THESIS APPROVAL

GRADUATE SCHOOL, KASETSART UNIVERSITY

Master of Science (Veterinary Parasitology)

DEGREE

Veterinary Parasitology — **FIELD**

Parasitology — **DEPARTMENT**

TITLE: Epidemiology of *Trypanosoma evansi* Infection of Dairy Cattle in Thailand

NAME: Miss Ketsarin Kamyingkird

THIS THESIS HAS BEEN ACCEPTED BY

_____ **THESIS ADVISOR**

(Associate Professor Sathaporn Jittapalapong, Ph.D.)

_____ **THESIS CO-ADVISOR**

(Assistant Professor Sirichai Wongnakpet, Ph.D.)

_____ **THESIS CO-ADVISOR**

(Mr. Marc Desquesnes, Ph.D.)

_____ **DEPARTMENT HEAD**

(Associate Professor Sathaporn Jittapalapong, Ph.D.)

APPROVED BY THE GRADUATE SCHOOL ON _____

_____ **DEAN**

(Associate Professor Gunjana Theeragool, D.Agr.)

THESIS

EPIDEMIOLOGY OF *TRYPANOSOMA EVANSI* INFECTION OF DAIRY CATTLE IN THAILAND

KETSARIN KAMYINGKIRD

A Thesis Submitted in Partial Fulfillment of
the Requirements for the Degree of
Master of Science (Veterinary Parasitology)
Graduate School, Kasetsart University
2009

Ketsarin Kamyingkird 2009: Epidemiology of *Trypanosoma evansi* Infection of Dairy Cattle in Thailand. Master of Science (Veterinary Parasitology), Major Field: Veterinary Parasitology, Department of Parasitology. Thesis Advisor: Associate Professor Sathaporn Jittapalapong, Ph.D. 76 pages.

Trypanosoma evansi is a tissue-blood parasite known as a haemoflagellate. It is causing "surra" that has spread in various geographical areas and widely distributed amongst the domestic and wild animals; it was even recently found in a human case in India. In cattle, *T. evansi* induces chronic infection more than acute; however it affects cattle production, especially dairy cows, since it is responsible for abortion and loss of weight and reducing milk production. Its economical and medical impacts are thus far from negligible. The diagnosis of surra is complicated since the parasitological methods are of low sensitivity. Therefore, the study of this disease in Thailand has been limited. The aim of this study was to determine the distribution of *T. evansi* infection in dairy cattle, to compare parasitological, serological and molecular technique for the diagnosis of *T. evansi* infection, and to measure the prevalence of *T. evansi* infection among dairy cattle in Thailand. Of 1,979 dairy cows tested by ELISA-*T. evansi*, 163 (8.2%) were seropositive, and 4/595 (0.7%) were positive by microscopic examination. By PCR examination, 55 out of 119 seropositive were positive; however all the 107 seronegative were still negative. Chaing Mai province located in the Northern region had the highest prevalence (P=21.3%) with 75% (6/8) of the farms infected. The statistic analysis indicated that regions, provinces and districts were associated with *T. evansi* infection ($p < 0.05$). Chi-squared test showed significant differences between age groups (p=0.02, $\chi 2$=7.71, df=2), and the seroprevalence was higher in older animals. These results may provide useful knowledge for establishing the prevention and control program for *T. evansi* infection of dairy cattle in Thailand.

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Student's signature Thesis Advisor's signature

ACKNOWLEDGEMENTS

For the successful completion of this work, I am deeply indebted to my committee members,

To Assoc. Prof. Dr. Sathaporn Jittapalapong, my advisor, for advice, encouragement, valuable suggestion for completely writing of thesis and his patience to me all the time.

To Assist. Prof. Dr. Sirichai Wongnakpet, my co-advisor, for every valuable suggestion, kindness and generous facilities. My research would not have been accomplished without the devoted and dedicated help from him.

To Adjunct Prof. Dr. Marc Desquesnes, my co-advisor, who expert for trypanosomes, for valuable guidance, helpful and his kindness.

I would like to thank the staff at Department of Parasitology, Faculty of Veterinary medicine, Kasetsart University and laboratory colleagues whom gave me for the generous help in everything and kindness suggestion. I do thank to my graduated friends from Veterinary Parasitology, Faculty of Veterinary Medicine, Kasetsart University, for their friendship, help, and cheerfulness.

This study was supported by funding from Graduate School.

Finally, I sincerely thank my parent who always gave me the chance to do everything, wish well, support and encouragement.

Ketsarin Kamyingkird

October 2009

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LIST OF ABBREVIATIONS

× *g*	=	Acceleration gravity
°C	=	Degree(s) Celsius
µg	=	Microgram(s)
µl	=	Microliter(s)
µm	=	Micrometer(s)
Ab	=	Antibody
Ag	=	Antigen
bb	=	Basal body
bp	=	Base pair(s)
CSF	=	Cerebrospinal fluid
DNA	=	Deoxyribonucleic acid
dNTP	=	Deoxynucleotide triphosphate
DW	=	Distilled water
EDTA	=	Ethylenediamine tetraacetic acid
et al	=	*et alli*
EtBr	=	Ethidium Bromide
fg	=	Free flagellum
g	=	Gram(s)
hrs	=	Hours

LIST OF ABBREVIATIONS (Continued)

IPTG	=	Isopropyl-1-thio-β-D-galactopyranoside
kt	=	Kinetoplast
L (l)	=	Liter(s)
LB	=	Luria Bertani (broth)
M	=	Mole
ME	=	Microscopic examination
mg	=	Milligram(s)
min	=	Minute(s)
ml	=	Milliliter(s)
mM	=	Micrometer(s)
ng	=	Nanogram(s)
nm	=	Nanometer(s)
Nu	=	Nuclease
PCR	=	Polymerase chain reaction
pH	=	Negative logarithm of hydrogen ion activity
rpm	=	Round(s) per minute
T. evansi	=	*Trypanosoma evansi*
T	=	Temperature
U/µl	=	Unit(s) per microliter
U/g	=	Unit(s) per gram
um	=	Undulating membrane

LIST OF ABBREVIATIONS (Continued)

UV	=	Ultraviolet
V	=	Volts
v/v	=	Volume by volume
w/v	=	Weight by volume
w/w	=	Weight by weight

EPIDEMIOLOGY OF *TRYPANOSOMA EVANSI* INFECTION OF DAIRY CATTLE IN THAILAND

INTRODUCTION

Trypanosomes are unicellular organism (Phylum Protozoa) belong to the order *Kinetoplastida*, the family *Trypanosomatidae*, the genus *Trypanosoma*. They are tissue blood parasites, are known as haemoflagellates. The subgenus *Trypanozoon*, the *brucei* group, comprises five members and one of them is *Trypanosoma evansi* causing 'surra'. Surra has spread in various geographical areas, and widely distributed amongst the domesticated and wild animals and causes the disease in horses, donkeys, mules , camels , Indian elephants , cattle , buffaloes , sheep , goats , dogs , cats, pigs, tapirs, deer, tiger, capybara, foxes, jackals, hyenas, mongoose (Gill, 1977) and bears (Muhammad *et al.*, 2007). Recently *T. evansi* was also isolated from humans in India (Joshi *et al.*, 2005).

T. evansi is normally transmitted by mechanical transmission involving bloodsucking Diptera, particularly members of the *Tabanidae* and *Stomoxys* (Lorne, 1986). Additionally, hematophagous bat might play a role as a natural host and vector of *T. evansi* in Latin America (Heitor *et al.*, 2005). This infection displays a few clinical signs such as fever, anaemia, weight loss, oedema, lymphadenomegaly, conjunctivitis, loss of appetite and sudden death (Laha and Sasmal, 2007). The most important symptom of the infection in cattle is abortion, which occurs suddenly without any clinical signs in the late stage of pregnancy (Loehr, 1986; Lun *et al*., 1993; Rowlands *et al*., 1995; Okech *et al*., 1996). Once cattle abort, the economic loss of the farmer would be incalculable since it happens in the late pregnancy and has an impact on a small-scale farm (Kashiwasaki *et al*., 1998). In Northeast Thailand, subclinical trypanosomosis caused low milk yield of newly introduced Holstein Friesian dairy cattle especially for the first year. (Pholpark *et al*., 1999).

Currently, surra is on the list of notifiable diseases (list B disease) of the OIE (World Organization for Animal Health) (Sarah *et al.*, 2006). *T. evansi* has the wide geographical distribution in South America, Africa and Asia particularly in Southeast Asia (Luckins, 1988), where it is an important constraint on the productivity of small farm holders in livestock (Reid, 2002). In Thailand, Trypanosomiasis has an effect on livestock productivity and herd health. Kashiwasaki *et al.* (1998) reported 80% of cattle in Loei province were seropositive for trypanosome by Ag-ELISA.

The diagnosis of *T. evansi* can be achieved by different methods. Athough, there are an application of various tests, but the problem remain exists (Davison *et al.*, 1999), such as the sensitivity of parasitological technique is poor because of the paucity and cyclical fluctuation of the parasitaemia. Serological diagnosis is hampered by the inability to distinguish a current infection from past exposure and the lack of specificity. However, Polymerase chain reaction (PCR) has proved to be a very specific and sensitive tool, increasing the sensitivity of trypanosome detection, with great impact on the epidemiological studies (Katakura *et al*., 1997; Davila *et al*., 2003; Heitor *et al*., 2005).

The decision to treat an infected animal is usually based on the presence and severity of clinical signs of disease, while most infected animals show subclinical signs. Chronically infected animals may survive for up to 3–4 years, causing heavy production losses such as lower milk and meat yields, abortions, premature births and inability to feed the young (Ngaira *et al.*, 2003). Diminazene, suramin, and quinapyramine have been used to treat animals with *T. evansi* infections, but some of them are ineffective. Laha and Sasmal (2007) found reinfection of *T. evansi* after treatment with quinapyramine. Therefore, to confirm *T. evansi* infection is beneficial for treatment and control. However, limited numbers of studies on *T. evansi* infection in dairy cattle in Thailand have been published. The aim of this study is to study the distribution of *T. evansi* infections in dairy cattle, to compare three techniques such as parasitological, serological and molecular diagnosis of *T. evansi* infection and to measure the prevalence of *T. evansi* infection among dairy cattle in Thailand.

OBJECTIVES

1. To study epidemiology of *T. evansi* infection of dairy cattle in Thailand.

2. To compare microscopic, serological and molecular technique in the detection of *T. evansi* infection in dairy cattle in Thailand

3. To evaluate the practical technique for diagnosis of *T. evansi* infection.

LITERATURE REVIEW

1. Overview of *Trypanosoma evansi*

1.1 History of *Trypanosoma evansi*

Trypanosomes were ranked high in medical and veterinary importance amongst parasites of humans and animals. Diseases caused by this pathogen had devastating effect on human life and livestock. Shortt (1951) comments that the infection of animals by trypanosomes as "more important than the human trypanosomosis when the whole picture was looked in perspective". The first pathogenic trypanosome was recognized by Evans (1880) from the blood of Indian horse and camel suffering from surra, then he sent the organism to Sureon-Major Lewis to identify in the name as *Herpetomonas lewisi* by Kent in 1880 and renamed as *Trypanosoma lewisi* by Laverane and Mesnil, 1901. Steel (1885) discovered again these parasites in the blood of mules in Burma and regarded as a spirochaete. Crookshank (1886) described the major characteristics, of trypanosomes such as undulating membrane and flagellum. Balbiani (1888) adopted the old generic name of *Trypanosoma*. Carter (1888) and Lingard (1893, 1894, 1895a, 1896a, 1899) confirmed the finding of Evans and Steel. In 1964, Hoare revised the classification of trypanosomes and according to the revised scheme, the causative organism of surra was known as *Trypanosoma* 'Trypanozoon' *evansi* (Steel 1885). *Trypanosoma evansi* is widely distributed and infected many animal such as camels, horses, donkey, mules, cattle, buffaloes, elephants, pigs, dogs, cats, tapirs, deer, goats, sheep, tiger and mongoose (Gill, 1977).

1.2 The etiological agent of surra

Evans (1880) examined and observed the 'eel-like' body , the blunted posterior end , the 'long slender lash' or free flagellum , the loop of the undulating membrane , the 'wonderfully active' movement , 'its visible length ordinarily appear on average to be about three or four times the diameter of a white corpuscle.

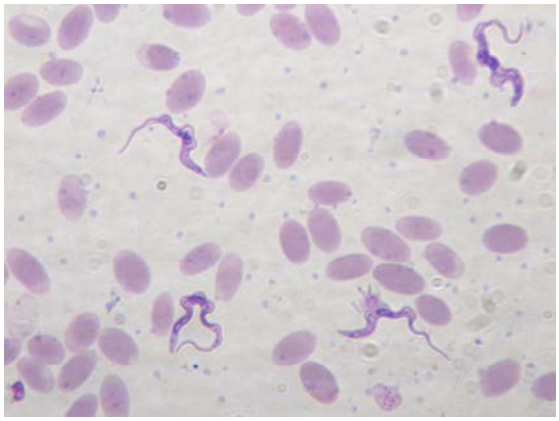


Figure 1 *Trypanosoma evansi* in thin blood smear stain with Modified Wright-Giemsa

Source : M. Desquesnes, 2009

1.2.1 Morphological Forms

Various morphological forms of kinetoplastids are observed. These forms are associated with different life cycle stages in host species. The different forms are principally distinguished by the position of the kinetoplastid in relation to the nucleus and the presence or absence of an undulating membrane. The four major morphological forms found in kinetoplastids which cause disease are: (1) Trypomastigote; the kinetoplast (kt) is located at the posterior end of the parasite. The flagellum emerges from the posterior end and folds back along the parasite's body; this attachment of the flagellum to the body forms an undulating membrane (um) that spans the entire length of the parasite and the free flagellum emerges from

the anterior end. This is considered the anterior end since the flagellum pulls the organism and the end with the free flagellum is the front in reference to the direction of movement. The undulating membrane functions like a fin and increases the motility of the organism. (2) Epimastigote**:** The kinetoplast (kt) is more centrally located, usually just anterior to nucleus (Nu). The flagellum (fg) emerges from the middle of the parasite and forms a shorter undulating membrane (um) than of trypomastigotes. Epimastigotes are noticeably less motile than trypomastigotes. (3) Promastigote: The kinetoplast (kt) is towards the anterior end and a free flagellum (fg) with no undulating membrane emerges. The end that the free flagellum emerges from in all three motile forms is designated as the anterior end because they move in that direction. (4) Amastigote: The parasite is more spherical in shape and has no free flagellum. A basal body (bb) and the base of the flagellum is still present. The kinetoplast (kt) is usually detectable as a darkly staining body near the nucleus (Nu). This form is a non-motile intracellular stage (Vickerman 1985).

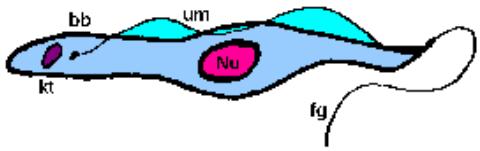
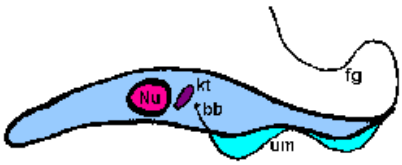
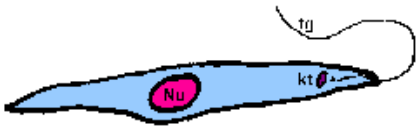
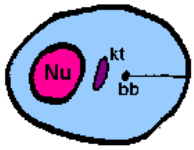
Trypomastigote	
Epimastigote	
Promastigote	
Amastigote	

Figure 2 Morphology of Trypanosome stages

Source: Vickerman (1985)

For *T. evansi*, unlike other trypanosomes, procyclic or developmental stages have not been observed in any vector (figure 3). In contrast, *T. evansi* reproduces only by binary fission in the mammalian host (Sarah *et al*., 2006).

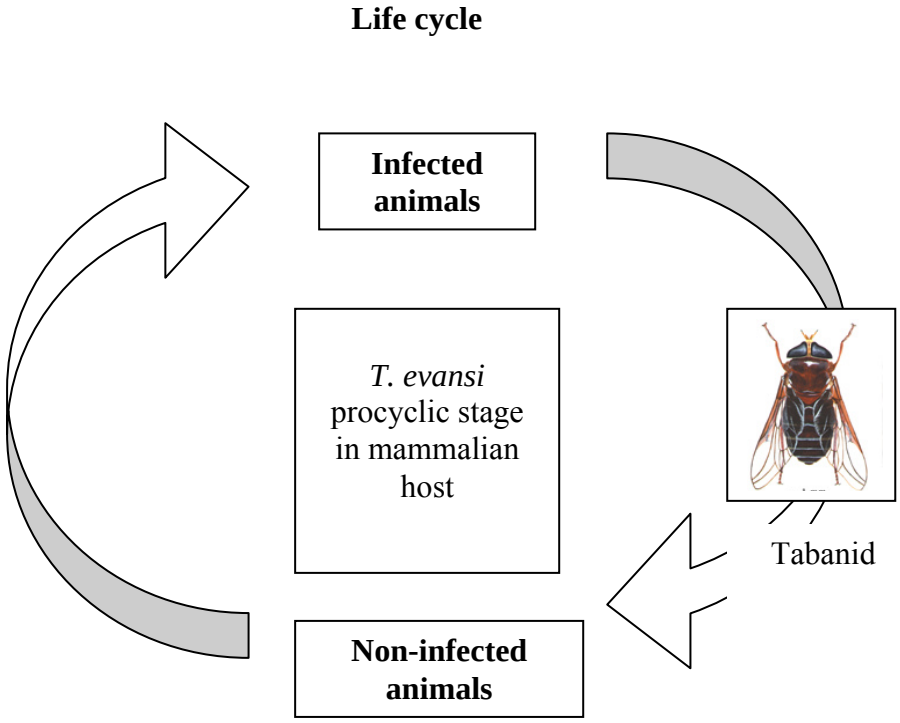


Figure 3 Life cycle of *Trypanosoma evansi*

1.3 Taxonomy of *T. evansi*

Trypanosomes are unicellular organism (Phylum Protozoa) belonging to the order Kinetoplastida, the family Trypanosomatidae, the genus *Trypanosoma* (see in Figure 4). They are tissue blood parasites (haemoparasites), which in the vertebrate host occur in the blood and tissue fluid and within that groups are known as haemoflagellates, as they progress activity by the movement of flagellum in the blood. The subgenus *Trypanozoon*, the brucei group, comprises five members: *T.brucei brucei*, *T. brucei gambiense*, *T. brucei rhodesisnse*, *T. evansi* and *T. equiperdum*, which exactly similar in morphology, but only *T. brucei gambiense* and *T. brucei*

rhodesiense are the cause of human sleeping sickness, *T. brucei brucei* is not infective to humans. *T. evansi* causes the disease, often called surra in Asia and by a variety of names elsewhere, such as *el debab* (northern Africa), *mal de caderàs* (Venezuala), *murrina* (central America), has spread as well as in tropical area (Gill, 1977).

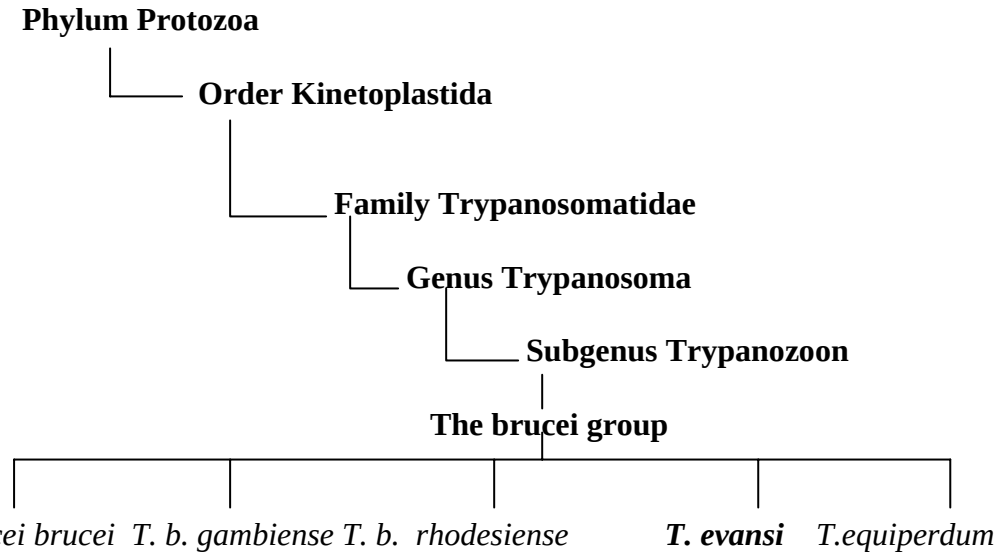


Figure 4 Taxonomy of *Trypanosoma evansi*

1.4 Host range of *T. evansi*

T. evansi is widely distributed amongst the domesticated and wild animals and it is the causative agent of diseases in horses, donkeys, mules, camels, the Indian elephant, cattle, buffaloes, sheep, goats, dogs, cats, pigs, tapirs, deer, tiger, capybara, foxes, jackals, hyenas and mongoose (Gill, 1977). Horses are most severely affected followed by mules and donkeys. The disease may be serious in draft Indian elephants (*Elephas maximus*). Cattle and buffaloes are frequently found with natural infections withnsubclinical signs. However, there are reports of fatal outbreak in some areas, particularly in the Far East (Lorne and Stephen, 1986). Recently *T. evansi* was isolated for the first time from a case of human trypanosomosis in India (Joshi *et al.*, 2005).

1.5 Transmission of Surra

T. evansi is normally transmitted from infected animals to naive animals by mechanical transmission involving bloodsucking Diptera, particularly members of the Tabanidae. It has lost its ability to undergo the developmental cycle in tsetse flies. Evans (1880) reported and concluded that *Tabanus* is the most potential vector of the transmission of surra in India. *Haematopota* and *Stomoxys* are also involved with the transmission of surra. The relative ability of different flies to act as vectors may be offset by the population of flies at any time. Rogers (1901) in classical investigations at Muktesar, India, experimented with the transmission of surra by horse-flies. Such flies fed on a rat failed to produce infection. However, there were no evidences of developmental stages of the parasite in these flies (Lorne and Stephen, 1986). Gill (1977) mentioned that 29 successful experimental transmissions have been reported in *Tabanus*, while several ones were obtained with *Haematopota* spp. Several demonstrations in experimental conditions were also reported for stomoxes by Gill (1977) with *Stomoxys calcitrans* and by others species as well as *Haematobosca squalida* (Mihok *et al.* 1995).

Mosquitoes and sand flies (Phlebotomus) although plentiful and frequently found feeding on animals show no evidence of ability to carry the disease mechanically. Similarly, lice, ticks or Lyperosia do not seem to play any role as vectors of T. evansi.

In Central and South America *T. evansi* synonyms; *T. hipicum* and *T. equinum* is usually transmitted by Tabanidae as mechanical vectors; however, the opportunity of transmission is considerably enhanced by the presence of a mammalian vector which relies on blood for its survival sush as the vampire bat (*Desmodus rotundus)*. This bat is widely distributed from northern Mexico to southern Argentina Morover, being an important transmitter of *T. evansi* to equines, this bat is also a dangerous host and vector of the virus of rabies. Hoare (1965) and Dunn (1932) had described the role played by the vampire bat in the transmission of trypanosomosis.

Hematophagous bat may play the role of a natural reservoir maintaining the multiplicative cycle of *T. evansi* in their blood and tissues (Heitor *et al*., 2005).

In the case of mechanical transmission by biting flies the trypanosome is passed from infected to clean host by infected blood in, or on, the mouth parts as the fly moves from one animal to another between interrupted blood meals. The parasites survive for only a short period of time on the contaminated proboscis. The vampire bat is a nocturnal feeder and gently raps the skin of its victim with it razor-sharp incisors until blood oozes from the wound. Anticoagulant in the bat's saliva prevents the blood from clotting, and the bat laps up the blood with its tongue, feeding may last up to half an hour and equal 50 ml. of blood. Animals, particularly those with a light hair coat, that have been attacked by a vampire bat are readily recognized by the dark of streak of dried blood trailing down from the site of the bite. The digestive tract of the vampire bat is adapted to the rapid digestion of blood which it is only source of sustenance and appear to allow the ready passage of trypanosomes both into the circulation of the bat which become infected and develops a parasitaemia, and the movement of the parasites from the bat's blood into the saliva at a subsequent feed. The parasite thus multiplies in the bat host, but does not undergo a cycle of development as it would in a true intermediate host. Bats may succumb to the infection or survive and recover (Gill, 1977).

1.6 Clinical signs of surra in domestic animals

This infection display typical signs in affected animals such as fever, anaemia, weight loss, oedema, lymphadenomegaly, conjunctivitis, loss of appetite and sudden death (Laha and Sasmal, 2007). The disease may be acute, or sometimes more chronic in horses. With the onset of fever and parasitaemia, the animal look dejected, its locomotory action is sluggishs, it may stumble at the fore legs and drag the hind legs. Ocular and nasal discharge is variable. Thirst may be abnormal, sometimes being very great. Appetite is capricious but some portion of the ration is usually consumed except when pyrexia is very high. The hair coat lacks luster and has a

staring appearance; perspiration was never seen. Invasion of the blood stream by trypanosomes is accompanied by pyrexia, the rectal temperature rising from 38.5 to 41.5 ˚C (101.3 to 107 ˚F). Parasitaemia may remain high for 4 to 6 days after which it declines and there is a remission of temperature. The animal appears to be much improved during the apyrexial and aparasitaemia periods, but is again obviously ill upon their return. Urticarial eruption of the skin appear early and irregularly. They are often localized, sometimes more general, and evanescent. The urine may be highly coloured and has been described as orange or 'dingy green' in colour. Faeces are generally normal, or are sometime said to be coated with mucus. In horses extensive subcutaneous oedema is often seen in infections caused by trypanosomes. Death often comes suddenly and quietly, or they may be some signs of delirium, but generally the animal is too weak to rise in the terminal stages. Lingard (1893) stated that Australian horses were more subject to the disease than Asian animals (Lorne, 1986). Laha and Sasmal (2007) observed that horses were suffering from long standing intermittent fever, edema of legs and ventral abdomen and weakness of hind legs. Splenomegaly, enlargement of lymph nodes and petechial haemorrhages in kidneys were found during post mortem examination of these died horses.

Surra in donkeys and mules is generally more chronic than in horses. Sign of disease and mortality attributed to the pathogenic effects of *T. evansi* vary from region to region and depending on the observer. Bennett (1933) considered the donkey to be a potentially dangerous reservoir of infection in the Sudan because it could harbour the parasite without showing any signs of the disease (Lorne, 1986).

Lingard (1899) said that surra will develop in cattle, but these animals are not especially susceptible and may recover spontaneously. The plains cattle of India, "exhibit a high degree of immunity, as a consequence, no doubt, of the ravages of the disease in former times, and to the present stock being the progeny of the survival of the fittest". Hill and plains cattle seldom or never succumb to uncomplicated surra from a spontaneous equine source. Mathias and Moungyai (1980) found a 24-day-old

calf to be infected by the parasite but not clinically ill (Loehr et al., 1985). Kashiwasaki *et al*. (1998) reported that cattle developed chronic infections rather than the initial stage of the infection. The most important symptom of the infection in cattle is abortion, which occurs suddenly without any clinical signs in the late stage of pregnancy (Loehr *et al*., 1986; Lun *et al*., 1993; Rowlands *et al*., 1995; Okech *et al*., 1996). Once cattle abort, the economic loss of the farmer would be incalculable since it happens in late pregnancy and only a small number of cattle are maintained in a small-scale farm (Kashiwasaki *et al*., 1998). In water buffalo, Surra is a chronic disease characterized by weight loss and infertility (Luckins, 1988; Davison *et al*., 1999) including abortion.

Pigs can be infected experimentally. The incubation period is frequently prolonged, and parasitaemia is usually only detectable by subinoculation of blood into susceptible animals. *T. evansi* is not highly pathogenic for pigs (Lorne, 1986). Otherwise, outbreaks with abortion and death have been observed Thailand (Teeraprasert *et al.*, 1984a; Bajyana Songa *et al.*, 1987), Malaysia (Arunusalam *et al.*, 1995) and Indonesia (Kraneveld and Mansjoer, 1947)

There are few reports of natural infections with *T. evansi* in dogs (Singh *et al*., 2000). However, Ian *et al*. (2004) reported that dogs in South America and Asia developed an acute disease with ocular, vascular and neurologic signs.Three cases of dogs infected with *T. evansi* in Iran, the animals were pyretic (temp. 40-41˚C) but other vital signs were normal and showed corneal opacity (Figure 5) was obvious in two of these cases (Morteza *et al*., 2007).

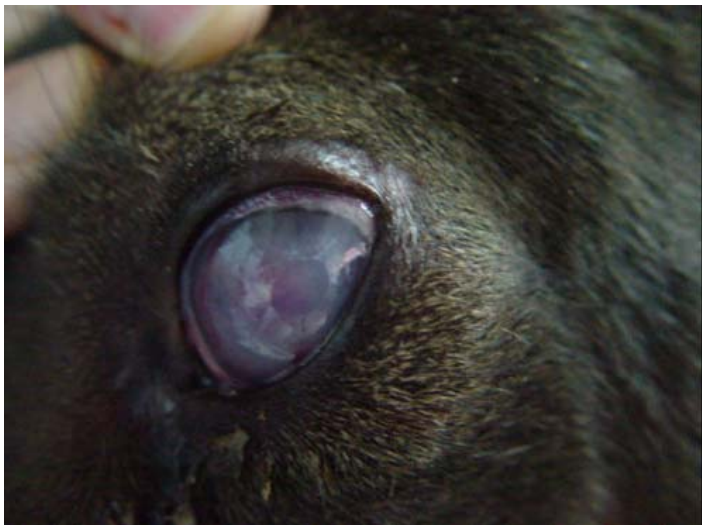


Figure 5 Corneal opacity in a dog infected with *T. evansi* in Iran

Source: Morteza *et al.*, (2007)

1.7 Pathogenesis of surra

The pathological effects caused by *T. evansi* in domestic animals have received little attention from invastigators. Nadim and Soliman (1967) reported decrease in erythrocyte counts, haemoglobin concentrations and lymphocyte and basophile counts, with increases in neutrophil, eosinophil and monocyte percentages, in natural infection in camels. Jatkar and Purohit (1971) found that pack cell volumes, red cell counts and haemoglobin values dropped, but there were increases in the total white blood cell counts, sedimentation rate and reticulocyte counts in the infected 20 camels with *T. evansi* compared blood values of 25 normal animals (Lorne, 1986). Three dogs cases reported in Iran showed low pack cell volume, serum analysis showed hyperproteinemia, however the values of alanine aminotransferase, aspartate aminotransferase and total billirubin were normal (Morteza *et al*., 2007).

Several factors are responsible for causing anaemia due to *T. evansi* infection, the production of haemolysin by trypanosomes resulting into haemolysis of RBCs,

extravascular destruction of RBCs, the erythrophagocytosis, immune mediated, depression of erythropoiesis and non-specific factors, which increase red cell fragility, may be responsible for anaemia (Laha and Sasmal,2007).

2. Diagnosis of *T. evansi*

The diagnosis of *T. evansi* can be achieved in different ways. Clinical diagnosis of trypanosomosis in mammalian hosts is difficult because the symptoms are evocative of the full range of haemoparasites or even other livestock disease and are not species-specific; labolatory diagnostic procedures are therefore necessary to confirm clinical suspicions and conduct epidemiological surveys (Desquesnes, 2004). Traditional diagnosis relies on the demonstration of the parasite in the wet blood preparation, stained smears and/or mouse inoculation (Heitor *et al*., 2005). The serological technique such as card agglutination test (CAT), indirect Fluorescent antibody test (IFAT) as well as the enzyme-linked immunosorbent assay (ELISA) was also used for the parasite detection. However, these tests do not distinguish between current infections and residual antibody from previous vaccinated and infected. (Chansiri *et al*., 2002). Despite the application of a variety of diagnostic tests, the diagnosis of *T. evansi* infection remains problematic and commonly used tests have important limitations (Davison *et al*., 1999).

2.1 Parasitological diagnosis

Trypanosomes detection can be conducted on blood, lymphnode, cerebrospinal fluid, general secretion, organ smear, etc. depending on the preparation of sample and practitioners. Direct microscopic examination, which is looking for the parasites on a fresh or a fixed sample most often blood sample. Direct examination of a fresh preparation necessary to used phase contrast microscope, this can identified the morphological and motility criteria of parasites, even this is low sensitivity. The definitive diagnosis is blood or lymph node smear, most often is blood sample, which collected preferably with anti-coagulant, is used to prepare a methanol fixed, Giemsa-

stained smear, observed under the microscope at 1000x magnification, it can be used to identified the subgenus of trypanosomes by morphological and morphometric features. Smear test are quite specific (at sub-genus level) but their sensitivity is very low, around 10^4-10^5 parasites/ml. Commonly is used for diagnosing human and animal trypanosomes, which is the examination of thick drop partially spread over the strip using another strip. The strip is quickly dried and then immersed in distilled water to lyse the red blood cells and eliminate the haemoglobin, then dried and stained for 30 minutes using 4% Giemsa in PBS.

2.1.1 Direct microscopic examination

This is the direct microscopic observation of the parasites on a fresh or fixed biological material, most often blood, soon after collection (2-4 hrs.).

2.1.1.1 Direct examination of a fresh preparation

This technique is to looking for the moving of the parasites in the biological material such as genital secretion, CSF, lymp node fluids or fresh blood by phase contrast microscope. It can allow to identify the parasites on the basis of morphology and motility criteria.

2.1.1.2 Blood or lymp node smears

This is the definitive diagnosis. Material aspired by ponction from an enlarged prescapular lymp node or a blood sample, collected with anti-coagulant, is used to fixed by ethanol, stained with Giemsa. Giemsa stained smear that is observed under a microscope (x1000) can allow to identify the subgenus on the basis of morphological and morphometric features. These test therefore relatively specific but very low sensitivity, around 10^4-10^5 parasites /ml.

2.1.2 Microscopic examination following concentration

2.1.2.1 Haematocrit centrifuge technique (HCT,Woo)

This technique is centrifugation of heparinized blood for 5 minutes at 13,000 rpm in a haematocrit tube, The tube is placed in the slot of a counting slide (capillary holder) and observed with a long focal lens. The capillary tube is partially rotated so as to examine the inside of the tube and thoroughly examined the buffy coat. This technique is quick and inexpensive and additionally shows when the animal is anaemic (packed cell volume).

2.1.2.2 Buffy coat method (BCM, Murray)

The BCM is another variation of HCT method in which the capillary tube is cut so as to extract the biological material located at the white blood cell/plasma junction. The fresh specimen is placed between a slide and cover slip, examined under the dark ground microscope. This method is can be applied to fixed and stain the material, but does not allow to examine the morphology of the parasite because the high-speed centrifugation alerates the morphology.

2.1.2.3 Quantitative buffy coat method (QBC)

This is an HCT that uses a capillary tube coated with acridine orange and potassium oxalate to make the parasites fluoresce, the tube is fitted with a float on a level with the buffy coat that flatten the parasited against the tube wall at the end of the centrifuge phase. As a result of these additions, it is easier to detect the parasites under a microscope. Its relevance for diagnosis of sleeping sickness in human is still under investigation. And the cost of the tube is expensive, so it’s not contemplated for veterinary use.

2.1.2.4 DEAE-Cellulose column filtration

This technique relies on the electrical properties of DEAE- cellulose at a given pH whereby blood cells are bound but not trypanosomes. In veterinary practice, this method is used more for isolating parasites on large capacity columns than for diagnostic purposes. In human, mincolumns have been developed for individual diagnosis, but they are still too expensive for veterinary use.

2.1.2.5 Silicone centrifugation system

It consists in depositing 1 ml of blood on a silicone mixture with a density of 1.075 and centrifuging it at 150 g for 5 minutes (Nessiem, 1994). The red blood cells sediment at the bottom of the tube whereupon the parasites can be retrieved from the supernatant for direct microscopic examination. Due to their cost, these techniques are more commonly used for isolating parasites than for diagnostic purposes.

2.1.3 Parasite culture

2.1.3.1 *In vitro*

For *T. evansi*, Baltz *et al.* have developed culture media that yield variable results depending on the strain. In these media, *T.equiperdum* produces blood stream forms of the parasite. Hirumi *et al.* have described a serum-free medium for the blood stream forms of *T. evansi* that is effective in maintaining the infectivity of the parasites for mice.

2.1.3.2 *In vivo*

The most widely used technique is intraperitoneal innoculation of infected biological material into mice, mainly the NMRI, Balb/C, CF1 and C3H strain. This is a method of choice for a sensitive diagnosis of *T. evansi*

infections since most parasite strains are highly virulent in mice and develop within three to five days. For excepting, in the case of *T. evansi*, *in vivo*-culture is not a widely used diagnostic tool (time-consuming, costly, uses live animals) although it is useful for isolating strains and large-scale production of parasites for antigen preparation (for ELISA).

2.1.4 Specificity and sensitivity

Specificity: trypanosome subgenera are identified on the basis of the morphological morphometric criteria proposed by Hoare, by microscopic examination of stained blood or lymp-node smear. Several parasites of characteristic shape and size must be observed which is possible only when the parasitemia is greater than 10^5 parasites/ ml. While these observations can be valuable in familiar epidemiological context, they are not always enough to identify the species. Even in ideal condition, it is impossible to distinguish *T. evansi* from *T.equiperdum* and *T.brucei*.

Sensitivity: In any case, the quickest and most economical technique is HCT and BCM, which is not require using live animal. For diagnosing *T. evansi* infection, the most sensitive technique is inoculating mice according to the survey of Monzon *et al.*

2.2 Serological diagnosis

Only two field diagnostic tests are available for trypanosomosis. One is the card agglutination test. A homogenous population of bloodstream trypomastigotes, with a defined predominant variable antigen type (VAT RoTAT 1.2), was used as the source of antigen. Consequently, the CATT sometimes gives false negative results, particularly in other trypanozoal infections, since such prevalent VATs do not occur in all species of trypanosomes. Moreover, the CATT also provides false positives, as it is based on agglutinating antibody detection. So, CATT alone cannot differentiate between infected individuals and some treated former patients

(Kashiwasaki *et al*., 2000). CATT/*T. evansi* is a quick and easy test which can be performed under field conditions. Depending on the cut-off score, the observed test sensitivity in the animal population described here varied from 86 to 100%, whereas test specificity varied from 96 to 98%. Aiming at maximal test sensitivity with a minor loss (2%) of test specificity, a cut-off score of is indicated for screening purposes (Gutierrez *et al*., 2000). Another test is a latex agglutination test for the detection of circulating trypanosomal antigens utilizing monoclonal antibodies. Suratex is designed for diagnosis of *T. evansi* infections. These products are claimed to be specific and sensitive, but the effectiveness of the tests has not yet been sufficiently demonstrated.

Probably, ELISA is one of the best serological methods to detect *T. evansi* because of the ability to measure specific antibodies and antigens in serum samples. Two types of ELISA methods are being used to detect infected animals which are antigen-ELISA (Ag-ELISA) and antibody-ELISA (Ab-ELISA) and both are characterised by a high sensitivity in camels and others species (Molinaa *et al*., 2000).

The information revealed by application of Ag-ELISA in conjunction with BCT could be utilized in planning and monitoring control programmes against camel trypanosomosis (Elmin *et al.*, 1998). Although parasitological procedures, such as direct microscopy and the haematocrit centrifuge technique (HCT) can be implemented at local laboratories, and an antigen-detection ELISA (Ag-ELISA) based on a combination of monoclonal (McAbs) and polyclonal antibodies (PcAbs) has already been introduced to regional diagnostic centres, detection of the parasite on a regular basis is hardly feasible because of a shortage of skilled personnel. (Kashiwasaki *et al*., 2000).

2.2.1 Antibody serology diagnosis

Although antibody detection cannot establish whether the

infection is active or took place in the past, it is extremely valuable as an epidemiological tool to assess the prevalence of infection.

2.2.1.1 Immunoglobulin M screening

2.2.1.1.1 Agglutination test

Micro agglutinations can be examined directly or under the microscope. This is the reaction between the suspected serum and a suspension of lysed parasites (by sonication) in a capillary tube.

2.2.1.1.2 CATT test

This is a variant of the agglutination test that uses whole, strained parasites (freeze-dried). A CATT test for *T. evansi* has been developed from Indonesian clone with the variable antigen type (VAT) that has been name RoTat 1.2, common to many *T. evansi* strains (isoVAT).

2.2.1.1.3 Indirect haemagglutination (IHA)

This method has been developed for *T. evansi* by Gill. Tanned red blood cell are sensitized with the trypanosome's soluble antigens and placed on micro-plates with successive dilutions of suspect sera (from 1/20 to 1/40,000). Agglutination is observed visually and the endpoint is set to the highest dilution that agglutinates. Controversal, this method still give false positive and impractical and also result in cross-reaction between species whereby *T. vivax* infections are detected using *T. evansi* antigen.

2.2.1.1.4 Enzyme linked immunosorbent assay (ELISA) IgM

In establishment of Desquesnes (2004), IgM concentrations observed during experimental infections are either stable or not. The value of the method has therefore yet to be established.

2.2.1.2 Indirect immunofluorescenes assay (IFA)

IFA was very widely used particularly for epidemiological surveys and has only been replaced by indirect ELISA technique (IgG detection) from the 1980s.

2.2.1.3 Immunoglobulin G detection by ELISA

The ELISA method described by Engval and Perlmann has been adapted for diagnosing many infectious diseases including *T. cruzi* induced trypanosomosis in humans and *T. evansi* , *T. equiperdum* , *T. vivax* , *T. congolense* trypanosomeses and others in animals.

2.2.1.4 Specificity, sensitivity and predictive value of antibody detection

Specificity: To interpret an epidemiological survey one need to consider the interference between the various pathogenic trypanosomes that may be present in the area of investigationsince detection of immunoglobulins directed against trypanosomes is not species-specific.

Sensitivity: All antibody detection test are very sensitive, and ELISA most of all. The latency of immune response requires a two week post infection time lack for positive seroconversion. If the infection suspected to

be recent, it is advisible to perform and additional parasitologial test (HCT) or repeat the test two weeks later.

2.2.2 Antigen serology diagnosis

Antibody detection by ELISA is highly sensitive but, not species-specific. A positive reaction to antibody detection does not mean that the infection exist at the time the sample was taken; positivity can persist between one and four months depending on the techniques used. Ideally an antigen detection test that is both sensitive and specific would solve these problems and allow comprehensive, detailed investigation to be conduct on the epidemiology of trypanosomes. Unfortunately, as will become apparent, none of the available method has so far proved fully satisfactory. Hyperimmune sera can be used to detect parasite antigens in samples tested with the indirect ELISA technique, but standardization of the method is difficult because plates are sensitized using the tested samples. Antigen detection can be done by mean of the immunecapture-ELISA technique that uses poly or monoclonal antibodies; the advantage of the monoclonal antibody is that they can be synthesized on a large scale and are more reproducible than the former. In all cases the ELISA plates are sensitized with the antibodies; once non-specific sites are blocked, the sera to be tested are incubated, the plates are washed, the same antibody or antibodies, conjugated to an enzyme, are incubated and then visualized by means of an appropriate subtrate/ chromogen complex. From the investigation of Ag-ELISA, the result showed poor performance and reached similar conclusion, which led to discard immunocapture-ELISA, during an international meeting at Nairobi in 1996 (Desquesnes 2004). Although there is an investigation of Ag-ELISA by Kashiwasaki *et al.* in 1998, in Loei province, Thailand was showed 80% of the cattle were positive for trypanosome antigens with low titre, and in the rainy season was 52.9% with high antigen levels (Kashiwasaki *et al.*, 1998).

2.3 Molecular diagnosis

Molecular tests to differentiate trypanosomes of the genus Trypanosoma

are preferably used because morphological discrimination of trypanosomes is difficult (Jittapalapong *et al*., 2007). Detecting a specific DNA segment provides a species-specific diagnosis of active infection by trypanosomes. DNA amplification by polymerase chain reaction (PCR), is able to show up the present of DNA segments, or partially known, sequences of bases. When the PCR is applied to diagnosis purposes, it can reveal the presence of the DNA of parasite. It is comparable to antigen detection techniques as much as it shows up and indicates that the infection is active (Desquesnes 2004). PCR has proved to be a very specific and sensitive tool, increasing the sensitivity of trypanosome detection, with great impact on the epidemiological studies, mainly in areas where cryptic infections occur (Katakura *et al*., 1997; Davila *et al*., 2003; Heitor *et al*., 2005).

PCR allows the amplification of a specific DNA segment for which, at least, 18–30 bases of the extremity sequences are known. Following 30–40 PCR cycles, the DNA will theoretically have been amplified approximately 1 billion times, and the resulting PCR product can be visualised on an agarose or polyacrilamide gel, after staining with ethidium bromide and exposing it under ultraviolet light. The specific size of the PCR product is evaluated by simultaneous migration of molecular size markers and a positive control. A negative control is run together to evidence any DNA contamination. In some instances, primers can amplify more than one tandem repeat which gives several specific bands), however a sample is positive when, at least, the single product is observed. The actual sequence amplified can be analysed by sequencing, or checked by DNA probing, which may increase the sensitivity to detect as little as 1 fg of DNA. Generally, once the specificity of the primers has been established, the size of the PCR product is sufficiently characteristic for diagnostic purposes.

Therefore, the isolate-specific finger printing allows the identification of the origin of the isolates found in the sampled herds. However, the technique has some limitations; the intensities of the bands, which vary with the quality and quantity of the target DNA, are difficult to interpret, and some weak bands may not be visible

despite the fact that the sequence is present in the sample. RAPD primers are generally designed so that they do not react with host DNA. However, the amplification of host DNA remains possible and cannot be controlled. DNA from the target species should not be contaminated with DNA from other species (e.g. host DNA + other micro-organism DNA) when using this technique because the patterns of bands obtained would not be specific to the parasite actually under study. For these reasons, typing or isolate differentiation on field samples containing parasite and host DNA are of a limited reliability (Desquesnes and Dávila, 2002).

2.3.1 PCR applied to trypanosomal detection

The first developments of PCR for trypanosome identification were published by Moser *et al.* in 1989. Masiga *et al.* have described oligonucleotides that are specific for Trypanozoon (TBR1 and TBR2) and *T. vivax* (TVW1 and TVW2) by rely on highly repetitive messages of parasitic DNA, which enhances their sensitivity (satellite DNA). Diall, followed by Ijaz *et al.* have described oligonucleotides that recognize *T. evansi* but do not react with *T. brucei* spp. *T. evansi* cannot be differentiated from T.equiperdum because the primers described for the former react with the latter erratically, and no techniques so far are able to distinguish *T. evansi* from *T. equiperdum*. In addition, the DNA probes and enzymatic digestions do not establish a clear-cut separation between these two species (Desquesnes 2004). However, recently, a PCR based on the amplification of NADH dehydrogenase subunit 5 (nad5) was developed to distinguish *T. evansi* from *T. equiperdum* and *brucei* (Li *et al*, 2007).

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

chromosomes of the nuclear DNA contain satellite DNA which has been the most favoured target in the development of species-specific primers able to detect very small amounts of parasite DNA. Such primers were developed for the main pathogenic trypanosomes: *Trypanozoon*, *T. vivax*, *T. evansi*, *T. simiae*, *T. congolense* savannah, forest, and Kilifi types, *T. godfreyi* and *T. cruzi* (Desquesnes and Dávila, 2002).

T. evansi, do not present maxi-circles in their kDNA network (Masiga and Gibson, 1990). Kinetoplastid mini-circle DNA sequences have been a useful target for development of specific primers for detection of *T. evansi* and *T. cruzi*.

Internal transcribed spacers (ITSs) of ribosomal DNA are suitable targets for PCR-based trypanosome diagnosis which are now under development (Desquesnes *et al.,* 2001). The PCR assay amplified the region that includes the internal transcribed spacer 1 (IST-1) between the 18S and 5.8S ribosomal RNA genes (rDNA) for differential diagnosis. The advantage of assay is that the relatively polymorphic ITS-1 is flanked by highly conserved rDNA, allowing detection of divergent species followed by more specific identification through amplicon sequence analysis. This assay can also be used to distinguish salivarian (*T. evansi*) and stercorarian (*T. lewisi*) infections with 520 and 623 bp amplicons, respectively (Jittapalapong *et al*., 2007).

The most popular PCR-based tool for isolate differentiation studies has been the Random Amplified Polymorphic DNA–RAPD technique for animal trypanosomes, successful sub-specific differentiation has been reported for *T. vivax.* Similarly, arbitrary primers which differentiate isolates of *T. evansi* have been described and were shown to be useful in the study of parasite population dynamics during an outbreak of Surra in Thailand (Watanapokasin *et a*l., 1998).

2.4 Diagnosis in vectors

Detecting of trypanosomes in vectors is useful diagnosing for

epidemiological survey, it can be the indicator to show that, where the vectors are positive for trypanosomes, wild fauna or livestock can be infected by trypanosomes too. PCR can be used in all kind of vectors (biological, cyclical and mechanical) but with various epidemiological meaning. It can be diagnose by parastological techniques or PCR depending on the vector and diagnose materials, and may require accurate species identification due to the potential presence of other Trypanosomatidae in the vector.

Table 1 The sensitivity and specificity of the various methods of *T. evansi* detection

Technique	Sensitivity (Parasites/ml)	Specificity	Advantages	Disadvantages
Direct examination	10^4-10^5	Weak	Simple, Cheap	Requires new samples, Weak sensitivity
Thick blood smear	10^4	Weak	Simple, Cheap	Posponed, Weak sensitivity
Thin blood smear	10^5	Detectable the sub-genus	Simple, Cheap, Specific	Delayed, Weak sensitivity
Hematocrite (Woo)	10^2-10^3	Detectable the sub-genus	Fast, sensitivity, specificity	Requires new samples
Buffy coat method (Murray)	10^2-10^3	Detectable the sub-genus	Sensitivity,sensitivity	Requires new samples, take time
Mouse innoculation	10-10^3	Species or sub-genus	sensitivity	Hazard, costly, live animal; delayed
PCR	1-20	Species or sub-genus	sensitivity	Costly

3. Epidemiological studies on *T. evansi*

T. evansi has the widest geographical distribution among pathogenic trypanosomes, being present in many countries of South America, Africa and Asia and endemic in most country of South-East Asia (Luckins, 1988). In mid-eastern Sudan, the prevalence of *T. evansi* infection in camels, 5.4% and 31.3% base on parasitological examination and Ag-ELISA respectively were reported by Elmin *et al.* (1998). Laha and Sasmal (2007) reported a high percentage (12.74%) of *T. evansi* infection in horses as observed by examination of Giemsa stained blood smears. Trypanosomosis caused by *T. evansi* is a significant constraint to livestock productivity and health in major areas of Thailand (Kashiwasaki *et al.*, 1998).There are the enzootic to Thailand, which is considered the primary agent of trypanosomosis among domestic animals in Asia and India. Eighty percent of the cattle in Loei province were positive for trypanosome antigens by Ag-ELISA in rainfall season and dropped to 32.3% in dry season (Kashiwasaki *et al*., 1998). Loehr *et al*. (1985) reported parasitological and serological prevalence of *T. evansi* infection in buffaloes from north-east, Thailand during the rainy and dry season were 15.4%, 23.6% and 3.7%, 17.1% respectively. The results indicate that infections occur much more frequently in the rainy season when fly activity is high. While *T. evansi* infection in rodents habitate in 3 provinces, Thailand was 1.5% and 0.4% was mixed infection with *T. lewisi* (Jittapalapong *et al*., 2007).

In Thailand, *T. evansi* transmitted by tabanids has been endemic since 1942, lossed a lot of horses used in Thai forces for the second worldwar (Komutbut, 1950) Infected animals could died within 3-4 hrs or up to 3-4 mths after infection. The treatment in that period used treatment of Mukteswar that injection 10 % of Naganol intrathecal for three times, however it would be risk for inflammation and toxic to animals (Komutbut, 1996). In 1986 there were reported of surra outbreak in a pig farm in Supanburi province. The animal has high fever (103-107˚F), weakness, bored with food, and red rash around buttlock, breast and abdomen. Of 61 pregnants died,

45 were aborted, from the detection by microscopic examination found 90% of sampling pigs were positive for *T. evansi* and treated with 3.5 mg/kg showed the good efficiency of drug (Siriwan *et al*., 1986). This parasite caused the disease in the dogs from the reported of Panjit of Kasetsart animal hospital cases reported, and also were reported from livestock such as buffalo, beef cows, dairy cows, horses and pigs for many time and many areas (Table 10).

4. Treatment and control of *T.evansi* infection

Surra currently is on the list of notifiable diseases of the OIE (World Organization for Animal Health). Reduction of the global economic impact of this disease on animal health requires dependable diagnostic tests, efficacious treatment, and diligent control programs. Historically, three drugs have been used to treat animals with *T. evansi* infections. These drugs include diminazene, suramin, and quinapyramine. Cymelarsan is the newest drug to be introduced in the last decade. The choice of drug, dosage, and route of administration vary by species affected, local preference, and presence or absence trypanosome drug resistance (Brun *et al.,* 1998).In one study, diminazene cleared the parasitemia of horses following natural infection. However, parasitemia returned in many animals within 1 week of the first treatment with diminazene or within 24 hours of the second treatment with diminazene. This suggests that diminazene affords little protective effect against repeated *T. evansi* infection or the drug is ineffective in totally eliminating the initial *T. evansi* infection (Tuntasuvan *et al*., 2003). Relapses have also been observed in horses following treatment with quinapyramine sulfate (Monzon *et al*., 2003). In summary, there is a growing body of evidence that *T. evansi* is becoming resistant to currently available drugs and may restrict their use or retard their effectiveness in the future. However, *in vitro* assays may help predict the effectiveness of drug treatment to given isolates of *T. evansi* (Sarah *et al*., 2006).

The decision to treat an animal usually is based on the presence and severity of clinical signs of disease. Given the occasional low sensitivity of certain diagnostic tests, subclinical infections (carriers) may present a dilemma when attempting to control *T. evansi* infections in herd outbreaks (Luckins, 1999).

Other measures (besides trypanocidal drug administration) such as minimizing exposure to fly populations may help control surra. Stables with suitable netting can be used to exclude fly populations. Smudge fires may also be used to repel flies. Fortunately, *T. evansi* cannot survive very long outside of its host. Therefore, animal carcasses from surra fatalities pose little threat of disease spread by insects, however, it is still at risk for canivores.

Laha and Sasmal (2007) found reinfection of T. evansi after treatment with quinapyramine and chloride. They preferred to the described of Joshi and Singh (2000) that it has been attributed to survival of the trypanosomes in cerebrospinal fluid as the drug could not penetrate the blood brain barrier.

Since 1992, a new drug was developed specifically for the control of *T. evansi*, infections, with a product derived from melarsomine (Cymelarsan®). To date, melarsomine seems to be the best product to cure this animal infected, however, dose and efficacy should be evaluated in various host species since the chemical was only registered for control in camels (as well as control of filaria in dogs). It should be evaluated in horses, cattle, buffalo etc. In bovines, goats and pigs, some preliminary works have already shown its efficacy but at higher doses than in camels (Lun *et* al., 1991; Zweygarth *et al* 1992; Payne *et* al, 1994; Dia & Desquesnes, 2003)

MATERIALS AND METHODS

Study areas, sampling and data collection

This study was performed in four regions of Thailand, between March and May 2006. Sampling focused on the main province for dairy cattle production. Dairy farms were selected by local veterinary services (Department of Livestock Development) insuring that both small and large units would be represented (see in table 2).

Sampling was stratified geographically by the country's four regions, and sample size was based on a mean expected prevalence of 10%. With a risk of 5% and an accuracy of 3%, the design required collecting at least 384 samples per region.

Cattle and calves (> 5 days) were randomly selected from dairy farms, which were randomly selected by local veterinary services (Department of Livestock Development). The number of samples from each farm was based on the total number of animals on the farm according to the following rules:
- If total number was less than six, all animals were sampled;
- If total number was more than 5 and less than 21, 6–20 animals were sampled;
- If total number was greater than 20, 20–30 animals were sampled.

The blood (5-10 ml) was collected from tail vein, separated 1-3 ml. in EDTA tube for thin blood smear, 3-5 ml. in clot tube for ELISA/*T. evansi* and 1-3 ml. in Sodium citrate for PCR.

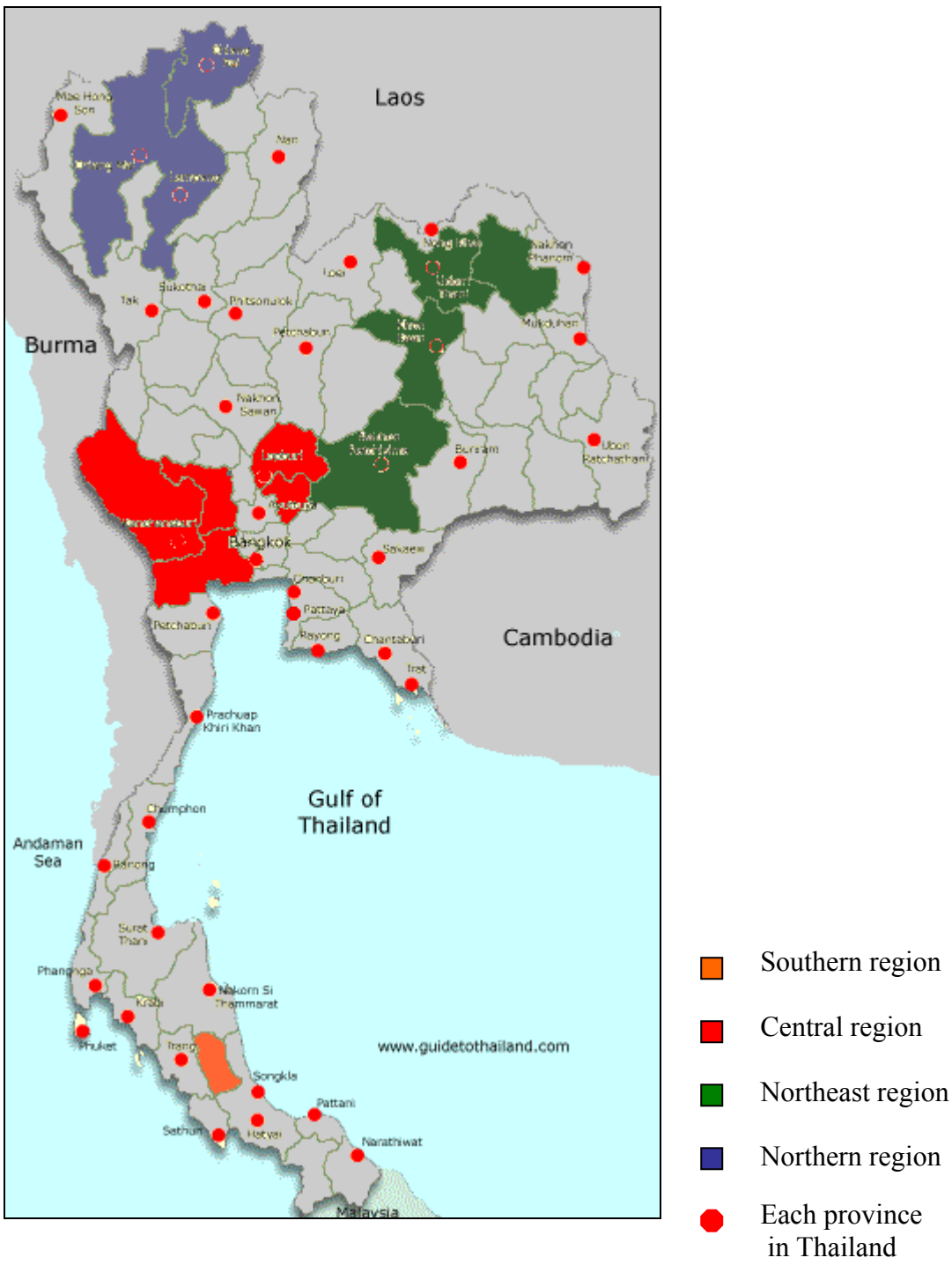
Data of cattle including sex (most of them are female), age, farm, province, region and abortion history of each farm were collected. All of the data information was investgated except abortion history due to the fact that most of the owner did not record this information. A questionnaire was designed to record this data.

Sex: Male or Female

Age: Young; 0-1year Adult; >1-5 Years Older; > 5 Years

Table 2 Number of animals and farms samples in each regions and provinces

Regions and Provinces	Number of animals/each region and province	Number of farms/each region and province
North-eastern	**460**	**58**
Udorn thani	121	13
Khon Khen	234	29
Sakon Nakhon	90	14
Nakorn Radcha Srima	15	2
Northern	**642**	**41**
Chiang Mai	150	8
Lum Pang	100	5
Chiang Rai	392	28
Southern	**187**	**14**
Pat ta lung	187	14
Central	**690**	**104**
Kanjanaburi	175	21
Radchaburi	112	20
Nakorn Patom	186	20
Saraburi	121	24
Lopburi	96	19
Total	**1979**	**217**



Southern region

Central region

Northeast region

Northern region

Each province
in Thailand

Figure 6 Map showing the thirteen provinces with sample collecting sites in four regions of Thailand

Diagnosis technique

1. The microscopic examination

By pipetting 3-5 µl of EDTA blood (maximum 3-6 hours after blood sampling), were dropped on the clean glass slide for thin blood smear. The slides were fixed in absolute methanol for 2 minutes, stained with Modified Wright-Giemsa for 5 minutes, destained with buffer for 10 minutes, washed the slide in running water, let dry and examined under the light microscope at 1000x magnification.

2. Serological examination (ELISA/*T. evansi*)

The ELISA procedure is derived from a previously described technique (Desquesnes *et al.* 2007). Briefly, Microtest 96-well Polysorp Nunc® immunoplates (Nunc, Roskilde, Denmark) were coated with 100 µl/well of *T. evansi* soluble antigen at 5 µg/ml protein concentration in carbonate buffer (pH 9.6) and incubated overnight at 4°C. The plates were rinsed once with phosphate buffer saline (PBS) and blocked with 150 µl/well of blocking buffer (PBS-7% skimmed milk) with permanent shaking (200 rpm) for 45 min at 37°C. The blocking buffer was discarded. Sera diluted 1:100 in blocking buffer were transferred in duplicate on the ELISA plate. After 30 min in a shaker-incubator at 37°C, 200 rpm, the plates were washed seven times with PBS 0.1% Tween 20® (Sigma,-Aldrich) (washing buffer, WB). Then 100 µl of peroxidase-conjugated anti-bovine IgG (A5295, Sigma-Aldrich), diluted 1:10,000 in blocking buffer, was added and the plates incubated for 30 min at 37°C with permanent shaking (200 rpm). After washing seven times with washing buffer, 100 µl of the complex substrate/chromogen 3,3´,5,5´-tetramethylbenzidine (TMB) (K blue® TMB substrate, Neogen Europe ltd, Scotland, UK) was added. The plates were shaken and then incubated in a dark room for 30 min. Optical density (OD) was measured at 630 nm in an ELISA reader (Dynex Technologies®, Virginia, USA).

The ELISAs were rerun for all samples in duplicate with three positive (PC) and three negative controls (NC) on each plate. The blank OD value was automatically deducted from each sample value. The results were expressed in relative percentage of positivity (RPP), as described previously (Desquesnes 2009), according to the following ratio:

$$\text{RPP of a sample} = \frac{\text{mean OD of the sample} - \text{mean OD of NC}}{\text{mean OD of PC} - \text{mean OD of NC}}$$

The cut-off value (COV) was determined based on the mean RPP of the batch of samplesfrom presumed noninfected farms:

COV (%) = mean RPPN (%) + (3 x standard deviation (%)).
COV (%) = 0% + (3x6%) = 18%

The sample status was positive when the RPP of a sample was greater than the 18%.

3. Molecular diagnosis

3.1 DNA extraction

In order to confirm the species identification and the presence of the parasite in blood samples, 119 seropositive samples (from two farms with seroprevalence rate > 60%) and 107 seronegative samples (from 14 farms considered non-infected) were submitted to DNA preparation and PCR analysis with specific primers for Trypanozoon (Masiga *et al.* 1992). DNA preparation was carried out with phenol-cloroform extraction by 100 µl of citrated blood samples that had been kept at −20°C.

3.2 PCR detection

PCR was analysis with specific primers for Trypanozoon (Masiga *et al.*1992). The amplifications were conducted in 10 µl reaction mixtures containing as final concentrations: 10 mM Tris–HCl, pH 8.3, 50 mM KCl, 1.5 mM MgCl2, 200 µM each of the four deoxynucleoside triphosphates (dNTPs), 0.83 µM of each primer,and 0.25 units of Taq DNA Polymerase (Taq DNA Polymerase, Invitrogen™). Samples were added under 1 µl volumes just before amplification. The amplifications were processed in a thermocycler Thermo™ under the following conditions: the initial denaturation step at 94°C was for 1 min, followed by 30 cycles of denaturation for 30 sec at 94°C, annealing for 1 min at 60°C and extension for 30 sec at 72°C. The terminal extension was allowed for 2 mins at 72°C and the samples were then maintained at 4°C until loading on a gel. Reaction mixture (11 µl) was loaded on a 2 % agarose gel (1st BASE™) in 100 ml of 90 mM Tris–HCl pH 8.3, 90 mM boric acid and 25 mM EDTA. Electrophoresis was processed for 1 h at 120 V. The gel was stained with ethidium bromide for 5 mins, destained with water for 15 mins and visualized under the ultraviolet transluminator. The sample was examined directly under UV lamp and in the photographs for substantial amounts of the specific product. When a product was visible at the expected molecular weight, 164 bp, the sample was considered positive.

Statistical analysis

Host individual factors including region, province, district, farm where the sample was collected and age group were determined for their significance in *T. evansi* infection using Chi-square test. Moreover, Fisher's exact test was used for confirmation when the expected frequencies were less than 5 samples. NCSS (Number Cruncher Statistical Systems, Kaysville, UT) program and WINKS SDA 6.0 program were used to analyze all data. A *P*-value of 0.05 was used to indicate statistical significance.

RESULTS

1. Microscopic examination (ME)

Due to the delay in the preparation of blood smear, only 595 of the sample was examined by this method and 4 blood samples are positives.

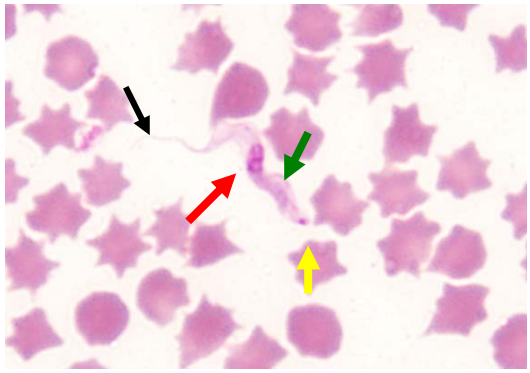


Figure 7 The long slender shape with free flagellum (black arrow) visible at the anterior end, undulating membrane (green arrow), nucleus (red arrow) and kinetoplast (yellow arrow) near the posterior end in the cattle blood smear (1000X)

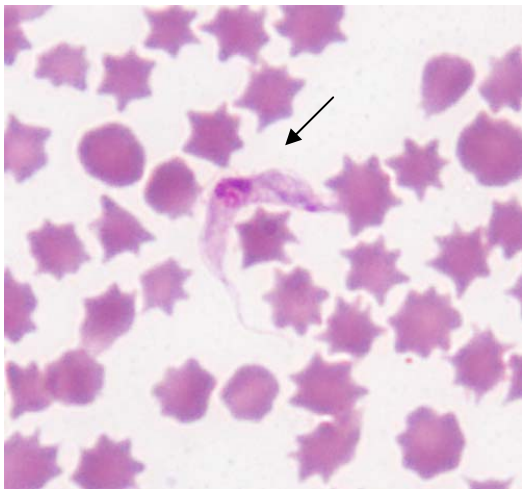


Figure 8 The Trypomastigote of *T. evansi* (arrows) in cattle blood smear (1000X)

2. Serological examination (ELISA/*T.evansi*)

A total of 1979 serum samples was collected, 163 (8.2%) were positive for *T. evansi* infection by ELISA test. Thirteen provinces such as Udon Thani, Khon Kaen, Sakon Nakhon, Nakhon Ratchasima, Chiang Mai, Chiang Rai, Lumpang, Phattalung, Kanchanaburi, Ratchaburi, Nakhon Pathom, Saraburi, and Lop Buri had 16.5%, 2.6%, 18.9%, 0.0%, 21.3%, 3.0%, 9.4%, 2.1%, 0.6%, 9.8%, 5.4%, 17.4 % and 1.0% were seropositive , respectively (Table 3). According to the region, in the north-eastern, northern, southern and central region had 43(9.3%), 72(11.2%), 4(2.1%) and 44(6.4%) were seropositive, repectively (Table 3). At the province level, individual prevalence ranged from 0.6-21.3%, and 4.8-75% of the farms were infected. There are variation of seropositive individual and farm according to the districts, the data show seropositive individual ranging from 0.6% up to 46.4% and seropositive farm range from 4.8% up to 100% (Table 4).

Table 3 The number and percentage of seropositive for *T. evansi* infection in each region and province of Thailand by individual and farm

Regions and Provinces	**Number of individual *T. evansi* seropositive (%)**	**Number of farm *T. evansi* seropositive (%)**
North-eastern	43/460 (9)	14/58 (24.1)
Udon Thani	20/121 (16.5)	3/13 (23.1)
Khon Kaen	6/234 (2.6)	3/29 (10.3)
Sakon Nakhon	17/90 (18.9)	8/14 (57.1)
Nakhon Ratchasima	0/15 (0)	0/2 (0)
Northern	72/642 (11.2)	19/41 (46.3)
Chiang Mai	32/150 (21.3)	6/8 (75)
Lumpang	3/100 (3)	2/5 (40)
Chiang Rai	37/392 (9.4)	11/28 (39.3)
Southern	4/187 (2.1)	3/14 (21.4)
Phattalung	4/187 (2.1)	3/14 (21.4)
Central	44/690 (6.4)	20/104 (19.2)
Kanchanaburi	1/175 (0.6)	1/21 (4.8)
Ratchaburi	11/112 (9.8)	5/20 (25)
Nakhon Pathom	10/186 (5.4)	6/20 (30)
Saraburi	21/121 (17.4)	7/24 (29.2)
Lop Buri	1/96 (1)	1/19 (5.3)
Total	163/1979 (8.2)	56/217 (25.8)

Table 4 The number and percentage of seropositive for *T. evansi* infection in each district and province of Thailand by individual and farm

Province	District	Number of seropositive animal (%)	Number of seropositive farm (%)
Udon Thani	Sri That	20/76 (26.3)	3/5 (60)
	Kud Jab	0/30 (0)	0/5 (0)
	Mueang	0/15 (0)	0/3 (0)
Khon Kaen	Mueang	1/111 (0.9)	1/12 (8.3)
	Nam Pong	0/50 (0)	0/7 (0)
	Ubonrat	2/48 (4.2)	1/6 (16.7)
	Kra Nuan	3/25 (12)	1/4 (25)
Sakon Nakhon	Mueang	17/90 (18.9)	8/14 (57.1)
Nakorn Ratchasima	Pak tong chai	0/15 (0)	0/2 (0)
Chiang Mai	Chai Pra Karn	32/150 (21.3)	6/8 (75)
Lumpang	Hang Chut	3/100 (3)	2/5 (40)
Chiang Rai	Mueang	0/80 (0)	0/4 (0)
	Mae lao	3/30 (10)	3/5 (60)
	Phan	1/65 (1.5)	1/4 (25)
	Terng	2/100 (2)	2/9 (22.2)
	Pa Ya Meng Rai	13/28 (46.4)	1/2 (50)
	Koon Tan	16/37 (43.2)	3/3 (100)
	Mae Sai	2/52 (3.8)	1/1 (100)
Phattalung	Maeung	4/187 (2.1)	3/14 (21.4)
Kanchanaburi	Ta Moang	1/56 (1.8)	1/10 (10)
	Ta ma ka	0/51 (0)	0/9 (0)
	Lao Kwan	0/68 (0)	0/2 (0)
Ratchaburi	Ban pong	1/45 (2.2)	1/8 (12.5)
	Potaram	9/42 (21.4)	3/7 (42.9)
	Chom bung	0/20 (0)	0/4 (0.0)
	Mueang	1/5 (20)	1/1 (100.0)
Nakhon Pathom	Kampang saen	9/151 (6)	5/13 (38.5)
	Mueang	1/35 (2.9)	1/7 (14.3)
Saraburi	Muak lek	21/121 (17.4)	7/24 (29.2)
Lop Buri	Pattananikom	1/96 (1)	1/19 (5.3)

3. Molecular diagnosis

According to the ELISA results, 119 seropositive and 107 seronegative animals (from presumed seronegative farms) were test by PCR with specific primer for Trypanozoon (TBR1 & 2) giving a PCR product of 164 bp (Figure 9). Fifty five samples from the seropositive group were confirmed positive by PCR, all the seronegative group were negative by PCR.

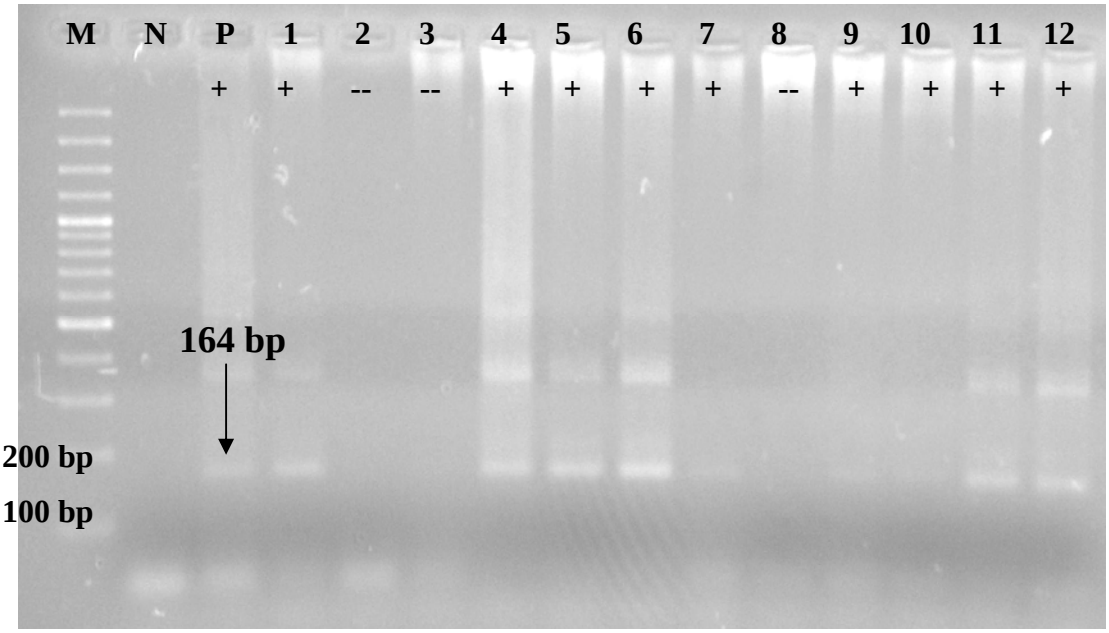


Figure 9 PCR amplification of *T. evansi* gene from dairy cows in Thailand **;** M ; molecular marker, N ; negative control (distilled water), P ; *T. evansi* positive control, lane 1-12 ; genomic DNA from cattle NO1, NO2, NO4, NO5, NO6, NO7, NO8, NO9, NO10, SN1, SN2, SN3 respectively.

4. Statictical analysis

The statistic analysis of *T. evansi* infection indicated that regions, provinces and districts were associated with *T. evansi* infection ($p < 0.05$) (Table 5). The highest and lowest seroprevalence individual of *T. evansi* infection is 11.2% and 6.4% in the northern and southern region, 21.3% and 0.6% in Chiangmai and Kanchanaburi, no individual infection in Nakhon Ratchasima. The highest and lowest seroprevalence farm of *T. evansi* infection is 46.3% and 19.2% in the northern and central region, 75% and 4.8% in Chiangmai and Kanchanaburi, no farm infection in Nakhon Ratchasima. Chi-squared test showed significant difference between age groups of dairy cattle and *T. evansi* infection (p=0.02, $\chi 2$=7.71, df=2) as shown in Table 5 & 6. The seroprevalence of each age 0-1, 1-5 and >5 years are 4.7%, 7.9% and 11.5% respectively. The geodistribution of *T. evansi* infection as show in Figure 10, the percentage and color indicated the seroprevalence in the area.

The prevalence of *T. evansi* infection was varied by technique such as ELISA (8.2%), microscopic examination (0.7%), and PCR (25.3% in a sub sample of seropositive) as show in Table 7. Test of agreement between two techniques such as ELISA and ME, ELISA and PCR were analyzed by kappa test data showed in table 8 and 9.

Table 5 Seroprevalence by ELISA-*T. evansi* in dairy cattle, Thailand by region, province, district and age group.

Parameter	ELISA	*p*-value
Region		0.0001
Northern	11.2% (72/642)	
Northeastern	9.3% (43/460)	
Central	6.4% (44/690)	
Southern	2.1% (4/187)	
Province		0.0000
Udon Thani	16.5% (20/121)	
Khon Kaen	2.6% (6/234)	
Sakon Nakhon	18.9% (17/90)	
Nakon Ratchasima	0.0% (0/15)	
Chiang Mai	21.3% (32/150)	
Lumpang	3.0% (3/100)	
Chiang Rai	9.4% (37/392)	
Phattalung	2.1% (4/187)	
Kanchanaburi	0.6% (1/175)	
Ratchaburi	9.8% (11/112)	
Nakon Pathom	5.4% (10/186)	
Saraburi	17.4% (21/121)	
Lopburi	1.0% (1/96)	
District		0.0000
Sri Tat	26.3% (20/76)	
Kud Jub	0.0% (0/30)	
Mueang Udon	0.0% (0/15)	
Mueang Khon Kaen	0.9% (1/111)	
Nam Pong	0.0% (0/50)	
Ubonrat	4.2% (2/48)	
Kra nuan	12.0% (3/25)	

Table 5 (Continued)

Parameter	ELISA	*p*-value
District		0.0000
Mueang Sakon	18.9% (17/90)	
Paktongchai	0.0% (0/15)	
Chaiprakan	21.3% (32/150)	
Hangchat	3.0% (3/100)	
Mueang Chiangrai	0.0% (0/80)	
Mae lao	10.0% (3/30)	
Phan	1.5% (1/65)	
Tern	2.0% (2/100)	
Praya mengrai	46.4% (13/48)	
Kuntan	43.2% (16/37)	
Mae sai	3.8% (2/52)	
Mueang Pattalung	2.1% (4/187)	
Ta Muang	1.8% (1/56)	
Tamaka	0.0% (0/51)	
Lao kuan	0.0% (0/68)	
Ban pong	2.2% (1/45)	
Photaram	21.4% (9/42)	
Chombung	0.0% (0/20)	
Mueang Radchaburi	20.0% (1/5)	
Kampangsan	6.0% (9/151)	
Mueang Nakonpathom	2.9% (1/35)	
Muak lek	17.4% (21/121)	
Pattananikom	1.0% (1/96)	
Age group		0.0212
0-1 year	4.7% (3/64)	
>1-5 years	7.9% (88/1113)	
>5 years	11.5% (72/628)	

Table 6 Statistic analysis of factors associated to *T. evansi* infection in dairy cattle of Thailand

Parameter	Statistic value		
	Chi-square	**Degree of freedom**	**Probability**
Region	20.64	3	0.0001
Province	119.32	12	0.0000
District	294.77	29	0.0000
Age group	7.71	2	0.0212

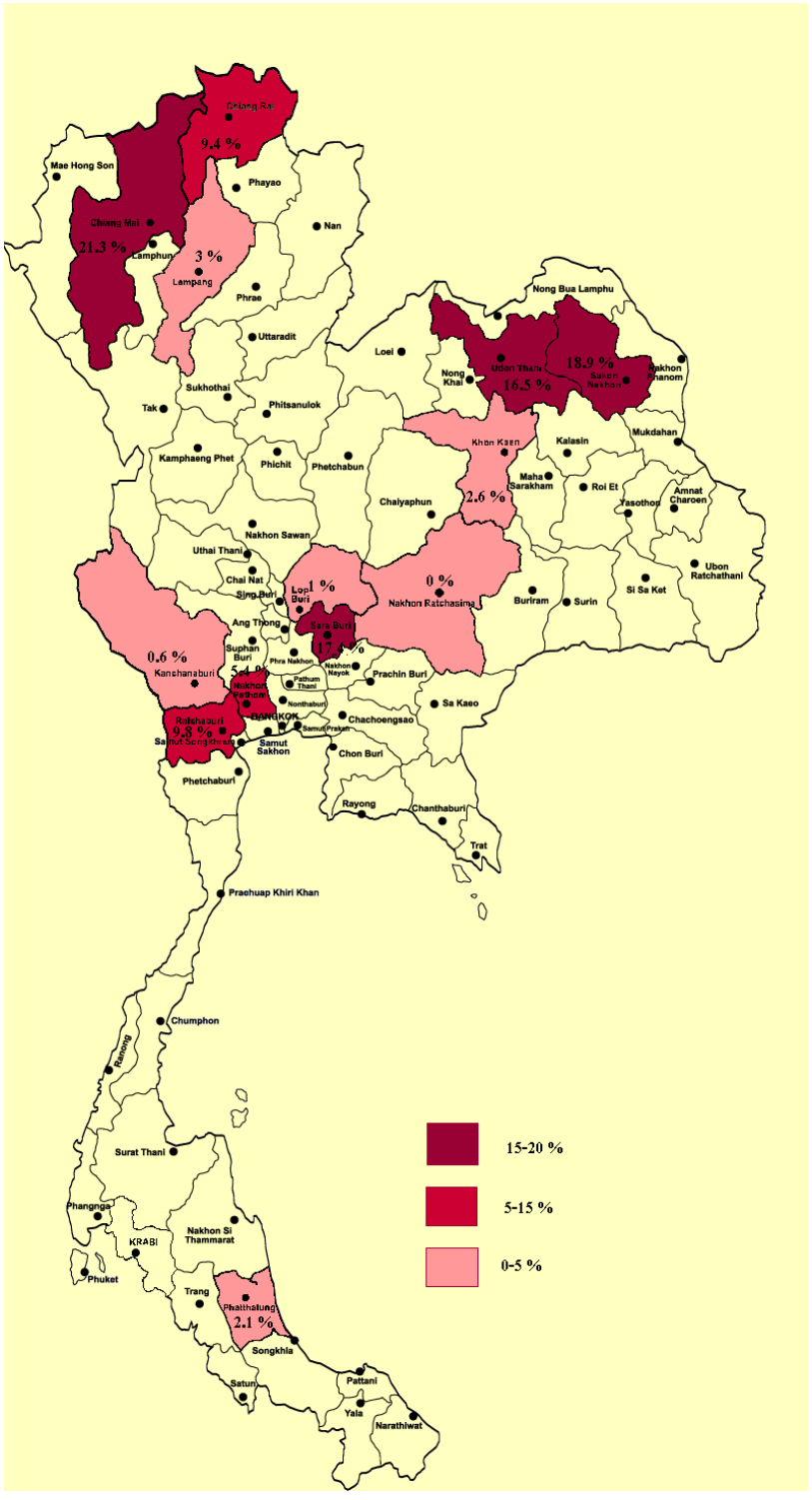


Figure 10 Seroprevalence of *T. evansi* in dairy cattle in 13 provinces of Thailand

Table 7 The number of animal, number of positive and percentage of positive test by ELISA compare with ME

Detection method	No. of animals	Total of positive animals	Prevalence	*p*-value	kappa
ELISA	1979	163	8.2%		
Micro-exam	595	4	0.7%	0.0001	0.090

Table 8 The Kappa test of ELISA and PCR comparison

Test		**ELISA**		**Total**
	Result	Number of Negative	Number of Positive	
PCR	Number of Negative	107	64	**171**
	Number of Positive	0	55	**55**
Total		**107**	**119**	**226**
Kappa test				**0.449**

Table 9 The Kappa test of ELISA and microscopic examination comparison

Test		**ELISA**		**Total**
	Result	Number of Negative	Number of Positive	
ME	Number of Negative	520	71	**591**
	Number of Positive	0	4	**4**
Total		**520**	**75**	**595**
Kappa test				**0.090**

DISCUSSION

Epidemiology of *Trypanosoma evansi* of dairy cattle in Thailand

Trypanosoma evansi has been studied for long time. This protozoa has spread in various geographical areas, and it is widely distributed in domestic and wild animals. Recently, the parasite was found in a human (Joshi *et al.*, 2005). It causes various acute symptoms in horses and camels, and causes chronic symptoms in cattle and buffaloes (Paikne and Dhake, 1972; Ogwu and Nuru, 1981; Well, 1981; Lohr *et al.*, 1986). Asia is the first region where cattle disease caused by *T. evansi* appears to be medically and economically important, it is the cause of low milk yield in newly introduced Holstein Friesian dairy cattle especially for the first year (Pholpark *et al*., 1999). Therefore it requires more investigations to determine why surra is more pathogenic in cattle in Asia than it is in Africa and Latin America (Desquesnes *et al.* 2009). There are reports of this disease in Thailand since 1942 (Table 10) in various hosts such as horse, pig, buffalo, dairy cattle and beef cattle in various provinces, deer, elephants, etc. but the whole picture in Thailand was no yet reported.

The present study showed that 8.2% of the samples from dairy farms were positive in ELISA. There is a significant link between area and the infection of *T. evansi* in dairy cattle in Thailand ($p < 0.05$). The Northern region gave the highest seroprevalence, 11.2% by individual and 46.3% by farm. At provincial level, Chiang Mai is the province in the Northern region that gave the highest seroprevalence, 21.3% by individual and 75% by farm. The provinces where has individual seroprevalence higher than 15% are distributed in three regions which are the northern, northeastern and central region. The seroprevalence has shown that trypanosomosis has spread in all the areas of Thailand at various levels. The seroprevalence of farms was higher than the seroprevalence of individuals. This means that trypanosomosis is largely distributed and is still a problem in the dairy cattle farms in Thailand. It might be that the disease can causes serious symptoms when animals are in the stress situations that also relate to the poor management (Kaewthamasorn *et al.* 2006).

Prevalence published by Kashiwasaki *et al.* in 1998, 52.8% in dry season and 80% in rainy season by Ag-ELISA are much higher than that observed in the present study. However, there were some mentioned about the Ag-ELISA that it has very hight sensitivity, but this technique had low specificity for Trypanosomes diagnosis and it has to be more evaluated. Nevertheless, they were tested with small number and repeatedly of animals which had clinical sign that is a reason why they got higher prevalence than our study. In addition, Lohr *et al*. (1985) reported parasitological and serological prevalence of *T. evansi* infection in buffaloes from northeastern region, Thailand with 3.7% and 17.1% during dry season. Whereas, this study was show 9.1% and 9.3% of parasitological and serological prevalence during dry season in northeastern region. One of the reasons why our prevalence is lower than that previously published is most probably due to the farming system investigated. Indeed, dairy farms have a better management of infections than beef cattle farming systems, it can then be speculated that the prevalence in beef farms would be higher than that observed in dairy farms; however it would require complementary specific epidemiological surveys.

The prevalence by parasitological examination in this study (0.7%) is lower than the studies by Elmin *et al.* (1998) in camels (5.4%), Laha and Sasmal (2007) in horses (12.7%) and Lohr *et al.* (1985) in buffaloes (15.4%), due to the various susceptibility of the hosts investigated, that causes the acute symptoms in camels and horses and chronic signs in cattle. In addition, the season of sampling can also affect the prevalence of trypanosomosis observed; previous studies have shown that the prevalence is higher during rainy season than the dry season (Lohr *et al*., 1985), this is directly related to the density of insects transmiting trypanosomosis such as tabanids and stomoxes. Future works should test by longitudinal studies for more information on seasonal variation with in various hosts.

Namely, investigations in beef cattle and buffalo should be carried out to establish the potential impact of surra in Thailand, which would be evaluated with the new models recently developed (Dargantes *et al* 2009).

Table 10 The reported of Trypanosomosis in Thailand

Year of outbreak	Month/season	Effective animal	Province	Diagnosis method	Prevalence	Reference
1942	-	Horse	-	Thick- Thin blood smear	-	Komutbut, 1950
1949	-	Horse	All regions	-	-	Komutbut, 1996
1974	December/ cool	Horse	Nakhon Ratchasima	-	-	Boonyawong *et al.*, 1975 in Phrikanahok *et al.*,2001
1981	October/ cool	Dairy cattle	Chiang Mai	-	-	Trisnarom,1981 in Phrikanahok *et al.*,2001
1983	August/ rainy	Buffalo	Surin	-	-	Timsard *et al.*, 1985 in Phrikanahok *et al.*,2001
1983	October/ cool	Beef cattle	Lampang	-	-	Chaichanapoonpol *et al.*, 1985 in Phrikanahok *et al.*, 2001
1984	June-August/ rainy	Pig	Pitsanuloke	-	-	Teeraprasert *et al.*, 1984 in Phrikanahok *et al.*,2001

The reported of Trypanosomosis in Thailand (continued)

Year of outbreak	Month/season	Effective animal	Province	Diagnosis method	Prevalence	Reference
1986	June-September/ rainy	Dairy cattle	Chiang Mai	-	-	Trisnarom, 1987 in Phrikanahok *et al*.,2001
1986	June-November/ rainy-cool	Pig	Supanburi	-	-	Siriwan *et al*., 1987
1989-1990	October-February/ cool-hot	Beef cattle	Phetchabun	-	-	Tuntasuwan, 1997 in Phrikanahok *et al*.,2001
2001	-	Buffalo	Lopburi	Ag-ELISA	22.64%	Suksaithaichana and Nawathong, 2001
2001	August-November/ rainy-cool	Dairy- Beef cattle	Singburi	IFAT	5.5%	Phrikanahok *et al*.,2001

The comparison of diagnosis method

Identification of trypanosomes has traditionally been based on microscopic observations (morphology, morphometry and motility of the parasite in host blood or tissues) (Hoare, 1972), however the technique is of low sensitivity. Nowadays, the diagnosis of *T. evansi* infection can be done by different ways. Due to the fact that the sampling was not only designed for trypanosomosis diagnosis, they were collected and stored at -20˚c before the diagnostic could be done; for this reason diagnosis techniques were adapted to frozen of fixed material. In this study we used three methods : microscopic examination of thin blood smear which allowed to show the presence of the parasite in 0.7% of the 595 smears observed indirect-ELISA *T. evansi* (Desquesnes *et al.* 2009) which allowed to establish the seroprevalence of the infection (8.2%) in the 1979 samples tested, and the PCR (Masiga *et al.*, 1992) which confirmed the species identification of *T. evansi* in a subset of 119 seropositive samples (showing 46% of PCR positive), as well as it confirmed the species specificity of the ELISA in a subset of 107 seronegative samples (showing 0% of PCR positive). These diagnosis methods are detecting various targets. Microscopical examination directly shows the presence of the parasite in the blood of the animals, however its sensitivity is very low and the specificity is also low when the parasitaemia is low. ELISA is detecting specifice IgG antibodies directed against *T. evansi*, thus it shows a present or past contact between the parasite and the immune system of the animal sampled; this is the best way to establish the level of contact parasite/host at the population level. PCR is detecting genomic DNA of *T. evansi* thus it allows to confirm the identity of *Trypanosoma evansi* (different from *T. theileri* for example) in the population; however, PCR is less sensitive than ELISA to establish the prevalence of the infection at population level. Moreover it is too expensive for application to all samples tested by ELISA. However, ELISA cannot establish whether the infection is active or took place in the past. Otherwise, it is extremely valuable as an epidemiological tool to assess the prevalence of infection (Desquesnes *et al.* 2009) as it is cheap and fast. ELISA was a useful tool for screening of the *Trypanosoma evansi* infection in animals within the short time and less cost when compare with PCR. We can see the whole picture of the disease by the results of

ELISA which showed how urgently the disease should be considered. TBR primers used in this study appeared to be the best primers to detect *T. evansi* in purified DNA, mice and cattle, and should then be preferred for epidemiological surveys. These results confirmed that TBR primers remain the gold standard for detection of trypanozoon and should therefore be included in subsequent evaluations of new diagnosis tools (Pruvot, *et al*., submitted).

Therefore, the kappa test was done for agreement comparison of ELISA with ME (kappa=0.090) and ELISA with PCR (kappa=0.449), that showed slight and moderate agreement respectively (interpreted from Anthony *et al*., 2005). Of 595 samples were tested by ME, 4 were positives and also were seropositive by ELISA, this means that ME is lower sensitivity than ELISA and also lower specificity due to the low number of parasite on the blood smear causing unidentified species of trypanosomes, while ELISA can identify. The 119 seropositive and 107 seronegative were confirmed by PCR, 55 (46.2%) were positive by PCR and none of sample from seronegative was positive by PCR (0%).This showed that ELISA does not give false positive. So, we can choose these two methods (ELISA and PCR) for trypanosomosis diagnosis depending on the capacity the labolatory can provide. Choosing only PCR for this disease would not be enough to confirm the infection due the fact that the parasite can be at very low level in the blood and even be hidden in extra-vascular biological compartiments such as joints, aqueous humor of the eye and nervous system, causing not detectable infection in the blood stream (Rottcher et al., 1987). So, using serodiagnosis with high sensitivity and specificity such as ELISA/ *T. evansi* like in this study, partially or fully combined with PCR to confirm the infection, appears to be the best way to implement epidemiological survey of such medium scale.

This study was the first reported and published (Desquesnes *et al.,* 2009) whole picture of trypanosomosis of dairy cattle of four regions in Thailand based on antibody detection (ELISA/ *T. evansi*), this may be useful for evaluation of the impact, treatment and control of this disease in Thailand.

CONCLUSION

The present study showed that 1979 dairy cows tested by ELISA-*T. evansi*, 163 (8.2%) were seropositive, and 4/595 (0.7%) were positive by microscopic examination. By PCR examination, 55 out of 119 seropositive were positive, and all the 107 seronegative were negative. The seroprevalence has shown that trypanosomosis has spread in all the areas of Thailand at various levels. Chaing Mai province located in the Northern region had the highest prevalence (P=21.3%) with 75% of the farms infected and decreasing by geographically from the Northern to Southern region. This means that trypanosomosis is largely distributed and is still a problem in the dairy cattle farms in Thailand.The statistic analysis indicated that regions, provinces and districts were associated with *T. evansi* infection ($p < 0.05$). Chi-squared test showed significant difference between age groups (p=0.02, $\chi 2$=7.71, df=2), seroprevalence being higher in older animals. These results may provide useful knowledge for prevention and control of *Trypanosoma evansi* infection of dairy cattle in Thailand.

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APPENDICES

Appendix A

The standard method

1. DNA extraction protocol; Phenol chloroform extraction of DNA and Ethanol precipitation (Sambrook and Russel, 2001)

1. **DNA extraction protocol;** Phenol chloroform extraction of DNA and Ethanol precipitation (Sambrook and Russel, 2001)

1. DNA was extracted from blood sample 100 µl mixed with denature solution 500 µl by shaken to 5-10 minutes.
2. Add chloroform 150 µl and DNA phenol (pH 7.9) 150 µl (chloroform : phenol=1:1), shaken for 10 minutes.
3. Centrifuge the mixture at 13,000 rpm for 5 minutes to separate the phases.
4. Collected the supernatant for 550-600 µl to the clean microtube (1.5 ml), carefully avoiding protein at the aqueous phenol interface at the last collecting.
5. Repeated the same protocol to clean the supernatant (step 2-4). In the second time, collected 400 µl of the supernatant and transfer to new microtube (1.5 ml).
6. Precipitated DNA by adding 1,000 µl (1 ml) of absolute ethanol (99.99%), invert gentally upside down and keep in -80 ˚c for 30 minutes or -20 ˚c for overnight.
7. Centrifuge at 13,000 rpm for 10 minutes. Remove the supernatant carefully.
8. To wash the DNA pallet with 75% ethanol. Centrifuge at 13,000 rpm for 5 minutes. Decant the supernatant, and dry the pallet by air.

Appendix B

Reagents and buffers for Phenol – Chloroform extraction and Ethanol precipitation

1. Denature solution (D-solution)

D-solution composed of 4M guanidine thiocyanate, 25mM sodium citrate pH 7, 10% N-lauroylsarcosine and sterilized millique water. The D-solution was transferred at dark bottle and stored at room temperature.

2. 10x Tris buffer (TE)

The stock 10x TE was prepared by dissolved 15.76 g of Tris-HCL and 3.72 g of EDTA in 500 ml of distilled water and adjusted pH to 8.0. The final volume was adjusted to 1,000 ml by distilled water. The 10x TBE was stored at 25°C. The 1x working solution was freshly prepared by diluting the stock 10x TE buffer with distilled water.

Appendix C

Reagents and buffers for agarose gel electrophoresis

Reagents and buffers for agarose gel electrophoresis

1. Gel loading buffer (loading dye)

The loading dye buffer composed of 0.25% bromphenol blue, 0.25% xylene cyanol, 30% glycerol and 35 ml of ultrapure distilled water. The loading dye solution was kept at 4°C.

2. Tris Borate EDTA buffer (10x TBE)

The stock 10x TBE was prepared by dissolved 108 g of Tris-base in 500 ml of distilled water. After the ingredient was completely dissolved, 55 g of Boric acid and 9.3 g of EDTA, were added into the solution. The final volume was adjusted to 1,000 ml by distilled water. The 10x TBE was stored at 25°C. The 1x working solution was freshly prepared by diluting the stock 10x TBE buffer with distilled water.

3. Working (1x TBE)

Fifty milliliter of 10X TAE was added to 950 ml of distilled water. This solution can be reused three times.

4. Ethidium Bromide (10 mg/ml)

One hundred milligram of Ethidium Bromide was dissolved to 100 ml of 1xTBE. The solution was transferred to dark bottle or aluminum foil wrap box and stored at room temperature.

Appendix D

The sample pictures of dairy cattle

Apppendix Figure D1 The area condition of dairy cattle farm in Udorn thani province, Norteastern region

Apppendix Figure D2 The milking house, where is the place for blood sampling.

Apppendix Figure D3 The milking house condition in Thailand

Apppendix Figure D4 The blood sampling from tail vein at the farm in Khonkhen province.

CIRRICULUM VITAE

NAME : Miss Ketsarin Kamyingkird

BIRTH DATE : July 7, 1983

BIRTH PLACE : Saraburi, Thailand

EDUCATION :

YEAR	INSTITUTE	DEGREE
2006	Kasetsart University	B. Sc.

WORK PLACE : March 2, 2009; Faculty of Veterinary Medicine, Kasetsart University

: March 15, 2005-February 15, 2009; Veterinary Diagnostic Labolatory

SCHOLARSHIP : Teacher's assiatance scholarship 2008

Thesis supporting scholarship 2009

PUBLICATION :

Pruvot, M., K. Kamyingkird, M. Desquesnes, N. Sarataphan and S. Jittapalapong. 2009. A comparison of 6 primer sets for detection of *Trypanosoma evansi* by polymerase chain reaction in rodents and livestock. (In press).

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