.

| Buffalo | 2G6 Ag-ELISA (PP) | | | Tr7 Ag-ELISA (PP) | | |
|---|---|---|---|---|---|---|
| | Serum | Serum + clot | Plasma | Serum | Serum + clot | Plasma |
| 1A | 6 | 26 | 6 | 9 | 14 | 13 |
| 2A | 12 | 11 | 16 | 13 | 9 | 10 |
| 3A | 21 | 19 | 19 | 11 | 8 | 7 |
| 4A | 77 | 58 | 69 | 87 | 103 | 98 |
| 5A | 47 | 52 | 50 | 136 | 112 | 105 |
| 6A | 23 | 16 | 18 | 20 | 17 | 21 |
| 7A | 32 | 41 | 50 | 18 | 15 | 14 |
| 8A | 4 | 5 | 6 | 30 | 20 | 20 |
| 9A | 33 | 43 | 72 | 61 | 85 | 95 |
| 10A | 9 | 7 | 9 | 10 | 8 | 8 |
| 11A | 10 | 10 | 11 | 66 | 52 | 49 |
| 12A | 2 | 3 | 4 | 18 | 12 | 15 |
| 13A | 27 | 25 | 32 | 33 | 23 | 29 |
| 14A | 5 | 40 | 8 | 19 | 13 | 13 |
| 15A | 5 | 60 | 6 | 11 | 8 | 10 |
| 16A | 7 | 6 | 10 | 42 | 31 | 29 |
| 17A | 10 | 12 | 20 | 37 | 30 | 32 |
| 18A | 15 | 21 | 54 | 66 | 57 | 58 |
| 19A | 5 | 6 | 6 | 13 | 14 | 16 |
| 20A | 18 | 16 | 13 | 14 | 13 | 14 |
| 21A | 11 | 17 | 15 | 113 | 90 | 83 |
| 22A | 3 | 3 | 7 | 14 | 17 | 20 |
| 23A | 6 | 6 | 6 | 31 | 47 | 45 |
| 24A | 15 | 18 | 27 | 9 | 7 | 13 |
| 25A | 52 | 85 | 65 | 66 | 77 | 77 |
| 26A | 9 | 9 | 12 | 45 | 37 | 42 |
| 27A | 30 | 32 | 38 | 60 | 53 | 77 |
| 28A | 68 | 72 | 80 | 28 | 26 | 26 |
| 29A | 39 | 34 | 5 | 11 | 16 | 16 |
| 30A | 13 | 13 | 19 | 22 | 27 | 31 |
| 31A | 195 | 151 | 78 | 28 | 20 | 19 |
| 32A | 200 | 169 | 187 | 62 | 71 | 65 |
| 33A | 41 | 52 | 64 | 79 | 58 | 65 |
| 34A | 19 | 34 | 35 | 127 | 134 | 131 |
| 35A | 13 | 14 | 21 | 20 | 15 | 20 |
| 36A | 9 | 5 | 6 | 11 | 10 | 13 |
| 37A | 2 | 4 | 4 | 13 | 9 | 10 |
| 38A | 23 | 27 | 24 | 45 | 36 | 31 |
| 39A | 44 | 49 | 83 | 105 | 77 | 65 |
| 40A | 4 | 3 | 4 | 67 | 65 | 66 |

**Table 5.5** Intraclass correlation coefficient estimates ($R$) with lower 95% confidence intervals calculated from the Ag-ELISA results of different types of samples from 40 buffaloes

| | Sample types compared | $R$ | |
|---|---|---|---|
| | | 2G6 Ag-ELISA | Tr7 Ag-ELISA |
| Visit 4 | Serum vs. plasma | 0.97 (≥0.95) | 0.96 (≥0.94) |
| Visit 5 | Serum vs. serum left on clot vs. plasma | 0.83 (≥0.79) | 0.92 (≥0.90) |

In the 2G6 Ag-ELISA, 0.5% normal mouse serum reduced the PP value of two out of five sera from the calves experimentally infected with *T. evansi* (Calf 917 on Day 19 and Calf 915 on Day 28) to below the 20 PP cut-off value. Two samples (Sample 16H and 15J) that gave false-positive results by the 2G6 Ag-ELISA without additional blocking agents, were negative when tested with any of the additional blocking agents. Four samples (Samples 10B, 15B, 8C and 9E) gave positive results without additional blocking agents, but only the PP values of Samples 8C and 9E were reduced below the 20 PP cut-off value (by the addition of 1% ovalbumin). By Cochran's test, there was no significant difference ($0.25 < P < 0.50$) between the proportions of positive and negative results obtained with or without additional blocking agents in either the 2G6 Ag-ELISA or Tr7 Ag-ELISA.

*5.3.5.2 Blocker study 2*

The effects of 1% bovine serum albumin and 0.5% normal mouse serum added either separately or together in the serum and conjugate diluents of the 2G6 Ag-ELISA and Tr7 Ag-ELISAs were compared (data shown in Table 5.7). The addition of 0.5% normal mouse serum reduced the PP value of the high-positive control (C++) to 55% of its original value obtained without additional blocking agents in the 2G6 Ag-ELISA and Tr7 Ag-ELISA. When 0.5% normal mouse serum was added with 1% bovine serum albumin, the high-positive control (C++) was reduced to 65% (2G6 Ag-ELISA) and 71% (Tr7 Ag-ELISA) of the respective original values. Of the six samples that gave false-positive results with the Tr7 Ag-ELISA without additional blocking agents, only Sample 3G was negative by Tr7 Ag-ELISA with either 0.5% normal mouse serum added alone or in combination with 1% bovine serum albumin.

**Figure 5.4** Effect of doubling serum dilutions on the optical densities of control sera and false-positive Australian buffalo serum Samples 1 to 4 tested by the 2G6 Ag-ELISA

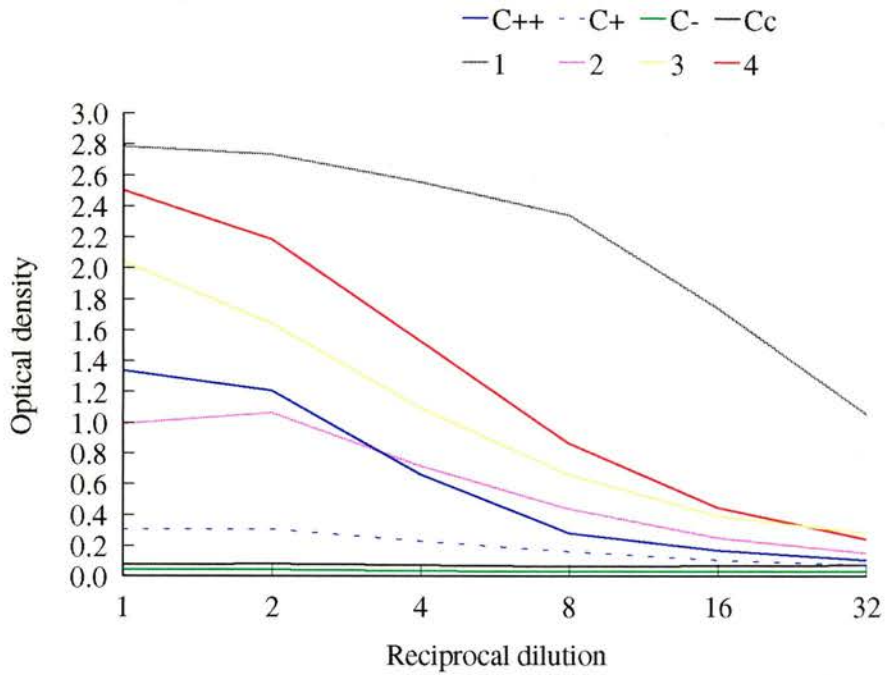


**Figure 5.5** Effect of doubling serum dilutions on the optical densities of false-positive Australian buffalo serum Samples 5 to 11 tested by the 2G6 Ag-ELISA

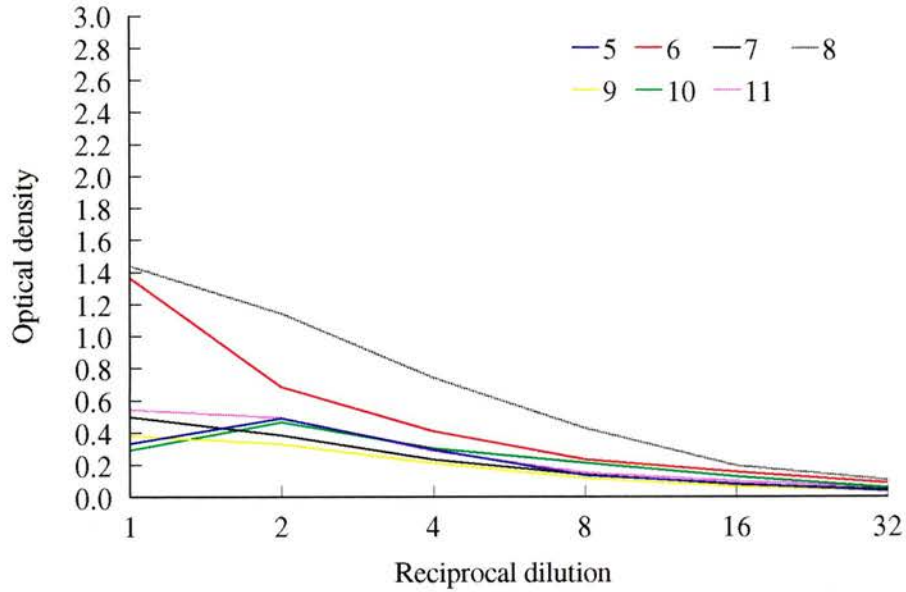


**Table 5.6** Blocker Study 1: Percent positivity (PP) values obtained with serum samples and controls with 2G6 Ag-ELISA and Tr7 Ag-ELISA with either 1% bovine serum albumin (BSA), 0.5% normal mouse serum (NMS) or 1% ovalbumin added to both serum and conjugate diluents.

| Sample | 2G6 Ag-ELISA (PP) | | | | Tr7 Ag-ELISA (PP) | | | |
|---|---|---|---|---|---|---|---|---|
| | No blocker | BSA | NMS | Ovalbumin | No blocker | BSA | NMS | Ovalbumin |
| C+ | 41 | 40 | 21 | 43 | 66 | 75 | 44 | 53 |
| C- | 10 | 3 | 5 | 4 | 6 | 4 | 5 | 3 |
| Cc | 14 | 1 | 5 | 2 | 8 | 1 | 3 | 3 |
| 10B | 16 | 11 | 15 | 15 | 34 | 25 | 33 | 23 |
| 15B | 6 | 3 | 4 | 4 | 27 | 33 | 24 | 22 |
| 5C | 6 | 2 | 5 | 3 | 12 | 7 | 9 | 6 |
| 8C | 6 | 2 | 6 | 2 | 26 | 22 | 32 | 19 |
| 12C | 9 | 2 | 5 | 3 | 8 | 3 | 4 | 5 |
| 15C | 13 | 6 | 6 | 7 | 15 | 13 | 10 | 9 |
| 17C | 9 | 3 | 9 | 4 | 8 | 6 | 6 | 6 |
| 2D | 11 | 2 | 9 | 4 | 11 | 11 | 11 | 6 |
| 9E | 10 | 4 | 10 | 7 | 21 | 21 | 25 | 19 |
| 10H | 15 | 6 | 13 | 7 | 11 | 3 | 6 | 3 |
| 16H | 21 | 1 | 2 | 6 | 3 | 4 | 5 | 4 |
| 15J | 25 | 19 | 19 | 20 | 8 | 4 | 5 | 2 |

C+: Low-positive control; C-: Negative control; Cc: Conjugate control.

**Table 5.6** Continued

| Sample | 2G6 Ag-ELISA (PP) | | | | Tr7 Ag-ELISA (PP) | | | |
|---|---|---|---|---|---|---|---|---|
| | No blocker | BSA | NMS | Ovalbumin | No blocker | BSA | NMS | Ovalbumin |
| Calf 915/ Day 28 | 26 | 22 | 13 | 21 | 34 | 37 | 19 | 33 |
| Calf 915/ Day 43 | 66 | 72 | 52 | 62 | 133 | 124 | 129 | 117 |
| Calf 915/ Day 50 | 52 | 57 | 33 | 59 | 77 | 97 | 62 | 77 |
| Calf 917/ Day 0 | 13 | 8 | 14 | 9 | 7 | 6 | 7 | 6 |
| Calf 917/ Day 19 | 20 | 14 | 26 | 14 | 53 | 64 | 71 | 38 |
| Calf 917/ Day 55 | 69 | 76 | 122 | 67 | 158 | 124 | 185 | 128 |

**Table 5.7** Blocker Study 2: Percent positivity (PP) values obtained with serum samples and controls with 2G6 Ag-ELISA and Tr7 Ag-ELISA with either 1% bovine serum albumin (BSA) or 0.5% normal mouse serum (NMS) added to both serum and conjugate diluents either separately or together.

| Sample | 2G6 Ag-ELISA (PP) | | | | Tr7 Ag-ELISA (PP) | | | |
|---|---|---|---|---|---|---|---|---|
| | No blocker | BSA | NMS | BSA + NMS | No blocker | BSA | NMS | BSA + NMS |
| C+ | 55 | 55 | 32 | 30 | 57 | 60 | 37 | 42 |
| C- | 4 | 3 | 5 | 4 | 4 | 4 | 3 | 4 |
| Cc | 6 | 1 | 3 | 2 | 5 | 1 | 2 | 2 |
| 9F | 20 | 9 | 13 | 13 | 3 | 2 | 2 | 5 |
| 11F | 14 | 6 | 10 | 8 | 128 | 119 | 127 | 121 |
| 17F | 58 | 15 | 34 | 15 | 5 | 3 | 2 | 4 |
| 3G | 11 | 6 | 9 | 7 | 28 | 28 | 6 | 10 |
| 4G | 27 | 18 | 44 | 26 | 6 | 5 | 5 | 7 |
| 5G | 39 | 55 | 12 | 11 | 6 | 7 | 6 | 6 |
| 3H | 142 | 128 | 9 | 7 | 17 | 15 | 7 | 10 |
| 4H | 20 | 4 | 5 | 5 | 29 | 30 | 25 | 26 |
| 5H | 14 | 1 | 3 | 2 | 14 | 17 | 20 | 21 |
| 6H | 20 | 3 | 1 | 1 | 16 | 11 | 10 | 8 |
| 7H | 11 | 2 | 3 | 2 | 24 | 26 | 28 | 33 |
| 11H | 19 | 6 | 8 | 8 | 34 | 15 | 34 | 27 |
| 13H | 22 | 4 | 2 | 1 | 2 | 3 | 2 | 7 |
| 14H | 27 | 33 | 41 | 47 | 47 | 46 | 56 | 56 |
| 19H | 15 | 10 | 12 | 12 | 3 | 30 | 25 | 26 |
| 20H | 25 | 22 | 9 | 9 | 3 | 3 | 6 | 4 |

C+: Low-positive control; C-: Negative control; Cc: Conjugate control.

Of the seven samples that gave false-positive results with the 2G6 Ag-ELISA without additional blocking agents, three samples (17F, 4G and 13H) were negative when tested with 1% bovine serum albumin, and four samples (5G, 3H, 13H and 20H) were negative when tested with 0.5% normal mouse serum with or without 1% bovine serum albumin. There was no significant difference between the proportions of positive and negative results obtained with the addition of any of these blocking agents alone or in combination, in either the 2G6 Ag-ELISA ($0.10 < P < 0.25$) or Tr7 Ag-ELISA $0.50 < P < 0.75$).

*5.3.5.3 Blocker study 3*

The effects of adding 1% bovine serum albumin in the conjugate diluent and/or 0.5% normal mouse serum in the serum diluent, and both blocking agents in the serum and conjugate diluents in the 2G6 Ag-ELISA were further assessed. This study differed from the previous two blocking studies in that imported Australian buffalo sera that gave false-positive results with the standardised 2G6 Ag-ELISA were used, and a larger number of samples from the calves experimentally infected with *T. evansi* were included.

Figures 5.6 and 5.7 show the effects of additional blocking agents on the PP values of sera from the calves experimentally infected with *T. evansi*. Without additional blocking agents, the Day 26 serum from Calf 915 and Day 19 and 26 sera from Calf 917 were negative by 2G6 Ag-ELISA. When tested with additional blocking agents several sera also had PP values below the 20 PP cut-off value: Day 31 and 33 sera from Calf 915 tested with the addition of 0.5% normal mouse serum in the serum diluent, Day 10 and 19 sera from Calf 915 and Day 31, 33 and 43 sera from Calf 917 tested with the addition of 1% bovine serum albumin in the conjugate diluent, were all negative. When both these blocking agents were added to their respective diluents, Day 10, 19, 31, 33 and 43 sera from Calf 915, and Day 31 and 33 sera from Calf 917, were also negative. When both these blocking agents were added to both the serum and conjugate diluents, Day 31, 33 and 43 sera from Calf 915 were negative.

There was a significant reduction in the proportion of sera from the experimentally-infected calves that were positive when tested by 2G6 Ag-ELISA with additional blocking agents ($0.001 < P < 0.01$).

Figure 5.8 shows the effect of additional blocking agents in the 2G6 Ag-ELISA on the PP values of false-positive Australian buffalo sera. Only three false-positive Australian buffalo sera were negative when tested with the 2G6 Ag-ELISA with 0.5% normal mouse serum in the serum diluent (Samples 7 and 22), with 1% bovine serum albumin in the conjugate diluent (Samples 9 and 22), with 0.5% normal mouse serum in the serum diluent and 1% bovine serum albumin in the conjugate diluent (Samples 7, 9 and 22) or with both these blocking agents in both the serum and conjugate diluents (Samples 7 and 22). All three samples (Samples 7, 9 and 22) had PP values near the cut-off value (36 PP, 23 PP and 26 PP, respectively) with the standardised 2G6 Ag-ELISA. Overall, the addition of 0.5% normal mouse serum and/or 1% bovine serum albumin in the serum diluent and/or conjugate diluent did not significantly reduce the proportion of false-positive results obtained with the Australian buffalo sera ($0.10 < P < 0.25$).

**Figure 5.6** The effect on the percent positivity values of sera from Calf 915 experimentally infected with *Trypanosoma evansi* TREU 1994 of adding 0.5% normal mouse serum (NMS) and/or 1% bovine serum albumin (BSA) in the 2G6 antigen-ELISA (NMS + BSA [1]: NMS in serum diluent and BSA in conjugate diluent; NMS + BSA [2]: NMS and BSA in both serum and conjugate diluents)

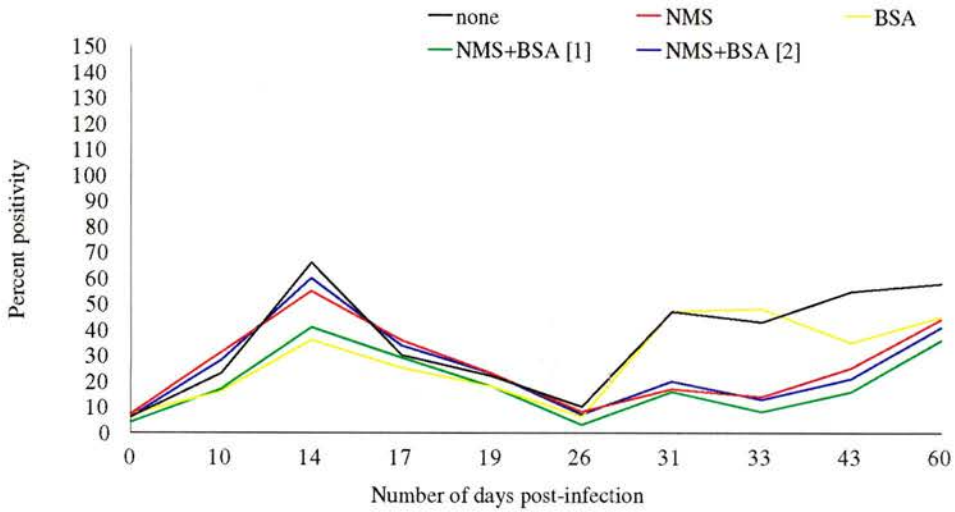


**Figure 5.7** The effect on the percent positivity values of sera from Calf 917 experimentally infected with *Trypanosoma evansi* TREU 1994 of adding 0.5% normal mouse serum (NMS) and/or 1% bovine serum albumin (BSA) in the 2G6 antigen-ELISA (NMS + BSA [1]: NMS in serum diluent and BSA in conjugate diluent; NMS + BSA [2]: NMS and BSA in both serum and conjugate diluents)

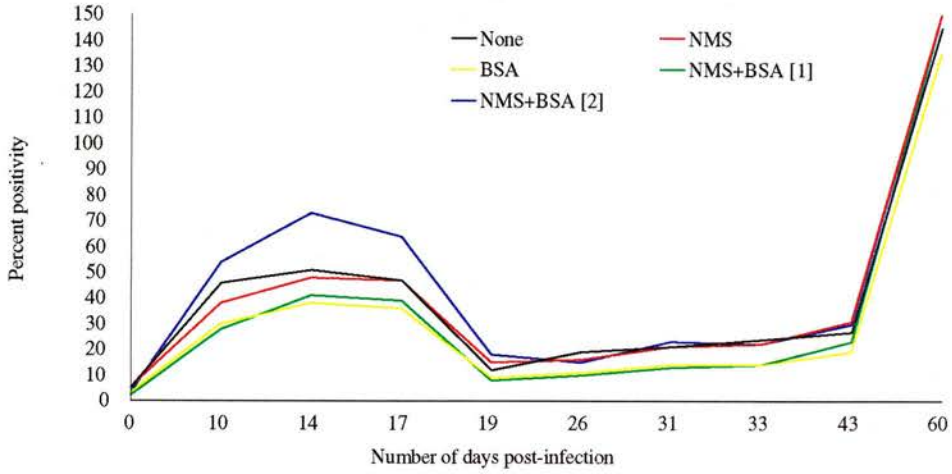
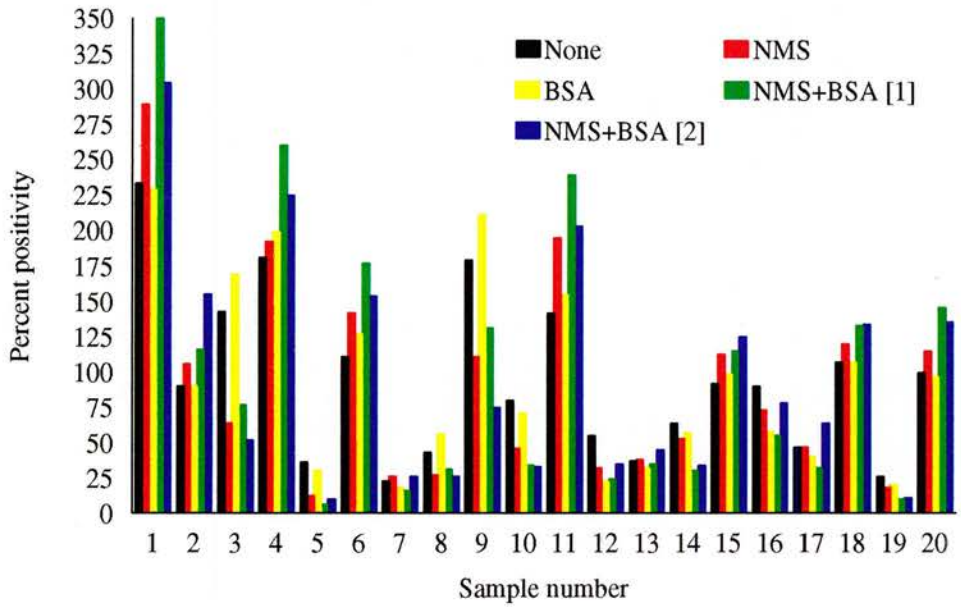


**Figure 5.8** The effect on the percent positivity values of false-positive Australian buffalo sera of adding 0.5% normal mouse serum (NMS) and/or 1% bovine serum albumin (BSA) in the 2G6 Ag-ELISA (NMS + BSA [1]: NMS in serum diluent and BSA in conjugate diluent; NMS + BSA [2]: NMS and BSA in both serum and conjugate diluents)



## 5.4 Discussion

The reliability and robustness of diagnostic tests are important characteristics which should be assessed prior to their establishment as routine diagnostic tests or application in epidemiological studies. These parameters were investigated for two *T. evansi* Ag-ELISAs, in particular in relation to working conditions in Indonesia where the tests were to be used in epidemiological studies on *T. evansi* infections in buffaloes.

The repeatability of the two *T. evansi* Ag-ELISAs was assessed by repeat testing two sets of weekly samples from 34 experimentally-infected buffaloes under the same operating conditions. The repeatabilities of both Ag-ELISAs were shown to be acceptable and the Tr7 Ag-ELISA had a higher intraclass correlation coefficient estimate ($R$) (0.99) than the 2G6 Ag-ELISA (0.90 to 0.95), on both occasions that repeatability was assessed. The repeatability estimates of each Ag-ELISA were similar for the two occasions that repeat testing was conducted; this was expected because both sets of samples were from the same buffaloes, but at different stages of infection, and therefore were not independent. Although assessment of the repeatability of diagnostic tests is commonly undertaken for medical investigations (Fleiss, 1986; Everitt, 1989), rarely is this parameter estimated for veterinary diagnostic tests. No publications on the evaluation of trypanosomosis Ag-ELISAs have reported estimates of the repeatability of these tests (Nantulya *et al.*, 1989b; Masake and Nantulya, 1991; Diall *et al.*, 1992; Bengaly *et al.*, 1995; Monzon *et al.*, 1995).

The collection of blood samples from livestock in Indonesia and other countries in Southeast Asia involves field trips of several days or weeks duration because target sites are often located in remote farming areas away from diagnostic laboratories. Previous work had shown that blood samples collected with anticoagulant remained positive with the MHCT and MI for up to 24 hours (Siswansyah *et al.*, 1987). However, the effects of different methods of sample processing and storage conditions for sera to be tested by *T. evansi* Ag-ELISAs had not been investigated. Sera that were separated without centrifugation and stored either overnight or for up to 14 days at 4°C (i.e., similar to field conditions) did not give significantly different

results compared to sera separated by centrifugation and stored at -20°C, when tested by the 2G6 Ag-ELISA. Previous workers found a rapid decline in the immune reactivity of recombinant *T. vivax* protein and suggested that false-negative results of field samples may be due to the instability of antigen detected by their *T. vivax* Ag-ELISA (Masake *et al,*. 1995a, b). However, in the present study, the *T. evansi* antigen detected by the 2G6 Ag-ELISA remained immunoreactive, even after one week at 25°C. The PP value of Buffalo 514 when tested after seven days at 4°C was unexpectedly high, but may have resulted from operator error in pipetting or incorrect identification of the sample. When triplicate samples of serum, serum left with blood clot and plasma were compared, differences were found between some of the results obtained with these types of sample which were tested by the same operator. The findings suggest that serum should be removed from the blood clot as soon as possible after clot retraction has occurred. In practical terms, sera should be separated from the blood clot in the field (without centrifugation) the day after blood collection and stored on ice for transportation to the laboratory; this method of sample preparation should give results that are comparable to sera separated by centrifugation and stored frozen. However, it would be advisable to pipette serum from well below the surface because serum components can layer in the tube during overnight storage and mixing is not possible prior to pipetting off serum without disturbing the blood clot.

The reproducibility of the assays between laboratories was not directly assessed and no long-term studies on the shelf-life of the reagents were carried out, but during the three-year project there was no observed decline in the assays performances which would be a source of systematic error. The performances of both Ag-ELISAs in Balitvet were comparable to their performances at the CTVM (data not shown). Similar results were obtained with chequerboard titrations conducted in the two laboratories and less than 5% of all Ag-ELISA plates were rejected because of the plate controls being outside the quality control limits established at the CTVM. The coating monoclonal antibody, conjugate, and serum controls were stored at either -20°C or -70°C and separate aliquots of these reagents were stored in different freezers to minimise loss in the event of an electrical failure. The plates were kept in

an air-conditioned laboratory and buffer chemicals were stored in air-tight containers with silica gel because of the high ambient temperature and humidity.

The effects of serum titration and the addition of blocking agents on diagnostic specificity were investigated because of the level of false-positive results obtained from non-exposed British cattle and Australian buffaloes. The standardised *T. evansi* Ag-ELISAs used a serum dilution of 1:2 for the 2G6 Ag-ELISA and 1:10 for the Tr7 Ag-ELISA. In the 2G6 Ag-ELISA, serum titration up to 1:32 did not eliminate all false-positive values of Australian buffalo sera, but with a 1:16 dilution the low-positive (C+) and high-positive (C++) controls had PP values below the cut-off value. The rates of decline of the ODs of the Australian buffalo sera were similar to the Ag-ELISA positive controls and may reflect the presence of antigenic components which have a similar affinity to the target trypanosomal antigens and are present at a high concentration in the false-positive sera (Kemeny 1991). Some Australian buffalo sera showed a more rapid decline in OD that could be due to antigenic components with a high affinity which are present at lower concentrations. Serum titration did not significantly improve the specificity of the *T. evansi* Ag-ELISAs, but reduced their sensitivity as shown by the decline of PP values below the cut-off value with some sera of the experimental calves.

Blocking agents are commonly added to ELISAs to reduce non-specific binding of ELISA reagents (e.g., conjugate) and serum immunoreactants. The standardised 2G6 Ag-ELISA and Tr7 Ag-ELISA use Tween 20 as a blocking agent in both serum and conjugate diluents and in the washing buffer. The addition of other blocking agents (normal mouse serum, bovine serum albumin, ovalbumin) was investigated because of the false-positive results obtained with negative sera, but none of these blockers significantly reduced the proportion of false-positive results. A disadvantage of these blocking agents, however, was the significant reduction in the number of sera of experimentally-infected calves that were positive by both Ag-ELISAs, and therefore the reduced sensitivity of the assays.

The present study did not determine the underlying causes of the false-positive results found with the non-exposed animals, but highlighted the need for further investigation, for example by selection of alternative monoclonal antibodies. Characterisation of the serum antigens detected by the two capture monoclonal antibodies in *T. evansi*-infected cattle and buffaloes would enable homology of the target antigens with common serum antigens to be estimated. Recently, Masake and her colleagues (1995b) characterised an antigen detected by a *T. vivax*-specific monoclonal antibody (Tv27) which was identified as a non-secretory protein located in the trypanosome cytosol and nucleus. However, the similarity between the antigenic determinants of this antigen and those of common serum antigens derived from other organisms was not reported.

# CHAPTER 6

# EVALUATION OF *TRYPANOSOMA EVANSI* ANTIGEN-ELISAS: II) ESTIMATION OF DIAGNOSTIC SENSITIVITY AND SPECIFICITY, KAPPA, PREDICTIVE VALUES AND TRUE PREVALENCE

## 6.1 Introduction

An ideal diagnostic test has both a diagnostic sensitivity and diagnostic specificity equal to 100% and will correctly classify all individuals according to disease or infection. Diagnostic sensitivity and specificity are indicators of the validity of a diagnostic test and are influenced by many factors including characteristics of test components, the handling and processing of samples and test operator error. It is, therefore, unsurprising that no diagnostic test has both a sensitivity and specificity of 100% when used to test large numbers of samples.

Estimation of diagnostic sensitivity and specificity is a prerequisite to the establishment of a diagnostic test for routine diagnosis or epidemiological studies (Thrusfield, 1995). With known estimates of these parameters, multiple test strategies can be employed to increase detection of affected animals (i.e., increase sensitivity) or to further confirm the positive status of test-positive animals and reduce the number of false-positives (i.e., increase specificity). With parallel testing, two or more tests are conducted at the same time and animals are classified as affected if they are positive by any of the tests. With serial testing, those animals that are positive to an initial test are tested again by a different test, and only animals that are test-positive by all sequential tests are considered positive (Thrusfield, 1995). Therefore, the advantage of multiple testing is that either sensitivity (by parallel testing) or specificity (by serial testing) can be increased.

Previous studies have estimated the sensitivity of trypanosomosis tests by testing animals from endemic trypanosomosis areas or animals experimentally infected with trypanosomes, and have estimated specificity by testing animals from non-endemic

areas. For example, an Ag-ELISA based on a monoclonal antibody against *T. rhodesiense* (a *T. brucei* group-specific monoclonal antibody) was used to test sera from camels in endemic areas of Mali and Kenya (Nantulya *et al.*, 1989b). The camels were classified as parasitaemic or non-parasitaemic by MI and/or the MHCT. Out of 20 MI-positive camels, 18 (90%) were positive and 16 of 17 MHCT-positive camels (94%) were positive by the Ag-ELISA. These authors suggested that parallel testing (i.e., testing by the MHCT and Ag-ELISA) could be used to increase sensitivity because some parasitaemic animals with putative, early infections did not have a detectable antigenaemia. In another study, Nantulya and colleagues (1989a) used the same Ag-ELISA to test 21 buffaloes naturally infected with *T. evansi*, and 19 buffaloes were found to be parasitaemic and antigenaemic, but three non-parasitaemic buffaloes were also antigenaemic. In Argentina, 60 horses from a region reported to be free of *T. evansi* were all negative when tested by an Ag-ELISA based on the same *T. brucei* group-specific monoclonal antibody and 74% of parasitaemic horses (by MHCT or MI) were found to be antigenaemic (Monzon *et al.*, 1995). This study also tested sera from three horses and two mules experimentally infected with *T. evansi* which were positive by Ag-ELISA throughout the post-infection monitoring period (35 to 127 days). The sensitivity and specificity of Ag-ELISAs based on *T. congolense*, *T. vivax* and *T. brucei* group-specific monoclonal antibodies were estimated by testing 1633 cattle in an trypanosomosis-endemic area and 50 cattle in a tsetse-free area of Burkina Faso (Bengaly *et al.*, 1995). The sensitivity of the Ag-ELISA that was estimated from 144 parasite-positive cattle (by BCT) was 75% (68, 82) and the specificity estimate was 98.9% (94, 100). However, the proportions of animals that were found to be infected by different trypanosome species differed between the BCT and the Ag-ELISA. For example, 39.6% of infections were found to be *T. brucei* and *T. congolense* mixed infections by Ag-ELISA, but only one animal was confirmed to have a *T. brucei* infection by BCT, suggesting that cross-reactions between the monoclonal antibodies and heterologous trypanosome species may have occurred.

For the diagnosis of *T. evansi* infections, the MI test has been considered by many workers to be the gold standard (Robson and Ashkar, 1972; Paris *et al.*, 1982),

particularly for the detection of *T. evansi* in chronically-infected animals (Luckins *et al.*, 1979). However, although MI was found to detect more *T. evansi*-infected horses (Monzon *et al.*, 1990) and buffaloes (Lohr *et al.*, 1986) than other parasitological tests, it failed to detect all *T. evansi*-infected animals. Nevertheless, the recent studies undertaken to estimate the sensitivity of trypanosomosis Ag-ELISAs (described above) relied on standard parasitological tests (e.g., MHCT and MI) to identify naturally-infected animals.

Previous studies on the application of Ag-ELISAs for *T. evansi* (and other *Trypanosoma* species) have suggested that these tests may be useful for the diagnosis of *T. evansi* infections in buffaloes in Indonesia and other countries in Southeast Asia. Therefore, the diagnostic sensitivities of two *T. evansi* Ag-ELISAs were estimated: 1) using buffaloes experimentally infected with *T. evansi*; and 2) using buffaloes that were confirmed to be naturally infected with *T. evansi,* by parasitological techniques. *Trypanosoma evansi* is endemic throughout most areas of Indonesia (Anon, 1993), and therefore the identification of non-exposed, naive buffaloes is difficult. Following preliminary estimation of the specificities of these tests using British cattle, sera from Australian buffaloes were obtained to estimate specificity using buffaloes (i.e., the target species) from a country not reported to have *T. evansi* (Anon, 1995). In addition, sera from cattle with serological evidence of *A. marginale* and *Babesia* species infections were also tested for cross-reactivity because these haemoparasites are endemic in Indonesia and other countries with *T. evansi*. For this purpose, sera from cattle in a trypanosomosis-free area of Peru that had been tested by *A. marginale* Ag-ELISA and *Babesia* antibody-detection ELISA in a previous study were used (Davison, 1991), because no suitable sera were available from Indonesia.

The diagnostic sensitivity and specificity of a test should not vary for a given cut-off value, if established using a representative sample of the target population, and these parameters can be used to estimate true prevalence values from test prevalence values. Diagnostic tests for trypanosomosis are used in different areas where the prevalence of infections in the target animal population is likely to vary substantially.

The local prevalence of trypanosomosis, however, will affect both the probability that an animal that is test-positive for *T. evansi* is truly infected (i.e., positive predictive value) and the probability that an animal that is test-negative for *T. evansi* is not infected (i.e., negative predictive value) (Smith, 1995). To demonstrate the effect of different prevalence values, theoretical true prevalence values from 10% to 90% were used to calculate a range of predictive values with the estimates of sensitivity and specificity obtained in this study. The Tr7 Ag-ELISA is based on the same *T. brucei* group-specific monoclonal antibody used in the Ag-ELISAs described in the studies above. The 2G6 Ag-ELISA has been used previously in several countries of Africa, Latin America and Southeast Asia (A.G. Luckins pers. comm.), and therefore it was considered important to assess agreement between these two Ag-ELISAs. Test agreement was quantified by calculation of *kappa* values defined as the proportion of agreement beyond chance exhibited by two or more tests (Thrusfield, 1995).

## 6.2 Materials and methods

The standardised 2G6 Ag-ELISA and Tr7 Ag-ELISA described in Chapter 3 were used to test the sera described in the following studies. All sera were stored at -20°C prior to testing at Balitvet.

### 6.2.1 Diagnostic sensitivity

*Indonesian buffaloes experimentally infected with the primary isolate* Trypanosoma evansi *Bakit 259 and secondary isolate* Trypanosoma evansi *Bakit 362*

The different stages of the experimental infection of buffaloes were: 1) the primary infection with *T. evansi* Bakit 259 (35 buffaloes); 2) chemotherapy; and 3) the secondary infection with *T. evansi* Bakit 362 (34 buffaloes) which are described in Chapter 4. Weekly blood samples were taken up to 105 days of the primary infection and up to 45 days of the secondary infection; 20 buffaloes were monitored up to 64 days of the secondary infection. Estimates of sensitivity were calculated for each week post-infection using the buffaloes shown to be infected with *T. evansi* by MHCT or MI in the primary infection (Group $259_{POS}$: n = 18; Group $259_{PREPOS}$: n = 6) and secondary infection (Group $362_{POS}$: n = 30).

*Indonesian buffaloes naturally infected with* Trypanosoma evansi

During the field work in Central Java described in Chapter 8, buffaloes were found to be naturally-infected with *T. evansi* by MHCT (n = 39) and by MI (n = 100). Estimates of sensitivity of the Ag-ELISAs were calculated using the 39 MI-positive buffaloes, 100 MHCT-positive buffaloes and both groups combined (n = 139).

6.2.2 Diagnostic specificity

*Australian buffaloes and cattle*

Sera from 263 Australian buffaloes and 80 Australian cattle were obtained from Michael Pearce, formerly of the Indonesia International Animal Science Research and Development Foundation (INI ANSREDEF), Bogor. The buffaloes were living in feral conditions in the Northern Territories, Australia, and the origin of the cattle within Australia was unknown. Estimates of specificity were calculated from the results, as described in Chapter 3, for the 2G6 Ag-ELISA and Tr7 Ag-ELISA, using 20 PP and 30 PP cut-off values.

*Indonesian buffaloes naturally infected with Trypanosoma theileri*

During the field work in Central Java, 60 buffaloes were found to be infected with *T. theileri* by MHCT based on the typical morphology and slow motility of this trypanosome (Hoare, 1972). Many of these buffaloes would be expected to also have been exposed to *T. evansi* because these trypanosomes can be transmitted by the same vectors, and four buffaloes were found to be concurrently infected with *T. evansi*. To reduce the likelihood of including buffaloes previously exposed to *T. evansi,* only those buffaloes found to be negative by both IgG ELISA and the CATT (n = 20) were included to determine whether or not there was evidence of cross-reactions in the Ag-ELISAs.

*British cattle experimentally infected with* Theileria *species or* Babesia *species*

Thirty-seven sera taken from British cattle 28 to 78 days after experimental infection with *Theileria annulata* (n = 21), *Theileria parva* (n = 13), *Babesia bigemina* (n = 2) or *Babesia bovis* (n = 1) were obtained from Professor Brown, CTVM, Edinburgh,

and were tested by the 2G6 Ag-ELISA and Tr7 Ag-ELISA. The proportion of sera that were negative by each Ag-ELISA was calculated, using a 20 PP cut-off value.

*Peruvian cattle naturally infected with* Anaplasma marginale *alone or with* Babesia bovis

In a previous study, sera from Peruvian cattle were tested for *A. marginale* by Ag-ELISA and for *B. bovis* by antibody-detection ELISA (Davison, 1991). Forty of the sera that were found to be positive for *A. marginale* were selected, of which 30 sera were also either weakly positive or positive by *B. bovis* antibody-ELISA. All the sera had been heat-treated at 56°C for 30 minutes to comply with UK importation regulations. The proportion of sera that were negative by each Ag-ELISA was calculated using a 20 PP cut-off value.

### 6.2.3 Estimation of true prevalence from test prevalence

Corrected estimates of true prevalence (P) were calculated for theoretical test prevalence values ($P^T$) of 10%, 30%, 50%, 70% and 90% using the following equation (Thrusfield, 1995):

$$P = \frac{P^T + \text{specificity} - 100}{\text{sensitivity} + \text{specificity} - 100}$$

P: Corrected estimate of true prevalence

$P^T$: Test prevalence

The diagnostic sensitivity estimated from the naturally-infected buffaloes (n = 139) and the diagnostic specificity estimated from the Australian buffaloes (n = 263), reported in this chapter, were used in this calculation using either a 20 PP or 30 PP cut-off value.

### 6.2.4 Positive and negative predictive values

Positive predictive values (PPV) and negative predictive values (NPV) of the 2G6 Ag-ELISA and Tr7 Ag-ELISA were calculated for theoretical true prevalence (P)

values of 10%, 30%, 50%, 70% and 90%, by the following equations (Lessard, 1994):

$$PPV = \frac{P \times \text{sensitivity}}{P \times \text{sensitivity} + (100 - P) \times (100\text{-specificity})},$$

$$NPV = \frac{(100 - P) \times \text{specificity}}{(100 - P) \times \text{specificity} + P \times (100 - \text{sensitivity})}.$$

6.2.5 Agreement between the two *Trypanosoma evansi* antigen-ELISAs

Agreement between the 2G6 Ag-ELISA and Tr7 Ag-ELISA was calculated from the results of 1880 buffaloes sampled in Central Java, as described in Chapter 8, and that were tested by both Ag-ELISAs. Test agreement was estimated by calculation of the *kappa* statistic (Thrusfield, 1995) with associated 95% confidence intervals calculated using the computer software EPISCOPE (Frankena *et al.*, 1990). The interpretation of *kappa* values was as follows: 0-0.20: slight; 0.21-0.40: fair; 0.41-0.60: moderate; 0.61-0.80: substantial; and 0.81-1.0: almost perfect agreement (Landis and Koch, 1977).

6.2.6 Data analysis

Optical density (OD) values were transformed to PP values as described in Chapter 3. The mean of duplicate sample PP values was categorised as positive or negative using either a 20 PP or 30 PP cut-off value, except for PP results from the primary *T. evansi* infection for which a mean PP value twice the respective mean Day 0 PP value was considered positive, for reasons given in Chapter 4. Point estimates of diagnostic sensitivity were calculated as the number of test-positive animals divided by the total number of *T. evansi*-infected animals tested, and expressed as a percentage. Point estimates of diagnostic specificity were calculated as the number of test-negative animals divided by the total number of non-exposed animals tested, and expressed as a percentage.

The proportions of test-positive or test-negative sera found in different sample groups with the two Ag-ELISAs were calculated, with associated 95% confidence intervals, using the software CIA with the exact binomial method, or if given the Normal

approximation where nP and n(1- P) were both greater than a threshold level defined by the software. Differences between proportions of test-positive or test-negative sera and estimates of sensitivity and specificity obtained with the two Ag-ELISAs were calculated with 95% confidence intervals using CIA. Positive differences between proportions were considered significant at the 5% level if the lower limit of the 95% confidence interval was above zero. To examine the effect of parallel testing, the proportions of positive and negative results were calculated when the results of the two Ag-ELISAs were categorised as follows: 1) a buffalo was considered positive, if positive by either of the two Ag-ELISAs; and 2) a buffalo was considered negative, if negative by both Ag-ELISAs.

## 6.3 Results

### 6.3.1 Diagnostic sensitivity

*Indonesian buffaloes experimentally infected with the primary isolate* Trypanosoma evansi *Bakit 259 and secondary isolate* Trypanosoma evansi *Bakit 362*

The proportions of buffaloes in Group $259_{POS}$ (i.e., those not found parasitaemic prior to study) and Group $259_{PREPOS}$ (i.e., those parasitaemic prior to study) that were positive by 2G6 Ag-ELISA at different stages of the primary infection are shown in Table 6.1. The highest proportion of buffaloes found positive by the 2G6 Ag-ELISA was 44% of Group $259_{POS}$ at 42 and 63 days post-infection, and 67% of Group $259_{PREPOS}$ between 49 to 91 days post-infection. Of the 11 buffaloes ($Group_{NEG}$) not shown to be parasitaemic during the primary infection, only two (18%) were positive by 2G6 Ag-ELISA on any sampling date.

The proportions of buffaloes in Group $259_{POS}$ and Group $259_{PREPOS}$ that were positive by Tr7 Ag-ELISA at different stages of the primary infection are shown in Table 6.2. The highest proportion of buffaloes found positive by the Tr7 Ag-ELISA was 39% of Group $259_{POS}$ at 42 days post-infection and 83% of Group $259_{PREPOS}$ at 98 and 105 days post-infection. Of the 11 $Group_{NEG}$ buffaloes, only two (18%) were positive by the Tr7 Ag-ELISA on any sampling date, and on 12 sampling dates no buffaloes were positive.

In Group $259_{POS}$, the sensitivity estimates obtained for the 2G6 Ag-ELISA generally were higher than estimates for the Tr7 Ag-ELISA. In Group $259_{PREPOS}$, more buffaloes were positive on more sampling dates and from an earlier time post-infection by Tr7 Ag-ELISA (highest sensitivity = 83%) than with the 2G6 Ag-ELISA (highest sensitivity = 67%).

**Table 6.1** Estimates of diagnostic sensitivity (%) with associated 95% confidence intervals (CI) (in brackets) for 2G6 Ag-ELISA‡ calculated for different stages of the primary infection with *Trypanosoma evansi* Bakit 259 of 24 Indonesian buffaloes

| Number of days post-infection | Group $259_{POS}$ (n = 18) | | Group $259_{PREPOS}$ (n = 6) | |
|---|---|---|---|---|
| | Sensitivity (%) | 95% CI | Sensitivity (%) | 95% CI |
| 7 | 6 | (0, 27) | 0 | (0, 46) |
| 14 | 6 | (0, 27) | 0 | (0, 46) |
| 21 | 11 | (1, 35) | 0 | (0, 46) |
| 28 | 22 | (6, 48) | 17 | (0, 64) |
| 35 | 11 | (1, 35) | 17 | (0, 64) |
| 42 | 44 | (22, 69) | 33 | (4, 78) |
| 49 | 39 | (17, 64) | 67 | (22, 96) |
| 56 | 28 | (10, 54) | 67 | (22, 96) |
| 63 | 44 | (22, 69) | 50 | (12, 88) |
| 70 | 28 | (10, 54) | 50 | (12, 88) |
| 77 | 28 | (10, 54) | 67 | (22, 96) |
| 84 | 22 | (6, 48) | 67 | (22, 96) |
| 91 | 33 | (13, 59) | 67 | (22, 96) |
| 98 | 33 | (13, 59) | 50 | (12, 88) |
| 105 | 22 | (6, 48) | 33 | (4, 78) |

‡: PP values greater than twice the respective Day 0 PP value were considered positive

**Table 6.2** Estimates of diagnostic sensitivity (%) with associated 95% confidence intervals (CI) (in brackets) for Tr7 Ag-ELISA‡ calculated for different stages of the primary infection with *Trypanosoma evansi* Bakit 259 of 24 Indonesian buffaloes

| Number of days post-infection | Group $259_{POS}$ (n = 18) | | Group $259_{PREPOS}$ (n = 6) | |
|---|---|---|---|---|
| | Sensitivity (%) | 95% CI | Sensitivity (%) | 95% CI |
| 7 | 6 | (0, 27) | 17 | (0, 64) |
| 14 | 0 | (0, 19) | 33 | (4, 78) |
| 21 | 11 | (1, 35) | 33 | (4, 78) |
| 28 | 28 | (10, 54) | 33 | (4, 78) |
| 35 | 11 | (1, 35) | 50 | (12, 88) |
| 42 | 39 | (17, 64) | 67 | (22, 96) |
| 49 | 17 | (4, 41) | 67 | (22, 96) |
| 56 | 17 | (4, 41) | 67 | (22, 96) |
| 63 | 22 | (6, 48) | 67 | (22, 96) |
| 70 | 17 | (4, 41) | 67 | (22, 96) |
| 77 | 11 | (1, 35) | 67 | (22, 96) |
| 84 | 11 | (1, 35) | 67 | (22, 96) |
| 91 | 11 | (1, 35) | 67 | (22, 96) |
| 98 | 17 | (4, 41) | 83 | (36, 100) |
| 105 | 6 | (0, 27) | 83 | (36, 100) |

‡: PP values greater than twice the respective Day 0 PP value were considered positive

Estimates of sensitivity of the 2G6 Ag-ELISA and Tr7 Ag-ELISA calculated for different stages of the secondary infection are shown in Table 6.3. Using the cut-off criteria described in the Materials and Methods, 33% of buffaloes were positive by the 2G6 Ag-ELISA and 67% were positive by the Tr7 Ag-ELISA prior to the secondary infection, whereas no buffaloes were considered positive prior to the primary infection. On most sampling dates, more buffaloes were positive by the Tr7 Ag-ELISA than the 2G6 Ag-ELISA. The difference between the proportion of buffaloes that were positive by Tr7 Ag-ELISA and by 2G6 Ag-ELISA was 26% (1, 38) at 15 days post-infection and 17% (-10, 36) at 43 days post-infection. For both Ag-ELISAs, the highest sensitivity

**Table 6.3** Estimates of diagnostic sensitivity (%) with associated 95% confidence intervals (CI) (in brackets), for 2G6 Ag-ELISA and Tr7 Ag-ELISA[‡] calculated for different stages of the secondary infection with *Trypanosoma evansi* Bakit 362 of 30 Indonesian buffaloes

| Number of days from infection* | 2G6 Ag-ELISA | | Tr7 Ag-ELISA | |
|---|---|---|---|---|
| | Sensitivity (%) | 95% CI | Sensitivity (%) | 95 %CI |
| 1 | 33 | (17, 53) | 50 | (31, 69) |
| 8 | 53 | (34, 72) | 53 | (34, 72) |
| 15 | 57 | (37, 75) | 83 | (65, 94) |
| 22 | 47 | (28, 66) | 73 | (54, 88) |
| 29 | 57 | (37, 75) | 83 | (65, 94) |
| 36 | 60 | (41, 77) | 87 | (69, 96) |
| 43 | 53 | (34, 72) | 70 | (51, 85) |
| 50 | 60 | (36, 81) | 95 | (75, 100) |
| 57 | 65 | (41, 85) | 100 | (83, 100) |
| 64 | 70 | (46, 88) | 95 | (75, 100) |

*:n = 30 (1 day to 43 days post-infection) and n = 20 (50 days to 64 days post-infection).[‡]: PP values greater than the 20 PP cut-off value were considered positive.

estimates were obtained with the samples collected 57 to 64 days post-infection, but only 20 of the 30 buffaloes were monitored through this period, and therefore included in these estimations.

*Indonesian buffaloes naturally infected with* Trypanosoma evansi

The diagnostic sensitivities of the 2G6 Ag-ELISA and Tr7 Ag-ELISA were estimated for buffaloes shown to be naturally infected with *T. evansi* by either MHCT (Table 6.4) or MI (Table 6.5) or a combination of these tests (Table 6.6). The diagnostic sensitivity of the Tr7 Ag-ELISA was higher than the sensitivity of the 2G6 Ag-ELISA in all three groups. The difference between the proportion of buffaloes found positive by the two Ag-ELISAs, using a 20 PP cut-off value, was 13% (1, 25) with the MHCT-positive buffaloes, 3% (-9, 12) with the MI-positive buffaloes and 10% (1, 19) with buffaloes that were either MI-positive or MHCT-positive. Using the

higher cut-off value of 30 PP, the respective values for these three groups were 18% (3, 31), 10% (-8, 22) and 16% (4, 26).

**Table 6.4** Estimates of diagnostic sensitivity (%), with associated 95% confidence intervals (CI) (in brackets), for the 2G6 Ag-ELISA and Tr7 Ag-ELISA using sera from Indonesian buffaloes (n = 100) shown to be naturally-infected with *Trypanosoma evansi* by the microhaematocrit technique.

| Test | 20 PP cut-off | | 30 PP cut-off | |
|---|---|---|---|---|
| | Sensitivity (%) | 95% CI | Sensitivity (%) | 95% CI |
| 2G6 Ag-ELISA | 67 | (57, 76) | 51 | (41, 61) |
| Tr7 Ag-ELISA | 80 | (71, 87) | 69 | (59, 78) |

**Table 6.5** Estimates of diagnostic sensitivity (%), with associated 95% confidence intervals (CI) (in brackets), for the 2G6 Ag-ELISA and Tr7 Ag-ELISA using sera from Indonesian buffaloes (n = 39) shown to be naturally-infected with *Trypanosoma evansi* by mouse inoculation.

| Test | 20 PP cut-off | | 30 PP cut-off | |
|---|---|---|---|---|
| | Sensitivity (%) | 95% CI | Sensitivity (%) | 95% CI |
| 2G6 Ag-ELISA | 79 | (64, 91) | 67 | (50, 81) |
| Tr7 Ag-ELISA | 82 | (67, 93) | 77 | (61, 89) |

**Table 6.6** Estimates of diagnostic sensitivity (%), with associated 95% confidence intervals (CI) (in brackets), for the 2G6 Ag-ELISA and Tr7 Ag-ELISA using sera from Indonesian buffaloes (n = 139) shown to be naturally-infected with *Trypanosoma evansi* by either the microhaematocrit technique or mouse inoculation (combined results from Tables 6.5 and 6.6).

| Test | 20 PP cut-off | | 30 PP cut-off | |
|---|---|---|---|---|
| | Sensitivity (%) | 95% CI | Sensitivity (%) | 95% CI |
| 2G6 Ag-ELISA | 71 | (63, 79) | 55 | (46, 63) |
| Tr7 Ag-ELISA | 81 | (75, 88) | 71 | (64, 79) |

Using parallel testing with the 2G6 Ag-ELISA and Tr7 Ag-ELISA and a 20 PP cut-off value, 92% (85, 97) of MHCT-positive buffaloes, 87% (73, 96) of MI-positive buffaloes and 91% (85, 95) of all the parasitaemic buffaloes were found to be

antigenaemic. With a 30 PP cut-off value, 86% (78, 92) of MHCT-positive buffaloes, 85% (70, 94) of MI-positive buffaloes and 86% (80, 91) of all the parasitaemic buffaloes were positive.

### 6.3.2 Diagnostic specificity

*Australian buffaloes and cattle*

The diagnostic specificity estimates obtained for both Ag-ELISAs are shown in Table 6.7. The difference between the point estimates of specificity obtained with the two Ag-ELISAs was 3% (-4, 10) with the Australian buffaloes and 32% (24, 33) with the Australian cattle, using a 20 PP cut-off value. At the higher cut-off value, the differences were 4% (-2, 10) and 30% (22, 30), respectively. Of the 66 buffaloes that gave a false-positive result at the higher cut-off value, only 11 buffaloes were positive by both Ag-ELISAs.

**Table 6.7** Estimates of diagnostic specificity (%), with 95% confidence intervals (CI) (in brackets), for the 2G6 Ag-ELISA and Tr7 Ag-ELISA, using imported sera from Australian buffaloes (n = 263) and cattle (n = 80).

| Ag-ELISA | Species | 20 PP cut-off | | 30 PP cut-off | |
|---|---|---|---|---|---|
| | | Specificity (%) | 95% CI | Specificity (%) | 95% CI |
| 2G6 | Buffalo | 75 | (70, 80) | 83 | (79, 88) |
| Tr7 | Buffalo | 78 | (73, 83) | 87 | (83, 91) |
| 2G6 | Cattle | 68 | (56, 78) | 70 | (59, 80) |
| Tr7 | Cattle | 100 | (96, 100) | 100 | (96, 100) |

Figures 6.1 and 6.2 show the frequency distributions of PP values obtained from non-exposed Australian buffaloes and *T. evansi*-infected buffaloes (MHCT-positive or MI-positive) by the 2G6 Ag-ELISA and Tr7 Ag-ELISA, respectively. A proportion of MHCT-positive (8%) and MI-positive (13%) buffaloes had PP values below 20 PP in both ELISAs. While 75% (70, 80) of Australian buffaloes had PP values less than 30 PP by both Ag-ELISAs, some buffaloes had PP values as high as 100 PP which is equivalent to the PP of the high positive control (C++). Using a 20 PP cut-off value,

**Figure 6.1** Frequency distribution of 2G6 Ag-ELISA percent positivity (PP) values of negative Australian buffalo sera (n=263), sera from Indonesian buffaloes positive by mouse inoculation (n = 39) (MI) and by the microhaematocrit test (n = 100) (MHCT)

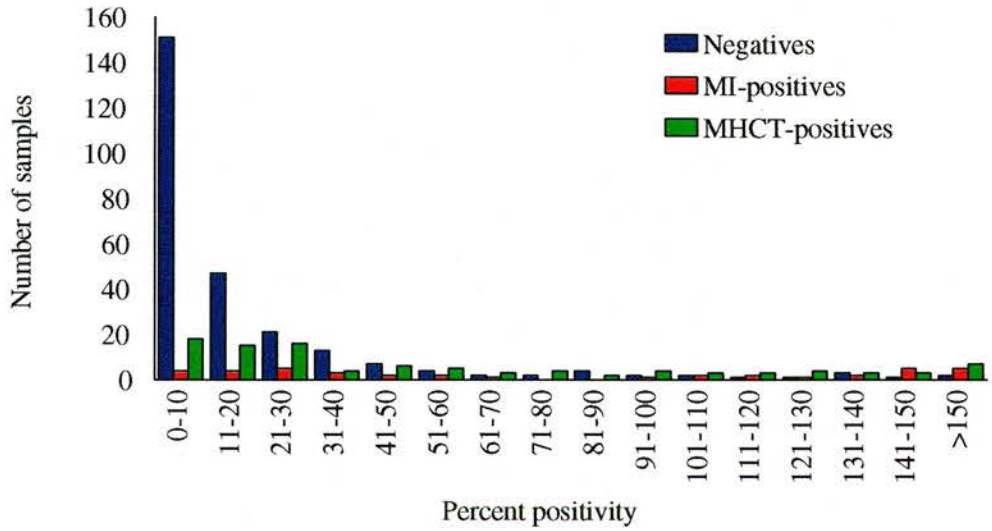
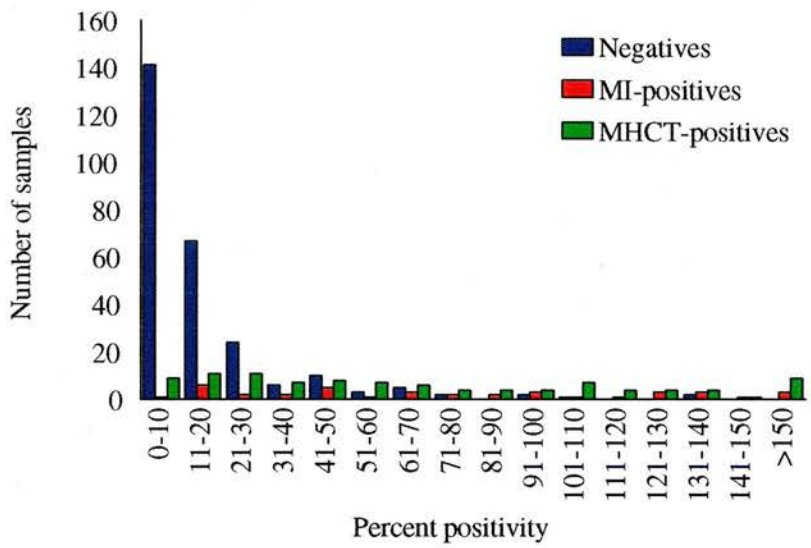



**Figure 6.2** Frequency distribution of Tr7 Ag-ELISA percent positivity (PP) values of negative Australian buffalo sera (n=263), sera from Indonesian buffaloes positive by mouse inoculation (n = 39) (MI) and by the microhaematocrit test (n = 100) (MHCT)



61% (55, 67) of Australian buffaloes were negative by both the Ag-ELISAs which is equivalent to the diagnostic specificity of these tests when used in parallel testing.

*Indonesian buffaloes naturally infected with* Trypanosoma theileri

Of 20 buffaloes shown to be infected with *T. theileri* and negative by *T. evansi* IgG ELISA and CATT, 75% (51, 91) were negative by 2G6 Ag-ELISA compared with only 35% (15, 59) by Tr7 Ag-ELISA, using the 20 PP cut-off value. The difference between these proportions of negative sera was 40% (-1, 64).

*British cattle experimentally infected with* Theileria *species or* Babesia *species*

Using a 30 PP cut-off value, only one serum sample from a *T. annulata*-infected calf and one serum sample from a *T. parva*-infected calf were positive by either the 2G6 Ag-ELISA or Tr7 Ag-ELISA (data shown in Table 6.8); these samples were not the same for the two Ag-ELISAs. Of the three *Babesia* spp.-positive serum samples, none was positive by 2G6 Ag-ELISA and only one *B. bovis*-positive serum sample was positive (42 PP) by Tr7 Ag-ELISA.

**Table 6.8** Percentage of sera from British cattle (n = 34) experimentally infected with *Theileria* species‡ that were negative by 2G6 Ag-ELISA and Tr7 Ag-ELISA, with associated 95% confidence intervals (CI) (in brackets)

| Test | 20 PP cut-off | | 30 PP cut-off | |
|---|---|---|---|---|
| | Negative (%) | 95% CI | Negative (%) | 95% CI |
| 2G6 Ag-ELISA | 94 | (81, 99) | 97 | (86, 100) |
| Tr7 Ag-ELISA | 94 | (81, 99) | 97 | (86, 100) |

‡:*Theileria annulata* (n = 21) and *T. parva* (n = 13)

*Peruvian cattle naturally exposed to* Anaplasma marginale *alone or with* Babesia bovis

Of 40 Peruvian cattle sera, by 2G6 Ag-ELISA 63% (46, 77) were negative using a 20 PP cut-off value and 70% (54, 83) were negative using a 30 PP cut-off value, and by Tr7 Ag-ELISA 80% (64, 91) and 93% (80, 98) were negative, using the same cut-off values, respectively. The difference between the proportion of negative sera was 17%

(-6, 36) at the lower cut-off value, and 23% (1, 34) at the higher cut-off value. All the sera that gave a PP value greater than 30 PP by either Ag-ELISA had previously been found to be positive for both *A. marginale* and *B. bovis*, except for two sera by 2G6 Ag-ELISA that had been found to be positive for *A. marginale* alone.

### 6.3.3 Estimation of true prevalence from test prevalence

True prevalence values calculated from a range of theoretical test prevalence values are shown in Table 6.9. Using a 20 PP cut-off value, a test prevalence of 50% gave the nearest point estimate of true prevalence by the 2G6 Ag-ELISA (P = 54%) and Tr7 Ag-ELISA (P = 47%). At the 30 PP cut-off value, the best point estimate of true prevalence was obtained when the test prevalence was 30%. At high test prevalence values, true prevalence was greater than test prevalence and at low test prevalence values, true prevalence was less than test prevalence. With test prevalence values of 10% and 90%, true prevalence values were outside the permissible range of 0 to 100%.

**Table 6.9** True prevalence values (P) calculated for a range of theoretical test prevalence values ($P^T$) using point estimates of sensitivity and specificity obtained for the two Ag-ELISAs using 20 PP and 30 PP cut-off values

| $P^T$ (%) | $P^‡$ (%) | | | |
|---|---|---|---|---|
| | 2G6 Ag-ELISA | | Tr7 Ag-ELISA | |
| | 20 PP | 30 PP | 20 PP | 30 PP |
| 10 | -33 | -18 | -20 | - 5 |
| 30 | 11 | 34 | 14 | 29 |
| 50 | 54 | 87 | 47 | 64 |
| 70 | 98 | 139 | 81 | 98 |
| 90 | 141 | 192 | 115 | 133 |

‡: Calculated using the following point estimates of diagnostic sensitivity:
20 PP cut-off value: 2G6 Ag-ELISA 71%; Tr7 Ag-ELISA 81%;
30 PP cut-off value: 2G6 Ag-ELISA 55%; Tr7 Ag-ELISA 71%;
and the following estimates of diagnostic specificity:
20 PP cut-off value: 2G6 Ag-ELISA 75%; Tr7 Ag-ELISA 78%;
30 PP cut-off value: 2G6 Ag-ELISA 83%; Tr7 Ag-ELISA 87%.

### 6.3.4 Positive and negative predictive values

The results in Table 6.10 show that as the value of true prevalence increased, the positive predictive value of both Ag-ELISAs increased whereas the negative predictive value decreased. Positive predictive values were higher with the higher cut-off value of 30 PP. The positive and negative predictive values calculated for the Tr7 Ag-ELISA were higher than corresponding values for the 2G6 Ag-ELISA. For example, with a prevalence of 50% and a 20 PP cut-off value, the positive predictive values of the 2G6 Ag-ELISA and Tr7 Ag-ELISA were 74% and 79%, respectively, and the corresponding negative predictive values were 72% and 80%. With prevalence values of 10%, the positive predictive values calculated were less than 50%.

**Table 6.10** Positive predictive values (PPV) and negative predictive values (NPV) calculated for a range of theoretical true prevalence values (P), using sensitivity and specificity estimates for the two Ag-ELISAs obtained using 20 PP and 30 PP cut-off values

| P (%) | 2G6 Ag-ELISA | | | | Tr7 Ag-ELISA | | | |
|---|---|---|---|---|---|---|---|---|
| | 20 PP | | 30 PP | | 20 PP | | 30 PP | |
| | PPV‡ | NPV‡ | PPV‡ | NPV‡ | PPV‡ | NPV‡ | PPV‡ | NPV‡ |
| 10 | 24 | 96 | 26 | 94 | 29 | 97 | 38 | 96 |
| 30 | 55 | 86 | 58 | 81 | 61 | 91 | 70 | 88 |
| 50 | 74 | 72 | 76 | 65 | 79 | 80 | 85 | 75 |
| 70 | 87 | 53 | 88 | 44 | 90 | 63 | 93 | 56 |
| 90 | 96 | 22 | 97 | 17 | 97 | 31 | 98 | 25 |

‡: Predictive values (%) were calculated using the following estimates of diagnostic sensitivity:
20 PP cut-off value: 2G6 Ag-ELISA 71%; Tr7 Ag-ELISA 81%;
30 PP cut-off value: 2G6 Ag-ELISA 55%; Tr7 Ag-ELISA 71%;
and the following estimates of diagnostic specificity:
20 PP cut-off value: 2G6 Ag-ELISA 75%; Tr7 Ag-ELISA 78%;
30 PP cut-off value: 2G6 Ag-ELISA 83%; Tr7 Ag-ELISA 87%.

### 6.3.5 Agreement between the two *Trypanosoma evansi* antigen-ELISAs

The *kappa* value, with a 95% confidence interval, was 0.20 (0.16, 0.24), indicating slight to fair agreement between the 2G6 Ag-ELISA and Tr7 Ag-ELISA.

## 6.4 Discussion

Diagnostic sensitivity and specificity, *kappa* and predictive values were estimated for the 2G6 Ag-ELISA and Tr7 Ag-ELISA. Diagnostic sensitivity was estimated using Indonesian buffaloes experimentally or naturally infected with *T. evansi*. The sensitivity of the Ag-ELISAs varied between different stages of the primary and secondary infections. The observed differences between sensitivity estimates may reflect underlying fluctuations in serum trypanosomal antigens that have been shown to occur in calves (data shown in Chapter 3) and buffaloes (data shown in Chapter 4) experimentally infected with *T. evansi*.

With the primary infection, the ranges of sensitivity found 7 to 105 days post-infection were 6% to 44% (2G6 Ag-ELISA) and 0% to 39% (Tr7 Ag-ELISA) with peak sensitivity at or after 42 days after infection. The sensitivities of both Ag-ELISAs were lower in the third month of the primary infection. With the secondary infection, the ranges of sensitivity found 8 to 64 days post-infection were 53% to 70% (2G6 Ag-ELISA) and 53% to 100% (Tr7 Ag-ELISA) with peak sensitivity also occurring after 42 days post-infection. No direct comparison could be made between the sensitivity estimates obtained with the primary and secondary infections because of the different cut-off criteria used (discussed in Chapter 4).

Using buffaloes shown to be naturally infected with *T. evansi* by MHCT or MI (n = 139), the sensitivity of the Tr7 Ag-ELISA was 81% (75, 88), significantly higher than the sensitivity of the 2G6 Ag-ELISA which was 71% (63, 79), using a 20 PP cut-off value. These estimates of sensitivity are comparable with the sensitivity of 74% reported for a *T. evansi* Ag-ELISA that was used to test naturally-infected MHCT-positive horses (Monzon *et al.*, 1995) and the sensitivity of 75% obtained for Ag-ELISAs used to detect tsetse-transmitted trypanosome infections in parasitaemic cattle (Bengaly *et al.*, 1995). In the present study, false-negative results were obtained with some MI-positive and MHCT-positive buffaloes by both Ag-ELISAs. As discussed in Chapter 4, other workers have suggested that false-negative Ag-ELISA results may be due to antigen-antibody complex formation (Nantulya and Lindqvist, 1989). However, this theory makes the assumptions that

sufficient antibodies are available to bind large amounts of circulating antigen and that dissociation of antigen and antibodies does not occur; such assumptions may not be valid. Furthermore, false-negative Ag-ELISA results could arise due to interference by other serum components, as previously reported with rheumatoid factor (Spencer, 1988), or due to operator error (e.g., dilution errors or mistaken identification of samples) when conducting the tests. However, at certain stages of *T. evansi* infections (e.g., early infection) it has been shown that antigens are not detectable in serum, even though animals are parasitaemic (as discussed in Chapter 4), and the combined application of parasitological tests and Ag-ELISAs has been advocated to ensure detection of early infections (Rae and Luckins, 1984; Bengaly *et al.*, 1995).

When used to test Australian buffalo sera (n = 263) with a 20 PP cut-off value, the specificity of the 2G6 Ag-ELISA was 75% (70, 80) and of the Tr7 Ag-ELISA was 78% (73, 83). These specificity estimates were similar to the preliminary estimates obtained from British cattle (described in Chapter 3) of 83% (78, 87) of the 2G6 Ag-ELISA and 78% (72, 82) for the Tr7 Ag-ELISA. However, when Australian cattle sera (n = 80) were tested, the Tr7 Ag-ELISA was significantly more specific (100% {96, 100}) than the 2G6 Ag-ELISA (68% {56, 78}). Australian buffaloes were considered to constitute a more representative negative population than British cattle because buffaloes were the target species for the application of the tests. The sample quality of the British cattle sera was superior to the Australian samples, which were of unknown status with regard to sample processing and storage conditions and some of which were haemolysed and/or contaminated. As expected, the specificity of both Ag-ELISAs was higher using the 30 PP cut-off value, but with a lower associated sensitivity underlining that the selection of optimal cut-off values usually involves a trade-off between these two diagnostic parameters (Smith, 1995).

Cross-reactions with *Babesia* species, *A. marginale* and *Theileria* species were not shown to occur commonly in the limited number of samples tested by Ag-ELISA, in agreement with the findings of previous workers (Nantulya *et al.*, 1987; Frame *et al.*, 1990) The high proportion of *T. theileri*-infected buffaloes that were

positive by the Tr7 Ag-ELISA (65%) needs be investigated further because this trypanosome is common in many countries. In a few samples, only one or two trypanosomes were observed in the microhaematocrit tube and were difficult to identify as *T. evansi* or *T. theileri* by their morphology and characteristic movement, and therefore it is possible that mis-classification of the trypanosomes occurred on some occasions. Previous workers did not report cross-reactions between *T. theileri* and a *T. brucei* Ag-ELISA (Delafosse *et al.*, 1995) that employed the same monoclonal antibody as the Tr7 Ag-ELISA.

The underlying cause of the false-positive results obtained is not known. As previously discussed in Chapter 3, the antigens detected by the 2G6 and Tr7 monoclonal antibodies of the Ag-ELISAs have not been fully characterised. The presence of homologous antigens from other organisms that are recognised by these monoclonal antibodies could produce false-positive reactions. Heterophilic antibodies, which have a broad specificity for immunoglobulins of other species, are also known to occur in some sera (Spencer, 1988). For example, anti-mouse antibodies could produce false-positive reactions by cross-linking between the Ag-ELISA monoclonal antibodies.

Using the sensitivity and specificity estimates of the two Ag-ELISAs obtained in this study, a theoretical test prevalence of 50% was a close estimate of true prevalence value using a 20 PP cut-off value, whereas a theoretical test prevalence of 30% was a close estimate of true prevalence using a 30 PP cut-off value. True prevalence was underestimated at higher test prevalence values (i.e., lack of sensitivity) and overestimated at lower test prevalence values (i.e., lack of specificity).

Using a 20 PP cut-off value, the ranges of positive predictive values calculated for theoretical true prevalence values of 10% to 90%, were 24% to 96% (2G6 Ag-ELISA) and 29% to 97% (Tr7 Ag-ELISA). Positive and negative predictive values of the Tr7 Ag-ELISA were higher than the corresponding 2G6 Ag-ELISA predictive values, and with both Ag-ELISAs these values were higher with a 30 PP cut-off value, particularly with low true prevalence values. With a true prevalence of

50% and a 20 PP cut-off value, the positive predictive values of the 2G6 Ag-ELISA and Tr7 Ag-ELISA were 74% and 79%, respectively. The usefulness of the Ag-ELISAs to detect *T. evansi*-infected buffaloes in areas with a low trypanosomosis prevalence, particularly at the level of the individual animal, therefore was questioned because the probability that a test-positive buffalo was truly *T. evansi*-infected was no greater than by chance alone, as indicated by a positive predictive value less than 50%.

Test agreement between the 2G6 Ag-ELISA and Tr7 Ag-ELISA was calculated because of the lack of a gold standard with a high sensitivity and specificity for the diagnosis of *T. evansi* infections. Only slight to fair agreement between the two Ag-ELISAs was found (*kappa* = 0.20 {0.16, 0.20}). However, *kappa* values are dependent both on both test agreement and the population of animals tested (Armitage and Berry, 1994). The sensitivity and specificity of two tests being compared will influence the concordance of test results, but even with a high level of concordance low *kappa* values may be obtained (Feinstein and Cicchetti, 1990). *Kappa* values are dependent on the prevalence of the factor of interest (e.g., *T. evansi* antigenaemia) and lower *kappa* values are associated with true prevalence values close to 0 or 100% (Thompson and Walter, 1988). The lack of observed agreement between the two Ag-ELISAs in this study may be due to differences in the detection of target antigens during *T. evansi* infections by these tests (as discussed in Chapter 4). The buffaloes sampled in Central Java would be expected to be heterogeneous in respect of their challenge by local strains of *T. evansi* (i.e., the number and duration of *T. evansi* infections). Furthermore, false-positive results were found in different buffaloes by the two Ag-ELISAs, thereby reducing test agreement, and to increase agreement the specificities of these Ag-ELISAs would have to be improved.

Diagnostic parameters were established for the 2G6 Ag-ELISA and Tr7 Ag-ELISA prior to their application in epidemiological studies in Indonesia. The two standardised *T. evansi* Ag-ELISAs were shown to have acceptable positive predictive values for testing buffaloes in areas with a trypanosomosis prevalence close to 50%.

However, the positive predictive values suggest that these tests would not be suitable for testing buffaloes in low prevalence areas (i.e., less than 30%). The evaluation of the 2G6 Ag-ELISA and Tr7 Ag-ELISA primarily involved their assessment as screening tests for the detection of *T. evansi* infections in buffaloes without clinical signs. A greater number of clinically-affected buffaloes would need to be included in future study populations to fully evaluate their use as clinical diagnostic tests (Smith, 1995). Furthermore, the results suggest that these Ag-ELISAs would be more useful as tests for groups of buffaloes than for individual buffaloes. Parallel testing using both the 2G6 Ag-ELISA and Tr7 Ag-ELISA improved sensitivity (91% {85, 95}), but gave unacceptably high numbers of false-positive results with a specificity of 61% (55, 67). Although there would be a reduction in sensitivity, serial testing may be a useful strategy to increase diagnostic specificity. These tests could be employed to test animals prior to import or export, particularly from low prevalence areas, when the identification of negative animals is important and a proportion of false-positive results may be acceptable. In addition, parallel testing with the MHCT and Ag-ELISA could be used to monitor buffaloes during outbreaks of trypanosomosis or naive buffaloes imported into endemic areas. A priority of future work should be the investigation of false-positive reactions and subsequent development of alternative Ag-ELISA systems with improved specificity.

# CHAPTER 7

# COMPARISON OF DIAGNOSTIC TESTS FOR *TRYPANOSOMA EVANSI* USING EXPERIMENTALLY-INFECTED AND NATURALLY-INFECTED BUFFALOES AND NON-EXPOSED CATTLE AND BUFFALOES

## 7.1 Introduction

Diagnostic tests have been used for many years for animal trypanosomoses and other animal diseases without proper validation. The application of trypanosomosis diagnostic tests in epidemiological studies without reference to test validity data has been commonly reported (e.g., Payne *et al.*, 1991c; Lun *et al.*, 1993; Singh *et al.*, 1995). In some studies, estimates of sensitivity and specificity had previously been established, but no indication of the precision of these estimates was given (e.g., Nantulya *et al.*, 1989b; Monzon *et al.*, 1995). Test validation is a prerequisite to the establishment and application of new diagnostic tests. However, when no test is available with a high diagnostic sensitivity and specificity that can be used as a gold standard (and therefore the true infection status of naturally-challenged animals is unknown), new tests are commonly compared with existing tests and the concordance of results measured in terms of relative sensitivity and specificity and test agreement quantified by calculation of *kappa* values (Smith, 1995).

Parasitological tests are limited by their inability to detect trypanosomes in animals with low levels of parasitaemia, especially in the chronic stages of infection (Luckins, 1992). To improve the detection of *T. evansi* infections, immunological tests have been developed that include the complement fixation test (CFT) (Caporale *et al.*, 1981), haemagglutination test (Jatkar and Singh, 1971), immunofluorescence antibody test (IFAT) (Luckins and Mehlitz, 1978; Katende *et al.*, 1987), antibody-detection ELISAs (Luckins, 1977; Rae *et al.*, 1989), and more recently a card agglutination test (CATT) (Bajyana Songa *et al.*, 1988). Moreover, several of these tests such as the IFAT, CFT and ELISA, can be used to test large numbers of samples at relatively low cost, and therefore have been widely used in serological surveys (Luckins, 1983; Payne *et al.*, 1991c; Shen *et al.*, 1985).

The major disadvantages of antibody-detection tests are the low level of detectable antibody in serum in the first few weeks of infection and the persistence of antibody responses for several weeks or months after effective chemotherapy. For example, Zebu cattle experimentally infected with *T. congolense* or *T. vivax* had elevated antibody responses 83 days after trypanocidal treatment (Luckins, 1977). Because of these limitations, Ag-ELISAs have been developed in an attempt to detect earlier trypanosome infections and to better differentiate between current infections and previous exposure (Nantulya *et al.,* 1989a; Nantulya and Lindqvist, 1989).

The diagnostic sensitivity and specificity of the two Ag-ELISAs have been estimated using both experimentally-infected and naturally-infected buffaloes and non-exposed buffaloes, respectively (see Chapter 6). Here, the diagnostic sensitivity and specificity of *T. evansi* IgM-specific and IgG-specific antibody-detection ELISAs and the CATT were estimated and compared with the Ag-ELISA parameters. The diagnostic parameters obtained for the three antibody-detection tests were used to calculate true prevalence for a range of theoretical test prevalence values and positive and negative predictive values. Agreement between the two Ag-ELISAs and the three antibody-detection tests was determined by calculation of *kappa* values.

## 7.2 Materials and methods

The *T. evansi* IgM ELISA, IgG ELISA and CATT described in Appendix I were used to test all the sera in the studies described below which were stored at -20°C prior to testing at Balitvet.

### 7.2.1 Diagnostic sensitivity

*Indonesian buffaloes experimentally infected with the primary isolate* Trypanosoma evansi *Bakit 259 and the secondary isolate* Trypanosoma evansi *Bakit 362*

Weekly blood samples were taken during the primary infection up to 105 days post-infection and during the secondary infection up to 45 days post-infection; 20 buffaloes that were the first group to be infected were monitored up to 64 days post-infection, as described in Chapter 4. Estimates of sensitivity were calculated for each week post-infection using the buffaloes shown to be infected with *T. evansi* in

the primary infection (Group $259_{POS}$: n = 18; Group $259_{PREPOS}$: n = 6) and secondary infection (n = 30).

*Indonesian buffaloes naturally infected with* Trypanosoma evansi

During the field work described in Chapter 8, buffaloes were found to be naturally-infected with *T. evansi* by MHCT (n = 39) and by MI (n = 100). All the sera from these buffaloes (n = 139) were tested by IgM ELISA, IgG ELISA and CATT, and estimates of sensitivity calculated.

### 7.2.2 Diagnostic specificity

*Australian buffaloes*

Sera from 114 buffaloes living in semi-feral conditions in the Northern Territories, Australia, were obtained from Michael Pearce, formerly of INI ANSREDEF, Bogor. All the sera from these buffaloes (n = 139) were tested by IgM ELISA, IgG ELISA and CATT, and estimates of specificity were calculated with associated 95% confidence intervals.

### 7.2.3 Estimation of true prevalence from test prevalence

Corrected estimates of true prevalence, P, were calculated for theoretical test prevalence values ($P^T$) of 10%, 30%, 50%, 70% and 90% using the calculation given in Chapter 6. The diagnostic sensitivities estimated for the buffaloes shown to be naturally-infected with *T. evansi* by either MHCT or MI (n = 139) and the diagnostic specificities estimated from Australian buffaloes (n = 263) were used, with cut-off values of 30 PP and 20 PP for the IgM ELISA and IgG ELISA, respectively.

### 7.2.4 Positive and negative predictive values

The positive predictive value (PPV) and negative predictive value (NPV) of the IgM ELISA, IgG ELISA and CATT were calculated for theoretical true prevalence (P) values of 10%, 30%, 50%, 70% and 90% using the method described in Chapter 6.

### 7.2.5 Agreement between *Trypanosoma evansi* antigen-detection tests and antibody-detection tests

To evaluate test agreement, 121 sera that had been collected from buffaloes in four villages in Central Java (Ponowareng, Wonosegoro, Wonokromo and Harjosari) were tested by 2G6 Ag-ELISA, Tr7 Ag-ELISA, IgM ELISA, IgG ELISA and CATT (the IgM ELISA was not included). These villages (see Chapter 8) were selected because a high prevalence was found in two of the villages (Ponowareng and Wonosegoro) and a low prevalence in the other two villages (Wonokromo and Harjosari), by the 2G6 Ag-ELISA.

Test agreement between each Ag-ELISA and the IgG ELISA and CATT was quantified by calculation of *kappa* values with associated 95% confidence intervals using the same method and interpretation described in Chapter 6. Overall agreement between the 2G6 Ag-ELISA, Tr7 Ag-ELISA, IgG ELISA and CATT was estimated using a method for multiple tests (Fleiss, 1981). The standard error of the multiple test *kappa* estimate was derived to test the hypothesis that the underlying value of *kappa* was zero by calculation of the associated $z$ value.

### 7.2.6 Data analysis

The OD values obtained with the IgM ELISA and IgG ELISA were transformed into PP values as described in Chapter 3. The mean of duplicate sample PP values was categorised as positive or negative using either a 20 PP (IgG ELISA) or 30 PP (IgM ELISA) cut-off value, except for PP results from the primary *T. evansi* infection for which a mean PP value twice the respective Day 0 mean PP value was considered positive (see Chapter 4). The CATT supplier's recommended interpretation of results was followed as described in Appendix 1. In the primary infection, all buffaloes that were negative by CATT on Day 0 and positive on the sampling date (score 1, 2 or 3) were included in the analysis, as well as buffaloes that were weakly positive (score 1) on Day 0 and were scored either 2 or 3 on the sampling date. Diagnostic specificities and sensitivities were calculated, with 95% confidence intervals, as described in Chapters 3 and 6, respectively.

The proportions of positive or negative sera found in different buffalo groups with the different tests were calculated, with associated 95% confidence intervals, using the software CIA with the exact binomial method, or if given the Normal approximation where nP and n(1- P) were both greater than a threshold level defined by the software. Differences between the proportions of test-positive or test-negative sera obtained by the different tests including differences between point estimates of sensitivity and specificity, were calculated with 95% confidence intervals using CIA. Positive differences between proportions were considered significant at the 5% level, if the lower limit of the 95% confidence interval was above zero.

## 7.3 Results

### 7.3.1 Diagnostic sensitivity

*Indonesian buffaloes experimentally infected with the primary isolate* Trypanosoma evansi *Bakit 259 and the secondary isolate* Trypanosoma evansi *Bakit 362*

The proportions of buffaloes in Group $259_{POS}$ (not found parasitaemic prior to study) and Group $259_{PREPOS}$ (parasitaemic prior to study) that were positive by IgM ELISA, IgG ELISA and CATT at different times after experimental infection with the primary *T. evansi* isolate Bakit 259 are shown in Tables 7.1, 7.2 and 7.3, respectively. The highest proportion of buffaloes found positive by IgM ELISA was 89% in Group $259_{POS}$ 49 days post-infection and 67% in Group $259_{PREPOS}$ at 42, 49 and 70 days post-infection. The highest proportion of buffaloes were found positive in Group $259_{POS}$ by IgG ELISA and CATT from 70 days post-infection (56% positive) and at 56 days post-infection (61% positive), respectively, and in Group $259_{PREPOS}$ at 84 days (50% positive) and from 35 days post-infection (33% positive), respectively. Of the 11 buffaloes not shown to be parasitaemic during the primary infection ($Group_{NEG}$), only four buffaloes (36%) were positive by IgM ELISA, one buffalo (9%) was positive by IgG ELISA and three buffaloes (27%) were positive by CATT on any sampling date.

With the primary infection, the sensitivity of the IgM ELISA was higher than the sensitivities of the IgG ELISA and CATT on most sampling days. The IgM ELISA had a sensitivity of 50% on Day 7 post-infection, whereas the sensitivity of the IgG

ELISA was higher in the later stages of infection. The sensitivity of the CATT was also higher later in infection, but unlike the IgG ELISA declined after 56 days post-infection. More buffaloes had low PP values by IgM ELISA on Day 0 (18/18 in Group $259_{POS}$; 5/6 in $259_{PREPOS}$) than with the IgG ELISA (10/18 in Group $259_{POS}$; 1/6 in $259_{PREPOS}$) or the CATT (15/18 in Group $259_{POS}$; 4/6 in $259_{PREPOS}$).

**Table 7.1** Estimates of diagnostic sensitivity with associated 95% confidence intervals (CI) (in brackets) for the IgM ELISA‡ at different stages of the primary infection of 24 Indonesian buffaloes with *Trypanosoma evansi* Bakit 259.

| Number of days post-infection | Group $259_{POS}$ (n = 18) | | Group $259_{PREPOS}$ (n = 6) | |
|---|---|---|---|---|
| | Sensitivity (%) | 95% CI | Sensitivity (%) | 95% CI |
| 7 | 50 | (26, 74) | 17 | (0, 64) |
| 14 | 66 | (41, 87) | 17 | (0, 64) |
| 21 | 50 | (26, 74) | 17 | (0, 64) |
| 28 | 33 | (13, 59) | 50 | (12, 88) |
| 35 | 56 | (31, 79) | 50 | (12, 88) |
| 42 | 72 | (47, 90) | 67 | (22, 96) |
| 49 | 89 | (65, 99) | 67 | (22, 96) |
| 56 | 50 | (26, 74) | 33 | (4, 78) |
| 63 | 33 | (13, 59) | 33 | (4, 78) |
| 70 | 56 | (31, 79) | 67 | (22, 96) |
| 77 | 56 | (31, 79) | 50 | (12, 88) |
| 84 | 28 | (10, 54) | 50 | (12, 88) |
| 91 | 33 | (13, 59) | 50 | (12, 88) |
| 98 | 28 | (10, 54) | 33 | (4, 78) |

‡: PP values greater than twice the respective Day 0 PP value were considered positive.

Estimates of diagnostic sensitivity of the IgM ELISA, IgG ELISA and CATT were calculated for different stages of the secondary *T. evansi* infection and are shown in Table 7.4. The sensitivities of both the IgG ELISA and CATT were 83% at 15 days post-infection. The highest estimates of sensitivity were found at 50 days

post-infection by IgM ELISA (90%), from 50 to 64 days post-infection by IgG ELISA (95%) and 15 to 29 days post-infection by CATT (83%).

**Table 7.2** Estimates of diagnostic sensitivity with associated 95% confidence intervals (CI) (in brackets) for the IgG ELISA‡ at different stages of the primary infection of 24 Indonesian buffaloes with *Trypanosoma evansi* Bakit 259.

| Number of days post-infection | Group $259_{POS}$ (n = 18) | | Group $259_{PREPOS}$ (n = 6) | |
|---|---|---|---|---|
| | Sensitivity (%) | 95% CI | Sensitivity (%) | 95% CI |
| 7 | 22 | (6, 48) | 17 | (0, 64) |
| 14 | 11 | (1, 35) | 17 | (0, 64) |
| 21 | 17 | (4, 41) | 0 | (0, 46) |
| 28 | 22 | (6, 48) | 17 | (0, 64) |
| 35 | 17 | (4, 41) | 17 | (0, 64) |
| 42 | 39 | (17, 64) | 33 | (4, 78) |
| 49 | 44 | (22, 69) | 33 | (4, 78) |
| 56 | 44 | (22, 69) | 33 | (4, 78) |
| 63 | 50 | (26, 74) | 33 | (4, 78) |
| 70 | 56 | (31, 79) | 17 | (0, 64) |
| 77 | 56 | (31, 79) | 33 | (4, 78) |
| 84 | 50 | (26, 74) | 50 | (12, 88) |
| 91 | 56 | (31, 79) | 33 | (4, 78) |
| 98 | 50 | (26, 74) | 33 | (4, 78) |

‡: PP values greater than twice the respective Day 0 PP value were considered positive.

Using the different cut-off criteria for the primary and secondary infections described earlier, different proportions of buffaloes were positive by the IgM ELISA (17%), IgG ELISA (63%) or the CATT (17%) prior to the secondary infection, whereas no buffaloes were considered positive prior to the primary infection.

*Indonesian buffaloes naturally infected with* Trypanosoma evansi

The diagnostic sensitivities of the IgM ELISA, IgG ELISA and CATT were estimated from buffaloes shown to be naturally infected with *T. evansi* by either MHCT (Table 7.5), MI (Table 7.6) or a combination of these tests (Table 7.7). For these buffalo groups, the sensitivity estimates of the CATT were 79% (70, 87), 77% (61, 89) and 78% (72, 85), respectively. The sensitivities of the two antibody-detection ELISAs were compared using a 20 PP cut-off value for the IgG ELISA and a 30 PP cut-off value for the IgM ELISA; the higher cut-off value was chosen for the IgM ELISA because of the low specificity (55%) obtained for this assay with the lower cut-off value (data given below). The sensitivity of the IgG ELISA was significantly higher than either the sensitivity of the IgM ELISA or of the CATT when estimated using the MHCT-positive buffaloes; the differences between sensitivities were 15% (6, 20) and 11% (1, 18), respectively. There was no difference between the sensitivities of the IgM ELISA and IgG ELISA and a difference of 10% (-6, 19) between the IgG ELISA and CATT sensitivities when estimated using the MI-positive buffaloes. When the MHCT-positive and MI-positive results were combined the IgG ELISA had the highest sensitivity (89%), and the differences between this and the sensitivities of the IgM ELISA and the CATT were 10% (4, 18) and 11% (3, 18), respectively.

**Table 7.3** Estimates of diagnostic sensitivity with associated 95% confidence intervals (CI) (in brackets) for the CATT[‡] at different stages of the primary infection of 24 Indonesian buffaloes with *Trypanosoma evansi* Bakit 259.

| Number of days post-infection | Group $259_{POS}$ (n = 18) | | Group $259_{PREPOS}$ (n = 6) | |
|---|---|---|---|---|
| | Sensitivity (%) | 95% CI | Sensitivity (%) | 95% CI |
| 7 | 22 | (6, 48) | 0 | (0, 46) |
| 14 | 11 | (1, 35) | 0 | (0, 46) |
| 21 | 22 | (6, 48) | 17 | (0, 64) |
| 28 | 28 | (10, 54) | 17 | (0, 64) |
| 35 | 44 | (22, 69) | 33 | (4, 78) |
| 42 | 56 | (31, 79) | 33 | (4, 78) |
| 49 | 56 | (31, 79) | 33 | (4, 78) |
| 56 | 61 | (36, 83) | 33 | (4, 78) |
| 63 | 44 | (22, 69) | 33 | (4, 78) |
| 70 | 39 | (17, 64) | 33 | (4, 78) |
| 77 | 28 | (10, 54) | 33 | (4, 78) |
| 84 | 11 | (1, 35) | 17 | (0, 64) |
| 91 | nd | | nd | |
| 98 | nd | | nd | |

nd: Not done; [‡]: Only a positive test in samples that were negative or weakly positive on Day 0 was classified as positive during infection, as described in the text.

**Table 7.4** Estimates of diagnostic sensitivity with associated 95% confidence intervals (CI) (in brackets) for the IgM ELISA, IgG ELISA and CATT‡ at different stages of the secondary infection of 30 Indonesian buffaloes with *Trypanosoma evansi* Bakit 362.

| Days from infection* | IgM ELISA | | IgG ELISA | | CATT | |
|---|---|---|---|---|---|---|
| | Sensitivity (%) | 95% CI | Sensitivity (%) | 95% CI | Sensitivity (%) | 95% CI |
| 1 | 10 | (2, 27) | 47 | (28, 66) | 10 | (2, 27) |
| 8 | 40 | (23, 59) | 63 | (44, 80) | 57 | (37, 75) |
| 15 | 60 | (41, 77) | 83 | (65, 94) | 83 | (65, 94) |
| 22 | 40 | (23, 59) | 83 | (65, 94) | 83 | (65, 94) |
| 29 | 50 | (31, 69) | 87 | (69, 96) | 83 | (65, 94) |
| 36 | 67 | (47, 83) | 87 | (69, 96) | 80 | (61, 92) |
| 43 | 63 | (44, 80) | 87 | (69, 96) | 73 | (54, 88) |
| 50 | 90 | (68, 99) | 95 | (75, 100) | 80 | (56, 94) |
| 57 | 80 | (56, 94) | 95 | (75, 100) | 80 | (56, 94) |
| 64 | 75 | (51, 91) | 95 | (75, 100) | 75 | (51, 91) |

*: n = 30 (1 day to 43 days post-infection) and n = 20 (50 days to 64 days post-infection);
‡: PP values greater than 30 PP (IgM ELISA) and 20 PP (IgG ELISA) and all positive CATT results were considered positive.

**Table 7.5** Estimates of diagnostic sensitivity, with associated 95% confidence intervals (CI) (in brackets), for the IgM ELISA and IgG ELISA using sera from Indonesian buffaloes (n = 100) shown to be naturally-infected with *Trypanosoma evansi* by the microhaematocrit technique.

| Test | 20 PP cut-off | | 30 PP cut-off | |
|---|---|---|---|---|
| | Sensitivity (%) | 95% CI | Sensitivity (%) | 95% CI |
| IgM ELISA | 86 | (78, 92) | 75 | (65, 83) |
| IgG ELISA | 90 | (82, 95) | 85 | (77, 91) |

**Table 7.6** Estimates of diagnostic sensitivity, with associated 95% confidence intervals (CI) (in brackets), for the IgM ELISA and IgG ELISA using sera from Indonesian buffaloes (n = 39) shown to be naturally-infected with *Trypanosoma evansi* by mouse inoculation.

| Test | 20 PP cut-off | | 30 PP cut-off | |
|---|---|---|---|---|
| | Sensitivity (%) | 95% CI | Sensitivity (%) | 95% CI |
| IgM ELISA | 92 | (79, 98) | 87 | (73, 96) |
| IgG ELISA | 87 | (73, 96) | 77 | (61, 89) |

**Table 7.7** Estimates of diagnostic sensitivity, with associated 95% confidence intervals (CI) (in brackets), for the IgM ELISA and IgG ELISA using sera from Indonesian buffaloes (n = 139) shown to be naturally-infected with *Trypanosoma evansi* by either the microhaematocrit technique or mouse inoculation (Table 7.5 and Table 7.6 results combined).

| Test | 20 PP cut-off | | 30 PP cut-off | |
|---|---|---|---|---|
| | Sensitivity (%) | 95% CI | Sensitivity (%) | 95% CI |
| IgM ELISA | 88 | (82, 93) | 78 | (72, 85) |
| IgG ELISA | 89 | (84, 94) | 83 | (77, 89) |

### 7.3.2 Diagnostic specificity

*Australian buffaloes*

The diagnostic specificity estimates of the IgM and IgG ELISA are shown in Table 7.8; the specificity of the CATT was 100% (97, 100). The difference between the specificities of the IgM ELISA and IgG ELISA was 4% (-5, 12) and between the IgG ELISA and CATT was 8% (3, 8). If a 20 PP cut-off value was also used for the IgM ELISA, the difference between the specificities of the two antibody-ELISAs was 37% (26, 48).

### 7.3.3 Estimation of true prevalence from test prevalence

True prevalence values were calculated from a range of theoretical test prevalence values and are shown in Table 7.9. A test prevalence value of 30% gave the closest estimate of true prevalence for the IgM ELISA (P = 28%) and IgG ELISA

(P = 27%). At test prevalence values equal to and above 50% by both the IgM ELISA and IgG ELISA and equal to and above 30% by the CATT, true prevalence values were higher than test prevalence values. Calculated true prevalence values were outside the permissible range of 0 to 100% with a 10% test prevalence by IgM ELISA and a 90% test prevalence by all three tests.

**Table 7.8** Estimates of diagnostic specificity, with 95% confidence intervals (CI) (in brackets), for the IgM ELISA and IgG ELISA, using imported Australian buffalo sera (n = 114)

| ELISA | 20 PP cut-off | | 30 PP cut-off | |
|---|---|---|---|---|
| | Specificity (%) | 95% CI | Specificity (%) | 95% CI |
| IgM | 55 | (46, 64) | 89 | (83, 94) |
| IgG | 92 | (86, 96) | 98 | (94, 100) |

**Table 7.9** True prevalence values (P) calculated for a range of theoretical test prevalence values ($P^T$) using point estimates of sensitivity and specificity obtained for three *Trypanosoma evansi* antibody-detection tests

| $P^T$ (%) | $P^‡$ (%) | | |
|---|---|---|---|
| | IgM ELISA* | IgG ELISA** | CATT |
| 10 | -1 | 2 | 13 |
| 30 | 28 | 27 | 38 |
| 50 | 58 | 67 | 64 |
| 70 | 88 | 77 | 90 |
| 90 | 118 | 101 | 115 |

*: Using a 30 PP cut-off value; **: Using a 20 PP cut-off value;
‡: Calculated using the following values of diagnostic sensitivity and specificity, respectively: IgM ELISA: 78%, 89%; IgG ELISA: 89%, 92%; CATT: 78%, 100%.

### 7.3.4 Positive and negative predictive values

The results in Table 7.10 show that as true prevalence values increase positive predictive values of the IgM ELISA and IgG ELISA increase, whereas negative predictive values decrease. With a true prevalence of 50%, the positive predictive values of the IgM ELISA, IgG ELISA and CATT were 88%, 92% and 100%, respectively. With true prevalence values between 10% and 90%, the CATT had the highest positive predictive value (100%) and the IgG ELISA had higher negative predictive values than the other tests.

**Table 7.10** Positive predictive values (PPV) and negative predictive values (NPV) calculated for various theoretical prevalence values (P) using sensitivity and specificity estimates obtained for three *Trypanosoma evansi* antibody-detection tests

| P (%) | IgM ELISA* | | IgG ELISA** | | CATT | |
|---|---|---|---|---|---|---|
| | PPV (%)‡ | NPV (%)‡ | PPV (%)‡ | NPV (%)‡ | PPV (%)‡ | NPV (%)‡ |
| 10 | 44 | 97 | 55 | 99 | 100 | 98 |
| 30 | 75 | 90 | 83 | 95 | 100 | 91 |
| 50 | 88 | 80 | 92 | 89 | 100 | 82 |
| 70 | 94 | 63 | 96 | 78 | 100 | 66 |
| 90 | 98 | 31 | 99 | 48 | 100 | 34 |

*: Using a 30 PP cut-off value; **: Using a 20 PP cut-off value;
‡:Calculated using the following values of diagnostic sensitivity and specificity, respectively: IgM ELISA: 78%, 89%; IgG ELISA: 89%, 92%; CATT: 78%, 100%.

### 7.3.5 Agreement between the *Trypanosoma evansi* antigen-detection tests and antibody-detection tests

*Kappa* values were calculated with 95% confidence intervals for the IgM ELISA, IgG ELISA and CATT using *T. evansi*-infected and non-exposed buffaloes sampled in four selected villages in Central Java, and are shown in Table 7.11. There was moderate agreement between the IgG ELISA and CATT ($k$ = 0.58) and fair agreement between the 2G6 Ag-ELISA and Tr7 Ag-ELISA ($k$ = 0.29) and between the Tr7 Ag-ELISA and CATT ($k$ = 0.26). Only slight agreement was found between the 2G6 Ag-ELISA and either the IgG ELISA or CATT or between the Tr7 Ag-ELISA and IgG ELISA. There was fair agreement between the five tests; the *kappa* value (0.27) for multiple tests was significantly greater than zero ($P < 0.001$).

**Table 7.11** *Kappa* values and associated 95% confidence intervals (CI) (in brackets) calculated for serum samples (n = 121) collected from buffaloes in four villages in Central Java and tested by different *Trypanosoma evansi* diagnostic tests

| Tests | *kappa* | CI |
|---|---|---|
| 2G6 vs. Tr7 | 0.29 | (0.11, 0.47) |
| IgG vs. CATT | 0.58 | (0.43, 0.74) |
| 2G6 vs. IgG | 0.17 | (-0.03, 0.38) |
| 2G6 vs. CATT | 0.19 | (0.02, 0.37) |
| Tr7 vs. IgG | 0.19 | (0.02, 0.37) |
| Tr7 vs. CATT | 0.26 | (0.07, 0.45) |
| All tests* | 0.27 | (SE ± 0.04) |

*: Using a method for multiple ratings per subject (Fleiss 1981); the standard error (SE) is given because the assumption of Normality was not made for a 95% CI to be included.

## 7.4 Discussion

Antibody-detection ELISAs have been used for many years for the diagnosis of trypanosome infections (Luckins, 1977), whereas Ag-ELISAs (Nantulya *et al.*, 1987; Frame *et al.*, 1990) and the CATT (Bajyana Songa *et al.*, 1987b) have been developed more recently. Diagnostic sensitivity and specificity, *kappa* and predictive values were estimated for the *T. evansi* IgM ELISA, IgG ELISA and CATT employed in this study, and compared with estimates obtained for the 2G6 Ag-ELISA and Tr7 Ag-ELISA given in Chapter 6.

Diagnostic sensitivities of the antibody-detection tests were estimated using Indonesian buffaloes either experimentally or naturally-infected with *T. evansi*. As found with the 2G6 Ag-ELISA and Tr7 Ag-ELISA (presented in Chapter 6), different point estimates of diagnostic sensitivity were obtained for the three antibody-detection tests at different stages of the experimental infections and were higher at or after 42 days post-infection. With buffaloes shown to be parasitaemic (Group $259_{POS}$) with the primary infection, the highest sensitivity of the IgG ELISA was found later during the infection (70 days) than the highest sensitivity estimate of the IgM ELISA (49 days) or CATT (56 days). Lower estimates of sensitivity were obtained for the IgM ELISA

and CATT in the later stages of infection, as with the Ag-ELISAs, which may reflect declining antibody responses. Lower IgM responses and weaker agglutination with the CATT were observed in the later stages of infection in the experimentally-infected calves (Chapter 3) and buffaloes (Chapter 4). The higher sensitivity of the IgM ELISA in the earlier stages of infection was expected because IgM responses precede IgG responses in trypanosome-infected cattle (Luckins, 1976a; Luckins, 1976b). The CATT also detects antibodies that appear early in infection, but which are predominantly IgG-specific antibodies against variable surface glycoproteins (Bajyana *et al.*, 1987b).

With the secondary *T. evansi* infection, the estimate of sensitivity of the CATT (83%) was significantly higher than the estimate of the IgM ELISA (60%) at 15 days post-infection. At this stage of infection, the sensitivity of the IgG ELISA (83%) was also high, but 63% of the buffaloes were positive by IgG ELISA before infection compared with only 17% by either IgM ELISA or CATT. The highest estimates of sensitivity for the IgM ELISA and IgG ELISA were obtained 50 to 64 days after infection compared with 15 to 29 days for the CATT. No comparisons were made between the sensitivity estimates obtained with the primary and secondary infections because of the different cut-off criteria used.

The observed differences between the point estimates of sensitivity obtained at different stages of the experimental *T. evansi* infections probably reflect fluctuations in serum antibodies in individual buffaloes which previously have been shown to occur in calves (data shown in Chapter 3) and buffaloes (data shown in Chapter 4) infected experimentally with *T. evansi*. In the experimentally-infected calves, IgG antibody responses increased more slowly than IgM antibody responses, but remained high for a longer period.

The experimentally-infected buffaloes were challenged with two isolates of *T. evansi* on separate occasions. By contrast, buffaloes naturally infected with *T. evansi* may be challenged on multiple occasions by one or more different strains of *T. evansi*. Therefore, the sensitivities of these tests were also estimated using 139 buffaloes from

Central Java shown to be naturally infected with *T. evansi* either by MHCT or MI. The sensitivity estimate obtained for the IgG ELISA (89% {84, 94}) was significantly higher than the estimates of the IgM ELISA (78% {72, 85}) or CATT (78% {72, 85}). By comparison, the estimates of sensitivity obtained with these buffaloes were 71% (63, 79) for the 2G6 Ag-ELISA and 81% (75, 88) for the Tr7 Ag-ELISA.

It was not possible to identify a population of buffaloes in Indonesia that had not been exposed to *T. evansi*, and therefore diagnostic specificity was estimated using Australian buffaloes. The specificity of the CATT (100% {97, 100}) was significantly higher than the specificity of the IgM ELISA (89% {83, 94}) or IgG ELISA (92% {86, 96}), using 30 PP and 20 PP ELISA cut-off values, respectively. If a 20 PP cut-off value was used for both antibody-ELISAs, the difference between their specificity estimates was 37% (26, 48), and for this reason the higher cut-off value of 30 PP was chosen for the IgM ELISA. The specificities estimated for the 2G6 Ag-ELISA (75% {70, 80}) and Tr7 Ag-ELISA (78% {73, 83}) were intermediate between the IgM ELISA and the IgG ELISA specificities obtained.

Underlying causes of the false-positive results observed with these antibody-detection tests is not known. Non-specific binding of assay components to the ELISA plate can occur, but the inclusion of various blockers did not prevent false positive reactions (data given in Chapter 3), suggesting that this was not the main source of these reactions. Furthermore, the detergent Tween 20 was included in the ELISA diluent buffers as a liquid phase blocker. Non-specific binding to ELISA reactants can result from non-immunological and immunological reactions (Spencer, 1988). Non-specific serum immunoglobulins may have bound to the crude trypanosomal antigen on the ELISA plate that were reactive with either the polyclonal anti-IgG (whole molecule) or anti-IgM (μ chain) conjugates used. Cross-reactions with antibodies against other haemoparasites were not investigated, but were not reported to be a problem in an earlier study (Luckins, 1977).

The diagnostic sensitivity and specificity estimates obtained in this study were used to obtain true prevalence values for a range of theoretical test prevalence values. A test prevalence of 30% gave the closest estimate of true prevalence for the IgM ELISA, IgG ELISA and a test prevalence of 10% gave the closest estimate of true prevalence for the CATT. True prevalence was underestimated at theoretical test prevalence values greater than or equal to 50% (IgM and IgG ELISAs) or 30% (CATT) and overestimated at lower test prevalence values. Some true prevalence values were outside the permissible range of 0 to 100%, indicating that the test prevalence values used in the calculations would not occur, if the estimates of sensitivity and specificity were valid for the target population.

Predictive values are useful indicators of the probabilities that a test-positive buffalo is infected with *T. evansi* and that a test-negative buffalo is uninfected, and depend on the prevalence of trypanosomosis as well as the test sensitivity and specificity. The positive predictive value of the CATT was calculated to be 100% for true prevalence values from 10% to 90% due to its high specificity, whilst positive predictive values ranged between 44% to 98% for the IgM ELISA and between 55% to 99% for the IgG ELISA. With a true prevalence of 50%, the positive predictive values of the antibody-detection tests were 100% (CATT), 92% (IgG ELISA) and 88% (IgM ELISA) compared with 80% and 74% found previously for the Tr7 Ag-ELISA and 2G6 Ag-ELISA, respectively. The IgG ELISA had the highest negative predictive values of the three antibody-detection tests. With a true prevalence of 50%, the negative predictive values of all five tests were 89% (IgG ELISA), 82% (CATT), 80% (IgM ELISA), 72% (2G6 Ag-ELISA) and 80% (Tr7 Ag-ELISA), and higher negative predictive values were obtained for lower true prevalence values.

Previously, only slight to fair agreement ($k$ = 0.20 {0.16, 0.24}) between the two Ag-ELISAs was found (Chapter 6) when 1880 buffaloes in Central Java were tested by both Ag-ELISAs. Test agreement between the IgG ELISA and CATT and between these antibody-detection tests and the two Ag-ELISAs were calculated by testing 121 buffaloes from four selected villages in Central Java. The number of buffaloes tested by the antibody-detection tests was limited by the amount of reagents

available, and for this reason the IgM ELISA was not included. Overall, there was fair agreement ($k = 0.27$) between the two Ag-ELISAs, IgG ELISA and CATT. Moderate agreement was found between the IgG ELISA and CATT ($k = 0.58$), but only slight agreement was found between the IgG ELISA and either Ag-ELISA ($k = 0.17$ and 0.19) and between the 2G6 Ag-ELISA and CATT ($k = 0.19$). However, the width of the associated 95% confidence intervals indicated the imprecision of the these *kappa* values. The lack of agreement between these tests may have resulted from differences between the serum antigen and antibody profiles of individual buffaloes (shown in Chapter 4); at certain stages of infection, specific antigens or antibodies are not detectable. Furthermore, it was observed that different samples gave false-positive results by these tests. It was not surprising that the best agreement was found between the IgG ELISA and CATT because both tests detect sub-populations of IgG antibodies. Moderate agreement between the same CATT and an IgG ELISA was also found when these tests were used for horses, cattle, dogs and capybaras naturally infected or uninfected with *T. evansi* in Brazil (Franke *et al.*, 1994). In the same study, poor agreement was found between the antibody-detection tests and an Ag-ELISA that employed the same monoclonal antibody as the 2G6 Ag-ELISA.

Antigen-detection and antibody-detection tests were developed because of the inability of standard parasitological techniques to detect sub-acute or chronic *T. evansi* infections in animals. The selection of a *T. evansi* diagnostic test for a particular purpose will depend on the diagnostic sensitivity and specificity of the test as well as the testing objectives. As shown above, trypanosomosis prevalence affects test predictive values and must be taken into consideration together with the costs associated with false-positive and false-negative results. The CATT was shown to have a high positive predictive value for a wide range of prevalence values and has the advantage that it could be applied under field conditions. Therefore, the CATT could be used to identify individual buffaloes with *T. evansi* infections for trypanocidal drug treatment which is expensive and therefore limited to smaller numbers of animals. Another advantage of the CATT and the Ag-ELISAs is that these tests can be readily adapted for testing different animal species (e.g., cattle, sheep, pigs, goats), although

for buffaloes the CATT had to be modified by the addition of rabbit anti-buffalo IgG antibody to prevent autoagglutination (Bajyana Songa *et al.*, 1987a). The high negative predictive values of the two Ag-ELISAs and three antibody-detection tests obtained for low prevalence values suggest that these tests could be used to screen animals from low prevalence areas, for example to check their negative status prior to movement within a country or export. In Indonesia, outbreaks of clinical trypanosomosis have occurred when new strains of *T. evansi* have been introduced into an area through animal movement and animals have been monitored using the MHCT and IgG ELISA (Payne *et al.*, 1990). Ag-ELISAs have been employed to monitor tsetse and trypanosomosis control programmes in Zanzibar (Mbwambo and Mpokwa, 1993) and Uganda (Okuna *et al.*, 1993). In the latter study, the prevalence by Ag-ELISA was less than 20% after six months of tsetse control and trypanocidal treatment activities in an area which had a prevalence of 40% prior to the programme. By contrast, the prevalence remained high (> 40%) in areas without control measures or where control measures were reported to be ineffective. However, prevalence was monitored by testing only 42 randomly selected cattle in each area, and therefore sampling variation may have biased the results.

Practical aspects of the application of diagnostic tests are important considerations for test selection. The CATT involved a subjective interpretation of the end-result (i.e., agglutination) and required a standardised shaking stage for which a special shaker was supplied with the kit. By comparison, the ELISA format allowed large numbers of samples to be tested rapidly using semi-automated equipment, but required trained operators and adequate laboratory facilities, and therefore could not be conducted under field conditions. In the future, adaptation of either the Ag-ELISAs or antibody-ELISAs for use as simple pen-side tests would be beneficial to increase their potential as field tests. A latex agglutination test (Suratex) has been developed using a monoclonal antibody against *T. evansi* (Nantulya, 1994). The sensitivity of this simplified antigen-detection test was estimated using parasite-positive camels and found to be 92% (MHCT-positive camels) and 88% (MI-positive camels), but its use with buffaloes has not been reported.

Multiple testing strategies, described in Chapter 6, can be used to increase either sensitivity or specificity. To increase sensitivity, parallel testing with the MHCT (to detect early infections) and IgG ELISA or Ag-ELISA could be used. The combination of the CATT which detects earlier IgG antibody responses and the IgG ELISA would be another option to increase sensitivity. Serial testing in which all samples would be tested initially with the IgG ELISA or CATT and then the positive samples tested with an Ag-ELISA would be useful in situations requiring a high specificity and where false-negative results are more acceptable. However, major disadvantages of multiple testing strategies are the increased cost per sample due to the higher input of labour and test reagents and the longer time taken to obtain the results.

# CHAPTER 8

# APPLICATION OF ANTIGEN-ELISAS AND OTHER DIAGNOSTIC TESTS FOR *TRYPANOSOMA EVANSI* IN EPIDEMIOLOGICAL STUDIES IN INDONESIA

## 8.1 Introduction

In Indonesia, epidemiological surveys for trypanosomosis have been conducted both in the field (Adiwinata and Dahlan, 1969; Siswansyah and Tarmudji, 1989; Payne, 1989; Payne *et al.,* 1991c; Partoutomo, 1993) and at abattoirs (Rukmana, 1979; Suhardono *et al.,* 1985). These surveys used common parasitological techniques (blood smear, MHCT and MI) and, in some cases antibody-detection ELISAs, to identify animals with trypanosomosis. However, the diagnostic tests used in these surveys were not fully evaluated in terms of their diagnostic sensitivity and specificity. Moreover, the precision of the prevalence estimates obtained was not given. The low sensitivity of the parasitological tests that were used could partly account for the low prevalence values obtained in some surveys. For example, Rukmana (1979) found only 1% of cattle and buffaloes infected with *T. evansi* using blood-smear examination and 10% infected by MHCT at an abattoir in Bandung, Java, but it is likely that the true prevalence was higher. Although more sensitive than blood smear examination, the MHCT is less sensitive than serological assays, as shown by Payne and colleagues (1991c) in their survey of cattle, buffaloes and horses on different islands of Indonesia. During an extensive longitudinal survey in the Eastern Islands (1990-1992) no cattle were found infected with *T. evansi* (B. Christie pers. comm.), despite previous reports of the parasite on these islands and serological evidence found in other studies (Payne *et al.*, 1991c). The need for more sensitive diagnostic tests for *T. evansi* to be developed and applied in epidemiological studies and control programmes has been recognised (Wilson, 1983; Luckins, 1988).

The aims of the studies described in this chapter were to: 1) evaluate the application of different diagnostic tests for *T. evansi*; and 2) estimate the prevalence and true

incidence of *T. evansi*-infections in buffaloes at selected field sites in Central Java. The work comprised both cross-sectional (prevalence) studies and longitudinal (incidence) studies and data were stratified by the age of buffalo and location. The relationship between the trypanosomosis status, body condition and PCV of buffaloes was also investigated, and background information was collected on local farming systems. A survey of markets in the five sampling districts was also included to determine the trypanosomosis status of buffaloes that were being traded locally. In addition, a retrospective study was conducted using sera previously collected from cattle and buffalo on Lombok Island (Utami, 1993).

## 8.2 Materials and methods

### 8.2.1 Cross-sectional studies to estimate the prevalence of *Trypanosoma evansi* infections in buffaloes

*Field locations and buffaloes sampled*

Field visits to five districts of northern Central Java were conducted every three months for a 12-month period. A total of five visits were made to locations in the districts of Batang, Pekalongan, Pemalang, Tegal and Brebes which are shown in Figure 2.1, Chapter 2. The visits were conducted in May 1994 (Visit 1), August 1994 (Visit 2), November 1994 (Visit 3), January 1995 (Visit 4) and May 1995 (Visit 1995). A sixth visit was conducted in July 1995 to Batang and Pekalongan districts only, to obtain extra samples from parasitaemic buffaloes for estimation of sensitivity (described in Chapter 6), but these samples were excluded from the prevalence studies. During each visit, a target of approximately 100 buffaloes were sampled per district at villages chosen by local government veterinary officers. The total numbers of buffaloes blood sampled in Visits 1 to 5 are shown in Table 8.1, and the buffalo populations per district are given with the proportion of villages where buffaloes were kept.

*Sample collection and diagnostic tests*

Buffaloes were blood sampled either in the animal houses or were presented by farmers at a central village area. From each buffalo, jugular blood was collected into

10 ml plain Vacutainers and 7 ml Vacutainers containing 15% EDTA $K_3$ anticoagulant. All the blood samples were kept on ice in a thermally-insulated box. Blood samples with anticoagulant were tested by MHCT (for *T. evansi* and *T. theileri*) later the same day and the PCVs were recorded. Blood samples without anticoagulant were kept on ice and allowed to clot overnight before the sera were pipetted off into new 2 ml plastic tubes (without centrifugation). Approximately 100 white mice were taken on each visit and approximately 50 mice were used in the incidence study and the remainder were used for the prevalence study. In each district, buffaloes were randomly selected for the MI test by selecting every $n^{th}$ buffalo; where n was the total number of buffaloes sampled divided by the number of mice available, per district. During Visit 5, 30 faeces samples were also collected from buffaloes in the incidence study to assess their infection status with *Fasciola gigantica* as a potential factor affecting PCV.

**Table 8.1** The buffalo population and numbers of villages (total number and number where buffaloes are kept) in the five districts visited in Central Java, shown with the numbers of villages in the study and numbers of buffaloes sampled.

| District | Buffalo population | Number of villages | Villages where buffaloes are kept | | Number of villages in the study | Number of buffaloes sampled |
|---|---|---|---|---|---|---|
| | | | Number | Proportion of all district villages (%) | | |
| Batang | 11,523 | 242 | 186 | 77 | 10 | 387 |
| Pekalongan | 18,369 | 274 | 192 | 70 | 12 | 422 |
| Pemalang | 25,496 | 216 | 205 | 95 | 12 | 611 |
| Tegal | 20,865 | 270 | 208 | 77 | 17 | 447 |
| Brebes | 16,690 | 291 | 230 | 79 | 8 | 520 |
| Totals | 92,943 | 1293 | 1021 | 79 | 59 | 2387 |

On return to the laboratory, serum samples were stored at -20°C and later tested by the standardised 2G6 Ag-ELISA and Tr7 Ag-ELISA (described in Chapter 3). For the prevalence study, only samples from Visits 1 to 3 were tested by the Tr7 Ag-ELISA because a limited amount of reagents was available.

*Data collection*

A field record sheet was completed with the following individual buffalo data: age, sex, owner's name, body score (BS), sample number, MHCT result, PCV, MI test performed/not performed. Additional information, including any history of ill-health, recent purchase, presence of Tabanids or *Stomoxys* spp., was recorded. Body condition was scored using the method for buffaloes described by Winugroho and Teleni (1993) using a body score scale of BS 3 (very poor) to BS 7 (very good). Blood samples were numbered using a code that included a district code (Batang: A, Pekalongan: B, Pemalang: C, Tegal: D, Brebes: E) and visit number.

### 8.2.2 Villages selected for estimation of *Trypanosoma evansi* seroprevalence by antibody-detection ELISAs and the card agglutination test

Since limited reagents were available for the antibody-detection tests, a total of 121 samples of buffaloes from four villages were tested by IgM ELISA, IgG ELISA and CATT, as well as by the two Ag-ELISAs. Two villages (Ponowareng and Wonosegoro) were chosen where at least one buffalo had been found to be parasitaemic and where > 50% of buffaloes sampled were antigenaemic by the 2G6 Ag-ELISA, and two villages (Harjosari and Wonokromo) were chosen where none of the buffaloes sampled was found to be parasitaemic and where < 25% of buffaloes sampled were antigenaemic by the 2G6 Ag-ELISA. Information on these four villages is given in Table 8.2.

**Table 8.2** Information on the numbers of buffaloes sampled in Ponowareng, Wonosegoro, Harjosari and Wonokromo and village-specific prevalence values by the two Ag-ELISAs.

| Village | District | Total number of buffaloes | Number of buffaloes sampled | Parasitaemic buffaloes found | Corrected prevalence (%) | |
|---|---|---|---|---|---|---|
| | | | | | 2G6 Ag-ELISA | Tr7 Ag-ELISA |
| Ponowareng | Batang | 55 | 31 | Yes | 72 | 88 |
| Wonosegoro | Batang | 155 | 42 | Yes | 85 | 83 |
| Wonokromo | Pemalang | 38 | 28 | No | 24 | 66 |
| Harjosari | Tegal | 500 | 21 | No | 17 | 68 |

### 8.2.3 Retrospective analysis of sera collected from buffalo and cattle on Lombok Island

Sera from an earlier trypanosomosis survey conducted in three districts of Lombok Island (Utami, 1993) were tested retrospectively by the 2G6 Ag-ELISA and Tr7 Ag-ELISA (n = 102 cattle; n = 56 buffaloes). The location of Lombok Island, which is situated to the east of Bali, is shown in Figure 2.1. The blood samples had been tested previously by MHCT and IgG ELISA and none of the cattle had been found to be parasitaemic, whereas one of the buffaloes was MHCT-positive. By IgG ELISA 41% of the cattle sera were positive and 50% of the buffalo sera were positive (Utami, 1993). Insufficient information was available on the number of sampling locations and the total number of village buffaloes, therefore 95% confidence intervals for simple random samples, rather than for two-stage cluster samples, were calculated.

### 8.2.4 Survey of buffaloes in five district markets

All five districts visited in Central Java for the prevalence survey hold livestock markets every five to seven days. In July 1995, 30 buffaloes were blood sampled in Batang market, and in September 1995 buffaloes were blood sampled at the markets in Pekalongan (n = 65), Pemalang (n = 50), Tegal (n = 56) and Brebes (n = 38). Field record sheets were used to collect data on the age, sex and body score of buffaloes sampled and on the numbers of buffaloes for sale by each farmer or agent. All the blood samples were tested by the MHCT, MI, 2G6 Ag-ELISA, IgG ELISA and CATT, and the PCVs were recorded.

### 8.2.5 Longitudinal study to estimate the true incidence rate of *Trypanosoma evansi* infections in buffaloes

The incidence study was conducted during the same field visits described above for the prevalence studies. A previous three-year longitudinal study had shown that up to 50% of buffaloes may not be available for sampling at later stages (Partoutomo, 1993). To identify approximately 50 test-negative buffaloes, more than 100 buffaloes were sampled because the study area was reported to have a high prevalence of *T. evansi* in buffaloes (Partoutomo, 1993). A target of 50 buffaloes was

chosen empirically for the longitudinal study as the maximum number of buffaloes that could be re-sampled during each visit because of the time required to collect and process samples and the number of mice that could be transported to the field for MI. To sample more than 100 buffaloes initially, it was necessary to include two villages: Kebumen in Batang district, where 50 buffaloes were sampled and Gejlig in Pekalongan district, where 53 buffaloes were sampled. All the buffaloes were individually identified by ear-tags, or in some buffaloes by painted-horn numbers when ear-tags were not permitted by the farmers. Buffaloes over one year of age were chosen because their owners intended to keep them for at least one year. From the initial 103 buffaloes sampled, 49 buffaloes that had no detectable parasitaemia by either the MHCT or MI and that were negative by the 2G6 Ag-ELISA and/or Tr7 Ag-ELISA were chosen for the incidence study. Insufficient buffaloes were negative by both the Ag-ELISAs, and therefore some buffaloes that were negative by only one Ag-ELISA were included.

Field record sheets were used to collect the individual buffalo data described in the prevalence studies. The sera were tested with the 2G6 Ag-ELISA and Tr7 Ag-ELISA, IgM ELISA, IgG ELISA and CATT. A buffalo was classified as newly infected with *T. evansi,* if found to be parasitaemic or if there was a two-fold increase in the initial antigen or antibody ELISA PP value, or if a strong positive CATT result (score 2 or 3) was found following a negative CATT result (score 0). True incidence rates were calculated as described below.

### 8.2.6 Informal farmer survey

When buffaloes were being sampled, farmers were asked about different aspects of local farming systems including animal management and health, for example the number of months per year that buffaloes provided draught power. The time that buffaloes were grazing, the types of housing and local fly challenge were recorded, as well as the movement of buffaloes to and from villages for work, sale or slaughter. Farmers were asked which health problems they considered most important in their buffaloes.

### 8.2.7 Data analysis

*Cross-sectional studies*

A cut-off value of 20 PP was chosen for both the Ag-ELISAs and the IgG ELISA, and a cut-off value of 30 PP was chosen for the IgM ELISA, as described in Chapters 6 and 7. District-specific, village-specific and age-specific prevalence values were estimated, together with overall prevalence estimates obtained by the different tests (test-specific prevalence), with associated 95% confidence intervals (Thrusfield, 1995). For calculation of 95% confidence intervals for district-specific prevalence values, a two-stage cluster sampling protocol was used to take account of the variation between villages as well as within villages. Ninety-five percent confidence intervals for test-specific prevalence values (biased estimates) and village-specific prevalence values were calculated by the method for single sample proportions using the software CIA with the exact binomial method, or if given the Normal approximation where nP and n(1- P) were both greater than a threshold level defined by the software. The distribution of the village-specific prevalence values did not have a Normal distribution when displayed in a dot-plot, and therefore 95% confidence intervals were considered approximate, and so standard errors were also quoted.

Point estimates of prevalence were compared by Chi-squared tests for 2 x 2 and 2 x $k$ tables with an adjustment for continuity using the software EPIINFO version 6.02[1]. Mean PCVs (and standard deviations) and median body scores were calculated for buffaloes sampled in the villages and the buffaloes sampled in the markets. Buffaloes were sub-divided into antigenaemic and non-antigenaemic buffaloes and into parasite-positive and parasite-negative buffaloes for these calculations. The PCVs were compared by a two-sample $t$-test using the software Minitab and both $P$ values and 95% confidence intervals of the difference between mean values are given, and $P$ values were considered significant at the 5% level.

Corrected estimates of true prevalence (P) were calculated using the point estimates of diagnostic sensitivity and specificity of the two Ag-ELISAs given in Chapter 6. Annual mean conversion rates were calculated by the method described by Thrusfield

(1995) using the corrected age-specific prevalence values obtained by the two Ag-ELISAs. To demonstrate the effect of age as a confounder for prevalence, an age-adjusted village-specific prevalence was calculated for Indrajaya, Brebes district, as an example, using the method of direct adjustment (Thrusfield, 1995).

*Longitudinal study*

For the longitudinal study, true incidence rates ($I$) were calculated because of the dynamic nature of the study population and the loss of some animals present at the start of the study precluded the accurate calculation of cumulative incidence. Incidence rates were calculated per animal-year at risk: the total number of test-positive buffaloes divided by the total number of months at risk, and multiplied by 12. The numerators were assumed to be Poisson variables (Kahn and Sempos, 1989) and 95% confidence intervals were taken from Poisson tables (Diem, 1967). Cumulative incidences ($CI$) were calculated for the 2G6 Ag-ELISA and Tr7 Ag-ELISA from the estimates of true incidence ($I$) using the equation: $CI = 1 - e^{-I}$ (Thrusfield, 1995), using the assumption that all animals remained positive after infection. The calculated one-year cumulative incidence was then compared with the prevalence data using the equation: $CI_n = 1 - (1 - CI_1)^n$, where n equals the number of years and $CI_n$ equals the cumulative incidence for a n-year period.

## 8.3 Results

### 8.3.1 Cross-sectional studies to estimate the prevalence of *Trypanosoma evansi* infections in buffaloes

A total of 2,387 buffaloes were sampled during the five visits to Central Java. The numbers of samples collected in each district and the number of samples analysed by each diagnostic test are given in Table 8.3. All samples were tested by the MHCT and 2G6 Ag-ELISA, 66% by Tr7 Ag-ELISA and 15% by MI. Of 360 MI tests, 17 (4.7%) gave no result because the mice died before the end of the 30-day monitoring period and without a parasitaemia being detected. More samples from Batang and Pekalongan districts were tested by MI because 103 buffaloes were tested in these districts in Visit 1 for selection of buffaloes for the incidence study.

**Table 8.3** Number of samples collected from each district and analysed by different *Trypanosoma evansi* diagnostic tests

| District | Diagnostic test | | | |
|---|---|---|---|---|
| | MHCT | MI | 2G6 Ag-ELISA | Tr7 Ag-ELISA |
| Batang | 387 | 110 | 387 | 233 |
| Pekalongan | 422 | 95 | 422 | 299 |
| Pemalang | 611 | 61 | 611 | 366 |
| Tegal | 447 | 47 | 447 | 301 |
| Brebes | 520 | 47 | 520 | 369 |
| All five districts | 2387 | 360 | 2387 | 1568 |

Overall, the point prevalence estimates obtained were 4% (3, 5) by MHCT, 9% (6, 12) by MI, 58% (56, 60) by 2G6 Ag-ELISA and 70% (68, 72) by Tr7 Ag-ELISA. Prevalence values obtained for each district by different diagnostic tests varied and are shown in Table 8.4. District-specific prevalence values obtained by the parasitological tests were lower (range 0 to 16%) than those found by either of the two Ag-ELISAs (range 39% to 86%). Prevalence values varied significantly between some districts; for example, by the 2G6 Ag-ELISA the prevalence found in Brebes was higher than in Pekalongan ($P < 0.001$) or Pemalang ($P < 0.001$). If 95% confidence intervals were calculated using a simple random sample method (that does not account for clustering of disease), smaller intervals were found than the 95% confidence intervals given in Table 8.4 (which were calculated using a two-stage cluster sample method). For example, the 95% confidence intervals for the prevalence found in Batang district by 2G6 Ag-ELISA were 53% to 63% (simple random method) and 42% to 75% (two-stage cluster sample method). In addition, due to practical constraints on the number of locations that could be visited during each field trip, the district-specific prevalence values were estimated using data from all the villages visited in each district. Therefore, these estimates would be confounded by seasonal effects because changes in the village-specific prevalence may have occurred during the one-year study period.

*Age-specific prevalence*

There was an age-dependent increase in prevalence with both Ag-ELISAs (see Table 8.5), except that with the Tr7 Ag-ELISA the prevalence decreased in buffaloes more than seven years of age. The distribution of the ages of buffaloes sampled is shown in the first column of Table 8.5; the majority of buffaloes sampled were over one year of age, although 255 buffaloes younger than this were sampled. By one year of age, 22% of buffaloes sampled were positive by Tr7 Ag-ELISA and 33% by 2G6 Ag-ELISA, and more than 50% of buffaloes sampled aged between three to five years old were positive by both Ag-ELISAs. The prevalence found by 2G6 Ag-ELISA in the village of Indrajaya, where 35% of the buffaloes sampled were less than three years of age, was age-adjusted to demonstrate the effect of age as a confounder: the prevalence was 74% before adjustment and 59% after age-adjustment which was below the lower 95% confidence limit of the unadjusted prevalence (68, 81). There was a decrease in the estimated annual rate of seroconversion (i.e., time to become antigenaemic) in older buffalo age groups (see Table 8.5).

*Village-specific prevalence*

Village-specific prevalence values were corrected using estimates of the diagnostic sensitivity and specificity of each test and are given in Tables 8.6 to 8.10. These prevalence values were not adjusted for age because the prevalence values of the different buffalo groups were the measures of interest and it was not considered appropriate to remove the effect of age as a confounder. Each table gives the results for all the villages visited in each district, and villages where at least one buffalo was found parasite-positive (by MHCT and/or MI) are denoted by the symbol +. Parasite-positive buffaloes were found in 32 of the 59 villages (54%) visited, including all the villages visited in Batang district.

**Table 8.4** Uncorrected district-specific prevalence values ($P^T$), with associated 95% confidence intervals (CI) (in brackets) and standard errors (SE), obtained using different diagnostic tests for *Trypanosoma evansi*

| District | Diagnostic test | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | MHCT | | | MI | | | 2G6 Ag-ELISA | | | Tr7 Ag-ELISA* | | |
| | $P^T$ (%) | CI | SE | $P^T$ (%) | CI | SE | $P^T$ (%) | CI | SE | $P^T$ (%) | CI | SE |
| Batang | 6 | (2, 10) | 0.02 | 16 | (6, 27) | 0.06 | 58 | (42, 75) | 0.09 | 65 | (41, 90) | 0.12 |
| Pekalongan | 6 | (3, 10) | 0.02 | 7 | (4, 11) | 0.02 | 39 | (29, 50) | 0.05 | 68 | (39, 96) | 0.29 |
| Pemalang | 3 | (0, 5) | 0.01 | 8 | (-5, 21) | 0.07 | 47 | (38, 56) | 0.05 | 61 | (58, 63) | 0.01 |
| Tegal | 1 | (0, 3) | 0.01 | 9 | (-2, 19) | 0.05 | 58 | (43, 74) | 0.08 | 69 | (42, 95) | 0.14 |
| Brebes | 4 | (1, 6) | 0.01 | 0 | | | 62 | (53, 72) | 0.05 | 86 | (75, 97) | 0.06 |

*: Tr7 Ag-ELISA results for samples from Visits 1-3 only; all other test results are for samples from Visits 1-5.

**Table 8.5** Corrected age-specific prevalence values (P) of antigenaemia obtained by two *Trypanosoma evansi* antigen-ELISAs shown with annual mean conversion rates

| Age group (years) | Number of buffaloes tested | P (%) | | Annual mean conversion rates* | |
|---|---|---|---|---|---|
| | | 2G6 Ag-ELISA | Tr7 Ag-ELISA | 2G6 Ag-ELISA | Tr7 Ag-ELISA |
| 0-0.5 | 48 | 13 | 12 | 24 | 23 |
| 0.5-1 | 207 | 33 | 22 | 33 | 22 |
| 1-2 | 390 | 35 | 47 | 19 | 27 |
| 2-3 | 403 | 37 | 49 | 28 | 20 |
| 3-5 | 548 | 62 | 54 | 18 | 14 |
| 5-7 | 442 | 61 | 54 | 13 | 11 |
| >7 | 401 | 68 | 36 | 11 | 5 |

*: Calculated using the highest age in each group (e.g., 0.5 was used for the age range 0-0.5 years; the mean age of 9.6 years was used for buffaloes over 7 years of age.

**Table 8.6** Batang district: Corrected village-specific prevalence values (P) of *Trypanosoma evansi* infections in buffaloes, with associated 95% confidence intervals (CI) (in brackets) obtained using two antigen-ELISAs

| Visit | Village | Total number of buffaloes | Sample size | 2G6 Ag-ELISA | | Tr7 Ag-ELISA | |
|---|---|---|---|---|---|---|---|
| | | | | P (%) | CI | P (%) | CI |
| 1 | Kebumen+ | 98 | 30 | 104 | (88, 100) | 86 | (69, 96) |
| 1 | Ponowareng+ | 55 | 31 | 72 | (52, 86) | 88 | (70, 96) |
| 1 | Siberuk+ | 63 | 27 | 74 | (54, 89) | 88 | (71, 98) |
| 1 | Wonosegoro+ | 155 | 42 | 85 | (72, 95) | 83 | (69, 93) |
| 2 | Kalimanggis+ | 155 | 44 | 59 | (43, 74) | 66 | (50, 80) |
| 2 | Sawangan+ | 90 | 11 | 85 | (48, 98) | 86 | (48, 98) |
| 3 | Wonotunggal+ | 200 | 48 | 46 | (31, 61) | 51 | (35, 65) |
| 4 | Cluwuk+ | 40 | 31 | 72 | (52, 86) | 61 | (42, 78) |
| 4 | Duren Ombo+ | 119 | 56 | 39 | (27, 53) | 59 | (45, 72) |
| 5 | Adinuso+ | 160 | 67 | 26 | (16, 38) | nd | |

nd: Not done; +: Village where at least one parasitaemic buffalo was found

**Table 8.7** Pekalongan district: Corrected village-specific prevalence values (P) of *Trypanosoma evansi* infections in buffaloes, with associated 95% confidence intervals (CI) (in brackets), obtained using two antigen-ELISAs.

| Visit | Village | Total number of buffaloes | Sample size | 2G6 Ag-ELISA | | Tr7 Ag-ELISA | |
|---|---|---|---|---|---|---|---|
| | | | | P (%) | CI | P (%) | CI |
| 1 | Gejlig+ | 180 | 66 | 46 | (33, 58) | 97 | (90, 100) |
| 1 | Wangandowo | 115 | 46 | 54 | (39, 69) | 63 | (48, 77) |
| 1 | Rowolaku+ | 75 | 18 | 41 | (17, 64) | 76 | (52, 94) |
| 2 | Kalijoyo+ | 70 | 33 | 46 | (28, 64) | 86 | (68, 95) |
| 2 | Tambakroto+ | 148 | 72 | 17 | (9, 27) | 19 | (11, 31) |
| 3 | S.Jomblang+ | 80 | 26 | 73 | (52, 88) | 93 | (75, 99) |
| 3 | Kalipancur | 125 | 38 | 37 | (22, 54) | 75 | (60, 89) |
| 4 | Banjarejo+ | 50 | 37 | 52 | (34, 68) | nd | |
| 4 | Karangsari+ | 50 | 41 | 41 | (26, 58) | nd | |
| 4 | Sankanjoyo | 16 | 12 | 17 | (2, 48) | nd | |
| 5 | Krasak Ageng+ | 63 | 29 | 14 | (4, 32) | nd | |
| 5 | Tegal Suruh+ | 19 | 4 | 54 | (7, 93) | nd | |

nd: Not done; +: Village where at least one parasitaemic buffalo was found

**Table 8.8** Pemalang district: Corrected village-specific prevalence values (P) of *Trypanosoma evansi* infections in buffaloes, with associated 95% confidence intervals (CI) (in brackets), obtained using two antigen-ELISAs

| Visit | Village | Total number of buffaloes | Sample size | 2G6 Ag-ELISA | | Tr7 Ag-ELISA | |
|---|---|---|---|---|---|---|---|
| | | | | P (%) | CI | P (%) | CI |
| 1 | Kabunan+ | 325 | 36 | 41 | (26, 59) | 66 | (49, 81) |
| 1 | Saradan+ | 46 | 25 | 85 | (64, 96) | 51 | (31, 72) |
| 1 | Penggarit | 172 | 50 | 67 | (53, 82) | 58 | (43, 72) |
| 2 | Wonogiri+ | 70 | 52 | 26 | (16, 41) | 61 | (47, 75) |
| 2 | Tegal Sari | 450 | 78 | 63 | (51, 74) | 61 | (50, 72) |
| 3 | Karangbrai+ | 120 | 70 | 41 | (30, 54) | 63 | (51, 74) |
| 3 | Sarwodadi | 36 | 23 | 50 | (31, 73) | 51 | (31, 73) |
| 3 | Wonokromo | 38 | 28 | 24 | (11, 45) | 66 | (44, 81) |
| 3 | Mojo | 78 | 4 | 54 | (7, 93) | 47 | (7, 93) |
| 4 | Pedurungan+ | 150 | 109 | 52 | (43, 62) | nd | |
| 4 | Surajaya | 400 | 96 | 33 | (24, 44) | nd | |
| 5 | Pegongsoran+ | 200 | 40 | 39 | (25, 57) | nd | |

nd: Not done; +: Village where at least one parasitaemic buffalo was found.

**Table 8.9** Tegal district: Corrected village-specific prevalence values (P) of *Trypanosoma evansi* infections in buffaloes, with 95% confidence intervals (CI) (in brackets), obtained using two antigen-ELISAs

| Visit | Village | Total number of buffaloes | Sample size | 2G6 Ag-ELISA | | Tr7 Ag-ELISA | |
|---|---|---|---|---|---|---|---|
| | | | | P (%) | CI | P (%) | CI |
| 1 | Dermasuci+ | 105 | 68 | 102 | (95, 100) | 100 | (95, 100) |
| 1 | Gembong Dadi+ | 135 | 11 | 24 | (6, 61) | 24 | (6, 61) |
| 1 | Harjosari | 500 | 21 | 17 | (5, 42) | 68 | (43, 85) |
| 2 | DukuhJatiKidul+ | 28 | 24 | 83 | (63, 95) | 97 | (79, 100) |
| 2 | Bedug | 4 | 4 | 54 | (7, 93) | 47 | (7, 93) |
| 2 | Grobog Kulon | 4 | 4 | 0 | (0, 60) | 90 | (40, 100) |
| 2 | Talok | 21 | 4 | 0 | (0, 60) | 47 | (7, 93) |
| 2 | Kagog | 2 | 2 | 163 | (16, 100) | 100 | (16, 100) |
| 2 | Slawi Wetan | 35 | 2 | 54 | (1, 99) | 47 | (1, 99) |
| 2 | Jenggawur | 6 | 3 | 91 | (29, 100) | 76 | (9, 99) |
| 2 | Suradadi | 60 | 43 | 41 | (27, 58) | 69 | (54, 83) |
| 2 | Jatimulya | 50 | 5 | 33 | (5, 85) | 0 | (0, 52) |
| 3 | Blubuk | 170 | 70 | 46 | (34, 58) | 54 | (16, 38) |
| 3 | Srengseng | 150 | 40 | 76 | (59, 87) | 95 | (83, 99) |
| 4 | BalopulangKulon | 160 | 55 | 52 | (39, 66) | nd | |
| 4 | Kaliwungu | 110 | 29 | 59 | (39, 77) | nd | |
| 5 | Pakulaut+ | 265 | 62 | 48 | (36, 61) | nd | |

nd: Not done; +: Village where at least one parasitaemic buffalo was found.

**Table 8.10** Brebes district: Corrected village-specific prevalence values (P) of *Trypanosoma evansi* infections in buffaloes, with 95% confidence intervals (CI) (in brackets), obtained using two antigen-ELISAs.

| Visit | Village | Total number of buffaloes | Sample size | 2G6 Ag-ELISA | | Tr7 Ag-ELISA | |
|---|---|---|---|---|---|---|---|
| | | | | P (%) | CI | P (%) | CI |
| 1 | Kutamendala | 267 | 99 | 63 | (52, 72) | 75 | (65, 83) |
| 2 | Indrajaya+ | 280 | 173 | 74 | (68, 81) | 93 | (88, 96) |
| 3 | Tanggeran+ | 120 | 97 | 61 | (50, 71) | 85 | (76, 91) |
| 4 | Pulosari | 76 | 19 | 26 | (9, 51) | nd | |
| 4 | Tengki | 70 | 9 | 41 | (14, 79) | nd | |
| 4 | Pagejugan | 80 | 12 | 17 | (2, 48) | nd | |
| 4 | Pasar Batang | 30 | 8 | 54 | (16, 84) | nd | |
| 5 | TembongRaya+ | 280 | 103 | 57 | (48, 67) | nd | |

nd: Not done; +: Village where at least one parasitaemic buffalo found.

In all five districts, village-specific point estimates of prevalence ranged from 0 to 100% by Ag-ELISA. By the 2G6 Ag-ELISA, 51% of the village-specific prevalence values were higher than 50%, and by Tr7 Ag-ELISA 82% of the village-specific prevalence values were higher than 50%. Of 39 villages tested by both Ag-ELISAs, 15 villages had prevalence values by the two Ag-ELISAs that differed by less than 10%. Only two villages (Tambakroto and Gembong Dadi) had prevalence values that were less than 30% by both Ag-ELISAs. In general, the Tr7 Ag-ELISA gave higher village-specific prevalence values than the 2G6 Ag-ELISA, and in a few villages the difference between prevalence values obtained by the two Ag-ELISAs was considerable. For example, the prevalence in Harjosari by 2G6 Ag-ELISA was 17%, but 68% by Tr7 Ag-ELISA, and in Wonokromo the prevalence values were 24% and 66%, respectively.

*Antigenaemia, parasitaemia and packed cell volume*

Overall, 19% of all buffaloes sampled had a low PCV (i.e. < 26%) and in some villages more than 25% of the buffaloes sampled had a low PCV including villages with a high prevalence (e.g., Kebumen, Sawangan, Ponowareng, Siberuk, Dukul Jati Kidul, Kutamendala) and a low prevalence (e.g., Gembong Dadi, Pulosari) of antigenaemia. The mean PCV of all buffaloes sampled was 30.1%, and the mean PCV of antigenaemic buffaloes was significantly lower, using either the 2G6 Ag-ELISA ($P < 0.0001$; {0.56, 1.45}) or the Tr7 Ag-ELISA ($P < 0.0001$; {0.45, 1.37}) than the mean PCV of non-antigenaemic buffaloes (see Table 8.11). The mean PCV of parasitaemic buffaloes was also significantly lower than of non-parasitaemic buffaloes ($P < 0.0001$; {1.95, 3.72}).

*Antigenaemia, parasitaemia and body score*

Of all the buffaloes sampled (n = 2387), 27% were found to have a low body score (BS 5 or less). Only 1.5% of buffaloes were in very poor condition (BS 3 or 4), but a higher proportion of these buffaloes ($P < 0.01$) were positive (91%; corrected value) than the overall prevalence (72 %; corrected value), by 2G6 Ag-ELISA. Of the two buffaloes with a very poor body score (BS 3), one was an 18 month-old male buffalo in Saradan, Pemalang district, with clinical signs of pyrexia and weakness, and was

parasitaemic with a PCV of 11% and antigenaemic (2G6 Ag-ELISA: 207 PP; Tr7 Ag-ELISA: 110 PP). There was no difference between the median body scores of antigenaemic and non-antigenaemic buffaloes, and between parasitaemic buffaloes and non-parasitaemic buffaloes, as shown in Table 8.11.

**Table 8.11** Mean packed cell volume (PCV) and median body score (BS) of 1880 village buffaloes that were tested by both the Ag-ELISAs and the parasitological tests (microhaematocrit test or mouse inoculation)

| Test status | Number of buffaloes | Mean PCV (SD)* | Median BS |
|---|---|---|---|
| 2G6 Ag-ELISA + | 970 | 29.5 (5.1) | 6 |
| 2G6 Ag-ELISA - | 910 | 30.5 (4.8) | 6 |
| Tr7 Ag-ELISA + | 1193 | 29.7 (5.1) | 6 |
| Tr7 Ag-ELISA - | 687 | 30.6 (4.8) | 6 |
| Parasite + | 124 | 27.4 (4.9) | 6 |
| Parasite - | 1756 | 30.2 (4.8) | 6 |

*: Standard deviation given in brackets

### 8.3.2 Village-specific prevalence values obtained by antibody-detection tests

Of the four villages where buffaloes were also tested by the antibody-detection tests, three villages (Harjosari, Wonosegoro and Ponowareng) had high corrected prevalence values by the IgG ELISA (96%, 109%, and 123%, respectively) and by the CATT (85%, 105%, and 111%, respectively). The corrected prevalence in Wonokromo was 56% by the IgG ELISA and 46% by CATT. Although both Harjosari and Wonokromo were selected as 'low prevalence' villages on the basis of the 2G6 Ag-ELISA results and the absence of parasite-positive buffaloes, corrected prevalence values were high in both villages by the Tr7 Ag-ELISA (68% and 66%, respectively).

### 8.3.3 Market study

In all the markets visited there was sufficient cooperation to obtain blood samples from the majority of buffaloes present. Most owners or agents were selling a single buffalo, but as many as 22 buffaloes were for sale by one agent, and some of the buffaloes had come directly from another local market. Of the 239 buffaloes sampled,

114 were female and 125 were male and their ages ranged from 9 months to 13 years. Parasitaemic buffaloes were found in all the markets visited and 24% of buffaloes sampled had a body score of five or less. Overall, 10% of buffaloes sampled were either MHCT-positive (5%) or MI-positive (5%) and in Tegal market two buffaloes were found to be infected with *T. theileri*. The corrected prevalence found in buffaloes sampled in the five markets was 39% by 2G6 Ag-ELISA, 56% by IgG ELISA and 47% by CATT (including seven weak-positives). The prevalence found by the 2G6 Ag-ELISA was significantly lower (39%) than the prevalence found by this test in the buffaloes sampled in the main prevalence study (72%) ($P < 0.001$). In addition, there were significant differences between the prevalence values obtained in different markets with the 2G6 Ag-ELISA which ranged from 15% (in Pekalongan) to 80% (in Pemalang) ($P < 0.001$).

A significantly higher proportion of market buffaloes (48%) had a low PCV than buffaloes sampled in the prevalence study (19%) ($P < 0.001$). Table 8.12 shows the mean PCVs and median body scores of market buffaloes found to be antigenaemic or non-antigenaemic and parasite-positive or parasite-negative. There was no significant difference between the mean PCVs of antigenaemic buffaloes and non-antigenaemic buffaloes ($P = 0.68$; {-0.97, 1.12}) nor between the mean PCVs of parasite-positive buffaloes and parasite-negative buffaloes ($P = 0.45$; {-1.04, 2.29}). Furthermore, no difference was found between the median body scores of these four groups of buffaloes.

**Table 8.12** Mean packed cell volume (PCV) and median body score (BS) of 239 market buffaloes that were positive or negative by the 2G6 Ag-ELISA or by parasitological tests (microhaematocrit test or mouse inoculation)

| | Mean PCV (SD)* | Median BS |
|---|---|---|
| 2G6 Ag-ELISA + | 25.6 (4.1) | 6 |
| 2G6 Ag-ELISA - | 25.5 (4.0) | 6 |
| Parasite-positive | 25.0 (3.7) | 6 |
| Parasite-negative | 25.6 (4.1) | 6 |

*: Standard deviation given in brackets

### 8.3.4 Retrospective analysis of sera collected from buffaloes and cattle on Lombok Island

The prevalence values obtained for the buffalo and cattle groups sampled on Lombok Island are given in Table 8.13. There were significant differences between the prevalence values obtained by the 2G6 Ag-ELISA in the three districts for both cattle ($0.05 < P < 0.01$) and buffaloes ($0.05 < P < 0.01$), and in Central Lombok a significantly higher prevalence was found with the cattle than the buffaloes sampled ($0.0001 < P < 0.0005$).

**Table 8.13** Corrected prevalence values (P) obtained by retrospective analysis by the 2G6 Ag-ELISA of buffalo and cattle sera collected from three districts of Lombok Island

| District | Number of sub-districts | Sample size | Number of animals < 1-year-old | Type of animal | P (%)* |
|---|---|---|---|---|---|
| West Lombok | 2 | 29 | 26 | Buffalo | 48 (29, 68) |
| | 4 | 41 | 22 | Cattle | 34 (20, 51) |
| Central Lombok | 1 | 27 | 12 | Buffalo | 19 (6, 38) |
| | 4 | 28 | 6 | Cattle | 71 (51, 52) |
| East Lombok | 0 | 0 | - | Buffalo | - |
| | 4 | 33 | 8 | Cattle | 30 (18, 52) |

*: 95% confidence intervals are given in brackets and were calculated using the method for simple random samples, as explained in the Materials and Methods.

### 8.3.5 Longitudinal study to estimate the true incidence rate of *Trypanosoma evansi* infections in buffaloes

During the longitudinal study, there was a steady decline in the number of buffaloes presented by farmers at each sampling visit. At the second visit, 36 of the 49 selected buffaloes were sampled, and by the fifth visit, only 25 buffaloes were sampled. Three new buffaloes were included in the study from the second visit because three buffaloes had been sold since Visit 1; thereafter no additional buffaloes were included. All the buffaloes except one were female, and were from 18 months to 12 years of age. The age distribution was similar to that of buffaloes sampled in the cross-sectional study (given in Table 8.5), except that no buffaloes less than six-months-old were sampled. During the study, nine buffaloes (18%) were sold, principally because their owners, who had planned to keep them for at least a year, were in need of money.

The individual buffaloes that were found positive by the different diagnostic tests are shown in Table 8.14. Of 27 buffaloes that became positive, 14 were positive by more than one test, and seven were positive by the parasitological tests, Ag-ELISAs or antibody-detection tests. Buffalo 84 was only found to be positive by the MHCT and MI. The body score was six or seven in 88-97% of the buffaloes sampled in Visits 1, 2, 3 and 5, but 21% of buffaloes sampled in Visit 4 had a body score of five or less. Of the buffaloes with a low body score, only Buffalo 73 was shown to be parasite-positive. The buffaloes sold all had a body score of six or seven and only Buffalo 74 was parasite-positive, at the sampling visit prior to their sale. None of the faeces collected from the study buffaloes in Visit 5 were positive for *Fasciola gigantica*.

*True incidence rates*

During the one-year monitoring period, three buffaloes in Kebumen and five buffaloes in Gejlig were found to be parasite-positive with the MHCT or MI. True incidence rates were calculated for the different diagnostic tests and are given in Table 8.15. Higher incidence rates were obtained using the Tr7 Ag-ELISA, IgM ELISA and CATT than the other tests, however, the width of the associated 95% confidence intervals indicates the imprecision of these estimates. The incidence rate of *T. theileri* obtained using the MHCT was 0.19 (0.04, 0.58) in Kebumen (three positive) and 0.06 (0.002, 0.34) in Gejlig (one positive) per animal-year at risk.

The one-year cumulative incidence ($CI_1$) was calculated to be 0.20 with the 2G6 Ag-ELISA ($I = 0.25$) and 0.36 with the Tr7 Ag-ELISA ($I = 0.44$). Using these values, the four-year cumulative incidence ($CI_4$) was calculated to be 0.59 with the 2G6 Ag-ELISA and 0.83 with the Tr7 Ag-ELISA.

**Table 8.14** Individual buffaloes that became infected with *Trypanosoma evansi* during the longitudinal study, as determined by different diagnostic tests, and the visit number when first found to be test-positive.

| | | Diagnostic test* | | | | | | |
|---|---|---|---|---|---|---|---|---|
| Buffalo number | Visit(s) when first positive** | MHCT | MI | 2G6 Ag-ELISA | Tr7 Ag-ELISA | IgM ELISA | IgG ELISA | CATT |
| 25 | 2 | | | | | + | | |
| 26 | 4 | | | | | | | + |
| 29 | 4 | | | | + | | | |
| 32 | 2,2,2 | | | + | + | | | + |
| 34 | 5 | | | | + | | | |
| 36 | 2 | | | | + | | | |
| 38 | 4 | | | | + | | | |
| 39 | 2 | | | | | + | | |
| 42 | 2 | | | | | | | + |
| 43 | 4,4,2 | + | + | | | + | | |
| 50 | 5,5 | | | | + | | + | |
| 53 | 2 | | | | | | + | |
| 57 | 4,4 | | | | | + | | + |
| 58 | 4 | | | | | | | + |
| 62 | 3 | | | | | | | + |
| 66 | 2 | | | + | | | | |
| 68 | 5,5 | | | | | + | | + |
| 73 | 3,4,3 | | + | + | | + | | |
| 74 | 3,3,3,3 | + | + | + | | | + | |
| 77 | 4,3 | | | + | | | | + |
| 78 | 3 | | | | | | | + |
| 80 | 5,5,2 | + | + | | | + | | |
| 82 | 2,5 | | | | | | + | + |
| 83 | 5,5 | | + | | | | + | |
| 84 | 5,5 | + | + | | | | | |
| 88 | 3,3,3,3,3,3 | | + | + | + | + | + | + |
| 89 | 3,3,4,4,3,3,4 | + | + | + | + | + | + | + |

*: The diagnostic criteria used for each test are explained in the Materials and Methods;

**: The visit numbers correspond to the visits when a buffalo was first found positive (+) by each test. For example, Buffalo 43 was first found positive by MHCT in Visit 4, MI in Visit 4 and IgM ELISA in Visit 2.

**Table 8.15** True incidence rates ($I$) of *Trypanosoma evansi* infection per animal-year at risk with associated 95% confidence intervals (CI) (in brackets) obtained using different diagnostic tests.

| Location | $I$ | | | | | |
|---|---|---|---|---|---|---|
| | MHCT/MI | 2G6 Ag-ELISA | Tr7 Ag-ELISA | IgM ELISA | IgG ELISA | CATT |
| Kebumen | 0.18 (0.04, 0.54) | 0.19 (0.04, 0.56) | 0.55 (0.24, 1.0) | 0.35 (0.11, 0.82) | 0.19 (0.04, 0.57) | 0.34 (0.11, 0.79) |
| Gejlig | 0.30 (0.10, 0.70) | 0.25 (0.07, 0.64) | 0.34 (0.11, 0.79) | 0.25 (0.07, 0.64) | 0.24 (0.06, 0.60) | 0.43 (0.17, 0.89) |
| Both villages | 0.24 (0.11, 0.48) | 0.22 (0.09, 0.45) | 0.44 (0.24, 0.76) | 0.30 (0.14, 0.56) | 0.22 (0.09, 0.44) | 0.39 (0.19, 0.65) |

## 8.4 Discussion

Buffaloes were blood sampled in 59 villages of five districts of Central Java to estimate the prevalence of *T. evansi* infections using the MHCT, MI and two Ag-ELISAs. In addition, the risk of *T. evansi* infections occurring in local buffalo populations was measured (i.e., true incidence rate) by a longitudinal study conducted in two of these villages. Currently, no gold standard is available for the detection of *T. evansi* infections (as distinct from clinical disease) in individual buffaloes and groups of buffaloes (Luckins, 1992). However, the diagnostic sensitivity and specificity of the two Ag-ELISAs used have been estimated, using both experimentally-infected and naturally-infected buffaloes and non-exposed buffaloes (data given in Chapters 6 and 7). Therefore, test prevalence values obtained in this study were corrected using these parameters to estimate true prevalence values.

The overall point estimates of test prevalence (i.e., uncorrected for sensitivity and specificity) were 4% (3, 5) by the MHCT, 9% (6, 12) by MI, 58% (56, 60) by 2G6 Ag-ELISA and 70% (68, 72) by Tr7 Ag-ELISA. Lower district-specific test prevalence values were obtained with the MHCT and MI (0-16%) than with the two Ag-ELISAs (39%-86%). In addition, there were significant differences between the district-specific prevalence values, for example the prevalence found in Brebes was higher than in Pekalongan or Pemalang ($P < 0.001$). The overall and district-specific test prevalence values were not corrected to demonstrate the range of values obtained directly by the different tests and because valid estimates of sensitivity for the MHCT and MI were not available. Although the sensitivities of these parasitological tests were estimated using experimentally-infected buffaloes (see Chapter 6), their estimation using naturally-infected buffaloes is problematic because of the difficulty of identifying truly positive buffaloes in the field. Previous studies that used parasitological tests also found low prevalence values of *T. evansi* infections in buffaloes and cattle sampled in other areas of Indonesia: 0-6.8% in seven districts of Kalimantan (Siswansyah and Tarmudji, 1989), 1% in three districts of Lombok Island (Utami 1993) and 3% in five areas of Java (Partoutomo *et al.*, 1994). One study found that 5.8% of buffaloes sampled in Central Java and 26% of buffaloes sampled in Lampung were MHCT-positive, but none of the buffaloes sampled on Sumba Island

or Kalimantan was MHCT-positive (Payne *et al.*, 1991c). However, much higher prevalence values were obtained when the sera were tested by an IgG ELISA (70%, Central Java; 87%, Lampung; 28.6%, Sumba Island; 10% Kalimantan).

Antigen-detection ELISAs have not been used before in large-scale surveys of *T. evansi* in Indonesia, and previous studies (using parasite-detection or antibody-detection tests) have not estimated prevalence in multiple villages within one district (or did not report village-specific prevalence data), but selected a limited number of sampling locations within an area. By contrast, in this study buffaloes were sampled in 8 to 17 villages per district. Corrected village-specific prevalence values (i.e., estimates of true prevalence) were found to range from 0 to 100%. However, the results obtained differed between the 2G6 Ag-ELISA and Tr7 Ag-ELISA. Of the 39 villages where buffaloes were tested by both Ag-ELISAs, only 15 villages had true prevalence estimates with an absolute difference of < 10%. In a few villages, the prevalence by the 2G6 Ag-ELISA varied markedly from the prevalence by the Tr7 Ag-ELISA; for example, the prevalence in Hardjosari was 17% by the 2G6 Ag-ELISA, but 68% by the Tr7 Ag-ELISA. Theoretically, correction of test prevalence values using valid estimates of sensitivity and specificity should eliminate differences between test prevalence values. The differences observed between the true village-specific prevalence values obtained by the two Ag-ELISAs may reflect the imprecision of the estimates of test parameters which could explain why corrected values outside the range 0 to 100% were found. Furthermore, the populations used in the estimation of sensitivity and specificity (described in Chapter 6) may not have been representative of the buffalo population tested in the prevalence studies.

There are several possible reasons for the variations found between village-specific prevalence values for *T. evansi* in the study areas. Age was shown to be a confounder: increasing prevalence was observeed with increasing age, in agreement with earlier studies (Payne *et al.*, 1991c; Partoutomo, 1993). To demonstrate the effect of age, the prevalence in Indrajaya (where 35% of buffaloes sampled were less than three years old) was adjusted for age and found to be lower (59%) after age-adjustment than without adjustment (74%). However, in most of the villages visited the majority

of buffaloes sampled were adult female buffaloes kept for draught power, although in a few villages (e.g., Indrajaya), younger buffaloes were kept for fattening. The number of buffaloes varied between villages, and the proximity of buffaloes to one another was dependent on different management practices. In the sampling locations the number of buffaloes owned by each farmer was usually between one to five which is similar to the number owned by farmers in other areas of Indonesia (Anon, 1986). Some buffaloes were kept in small groups in traditional stalls adjacent to their owner's house, but in many villages buffaloes were kept in large communal animal houses (*kandangs*) in groups up to 100 buffaloes and were grazed together. Although exercise did not increase parasitaemias in *T. evansi*-infected buffaloes in one study (Payne *et al.*, 1991a), the effect on antigenaemia is not known, and during working periods (one to six months of the year) buffaloes would be exposed to biting flies for longer periods during the day, and therefore potentially would have an increased challenge. Some buffaloes were moved to local villages to be rented by other farmers which could have exposed them to different strains of *T. evansi*. Many aspects of management may therefore have influenced the potential for transmission of *T. evansi* by biting flies between infected and susceptible buffaloes.

Furthermore, populations of biting flies (e.g., Tabanids and *Stomoxys* species) are dependent on seasonal factors and local habitats (Chainey, 1993); for example, in some areas teak plantations were reported by farmers to have high numbers of Tabanids which prevented buffaloes from grazing nearby at certain times of year. The *T. evansi* infection rates of *Tabanus rubidus* were shown to vary between different villages in West Java (Hartini and Aziz, 1991) and the efficiency of transmission differs between Tabanid species (Foil, 1989), but little information is available on the fly populations currently present in Indonesia. In addition, strains of *T. evansi* differ in their pathogenicity (Luckins, 1988), and newly purchased animals are a potential source of different strains; therefore, the rate of turnover of livestock in a village may influence prevalence. Parasitaemic buffaloes are a source of *T. evansi* to susceptible hosts, but the role of naturally-infected antigenaemic buffaloes that do not have a detectable parasitaemia is not known. However, antigenaemic cattle that were

experimentally infected with *T. brucei*, but did not have a detectable parasitaemia, were shown to be infective for tsetse flies (Masake *et al.*, 1995a).

When cattle and buffalo sera from Lombok Island were tested retrospectively by the 2G6 Ag-ELISA, prevalence values in both cattle and buffaloes differed significantly between the three districts ($P < 0.01$). By contrast with the prevalence data from Central Java, a higher prevalence was found in buffaloes in West Lombok (50%), where most of the buffaloes sampled were less than one year of age, than in buffaloes in Central Lombok (17%), where less than half the buffaloes sampled were less than one year of age. Previous estimates of prevalence obtained by testing the same sera by an IgG ELISA were 46% cattle and 57% buffaloes (Utami, 1993), but these estimates had not been corrected for the sensitivity and specificity of the test.

It was considered inappropriate to remove the effect of age as a confounder by standardization of all the prevalence data because estimates of true village-specific prevalence values were considered to be more meaningful measures of the occurrence of *T. evansi* infections in the buffalo populations sampled. Overall, of the buffaloes sampled up to one year of age, 33% were positive by the 2G6 Ag-ELISA and 22% were positive by the Tr7 Ag-ELISA compared with 62% (2G6 Ag-ELISA) and 54% (Tr7 Ag-ELISA) of the buffaloes sampled that were three to five years old. Although self-cure has been reported in experimentally-infected animals (Williams *et al.*, 1991; Onah, 1992), it has not been proven to occur in natural infections (Luckins, 1988). The age-specific prevalence values obtained here support the theory that buffaloes are likely to remain infected for many years after natural challenge (possibly with multiple re-challenge infections) and that infected buffaloes are able to survive with *T. evansi* infections long-term. However, there was a decrease in prevalence obtained by the Tr7 Ag-ELISA in buffaloes more than seven years of age. This may be explained by the lower detection of *T. evansi*-infections by the Tr7 Ag-ELISA in older buffaloes (e.g., due to a decline in detectable antigen in chronic infections) or by the removal of infected buffaloes from the population by premature culling or death. This latter explanation is not supported by the results obtained with the 2G6 Ag-ELISA: the highest prevalence was found by this Ag-ELISA in buffaloes more than seven years

old. Annual mean conversion rates for antigenaemia detected by both Ag-ELISAs decreased in the older age groups, which would suggest that antigenaemic buffaloes were being removed from the village populations, but the assumption that *T. evansi* infections produce a life-long antigenaemia for the calculation of these conversion rates may not be strictly valid. Fluctuations in antigenaemia have been observed in experimentally-infected buffaloes (see Chapter 4), but the situation in buffaloes under various degrees of natural challenge is not known.

Clustering of trypanosomosis appeared to occur in the sampling areas, suggested by the narrower confidence intervals calculated assuming simple random sampling, and this observation has important implications for future surveys. The between-cluster variance found in this study could be used to determine future sample size requirements. Many surveys are designed and conducted according to convenience and the financial restrictions imposed on sample collection, without consideration of the effect of inter-cluster and intra-cluster variation on the precision of the results (Smith, 1995). There were restrictions in this study on the selection of locations, but inter-village variation was acknowledged by calculation of confidence intervals for two-stage cluster samples.

During the one-year period of the field work, clinical signs of acute trypanosomosis (e.g., pyrexia and weakness) were observed only in a few buffaloes, but 19% of buffaloes had a PCV less than 26%, and 27% of buffaloes had a body score less than six, which are characteristic changes of chronic trypanosomosis and other debilitating diseases. Previous work has shown that *T. evansi* infections in buffaloes can be associated with lower PCVs (Payne *et al.*, 1991a) and body weight gain (Payne *et al.*, 1994b). Buffaloes that were found to be parasitaemic with a normal PCV (27%-35%) (Thahar *et al.*, 1983) and without a detectable antigenaemia probably had a recent infection. The mean PCV of parasitaemic buffaloes was lower than the mean PCV of non-parasitaemic buffaloes, and the mean PCV of antigenaemic buffaloes was also lower than the mean PCV of non-antigenaemic buffaloes. Although statistically significant, the magnitude of the differences was not considered to be of clinical significance particularly with respect to antigenaemia. Other diseases including

helminthosis, babesiosis, anaplasmosis and mineral deficiencies are known to adversely affect PCV. Fascioliosis is considered an important cause of low PCVs in adult buffaloes in Indonesia (J. Roberts pers. comm.). However, faecal samples collected from 30 buffaloes during Visit 5 were all negative, and *Babesia* spp. and *A. marginale* were not detected in blood smears taken from buffaloes with a low PCV.

In buffaloes, a poor body score (score four or less) is associated with reproductive problems and reduced work output (Winugroho and Teleni, 1993). In this study, 1.5% of buffaloes sampled had a body score of four or three, and of these buffaloes 91% were antigenaemic. However, this finding, and the association between *T. evansi* infections and PCV described above, need to be interpreted with caution because apparent correlations between group variables may differ from underlying individual animal correlations and aetiology; a concept known as 'ecological fallacy' (Piantadosi *et al.*, 1988). Furthermore, there was no difference between the median body scores of parasitaemic and non-parasitaemic buffaloes, and of antigenaemic and non-antigenaemic buffaloes. Nevertheless, such observations can highlight possible areas for future research which could include studies to evaluate the impact of intervention strategies on the health status of the target population (Kleinbaum *et al.*, 1982). For example, the mean PCV of naturally-infected Bali cattle in a feedlot increased after trypanocidal treatment (Payne *et al.*, 1994b) and the effect of treatment on the PCV and body score of buffaloes in the villages found with a high prevalence in this study could be investigated. Given that most buffaloes in Indonesia are affected by more than one disease, an integrated approach to future studies would be a logical procedure to determine the importance of different diseases.

The market surveys suggested that buffaloes infected with *T. evansi* were being traded locally, and therefore may represent an important source of infection for susceptible buffaloes in markets and in villages. The majority of buffaloes in the markets were adult buffaloes which are more likely to have *T. evansi* infections than younger buffaloes. A rapid turnover of buffaloes has been reported previously in West Java (Pearce, 1994) where they are kept principally for fattening and investment. By

contrast, in Central Java buffaloes tend to be kept longer because of their use for draught power, but they are sold if they are not required for work or if the farmer needs capital. Furthermore, farmers reported in Central Java that sick buffaloes are commonly sold within days (at a reduced price) because of the risk of substantial financial loss if they die, and this practice was reported to occur in West Java (Pearce, 1994). Although a lower prevalence was found with the 2G6 Ag-ELISA in the market buffaloes than in the buffaloes sampled in the villages, 10% of the market buffaloes were found to be parasitaemic, 39% antigenaemic, 56% positive by IgG ELISA and 47% positive by CATT. A higher proportion of the market buffaloes (48%) than the village buffaloes (19%) that were sampled had a low PCV, although most of the buffaloes in good condition and sick buffaloes were not taken to market, but were reported to be slaughtered locally at emergency abattoirs.

True incidence rates have not previously been determined for *T. evansi* infections in buffaloes, and rarely for other trypanosome infections. Higher incidence rates per animal-year at risk were obtained with the Tr7 Ag-ELISA (0.44 {0.24, 0.76}) and CATT (0.39 {0.19, 0.65}) than with the parasitological tests (0.24 {0.11, 0.48}), 2G6 Ag-ELISA (0.22 {0.09, 0.45}), IgM ELISA (0.30 {0.14, 0.56}) and IgG ELISA (0.22 {0.09, 0.44}). However, the estimates were imprecise due to the small sample sizes that were determined by practical constraints and, unlike prevalence values, these rates were not corrected to account for the differing sensitivity and specificity of the tests used. Within these limitations, the results suggest that on average buffaloes in the study population are likely to become infected within four years, if the incidence rate equals 0.25 per animal-year at risk. The calculated four-year cumulative incidence (0.59) was close to the prevalence obtained in buffaloes three to five years old (62%), using the 2G6 Ag-ELISA, but was higher (0.83) than the corresponding prevalence (54%), using the Tr7 Ag-ELISA.

Four buffaloes were also found to be infected with *T. theileri*, which is the only other trypanosome species that infects buffaloes in Indonesia, during the longitudinal study. Since *T. theileri* and *T. evansi* have common fly vectors, mixed infections with these trypanosomes can occur and were observed in a few buffaloes. Although none of

these four *T. theileri*-infected buffaloes was shown to be parasitaemic with *T. evansi*, two of the buffaloes were positive by Ag-ELISA or antibody-detection tests which are considered to be species-specific (Delafosse *et al.*, 1995). No other studies are known to have reported incidence rates for *T. evansi* infections in Indonesian livestock. However, imported Australian buffaloes were monitored in four areas of Java, and, by thirteen weeks after their arrival 31% of buffaloes (n = 45) were parasitaemic in one area, but only 25% were positive by an IgG ELISA, indicative of early infections. In Ethiopia, the cumulative incidence of *T. congolense* infections in cattle was reported to vary from 10.1% to 23.8% per year (Rowlands *et al.*, 1993), but the definition of a new infection used (i.e., 'an infection that was detected in an animal which was aparasitaemic and had a PCV > 26% in at least the two previous months') would have included a proportion of infected animals because only the BCT was conducted to detect *T. congolense* infections which has a low sensitivity, and no serological tests were used.

Incidence rates can accommodate populations of a dynamic nature (i.e., as in this study where buffaloes were not available for the full monitoring period). Although, cumulative incidence can be a more meaningful measurement than incidence rate because it can be interpreted at the level of the individual animal, it was not possible to calculate directly because of changes in the study population. Furthermore, the selection of truly negative buffaloes was difficult because of the high proportion of buffaloes that had already been exposed to *T. evansi* in the study locations.

By contrast with most earlier surveys, the precision of prevalence and incidence data was indicated here by the inclusion of associated 95% confidence intervals. However, the village-specific prevalence values were shown not to be Normally distributed and the selection of villages and individual buffaloes was not strictly random, therefore the confidence intervals should be regarded as approximate. During the study, buffaloes were sampled in only 5.8% of all villages in the five districts (i.e., the village sampling fraction was small). The study area in Central Java has a high density of buffaloes, which are kept in almost 80% of villages. The successful collection of large numbers

of blood samples was achieved through excellent cooperation with regional and district government veterinary officers, village leaders and farmers. Previous studies in Central Java reported problems in obtaining permission from farmers to blood sample their buffaloes (Payne *et al.*, 1991d) which severely restricted sample sizes. The selection of villages by local veterinary officers is likely to bias prevalence values. For example, villages considered to have a 'trypanosomosis problem' would have been favoured. However, many villages were visited on account of the high level of cooperation offered by farmers and village leaders. Other important factors that influenced village selection included their accessibility by road, the total number of village buffaloes and the type of housing (large numbers of samples could be rapidly collected from buffaloes in *kandangs*). When the owners of buffaloes were present at the time of sampling, the buffalo ages recorded were considered to be reasonably accurate (i.e., ± six months for adult buffaloes and ± one month for young buffaloes), but in some villages the owners were absent and ages were estimated by other farmers or local officials.

*Trypanosoma evansi* infections have been reported in buffaloes and cattle throughout most of Indonesia (Anon, 1993) and there is regular movement of livestock between islands. In some areas, only serological evidence of infection has been reported, and in surveys that employed different diagnostic tests much higher prevalence values were obtained using serological tests than with parasitological tests (Payne *et al.*, 1991c; Utami, 1993). In this study, the prevalence in buffaloes in five districts of Central Java was high (58% to 70%) as determined by Ag-ELISAs, and there was variation between the prevalence values found in different districts and villages. The results underlined the dependency of such studies on the sensitivity and specificity of the diagnostic tests that are used, particularly if uncorrected test prevalence values alone are reported.

The estimation of the prevalence and incidence of *T. evansi* infections in buffaloes is important for several reasons. Government veterinary services have limited budgets for animal health programmes; therefore diseases must be ranked in order of importance for allocation of finances for control programmes and research. In

Indonesia, the current decentralisation of veterinary services to regional level highlights the importance of information on *T. evansi* (and other diseases) at the local level. Prevalence estimation is an important component of control programmes, both in the initial stages to quantify the number of *T. evansi*-infected animals, and later to monitor the efficacy of control strategies. The risk of new *T. evansi* infections is pertinent to 'naive' animals imported from countries without *T. evansi* (e.g., Australia) and non-infected animals that are moved between islands within Indonesia. For example, buffaloes bred on Sumbawa Island are transported to Java and Irian Jaya, and Bali cattle are widely distributed by the government transmigration programme (Anon, 1986) which aims to relocate people from over-populated areas of Java to the outer islands and to provide them with livestock as a source of income. The monitoring of imported livestock with both parasitological and serological tests has previously been conducted (Payne *et al.*, 1991d) to identify new *T. evansi* infections for strategic treatment and to monitor the efficacy of prophylactic treatment with trypanocidal drugs. The finding of *T. evansi*-infected buffaloes in markets suggests that the strategic treatment of market buffaloes or newly purchased buffaloes may reduce the risk of introducing different strains of *T. evansi* and of outbreaks of clinical trypanosomosis. In addition, monitoring buffaloes at markets would be useful to assess the local movement of *T. evansi*-infected livestock. The spread of *T. evansi* during an outbreak of trypanosomosis on Madura Island, in which 50% of the buffaloes sampled were parasitaemic, was attributed to the introduction of horses from Java that became infected with *T. evansi* (possibly after their arrival) and were sold through a local market (Payne *et al.*, 1990). Therefore, surveillance of animals being sold through markets and targeted treatment of infected animals may be an effective strategy to reduce the likelihood of similar outbreaks. Applications of diagnostic tests for *T. evansi* are discussed further in the following chapter.

# CHAPTER 9

# GENERAL DISCUSSION

## 9.1 Introduction

*Trypanosoma evansi* is the most widely geographically distributed pathogenic trypanosome, infecting domesticated livestock in Asia, Africa and South America (Luckins, 1992). As yet, however, no standardised test with a high diagnostic sensitivity and specificity for *T. evansi* infections is available, and many of the diagnostic tests that have been used either for routine clinical diagnosis or in epidemiological studies have not been properly evaluated. Parasitological tests and antibody-detection tests have commonly been applied, but *T. evansi* is often not detectable in sub-acute or chronic infections; moreover, antibody-detection tests indicate exposure rather than current infection. Antigen-detection ELISAs, that are reported to be better indicators of current *T. evansi* infections, have been developed. Previous studies evaluated *T. evansi* Ag-ELISAs using camels in Africa (Nantulya *et al.*, 1989b; Waitumbi and Nantulya, 1993) and horses in South America (Monzon *et al.*, 1995), but these assays have not been fully evaluated with buffaloes, and furthermore Ag-ELISAs based on different monoclonal antibodies have not been compared. The objective of this study, therefore, was to evaluate two *T. evansi* Ag-ELISAs, in terms of their reliability and validity, using buffaloes in Southeast Asia where *T. evansi* is endemic and livestock are important for draught power, meat and investment. The Ag-ELISAs were standardised in the UK prior to their establishment in Indonesia and estimation of their diagnostic sensitivities and specificities using experimentally-infected and naturally-infected buffaloes. No gold standard is currently available for *T. evansi* infections. Therefore the Ag-ELISAs were compared with two parasitological tests (MHCT and MI) and three antibody-detection tests (IgM ELISA, IgG ELISA and CATT).

## 9.2 Standardisation and quality assurance of ELISAs

For standardisation, international guidelines for antibody-detection ELISAs were adapted for the Ag-ELISAs (Wright *et al.*, 1993). In particular, ODs were expressed

as a positivity index (percent positivity: PP) of the high positive control (C++) on each ELISA plate to minimise the effects of plate-to-plate and day-to-day variation. In addition, control limits were determined for the four Ag-ELISA controls (Cc, C-, C+ and C++) and used to accept or reject each plate. Although not used in this project, internal laboratory controls are increasingly being used to monitor ELISA performance with computer software packages. For example, in Canada a commercial quality control computer programme was used to monitor an antibody-detection ELISA for *Brucella abortus* (Wright, 1987) and in Thailand Shewhart-CUSUM control charts were used for hog cholera and foot and mouth disease virus Ag-ELISAs (Blacksell *et al.*, 1996). Blacksell and colleagues suggested that at least ten tests should be conducted to provide reference control data. With the *T. evansi* Ag-ELISAs in this study, six replicate tests were conducted to establish control limits, and less than 5% of subsequent test plates were rejected using these limits. ELISA data are commonly analysed using parametric statistics, but ELISA data frequently do not have a Normal distribution even after log-transformation. Non-parametric analysis is therefore more appropriate. For this reason, the 90th and 10th percentile values of the *T. evansi* Ag-ELISA data were selected as the upper and lower control limits, respectively.

Quality control is an essential feature of ELISAs because of their inherent variability, and provides confidence in the results obtained. Sources of error can be random (e.g., dilution errors) or systematic (e.g., degradation of reagents). Various measures have been recommended to minimise such variation: 1) the use of large reagent batches stored under optimal conditions; 2) technical training of operators; 3) good laboratory management (e.g., to avoid contamination of reagents). The *T. evansi* Ag-ELISAs were shown to have a high repeatability, and comparable results were obtained with sera from blood samples either processed in the laboratory or under field conditions. In medical diagnostics, assessment of the repeatability and robustness of ELISAs is commonplace (Kemeny, 1991), but rarely are these aspects of test performance reported for veterinary diagnostics, and whilst human diagnostics are subject to the European Union Medical Devices Directive, veterinary diagnostics are not (Tribe, 1997).

## 9.2 Determination of cut-off values

The categorisation of animals as positive or negative by Ag-ELISAs (and other tests) is dependent on the cut-off value chosen. To establish this value, a frequency distribution of results from positive and negative animals is commonly plotted and a cut-off value is chosen that gives acceptable proportions of false-negative and false-positive results for the purpose of the test (Wright *et al.*, 1993). Conventionally, cut-off values can be calculated from the mean of a negative population plus two or three standard deviations, or twice the negative mean value (de Savigny and Voller, 1980). Cut-off values of 20 PP and 30 PP were chosen empirically for the two *T. evansi* Ag-ELISAs as the values that gave the best estimates of sensitivity and specificity, and the 20 PP cut-off value was used for the epidemiological studies.

If the negative population used to establish a cut-off value is not representative of the target population, lower than optimal cut-off values may be selected. For example, 'conventional' cut-off values obtained from non-exposed dogs, cattle and horses in Germany for a *T. evansi* Ag-ELISA and antibody-detection ELISA were much lower than cut-off values derived by a computer-assisted analysis of mixtures (Franke *et al.*, 1994). This analysis is performed by a computer programme (C.A.MAN) that models the observed distribution of ELISA values in the test population and selects an 'intrinsic cut-off value' that differentiates between subpopulations of low and high responders, without the need to use a non-endemic population. For example, 18.6% of dogs sampled in Brazil were positive by *T. evansi* antibody-detection ELISA using a C.A.MAN-derived cut-off value compared with 51.4% using a 'conventional' cut-off value (mean + 3SDs) (Franke *et al.*, 1994). Another method is receiver operating characteristics analysis (ROC) which graphically presents the effect of different cut-off values on sensitivity and specificity; ROC was used in the evaluation of antibody-detection tests for brucellosis (Nielsen *et al.*, 1996a).

An advantage of using two-level cut-off values that incorporate an inconclusive band between positive and negative thresholds (e.g., <20 PP: negative; 20-30 PP: inconclusive; >30 PP: positive) is that either the higher or lower cut-off value can be

chosen by the test-user to maximise either specificity or sensitivity, respectively. Cut-off values should always be stated with test data because of the dependency of the results on these values, but are frequently omitted. For example, 96% of 1448 cattle tested in Indonesia were found to be positive by a *B. bovis* antibody-detection ELISA, but no cut-off value or estimates of sensitivity or specificity of the test were given (Sukanto *et al.*, 1993).

### 9.3 Estimation of diagnostic sensitivity and specificity

To estimate the diagnostic sensitivity and specificity of a test, randomly selected infected and uninfected animals should be tested from the target population. However, as discussed in earlier chapters, the identification of *T. evansi*-infected and uninfected buffaloes in Indonesia is problematic. Therefore, sensitivity was estimated using experimentally-infected buffaloes and parasitaemic, naturally-infected buffaloes, whereas specificity was estimated using non-exposed British cattle and Australian buffaloes. The experimentally-infected buffaloes were positive by the MHCT on fewer occasions than by the Ag-ELISAs or antibody-detection tests; the sensitivity of the MHCT is limited by the level of parasitaemia, and the lowest detectable level was shown to be $5 \times 10^2$ trypanosomes per ml blood (Paris *et al.*, 1982). Of 240 weekly samples from the 30 buffaloes with the secondary infection, the proportions found to be positive were 32% (26, 38) by MHCT, 55% (47, 62) by MI, 54% (47, 62) by the 2G6 Ag-ELISA, 76% (69, 82) by the Tr7 Ag-ELISA, 54% (47, 62) by IgM ELISA, 82% (76, 87) by IgG ELISA and 76% (69, 82) by CATT. However, these proportions were affected by the number of buffaloes that were test-positive prior to infection; for example, more buffaloes were positive pre-infection by the Tr7 Ag-ELISA and IgG ELISA than by the other tests. The higher sensitivities of the Ag-ELISAs compared with the MHCT are in agreement with the findings of two previous studies. In one study, seven cattle were experimentally infected with *T. congolense* (82.5% positive by Ag-ELISA and 19.7% by MHCT; Masake and Nantulya, 1991), and in another study four cattle were experimentally infected with *T. brucei* (81.1% positive by Ag-ELISA and 16.3% by BCT; Masake *et al.*, 1995a).

By comparison, using the 139 naturally-infected Indonesian buffaloes, the diagnostic sensitivity of the Tr7 Ag-ELISA was 81% (75, 88) which was significantly higher than the sensitivity of the 2G6 Ag-ELISA (71% {63, 79}), and the IgG ELISA sensitivity was 89% (75, 88) which was significantly higher than the sensitivities of the IgM ELISA or CATT which were both 78% (72, 85). These sensitivity estimates from parasite-positive buffaloes correspond to the estimates of studies that used MHCT-positive camels (89% antigenaemic: Waitumbi and Nantulya, 1993), MI-positive camels (83.3% antigenaemic: Diall *et al.*, 1992) or horses shown to be naturally-infected with *T. evansi* by blood smear, MHCT or MI (74% antigenaemic: Monzon *et al.*, 1995). In another study using different Ag-ELISAs based on species-specific monoclonal antibodies for tsetse-transmitted trypanosomes, 75% of 144 MHCT-positive cattle were antigenaemic (Bengaly *et al.*, 1995), but the proportion of trypanosome species found by the parasitological tests did not correspond to the Ag-ELISA results. With cattle in Uganda, Ag-ELISAs also indicated a higher proportion of mixed trypanosome infections compared with the BCT used (Okuna *et al.*, 1993), suggesting that cross-reactions between heterologous monoclonal antibodies and trypanosomes occurred, if mixed infections were not missed by the parasitological tests. The buffaloes sampled in Central Java potentially would have been exposed to multiple challenges, possibly with different strains of *T. evansi*, and have had infections of varying duration. Although testing naturally-infected buffaloes may have been preferable to experimentally-infected buffaloes to estimate sensitivity, the selection of only parasiteamic buffaloes would have biased the results because the full range of infected buffaloes in the population was not included (Martin, 1984). The use of parasitaemic animals only for the *T. evansi* Ag-ELISAs may underestimate sensitivity because parasitaemias are often detectable in early infections prior to the appearance of antigens in the circulation, whereas the use of experimentally-infected animals may overestimate sensitivity if later stages of infection are not monitored when antigen responses may decline.

Several problems were encountered in the estimation of sensitivity using the experimentally-infected buffaloes. Although only MHCT-negative buffaloes were purchased, some buffaloes were found to be parasitaemic after their arrival at Balitvet, and in some buffaloes serum antigens and antibodies persisted after Cymelarsan treatment. It was not possible to screen buffaloes with the Ag-ELISAs or antibody-detection tests prior to purchase and, even with screening, it would have been difficult to identify 35 non-exposed buffaloes because of the high prevalence of *T. evansi* infections. However, the buffaloes used for the experimental infections were considered to be representative of the target population. Important findings were: 1) the low sensitivity of the MHCT compared with the other tests; 2) some infected buffaloes were found to be parasitaemic on one or two occasions only; 3) in contrast to other studies (Masake *et al.*, 1995a), parasitaemias did not always precede antigenaemia; 4) there was marked individual variation between buffaloes in parasitaemia, antigen and antibody profiles; 5) antigen and antibody responses fluctuated during infection (sometimes below the cut-off values); and 6) antigen and antibody responses persisted after treatment in some buffaloes. Persistence of antigenaemia up to 30 days after treatment was reported in 20% of treated camels (Waitumbi and Nantulya, 1993). Serum antigen may persist if trypanosomes are present in 'cryptic' tissue sites which are not penetrated by trypanocides or if there is inadequate clearance of serum antigen from the peripheral circulation by the host's immune system.

In contrast to previous studies, a significant proportion of sera from the non-exposed cattle and buffaloes gave false-positive reactions with both the *T. evansi* Ag-ELISAs. Aparasitaemic camels in two non-endemic areas of Africa (Nantulya *et al.*, 1989b; Diall *et al.*, 1992), non-exposed cattle in Germany (Bengaly *et al.*, 1995) and aparasitaemic horses in a non-endemic area of Argentina (Monzon *et al.*, 1995) were all negative by *T. evansi* Ag-ELISAs. In the present study, only with the CATT were no false-positive results found (100% specificity). The specificity of the Tr7 Ag-ELISA was found to be the same with the British cattle and Australian buffaloes (78% {73, 83}), whereas the specificity of the 2G6 Ag-ELISA was found to be higher with the British cattle (83% {83, 94}) than the Australian buffaloes (75% {70, 80}).

The cattle sera had been collected more recently and were of better quality than the buffalo sera, and contaminant bacterial enzymes in the latter may have interfered with the 2G6 Ag-ELISA (Spencer, 1988). Although the collection and storage conditions of the buffalo sera were unknown, these sera were from the target animal species.

Although false-positive results have not previously been considered a problem with *T. evansi* Ag-ELISAs, these reactions are known to occur in other parasite ELISA systems (Venkatesan and Wakelin, 1993). Tween 20 was the only blocker included in the two *T. evansi* Ag-ELISAs because the addition of other blockers (e.g., normal mouse serum, bovine serum albumin, ovalbumin) did not reduce the proportion of false-positive results to an acceptable level, but did adversely affect sensitivity. False-positive results can be caused by either specific binding of assay reactants (e.g., with homologous antigens) or by non-specific binding that can involve non-immunological or immunological reactions (Kemeny and Challacombe, 1988). Heterophilic antibodies have been reported to interfere with Ag-ELISAs based on monoclonal antibodies by cross-linking capture and detector monoclonal antibodies (Boscato and Stuart, 1988). Furthermore, naturally-occurring antibodies, principally of the IgM isotype, that react with trypanosomes and other protozoa can give false-positive reactions in antibody-detection tests by their recognition of coating antigen (Konishi, 1993). Future work with the two *T. evansi* Ag-ELISAs could investigate the role of non-specific binding versus specific binding in the false-positive reactions; for example, by the addition of coating monoclonal antibody in the serum diluent to block specific reactions.

Negative test results for *T. evansi*-infected animals have been reported previously with *T. evansi* Ag-ELISAs and were observed in this study. Several explanations for these false-negative results have been proposed, for example: 1) serum antigen is not available for binding because it is bound within immune complexes (Masake *et al.*, 1995a); 2) the amount of serum antigen, which is related to the number of dying trypanosomes in the circulation, may be low in early infections (Nantulya and Lindqvist, 1989; Waitumbi and Nantulya, 1993); 3) some animals may be falsely identified as infected by parasitological tests (e.g., by mis-identification of

other trypanosomes such as *T. theileri*); or 4) idiotypic antibodies that recognise determinants of the Ag-ELISA monoclonal antibodies may interfere with antigen-binding (Pesce and Michael, 1988). In an *Onchocerca gibsoni* Ag-ELISA, sensitivity was increased by boiling sera with sodium EDTA to dissociate antigen-antibody complexes (More and Copeman, 1991).

### 9.4 Agreement between diagnostic tests

Test agreement (*kappa* value) was calculated as an indicator of test validity because of the lack of a gold standard for *T. evansi* infections (Thrusfield, 1995). Moderate agreement was found between the IgG ELISA and CATT, but only slight to fair agreement between the two Ag-ELISAs, and between the Ag-ELISAs and the antibody-detection tests, a finding similar to another study that compared a *T. evansi* Ag-ELISA and IgG ELISA (Franke *et al.*, 1994). However, *kappa* values depend on the prevalence of infection as well as diagnostic sensitivity and specificity, and low *kappa* values are obtained with either high or low prevalence values (Thompson and Walter, 1988). In this study, the prevalence was not close to either 0 or 100%, but underlying differences in the profiles of antigenaemia (found in the experimentally-infected buffaloes) and the observation that different buffaloes gave false-positive results by each Ag-ELISA may have contributed to the low test agreement. Furthermore, high concordance between tests can occur even with low *kappa* values (Feinstein and Cicchetti, 1990) and *kappa* values can be adjusted to account for bias and prevalence (Byrt *et al.*, 1993).

### 9.5 Predictive values and calculation of true prevalence

Estimation of diagnostic sensitivity and specificity enables confidence in the accuracy of positive and negative test results to be assessed by calculation of predictive values, and also calculation of true prevalence. With the *T. evansi* Ag-ELISAs, true prevalence was underestimated at test prevalence values above 50% (lack of sensitivity) and overestimated at test prevalence values lower than 50% (lack of specificity). With theoretical test prevalence values 10% to 90%, the positive predictive values were 24%-96% for the 2G6 Ag-ELISA, 29%-97% for the Tr7 Ag-ELISA, 44%-98% IgM ELISA, 55%-99% IgG ELISA and were all 100% for the

CATT. Predictive values are probability values that are a function of prevalence as well as the sensitivity and specificity of a test, and therefore indicate how well a test will function in different populations (Martin, 1984). Predictive values outside the permissible range 0 to 100% were obtained, suggesting that the estimates of sensitivity and specificity were not entirely accurate, possibly because of differences between the positive and negative populations used in their estimation and the target population. A disadvantage of predictive values is that the sensitivity and specificity of a test are unstable and related to the strength of the test signal (e.g., a high OD is more likely to be positive than an OD near the cut-off value). For this reason, the use of likelihood ratios has been advocated, where the positive likelihood ratio describes the post-test odds of disease relative to the pre-test odds of disease, if a positive result is obtained (Gambino, 1989). Therefore, likelihood ratios are more stable indicators of test performance, particularly for ELISA systems, which are not affected by prevalence (Smith, 1995).

### 9.6 *Trypanosoma evansi* infections in buffaloes in Indonesia

*Trypanosoma evansi* was first identified in Indonesia in a horse in Central Java (Penning, 1900) and outbreaks of surra occurred in this province in 1968-1969 in which 1,870 buffaloes, 783 cattle and 22 horses died (Adiwinata and Dachlan, 1969). Over the past 30 years, no studies have estimated the prevalence of *T. evansi* infections in multiple villages within one district, or the true incidence rate and none has reported prevalence values with associated precision. Furthermore, this study was the first to employ two *T. evansi* Ag-ELISAs in large-scale studies in Southeast Asia. Overall, only 4% of the buffaloes sampled were positive by the MHCT and 9% by MI compared with 58% by the 2G6 Ag-ELISA and 70% by the Tr7 Ag-ELISA. Payne and colleagues (1991c) also found fewer positive buffaloes by MHCT (5.8%) than with an antibody-detection ELISA (48%). Prevalence varied between the five districts and between villages visited in Central Java. True village-specific prevalence ranged from 0 to 100% and there were marked differences between the prevalence values obtained by the two Ag-ELISAs. In addition, prevalence in cattle and buffaloes was found to vary between districts of Lombok Island.

Clustering of *T. evansi* infections in buffaloes was suggested by the wider 95% confidence intervals of prevalence values obtained by cluster analysis compared with those obtained by simple random sample analysis, and by the significant differences found between village-specific prevalence values. Many factors may contribute to clustering of trypanosomosis, for example the proximity of infected and susceptible buffaloes (in animal houses or when grazing) and the duration of exposure to local biting fly populations (e.g., during work periods). Although the non-random selection of the sampling locations by local veterinary services may have biased the results, it is known that Tabanid species differ in their ability to transmit *T. evansi* (Foil, 1989) and *T. evansi* infection rates of Tabanids vary between villages on Java (Hartini and Aziz, 1991). Clustering of trypanosomosis was also shown to occur in Colombia, where the prevalence of *T. vivax* infections in cattle varied between 0-24% by parasitological tests, and, as well as incidence, varied between the herds sampled (Otte *et al.*, 1994). In the latter study, multiple logistic regression demonstrated that *T. vivax* infections were associated with low-lying swampy areas (odds ratio 3.7) and fly populations (odds ratio 1.6). However, *T. vivax* infections would have been missed because only the MHCT and MI were used, and therefore it is likely that a significant proportion of infected cattle would have been wrongly categorised as uninfected.

Otte and colleagues (1994) also calculated the cumulative infection rate of calves from birth to 10 months of age which ranged from 0 to 74%, and in another study in Ethiopia the cumulative incidence of *T. congolense* infections in cattle were found to range from 10.1% to 23.8% by BCT (Rowlands *et al.*, 1993). By contrast, true incidence rates of *T. evansi* infections in adult buffaloes in Central Java were found to be 0.22 (0.09, 0.45) to 0.44 (0.24, 0.76) per animal-year at risk, depending on the diagnostic test. Although true incidence rates of *T. evansi* infections in Indonesia have not previously been calculated, 31% of buffaloes imported into Central Java were found to be parasitaemic during the first 13 weeks after arrival (Payne *et al.*, 1991d) and five out of nine buffalo calves became infected during the first 12 months of life (Payne *et al.*, 1991b). As found in earlier studies, the prevalence of *T. evansi* infections was found to be age-dependent (Payne *et al.*, 1991c; Partoutomo, 1993) and calculation of seroconversion rates for the 2G6 Ag-ELISA data suggested that

infected buffaloes were not being removed from the study population. However, infected buffaloes may have been traded within this population, for example the premature sale of sick, *T. evansi*-infected buffaloes.

The surveys of local markets provided important information on the *T. evansi*-infection status of buffaloes being traded in the five districts visited. Overall, 10% of the market buffaloes sampled were parasitaemic and 39% were antigenaemic. Although abattoir surveys have been conducted (Rukmana, 1979; Suhardono *et al.*, 1985), this is the first known report of market surveys in Indonesia. The findings suggest that market buffaloes are a potential source of *T. evansi* and these buffaloes should be targeted for trypanocidal treatment to reduce the risk of introducing different strains of *T. evansi* into village buffalo populations.

### 9.7 Evaluation of *Trypanosoma evansi* diagnostic tests

The evaluation of diagnostic tests is important prior to their application to: 1) give confidence in test results; 2) provide test performance data on which to base selection of an appropriate test for a particular application; and 3) enable results from different laboratories to be compared. This study has examined the methodology of test evaluation and has highlighted problems encountered in the evaluation of diagnostic tests for *T. evansi*, many of which are relevant to other animal diseases. Several sources of bias exist in the evaluation of diagnostic tests (Smith, 1995) and would have been present in this study. Firstly, parasitological tests were used to identify buffaloes naturally infected with *T. evansi*, and therefore relative rather than absolute estimates of sensitivity were obtained. Secondly, the experimental buffaloes and Australian buffaloes would not have been fully representative of buffaloes in Southeast Asia (the target population). Thirdly, the infection status of the experimentally-infected buffaloes and Australian buffaloes was known to the test-operator, although samples from buffaloes sampled in Central Java were coded and test-operators were not aware of other test results.

## 9.8 Evaluation of ELISAs for other animal diseases

The problems that were encountered in the evaluation of the *T. evansi* diagnostic tests, as discussed above, are common to evaluation studies for other diagnostic tests. In particular, the definition of the target population and the selection of sufficient numbers of animals that are representative of this population are major sources of bias (Smith 1995). Ideally, the health status of animals should be determined by an established test based on different biological parameters (e.g., comparison of a serological test with a parasitological test or culture), but these tests will not be capable of detecting all truly infected animals and moreover, a newly developed test would be expected to be more sensitive than an established test (Martin 1977). The estimation of specificity using animals in endemic areas that are negative by multiple tests is likely to underestimate its true value, but animals living in non-endemic areas may be less representative. A study to evaluate different assays for detecting *Mycobacterium bovis* infections in Australian cattle exemplifies these limitations: 1) sensitivity estimates were based on only 22 (out of 1362) cattle that were culture-positive and 2) the test specificities may have been underestimated because the cattle were from infected herds and not all infected cattle would have been detected by culture (Wood *et al.*, 1992).

It is likely that after evaluation tests may be applied to different populations (i.e., the target population may change) and this can affect the sensitivity and specificity of the test. For example, cross-reacting organisms may be present that give false-positive results or inherent characteristics of the population may be different. For example, the sensitivity of a *M. bovis* antibody-detection ELISA was found to be higher in male than female badgers (Clifton-Hadley et al. 1995), possibly due differences in their humoral responses.

For further comparison, the evaluation and application of ELISAs for *Brucella abortus* (Nielsen *et al.*, 1996a), *Anaplasma marginale* (Nielsen *et al.*, 1996b) and *Babesia bovis* (de Echaide *et al.*, 1995) will be considered. *Brucella abortus* and *A. marginale* have been eradicated from Canada, but animals continue to be tested for import/export regulations and by government surveillance programmes to enable the

rapid implementation of control programmes in the event of new infections occurring. For this purpose, diagnostic tests that have a high sensitivity and specificity are required that can assay large numbers of samples quickly and economically. Evaluation of antibody-detection ELISAs for *Br. abortus* and *A. marginale* have been conducted to support the replacement of established tests including the CFT which is used as a confirmatory test (see Table 9.1). As with *T. evansi*, the identification of naturally-infected animals is difficult because low parasitaemias are characteristic of *A. marginale* and *B. bovis* infections, and *Br. abortus* is difficult to culture. In these studies, positive cattle comprised naturally-infected cattle shown to be positive by blood smear examination (*A. marginale* and *B. bovis*) or IFAT (*B. bovis*) or culture (*Br. abortus*) and experimentally-infected cattle (*A. marginale* and *B. bovis*) and vaccinated cattle (*A. marginale*). However, as with the *T. evansi* studies, these positive cattle were only representative of a sub-population of infected cattle within the target population. Precise estimates of sensitivity and specificity were obtained because large numbers of cattle were tested, and separate estimates of specificity were reported for the *Br. abortus* and *A. marginale* ELISAs that were obtained after re-testing false-positive sera. With the *T. evansi* Ag-ELISAs, a proportion of false-positive sera were retested in the blocking studies and consistently gave positive PP values, but not all the false-positive sera were re-tested. As with the *T. evansi* Ag-ELISAs, the specificity of the *B. bovis* ELISA was considered to be low.

**Table 9.1** Recent publications on the evaluation of ELISAs for the diagnosis of *Brucella abortus*, *Anaplasma marginale* and *Babesia bovis*

| ELISA(s) | Number of animals | | Sensitivity* (%) | Specificity* (%) | Reference |
|---|---|---|---|---|---|
| | Positive | Negative | | | |
| Indirect *B. bovis* ELISA | 500 | 500 | 98 (96, 99) | 95 (93, 97) | de Echaide *et al.*, 1995 |
| Indirect and competitive *Br. abortus* ELISAs | 424 | 15,716 | 98.6-100** | 95.9-99.9** | Nielsen *et al.*, 1996a |
| Indirect *A. marginale* ELISA | 1726 | 1842 | 87.3 (85.7, 88.9) | 98.4 (97.8, 99.0) | Nielsen *et al.*, 1996b |

*: 95% confidence intervals given in brackets for *A. marginale* and *B. bovis* ELISAs
**: Range of estimates obtained for the types of ELISAs employed

## 9.9 Applications of *Trypanosoma evansi* diagnostic tests

Diagnostic tests for *T. evansi* have many potential applications, for example as screening tests in surveillance or control programmes, as clinical diagnostic tests or as epidemiological research tools, and therefore have an important role in decision-making processes. In Indonesia, the current decentralisation of government veterinary services suggests that provincial and district veterinary services will need to assess the importance of animal diseases locally to rationalise the allocation of limited financial resources. Therefore, properly designed and conducted studies are required to estimate disease morbidity and mortality rates and associated economic losses, at both local and national levels. Internationally, the Office International des Epizooties (OIE) is promoting the standardisation of animal disease diagnosis to: 1) improve the quality of data collected on disease occurrence; 2) standardise research results; 3) improve the control of animal diseases; and 4) harmonise import/export regulations (Reichard, 1992).

The application of Ag-ELISAs to monitor control programmes for tsetse-transmitted trypanosomoses has been reported in Uganda (Okuna *et al.*, 1993) and Zanzibar (Mbambo and Mpokwa, 1993). In both studies parasitological tests (BCT or MHCT, respectively) were used in parallel with Ag-ELISAs, and in areas with effective control measures no cattle were found to be parasite-positive several months later, but 9.8-14.3% of the cattle remained antigenaemic. The reasons for these findings were suggested to be drug resistance or inadequacies of control measures. However, it is likely that some of the positive results were false-positives, but no other confirmatory tests were conducted. In Indonesia, the evaluation of *T. evansi* tests has demonstrated that, whilst the two *T. evansi* Ag-ELISAs could be usefully applied in high prevalence areas, antibody-detection tests (in particular, the IgG ELISA or CATT) would be more appropriate to test buffaloes in low prevalence areas or to confirm the negative-status of buffaloes prior to export or movement within Indonesia. However, the risk of missing disease in test-negative animals depends not only on the prevalence of disease and test sensitivity, but increases with the number of animals tested (Marchevsky *et al.*, 1989). The *T. evansi* Ag-ELISAs should be used to test groups of animals rather than individual animals, whereas the

high positive and negative predictive values of the CATT for a wide range of prevalence values suggest that this test could be used to test individual buffalo, for example prior to costly trypanocidal treatment. An advantage of the CATT is that the test could be conducted in the field, but the interpretation of the results is subjective and larger numbers of samples are less readily tested than by ELISA.

In Indonesia, there are no current large-scale government surveillance or control programmes for *T. evansi.* However, cattle and buffaloes are screened by blood smear examination before movement between islands, for example for distribution in transmigration programmes, and in the past parasitological and serological tests have been used to monitor imported buffaloes (Payne *et al.,* 1991d) and outbreaks of clinical trypanosomosis (Payne *et al.,* 1990). Trypanosomosis is ranked as the eleventh most economically important animal disease by the Government of Indonesia (Soehadji, 1994) and associated economic losses have been estimated to be US$ 22.4 million (total loss = {mortality rate x mean body weight x price/kg x population at risk} + {morbidity rate x weight loss x mean body weight x price/kg x population at risk}) (Ronohardjo *et al.,* 1986). However, this calculation does not include other costs of *T. evansi* infections (e.g., the financial loss of individual farmers who sell sick animals at lower market prices, reduced work output or fertility or cost of trypanocides), many of which are difficult to estimate and for which no accurate data exist. Furthermore, the assumption is made that all Indonesian cattle and buffaloes are at risk of *T. evansi* infection which, if not valid, would greatly overestimate total losses due to the size of livestock populations. Data on case-fatality rates and morbidity are not available for most areas of Indonesia (Anon, 1986) or have been obtained by parasitological tests which would underestimate these values because of their low diagnostic sensitivity.

The calculation of national losses considers the loss to the public sector whereas the potential loss to individual farmers (who on average own two to four buffaloes) should also be recognised. A farmer survey in West Java found that the average loss due to the sale of sick buffaloes was equivalent to 10% of the annual income of some farmers (Pearce, 1994). Although only a few buffaloes were observed with clinical

signs of trypanosomosis during field work in Central Java, a wide range of clinical signs are known to occur in *T. evansi*-infected buffaloes (Damayanti, 1991) which may lead to the premature sale of buffaloes.

## 9.10 Conclusion

Diagnostic tests for *T. evansi* with a high sensitivity and specificity are prerequisites for future studies on the epidemiology of surra and the development and monitoring of strategic control programmes. As yet, no gold standard is available, but the *T. evansi* Ag-ELISAs and antibody-detection tests employed in this study have many advantages over commonly used parasitological tests in terms of their diagnostic sensitivity and ability to rapidly test large numbers of samples. Multiple testing strategies could be used to increase either sensitivity or specificity, for example serial testing with Ag-ELISAs and antibody-detection tests to increase specificity. Further modification of the *T. evansi* Ag-ELISAs is required to improve their specificities and to evaluate their use as clinical diagnostic tests. The Tr7 Ag-ELISA had a significantly higher sensitivity than the 2G6 Ag-ELISA, but specificity estimates of the two Ag-ELISAs were similar, and the possibility of cross-reactions with *T. theileri* with the Tr7 Ag-ELISA needs to be further investigated. The estimates of sensitivity and specificity obtained in this study were used to determine predictive values which indicate the probability of that test-positive or test-negative buffaloes are truly infected or uninfected with *T. evansi*, respectively, and to calculate true prevalence values. The selection of diagnostic tests for *T. evansi* depends not only on test validity parameters, but also on the prevalence of *T. evansi* in the test population, the main purposes of the testing and practical considerations.

## REFERENCES


Adiwinata, R.T. and Dachlan, A., 1969. A brief note on surra in Indonesia. ELVEKA, Folia Veterinariae, 3: 10-15.

Akol, G.W.O. and Murray, M., 1983. *Trypanosoma congolense*: Susceptibility of cattle to cyclical challenge. Experimental Parasitology, 55: 386-393.

Anon, 1986. A review of the livestock sector in the Republic of Indonesia. Volumes 1 and 2. Winrock International Institute for Agricultural Development, USA.

Anon, 1989. Buku Statistik Kesehatan Hewan (Book of Livestock Statistics). Direktorat Jenderal Peternakan, Jakarta.

Anon, 1990. Annual report of the Research Institute for Veterinary Science (Balitvet), Bogor, Indonesia, 1989/1990, pp. 20-22.

Anon, 1993. Bulletin Epidemiologi Veteriner. Direktorat Bina Kesehatan Hewan, Direktorat Jenderal Peternakan, Jakarta. pp. 16, 28.

Anon, 1994. Report of the 15th Annual Meeting of the OIE *ad hoc* Group on Non-Tsetse-Transmitted Animal Trypanosomoses (NTTAT), L. Touratier (Editor), held in Paris, France, 18th May, 1994, at the OIE Headquarters.

Anon, 1995. Animal Health Year Book 1994, FAO-OIE-WHO. Food and Agriculture Organization, Rome.

Armitage, P. and Berry, G., 1994. Statistical Methods in Medical Research. Third edition. Blackwell Science Ltd., Oxford.

Ashkar, T. and Ochilo, M., 1972. The application of the indirect fluorescent antibody test to samples of dried sera and blood from cattle in the Lambwe Valley, South Nyanza, Kenya. Kenya Bulletin, WHO, 47: 769-772.

Authie, E., Duvallet, G., Robertson, C. and Williams, D.J.L., 1993a. Antibody responses to a 33kDa cysteine protease of *Trypanosoma congolense*: relationship to 'trypanotolerance' in cattle. Parasite Immunology, 15: 465-474.

Authie, E., Muteti, D.K. and Williams, D.J.L., 1993b. Antibody responses to invariant antigens of *Trypanosoma congolense* in cattle of differing susceptibility to trypanosomiasis. Parasite Immunology, 15: 101-111.

Bailey, N.T.J., 1981. Statistical Methods in Biology. Second edition. Hodder and Stoughton, London.

Bajyana-Songa, E., Hamers, R. and Songa, E.B., 1988. A card agglutination test (CATT) for veterinary use based on an early VAT RoTat 1/2 of *Trypanosoma evansi*. Annales de la Societe Belge de Medecine Tropicale, 68: 233-240.

Bajyana-Songa, E., Hamers-Casterman, C., Hamers, R., Pholpark, M., Pholpark, S., Leidl, K., Tangchaitrong, S., Chaichanopoonpol, I., Vitoorakool, C., Thirapatsakum, T. and Songa, E.B., 1987a. The use of the card agglutination test (Testryp CATT) for the detection of *Trypanosoma evansi* infection: a comparison with other trypanosomiasis diagnostic tests under field conditions in Thailand. Annales de la Societe Belge de Medecine Tropicale, 67: 137-148.

Bajyana-Songa, E., Kageruka, P. and Hamers, R., 1987b. The use of the card agglutination test (Testryp CATT) for the serodiagnosis of *Trypanosoma evansi* infection. Annales de la Societe Belge de Medecine Tropicale, 67: 51-57.

Basu, B.C., 1945. Distribution and seasonal incidence of surra in India. Indian Journal of Veterinary Science, 15: 277-280.

Bengaly, Z., Kanwe, A.B. and Duvallet, G., 1995. Evaluation of an antigen-detection ELISA for the diagnosis of trypanosomiasis in naturally infected cattle. Tropical Medicine and Parasitology, 46: 284-286.

Bennett, S.C.J. and Kenny, P.A.C., 1928. Mercuric chloride as a diagnostic agent for trypanosomiasis in camels. Journal of Comparative Pathology and Therapeutics, 40: 341-353.

Bishop, S., 1992. The use of the enzyme-linked immunosorbent assay in the diagnosis of *Trypanosoma equiperdum* and *Trypanosoma evansi*. MSc Thesis, University of Edinburgh.

Bishop, S., Rae, P.F., Phipps, L.P., Boid, R. and Luckins, A.G., 1995. *Trypanosoma equiperdum*: Detection of trypanosomal antibodies by enzyme-linked immunosorbent assay. British Veterinary Journal, 151: 715-720.

Blacksell, S.D., Cameron, A.R., Chamnanpood, C., Chamnanpood, P., Tatong, D., Monpolsiri, M. and Westbury, H.A., 1996. Implementation of internal laboratory quality control procedures for the monitoring of ELISA performance at a regional veterinary laboratory. Veterinary Microbiology, 51:1-9.

Boid, R., 1988. Isoenzyme characterisation of 15 stocks of *Trypanosoma evansi* isolated from camels in the Sudan. Tropical Medicine and Parasitology, 39: 45-50.

Boid, R. and Mleche, W.C.H., 1985. Isoenzyme analysis of stocks of trypanosomes isolated from cattle in Indonesia. Research in Veterinary Science, 39: 388-389.

Borst, P. and Cross, G.A.M., 1982. Molecular basis for *Trypanosoma* antigenic variation. Cell, 29: 291-303.

Borst, P., Fase-Fowler, F. and Gibson, W.C., 1987. Kinetoplast DNA of *Trypanosoma evansi*. Molecular Biochemical Parasitology, 23: 31-38.

Boscato, L.M. and Stuart, M.C., 1988. Heterophilic antibodies: a problem for all immunoassays. Clinical Chemistry, 34: 27-33.

Bose, R., Friedhoff, K.T., Olbrich, S., Buscher, G. and Domeyer, I., 1987. Transmission of *Trypanosoma theileri* to cattle by *Tabanidae*. Parasitological Research, 73: 421-424.

Bosompem, K.M., Masake, R.A., Assoku, R.K.G., Opiyo, E.A. and Nantulya, V.M., 1996. Field evaluation of a dot-ELISA for the detection and differentiation of trypanosome species in infected tsetse flies (*Glossina* spp.). Parasitology, 112: 205-211.

Bourn, D. and Scott, J.M., 1978. The successful use of work oxen in agricultural development of tsetse-infested land in Ethiopia. Tropical Animal Health and Production, 10: 191-203.

Byrt, T., Bishop, J. and Carlin, J.B., 1993. Bias, prevalence and kappa. Journal of Clinical Epidemiology, 46: 423-429.

Caporale, V.P., Biancifiori, F., Frescura, F., di Matteo, A., Nannini, D. and Urbani, G., 1981. Comparison of various tests for the serological diagnosis of *Trypanosoma equiperdum* infection in the horse. Comparative Immunology and Microbiology of Infectious Diseases, 4: 243-246.

Casali, P., Burastero, S.E., Nakamura, M., Inghirami, G. and Notkins, A.L., 1987. Human lymphocytes making rheumatoid factors and antibodies to ssDNA belong to the Leu 1+ B-cell subset. Science, 236: 77-81.

Casali, P. and Notkins, A.L., 1989. CD5+ lymphocytes, polyreactive antibodies and the human B-cell repertoire. Immunology Today, 10: 364-368.

Chainey, J.E., 1993. Horse-flies, deer-flies and clegs (*Tabanidae*). In: R.P. Lane and R.W. Crosskey, (Editors). Medical Insects and Arachnids. Chapman and Hall, London, pp. 310-332.

Clarkson, M.J., Penhale, W.J. and McKenna, R.B., 1975. Progressive serum protein changes in experimental infection of calves with *Trypanosoma vivax*. Journal of Comparative Pathology, 85: 397-410.

Cochran, W.G., 1950. The comparison of percentages in matched samples. Biometrika, 37: 256-266.

Cross, G.A.M., 1975. Identification, purification and properties of variant-specific glycoprotein antigens constituting the surface coat of *Trypanosoma brucei*. Parasitology, 71: 393-417.

Cross, G.A.M., 1990. Cellular and genetic aspects of antigenic variation in trypanosomes. Annual Review of Immunology, 8: 83-110.

Damayanti, R., 1991. Studies of the pathology of *Trypanosoma evansi* infection in the buffalo (*Bubalus bubalis*). MSc Thesis. James Cook University, North Queensland, Australia.

Damayanti, R., 1993. The pathology of natural *Trypanosoma evansi* infection in the buffalo (*Bubalus bubalis*). Penyakit Hewan, 25: 34-39.

Davison, H.C., 1991. The application of the enzyme-linked immunosorbent assay in the serodiagnosis of bovine babesiosis and anaplasmosis. MSc Thesis. University of Edinburgh.

de Echaide, S.T., de Echaide, I.E., Gaido, A.B., Mangold, A.J., Lugaresi, C.I., Vanzini, V.R. and Guglielmone, A.A., 1995. Evaluation of an enzyme-linked immunosorbent assay kit to detect *Babesia bovis* antibodies in cattle. Preventive Veterinary Medicine, 24: 277-283.

Delafosse, A., Bengaly, Z. and Duvallet, G., 1995. Absence d'interaction des infections a *Trypanosoma theileri* avec le diagnostic des trypanosomoses animales par detection des antigenes circulants. Revue d'Elevage et de Medecine Veterinaire des Pays Tropicaux, 48: 18-20.

Dempsey, W.A. and Mansfield, J.M., 1983a. Lymphocyte function in experimental African trypanosomiasis. V. Role of antibody, and the mononuclear phagocyte system in variant-specific immunity. Journal of Immunology, 130: 405-411.

Dempsey, W.L. and Mansfield, J.M., 1983b. Lymphocyte function in experimental African trypanosomiasis. VI. Parasite-specific immunosuppression. Journal of Immunology, 130: 2896-2898.

de Savigny, D. and Voller, A., 1980. The communication of ELISA data from laboratory to clinician. Journal of Immunoassay, 1:105-128.

Diall, O., Nantulya, V.M., Luckins, A.G., Diarra, B. and Kouyate, B., 1992. Evaluation of mono- and polyclonal antibody-based antigen detection immunoassays for diagnosis of *Trypanosoma evansi* infection in the dromedary camel. Revue d'Elevage et de Medecine Veterinaire des Pays Tropicaux, 45: 149-153.

Dieleman, E.F., 1983. Trypanosomiasis in Indonesia; a review and a report of studies on chemotherapy in experimentally-infected mice. Research Institute for Animal Diseases, Bogor, Indonesia, pp. 1-109.

Diem, K., 1962. Documenta Geigy Scientific Tables. Sixth edition. Geigy Pharmaceutical Co. Ltd, Manchester, p.107.

Dijkhuizen, A.A., Huirne, R.B.M. and Jalvingh, A.W., 1994. Economic analysis of animal diseases and their control. Kenya Veterinarian, 18: 13-14.

Doherty, M.L., Windle, H., Voorheis, H.P., Larkin, H., Casey, M., Clery, D. and Murray, M., 1993. Clinical disease associated with *Trypanosoma theileri* infection in a calf in Ireland. Veterinary Record, 132: 653-656.

Dolan, R.B., 1987. Genetics and trypanotolerance. Parasitology Today, 3: 137-143.

Doyle, J.J., 1977. Antigenic variation in the salivarian trypanosomes. In: L. H. Miller, P. A. Pino and J. McKelvey, (Editors). Immunity to Blood Parasites of Animals and Man. Plenum Press, New York, pp. 27-29.

Evans, G., 1881. On a horse disease in India known as "surra", probably due to a haematozoon. Veterinary Journal and Annals of Comparative Pathology, 13: 1-11, 82-88, 180-200, 326-333.

Evans, G., 1882. On a horse disease in India known as "surra", probably due to a haematozoon. Veterinary Journal and Annals of Comparative Pathology, 14: 97-110, 181-200.

Everitt, R.S., 1989. Statistical Methods for Medical Investigations. Oxford University Press, New York/Edward Arnold, London.

Feinstein, A.R. and Cicchetti, D.V., 1990. High agreement but low kappa: The problems of two paradoxes. Journal of Clinical Epidemiology, 43: 543-549.

Fiennes-T-W, R.N., 1954. Haematological studies in trypanosomiasis of cattle. Veterinary Record, 66: 423-434.

Fleiss, J.L., 1981. Statistical Methods for Rates and Proportions. John Wiley and Sons, New York, 2nd Edition, pp. 1-311.

Fleiss, J.L., 1986. The Design and Analysis of Clinical Experiments. John Wiley & Sons, New York.

Foil, L.D., 1989. Tabanids as vectors of disease agents. Parasitology Today, 5: 88-96.

Frame, I.A., Rae, P.F., Boid, R. and Luckins, A.G., 1990. Antigen detection ELISA for *Trypanosoma evansi* using group-specific monoclonal antibody. Transactions of the Royal Society of Tropical Medicine and Hygiene, 84: 893.

Franke, C.R., Greiner, M. and Mehlitz, D., 1994. Investigations on naturally occurring *Trypanosoma evansi* infections in horses, cattle, dogs and capybaras (*Hydrochaeris hydrochaeris*) in Pantanal de Pocone (Mato Grosso, Brazil). Acta Tropica, 58: 159-169.

Frankena, K., Noordhuizen, J.P., Willeberg, P. and van Voorthuysen, P.F., 1990. EPISCOPE: Computer programmes in veterinary epidemiology, Veterinary Record, 126: 573-576.

Gambino, R., 1989. The misuse of predictive value - or why you must consider the odds. Laboratory Report, 11:65-72.

Gardner, M.J. and Altman, D.G., 1989. Statistics with Confidence - Confidence Intervals and Statistical Guidelines. British Medical Journal, London.

Gibson, W., Marshall, T.F. and Godfrey, D.G., 1980. Numerical analysis of enzyme polymorphism: A new approach to the epidemiology and taxonomy of trypanosomes of the subgenus *Trypanozoon*. Advances in Parasitology, 18: 175-246.

Gibson, W.C., Wilson, A.J. and Moloo, S.K., 1983. Characterisation of *Trypanosoma (Trypanozoon) evansi* from camels in Kenya using isoenzyme electrophoresis. Research in Veterinary Science, 34: 114-118.

Gichuki, C.W., Nantulya, V.M. and Sayer, P.D., 1994. *Trypanosoma brucei rhodesiense*: Use of an antigen detection enzyme immunoassay for evaluation of response to chemotherapy in infected vervet monkeys (*Cercopithecus aethiops*). Tropical Medicine and Parasitology, 45: 237-242.

Goodwin, L.G., 1970. The pathology of African trypanosomiasis. Transactions of the Royal Society of Tropical Medicine and Hygiene, 64: 797-812.

Gray, A.R. and Luckins, A.G., 1976. Antigenic variation in salivarian trypanosomes. In: W. H. R. Lumsden and D. A. Evans, (Editors). Biology of the *Kinetoplastida*: Volume 1. Academic Press Inc, New York, pp. 493-496.

Grencis, R.K., 1990. Antigen uptake, processing and presentation. In: J.M. Behnke, (Editor). Parasites: Immunity and Pathology. Taylor and Francis Ltd, London, First edition, pp. 63-85.

Hamilton, R.G. and Adkinson, N.F., 1988. Quantitative aspects of solid phase immunoassays. In: D.M. Kemeny and S.J. Challacombe, (Editors). ELISA and Other Solid Phase Immunoassays: Theoretical and Practical Aspects. Wiley Medical Publications. John Wiley and Sons, London, pp. 57-84.

Hardjosubroto, W., 1985. Breed evaluation of large ruminants in Indonesia. In: Evaluation of Large Ruminants for the Tropics. Proceedings of an International Workshop held at CSIRO, Rockhampton, Queensland, Australia, 19-23 March 1984 (ACIAR series No. 5), ACIAR, Canberra.

Hartini, S. and Aziz, J., 1991. *Trypanosoma evansi* in *Tabanus rubidus* in Jonggol, West Java. Malayan Veterinary Journal, 3: 31-32.

Hoare, C.A., 1964. Morphological and taxonomic studies on mammalian trypanosomes X. Revision of the systematics. Journal of Protozoology, 11: 200-210.

Hoare, C.A., 1972. The Trypanosomes of Mammals. A zoological monograph. Blackwell Scientific Publications, Oxford.

Holmes, P.H., Mamo, E., Thomson, A., Knight, P.A., Lucken, R., Murray, P.K., Murray, M., Jennings, F.W. and Urquhart, G.M., 1974. Immunosuppression in bovine trypanosomiasis. Veterinary Record, 95: 86-87.

Hunter, A.G. and Luckins, A.G., 1990. Diseases caused by protozoa: Trypanosomosis. In: M. M. H. Sewell and D. W. Brocklesby, (Editors). Handbook on Animal Diseases in the Tropics. Bailliere Tindall, London, 4th Edition, pp. 204-224.

Hutabarat, T.S.P.N. and Holden, S.J. 1991. Inclusion of economic information in national animal health planning in Indonesia. In: Proceedings of the Sixth International Symposium on Veterinary Epidemiology and Economics, Ottawa, 12-16 August 1991. Editor Martin S.W. pp. 201-203.

Ijagbone, I.F., Staak, C. and Reinhard, R., 1989. Fractionation of trypanosome antigens for species-specific sero-diagnosis. Veterinary Parasitology, 32: 292-299.

Ilemobade, A.A., Adegboye, D.S., Onoviran, O. and Chima, J.C., 1982. Immunodepressive effects of trypanosomal infection in cattle immunized against contagious bovine pleuropneumonia. Parasite Immunology, 4: 273-282.

Imboden, M., Muller, N., Hemphill, A., Mattioli, R. and Seebeck, T., 1995. Repetitive proteins from the flagellar cytoskeleton of African trypanosomes are diagnostically useful antigens. Parasitology, 110: 249-258.

Jatkar, P.R. and Singh, M., 1971. Diagnosis of surra in camels by the passive haemagglutination test. British Veterinary Journal, 127: 283-288.

Jones, T.W., 1987. The epizootiology of trypanosome infection of livestock in Indonesia. Report on a liaison visit for research scheme R3936, August 17 to September 9, 1987.

Jones, T.W. and McKinnell, C., 1985. Antigenic variation in *Trypanosoma evansi*: a comparison of the predominant variable antigen type repertoire of stocks from Sudan. Tropical Medicine and Parasitology, 36: 205-209.

Kahn, H.A. and Sempos, C.T., 1989. Statistical Methods in Epidemiology. Monographs in Epidemiology and Biostatistics. Volume 12. First edition. Oxford University Press, Oxford.

Kassai, T., Cordero del Campillo, M., Euzeby, J., Gaafar, S., Hiepe, Th. and Himonas, C.A., 1988. Standardised nomenclature of animal parasitic diseases (SNOAPAD). Veterinary Parasitology, 29: 299-326.

Katende, J.M., Musoke, A.J., Nantulya, V.M. and Goddeeris, B.M., 1987. A new method for fixation and preservation of trypanosomal antigens for use in the indirect immunofluorescence antibody test for diagnosis of bovine trypanosomiasis. Tropical Medical Parasitology, 38: 41-44.

Kemeny, D.M., 1991. A Practical Guide to ELISA. Pergamon Press, Oxford, First Edition pp. 1-115.

Kemeny, D.M. and Chantler, S., 1988. An introduction to ELISA. In: D.M. Kemeny and S.J. Challacombe, (Editors). ELISA and Other Solid Phase Immunoassays: Theoretical and Practical Aspects. Wiley Medical Publications. John Wiley and Sons, London, pp. 1-30.

Kemeny, D.M., and Challacombe, S.J., 1988. Microtitre plates and other solid phase supports. In: D.M. Kemeny and S.J. Challacombe, (Editors). ELISA and Other Solid Phase Immunoassays: Theoretical and Practical Aspects. Wiley Medical Publications. John Wiley and Sons, London, pp. 31-56.

Killick-Kendrick, R., 1968. The diagnosis of trypanosomiasis of livestock; a review of current techniques. Veterinary Bulletin, 38: 191-197.

Kleinbaum, D.G., Kupper, L.L. and Morgenstern, H., 1982, Epidemiologic Research. Principles and Quantitative Measures. Lifetime Learning Publications, Belmont.

Kobayashi, A., Tizard, I.R. and Woo, P.T.K., 1976. Studies on the anaemia in experimental African trypanosomiasis. II The pathogenesis of the anemia in calves infected with *Trypanosoma congolense*. American Journal of Tropical Medicine and Hygiene, 25: 401-406.

Konishi, E., 1993. Naturally occurring antibodies that react with protozoan parasites. Parasitology Today, 9:361-364.

Kraemer, H.C. and Bloch, D.A., 1988. Kappa coefficients in epidemiology: an appraisal of a reappraisal. Journal of Clinical Epidemiology, 41: 959-968.

Kraneveld, F.C., 1931. Einzelne Ubertragungsversuche von *Trypanosoma theileri* Laveran 1902 mit Tabaniden. Nederlandsch-Indische Bladen voor Diergeneeskunde, 43: 132-139.

Kraneveld, F.C. and Mansjoer, M., 1952. Onderzoekingen over de gevoeligheid voor surra. II. Het verloop der ziekte bij enkele in het wild levende dieren in Indonesia. Hemera Zoa, 59: 117-146.

Kukla, B.A., Majiwa, P.A.O., Young, J.R., Moloo, S.K. and Ole-Moiyoi, O.K., 1987. Use of species-specific DNA probes for detection and identification of trypanosome infection in tsetse flies. Parasitology, 95: 1-16.

Landis, J.R. and Koch, G.C., 1977. The measurement of observer agreement for categorical data. Biometrics, 33: 159-174.

Lanham, S.M. and Godfrey, D.G., 1970. Isolation of salivarian trypanosomes from man and other mammals using DEAE-cellulose. Experimental Parasitology, 28: 521-528.

Leese, A.S., 1912. Biting flies and surra. Journal of Tropical Veterinary Science, 7(1): 19-32.

Lessard, P. 1994. African Animal Trypanosomoiasis - Epidemiology. A training course held in ILRAD, Kenya, February 1994, by the International Atomic Energy Agency, Vienna.

Liu, M.K., Pearson, T.W., Sayer, P.D., Gould, S.S., Waitumbi, J.N. and Njogu, A.R., 1988. Serodiagnosis of African sleeping sickness in vervet monkeys by detection of parasite antigens. Acta Tropica, 45: 321-330.

Lohr, K.F., Pohlpark, S., Srikitjakarn, L., Thaboran, P., Betterman, G. and Staak, C., 1985. *Trypanosoma evansi* infection in buffaloes in north-east Thailand. I. Field investigations. Tropical Animal Health and Production, 17: 121-125.

Lohr, K.F., Pholpark, S., Siriwan, P., Leesirikul, N., Srikitjakarn, L. and Staak, C., 1986. *Trypanosoma evansi* infection in buffaloes in north-east Thailand. II. Abortions. Tropical Animal Health and Production, 18: 103-108.

Losos, G.J. and Ikede, B.O., 1972. Review of the pathology of diseases in domestic and laboratory animals caused by *Trypanosoma congolense*, *T. vivax, T. brucei, T. rhodesiense* and *T. gambiense.* Journal of Veterinary Pathology, 9: 1-15.

Luckins, A.G., 1972. Adoptive immunity in experimental trypanosomiasis. Transactions of the Royal Society of Tropical Medicine and Hygiene, 66: 346-347.

Luckins, A.G., 1976a. The immune response of zebu cattle to infection with *Trypanosoma congolense* and *T. vivax.* Annals of Tropical Medicine and Parasitology, 70: 133-145.

Luckins, A.G., 1976b. Observations on serum immunoglobulin levels in cattle infected with *Trypanosoma brucei*, *T. vivax* and *T. congolense*. Annals of Tropical Medicine and Parasitology, 70: 479-480.

Luckins, A.G., 1977. Detection of antibodies in trypanosome-infected cattle by means of a microplate enzyme-linked immunosorbent assay. Tropical Animal Health and Production, 9: 53-62.

Luckins, A.G., 1983. Development of serological assays for studies on trypanosomiasis of livestock in Indonesia. Report for consultancy undertaken January 26 to February 27, 1983. CTVM, Edinburgh, pp. 1-68.

Luckins, A.G., 1988. *Trypanosoma evansi* in Asia. Parasitology Today, 4: 137-141.

Luckins, A.G., 1991. Antigen detection ELISA for *Trypanosoma evansi* using group specific monoclonal antibodies. In: Improving the Diagnosis of Trypanosomiasis and Other Vector-borne Diseases of African Livestock using Immunoassay Methods. Third Research Co-ordination Meeting held in Abidjan, May 20-25, 1991, by the International Atomic Energy Agency, Vienna.

Luckins, A.G., 1992. Diagnostic methods for trypanosomiasis in livestock. World Animal Review, 71: 15-20.

Luckins, A.G., Boid, R., Rae, P.F., Mahmoud, M.M., El Malik, K.H. and Gray, A.R., 1979. Serodiagnosis of *Trypanosoma evansi* in camels in the Sudan. Tropical Animal Health and Production, 11: 1-12.

Luckins, A.G., Gray, A.R. and Rae, P.F., 1978. Comparison of the diagnostic value of serum immunoglobulin levels, an enzyme-linked immunosorbent assay and a fluorescent antibody test in experimental infections with *Trypanosoma evansi* in rabbits. Annals of Tropical Medicine and Parasitology, 72: 429-441.

Luckins, A.G., Hopkins, J., Rae, P.F. and Ross, C.A., 1990. Stability of metacyclic variable antigen types (M-VATS) during the early stages of infection with *Trypanosoma congolense*. Acta Tropica, 47: 129-136.

Luckins, A.G., McIntyre, N. and Rae, P.F., 1992. Multiplication of *Trypanosoma evansi* at the site of infection in skin of rabbits and cattle. Acta Tropica, 50: 19-27.

Luckins, A.G. and Mehlitz, D., 1976. Observations on serum immunoglobulin levels in cattle infected with *Trypanosoma brucei, T. vivax and T. congolense*. Annals of Tropical Medicine and Parasitology, 70: 479-480.

Luckins, A.G. and Mehlitz, D., 1978. Evaluation of an indirect fluorescent antibody test, enzyme-linked immunosorbent assay and quantification of immunoglobulins in the diagnosis of bovine trypanosomiasis. Tropical Animal Health and Production, 10: 149-159.

Lumsden, W.H.R., Herbert, W.J. and McNeillage, G.J.C., 1973. Techniques with Trypanosomes. Churchill Livingstone, London.

Lumsden, W.H.R., Kimber, C.D. and Strange, M., 1977. *Trypanosoma brucei*: detection of low parasitaemias in mice by a miniature anion-exchange/centrifugation technique. Transactions of the Royal Society of Tropical Medicine and Hygiene, 71: 421-424.

Lun, Z.-R., Allingham, R., Brun, R. and Lanham, S.M., 1992. The isoenzyme characteristics of *Trypanosoma evansi* and *Trypanosoma equiperdum* isolated from domestic stocks in China. Annals of Tropical Medicine and Parasitology, 86: 333-340.

Lun, Z-R. and Desser, S.S., 1995. Is the broad range of hosts and geographical distribution of *Trypanosoma evansi* attributable to the loss of maxicircle kinetoplast DNA? Parasitology Today, 11: 131-133.

Lun, Z-R., Fang, Y., Wang, C-J. and Brun, R., 1993. Trypanosomiasis of domestic animals in China. Parasitology Today, 9: 41-45.

Lun, Z.R., Min, Z.P., Huang, D., Liang, J.X., Yang, X.F. and Huang, Y.T., 1991. Cymelarsan in the treatment of buffaloes naturally infected with *Trypanosoma evansi* in South China. Acta Tropica, 49: 233-236.

MacAskill, J.A., Holmes, P.H., Whitelaw, D.D., McConnell, I., Jennings, F.W. and Urquhart, G.M., 1980. Immunological clearance of 75Se-labelled *Trypanosoma brucei* in mice. Immunology, 40: 629-635.

Mahmoud, M.M. and Gray, A.R., 1980. Trypanosomiasis due to *Trypanosoma evansi* (Steel, 1885) Balbani, 1888. A review of recent research. Tropical Animal Health and Production, 12: 35-47.

Maizels, R.M., 1990. Parasite antigens. In: J. M. Behnke, (Editor) Parasites: Immunity and Pathology. Taylor and Francis Ltd, London, First Edition, pp. 23-39.

Majiwa, P.A.O., Maina, M., Waitumbi, J.N., Mihok, S. and Zweygarth, E., 1993. *Trypanosoma (Nannomonas) congolense*: molecular characterisation of a new genotype from Tsavo, Kenya. Parasitology, 106: 151-162.

Mansfield, J.M., 1994. T-cell responses to the trypanosome variant surface glycoprotein: A new paradigm? Parasitology Today, 10: 267-270.

Marchevsky, N., Held, J.R. and Garcia-Carrillo, C., 1989. Probability of introducing diseases because of false negative test results. American Journal of Epidemiology, 130:611-614.

Martin, S.W., 1984. Estimating disease prevalence and the interpretation of screening test results. Preventive Veterinary Medicine, 2: 463-472.

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

Masake, R.A. and Nantulya, V.M., 1991. Sensitivity of an antigen-detection enzyme immunoassay for diagnosis of *Trypanosoma congolense* infections in goats and cattle. Journal of Parasitology, 77: 231-236.

Masake, R.A., Onesmo, K., Yoi, Ole-Moi., Urakawa, T., Hirumi, H., Majiwa, P.A.O., Wells, C.W., Minja, S.H., Makau, J.M. and Nantulya, V.M., 1995b. Immunological characterisation and expression in *Escherichia coli* and baculovirus systems of a *Trypanosoma vivax* antigen detected in the blood of infected animals. Experimental Parasitology, 81: 536-545.

Masiga, D.K., McNamara, J.J. and Gibson, W.C., 1996. A repetitive DNA sequence specific for *Trypanosoma (Nannomonas) godfreyi*. Veterinary Parasitology, 62: 27-33.

Matthews, J.N.S., Altman, D.G., Campbell, M.J. and Royston, P., 1990. Analysis of serial measurements in medical research. British Medical Journal, 300: 230-235.

Mbwambo, H.A. and Mpokwa, M.H., 1993. Antigen-detection enzyme-linked immunoassay (Ag-ELISA) as an aid to the diagnosis of animal trypanosomiasis in Tanzania. In: Improving the Diagnosis and Control of Trypanosomiasis and Other Vector-borne Diseases of African Livestock using Immunoassay Methods. IAEA-TECDOC-707. International Atomic Energy Agency, Vienna. pp. 79-86.

McCullough, K.C., 1993. The application of biotechnology to the diagnosis and control of animal diseases. Revue Scientifique et Technique, Office International des Epizooties, 12: 325-354.

Mihok, S., Maramba, O., Munyoki, E. and Kagoiya, J., 1995. Mechanical transmission of *Trypanosoma* species by African *Stomoxyinae* (Diptera: *Muscidae*). Tropical Medicine and Parasitology, 46: 103-105.

Molyneux, D.H. and Ashford, R.W., 1983. The biology of *Trypanosoma* and *Leishmania*. In: Parasites of Man and Domestic Animals. First Edition. Taylor and Francis, London, pp. 1-267.

Monzon, C.M., Jara, A. and Nantulya, V.M., 1995. Sensitivity of antigen ELISA test for detecting *Trypanosoma evansi* antigen in horses in the subtropical area of Argentina. Journal of Parasitology, 8: 806-808.

Monzon, C.M., Mancebo, O.A. and Roux, J.P., 1990. Comparison between six parasitological methods for diagnosis of *Trypanosoma evansi* in the subtropical area of Argentina. Veterinary Parasitology, 36: 141-146.

More, S.J. and Copeman, D.B., 1991. Antigen detection ELISAs: pretreatment of serum to reduce interference by specific host antibodies. Tropical Medicine and Parasitology, 42:91-94.

Morrison, W.I., Black, S.J., Paris, J., Hinson, C.A. and Wells, P.W., 1982. Protective immunity and specificity of antibody responses elicited in cattle by irradiated *Trypanosoma brucei*. Parasite Immunology, 4: 395-407.

Morrison, W.I., Murray, M. and Akol, G.W.O., 1985. Immune responses of cattle to African trypanosomes. In: I. Tizard, (Editor). Immunology and Pathogenesis of Trypanosomiasis. CRC Press, Boca Raton, Florida, pp. 104-124.

Morrison, W.I., Murray, M., Whitelaw, D.D. and Sayer, P.D., 1983. Pathology of infection with *Trypanosoma brucei*: disease syndrome in dogs and cattle resulting from severe tissue damage. Contributions to Microbiology and Immunology, 7: 103-113.

Mulligan, H.W. and Potts, W.H., (Editors). 1970. The African Trypanosomiases. Allen and Unwin, London.

Murray, M., 1977. An improved parasitological technique for the diagnosis of African trypanosomiasis. Transactions of the Royal Society of Tropical Medicine and Hygiene, 71: 325-326.

Murray, M. and Dexter, T.M., 1988. Anaemia in bovine African trypanosomiasis: A review. Acta Tropica, 45: 389-435.

Murray, M., Morrison, W.I. and Whitelaw, D.D., 1982. Host susceptibility to African trypanosomiasis: Trypanotolerance. Advances in Parasitology, 21: 1-67.

Naessens, J. and Williams, D.J.L., 1992. Characterisation and measurement of CD5+ B cells in normal and *Trypanosoma congolense*-infected cattle. European Journal of Immunology, 22: 1713-1718.

Nantulya, V.M., 1986. Immunological approaches to the control of animal trypanosomiasis. Parasitology Today, 2: 168-173.

Nantulya, V.M., 1989. An antigen-detection enzyme immunoassay for the diagnosis of *rhodesiense* sleeping sickness. Parasite Immunology, 11: 69-75.

Nantulya, V.M., 1990. Trypanosomiasis in domestic animals: The problem of diagnosis. Revue Scientifique et Technique, Office International des Epizooties, 9: 357-367.

Nantulya, V.M., 1991. Molecular diagnosis of parasites. Experientia, 47: 142-145.

Nantulya, V.M, 1994. Suratex: A simple latex agglutination antigen test for diagnosis of *Trypanosoma evansi* infections (surra). Tropical Medicine and Parasitology, 45: 9-12.

Nantulya, V.M., Bajyana Songa, E. and Hamers, R., 1989a. Detection of circulating trypanosomal antigens in *Trypanosoma evansi*-infected animals using a *T. brucei* group specific monoclonal antibody. Tropical Medicine and Parasitology, 40: 263-266.

Nantulya, V.M. and Lindqvist, K.J., 1989. Antigen-detection enzyme immunoassays for the diagnosis of *Trypanosoma vivax, T. congolense* and *T. brucei* infections in cattle. Tropical Medicine and Parasitology, 40: 267-272.

Nantulya, V.M., Lindqvist, K.J., Diall, O. and Olaho-Mukani, W., 1989b. Two simple antigen-detection enzyme immunoassays for the diagnosis of *Trypanosoma evansi* infections in the dromedary camel (*Camelus dromedarius*). Tropical Medicine and Parasitology, 40: 415-418.

Nantulya, V.M. and Moloo, S.K., 1989. Recent developments in trypanosomiasis. International Journal of Animal Sciences, 4: 71-84.

Nantulya, V.M., Musoke, A.J., Rurangirwa, F.R., Barbet, A.F., Ngaira, J. and Katende, J.M., 1982. Immune depression in African trypanosomiasis. The role of antigenic competition. Clinical Experimental Immunology, 47: 234-241.

Nantulya, V.M., Musoke, A.J., Rurangirwa, F.R., Saigar, N. and Minja, S.H., 1987. Monoclonal antibodies that distinguish *Trypanosoma congolense, T. vivax* and *T. brucei*. Parasite Immunology, 9: 421-431.

Nielsen, K., Kelly, L., Gall, D., Balsevicius, S., Bosse, J., Nicoletti, P. and Kelly, W., 1996a. Comparison of enzyme immunoassays for the diagnosis of bovine brucellosis. Preventive Veterinary Medicine, 26:17-32.

Nielsen, K., Smith, P., Gall, D., de Echaide, S.T., Wagner, G. and Dajer, A., 1996b. Development and validation of an indirect enzyme immunoassay for detection of antibody to *Anaplasma marginale* in bovine sera. Veterinary Parasitology, 67:133-142.

Nielsen, K., Sheppard, J., Holmes, W. and Tizard, I., 1978. Experimental bovine trypanosomiasis. Changes in the catabolism of serum immunoglobulins and complement components in infected cattle. Immunology, 35: 811-816.

Nieschulz, O., 1926. Zoologische bijdragen tot het surraprobleem. XI. Enkele proeven met *Haematopota truncata* Schuurm. Stekh., *Haematopota irrorata* Macq. en *Tabanus brunnipes* Schuurm. Stekh. Nederlandsch-Indische Bladen voor Diergeneeskunde, 39: 226-238.

Nieschulz, O., 1927. Zoologischebijdragen tot het surra-probleem. VIII. Over tabaniden broedplaatsen op Java en Sumatra. Nederlandsch-Indische Bladen voor Diergeneeskunde, 39: 1-46, 371-390.

Nieschulz, O., 1928. Zoologische bijdragen tot het surraprobleem. Nederlandsch-Indische Bladen voor Diergeneeskunde, 40: 249-307.

Nieschulz, O., 1929. Zoologische bijdragen tot het surraprobleem. XXVI. Over den incubatietijd bij surrainfecties. Nederlandsch-Indische Bladen voor Diergeneeskunde, 41: 253-264.

Nieschulz, O. and Ponto, S.A.S., 1927. Zoologische bijdragen tot het surraprobleem. XVIII. Over meervardige infecties met *Tabanus striatus* Fabr. Nederlandsch-Indische Bladen voor Diergeneeskunde, 39: 364-370.

O'Sullivan, K., 1995. An application of antigen-detection enzyme-linked immunosorbent assay to estimate the prevalence of *Trypanosoma evansi* in buffaloes in Central Cambodia. MSc Thesis. University of Edinburgh.

Ogwu, D. and Nuru, S., 1981. Transplacental transmission of trypanosomes in animals and man: A review. Veterinary Bulletin, 51: 381-384.

Okuna, N.M., Mayende, J.S.P. and Magona, J., 1993. The validation of an Ag-ELISA for the diagnosis of trypanosomiasis in cattle in Uganda and its use in assessing the efficacy of a control programme. In: Improving the Diagnosis and Control of Trypanosomiasis and Other Vector-borne Diseases of African Livestock using Immunoassay Methods. IAEA-TECDOC-707. International Atomic Energy Agency, Vienna. pp. 69-77.

Olaho-Mukani, W., Nyang'ao, J.M.N. and Ouma, J.O., 1996. Use of Suratex for field diagnosis of patent and non-patent *Trypanosoma evansi* infections in camels. British Veterinary Journal, 152: 109-111.

Onah, D., 1992. *Trypanosoma evansi*: Immune responses and immunosuppression during experimental infection in sheep. PhD Thesis. University of Edinburgh.

Otte, J., 1989. The epidemiology of *Trypanosoma vivax* and its effect on cattle productivity in the northern tropical zone of Colombia. PhD Thesis. University of Reading.

Otte, M.J., Abuabara, J.Y. and Wells, E.A., 1994. *Trypanosoma vivax* in Colombia: Epidemiology and production losses. Tropical Animal Health and Production, 26: 146-156.

Paikne, D.L. and Dhake, P.R., 1972. Abortion due to *Trypanosoma evansi* in a she buffalo. Indian Veterinary Journal, 49: 1091-1092.

Paling, R.W., Moloo, S.K., Scott, J.R., McOdimba, F.A., Logan-Henfrey, L., Murray, M. and Williams, D.J.L., 1991. Susceptibility of N'Dama and Boran cattle to tsetse-transmitted primary and rechallenge infections with a homologous serodeme of *Trypanosoma congolense*. Parasite Immunology, 13: 413-425.

Panyim, S., Viseshakul, N., Luxananil, P., Wuyts, N. and Chokesajjawatee, N., 1992. A PCR method for highly sensitive detection of *Trypanosoma evansi* in blood samples. In: Resistance or Tolerance of Animals to Disease, Veterinary Epidemiology and Diagnostic Methods. Proceedings of a meeting held in November 1992, Rethymno, Crete, 138-143.

Paris, J., Murray, M. and McOdimba, F., 1982. A comparative evaluation of the parasitological techniques currently available for the diagnosis of African trypanosomiasis in cattle. Acta Tropica, 39: 307-316.

Parish, N.M., Morrison, W.I. and Pearson, T.W., 1985. Identification of an antigen specific to *Trypanosoma congolense* by using monoclonal antibodies. Journal of Immunology, 134: 593-597.

Partoutomo, S., 1993. Studies on the epidemiology of *Trypanosoma evansi* in Java. PhD Thesis. James Cook University, Queensland, Australia.

Partoutomo, S., Soleh, M., Politedy, F., Day, A., Stevenson, P., Wilson, A.J., Copeman, D.B. and Owen, L., 1994. The epidemiology of *Trypanosoma evansi* and *Trypanosoma theileri* in cattle and buffalo in small holder farms in Java. Penyakit Hewan, 26: 41-46.

Payne, R.C., 1989. Studies on the epidemiology of *Trypanosoma evansi* in the Republic of Indonesia. MPhil Thesis. University of Edinburgh.

Payne, R.C., 1992. Studies on the epidemiology of *Trypanosoma evansi* in the Republic of Indonesia. Final report to the Overseas Development Administration, London.

Payne, R.C., Djauhari, D., Partoutomo, S., Jones, T.W. and Pearson, R.A., 1991a. *Trypanosoma evansi* infection in worked and unworked buffaloes (*Bubalus bubalis*) in Indonesia. Veterinary Parasitology, 40: 197-206.

Payne, R.C., Sukanto, I.P., Bazeley, K. and Jones, T.W., 1993. The effect of *Trypanosoma evansi* infection on the oestrous cycle of Friesian Holstein heifers. Veterinary Parasitolgy, 51: 1-11.

Payne, R.C., Sukanto, I.P., Djauhari, D. and Jones, T.W., 1991b. *Trypanosoma evansi* infection in bovine and buffalo calves in Indonesia. Veterinary Parasitology, 38: 253-256.

Payne, R.C., Sukanto, I.P., Djauhari, D., Partoutomo, S., Wilson, A.J., Jones, T.W., Boid, R. and Luckins, A.G., 1991c. *Trypanosoma evansi* infection in cattle, buffaloes and horses in Indonesia. Veterinary Parasitology, 38: 109-119.

Payne, R.C., Sukanto, I.P., Graydon, R., Saroso, H. and Jusuf, S.H., 1990. An outbreak of trypanosomiasis caused by *Trypanosoma evansi* on the island of Madura, Indonesia. Tropical Medicine and Parasitology, 41: 445-446.

Payne, R.C., Sukanto, I.P., Partoutomo, S. and Jones, T.W., 1994a. Efficacy of Cymelarsan treatment of suramin resistant *Trypanosoma evansi* in cattle. Tropical Animal Health and Production, 26: 92-94.

Payne, R.C., Sukanto, I.P., Partoutomo, S. and Polytedi, F., 1992. Experimental infection of Friesian Holstein calves with an Indonesian isolate of *Trypanosoma evansi*. Tropical Medicine and Parasitology, 43: 115-117.

Payne, R.C., Sukanto, I.P., Partoutomo, S., Sitepu, P. and Jones, T.W., 1994b. Effect of suramin treatment on the productivity of feedlot cattle in a *Trypanosoma evansi* endemic area of Indonesia. Tropical Animal Health and Production, 26: 35-36.

Payne, R.C., Waltner-Toews, D., Djauhari, D. and Jones, T.W., 1991d. *Trypanosoma evansi* infection in swamp buffalo imported into Central Java. Preventive Veterinary Medicine, 11: 105-114.

Pearce, M.C., 1994. Consultant's Report (M2-94-01) for MCF Project Extension of ODA Animal Health Project April 1993-March 1994. Indonesia International Animal Science Research and Development Foundation, pp.1-41.

Penning, C.A., 1900. Over het voorkomen van anaemia perniciosa of infectiosa of surra onder de paarden in Nederlandsch-Indie. Veeartsenijkundige Bladen voor Nederlandsch-Indie, 12: 123-128.

Pesce, A.J. and Michael, J.G., 1988. The use of ELISA in the characterisation of protein antigen structure and immune response. In: D.M. Kemeny and S.J. Challacombe, (Editors). ELISA and Other Solid Phase Immunoassays: Theoretical and Practical Aspects. Wiley Medical Publications. John Wiley and Sons, London, pp. 181-196.

Petchpoo, W., Tan-ariya, P., Boonsaeng, V., Brockelman, C.R., Wilairat, P. and Panyim, S., 1992. A specific DNA probe which identifies *Babesia bovis* in whole blood. Veterinary Parasitology, 42: 189-198.

Piantadosi, S., Byar, D.P. and Green, S.B., 1988. The ecological fallacy. American Journal of Epidemiology, 127: 893-904.

Pinder, M., Bauer, J., Van Melick, A. and Fumoux, F., 1988. Immune responses of trypanoresistant and trypanotolerant cattle after cyclic infection with *Trypanosoma congolense*. Veterinary Immunology and Immunopathology, 18: 245-257.

Rae, P.F. and Luckins, A.G., 1984. Detection of circulating trypanosomal antigens by enzyme immunoassay. Annals of Tropical Medicine and Parasitology, 78: 587-596.

Rae, P.F., Thrusfield, M.V., Higgins, A., Aitken, C.G.G., Jones, T.W. and Luckins, A.G., 1989. Evaluation of enzyme immunoassays in the diagnosis of camel (*Camelus dromedarius*) trypanosomiasis: a preliminary investigation. Epidemiology and Infection, 102: 297-307.

Randall, R. and Schwartz, S.C., 1936. A survey for the incidence of surra in the Philippine Islands. Veterinary Bulletin (Supplement to the US Army Medical Bulletin), 30: 99-108.

Ray, D., Biswas, G. and Sen, G.P., 1992. *Trypanosoma* infection in cattle and buffalo. Indian Journal of Animal Sciences, 62: 420.

Reichard, R., 1992. Diagnostic methods for disease eradication campaigns: role of the Office International des Epizooties. In: Resistance or Tolerance of Animals to Disease and Veterinary Epidemiology and Diagnostic Methods. Proceedings of a meeting held November 1992, in Rethymno, Crete, pp 89-91.

Reinitz, D.M. and Mansfield, J.M., 1990. T-cell-independent and T-cell-dependent B cell responses to exposed variant surface glycoprotein epitopes in trypanosome-infected mice. Infection and Immunolgy, 58: 2337-2342.

Robson, J. and Ashkar, T.S., 1972. Trypanosomiasis in domestic livestock in the Lambwe Valley area and a field evaluation of various diagnostic techniques. Bulletin of the World Health Organization, 47: 727-734.

Roelants, G.E., Tamboura, I., Sidiki, D.B., Bassinga, A. and Pinder, M., 1983. Trypanotolerance: An individual not a breed characteristic. Acta Tropica, 40: 99-104.

Rogers, L., 1901. The transmission of *Trypanosoma evansi* by horse flies, and other experiments pointing to the probable identity of surra of India and nagana or tsetse-fly disease of Africa. Proceedings of the Royal Society of London, B, 68: 163-170.

Ronohardjo, P., Wilson, A.J. and Hirsts, R.G., 1985. Current livestock disease status in Indonesia. Penyakit Hewan, 17: 317-326.

Ronohardjo, P., Wilson, A.J., Partoutomo, S. and Hirst, R.G., 1986. Some aspects of the epidemiology and economics of important diseases of large ruminants in Indonesia. In: Proceedings of the Fourth International Symposium on Veterinary Epidemiology and Economics, Singapore, 18-22 November 1985, pp. 303-305.

Rowlands, G.J., Mulatu, Woudyalew., Authie, E., d'Ieteren, G.D.M., Leak, S.G.A., Nagda, S.M. and Peregrine, A.S., 1993. Epidemiology of bovine trypanosomiasis in the Ghibe valley, southwest Ethiopia. 2. Factors associated with variations in trypanosome prevalence, incidence of new infections and prevalence of recurrent infections. Acta Tropica, 53: 135-150.

Rowlands, G.J., Mulatu, Woudyalew., Authie, E., d'Ieteren, G.D.M., Leak, S.G.A. and Nagda, S.M., 1994a. Effects of trypanosomiasis on growth and mortality of young East African Zebu cattle exposed to drug-resistant trypanosomes. Preventive Veterinary Medicine, 21: 87-101.

Rowlands, G.J., Mulatu, Woudyalew., Authie, E., d'Ieteren, G.D.M., Leak, S.G.A. and Nagda, S.M., 1994b. Effects of trypanosomiasis on reproduction of East African Zebu cows exposed to drug-resistant trypanosomes. Preventive Veterinary Medicine, 21: 237-249.

Ruitenberg, E.J., van Amstel, J.A., Brosi, B.J.M. and Steerenberg, P.A., 1977. Mechanisation of the enzyme-linked immunosorbent assay (ELISA) for large scale screening of sera. Journal of Immunological Methods, 16: 351-359.

Rukmana, M.P., 1979. Microhaematocrit method as a new technology in diagnosing surra and its relevancy to livestock economics. PhD Thesis. Universitas Padjadjaran, Indonesia.

Rurangirwa, F.R., Musoke, A.J., Nantulya, V.N. and Tabel, H., 1983. Immune depression in bovine trypanosomiasis: effects of acute and chronic *Trypanosoma congolense* and chronic *Trypanosoma vivax* infections on antibody responses to *Brucella abortus* vaccine. Parasite Immunology, 5: 267-276.

Seifi, H.A., 1995. Clinical trypanosomosis due to *Trypanosoma theileri* in a cow in Iran. Tropical Animal Health and Production, 27: 93-94.

Shak, S., Davitz, M.A., Wolinsky, M.L., Nussenzweig, V., Turner, M.J. and Gurnett, A., 1988. Partial characterisation of the cross-reacting determinant, a carbohydrate epitope shared by decay accelerating factor and the variant surface glycoprotein of the African *Trypanosoma brucei*. Journal of Immunology, 140: 2046-2050.

Shen, J., Fang, W.M., Sun, J.L., Zheng, R.J., Wu, K.K., Wang, X.G. and Tao, Z.F., 1985. Diagnosis of *Trypanosoma evansi* infection in buffaloes with ELISA. Chinese Journal of Veterinary Medicine, 11: 7-8.

Siegel, S. and Castellan, N.J., 1988. Nonparametric Statistics for the Behavioural Sciences. Second edition, McGraw-Hill, New York.

Sigit, S.H., Partosoedjono, S. and Saleh Akib, M., 1983. Laporan penelitian inventarisasi dan pemetaan parasit Indonesia tahap pertama: ektoparasit (Research report on the identification and distribution of Indonesian parasites. Part I: Ectoparasites). Proyek peningkatan dan pengembangan perguruan tinggi (University Development Project). Institut Pertanian, Bogor.

Sileghem, M., Flynn, F.N., Darji, A., de Baetselier, P. and Naessens, J., 1994. African Trypanosomes. In: F. Kierszenbaum, (Editor) Parasitic Infections and the Immune System. Academic Press Inc., London, First Edition, pp. 1-37.

Simpson, L., 1986. Kinetoplast DNA in trypanosomid flagellates. International Review of Cytology, 99: 119-179.

Singh, B., Kalra, I.S., Gupta, M.P. and Nauriyal, D.C., 1993. *Trypanosoma evansi* infection in dogs: seasonal prevalence and chemotherapy. Veterinary Parasitology, 50: 137-141.

Singh, Veer., Chaudhari, S.S., Kumar, S. and Chhabra, M.B., 1995. Polyclonal antibody-based antigen-detection immunoassay for diagnosis of *Trypanosoma evansi* in buffaloes and horses. Veterinary Parasitology, 56: 261-267.

Siswansyah, D.D., Dahlan, M, and Payne, R.C., 1987. Survival time of *Trypanosoma evansi* in samples of blood taken from infected buffaloes. Penyakit Hewan, 19:24-25.

Siswansyah, D.D. and Tarmudji, P., 1989. Prevalensi trypanosomiasis pada pemeriksaan darah sapi dan kerbau di Kalimantan Selatan dengan metode mikrohematokrit (Prevalence of trypanosomiasis obtained by examination of blood samples from cattle and buffaloes in South Kalimantan by the microhaematocrit test). Penyakit Hewan, 21: 118-122.

Smith, R.D., 1995. Veterinary Clinical Epidemiology - A Problem Oriented Approach. Second edition. CRC Press, Boca Raton, Florida.

Soehadji, P., 1994. Pembinaan kesehatan hewan dan pengamanan bahan pangan asal ternak (Animal health and safety of animal-derived food). In: Proceedings of the Biotechnology seminar held by Balitvet at Cisarua, Bogor, March 22-24 1994, pp.1-10.

Spencer, T.L., 1988. Blockers, diluents and anomalous reactions in ELISA. In: Burgess, G.W (Editor). ELISA Technology in Diagnosis and Research. James Cook University of North Queensland, Townsville, Australia, pp. 51-62.

Stephen, L.E., 1986. Trypanosomiasis: A Veterinary Perspective. Pergamon Press, Oxford, pp. 3-14, 184-214.

Stites, D.P. and Rodgers, R.D.P., 1991. Clinical laboratory methods for detection of antigen and antibodies. In: D.P. Stites and A.I. Terr (Editors). Basic and Clinical Immunology. Seventh Edition. Appleton and Lange, East Norwalk, pp. 217-262.

Suhardono, P., Partoutomo, S., Stevenson, P. and Wilson, A.J., 1985. Prevalence of infection with *Trypanosoma spp.* in cattle and buffaloes examined at an abattoir in Bogor during 1982-1983. Penyakit Hewan, 17: 1-4.

Sukanto, I.P., Payne, R.C. and Partoutomo, S., 1993. Bovine babesiosis in Indonesia. Preventive Veterinary Medicine, 16:151-156.

Sztein, M.B. and Kierszenbaum, F., 1993. Mechanisms of development of immunosuppression during *Trypanosoma* infections. Parasitology Today, 9: 424-428.

Thahar, A., Moran, J.B. and Wood, J.T., 1983. Haematology of Indonesian large ruminants. Tropical Animal Health and Production, 15:76-82.

Thompson, W.D. and Walter, S.D., 1988. A reappraisal of the kappa coefficient. Journal of Clinical Epidemiology, 41: 949-958.

Thrusfield, M., 1995. Veterinary Epidemiology. Blackwell Science, Oxford, Second Edition pp. 266-284.

Topacio, T. and Acevedo, R.A., 1938. A survey of the incidence of surra in Philippine cattle and carabaos by complement fixation test. Philippine Journal of Animal Industry, 5(6): 597-604.

Touratier, L., 1993. Informe de la Decimotercera reunion international del Grupo *ad hoc* de la OIE sobre tripanosomosis animales no transmitidas por glosinas. Revue Scientifique et Technique, Office International des Epizooties, 12: 261-272.

Trail, J.C.M., d'Ieteren, G.D.M., Maille, J.C., Yangari, G. and Nantulya, V.M., 1992. Use of antigen-detecting enzyme immunoassays in assessment of trypanotolerance in N'Dama cattle. Acta Tropica, 50: 11-18.

Tribe, G.W., 1997. Diagnostic aids. Veterinary Record (Letter), 140:240.

Utami, S., 1993. Survey of *Trypanosoma* species in cattle, buffaloes and horses in three districts of Lombok. Report for the Eastern Islands Veterinary Services Project and the Indonesian Livestock Services of East Nusa Tenggara Province, pp1-18.

Urquhart, G.M. and Holmes, P.H., 1987. African trypanosomiasis. In: E. J. L. Soulsby, (Editor). Immune Responses in Parasitic Infections, Immunology, Immunopathology and Immunoprophylaxis. Volume 3. CRC Press, Boca Raton, Florida, pp. 1-22.

Van der Ploeg, L.H.T., Valerio, D., De Lange, T., Bernards, A., Borst, P. and Grosveld, F.G., 1982. An analysis of cosmid clones of nuclear DNA from *Trypanosoma brucei* shows that the genes for variant surface glycoproteins are clustered in the genome. Nucleic Acids Research, 10: 5905-5923.

Van Meirvenne, N. and Le Ray, D., 1985. Diagnosis of African and American trypanosomiases. British Medical Bulletin, 41: 156-161.

Venkatesan, P. and Wakelin, D., 1993. ELISAs for parasitologists: or lies, damned lies and ELISAs. Parasitology Today, 9:228-232.

Venus, E.B. and Dumag, P.U., 1967. Incidence of surra infection among carabaos examined in Nueva Ecija and Pampanga. Philippine Journal of Animal Industry, 22: 177-181.

Vickerman, K., 1969. On the surface coat and flagellar adhesion in trypanosomes. Journal of Cell Science, 5: 163-194.

Vickerman, K., 1978. Antigenic variation in trypanosomes. Nature, 273: 613-617.

Vickerman, K. and Luckins, A.G., 1969. Localisation of variable antigens in the surface coat of *Trypanosoma brucei* using ferritin conjugated antibody. Nature, 224: 1125-1126.

Vickerman, K. and Tetley, L., 1977. Recent ultrastructural studies on trypanosomes. Annales de la Societe Belge de Medecine Tropicale, 57: 441-455.

Voller, A., Bartlett, A. and Bidwell, D.E., 1976. Enzyme immunoassays for parasitic diseases. Transactions of the Royal Society of Tropical Medicine and Hygiene, 70: 98-106.

Waithanji, E.M., Nantulya, V.M. and Mbiuki, S.M., 1993. Use of antigen-capture tube enzyme-linked immunosorbent assay for the diagnosis of *Trypanosoma evansi* infections in dromedary camels (*Camelus dromedarius*). Revue Scientifique et Technique, Office International des Epizooties, 12: 665-672.

Waitumbi, J.N. and Murphy, N.M., 1993. Inter- and intra-species differentiation of trypanosomes by genomic fingerprinting with arbitrary primers. Molecular Biochemical Parasitology, 58: 181-186.

Waitumbi, J.N. and Nantulya, V.M., 1993. A comparison of the antigen detection ELISA and parasite detection for the diagnosis of *Trypanosoma evansi* infections in camels. Veterinary Parasitology, 49: 159-178.

Waitumbi, J.N. and Young, J.R., 1994. Electrophoretic karyotyping is a sensitive epidemiological tool for studying *Trypanosoma evansi* infections. Veterinary Parasitology, 52: 47-56.

Watson, E.A., 1920. Dourine in Canada 1904: History, research and suppression. Department of Agriculture. Dominion of Canada.

Wells, E.A., 1984. Animal trypanosomiasis in South America. Preventive Veterinary Medicine, 2: 31-41.

Wernery, U., Seifert, H.S.H., Billah, A.M. and Ali, M., 1991. Predisposing factors in enterotoxaemias of camels (*Camelus dromedarius*) caused by *Clostridium perfringens* type A. Revue d'Elevage et de Medecine Veterinaire des Pays Tropicaux, 44: 147-152.

Williams, D.J.L., Naessens, J., Scott, J.R. and McOdimba, F.A., 1991. Analysis of peripheral leucocyte populations in N'Dama and Boran cattle following a rechallenge infection with *Trypanosoma congolense*. Parasite Immunology, 13: 171-185.

Williams, D.J.L., Taylor, K., Newson, J., Gichuki, B. and Naessens, J., 1996. The role of anti-variable surface glycoprotein antibody responses in bovine trypanotolerance. Parasite Immunology, 18: 209-218.

Wilson, A.J., 1969. Value of the indirect fluorescent antibody test as a serological aid to diagnosis of *Glossina*-transmitted bovine trypanosomiasis. Tropical Animal Health and Production, 1: 89-95.

Wilson, A.J., 1983. Some observations on the epidemiology of the animal trypanosomiases with particular reference to *T. evansi*. In: R. S. F. Campbell (Editor). Veterinary Epidemiology. AUIDP, Canberra, pp. 201-210.

Wilson, A.J., Paris, J. and Dar, F.K., 1975. Maintenance of a herd of breeding cattle in an area of high trypanosomiasis challenge. Tropical Animal Health and Production, 7: 63-71.

Wilson, A.J., Paris, J., Luckins, A.G., Dar, F.K. and Gray, A.R., 1976. Observations on a herd of beef cattle maintained in a tsetse area. II. Assessment of the development of immunity in association with trypanocidal drug treatment. Tropical Animal Health and Production, 8: 1-12.

Winugroho, M. and Teleni, E. 1993. Feeding and breeding strategies of draught animals. In: Teleni, E., Campbell, R.S.F. and Hoffman, D. (Editors). Draught Animal Systems and Management: An Indonesian Study. Australian Centre for International Agricultural Research (ACIAR), Canberra, Australia. pp. 60-76.

Woo, P.T.K., 1969. The haematocrit centrifuge for the detection of trypanosomes in blood. Canadian Journal of Zoology, 47: 921-923.

Wright, P.F., 1987. Enzyme immunoassay: Observations on aspects of quality control. Veterinary Immunology and Immunopathology, 17:441-452

Wright, P.F., Nilsson, E., Van Rooij, E.M.A., Lelenta, M. and Jeggo, M.H., 1993 Standardisation and validation of enzyme-linked immunosorbent assay techniques for the detection of antibody in infectious disease diagnosis. Revue Scientifique et Technique, Office International des Epizooties, 12: 435-450.

Zweygarth, E., Sabwa, C. and Rottcher, D., 1984. Serodiagnosis of trypanosomiasis in dromedary camels using a card agglutination test set (TESTRYP CATT). Annales de la Societe Belge de Medecine Tropical, 64: 309-313.

## APPENDIX I

## ADDITIONAL PROTOCOLS

### A. Preparation of buffers

1) Stock phosphate buffered saline (PBS; x 10 concentration, pH 7.2) and washing buffer (PBS/0.05T).

| | |
|---|---|
| Disodium hydrogen phosphate ($Na_2HPO_4.12H_2O$) | 28.9g |
| Sodium chloride (NaCl) | 80.0g |
| Potassium dihydrogen phosphate ($KH_2PO_4$) | 2.0g |
| Potassium chloride (KCl) | 2.0g |

To make up the stock solution, the reagents given above were dissolved in 1000 ml distilled water, the pH was adjusted to pH 7.2 with 1 M NaOH and the solution was stored at 4°C. To make up 4000 ml washing buffer (PBS/0.05T), the PBS stock solution was left to reach room temperature (crystallisation occurs at 4°C) and then 400 ml were added to 3600 ml distilled water with 2 ml polyoxyethylene sorbitan monolaurate (Tween 20). The washing buffer was mixed well before use and kept at room temperature for a maximum of four weeks.

2) Phosphate buffered saline glucose (PSG; pH 8.0)

Phosphate saline stock (PS stock)

| | |
|---|---|
| Disodium hydrogen phosphate, anhydrous ($Na_2 HP0_4$) | 14.38 g |
| Sodium dihydrogen phosphate ($NaH_2 P0_4.2H_20$) | 0.78 g |
| Sodium chloride (NaCl) | 4.25 g |

The reagents were made up to 1000 ml with warm distilled water. To prepare a working dilution of phosphate saline (PS), six parts of PS stock were mixed with four parts of distilled water. Phosphate buffered saline glucose (PSG) was prepared by adding glucose to the PS working dilution to give a 1% solution (i.e., 1 g per 100 ml).

3) Preparation of diethylaminoethyl cellulose (DEAE-52)

One hundred grams of diethylaminoethyl cellulose (DEAE-52; Whatman Laboratories Sales Ltd) were suspended in 400 ml PSG and the pH was adjusted to pH 8.0 with 1 M orthophosphoric acid. The DEAE-52 was then washed four times using 400 ml PSG per wash. The DEAE-52 was stored overnight at 4°C or, for longer storage, at -20°C.

## B. Separation of bloodstream trypanosomes by anion exchange chromatography (Lanham and Godfrey, 1970)

The DEAE-52 column was prepared by packing equilibrated DEAE-52 cellulose in a mounted 20 ml syringe or glass column after partially plugging the outlet with sterile glass wool. A paper filter disc (Whatman Laboratory Sales Ltd) was placed on top of the DEAE-52 and the column washed with 50 ml of sterile PSG. Heparinised blood collected from mice with high parasitaemias was poured onto the DEAE-52 column, and eluted by addition of further PSG. Trypanosomes passing through the column were collected in the eluate in a sterile plastic universal bottle kept on ice. The eluate was centrifuged at 2260 r.p.m. for 20 minutes at 4°C. The pellet of trypanosomes was washed three times with 25 ml sterile PSG, and the trypanosomes were counted using an improved Neubauer haemocytometer chamber and adjusted to $10^5$-$10^7$ trypanosomes per ml of sterile PSG.

## C. *Trypanosoma evansi* antibody-detection ELISAs

Sonicated trypanosomal antigen was prepared as described by Luckins (1977) and the same protocol was used for both the IgM ELISA and IgG ELISA. Reagent volumes of 100 µl per well were used throughout, except for sulphuric acid which was added in 50 µl volumes per well.

1) Immulon 1 microtitre plates (M129A; Dynatech) were coated with 1 µg/ml of TREU 1994 *T. evansi* antigen (Luckins, 1977) diluted in 0.05 M, pH 9.6, carbonate-bicarbonate buffer (C-3041; Sigma). The plate was covered and shaken briefly on an incubator/shaker (Dynatech) at ambient temperature before storage at 4°C overnight.

2) The following day the plate was washed (three by one minute cycles) using an automatic ELISA plate washer (Dynatech) with phosphate buffered saline, pH 7.4, containing 0.05% Tween 20 (PBS/0.05T). The serum diluent (PBS/0.05%T) was prepared from PBS tablets (P-4417; Sigma) with the addition of Tween 20. Test serum samples were added to duplicate wells at 1:800 for the IgG ELISA and at 1:250 for the IgM ELISA. Positive (C+) and negative (C-) controls were included in Column 2 on each plate (four wells per control). The conjugate control (Cc) which was serum diluent (PBS/0.05%T) only was added to all wells of Column 1. The IgM ELISA positive control (C+) was a pool of sera taken from the experimental Calves 915 and 917 14 to 28 days post-infection. The IgG ELISA positive control (C+) was a pool of sera taken from the same calves 33 to 40 days post-infection. Pools of pre-infection calf sera were used for the negative controls (C-). Test serum samples were initially diluted 1:8 with PBS without Tween 20 and stored at -20°C until use, after further dilution with PBS/0.05%T. The plate was covered and shaken on the incubator/shaker at 37°C for 30 minutes.

3) The plate was washed using the same wash cycle as before. For the IgG ELISA, rabbit anti-bovine IgG (whole molecule, affinity isolated) antibody conjugated with horseradish peroxidase (HRPO) (A-5295; Sigma) was used at 1:16,000. For the IgM ELISA, sheep anti-bovine IgM (μ chain) antibody conjugated with HRPO (PP203; The Binding Site) was used at 1:4000. Both conjugates were stored at -20°C with glycerol (1:2 dilution) and were diluted with PBS/0.05%T immediately prior to use. The diluted conjugate was added to all wells of the plate which was then covered and shaken on the incubator/shaker at 37°C for 30 minutes.

4) The plate was washed using the same wash cycle as before. For each plate, one 10 mg tetramethylbenzidine dihydrochloride tablet (TMB) (T-3405; Sigma) was dissolved in 10 ml of freshly prepared 0.05 M, pH 5.0, phosphate-citrate buffer containing 0.03% sodium perborate (S-P4922; Sigma). The substrate solution was added to all wells of the plate which was then covered and shaken for 10 minutes on the incubator/shaker at 37°C.

5) The enzymatic reaction was stopped by the addition of 50 µl of 2 M sulphuric acid to each well. The plate was read immediately using a Titertek Multiskan ELISA plate reader (Life Sciences International (UK) Ltd) with a 450nm filter, and the mean OD of Column 1 was used to blank all the OD values.

### D. *Trypanosoma evansi* card agglutination test (CATT)

The CATT kits were obtained from Professor N. Van Meirvenne, Institute of Tropical Medicine[1]. The CATT uses as antigen, bloodstream-form trypanosomes (RoTat 1.2) of a variable surface antigen type (VAT) that is common to all *T. evansi* stocks so far examined. The trypanosomes have been fixed with formaldehyde, stained with Coomassie blue and freeze-dried, and are reported to be stable at 5-10°C for more than one year. Each vial contains sufficient antigen for approximately 50 tests. Cross-reactions with other salivarian trypanosomes has been reported, but were not considered a problem in this study because of the absence of salivarian trypanosomes other than *T. evansi* in Indonesia.

Positive (freeze-dried goat antiserum) and negative (freeze-dried bovine serum albumin) controls containing 0.10% sodium azide as a preservative were provided in the kit. Phosphate buffered saline (PBS), pH 7.2, containing 0.10% sodium azide was used to reconstitute the antigen (2.5 ml PBS/vial), positive and negative controls (0.5 ml/vial) and for dilution of test samples. The recommended dilution for test samples was 1:4 to 1:8, depending on the animal species and preliminary serial dilutions were conducted to determine the optimal dilution for buffalo sera.

[1] Institute of Tropical Medicine, Laboratory of Serology, Nationalstraat 155, B-2000 Antwerp, Belgium.

CATT protocol

1) The reagents were allowed to warm to room temperature and then reconstituted with PBS, as described above.

2) The test samples were diluted 1:8 using a 96-well plastic microtitre plate (M29A; Dynatech) by adding 20 µl serum to 140 µl PBS. The dilutions were mixed using a plate shaker for one minute. Usually samples were prepared in advance and stored at -20°C for subsequent testing in batches.

3) The test was conducted on plastic cards marked out with a series of circles in which the reagents were placed. Each card was used to test 10 samples and one drop of antigen and 25 µl diluted test sample were added to each circle. Positive and negative controls were included on the first card of each test run. The antigen and serum dilutions were mixed within individual circles with a plastic stirrer that was wiped clean between each sample.

4) The reactants were then mixed thoroughly for five minutes by placing the card on a special rotator provided by the kit supplier. The results were read immediately and recorded as score 0 (negative: no agglutination), score 1 (weakly positive: slight agglutination), score 2 (positive: strong agglutination) or score 3 (positive: very strong agglutination).

## APPENDIX II

## ADDRESSES OF MANUFACTURERS/SUPPLIERS

Becton-Dickinson
Between Towns Road
Cowley
Oxford OX4 3LY

Cambridge Veterinary Sciences
Henry Crabb Road
Littleport
Ely
Cambridgeshire CB6 1SE

Ciba Animal Health
Whittlesford
Cambridge CB2 4QT

Dynatech Laboratories Ltd.
Daux Road
Billingshurst
West Sussex RH14 9SJ

Hoechst Roussel Vet Limited
Walton Manor
Walton
Milton Keynes MK7 7AJ

Life Sciences International (UK) Ltd.
Unit 5, The Ringway Centre
Edison Road
Basingstoke
Hampshire RG21 6YH

Mallinckrodt Veterinary Ltd.
Breakspear Road South
Harefield
Uxbridge
Middlesex UB9 6LS

MSD AGVET
Division of Merck Sharp & Dohme Ltd.
Hertford Road
Hoddesdon
Hertfordshire EN11 9BU

Pierce and Warriner (UK) Ltd.
44 Upper Northgate Street
Chester CH1 4EF

Rhone Merieux Ltd.
Spire Green Centre
Harlow
Essex CM19 5TS

Ruddweigh Pty
Guyra
Australia

Scottish Antibody Production Unit (SAPU)
Law Hospital
Carluke
Lanarkshire

Sigma Chemical Company
3050 Spruce Street
PO Box 14508
St Louis
MO, 63178, USA

The Binding Site Ltd
97 Vincent Drive
Edgbaston
Birmingham B15 2SQ

Whatman Laboratory Sales Ltd
PO Box 6
Twyfords
Reading
Berkshire RG10 9NL