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Research article

Prevalence of *Trypanosoma evansi* infections in buffaloes, beef and dairy cattle in Sakon Nakhon province using molecular and serological assays

Eukote Suwan[a], Piangjai Chalermwong[a], Suchada Choocherd[a], Sakulchit Wichainchot[a], Chanapath Thabthimsri[a], Phirabhat Saengsawang[b], Marc Desquesnes[c,d], Wanwisa wachoom[e], Kannika Wongpanit[f], Sathaporn Jittapalapong[a,g,*]

[a] *Department of Veterinary Technology, Faculty of Veterinary Technology, Kasetsart University, Bangkok 10900, Thailand*

[b] *Akkhraratchakumari Veterinary College, Walailak University, Nakhon Si Thammarat 80161, Thailand*

[c] *The French Agricultural Research Centre for International Development , Unité mixte de recherche– Interactions Hôte-Vecteur-Parasite-Enrironnement dans les Maladies Tropicales Négligées dues aux Trypanosomatidés , Kasetsart University Research and Development Institute, Kasetsart University, Bangkok 10900, Thailand*

[d] *Interactions Hôte-Vecteur-Parasite-Enrironnement dans les Maladies Tropicales Négligées dues aux Trypanosomatidés, University of Montpellier, The French Agricultural Research Centre for International Development, Institute of Research for Development, F-34398 Montpellier, France*

[e] *Nawa District Livestock Office, Nakhon Phanom 48180, Thailand*

[f] *Department of Agriculture and Resources, Faculty of Natural Resources and Agro-Industry, Chalermphrakiat Sakon Nakhon Province Campus, Kasetsart University, Sakon Nakhon 47000, Thailand*

[g] *Center for Advanced Studies for Agriculture and Food, Kasetsart University Institute for Advanced Studies, Kasetsart University, Bangkok 10900 Thailand (CASAF, NRU-KU, Thailand)*

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Abstract

Importance of the work: *Trypanosoma evansi* is a vector-borne pathogen, responsible for *T. evansi* infection (surra) which has a major impact on animal health in Southeast Asia, including Thailand.
Objectives: To update the current situation regarding trypanosome infections of cattle and buffaloes in Sakon Nakhon province, Thailand.
Materials & Methods: Blood samples were collected from buffaloes (455) and beef (1,077) and dairy (227) cattle in eight districts of Sakon Nakhon province and tested for trypanosome infections using *T. evansi* indirect-enzyme-linked immunosorbent assay (ELISA) and TRYP1 polymerase chain reaction (PCR).
Results: Among the 1,759 samples, there were 77 positives (4.38%; 77/1,759) from *T. evansi* indirect-ELISA and 8 positives (0.45%; 8/1,759) from TRYP1 PCR, of which two samples were positives for both tests. The ITS1 sequencing analysis revealed high *T. evansi* sequence identities (97.22–100%). Factors associated with *T. evansi* infections were age, species, farm size and district. The highest prevalence was in buffaloes (9.67%; 44/455), followed by beef cattle (3.25%; 35/1,077) and dairy cattle (1.76%; 4/227). The significant associations with *T. evansi* infection were an animal age of 1–3 yr (6.82%; 53/777; $p = 0.00$), a medium herd size (5.99%; 13/217; $p = 0.03$) and farm located in Phanna Nikhom district (21.33%; 32/150; $p = 0.00$).
Main finding: Cattle and buffaloes in Sakon Nakhon province had a low prevalence (less than 5%) for current infection by *T. evansi*. However, the prevalence of trypanosome infections might be increasing due to the high densities of blood-sucking flies.

* Corresponding author.
E-mail address: fvetspj@ku.ac.th (S. Jittapalapong)

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Introduction

Trypanosomes are blood protozoa, found in both domestic and wild animals that are mechanically transmitted by blood-sucking flies, such as tabanids and *Stomoxys* spp. (Desquesnes et al., 2013a). Trypanosomosis is known as an economically important disease that causes high mortality, lower milk and meat production and lower reproductive performance, as well as immunosuppression in livestock (Dargantes et al., 2009; Desquesnes et al., 2013b). *T. evansi* has been reported to be occasionally zoonotic in India, Sri Lanka, Egypt and Vietnam (Truc et al., 2013; Chau et al., 2016).

Many diagnostic assays have been developed and used to detect *T. evansi* infections, including blood smear examination, the haematocrit centrifuge technique, mouse inoculation, the card agglutination test (*T. evansi*), a latex agglutination test (*T. evansi*), indirect enzyme-linked immunosorbent assay (ELISA) and polymerase chain reaction (PCR) (Monzón et al., 1990; Verloo et al., 2000; Migri et al., 2016). Frequently, indirect ELISA has been used to detect *T. evansi* infection in cattle and buffaloes in Indonesia (Payne et al., 1991) and India (Sinha et al., 2006). In Thailand, *T. evansi* infections have been reported in rodents (Milocco et al., 2013, prevalence = 0.7%), elephants (Camoin et al., 2018, prevalence = 2.1%) and horses (Camoin et al., 2019, prevalence = 0.4%), as well as in dairy cattle (Desquesnes et al., 2009, prevalence = 25.0%), and buffaloes (Kocher et al., 2015, prevalence = 12.2%).

Northeast Thailand has the largest population of buffaloes and beef cattle (Department of Livestock Development, 2022). These livestock have been reported to be significantly clinically and economically affected by surra, a disease due to *T. evansi* in various provinces in Northeast Thailand, with Khon Kaen province having a prevalence of 51.5% in beef cattle, while Nakhon Phanom had 39.4% in buffaloes and Sakon Nakhon had 6.1% in dairy cows (Pholphak and Pholphak, 2013). Sakon Nakhon province is the major source of high-quality beef in Thailand, where there are more than 5,000 household members (Department of Intellectual Property, 2018).

The aim of this study was to determine the prevalence of *T. evansi* infections in beef and dairy cattle and in buffaloes in Sakon Nakhon province using indirect ELISA and a PCR technique. The PCR positives were further investigated using species-specific primer PCR to identify the species.

Materials and Methods

Sample collection

A cross-sectional study design was conducted, with the sample size calculated using the formula of Yamane (1973), based on the number of animals obtained from Department of Livestock Development (2021). During October–November 2021, 1,759 blood samples were collected, consisting of 1,077 beef cattle (941 females and 136 males), 455 buffaloes (360 females and 95 males) and 227 dairy cattle (227 females) from 356 farms in 8 districts of Sakon Nakhon (Akat Amnuai, Khok Sri Suphan, Kusuman, Mueang, Phanna Nikhom, Sawang Daen Din, Wanon Niwat and Waritchaphum). The ages of the animals were divided into three groups (<1 yr, 1–3 yr and >3 year). The farm size based on the number of animals per farm were defined as small (1–10 head/farm), medium (11–50 head/farm) and large (>50 head/farm), according to Department of Livestock Development (2018). A blood samples (10 mL) was collected from the jugular vein of each sampled animal, stored in sterile and ethylene-diamine-tetraacetic acid (EDTA) tubes and transported within 24 hr to the Faculty of Veterinary Technology, Kasetsart University, Bangkok, Thailand. Sera were separated and stored at -20°C until used. Whole blood samples in EDTA were kept at 4°C for analysis using parasitological and hematological techniques and stored at -20°C for DNA preparation in the molecular process.

The intensity of the contact between livestock and trypanosome was estimated using the ELISA antibody detection method, as recommended by World Organization for Animal Health (2013). Prevalence of active infections was estimated using ITS1 ribosomal DNA amplification and Giemsa-stained blood smear examination, as described below.

Indirect-enzyme-linked immunosorbent assay for T. evansi antibody detection

The soluble antigen of *T. evansi* was modified according to Desquesnes et al. (2009). Briefly, a cryopreserved Thai isolate of *T. evansi* was intraperitoneally inoculated in 2 Wistar rats (*Rattus norvegicus*). After their parasitemia reached 1×10^8 parasites/mL, the rats were anaesthetized with chloroform and blood was collected. The buffy-coat and lower part of the plasma were harvested using 10,000×*g* centrifugation for 10 min and applied on diethyl-aminoethyl-cellulose (DEAE-cellulose®; Sigma-Aldrich; USA) to separate any parasites from

the blood cells. The parasites were washed twice in phosphate saline glucose at pH 8.0 using 10,000×*g* centrifugation for 10 min. The pellet was collected and subjected to the freeze-thaw method (five cycles of freezing for 2 min in liquid nitrogen followed by 5 min thawing in an incubator at 37°C) before being lysed using sonication (60% active cycle with output power 7 s for 2 min). The lysate was collected using 10,000×*g* centrifugation for 10 min at 4°C and stored at -80°C until used.

The indirect-ELISA procedure was derived as described for dairy cattle (Desquesnes et al., 2009) and buffaloes (Kocher et al., 2015), with some modifications. Briefly, 10 µg/mL of *T. evansi* soluble antigen in carbonate buffer at pH 9.6 was coated on Microtest 96-well Polysorp Nunc® Immuno Plates (Nunc; Denmark) and incubated at 37°C for 2 hr. The plates were blocked with blocking buffer (7% skim milk in 1× phosphate buffer saline (PBS), pH 7.4 (Wako Pure Chemical Industries; Japan) and shaken at 37°C and 300 revolutions per minute (rpm) for 1 hr. After discarding the blocking buffer, 1:100 diluted sera in blocking buffer were applied in duplicate on the ELISA plate. Each plate was shaken at 37°C and 300 rpm for 30 min before being washed seven times with washing buffer (1× PBS, pH 7.4 with 0.1% Tween 20). Then, 1:10,000 diluted peroxidase-conjugated anti-bovine IgG (Sigma-Aldrich; USA) in blocking buffer was added. The plate was incubated at 37°C and 300 rpm for 30 min and washed seven times with washing buffer. The substrate/chromogen complex 3,3',5,5'-tetramethylbenzidine (TMB) (SureBlue TMB; USA) was added; then, the plate was incubated in a dark room at room temperature for 30 min. The optical density (OD) was measured at 655 nm using a microplate absorbance reader (Bio-Rad Laboratories; USA).

ELISA was performed in duplicate for all samples, with three positive controls and three negative controls on each plate. Selection of reference samples and the cut-off value were as described by Desquesnes et al. (2009). The blank OD value was automatically deducted from each sample value and the results were expressed as a relative percentage of positivity (RPP) according to Equation 1:

$$\text{Sample RPP} = \frac{\text{Mean OD of sample-Mean OD of negative control}}{\text{Mean OD of positive control-Mean OD of negative control}} \quad (1)$$

Polymerase chain reaction for T. evansi DNA detection

Trypanosoma evansi genomic DNA was extracted from whole blood samples using an E.Z.N.A.® Tissue DNA Kit (Omega Bio-Tek; USA) according to the manufacturer's instructions and kept at -20°C until used. A trypanosome-specific primer (TRYP1) was used to amplify the internal transcribed spacer 1 (ITS1, situated between the 18S and 5.8S genes) of the ribosomal DNA (Pruvot et al., 2010). The PCR reactions consisted of 1× buffer, 200 µM of each dNTP, 1.5 mM $MgCl_2$, 1 µM of each primer, 0.5 unit of *Taq* DNA polymerase (Thermo Fisher Scientific; USA) and 5% dimethyl sulfoxide. The PCR conditions were: 94°C for 3 min, followed with 35 cycles of 94°C for 1 min, 60°C for 1 min, 72°C for 1 min and 72°C for 10 min. The PCR products were verified using 1.2% agarose gel electrophoresis. The TRYP1-positive samples were sent for sequencing (Macrogen; South Korea), and species investigation was conducted using RoTat1.2 (*T. evansi* species-specific primers), Lew1 (*T. lewisi* species-specific primers) and TCZ (*T. cruzi* species-specific primers), as shown in Supporting Information Table S1). The PCR reaction was performed as described above and the PCR conditions were: 94°C for 1 min, followed with 35 cycles of 94°C for 30 s, 60°C for 1 min, 72°C for 30 s and 72°C for 5 min.

Hematological examination

All blood samples in EDTA tubes were kept at 4°C and sent to the Veterinary Diagnostic Laboratory Center, Kasetsart University, Bangkok, Thailand for Giemsa-stained thin blood smear, a manual differential count and complete blood count tests. Common blood components and features were analyzed for erythrocytic count (RBC), hemoglobin concentration, hematocrit level, mean corpuscular volume (MCV), mean corpuscular hemoglobin (MCH), mean corpuscular hemoglobin concentration, platelet (PLT) count, leukocyte count, neutrophils (BandNEU), segment neutrophils (SegNEU), lymphocytes (LYMP), monocytes (MONO), eosinophils (EOSIN) and basophils (BASO).

Statistical analysis

An estimation of prevalence was directly inferred from both the ELISA and PCR results and a χ^2 test was applied to analyze which factors were associated with trypanosome infections based on a comparison between potential risk factors and the outcome variable (*T. evansi*-positive and *T. evansi*-negative animal status). Logistic regression was carried out using variables that showed significance in the univariable analysis; all these were included in

a stepwise variable selection with a linear model function. The backward direction was used as the option for model selection, not significant interactions were removed in the backward process. The algorithm of the statistical program performed model selection, based on the Akaike information criterion (AIC) until the model with the lowest AIC was found and used as the final model. Then, the overall model fitting of the final model was tested based on a likelihood ratio test. A comparison of hematological values was evaluated between *T. evansi*-positive and *T. evansi*-negative groups using the Mann-Whitney U test. All statistical analyses were performed using the R programming language version 4.0.4 (R Core Team, 2021), with $p < 0.05$ considered significant.

Ethics statement

This study was approved by the Ethics Committee of Kasetsart University, Bangkok, Thailand (Approval no. ACCKU64-VTN-017).

Results

Prevalence of T. evansi infection

Amongst the 1,759 animals from 356 farms from 8 different districts in Sakon Nakhon province (Fig. 1), *T. evansi*-indirect ELISA detected seropositive animals at a rate of 4.38% (77/1,759)

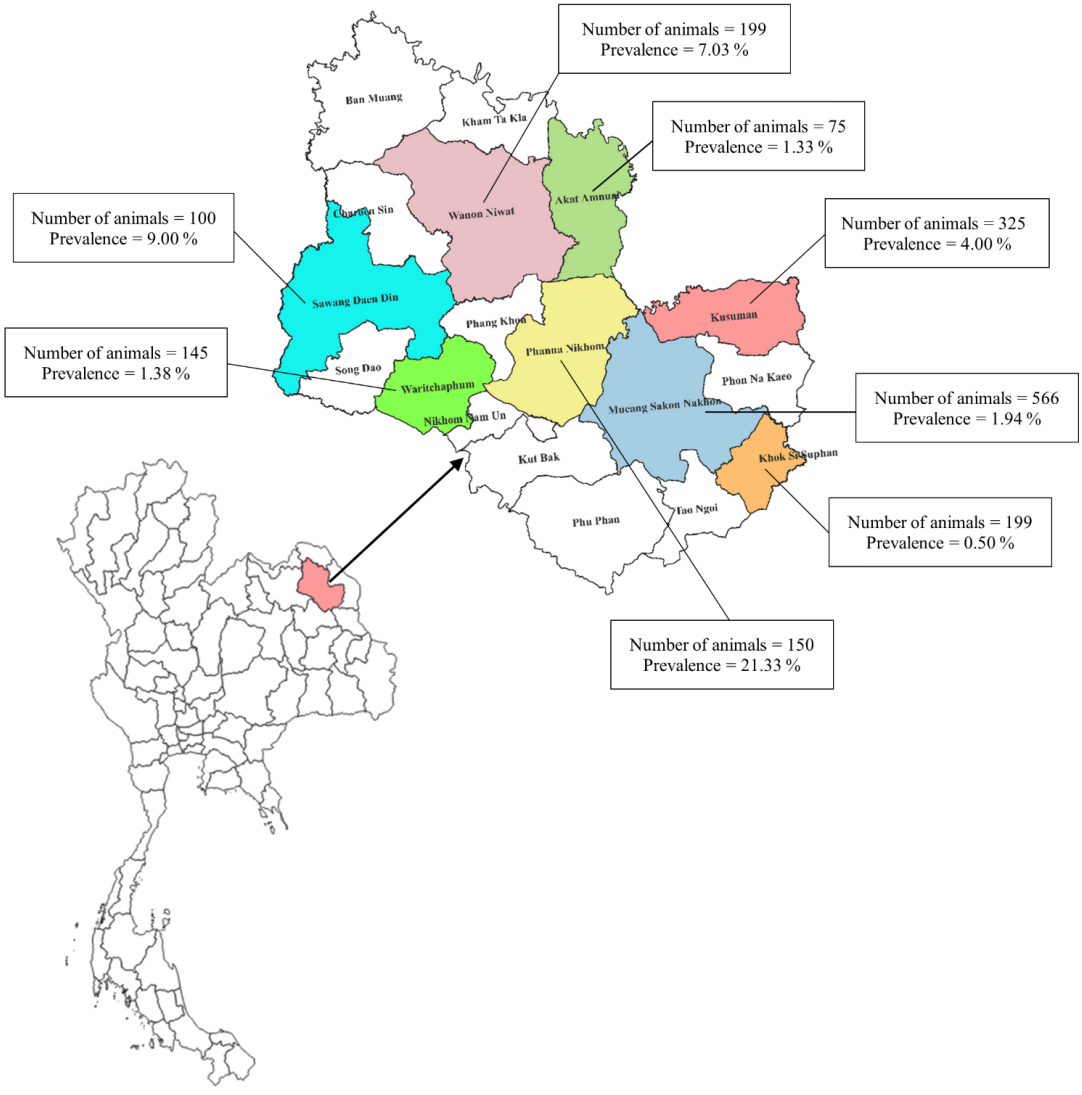

Fig. 1 Geographical representation of current status of *Trypanosoma evansi* infections of buffaloes and beef and dairy cattle in Sakon Nakhon province (in red), Thailand

and farm prevalence at 11.80% (42/356), while molecular assay based on TRYP1 PCR detected prevalence at 0.45% (8/1,759) and farm prevalence at 1.12% (4/356), as shown in Table 1 and Supporting Information Table S2. There were two samples that were positive by both PCR and ELISA. In the study of associations between *T. evansi* infections and the risk factors under univariable analysis, no significant differences were found between sex. Animals aged >1–3 yr had the highest prevalence at 6.82% (53/777; $p = 0.00$) compared to animals aged less than or equal to 1 yr (1.11%) and greater than 3 yr (3.49%). Among each type of animal, buffaloes had the highest prevalence at 9.67% (44/455), followed by beef cattle at 3.25% (35/1,077) and dairy cattle at 1.76% (4/227; $p = 0.00$). The medium-sized farms had the highest positives (5.99%; 13/217; $p = 0.03$) compared to small and large farms at 5.40% and 2.45%, respectively. The Phanna Nikhom district (all samples were from buffaloes) had the highest prevalence at 21.33% (32/150), followed by 9 % (9/100) in Sawang Daen Din district for beef cattle and 1.38% (2/145; $p = 0.00$) for Waritchaphum dairy cattle.

Based on the logistic regression analysis, four variables remained in the final model—age, animal species, farm size and district; these significantly related to *T. evansi* infection (AIC = 573.30). The result from the likelihood ratio test indicated a good model fit ($\chi^2 = 123.59$, $p = 0.00$). The results of *T. evansi* infection showed that based on animal age, the risk for the group aged 1–3 yr was six times higher than for the group aged less than 1 yr. In addition, buffaloes had three times higher infection level compared to beef cattle. The odds of *T. evansi* infection in medium-sized farms were 1.13 times higher than for small-sized farms. The animals in Phanna Nikhom, Sawang Daen Din, Wanon Niwat and Kusuman districts had higher infection risks at 17.99 times, 6.64 times, 5.07 times and 2.80 times, respectively, compared to the Waritchaphum breed.

All eight positives were investigated using species-specific primers and sequencing. Positive bands were observed from *T. evansi*-specific primers (RoTat1.2), while none of them produced bands with the Lew1 and TCZ primers. Sequence analysis using Clustal Omega (https://www.ebi.ac.uk/Tools/msa/clustalo/) revealed 97.22–100% sequence identities, with four samples (three buffalo samples from Phanna Nikhom district and a beef cattle sample from Wanon Niwat district) shared 99.07–99.63% sequence identities with *T. evansi* (accession no. D89527.1), while the other samples (three buffalo samples

Table 1 Associations between *Trypanosoma evansi* infection and risk factors

Variable	Number of animals	*T. evansi* infection		Univariable analysis		Logistic regression analysis	
		Positive		χ^2	*p* value	Odds ratio	*p* value
		n	%				
Sex				0.23	0.64	-	-
Female	1,528	74	4.84				
Male	231	9	3.90				
Age				15.54	0.00		
≤ 1 year old	180	2	1.11			Ref.	
> 1–3 years old	777	53	6.82			5.94	0.04
> 3 years old	802	28	3.49			3.11	0.14
Type				34.40	0.00		
Beef cattle	1,077	35	3.25			Ref.	
Buffalo	455	44	9.67			3.18	0.00
Dairy cattle	227	4	1.76			0.55	0.14
Farm unit				7.04	0.03		
Small	1,093	59	5.40			Ref.	
Medium	217	13	5.99			1.13	0.00
Large	449	11	2.45			0.45	0.98
Area				122.00	0.00		
Waritchaphum	145	2	1.38			Ref.	0.54
Akat Amnuai	75	1	1.33			1.03	0.38
Khok Sri Suphan	199	1	0.50			0.38	0.04
Kusuman	325	13	4.00			2.80	0.98
Mueang	566	11	1.94			1.33	0.00
Phanna Nikhom	150	32	21.33			17.99	0.00
Sawang Daen Din	100	9	9.00			6.64	0.00
Wanon Niwat	199	14	7.03			5.07	

Ref. = Reference

from Phanna Nikhom district and a beef cattle sample from Wanon Niwat district) shared 100% identities with *T. evansi* (accession no. U75507.1), as shown in Fig. S1.

Parasitological and hematological assays

All 635 risk animals from the infected farms with 36.10% prevalence were further investigated using parasitological and hematological assays. Based on the light microscope examination, there were approximately 4×10^2–2.9×10^7 parasites/mL of blood (0.002–18.9 parasites/field) and the *T. evansi* morphology observed included a size of approximately 25–35 µm with a small kinetoplast, a thin posterior extremity, a central nucleus and a large undulating membrane (Desquesnes et al., 2013a).

The hematological comparison between the *T. evansi*-positive and *T. evansi*-negative animals showed a significant difference in blood parameters ($p < 0.05$), as provided in Fig. 2.

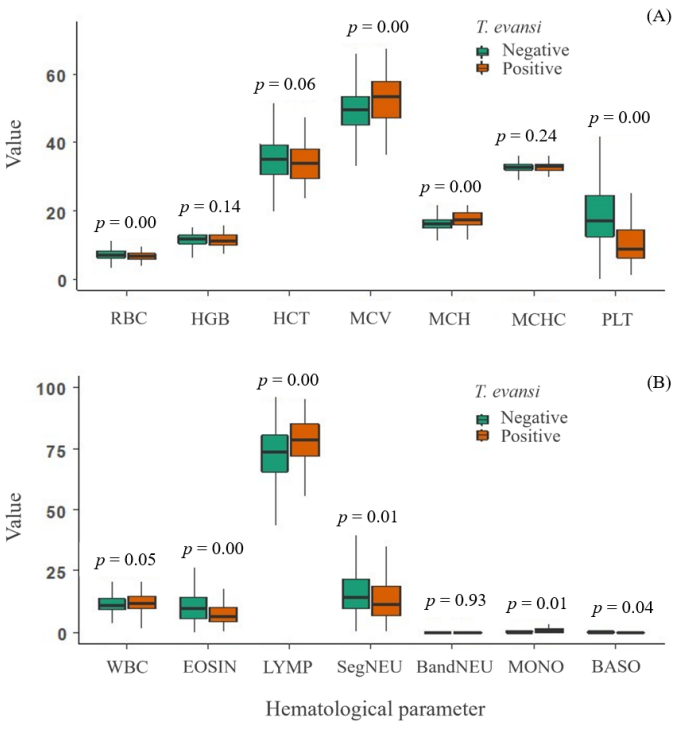

Fig. 2 Box plot of hematological parameters of animals on positive farms: (A) erythrocytes and thrombocytes; (B) leukocytes, where RBC = red blood cell, HGB = hemoglobin; HCT = hematocrit, MCV = mean corpuscular volume, MCH = mean corpuscular hemoglobin, MCHC = mean corpuscular hemoglobin concentration, PLT= platelets, WBC = white blood cells, BandNEU = band neutrophils, SegNEU = segmented neutrophils, LYMP = lymphocytes, MONO = monocytes, EOSIN = eosinophils and BASO = basophils

Amongst the erythrocyte parameters, the RBC value was significantly decreased in *T. evansi*-positive animals as well as the thrombocyte parameter and the PLT count, compared to negative animals. In contrast, the values of MCV and MCH were significantly higher in positive animals. The comparison of leukocyte parameters (the percentage of SegNEU, EOSIN and BASO) significantly reduced, while LYMP and MONO significantly increased in the positive animals. Only BandNEU did not have a significant difference ($p = 0.93$), as shown in Table S3.

Discussion

Indirect-ELISA and PCR were used to detect *T. evansi* infections in buffaloes, beef, and dairy cattle in Sakon Nakhon province. The results indicated that the factors of age, animal type, farm size and district were significantly associated with *T. evansi* infections. Buffaloes had the highest infected number at 9.67%, followed by beef cattle at 3.25% and dairy cattle at 1.76% compared to the other studies that reported on the seroprevalence of buffaloes (8%), beef cattle (6%), and dairy cattle (19%) in Sakon Nakhon district (Desquesnes et al., 2009; Kocher et al., 2015). The results from the current study might indicate that buffaloes are a probable robust reservoir for *T. evansi* and are responsible for the high risk of *T. evansi* infection in animals that are raised in the same area (Kocher et al., 2015). Dairy cattle and a large farm size had the lowest infection levels, perhaps due to intensive management (such as health care procedures and vaccination and deworming programs) to prevent diseases, which could adversely impact all animals in a herd. The dairy cow management was quite intensive since these cows were kept inside in stalls, except during their dry period. For large-sized farms, the Phon Yang Kham Cooperative, one of the professional beef producers in Thailand, located in Sakon Nakhon has a modern farm management system that efficiently prevents arthropod-borne diseases. Considering the area and animal type factors, the highest infected area was Phanna Nikhom district (21.33%) and buffaloes had the highest prevalence (9.67%). Buffaloes are considered as a reservoir species for trypanosome infections since they are reared, used in agricultural practice, and live longer than beef cattle (Kocher et al., 2015). Normally, buffaloes are a native breed in Thailand so that they can resist this infection and might not have severe clinical signs when infected. Therefore, buffaloes have an important role as reservoirs or carriers of this disease.

Normally, buffaloes and beef cattle are reared and maintained in the field rather than in stalls because of two factors. First, buffaloes have been used in conventional agricultural practice and have been kept outside to lower their cost. Second, some beef cattle are normally used as for transportation; therefore, they are kept outside all day. Most beef cattle will be reared on public grassland or in the farmyard until dark. Therefore, buffaloes might be at most risk to attack by blood-sucking flies in the nearby environment, since they are outside during the day. Beef cattle might be the second most-risk group because they share the same conditions as buffaloes.

Animals aged 1–3 yr had the highest *T. evansi* infection (6.82%) compared to the other age groups (1.11% and 3.49%). Notably, only 2 of 180 animals aged less than 1 yr were identified as infected, probably due to the fact that young animals are poorer attractants to the biting flies acting as vectors.

The prevalence of trypanosome infections was correlated with a high availability of biting flies since *Tabanus* spp. plays an important role in *T. evansi* outbreaks in Thailand. More than 45 *Tabanus* species were identified and described, in which *Tabanus striatus* is the most common species and has been found in North and Central Thailand, followed by *T. megalops* has been found in Central Thailand, while *T. rubidus* has been found all over the country (Ito, 1996; Changbunjong et al., 2018; Wongthangsiri et al., 2019). The seasonal effect on *T. evansi* infection due to the *Tabanus* activity has been described by Kashiwazaki et al. (1998) using serological and parasitological assays.

All PCR positives were found on the same farms with indirect-ELISA positives, as a couple of techniques were successful in detecting and monitoring the current situation of trypanosome infection in animals. Both PCR and light microscopy revealed the same results of detection, which are probably related to the amount of parasitemia (1×10^2 trypanosomes per mL of blood) that could be observed and detected in the samples (Konnai et al., 2009). The comparison of blood parameters showed that the counts of the erythrocyte, platelet, neutrophil, eosinophil and basophil cells significantly decreased in positive animals. In contrast, MCV, MCH, lymphocyte and monocytes significantly increased in these positive animals. According to the results, anemia, thrombocytopenia, polynuclear leukopenia and lymphocytosis were the most important hematological changes associated with trypanosome infections, which agreed with the studies of Kagira et al. (2006) and Yusuf et al. (2013). The prevention of future outbreaks of trypanosomosis will require monitoring blood-sucking flies in the nearby environment, routine checks of blood hemogram on the farm and the prompt treatment of infected animals.

Conflict of Interest

The authors declare that there are no conflicts of interest.

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