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THE DIAGNOSIS OF *TRYPANOSOMA EVANSI* AND ITS IMMUNOSUPPRESSIVE EFFECT IN WATER BUFFALOES AND PIGS

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Come to the field and work by my side
Hark at me, buffalo
Ploughing and sowing is our job,
Working together we shouldn't spare any effort
Work until the rice is ripe,
You'll have grass in plenty then
(Old Vietnamese folksong)

Aan Barbara en onze drie zonen: Bram, Gijs en Job

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Wicher

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INTRODUCTION

Trypanosoma evansi, the cause of Surra, is pathogenic to most domestic and wild animals but its effect on the host varies according to the virulence of the trypanosome strain, the host species, general stress, other infections and local epizootiological conditions. Surra is endemic in many areas of South-East Asia, where water buffaloes, cattle and pigs are an important income for subsistence farmers. As a result of its adaptation to the mechanical mode of transmission, *T. evansi* is the most widely distributed of the pathogenic animal trypanosomes and has spread to different ecological regions in Africa, the Americas, parts of Europe and Asia. Various species of domesticated livestock can be infected with *T. evansi*: horses, camels, buffaloes, cattle and pigs. In principal all blood sucking flies can transmit the disease, though the ubiquitous 'horse' fly, *Tabanus,* and 'stable' fly, *Stomoxys* are most renowned. In South-America next to flies also vampire bats, during certain seasons can transmit the disease.

The nature of Surra in especially South(-East) Asia has changed considerably over the years. Initially it was an acute disease primarily affecting horses and camels but gradually it developed into a more chronic ailment affecting principally livestock, although acute outbreaks in the various species are still reported. Despite mortality rates in livestock are low, Surra can have a detrimental effect on the performance of animals: fertility, growth rates and feed conversion, can be affected, while on top the immune system is depressed. Especially for mixed farming systems in which livestock is primarily used for draught power it has been well documented that Surra limits the use and performance of draught animals. Moreover as a result of the added stress of work and frequent marginal planes of nutrition, animals may succumb.

With less clinical cases reported it appeared as if the incidence of the disease dropped. This decrease could among others be explained by limited sensitivity of diagnostic tests. Since Surra has become endemic the number of chronic cases has increased and traditional parasitological techniques, with their inherent limitations often cannot detect the presence of trypanosomes and consequently diagnosis is often not made. Once more sensitive serological assays are applied in endemic areas prevalence rates go up significantly. Accurate sensitive diagnostic tools such as, the Card

Agglutination Tests (CATT) and the Enzyme Linked Immunosorbent Assay (ELISA), are therefore a prerequisite to assess the spread and importance of Surra. Other reasoning for the observed declined prevalence rates can be found in a certain degree of selection in both animal and parasite populations over time, while in certain areas effective disease control measures were implemented.

Not being anymore a killer disease for livestock, interest on Surra research decreased, albeit in 1983 an international working group of *T. evansi* was established by the Office Internationale des Epizooties (OIE) with the mandate to review and stimulate research, promote co-ordination between institutes and to provide a platform to discuss recent developments in the diagnosis, epizootiology, treatment, immunology and economic significance of the parasite.

Over the last decades no major progress was made in the control of Surra, and farmers still depend largely on the use of chemotherapeutic drugs. Due to the antigenic diversity of the parasites and their ability to vary antigens during the course of infection, the prospect of developing a vaccine against *T. evansi* will remain, like for tsetse-transmitted trypanosomes, a remote dream. Vector control to prevent transmission of disease has been, on a small scale, rather successful, in controlling tsetse-transmitted trypanosomes. It should however be noted that tsetse biology has been studied in depth and this knowledge was used in the development of effective control methods, such as traps and insecticide treated animals. Vector control seems not the solution for Surra as a range of non-related biting flies should be targeted, each with its own biology, while unlike tsetse flies most 'other' flies are proliferate breeders, and as such vector populations are by definition difficult to control.

CHAPTER 1
LITERATURE REVIEW ON *TRYPANOSOMA EVANSI*

Trypanosoma evansi, the aetiological agent of the disease known as Surra, affects a wide range of domestic animals and as a result of its adaptation to the mechanical mode of transmission is widely distributed (Hoare, 1972; Stephen, 1986; Urquhart and Holmes, 1987). In South-East Asia it is considered the only pathogenic *Trypanosoma* species affecting cattle, horses, water buffaloes and pigs (Luckins, 1988; Reid, 2002). During the past two decades there have been several reviews, that provide a comprehensive overview of previous *T. evansi* research e.g Mahmoud and Gray, 1980; Luckins, 1988; Lun *et al.,*1994; Sileghem *et al.,*1994; Reid, 2002. In the present review one emphasis will be on the diagnosis and immune responses to *T. evansi.*

1.1. Introduction

1.1.1 Historical background

Although various scientists had observed 'motile elongated mircoroganisms' in the blood of fish and amphibians it was Gruby who in 1843 created the generic name *Trypanosoma*. The first pathogenic mammalian species of *Trypanosoma: Trypanosoma evansi* was discovered in 1880 by Evans in India, when investigating a disease of horses known by the local Indians as 'Surra' (meaning low and rotten) (Evans, 1881 & 1882). In the same work he also demonstrated experimentally that *T. evansi* was most likely transmitted by haematophagous flies.

1.1.2 Classification

According to the classification proposed by the Committee on Systematics and Evolution of the Society of Protozoologists, cited by Mehlhorn (2000), the systematic position of pathogenic trypanosomes is as follows:

Sub kingdom	Protozoa
Phylum	Sarcomastigophora
Subphylum	Mastigophora
Class	Zoomastigophorea
Order	Kinetoplastida
Suborder	Trypanosomatina
Family	Trypanosomatidae
Genus	Trypanosoma

Mammalian trypanosomes can be divided into two major groups the Stercoraria and Salivaria (Hoare, 1964) on the basis of their invertebrate cycle and preferred host species. The Stercoraria contain species in which the entire development is confined to the gut of the vector and infective metatrypanosomes can be found in the faeces of the insect. The two most well known species of Stercoraria are *T. cruzi,* the pathogenic trypanosome causing Chagas disease in Latin America, and *T. theileri,* which is usually non pathogenic (Stephen, 1986). Salivarian trypanosomes complete their cyclical development in the 'anterior station' of the vector and infective stages are transmitted to the mammalian host through the bite of an infected fly. *T. evansi* and most of the pathogenic African trypanosomes are classified as salivarian trypanosomes, though *T. evansi* has no cyclical development in the insect vector. Like *T. brucei* and *T. equiperdum, T. evansi* is classified in the sub-genus *Trypanozoon*. Other important sub-genera of veterinary importance in the salivarian group are *Nannomonas,* with *T. congolense* and *T. simiae,* and *Duttonella,* with *T. vivax.*

Table 1 lists the trypanosomes of most veterinary importance, their main characteristics, host preferences, mode of transmission and distribution.

Table 1.1 Trypanosomes of veterinary importance

Species	Group	Hosts				Transmission	Distribution
		Carnivore	Camels/Horse	Ruminants	Pigs		
T. vivax	Salivaria	+	+	+++	-	Tsetse/Mechanically	Sub-Saharan Africa/ ##
T. congolense	Salivaria	+	+	+++	+	Tsetse	Sub-Saharan Africa
T. brucei	Salivaria	+	+	+++	++	Tsetse	Sub-Saharan Africa
T. suis	Salivaria	-	-	-	+++	Tsetse	Sub-Saharan Africa
T. simiae	Salivaria	-	-	-	+++	Tsetse	Sub-Saharan Africa
T. equiperdum	Salivaria	-	+++	-	-	Coitus	(Sub) Tropics
T. evansi	Salivaria	+++	+++	++	+	Mechanically	Africa, Asia, L-America
T. theileri	Stercoaria	-	-	+++	-	Faeces of vector	Global
T. cruzi	Stercoaria	+++	+	+	+	Faeces of bugs	L-America

Adapted from Desquesnes and Davilla 2002,

##, mechanical transmitted *T. vivax* also in Latin-America

1.1.3 Morphology

Unlike other members of the sub-genus *Trypanozoon*, *T. evansi* is monomorphic, although sporadically pleiomorphic strains have been described (Hoare, 1972). With the exception of the slender forms of *T. brucei* one can readily differentiate *T. evansi* from other trypanosomes: *T. evansi* has a small terminal kinetoplast, an elongated nucleus and a long free flagellum (Stephen, 1986). A considerable variation exists in the dimensions of *T. evansi* but its overall length distribution is 15-34 μm with a mean length of 24 μm (Hoare, 1956). Unlike tsetse-borne trypanosomes, whose life cycle involves a defined cycle of development in the insect vector prior to natural transmission, *T. evansi* does not undergo any cyclical development in an intermediate host before transmission. Multiplication of the parasite is restricted to the vertebrate host in which they proliferate by longitudinal binary fission in the trypomastigote stage.

1.1.4 Characterisation

It was postulated by Hoare (1972) that *T. evansi* derived from a *T. brucei* population that was adapted to non-cyclical transmission. Camels at the northern edges of the sub-Saharan tsetse belt, infected with *T. brucei,* would have travelled to non- tsetse areas where other blood-sucking insects would have propagated the infection. Caravans with infected camels, disseminated infections to the Middle-East from where it subsequently spread, most likely through horses, into India and finally to South-East Asia, where it has been present for approximately 100 years (Gibson *et al.,*1980).

Various methods exist to characterise *T. evansi* isolates. Morphologically, *T. evansi* strains are indistinguishable, and it was also noted that the banding patterns of soluble isoenzymes (zymodemes) of geographically separated *T. evansi* isolates had a homogenous pattern (Gibson *et al.,*1983; Boid, 1988). Even isolates from various host species, like buffaloes and cattle, with marked differences in the biology had similar zymodeme patterns (Stevens *et al.,*1989; Lun *et al.,*1992a).

The kinetoplast DNA which consists of mitochondrial DNA is organised in structures called maxicircles and minicircles (Simpson, 1986). Maxicircles which play an important role in cyclic transmission in tsetse flies (Borst *et al.,*1987) are absent in mechanically transmitted *T. evansi*, while the sequence of the minicircle kDNA of

various isolates of *T. evansi* is consistent. Isolates of *T. evansi*, which were indistinguishable by isoenzyme patterns, were found to fall into several karyotype groups (Lun *et al*.,1992b). This relative homology in the isoenzyme banding patterns and consistent karyotype, corroborate the earlier suggestion that *T. evansi* originates from *T. brucei* (Borst *et al.,*1987) and possibly from a mutated *T. brucei* deprived of its maxicircle (Lun and Desser, 1995).

Molecular characterisation of the chromosome DNA by pulsed-field gradient gel electrophoresis seems a good discriminating tool to distinguish between various *T. evansi* isolates (Borst *et al.,*1987). The technique is based on the use of restriction enzymes that cut the chromosome DNA in different fragments, which sizes are subsequently characterised by electrophoresis. The technique has been used to discriminate various parasite strains in epidemiological studies (Waitumbi and Young,1994). Using this technique it was also demonstrated that Chinese isolates were identical but distinct from isolates from South America, Africa and the Philippines (Zhang and Baltz, 1994)

The current most widely used PCR based tool to characterise isolates is the random-amplified polymorphic DNA (RAPD) technique (Waitumbi and Young, 1993). The method has the advantage of being simple, fast and sensitive since it does not require prior DNA sequence information. Also for *T. evansi* a number of arbitrary primers have been identified that can differentiate between different isolates (Basagoudanavar *et al.,*1999). This so-called fingerprinting technique was used in the study of parasite population dynamics during an outbreak in Thailand, and it was demonstrated that the parasites causing the epidemic originated from two different sources (Watanapokasin *et al.,*1998). Since this technique needs pure parasite DNA, typing of field isolates containing host DNA is impossible and prior analysis isolation of parasites for instance through an anion exchange column is needed

1.2 Clinical signs and Pathology

There is a considerable degree of variation in the severity of the pathological effects of *T. evansi*. This variation is ascribed to the difference in virulence of individual strains, susceptibility of the host, local epizootiological conditions and stress factors (Hoare, 1972). The earliest clinical sign of infection with *T. evansi* in any host is the development at the fly bite of a chancre: a cutaneous swelling in which the first trypanosomes multiply (Luckins *et al.*, 1992). This initial replication increases the

establishment of infection, while at this spot also the first interactions take place between the host immune system and the trypanosomes (Dwinger *et al.,* 1989). After formation of a chancre, trypanosomes invade the blood stream, which is accompanied by pyrexia. The parasitaemia may remain high for 4-6 days after which it declines with remission of the temperature. Anaemia is reported the primary cause of death of infected animals (Logan-Henfrey, *et al.,*1992). The cause of anaemia is believed to vary with the stage of infection. In the initial stages the anaemia is most likely caused by extravascular haemolysis, in especially the spleen, liver and lymph nodes and is auto-immune mediated and could result from the deposition of immune complexes on the surface of the erythrocyte (Rickman, 1983). Later in infection insufficient haemopoiesis may contribute to anaemia (Dargie, 1979a & 1979b).

Besides anaemia, other commonly ascribed clinical symptoms are fever, marked depression and dullness, followed by oedema, cachexia and death. There is often a tendency to ascribe the occurrence of severe disease to camels and horses, and milder forms of disease to cattle and water buffaloes. In water buffaloes, Surra evolved over the years from an acute- into a more chronic disease characterized by weight loss and infertility (Luckins, 1988; Thu *et al.,*1998; Davison *et al.,*1999) including abortion (Lohr *et al.,*1986) and anoestrus (Payne *et al.,*1993). However, epidemics of acute Surra in water buffaloes with high case-fatality are still described in Vietnam, China and the Philippines (Luckins,1988; Lun *et al.,*1993; Thu *et al.,*1998; Reid, 2002). In cattle, nervous symptoms such as excitation, aggressive behaviour and convulsions have been described (Tuntasuvan *et al.,*1997). In experimentally infected buffaloes most pathological findings at necropsy were non-specific such as pneumonia, congested liver and spleen, hydropericardium, and hyperplastic bone marrow (Damayanti *et al.,*1994). In deer naturally infected with *T. evansi* congested blood and petechial haemorrhages were seen in the brain at post-mortem (Tuntasuvan *et al.,*2000).

Clinical symptoms of Surra in pigs are seldom observed and it is possible that infections are mostly unnoticed (Stephen, 1986). In Vietnam for instance Surra has never been reported in pigs. Also in fattening pigs experimentally infected with *T. evansi* in Vietnam (Phan Dic Lan, personal communication) and Indonesia (Reid *et al.,*1999) no visual indications of disease could be observed. In Thailand,

(Teeraprasert *et al.,*1984a; Bajyana Songa *et al.,*1987) Malaysia, (Arunusalam *et al.,*1995) and Indonesia (Kraneveld and Mansjoer, 1947) outbreaks associated with abortion and death in sows have been observed. Natural infected sows in Thailand had typical skin rashes on ears and lateral sides of the body and abortion was observed most frequently at the early stage of pregnancy (Teeraprasert *et al.,*1984a). In addition nervous signs such as convulsions and circling movement were observed in the infected sows. Natural infected weaning and fattening pigs showed no symptoms (Teeraprasert *et al.,* 1984a). Experimental infection of sows at 1-2 month pregnancy resulted in abortion after approximately 4 days (Teeraprasert *et al.,*1984b). In India, acute infections were observed in *T. evansi* infected pigs with symptoms similar to Classical Swine Fever (Gill *et al.,* 1987).

1.3 Epidemiology

1.3.1 Hosts and reservoirs

Surra, seems a newcomer to South-East Asia and the epidemics reported from this region, at the beginning of the 20th century, are indicative that Surra only spread into the area within the last 100 years (Luckins, 1998). Acute epidemic outbreaks were also observed in South America, only here the peak of the epidemics occurred in the mid 19th century.

T. evansi infects a wide host range; among domesticated animals it affects camels, water buffaloes, cattle, sheep, goats, horses, donkeys, the Indian elephant, dogs, cats and pigs (Stephen, 1986). Besides it affects a wide range of wild animals such as; wild pig, Rusa deer, hog deer, tigers and wallabies (Stephen, 1986; Reid *et al.,*1999; Tuntasuvan *et al.,*2000; Reid *et al.,*2001b). The principle host varies between the geographical regions: in Africa camels are the most important host (Dia *et al.,*1997), in South America horses are most affected (Monzon *et al.,*1995) while in Asia a range of hosts can become affected including cattle, camels, buffalo and pigs (Luckins, 1988). For Africa and Latin America there seems little indication that other domesticated livestock species are also commonly infected, although in Africa there was serological evidence that goats and sheep were infected (Boid *et al.,*1981) while in Latin America, infection in cattle was noted (Francke *et al.,*1994).

For wild animals in South-East Asia *T. evansi* infections are just as exotic as for domesticated species, unlike the situation in sub-Saharan Africa where wild animals

have been exposed to trypanosomes for millennia prior to the introduction of domesticated cattle and thus evolved to develop effective protection mechanisms. Since many wild animals species in Asia are still highly susceptible to *T. evansi* it seems unlikely that wildlife would develop chronic, persistent infections and thereby form a reservoir for Surra (Hoare, 1972). In experimental infections of pig and Rusa deer in Indonesia however, it was demonstrated that animals were persistently infected without displaying clinical symptoms, pigs supposedly a model for the wild boar (Reid *et al.,*1999). Recently, in Latin America it was found that both capybara and coati could play an important role in enzootic areas as reservoir hosts (Franke *et al.,*1994; Herrera *et al.,*2001). However there is no clear indication that wild animals play a role in the epidemiology of *T. evansi* in the densely populated, intensively farmed regions in South-East Asia where the ecological conditions preclude close contact between domesticated and game animals (Luckins, 1998).

Over the last decades the apparent incidence of trypanosomosis in South-East Asia has dropped significantly. Various explanations exist: with increased mobility of man and livestock, infections have now spread widely and in most livestock populations infection has become endemic. After the initial acute epidemic stage, infection in most herds gradually became endemic, with sporadic relapses due to stress factors such as: feed shortage, pregnancy or work. Once endemic, Surra is characterized by weight loss and infertility (Luckins, 1988; Thu *et al.,*1998; Davison *et al.,*1999). Acute disease with high mortality can still be noted in naïve herds when infected livestock is introduced, or if naïve animals are imported into an endemic area. Recently Surra was introduced on to the Canary Islands by importation of infected camels (Molina *et al.,*2001) while in the eighties the importation of feral buffaloes from Australia to Indonesia led to losses among these animals due to *T. evansi* infections or mixed *Anaplasma / T. evansi* infections (Payne *et al.,*1991a).
The observed drop of incidence could also be due to natural selection processes in both parasite and host populations such that parasites now have a lower pathogenicity and highly susceptible animals have been selected out.

To study the behaviour of trypanosomes in a herd, Wilson (1983) listed 3 main parameters: animal population(s), vector population(s) and the characteristics of the *T. evansi* strain involved. As each variable includes a wide range of determinants,

predicting the spread of infection becomes a very difficult task. In the development of the quantitative epidemiology and improved modelling systems our knowledge of how infections behave in populations by identifying association and risk factor has improved significantly. In Colombia mechanically transmitted *T. vivax* infection was strongly associated with *Tabanid* activity and pastures located in low marshlands (Otte *et al.,*1994). In Ethiopia tsetse transmitted trypanosome infections were associated with season, herd, age and sex (Rowlands *et al.,*1993). For *T. evansi,* a data set from Indonesia was analysed and it was postulated that buffalo could spontaneously recover from infection and be re-infected nearly every year (Coen *et al*.,2001)

1.3.2 Transmission

T. evansi is transmitted mechanically by haematophagous flies such as: *Chrysops, Haematopota, Stomoxys* and *Tabanus* (Stephen, 1986). *Tabanus* is considered the most important vector in South-East Asia and its abundance in the rainy seasons is associated with an increased prevalence of infection with *T. evansi* in especially water buffaloes (Luckins, 1988). The most elaborate work on transmission of *T. evansi* by various flies was conducted at the beginning of the twentieth century by Nieschulz in Indonesia and reviewed by Dieleman (1986). Nieschulz, in a number of experimental studies, demonstrated that over 25 *Tabanus* species could transmit the disease. Not only the structure of its mouthparts but more importantly the feeding behaviour of the fly makes it an efficient vector (Luckins, 1988). *Tabanus* species may consume volumes up to 200 µl (Hoare, 1972), and are determined feeders, quickly changing hosts. Although *Chrysops, Haematopota* and *Stomoxys* have been described in a number of Asian countries including Indonesia and Vietnam (Dieleman, 1986; Lan, 1996), their role in the transmission is considered of less importance (Foil, 1989). Although under experimental conditions it was demonstrated that *Stomoxys* could transmit *T. evansi* to mice (Mihok *et al.,*1995). In general, the dynamics of mechanical transmission are not well understood and more work needs to be done on the relationships between the host, duration of infection and level of parasitaemia, the period between feedings and the efficiency of various vector species.

In Latin America, transmission is not only through insects but the vampire bat (*Desmodus rotundus*) also plays an important role (Stephen, 1986). These bats are

ideal vectors, when feeding on hosts such as cattle with very scanty parasitaemia, their infection is ensured by the large amount of blood that they consume (50 ml), while the subsequent proliferation of the trypanosomes in their own blood increases the chances of successful transmission to new hosts. The role of bats is thus twofold that of host and vector (Hoare, 1965). Carnivores can become infected with *T. evansi* by the ingestion of raw meat from infected animal carcasses (Mulligan, 1970).

Iatrogenic transmission has been described for anaplasmosis (Guglielmone, 1995) and although not yet verified for *T. evansi* might play also a role in the transmission of *T. evansi*.

1.3.3 Economic importance

In earlier days the economic impact of *T. evansi* was clear as large numbers of animals succumbed each year in epidemics and large amounts of trypanocidal drugs were used. Nowadays where infection has become less pathogenic and an endemic situation occurs, more animals are chronically infected, mortality rates have dropped dramatically and disease often goes unnoticed (Luckins, 1988). Hence there is a tendency to under-estimate the prevalence of Surra, as animal health staff tend to emphasize the importance of more acute infectious diseases e.g. haemorrhagic septicaemia in water buffaloes or hog cholera in pigs.

To estimate the indirect costs of *T. evansi* infections firstly the prevalence rate should be determined accurately for which accurate diagnostic tools are necessary, and secondly the impact on various productivity parameters needs to be defined. In a limited number of studies it was demonstrated that *T. evansi* infections reduced weightgain by nearly 8 kg in feedlot cattle over a three months period (Payne *et al.,*1994a). Also, the impact of infection on the performance of working buffaloes was quite dramatic, with 30 % less output (Pearson *et al.,*1999). The impact on reproductive performance is two fold. Firstly infections have been associated with abortions in water buffalo, pigs and cattle (Lohr *et al.,*1986; Bayjana Songa *et al.,*1987; Arunasalam *et al.,*1995, Kashiwazaki *et al.,*1998), secondly, chronic infections lead to weight loss and subsequently to cessation of the oestrus cycle (Payne *et al.,*1993). In Thailand it was demonstrated that cattle with *T. evansi* infection had a reduced milk yield compared to uninfected animals (Kashiwazaki *et al.,*1998; Pohlpark *et al.,*1999).

1.4 Immune response

When attacked by the hosts immune system, parasites use various techniques to ensure their survival (Borst, 1991). For trypanosomes, both antigenic variation and immunosuppression have been described extensively (Sileghem *et al.,*1994; Taylor, 1998). Since most of the research on interactions between the trypanosomes and the immune system was conducted on tsetse-transmitted trypanosomes this review will describe general aspects of the immunology of trypanosomes and when applicable the specific characteristic of *T. evansi* infections will be mentioned.

1.4.1 Antigenic variation

To understand the immune response against trypanosomes it is important to realize that the entire surface of the trypanosome is covered with a dense protein coat that consists of a single protein species known as the variant surface glycoprotein (VSG). Trypanosomes posses the ability to undergo antigenic variation, which is a process whereby the parasites sequentially express and shed a series of different VSGs (Vickerman, 1978) that enables them to evade the host's protective immune responses. VSG's are capable of inducing protective immunity, and the immune response to each variant, although rapid and highly efficient in destroying any trypanosomes that possess that particular antigen, is invariably too late to affect trypanosomes which have altered their surface antigenic identity.

The highly immunogenic VSG determines the antigenic characteristics of the trypanosomes, known as variable antigen types (VATs, Vickerman and Luckins, 1968). During an infection, although an immune response is elicited against a particular VAT, the trypanosomes are able to express new and immunologically distinct VSGs which generate different VATs and result in persistent infection (Vickerman, 1978). Clinically, this is manifested as fluctuating or successive waves of parasitaemia with the parasite staying one step ahead of the immune response. Each parasitaemic peak is a mixture of a mainly predominant VAT population and a small fraction of switched minor VAT populations (Borst, 1991). The switching of the exposed variant-specific antigen in a subfraction of the parasite population and the expression of the surface antigen genes occur in a defined order, which avoids simultaneous gross population heterogeneity. This in turn avoids rapid induction of antibodies against all members of the surface antigen repertoire which otherwise would ensure the ablation of the entire parasite population by the antibodies (Borst,

1991). Although *Trypanozoon* organisms posses genes to express hundreds of VAT's (Van der Ploeg *et al.,*1982) in the early stage of infections only a few VAT's are expressed consistently by stocks of different origin. These so called predominant VATs can be defined as VATs that are more frequently activated than others. In addition, they have a strong tendency to reappear at any stage of the infection and antibodies against these predominant VATs often persist in chronically infected animals (Van Meirvenne, 1987). Based on these characteristics, predominant VATs are considered a good source of antigen to develop diagnostic antibody detection tests. *Trypanosoma evansi* RoTat 1.2 which was isolated from a buffalo in Indonesia is such a predominant VAT (Bayjana Songa *et al.,*1987). Antibodies against the RoTat 1.2 VAT were elicited by 10 different *T. evansi* stocks and clones from different geographical areas within 5 weeks of inoculation when the parasites were injected in rabbits (Verloo *et al.,*2001).

1.4.2 Immunology

As most trypanosomes live free in the blood of the vertebrate host and have no intracellular stages, most times they can be a direct target for antibody mediated destruction and antibodies are therefore of major importance in the control of parasitaemia in the infected host. If trypanosomes are located in areas which cannot be targeted by antibodies like the brain (Tuntasuvan, 2000) they are protected from this humoral immune response.

The pre-eminent importance of antibodies was demonstrated in animal that were inoculated with irradiated trypanosomes and which became resistant to subsequent challenge with the homologous trypanosomes (Morrison *et al.,*1982). The immune responses generated in the immune cattle were primarily against the exposed VSG's, and it was concluded that these VAT-specific antibodies were the most vital part of the immune system that can alter the course of infection (Akol and Murray, 1983).

Humoral immunity is rapid and strong but because of antigenic variation is effective only against the VAT that elicited it (Morrison, *et al.,*1985). The typical primary humoral immune response of the mammalian host is somewhat modified during infection in that IgM production is greatly enhanced and prolonged. This serum hypergammaglobulinaemia of mainly the IgM isotype has been described in a range of hosts including cattle (Luckins, 1976; Luckins and Mehlitz 1978a: Morrison *et al.,*1985) and forms the basis of a number of serological tests such as the card

agglutination test (CATT, Bayjana Songa and Hamers, 1988). Whether these elevated IgM levels are largely non-specific possibly resulting from polyclonal activation of B cells (Hudson *et al.,*1976) or parasite specific (Musoke *et al.,*, 1981) has been under long debate. Onah (1998a) demonstrated that the B cells in *T. evansi* infected sheep were for 70 % of the CD5+ type, which is in line with previous findings with *T. congolense* in cattle (Williams *et al.,*1991). Since CD5+ B cells are known to produce non-specific low–affinity IgM (Casali and Notkins 1989) the situation in sheep and cattle seems similar to that in mice, in that CD5+ B cells produce antibodies that are non-restrictive, non-specific and react with self-components (Minoprio *et al.*, 1991).

Because of the strong evidence that antibodies mediate protection against trypanosome infections, limited effort has been made to investigate cell-mediated immune responses to trypanosomes. It seems unlikely that effector T cells play a direct role in attacking the trypanosomes. But significant changes were noted in regulatory T cells and T cell functions, which have both their effect on the immune response following infection with *T. evansi*. Reinitz and Mansfield (1990) demonstrated in mice that the VSG-generated antibodies represented a mixture of T-cell dependent and T-cell independent antibodies directed at different exposed VSG epitopes.

In a recent study on *T. evansi* in sheep Onah and collegues (1998b) demonstrated that in sheep in which there was a moderate B cell increase, a normal CD4+ and reduced CD8+ T-cell population, produced high levels of neutralizing antibodies. Conversely, sheep with a marked increase in circulating B cells and reduced subsets of both CD4+ and CD8+ T cell produced lower parasite-specific antibody levels and failed to eliminate trypanosomes. It was concluded that although T cell subsets play no direct role in trypanosome killing, alterations in their relative numbers by an ongoing infection affects the level and pattern of antibody responses and therefore, the dynamics of the infection.

Another aspect of the cellular immune response to trypanosomes is the development of a skin reaction at the site of inoculation of the infectious trypanosomes, which usually starts at about 5 to 7 days and peaks at about 9 to 12 days after the inoculation (Luckins *et al.,*1992). The immunological role of the chancre is not clear but there are indications that trypanosome-infected animals do not develop a chancre upon homologous challenge (Dwinger *et al.,*1989), while animals of low susceptibility develop smaller chancres. Furthermore, immunisation of goats with tsetse-transmitted

T. congolense followed by drug treatment on day 13 p.i. after chancre development, gave rise to the development of protection to homologous challenge (Taiwo *et al.,*1990). In contrast, immunisation by intravenous inoculation of the metacyclics, or treatment of the tsetse infected goats prior to development of chancre, failed to induce protection to homologous challenge (Taiwo *et al.,*1990). Thus chancre formation and the cellular changes which occur are vital for the induction of comprehensive immune recognition of metacyclic variable antigen repertoire deposited in the skin by infected tsetses, and hence the development of protective immunity. For *T. evansi* only limited research has been conducted on chancres (Luckins, 1992). Akol and colleagues (1986) speculated that the wide variability in susceptibility to *T. evansi* in various hosts could be linked to their ability to regulate multiplication in the skin, in a similar way to that which occurs in trypanotolerant cattle

1.4.3 Immunosuppression

Pathogenic trypanosomes induce a generalised immunosuppression of both humoral antibody response and T cell-mediated immune responses. Immunosuppression has a three fold effect: (1) together with antigenic variation it ensures survival and dissemination of the parasites in the host, (2) predisposes hosts to secondary infections and (3) compromises effective immune responses to vaccine antigens (Sileghem and Flynn, 1992, Sileghem *et al.,*1994). As a result, in the long term, the host's immune responses fail and it succumbs to either the overwhelming parasite load, especially in rodents, or to secondary infection.

Various studies have shown that polyclonal B cell activation, generation of suppressor T cells and macrophages and altered antigen handling and presentation are all mechanisms that could be involved in trypanosome mediated immunosuppression (reviewed by Sileghem *et al.,*1994). It seems that macrophages are central to immunosuppression and that upon activation of these cells a variety of factors and cytokines are released which cause a range of effects such as B-cell activation and T-cell suppression (Askonas, 1985, Sileghem *et al.,*1994).

Most of the work so far was conducted on tsetse-transmitted trypanosomes and primarily on rodents. It was initially assumed that immunosuppression might not be a feature of livestock trypanosomes, especially as the enlargement and destruction of lymph node architecture in large animals is not as dramatic as in rodent trypanosomosis. Studies on immunosuppression in livestock evolved from describing

differences in disease prevalence and pathogenicity of various micro-organisms in trypanosome-infected and non-infected animals (Rurangirwa *et al.,*1979; Bayjana Songa *et al.,*1987; Wernery *et al.,*1991; Kauffmann *et al.,*1992) to studies monitoring immune responses after vaccination (Whitelaw *et al.,*1979; Ilemobade *et al.,*1982; Rurangirwa *et al.,*1983; Ikeme *et al.,*1984).

Onah in a number of studies, (Onah *et al.,*1996, 1997, 1998a, 1998c, 2000) confirmed that *T. evansi* induces haematological changes and immunosuppression similar to those seen in animals infected with tsetse-transmitted *Trypanosoma* species. In sheep *T. evansi* induced increased production of CD5+ B cells in the peripheral circulation and cells showing immature and/or dying characteristics such as the co-expression of the CD4 and CD8 surface antigens in prefemoral efferent lymph (Onah *et al.,*1998b). *T. evansi* also significantly suppressed mitogen induced proliferation in peripheral blood mononuclear cells upon stimulation with: trypanosomal and Pasteurella antigen (Onah *et al.,*1998c). Finally, as in *T. congolense* infected cattle, macrophages were held mainly responsible for suppressed T cell proliferation upon mitogenic stimulation in *T. evansi*-infected sheep (Onah *et al.,* 2000). Onah confirmed that infection in sheep increased susceptibility to other pathogens and compromised vaccination responses. No comprehensive studies on immunosuppression have been conducted, with water buffalo or pigs, which are of major importance in South-East Asia.

1.5 Diagnosis

In order to obtain information on the epidemiological importance of Surra, diagnosis is of utmost importance. At the individual level, diagnosis is important to target animals for treatment, while at the herd level it can be used to study the spread of disease within the population. Currently, diagnosis for single animals still depends on clinical signs and traditional parasitological techniques such as Giemsa-stained slides and micro haematocrite concentration technique (MHCT). It is likely that the diagnosis for individual animals is often missed as clinical symptoms are not pathognomonic and the diagnostic sensitivity of traditional parasitological techniques is low.

Each diagnostic test has a number of important characteristics: sensitivity (proportion of infected animals testing positive), specificity (proportion of non-infected animals testing negative), reproducibility (ability of test to produce consistent results when

preformed in different laboratories), repeatability (ability of test to produce consistent results, when test is run on various occasions under identical conditions). Other important features are; ease of interpretation, user friendliness, quick results and low cost. As these features vary for each diagnostic test or are often not known, the choice of which tests is not an easy task. The requirements of trypanosomosis diagnostic tests depend on a range of factors such as: trypanosome species, host species, epidemiological parameters as estimated prevalence and the reason for diagnosing (quarantine, survey, diseased animal) (Martin, 1977; Thrusfield, 1997; Jacobsen, 1998)

Diagnostic tests for trypanosomes can be divided into 2 groups: (1) direct tests that detect either whole parasites, parasite antigens or the parasites' DNA (2) and indirect tests that detect the antibody response of the host to the infection.

1.5.1 Parasitological tests

Parasitological tests have the advantage to demonstrate the parasite and are consequently 100% specific, and in addition the trypanosome species can be identified on morphological characteristics. Direct techniques examine blood without any concentration step i.e. wet blood films and stained thin and thick smears (Kageruka and van Meirvenne, 1996). The major disadvantage of these tests is the low sensitivity as only parasite levels above 100,000 per ml can be detected (Paris *et al.,*1982).

In the MHCT, trypanosomes are concentrated at the buffy coat of a capillary tube after centrifugation and capillaries can directly be examined under the microscope (Woo, 1970). A modification of the MHCT is the Buffy Coat Technique (BCT): where the buffy coat is expelled on a glass slide and subsequently examined under dark ground (Murray *et al.,* 1977). Although the BCT is more sensitive, MHCT is the test of choice for detection of animal trypanosomosis under field conditions (Molyneux, 1975). For human sleeping sickness the miniature mini-anion exchange centrifugation technique (MAECT), is the preferred parasitological test, able to pick up parasites at levels of 18 trypanosomes per ml of blood (Lumsden *et al.,*1977). In the MAECT a phosphate buffered saline glucose buffer (PSG) is used which negatively charges the host blood cells which are subsequently absorbed into the anion-exchange column, while the trypanosomes are eluted, retaining their viability

(Lanham *et al.,*1970). The most sensitive method for detecting parasites is the mouse inoculation technique, in which whole blood (0,3 – 0,5 ml) is injected intra-peritoneally (Godfrey *et al.,*1962). The blood of the mice is subsequently monitored 3 times per week, by wet blood film examination. Theoretically 1 trypanosome per injected volume can be picked up. Most of the parasitological tests were validated for the sub-Saharan trypanosome species (Paris *et al*. 1982), but such studies are nearly absent for *T. evansi,* except for a study that compared several parasitological tests in horses (Monzon *et al.,*1990) and a recent study that further optimised the existing tests using mice (Reid *et al.,*2001a)

1.5.2 Serological tests

Antigen detection

Antigen detection tests have some potential advantages over antibody detection assays. Firstly antigen detection assays have the ability to detect active trypanosome infections as indicated by antigenaemia and secondly after chemotherapy clearance of the trypanosomes can be monitored as indicated by disappearance of antigenaemia (Nantulya et al.,1987).

Several authors reported on the use of *T. evansi* antigen detection ELISAs to test camels (Nantulya *et al.,*1989, Diall *et al.,*1992), horses (Monzon *et al.,*1995), cattle (Kashiwazaki *et al.,*1998) and buffaloes (Nantulya *et al.,*1989, Davison *et al.,*2000). However, proper evaluation of the diagnostic parameters of *T. evansi* antigen detection ELISAs showed poor results (Davison *et al.,*1999, Thammasart *et al.,*2001).

Other, monoclonal based antibody- antigen detections tests which have been developed for *Trypanozoon* infections are a dipstick colloidal dye immunoassay (Kashiwazaki *et al.,* 1994) an antigen-capture tube ELISA (Waithanji *et al.,*1993) and a latex agglutination assay (Suratex, Nantulya, 1994). The commercial Suratex assay, an indirect latex agglutination, in which latex particles have been sensitized with a specific monoclonal antibody against a somatic antigen, has been partially validated using experimentally infected camels, and initial results were promising (Olahu Mukani *et al.,*1996).

Antibody detection

Antibody detection assays have major disadvantages as the presence of antibodies does not necessarily indicate that parasites are present and the assays cannot be used

to confirm efficacy of therapy as antibodies remain elevated for long periods (Luckins *et al.,*1979). In addition, depending on the capture antigen employed, cross-reactions with antibodies against other micro-organisms can be a major draw-back in the interpretation of the results.

The early methods to detect humoral antibodies to *T. evansi* include complement fixation, indirect haemagglutination, slide agglutination and precipitation tests (Kageruka and van Meirvenne, 1996). Later on, antibody ELISAs (Luckins *et al.,*1978b) and the Indirect Fluorescent Antibody Test (IFAT, Luckins 1979) were developed. In the IFAT the antigen consist of acetone fixed whole trypanosomes on a microscope slide. The IFAT is still in use, but has drawbacks since, heterologous cross-reactions are not uncommon, test interpretation is operator dependant and test procedures are rather laborious. The ELISA has several advantages over other assays: capacity to test large numbers of samples, a quantitative result instead of subjective qualitative interpretation and often better test parameters. ELISA tests have been used for different target species employing various antigens. Most of the current antibody ELISAs employ relatively crude antigen preparations derived from bloodstream form trypanosomes grown in rodents. Only recently the DNA coding for RoTat 1.2 VSG-antigen was expressed in a *Baculo* system and recombinant antigen was obtained with potential use, in probable better diagnostic assays (Urukawa *et al.,*2001).

The CATT (Magnus 1978) is a rapid direct agglutination test which uses fixed, Coomassie blue stained, freeze-dried trypanosomes of certain predominant VAT's. The CATT/*T. evansi is* based on VAT RoTat 1.2 (Bajyana Songa and Hamers 1988). The CATT is quick and easy to perform, and for prevailing conditions in Indonesia was found to be the best antibody detection test in water buffaloes (Davision *et al.,*1999). Among the drawbacks of the CATT are the subjective test interpretation and the need for serum, which is not readily available under field conditions.

To obtain more specific antigen the variable surface glycoprotein (VSG) of *T. evansi* VAT RoTat 1.2 was purified and subsequently covalently coupled to latex particles (Verloo *et al.,*1998) based on the method of Lejon *et al.* (1998). Handling and test interpretation are similar to those used in the CATT/*T. evansi.* With its purified antigen the latex agglutination assay (LATEX) should potentially have better test characteristics than the CATT but until now, it has not been validated.

The diagnostic principle of the immune trypanolysis test is based on the progressive accumulation of protective antibodies to a range of VATs that are successively

experienced in the course of infection (Van Meirvenne, 1987). For use in immune trypanolysis the VAT employed should be one of the early predominant ones. Serum with specific antibodies against a VAT in the presence of complement will cause lysis of life trypanosomes of that VAT. As non-specific lysis may sometimes occur, all serum samples that test positive, should be tested subsequently with a VAT that is never expressed by *T. evansi*. Van Meirvenne and colleagues (1995) concluded that the immune trypanolysis assay could serve as a reference antibody test for all serological assays with that specific VAT, since both the diagnostic specificity and sensitivity are exceptionally high.

Except for the CATT/*T. evansi,* none of the serological test based on RoTat 1.2 have yet been validated, which is a prerequisite for their use in the field.

1.5.3 Molecular diagnosis

With the introduction of molecular diagnostic techniques, several diagnostic assays based on the detection of trypanosomal DNA by PCR have been developed (Wuyts *et al.,*1995; Clausen *et al.,*1998, Donelson *et al.,*1998, Omanwar *et al.,*1999). PCR is reported to be more sensitive than conventional parasitological techniques in a number of hosts and has the advantage that it can identify parasites at the species level (Wuyts *et al.,*1995; de Almeida *et al.,*1997; Desquesnes,1997; Masake *et al.,*1997; Desquesnes and Davila ,2002). In the absence of contamination, the positive predictive values of a PCR are very high, and detection of trypanosomal DNA is synonymous with active infection, as it has been demonstrated that trypanosomal DNA is eliminated within 1-2 days after treatment with diminazene aceturate (Clausen *et al.,*1999). However, diagnosis by regular PCR is still relatively complicated and expensive, especially when used as a diagnostic tool in hosts like African cattle potentially infected with multiple species of trypanosomes. With the introduction of PCR-ELISA (Chansiri *et al.,*2002) and techniques which permit to distinguish between various *Trypanosoma* taxa (even in mixed infections) with one single PCR (Desquesnes *et al.,* 2001), the role of PCR in epidemiological studies will expand.

Various specific primers for detection of *Trypanosoma evansi* based on kinetoplastid mini-circle DNA sequences have been identified (Masiga and Gibson, 1990; Artama *et al.,* 1992) unlike the situation with *T. equiperdum* for which no specific primers have yet been described (Desquesnes and Davila, 2002).

1.6 Control

Control of Surra can be achieved in two ways: (1) prevention of transmission or (2) treatment of affected animals. Several options are at hand to prevent transmission of *T. evansi*. The most obvious method is the application of insecticides, which kill potential transmitters of the infection. Insecticides can be used on the animal, on possible resting sites or on places where insects prefer to breed. Due to the size and the behaviour of most *Tabanus* species, insecticides in general have low efficacy. Further the price of the chemicals limits their regular use. Another approach is changing the environment so that conditions become unfavourable for insect breeding (e.g drainage systems) and the risk of animals becoming infected can be reduced. Unfortunately there is only scarce information on the ecology of the various *Tabanus* species and other potential vectors, but with the information available it is already clear that no single approach can be followed to attain control of all the potential vectors (Stephen, 1986). The only method currently used to prevent transmission of Surra with some success is to avoid contact between the vector and the host. This can be achieved by confinement of animals in stables during the days, as Tabanids prefer to feed in sunlight, or by the introduction of mosquito netting as in commercial pig farms. Historically smudge fires (fires that produce lots of smoke) have been used to reduce attacks of biting flies, and in rural areas it is still common practice.

Chemotherapy has been used for decades to control Surra. Suramin was one the earliest drugs, and has been very successful despite its relative toxicity and its intravenous route of administration. Due to increasing resistance (Partoutomo *et al.,*1986) and commercial reasons of its producer, the manufacturing of suramin ceased, and farmers shifted to other drugs.

Diminazene aceturate has proven to be very successful in the treatment of most trypanosomes in a range of animal species. The product is however toxic for camels. Diminazene aceturate has been used successfully in the treatment of Surra in buffalo and cattle (Mahmoud and Gray, 1980). Recent studies in horses indicate that the treatment is not always successful in this species (Tuntasuvan *et al., 2003).* Melarsomine (Cymelarsan®) is the most recent drug developed and has proven to be highly efficacious in the treatment of infected camels (Zelleke *et al.,*1989). Despite its high price the product is increasingly used in the treatment of buffalo and cattle,

especially since it is also effective against suramin-resistant trypanosomes (Lun *et al.,*1991; Payne *et al.,*1994b).

Isometamidium is used mainly in the prevention of tsetse-transmitted trypanosomosis. Its usefulness in the treatment of *T. evansi* infections is not clear. In Indonesia it had very limited effect in experimentally infected cattle and buffaloes (Dieleman, 1986), while in Vietnam it has been used successfully in both cattle and buffaloes (My *et al.,*1998). Additional research suggested that *T. evansi* might be innately resistant to this compound (Brun and Lun, 1994). Quinapyramine is the drug of choice in India, but in other countries its use is limited as its efficacy is considered low. The medium-term outlook for the control of Surra is likely to remain dependent on chemotherapeutic intervention, principally with diminazene aceturate, while for problematic cases melarsomine might be the product of choice (Payne *et al.,*1994b).
Chemoprophylaxis is not a common practise in the prevention of Surra. Isometamidium which is widely used in nagana, has only limited prophylactic activity against Surra.

1.7 *Trypanosoma evansi* in Vietnam

Livestock composes 25 % of the agriculture output value in northern Vietnam and is almost entirely in the hand of small farmers, as part of a system integrated with crops and fish (Ly, 1996). During the last few years pig numbers have increased steadily to 20 million head while the buffalo population has remained stable at nearly 3 million (Table 1.1). Buffalo are primarily raised for draught purposes, while pigs are raised for their meat. The cattle population has more than doubled over the last 20 years. In southern Vietnam due to agriculture mechanisation, buffaloes have nearly disappeared; people increasingly rear more productive cattle (Ly, 1996). In contrast in the north of Vietnam the cattle population remains small, since cattle are considered unsuitable for land tillage.
Surra was first detected in Vietnam in mules in 1886 (Luckins, 1988). In the 1960, 20.000 buffaloes succumbed due to Surra infections (Doan and My, 1985). In the seventies of the last century at the time of farmer cooperatives, large outbreaks were reported from the north of Vietnam. After their labour in the rice fields of the Red River delta water buffaloes were transferred to the mountains to be relocated the next ploughing season.

Table 1.2 Evolution of human and livestock populations in Vietnam in the period from 1965 to 2000

	1965	1970	1975	1980	1985	1990	1995	2000	*Change 1965-2000*
Human population (mill persons)									
Urban	6.3	7.9	0.0	10.2	11.6	13.0	14.2	15.4	*+144%*
Rural	31.8	35.1	39.0	42.8	47.5	53.0	58.7	62.7	*+79%*
Total	38.1	43.0	48.0	53.0	59.1	66.1	72.8	78.1	*+104%*
Livestock populations (mill head)									
Cattle	1.9	1.6	1.5	1.7	2.6	3.1	3.6	4.1	*+115%*
Buffalo	2.3	2.3	2.2	2.3	2.6	2.9	3.0	2.9	*+26%*
Pigs	8.3	9.5	8.7	10.0	11.8	12.3	16.3	20.2	*+143%*
Poultry	65.1	64.4	58.8	64.5	91.2	107.4	142.1	191.8	*+194%*

*Note * includes both chickens and ducks. Ducks constitute about 25-30% of the national poultry flock.*
Source: FAO on-line database, http://apps1.fao.org/

Due to this extensive travelling, mingling of animals from various origins and shortage of fodder at the end of the winter trypanosomosis killed large numbers of water buffaloes. In the eighties, when water buffaloes remained all year round in the delta, acute outbreaks halted (Pham,1978; My *et al.,*1998; Thu *et al.,*1998) and Surra became increasingly endemic. Prevalence rates between the geographical areas started to vary, in the mountain areas infections rates were approximately 10 %, while in the delta areas infections rates went up to 60% (Pham, 1985). In the mid eighties an acute outbreak was reported in an imported herd of Murrah buffaloes from India, a breed of riverine buffaloes, with frequent abortions, and an incidence rate of over 50% (Bui and Le, 1986). Also in swamp buffaloes abortion has frequently been observed (Khai, 1995; My *et al.,*1994, My *et al.,*1998).
Over the last 30 years suramin has been the drug of choice in Vietnam, while more recently diminazene aceturate and isometamidium have been introduced. For isometamidium some resistance has already been observed, whereas all *T. evansi* isolates were still sensitive to suramin and diminazene aceturate (My *et al.,*1998).

Now that *T. evansi* has become endemic and is often unnoticed in most areas in South East Asia, and with the circumstantial evidence that poor protection after vaccination against bacteriological and virological occurs most often in endemic areas there is a strong need (1) to obtain more sensitive validated diagnostic tools to conduct epidemiological surveys and (2) to investigate the possible interference of *T. evansi* in

the development of protective immunity following vaccination of water buffaloes against haemorrhagic septicaemia and pigs against classical swine fever.

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OBJECTIVES OF THE STUDY

The two main objectives of the work were (1) to develop, optimise and validate diagnostic tests for *Trypanosoma evansi* infections in water buffaloes and pigs and (2) to determine if *T. evansi* infections in water buffaloes and pigs had a negative influence on the development of protective immunity following vaccination.

In the **first part** of this thesis the optimisation and evaluation of a number of serological tests based on the RoTat 1.2 antigen of *T. evansi* i.e. card agglutination test (CATT), latex agglutination test (LATEX), ELISA, the immune trypanolysis test (T.L.), parasitological tests and a PCR for water buffaloes are described (Chapters 2 to 5). The serological assays were initially developed for use with serum samples, but later other substrates were evaluated including fresh blood for the CATT and LATEX and fluid eluted from blood dried on filter-paper discs, for the ELISA and T.L. The diagnostic tests were evaluated in both experimental infections and under field conditions. The influence of storage conditions of test samples on test result was also investigated to optimise the manner in which blood samples might be stored and the time interval required to process samples after collection. Each of antibody assays was then optimised for use in pigs (Chapter 6). After initial validation in experimentally infected pigs, the assays were employed in a survey in Northern Vietnam and in North-Eastern Thailand.

The **second part** of this thesis determines the possible implication of *T. evansi* infections on the immuno-responsiveness of water buffaloes and pigs. In particular the effects of experimentally induced infection on the development of specific antibody responses to vaccination against haemorrhagic septicaemia, and concomitant immunization with human serum albumin as a control antigen was investigated in buffaloes (Chapter 7). The possible interference of *T. evansi* with the development of immunity after vaccination against classical swine fever in pigs was also investigated (Chapter 8).

The **third** and last part consists of a general discussion in which the practical implications of the results are discussed

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PART 1

Diagnosis of *Trypanosoma evansi* in water buffaloes and pigs

CHAPTER 2
COMPARISON OF SEROLOGICAL TESTS FOR *TRYPANOSOMA EVANSI* INFECTIONS IN WATER BUFFALOES FROM NORTH VIETNAM[1]

2.1 Introduction

In the present study, a collection of water buffalo serum samples originating from the North of Vietnam was used for evaluation of the card agglutination test (CATT), latex agglutination test (LATEX), immune trypanolysis (T.L.) and Enzyme-linked immunosorbent Assay (ELISA). Diagnostic sensitivity and specificity for each assay was calculated using samples that had been confirmed positive or negative on the basis of mouse inoculation.

According to Van Meirvenne *et al.* (1995) the *T.b. gambiense* T.L. can serve as a reference antibody test, confined to laboratory use, for identifying *T.b. gambiense* infected humans. The use of the T.L. *T.b. gambiense* as proposed by Van Meirvenne *et al.,* (1995) was complicated since no predominant antigens were yet identified and the use of a set of VATs was suggested. This unlike the situation for *T. evansi* where the predominant antigen RoTat 1.2 had been identified (Bajyana Songa and Hamers 1988) which seemed to be appropriate for the development of a T.L. using a single VAT.

In this study the T.L. with this predominant *T. evansi* RoTat 1.2 VAT (Bajyana Songa and Hamers 1988, Verloo *et al*., 1997, Verloo *et al.,* 2001) was used as a reference test. By calculating agreement between test results of the T.L. and either CATT/*T evansi*, LATEX/*T. evansi* or ELISA/*T. evansi* it is possible to evaluate how well each of these three assays can predict the presence of anti Rotat 1.2 antibodies and thus indicating ongoing or past infection with *T. evansi.*

[1] Based on; Verloo D., Holland W., My L.N., Thanh N.G., Tam P.T., Goddeeris B., Vercruysse J., Büscher P. 2000. Comparison of serological tests for *Trypanosoma evansi* natural infections in water buffaloes from North Vietnam. Vet. Parasitol. 92, 87-96

2.2 Materials and methods

2.2.1 Field sampling

Samples were collected from 415 water buffaloes (*Bubalus bubalis*) presented for examination in a number of villages. The villages, selected on the basis of a recent history of confirmed *T. evansi* outbreaks, were located in Hoa Binh and Thai Nguyen province in the North of Vietnam. From each water buffalo, two blood samples were taken: one plain blood sample and one with heparin since this is the anticoagulant best tolerated by the parasite (Sarmah *et al*.,1986). During transport to the National Institute of Veterinary Research in Hanoi the heparinized blood was stored in a cooling box; plain blood was kept at ambient temperature to enable clotting thereafter it was centrifuged and the serum collected. The serum was stored at -20°C.

2.2.2 Parasitological examination

All 415 samples were checked using the mouse inoculation technique (M.I.) by injecting 0.25-0.5 ml of heparinized blood intraperitoneally into mice which had received 200 mg/kg cyclophosphamide just before inoculation. The mice were laboratory bred, of mixed sex and of no particular breed. Initially, only one mouse was inoculated per sample but after massive deaths of unknown aetiology, one mouse was inoculated immediately after sampling and another one upon return in the laboratory. The inoculated mice were bled from the tail and a wet blood film was prepared onto a clean slide and covered with a coverslip (25x25 mm) to spread the blood as a monolayer of cells. For each preparation, a total of 100 fields were examined by light microscopy (100x or 200x) to detect motile trypanosomes. Mice were checked 2-3 times a week for one month.

In addition all 415 samples were checked using the micro-haematocrit centrifugation technique (MHCT, Woo, 1969): two capillaries were filled with the heparinized blood, centrifuged for 5 minutes at 12.000 g and the buffy coats examined for the presence of trypanosomes at 10x10 magnification using a slide capillary holder (Van Meirvenne, 1999).

2.2.3 Serological examination

CATT/T. evansi

The CATT/*T. evansi* is a rapid direct agglutination test which uses formaldehyde fixed, Coomassie Blue stained, freeze-dried trypanosomes of *T. evansi* VAT RoTat

1.2 (Bajyana Songa and Hamers, 1988). Two-fold serial serum dilutions in buffer (KH_2PO_4 0.43 g/l; Na_2HPO_4 1.85 g/l; NaCl 7.2 g/l; NaN_3 1 g/l; pH 7.2) are tested. Twenty five µl of serum dilution are mixed with 45 µl of reagent on a reaction zone of the agglutination card. The card is rocked for 5 minutes on a flat bed rotator at 70 rpm. A reaction is scored positive when macroscopic agglutination is visible. The end titre is defined as the highest dilution of the test serum still showing a positive result.

LATEX/T. evansi

LATEX/*T. evansi* (Verloo *et al*.,1997; Verloo *et al*.,1998) is a rapid indirect agglutination test in which the antigen consists of purified variable surface glycoprotein (VSG) of *T. evansi* VAT RoTat 1.2 covalently coupled to latex particles. The method used for VSG purification and coupling is based on that of Lejon *et al*. (1998). The reagent is stabilized by lyophilisation and rehydrated with deionized water before use. Serum dilutions are prepared as for CATT. Twenty µl of twofold dilutions are mixed with 20 µl of reagent on a test card. Further manipulations and reading are the same as described above for CATT/*T. evansi.*

Immune trypanolysis

Immune trypanolysis (T.L.) was performed according to Van Meirvenne *et al*. (1995) with *T. evansi* VAT RoTat 1.2 and with *T. brucei brucei* VAT AnTat 1.1. The latter serves as a negative control for the anti RoTat 1.2 positives since this VAT is never expressed by *T. evansi* trypanosomes. The sera were tested at a 1/4 dilution. Live trypanosomes obtained from a RoTat 1.2 parasitaemic mouse were incubated for 60 minutes with test serum in the presence of guinea pig serum as the source of complement. If variant specific antibodies are present in the serum, lysis of the RoTat 1.2 trypanosomes is observed microscopically. When 50% or more of the trypanosomes are lysed, the sample is considered positive for the presence of anti RoTat 1.2 antibodies, indicating current or past infection.

ELISA/T. evansi

The ELISA was based on that described by Büscher *et al*. (1995). For coating the ELISA plate (Nunc-Immuno™ Plate, Maxisorp™ Surface), purified VSG of *T. evansi*

VAT RoTat 1.2 was diluted in PBS (0,01M; pH 7,4; NaCl 8,2g/l; $NaH_2PO_4.H_2O$ 0,2g/l; $Na_2HPO_4.2H_2O$ 1,44g/l) to a concentration of 2 µg/ml. Then, 150 µl/well of antigen solution was dispensed in the plate for overnight incubation at 4°C. Half of the wells were left empty as antigen negative control. Further manipulations were done at room temperature. After removing the antigen solution, the plate was blocked by filling each well with 350 µl of PBS-Blotto for 2 hours (0,01M; pH 7,4; NaCl 11,7g/l; $NaH_2PO_4.H_2O$ 0,2g/l; $Na_2HPO_4.2H_2O$ 1,44g/l; NaN_3 0,5g/l; skimmed milk powder (Regilait) 10g/l). Washing was done 3 times with a 1 minute interval by filling each well with 350 µl of PBS-Tween (0,01M, pH 7,4; NaCl 8,2g/l; $NaH_2PO_4.H_2O$ 0,2g/l; $Na_2HPO_4.2H_2O$ 1,44g/l; Tween-20 0,5ml/l). Serum was diluted 1/400 in PBS-Blotto and added in duplicate to both the antigen containing and the antigen negative control wells at 50 µl per well. A strong positive, a weak positive and a negative control serum were added in duplicate to each plate. The plate was incubated for 30 minutes and washed 5 times. Rabbit anti bovine IgG (whole molecule)-peroxidase Sigma (A8917), diluted 1/10000 in PBS-Tween, was added at 150 µl/well and incubated for 45 minutes. Thereafter, the plate was washed 6 times and the wells then filled with 150 µl/well of the substrate chromogen solution (1 ABTS tablet (2,2'-azinobis(3-ethyl-benzothiazoline)-6-sulfonic acid), Boehringer 1112422, 50 mg/tablet, dissolved in 100 ml ABTS buffer) for 1 h. The plates were shaken and optical density (O.D.) read at 415 nm with a Labsystems Multiskan MS ELISA reader. The corrected O.D. of each sample and of the serum controls was calculated by subtracting the mean O.D. of the two antigen negative wells from the mean O.D. of the two corresponding antigen containing wells. These corrected O.D.'s were expressed as percentage of the O.D. obtained with the strong positive control (percent positivity, P.P.) included in each plate. All ELISA tests were executed by the same person and in the same laboratory. If for a given sample the difference between the two raw O.D.'s was more than 25 percent of their mean, the results were rejected and the sample retested.

2.2.4 Negative controls

Due to the lack of water buffalo sera from non endemic regions, twelve serum samples from healthy cattle (*Bos taurus*) obtained in Belgium were used in each assay.

2.3 Results

2.3.1 Parasitological examination

Mouse inoculation: Eight of the 415 blood samples induced patent parasitaemia in M.I. within eight days. No additional positive results were obtained after these eight days.

Microhaematocrit centrifugation: None of the 415 blood samples (including the 8 M.I. positives) examined with this technique, were positive.

2.3.2 CATT/*T. evansi* and LATEX/*T. evansi*

Testing of the samples started at a 1/4 dilution for the CATT/*T. evansi* and at 1/8 for the LATEX/*T. evansi* since at lower dilutions false positive reactions were observed with the serum from negative control animals. All 415 samples were tested and diagnostic sensitivity (proportion of parasitologically confirmed animals that were positive in the serological tests) and specificity (proportion of apparently uninfected animals that yield negative results in these tests) were calculated for the different serum dilutions and are presented in Table 2.1. At dilution 1/8 CATT/*T. evansi* combines maximum sensitivity with high specificity. In LATEX/*T. evansi*, the best combination of sensitivity and specificity was also obtained at 1/8 dilution although about 18% of the tested samples gave apparently false positive results.

2.3.3 ELISA/*T. evansi*

The between-run repeatability of the ELISA was estimated on the day to day variation of the strong positive control and visualized in a Levey-Jennings chart (Figure 2.1). The lines representing ±1, ± 2 and ± 3 times the standard deviation were used as measure of dispersion (MacWilliams and Thomas, 1992). All values remained within ± 2 standard deviation of the mean of all runs, indicating acceptable between-run repeatability (Jacobson, 1998).

By adding two times the standard deviation to the mean P.P. value of the negative controls (18 P.P.) a cut-off value of 30 P.P. was obtained (*i.e.* result with a P.P. ≥ 30 was considered positive). At this cut-off value the diagnostic sensitivity and specificity of the ELISA was 100% and 83% respectively. Increasing the cut-off to 40

P.P. and 50 P.P. did not change the sensitivity but increased the specificity to 90% and 95% respectively (Table 2.1).

Table 2.1 *Diagnostic sensitivity and specificity for CATT/T. evansi and LATEX/T. evansi at different serum dilutions and for ELISA/T. evansi at different cut-off levels (n=8).*

	CATT/*T. evansi* Serum dilution				**LATEX/*T. evansi*** Serum dilution				**ELISA/*T. evansi*** Cut-off (P.P.)		
	1/4	1/8	1/16	1/32	1/8	1/16	1/32	1/64	30%	40%	50%
Sn	100%	100%	75%	38%	100%	88%	38%	13%	100%	100%	100%
Sp	90%	98%	99%	99%	82%	92%	98%	99%	83%	90%	95%

Sn = diagnostic sensitivity; Sp = diagnostic specificity

2.3.4 Immune trypanolysis

RoTat 1.2: None of the twelve negative bovine sera induced lysis. All 8 parasitologically confirmed animals were positive in the T.L. test resulting in a diagnostic sensitivity of 100%. Another 82 animals were positive in the T.L. test while the remaining 325 animals were negative.

AnTat 1.1: None of the T.L. positives with RoTat 1.2 induced lysis of the *T.b.brucei* AnTat 1.1 clone.

Table 2.2 *Proportion of positive results of CATT/T. evansi, LATEX/ T. evansi and ELISA/T. evansi at different dilutions and cut-off levels within the groups of RoTat 1.2 immune trypanolysis test positive and negative sera*

	CATT/T. evansi Serum dilution		**LATEX/T. evansi** Serum dilution	**ELISA/T. evansi** Cut-off		
	1/4	1/8	1/8	30%	40%	50%
T.L. positives	28	16	69	59	41	29
T.L. negatives	8	1	7	7	3	1

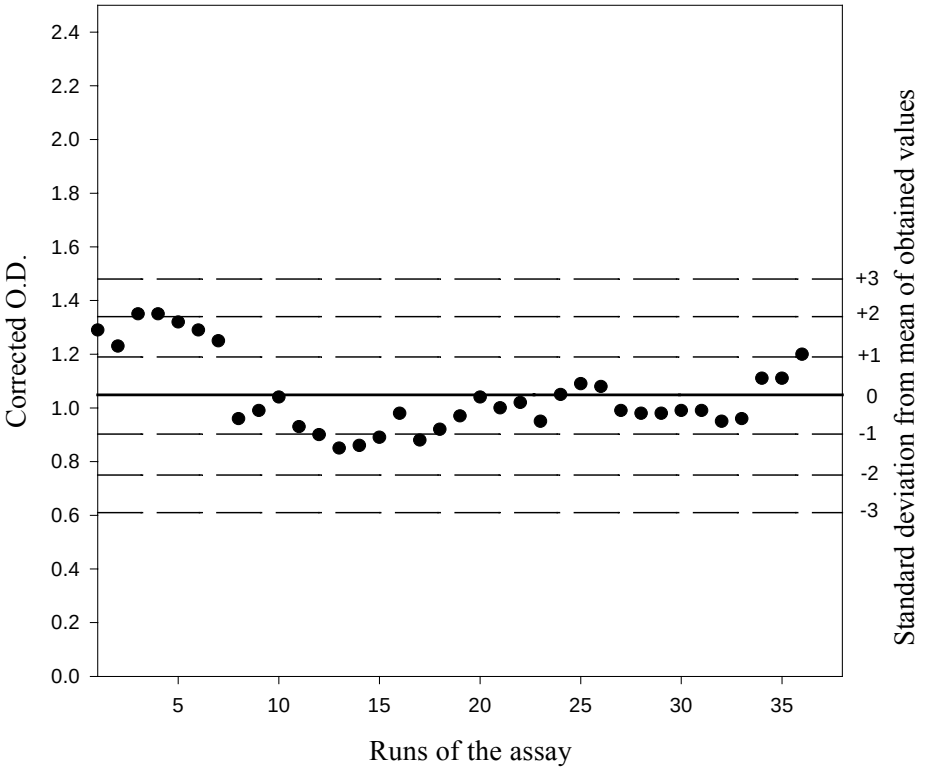
T.L. positives = RoTat 1.2 immune trypanolysis positives; T.L. negatives = RoTat 1.2 immune trypanolysis negatives

2.3.5 Concordance between immune trypanolysis and the other serological tests

Concordance between the results of the T.L. and those of the other assays gave additional information about the sensitivity and specificity of the latter assays for the presence of anti RoTat 1.2 antibodies. Table 2.2 presents the proportion of samples

within the T.L. positive and T.L. negative groups which were positive in CATT/*T. evansi*, LATEX/*T. evansi* and ELISA/*T. evansi*, considering only those serum dilutions and cut-off values that yielded maximum diagnostic sensitivity.

Figure 2.1 *Levey-Jennigs chart of the ELISA/T. evansi. For each of the 36 assay runs the corrected O.D. of the strong positive control is plotted. The solid horizontal line represents the mean corrected O.D. of the strong positive control (O.D.=1.049). The dashed horizontal lines represent ±1, ±2 and ±3 times the standard deviation (S.D.=0.146) above and below the mean.*



2.4 Discussion

Compared to the MHCT, the higher sensitivity of the M.I. test for detecting *T. evansi* is obvious and is in agreement with the findings of Lohr *et al*. (1986) in water buffaloes and by Monzon *et al*. (1990) in horses. It should be noted however, that the blood was examined after returning from the field after a maximum 6 hours and that no data were available on the viability and motility of *T. evansi* in heparinized water buffalo blood as a function of time and temperature. Reports on the survival time of African trypanosomes (Paris *et al*. 1982) in properly refrigerated (4°C) cattle blood and *T. vivax* in refrigerated blood of small ruminants (Ekwuruke *et al*., 1985) recommend that samples should be examined within 6 and 24 hours, respectively.

Although all mice were checked regularly for up to 1 month after inoculation, all parasitologically positive mice had been recognized within eight days after inoculation. This observation questions the need for keeping the mice over a longer period albeit that some *T. evansi* strains may replicate very slowly in mice (Monzon *et al*., 1990).

For the evaluation of serodiagnostic tests, sufficient numbers of true infected and true non-infected animals need to be tested in order to obtain precise estimates of sensitivity and specificity (Martin, 1977; Thrusfield, 1997; Jacobson, 1998). Due to the low sensitivity of parasitological examinations, the identification of true non-infected animals from within an endemic region can be problematic. Furthermore, in this study the number of parasitologically confirmed animals was too low to obtain a precise estimate of the diagnostic sensitivity (Jacobson, 1998).

Nevertheless, the results presented here illustrate the potential value of the antibody detection tests of which LATEX/*T. evansi* and ELISA/*T. evansi* have only recently been introduced. For instance, all parasitologically confirmed animals were positive in T.L. and in CATT/*T. evansi*, LATEX/*T. evansi* and ELISA/*T. evansi* at serum dilutions or cut-off values associated with acceptable specificity.

Similar results were obtained with naturally infected camels in Niger (Verloo *et al*.,1998). In another study, it was observed that RoTat 1.2 specific antibodies always appeared within one month after infection in rabbits experimentally infected with stocks of *T. evansi* originating from different parts of the world (Verloo *et al.,* 1997, Verloo *et al.,* 2001). Taken together, these observations corroborate the statement of Bajyana Songa and Hamers (1988), that RoTat 1.2 is a widespread predominant *T. evansi* VAT*, i.e.* a VAT that appears soon after infection and that is expressed by

many, if not all, *T. evansi* serodemes. In addition, since none of the sera that were positive in RoTat 1.2 T.L., showed lysis of AnTat 1.1, a *T. b. brucei* VAT that is not expressed by *T. evansi*, we may conclude that water buffalo serum, under the given conditions, does not contain active trypanolytic factors other than the VAT specific antibodies.

When the diagnostic specificity is calculated on the basis of the parasitological data, it is highest for CATT/*T. evansi* at 1/8 serum dilution (98%), followed by ELISA/*T. evansi* with 50 P.P. cut-off (95%). Under these conditions, both tests allow identification of all patent infections without revealing too many sero-positives that cannot be confirmed parasitologically. At 1/8 serum dilution, LATEX/*T. evansi* detected all parasitologically positive animals but specificity was only 82%. The 18% false positives can be explained by the presence of anti-RoTat 1.2 antibodies in the serum of animals with non-patent infections. In fact, as for CATT/*T. evansi* and ELISA/*T. evansi*, few non parasitologically confirmed animals were positive in these tests without confirmation by T.L. (1-8%, Table 2.2). It is possible that in CATT/*T. evansi*, LATEX/*T. evansi* and ELISA/*T. evansi* tests, non-VAT specific epitopes, which are exposed on the VSG molecules but not on the living trypanosomes used in T.L., do cross-react with antibodies induced by infections with other micro organisms although it is not excluded that they might be due to cryptic infection with *T. evansi*. Concordance analysis between T.L. and the other tests indicate further that T.L. can detect low levels of anti-RoTat 1.2 antibodies. Indeed, only 16-69% of the T.L. positives were also positive in the other tests, depending on the applied cut-off value. In this respect, LATEX/*T. evansi* and ELISA/*T. evansi* show greater concordance with T.L. than CATT/*T. evansi*.

The fact that the T.L. with RoTat 1.2 in relation to confirmed parasitological positive animals has a diagnostic sensitivity of 100%, and that the test can detect low antibody levels, combined with the predominant character of RoTat 1.2 suggests that T.L. could be used for checking the appearance of RoTat 1.2 antibodies as a marker of infection in the M.I. test for *T. evansi*.

Based on the mouse inoculation results, the apparent prevalence of *T. evansi* in the examined water buffalo population was 1.9%. During a survey conducted in 1996 in the same region, Vuong (1997) found about 1% of 322 buffaloes positive by M.I. Thu *et al*. (1998) reported prevalences of 15 to 21% but these were associated with epidemic outbreaks of disease, which was not the case during this survey. In contrast

with the low parasitological prevalence, anti-RoTat 1.2 antibodies were detected in 22% of the animals by T.L., indicating evidence of infection with *T. evansi*. Although it is possible that some of them were free of infection owing to treatment or to self-cure, this high seroprevalence illustrates a widespread occurrence of *T. evansi* in the North of Vietnam that is not necessarily associated with severe outbreaks of disease. Epidemics of Surra may result from the introduction of *T. evansi* strains into new regions through the movement of infected livestock (Luckins, 1988; Payne *et al*., 1990). Apart from the T.L. test which is not generally applicable, the LATEX/*T. evansi* and ELISA/*T. evansi* with a 30 P.P. cut-off, thanks to their superior capacity of detecting *T. evansi* specific antibodies, would be suitable epidemiological tools for detecting both active infections and persisting *T. evansi* specific antibodies. Taking into account appropriate cut-off values, a negative result in these tests would mean that there is a low risk that the animal had ever been infected with *T. evansi*. The CATT/*T. evansi* and the ELISA/*T. evansi* with a 50 P.P. cut-off on the other hand, would seem to be more appropriate to identify true positive buffaloes.

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CHAPTER 3
EVALUATION OF FRESH AND DRIED BLOOD SPOTS IN SEROLOGICAL TESTS FOR *TRYPANOSOMA EVANSI* IN EXPERIMENTALLY INFECTED WATER BUFFALOES[2]

3.1 Introduction

Several antibody detection techniques have been developed for the diagnosis of trypanosomosis but until now have only been applied to serum samples. Similar tests such as the CATT/*Trypanosoma brucei gambiense* (Magnus *et al.,*1978) and LATEX/*T.b. gambiense* (Büscher *et al.,*1999) exist for sleeping sickness or human African trypanosomiasis caused by *T.b. gambiense*. Their application is greatly facilitated by the testing of whole blood instead of serum, the latter not being readily available in the field. If tests can be used next to the patient, positive test results can be followed instantly by treatment and such improve the prognosis of the patient. In addition costs for logistics will be reduced since a repeated visit for treatment will not be needed.

Furthermore, when large numbers of samples have to be collected, *e.g.* for epidemiological surveys, testing in ELISA/*T. evansi* of blood spots on filter paper instead of serum may be advantageous because collection of blood on filter paper is relatively easy and cost-effective, particularly in sub-optimal field conditions (Esposito *et al.,* 1990; Hopkins *et al.,* 1998).

Hence, the present study was undertaken to compare the use of filter paper blood-spot disks (confetti) versus serum, in the indirect antibody ELISA and the immune trypanolysis (T.L.) and the use of whole blood versus serum in card agglutination test (CATT) and latex agglutination test (LATEX). Each of these tests contains the Variable Surface Glycoprotein (VSG) of the predominant Variable Antigen Type (VAT) RoTat 1.2 (Bajyana Songa and Hamers, 1988) as major antigen. Blood and serum test samples were obtained from experimentally infected water buffaloes.

Accuracies of these tests were estimated according to two parameters for infection status: 1° the parasitological status of the animal, assessed by mouse inoculation (M.I.) and 2° the presence of RoTat 1.2 VAT lytic antibodies as determined by T.L.

[2] Based on: Holland W.G., Thanh N.G., My L.N., Magnus. E, Verloo D., Büscher P., Goddeeris B., Vercruysse J. 2002. Evaluation of plain and blotted blood in serological tests for *Trypanosoma evansi* in experimentally infected water buffaloes. Act. Trop. 81(2), 159 – 165

on serum. This test is considered to be a 100 % specific reference antibody test for the presence of antibodies against this specific VAT (Van Meirvenne *et al.,* 1995; see Chapter 2).

3.2 Material and methods

3.2.1 Animals and experimental design

Twenty water buffaloes, 4-8 months old, were purchased from an area with no recent history of *T. evansi* outbreaks. The absence of *T. evansi* parasites was verified by M.I. inoculation while the absence of specific antibodies was confirmed with ELISA/*T. evansi* (see Chapter 2). Upon arrival, all animals were treated with 10 mg/kg mebendazole and received an injection of 3.5 mg/kg diaminazene aceturate (Berenil - Intervet). The water buffaloes were housed in fly proof stables and fed on grass. After one week of acclimatization, the buffaloes were divided randomly into two equal groups. Group B remained uninfected while animals of Group A were injected intravenously with 1.10^5 trypanosomes of WHITMAS 101298, isolated in 1998 from a water buffalo in Ha Tay province, Vietnam. In weeks 1 – 4, animals were bled twice weekly thereafter only once weekly. Mouse inoculation, LATEX and CATT tests on whole blood were conducted within 2 hours after sample collection. For the ELISA and T.L. with blood-spots, whole blood was collected onto 7.5 cm discs of Whatman No.4 filter paper which were air dried and stored in a cool place in sealed plastic bags with silica gel desiccant. Blood for serum collection was allowed to clot at room temperature and kept overnight at 4 °C. The next day, blood samples were centrifuged and serum was collected and stored at -20 °C for further analysis.

3.2.2 Diagnostic tests

Mouse inoculation (M.I.)

To confirm the presence of parasites in infected buffaloes and to verify the absence of parasites in non-infected buffaloes, blood samples were checked with the mouse inoculation technique by injecting 0.25-0.5 ml of heparinized blood intraperitoneally into mice.

Immune trypanolysis (T.L.)

Immune trypanolysis was performed according to the description in Chapters 2.2.3, using *T. evansi* VAT RoTat 1.2. Sera were tested at a 1/4 dilution in guinea pig serum

(GPS, GIBCO 19195-015). The test with blood-dotted filter-paper was a modification of the test with serum. A confetti of 6 mm diameter was punched out from the blood-dotted filter-paper and put in a flat-bottom plate after which 20 µl GPS per well was added. The plate was covered and incubated for 1 hour at 4°C with continuous shaking. A trypanosome-suspension (5 trypanosomes/microscopic field, 400x) in GPS was prepared from the blood of mouse inoculated with the RoTat 1.2 stabilate, and 10 µl of this suspension was added to each well, with the confetti's still in place. Plates were shaken for 1 min. and incubated for 90 min. at room temperature and again shaken after 30, 60 and 90 minutes.

CATT/T. evansi

The CATT/*T. evansi* was preformed as described in Chapter 2.2.3. For both blood and serum the tests procedures were similar.

LATEX/T. evansi

The LATEX/*T. evansi* with serum and blood was run as described earlier in Chapter 2.2.3.

ELISA/T. evansi

In ELISA/*T. evansi* (see Chapter 2.2.3), serum samples were tested at 1/400 dilution in PBS-Blotto while the following procedure was followed with blood collected on filter paper. From each sample filter paper, two confetti of 6 mm diameter were punched out and added to 2.0 ml of PBS-Blotto/Tween (PBS-Blotto with Tween-20 0,5ml/l) for overnight incubation at 4 ºC. 100 µl of confetti elution was added in duplicate to an ELISA plate which had been coated overnight with 2 µg/ml of purified VSG of *T. evansi* VAT RoTat 1.2 in PBS. Test procedures and test assessment were conducted as described in Chapter 2.2.3. The corrected O.D.'s were expressed as percentages of the O.D. obtained with the strong positive control (percent positivity, P.P.) included in each plate. For both ELISA's the same positive control serum was used with mean corrected optical density of 1.14 (SD ± 0.30) and 1.04 (SD ± 0.29) for ELISA-Serum and ELISA-Confetti respectively. Cut-off values for both ELISA's; ELISA-Serum P.P. = 32 P.P. and ELISA-Confetti P.P. = 26, were calculated by adding two times the standard deviation to the mean of the P.P. values obtained in the non-infected group

3.2.3 Statistics

For each sampling round, the proportion of test positive animals per test was calculated for the infected group and the proportion of test negative animals for the non-infected group. For the non- infected group the mean of the proportions was calculated for the whole period.

Test agreement between the serum and blood versions of CATT, LATEX, ELISA and T.L. was estimated by calculation of the *kappa* values with associated 95 % confidence intervals. In addition agreement between results obtained in T.L., which is considered as a reference test for the presence of anti RoTat 1.2 antibodies, and those obtained in the other assays was estimated by calculation of the *kappa* value.

3.3 Results

Unfortunately one animal of the control and one animal of the infected group died during the experiment. No immediate cause for their death could be identified and consequently their data were omitted from the experiment.

3.3.1 Parasitological results

All infected water buffaloes were positive in the M.I. on at least one occasion. All mice inoculated with the blood of non-infected buffaloes remained negative during the one month follow-up.

3.3.2 Serological results

In total, 147 blood and serum samples were collected from non-infected and 171 from infected buffaloes and tested subsequently by all assays. The proportion of sero-positive animals in the infected group per sampling occasion is presented in Table 3.1. The proportion of sero-negative animals in the non-infected group per sampling occasion is presented in Table 3.2.

Infected group

During the first three weeks none of the animals tested positive in T.L.-Serum or T.L.-Confetti. In all other tests the number of positive results fluctuated during this period (Table 3.1). At week 6 P.I. the T.L.-Serum test was the first to identify all animals positive, the T.L.-Confetti followed at week 7 P.I. while the rest of the tests followed from week 8. From that point on, all infected animals remained positive to all tests (Table 3.1).

Table 3.1 *Proportion of sero-positive animals for the different diagnostic tests in the infected group during the experiment.*

Week	T.L.-Serum	T.L.-Confetti	CATT-Serum	CATT-Blood	LATEX-Serum	LATEX-Blood	ELISA-Serum	ELISA-Confetti
1	0	0	72	0	28	11	0	6
2	0	0	22	0	22	28	17	11
3	0	0	33	11	22	17	11	11
4	22	0	72	28	50	28	22	17
5	89	67	67	56	56	56	44	56
6	100	89	89	89	89	89	67	78
7	100	100	89	89	89	89	78	78
8	100	100	100	100	100	100	100	100
9	100	100	100	100	100	100	100	100
10	100	100	100	100	100	100	100	100
11	100	100	100	100	100	100	100	100
12	100	100	100	100	100	100	100	100
13	100	100	100	100	100	100	100	100
14	100	100	100	100	100	100	100	100
15	100	100	100	100	100	100	100	100

Non-infected group

The T.L. tests were the only tests that never yielded false positive results during the experiment. With all other tests, some false positive results were obtained throughout the whole experiment period (Table 3.2). The mean values calculated over the whole period indicated that using blood, the agglutination assays yielded slightly more false positives than with serum. In contrast, the ELISA on serum yielded more false positives compared with ELISA-Confetti.

Agreement between the serum and blood versions of a test

The *kappa* values with their 95 % confidence interval for test agreement between serum and confetti versions of the tests were, in increasing order, 0.75 ± 0.11 for the CATT; 0.80 ± 0.11 for the ELISA; 0.84 ± 0.11 for the LATEX and 0.93 ± 0.11 for the T.L.

Table 3.2 *Proportion of sero-negative animals for the different tests in the non-infected group during the experiment.*

Week	T.L.-Serum	T.L.-Confetti	CATT-Serum	CATT-Blood	LATEX-Serum	LATEX-Blood	ELISA-Serum	ELISA-Confetti
1	100	100	100	80	80	100	100	100
2	100	100	90	100	90	70	80	90
3	100	100	90	90	90	90	100	100
4	100	100	80	80	80	80	100	100
5	100	100	90	90	100	90	100	100
6	100	100	100	70	100	100	100	100
7	100	100	100	100	100	100	100	100
8	100	100	100	100	100	100	89	89
9	100	100	100	89	100	100	100	100
10	100	100	100	100	100	67	100	100
11	100	100	100	100	100	78	100	89
12	100	100	100	89	100	89	100	89
13	100	100	100	100	100	100	88	100
14	100	100	100	100	100	88	88	100
15	100	100	100	100	100	75	88	100
Mean	100	100	96.6	92.5	96	88.5	95.5	97.1

Agreement between T.L.-Serum and the other serological tests

The *kappa* values with their 95 % confidence interval for test agreement between the T.L.-Serum and the other tests were, in increasing order, 0.70 ± 0.10 for the CATT-Serum; 0.75 ± 0.11 for the LATEX-Blood; 0.77 ± 0.11 for the LATEX-Serum; 0.81 ± 0.10 for the CATT-Blood; 0.81 ± 0.11 for the ELISA-Serum and 0.84 ± 0.11 for the ELISA-Confetti.

3.4 Discussion

The main objective of this study was to investigate whether whole blood could be substituted for serum in the CATT and the LATEX for the sero-diagnosis of *T. evansi* in buffaloes. Similarly blood spots on filter paper were compared to sera for use in the indirect ELISA and T.L.

Well aware of the inherent limitations of using experimental infections, it was decided to use experimentally infected animals owing to the lack of suitable reference material and the absence of an accurate ‘gold standard’ (Luckins, 1992). Because the

subsequent observations are not independent and experimental infection studies often suffer from small numbers of tested animals, the individual animal variation might become important and observed *kappa* values should be interpreted with caution. However, it was decided, to use the *kappa* values to compare the performance of the tests during the infection as all test were executed in parallel during the same period. Further limitations of experimental infections are the short monitoring period compared to long-standing natural infections that can evolve from acute to chronic stages. Finally, severity of infection and associated clinical signs may depend on the strain of parasite chosen (Jacobsen, 1998).

The animals studied originated from an area with no recent history of *T. evansi* and tested negative in the M.I. and the ELISA-Serum (data not shown). Moreover, the *T. evansi*-infected buffaloes only became sero-positive with the T.L. test after three weeks, suggesting the absence of a previous exposure to *T. evansi*. Although T.L. remained negative during the first three weeks, positive results were observed with CATT, LATEX and ELISA in various degrees during that period. This could be explained by reactions with trypanosome specific VSG epitopes, which are not exposed at the surface of living trypanosomes as used in T.L. (see Chapter 2.2.3).

In dogs and goats experimentally infected with *T. evansi* (Bayjana Songa *et al.,* 1987) positive CATT/*T. evansi* results were described after two weeks of infection. In the present study however the CATT-Serum test yielded an unexpectedly high number of sero-positives (72%) during the first week decreasing, to 22% the second week. It is likely therefore that some unexplained aspecific reactions occurred during that first week which is also the reason why *kappa* value for the agreement between CATT-Serum and T.L.-Serum was lower than for the other tests. All tests with the exception of T.L.-Serum and T.L.-Confetti, tended to give some false positive results in the non-infected population at different times and for different animals (individual data not shown).

The best agreement between the serum and blood (confetti) versions is shown in the order T.L., LATEX, ELISA and CATT (*kappa* values respectively 0.93, 0.84, 0.80 and 0.75). The earlier described aspecific reaction of the CATT-Serum during the first week of infection is responsible for the lower *kappa* value. For the ELISA-Confetti the overall results (best agreement with T.L.-Serum) are even better than the ELISA-Serum which is in agreement with a previous study by Hopkins *et al.* (1998) who compared eluted bloodspots and serum in an antibody detection ELISA for

tsetse-transmitted trypanosomosis and who demonstrated a lower cut-off value and a higher specificity and sensitivity using eluted bloodspots. In our study it appeared that during weeks 4 - 6, which is the period antibody levels rapidly increase, the (analytical) sensitivity of the T.L.-Confetti is slightly lower than that of T.L.-Serum.

It is concluded that serum can be replaced with fresh blood for the agglutination assays or blood on filter paper for the ELISA and T.L., which facilitates the work on the field.

Further simplification of the agglutination test protocols as has been done for the CATT/*T.b. gambiense* for human sleeping sickness (Laveissière and Penchenier, 2000) will allow pen side testing even under sub-optimal conditions that are likely as to prevail in many parts of the regions in which Surra is endemic.

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CHAPTER 4
A COMPARATIVE EVALUATION OF PARASITOLOGICAL TESTS AND A PCR FOR *TRYPANOSOMA EVANSI* DIAGNOSIS IN EXPERIMENTALLY INFECTED WATER BUFFALOES[3]

4.1 Introduction

With the introduction of molecular diagnostic techniques, several diagnostic assays based on the detection of trypanosomal DNA by PCR have been developed but not yet fully validated (Wuyts *et al*., 1995; Clausen *et al.,* 1998, Donelson *et al.,* 1998; Omanwar *et al.,* 1999). For various trypanosome species in a number of hosts, PCR has been reported to be more sensitive than conventional parasitological techniques (Wuyts *et al.,*1995; de Almeida *et al.,*1997, Desquesnes, 1997; Masake *et al.,*1997). However, no similar information is currently available for *Trypanosoma evansi* infections in water buffaloes.

As described in Chapter 2, specific antibodies against *T. evansi* were revealed in > 20 % of all animals whereas only 1.9 % of the animals showed patent infection through mouse inoculation and no animal was found positive with the microhaematocrit centrifugation technique (MHCT). Discordance between serological and parasitological results can be explained by the poor sensitivity of the parasitological techniques and, since the viability of trypanosomes is limited in time, by the extended interval between sample collection and parasite detection.

Hence, the present study was conducted to determine the diagnostic sensitivity of the currently employed parasitological techniques for the detection of *T. evansi* in experimentally infected water buffaloes *i.e*. MHCT, buffy coat method, wet blood film clarification using sodium-dodecyl sulfate solution, mini-anion exchange centrifugation technique and mouse inoculation (M.I.). In addition, the sensitivity of a PCR based on the ESAG 6/7 primer set (Braem, 1999) was evaluated. Finally, the effect of time and temperature on the ability of the MHCT test to detect *T. evansi* in heparinized blood of water buffalo was investigated.

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[3] Based on: Holland W.G., Claes F., My L.N., Thanh N.G., Tam P.T., Verloo D., Buscher P., Goddeeris B., Vercruysse J. 2001. A comparative evaluation of parasitological tests and a PCR for *Trypanosoma evansi* diagnosis in experimentally infected water buffaloes. Vet. Parasitol., 97: 1, 23-3

4.2 Materials and methods

4.2.1 Animals and experimental design

Twenty water buffaloes (*Bubalus bubalis*) 4 - 8 months old, of mixed sexes, were purchased from an area with no recent history of *T. evansi* outbreaks. The absence of *T. evansi* infection in the animals was verified by M.I. and by the absence of specific antibodies in ELISA/*T. evansi* (see Chapter 2). At arrival, all animals were treated with 10 mg/kg mebendazole and an injection of 3.5 mg/kg of diminazene aceturate (Berenil - Intervet). The animals were housed in a fly proof stable, in pens of 3-5 animals and fed on a diet primarily of grass. Ten animals were intravenously inoculated with 1.0 x 10^5 trypanosomes of the field isolate WHITMAS 101298, which had been isolated in 1998 from a buffalo in Ha Tay province, Vietnam. The other 10 buffaloes served as negative controls. Heparinized blood was collected twice weekly until week 4 and once weekly from week 5 until week 15. One hundred and eighty µl of each heparinized blood sample were mixed with an equal volume of the Qiagen AS-1 storage buffer (Westburg, Leusden, the Netherlands) and stored at room temperature until further processing. At week 16, the water buffaloes were treated with 3.5 mg/kg, diminazene aceturate and sampled daily until three days after treatment.

4.2.2 Diagnostic tests

Wet blood film clarification with Sodium Dodecyl Sulphate (SDS)

In the wet blood film clarification, the red blood cells are lysed by sodium dodecyl sulfate (SDS) to facilitate detection of motile trypanosomes (Van Meirvenne, 1999). The method requires SDS solution (1 % SDS dissolved in Tris-glucose-saline, pH 7.5), inoculating loops (10 µl), slides and coverslips (25x25 mm), and a drop of fresh plain heparinized blood. Ten microlitres of heparinized blood were put on a slide and approximately 10 µl of SDS solution were added using an inoculation loop and mixed gently. A coverslip was applied and the entire preparation was examined microscopically at low magnification (100x).

Micro-haematocrit Centrifugation Technique (MHCT)

See Chapter 2.2.2

Buffy Coat Technique (BCT)

One capillary of the blood, prepared as for the MHCT (see above) was scratched and broken 1 mm below the buffy-coat layer, the upper part thus containing the top layer of red blood cells, white blood cells and the plasma. The contents of this piece were partially expelled onto a slide, covered with a coverslip (18x18 mm) and examined under ordinary illumination (Murray *et al.,* 1977). The slide was examined for 3 minutes at a 100x magnification.

Mini-Anion Exchange Centrifugation Technique (MAECT)

In the MAECT a phosphate buffered saline glucose buffer (PSG) is used which negatively charges the host blood cells which are subsequently absorbed onto an anion-exchange column with diethylamino-ethylcellulose (DEAE-cellulose), while the trypanosomes are eluted, retaining their viability. The MAECT protocol was based that of Lanham and Godfrey (1970). PSG composition was as follows: Na_2HPO_4 5,77g/l; NaH_2PO_4 0,33g/l; NaCl 1,82g/l; 15g/l glucose; pH = 8.

Mouse Inoculation (M.I.)

A single mouse was injected and monitored as described in Chapter 2.2.2.

Polymerase Chain Reaction (PCR)

The commercially available QIAamp DNA blood mini kit (Westburg, Leusden, The Netherlands) was used in combination with the Qiagen AS-1/AS-2 buffers. In total, 180 µl of blood was used for extraction resulting in 200 µl of extracted DNA in TE buffer (100µg DNA ml^{-1}). Manipulation was performed according to the QIAmp DNA blood minikit handbook.

Twenty µl of extracted material was mixed with 30 µl of a PCR-mix consisting of: 1,5 U Taq DNA recombinant polymerase (Gibco BRL, UK), 10 x PCR buffer (Gibco BRL, UK), 3,0 mM $MgCl_2$ (Gibco BRL, UK), 200 µM of each of the four dNTPs (Roche, Mannheim, Germany) and 0,5 µM of each ESAG 6/7 primer;

ESAG 6/7 Forward 5' ACA TTC CAG CAG GAG TTG GAG 3', 21-mer,

ESAG 6/7 Reverse 5' CAC GTG AAT CCT CAA TTT TGT 3', 21-mer.

The ESAG 6/7 primers, which are *Trypanozoon* specific (Braem, 1999), were used for amplification of a 237 bp fragment from *T. evansi* genomic DNA. Amplification was

carried out in a Hybaid Omn-E thermocycler. The PCR mixture was overlaid with 45 µl mineral oil (Sigma, USA) to prevent evaporation. Cycling conditions were as follows: a first denaturation step of 4 minutes at 94 ºC was followed by thirty five cycles consisting of 1 minute denaturation at 94 ºC, 1 minute primer-template annealing at 55 ºC and 1 minute polymerization at 72 ºC. The last extension step of 5 minutes at 72 °C was performed to polymerize all remaining single strand DNA fragments (ssDNA).

Twenty µl of the PCR product were electrophoresed on a 2 % agarose gel (25 minutes at 100V) with a 100 bp marker (Gibco BRL, UK as size marker). The gels were stained with ethidium bromide (2 µl/50 ml gel) and analyzed on a U.V. transilluminator. Detection limits of the PCR were estimated at 100 trypanosomes ml^{-1} using spiked mouse blood (Braem, 1999) while in the present work the detection limits were 200 trypanosomes ml^{-1} when spiked water buffalo blood was used, i.e. 5 trypanosomes per PCR reaction (data not shown).

4.2.3 Diagnostic sensitivity and specificity

Diagnostic sensitivity of each parasitological test was first calculated for each individual infected animal as the percentage of positive samples over the 19 occasions of sampling. Combined estimates of sensitivity (CE_{se}) for each technique were obtained as the means of these individual values. In addition, 95 % confidence intervals (CI) for CE_{se} were calculated using MS-Excel©. The values of CE_{se} obtained within this study should not be considered as point estimates for accuracy of the used tests but should be interpreted as a measure for qualitative comparison of the different test systems used.

The diagnostic sensitivity of a test was also calculated for each week post-infection using $Se_s = n_{sp}/n_s \times 100$ where n_{sp} is the number samples from the *s*th week post-infection having a positive result from a total of n_s samples collected in that week. The value of Se_s which is a measure for the accuracy of a test at a given time post infection, can be used to compare the different test systems at that given time.

To estimate the specificity of the PCR, 93 samples were collected from 8 uninfected buffaloes over a period of 16 weeks.

4.2.4 The influence of time and temperature on the detection of *T. evansi* in heparinized water buffalo blood with MHCT

Blood samples from water buffaloes were spiked with *T. evansi* positive mouse blood to obtain known levels of parasitemia. These samples were examined with the MHCT after 30 minutes, 1 hour and thereafter each hour until 8 hours after collection. Parasitemia was scored using a semi-quantitative system similar to the scoring system as used by Paris *et al.,* (1982) for the BCT test. This scoring system uses the same logaritmic scale from 0 to 5 and provides a reproducible estimate of the number of trypanosomes per ml (Table 4.1).

Four storage conditions were compared: blood maintained at 4 ºC, left at ambient temperature (20 - 27 ºC), at 37 ºC and in the direct sunlight. The experiment was conducted 8 times on different days.

Table 4.1 *Parasitemia scoring system for T. evansi in the Micro-haematocrit Centrifugation Technique (MHCT)*

Score	trypanosomes per capillary	estimated parasitemia (trypanosomes per ml)
5+	swarming buffy-coat	$> 10^6$
4+	> 100	$> 10^5 - 10^6$
3+	11 - 100	$> 10^4 - 10^5$
2+	2 - 10	$> 10^3 - 10^4$
1+	1	$2.5 \times 10^2 - 10^3$
0	0	$< 2.5 \times 10^2$

4.3 Results

Unfortunately one animal of the control (week 9) and one animal of the infected group died during the experiment. No immediate cause for their death could be identified and consequently their data were omitted from the experiment. Each of the 9 *T. evansi* infected buffaloes developed a detectable infection within the 15 weeks post infection. All infected buffaloes were positive on at least one occasion by each diagnostic test. In weeks 13 and 14 DEAE-cellulose was not available and the MAECT was not performed. Furthermore 2 mice died one day after inoculation (week 9 and 10) and no results were obtained and finally, 6 samples stored for PCR were clotted and could not be processed. No parasites were found and no positive PCR reactions occurred in any of the 93 samples collected from the non-infected buffaloes.

4.3.1 Diagnostic sensitivity of the parasitological tests

For each technique the CE_{se} and the 95 % CI for the 15 weeks period was calculated (Table 4.2), with PCR having the highest (78.2 % ± 0.1 %) and SDS the lowest CE_{se} (25.1 % ± 7.7 %). The average pre-patent period of detectable infection varied according to the technique used. For M.I., the mean pre-patent period was 14.4 days with a range from 3 - 28 days while for the SDS test this was 34.5 days with a range from 24 - 66 days (Table 4.2). The narrowest range of the pre-patent period was 17 - 24 days for PCR, with a mean of 17.4 days.

Table 4.2 *Comparative study of 6 diagnostic techniques in blood samples from 9 experimentally infected buffaloes with T. evansi*

Test	No. test positives (total # tests)	CE_{se} ± 95 % CI	Day first positive (mean)	Day first positive (time range)
PCR	129 (165)	78.2 ± 0.1	18.6	17-24
MI	125 (169)	74.0 ± 9.7	14.4	3-28
MHCT	119 (171)	69.6 ± 8.5	22.5	14-45
MAECT	98 (153)	64.1 ± 10.2	24.6	14-45
BCT	66 (171)	38.6 ± 9.2	31.2	17-45
SDS	43 (171)	25.1 ± 7.7	34.5	24-66

PCR: Polymerase chain reaction; M.I.: Mouse Inoculation; MHCT: Micro haematocrite centrifugation technique; MAECT: Mini anion-exchange centrifugation technique; BCT: Buffy coat technique; SDS: clarification using SDS solution; CE_{se}: Combined estimate of sensitivity; 95 % CI; 95 % confidence interval.

For each test, the Se_s per week was calculated (Table 4.3). The Se_s of the PCR increased to 100 % in the 3rd week post-infection (PI) and remained at this level until week 15. M.I. reached 100 % in week 6 PI while for MHCT and MAECT these high levels were reached only in the 7th week, fluctuating little. The Se_s of BCT and SDS fluctuated with maxima of 78 % and 67 % respectively. The number of days before the inoculated mice developed a detectable infection varied between a mean of 17.8 days (range 12 – 29, n=4) and 4.8 days (range 4 – 7, n=9) with buffalo blood collected on of day 3 PI and day 52 PI, respectively. In mice inoculated with buffalo blood yielding a negative result with the MHCT test (which was the second most sensitive parasitological test after M.I.), the mean number of days to develop a detectable infection was 13.1 days (range 6 - 29 days, n=23) whereas in mice inoculated with buffalo blood with a positive MHCT result this interval was 5.3 days (range 3 – 11,

n=113). None of the samples collected after treatment with diminazene aceturate were positive by PCR.

Table 4.3 *Sensitivity (%) of six diagnostic methods, in detecting T. evansi in experimentally infected water buffaloes calculated per week (Se_s)*

Week PI	SDS	BCT	MAECT	MHCT	MI	PCR
1	0	0	0	0	22	0
2	0	0	11	6	23	0
3	0	11	39	44	78	100
4	22	28	78	72	94	100
5	67	44	67	89	78	100
6	22	44	67	89	100	100
7	67	78	100	100	100	100
8	44	67	100	100	100	100
9	56	68	100	100	100	100
10	33	68	100	100	100	100
11	22	56	100	100	89	100
12	33	75	100	89	100	100
13	11	22	na	100	100	100
14	44	78	na	100	100	100
15	33	44	100	100	100	100

Week PI: week post infection; SDS: clarification using SDS solution; BCT: Buffy coat technique; MAECT: Mini anion-exchange centrifugation technique; MHCT: Micro-haematocrit centrifugation technique; MI: Mouse inoculation; PCR: Polymerase chain reaction; na: not available.

4.3.2 The influence of time and temperature on the ability to detect *T. evansi* in heparinized water buffalo blood using MHCT

The survival time of *T. evansi*, in heparinized water buffalo blood, at a given temperature was determined by the time interval giving repeated positive results with the MHCT (Table 4.4). In blood kept in direct sunlight parasites became undetectable within 30 minutes. In blood with a low parasitemia (1+ and 2+) no parasites could be detected anymore after 3-5 hours, irrespective whether the blood was stored at 4°, 37° or at ambient temperatures. Blood samples with a high parasitemia (5+) were still positive after 8 hours. Blood samples with an intermediate parasitemia (4+, 3+) showed intermediate results. In general the survival time tended to be longer when blood was kept at 4° C, as illustrated by the total of all scores at each temperature (Table 4.4).

Table 4.4 *Influence of storage conditions on different parasitemia levels of T. evansi blood as determined by MHCT in heparinized water buffalo*

A. Storage at 4°C

Time (h)	Parasitemia level				
	5+	4+	3+	2+	1+
1/2	5+	4+	3+	1+	1+
1	5+	4+	3+	1+	1+
2	5+	3+	2+	1+	1+
3	5+	3+	2+	1+	1+
4	5+	3+	1+	1+	0
5	5+	3+	1+	1+	0
6	5+	3+	0	0	0
7	5+	3+	1+	0	0
8	5+	3+	0	0	0
	Total score = 97				

B. Storage at 20-27°C

Time (h)	Parasitemia level				
	5+	4+	3+	2+	1+
1/2	5+	3+	3+	1+	1+
1	5+	3+	3+	1+	1+
2	5+	3+	1+	1+	1+
3	5+	3+	1+	1+	1+
4	5+	2+	1+	1+	1+
5	5+	3+	1+	1+	0
6	5+	2+	1+	0	0
7	5+	2+	1+	0	0
8	4+	2+	0	0	0
	Total score = 90				

C. Storage at 37°C

Time (h)	Parasitemia level				
	5+	4+	3+	2+	1+
1/2	5+	3+	3+	1+	1+
1	5+	3+	3+	1+	1+
2	5+	3+	3+	1+	1+
3	5+	3+	0	1+	0
4	5+	2+	0	1+	0
5	4+	2+	0	0	0
6	4+	3+	0	0	0
7	3+	3+	0	0	0
8	3+	3+	0	0	0
	Total score = 81				

D. Storage in direct sun light

Time (h)	Parasitemia level				
	5+	4+	3+	2+	1+
1/2	nd	0	0	0	0
1	nd	0	0	0	0
2	nd	0	0	0	0
3	nd	0	0	0	0
4	nd	0	0	0	0
5	nd	nd	nd	nd	nd
6	nd	nd	nd	nd	nd
7	nd	nd	nd	nd	nd
8	nd	nd	nd	nd	nd
	Total score = 0				

nd: not done

4.4 Discussion

Of the parasitological techniques, M.I. possessed the highest CE_{se} (74 %) which is in line with observations by Monzon *et al.,* in horses (1990), by Pathak *et al.,* (1998) in camels, by Wuyts *et al.,* (1995) in cattle and by Lohr *et al.,* (1986) and Davison (1997) in buffaloes. However, the CE_{se} of the M.I. (74 %) and the MHCT (69.6 %) obtained in this study, albeit likely to be overestimated, are considerably higher than those reported by Davison (1997) (respectively 28 % and 9 %) in Indonesia on experimentally infected buffalo under similar conditions.

Although the buffaloes in Davison's study were significantly older (18 - 24 months) than those used in the present study (4 - 8 months), age is not a likely explanation for the discrepancies since, in general, calves are considered more resistant to trypanosome infections than adult animals (Stephen, 1986). A more likely explanation is that over 25 % of the buffaloes used by Davison (1997), were initially parasitologically positive and therefore were treated before starting the experiment. These animals most probably carried antibodies against *T. evansi,* which could have influenced the parasitemia during the experimental infection.

The PCR in this experiment proved consistently to be the most sensitive diagnostic test, except for the first two weeks when M.I. was superior. These findings agree with those of de Almeida *et al.,* (1997) with *T. vivax* in goats, who found twice as many positive results with PCR than with the combination of BCT and MHCT. In cattle with single *T. vivax* or mixed *T. vivax* / *T.b. brucei* infections, PCR was almost twice as sensitive compared with the parasitological techniques (Masake *et al.,* 1997; Clausen *et al.,* 1998). Studies on *T. evansi* in cattle (Wuyts *et al.,* 1995) and in mice (Ijaz *et al.,* 1998) also showed the PCR to be highly sensitive.

The M.I. test detected trypanosomes within the period (14.4 days) of infection of all six tests followed closely by the PCR (18.6 days). In cattle experimentally infected with *T.b. brucei*, *Trypanozoon* specific PCR products could be detected as early as day 1 after infection (Clausen *et al.,* 1998) while in cattle infected with *T. vivax* (Masake *et al.,*1997) and *T. evansi* (Wuyts *et al.,*1994) PCR became positive after 5 and 2 days, respectively. It should be mentioned that the time when PCR signals first appear and when parasitological tests turn positive, both depend on the number and virulence of the inoculated trypanosomes. In our study with 10 buffaloes, inoculated with a relatively low number of trypanosomes, PCR turned positive on day 17. One animal

was detected positive at PCR at day 24 but some of its earlier stored samples were clotted and were therefore not processed.

In this study 85 % of the positive mice developed a patent infection within one week, which is in contrast with earlier observations of Monzon *et al.,* (1990) and Verloo *et al.* (Chapter 2) who reported 98 % and 100 %, respectively. Since in this study one mouse turned positive only after 29 days, it is suggested that the mice be examined for at least 30 days PI.

The Se_s of MHCT, MAECT and M.I. reached the 100 % level after 7, 7 and 6 weeks respectively while for PCR, Se_s became 100 % within the 3rd week. The Se_s of SDS and BCT never reached 100 % but fluctuated during the 15 weeks period, the latter almost consistently testing more animals' positive than SDS.

The scoring system proposed by Paris *et al.,* (1982) and applied on the MHCT provided a reproducible estimate of the parasitemia when conducted with *T. evansi* and buffalo blood, which contrasts with earlier findings of African trypanosomes in cattle where little correlation was found. Woo and Rogers (1974) observed however that 85 % of *T. evansi* present in a blood sample was visible in each capillary indicating a strong relationship between the number of trypanosomes observed and the number present.

In a recent survey in buffaloes in the north of Vietnam, specific antibodies against *T. evansi* were detected in over 20 % of all samples (see Chapter 2) whereas only 1.9 % of the animals showed patent infection through mouse inoculation and no animal was found positive with the MHCT. One of the of the reasons for these findings might have been the relatively long interval between sampling and laboratory processing, which sometimes exceeded 6 hours. In the present study a distinct influence of the time interval on the outcome of the MHCT test can be seen. MHCT test results can become negative after 3 hours particularly in samples with low parasitemias. This is in line with the earlier observations of Paris *et al.,* (1982) who advised that blood samples with *T. vivax*, *T. congolense* and *T.b. brucei* should be tested within 6 hours after sampling. Ekwuruke *et al.* (1985) allowed a longer period, with *T. vivax* although in their study the parasitemia was not quantified. Interestingly the storage temperature had less influence on the MHCT test result, as long as samples were not exposed to direct sunlight. For optimal conditions parasitological tests should be done within 3 hours after sampling, and if possible, kept at 4 °C.

At the end of the experiment, the infected buffaloes were treated with diminazene aceturate and blood samples were collected for another 3 days. PCR on these samples was negative starting the first day after treatment, which confirms earlier findings with *T. evansi* in cattle (Wuyts *et al.,*1994) although longer intervals (3 - 4 days) have been reported (Clausen *et al.,* 1999) after drug treatment of *T.b. brucei* infected cattle. Possible explanations for these discrepancies could lie in the sensitivity of different PCRs, the trypanosome species, the various hosts and the parasitemia level before treatment. Furthermore, with the given data it cannot be excluded that diminazene aceturate or its metabolites interfere with the PCR reaction. This should be investigated before proposing to use PCR for monitoring trypanocidal drug efficacy.

The PCR technique as used in this study was, except for early infections, consistently the most sensitive diagnostic technique. PCR also has some major advantages over the parasitological techniques. Sample processing does not have to be done within 3 hours after collection as recommended for MHCT, but can be delayed at least 180 days (data not shown), thus enabling testing under controlled laboratory conditions. Sample storage is easy and does not require a cold chain. Results on confirmed negative samples also indicate the specificity of the PCR is satisfactory. The ESAG6/7 primer, which is Trypanozoon specific, does not give cross-reactions with e.g. *T. theileri* (Braem, 1999) and due to absence of other species of pathogenic trypansome, cross-reactions in water buffalo in this region are not considered important. No data for this primer set are yet available on possible cross-reactions with other related microorganisms such as *Anaplasma* spp., *Theileria* spp. and *Babesia* spp. Once the specificity of the PCR is confirmed for these micro-organisms, the technique may be recommended for the detection of *T. evansi* infections in buffaloes. PCR will especially be most useful when large numbers of animals need to be sampled during field surveys in remote areas, where storage of samples is problematic. However, the requirements for infrastructure, equipment, reagents and technical skills remain an obstacle for use of PCR in many laboratories in those countries where *T. evansi* is endemic.

In the meantime, this study demonstrates that M.I., MHCT and MAECT are highly sensitive except in recent infections, and may be used successfully in the field for detection of *T. evansi* in water buffalo.

4.5 References

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CHAPTER 5
PREVALENCE OF *TRYPANOSOMA EVANSI* IN WATER BUFFALOES IN REMOTE AREAS IN NORTHERN VIETNAM USING PCR AND SEROLOGICAL METHODS[4]

5.1 Introduction

In Vietnam several surveys were undertaken to estimate the prevalence of *Trypanosoma evansi* in livestock (Vuong, 1997; Thu *et al.,* 1998; My *et al.,* 1999; see Chapter 2) but until now little is known on the importance of *T. evansi* in remote inaccessible areas.

The present study was undertaken under field conditions to confirm the value of diagnostic methods, which had only been tested in experimentally infected animals and to determine the importance of *T. evansi* in remote areas with no disease reporting system in place. The tests evaluated were an ELISA and an immune-trypanolysis test (T.L.) using elution fluid from filter paper blood spots, a LATEX test using whole blood as a pen-side diagnostic test and a PCR on blood samples preserved in a special storage buffer, to enable later processing in the laboratory (see Chapters 3 and 4).

5.2 Materials and methods

In May 2000, 265 blood samples were collected from water buffaloes (*Bubalus bubalis*), from three remote mountainous provinces in the North of Vietnam with large populations of water buffaloes namely Hoa Binh, Bac Can and Cao Bang. Villages were selected randomly from each province and the number of water buffaloes in each village was estimated and at least 50 % of them were sampled and checked for clinical signs. Heparinized blood samples were collected, preferably from the ear-vein, and information on the previous use of trypanocidal drugs in each animal was obtained. One hundred and eighty microlitres of the blood sample was mixed immediately with an equal volume of Qiagen AS-1 storage buffer (Westburg, Leusden, the Netherlands) using a micropipette and stored at ambient temperatures until further processing (see Chapter 4). The LATEX-blood test at blood dilution 1/8

[4] Based on; Holland W.G. , Thanh N.G., My L.N., Do T.T., Goddeeris B.M., Vercruysse J. 2004 Prevalence of *Trypanosoma evansi* in water buffaloes in remote areas in Northern Vietnam using PCR and serological methods. Trop. Anim. Hlth Prod. In press

was conducted within 15 min. after sample collection using an orbital shaker run off a car battery. For the T.L. and ELISA/*T. evansi*, blood drops were put onto 7.5 cm discs of Whatman No.4 filter paper with a micropipette, air dried, stored in sealed plastic bags with silica gel desiccant and stored in a cool place upon return from the field. Test procedures for the PCR were as described previously (see Chapter 4.2.2). To achieve complete lysis of samples, which had been clotted due to insufficient mixing, the amount of proteinase-K was doubled and the incubation period was prolonged up to 60 minutes. The T.L., the ELISA on blood-eluted filter-paper and the LATEX-blood test were conducted according to Holland and colleagues (see Chapter 3.2.2).

5.3 Results

The prevalence of *T. evansi* in each of the 9 areas, based on T.L. data and PCR positive test results, is shown in Table 5.1. The LATEX-blood test, tested 27 samples positive (10.2 %) and the ELISA/*T. evansi* with a cut-off P.P. of 26 (percent positivity = percentage of the O.D. obtained with the strong positive control) tested 59 animals positive suggesting a prevalence of 22.3 %. None of the apparently positive animals showed any clinical signs of disease. In the previous 5 years, trypanocidal drugs had been used in only three animals, all of which tested negative by each serological assay.

Table 5.1 *T. evansi prevalence estimated by T.L. and PCR per commune*

Province	Commune	Total samples	TL positive	Prevalence (%)	PCR positive	Prevalence (%).
Hoa Binh	Dan Chuon	16	4	25	1	6
"	Bung	15	1	7	1	7
"	Bai Duong	15	9	60	1	7
"	Xan	30	13	43	7	23
"	Vo	32	5	16	0	0
Bac Can	Dai Sao	40	4	10	2	5
"	Khuoi Cu	36	5	14	0	0
Cao Bang	Nam Tuan	45	0	0	2	4
"	Binh Lang	36	1	3	1	3
Totals		265	42	16	15	5

The estimate of specificity (the proportion of PCR-negative animals that tested negative with a serological test) and estimate of sensitivity (the proportion of PCR-positive animals that tested positive with a serological test) of the T.L. was 88 % and 87 %, for the ELISA/*T. evansi* 82 % and 87 % and for the LATEX-blood test, both 94 %. Test concordance for positive results between the T.L. and the ELISA/*T. evansi* or LATEX-blood test were: 98 % and 58 %, respectively, while the agreement for negative results between T.L. and ELISA/*T. evansi* or LATEX-blood test were 92 % and 99 %, respectively.

5.4 Discussion

Of the 15 animals found to give positive PCR reactions, 13 were confirmed by all three serological tests. One was not confirmed by any serological assay and one animal was positive with LATEX-blood only. As no specificity problems with the ESAG 6/7 primers have been recorded (Braem, 1999; see Chapter 4), these results suggest that two PCR positive animals were infected only a few weeks prior to sampling as no sero-conversion was detectable by T.L.. Due to the inherent detection limits of the PCR (see chapter 4) and the fluctuating levels of parasitemia (Nantulya, 1990), the number of ongoing infections will be underestimated by PCR. The latter is corroborated by the higher percentage (15.8 %) of animals with anti-Rotat 1.2 antibodies as found by T.L., indicating previous contact with this specific VSG (Van Meirvenne *et al*. 1995; see Chapter 2). There may have been persistence of antibodies in self-cured animals, although self-cure for *T. evansi* in buffaloes has not been documented. Although the LATEX seemed to lack the ability to test all apparent serological animals positive, it still detected almost twice as many animals positive as the PCR. Further it appeared to be a highly specific and therefore the LATEX on whole blood can be recommended as a suitable pen-side diagnostic test.

The T.L. results indicate that areas of high prevalence are located in Hoa Binh and areas of low prevalence in all three provinces. Communes with low- and high prevalence are sometimes located less than 10 km one from another. These findings support earlier observations that the prevalence of Surra depends on local conditions such as a suitable climate for the vectors, the presence of reservoir animals and the availability of forage (Stephen, 1986).

This possible great variation in prevalence rates in adjacent population is also important to recognize if (chemotherapeutic) control programs are proposed. Prior to

any intervention proper assessment of the prevalence in the various sub-populations is a necessity. The results of this study indicate that the ELISA/*T. evansi* using eluted blood with P.P. 26 could be an important tool in these surveys. In addition the LATEX test using whole blood can be used as pen-side diagnostic test.

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CHAPTER 6
EVALUATION OF DIAGNOSTIC TESTS FOR *TRYPANOSOMA EVANSI* IN EXPERIMENTALLY INFECTED PIGS AND SUBSEQUENT USE IN FIELD SURVEYS IN NORTH VIETNAM AND THAILAND[5]

6.1 Introduction

Trypanosoma evansi (Surra) is endemic in most countries of South-East Asia (Luckins, 1988) and disease reports are often limited to water buffaloes and cattle with an occasional outbreak in horses. Although *T. evansi* is not highly pathogenic for pigs (Stephen, 1986) outbreaks with abortion and death have been observed in Thailand (Teeraprasert *et al.*,1984a; Bajyana Songa *et al.,*1987), Malaysia (Arunusalam *et al.,* 1995) and Indonesia (Kraneveld and Mansjoer, 1947). In India, acute infections were observed in pigs with symptoms similar to Classical Swine Fever (Gill *et al*., 1987). As the number of reports on *T. evansi* infections in pigs is limited one might conclude that Surra is of limited importance. However, recently the immunosuppressive effect of Surra on pigs has been described (see Chapter 8) and it could well be that vaccination failures noted in pigs in trypanosome endemic areas are due to chronic *T. evansi* infections. To further investigate the possible impact of immunosuppression, validated diagnostic tests are a prerequisite.

As validated serological assays for the diagnosis of *T. evansi* for pigs are not available an initial evaluation of the latex agglutination test (LATEX), card agglutination test (CATT), ELISA and the immune trypanolysis (T.L.) tests was carried out using serum samples from experimentally infected pigs with *T. evansi*. Moreover the tests were used in a field survey to determine the prevalence of *T. evansi* in pigs from northern Vietnam and on breeding farms in Thailand. Estimates of sensitivity and specificity for the tests under field conditions were calculated on the basis of T.L. test results, since both the diagnostic specificity and sensitivity of T.L. are high.

[5] Based on: Holland W.G., Thanh N.G., Do T.T., Sangmeendet S , Vercruysse J., Goddeeris B. Evaluation of diagnostic tests for *Trypanosoma evansi* in experimental infected pigs and subsequent use in field surveys in north Vietnam and Thailand. To be submitted to Vet. Parasitol.

6.2 Materials and methods

6.2.1 Experimental infections

A total of 14 certified parasitological and serological negative piglets of mixed sex, 6 weeks of age, were infected with 10^6 *T. evansi* bloodstream trypomastigotes of the isolate WHITMAS 101298 (see Chapter 3) (group A). A second group of 7 animals (group B) remained uninfected. Plain, and heparinized blood was collected weekly from each animal until week 13 post infection (P.I.). Heparinized blood from all pigs was examined using the micro-haematocrit centrifugation technique (MHCT) and if negative the blood sample was inoculated into mice (M.I.), since M.I. is a more sensitive test (Paris *et al*.,1982). Blood for serum collection was allowed to clot at room temperature and kept overnight at 4°C. The next day blood samples were centrifuged and serum was removed and stored at -20°C for further serological analysis.

6.2.2 Field samples

Four hundred and thirty-seven serum samples were collected from mainly fattening pigs, of small holder farmers from Bac Can, Bac Ninh and Nghe Anh province in the northern half of Vietnam, an area with a history of Surra problems in cattle and water-buffaloes.

Seventy-seven plain and heparinized blood samples were collected, from breeding stock at 5 different farrowing farms around the city of Khon Kaen in North-eastern Thailand, a *T. evansi* endemic area. The heparinized blood was examined using the MHCT. On each farm the following symptoms were observed with breeding sows to a varying degree: abortion, a typical skin rash with erythrema and point bleedings.

6.2.3 Diagnostic tests

Micro haematocrit centrifugation technique – For further details see Chapter 4.2.2

Mouse inoculation - For further details see Chapter 4.2.2

CATT - Testing started at a serum dilution of 1/4 and if a positive result was obtained, additional twofold serum dilutions were tested. For further details see Chapter 2.2.3.

LATEX - Testing started at a serum dilution of 1/4. For further details see Chapter 2.2.3.

Immune trypanolysis – Immune trypanolysis (T.L.) was performed according to Van Meirvenne *et al.* (1995) with *T. evansi* VAT RoTat 1.2. The sera were tested at a 1/4 dilution. Fur further details see Chapter 2.2.3.

ELISA - The ELISA/ *T. evansi* was an adaptation of the technique described in Chapter 2. For coating, the purified VSG antigen of *T. evansi* RoTat 1.2 was diluted in PBS (pH 7.2) to a concentration of 2 µg /ml and 150µl added to each well. After overnight coating at 4°C the plates were blotted for two hours with PBS-blotto and control and test sera were diluted 1/400 in PBS-blotto. After incubation and subsequent washing, rabbit anti-pig IgG peroxidase conjugate (Sigma, A-5670) diluted 1/15.000 in PBS-tween was added. After incubation and several washing steps ABTS was added as a substrate chromogen solution. After 45 minutes incubation at room temperature the plate was read photometrically at 415 nm. The O.D. of each sample tested was expressed as a percentage of a well-defined reference positive sample to give a uniform and continuous scale of percent positivity (P.P.). The cut-off value P.P. = 17, was calculated by adding three times the standard deviation to the mean of the O.D. values obtained in the non-infected group during the experiment. Test parameters were also calculated for cut-off P.P. = 22, since this P.P fitted best with samples from Thailand.

6.2.4 Statistics

Experimental infections

For each weekly sampling round, the proportion of positive animals per test was calculated for the infected group and the proportion of negative animals for the non-infected group. In addition, the mean of the proportions of the non- infected group was calculated for the whole period.

Field samples

In the absence of a gold standard, test results of CATT, LATEX and ELISA were related to T.L, which is considered the reference test for presence of RoTat 1.2 antibodies. Concordance between the results of the T.L. and those of the other assays gives information about the sensitivity and specificity of the latter assays and was

determined by calculating the fraction of samples within the T.L. positive and T.L. negative groups that are positive in CATT/*T. evansi*, LATEX/*T. evansi* and ELISA/*T. evansi*.

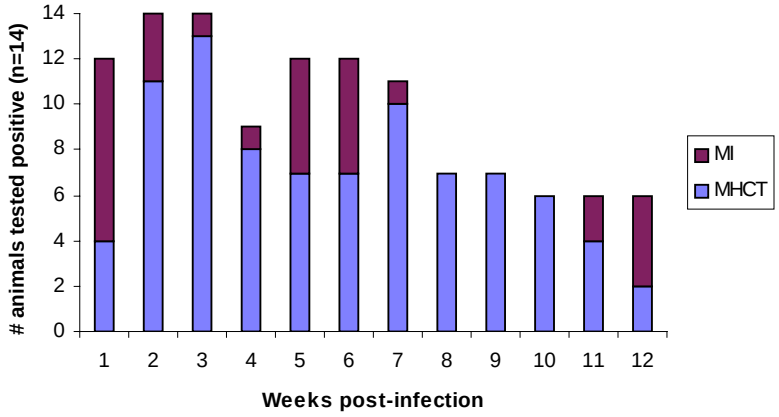
6.3 Results

6.3.1 Experimental infections

Parasitological results

In total 168 samples from 14 *T. evansi* infected pigs were examined over a 12 weeks period. In total 116 samples (69 %) were confirmed parasitological positive: 86 were positive with the MHCT and of the 82 samples negative with MHCT another 30 were confirmed positive with mouse inoculation. All 14 infected pigs were parasitologically positive two and three weeks post infection (P.I.). Afterwards the weekly-prevalence level decreased to levels of 40-50 % at weeks 9 to 12 (Fig. 6.1).
During the study no clinical signs were observed,

Fig. 6.1 *Number of T. evansi experimental infected pigs (n=14), which were parasitologically positive for MHCT and M.I. when analysed on a weekly basis. M.I. was only performed on samples that were negative in the MHCT*



Serological results

All samples from the infected pigs together with 84 samples collected from non-infected animals were subsequently tested using all 4 serological assays. In the first week P.I., all infected animals tested positive with CATT/*T. evansi* plus LATEX/*T. evansi* at dilution 1/4 and in the ELISA/*T. evansi* (P.P. = 17). For both agglutination tests at dilution 1/4 the percentage of sero-positive animals for each sampling round,

remained stable throughout the 12-week period with levels between 70 and 100 %. When serum was diluted 1/8 or 1/16 in CATT/*T. evansi* and LATEX/*T. evansi*, the proportion of sero-positive animals started to decrease in week 3 P.I. and these proportions decreased gradually until week 12 (Tables 6.1 and 6.2). The T.L. tested the first pig positive in week 2, while the last infected animal was confirmed positive with T.L. in week 4. Once the T.L. was positive for an animal it remained positive the subsequent weeks.

Table 6.1 *Proportion of seropositive animals for the CATT/T. evansi and ELISA/T. evansi at different dilutions and cut-off's in the infected group (n=14) and proportion of sero-negative animals for these tests in the non-infected (n=7) group*

	Percentage sero-positive in infected group					**Percentage sero-negative in non-infected group**				
	CATT			**ELISA**		**CATT**			**ELISA**	
Week	**1/4**	**1/8**	**1/16**	**PP 17**	**PP 22**	**1/4**	**1/8**	**1/16**	**PP 17**	**PP 22**
1	100	100	100	100	93	100	100	100	100	100
2	100	100	100	100	100	100	100	100	100	100
3	100	100	86	100	93	86	100	100	100	100
4	100	71	43	100	93	100	100	100	100	100
5	93	71	29	100	93	100	100	100	100	100
6	93	50	14	100	86	86	86	86	100	100
7	71	29	0	100	100	100	100	100	100	100
8	93	14	7	100	100	86	100	100	100	100
9	100	29	0	100	100	86	100	100	100	100
10	86	21	7	100	100	57	100	100	100	100
11	93	43	7	100	100	86	100	100	100	100
12	100	64	29	100	100	43	71	100	100	100
Mean						86	96	99	100	100

For the non-infected group the percentage of negative animals with CATT/*T. evansi* and LATEX/*T. evansi* was calculated at each of the three serum dilutions over the 12

weeks period (Tables 6.1 and 6.2). At dilution 1/4 the specificity of both tests in weeks 10 –12 decreased to low levels. For the T.L. and the ELISA/*T. evansi* with the calculated cut-off P.P. of 17 and 22 all samples collected from non-infected animals always tested negative.

Table 6.2 *Proportion of sero-positive animals for the LATEX/T. evansi and T.L. at different dilutions in the infected group (n=14) and proportion of sero-negative animals for these tests in the non-infected (n=7) group.*

	Percentage sero-positive in infected group				**Percentage sero-negative in non-infected group**			
	LATEX				**LATEX**			
Week	**1/4**	**1/8**	**1/16**	**TL**	**1/4**	**1/8**	**1/16**	**TL**
1	100	100	100	0	100	100	100	100
2	100	100	100	14	100	100	100	100
3	100	100	71	79	86	100	100	100
4	71	57	36	100	100	100	100	100
5	71	64	36	100	100	100	100	100
6	93	86	21	100	86	86	86	100
7	86	64	7	100	86	86	86	100
8	93	29	7	100	86	100	100	100
9	100	36	7	100	100	100	100	100
10	100	36	7	100	43	71	100	100
11	100	57	14	100	71	86	100	100
12	100	57	29	100	43	57	86	100
Mean					83	90	96	100

6.3.2 Field samples

Thailand

Of the 77 samples collected from breeding stock of five different farms, 2 animals from the same farm were found positive with MHCT and 28 adult animals were sero-positive with the T.L. indicating presence of anti-Rotat 1.2 antibodies. Each farm contained sero-positive animals. Parasitological animals were sero-positive with each

serological assay at any dilution.. To evaluate the performance of the other tests, concordance between the T.L. and the other assays was assessed. At a dilution of 1/4 the proportion of CATT/*T. evansi* and LATEX/*T. evansi* negative results within the T.L. negative groups were 63 % and 59 % respectively (Table 6.3). At higher serum dilutions these proportions increased, however the proportion of positive results within the T.L. positive group, especially for the LATEX/*T. evansi* test dropped dramatically. The percentage of ELISA-negative animals within T.L. negative group at the earlier calculated P.P =17 was 57 %. Shifting the P.P. to 22 increased the percentage to 98 % maintaining the 100 % correlation with the T.L. positive samples.

Table 6.3 *Percentage of positive and negative animals in the CATT/T. evansi, LATEX/T. evansi and ELISA/T. evansi at different dilutions or cut-off levels within the groups of RoTat 1.2 immune trypanolysis positives and negatives, respectively*

	CATT			**LATEX**			**ELISA**	
	1/4	**1/8**	**1/16**	**1/4**	**1/8**	**1/16**	**P.P. 17**	**P.P. 22**
T.L. negative Thailand	63	93	100	59	96	100	57	98
T.L. positive Thailand	100	97	70	97	57	20	100	100
T.L. negative Vietnam	30	78	95	48	88	97	89	97
T.L. positive Vietnam	na	na	na	na	na	na	na	na

na = not available

Vietnam

Of the total 437 pig sera tested, no positive T.L. sample was found, indicating absence of RoTat 1.2 antibodies. For both CATT/*T. evansi* and LATEX/*T. evansi* large numbers of apparent positive results were found at 1/8 and especially at 1/4 dilution, suggesting false positive results. As with the samples originating from Thailand raising the P.P. in the ELISA/*T. evansi* from 17 to 22 increased the correlation with the negative samples of the T.L. test from 89 to acceptable levels of 97 %.

6.4 Discussion

The main objective of this study was to evaluate the usefulness of established diagnostic tests for the diagnosis of *T. evansi* in pigs. Some of the serological tests

prior to this study had been used successfully in water buffaloes (Davison *et al.,* 1999; see Chapter 3) and camels (Dia *et al.,* 1997). Except for limited field trials with CATT/*T. evansi* (Bajyana Songa and Hamers, 1988; Reid and Copeman, 2000) none of these antibody assays were evaluated for pigs.

All experimentally infected pigs were confirmed parasitologically positive within the first 2 weeks after infection. At the end of the study fewer peaks of parasitemia could be detected, and it appeared that the animals were more capable of controlling the parasitemia once the infection progressed (Reid *et al*., 1999). In these young pigs no clinical symptoms were observed which is in line with previous observations in Thailand (Teeraprasert *et al.*, 1984a).

During the experimental period all animals became serologically positive within the first week, except for the T.L. Such early reactions in agglutination assays were also observed using the same trypanosome isolate in experimentally infected buffaloes (see Chapter 3). Such apparently non-specific reactions were also found using the CATT LiTat 1.3 in pigs, a test normally used for sero diagnosis of *T. brucei gambiense* in humans (Noireau *et al.,* 1991; Büsher, personal communication). It further appeared that once the experimental infection progressed, the CATT/*T. evansi* and LATEX/*T. evansi* lost their apparent diagnostic specificity as well as the capacity to test infected animals positive. Dilution of sera to 1/8 and 1/16 resulted in a moderate increase of specificity, but a drastic decrease in ability to test infected animals positive. It appeared that under the prevailing conditions the CATT/*T. evansi* and LATEX/*T. evansi*, had limited value

The ELISA/*T. evansi* proved to be more reliable than the agglutination assays in sensitivity as well as specificity. The T.L. performed as anticipated with all animals testing positive within 4 weeks after infection and no positive reactions in the non-infected group. Based on these results and earlier studies (Van Meirvenne *et al.,* 1995, see Chapter 2), it was justified that the T.L. could also serve in pigs as a reference antibody test for the detection of anti RoTat 1.2 antibodies.

Having this reference test, two surveys were conducted in pig in Vietnam and Thailand. In the Vietnam study no pigs were detected positive in the T.L., despite the fact that outbreaks in buffaloes in these areas had been reported and confirmed by parasitological testing (non-published reports) and most samples were collected at the end of the rainy season, the season with highest transmission rates (Luckins, 1988). The apparent absence of any infected pig in this endemic area is remarkable and it

could be speculated that *T. evansi* strains originating from buffaloes and cattle are not adapted to infect pigs. It might also be that vectors have a strong host preference. It could also be speculated that small-holders keep their pigs in dark half open sheds which prevent Tabanidae, which prefer to feed in bright sun light, from biting them.

Since samples were not collected at random, no further statistical analysis was done on these data.

In Thailand however, *T. evansi* serologically positive sows were detected in each farm. Although all farms reported reproductive problems as stillbirth and abortion, it is unclear whether *T. evansi* infection accounted for these problems although the observed fertility problems gradually disappeared when all sows were curatively treated with diminazene aceturate (Sangmeenendet, personal communication). In addition experimental infection of sows at 1-2 month pregnancy, induced abortion after approximately 4 days (Teeraprasert *et al.* 1984b). It should be noted that in the same areas there was a strong indication that abortion in water buffaloes was invoked by *T. evansi* infection (Lohr *et al.*, 1986).

The apparent specificity of the ELISA under Thai conditions at P.P. = 17 appeared to be rather low, but could be drastically improved by raising the cut-off to P.P. = 22. If this cut-off was used on the samples of the experimentally infected pigs and on the Vietnamese field surveys, it appeared that also for these sample test parameters were more than acceptable. The ELISA/*T. evansi* at cut-off P.P. = 22, can therefore be recommended.

In conclusion this study indicates that the agglutination assays for the serological diagnosis of Surra in pigs lack sensitivity and/or specificity. Conversely the ELISA/*T. evansi* appears to fulfil the requirements of a good test for epidemiological surveys. Finally the absence of RoTat 1.2 antibodies in the Vietnamese pigs sampled, indicate that *T. evansi* at least in the areas examined is most likely not a significant disease.

6.5 References

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PART 2

Immunosuppression of *Trypanosoma evansi* in water buffaloes and pigs

CHAPTER 7
THE INFLUENCE OF *TRYPANOSOMA EVANSI* INFECTION ON THE IMMUNO-RESPONSIVENESS OF EXPERIMENTALLY INFECTED WATER BUFFALOES[6]

7.1 Introduction

Trypanosomosis and haemorrhagic septicaemia are considered important diseases in ruminants in Vietnam, causing significant losses to the livestock sector (Luckins, 1988; Phuong, 1993b; My *et al.*, 1994; Thu and My, 1995). Both diseases manifest themselves mostly in outbreaks with infection rates varying over time and place. *Trypanosoma evansi* infections occur especially during the spring-winter season when conditions are harsh due to shortage of forage and low temperatures (Luckins, 1988) while haemorrhagic septicaemia outbreaks are related to cold weather and heavy rainfall (De Alwis, 1999). To prevent haemorrhagic septicaemia, various types of *Pasteurella multocida* vaccines have been developed in Vietnam (Phuong, 1993a, b). Depending on the vaccine and adjuvant (aluminium hydroxide or oil), protection can be induced for over 6 months. Based on field experience within Vietnam, vaccines are effective and protect buffaloes against clinical outbreaks of pasteurellosis in most areas. However, in some areas such as in Hoa Binh province in 1990, buffaloes were reported to die with symptoms of acute pasteurellosis despite being vaccinated. It was found that buffaloes in those areas with poor protection after vaccination also had high infection levels with *T. evansi*. It was therefore postulated that the immunosuppressive activity of *T. evansi* may interfere with the immune response against *Pasteurell*a vaccination.

This study aimed to determine if an experimentally induced *T. evansi* infection in buffaloes interfered with the specific antibody responses upon vaccination against *P. multocida*, and concomitant immunization with human serum albumin as a control antigen.

[6] Based on; Holland W.G., My L.N., Dung T.V., Thanh N.G., Tam P.T., Vercruysse J., Goddeeris B.M. 2001. The influence of *Trypanosoma evansi* infection on the immuno-responsiveness of experimentally infected water buffaloes. Vet. Parasitol. 102, 225-234.

7.2 Materials and methods

7.2.1 Animals and experimental design

Twenty buffaloes, 4-8 months old were purchased from an area with no recent history of *T. evansi* outbreaks and where haemorrhagic septicaemia vaccination is practised twice a year. Animals were verified for the absence of *T. evansi* parasites by mouse inoculation (M.I.) and for the absence of specific trypanosome antibodies with the ELISA/*T. evansi* RoTat 1.2 (see Chapter 2). Animals had low levels of specific antibodies against *P. multocida* as determined in a *P. multocida* indirect antibody ELISA (see later). Upon arrival, all animals were treated with 10 mg/kg mebendazole and 3.5 mg/kg diminazene aceturate (Berenil – Intervet). The buffaloes were divided randomly into two equal groups, one for *T. evansi* infection (group A) and another as a non-infected control group (group B). Animals were housed by group, in a fly proof stable on a diet primarily of grass. After one week, animals of group A were inoculated with 1.10^5 bloodstream trypamastigotes of the field isolate WHITMAS 101298, which had been isolated in 1998 from a buffalo in Ha Tay province, Vietnam. Four weeks after infection of group A, animals of both groups were vaccinated subcutaneously in the neck site, with a locally produced aluminium-precipitated *P. multocida* vaccine. Prior to vaccination, the efficacy of the vaccine was tested in mice (see later). The skin at the inoculation site was shaved before vaccination for hygienic reasons and in order to localise the inoculation site for subsequent measurement of the local inflammatory reaction. Four weeks after the first injection, a booster vaccination was applied on the contra-lateral neck site. Simultaneously with the two *P. multocida* vaccinations, all animals received an intra muscular immunisation in the thigh muscles with 1 mg human serum albumin (HSA, Sigma A-3782): 1 mg of HSA was dissolved in 0.5 ml saline and mixed with 0.5 ml Freund's incomplete adjuvant.

Animals of both groups were sampled once a week to confirm the presence or absence of parasites and to examine levels of antibodies against *P. multocida* and HSA. The local inflammatory response at the *Pasteurella* vaccination site was assessed by skin palpation and measurement of cutaneous/subcutaneous thickness, starting the day prior to vaccination until 14 days post-vaccination.

7.2.2 *Pasteurella multocida* vaccine

The aluminium-precipitated *P. multocida* vaccine was produced from a culture of *P. multocida* somatic serotype 6B (Iran strain). The bacteria were grown in liquid media using the Sparger aeration method. The culture was inactivated by the addition of 0.5 % formalin after the density of bacterin was measured and adjusted to 10^8 colony-forming units (CFU) ml^{-1}. Potash alum was added to give a final concentration of 1 % (De Alwis, 1999).

The efficacy of the vaccine was tested in the active mouse protection test (AMPT). A group of mice was vaccinated twice with an interval of 3 weeks with 0.2 ml of the *P. multocida* vaccine while a control group was injected with 0.2 ml saline. One week after the last vaccination both groups were challenged with 100 CFU of the *P . multocida* serotype 6B P52 strain. While none of the 20 vaccinated mice died, 17 out of 20 in the control group died within 48h with positive blood isolation of *P. multocida.*

7.2.3 Detection of *Trypanosoma evansi* infections

To check animals for infection with *T. evansi*, heparinized blood was inoculated into mice (see Chapter 2.2.2). Antibodies against *T. evansi* were measured with the ELISA/*T. evansi* based on *T. evansi* VAT RoTat 1.2 (see Chapter 2.2.3).

7.2.4 *Pasteurella multocida* indirect antibody ELISA

P. multocida antibody levels were monitored by an indirect antibody ELISA. The procedure of the indirect antibody ELISA is a slight modification of the assay described by Johnson *et al.* (1988). The coating antigen was produced by boiling a formalin-killed culture (10^8 CFU/ml) of the *P. multocida* somatic serotype 6B for 60 minutes. The solution was centrifuged for 30 min. and the supernatant containing the crude lipopolysaccharides (LPS) antigens was collected. Microtitre plates (Nunc-Immuno™ Plate, Polysorp™ Surface) were coated with 100 µl/well of the LPS-antigen diluted 1/200 in PBS (0,01M; pH 7,4; NaCl 8,2g/l; $NaH_2PO_4.H_2O$ 0,2g/l; $Na_2HPO_4.2H_2O$ 1,44g/l) and incubated overnight at 4 ºC. The plates were blocked for one hour with PBS-0.2 % Tween 20 (0,01M, pH 7,4; NaCl 8,2g/l; $NaH_2PO_4.H_2O$ 0,2g/l; $Na_2HPO_4.2H_2O$ 1,44g/l; Tween-20 2.0ml/l) at 37 ºC and washed 3 times with PBS-Tween.

Serial two-fold dilutions of sera were added in duplicate to the wells starting at a dilution of 1/50 (100 µl/well) and the plates were incubated for 1 hour at 37 ºC. After 5 washes, rabbit anti-bovine IgG (whole molecule)-peroxidase (Sigma A8917), diluted 1/8000 in PBS-Tween, was added at 150 µl/well and incubated for 45 minutes at 37 ºC. As controls, a positive and a negative serum and a conjugate control were added in duplicate to each plate. Plates were washed 6 times with PBS-Tween, filled with 150 µl/well of the substrate chromogen solution (1 ABTS tablet (2,2'-azinobis(3-ethyl-benzothiazoline)-6-sulfonic acid), Boehringer 1112422, 50 mg/tablet, dissolved in 100 ml ABTS buffer) and incubated for 1h. The plates were shaken and optical densities (O.D.) were read at 415 nm (Labsystems Multiskan MS ELISA reader). The ELISA titer was expressed as the reciprocal of the highest dilution of serum that gave an equal or lower absorbance to that of the day 0 serum of that particular animal at dilution 1/50.

7.2.5 HSA -indirect antibody ELISA

ELISA plates (Nunc-Immuno^{TM} Plate, Maxisorp^{TM} Surface) were coated overnight at 4° C with 150 µl/well of HSA (Sigma A-3782) diluted in PBS to a concentration of 2 µg/ml. Plates were blocked by 350 µl/well of PBS-Blotto (0,01M; pH 7,4; NaCl 11,7g/l; $NaH_2PO_4.H_2O$ 0,2g/l; $Na_2HPO_4.2H_2O$ 1,44g/l; NaN_3 0,5g/l; skimmed milk powder (Regilait) 10g/l) for 2 hours. Washing was done 3 times with 350 µl of PBS - 0.05 % Tween 20 (0,01M, pH 7,4; NaCl 8,2g/l; $NaH_2PO_4.H_2O$ 0,2g/l; $Na_2HPO_4.2H_2O$ 1,44g/l; Tween-20 0,5ml/l). Serum was diluted 1/600 in PBS-Blotto and added in duplicate at 50 µl/well. A negative control serum and a conjugate control were added in duplicate to each plate. For each serum sample an antigen-negative well was included for subtraction of the OD from the mean OD of antigen-positive wells. The plate was incubated for 30 minutes on a shaking machine and all subsequent manipulations were performed as described in the *P. multocida* ELISA.

The corrected O.D. of each sample was calculated as follows;

$O.D._{nx}$ = (mean $O.D._{nx\text{-}a} - O.D._{nx\text{-}c}$) - (mean $O.D._{n0\text{-}a} - O.D._{n0\text{-}c}$). $O.D._{nx}$ represents the corrected O.D. of buffalo 'n' at day 'x' post immunisation and $O.D._{nx\text{-}a}$ and $O.D._{nx\text{-}c}$ are the means of the O.D. readings of the two antigen-containing and the O.D. reading of the antigen-negative well, respectively, of that buffalo at day 'x'. $O.D._{n0\text{-}a}$ and $O.D._{n0\text{-}c}$ are the means of the O.D. readings of the two antigen-containing- and the O.D. reading

of the antigen-negative wells, respectively, of that buffalo at day 0. In order to detect significant differences between the two groups, the mean of the O.D.'s of each group was calculated and analysed using ANOVA.

7.2.6 Measurement of the local subcutaneous inflammatory response (SIR)

The subcutaneous inflammatory response (SIR) at the inoculation site was measured daily with a pair of vernier calipers for two weeks after both the primary and secondary vaccine administration (Onah *et al*.,1998). The change in SIR was calculated using the formula $SIR_i = M_i - ((M_{-1} + M_0)/2)$, where SIR_i is the increase of the local inflammation reaction in mm on the *i*th day after vaccination and M_i , M_{-1} and M_0 are measurements on the *i*th day after vaccination, the day prior vaccination and on the day of vaccination, respectively. The mean of both groups was calculated and compared with ANOVA.

7.3 Results

7.3.1 *T. evansi* screening and infection

All 20 buffaloes tested negative by mouse inoculation and by the ELISA/*T. evansi* prior to the start of the experiment. Unfortunately one animal of the control and one animal of the infected group died during the experiment. No immediate cause for their death could be identified and consequently their data were omitted from the results.

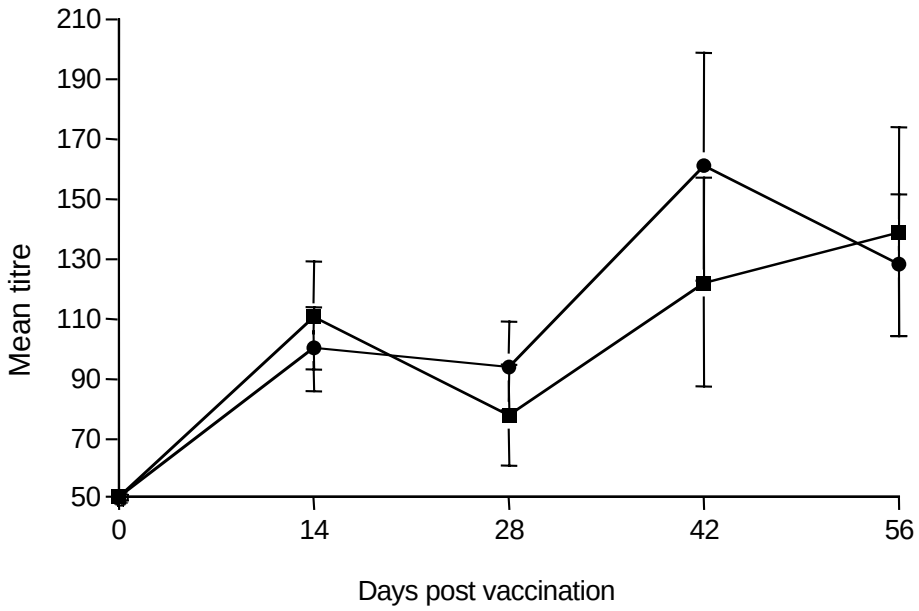
All animals inoculated with *T. evansi* were confirmed infected by both the M.I. and the ELISA/*T. evansi*, while all animals of the control group remained negative. The average period to detect infection in the nine buffaloes with the M.I. was 14,4 days with a range of 3 to 28 days. *T. evansi*-specific antibodies were on an average first observed after 27,2 days with a range of 7 to 45 days. From 5 weeks and 10 weeks post-infection onwards, all animals tested positive continuously in the M.I. and ELISA/*T. evansi*.

7.3.2 *Pasteurella*-specific antibody responses

Following primary *P. multocida* vaccination the uninfected and infected animals displayed a moderate two fold increase in specific antibodies titres on day 14 (Fig. 7.1) followed by a gradual decrease in both groups until the time of the secondary vaccine administration on day 28. The secondary antibody responses in infected and uninfected animals appeared higher and persisted longer than the primary responses.

No significant differences were observed between the mean titres of the respective groups although there was a tendency for higher responses in the uninfected group.

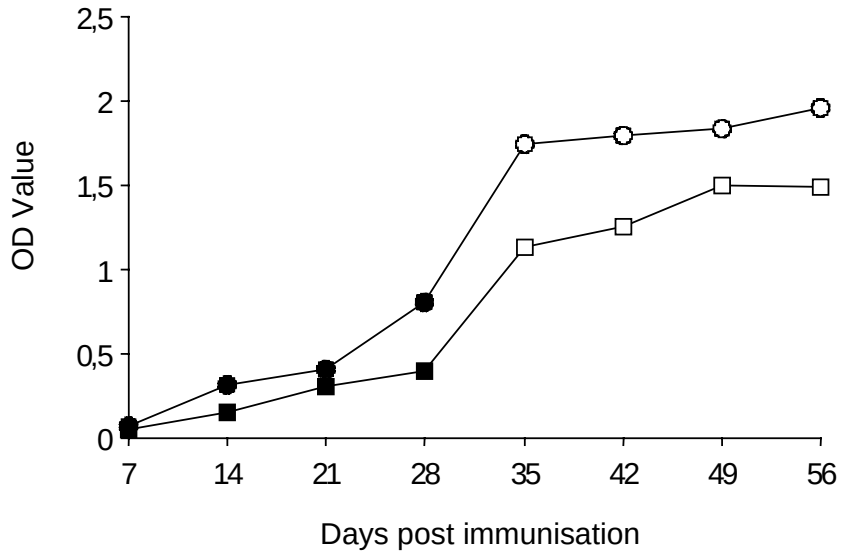
Figure 7.1 *Pasteurella multocida antibody titres after vaccination at day 0 and day 28 with P. multocida vaccine in T. evansi infected (□) and uninfected buffaloes (O)*



7.3.3 HSA-specific antibody responses

All 18 buffaloes sero-converted against HSA as measured by ELISA. After primary immunisation the mean O.D.'s of the infected and the uninfected group increased gradually in both groups with a non-significant higher level in the non-infected group (Fig. 7.2). Following the booster immunisation, the mean O.D.'s for both groups increased abruptly, and this increase in the uninfected group was at all time points significantly higher than in the infected group.

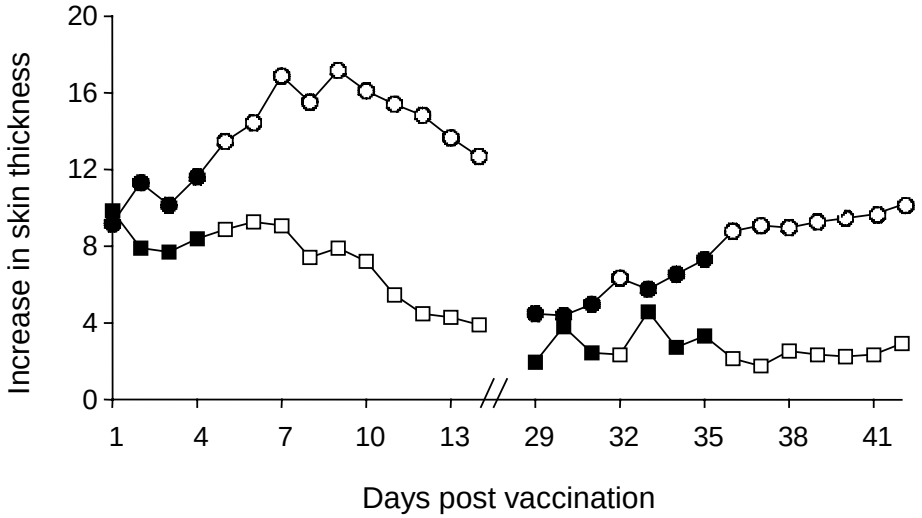
***Figure 7. 2**. HSA-specific antibody responses (corrected O.D.) of T. evansi infected (□) and uninfected buffaloes (O) after primary (day 0) and secondary (day 28) HSA immunisation. Open symbols indicate statistically significant differences (P<0.05)*



7.3.4 Local cellular immune response upon Pasteurella vaccination

In both groups the primary vaccination induced a classical inflammatory response during the first 3 - 4 days, although these symptoms were less profound in the infected group. The swelling during the first 4 days did not vary significantly between the two groups (Fig. 7.3). This acute phase was followed by a painless induration of the skin, which was most prominent in the uninfected group with a peak in sub-cutaneous swelling at 7-9 days following vaccination. For the infected group no distinct peak could be distinguished and a gradual decrease in sub-cutaneous swelling was seen after 5 days. The difference of the mean (Student's t-test) was significant after 4 days. Following the booster vaccination the acute inflammatory response in both groups was less acute than in the primary response. The increase in skin thickness of the infected group was only marginal. Conversely, a slight increase in skin thickness was observed in the uninfected group, which 8 days after the boost became significantly different from that of the infected group.

***Figure 7.3**. Average increase in subcutaneous swelling after primary (day 0) and secondary (day 28) subcutaneous Pasteurella multocida vaccination in T. evansi infected (□) and uninfected buffaloes (O). Open symbols indicate statistically significant differences (p<0.05)*



7.4 Discussion

This study clearly demonstrates that an active *Trypanosoma* infection lowers the immunoresponsiveness of animals to concurrent immunisation with antigens: antibody responses against the test antigen HSA were significantly reduced in the infected animals as well as the local immune responses at the site of Pasteurella vaccination. However, no significant differences in antibody responses against *P. multocida* were observed.

These observations partly support findings that trypanosome infections cause suppression of antibody responses upon vaccination described by a number of authors (Whitelaw *et al*., 1979; Ilemobade *et al*., 1982; Rurangirwa *et al*., 1983; Ikeme *et al*., 1984, Onah *et al*., 1998). However, from the results obtained in this study no conclusive picture emerges whether *T. evansi*-induced immunosuppression interferes with the development of protective immunity following *P. multocida* vaccination. In both the infected and uninfected group the primary and secondary humoral immune responses to *P. multocida* vaccination were low and displayed similar kinetics. This in contrast with earlier findings in Vietnam where aluminium-precipitated instead of oil-

adjuvated vaccine was used (Phuong *et al*., 1996). The reason for the low humoral response induced by the vaccine, even in the non-infected group, is unclear. The vaccine was apparently of good quality as it induced good protection in mice as demonstrated by the AMPT. However, some studies indicate that the correlation between the AMPT and the protection induced in buffaloes disappears when low concentrations of bacteria per vaccine dose are used (Gomis *et al*., 1989). The poor induction of antibody responses in our study might possibly be due to the presence of already circulating antibodies in the experimental animals at the onset of the experiment. Indeed, the young animals which were 6 months of age and originated from Pasteurella-endemic areas where vaccination is practised, had residual levels of maternal *Pasteurella*-specific antibodies which could inhibit the specific activation of B cells and concomitant increase of serum antibodies.

In a similar type of study where *T. evansi*-infected cattle were vaccinated against haemorrhagic septicaemia, no significant relation between haemagglutinating antibody titres and trypanosome infection was detected (Ikeme *et al*., 1984). The authors explained the absence of immunosuppression by the low levels of parasitemia in the infected animals. This association of low levels of immunosuppression with low parasitemia levels has also been supported by a number of other authors (Whitelaw *et al*., 1979; Baltz *et al*., 1981; Rurangirwa *et al*., 1983).

The *T. evansi*-infected buffaloes displayed a significant reduction of the subcutaneous inflammatory response at the vaccination site. A local inflammatory response by subcutaneous vaccination is mediated by macrophages and T cells. A decline in the number of T cells by *T. evansi* infection has been well documented in sheep (Onah *et al*., 1998). It is therefore possible that suppressed local inflammatory responses arise from reduced T cell levels. Suppression of delayed type hypersensitivity (DTH) reactions to tuberculin has been associated with trypanosomosis in rabbits and rats (Mansfield and Wallace 1974; Mackenzie *et al*., 1979). T cell suppression during tsetse-transmitted trypanosomosis has been investigated extensively in mice (Sileghem *et al*., 1991) and cattle (Sileghem and Flynn 1992). In both species T cell proliferation was profoundly suppressed upon mitogenic stimulation and mediated by macrophage-like suppressor cells, leading to suppressed interleukin 2 secretion and impaired expression of the interleukin 2 receptor. However, the role of the various T cells in both animal species was not similar (Sileghem *et al*., 1994). Also, in *T. evansi*-infected sheep, macrophages were held mainly responsible for suppressed T cell

proliferation upon mitogenic stimulation and the underlying pathways resembled those in *T. congolense* infections in cattle (Onah *et al.*, 2000).

By various authors it was demonstrated that a relationship exists between *P. multocida* ELISA antibody levels and active protection after challenge (Chandrasekaran *et al*., 1994b; Verma and Jaiswal, 1997). The role of cell-mediated immune responses has not received much attention in research on haemorrhagic septicaemia. However, for effective protection against haemorrhagic septicaemia both humoral and cell-mediated immune responses are required (Chandrasekaran *et al*., 1994a; Verma *et al*., 1997), moreover, a good helper T cell response is still required for an optimal B cell response. In one experiment, where ultrasonicated *P. multocida* bacteria were inoculated intradermally, an apparent correlation was observed between the DTH and the level of protection in buffaloes, which had been vaccinated with various types of *P. multocida* vaccine (Chandraskaran *et al*., 1994b). Thus, based on the present findings it might be concluded that *T. evansi* impairs local inflammatory reactions. This could be explained by reduced levels or reduced reactivity of certain T cell phenotypes. This reduced skin induration would suggest diminished levels of protection against *P. multocida* (Chandrasekaran *et al*. 1994a)

In conclusion, the present results indicate that the capacity to mount immune responses to heterologous antigens is suppressed in *T. evansi*-infected water buffaloes and consequently that *T. evansi* infection might interfere with the development of protective immunity against heterologous vaccinations. This immunosuppression possibly explains the Vietnamese claim of poor protection of Pasteurella-vaccinated water buffaloes in *T. evansi*-endemic areas. As a result, this immunosuppression has important consequences on vaccination programmes in *T. evansi*-endemic areas and it might be a pre-requisite for prior treatment with trypanocidal drugs before heterologous vaccination is applied.

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CHAPTER 8
THE EFFECT OF A *TRYPANOSOMA EVANSI* INFECTION ON PIG PERFORMANCE AND VACCINATION AGAINST CLASSICAL SWINE FEVER[7]

8.1 Introduction

A similar situation to that described in water buffaloes where vaccination failures were observed against haemorrhagic septicaemia (see Chapter 7), also seems to occur in pigs. Recently, a large number of cases of insufficient protection induced by different commercial classical swine fever (CSF) vaccines were reported in Vietnam: vaccinated pigs died with clear symptoms of acute CSF despite being vaccinated. CSF is considered to be one of the most important infectious disease affecting pigs in many countries in Asia and elsewhere, with numerous outbreaks all year round (Dung and Blacksell, 2000).

Many possibilities for vaccination failures do exist e.g vaccine quality, vaccination technique, and quality of cold chain. Although vaccination failures due to trypanosome infections have been described in ruminants (Whitelaw *et al.,* 1979; Ilemobade *et al.,* 1982; Rurangirwa *et al.,* 1983; Ikeme *et al.,* 1984; Onah *et al.*, 1997 & 1998, see Chapter 7), so far no investigations have been carried out on the possible immunosuppressive role of trypanosome infections in pigs.

The main objective of the present study was to determine if *T. evansi* induces immunosuppression in pigs. *T. evansi* infected and non-infected pigs were vaccinated against CSF and subsequently challenged with virulent CSF virus. Fever, survival rate, leucocyte and thrombocyte counts and specific CSF antibody titres were monitored. All animals received also two immunizations with human serum albumin (HSA) as a control antigen and antibody titres against HSA were monitored. Additionally, the pathological effects of *T. evansi* in pigs were studied. Several studies have been undertaken to investigate the influence of *T. evansi* infection on production in livestock (Payne *et al.,* 1991, 1992, 1993; Pohlpark *et al.,* 1999). However no such studies have been conducted in pigs therefore to further elaborate on the possible pathological role

[7] Based on; Holland W.G., Do T.T., Huong N.T., Dung N.T., Thanh N.G., Vercruysse J., Goddeeris B.M. 2003. The effect of *Trypanosoma evansi* infection on pig performance and vaccination against classical swine. Vet. Parasitol. 111, 115-123

of *T. evansi* in pigs, daily weight gain, feed conversion and packed cell volume (PCV) of infected and non-infected pigs were monitored after experimental infection.

8.2 Materials and methods

8.2.1 Animals and experimental design

A total of 21 piglets of mixed sex, 5 weeks of age, and confirmed negative for *T. evansi* and CSF virus-specific antibodies (ELISA as described in Chapter 2 and Colijn *et al.,* 1997) were selected. After 3 days acclimatisation, animals were allotted into 3 equal groups (n = 7). The three groups were housed separately and animals were fed equally on a restricted diet of commercial available concentrate and the daily intake recorded. Groups A and C were infected with 1 x 10^6 *T. evansi* bloodstream trypamastigotes of the isolate WHITMAS 101298 (see chapter 4) while Group B was the non-infected control group. Four weeks after infection with *T. evansi* animals of Group A and control Group B were vaccinated intra-muscular in the right neck, with the commercial live CSF vaccine Pestiffa® C strain (Lot no. L73452, Merial-France). Further, all 3 groups were immunised intra-muscular in the left neck site with 1 mg human serum albumin (HSA, Sigma A-3782). For this, 1 mg of HSA was dissolved in 0.5 ml saline and subsequently mixed with 0.5 ml Freund's incomplete adjuvant. Four weeks after the first immunisation all animals received a booster with HSA. Eight weeks after the CSF vaccination (12 weeks after *T. evansi* infection) all 3 groups were challenged intra-nasally with heparinized whole blood of a CSF viraemic pig which prior was experimentally infected with a CSF strain isolated from Vietnam (Ky Son district, Nghe An province).

8.2.2 Sampling procedures

Plain and heparinized blood samples from all pigs were collected weekly until the CSF virus challenge to confirm the presence or absence of parasites, to determine the PCV and the levels of antibodies against CSF virus and HSA. *T. evansi*-infected animals were observed daily for fever, anorexia and survival rate. All animals were weighed every fortnight to measure weight gain, and feed conversion.

After challenge with the CSF virus, blood samples were collected in EDTA at days 0, 2, 4, 7, 10 and 14 post-challenge to determine the number of leucocytes and

thrombocytes. After challenge, animals were examined daily for body temperature (fever > 40 °C) and clinical symptoms of CSF e.g. anorexia, paresis.

8.2.3 Laboratory procedures

Detection of T. evansi infections

To check animals for the presence of *T. evansi*, heparinized blood was examined by the microhaematocrit centrifugation technique (MHCT) and the PCV was recorded at that same time (Van Meirvenne, 1999). If the MHCT was negative, heparinized blood (0.25 - 0.5 ml) was inoculated intraperitoneally into laboratory bred white mice. The inoculated mice were bled from the tail and a wet blood film was prepared to detect parasitemia. Mice were checked 2-3 times a week for one month.

Antibodies against *T. evansi* were measured by ELISA on *T. evansi* VAT RoTat 1.2 as described in Chapter 2 with minor modifications: test sera were diluted 1/400 in PBS-blotto and rabbit anti-pig IgG peroxidase (Sigma, A-5670) diluted 1/15000 in PBS-Tween 20 was used as a conjugate.

CSF-specific antibodies

Sera were tested with the Ceditest® CSF virus, a complex-trapping blocking ELISA that uses two monoclonal antibodies directed against the recombinant CSFV E2 antigen (Colijn *et al.,* 1997). ELISA results were expressed as the percentage inhibition (PIH = 100 x (1 - ([O.D. test serum - mean O.D. positive reference serum] / [mean O.D. negative reference serum - mean O.D. positive reference serum])) and a cut-off value of 30 % was applied: samples > 30 % were considered positive, samples ≤ 30 % were considered negative. All sera were tested undiluted and diluted twofold in PBS (1/2 to 1/8). The titre of a test serum was the inverse of the highest dilution with PIH > 30 %. In order to detect significant differences between the two groups, the mean titre of each group was calculated and analysed by ANOVA.

HSA-indirect antibody ELISA

The ELISA previously described in Chapter 7 was modified slightly. ELISA plates were coated overnight with HSA (Sigma A-3782) diluted in PBS to a concentration of 2 µg/ml and the next day blocked and subsequently washed. All test sera collected post-immunisation were diluted in PBS starting at 1/1000 and subsequent two fold

dilutions were prepared. After washing, rabbit anti-pig IgG (whole molecule)-peroxidase (Sigma A-5670) diluted 1/10.000 in PBS was added. ABTS solution was used as a substrate chromogen. The titre of a test serum was the inverse of the dilution having an O.D. higher or equal to the calculated cut-off. The latter consisted of the mean O.D. of all pre-immunisation sera (n = 21) at dilution 1/1000, plus 3 times the standard deviation (Cut-off = O.D. 0.22). In order to detect significant differences between the two groups, the mean titre of each group was calculated and analysed by ANOVA.

Leucocyte and thrombocyte counts

The number of leucocytes and thromobocytes in EDTA blood samples was determined in a Sysmex® KX21 Coulter counter. Leucopaenia was defined as $< 8 \times 10^6$ cells/ml and thrombocytopaenia as $< 200 \times 10^6$ cells/ml.

Fluorescence antibody test

To confirm the presence of CSF virus antigen in necropsied material, the fluorescence antibody test (FAT) was used as described by OIE (1996).

8.3 Results

8.3.1 *T. evansi* screening and infection

All *T. evansi*-injected animals (group A and C) were confirmed parasitologically positive from the first week post-infection onwards, while all animals of the control group remained negative, throughout the experiment. From nine weeks post-infection onwards some infected animals could no longer be confirmed parasitologically positive. All pigs of Group A and C became serologically positive (*T. evansi*-specific antibodies) from day 7 post-infection onwards, while animals of Group B remained negative. No clinical signs of *T. evansi* infection were observed, during the 12 weeks infection period.

8.3.2 Growth, feed conversion and PCV

No significant differences in daily weight gain and feed conversion between infected and non-infected animals were observed. The 14 pigs infected with *T. evansi* (Groups A and C) gained on average 19.6 kg, while the non-infected pigs (Group B) gained 20.4 kg over the same period until challenge with the CSF virus. The infected and non-

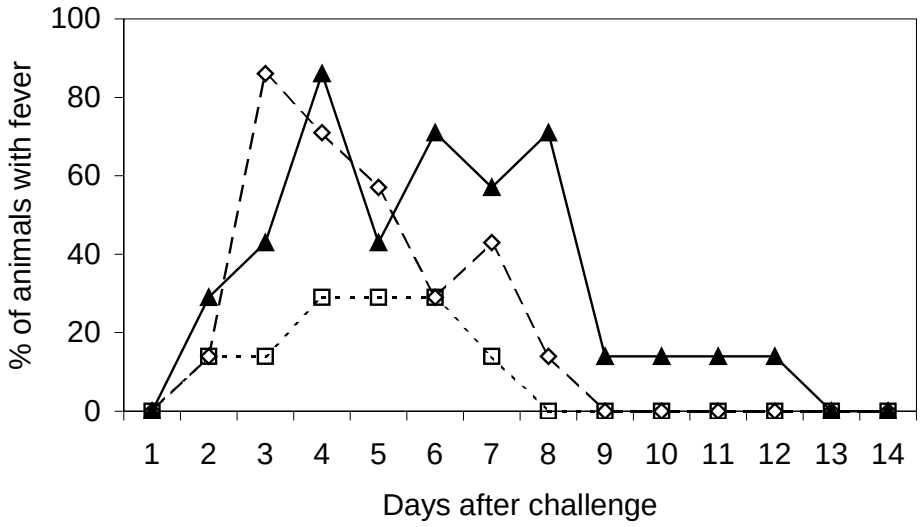
infected groups had over the 12 weeks period feed conversions of 2.85 and 2.80 respectively.

During the 12-week monitoring period no significant differences in packed cell volume between the groups could be observed: the mean PCV levels remained stable at 35, with no major fluctuations in any group.

8.3.3 Clinical symptoms after CSF virus challenge

One animal from Group B succumbed after chronic diarrhoea (*Clostridia* spp.) on the day of challenge. After the CSF virus challenge, 3 out of 7 animals in the non-vaccinated, *T. evansi*-infected group C were found recumbent and were subsequently euthanised on day 2, 7 and 8 respectively. For these three pigs the FAT on spleen samples were positive for CSF. The other 4 animals in this group lost their appetite and were dull for a number of days. In the vaccinated, *T. evansi*-infected group A, 4 animals had reduced appetite for 2–5 days after challenge. In the vaccinated, uninfected B group, however, no symptoms of CSF were observed.

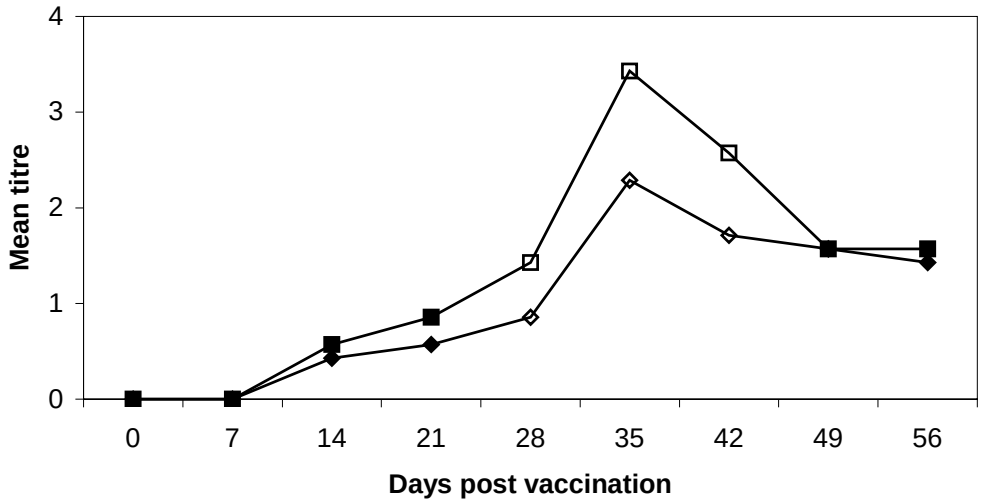
Figure 8.1 *Percentage (%) of animals per day with temperatures* > 40°C *after CSFV challenge. T. evansi infected and CSF vaccinated (◊), only CSF vaccinated (□), only T. evansi infected (▲).*



Body temperature was recorded daily for 14 days after CSF challenge. The percentage of recordings of animals with fever (> 40°C) over this 14 day period was 22, 11 and 42

for Groups A, B and C, respectively. The daily percentages of animals with fever are represented in Figure 8.1. Upon CSF challenge, more animals with fever were detected in the *T. evansi*-infected groups A and C than in the non-infected group B, and could be detected for a longer time. For the CSF-vaccinated groups, more animals with pyrexia were detected in the *T. evansi*-infected Group A than in the non-infected Group B. Also these animals in group A were pyrexia for a longer time (Figure 8.1).

Figure 8.2 *CSF antibody titres determined by the Ceditest® CSFV, of T. evansi infected(◊) and uninfected pigs (□) after vaccination with Pestiffa® C strain. Open symbols indicate statistically significant differences (P<0.05)*



8.3.4 Leucocyte and thrombocyte counts

In Group A, no leucopaenia could be detected throughout the 14 day monitoring period after the CSF virus challenge. One animal of Group B had leucopaenia on day 4 and recovered on day 7. In Group C, three animals were found with leucopaenia; one on day 4, one on day 7 which both succumbed the same day while the third animal was found with leucopaenia on day 10 but this one recovered fully on day 14. Thrombocyte counts prior challenges were similar between the infected and non-infected group. Thrombocytopenia was found in two animals in Group A on days 2 and 4, one animal only on day 2 and one animal only on day 4. In Group B low thrombocyte levels were found, in one animal on days 2 and 4, one animal on days 4

and 7 and one animal only on day 2. In Group C low thrombocytopaenia was found in two animals on days 2 and 4 one of which one died, one animal on days 2, 4 and 7 which subsequently succumbed and finally, one animal on days 4 and 7 which also died.

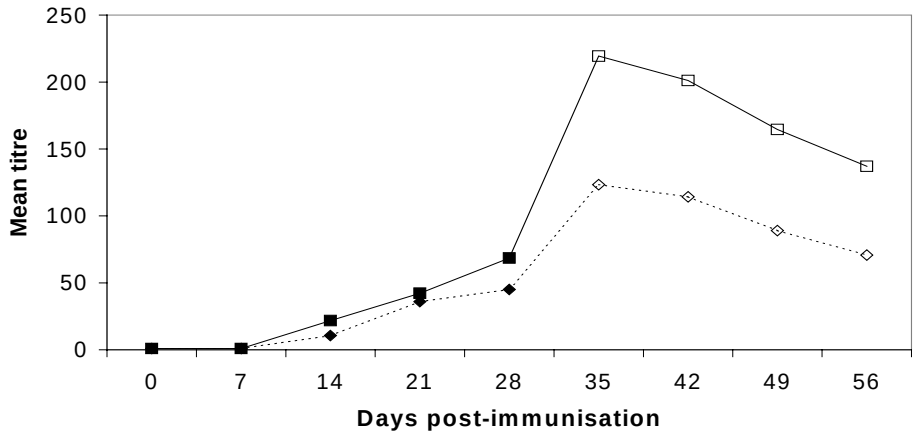
8.3.5 CSF-specific antibodies

All pigs vaccinated with the CSF vaccine (Groups A and B) sero-converted as measured by the Ceditest® CSFV. CSF-specific antibody titres increased gradually from day 14 onwards to maximum levels at day 35. On days 28, 35 and 42 post-vaccination, titres were significantly higher in the non-infected Group B compared to the infected Group A (Figure 8.2). Animals of the non-vaccinated control group remained sero-negative until the day of CSF-challenge.

8.3.6 HSA indirect antibody ELISA

Upon HSA-immunization, all 21 pigs sero-converted against HSA as measured by ELISA. After primary immunisation the mean titres of the *T. evansi*-infected Groups A and C and the uninfected Group (B) increased gradually in all groups with a non-significant higher level in Group B (figure 8.3). Following the booster immunisation at day 28, the mean titres for both infected and non-infected groups increased abruptly. However, this increase was at all time points significantly higher in the uninfected Group B compared to the infected Groups A and C.

Figure 8.3 *HSA-specific antibody titres of T. evansi infected (◊) and uninfected pigs (□) after primary (day 0) and secondary (day 28) HSA immunisation. Open symbols indicate statistically significant differences (P<0.05)*



8.4 Discussion

The experimental infections of pigs with the *T. evansi* buffalo strain WHITMAS 101298 did not induce any major clinical signs: growth performance, feed conversion and PCV levels were not affected compared with the uninfected control pigs. The absence of clinical symptoms or any influence on growth, feeding or PCV levels confirms findings by others (Dr. Phan Dich Lan, Vietnam, personal communication; Reid *et al.,* 1999) that *T. evansi* experimental infection in young pigs has only a limited pathology. Other studies, however, investigating the influence of natural *T. evansi* infection on pig production, demonstrated fertility problems (Arunasalam *et al.,* 1995; Bajyana Songa *et al.,* 1987). It should however be noted that all experimental infections including the present study, were carried out with buffaloes isolates on young animals.

Challenge with the CSF virus was lethal for three out of seven non-vaccinated pigs while the remaining four animals had fever for at least three days post-challenge. Also, the percentage of recordings of thrombocytopaenia in the non-vaccinated group was nearly twice (32 %) those of the vaccinated groups A and B (17 %). Conversely, leucopaenia appeared not to be of major importance for the challenged, non-vaccinated group. On the contrary, all the vaccinated animals survived the virus challenge, confirming the protective value of the vaccine that prevents mortality and appearance of major clinical symptoms. However, some animals in the *T. evansi*–infected group A showed loss of appetite for 2–5 days post challenge.

The present study demonstrates that *T. evansi* infection lowers the immunoresponsiveness of fattening pigs to concurrent immunisations. Indeed, antibody responses against both the test antigen HSA and the CSF vaccine were significantly reduced in *T. evansi*-infected animals compared with uninfected animals. This reduced response against the CSF vaccine appears to be accompanied with a less well-developed protection against CSF. A more profound loss of appetite post-challenge and twice the number of days with fever were observed in the *T. evansi*-infected group as compared to the non-infected group. However, no differences in leucopaenia, thrombocytopaenia or survival rates were observed between the groups.

These data support the findings in ruminants on the immunosuppressive nature of trypanosome infections (Whitelaw *et al.,*1979; Ilemobade *et al.,* 1982; Rurangirwa *et al.,* 1983; Ikeme *et al.,* 1984, Onah *et al.,* 1998, see Chapter 7). In mice and cattle (Sileghem *et al.,* 1991; Sileghem and Flynn, 1992), it has been demonstrated that

Trypanosoma infections reduce T cell responsiveness. In both species, T cell proliferation was profoundly depressed upon mitogenic stimulation and mediated by macrophage-like suppressor cells, leading to a suppressed interleukin-2 secretion and impaired expression of the interleukin-2 receptor. A possible explanation for the reduced antibody responses in infected pigs might thus reside in a T cell suppression induced by the concurrent *T. evansi* infection. Indeed, the induction of a good antibody response needs an optimal T helper cell response as the latter provide help to the B cell through delivery of cytokines.

In conclusion, these results suggest that the capacity to mount immune responses against heterologous antigens may be suppressed in *T. evansi*-infected fattening pigs and consequently that *T. evansi* infection might interfere with the development of protective immunity upon heterologous vaccinations. This immunosuppression may explain the accounts of poor protection of CSF-vaccinated pigs reported in *T. evansi*-endemic areas of Vietnam. It is suggested that in *T. evansi*-endemic areas prior treatment of pigs with trypanocidal drugs may enhance the efficacy of CSF vaccination.

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PART 3

General discussion and summary

CHAPTER 9
GENERAL DISCUSSION

In the first part of this thesis the diagnostic techniques that can assist in the measurement of the prevalence or incidence of infection with *Trypanosoma evansi* were evaluated and validated, while in the second part of this work experiments were performed to determine whether Surra has an immunosuppressive effect like nagana in sub-Saharan Africa (Taylor, 1998).

This final chapter will assess the findings of the previous chapters in the light of the overall objective of this thesis: to make an assessment on the importance of Surra in water buffaloes and pigs in Vietnam. To make this appraisal three questions need answering: 1) which is the best diagnostic tool to perform prevalence studies; 2) how widespread is Surra in buffaloes and pigs and 3) what has the present study contributed to a better understanding of the impact of *Trypanosoma evansi* in pigs and buffaloes in Vietnam.

9.1 Diagnosis of Surra

To have a systemic approach to diagnosis, standardisation is of utmost importance. Not only do all test reagents have to be standardised, but also the positive and negative reference sera plus the test protocol (Wright *et al*., 1993). Once assays are conducted in a standardized way, test results by different laboratories at different dates become comparable and data on prevalence and economic impact become compatible.

The choice which diagnostic tests to use, depends on factors such as the approximate prevalence in the population, purpose of testing and test parameters such as specificity and sensitivity. To estimate the prevalence of the disease, profound knowledge on the behavior of the disease in the individual animal and on herd level is needed while in addition a good understanding of the possible differential diagnosis is required. The diagnostic tests for *T. evansi* can serve a range of purposes such as screening in surveillance programs, diagnosis to confirm clinical infection, epidemiological studies or verification of absence of infection from certain regions or during quarantine. For this last purpose, it is necessary to reduce the likelihood that infected animals are classified as uninfected, requiring a test sensitivity of 100 %, while specificity is of less importance. If a test is used to confirm infection in a suspected case: ease of use, quick

results combined with good levels of both sensitivity and specificity are considered important (Thrushfield, 1997).

Most tests evaluated in these studies were developed with a clear goal. The latex agglutination assay (LATEX) and the card agglutination test (CATT) as discussed in Chapter 3 and 5 were developed with the aim of pen-side diagnosis. Although test concordance for positive results between the T.L. and the LATEX/*T. evansi* test was only 58 %, (see Chapter 5), the LATEX using fresh blood confirmed nearly twice as many animals positive as the PCR, and therefore is a useful test for pen-side diagnosis.

The PCR technique as described in Chapters 4 and 5 was a sensitive and highly specific diagnostic tool and in addition had a number of advantages over conventional parasitological techniques such as the microhaematocrit centrifugation technique (MHCT) and mouse inoculation (M.I.). The major disadvantage of the PCR at present is the high cost per test sample. It can be expected that especially labour costs with the introduction of PCR-ELISA (Chansiri *et al.,*2002) will be reduced. The main purpose of the T.L. as reported in Chapter 2, 3, 4 and 5 is in epidemiological research, being the 'gold standard' for the presence of anti-RoTat1.2 antibodies (Bayjana Songa and Hamers, 1988; Van Meirvenne *et al.,* 1995).

Since Surra has become a more chronic infection during its spread in South- East Asia the need for diagnostic assays with well defined test parameters and capacity to test large numbers of animals for the estimation of regional and national occurrence of Surra seems more urgent than ever (Luckins, 1998a). The ELISA/*T. evansi* based on RoTat 1.2 antigen as evaluated in both pigs and water buffaloes is such a robust and validated test. Recently, recombinant RoTat 1.2 antigen has been produced and it can be expected that the specificity of the test with the recombinant antigen will improve since significantly fewer cross–reactions are expected to occur (Urukawa *et al.*, 2001).

However it could be speculated that if strains exist in which RoTat 1.2 does not occur or does not appear until late in the course of infection, the sensitivity of a serological assay based on that antigen will be reduced. However until now such strains have not been isolated whereas *T. evansi* isolates from three continents were found to produce RoTat 1.2 antibodies in rabbits within 32 days after inoculation, confirming its widespread and predominant character (Verloo *et al.,* 2001).

It is important to emphasize that the validation of the tests as used in this thesis has not been completed. Jacobson (1998) discussed in depth the process of test validation and

various critical points. If we compare our validation process against the ideal process described by Jacobson (1998), several critical comments can be made: how well do the positive control groups of acute experimentally infected young water buffaloes or pigs represent the target population of natural infected water buffaloes and pigs respectively from north Vietnam? It is also necessary to look at assay validation as a continuous and incremental process, since validation does not stop with a time-limited series of experiments with a small range of reference sera. It requires rather a constant vigilance and maintenance along with reassessment of its performance characteristics for each unique population of animals. Test parameters that were obtained from experimentally infected Vietnamese piglets of mixed breed, should be used with caution when applied in farrowing sows from commercial units in Thailand (see Chapter 8). It should also be noted that the confidence people have for a test increases each time a test is used e.g. the trust people have in a new test such as the LATEX/*T. evansi* for use in water buffaloes is less, than they have in the CATT/*T. evansi* which had prior been evaluated in Thailand (Bayjana Songa and Hamers,1988) and Indonesia (Davison *et al.,* 2000).

It has been suggested that the predictive value of testing is increased by using diagnostic tests in parallel (Davison, 1997). Since the serologic tests that have been evaluated in this study are all based on the RoTat 1.2 antigen, the combined use of these tests will not increase the predictive value. Parallel testing with an antigen or parasite detection assay is more likely to increase the likelihood that animals are properly diagnosed.

9.2 Prevalence of Surra

In the year 2000, ten countries officially reported Surra to the Office International des Epizooties, while seven more countries could be added to this list by extracting information from scientific publications (Touratier, 2002). That the list is complete seems unlikely, since assumed endemic countries like China, Malaysia, Iran, Bangladesh and Saudi Arabia are not included.

Within this study a number of surveys were conducted in both buffaloes and pigs with the ELISA based on RoTat 1.2 as a capture antigen. In 1998 in Hoa Binh and Thai Nguyen province (Chapter 2), 20 % of water buffaloes were found serologically positive, while in 2000 in various districts of Hoa Binh, Bac Can and Cao Bang province the sero- prevalence varied between 0 and 60 %, depending on the location (Chapter 5). No trypanosomal antibodies were detected in Vietnamese pigs while in

northern Thailand, pigs were serologically positive on 5 commercial farrowing farms. In most Asian countries epidemiological data are often based on clinical observations or on the number of treatments given to sick animals, as in the Philippines (Manuel, 1998). In a few countries irrespective of outbreaks, surveys were conducted in a more systematic way. In Thailand prevalence rates for buffaloes were estimated at around 20 % while for pigs the estimate was 5 % (Tuntasuvan and Luckins, 1998). In China a large number of surveys have been conducted primarily in water buffaloes (Lun *et al.,* 1993). Infection rates varied between the areas and between the various seasons and ranged from 3 to 86 %. The largest number of outbreaks however was observed during the winter season, when animals were on nutritional stress, as also previously found in Vietnam (Thu *et al.,* 1998). In Indonesia, in a market study, 47 % of buffaloes was confirmed positive with CATT, while with an antigen ELISA in a cross sectional study, 59 villages were surveyed and prevalence rates varied from 26 to 97 % (Davison *et al.,* 2000). Although several suggestions were made for the observed inter-village variation, no analysis was made except for the confounding effect of age. Prevalence rate of the group below 6 months of age was 23 %, while the prevalence peaked to 68 % for the group 37 – 60 months (Davison *et al.,* 2000). It has also been suggested that Indonesian buffalo could spontaneously recover from infection and get re-infected nearly every year (Coen *et al.,* 2001.). However, further study is needed to confirm these findings.

In Latin-America outbreaks of Surra in horses coincided with the onset of the rainy season and the increased movement of livestock (Silva *et al.,* 1995). Additionally there were speculations on the prominent role of the capybaras as a reservoir host (Franck *et al.,* 1994)

In Africa, prevalence among camels was related to regional characteristics such as number of watering points, existence of wooded areas and migratory patterns of animals. Also was there a between herd variation which could be accounted for by the amount of trypanocidal drugs applied, and finally infection among young animals was rare (Dia *et al.,* 1997; Ngaira *et al.*, 2002).

9.3 Impact of *T. evansi* infections

During the experimental infections and the various field surveys, described in this thesis, few clinical signs of disease could be observed in either pigs or water buffaloes. In corroboration with other studies these findings support that Surra has evolved into a

subclinical disease (Luckins, 1988; Thu *et al.,* 1998; Davison *et al.,* 1999), and that even in case of epidemics, clinical cases are mild (Ozawa, 1998).

Prevalence estimates are frequently based on results obtained from studies carried out in response to (sub)-clinical epidemics. Often only parasitological tests like Giemsa-stained blood-smears are used in such studies (Reid, 2002). With fewer clinical outbreaks reported, inadequate diagnostic tests and an incomplete picture of the impact of *T. evansi* on productivity parameters, the attitude of national governments to control the disease and research groups to implement research on Surra is affected in a negative way. Even in case of massive epidemics with hundreds of animals killed and massive amounts of trypanocidal drugs applied, hardly any evaluation is made to determine the financial consequences of the disease. In the case of sub-clinical infections, where animals can be infected for a longer period of time, such an economic evaluation is nearly always absent. Recently a longterm study was undertaken in Indonesia in which an infected and non-infected group of water buffaloes were monitored for nine months (Luckins, 1998b). Herd productivity was reduced in the infected group by 22 % and this could primarily be attributed to mortality losses and emergency sales, while weight gain of infected animals was hardly affected. In this study also the threshold for mass treatment of a herd was calculated and set at > 40 % sero-prevalence, which would have meant that based on the results of the surveys described in Chapter 5, two areas would have been short listed for mass treatment.

In a limited number of studies it was demonstrated that *T. evansi* infections reduced weight gain by nearly 8 kg in feedlot cattle over a three months period (Payne *et al.,* 1994a), while in experimentally infected Holstein Friesian calves a reduced weight gain of 16 kg was recorded (Payne *et al.,* 1992). *T. evansi* also has a significant impact on the performance of working buffaloes (Pearson *et al.,*1999), abortion rates (Teeraprasert, 1984; Lohr *et al.,*1986; Bayjana Songa *et al.,*1987; Arunasalam *et al.,*1995; Kashiwazaki *et al.,*1998), cessation of the oestrus cycle (Payne *et al.,*1993) and milk yield (Pohlpark *et al.,*1999).

In Chapters 7 and 8 the impact of Surra on the immune responsiveness following vaccination was also analysed. How important this immune responsiveness will be in practice is not yet clear, although at least in pigs vaccinated against Classical Swine Fever, it has been demonstrated that protective immunity was reduced upon challenge

with a field strain. On the other hand, we should conclude that with the current management practices on commercial farrowing farms in endemic areas of Thailand, good technical results can be achieved (Dr. Sangmeendet pers. com.) and immunosuppression does not appear to have a huge impact. Of course it is important to realize that commercial bred sows are under limited stress, and that especially their nutritional status is optimal. It should however be borne in mind that *T. evansi* outbreaks on farrowing farms can lead to heavy losses due to mortality, abortion and culling. Siriwan *et al.* (1989) calculated the economic loss for a Surra outbreak of 6 months on an intensive farrowing farm of 380 sows at 8500 US$. In total 61 animals died during this outbreak.

Unfortunately the water buffaloes of Chapter 7 were not challenged with *Pasteurella multocida* and protective immunity was not directly monitored. However results strongly indicate the immunosuppressive effect of *T. evansi* in water buffaloes. With an emerging dairy industry in the region high yielding cattle become increasingly important and studies to monitor the immunosuppressive effect of *T. evansi* on dairy cattle will be needed.

Just recently FMD vaccination failures in cattle were reported from Cambodia. If *T. evansi* induced immunosuppression is the basis for these noted vaccination failures, it will have major implications for the regional South-east Asian Foot-and-Mouth-Disease (SEAFMD) eradication program (Touratier personal communication).

As with other parasitic diseases such as haemonchosis and fasciolosis (Abbott *et al.,* 1986; Torgerson and Claxton, 1999) the impact of Surra increases rapidly when animals come under severe stress, especially nutritional stress. Therefore, animals most prone to the immunosuppressive effects are the ruminants kept by subsistence farmers during the winter/spring season when there is a shortage of forage and animals are intensively used for land tillage (Luckins, 1988).

9.4 Conclusions

With the various RoTat 1.2 assays and especially the ELISA/*T. evansi*, adequate diagnostic tools have been identified to assess the prevalence and/or incidence of Surra in a herd. However a clear picture on the overall impact of the disease on livestock production has not yet been achieved. To make an appraisal of this impact two routes

can be followed; an approach in which each productivity parameter is examined separately, or a holistic approach in which two identical populations, except for their *T. evansi* infection status, are monitored for at least one production cycle. Although this differentiate approach can quantify the economic impact of each parameter separately and is of great value in understanding the importance of the different pathophysiological effects of the disease, it doesn't provide an answer to the overall impact. A productivity trial, if planned meticulously, is more likely to provide this answer.

The obvious starting point should be farrowing farms and feedlots in the region since conditions are well controlled and in- and outputs are in detail recorded (Teeraprasert *et al.,* 1984).

Once this economic appraisal is completed, it should be possible to calculate the financial impact of the disease at various prevalence rates. National or regional governments are then in a position to conduct surveys on the prevalence of infection and assess the economic impact of Surra in an area.

With less clinical cases recorded over the years, it is tempting to believe that *T .evansi* is of minor importance in the region but only once the economic importance of Surra is clarified such conclusions can safely be made.

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SUMMARY

This thesis consists of four parts: a general section including an introduction, a literature review and the objectives of the study. In Part 1 the personal investigations on the diagnosis of Surra in water buffaloes and pigs are described while in Part 2 the studies on the immunosuppressive effect of *T. evansi* are explained. In the final Part 3, the general discussion, results are put in a wider perspective.

In Chapter 1, the important aspects of the life history of trypanosomes including topics on the epidemiology, transmission and economics of the disease are reviewed. Emphasis in this literature review is on the immune response of the host, the various methods for diagnosis, and on the parasite's mechanism to derail the hosts' immune system leading to immunosupression. The first chapter finishes with describing various control methods for Surra and highlights the little progress that been made in developing new possibilities for control. The main question coming out of this review was: how important is Surra for the livestock population in the area. To make this assessment, two questions need to be answered: (1) what is the prevalence of Surra in the various populations (2) and what is the impact of the disease on the various sub-populations. To answer the first question well validated, accurate and robust diagnostic tests are needed. The first part of this thesis therefore aims at developing, validating and optimising existing and new test for the diagnosis of Surra in water buffaloes and pigs and use these in a number of survey. The second question is more complex, since the infection has an effect on a number of productivity parameters. To assist in providing an answer to the second question, in Part 2 of this study the possible interference of *T. evansi* with the development of protective immunity after vaccination is investigated.

In Chapter 2 a collection of 415 water buffalo serum samples originating from the North of Vietnam was used for evaluation of different diagnostic antibody detection methods: direct card agglutination test (CATT), indirect card agglutination test (LATEX) and antibody detection ELISA. All animals (n=8) confirmed parasitologically positive by mouse inoculation (M.I.) were positive in all serological tests and the CATT showed the highest diagnostic specificity (98 %) followed by the ELISA (95 %) and the LATEX (82 %). The immune trypanolysis (T.L.) assay with

the predominant *T. evansi* RoTat 1.2 variable antigen type was introduced as a reference test. In total 20 % of the samples (n=415) was found sero-positive. Concordance of the variant specific T.L. test with the other tests was calculated and revealed that few (1-8 %) false positive results were actually due to nonspecific reactions and that LATEX and ELISA detected more immune trypanolysis positives than the CATT. It was concluded that, ELISA with a percent positivity (P.P.) cut- off of 30 and the LATEX, thanks to their superior capacity of detecting *T. evansi* specific antibodies, would be suitable as epidemiological tools detecting both active infections and persisting *T. evansi* specific antibodies. The CATT and the ELISA with a cut-off of P.P. = 50 on the other hand, seems more appropriate to detect true infected buffaloes.

In Chapter 3 investigations were made if fresh whole blood could substitute for serum in the CATT and the LATEX in buffaloes. Likewise blood spots on filter paper were compared to sera for use in the ELISA and T.L. Due to lack of reference material, samples were collected weekly from experimentally *T. evansi* infected- and non-infected water buffaloes. To estimate test agreement between serum and blood (confetti) versions of the tests, *kappa* values were calculated; 0.75 for the CATT; 0.80 for the ELISA; 0.84 for the LATEX and 0.93 for the T.L. In addition *kappa* values were computed to assess agreement between results obtained in the reference T.L. test on serum and those obtained in the other assays; 0.70 for the CATT-Serum; 0.75 for the LATEX-Blood; 0.77 for the LATEX-Serum; 0.81 for the CATT-Blood; 0.81 for the ELISA-Serum and 0.84 for the ELISA-Confetti. Based on the high *kappa* values as calculated, we conclude that serum can be replaced by fresh blood for the agglutination assays or blood on filter paper for the T.L. and ELISA at P.P. 26.

Chapter 4 is concerned with the comparison of 5 parasitological methods and a polymerase chain reaction (PCR) for diagnostic sensitivity in experimentally infected water buffaloes over a period of 15 weeks. The combined estimates of sensitivity (CE_{se}) of the PCR proved to be highest at 78.2 %, closely followed by M.I., the micro haematrocrite centrifugation technique (MHCT) and the mini-anion exchange centrifugation technique (MAECT) with CE_{se} of respectively; 74.0, 69.6 and 62.4 %. The CE_{se} of the buffy coat technique (BCT) at 38.6 % and the sodium dodecyl sulphate clarification technique (SDS) at 25.1 % were considerably lower. PCR

detected consistently all buffaloes infected from week 3 post-infection (P.I.) onwards. For M.I. this occurred after 5 weeks P.I. while for MHCT and MAECT these sustainable high levels were reached in the 7th week P.I. The influence of time and temperature on the viability of *T. evansi* in heparinized blood from water buffalo was also studied. In general survival time tends to be longer when blood is kept at 4° C. In samples kept in direct sun light parasites became undetectable with the MHCT after 30 minutes. After treatment of the water buffaloes with diminazene aceturate, the PCR signal disappeared within 24 hours.

In Chapter 5 the prevalence of *T. evansi* in water buffaloes was investigated in remote mountainous areas of northern Vietnam from where no disease reports were yet received. It was demonstrated that the ELISA-confetti the LATEX using fresh blood and the diagnostic PCR on room temperature preserved blood samples were appropriate diagnostic tests under such conditions. The sero-prevalence varied widely between areas, low (7 %) and high prevalence (60 %) regions adjacent to another. This supports earlier observations that the prevalence of Surra depends on local conditions. Overall prevalence was 16 %

The sixth Chapter is concerned with the evaluation of the usefulness of established diagnostic test for diagnosis of *T. evansi* in pigs. Serological tests were initially evaluated in experimental infected fattening pigs, while afterwards the tests were used in two field surveys. Results of both agglutination assays: CATT and LATEX at different serum dilutions didn't give consistent results in the various sub-populations. The ELISA at P.P 22 combined an apparent specificity of 100 % with sensitivity higher as 97 %. Of the 437 samples collected of small-holder pigs from the north of Vietnam no positive pigs were detected with the T.L. In the second survey samples were collected from five farrowing farms in northern Thailand with a history of Surra. On each farm positive sows were detected and the overall prevalence was 36 %.

The second part of this thesis is focused on immunosuppressive studies and so in Chapter 7 the immuno-suppressive capacity of *T. evansi* was investigated. Infected and non-infected buffaloes were vaccinated against *Pasteurella multocida* (haemorrhagic septicaemia) and simultaneously immunised with a control antigen, human serum albumin (HSA), and the immune responses against these heterologous

antigens were monitored. The antibody responses against HSA were significantly reduced in *T. evansi* infected animals, but no conclusive data were obtained on the antibody responses against *P. multocida*. Conversely, the local inflammatory response at the site of *Pasteurella* vaccination, as measured by increase in size, was significantly reduced in *T. evansi*-infected animals. The results indicated that the inductive capacity to mount humoral and cell-mediated immune responses against heterologous antigens was suppressed in *T. evansi*-infected animals. Consequently, *T. evansi* infection could interfere with the development of protective immunity upon heterologous vaccinations and could explain the poor protection of *Pasteurella*-vaccinated buffaloes in *T. evansi*-endemic areas of Vietnam.

In Chapter 8, a similar type of study was conducted with pigs. In *T. evansi* infected and non-infected animals, induction of immune responses by vaccination against classical swine fever (CSF) and by immunization with HSA was assessed. Antibody responses against both the test antigen HSA and the CSF vaccine were significantly reduced in *T. evansi*-infected animals as compared to uninfected animals. Moreover, the reduced response against the CSF vaccine appeared to be accompanied by a less well-developed protection against CSF with higher fever responses. This immunosuppression could explain the accounts of poor protection of CSF-vaccinated pigs reported in *T. evansi*-endemic areas of Vietnam, and suggested that treatment with trypanocidal drugs to improve the efficacy of CSF vaccination may be justified.

In the general discussion, Chapter 9, focus lays on the overall objective of this thesis i.e. to provide additional tools to answer the central question: what is the importance of Surra in water buffaloes and pigs in Vietnam. To make this appraisal three sub-questions were posed: 1) which is the best diagnostic tool to perform prevalence studies; 2) how widespread is Surra in buffaloes and pigs and 3) what is the impact of *T. evansi* infection on production. From the results we can conclude that the ELISA Rotat 1.2 is an excellent tool to estimate the sero-prevalence in an area, and that prevalence in water- buffaloes varies considerable but overall prevalence is estimated at around 25 - 35 %. The final questions on the economical impact needs further investigation and suggestions for additional research are made.

SAMENVATTING

Dit proefschrift bestaat uit 4 onderdelen: een algemeen gedeelte met een introductie, een literatuur studie en de beschrijving van de doelstellingen van de studie. In Deel 1 worden de verschillende studies beschreven ter verbetering van de diagnostiek van Surra in water buffels en varkens terwijl in Deel 2 de onderzoeken naar het immuun suppressief karakter van *Trypanosoma evansi* worden besproken In het laatste Deel 3 worden resultaten, in een algemene discussie, in een wijder perspectief geplaatst.

In Hoofdstuk 1, de literatuur studie, worden verschillende aspecten van de biologie van trypanosomen belicht, inclusief de epidemiologie, transmissie en economische aspecten. De nadruk in deze literatuur studie ligt op de immunologische respons van de gastheer en op de mechanismen welke de parasiet ter beschikking heeft om het immuun systeem te onderdrukken. Daarnaast worden meerdere diagnostische methodes met de voor- en nadelen besproken. Hoofdstuk 1 eindigt met de beschrijving van de verschillende bestrijdings methodes voor Surra en belicht de beperkte vooruitgang welke is geboekt op dit gebied. De centrale vraag welke naar voren komt uit de literatuur studie is: hoe schadelijk is Surra werkelijk voor de veehouderij in Zuidoost Azië. Om tot een antwoord te komen dienen 2 subvragen zich aan: wat is de prevalentie van de ziekte en wat is het gevolg van de infectie in de verschillende dier species. Om de eerste vraag te kunnen beantwoorden zijn gevalideerde, accurate en robuuste diagnostische testen een eerste voorwaarde. Het eerste gedeelte van dit proefschrift behandelt daarom de ontwikkeling, optimalisering en validatie van diagnostische testen en het gebruik van deze testen in een aantal gelimiteerde prevalentie studies. Om een bijdrage te leveren in de beantwoording van de tweede vraag wordt in Deel 2 van deze studie de mogelijke interferentie van *T. evansi* op de immuniteits opbouw na vaccinatie onderzocht.

In Hoofdstuk 2 worden de directe kaart agglutinatie test (CATT) een indirecte latex agglutinatie test (LATEX) en een ELISA geëvalueerd met behulp van 415 stalen welke werden verzameld van water buffels uit noord Vietnam. Alle dieren welke parasitologisch positief waren met de muis inoculatie (M.I.) waren tevens positief in alle andere testen. De CATT had de hoogste diagnostische specificiteit (98 %), gevolgd door de ELISA (95 %) en de LATEX (82 %). De immune trypanolysis test

(T.L.) met het predominante *T. evansi* type, RoTat 1.2 werd geïntroduceerd als referentie test. In totaal werden 20 % van de stalen positief bevonden met deze test. Overeenkomsten tussen de resultaten met de T.L. en de andere testen werden berekend. Het bleek dat 1-8 % van de vals positieve resultaten door aspecifieke reacties werd veroorzaakt. Verder bleek dat de LATEX en ELISA meer T.L. positieve resultaten konden conformeren dan de CATT. Tevens werd geconcludeerd dat de LATEX en de ELISA, met een cut-off van 'percentage positivity' (P.P.) 30, dankzij de eigenschap om specifieke *T. evansi* antilichamen te kunnen detecteren, geschikt zouden zijn voor epidemiologische studies, om actieve en persisterende infecties op te kunnen sporen. De CATT en de ELISA met een cut-off van P.P. = 50 zouden meer geschikt zijn om werkelijk geïnfecteerde dieren op te sporen.

In Hoofdstuk 3 wordt het onderzoek beschreven of vers bloed, serum zou kunnen vervangen in de CATT en LATEX tests in water buffels. Tevens werd op filterpapier opgedroogd bloed vergeleken met serum in ELISA and T.L. tests. Omdat ons geen referentie materiaal ter beschikking stond, werden wekelijks stalen verzameld van buffels welke experimenteel geïnfecteerd waren met *T. evansi* plus een controle groep van niet geïnfecteerde dieren. Om de overeenstemming te kunnen bepalen tussen de uitslagen van serum en volbloed versies van een test werden *kappa* waardes bepaald*:* 0,75 voor de CATT: 0,80 voor de ELISA: 0,84 voor de LATEX en 0,93 voor de T.L. Tegelijkertijd werden *kappa* waardes berekend om overeenkomsten tussen test resultaten te analyseren voor de T.L. referentie test met serum en de andere testen: 0.70 voor de CATT-Serum: 0,75 voor de LATEX-Bloed: 0,77 voor de LATEX–Serum: 0,81 voor de CATT-Bloed: 0,81 voor de ELISA-Serum en 0,84 voor de ELISA-Confettie. Door de hoge *kappa* waarde, kunnen we concluderen dat vers bloed serum kan vervangen in het gebruik van agglutinatie testen en dat bloed opgedroogd op filterpapier, serum kan vervangen in de T.L. test en de ELISA met P.P. = 26.

In Hoofdstuk 4 werden vijf parasitologische tests en een 'polymerase chain reaction' (PCR) onderling vergeleken voor test gevoeligheid in een groep van experimenteel geïnfecteerde waterbuffels voor een periode van 15 weken. De gecombineerde schatting van sensitiviteit (CE_{se}) van de PCR was het hoogst (78,2 %), gevolgd door de M.I., de 'micro haematrocrite centrifugation technique' (MHCT) en de 'mini-anion ,exchange centrifugation technique' (MAECT) met een CE_{se} van respectievelijk; 74,0,

69,6 en 62,4 %. De CE_{se} van de 'buffy coat test' (BCT) met 38,6 % en de sodium dodecyl sulphate clarification techniek (SDS) met 25,1 % waren beduidend lager. De PCR detecteerde alle buffels doorlopend positief vanaf de derde week na infectie. Hetzelfde deed de M.I. vanaf de vijfde week na infectie, terwijl voor de MHCT en de MAECT deze niveaus werden bereikt op week 7 na infectie. Ook werd de invloed bestudeerd van tijd en temperatuur op de overlevingsduur van *T. evansi* in gehepariniseerd bloed van water buffels. De overlevingsduur bij 4° C is langer dan bij kamer temperatuur. In bloedstalen bewaard in direct zonlicht, waren al na 30 minuten geen parasieten meer te vinden. Na behandeling van de waterbuffels met diminazene aceturaat, verdween het PCR signaal binnen 24 uur.

In Hoofdstuk 5, werd de sero-prevalentie van *T. evansi* infecties in water buffels kuddes bepaald in afgelegen bergachtige gebieden van noord Vietnam, waar tot op heden geen informatie met betrekking tot dierziektes van bekend was. De seroprevalentie binnen de verschillende gebieden varieerde sterk, met lage- en hoge prevalentie gebieden naast elkaar gelegen. Dit stemde overeen met eerdere observaties dat de prevalentie van Surra sterk afhankelijk was van locale omstandigheden. Tevens werd aangetoond dat de ELISA-Confettie, de LATEX met vers bloed en de PCR goed werkten onder de gegeven omstandigheden.

In het zesde Hoofdstuk werd de bruikbaarheid van de verschillende diagnostische testen geëvalueerd voor de diagnose van Surra in varkens. Serologische tests werden eerst geëvalueerd in experimenteel geïnfecteerde varkens, en naderhand werden de tests gebruikt in veld studies. De resultaten van de beide agglutinatie tests: CATT en LATEX bij verschillende serum verdunningen, waren niet consistent in de verschillende subpopulaties. De ELISA met een 'cut-off' van P.P. = 22, combineerde een diagnostische specificiteit van 100 % met een sensitiviteit van 97 %. In totaal werden 437 stalen verzameld van varkens van kleine boeren uit het noorden van Vietnam, hiervan werd geen enkel varken positief bevonden met de T.L. In een tweede onderzoek in Noord Thailand werden 5 vermeerderingsbedrijven bezocht met een geschiedenis van Surra. Op elk bedrijf werden serologisch positieve dieren gevonden en de algehele sero-prevalentie was 36 %.

Het tweede gedeelte van dit proefschrift richtte zich op studies over het mogelijk immunosuppressief karakter van *T. evansi*. In hoofdstuk 7 werden *T. evansi* geïnfecteerde buffels gevaccineerd tegen *Pasteurella multocida* (haemorrhagische septicaemie) en tegelijkertijd geïmmuniseerd met een controle antigeen, humaan serum albumine (HSA). De immuunrespons tegen beide antigenen werd bestudeerd. De antilichaamrespons tegen het HSA was significant lager bij de *T. evansi* geïnfecteerde dieren. Er konden geen conclusies worden getrokken uit de antilichaam reactie tegen *P. multocida*. De grootte van de locale ontstekingsreactie op de plek van de *P. multocida* vaccinatie, was significant kleiner in de *T. evansi* geïnfecteerde dieren dan in de controles. De resultaten geven een indicatie dat zowel de niet-celgebonden als de celgebonden immuniteitsopbouw na vaccinatie verminderd was in *T. evansi* geïnfecteerde dieren. Het is dus mogelijk dat een *T. evansi* infectie interfereert met de ontwikkeling van beschermende immuniteit na vaccinatie. Dit zou tevens een verklaring kunnen zijn voor de matige bescherming van *Pasteurella* gevaccineerde water buffels in *T. evansi* endemisch gebied.

In Hoofdstuk 8 wordt een soortgelijke studie uitgevoerd, echter nu in varkens. In *T. evansi* geïnfecteerde en controledieren, werd de immunerespons gemeten na vaccinatie tegen zowel Klassieke Varkenspest (KVP) als tegen HSA. Antilichaam reacties tegen zowel het HSA als het KVP vaccine waren significant lager in de *T. evansi* geïnfecteerd dieren vergeleken met de controle dieren. De verlaagde antilichaam opbouw na KVP vaccinatie werd gecombineerd met een verminderde bescherming tegen het KVP virus bij kunstmatige besmetting, in combinatie met hogere lichaamstemperaturen. De immuunsuppressie zou de verminderde bescherming kunnen verklaren van KVP geïmmuniseerde dieren in *T. evansi* endemische gebied. De suggestie wordt gedaan dat gelijktijdige behandeling van een trypanocide bij de varkenspest vaccinatie de bescherming zou bevorderen.

In de algemene discussie in hoofdstuk 9, ligt de nadruk op de algehele doelstelling van dit proefschrift, het verwerven van instrumenten om de centrale vraag te kunnen beantwoorden: wat is het belang van Surra in water buffels en varkens in Vietnam. Voor de beantwoording van deze vraag behoeven drie subvragen beantwoording: ten eerste wat is de beste diagnostische test om prevalenties studies uit te voeren, ten tweede wat is de verspreiding van Surra in waterbuffels en varkens en als laatste de

vraag wat is het effect van een Surra infectie op de productiviteit van dieren. De ELISA test met het RoTat 1.2 antigeen blijkt een uitstekende test om prevalentie studies in een gebied uit te voeren. Voor buffels lijkt de prevalentie per gebied nogal te verschillen. Ter beantwoording van de laatste vraag over het economisch belang van Surra zijn tot op heden onvoldoende gegevens beschikbaar en suggesties voor verdere studie worden gegeven.

List of abbreviations

ABTS	2,2'-azinobis(3-ethyl-benzothiazoline)-6-sulfonic acid
AMPT	active mouse protection test
ANOVA.	Analysis of variance
BCT	Buffy coat test
bp	Base pair
CATT	Card Agglutination Test
CE_{se}	Combined estimates of sensitivity
CFT	Complement Fixation Tests
CFU	Colony-forming units
CSF	Classical swine fever
CSFV	Classical swine fever virus
CI	Confidence intervals
DEAE	Diethylamino ethyl cellulose
DNA	Deoxyribonucleic acid
dNTP	Deoxynucleotides triphosphates
DTH	Delayed type hypersensitivity
EDTA	Ethylene diaminetetraacetic acid
ELISA	Enzyme-linked immunosorbent Assay
FAT	Fluorescence antibody test
FMD	Foot-and-mouth-disease
g	Gravity
GPS	Guinea pig serum
h	Hour
HAS	Human serum albumin
IFAT	Indirect Fluorescent Antibody Test
kDa	Kilo dalton
kDNA	Kinetoplast deoxyribonucleic acid
LATEX	Latex Agglutination Assays
LPS	lipopolysaccharides
MAECT	Mini anion-exchange centrifugation technique
MHCT	Microhaematocrit centrifugation technique
MI	Mouse inoculation
na	Not available
nd	Not done

nm	Nano meter
OD	Optical density
OIE	Office International des Epizooties
PBS	Phosphate-buffered saline
PSG	Phosphate-buffered saline glucose
PCR	Polymerase chain reaction
PCV	Packed cell volume
PI	Post infection
PIH	Percent inhibition
PP	Percent Positivity
RAPD	Random- Amplified Polymorphic DNA
rpm	revolutions per minute
SD	Standard deviation
SDS	Sodium Dodecyl Sulphate
SIR	Subcutaneous inflammatory response
Sn	Sensitivity
Sp	Specificity
SsDNA	Single stranded Deoxyribonucleic acid
TL	Immune trypanolysis test
UV	Ultra violet
VAT	Variabel Antigen type
VSG	Variant Surface Glycoprotein

RESUME

Wicher was born in Arnhem – Netherlands the 27th of August 1963, where he also visited primary and secondary school. After one year at the Medical Faculty in Utrecht he shifted in 1982 to the Veterinary Faculty at the same university where he graduated as Doctor Veterinary Medicine in 1990. During his final year he spend 6 months in Nigeria at a recent established dairy farm where he developed and implemented strategies for the treatment and prevention of infectious and parasitological diseases in dairy cattle. Immediately after graduation Wicher worked for almost 2 years in a number of mixed veterinary practices in the Netherlands, until he got an offer to work as an Associate Professional Officer with the FAO. Just prior to this appointment he worked for two months in Algeria to assist a Dutch cattle export company in the quarantine procedures of pregnant dairy heifers. The initial FAO project where he started to work in 1992 was a tsetse eradication project in Zanzibar – Tanzania. During this period he was responsible for the organization and implementation of veterinary field and laboratory activities to monitor incidence and prevalence of trypanosome infections. In 1995 Wicher was transferred to the International Atomic Energy Agency in Vienna. During this assignment he assisted in the preparation of regional and national disease control / eradication programs, developed training materials and organized and lectured at various ELISA training courses. After repatriation in 1997, Wicher assisted in the Classical Swine fever eradication programs in the Netherlands, and took up a temporary assignment with the ID -Lelystad business development group. In 1998 his employment with the Veterinary Faculty in Ghent started, and soon he was send overseas to become project leader of the Belgian -Vietnamese veterinary project at the National Institute of Veterinary Research – Hanoi, Vietnam. This research project focused on four areas: chronic classical swine fever; gastro-intestinal parasites in ruminants; cysticercosis in swine and *Trypanosom evansi*. The *T. evansi* research was the basis of this PhD thesis. Since 2001 Wicher works at Intervet International Boxmeer – Netherlands, where he responsible for the worldwide technical support and marketing of antiparasitics products for ruminants.

Wicher is geboren op 27 augustus 1963 in Arnhem – Nederland. In zijn geboorte stad is hij zowel naar de lagere als middelbare school geweest. Na een jaar als medisch student, werd hij in 1982 ingeloot voor de studie diergeneeskunde in Utrecht, waar hij in 1990 als Dierenarts afstudeerde. In zijn laatste studie jaar heeft hij stage gelopen in Nigeria op een recent opgestart melkveebedrijf, waar hij behandelingsschema's ontwikkelde en uitvoerde voor de behandeling en preventie van de belangrijkste infectieuze en parasitaire ziekten bij melkvee. Na zijn afstuderen heeft hij bijna 2 jaar in verschillende gemengde dierenartspraktijken in Nederland gewerkt, totdat er een aanbieding vanuit FAO lag om als assistent deskundige te komen werken. Net voor deze uitzending heeft Wicher 2 maanden in Algerije gewerkt bij een vee exporteur, hij assisteerde in quarantaine procedures voor drachtige melkvee vaarzen. De eerste FAO aanstelling was bij een Tsetse / Trypanosomosis eradicatie project in Zanzibar – Tanzania, waar Wicher verantwoordelijk was voor de veterinaire activiteiten binnen het project. In 1995 werd hij overgeplaatst naar het Internationaal Atoom Energie Agentschap in Wenen. Hier werd Wicher betrokken bij nationale en regionale dierziekte bestrijding programma's, ontwikkelde trainingsmaterialen en organiseerde meerdere trainingscursussen in de epidemiologie en ELISA technieken. Na repatriëring heeft hij voor een korte periode gewerkt bij de varkenspest bestrijding in Nederland, en tevens voor een half jaar bij het ID-DLO in Lelystad waar hij te werk was gesteld bij 'Bussiness Development". In 1998 ging Wicher werken bij de Diergeneeskunde Faculteit in Gent, waar hij na een korte inwerk periode vertrok naar Vietnam, alwaar hij project leider werd van het Belgisch-Vietnamees project op het Nationaal Instituut voor Diergeneeskundig Onderzoek – Hanoi, Vietnam. Dit onderzoeksproject had vier aandachtsgebieden: varkenspest, maagdarmwormen, cysticercose bij het varken, en *Trypanosoma evansi* in vee. Het onderzoek op *T. evansi* is de basis voor dit proefschrift. Sinds 2001, werkt Wicher bij Intervet International waar hij verantwoordelijk is voor wereldwijde marketing en technische ondersteuning van anti parasitaire producten voor herkauwers.