

# ***Trypanosoma evansi* infection in mice and sow: cryopreservation of *T. evansi*, its infectivity and subsequent clinical signs and pathological findings**

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## **Abstract**

*Trypanosoma evansi* causes a disease known as surra. Surra is endemic and cause economic loss in many parts of South-East Asia including Thailand. The aims of the present study were to: 1) compare the effect glycerol and DMSO as cryoprotectant for *T. evansi* 2) study the infectivity of cryopreserved *T. evansi* in mice and pregnant sows. For experiments I-III, the mice were injected with 0.5 ml-of blood from infected beef cattle, located in Kanchanaburi and examined for parasitemia on day 3 post infection. After the mice showed parasitaemia, they were anesthetized/blooded for cryopreservation with freezing medium (Sodium citrate + PBS + Blood + Glycerol or DMSO) by using control rate freezer. Post-thaw motility cryopreserved *T. evansi* was evaluated under a phase contrast microscope. The vital organs from mice that died after parasitemia were collected and fixed in neutral buffer saline, stained with haematoxylin-eosin and examined under light microscope. For experiment IV, early pregnant sows were infected with  $4 \times 10^6$  *T. evansi* via ear vein, directly after thawing. Their body temperature, parasitemia and clinical signs were observed. The results reveal that using glycerol as cryoprotectant yields a superior motility than DMSO. The mice showed parasitemia and inflammation of vital organs, clinical and pathological signs such as lethargy, panting and drowsiness while pregnant sows had parasitaemia during 3-14 days post infection, clinical signs such as plaques hemorrhage and necrotic lesion on the skin of udder and an abortion occurred on day 11 post infection. In conclusion, cryopreserved *T. evansi* can cause clinical signs, clinical pathology and pathology in mice and also in pregnant sows which provides the feasibility of long term storage for further studies.

**Keywords:** *Trypanosoma evansi*, cryopreservation, abortion, sows

# การติดเชื้อ *Trypanosoma evansi* ในหนูทดลองและในสุกรนาง: การแช่แข็ง *T. evansi* ความสามารถในการก่อโรค อาการทางคลินิก และพยาธิสภาพ

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## บทคัดย่อ

เชื้อ *Trypanosoma evansi* เป็นเชื้อที่ก่อให้เกิดโรคที่เรียกกันว่า ซูร์รา (Surra) ซึ่งโรคนี้เป็นโรคประจำถิ่นที่มีการระบาดและก่อให้เกิดการสูญเสียทางเศรษฐกิจอยู่ในเอเชียตะวันออกเฉียงใต้ รวมทั้งประเทศไทยด้วย การทดลองครั้งนี้มีวัตถุประสงค์เพื่อ 1) เปรียบเทียบผลของการแช่แข็งเชื้อ *T. evansi* โดยใช้ glycerol และ DMSO 2) ศึกษาความสามารถในการก่อโรคของเชื้อ *T. evansi* ที่ผ่านการแช่แข็งแล้วในหนูทดลองและในสุกรนาง สำหรับการทดลองที่ 1-3 เลือดของวัวเนื้อจากจังหวัดกาญจนบุรีที่มีเชื้อ *T. evansi* ปริมาตร 0.5 มิลลิลิตร จะถูกฉีดเข้าไปในช่องท้องหนูทดลองปริมาตร 0.5 มิลลิลิตร และทำการตรวจดูระดับของตัวเชื้อในกระแสเลือดของหนูทดลอง โดยเริ่มสังเกตจากวันที่ 3 หลังการฉีดเชื้อ ถ้าพบว่าหนูทดลองมีภาวะเลือดมีปรสิต (Parasitaemia) หนูจะถูกวางยาสลบและทำเมตตามาคเพื่อนำเลือดไปผ่านการแช่แข็งด้วยสารละลายที่ประกอบด้วย Sodium citrate + PBS + Blood + Glycerol or DMSO จากนั้นนำมาละลายและประเมินการเคลื่อนที่โดยใช้กล้องจุลทรรศน์ชนิดตัดแสง อวัยวะของหนูที่ตายจากการติดเชื้อจะนำมาแช่ฟอร์มาลิน ผ่านกระบวนการซิมซอบด้วยพาราฟิน และย้อมสีเพื่อดูลักษณะจุลพยาธิวิทยา ด้วยกล้องจุลทรรศน์แสงสว่าง สำหรับการทดลองที่ 4 สุกรที่ตั้งท้อง ไม่เกิน 1เดือน จะถูกฉีดเข้าไปในกระแสเลือดที่ใบหูจำนวน  $4 \times 10^6$  ตัว จากนั้นทำการบันทึกอุณหภูมิร่างกาย ระดับตัวเชื้อในกระแสเลือด และอาการทางคลินิก ผลการทดลองพบว่า ในหนูทดลองพบภาวะ Parasitaemia นอนซึม ขาไม่มีแรง และพบการอักเสบของอวัยวะสำคัญ ในสุกรนางพบภาวะ Parasitaemia ในช่วง 3-14 วันหลังฉีดเชื้อ และพบอาการทางคลินิก เช่น plaques hemorrhage และ necrotic lesion ที่บริเวณเต้านม และแท้งที่ 11 วันหลังฉีดเชื้อ จากผลการทดลองสรุปได้ว่า เชื้อ *T. evansi* ที่ผ่านการแช่แข็ง สามารถก่อโรคในหนูทดลองและแม่สุกรนางที่ตั้งท้องได้ ซึ่งแสดงถึงความเป็นไปได้ในการเก็บเชื้อ *T. evansi* ด้วยวิธีการแช่แข็งเพื่อนำไปศึกษาวิจัยต่อไป

คำสำคัญ: *Trypanosoma evansi* การแช่แข็ง การแท้ง สุกรนาง

## Introduction

*Trypanosoma evansi* causes a disease known as surra. Surra is endemic in many parts of South-East Asia including Thailand. In Kanchanaburi province where our university campus located, there is a high incidence of *T. evansi* infections in beef and dairy cattle.

Trypanosome infection has been found in livestock 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 farm. Acute and chronic signs have been observed. Skin rashes are usually observed. High rate abortion occurs at 1-2 months after the outbreak. Deaths and neurological signs sometimes followed by abortion. However, infected weaning and fattening pigs do not show any clinical signs with high levels of parasitaemia (Teeraprasert et al. 1984a; Sirivan et al. 1987; Taweanan et al. 2001). These healthy carriers allow the parasite to easily spread throughout the populations. It's accepted that mice are a good laboratory animal model for experimental infection and also for propagation of *T. evansi* before further experiments (e.g. cryopreservation, experimental infection model).

Cryopreservation of this particular protozoan has been done only in species of *T. vivax* (Ndao et al. 2004) and *T. cruzi* (Filardi and Brener, 1975). To our knowledge, no study has been reported on cryopreservation of

*T. evansi*. Nevertheless, in order to better understand the epidemiological situation for further prevention and control of Surra in Thailand, studies on the cryopreservation of *T. evansi*, clinical pathology of *T. evansi* infected mice and sows are needed. Therefore, the specific aims of the present study were as follows: to compare freezing medium for cryopreservation of *T. evansi*; to perform an experimental infection in mice and pregnant sows; to perform histological examination of vital organs of Trypanosome-infected-mice; to observed the clinical sign of infected sows.

## Materials and Methods

The research proposal of this project was approved by the Faculty of Veterinary Science-Animal Care and Use Committee (FVS-ACUC)-Mahidol University, no. MUVS-2008-20.

### Experiment I

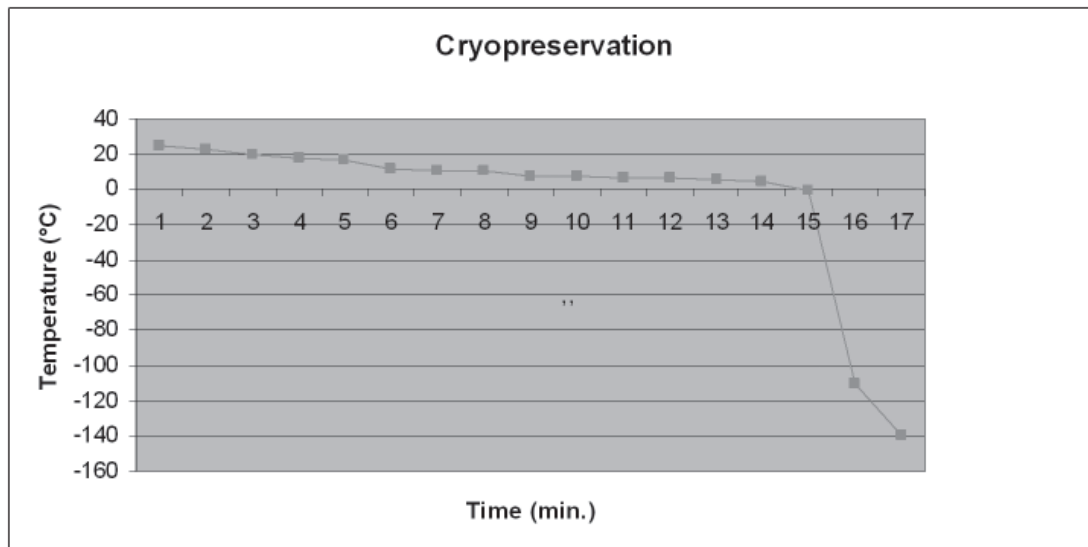
*T. evansi* was obtained from naturally infected beef-cattle. Blood was sampled from jugular vein and kept in EDTA tube for propagation in mice. The mice (*Mus musculus*) were injected with 0.5 ml-blood and examined for parasitemia, starting on day 3. For the mice that showed parasitemia (around days 5-7 post infection). They were anesthetized and bled for freezing with freezing medium which consist of Sodium citrate+PBS+Blood+Glycerol or DMSO (the method was modified after Ndao et al. (2004) (Table 1).

**Table 1** Composition of freezing medium for *Trypanosoma evansi*

Chemical	Volume/Quantity
Sodium citrate 4.5%	1 ml
Blood sample from infected mice	5-10 drops
Glycerol or DMSO	0.45 ml
Phosphate buffered saline (PBS)	1.05 ml
<b>Total volume</b>	<b>3 ml</b>

Trypanosome-samples were loaded into medium-straw or cryotube. The freezing was performed by using a control rate freezer (Icecube 14s, Sylab, Purkersdorf, Austria) (Fig. 1). In 18 minutes, the sample was brought down to a temperature of -140°C in the vapour phase of liquid nitrogen. In detailed, the freezing

rate was 3°C/min from +5° to -5°C, 1 min of holding time, and thereafter 50°C/min from -5° to -140°C. Thereafter, the samples were transferred to canisters containing LN<sub>2</sub> (-196°C) for storage. Post-thawing motility was performed at 37°C in a water baht, for 1 min and evaluated under phrase-contrast microscope.



**Figure 1** Freezing curve from a control rate freezer (Sylab IceCube 14S)

### Experiment II

This experiment was design to test the infectivity of frozen trypanosome-samples in mice before experimental infection in sows.

In 2005, we obtained trypanosome from naturally infected beef-cattle in Kanchanaburi and froze in cryotubes with Sodium citrate + PBS + Blood + Glycerol-extender, using a control rate freezer. After freezing as described in Experiment I, cryotubes were kept in liquid nitrogen tank for further experimental infection in sows. Prior to inoculation, this sample was thawed and kept in a water bath of 37°C for 1 min, hereafter the motility was checked by microscopic examination. After 4 years the motility was around 90%. Five mice were injected with 0.2 ml of trypanosome-samples and examined for clinical signs/parasitemia on day 3 post-infection.

### Experiment III

Organs (i.e. liver, spleen, kidney and testis) from mice that died from high parasitemia or euthanized by using pentobarbital sodium, were fixed in 10% neutral buffer formalin (NBF), embedded in paraffin, stained with Haematoxylin-Eosin, and examined under light microscope.

### Experiment IV

To obtain an infective dose to infect the sows with *T. evansi*, mice were infected by an intraperitoneal injection with *T. evansi* as described earlier in Experiment II. After a 30 day acclimatization period, 3 crossbred healthy sows from *T. evansi* free farm were housed individually in a fly-proof and tick-free concrete pen. Oestrous detection was performed daily. Sows were inseminated with fresh semen at 24 h and 36 h after

standing oestrous. The sows were subjected to pregnancy diagnosis twice on day 18 and 24 after insemination by using real time ultrasound. 25-day-pregnant sows were separated into 3 groups: the first sow (A) was infected with  $4 \times 10^6$  *T. evansi*, directly after thawing, the second sow (B) was infected with  $4 \times 10^6$  *T. evansi* after propagation in the mouse and the third sow (C, Control) was injection with sterile mouse's blood. The blood samples were collected daily during the first week post infection, and thereafter every 2 days for 14 days, parasitaemia was monitored by the micro-haematocrit centrifuge technique (MHCT) and thin smear. Clinical signs were monitored daily.

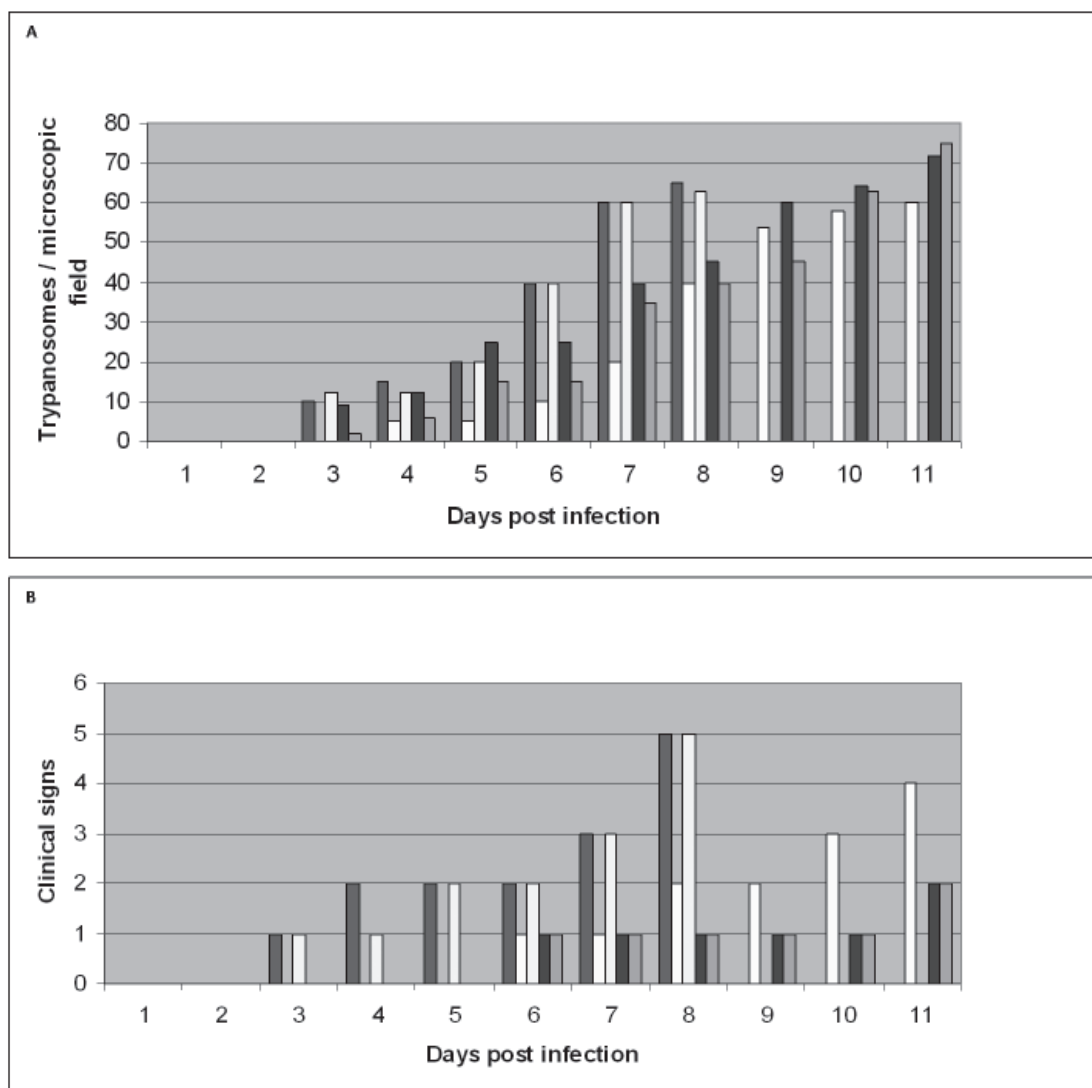
## Results

### Experiment I

The percentage of motility in Glycerol-freezing-extender was higher (80% versus 60%) than in DMSO-freezing-extender.

### Experiment II

Mice showed clinical signs (off feed and low activity) and high parasitemia on day 5 post-infection, and on day 8 for the others. The high-parasitemia mice (presence of trypanosome 70% in microscopic field) died one day after showing high-parasitemia. The correlation of clinical signs such as lethargy, panting, drowsiness and parasitemia is presented in Figure 2.



**Figure 2** Post-infection parasitemia (A) and clinical signs (B) of infected mice (each bar represent each mouse)

### Experiment III

In the liver, focal coagulative necrosis (indicating of anemia), megakaryocytes (indicating of regenerative anemia), necrosis of hepatocytes, diffuse fatty degeneration, atrophy of hepatic cords, infiltration of inflammatory cells in portal triad, and *T. evansi* in vessel were found. In the spleen, multi-focal necrosis of lymphoid cells in white pulp, megakaryocytes in red and white pulps, connective tissue framework, congestion, necrotic debris, and lymphoid depletion were observed. In kidney, mild glomerulitis and *T. evansi* in vessel were

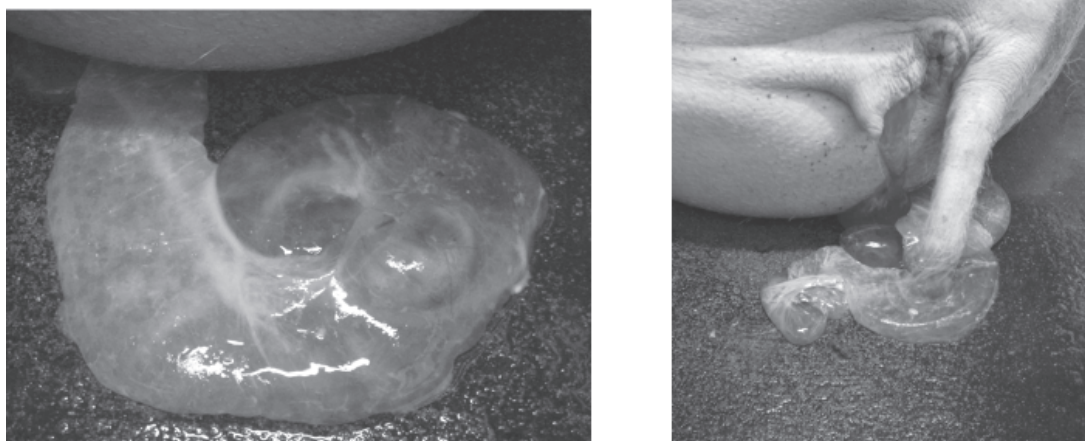
observed. There is no distinct pathological changed in testis.

### Experiment IV

Sow-A and -C have no clinical signs and clinicopathological changes in which no trypanosome was observed in the blood. While sow-B showed clinical sign (Figure 2) and parasitaemia during 3-14 days post infection and an abortion occurred on day 11 post infection.



**Figure 3** Clinical signs of plaques hemorrhage and necrotic lesion on the skin of udder



**Figure 4** Clinical signs of abortion and aborted fetus on day 11 post infection

## Discussion

For the cryopreservation of *T. evansi* from infected animal (mice and beef cattle), glycerol provided a superior motility of *T. evansi* than that of DMSO which is in agreement with the cryopreservation of *T. vivax* by Ndao et al. (2004). To our knowledge, we are the first group who reported the successful of cryopreservation of *T. evansi* from infected beef and mice by using control rate freezer and storage the cryopreserved *T. evansi* for 4 year and proved that their infectivity still remained.

The isolation of *T. evansi* used in the present study was obtained from a naturally infected beef cattle case of surra from Kanchanaburi province 4 year before commencing the experiment. This was assumed to be a representative strain of trypanosome that causing significant mortality and morbidity in livestock animals in the western and central parts of Thailand over the past decade. The present results suggested that after being cryopreserved and passaged twice in mice, *T. evansi* are able to infect mice and sows and induce clinical signs. In mice, most of them developed clinical signs and a high parasitemia by day 5-7 after inoculation, and die with nervous sign which is longer (3 days) than the study by Tuntasuvan et al. (2000). This difference might be the reason that the present study used cryopreserved *T. evansi*, not directly from infected animal. In sows, however, less severity of clinical signs were observed in this study when compared with the report by Teeraprasert et al. (1984a,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. (1984b) reported that *T. evansi* isolated from a clinical case passaged in mice and used  $2 \times 10^6$  *T. evansi* to infect the sows having parasitemia 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 cryopreserved strain of *T. evansi*, in this study, could cause the disease after being passaged in mice. However, the prolong prepatent period and less virulence were observed in this study. In addition, fever was observed only one day before abortion or at the abortion day. In accordance with the experimental infection study in goat (Dargantes et al. 2005), the degree of parasitaemia in pig is not paralleled with the body temperature, indicating that the number of trypanosomes in the blood circulation might not related to the concentration of pyrogenic cytokines as reported in hog deer (Tuntasuvan et al. 2000).

## Conclusion

Cryopreserved *T. evansi* can cause clinical signs, clinical pathology and pathology in mice and also in pregnant sows which provides the feasibility of long term storage for further studies.

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## References

- Dargantes, A.P., Reid, S.A. and Copeman, D.B. 2005. Experimental *Trypanosoma evansi* infection in the goat. I. clinical signs and clinical pathology. J. Comp. Path. 133. 261-266.
- Desquesnes, M., Kamyinkird, K., Pruvot, M., Kengradomkij, C., Bossard, G., Sarataphan, N. and Jittapalapong, S. 2009. Antibody-ELISA for *Trypanosoma evansi*: Application in a serological survey of dairy cattle, Thailand, and validation of a locally produced antigen. Prev. Vet. Med. 90. 233-241.

- Filardi, L.S. and Brener, S. 1975. Cryopreservation of *Trypanosoma cruzi* blood stream form. J. Protozool. 22: 398-401.
- Holland, W.G., Thanh, N.G., Do, T.T., Sangmaneeet, S., Goddeeris, B. and Vercruyse, J. 2005. Evaluation of diagnostic tests for *Trypanosoma evansi* in experimentally infected pigs and subsequent use in field surveys in north Vietnam and Thailand. Trop. Anim. Health. Prod. 37. 457-467.
- Indrakamhang, P. 1998. *Trypanosoma evansi* infection in livestock in Thailand. J. Protozool. Res. 8, 153-161.
- Ndao, M., Magnus, E., Buscher, P. and S. Geerts. 2004. *Trypanosoma vivax*: a simplified protocol for *in vitro* growth, isolation and cryopreservation. Parasite, 11, 103-106.
- Sirivan, C., Sarataphan, N., Bunyahotra, R., Mekamol, C., Mepeuch, Y. and Asawamasakda, C. 1987. Trypanosomiasis in pigs. 1. Occurrence of trypanosomiasis in a pig farm in Suphanburi. Proceeding of the 6<sup>th</sup> annual livestock conferences, Department of Livestock Development. Bangkok, Thailand. May 18-20, 84-97.
- Srivastava R.P. and S.S. Ahluwalia. 1972. Clinical observation on pigs experimentally infected with *Trypanosoma evansi*. Ind. Vet. J. 42. 1184-1186.
- Taweean, W., Jiwkanon, J. and P. Karnbutra. 2001. Case report: an outbreak of *Trypanosoma evansi* in pigs in Khon Kaen. KKU. Vet. J. 11. 42-46.
- Teeraprasert, E., Chaichanapunpol, I., Trongwongsa, L., Sakdasirisathapom, A., Wongkasemchit, S., Udomphant, S., Suvanvasi, P., Piniij, N. and Tungchaitong, S. 1984a. A case report of *T. evansi* in pigs. Proceeding of the 11th annual veterinary sciences conferences, The Thai veterinary medical association under royal patronage. Bangkok, Thailand. December 12-14: 53-64.
- Teeraprasert, E., Trongwongsa, L., Chaichanapunpol, I., Sakdasirisathapom, A., Wongkasemchit, S., Udomphant, S., Suvanvasi, P., Piniij, N. and Tungchaitong, S. 1984b. The experimental infection of *T. evansi* in a sow. Proceeding of the 11<sup>th</sup> annual veterinary sciences conferences, The Thai veterinary medical association under royal patronage. Bangkok, Thailand. December 12-14: 65-72.
- Tuntasuvan, D. and Luckins, A.G. 1998. Status of Surra in livestock in Thailand. J. Protozool. Res. 8: 162-170.
- Tuntasuvan, D., Mimapan, S., Sarataphan, N., Trongwongsa, L., Intraraksa R. and A.G. Luckins. 2000. Detection of *Trypanosoma evansi* in brain of the naturally infected hog deer by streptavidin-biotin immunohistochemistry. Vet. Parasitol. 87. 223-230.