International Journal of Agricultural Technology 2022Vol. 18(1):205-214
Available online http://www.ijat-aatsea.com
ISSN 2630-0192 (Online)

A molecular survey of *Theileria* spp. in Ruminants in the Thailand-Cambodia border region

Kaewhom, P.[1] and Srikijkasemwat, K.[2*]

[1]Faculty of Agricultural Technology, Burapha University, Sa Kaeo Campus, Sa Kaeo, Thailand; [2]Department of Animal Production Technology and Fisheries, Faculty of Agricultural Technology, King Mongkut's Institute of Technology Ladkrabang, Bangkok, Thailand.

Kaewhom, P. and Srikijkasemwat, K. (2022). A molecular survey of *Theileria* spp. of Ruminant in Eastern border area of Thailand-Cambodia. International Journal of Agricultural Technology 18(1):205-214.

Abstract Theileriosis is caused by *Theileria* spp. Parasites which transmitted by various species of ticks including Amblyomma, Haemaphysalis, Hyalomma and Rhipicephalus. This pathogen can be found in red blood cells and white blood cells of farmed animals, including cattle, buffaloes, goats and sheep. *Theileria* spp. leads to a high morbidity in small ruminants, economic loss in agricultural production and time spent on infection prevention. It is necessary to detect the infection in a farm, as there are generally no clearly recognisable clinical signs. Our results revealed that infected animals displayed 230 bp DNA fragments, which is the length of the V4 region of the 18S rRNA gene of *Theileria* spp. The overall prevalence of *Theileria* spp. in ruminants farmed in Sa Kaeo Province, the border between Thailand and Cambodia, was 9% (29/314). The prevalence of *Theileria* spp. in meat cattle (6%), buffaloes(6%), meat goats (6%) and crossbred meat sheep (6%). It was also found that the prevalence of *Theileria* spp. was linked to neither species nor gender ($p>0.05$). Polymerase Chain Reaction was used to analyse *Theileria* spp. since it is very sensitive, specific and fast. Our analysis can be used to prevent and control the spread of *Theileria* infection in ruminants. Furthermore, this information may guide implemention of a policy on animal movement within the border regions.

Keywords: Infection prevalence, Ruminant, Theileriosis, *Theileria* parasites, PCR technique

Introduction

Theileriosis is caused by *Theileria* spp. belonging to the family Theileriidae. *Theileria* is transmitted by various ticks, including Amblyomma, Haemaphysalis, Hyalomma and Rhipicephalus (Mans *et al*., 2015). These Ixodid ticks have complex life cycles in both vertebrate and invertebrate hosts.

Theileria is a single cell intracellular protozoa which has several forms in round, oval or rod shaped. *Theileria* undergoes exoerythrocytic merogony in lymphocytes, histiocytes, erythroblasts and other cells of internal organs.

[*] **Corresponding Author**: Srikijkasemwat, K.; **Email:** kanokrat.sr@kmitl.ac.th

Theileria infects both domestic and wild ruminants, including cattle, buffalos, sheep and goats. Some *Theileria* are pathogenic strains, e.g. *T. parva* and *T. annulata*, which cause acute lymphoproliferative diseases in cattle, resulting in high morbidity. Others are non-pathogenic, e.g. *T. sergenti*, *T. buffeli*, *T. orientalis* (Uilenberg, 1981; Fujisaki *et al*., 1994; Chansiri *et al*., 1999). *Theileria* can cause East Coast Fever (ECF) and tropical theileriosis in cattle and malignant ovine theileriosis in sheep and goats. There have been several studies of the spread of *Theileria* spp. in many ruminants, e.g. beef cattle (Moumouni *et al*., 2015), dairy cattle (Pulforda *et al*., 2016), buffaloes (Altangerel *et al*., 2011; Chaisi *et al*., 2011), horses (Moretti *et al*., 2010), deer (Chae *et al*., 1999; Hana *et al*., 2009) and goats and sheep (Cao *et al*., 2013; Irshad *et al*., 2010).

In Thailand, *Theileria* was detected for the first time in 1971 in Thai native cattle in the southern part of the country (Aranyakanon, 1971). Several years later, it was found in dairy cattle in central Thailand (Jittapalapong and Leowijuk, 1988).

Theileria infections cause health problems in farmed animals, leading to significant economic loss, since they do not display clear signs of infection by this pathogen. Observing the symptoms of infected animals is not only difficult, but it is also inaccurate. Hence, specialists are required. Blood parasites are diagnosed by a combination of clinical examinations and appropriate laboratory testing. *Theileria* spp. can be investigated by checking for *Theileria* parasites in Giemsa-stained blood smears: this is inexpensive, but requires specialists. However, the pathogens are usually found in the blood of the animals, when they are acutely infected. Detection by molecular techniques appears to be a more effective method, since genomic material of the parasites can be detected: these techniques are very sensitive and specific, but also fast. A key advantage is that it is able to detect the pathogens in carriers, because they harbour only small numbers of pathogens and show no clinical signs, making it is generally difficult to detect the pathogens by other methods.

The present study was designed to assess the prevalence of *Theileria* spp. in ruminants, showing no clinical signs for Theileriosis, farmed in several areas in Sa Kaeo Province, Thailand (13°49’14”_N_102°03’32”_E_) which borders Cambodia. The gateway to Cambodia is extensively used by international commercial transport and tourism. Many animals are exchanged between countries in this area, so it is vital to detect the presence of the pathogen at the border, prior to an uncontrolled spread of the infection to other areas. Early detection is essential for controlling infections, within a herd, and it can prevent further spread to other areas. Moreover, associated risk factors should be

studied whether gender or species of the ruminants may have an influence on the infection of *Theileria* spp.

Materials and methods

Blood sample collection and analysis

Blood samples were collected from 314 ruminants, without clinical signs for Theileriosis, from randomly selected herds in several areas in Sa Kaeo Province, during summer, March-May, 2020. Blood was taken from jugular vein of 64 beef cattle (28 females and 36 males), 49 buffaloes (20 female and 29 male), 110 meat goats (72 females and 38 males) and 91 crossbred meat sheep (29 females and 62 males). The cows and buffaloes were older than 2 years. The meat goats weighed between 30 and 60 kg, and the sheep weighed from 15 to 25 kg. The blood samples were collected in the tubes, with Ethylene Diamine Tetra-acetic Acid (EDTA). to prevent blood clotting and they were stored at -20 ℃, until DNA was extracted, using acid phenol extraction (Chomczynsky and Sacchi, 1987), then the V4 region of the 18S rRNA gene was amplified using a *Theileria* genus specific primer. The PCR amplification of the forward primer (5'-GGTAATTCCAGCTCCAATAG-3') and the reverse primer (5'-ACCAACAAAATAGAACCAAAGTC-3') was carried out in a total volume of 100 ml. The final reaction conditions were 10 ml DNA templates, 10 ml buffer (20 mM Tris-HCl (pH8.4), 50 mM KCl), 20 mM dNTPs, 200 mM MgCl2, 0.5 picomoles of sense and anti-sense primer and 5.0 Unit DNA polymerase (Invitrogen â, USA). PCR used a Primus 96 plus thermocycler. Initially, the DNA templates were denatured (94 ℃, 4 min). Then the 35 PCR cycles followed, with each cycle having three steps - denaturation (94 ℃, 45 sec), annealing (51 ℃, 45 sec) and extension (72 ℃, 45 sec). Then a final terminal extension step followed (72 ℃, 10 min) to ensure a high yield of PCR products. The products were then analysed by agarose gel electrophoresis (potential difference 100 V, 30 min) with the RedSafe™ dye added and visualised under UV light. The DNA marker used to match the PCR products was 50 ng 100 bp DNA ladder (Invitogent™).

Evaluation of the prevalence of Theileria spp.

For all samples, the V4 region of the 18S rRNA gene was amplified, revealing the expected DNA ~230 bp fragments. A positive result indicated the presence of *Theileria* DNA in the sample. The prevalence of *Theileria* spp. in cows, buffaloes, goats and sheep was then calculated, and reported as a

percentage. A χ^2 test determined whether the prevalence was associated with the species and gender of the ruminants.

Results

Molecular analysis of Theileria spp.

314 blood samples from healthy ruminants with no signs of disease were examined. For the infected animals, DNA fragments of 200-300 bp were detected in agarose gel (Figure 1 and Figure 2).

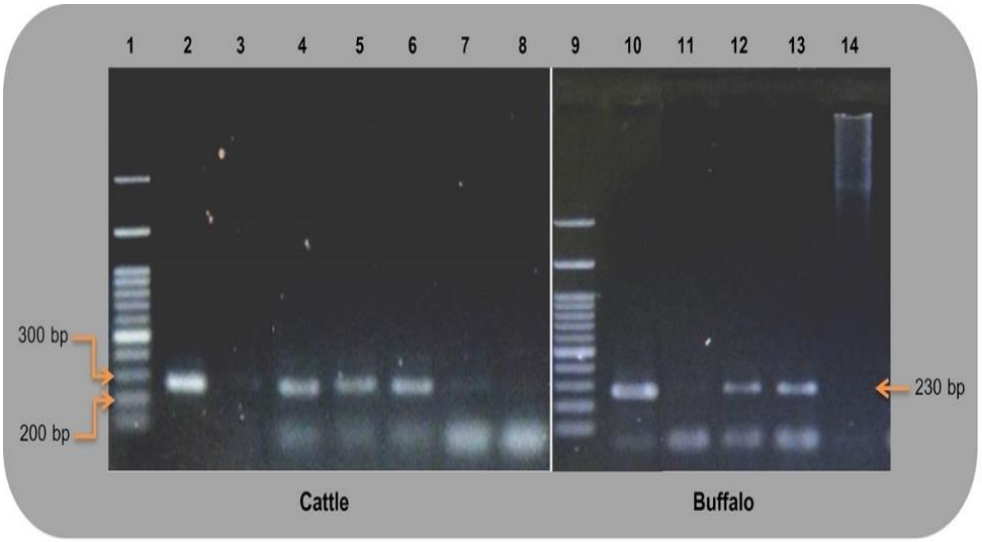

Figure 1. Analysis of PCR products of the 18S rRNA gene of *Theileria* spp. from cattle and buffalo on agarose. Lanes 1 and 9 = DNA marker (100 bp), lanes 2 and 10 = positive control, lanes 3 and 11 = negative control, lanes 4-8 = PCR products of cattle samples and lanes 12-14 = PCR products of buffalo samples

Prevalence of Theileria in ruminants and associated risk factors

The prevalence of *Theileria* was in cows (6%), buffaloes (6%), goats (14%) and sheep (6%) (Table1). The overall prevalence of *Theileria* spp. in ruminants was 9%. Neither species nor gender was associated with *Theileria* prevalence (Table 2).

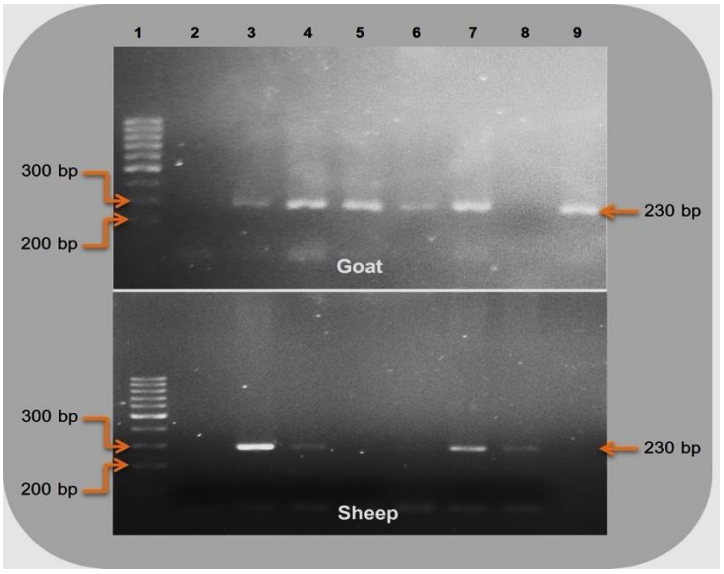

Figure 2. Analysis of PCR products of the 18S rRNA gene of *Theileria* spp. from goat and sheep. Lane 1 = DNA marker (100 bp), lane 2 = negative control, lane 3 = positive control, lanes 4-9 = PCR products of goat and sheep samples

Table 1. Prevalence of *Theileria* spp. in ruminant blood from Eastern border area of Thailand-Cambodia

Ruminant	Sample (n)	positive	Prevalence (%)
Species			
Cattle	64	4	6.2
Buffaloes	49	3	6.1
Goat	110	16	14.6
Sheep	91	6	6.6
Total	314	29	9.2

Table 2. Analysis of blood collection factors to the prevalence of *Theileria* spp.

Factor	Sample (n)	Positive(%Prevalence)	Statistic value		
			χ^2	df	P-value
Species			5.7047	3	0.1269
Cattle	64	4 (6.2)			
Buffalo	49	3 (6.1)			
Goat	110	16 (14.6)			
Sheep	91	6 (6.6)			
Sex					
Female	149	16 (10.7)	0.7637	1	0.3822
Male	165	13 (7.9)			
Total	314	29 (9.2)			

Discussion

Giemsa-stained blood smears can rapidly detect blood parasites, but it is slow when investigating many samples. Besides this, infected animals, without clinical signs, may have small and insufficient numbers of pathogens, for detection. Molecular techniques are therefore an appropriate method to prevent the spread by early detection, especially during the rainy season, when animals encounter more ticks, leading to uncontrollable spreads of pathogens, through transmission by ticks.

Blood samples were collected from 314 ruminants showing no clinical signs. DNA was extracted, followed by amplification of the V4 region of the 18S rRNA gene. *Theileria* spp. was detected in the blood sampled, and 200-300 bp PCR products were found as expected.

In Thailand, there have been many studies on blood protozoan diseases in farmed animals, particularly bovine theileriosis, caprine anaplasmosis and babesiosis. However, the only previous study of theileriosis in ruminants was published in Thai (Kaewhom and Thitasarn, 2017) in the border region between Thailand and Cambodia. From Sa Kaeo Province (13°49′14″N 102°03′32″E), the border province, animals are imported and exported regularly. Therefore, the infection of animals in this area can lead to an uncontrollable spread of disease in the country.

The weather in Thailand is hot and humid throughout the year. Additionally, both geographical location and climatic conditions of Sa Kaeo are very conducive to various species of ticks. Thus, it is an excellent environment for the spread of Theileriosis as *Theileria* are transmitted by ticks.

We showed that the prevalence of *Theileria* in ruminants in this area was 9 %. Goats had the highest prevalence (14%). After further investigation, it was found that goat farm management had lower standards than cattle farms. The owners of cattle farms seemed to have been educated and trained to manage their farms better than the goat farm owners, since goat farming is relatively newer to Thai farmers than cattle farming. The number of goat farms increased over the past ten years in this area, after the government encouraged the farmers. These goat farms were generally small, with an average of 7 – 15 goats in each farm. The goats feed on natural grass and weeds. They are let out to graze freely around farm houses in communal land or along roadsides. This increases the chance of transferring ticks between neighbours leading to a spread of disease. A cut-and-carry system is used only in the rainy season. In contrast, cattle are fed with high-energy concentrates and rice straw or roughage, within their barns, or grazed on their own land. Additionally, cattle farmers control pests, so that they are less likely to pass ticks to each other.

The prevalence of *Theileria* spp. in ruminants living in this area, however, was relatively lower than in other studies. Srikijkasemwat and Kaewhom (2019) found *Trypanosoma evansi* infection in cattle and buffaloes in Sa Kaeo at 39%. Furthermore, it was still lower than incidence of *Theileria* in sheep and goats in Eastern Turkey, where *T. Ovis* was found to be 18% (120/656) of sheep and 2% (4/139) of goats (Altay *et al.*, 2005), whereas 58% (398/677) of sheep and 11% (16/142) of goats were infected by *Theileria* (Altay *et al*., 2007). Aktas *et al*. (2005) found that *Theileria* spp. infection in sheep in Turkey at 41% (90/218). Kursat *et al*. (2007) detected blood parasites in 38% of small ruminants, whereas the prevalence of *Theileria* was 36%. Gebrekidan *et al*. (2014) measured the incidence of *T. ovis* and *T. separata* in sheep in northern Ethiopia, finding that out of 160 sheep, 147 were infected by *T. ovis* (92%), and 3 were infected by *T. separata* (2%). In another *Theileria* study in China, DNA was extracted from blood of 198 sheep: using nested polymerase chain reaction (nPCR), the 18S rRNA genes were amplified and prevalences of *Theileria* were in Yanji (80%), Nongan (40%), Longjing (37%), Toudao (24%) and Jinchang (32%) (Cao *et al*., 2013).

Theileria incidence has been studied in many areas, but not many have investigated risk factors associated with theileriosis in ruminants. Therefore, we investigated the prevalence of *Theileria* spp., and also the factors that increase the likelihood of infection.

This study revealed that the ruminant species and gender did not significantly affect the probability infection by *Theileria* (***p***>0.05). Similarly, Chunhavaranon *et al*. (2017) investigated the risk factors associated with infection by *Babesia* spp. in goats and sheep, including species, area, age and gender. They showed that species, area and gender did not affect the incidence of *Babesia*, whereas age did (***p***=0.019).

The prevalence of *Theileria* in goats was greater than for other ruminants, so this suggested that the standard of goat farming should be raised. Associated organisations should educate goat farmers to improve farm standards. A better farm management will help to prevent other diseases and avoid economic loss. Furthermore, a further study of the molecular sequence of *Theileria* spp. should be used to evaluate the spread of the pathogen.

This study of prevalence of *Theileria* spp. and associated risk factors in ruminants in the border area between Thailand and Cambodia was therefore a pilot study. The results can be used to prevent, prepare and control the infection spread. Local authorities may develop policies on animal movement between Thailand and Cambodia.

However, we note that we did not have age data in our samples and that Chunhavaranon *et al*. (2017) did observe an age effect, so this should be checked in further work.

Acknowledgements

We thank the Faculty of Agricultural Technology, Burapha University, Sa Kaeo Campus for providing equipment. A sincere thank you to Tuantong Ard-rugsa, animal husbandry officer from The Watthana Nakhon District Livestock Office for the blood sample collection. We thank A/Prof John Morris for removing all unnecessary words and simplifying our early drafts.

References

Aktas, M., Altay, K. and Dumanli, N. (2005). Survey of Theileria parasites of sheep in eastern Turkey using polymerase chain reaction. Small Ruminant Research, 60:289-293.

Altangerel, K., Sivakumar, T., Inpankaew, T., Jittapalapong, S., Terkawi, M. A., Ueno, A., Xuan, X., Igarashi, I. and Yokoyama, N. (2011). Molecular Prevalence of Different Genotypes of *Theileria orientalis* Detected from Cattle and Water Buffaloes in Thailand. The Journal of Parasitology, 97:1075-1079.

Altay, K., Dumanli, N., Holman, P. J. and Aktas, M. (2005). Detection of *Theileria ovis* in naturally infected sheep by nested PCR. Parasitology, 127:99-104.

Altay, K., Aktas, M. and Dumanli, N. (2007). Theileria Infections in Small Ruminants in the East and Southeast Anatolia Türkiye. Turkish Journal of Parasitology, 31:268-271.

Aranyakanon, P. (1971). Splenectomized cattle for the study of blood parasite. Thai Veterinary Medical Association, 22:4 9.

Cao, S., Zhang, S., Jia, L., Xue, S., Yu, L., Kamyingkird, K., Moumouni, P. F. A., Moussa, A. A. E. M., Zhou, M. Zhang, Y., Terkawi, M. A., Masatani, T., Nishikawa, Y. and Xuan, X. (2013). Molecular detection of Theileria species in sheep from northern China. Journal of Veterinary Medical Science, 75:1227-1230.

Chae, J. S., Waghela, S. D., Craig, T. M., Kocan, A. A., Wagner, G. G. and Holman, P. J. (1999). Two Theileria cervi SSU rRNA gene sequence types found in isolates from white-tailed deer and elk in North America. Journal of Wildlife Diseases, 35:458-465.

Chaisi, M. E., Sibeko, K. P., Collins, N. E., Potgieter, F. T. and Oosthuizen, M. C. (2011). Identification of *Theileria parva* and *Theileria* sp. (buffalo) 18S rRNA gene sequence variants in the African Buffalo (*Syncerus caffer*) in southern Africa. Veterinary Parasitology, 182:150-162.

Chansiri, K., Kawazu, S., Kamio, T., Terada, Y., Fujisaki, K., Philippe, H. and Sarataphan, N. (1999). Molecular phylogenetic studies on Theileria parasites based on small subunit ribosomal RNA gene sequences. Veterinary Parasitology, 83:99-105.

Chomczynsky, P. and Sacchi, N. (1987). Single-step method of RNA isolation by acid guanidinium thiocyanate phenol choroform extraction. Analytical Biochemistry, 162:156-159.

Chunhavaranon, J., Chantarasiriwatthana, A., Fongkerd, S., Pradabsook, P., Simking, P., Saengow, S., Panphan, S., Sunanta, C. and Cheepnurat, K. (2017). The Prevalence Study of *Babesia* spp. by Molecular Techniques in goats and sheeps, Chiang Rai province, Thailand. Agricultural Sciences Journal, 48:961-967.

Fujisaki, K., Kawazu, S. and Kamio, T. (1994). The taxonomy of the bovine *Theileria* spp. Parasitology Today, 10:31 33.

Gebrekidan H., Asrat, H., Aysheshm, K., Iva, R., Carla, M., Dalit, T. F., Alon, W. and Gad, B. (2014). Theileria infection in domestic ruminants in northern Ethiopia. Veterinary Parasitology, 200:31-38.

Hana, J. I., Janga, H. J., Leeb, S. J. and Naa, K. J. (2009). High prevalence of *Theileria* spp. in wild Chinese Water Deer (Hydropotes inermis argyropus) in South Korea. Parasitology, 164:311-314.

Irshad, N., Qayyum, M., Hussain, M. and Qasim, K. M. (2010). Prevalence of Tick Infestation and Theileriosis in Sheep and Goats. Pakistan Veterinary, 30:178-180.

Jittapalapong, S. and Leowijuk, C. (1988). Survey of blood protozoa of dairy cow in Nongpho. The 26th Kasetsart University Annual Conference Report, pp.117-122.

Kaewhom, P. and Thitasarn, W. (2017). The Prevalence of *Theileria* spp. of Goat in Watthana Nakhon District, Sa Kaeo Province. Journal of Mahanakorn Veterinary Medicine, 9:49-61 (in Thai).

Kursat, A., Dumanli, N. and Aktas, M. (2007). Molecular identification, genetic diversity, and distribution of *Theileria* and *Babesia* species infecting small ruminants. Parasitology, 147:161-165.

Mans, B. J., Pienaar, R. and Latif, A. A. (2015). A review of Theileria diagnostics and epidemiology. International Journal for Parasitology: Parasites and Wildlife, 4:104-118.

Moretti, A., Mangili, V., Salvatori, R., Maresca, C., Scoccia, E., Torina, A., Moretta, I., Gabrielli, S., Tampier, M. P. and Pietrobelli, M. (2010). Prevalence and diagnosis of *Babesia* and *Theileria* infections in horses in Italy: A preliminary study. The Veterinary Journal, 184:346-350.

Moumouni, P. F. A., Aboge, G. O., Terkawi, M. A., Masatani, T., Cao, S., Kamyingkird, K., Jirapattharasate, C., Zhou, M., Wang, G. Liu, M., Iguchi, A., Vudriko, P., Ybanez, A. P., Inokuma, H., Shirafuji-Umemiya, R., Suzuki, H. and Xuan, X. (2015). Molecular detection and characterization of Babesia bovis, Babesia bigemina, Theileria species and Anaplasma marginale isolated from cattle in Kenya. Parasites & Vectors, 8:496.

Pulforda, D. J., McFaddena, A. M. J., Hamiltonb, J. S. and Donaldc, J. (2016). Investigation of the index case herd and identification of the genotypes of Theileria orientalis associated with outbreaks of bovine anaemia in New Zealand in 2012. The Veterinary Journal, 64:21-28.

Srikijkasemwat, K. and Kaewhom, P. (2019). Prevalence and Genetic Diversity of Trypanosoma evansi Infections Causing Abortions among Cattles and Buffaloes in Eastern Border Area of Thailand-Cambodia. International Journal of Agricultural Technology, 15:1021-1032.

Uilenberg, G. (1981). Theilerial species of domestic livestock. In: Irving A. D., Cunningham M. P., Young A. S., ed. Advances in the Control of Theileriosis, Martinus Nijhoff Publishers, pp.4-37.

(Received: 29 August 2021, accepted: 25 December 2021)