การตอบสนองโปรอินเฟลมมาทอรี ไซโตไคน์ ชนิดที่สำคัญในสุกรแม่พันธุ์อุ้มท้องหลังการทดลอง
ติดเชื้อทริปปาโนโซมา อีแวนซาย

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MAJOR PROINFLAMMATORY CYTOKINE RESPONSES IN PREGNANT SOWS AFTER

EXPERIMENTAL INFECTION WITH *TRYPANOSOMA EVANSI*

Miss Siriporn Tantawet

A Thesis Submitted in Partial Fulfillment of the Requirements

for the Degree of Master of Science Program in Veterinary Pathobiology

Department of Veterinary Pathology

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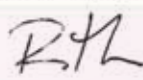Copyright of Chulalongkorn University

Thesis Title	MAJOR PROINFLAMMATORY CYTOKINE RESPONSES IN PREGNANT SOWS AFTER EXPERIMENTAL INFECTION WITH *TRYPANOSOMA EVANSI*
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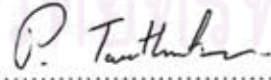
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สิริพร ตัณฑเวส: การตอบสนองโปรอินเฟลมมาทอรี ไซโตไคน์ ชนิดที่สำคัญในสุกรแม่พันธุ์อุ้มท้องหลังการทดลองติดเชื้อทริปปาโนโซมา อีแวนซาย (MAJOR PROINFLAMMATORY CYTOKINE RESPONSES IN PREGNANT SOWS AFTER EXPERIMENTAL INFECTION WITH *TRYPANOSOMA EVANSI*) อ. ที่ปรึกษาวิทยานิพนธ์หลัก: ศ.น.สพ.ดร. รุ่งโรจน์ ธนาวงษ์นุเวช, อ. ที่ปรึกษาวิทยานิพนธ์ร่วม: ผศ.น.สพ.ดร. ปิยนันท์ ทวีถาวรสวัสดิ์, 97 หน้า.

เนื่องจากการระบาดของโรคทริปปาโนโซเมียสิสหรือโรคเซอร่าในฝูงสุกรนั้นสามารถพบอุบัติการณ์ได้ทั่วประเทศไทย โดยก่อให้เกิดอาการทางคลินิกที่หลากหลาย การศึกษาในครั้งนี้จึงมุ่งเน้นที่จะทดสอบและวัดผลการเปลี่ยนแปลงของระดับ Proinflammatory cytokine ชนิด TNF-α IL-1β IL-6 และ Prostaglandin ชนิด PGE$_2$ PGF$_{2\alpha}$ รวมถึงอาการทางคลินิก และพยาธิวิทยาคลินิกของสุกรพันธุ์อุ้มท้องหลังทดลองติดเชื้อ *T. evansi* โดยผลการศึกษาในครั้งนี้พบว่าสุกรกลุ่มทดลองที่มีการติดเชื้อ แสดงอาการป่วยหลังการติดเชื้อ ได้แก่ แท้ง ไข้สูง เกิดผื่นแดง จุดเลือดออก ปื้นเลือดออกบริเวณผิวหนัง มีการเพิ่มสูงขึ้นของจำนวน Monocyte ในกระแสเลือด รวมถึงมีการเพิ่มขึ้นของระดับเอนไซม์ ALT และ AST ในซีรั่ม และยังพบว่าเชื้อ *T. evansi* สามารถติดต่อจากแม่สุกรไปยังลูกสุกรผ่านทางรกและสายสะดือได้ ในขณะที่ผลการตรวจวัดระดับของ Proinflammatory cytokine ในสุกรกลุ่มทดลองติดเชื้อและกลุ่มควบคุมนั้นไม่มีความแตกต่างกัน อีกทั้งระดับของ Prostaglandin ที่ตอบสนองในขณะที่มีการติดเชื้อนั้นก็ไม่สัมพันธ์กับอาการทางคลินิกในส่วนของการแท้งและมีไข้สูงที่เกิดขึ้น ส่วนผลการวินิจฉัยการตรวจยืนยันการติดเชื้อ *T. evansi* โดยวิธีการทางซีรั่มวิทยาโดยใช้ CATT/*T. evansi* ร่วมกับ Ab-ELISA/ *T. evansi* พบว่าสุกรที่ติดเชื้อมีการตอบสนองของภูมิคุ้มกันต่อเชื้อ และเมื่อทำการตรวจหาสารพันธุกรรมของเชื้อโดยวิธีการ PCR นั้นก็พบการติดเชื้อทริปปาโนโซมในสุกรทุกตัวที่ได้รับการฉีดเชื้อ *T. evansi* ชนิดแช่แข็งสัมพันธ์กับอาการทางคลินิกที่เกิดขึ้น จากการศึกษาในครั้งนี้จึงสามารถสรุปได้ว่า การตรวจวินิจฉัยโรคเซอร่าในสุกรพันธุ์นั้นต้องใช้การวินิจฉัยจากการตรวจพบเชื้อในกระแสเลือด อาการทางคลินิก พยาธิวิทยา พยาธิวิทยาคลินิก ร่วมกับการวินิจฉัยทางห้องปฏิบัติการซึ่งได้แก่การตรวจการตอบสนองของภูมิคุ้มกันต่อเชื้อ และการตรวจพบสารพันธุกรรมต่อเชื้อเป็นการยืนยันการติดเชื้อ

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SIRIPORN TANTAWET: MAJOR PROINFLAMMATORY CYTOKINE RESPONSES IN PREGNANT SOWS AFTER EXPERIMENTAL INFECTION WITH *TRYPANOSOMA EVANSI*. ADVISOR: PROF. ROONGROJE THANAWONGNUWECH, Ph.D., CO-ADVISOR: ASST. PROF. PIYANAN TAWEETHAVONSAWAT, Ph.D., 97 pp.

In Thailand, trypanosomiasis or surra outbreaks in pigs has been reported throughout Thailand with varying clinical manifestations. Therefore, the aim of this study is to evaluate levels of proinflammatory cytokine (TNF-α, IL-1β and IL-6) and prostaglandin (PGE$_2$, PGF$_{2\alpha}$) response, clinical and clinico-pathology in pregnant sows after experimental infection with *T. evansi*. In the present study, infected sows showed clinical and clinico-pathology signs including abortion, fever, petechial and plaque haemorrhage, skin rash, anaemia, monocytosis and elevation of AST and ALT enzymes. The results demonstrated that transplacental transmission is another route of *T. evansi* from sows to their fetuses. However, the data on the levels of proinflammatory cytokine (TNF-α, IL-1β and IL-6) response could not differentiated between trypanosomes infected sows and control sows. In the present study, the alteration of PGE$_2$ and PGF$_{2\alpha}$ in experimental sows showed no significative correlation with clinical signs such as fever and abortion. Whereas, the results of CATT/*T. evansi*, Ab-ELISA/*T. evansi* and PCR diagnosis were confirmed parasitologically positive in all infected sows. To summary, this study indicated that parasitaemia, clinico-pathological signs, serological assays and PCR method are the gold standard for the diagnosis of surra in pigs.

Department : Veterinary Pathology	Student's Signature SIRIPORN TANTAWET
Field of Study : Veterinary Pathobiology	Advisor's Signature
Academic Year : 2010	Co-advisor's Signature

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LIST OF ABBREVIATIONS

Ab-ELISA   Antibody-detection enzyme linked immunosorbent assay

ALP   Alkaline Phosphatase

ALT   Alanine amine transferase

APCs   Antigen presenting cells

APP   Acute phase proteins

APR   Acute phase response

AST   Aspartate alanine transferase

AT   African trypanosomiasis

BBB   Blood-brain barrier

BCSFB   Blood-cerebrospinal fluid barrier

BSF-2   B cell-stimulatory factor 2

BUN   Blood urea nitrogen

CAFs   Complement-activating factors

CATT   Card agglutination test for trypanosomiasis

CNS   Central nervous system

CSF   Classical swine fever

$CD4^+$ Cluster of differentiation 4^+

$CD8^+$ Cluster of differentiation 8^+

COX   Cyclooxygenase enzymes

COX-2   Cyclooxygenase-2

CPs   Cysteine peptidases

DCs   Dendritic cells

DIC   Disseminated intravascular coagulation

DPI   Day post infection

EP   Endogenous pyrogen

GIP   Glycosyl-inositol-phosphate

HB   Haemoglobin

HPA   Hypothalamo-pituitary-adrenal

HPGF	Hybridoma/plasmacytoma growth factor
HSF	Hepatocyte-stimulating factor
IFAT	Immunofluorescent antibody test
IFN-$\beta 2$	Interferon-$\beta 2$
IFN-γ	Interferon gamma
IgG	Immunoglobulin G
IgM	Immunoglobulin M
IL-1	Interleukin-1
IL-1α	Interleukin-1α
IL-1β	Interleukin-1β
IL-2	Interleukin-2
IL-4	Interleukin-4
IL-5	Interleukin-5
IL-6	Interleukin-6
IL-8	Interleukin-8
IL-10	Interleukin-10
K_3-EDTA	Tripotassium hydrogen ethylenediaminetetraacetate
LAF	Lymphocyte-activating factor
LEM	Leukocyte endogenous mediator
LGL	Large granular lymphocytes
LN_2	Liquid nitrogen
MCF	Mononuclear cell factor
MCH	Mean corpuscular haemoglobin
MCHC	Mean corpuscular haemoglobin concentration
MCV	Mean corpuscular volume
MGI-2	Monocyte granulocyte inducer type 2
MHC	Major histocompatibility complex
MHCT	Microhaematocrit technique
NF-κB	Nuclear factor κB
NK cells	Natural killer cells

NOS2	Nitric oxide synthase 2
OPs	Oligopeptidases
PAMPs	Pathogen-associated molecular patterns
PCR	Polymerase chain reaction
PCV	Packed cell volume
PGs	Prostaglandins
PGE_2	ProstaglandinE$_2$
$PGF_{2\alpha}$	ProstaglandinF$_{2\alpha}$
PRRSV	porcine reproductive and respiratory syndrome virus
RBC	Red blood cell count
RPP	relative percentage of positivity
SPF	Specific pathogen free
T. brucei	*Trypanosoma brucei*
T. congolense	*Trypanosoma congolense*
T. evansi	*Trypanosoma evansi*
Th1	Helper 1 T cells
Th2	Helper 2 T cells
TLR	Toll-like receptors
TLTF	Trypanosome lymphocyte triggering factor
TNF-α	Tumor necrosis factor α
T. simiae	*Trypanosoma simiae*
VSGs	Variant surface glycoproteins
WBC	Total leucocyte count

CHAPTER I

INTRODUCTION

Background and Rationale

Trypanosomiasis or surra is caused by *Trypanosoma evansi*. It can be found in several animal species such as cattle, buffalo, swine, horse, camel, elephant, mule, sheep, goat and dog. This parasite is widespread by mechanical vectors including *Tabanus* spp., Stable flies (*Stomoxys calcitrans*), *Chrysops* spp. and *Haematopota* spp. (Indrakamhang, 1998; Sukhumsirichart et al., 2000). Not only transmission by biting flies, but ingestion of fresh meat from infected carcasses is also another source of transmission (Enwezor and Sackey, 2005). Moreover, there is a case report of human (an Indian farmer) trypanosomiasis due to *T. evansi*, caused by transmission of blood from an infected animal (Joshi et al., 2005). In Thailand, surra was first reported in mule from Ratchaburi province, since 1949 (Sananraksat, 1949). Following the first case, many outbreaks in various kinds of animals was found throughout Thailand. Clinical signs were varied from asymptomatic to symptomatic clinical signs leading to abortion and dead. The economic loss is enormous due to production reduction and cost of treatment. Subsequently, this disease was regulated as a notifiable disease under the Animal Epidemic Act A.D. 1956. Currently, the severe cases caused by surra are not frequently seen, but they do sporadically occur. Trypanosome infection has been found in livestock and domestic animal throughout Thailand, with a distinct peak of infection during the rainy season when the climatic condition is suitable for the vector development (Indrakamhang, 1998; Tuntasuvan and Luckins, 1998; Desquesnes et al., 2009). In swine production, the breeding pig and pregnant sows are quite susceptible, especially in the conventional pig farming located near cattle farming. Acute and chronic signs have been observed. Skin rashes are usually observed. High rate abortion occurs at 1-2 months after the outbreak (Sirivan et al., 1987). Deaths and neurological signs sometimes rare followed by abortion. However, infected weaning and fattening pigs do not show any clinical signs with high levels of parasitaemia (Teeraprasert et al., 1984[a]; Taweenan et al., 2001). These healthy carriers allow the parasite to easily spread

throughout the populations. Investigations in Thailand in pig and other host species are needed to better understand the epidemiological situation for a proper prevention and control.

There are no pathognomonic signs of trypanosomiasis, thus laboratory diagnosis has to be carried out to confirm infection. Parasitological and serological diagnoses are poorly sensitive and in most situations *T. evansi* is under-diagnosed and the level of infection is greater than reported. On the other hand, some tests are more sensitive such as enzyme linked immunosorbent assay for *T. evansi* (ELISA/*T. evansi*) and card agglutination test for *T. evansi* (CATT/*T. evansi*). However, it still cannot distinguish current infection from cured infection. Currently, non-validation, standardization, application and development of available tests are insufficient making difficulty in field diagnosis (Holland et al., 2001; Enwezor and Sackey, 2005; Desquesnes et al., 2009).

The major symptoms of trypanosomiasis in pigs and breeding pigs are anemia, and intermittent high fever. Infertility and abortion may also be seen. Infection with trypanosomes causes significant immunosuppression. Concurrent infections may complicate this disease. This hemo-protozoan parasite is resistant to host innate immune responses mediated by complement and trypanosome evades the adaptive immune response by a mechanism known as antigenic variation. Despite successful evasion of antibody-mediated clearance by antigenic variation, the parasite is killed by adaptive immune responses, such as tumor necrosis factor α (TNF-α). Accordingly, the continual release of TNF-α (cachexin) from activated macrophages causes the chronic wasting disease associated with *T. evansi* (Raper et al., 2001; Pays, 2006). Lymphopenia is frequently reported and the underlying causes have not been directly addressed so far (Antoine-Moussiaux et al., 2009[a,b]). These data suggest that disease occurs because of the host's reaction to the infection or because the invader inadvertently causes significant damage to its host. The acute phase response (APR) is the answer of host to protect itself. APR belongs to the innate immunity and is mediated by proinflammatory cytokines mainly TNF-α, interleukin-6 (IL-6) and interleukin-1β (IL-1β). As a result, a systemic reaction is induced, showing clinical signs like fever, metabolic and hormonal imbalance. Currently, inflammatory cytokines detection is used

in clinical medicine to monitor states and progression of infection in many species. Furthermore, a family of prostaglandin derivatives including prostaglandinE$_2$ (PGE$_2$) and prostaglandinF$_{2\alpha}$ (PGF$_{2\alpha}$) are also play an important role in inflammatory reactions especially pregnant pigs (Antoine-Moussiaux et al., 2009[a]; Piñeiro et al., 2009; Skipor et al., 2010; Thengchaisri et al., 2010). Teeraprasert et al. (1984[b]) did the experimental infection in 1 ½ month pregnant sows with *T. evansi*, resulting in sow abortion at 100 hours post-infection with other surra associated clinical manifestations. However, major proinflammatory cytokines and prostaglandin responses level in pig after *T. evansi* infection have not previously been investigated. Clinical signs, clinical pathology and pathology outcome could not be expected due to antigenic variation and adaptation of *T. evansi*, especially in healthy resistance host. Moreover, *T. evansi* has not been studies extensively in experimental animal infection models, particularly in pigs. Data related to the proinflammatory responses of this parasite have not been established. To fill this gap, there is the need to determine clinical signs, clinical pathology, pathology, major proinflammatory cytokine and prostaglandin responses in pregnant sows after experimental infection with *T. evansi*.

Objectives

1. To study the pattern of major proinflammatory cytokine responses in 30-day-pregnant sows after *T. evansi* infection including tumor necrosis factor α (TNF-α), interleukin-1β (IL-1β) and interleukin-6 (IL-6).

2. To study the pattern of prostaglandin responses in 30-day-pregnant sows after *T. evansi* infection including prostaglandinE$_2$ (PGE$_2$) and prostaglandinF$_{2\alpha}$ (PGF$_{2\alpha}$).

3. To study the effects of *T. evansi* infection in 30-day-pregnant sows in terms of clinical signs, clinical pathology and pathology.

Expected output

1. Obtain the pattern of major proinflammatory cytokine and prostaglandin responses data in 30-day-pregnant sows after *T. evansi* infection.

2. Obtain the clinical signs, clinical pathology and pathology of *T. evansi* in 30-day-pregnant sows.

3. Obtain the diagnostic tool for *T. evansi* in pigs.

CHAPTER II

LITERATURE REVIEW

An overview of trypanosomiasis

Trypanosoma brucei evansi (T. evansi) is a haemo-protozoan parasite which is responsible for surra, trypanosomosis or trypanosomiasis in mammals. T. evansi infection has been reported in various kinds of animals such as elephant, horse, mule, donkey, cattle, buffalo, sheep, goat, pig, dog, and some wild animals (Indrakamhang, 1998; Tuntasuvan and Luckins, 1998). Mechanical transmission, which does not require the biological cycle in the insect vectors, allows T. evansi affecting both humans and livestocks (Joshi et al., 2005). Tabanus spp., Stomoxys calcitrans, Chrysops sp. and Haematopota sp. are the insect vectors of T. evansi. However, ingestion of meat from infected carcasses and vampire bats biting can also result in infection (Indrakamhang, 1998; Tuntasuvan and Luckins, 1998; Enwezor and Sackey, 2005; Pays, 2006). Clinical signs caused by infection with T. evansi have been widely described, including anaemia, edema, nervous signs, paralysis of the hindlimbs, intermittent or high fever, loss of condition, abortion, infertility and possible death. Although there are many experimental studies in a variety of hosts, to date pathognomonic lesions of T. evansi infection have not been established. Moreover, many stress factors, pregnancy, immunosuppression by other infections and host-specific factors can enhance different clinical manifestations of T. evansi infected animals (Murray et al., 1990; Indrakamhang, 1998; Enwezor and Sackey, 2005). In addition, these organisms can cause prolong chronic infection due to their antigenic variation ability. During the bloodstream stage, the parasite surface is entirely covered with a monolayer made of 10^7 copies of the single variant surface glycoproteins (VSGs), whose antigenic specificity continuously changes. This adaptation of parasite to the host allow them escaping the antibody response and causing long-lasting chronic infections (Gupta, 2005; Pays, 2006). Because of VSGs coat switch, the development of a vaccine or a trypanocide-drug has long been frustrated (Antoine-Moussiaux et al., 2009[a]).

In Thailand, surra was first detected in mules in Ratchaburi province since 1949. Following this first case, many outbreaks in livestock animals including pigs throughout Thailand occurred. Economic losses from the infected animals are considerable. Clinical signs observed vary forms of asymptom to abortion and dead. Pigs with mild or subclinical infection pose a serious public health risk in term of the fact that pigs are potential reservoirs for *T. evansi* infective for humans and other livestock animals (Tuntasuvan and Luckins, 1998; Joshi et al., 2005; Anene et al., 2011). Prevalence rate for this disease in a serological survey of dairy cattle throughout Thailand showed approximately 25 % of survey cattle were exposed to *T. evansi* (Desquesnes et al., 2009).

Porcine trypanosomosis

Pigs are commonly infected by *Trypanosoma brucei*, *Trypanosoma congolense*, *Trypanosoma simiae* and *Trypanosoma evansi* (Teeraprasert et al., 1984[a]; Anene et al., 2011). In Thailand, pathogenic trypanosomes isolated from pigs have been reported as *T. evansi* due to climatic condition is suitable for the vectors development. Tabanids and stable flies are probably the important vectors. There were no significant different of *T. evansi* total length isolated from pigs, cattle, dogs and horses (Sarataphan et al., 1987). Pigs, especially pregnant sows are severely affected by *T. evansi* infection. The clinical signs in pigs infected with *T. evansi* are depression, inappetence, intermittent fever, skin rashes and petechial hemorrhages particularly on the skin of the ears, udder, legs, scrotum, and lateral sides of the body. Some pregnant sows aborted and death. It has been reported that during June - November 1986, *T. evansi* infected breeding sows in Suphanburi resulted in abortion at the period of 1-2 months, 2-3 months and over 3 months pregnancy as 84.44 %, 11.11 %, 4.44 %, respectively (Sirivan et al., 1987). Abortion can occur at any stage of pregnancy, but more frequently at 1-2 months. Death has also been observed after sow abortion. In addition, *T. evansi* was found in amniotic fluid of aborted fetus in naturally infected sows. Transplacental is another way of transmission (Teeraprasert et al., 1984[a]; Sirivan et al., 1987). Some infected pigs showed nervous signs such as head pressing, convulsion, circling movement and were

dead eventually. Hence, in acute, clinical disease, central nervous system disorder and even death can occur. In chronic infection, working capacity and productivity of animals are more likely to be affected. Infected weaning and fattening pigs show no clinical signs. This makes it urgent to treat and control trypanosomiasis in pigs. Since subclinical pigs are being potential reservoirs of *T. evansi* and may pose a serious public health risk. Whenever outbreak occurs, health risk and economic loss is unavoidable. Trypanosomiasis reduces piglet production in infected sows and the cost of treatment with antitrypanocidal drugs is high. Moreover, sometimes, drug used is not effective and *T. evansi* can be detected from infected animals 2 weeks post treatment. Whole herd treatment and vectors control are recommended (Teeraprasert et al., 1984[a,b]; Sirivan et al., 1987; Indrakamhang, 1998; Tuntasuvan and Luckins, 1998; Anene et al., 2011). Effective diagnostic tools and treatments in porcine trypanosomosis are still needed and host-parasite interaction should be investigated for more information.

Properties and pathogenicity of *Trypanosoma evansi*

Trypanosomoses are diseases mainly found in intertropical zone of Africa, South America and Asia. There are many trypanosomes that are pathogenic for mammals, including man. African trypanosomiasis (AT) is of importance in cattle and can cause serious losses in pigs, camels, sheep, goats, dogs, cats, camels, horses, and monkeys (World Organisation for Animal Health, 2010). Infection of animal by AT resulting in subacute, acute, or chronic disease characterized by intermittent fever, anemia, occasional diarrhea, and rapid loss of condition and mostly resulting in death if untreated. Rats, mice, guinea pigs, and rabbits are useful laboratory species. Wild pigs are susceptible and can also serve as carriers of trypanosomes (World Organisation for Animal Health, 2010). Trypanosomosis is also mechanically transmitted by biting flies through the transfer of blood from one animal to another (Maré, 2004; Enwezor and Sackey, 2005).

In Asia, including Thailand, *T. evansi* had already been discovered as the causative agent of surra, a disease affecting various kinds of animals, and its morphological similarity to *T. brucei*. In the case of African trypanosomes, taxonomy has

been driven by utilitarian consideration such as the type of disease caused, host range and geographical distribution (Table 2.1 and Table 2.2). While molecular biology was initially exploited to provide markers for these species and facilitate diagnosis.

Table 2.1 Pathogenic trypanosomes (Gibson, 2007)

Species	Year of discovery	Original host	Disease
Trypanosoma evansi	Balbiani (1888)	Equines, camels	Surra
Trypanosoma brucei	Plimmer and Bradford (1899)	Cattle, dogs, horses	Nagana
Trypanosoma equiperdum	Doflein (1901)	Horse	Dourine
Trypanosoma gambiense	Dutton (1902)	Human	Trypanosoma fever, Gambian sleeping sickness
Trypanosoma rhodesiense	Stephens and Fantham (1910)	Human	Rhodesian sleeping sickness

Table 2.2 Revised taxonomy of *Trypanosoma evansi* (Gibson, 2007)

Trypanosoma evansi: Scientific classification	
Kingdom	Protista
Phylum	Euglenozoa
Class	Kinetoplastea
Order	Trypanosomatida
Family	Trypanosomatidae
Genus	*Trypanosoma*
Species	*T. evansi*

T. evansi can infect various hosts causing species-specific pathology. In susceptible hosts, initial replication of trypanosomes is at the site of inoculation in the

skin causing swollen and sore. Trypanosomes then spread to the local lymph nodes and blood. Antibody to the VSGs coat of the trypanosome kills the trypanosome resulting in the development of immune complexes. However, antibody does not completely clear the infection. The trypanosome has genes that can code for many different surface-coat glycoproteins and change its surface glycoprotein (VSGs) to evade the antibody (Pays, 2006). Hence, persistent infection results in continuing cycles of trypanosome replication, antibody production, immune complex development, host immunoresponsiveness and switching to new surface-coat glycoproteins (Gray and Gill, 1993).

Immune mediated lesions are significant in trypanosomiasis, and it has been suggested that many of the lesions including skin rash, skin edema, anemia, vasculitis, disseminated intravascular coagulation (DIC) and glomerulonephritis in these diseases may be the result of the deposition of immune complexes. The most significant and complicating factor in the pathogenesis of trypanosomiasis is the profound immunosuppression that occurs following *T. evansi* infection. This marked immunosuppression lowers the host's resistance to other infections and thus results in secondary disease, which greatly complicates both the clinical and pathological features of trypanosomiasis. Holland et al. (2003) demonstrated that the presence of *T. evansi* infection lowered the immunoresponsiveness of fattening pigs and consequently this immunosuppression might explain the poor protection of classical swine fever (CSF)-vaccinated pigs reported in *T. evansi* endemic area. Even though, *T. evansi* is not established as an important parasite in pigs, it may interfere with other pathogens or vaccinations by its immunosuppressive ability. Furthermore, the report in Nigeria showed trypanosomosis as a possible cause of anaemia among pigs in this area, it is supported by significant depression of packed cell volume (PCV) of the infected pigs. Anaemia is a major component of the pathology of surra. In addition, the results of enzyme assays show elevation of aspartate alanine transferase (AST) and alanine amine transferase (ALT), and a fall in alkaline phosphatase (ALP) levels. These data attributed to the invasiveness of parasites in several organs such as liver, kidney, muscle and heart causing damage to cells and release of enzymes into the blood (Anene et al.,

2011). In the late states, persistent anaemia lead to anoxic condition could be observed. An increase in cardiac output and a decrease in circulation time are obvious. The central nervous system (CNS) is a consequence of cerebral anoxia.

Furthermore, *T. evansi* can cross the blood-brain barrier (BBB) and blood-cerebrospinal fluid barrier (BCSFB) resulting in lesions and demyelination. Progressively, infected animals die after neuro-endocrine and circadian rhythms disturbances, meningoencephalitis, and coma. The mechanisms and situation in the kinetics of the disease remain unclear. A hypothesis to explain BBB and BCSFB disruption during African trypanosomes infection is that inflammatory mediators, such as TNF-α, or IL1-β, and IL-6, produced in the CNS or blood stream, may modify endothelial cell activation. *In vitro* experiments showed the synthesis of inflammatory mediators and adhesion molecules during natural or experimental trypanosomosis, and showed that parasites might have a direct effect on endothelial cells due to the pathological effects of trypanosomal factors (Girard et al., 2005; Antoine-Moussiaux et al., 2009[a]).

Trypanosomal virulent factors involving in AT include VSGs, glycosyl-inositol-phosphate (GIP) anchors, cysteine peptidases (CPs), oligopeptidases (OPs), sialidases, peptidase inhibitors, complement-activating factors (CAFs), trypanosome lymphocyte triggering factor (TLTF) and B-cell mitogen-like molecule occupy a central role in trypanosomiasis pathogenesis. The mechanisms remain unclear and vary depending on specific host-parasite interaction. For example, the VSG switching contributes to immune evasion mechanism and immune suppression is a classical feature of trypanosomes infection. This pathogenesis is partly related to macrophage over activation and uncontrolled production of cytokines, such as TNF-α, which induces immune suppression, anemia, organ lesions, and weight loss. Moreover, in parasite lysis phase, the toxic metabolites produced by dying trypanosomes might cause fever. Haemorrhage and serous exudation could be caused by haemolysis involving the expanded mononuclear phagocytic system. In addition, OPs are strongly thought to be responsible for the observed endocrine dysfunction causing abortion in pregnant animals (Antoine-Moussiaux et al., 2009[a]). Although there are many experimental reports demonstrating different clinical signs showed by the end host, it has not currently been

possible to establish the model. Precise knowledge of host-parasite interactions and parasitic factors involved in pathogenesis are needed (Gray and Gill, 1993; Maina et al., 2004; Antoine-Moussiaux et al., 2009[a]).

Immune responses in trypanosomes infection

The body's defenses compose of physical barriers, non-specific or innate immunity and specific or acquired immunity. An initial immune response to foreign antigen requires innate immune responses including macrophages, neutrophils, eosinophils, natural killer cells (NK cells) and inflammation. Some of these innate cells act as antigen presenting cells (APCs). They present an antigen on its cell surface to B cells, and then the B cells are signaled to proliferate and to produce antibodies that specifically bind to that antigen. Specific or acquired immune responses occurred after stimulating by specific antigens. Regarding its specificity and memory, the important cells are B cells and T cells (Tizard, 2009).

Infectious disease due to protozoan parasites causing vast morbidity and mortality, contributes to political, social and economic mutability, especially in developing countries. In the severe cases, disease occurs because of the host's reaction to the infection or because the parasites inadvertently causes serious damage to the host. Well-developed parasites have evolved that their presence in the host is hardly noticed. They always block or delay the innate and acquired immune responses of their host so that they may persist for sufficient time to multiply. Some parasites may simply delay host immune responses, but well-adapted parasites may plan to survive for the life time of their host, and will be protected from immunological attack by complicated and specific evasive strategies. In addition, many parasites take advantage on using the host's metabolic or control pathways for their own purpose. During the early stages of infection, the host innate immune system must rapidly detect and response to protozoan parasite infection. The innate, non-specific defend mechanisms is mediated by proinflammatory cytokines mainly TNF-α, IL-6 and IL-1 that act as mediators between the injured tissue and the target organs that coordinate the responses. As a result, a systemic reaction is induced, including fever, anorexia, metabolic and hormonal

alteration (Piñeiro et al., 2009; Tizard, 2009). In addition, studies in animal model infection suggest that the disease severity is associated with inflammatory responses. In mice studied model suggest that component of GPI anchor of trypanosome VSGs molecule when cast induce macrophage activation, and the early phase of AT infections are characterized by up-regulated synthesis of the cluster of differentiation 4^+ (CD4$^+$) helper 1 T cells (Th1) and proinflammatory cytokines, such as interferon gamma (IFN-γ) and TNF-α. Subsequently, AT prolonged survival depends on change of host's cytokine profile and helper 2 T cells (Th2) pattern. However, little is known about the role of cytokines in the pathogenesis of AT including *T. evansi*. A better knowing of immunological responses in AT will be essential in identifying the precise role of inflammatory responses in the pathophysiology of trypanosomes infection (Inoue et al., 1999; Maina et al., 2004).

Trypanosoma evansi is a flagellate extracellular protozoan parasite, causing surra in animals and humans. Generally, antibody-dependent immune responses are important to control extracellular parasites. In the presence of T-cells, VSGs specific B-cell responses greatly enhance, both T-cell-dependent and T-cell-independent B-cells. The data suggest that helper T cell plays an important role in controlling trypanosomes infection. After naïve helper T cells recognize major histocompatibility complex (MHC)-binding antigenic peptide and differentiate into functionally distinct helper T cell subsets regarding Th1 and Th2. The diversion of cytokine production pattern is characterized by Th1 and Th2. Roughly, the Th1 is labeled by its production of interleukin 2 (IL-2), IFN-γ and lymphotoxin, and promotes cell-mediated immune responses. The Th2 produces interleukin-4 (IL-4), interleukin-5 (IL-5), interleukin-6 (IL-6) and interleukin-10 (IL-10), and promotes humoral immune responses. Efficient control of parasitic load and host survival rely on T cell-mediated immunity via helper T cell-dependent protective antibody responses and macrophage activation. Furthermore, the significant of Th1/Th2 balance has been demonstrated in experimental trypanosomiasis (Assoku, 1975; Uche et al., 1992; Abrahamsohn, 1998; Inoue et al., 1999; Maina et al., 2004; Baral et al., 2007; Antoine-Moussiaux et al., 2009[a]). The association between antibody pattern and the nature of *T. evansi* infection suggesting that the immune

system may play an important role in determining the way in which individual animals respond to the *T. evansi* infection. The serum from resistant animal had antibodies such as immunoglobulin G (IgG) and immunoglobulin M (IgM) to many trypanosome components, with the antibodies appearing early in infection and persisting throughout the course of the infection. On the other hand, the susceptible animals had antibodies to fewer trypanosome components. The antibodies were detected later in infection and were not maintained throughout the course of infection. Moreover, trypanotolerant animals produce more IL-4 and less IL-6 than susceptible animals. This fact indicates that the immunoresponsiveness of animals to *T. evansi* infection is under genetic control. The observations have been reported in the cases of trypanotolerance in cattle which is influenced by inheritance and *T. evansi*-resistant breeds of other animals (Uche et al., 1992; Tizard, 2009). In addition, experimental mouse models showed only IgM antibodies contribute significantly to parasite control, despite *T. evansi* infection causing induction of TNF-α, IFN-γ and nitric oxide (NO) during both acute and chronic phase of infection. In acute phase of the disease, lymph nodes and spleen are notably reactive, with plasma cells predominating. Leucocytosis, neutrophilia and eosinophilia have been reported in *T. evansi* infection of camels (Enwezor and Sackey, 2005). This may account for the generalized lymphoid tissue hyperplasia characteristic of *T. evansi* infection, while in the chronic stage; depletion of lymphoid cells can be seen. Hence, overreaction and uncontrolled production of the antibody found in infected animals have the directed effect against the parasites causing pathological effects such as haemolytic effect, anaemia, tissue damages in vital organs, microvascular obstruction and microvascular dilatation due to circulating and tissue-mediated immune complex. A state of immunosuppression associated with many factors later develops (Enwezor and Sackey, 2005; Baral et al., 2007; Antoine-Moussiaux et al., 2009[a]). The action of trypanosomal virulent factors such as VSGs and trypanosome enzymes have all been implicated and demonstrated in natural and experimental infected animals. For example, a non-cytosolic protein of *T. evansi* induces CD45-dependent lymphocyte death (Antoine-Moussiaux et al., 2009[b]). Most infected cases are suffering from

secondary infections and vaccination failure, consequently production loss and death if untreated (Holland et al., 2003).

Proinflammatory cytokine responses in trypanosomes infection

Cytokines are a large family of more than 100 small proteins that function as short-range mediators involving in essential all biological processes. Currently, many cytokines have been found to be useful in clinical medicine. Those are used in various purpose such as detecting inflammatory states, monitoring the progression of infectious disease and therapeutic target in chronic diseases.

Sentinel cells including macrophages, dendritic cells (DCs), and mast cells are activated when pathogen-associated molecular patterns (PAMPs) or alarmins bind to their receptors. Consequently, they respond by synthesizing and secreting a mixture of cytokines and other molecules that trigger inflammation during activating acquired immunity. When exposed to infectious agents or their PAMPs, sentinel cells synthesize and secrete many different proteins including the major proinflammatory cytokines IL-1, TNF-α, as well as IL-6 and others. They synthesize nitric oxide synthase 2 (NOS2). Besides, they also synthesize the enzyme cyclooxygenase-2 (COX-2) that generates the inflammatory lipids prostaglandins and leukotrienes. When released in sufficient quantities, these molecules cause fever and sickness behavior and promote an acute phase response (APR). If they detect the presence of damaged or foreign DNA, they trigger DCs to secrete the cytokines known as interferons.

Major proinflammatory cytokine responses

Tumor Necrosis Factor-α (TNF-α): the other names of TNF-α are cachectin, macrophage cytotoxin, necrosin, cytotoxin, haemorrhagic factor, macrophage cytotoxic factor and differentiation-inducing factor. TNF-α is a 25 kDa trimeric protein produced by macrophages, mast cells, T cells, endothelial cells, B cells and fibroblasts. It is a potent paracrine and endocrine mediator of inflammatory and immune functions. It is also known to regulate growth and differentiation of a wide variety of cells types. Many of the actions of TNF-α occur in combination with other cytokines as part of the cytokine

network. TNF-α has turned out to be a cytokine with very diverse biological activities that might explain its role in various physiological and pathological phenomenon, such as infection, inflammation, immunomodulation, cancer, cachexia, and lethal septic shock. TNF-α also causes the secretion of various cytokines including IL-1, IL-6 and IL-8. Moreover, TNF-α rapidly induces neutrophil adherence to endothelial cells, activates phagocytosis, enhances specific antibody-dependent cellular cytotoxicity and it also has an antiparasitic activity. In cases of overreaction of host or deficiency of a natural autoregulatory network, several deleterious effects of TNF-α can be observed. Associated pathological disorders include pathophysiology of sepsis, cachexia, cerebral malaria, multiple sclerosis, rheumatoid arthritis, cancer and other inflammation responses (Mire-Sluis and Thorpe, 1998; Fitzgerald et al., 2001; Tizard, 2009).

Interleukin-1 (IL-1): The other names of IL-1 are lymphocyte-activating factor (LAF), endogenous pyrogen (EP), leukocyte endogenous mediator (LEM), mononuclear cell factor (MCF) and catabolin. IL-1 has a very wide range of biological activities on many different target cell types including B cells, T cells and monocytes. IL-1 is divided into two polypeptide mediators; IL-1α and IL-1β. IL-1β is produced as a large pro-protein that is cleaved by caspase-1 to form the active molecule. IL-1β is produced tenfold to fiftyfold more than IL-1α, and while IL-1β is secreted, IL-1α remains attached to the secreting cell. Therefore, IL-1α acts only on target cells that come into direct contact with the macrophage. Furthermore, transcription of IL-1β mRNA occurs within 15 minutes after ligand binding. IL-1β production reaches a peak 3 to 4 hours later and levels off for several hours before declining. A wide variety of cells secrete IL-1, such as monocytes, tissue macrophages, Langerhans cells, DCs, T lymphocytes, B lymphocytes, NK cells, large granular lymphocytes (LGL), vascular endothelium, smooth muscle, fibroblasts, thymic epithelia, astrocytes, microglia, glioma cells, keratinocytes and chondrocytes. During severe infections, some IL-1β circulates in the bloodstream, where (in association with TNF-α) it is responsible for sickness behavior. Moreover, it acts on the brain to cause fever, lethargy, malaise, and lack of appetite. It acts on muscle cells to mobilize amino acids causing pain and fatigue. It acts on hepatocytes to induce the production of new proteins, called acute phase proteins (APP) assisting in

the defense of the body. Collectively, IL-1 is a key mediator of the series of host responses to infection and inflammation known as acute phase responses (APR) cooperating with TNF-α and IL-6. In addition, animal model studies have indicated other pathologies where blockade of IL-1 might be beneficial, for vasculitis, DIC and autoimmune diseases. Inhibition of IL-1 and inflammation by an important regulator of IL-1 activity, called IL-1RA, anti-IL-1 or anti-IL1R antibodies are protective in various animal models. It reduces mortality in septic shock and has anti-inflammation effects (Mire-Sluis and Thorpe, 1998; Fitzgerald et al., 2001; Tizard, 2009).

Interleukin-6 (IL-6): It is also called Interferon-β2 (IFN-β2), B cell-stimulatory factor 2 (BSF-2), hybridoma/plasmacytoma growth factor (HPGF), hepatocyte-stimulating factor (HSF), monocyte granulocyte inducer type 2 (MGI-2), cytotoxic T cell-differentiation factor and thrombopoietin. IL-6 was identified in 1986 as a B cell regulatory factor and is now recognized as mediating pleiotropic functions including effects on the maturation and activation of B and T cells, macrophages, osteoclasts, chondrocytes, and endothelial cells and broad effects on hematopoiesis in the bone marrow. It is produced by lymphoid cells (T and B cells), nonlymphoid cells, such as macrophages, mast cells, bone marrow stromal cells, fibroblasts, keratinocytes, mesangium cells, astrocytes and endothelial cells. Its production is stimulated by bacterial endotoxins, IL-1 and TNF-α. IL-6 affects both inflammation and acquired immunity. It is a major mediator of APR and septic shock. It has been suggested that IL-6 regulates the transition from neutrophil-dominated process early in inflammation to a macrophage-dominated process later on. Analysis of IL-6 as a clinical marker of inflammation or infection has shown to correlate with other indices of disease activity, and it is now used for this purpose in combination with other clinical tests. IL-6-dependent pathology could determine as over-expression of IL-6 in a variety of acute inflammation or infection and autoimmune conditions (Mire-Sluis and Thorpe, 1998; Fitzgerald et al., 2001; Brennan and McInnes, 2008; Tizard, 2009).

The patterns of cytokines elicited by different parasite strains suggest that they may use similar survival strategies in the mammalian hosts. Therefore, the activation mechanisms were demonstrated similarly in different parasite strains. Further

characterization and comparison of the nature of the activating molecules are necessary.

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┌─────────────────────────────────┐
│  Pathogenic agents              │
│      ● Protozoan parasite       │
│      ● Virus                    │
│      ● Bacteria                 │
│      ● Fungi and yeast          │
└─────────────────────────────────┘
                ⇩
        ┌──────────────────────┐
        │  Infection / Intrusion │
        └──────────────────────┘
                ⇩
        ┌──────────────────────┐
        │  Innate immune system │
        └──────────────────────┘
                ⇩
      (  Inflammatory cytokines  )
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Local response	Systemic response	Central response
● Capillary dilatation	● Stimulation of hematopoiesis	● Fever
● Macrophages attraction/activation	● Production of APP*	● Anorexia, adispia
● Diapedesis	● Increase of lipolysis	● Reduced social interactions
● Phagocytosis	● Muscular proteolysis	● Decreased sexual activity
		● HPA* axis activation

* APP = Acute phase proteins, HPA = Hypothalamo-pituitary-adrenal

Figure 2.1 The local, systemic and central component of acute phase reaction during infection or intrusion of pathogenic agents (adapted from Pecchi et al., 2009).

Prostaglandins and sickness behavior

Previous studies have shown that prostaglandins play a key role in the development of sickness behavior observed during inflammatory states. In general, ProstaglandinE$_2$ (PGE$_2$) is produced in the brain by a variety of inflammatory signals.

Arachidonic acid is converted into prostaglandins (PGs) by cyclooxygenase enzymes (COX). Sickness behavior has been extensively studies, and it has been demonstrated that strategies aiming at inhibiting COX limit anorexia, weight loss and fever in sick animals. However, inhibiting COX activity may lead to negative gastric or cardiovascular effects, due to COX play a role in the synthesis of others prostanoids. In addition, COX-2, an isoform of cyclooxygenase, has been shown to be expressed in macrophages, epithelial cells, and fibroblasts after exposure to several proinflammatory stimuli such as cytokines and growth factors, leading to the release of the other kind of prostaglandins called prostaglandin$F_{2\alpha}$ ($PGF_{2\alpha}$). A previous study has shown that plasma $PGF_{2\alpha}$ levels are raised in autoimmune diseases and chronic inflammatory diseases. Due to oxidative modification of arachidonic acid, through both non-enzymatic lipid peroxidation and enzymatic (cyclooxygenase) pathways, is involved in endotoxin induced inflammation in septic shock, hepatotoxin induced oxidative injury and cerebral oxidative injury after resuscitation from cardiac arrest. These suggest that oxidative injury and inflammation are closely associated in various syndromes (Basu et al., 2001; Pecchi et al., 2009).

Acute infections and other immune challenges trigger in the host defense response called acute phase reaction comprising immune, physiological and behavioral changes. Fever stimulates proliferation of immune cells and limits pathological agent growth and proliferation. Understanding the molecular and cellular mechanisms underlying the triggering of sickness behavior is necessary to design therapeutic approaches in such infection and inflammation cases. During infection, cells of innate immune system located in the periphery including macrophages, neutrophils, DCs, lymphocytes and NK cells or in the CNS recognize specific components of micro-organisms called PAMP, through the expression of specific receptors, the toll-like receptors (TLR). The activation of these receptors leads to the recruitment of downstream signaling pathways including the nuclear factor κB (NF-κB) which in turn induce the synthesis and release of proinflammatory cytokines such as IL-1β, TNF-α and IL-6. These soluble mediators coordinate the local and systemic inflammatory response to micro-organism products. They also act on the brain in inducing the CNS symptoms specific to sickness. Among these proinflammatory cytokines, IL-1β seems to

be the most potent in inducing sickness behavior. Furthermore, in chronic inflammatory anorexia observed during cancer and chronic infectious diseases, most strategies aiming at inhibiting the action of TNF-α and IL-6 promoting the increase in feed intake. Interestingly, observation in various experimental models seems to be related to the redundant and overlapping actions of cytokines, which could permit unusually extensive developmental compensation. Hence, inflammatory cytokines can replace each other to a certain extent in mediating inflammation-induced sickness behavior (Gazzinelli and Denkers, 2006; Pecchi et al., 2009).

In addition, in pigs, the uterus of cycling and pregnant pigs as well as developing embryos are the source of PGE_2 and $PGF_{2\alpha}$. Both PGE_2 and $PGF_{2\alpha}$ are known as vasoactive factors in the reproductive organs (Skipor et al., 2010). PGs influence uterine smooth muscle tone and cause the mechanical compression of the uterine vasculatures changing blood flow in supplying arteries. PGs also have both positive and negative effects on reproduction. PGs affect ovulation, luteal regression, the implantation and maintenance of pregnancy, parturition and postpartum physiology. Various studies demonstrated that effects of PGE_2 and $PGF_{2\alpha}$ play an important role in female reproductive organs (Weems et al., 2006; Norrby, 2010). The different distribution of PGs level, not only in reproductive organs but also in bloodstream may cause the effect on reproduction. For example, $PGF_{2\alpha}$ administered intramuscularly to pregnant sows, at a single dose of 10 mg, can induce either abortion or parturition in pregnant sows (Weems et al., 2006).

Diagnosis of *Trypanosoma evansi* in animals

There are no pathognomonic signs and lesions of surra and thus laboratory diagnosis has to be carried out to confirm infection. Commonly, this involves parasitological and serological diagnosis. Parasitological diagnosis in pigs is carried out directly by microscopic examination of blood or buffy coats and/or rodent inoculation. However, owing to fluctuating levels of parasitaemia, especially during the chronic stages of infection, the test has a poor sensitivity. Serological techniques, such as immunofluorescent antibody test (IFAT), antibody-detection enzyme linked

immunosorbent assay (Ab-ELISA) and card agglutination test for trypanosomiasis (CATT) have been used extensively, although sensitive, these tests cannot distinguish current infection from cured infection. It can infer that in most situations *T. evansi* is under-diagnosed and the level of infection is greater than reported. Since validated serological tests for the diagnosis of *T. evansi* for pigs are not available, an initial evaluation of CATT and Ab-ELISA was carried out using serum sample from experimentally infected pigs. Thereafter, the serological tests were used in field surveys coupled with highly specific and sensitive polymerase chain reaction (PCR). The PCR is a technique used for DNA amplification *in vitro* and has already been applied to identification of *T. evansi* in various animals and vectors (Sukhumsirichart et al., 2000; Holland et al., 2001). The advantages of combination of tests are very low false-positives and insensitive to other haemoparasite protozoans. These tests are suitable for tracing and surveillance for *T. evansi* infection in epidemiological survey and experimental infection. In addition, they are also useful for designing rational trypanosomosis control programe in the endemic areas (Masiga et al., 1992; Reid and Copeman, 2003; Enwezor and Sackey, 2005; Holland et al., 2005; Desquesnes et al., 2009).

In pigs, *T. evansi* has not been studies extensively. There are no pathognomonic signs and lesions of surra in sows. Vertical transmission is unclear. Clinical signs, clinical pathology and pathology could not be expected due to antigenic variation and adaptation of *T. evansi*. Moreover, major proinflammatory cytokines and prostaglandin responses level in pig after *T. evansi* infection have not been established before. Hence, the present study was conducted to determine clinical signs, clinical pathology, pathology, major proinflammatory cytokine and prostaglandin responses in pregnant sows after experimental infection with *T. evansi*.

CHAPTER III

MATERIALS AND METHODS

The research was done as the following framework.

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┌─────────────────────────────────────────┐
│    Collected and cryopreserved T. evansi │
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                    ↓
┌─────────────────────────────────────────┐
│    Experimental infection in pregnant sow│
└─────────────────────────────────────────┘
                    ↓
┌─────────────────────────────────────────┐
│             Collecting the data          │
└─────────────────────────────────────────┘
                    ↓
┌──────────────┐  ┌──────────────┐  ┌──────────────┐
│ Proinflammatory│ │   Antibody   │  │ Clinical signs│
│   cytokines   │ │   responses  │  │ and pathology │
└──────────────┘  └──────────────┘  └──────────────┘
                    ↓
┌─────────────────────────────────────────┐
│      Evaluating data by statistical analysis │
└─────────────────────────────────────────┘
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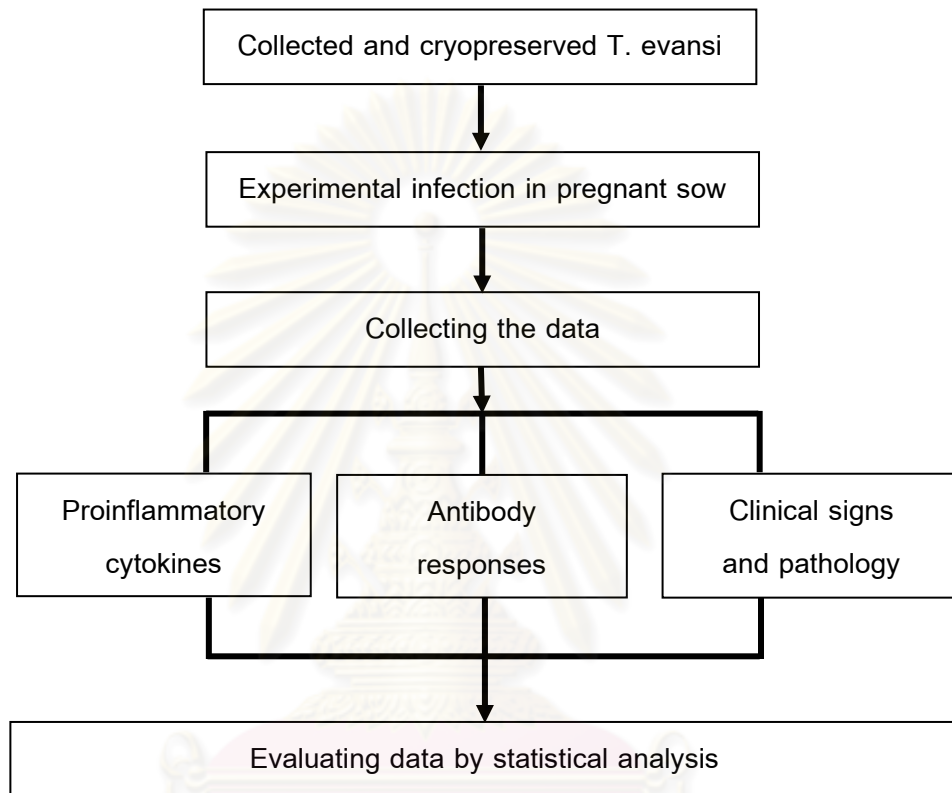
Figure 3.1 Conceptual framework of the research

Experimental sows

Six clinically healthy crossbred sows (Large white x Landrace) aged 4-4.5 years were purchased from a conventional breeding farm, free from *T. evansi* infection. Sows were housed in a fly-proof and tick-free concrete pen and treated on arrival with ivermectin (IVOMEC® 1%, Merial, Australia) at the dose 300 µg/kg bodyweight and orally fenbendazole powder mix directly into the feed at the dose 5 mg/kg bodyweight (Panacur® Powder 4%, Intervet, United States) to ensure being free from helminths and ectoparasites. Auricular thin blood smear was examined for trypanosomes by the microhaematocrit technique (MHCT) at the time of purchase and then weekly during a 30-day acclimatization period, to confirm that the animals remained free of infection.

Rectal temperatures and haematology were also monitored. The sows were kept in individual crates and fed twice daily with commercial diet formulating for pregnant sows. Water was available *ad libitum*.

After acclimatization period, oestrous detection was carried out by examination of the vulva for reddening and swelling as well as by control of the standing reflex in the presence of a boar. The oestrous detection was performed daily. Sows were inseminated with fresh semen, using a dose of $3x10^9$ spermatozoa in 80 ml of BTS extender at 24 h and 36 h after standing oestrous. The sows were subjected to pregnancy diagnosis (trans abdominal) twice on Days 18 at the first time and on 24 day after insemination by using real time ultrasound (50STringa, sector probe with 5 MHz, ESAOTE Pie Medical, The Netherlands) with return to oestrous detection by standing reflex test in the presence of a boar on Days 18-24 (Kaeoket et al., 2002).

T. evansi collection and cryopreservation

T. evansi originally isolated from a clinical milk cow case of surra in Kanchanaburi province was passaged twice in mice and collected the whole blood during high parasitaemia for cryopreservation. The concentration of *T. evansi* in blood from infected mouse was determined with Wright-Giemsa stained thin blood smears and Neubauer haemocytometer. Shortly after collection, *T. evansi* infected blood was diluted with cryoprotectant 3 ml composed glycerol 0.45 ml, mice blood 0.5 ml, phosphate buffered saline (PBS) 1.05 ml and sodium citrate 4.5% 1 ml to a concentration of 8×10^6 *T.evansi*/ml (Ndao et al., 2004; unpublished data by Taweethavornsawat and Kaeoket, 2005). And then 0.5 ml. processed blood was loaded into 1.5 ml cryotube. The cryotubes were sealed before being placed in a controlled rate freezer (Icecube 14s, Sylab, Purkersdorf, Austria). The freezing rate was 3° C/min from $+5^{\circ}$ to -5° C, 1 min of holding time, and thereafter 50° C/min from -5° to -140° C. Then, the cryotubes were plunged into LN_2 (-196° C) for storage.

Experimental infection

Thawing procedure: Thawing was achieved by immersing the cryotubes in water at 70° C for 1 min. Immediately after thawing, the post-thawed *T. evansi* was incubated in a 37° C water-bath for 30 min and evaluating *T. evansi* quality in terms of motility and viability before inoculating into mice. Inocula (0.5 ml) containing about 4 x 10^6 *T. evansi* were used to infect mice. Three mice were inoculated *T. evansi* intraperitoneally.

Isolation of *T.evansi*: After sub-inoculation into mice, parasitaemia was estimated daily during day 3rd to 10th after infection by the count of trypanosomes in a hundred microscopical fields of wet blood film under 22 x 22 coverslip using 400 magnification. Mice blood was collected when mice showed high parasitaemia presence of *T. evansi* more than 80-90% in microscopical fields. Mice with high parasitemia were anesthetized with ether and tripotassium hydrogen ethylenediaminetetraacetate (K$_3$-EDTA) blood was collected from the hearts. The numbers of *T. evansi* were determined in pooled blood with wet blood film and Neubauer haemocytometer according to the methodology previously described. The results were expressed as parasites/ml. Then the concentration of *T. evansi* in blood was diluted in sterile normal saline solution (0.90% w/v of NaCl). Inocula (0.5 ml) containing about 4 x 10^6 trypanosomes were used to infect the experimental sows.

Experimental infection in sows: Twenty five-day-pregnant-sows were randomly divided into two groups. In the treatment group, three sows were each inoculated intravenously with 4 x 10^6 trypanosomes (i.e. T1, T2 and T3). Similarly, three sows were each inoculated intravenously with diluted sterile mouse blood in normal saline 0.5 ml (i.e. C1, C2 and C3) in the control group.

Collection of blood samples

Two milliliters of blood were collected from sows daily during the first week after infection and thereafter every 2 days until the end of experiment (abortion or death) by venipuncture of peripheral ear vein and used for parasitaemia determination. Whole blood was preserved in K$_3$-EDTA anticoagulant tubes. After daily used for parasitaemia and haematology determination, whole blood was aliquoted into 1 ml eppendrofs and

kept at -80° C until used for PCR. In term of daily Haematology determination, blood samples were analysed for red blood cell count (RBC), total leucocyte count (WBC), haemoglobin (HB), mean corpuscular volume (MCV), mean corpuscular haemoglobin concentration (MCHC), mean corpuscular haemoglobin (MCH) and platelet count using ABX Micros ABC Vet automated hematology analyzer (HORIBA, Ltd), leucocyte differential counts using smears stained with Wright-Giemsa, packed cell volume (PCV) by microhaematocrit centrifugation method.

For serology, 10 ml blood samples were collected from the jugular vein of pregnant sows on days 0, 1, 3, 5, 7, 9, 11, 13, 15 after infection. Blood samples were allowed to clot for 2 hours at room temperature (25° C) before centrifuging for 20 minutes at 1000 x g. Sera were used for biochemical enzyme tests including ALT, ALP, AST, blood urea nitrogen (BUN) and creatinine. Biochemical enzymes were assayed by the test strip for the quantitative determination of ALT, ALP, AST, BUN and creatinine in serum using Reflotron® dry chemistry test strips and Reflotron® clinical chemistry analyzer (Roche Diagnostics Ltd, UK) according to the manufacturers recommendation. After routine biochemical enzyme testing, sera were aliquoted into 1 ml eppendrofs and stored at -80° C until used. Sera were preserved for proinflammatory cytokine assays (IL-1β, IL-6 and TNF-α), prostaglandin assays (PGE$_2$ and PGF$_{2\alpha}$), CATT/*T. evansi* and Ab-ELISA/*T. evansi* assay.

At the end of experiment, infected sow was treated by diminazene aceturate 3.5 mg/kg bodyweight twice 5 days apart. After that, all survivors were Euthanized (killed) and burned in animal carcass incinerator.

Clinical observation and parasitaemia evaluation

All animals were clinically examined daily. Rectal body temperature was monitored twice daily (6 am and 4 pm) with a digital thermometer and every clinical alteration was recorded. Parasitaemia was estimated by the MHCT (3 capillary tubes for each sample). Parasitaemia was scored according to the mean number of trypanosomes counted per capillary tube at the interface of the buffy coat and plasma, as follows: 0, none; 1, 1-10 *T. evansi*; 2, 11-20 *T. evansi*; 3, 21-30 *T. evansi*; 4, 31-40 *T.*

evansi; 5, >40 *T. evansi* (Woo, 1970; Holland et al., 2001; Reid et al., 2001). In addition, direct microscopic examination of blood and buffy coats smears stained with Wright-Giemsa were determined using a X10 eyepiece and X40 or X100 objective. The thin blood smear results were expressed as parasites/200RBC and were scored as follows: 0, none; 1, 1-10 *T. evansi*/200RBC; 2, 11-20 *T. evansi*/200RBC; 3, 21-30 *T. evansi*/200RBC; 4, 31-40 *T. evansi*/200RBC; 5, >40 *T. evansi*/200RBC.

Gross and microscopic examination

Gross pathological study: Aborted fetuses were collected. Gross pathology and crown-lump-length were evaluated. Blood from umbilical cord and amniotic fluid were collected by sterile technique and aliquoted into 1 ml eppendrofs and stored at -80° C until tested for PCR. Blood from umbilical cords and amniotic fluid were collected for finding the evidences of transplacental transmission of *T. evansi* in pigs.

Histopathological study: Samples including aborted fetus, placenta and umbilical cords were fixed in 10% buffered formalin and processed routinely. Tissues were embedded in paraffin wax, sectioned at 4 µm thickness and stained by Hematoxylin and Eosin (H & E). Sections were observed under light microscope for *T. evansi* infection in the interstitial tissues and visible histopathologic lesions of all collected samples.
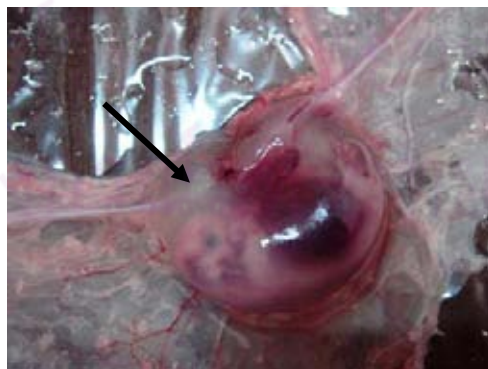
Figure 3.2 Location of amniotic fluid collection in aborted fetus (arrow)

Proinflammatory cytokine assay

TNF-α: TNF-α level was assessed by using enzyme immunoassay kits according to the manufacturer's protocol (R&D systems, Minneapolis, USA). This assay employs the quantitative sandwich enzyme immunoassay technique. A monoclonal antibody specific for porcine TNF-α was pre-coated onto a microplate. Duplicated samples are pipetted into the wells and porcine TNF-α protein is bound by the immobilized antibody. After washing the unbound substances, an enzyme-linked monoclonal antibody specific for porcine TNF-α is added to the wells. Following a wash to remove the unbound antibody-enzyme reagent, a substrate solution is added to the wells. The enzyme reaction yields a blue product and turns yellow when the stop solution is added. The intensity of the color measured is in proportion to the amount of porcine TNF-α bound in the initial step. The sample values are then compared with the standard curve.

IL-1β: IL-1β level was assessed by using enzyme immunoassay kits according to the manufacturer's protocol (R&D systems, Minneapolis, USA). This assay employs the quantitative sandwich enzyme immunoassay technique. A monoclonal antibody specific for porcine IL-1β was pre-coated onto a microplate. Duplicated samples are pipetted into the wells and porcine IL-1β protein is bound by the immobilized antibody. After washing the unbound substances, an enzyme-linked polyclonal antibody specific for porcine IL-1β is added to the wells. Following a wash to remove the unbound antibody-enzyme reagent, a substrate solution is added to the wells. The enzyme reaction yields a blue product and turns yellow when the stop solution is added. The intensity of the color measured is in proportion to the amount of porcine IL-1β bound in the initial step. The sample values are then compared with the standard curve.

IL-6: IL-6 level was assessed by using enzyme immunoassay kits according to the manufacturer's protocol (R&D systems, Minneapolis, USA). This assay employs the quantitative sandwich enzyme immunoassay technique. A polyclonal antibody specific for porcine IL-6 was pre-coated onto a microplate. Duplicated samples are pipetted into the wells and porcine IL-6 protein is bound by the immobilized antibody. After washing the unbound substances, an enzyme-linked polyclonal antibody specific for porcine IL-6

is added to the wells. Following a wash to remove the unbound antibody-enzyme reagent, a substrate solution is added to the wells. The enzyme reaction yields a blue product and turns yellow when the stop solution is added. The intensity of the color measured is in proportion to the amount of porcine IL-6 bound in the initial step. The sample values are then compared with the standard curve.

Prostaglandin assay

$\underline{PGE_2}$: PGE_2 level was assessed by a competitive binding technique using enzyme immunoassay kits according to the manufacturer's protocol (R&D systems, Minneapolis, USA). In brief, duplicated samples were incubated onto the microplate competing with fixed amount of alkaline phosphatase-labeled PGE_2. Excess conjugate and unbound sample were washed out before adding a substrate solution to find out the bound enzyme activity. Immediately after color development, the microplate reader absorbance was read at 450 nm. The color intensity is inversely proportional to the serum PGE_2 concentration. A standard curve was prepared by plotting the mean absorbance for each standard on a linear y-axis againt the concentration on a logarithmic x-axis and draw the best fit curve through the points on the graph. And then concentration of PGE_2 was read from the standard curve.

$\underline{PGF_{2\alpha}}$: $PGF_{2\alpha}$ level was assessed by a competitive binding technique using enzyme immunoassay kits according to the manufacturer's protocol (Assay designs, Inc., Ann Arbor, USA) In brief, duplicated samples were incubated onto the microplate competing with fixed amount of alkaline phosphatase-labeled $PGF_{2\alpha}$. Excess conjugate and unbound sample were washed out before adding a substrate solution to find out the bound enzyme activity. Immediately after color development, the microplate reader absorbance was read at 405 nm. The yellow color intensity is inversely proportional to the serum $PGF_{2\alpha}$ concentration. A standard curve was prepared by plotting the mean absorbance for each standard on a linear y-axis againt the concentration on a logarithmic x-axis and draw the best fit curve through the points on the graph. And then concentration of $PGF_{2\alpha}$ was read from the standard curve.

PCR diagnosis

Briefly, one hundred microlitres of amniotic fluid or buffy coat collected from the centrifugation of 1 ml of blood were subjected to DNA purification using the phenol chloroform method (Sambrook and Russell, 2001). PCR was carried out in 10-µl master mixed volumes with 1 µl of sample, using TBR primers according to a protocol previously described (Masiga et al., 1992; Pruvot et al., 2010). Care was taken to work by ascending concentration to avoid contamination.

TBR1/2 primers (Masiga et al., 1992) were used to amplify a 164bp highly repeated sequence of mini-chromosome satellite DNA. It was the first PCR primer set described and largely used for detection of *Trypanozoon* DNA. The nucleotide strands of TBR1/2 primers are:

TBR1: 5' GAATATTAAACAATGCGCAG 3'	(forward)
TBR2: 5' CCATTTATTAGCTTTGTTGC 3'	(backward)

The sizes of specifically amplified products are 164bp.

After samples were prepared as the following, 1 µl of sample mixed with a reaction mixture containing 10-µl master mixed composing of:

1 x 10 mM Tris; 50 mM KCl	1.10 µl	(buffer)
200 µM dNTP with stock solution dNTP	0.88 µl	(dNTP)
1.5 mM $MgCl_2$	0.33 µl	($MgCl_2$)
1 µM Primer 1	0.55 µl	(TBR1)
1 µM Primer 2	0.55 µl	(TBR2)
0.5 unit TagPol per 11 µl of MM	0.11 µl	(TagPol)
Distilled water	6.48 µl	(distilled water)

Then PCR reactions run in PCR Px2 Thermal Cycler (Thermo Electron Corporation; Waltham, Massachusetts, USA) with a condition as follow:

	Temperature ($^\circ$ C)	Time (Second)	
Innitial denaturation	94	60	
Denaturation	94	30	x 30 cycles
Annealing	60	60	x 30 cycles

Extension	72	30	x 30 cycles
Termination	72	120	
Holding	4	∞	

After PCR cycling, PCR products including samples, positive controls (infected mouse blood) and negative controls (distilled water) were subjected for electrophoresis 1 hour at 120 V in 2% agarose gels, together with Generuler® 100-bp DNA ladder plus (Fermentas, Invitrogen Corporation, Van Allen Way, Carlsbad, CA). Gels were stained with ethidium bromide and visualized under UV light (λ = 302 nm, DyNA Light, Labnet) for examination.

Serological examination

The card agglutination test for trypanosomes (CATT/*T. evansi*) (antigen lot 10A1B2) was carried out on sera diluted 1:4 as described by the manufacturer (Prince Leopold Institute of Tropical Medicine, Belgium). The CATT/*T. evansi* is a rapid direct agglutination test using freeze-dried trypanosomes of *T. evansi* VAT Rotat 1.2 fixed with formaldehyde and stained with Coomassie blue (Bajyana Songa and Hamers, 1988). Testing starts at a dilution of 1:4. If a test proved positive, additional two-fold serum dilutions were tested to obtain the final antibody titer. The results of CATT/*T. evansi* (+/-) were scored as follow; negative(-) = 0, positive(+) = 1, positive(++) = 2, positive(+++) = 3.

The Ab-ELISA/*T. evansi* procedure was done as previously described (Desquesnes et al., 2007; Desquesnes et al., 2009). 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) overnight at 4 °C. Plates were blocked with 150 µl/well of blocking buffer: phosphate buffered saline (PBS) with 0.1% Tween 20® (Sigma-Aldrich) and 7% Wako skim milk powder (ref: 190-12865, Wako Pure Chemical Industries Ltd., Osaka, Japan), under permanent shaking (150 rpm) for 45 min at 37 °C. After discarding the blocking buffer, sera diluted 1:100 in blocking buffer were transferred in duplicate on the ELISA plates. After 30 min incubation in a shaker-incubator (37 °C, 150 rpm), the plates were washed

seven times with washing buffer (PBS-0.1% Tween 20[®]). 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 (37 °C, 150 rpm). After discarding sera, the plates were washed seven times and added to 100 µl of the complex substrate/chromogen 3,3′,5,5′-tetramethylbenzidine (TMB) (Sureblue[®] TMB Microwell Peroxidase Substrate, Gaithersburg, Maryland, USA). Plates were incubated in a dark room for 30 min. Optical density (OD) was measured at 630 nm in an ELISA reader (Dynex Technologies[®], Chantilly, VA, USA). ELISA *T. evansi* results were expressed in relative percentage of positivity (RPP) *versus* three negative and three positive controls, according to the following ratio:

$$\text{RPP of a sample} = \frac{\text{Mean OD of the sample} - \text{Mean OD of the negative controls}}{\text{Mean OD of the positive controls} - \text{Mean OD of the negative controls}}$$

Positive results were confirmed when the RPP was above 20%.

Statistical analysis

The statistical analysis was performed using the Statistical Analysis Systems software package (Version 9.0, SAS Institute Inc., 1996, Cary, N.C., USA). Descriptive statistics were used to describe parasitaemia, clinical signs and clinical pathology. The difference between treatment group and control group were compared using unpaired t-test. Pearson's correlation was used to evaluate the correlation among cytokine responses, biochemical enzymes, prostaglandins and body temperature. Differences among groups were tested by one-way analysis of variance, including data on body the temperature of sows; level of TNF-α, IL-1β, IL-6, PGE$_2$, PGF$_{2\alpha}$, BUN, creatinine, ALT, AST, ALP; and haematology values. The significance level was set at $P<0.05$.

Ethical approval

All research procedures were approved by the Faculty of Veterinary Science-Animal Care and Use Committee (FVS-ACUC) Mahidol University, number MUVS-2010-30.

CHAPTER IV

RESULTS

Parasitaemia

Table 4.1 Parasitaemia scores using MHCT and Wright-Giemsa stained thin blood smear of infected T1 sows.

Sow	Day	Parasitaemia scores	
		MHCT**	Blood smear***
T1	0	0	0
	1	0	0
	2	0	0
	3	1	0
	4	1	1
	5	2	1
	6	3	1
	7	2	1
	9	ND	ND
	11*	2	1
	13	2	1
	15	0	0

ND = No data

* Abortion day

** Parasitaemia scores was determined as follows; 0, none; 1, 1-10 *T. evansi*; 2, 11-20 *T. evansi*; 3, 21-30 *T. evansi*; 4, 31-40 *T. evansi*; 5, >40 *T. evansi*.

*** Parasitaemia scores was determined as follows; 0, none; 1, 1-10 *T. evansi*/200RBC; 2, 11-20 *T. evansi*/200RBC; 3, 21-30 *T. evansi*/200RBC; 4, 31-40 *T. evansi*/200RBC; 5, >40 *T. evansi*/200RBC.

Table 4.2 Parasitaemia scores using MHCT and Wright-Giemsa stained thin blood smear of infected T2 sows.

Sow	Day	Parasitaemia scores	
		MHCT**	Blood smear***
T2	0	0	0
	1	0	0
	2	0	0
	3	0	0
	4	0	0
	5	0	0
	6	0	0
	7	1	0
	9	1	1
	11	5	1
	13	5	5
	14*	0	0
	15	1	0

* Abortion day

** Parasitaemia scores was determined as follows; 0, none; 1, 1-10 *T. evansi*; 2, 11-20 *T. evansi*; 3, 21-30 *T. evansi*; 4, 31-40 *T. evansi*; 5, >40 *T. evansi*.

*** Parasitaemia scores was determined as follows; 0, none; 1, 1-10 *T. evansi*/200RBC; 2, 11-20 *T. evansi*/200RBC; 3, 21-30 *T. evansi*/200RBC; 4, 31-40 *T. evansi*/200RBC; 5, >40 *T. evansi*/200RBC.

Table 4.3 Parasitaemia scores using MHCT and Wright-Giemsa stained thin blood smear of infected T3 sows.

Sow	Day	Parasitaemia scores	
		MHCT**	Blood smear***
T3	0	0	0
	1	0	0
	2	0	0
	3	0	0
	4	0	0
	5	0	0
	6	0	0
	7	0	0
	9	5	1
	11*	1	1
	13	0	0
	15	1	1

* Abortion day

** Parasitaemia scores was determined as follows; 0, none; 1, 1-10 *T. evansi*; 2, 11-20 *T. evansi*; 3, 21-30 *T. evansi*; 4, 31-40 *T. evansi*; 5, >40 *T. evansi*.

*** Parasitaemia scores was determined as follows; 0, none; 1, 1-10 *T. evansi*/200RBC; 2, 11-20 *T. evansi*/200RBC; 3, 21-30 *T. evansi*/200RBC; 4, 31-40 *T. evansi*/200RBC; 5, >40 *T. evansi*/200RBC.

Trypanosomes were first detected by MHCT in the blood of all infected sows between 3-9 days after infection. Accordingly, recurring peaks and troughs of parasitaemia in each infected animal was regular (Tables 4.1, 4.2 and 4.3). No trypanosomes were found in any of the uninfected control sows (C1, C2 and C3).

Individual variation of the peak of parasitaemia in infected sows was observed, and the parasitaemia scores showed fluctuation of the *T. evansi* in blood (Figures 4.1,

4.2 and 4.3). Interestingly, no trypanosomes were detected in the blood by microscopic examination when T2 sow showed abortion at day 14.
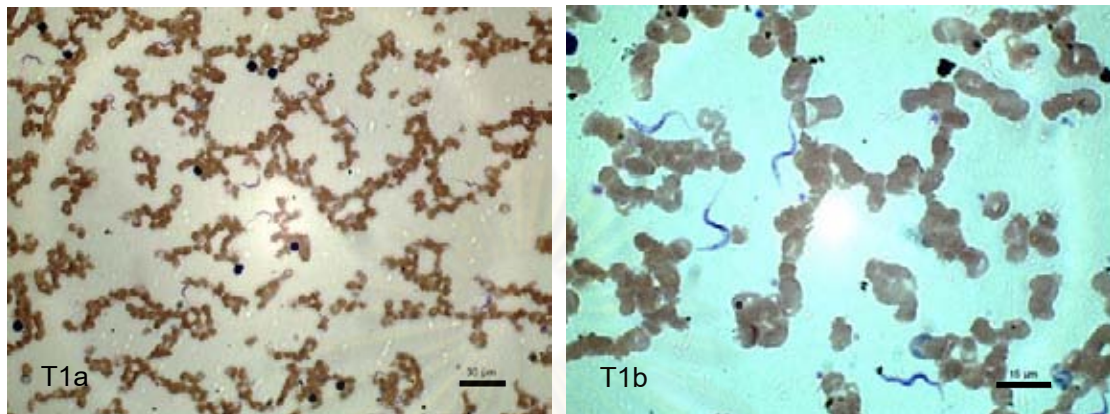
Figure 4.1 *T. evansi* in Wright-Giemsa stained thin blood smear of T1 sow score 1 including T1a (bar = 30 μm) and T1b (bar = 15 μm) at day 12 after inoculation.
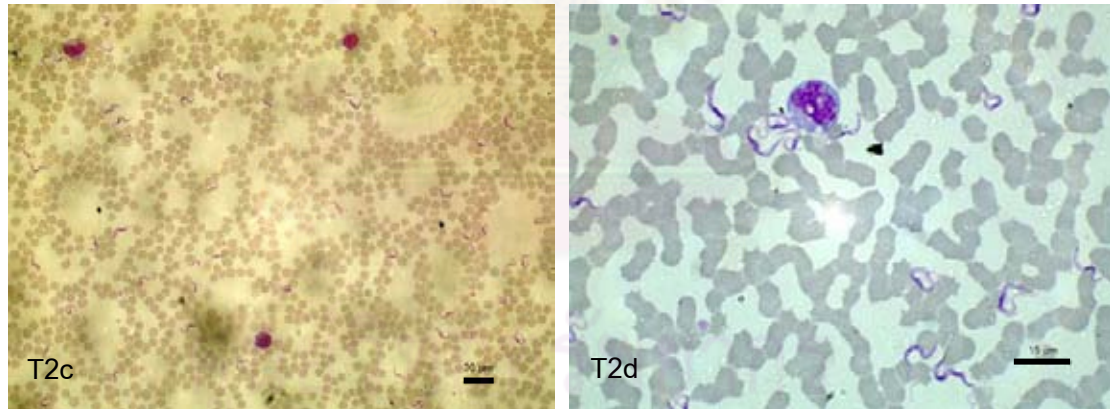
Figure 4.2 *T. evansi* in Wright-Giemsa stained thin blood smear of T2 sow score 5 including T2c (bar = 20 μm) and T2d (bar = 15 μm) at day 13 after inoculation.
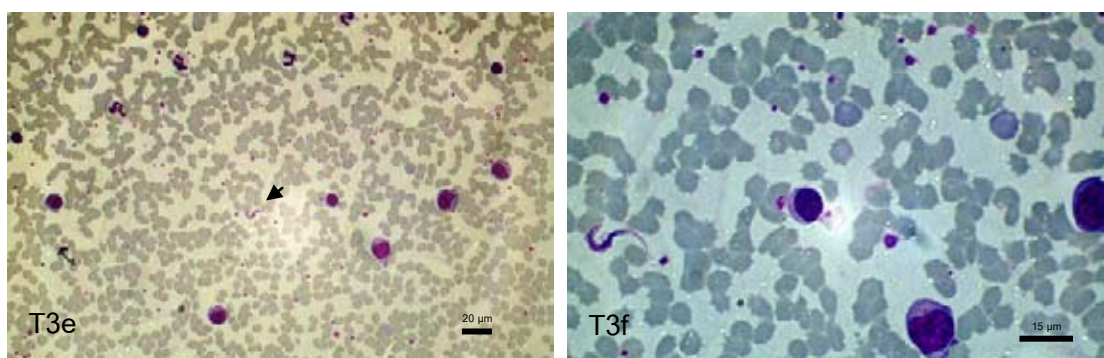
Figure 4.3 *T. evansi* in Wright-Giemsa stained thin blood smear of T3 sow score 1 including T3e (bar = 20 μm) and T3f (bar = 15 μm) at day 15 after inoculation.

Clinical signs

All infected sows showed varying degree of clinical signs including weakness, loss appetite, abortion, a typical skin rash with erythema and point bleedings, petechial or plaque hemorrhage on the skin of udder and ventral part of the body. Skin lesion could be seen as the small light pink to dark purple area (Figures 4.5, 4.6 and 4.7). The infected sows were aborted during 11-14 day after infection. The infected sows (T1, T2 and T3) had no appetite during 2-4 day before abortion, and 1 day before abortion the rectal temperature increased to 39.5°-40.3° C (Figure 4.4). Crown-lump-length of aborted fetuses was range 1.5 – 1.7 cm.

Table 4.4 Clinical signs of experimental *T.* evansi-infected sows

Sows	First parasitaemia (dpi)	Inappetance (dpi)	Abortion (dpi)	Skin lesion (dpi)
T1	3	8-11	11	10-15
T2	7	10-14	14	3-23*
T3	9	10-12	11	6-21

* T2 showed the most severe skin lesion, especially during days 9-23.
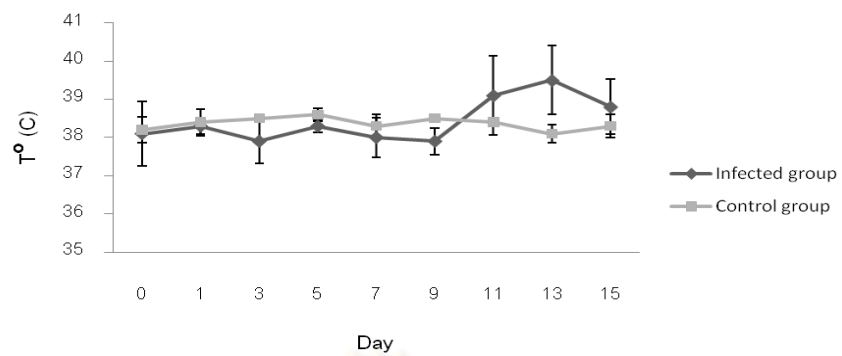
Figure 4.4 Rectal temperature ($^{\circ}$ C) of infected and control sows along the experimental period.
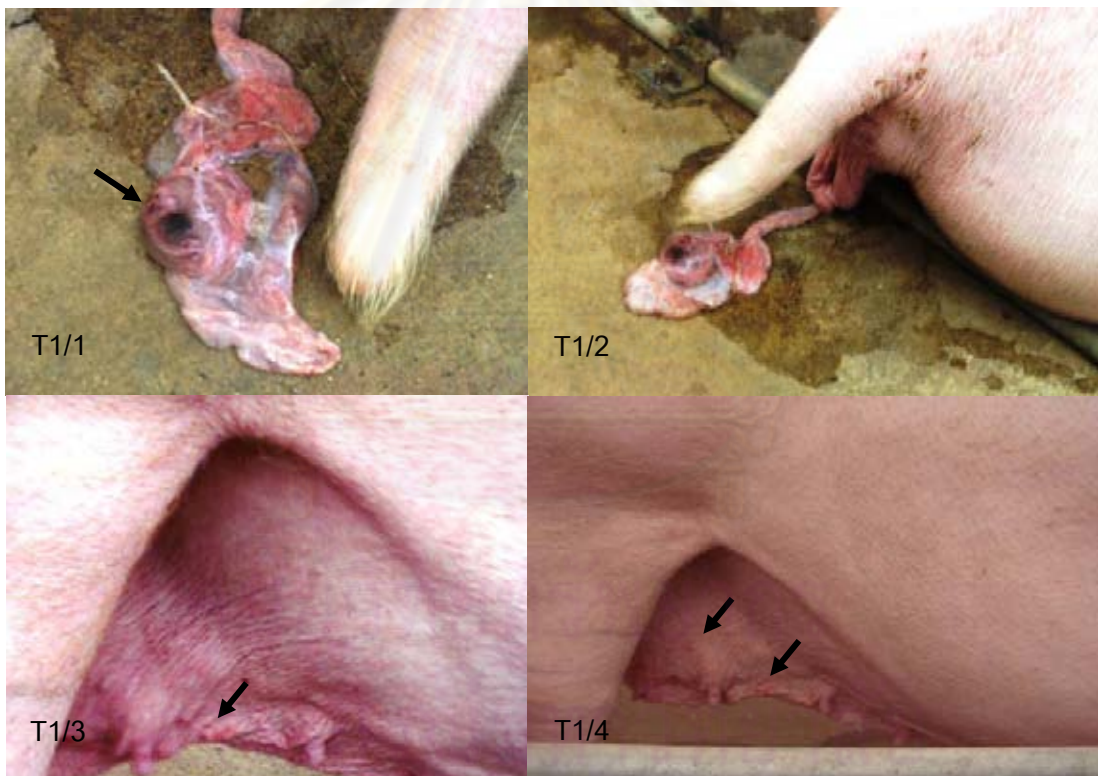
Figure 4.5 Clinical signs of T1 sow: aborted fetus on day 11 (T1/1), abortion on day 11 (T1/2), skin rash and petechial hemorrhage during days 9-10 (T1/3), skin lesion recovery on day 12 with small area of papules and petechial hemorrhage (T1/4).

Figure 4.6 Clinical signs of T2 sow: abortion and aborted fetus on day 14 (T2/1,T2/2 and T2/3), skin rash and petechial hemorrhage (T2/5), plaques hemorrhage on the skin of udder (T2/4 and T2/6), weight loss (T2/7), skin lesion recovery at day 7 after treatment (T2/8).

Figure 4.7 Clinical signs of T3 sow: abortion and aborted fetus on day 11 (T3/1 and T3/2), skin rash, pruritus, petechial and ecchymotic hemorrhage on the skin of udder (T3/3 and T3/4).

Histopathological study

      Sections of aborted fetus, placenta and umbilical cords were observed under light microscope for the presence of *T. evansi* organisms in the interstitial tissues. In this study, *T. evansi* was not observed in the interstitial tissues of all collected samples, while congestion and haemorrhage were found (Figures 4.8, 4.9 and 4.10).
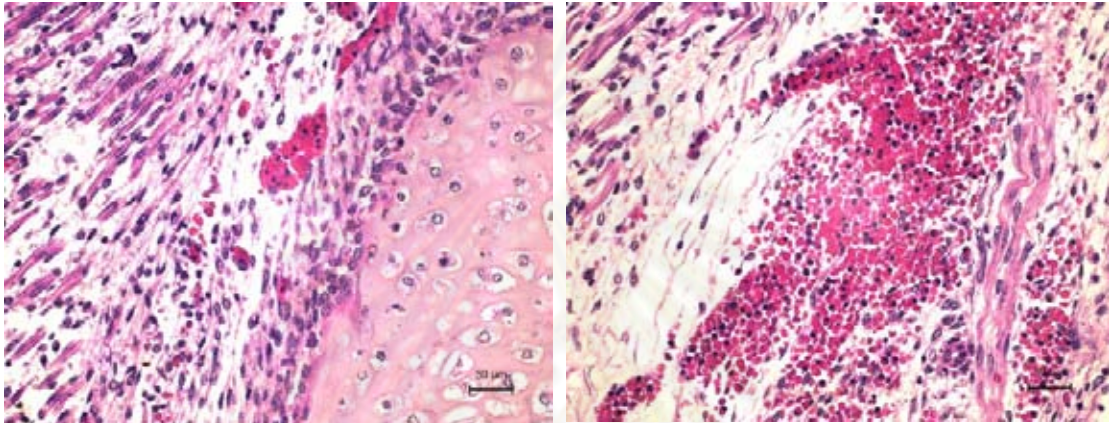
Figure 4.8 Section of fetuses from aborted sows. Congestion and haemorrhage were observed in the interstitial tissues. (H&E stain, Bar = 30 µm)
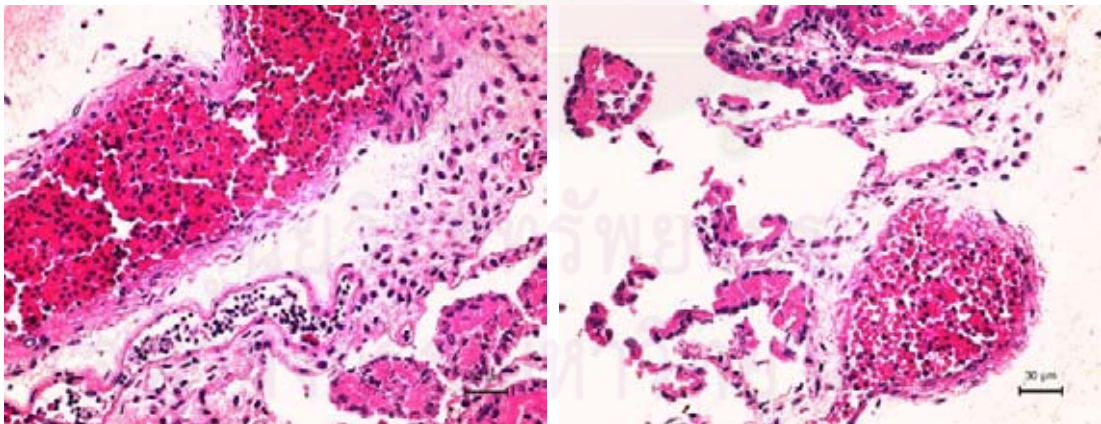
Figure 4.9 Section of placenta from aborted sows. Congestion was observed. (H&E stain, Bar = 30 µm)
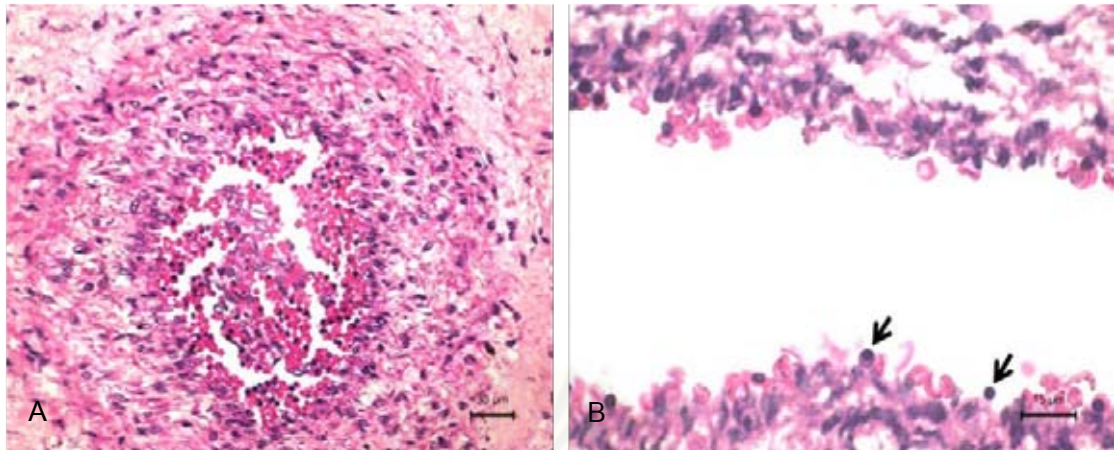
Figure 4.10 Section of umbilical cords from aborted sows. A, Congestion was observed (Bar = 30 µm). B, A few lymphocytes (arrow) were found in umbilical cord lumen (Bar = 15 µm). H&E stain.

Haematology

There was a progressive decline of RBC, PCV and haemoglobin (HB) of all infected sows, starting from 1 week after inoculation through the end of the trial (Figure 4.11). Comparison of RBC, MCV, MCHC and HB between groups, when the number of animals was sufficient for statistical analysis, revealed significant differences between these infected and non-infected control groups ($P<0.05$) (Figure 4.11, Appendix A). Total RBC in the infected group was progressive declined and slightly anemia was observed on 15 dpi, while mean PCV, MCV, MCH, MCHC and HB values were within the normal ranges.

Total leucocyte counts in infected group decreased during 3-5 dpi, thereafter the WBC increased progressively at 2 weeks after inoculation (Figure 4.12). The leucocyte classification and numbers were fluctuated in the infected group (Figure 4.12). Monocytosis was observed in the infected group on 15 dpi and was significantly higher than those of the control group ($P<0.05$). However, no alteration was observed in total leucocyte counts and classification. A decrease in blood platelets from days 11 to 15 of infection was observed. Consequently, thrombocytopenia was seen in the *T. evansi* infected sow which was compatible with the clinical signs such as petechial and ecchymotic hemorrhage of skin.
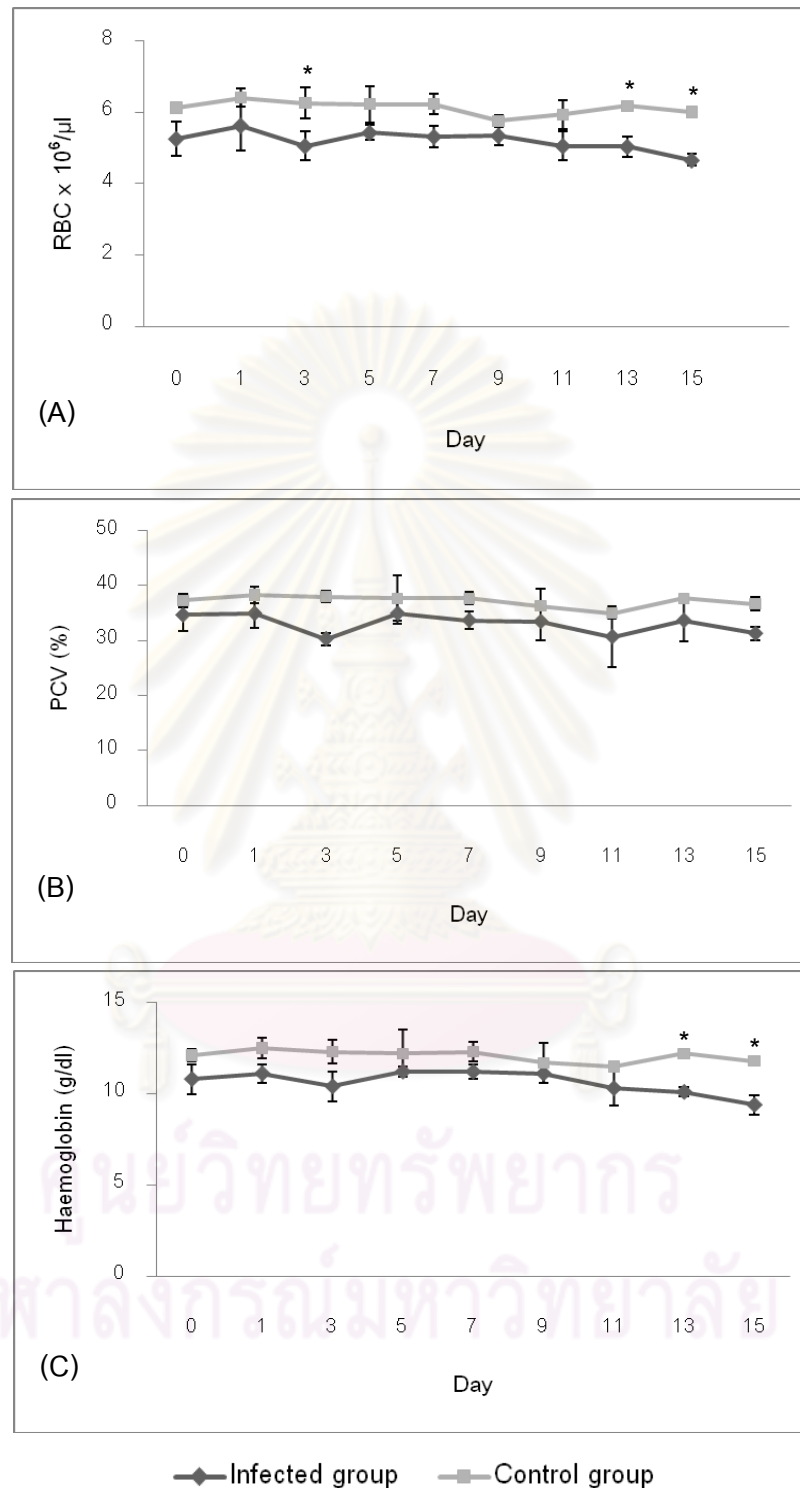
Figure 4.11 Mean ± standard deviation (SD) of the haemogram of pregnant sows experimentally infected by *T. evansi*: (A) = RBC, (B) = PCV, (C) = Haemoglobin.

* = *P* < 0.05
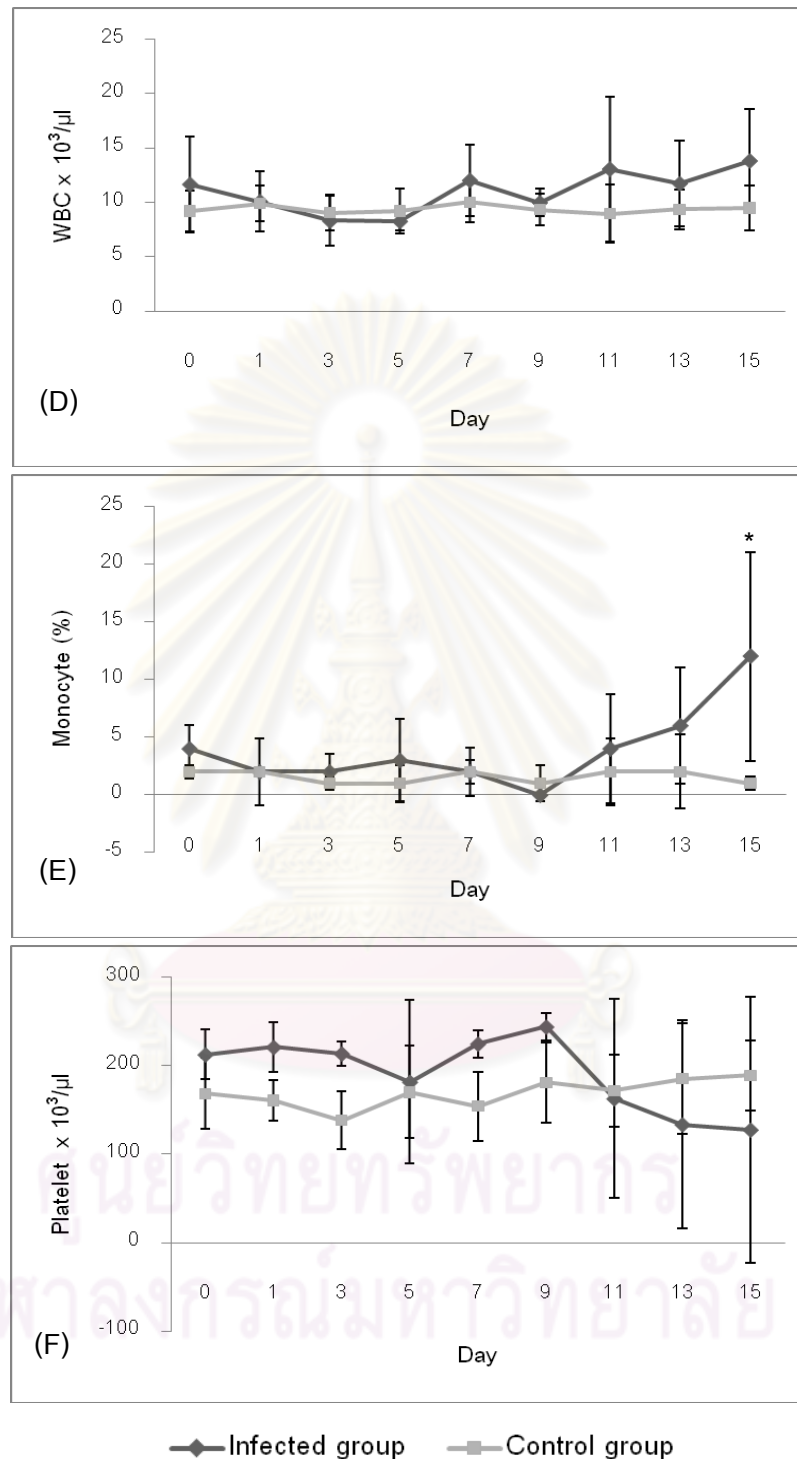
Figure 4.12 Mean ± standard deviation (SD) of the leucogram of pregnant sows experimentally infected by *T. evansi*: (D) = WBC, (E) = Monocyte, (F) = Platelet count.
* = *P* < 0.05

Serum biochemistry values

The results of serum biochemical tests including ALT, AST, ALP, BUN, creatinine and total protein are presented in Figures 4.13 and 4.14. No alteration was observed among groups, while only T3 sow showed the elevation of ALT and AST values on days 13 and 15 postinfection. An increase in ALT and AST of T3 sow was 7-10 times higher than the normal range. The ALT values of T3 sow on days 13 and day 15 postinfection were 1233 and 1098, and the AST values were 744 and 614, respectively.
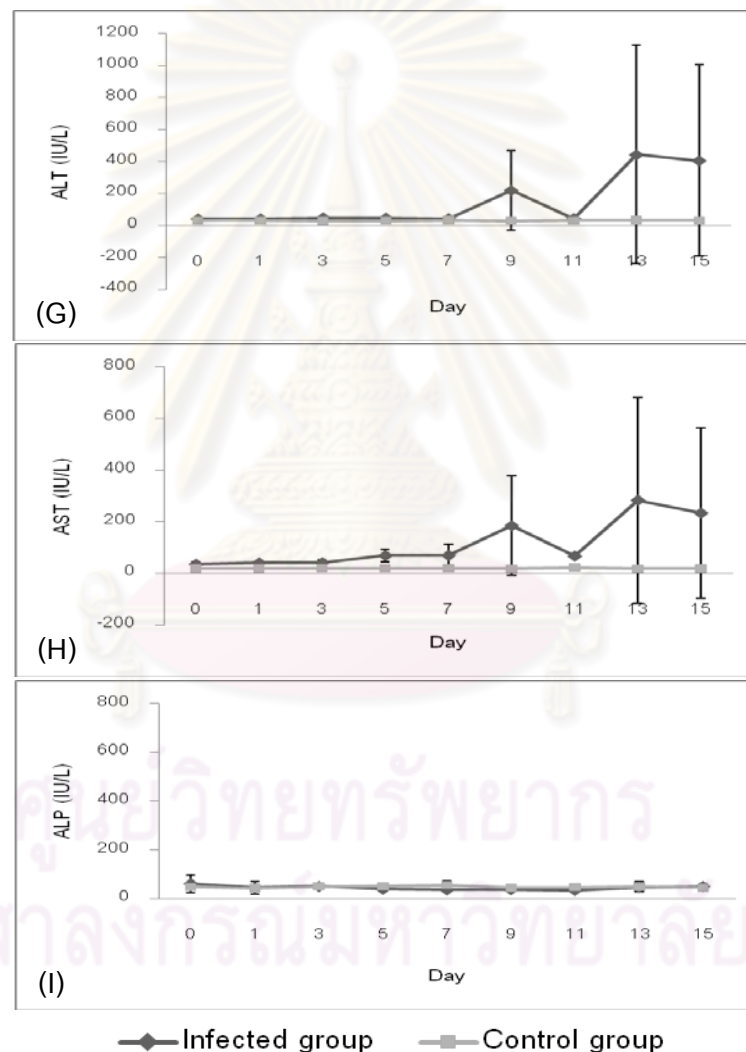
Figure 4.13 Mean ± standard deviation (SD) of the serum biochemical values of experimented sows: (G) = ALT, (H) = AST and (I) = ALP.
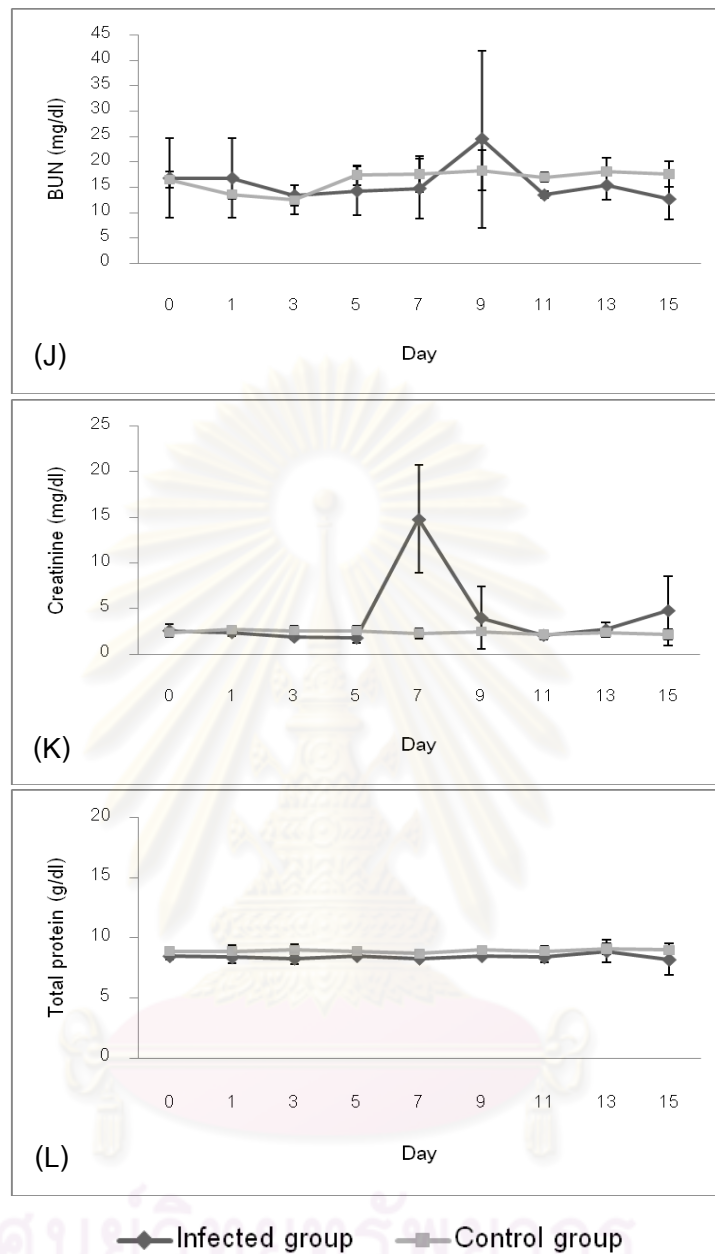
Figure 4.14 Mean ± standard deviation (SD) of the serum biochemical values of experimented sows: (J) = BUN, (K) = creatinine and (L) = Total protein.

Proinflammatory cytokine assays

The results of the present study showed no significant changes and differences in production of all three cytokines (TNF-α, IL-1β, IL-6) tested by sera between infected and control groups, although mild alteration was observed (Figure 4.15).
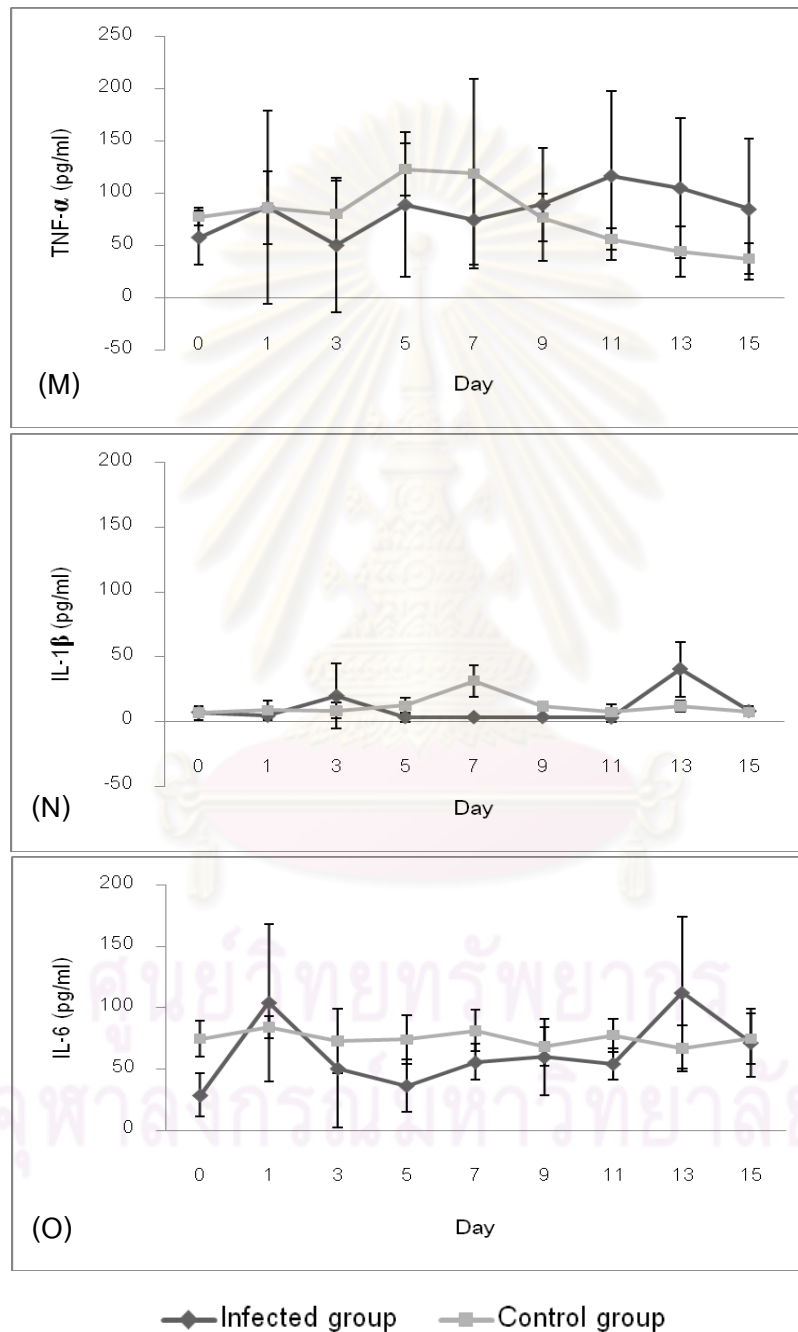
Figure 4.15 Mean $\pm$ standard deviation (SD) of the cytokine levels of experimented sows: (M) = TNF-α, (N) = IL-1β and (O) = IL-6.

Prostaglandin assays

The level of systemic prostaglandins (PGE_2 and $PGF_{2\alpha}$) in control group was higher than infected group, although the control group has no clinical signs such as fever and abortion. Interestingly, all sows in the infected group had fever and abortion, and fluctuation level of systemic $PGF_{2\alpha}$ along the experimental period was observed (Figure 4.16). It should be noted that PGE_2 levels showed significant correlation to $PGF_{2\alpha}$ ($R^2 = 0.89016$, $P<0.0001$).
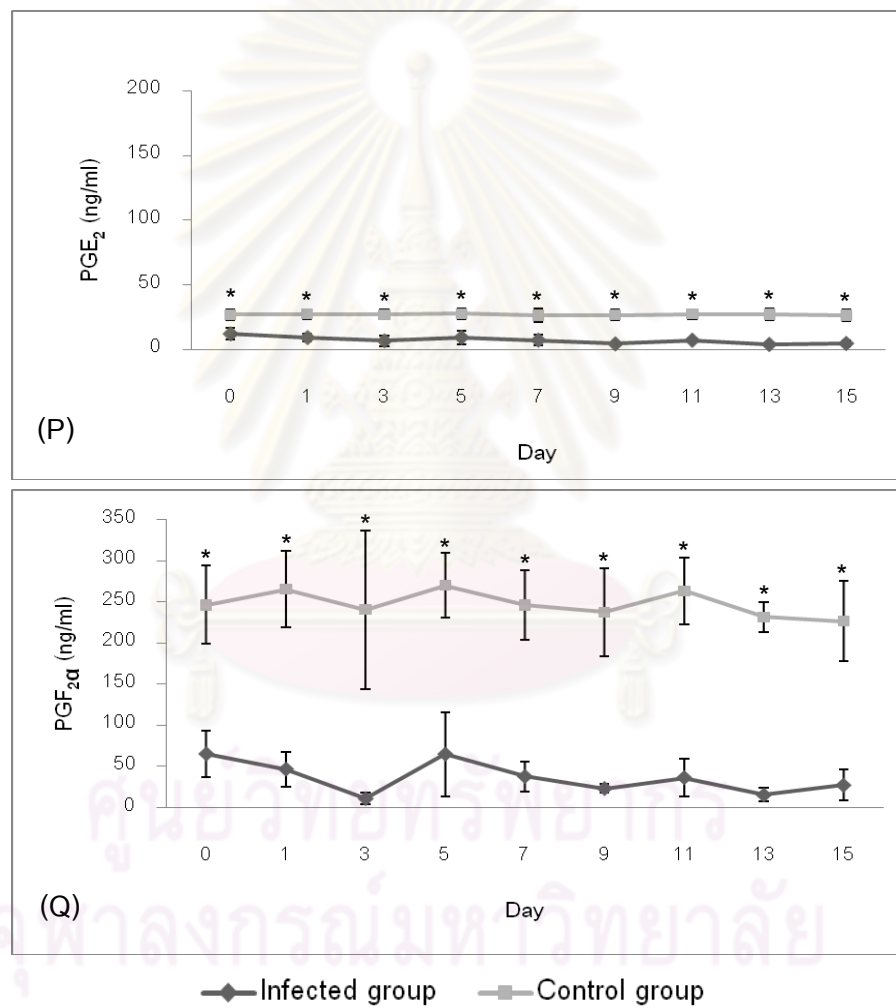
Figure 4.16 Mean ± standard deviation (SD) of prostaglandins levels of pregnant sows experimentally infected with *T. evansi*: (P) = PGE_2 and (Q) = $PGF_{2\alpha}$. * = $P < 0.05$

PCR diagnosis

The results revealed that all 3 pregnant sows in *T. evansi* infected group were positive by PCR identification of trypanosomes, while the results of control group were all negative (Table 4.3).

Table 4.5 Results of PCR assay in 6 experimental sows

Sows	PCR		
	Blood	Blood from umbilical cords	Amniotic fluid
T1	1	ND	1
T2	1	ND	1
T3	1	1	1
C1	0	0	0
C2	0	0	0
C3	0	0	0

ND = No data

Interestingly, *T. evansi* were found not only in blood of infected sows but also found in amniotic fluid of aborted fetuses. Moreover, blood collected from aborted fetuses' umbilical cords of T3 sow was also positive for *T. evansi* (Figures 4.17, 4.18 and 4.19).
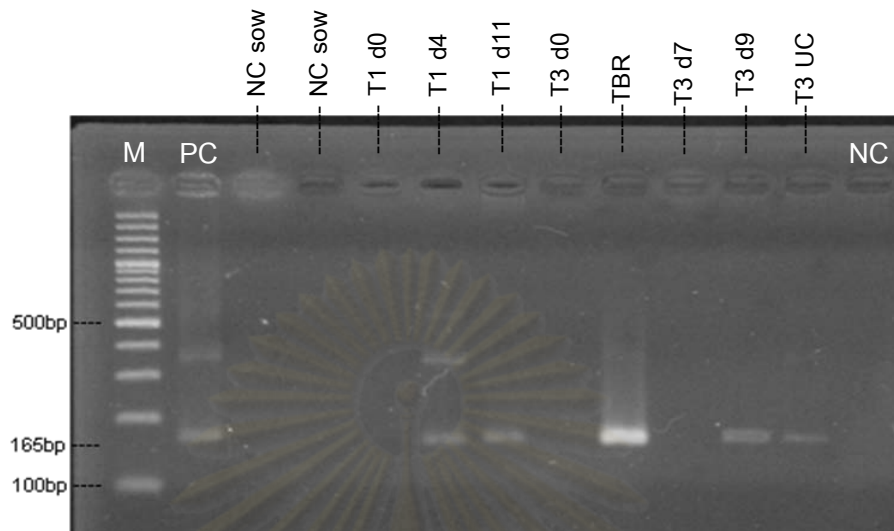
Figure 4.17 PCR detection of *T. evansi* using TBR1/2 primer sets. Lane M represents marker 100 bp plus (Fermentas); lane PC represents positive control; lane NC sow represents negative control sow; lane T1 d0 represents negative blood of T1 sow on the first day of *T. evansi* infection; lane T1 d4 represents positive blood of T1 sow on 4 dpi; lane T1 d11 represents positive blood of T1 sow on 11 dpi (abortion day); lane T3 d0 represents negative blood of T3 sow on 0 dpi; lane TBR represents positive *T. evansi* cloned TBR product; lane T3 d7 represents negative blood of T3 sow on 7 dpi; lane T3 d9 represents positive blood of T3 sow on 9 dpi; lane T3 UC represents positive umbilical cords blood from aborted fetuses of T3 sow on 11 dpi (abortion day); lane NC represents negative control.
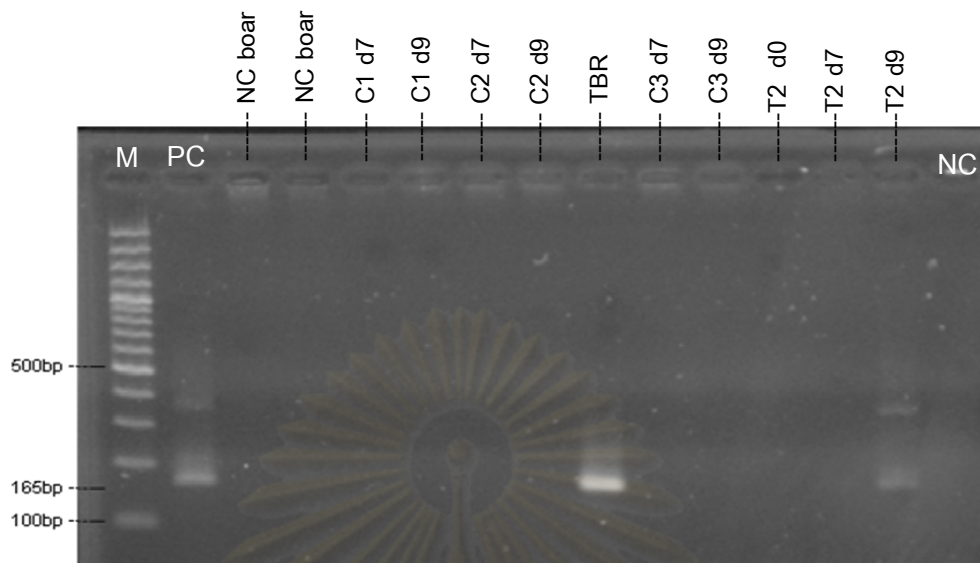
Figure 4.18 PCR detection of *T. evansi* using TBR1/2 primer sets. Lane M represents marker 100 bp plus (Fermentas); lane PC represents positive control; lane NC boar represents negative control boar; lane C1 d7 represents negative blood of C1 sow on day 7th; lane C1 d9 represents positive blood of C1 sow on day 4th; lane C2 d7 represents negative blood of C2 sow on day 7th; lane C2 d9 represents negative blood of C2 sow on day 9th; lane TBR represents positive *T. evansi* cloned TBR product; lane C3 d7 represents negative blood of C3 sow on day 7th; lane C3 d9 represents negative blood of C3 sow on day 9th; lane T2 d0 represents negative blood of T2 sow on the first day of *T. evansi* infection; lane T2 d7 represents negative blood of T2 sow on 7 dpi; lane T2 d9 represents positive blood of T2 sow on 9 dpi; lane NC represents negative control.
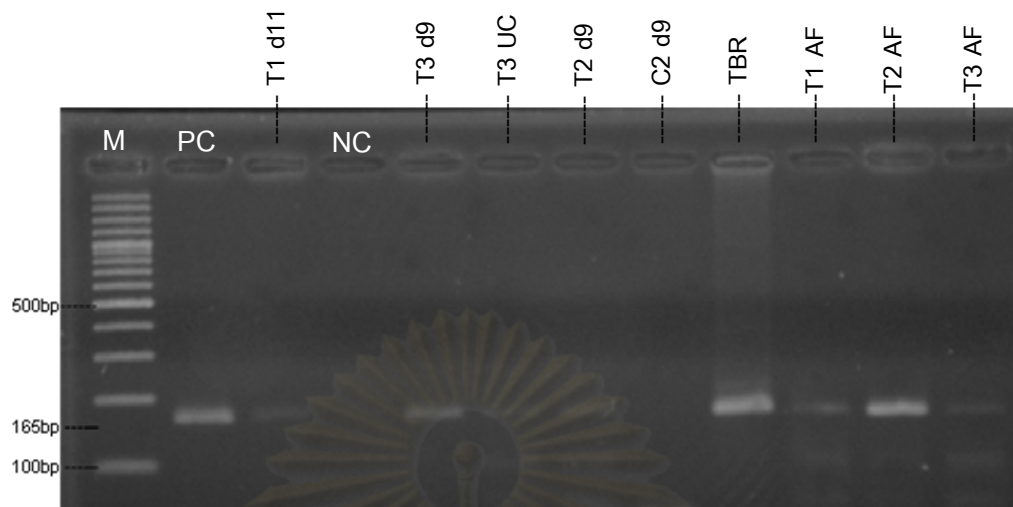
Figure 4.19 PCR detection of *T. evansi* using TBR1/2 primer sets. Lane M represents marker 100 bp plus (Fermentas); lane PC represents positive control; lane T1 d11 represents positive blood of T1 sow on 11 dpi (abortion day); lane NC represents negative control; lane T3 d9 represents positive blood of T3 sow on 9 dpi; lane T3 UC represents positive umbilical cords blood from aborted fetuses of T3 sow on 11 dpi (abortion day); lane T2 d9 represents positive blood of T2 sow on 9 dpi; lane C2 d9 represents negative blood of C2 sow on day 9th; lane TBR represents positive *T. evansi* cloned TBR product; lane T1 AF represents positive amniotic fluid of T1 sow; lane T2 AF represents positive amniotic fluid of T2 sow; lane T3 AF represents positive amniotic fluid of T3 sow.

Serological examination

CATT/*T. evansi*

A rapid direct agglutination test showed the positive results from sera of infected sows and negative results in uninfected control group (Table 4.6). The positive results in infected sows indicated that the infected sow had the antibody response after experimental *T. evansi* infection, whereas uninfected control sows were still clear from *T. evansi* infection.

Table 4.6 Direct agglutination test of sera by CATT/*T. evansi* in experimental sows.

DPI	CATT +/- (Score 0-3)*					
	T. evansi infection			Control		
	T1	T2	T3	C1	C2	C3
0	0	0	0	0	0	0
1	ND	ND	ND	ND	ND	ND
3	ND	ND	ND	ND	ND	ND
5	0	1	0	0	0	0
7	0	1	0	0	0	0
9	ND	2	0	0	0	0
11	1	2	3	0	0	0
13	2	3	3	0	0	0
15	ND	3	3	0	0	0

* Negative (-) = 0, positive (+) = 1, positive (++) = 2, positive (+++) = 3

ND = No data

Ab-ELISA/*T. evansi*

In the present study (Figure 4.17), antibody-enzyme linked immunosorbent assay for *T. evansi* (Ab-ELISA/*T. evansi*) was applied to confirm infection status of experimental sows. All sows in *T. evansi* infected group (T1, T2 and T3) became seropositive by Ab-ELISA/*T. evansi* at day 9 day after infection and the results were negative in the non-infected group (C1, C2 and C3).
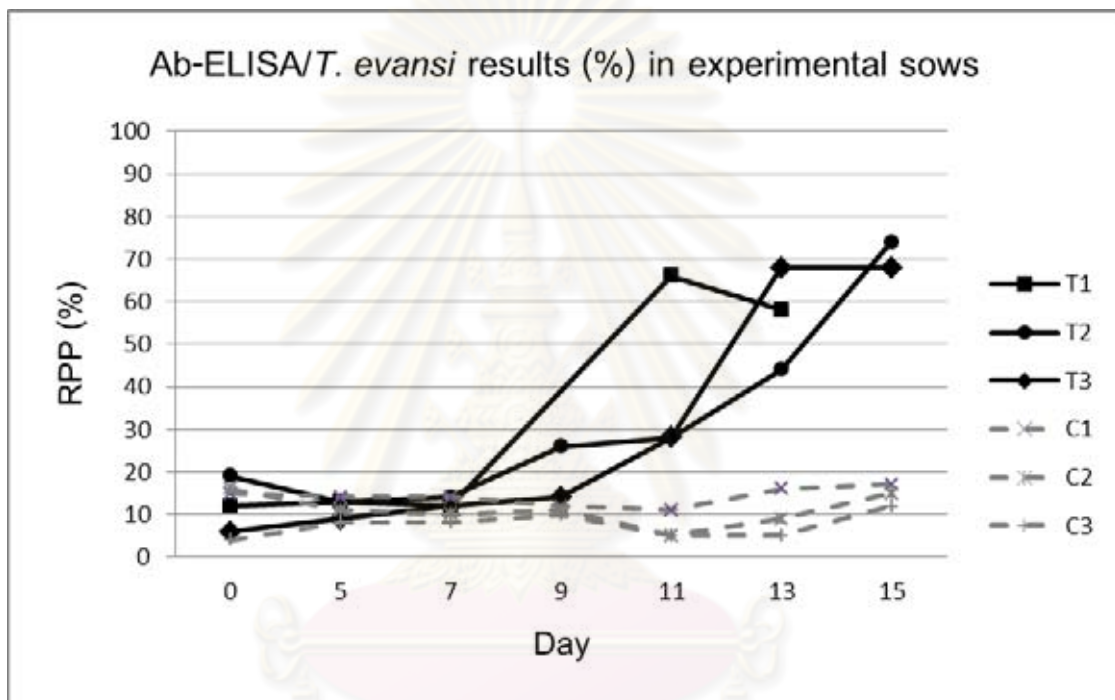
Figure 4.20 Relative percentage of positivity (RPP) of samples by Ab-ELISA/*T. evansi*, results were considered positive when the RPP was above 20%.

CHAPTER V

DISCUSSION

Discussion

These findings suggested that levels of IL-1β, IL-6 and TNF-α in sera were not related to the clinical signs and pathogenesis of *T. evansi* infection in pregnant sows. The number of trypanosomes in the blood circulation and tissues is not related to the concentration of pyrogenic cytokines induced. In addition, the alteration of PGE_2 and $PGF_{2\alpha}$ levels in experimental sows was not related to the clinical signs such as fever and abortion. Generally, the surge or presence of cytokines and prostaglandins in blood collected from healthy, pregnant sows can be found at different stages of pregnancy (Hebisch et al., 2004; Weems and Randal 2006; Skipor et al., 2010). Cytokines are involved in many patho-physiological processes of the body (Tizard, 2009). Also, the production of monocytic cytokines such as TNF-α, IL-1β, IL-6 have also been proposed to play an important role in the acute infection and also involved in the regulation of the parturition (Raghupathy et al., 2000; Piñeiro et al.,2009; Skipor et al., 2010). In addition, various studies suggested that IL-1β, IL-6, TNF-α, PGE_2 and/or $PGF_{2\alpha}$ in blood and sera could be used as markers for identification of various infectious disease (Pecchi et al., 2009; Piñeiro et al.,2009). These proinflammatory cytokines could also be produced in various scenarios (Bakhiet et al., 1996; Abrahamsohn, 1998; Inoue et al., 1999; van Reeth and Nauwynck, 2000; Maina et al., 2004). In contrast, increased serum levels of IL-6 and/or TNF-α were found both in *T. evansi* infected and control groups. The levels of IL-1β response in this study were unaltered during all the periods of collection in infected and non-infected groups. No alteration or correlation was observed in the levels of proinflammatory cytokine responses in this study. In addition, no correlation among clinical signs, parasitaemia and cytokine levels was observed in this study. Taken together, the present results suggested that levels of IL-1β, IL-6 and TNF-α could not be used for the explanation of *T. evansi* pathogenesis in pregnant sows. In pigs, systemic and local PGE_2 and $PGF_{2\alpha}$ play major roles in luteolysis and uterine contractility. The studies using porcine endometrial cell culture have demonstrated that TNF-α is very effective stimulators of $PGF_{2\alpha}$ production and could play a role in the induction of

luteolysis in pig (Norrby, 2010). Furthermore, a number of studies have shown that prostaglandins play an important role in the development of sickness behavior during inflammatory stages (Pecchi et al., 2009). However, in this study, the alteration of proinflammatory cytokines, PGE_2 and $PGF_{2\alpha}$ in the experimental sows showed no correlation with the clinical signs. It seems that the production and effect of the cytokines may not reflect the blood levels. On the contrary, there was an evidence of the increase in blood proinflammatory cytokines in normal term pregnancy (Raghupathy et al, 2000; Hebisch et al., 2004). It should be mentioned that the times and frequency of sample collection might influence the results (Hebisch et al., 2004). Moreover, laboratory techniques and the species specific of the commercial test kits used in this study should be taken into the consideration.

The isolation of *T. evansi* used in the present study was obtained from a naturally infected bovine case of surra from Kanjanaburi province. It was assumed to be a representative strain causing significant mortality and morbidity in livestock animals in the central part of Thailand over the past decade. The present results suggested that after being cryopreserved and passaged twice in mice, *T. evansi* was still able to infect sows and induced clinical signs. However, less severity was observed in this study when compared with the report by Teeraprasert et al. (1984[a,b]). The clinico-pathological signs in this study showed a prepatent period ranged from 3 – 9 days in the infected sows, and abortion occurred during 11-14 dpi. Teeraprasert et al. (1984[b]) reported that *T. evansi* isolated from a clinical case passaged in mice and 2 x 10^6 *T. evansi* were used to infect the sows showing parasiteamia at 18 hours post infection. In addition, skin lesion occurred at 3 days and the experimental sows aborted at 4 days post infection. The clinical signs including skin rash, plaque and petechial haemorrhage, fever and vaginal discharge were observed. The results indicated that a cryopreserverved strain of *T. evansi,* in this study, could still cause the disease after being passaged in mice. However, the prolong prepatent period and less virulency were observed in this study.

Evaluation of the peripheral thin blood smears showed the first detection of parasitaemia recorded at 4 dpi (1-10 trypanosomes per 200 RBC at 1000x magnification) while the MHCT showed the first detection at 3 dpi. Comparing two methods, the MHCT is more sensitive than the thin blood smears method. In addition,

the cyclical peaks and troughs of parasiteamia following the infection were not correlated with the fluctuations of temperature. Fever was observed only one day before abortion or at the abortion day. In pigs, the degree of parasitaemia is not paralleled with the temperature, implying that the number of trypanosomes in the blood circulation and tissues, is not related to the concentration of pyrogenic cytokines induced. Frequency and timing of sera collected and types of test kits used should be considered in this study. All infected sows showed irregular recurring of parasitaemia, presumably reflecting the development and demise of successive VSGs by host defense mechanism (Antoine-Moussiaux et al., 2009[a]). The erratic pattern of parasitaemia was previously described in pigs (Teeraprasert et al., 1984[b]; Holland et al., 2005) possibly due to the time and ability at which antibody is produced by the host against each antigenic variant glycoproteins (Antoine-Moussiaux et al., 2009[a]). The absence of detectable parasites in the blood in some periods between successive peaks of parasitaemia by MHCT or PCR demonstrates the unreliability of parasiteamia in diagnosis of surra as well as the limited value for diagnostic methods as their sensitivity and specificity decreased by the time after infection (Dargantes et al., 2005[a]; Holland et al., 2005). At the end of the study, fewer peaks of parasitaemia were observed demonstrating that the animals were capable of controlling the parasiteamia when the infection progressed. There are supporting reports showed that pigs can serve as a reservoir of *T. evansi* (Teeraprasert et al., 1984[a,b]; Indrakamhang, 1998; Tuntasuvan and luckins, 1998; Holland, 2005).

In this study, anaemia was normocytic and normochromic type observed at 15 dpi. Anaemia could have caused due to internally haemorrhage, intravascular haemolysis, haemodilution or erythrophagocytosis of immune-altered erythrocytes. The sign of anaemia was observed compatible with monocytosis at 15 dpi and progressive skin haemorrhage during 9-23 dpi (Aquino et al., 1999; Witola and Lovelace, 2001; Dargantes et al., 2005[a,b], Enwezor and Sackey, 2005). This suggests massive destruction of red blood cells by trypano-haemolytic factors and mononuclear phagocyte system. Moreover, immune complexes bound to red blood cells, together with mechanical damage to the red blood cells by *T. evansi* may trigger phagocytosis by the local and systemic macrophage system. Trypano-haemolytic factors released by lysed trypanosomes may also contribute to the destruction of red blood cells and

endothelial cells. In late term of infection or chronic infection, progressive anaemia could be observed (Witola and Lovelace, 2001; Dargantes et al., 2005[a,b], Antoine-Moussiaux et al., 2009[a]). A decrease in total leucocyte counts (WBC) during 3-5 dpi in infected group was observed and then a progressive rise in WBC during the 2nd week after infection was demonstrated. Although the fluctuation of WBC was observed in this study, the values remained within the normal range. Trypanosomes could induce immunosuppression in early phase of infection. Later the host immune system is kicking off to limit *T. evansi* propagation, resulting in trypano-tolerance and latent infection in pigs. In addition, these changes occur as a result of an increase in the activity of the mononuclear phagocytic system (Enwezor and Sackey, 2005; Antoine-Moussiaux et al., 2009[b]; Tizard, 2009). In this study, monocytosis occurred in infected group at 15 dpi ($P<0.05$) and tissue injury was observed relating to an increase in the circulating ALT and AST levels during 13-15 dpi. An increased in ALT and AST levels was attributed to the invasiveness and sequestration of the parasites in several organs especially in the liver, kidney, muscle and heart (Dargantes et al., 2005[b]) causing damage to cells and releasing of enzymes into the blood. Releasing substances from dying trypanosomes are another possible source of increase in ALT and AST levels (Costa et al., 2010; Anene et al., 2011). In acute phase of the disease, lymph nodes and spleen are remarkably reactive, while in the late stages immune system becomes depleted of lymphoid cells, resulting in alterations in total leucocyte counts of peripheral blood (Enwezor and Sackey, 2005; Anene et al., 2011). Collectively, this study provides clinical and clinico-pathological signs aiding in the diagnosis of *T. evansi* infection in pregnant sows. Therefore, inappetence, fever, skin rash, petechial and plaque haemorrhage especially on the ventral part of the body, abortion, anaemia, monocytosis and increasing in ALT and AST levels are suggestive of surra in pregnant sows.

All experimentally infected sows were confirmed serologically using CATT/*T. evansi*, Ab-ELISA/*T. evansi* and parasitologically positive using PCR method within the first two weeks after infection. Serologically positive sows at 5-15 dpi were detected on infected group, while test were all negative in the control group. The CATT/*T. evansi* and the Ab-ELISA/*T. evansi* were both used for the immunoglobulin detection in trypanosomiasis. Then, PCR-based assay was applied to detect and to confirm *T.*

evansi presence in blood and amniotic fluid. All sensitive tests used in this study are useful for diagnosis of *T. evansi* in any possible carrier. Interestingly, trypanosomes were detected by PCR in the blood and amniotic fluid of all infected sows. *T. evansi* was also observed in umbilical cords blood of fetuses from T3 sow. This is the first experimental study demonstrating *T. evansi* transmission via transplacental infection. No *T. evansi* was detected in the control sows.

Conclusions

1. Cryopreserved *T. evansi* is able to cause clinical signs, clinical pathology and pathology in 30-day-pregnant sows.

2. Transplacenta is another transmission route of *T. evansi* from sows to their fetuses.

3. Major proinflammatory cytokine and prostaglandin responses in this study were not related to the pathogenesis of experimental *T. evansi* infection in 30-day-pregnant sows.

Additional comments

Further studies may be needed using more sows or specific pathogen free (SPF) sows in order to achieve baseline levels of proinflammatory cytokines. In addition, test validation using commercial ELSAs should be done.

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APPENDICES
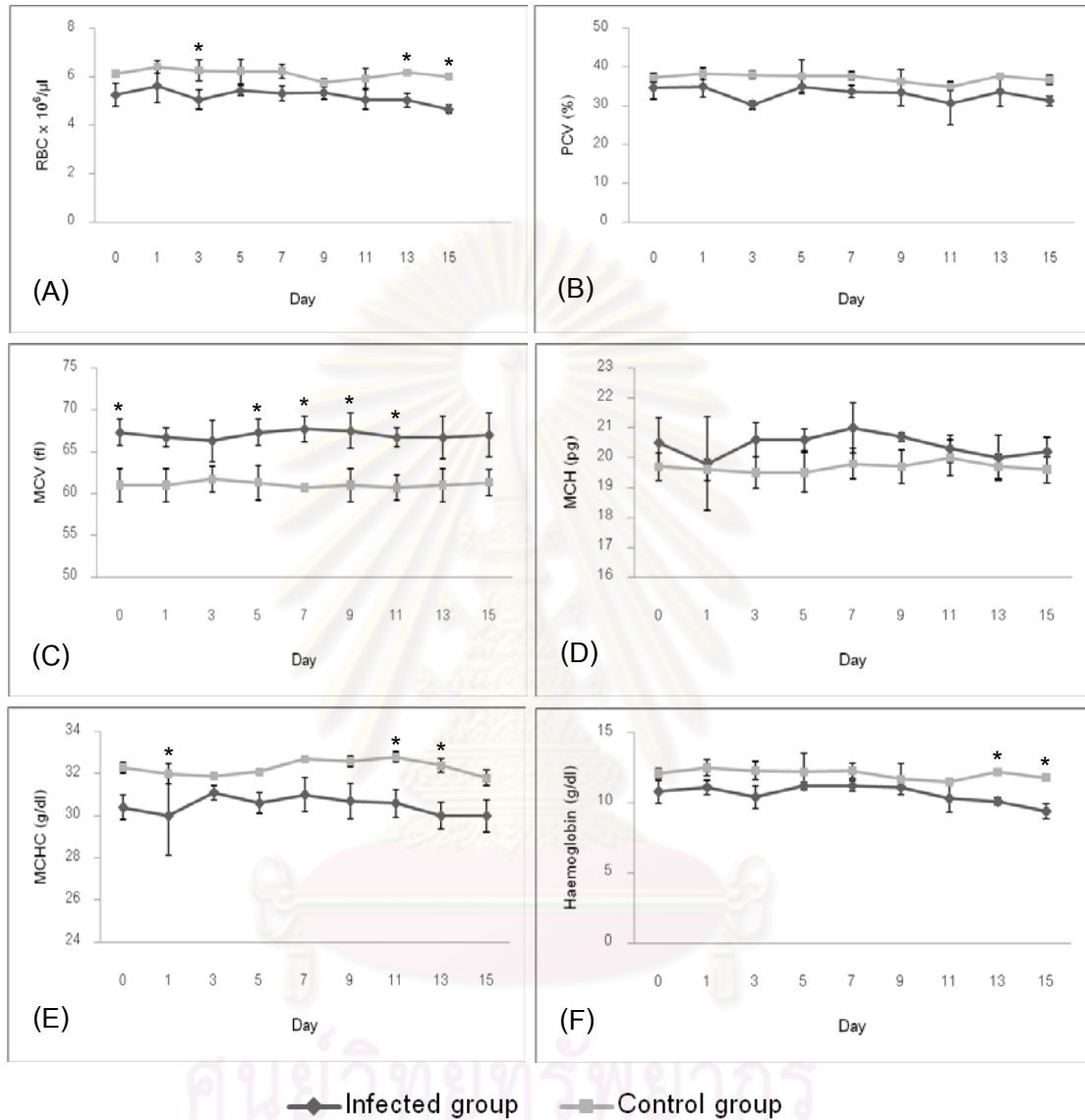
APPENDIX A

Mean ± standard deviation (SD) of the haematology of pregnant sows experimentally infected by *T. evansi*: (A) = RBC, (B) = PCV, (C) = MCV, (D) = MCH, (E) = MCHC, (F) = Haemoglobin. * = $P < 0.05$

APPENDIX B
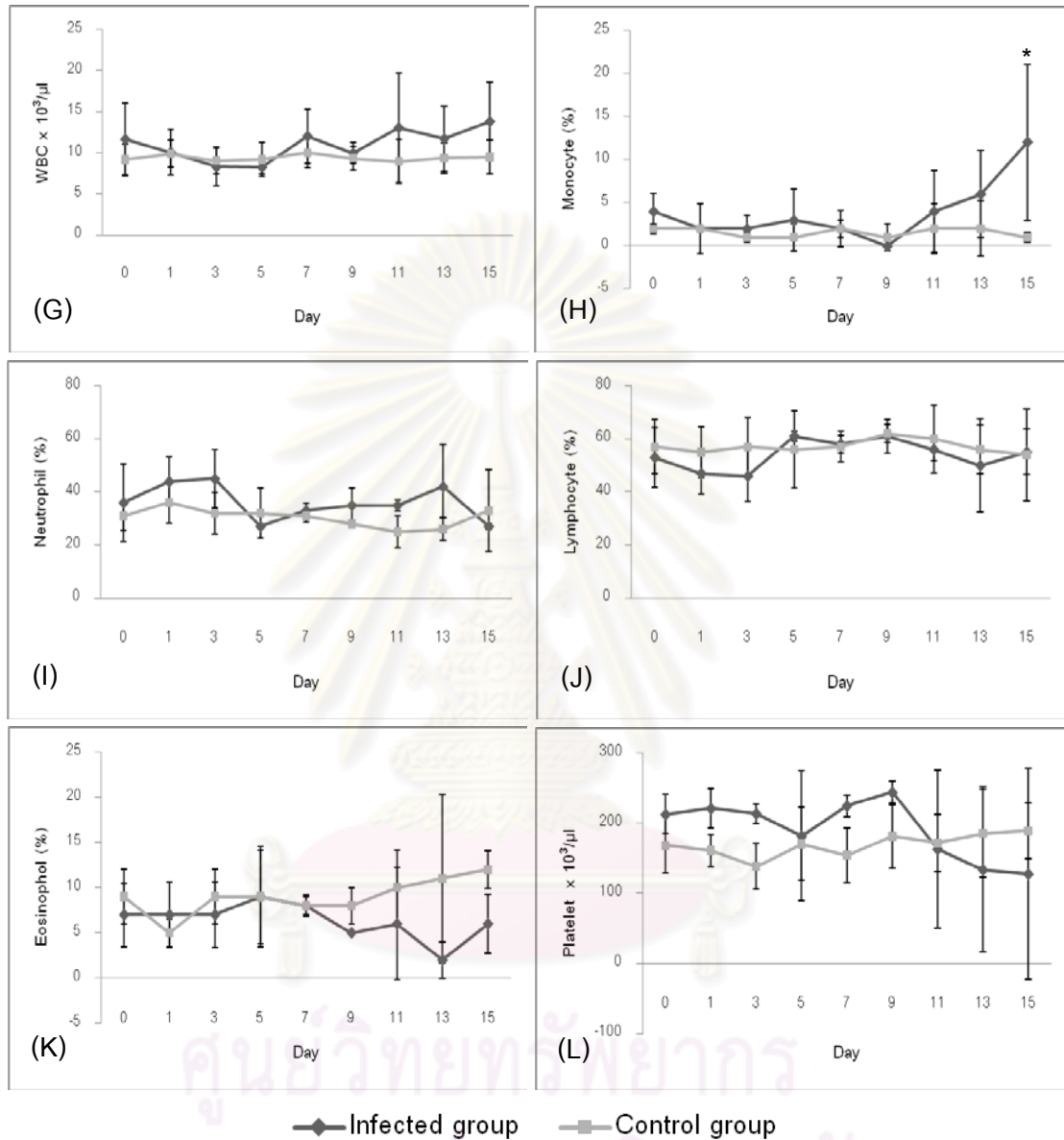
Mean ± standard deviation (SD) of the haematology of pregnant sows experimentally infected by *T. evansi*: (G) = WBC, (H) = Monocyte, (I) = Neutrophil, (J) = Lymphocyte (J), (K) = Eosinophil, (L) = Platelet count. * = *P* < 0.05

APPENDIX C

Porcine TNF-α/TNFSF1A Immunoassay (Quantikine®, R&D Systems, USA)

Materials provided

Porcine TNF-α Microplate (Part 890868) - One 96 well polystyrene microplate (12 strips of 8 wells) coated with a monoclonal antibody specific for porcine TNF-α.

Porcine TNF-α Conjugate (Part 890870) - 12 mL of a monoclonal antibody specific for porcine TNF-α conjugated to horseradish peroxidase with preservatives.

Porcine TNF-α Standard (Part 890869) - 3 vials (3 ng/vial) of recombinant porcine TNF-α in a buffered protein base with preservatives; lyophilized.

Porcine TNF-α Control (Part 890188) - 3 vials of recombinant porcine TNF-α in a buffered protein base with preservatives; lyophilized. The concentration range of porcine TNF-α after reconstitution is shown on the vial label. The assay value of the Control should be within the range specified on the label.

Assay Diluent RD1-63 (Part 895352) - 12.5 mL of a buffered protein solution with preservatives.

Calibrator Diluent RD5T (Part 895175) - 21 mL of a buffered protein solution with preservatives. For cell culture supernate samples.

Calibrator Diluent RD6-33 (Part 895349) - 21 mL of diluted animal serum with preservatives. For serum/plasma samples.

Wash Buffer Concentrate (Part 895024) - 50 mL of a 25-fold concentrated solution of a buffered surfactant with preservative.

Color Reagent A (Part 895000) - 12.5 mL of stabilized hydrogen peroxide.

Color Reagent B (Part 895001) - 12.5 mL of stabilized chromogen (tetramethylbenzidine).

Stop Solution (Part 895174) - 23 mL of a diluted hydrochloric acid solution.

Plate Covers (Part 640197) - 4 adhesive plate sealers.

Other supplies required

- Microplate reader capable of measuring absorbance at 450 nm, with the correction

- wavelength set at 540 nm or 570 nm.

- Pipettes and pipette tips.

- Deionized or distilled water.

- Squirt bottle, manifold dispenser, or automated microplate washer.

- 1000 mL graduated cylinder.

- Polypropylene tubes.

Sample collection and storage

Serum - Allow samples to clot for 2 hours at room temperature or overnight at 2 - 8° C before centrifuging for 20 minutes at 1000 x g. Remove serum and assay immediately or aliquot and store samples at $\leq$ -20° C. Avoid repeated freeze-thaw cycles.

Reagent preparation

Bring all reagents to room temperature before use.

Porcine TNF-α Kit Control - Reconstitute the Kit Control with 1.0 mL deionized or distilled water. Assay the Control undiluted.

Wash Buffer - If crystals have formed in the concentrate, warm to room temperature and mix gently until the crystals have completely dissolved. To prepare enough Wash Buffer for one plate, add 25 mL Wash Buffer Concentrate into deionized or distilled water to prepare 625 mL of Wash Buffer.

Substrate Solution - Color Reagents A and B should be mixed together in equal volumes within 15 minutes of use. Protect from light. 100 _L of the resultant mixture is required per well.

Porcine TNF-α Standard - Reconstitute the porcine TNF-α Standard with 2.0 mL of Calibrator Diluent RD5T (for cell culture supernate samples) or Calibrator Diluent RD6-33 (for serum/plasma samples). Do not substitute other diluents. This reconstitution produces a stock solution of 1500 pg/mL. Allow the standard to sit for a minimum of 5 minutes with gentle mixing prior to making dilutions. Use polypropylene tubes. Pipette 200 μL of the appropriate Calibrator Diluent into each tube.Use the stock solution to produce a dilution series (below). Mix each tube thoroughly before the next transfer. The undiluted porcine TNF-α Standard serves as the high standard (1500 pg/mL). The appropriate Calibrator Diluent serves as the zero standard (0 pg/mL).
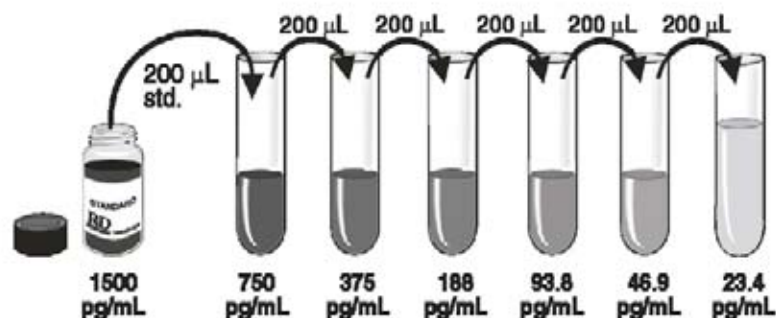
	200 µL std.	200 µL	200 µL	200 µL	200 µL	200 µL
1500 pg/mL	750 pg/mL	375 pg/mL	188 pg/mL	93.8 pg/mL	46.9 pg/mL	23.4 pg/mL

Assay procedure

Bring all reagents and samples to room temperature before use. It is recommended that all samples, standards, and control be assayed in duplicate.

1. Prepare reagents, samples, and standard dilutions as directed by the previous sections.

2. Remove excess microplate strips from the plate frame, return them to the foil pouch containing the desiccant pack, and reseal.

3. Add 50 µL of Assay Diluent RD1-63 to each well.

4. Add 50 µL of Standard, Control, or sample per well. Mix by gently tapping the plate frame for 1 minute. Cover with the adhesive strip provided. Incubate for 2 hours at room temperature. A plate layout is provided to record standards and samples assayed.

5. Aspirate each well and wash, repeating the process four times for a total of five washes. Wash by filling each well with Wash Buffer (400 µL) using a squirt bottle, manifold dispenser, or autowasher. Complete removal of liquid at each step is essential to good performance. After the last wash, remove any remaining Wash Buffer by aspirating or decanting. Invert the plate and blot it against clean paper towels.

6. Add 100 µL of porcine TNF-α Conjugate to each well. Cover with a new adhesive strip. Incubate for 2 hours at room temperature.

7. Repeat the aspiration/wash as in step 5.

8. Add 100 µL of Substrate Solution to each well. Incubate for 30 minutes at room temperature. **Protect from light.**

9. Add 100 µL of Stop Solution to each well. Gently tap the plate to ensure thorough mixing.

10. Determine the optical density of each well within 30 minutes, using a microplate reader set to 450 nm. If wavelength correction is available, set to 540 nm or 570 nm. If wavelength correction is not available, subtract readings at 540 nm or 570 nm from the readings at 450 nm. This subtraction will correct for optical imperfections in the plate. Readings made directly at 450 nm without correction may be higher and less accurate.

Calculation of results

Average the duplicate readings for each standard, control, and sample and subtract the average zero standard optical density. Create a standard curve by reducing the data using computer software capable of generating a four parameter logistic (4-PL) curve-fit. As an alternative, construct a standard curve by plotting the mean absorbance for each standard on the y-axis against the concentration on the x-axis and draw a best fit curve through the points on the graph. The data may be linearized by plotting the log of the porcine TNF-α concentrations versus the log of the O.D. and the best fit line can be determined by regression analysis. This procedure will produce an adequate but less precise fit of the data. If samples have been diluted prior to assay, the concentration read from the standard curve must be multiplied by the dilution factor.

Typical data

These standard curves are provided for demonstration only. A standard curve should be generated for each set of samples assayed.

(pg/mL)	OD	Average	Corrected
0	0.046 0.047	0.046	—
23.4	0.083 0.089	0.086	0.040
46.9	0.118 0.119	0.118	0.072
93.8	0.183 0.192	0.187	0.141
188	0.333 0.334	0.333	0.287
375	0.592 0.622	0.607	0.561
750	1.116 1.134	1.125	1.079
1500	1.993 2.108	2.050	2.004

APPENDIX D

Porcine IL-1β/IL-1F2 Immunoassay (Quantikine®, R&D Systems, USA)

Material provided

Porcine IL-1β Microplate (Part 890862) - One 96 well polystyrene microplate (12 strips of 8 wells) coated with monoclonal antibody specific for porcine IL-1β.

Porcine IL-1β Conjugate (Part 890864) - 21 mL of a polyclonal antibody against porcine IL-1β conjugated to horseradish peroxidase with preservatives.

Porcine IL-1β Standard (Part 890863) - 3 vials (5 ng/vial) of recombinant porcine IL-1β in a buffered protein base with preservatives; lyophilized.

Porcine IL-1β Control (Part 890145) - 3 vials of recombinant porcine IL-1β in a buffered protein base with preservatives; lyophilized. The concentration range of porcine IL-1β after reconstitution is shown on the vial label. The assay value of the Control should be within the range specified on the label.

Assay Diluent RD1-63 (Part 895352) - 12.5 mL of a buffered protein solution with preservatives.

Calibrator Diluent RD5-27 (Part 895395) - 21 mL of a buffered protein solution with preservatives.

Wash Buffer Concentrate (Part 895024) - 50 mL of a 25-fold concentrated solution of a buffered surfactant with preservative.

Color Reagent A (Part 895000) - 12.5 mL of stabilized hydrogen peroxide.

Color Reagent B (Part 895001) - 12.5 mL of stabilized chromogen (tetramethylbenzidine).

Stop Solution (Part 895174) - 23 mL of a diluted hydrochloric acid solution.

Plate Covers (Part 640197) - 4 adhesive plate sealers.

Other supplies required

- Microplate reader capable of measuring absorbance at 450 nm, with the correction
- wavelength set at 540 nm or 570 nm.
- Pipettes and pipette tips.
- Deionized or distilled water.

- Squirt bottle, manifold dispenser, or automated microplate washer.

- 100 mL and 1000 mL graduated cylinders.

- Polypropylene tubes for serial dilution.

Sample collection and storage

Serum - Allow samples to clot for 2 hours at room temperature or overnight at 2 - 8° C before centrifuging for 20 minutes at 1000 x g. Remove serum and assay immediately or aliquot and store samples at $\leq$ -20° C. Avoid repeated freeze-thaw cycles.

Reagent preparation

Bring all reagents to room temperature before use.

Porcine IL-1β Kit Control - Reconstitute the Kit Control with 1.0 mL deionized or distilled water. Assay the Control undiluted.

Wash Buffer - If crystals have formed in the concentrate, warm to room temperature and mix gently until the crystals have completely dissolved. To prepare enough Wash Buffer for one plate, add 25 mL Wash Buffer Concentrate into deionized or distilled water to prepare 625 mL of Wash Buffer.

Substrate Solution - Color Reagents A and B should be mixed together in equal volumes within 15 minutes of use. Protect from light. 120 µL of the resultant mixture is required per well.

Porcine IL-1β Standard - Reconstitute the porcine IL-1β Standard with 4.0 mL of Calibrator Diluent RD5-27. Do not substitute other diluents. This reconstitution produces a stock solution of 1250 pg/mL. Allow the standard to sit for a minimum of 5 minutes with gentle mixing prior to making dilutions. Use polypropylene tubes. Pipette 300 µL of Calibrator Diluent RD5-27 into each tube. Use the stock solution to produce a dilution series (below). Mix each tube thoroughly before the next transfer. The undiluted porcine IL-1β Standard serves as the high standard (1250 pg/mL). Calibrator Diluent RD5-27 serves as the zero standard (0 pg/mL).

Assay procedure

Bring all reagents and samples to room temperature before use. It is recommended that all samples, standards, and control be assayed in duplicate.

1. Prepare reagents, samples, and standard dilutions as directed by the previous sections.

2. Remove excess microplate strips from the plate frame, return them to the foil pouch containing the desiccant pack, and reseal.

3. Add 50 µL of Assay Diluent RD1-63 to each well.

4. Add 100 µL of Standard, Control, or sample per well. Mix by gently tapping the plate frame for 1 minute. Cover with the adhesive strip provided. Incubate for 2 hours at room temperature. A plate layout is provided to record standards and samples assayed.

5. Aspirate each well and wash, repeating the process four times for a total of five washes. Wash by filling each well with Wash Buffer (400 µL) using a squirt bottle, manifold dispenser, or autowasher. Complete removal of liquid at each step is essential to good performance. After the last wash, remove any remaining Wash Buffer by aspirating or decanting. Invert the plate and blot it against clean paper towels.

6. Add 200 µL of porcine IL-1β Conjugate to each well. Cover with a new adhesive strip. Incubate for 2 hours at room temperature.

7. Repeat the aspiration/wash as in step 5.

8. Add 120 µL of Substrate Solution to each well. Incubate for 30 minutes at room temperature. Protect from light.

9. Add 120 µL of Stop Solution to each well. Gently tap the plate to ensure thorough mixing.

10. Determine the optical density of each well within 30 minutes, using a microplate reader set to 450 nm. If wavelength correction is available, set to 540 nm or 570 nm. If wavelength correction is not available, subtract readings at 540 nm or 570 nm from the readings at 450 nm. This subtraction will correct for optical imperfections in the plate. Readings made directly at 450 nm without correction may be higher and less accurate.

Calculation of results

Average the duplicate readings for each standard, control, and sample and subtract the average zero standard optical density. Create a standard curve by reducing the data using computer software capable of generating a four parameter logistic (4-PL) curve-fit. As an alternative, construct a standard curve by plotting the mean absorbance for each standard on the y-axis against the concentration on the x-axis and draw a best fit curve through the points on the graph. The data may be linearized by plotting the log of the porcine IL-1_ concentrations versus the log of the O.D. and the best fit line can be determined by regression analysis. This procedure will produce an adequate but less precise fit of the data. If samples have been diluted prior to assay, the concentration read from the standard curve must be multiplied by the respective dilution factor.

Typical data

This standard curve is provided for demonstration only. A standard curve should be generated for each set of samples assayed.

(pg/mL)	O.D.	Average	Corrected
0	0.081 0.084 0.105	0.082	—
19.5	0.119 0.144	0.112	0.030
39.1	0.153 0.216	0.148	0.066
78.1	0.220 0.364	0.218	0.136
156	0.406 0.657	0.385	0.303
312	0.690 1.198	0.674	0.592
625	1.241 2.165	1.220	1.138
1250	2.280	2.222	2.140
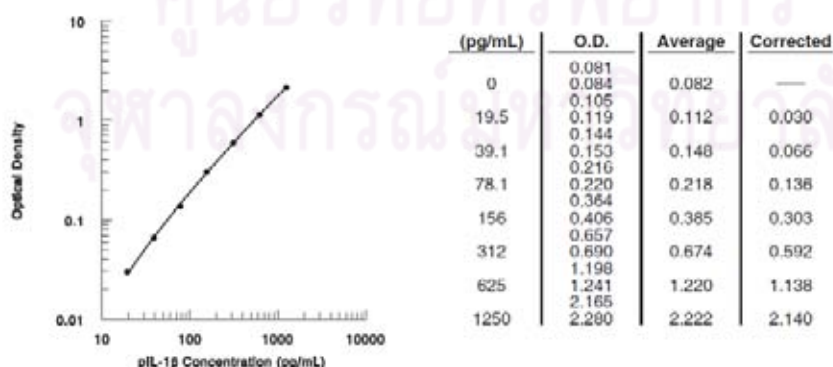
APPENDIX E

Porcine IL-6 Immunoassay (Quantikine®, R&D Systems, USA)

Material provided

Porcine IL-6 Microplate (Part 890865) - 96 well polystyrene microplate (12 strips of 8 wells) coated with polyclonal antibody specific for porcine IL-6.

Porcine IL-6 Conjugate (Part 890867) - 23 mL of a polyclonal antibody against porcine IL-6 conjugated to horseradish peroxidase with preservatives.

Porcine IL-6 Standard (Part 890866) - 3 vials (5 ng/vial) of recombinant porcine IL-6 in a buffered protein base with preservatives; lyophilized.

Porcine IL-6 Control (Part 890187) - 3 vials of recombinant porcine IL-6 in a buffered protein base with preservatives; lyophilized. The concentration range of porcine IL-6 after reconstitution is shown on the vial label. The assay value of the Control should be within the range specified on the label.

Assay Diluent RD1-63 (Part 895352) - 12.5 mL of a buffered protein solution with preservatives.

Calibrator Diluent RD5T (Part 895175) - 21 mL of a buffered protein solution with preservatives. For cell culture supernate samples.

Calibrator Diluent RD6-32 (Part 895336) - 21 mL of diluted animal serum with preservatives. For serum/plasma samples.

Wash Buffer Concentrate (Part 895024) - 50 mL of a 25-fold concentrated solution of a buffered surfactant with preservative.

Color Reagent A (Part 895000) - 12.5 mL of stabilized hydrogen peroxide.

Color Reagent B (Part 895001) - 12.5 mL of stabilized chromogen (tetramethylbenzidine).

Stop Solution (Part 895174) - 23 mL of a diluted hydrochloric acid solution.

Plate Covers (Part 640197) - 4 adhesive plate sealers.

Other supplies required

- Microplate reader capable of measuring absorbance at 450 nm, with the correction
- wavelength set at 540 nm or 570 nm.

- Pipettes and pipette tips.

- Deionized or distilled water.

- Squirt bottle, manifold dispenser, or automated microplate washer.

- 100 mL and 1000 mL graduated cylinders.

- Polypropylene tubes for serial dilution.

Sample collection and storage

Serum - Allow samples to clot for 2 hours at room temperature or overnight at 2 - 8° C before centrifuging for 20 minutes at 1000 x g. Remove serum and assay immediately or aliquot and store samples at $\leq$ -20° C. Avoid repeated freeze-thaw cycles.

Note: Grossly hemolyzed or lipemic samples may not be suitable for measurement of porcine IL-6 with this assay.

Sample preparation

Serum and plasma samples require a 2-fold dilution into Calibrator Diluent RD6-32 prior to assay. A suggested 2-fold dilution is 125 µL sample + 125 µL Calibrator Diluent RD6-32.

Reagent preparation

Bring all reagents to room temperature before use.

Porcine IL-6 Kit Control - Reconstitute the Kit Control with 1.0 mL deionized or distilled water. Assay the Control undiluted.

Wash Buffer - If crystals have formed in the concentrate, warm to room temperature and mix gently until the crystals have completely dissolved. To prepare enough Wash Buffer for one plate, add 25 mL Wash Buffer Concentrate into deionized or distilled water to prepare 625 mL of Wash Buffer.

Substrate Solution - Color Reagents A and B should be mixed together in equal volumes within 15 minutes of use. Protect from light. 120 µL of the resultant mixture is required per well.

Porcine IL-6 Standard - Reconstitute the Porcine IL-6 Standard with 2.0 mL of Calibrator Diluent RD5T (for cell culture supernate samples) or Calibrator Diluent RD6-32

(for serum/plasma samples). Do not substitute other diluents. This reconstitution produces a stock solution of 2500 pg/mL. Allow the standard to sit for a minimum of 5 minutes with gentle mixing prior to making dilutions. Mix each tube thoroughly before the next transfer. Use polypropylene tubes. Pipette 300 µL of the appropriate Calibrator Diluent into each tube. Use the stock solution to produce a dilution series (below). The undiluted porcine IL-6 Standard serves as the high standard (2500 pg/mL). The appropriate Calibrator Diluent serves as the zero standard (0 pg/mL).
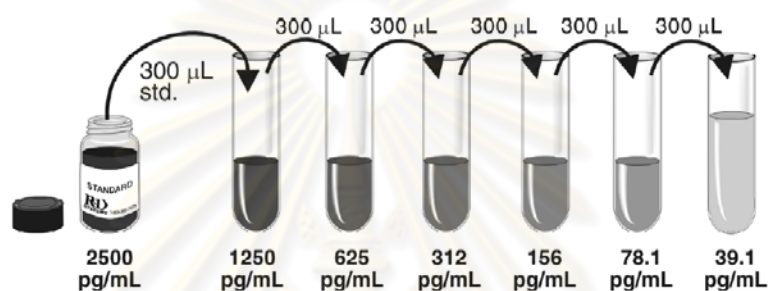
Assay procedure

Bring all reagents and samples to room temperature before use. It is recommended that all samples, standards, and control be assayed in duplicate.

1. Prepare reagents, samples, standards, and control as directed by the previous sections.

2. Remove excess microplate strips from the plate frame, return them to the foil pouch containing the desiccant pack, and reseal.

3. Add 50 µL of Assay Diluent RD1-63 to each well.

4. Add 100 µL of Standard, Control, or sample* per well. Mix by gently tapping the plate frame for 1 minute. Cover with the adhesive strip provided. Incubate for 2 hours at room temperature. A plate layout is provided to record standards and samples assayed.

5. Aspirate each well and wash, repeating the process four times for a total of five washes. Wash by filling each well with Wash Buffer (400 µL) using a squirt bottle, pipette, manifold dispenser, or autowasher. Complete removal of liquid at each step is essential to good performance. After the last wash, remove any remaining Wash Buffer by aspirating or decanting. Invert the plate and blot it against clean paper towels.

6. Add 200 µL of Porcine IL-6 Conjugate to each well. Cover with a new adhesive strip. Incubate for 2 hours at room temperature.

7. Repeat the aspiration/wash as in step 5.

8. Add 120 µL of Substrate Solution to each well. Incubate for 30 minutes at room temperature. Protect from light.

9. Add 120 µL of Stop Solution to each well. Gently tap the plate to ensure thorough mixing.

10. Determine the optical density of each well within 30 minutes, using a microplate reader set to 450 nm. If wavelength correction is available, set to 540 nm or 570 nm. If wavelength correction is not available, subtract readings at 540 nm or 570 nm from the readings at 450 nm. This subtraction will correct for optical imperfections in the plate. Readings made directly at 450 nm without correction may be higher and less accurate. *Porcine serum and plasma samples require a 2-fold dilution. See Sample Preparation.

Calculation of results

Average the duplicate readings for each standard, control, and sample and subtract the average zero standard optical density. Create a standard curve by reducing the data using computer software capable of generating a four parameter logistic (4-PL) curve-fit. As an alternative, construct a standard curve by plotting the mean absorbance for each standard on the y-axis against the concentration on the x-axis and draw a best fit curve through the points on the graph. The data may be linearized by plotting the log of the porcine IL-6 concentrations versus the log of the O.D. and the best fit line can be determined by regression analysis. This procedure will produce an adequate but less precise fit of the data. Because serum and plasma samples have been diluted prior to assay, the concentration read from the standard curve must be multiplied by the dilution factor.

Typical data

These standard curves are provided for demonstration only. A standard curve should be generated for each set of samples assayed.

(pg/mL)	O.D.	Average	Corrected
0	0.065 0.068 0.102	0.066	—
39.1	0.101 0.128	0.102	0.036
78.1	0.134 0.194	0.131	0.065
156	0.191 0.319	0.192	0.126
312	0.322 0.563	0.320	0.254
625	0.561 1.030	0.562	0.496
1250	1.044 1.819	1.037	0.971
2500	1.859	1.839	1.773
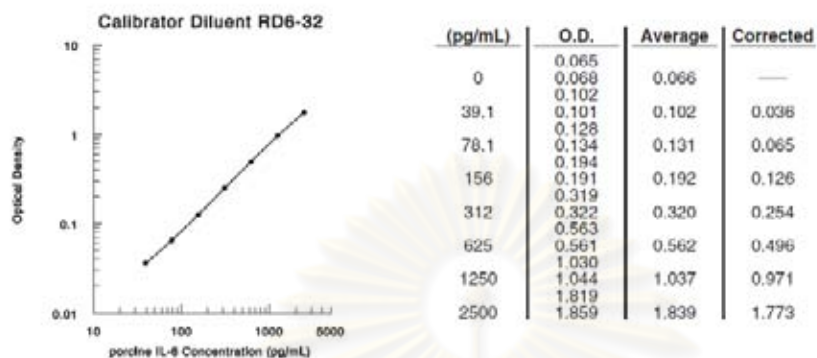
APPENDIX F

PGF$_{2\alpha}$ EIA kit (EnzoLifeSciences, UK)

Materials Supplied

      1. Donkey anti-Sheep IgG Microtiter Plate, One Plate of 96 Wells, Catalog No. 80-0045 A plate using break-apart strips coated with donkey antibody specific to sheep IgG.

      2. PGF$_{2\alpha}$ EIA Conjugate, 5 mL, Catalog No. 80-0027 A blue solution of alkaline phosphatase conjugated with PGF$_{2\alpha}$.

      3. PGF$_{2\alpha}$ EIA Antibody, 5 mL, Catalog No. 80-0639 A yellow solution of a polyclonal sheep antibody to PGF$_{2\alpha}$.

      4. Assay Buffer, 30 mL, Catalog No. 80-0010 Tris buffered saline, containing proteins and sodium azide as preservative.

      5. Wash Buffer Concentrate, 30 mL, Catalog No. 80-1286 Tris buffered saline containing detergents.

      6. Prostaglandin F$_{2\alpha}$ Standard, 0.5 mL, Catalog No. 80-0029 A solution of 500,000 pg/mL PGF$_{2\alpha}$.

      7. pNpp Substrate, 20 mL, Catalog No. 80-0075 A solution of p-nitrophenyl phosphate in buffer. Ready to use.

      8. Stop Solution, 5 mL, Catalog No. 80-0247 A solution of trisodium phosphate in water. Keep tightly capped. Caution: **Caustic.**

      9. PGF$_{2\alpha}$ Assay Layout Sheet, 1 each, Catalog No. 30-0121

      10. Plate Sealer, 1 each, Catalog No. 30-0012

Storage

      All components of this kit are stable at 4° C until the kit's expiration date.

Materials Needed but Not Supplied

      1. Deionized or distilled water.

      2. Precision pipets for volumes between 5 μL and 1,000 μL.

      3. Repeater pipets for dispensing 50 μL and 200 μL.

      4. Disposable beakers for diluting buffer concentrates.

      5. Graduated cylinders.

      6. A microplate shaker.

7. Adsorbent paper for blotting.

8. Microplate reader capable of reading at 405 nm, preferably with correction between 570 and 590 nm.

Sample Handling

The $PGF_{2\alpha}$ EIA is compatible with $PGF_{2\alpha}$ samples in a wide range of matrices. Samples diluted sufficiently into Assay Buffer can be read directly from the standard curve. However, the end user must verify that the recommended dilutions are appropriate for their samples. Samples containing sheep IgG may interfere with the assay. Samples in the majority of tissue culture media, including those containing fetal bovine serum, can also be read in the assay, provided the standards have been diluted into the tissue culture media instead of Assay Buffer. There will be a small change in binding associated with running the standards and samples in media. Users should only use standard curves generated in media or buffer to calculate concentrations of $PGF_{2\alpha}$ in the appropriate matrix. For tissue, urine and plasma samples, prostaglandin synthetase inhibitors, such as, indomethacin or meclofenamic acid at concentrations up to 10 µg/mL should be added to either the tissue homogenate or urine and plasma samples. Some samples normally have very low levels of $PGF_{2\alpha}$ present and extraction may be necessary for accurate measurement. A suitable extraction procedure is outlined below:

Materials Needed

1. $PGF_{2\alpha}$ Standard to allow extraction efficiency to be accurately determined.

2. 2M hydrochloric acid, deionized water, ethanol, hexane and ethyl acetate.

3. 200 mg C18 Reverse Phase Extraction Columns.

Procedure

1. Acidify the plasma, urine or tissue homogenate by addition of 2M HCl to pH of 3.5. Approximately 50 µL of HCl will be needed per mL of plasma. Allow to sit at 4° C for 15 minutes. Centrifuge samples in a microcentrifuge for 2 minutes to remove any precipitate.

2. Prepare the C18 reverse phase column by washing with 10 mL of ethanol followed by 10 mL of deionized water.

3. Apply the sample under a slight positive pressure to obtain a flow rate of about 0.5 mL/minute. Wash the column with 10 mL of water, followed by 10 mL of 15% ethanol, and finally 10 mL hexane. Elute the sample from the column by addition of 10 mL ethyl acetate.

4. If analysis is to be carried out immediately, evaporate samples under a stream of nitrogen. Add at least 250 µL of Assay Buffer to the dried samples. Vortex well then allow to sit five minutes at room temperature. Repeat twice more. If analysis is to be delayed, store samples as the eluted ethyl acetate solutions at -80° C until the immunoassay is to be run. Evaporate the organic solvent under a stream of nitrogen prior to running assay and reconstitute as above. Please refer to references 12-15 for details of extraction protocols.

Procedural Notes

1. Do not mix components from different kit lots or use reagents beyond the kit expiration date.

2. Allow all reagents to warm to room temperature for at least 30 minutes before opening.

3. Standards can be made up in either glass or plastic tubes.

4. Pre-rinse the pipet tip with reagent, use fresh pipet tips for each sample, standard and reagent.

5. Pipet standards and samples to the bottom of the wells.

6. Add the reagents to the side of the well to avoid contamination.

7. This kit uses break-apart microtiter strips, which allow the user to measure as many samples as desired. Unused wells must be kept desiccated at 4 °C in the sealed bag provided. The wells should be used in the frame provided.

8. Care must be taken to minimize contamination by endogenous alkaline phosphatase. Contaminating alkaline phosphatase activity, especially in the substrate solution, may lead to high blanks. Care should be taken not to touch pipet tips and other items that are used in the assay with bare hands.

9. Prior to addition of substrate, ensure that there is no residual wash buffer in the wells. Any remaining wash buffer may cause variation in assay results.

Reagent Preparation

1. PGF$_{2\alpha}$ Standard

Allow the 500,000 pg/mL PGF$_{2\alpha}$ standard solution to warm to room temperature. Label eight 12 x 75 mm glass tubes #1 through #8. Pipet 900 μL of standard diluent (Assay Buffer or Tissue Culture Media) into tube #1. Pipet 750 μL of standard diluent into tubes #2 through #8. Add 100 μL of the 500,000 pg/mL standard to tube #1. Vortex thoroughly. Add 250 μL of tube #1 to tube #2 and vortex thoroughly. Add 250 μL of tube #2 to tube #3 and vortex. Continue this for tubes #4 through #8.

The concentration of PGF$_{2\alpha}$ in tubes #1 through #8 will be 50,000, 12,500, 3,125, 781.25, 195.31, 48.83, 12.2, and 3.05 pg/mL respectively. See PGF$_{2\alpha}$ Assay Layout Sheet for dilution details. Diluted standards should be used within 60 minutes of preparation.

2. Wash Buffer

Prepare the Wash Buffer by diluting 5 mL of the supplied concentrate with 95 mL of deionized water. This can be stored at room temperature until the kit expiration date, or for 3 months, whichever is earlier.

Assay Procedure

Bring all reagents to room temperature for at least 30 minutes prior to opening. All standards and samples should be run in duplicate.

1. Refer to the Assay Layout Sheet to determine the number of wells to be used and put any remaining wells with the desiccant back into the pouch and seal the ziploc. Store unused wells at 4° C.

2. Pipet 100 μL of standard diluent (Assay Buffer or Tissue Culture Media) into the NSB and the Bo (0 pg/mL Standard) wells.

3. Pipet 100 μL of Standards #1 through #8 into the appropriate wells.

4. Pipet 100 μL of the Samples into the appropriate wells.

5. Pipet 50 μL of Assay Buffer into the NSB wells.

6. Pipet 50 μL of blue Conjugate into each well, except the TA and Blank wells.

7. Pipet 50 μL of yellow Antibody into each well, except the Blank, TA and NSB wells.

NOTE: Every well used should be Green in color except the NSB wells which should be Blue. The Blank and TA wells are empty at this point and have no color.

8. Incubate the plate at room temperature on a plate shaker for 2 hours at ~500 rpm. The plate may be covered with the plate sealer provided, if so desired.

9. Empty the contents of the wells and wash by adding 400 µL of wash solution to every well. Repeat the wash 2 more times for a total of 3 Washes.

10. After the final wash, empty or aspirate the wells, and firmly tap the plate on a lint free paper towel to remove any remaining wash buffer.

11. Add 5 µL of the blue Conjugate Solution to TA wells.

12. Add 200 µL of the pNpp Substrate solution to each well. Incubate at room temperature for 45 minutes without shaking.

13. Add 50 µL of Stop Solution to each well.

14. Blank the plate reader against the Blank wells, read the optical density at 405 nm, preferably with correction between 570 and 590 nm. If the plate reader is not able to be blanked against the Blank wells, manually subtract the mean optical density of the Blank wells from all readings.

Calculation of Results

Several options are available for the calculation of the concentration of $PGF_{2\alpha}$ in the samples. We recommend that the data be handled by an immunoassay software package utilizing a 4 parameter logistic curve fitting program. If this sort of data reduction software is not readily available, the concentration of $PGF_{2\alpha}$ can be calculated as follows:

1. Calculate the average net Optical Density (OD) bound for each standard and sample by subtracting the average NSB OD from the average OD bound:

Average Net OD = Average Bound OD - Average NSB OD

2. Calculate the binding of each pair of standard wells as a percentage of the maximum binding wells (Bo), using the following formula:

Percent Bound = $\dfrac{\text{Net OD} \times 100}{\text{Net Bo OD}}$

3. Using Logit-Log paper plot Percent Bound versus Concentration of $PGF_{2\alpha}$ for the standards. Approximate a straight line through the points. The concentration of $PGF_{2\alpha}$ in the unknowns can be determined by interpolation.

Typical Results

The results shown below are for illustration only and should not be used to calculate results from another assay.

Sample	Mean OD (-Blank)	Average Net OD	Percent Bound	$PGF_{2\alpha}$ (pg/mL)
Blank OD	(0.071)			
TA	1.021	1.021		
NSB	0.008	0.000	0.0%	
Bo	0.689	0.681	100%	0
S1	0.020	0.012	2.9%	50,000
S2	0.050	0.042	7.4%	12,500
S3	0.121	0.113	17.7%	3,125
S4	0.220	0.212	32.1%	781
S5	0.347	0.339	50.5%	195
S6	0.516	0.508	74.9%	48.8
S7	0.642	0.634	93.2%	12.2
S8	0.680	0.672	98.7%	3.05
Unknown 1	0.507	0.499	73.6%	182
Unknown 2	0.110	0.102	16.0%	5,852

Typical Standard Curve

A typical standard curve is shown below. This curve must not be used to calculate $PGF_{2\alpha}$ concentrations; each user must run a standard curve for each assay.
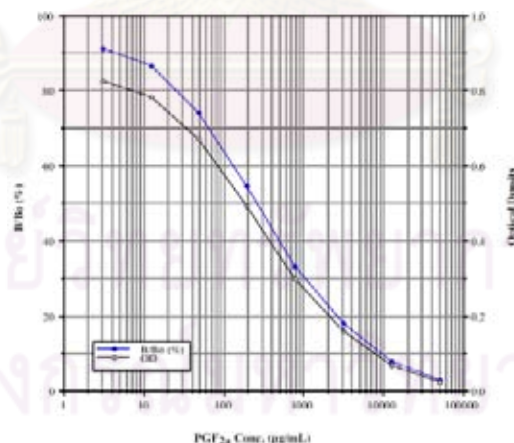
Typical Quality Control Parameters

Total Activity Added = 1.021 x 10 = 10.21

%NSB = 0.08%, %Bo/TA = 6.70%

Quality of Fit = 0.9999 (Calculated from 4 parameter logistic curve fit)

20% Intercept = 2,041 pg/mL, 50% Intercept = 230 pg/mL

80% Intercept = 33 pg/mL

APPENDIX G

PGE$_2$ Assay (Parameter™, R&D Systems, USA)

Material provided

Description	Part #	Cat. # KGE004B	Cat. # SKGE004B
Goat Anti-mouse Microplate - 96 well polystyrene microplate (12 strips of 8 wells) coated with a goat anti-mouse polyclonal antibody.	892575	1 plate	6 plates
PGE$_2$ Conjugate - 6 mL/vial of PGE$_2$ conjugated to horseradish peroxidase with red dye and preservatives.	893375	1 vial	6 vials
PGE$_2$ Standard - 25,000 pg/vial of PGE$_2$ in buffer with preservatives; lyophilized.	893377	1 vial	6 vials
Primary Antibody Solution - 6 mL/vial of a mouse monoclonal antibody to PGE$_2$ in buffer with blue dye and preservatives.	893376	1 vial	6 vials
Calibrator Diluent RD5-56 - 21 mL/vial of a buffered protein base with preservatives.	895612	2 vials	12 vials
Wash Buffer Concentrate - 21 mL/vial of a 25-fold concentrated solution of buffered surfactant with preservatives.	895003	1 vial	6 vials
Color Reagent A - 12.5 mL/vial of stabilized hydrogen peroxide.	895000	1 vial	6 vials
Color Reagent B - 12.5 mL/vial of stabilized chromogen (tetramethylbenzidine).	895001	1 vial	6 vials
Stop Solution - 11 mL/vial of 2 N sulfuric acid.	895926	1 vial	6 vials
Plate Covers - Adhesive strips.	—	4 strips	24 strips

Other supplies required

- Microplate reader capable of measuring absorbance at 450 nm, with the correction wavelength set at 540 or 570 nm.
- Pipettes and pipette tips.
- Deionized or distilled water.
- Squirt bottle, manifold dispenser, or automated microplate washer.
- 500 mL graduated cylinder.
- Horizontal orbital microplate shaker (0.12" orbit) capable of maintaining a speed of 500 ± 50 rpm.
- Test tubes for dilution.
- Indomethacin (Sigma, Catalog # I7378 or equivalent).
- PGE2 Controls (optional; available from R&D Systems).

Precautions

This assay is temperature sensitive. Room temperature is defined as 18 - 23° C.

The Stop Solution provided with the kit is an acid solution. Wear eye, hand, face, and clothing protection when using this material. Care should be taken when handling the PGE_2 Standard because of the known and unknown effects of prostaglandins.

Sample collection and storage

Samples containing mouse IgG may interfere with this assay.

Serum* - Use a serum separator tube (SST) and allow samples to clot for 30 minutes before centrifugation for 15 minutes at 1000 x g. Remove serum and assay immediately or aliquot and store samples at $\leq$ -20° C. Avoid repeated freeze-thaw cycles.

Note: Citrate plasma has not been validated for use in this assay. Do not use lipemic samples.

*To inhibit prostaglandin synthesis by COX-2, indomethacin should be added to serum and plasma collection tubes immediately following draw (to a final concentration of approximately 10 μg/mL).

Sample preparation

All samples require a 3-fold dilution. A suggested 3-fold dilution is 150 μL sample + 300 μL of Calibrator Diluent RD5-56.

Reagent preparation

Bring all reagents to room temperature before use.

Wash Buffer - If crystals have formed in the concentrate, warm to room temperature and mix gently until the crystals have completely dissolved. Dilute 20 mL of Wash Buffer Concentrate into deionized or distilled water to prepare 500 mL of Wash Buffer.

Substrate Solution - Color Reagents A and B should be mixed together in equal volumes within 15 minutes of use. Protect from light. 200 μL of the resultant mixture is required per well.

PGE_2 Standard - Reconstitute the PGE_2 Standard with 1.0 mL of deionized or distilled water. This reconstitution produces a stock solution of 25,000 pg/mL. Mix the standard to ensure complete reconstitution and allow the standard to sit for a minimum of 15 minutes with gentle agitation prior to making dilutions. Pipette 900 μL of Calibrator

Diluent RD5-56 into the 2500 pg/mL tube, and 500 µL of Calibrator Diluent RD5-56 into the remaining tubes. Use the 25,000 pg/mL standard stock to produce a dilution series (below). Mix each tube thoroughly and change pipette tips between each transfer. The 2500 pg/mL standard serves as the high standard and Calibrator Diluent RD5-56 serves as the zero standard (B0) (0 pg/mL).
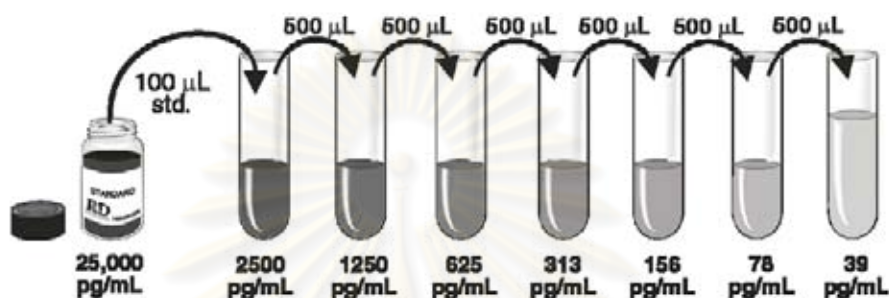
Assay procedure

Bring all reagents and samples to room temperature (18 - 23° C) before use. It is recommended that all samples, controls, and standards be assayed in duplicate.

1. Prepare all reagents, working standards, and samples as directed in the previous sections.

2. Remove excess microplate strips from the plate frame, return them to the foil pouch containing the desiccant pack, and reseal.

3. Add 200 µL of Calibrator Diluent RD5-56 to the NSB wells.

4. Add 150 µL of Calibrator Diluent RD5-56 to the zero standard (B0) wells.

5. Add 150 µL of Standard, control, or sample* to the remaining wells.

6. Add 50 µL of the Primary Antibody Solution to each well (excluding the NSB wells). All wells except the NSB wells will now be blue in color.

7. Securely cover with a plate sealer, and incubate for 1 hour at room temperature on a horizontal orbital microplate shaker (0.12" orbit) set at 500 ± 50 rpm. A plate layout is provided to record standards and samples assayed.

8. Do not wash the plate. Add 50 µL of PGE2 Conjugate to each well. All wells except the NSB wells will now be violet in color.

9. Cover with the adhesive strip provided, and incubate for 2 hours at room temperature on the shaker.

10. Aspirate each well and wash, repeating the process three times for a total of four washes. Wash by filling each well with Wash Buffer (400 μL) using a squirt bottle, manifold dispenser, or auto-washer. Complete removal of liquid at each step is essential to good performance. After the last wash, remove any remaining Wash Buffer by aspirating or decanting. Invert the plate and blot it against clean paper towels.

11. Add 200 μL of Substrate Solution to each well. Incubate for 30 minutes at room temperature on the benchtop. Protect from light.

12. Add 100 μL of Stop Solution to each well. The color in the wells should change from blue to yellow. If the color in the wells is green or the color change does not appear uniform, gently tap the plate to ensure thorough mixing.

13. Determine the optical density of each well within 30 minutes, using a microplate reader set to 450 nm. If wavelength correction is available, set to 540 or 570 nm. If wavelength correction is not available, subtract readings at 540 or 570 nm from the readings at 450 nm. This subtraction will correct for optical imperfections in the plate. Readings made directly at 450 nm without correction may be higher and less accurate. *Samples require dilution. See Sample Preparation section.

Calculation of results

Average the duplicate readings for each standard, control, and sample then subtract the average NSB optical density. Create a standard curve by reducing the data using computer software capable of generating a four paramater logistic (4-PL) curve-fit. As an alternative, construct a standard curve by plotting the mean absorbance for each standard on a linear y-axis against the concentration on a logarithmic x-axis and draw the best fit curve through the points on the graph. Do not include the B0 in the standard curve. If desired, % B/B0 can be calculated by dividing the corrected OD for each standard or sample by the corrected B0 OD and multiplying by 100.

Calculate the concentration of PGE_2 corresponding to the mean absorbance from the standard curve. Since samples have been diluted, the concentration read from the standard curve must be multiplied by the dilution factor.

Typical data

This standard curve is provided for demonstration only. A standard curve should be generated for each set of samples assayed.

pg/mL	O.D.	Average	Corrected	% B/B₀
NSB	0.009 / 0.009	0.009	—	—
0(B₀)	1.404 / 1.419	1.412	1.403	—
39	1.304 / 1.333	1.319	1.310	93
78	1.212 / 1.250	1.231	1.222	87
156	1.051 / 1.073	1.062	1.053	75
313	0.910 / 0.939	0.925	0.916	65
625	0.657 / 0.707	0.682	0.673	48
1250	0.448 / 0.452	0.450	0.441	31
2500	0.249 / 0.265	0.257	0.248	18
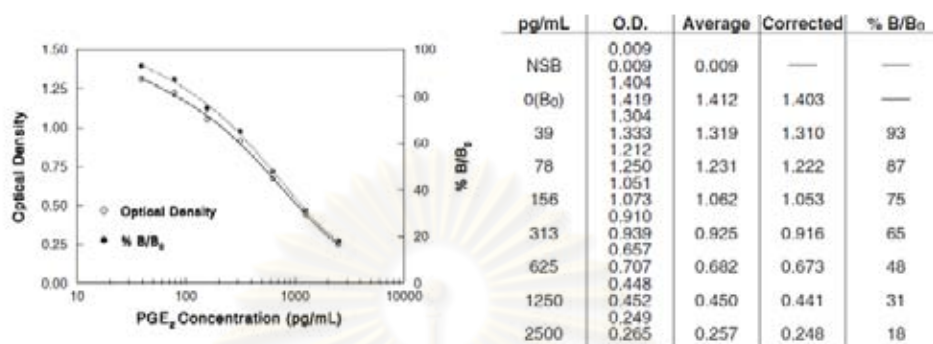
APPENDIX H

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.

9. Dissolve DNA pallet with 40-50 µl of TE buffer and storage in -20° C as DNA template

APPENDIX I

Indirect-ELISA PROTOCOL for *T. evansi* (provided by CIRAD-Bios, Dr M Desquesnes)

Material provided

    Polysorp Nunc microtiter plates®

    Carbonate buffer for coating (CB)

    Phospate buffered saline pH 7.4 (PBS)

    Washing buffer (WB) is PBS 0.1% tween 20®

    Blocking buffer (BB) is WB with 5% skimmed milk

    Anti-IgG cattle SIGMA®

    T. evansi antigen

Reagants

    Na_2CO_3

    $NaHCO_3$

    Na_2HPO_4 anhydrous

    NaH_2PO_4 anhydrous

    NaCl anhydrous,

    Hcl 1M

    NaOH 3M

    H3PO4 1M

    Tween 20

    Skimmed milk powder

    Conjugate anti-bovine (Sigma A 5295),

    Antigen: 0.5-2mg/ml concentration; Keep at -80° C

Coating buffer: Bring buffers at room temperature 30-15 min before using them.

    Na_2CO_3 1.58g

    $NaHCO_3$ 2.93g

    H_2O qsf 1 liter   adjust pH 9.6

PBS X 10 (Stock solution):

    Na_2HPO_4 anhydrous   59.64 g or $Na_2HPO_4 \cdot 7H_2O$ 112.60g or $Na_2HPO_4 \cdot 12H_2O$ 150.42 g

NaH$_2$PO$_4$ anhydrous   9.6 g

NaCl anhydrous   438.75 g

Distilled water   SQF 5 liters

PBS pH 7.4 (PBS 7.4 Working solution):

Dilute PBS X10 in 1:10 in distilled water; shake 20 minutes ; set the pH at 7.4 (HCl or NaOH).

(for two plates : 2 litres amongst which 1 is added with tween for WB 0.1%)

Washing buffer 0.1% (WB) :

1 litre of PBS 7.4 + 1 ml Tween 20$^®$ (1 litre)

Blocking Buffer (BB): (100 ml)

7 gram skimmed milk powder/100ml of WB

Peroxidase conjugate anti-bovin SIGMA$^®$ (ref A. 5295)

place 1 µl / 10 ml of BB

Conserved the stock at – 80$^°$ C

(1µ)

positive and negative control sera

Conserved at –20$^°$ C (or –80$^°$ C for the stock)

(4µ)

TMB: keep the stock at 4$^°$ C, but take out the necessary quantity and put it at room temperature 15 min before using it.

Reference samples:

Wells A1 A2: empty (plate density)

Wells B1-B2: no serum sample (blocking, conjugate and substrate control)

Wells C1-C2: positive control 1

Wells D1-D2: negative control 1

Wells E1-E2: positive control 2

Wells F1-F2: negative control 2

Wells G1-G2: positive control 3

Wells H1-H2: negative control 3

40 test samples are deposited in duplicate horizontally on one microtiter plate

Protocol:

Coating: 100 µl/well at a protein concentration of 5 µg/ml in CB, overnight at 4° C or 2Hrs at 37° C. (For Antigen 6.8 mg/ml we used 7.4 µl/10 ml of CB)

Rincing once with PBS, then discard

Blocking with 150 µl of BB/well, 45 min at 37° C; then discard

Rincing once with WB before transferring

Pre-dilution of sera 1:50 in BB (3 µl/147 µl of BB)

Transfer and dilution of sera: Place 50 µl of BB in each well and add 50 µl of serum for a final dilution of 1:100

Incubation : 30 min. at 37° C, permanent shaking (2rpm)

Washing X 7 with WB

Conjugate diluted 1:10,000 in BB: add 100 µl per well

Incubation 30 min. at 37° C, permanent shaking

Washing X 7 with WB

Substrate: 100 µl/well of TMB (place TMB at room temperature 15 min before using it)

Incubation in a dark room (if possible with permanent shaking) for about 30 minutes

Reading of the plates at 620nm wavelength

Expression of results is done in relative percentage of positivity (RPP) as follows:

$$RPP = \frac{DO\ sample\ -\ DO\ ref.negative}{DO\ ref.\ positive\ -\ DO\ ref.\ negative}$$

BIOGRAPHY

SIRIPORN TANTAWET, D.V.M.

Date of birth: June 2nd, 1982

Place of birth: Phuket province

Education:

2000-2005	Doctor of Veterinary medicine (D.V.M.), Mahidol University
1994-1999	Ammartpanichnukul school, Krabi, Thailand
1988-1993	Tesaban 3 (Tahdang) school, Krabi, Thailand

Work experience:

2006-2007	Technical Sale Service, BioScience Animal Health Co., Ltd.
2008	Teaching assistant at Faculty of Veterinary Science, Mahidol University
2009	Instructor at Faculty of Veterinary Science, Mahidol University

Publication:

Tantawet, S., Chanapiwat, P., Kongsuk, C. and Kaeoket, K. 2005. Induce farrowing in sows by using Cloprostenol (Planate$^{®}$): studies on behavioral changes, timing of farrowing, duration of parturition, total born litter size and stillbirth rate. Proceeding of the 2nd APVS congress, Manila, Phillipines. September 19-21: 128-129.

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