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Prevalence and risk factors of *Babesia* infections in cattle trespassing natural forest areas in Salakpra Wildlife Sanctuary, Kanchanaburi Province

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ABSTRACT

Bovine babesiosis is a major tick – borne disease in livestock development since this disease has an effect on animal health led to economic losses due to increase mortality and lower production. Babesiosis in cattle is caused by *Babesia bovis* and *B. bigemina* and transmitted by tropical cattle tick, *Rhipicephalus (Boophilus) microplus*. The objective of this study was to investigate the prevalence of *Babesia* infection in the cattle invading natural forest area of Salakpra Wildlife Sanctuary (SWS) in Kanchanaburi province using molecular assays. A total of 244 cattle blood samples were collected from SWS areas located among 3 districts of Kanchanaburi Province including Mueang, Si Sawat, and Bo Phloi and examined by polymerase chain reaction (PCR) using SBP2 gene to identify *B. bovis* and *rap – 1* gene for *B. bigemina*. Factors associated with *Babesia* infection including location, age, sex, herd size, seasonal management, insect abundant, forage density, deworming, domestic pet in household, tick infestations in cattle and barn, and the grazing area management was statistically analyzed. The overall infection of *Babesia* spp. of cattle in SWS was 46.7% (114/244), comprising *B. bovis* (5.3%, 13/244), *B. bigemina* (38.9%, 95/244) and mixed (*B. bovis* and *B. bigemina*) (2.4%, 6/244). Mueang District had the highest infection at 47.9% (45/94). The potential significant factors associated with *Babesia* infections were herd size and age ($p < 0.05$). The high infection of *Babesia* spp. in SWS indicated the possible risk of the wild to get infections via ticks contaminated in this areas.

Key word: prevalence, *B. bovis*, *B. bigemina*, cattle, PCR, Salakpra Wildlife Sanctuary

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INTRODUCTION

Babesiosis is a major tick – borne disease of livestock worldwide particularly in tropic and subtropic areas. This disease is caused by *Babesia* spp. resulting in economic losses of developing countries due to its effect on animal health and production. A distribution of babesiosis is varied on the spreading of cattle tick (*I. microplus*) in the nearby environment [1].

The conventional diagnosis of bovine babesiosis is based on the identification of parasites in the stained blood smear under the light microscope. However, microscopic examination has low sensitivity and is not practical in epidemiological investigations due to its time consuming and the result depends much on the degree of parasitemia [2]. Currently, the molecular tool has been developed and seems very useful in the epidemiological studies because of its high sensitivity and specificity in a detection of the parasite's DNA [3-5]. In particular, genetic and antigenic differences were observed among merozoite surface antigens (MSAs) and some merozoite surface antigens, such as MSA-1, MSA-2a1, MSA-2a2, MSA-2b, and MSA-2c, had been well characterized and considered as vaccine candidate antigens [6-8]. Spherical body protein 2 (SBP-2) was also a candidate for many applications. The *rap - 1* α gene of *B. bigemina* is highly conserved and potent in identifying specific *Babesia* [9].

Bovine babesiosis has been reported in all regions of Thailand since 1980 [10-16]. Kanchanaburi is in the Western Thailand where the largest protected forest areas are located. Salakpra Wildlife Sanctuary (SWS) covers 4 districts of Kanchanaburi where some areas are invaded by the nearby villagers and their animals. The increasing of livestock rearing and urbanization are important factors associated with deforestation and forestry decay [17,18]. The high number of trespassing cattle over forestry areas has affected the food resource and habitats of wildlife. Moreover, these cattle might be the potential risk to threatening the wildlife's health because they can be potential reservoirs of some

major tick-borne diseases including babesiosis [19,20]. Therefore, the objective of this study was to investigate the molecular prevalence and factors associated with *Babesia* infections of cattle resided in the Salakpra Wildlife Sanctuary.

MATERIALS AND METHODS

1. Blood samples collection and microscopic examination

A total of 244 cattle blood samples were collected from Salakpra Wildlife Sanctuary in Kanchanaburi including Mueang Si Sawat, and Bo Phloi Districts. The sample size was calculated from Winepiscop program using randomization model (Multistage sampling) which was based on total population at 12,000 individual, the expected prevalence of 30%, absolute precision of $\pm 5\%$ and 95% confidence interval. Ten ml of blood was drawn from jugular vein, transferred to sodium citrate tubes and stored at -40°C until the laboratory analysis.

The thin blood smear was used for microscopic examination. The blood smears were stained by Wright & Giemsa stain and detected for the infected erythrocyte under the light microscope.

2. Data Collection

Cattle were thoroughly examined for health profiles. The information regarding age, sex, health condition, and location are recorded. In addition, the questionnaire was designed to record these data including herd sized (small: 1-40 cattle/farm; medium: 41-80 cattle/farm; large: >80 cattle/farm), seasonal management (Summer/Raining: roaming/in house), insect abundant in barn (low density: 0-10/cattle; moderate density: 11-20/cattle; high density: >20/cattle), Forage density pattern (low density: 1-10 trees/5 m²; moderate density: 10-20 trees/ 5 m²; high density: over 20 trees/ 5 m²) deworming, domestic pet in household(found or not found), the tick infestation on the animal, the tick existence in barn, and the using of grazing areas (selected or public areas). All factors were statistical analyzed to identify the significant effect on the distribution pattern of *Babesia* infection among cattle.

3. DNA extraction

One hundred μ l of each sample was added into microtube and mixed with 500 μ l of D-solution. Then, DNA was extracted by using a phenol-chloroform technique and the purified DNA was stored at -20 °C until used. DNA preparation and molecular analysis were processed at the Molecular Laboratory Unit, Department of Veterinary Parasitology, Faculty of Veterinary Medicine, Kasetsart University.

4. PCR and DNA Sequencing

For *B. bovis* identification, cattle blood DNA was assayed with the screening PCR method previously described [1]. The 20 μ l reactions contained 1 \times buffer (10 mM Tris-HCl pH 8.8, 50 mM KCl and 0.1% Triton X-100), 1.5 mM MgCl₂, 1.0 pmol of each primer, 0.2 mM of each dNTP, 0.75 unit of Taq DNA polymerase (DyNAzyme, FINNZYMES) and 1 μ g of DNA template. The primers BbovisSBP1 (5' CGAATTCTGGAAGTGGATCTCATGCAACC-3') and BbovisSBP2 (5' ATCTCGAGTCACGAGCACTCTACGGCTTGAG-3') which amplified approximately 1,236 nucleotides of the SBP2 gene are the 1st primer. The PCR conditions were: pre - denaturation at 95°C for 5 min, then 35 cycles of denaturation at 94°C for 1 min, annealing at 64°C for 1 min and extension at 72°C for 2 min and followed by final extension at 72°C for 10 min. The amplified PCR product of 0.5 ml was used for the subsequent nPCR with a limited annealing temperature at 58°C and 1 min at 72°C. in a MyCyclerTM Thermal Cycler (BioRad Laboratories, USA). The primer BbovisSBP2 3 (5'-CGAATCTAGGCATATAAGGCAT-3') and BbovisSBP2 4 (5'-ATCCCCCTC CTAAGGT TGGCTAC - 3') are the nested primer.

For a detection of *B. bigemina*, PCR assay was modified following the previous study [5]. The 20 μ l reaction contained 1 \times buffer (10 mM Tris-HCl pH 8.8, 50 mM KCl and 0.1% Triton X-100), 1.5 mM MgCl₂, 2 pmol of each primer, 0.2 mM of each dNTP, 1.0 unit of Taq DNA polymerase (DyNAzyme, FINNZYMES) and 1 μ g of DNA template. The primer Bbigrap - 1 α F1 (5'

GAGTCTGCCAAATCCTTAC 3') and Bbigrap - 1 α R1 (5' TCCTCTACAGCTGC TTG 3') were used to amplified approximately 879 bp of the *B. bigemina* rap - 1 α gene. The PCR conditions were: pre - denaturation at 94°C for 5 min, then 35 cycles of denaturation at 94°C for 1 min, annealing at 55°C for 1 min and extension at 72°C for 1 min and followed by final extension at 72°C for 10 min. The nested primers were Bbigrap- 1 α F2 (5'-AGCT TGCTTCACAAGT GCC-3') and Bbigrap - 1 α R2 (5'-TTGGTGCTTGACCG ACGACAT-3') and the nested PCR protocol was repeated using the same protocol. The nested PCR product was sized at 419 bp. The positive control was the infected cattle gDNA sample for *B. bovis* and *B. bigemina* and the negative control was deionized water.

5. Statistical Analysis

Number Cruncher Statistical System (NCSS) ver. 2000 (Kaysville, UT) program was used to assess differences in the prevalence and intensity of infection. The univariate analysis was also undertaken to investigate environmental variables correlated with the infection patterns, based on the probability that individual cattle was infected, by using Chi - square calculation which p -value ≤ 0.05 were considered significant. The multivariate analysis was estimated by using logistic regression models which the Odds - ratios (OR) and 95% interval also provided from this model by using Wald test algorithm.

RESULTS

The positive DNA was shown at approximately 580 bp for *B. bovis* SBP2 gene and 412 bp for *B. bigemina* rap - 1 α gene under UV light (Fig. 1). A total of 114 *Babesia*-positive cattle among 36 farms was distributed in the SWS areas as shown in Fig. 2. The highest infection was found at Mueang District (47.9%; 45/94) while Sri Sawat had the lowest prevalence (44.0%; 11/25). The overall *Babesia* infection of cattle in SWS was 46.7% (114/244) for individual prevalence and 88.9% (32/36) for herd prevalence. The prevalence of *B. bovis* infection is 5.3% (13/244) and was mostly found in Sri Sawat District (16.0%; 4/25). The

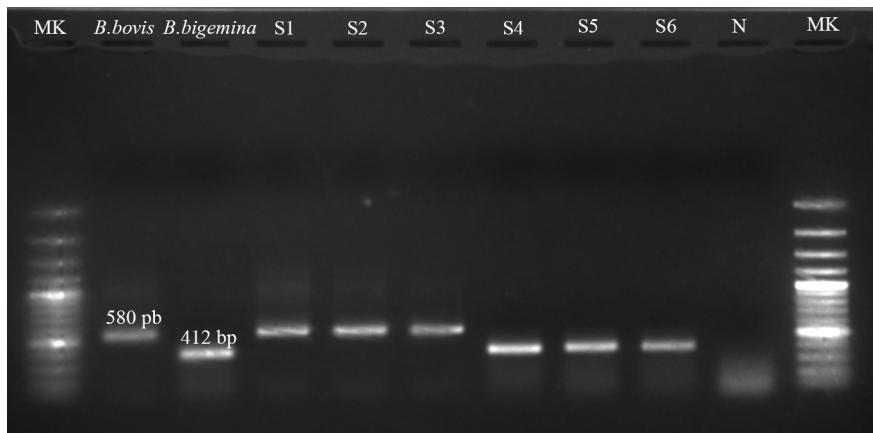


Fig 1 DNA bands of *B. bovis* and *B. bigemina* infection. S1 – S3 : *B. bovis* positive sample (580 bp), S4 – S6: *B. bigemina* positive sample (412 bp), N: negative sample, MK: DNA marker 100 pb plus

prevalence of *B. bigemina* infection was 38.9% (95/244) and was frequently distributed in Bo Phloi District (45.6%; 57/125). In addition, the mixed infection between *B. bigemina* and *B. bovis* was also found at 2.5% (6/244). The univariate analysis of the associated factors such as location, age, sex, herd size, seasonal management, insect density, forage density, deworming, pet in farm, tick infestation of cattle, the tick existence in house, and the using of grazing areas were shown in Table 1. Some potential factors associated with *Babesia* infections including herd sizes, age, and forage density division were statistical significance ($p<0.05$). However, the multivariate analysis using logistic regression models significantly showed that the infections were affected by the age of animal (between 1 – 5 years) and the herd size (40 – 80 cattle/farm) (Table 2).

DISCUSSIONS

The result of this study demonstrated that 5.3% and 38.9% of cattle in SWF areas were infected with *B. bovis* and *B. bigemina*, respectively. However, the microscopic examination only presented 2.4% of *Babesia* spp. in the stained blood smear without species identification. Specific genes such as spherical body protein 2 (SBP-2) and rhoptry-associated protein 1 (*rap-1*) gene are used to improve their specificity and sensitivity

to identify *B. bovis* and *B. bigemina*, respectively, being beneficial for epidemiological investigation [21-25]. The genetic variation of subpopulation within *B. bovis* or *B. bigemina* was also shown distinct biological characteristic [26].

For the factors associated with *Babesia* infections, the age of animals was significantly potential related with the infection ($p<0.05$). In this study, the animal age (between 1 – 5 years) showed the significant resistance against *Babesia* infection (Odds ratio = 0.32, CI= 0.14 – 0.72, $p = 0.005$), as shown as 5.4% and 33.3% infection by *B. bovis* and *B. bigemina*, respectively. This might be the evidence of passive immunity in the endemic area. After birth, calves have received passive immunity via the colostrum from pre-immunized mothers. Evidently, the young animals have been reported to be more susceptible to *Babesia* infection than animals without passive immunity [27-28]. Moreover, the older animals (>5 years) probably have the longer exposure to the pathogen and developed the protective immunity compared to the young animals [29]. In the endemic areas, mild *Babesia* infections of cattle were normally occurred and induced immunity against babesiosis particularly in native animals. This immunity was also correlated with the herd immunity. The differences of actual prevalence among herds were depended on the diagnostic

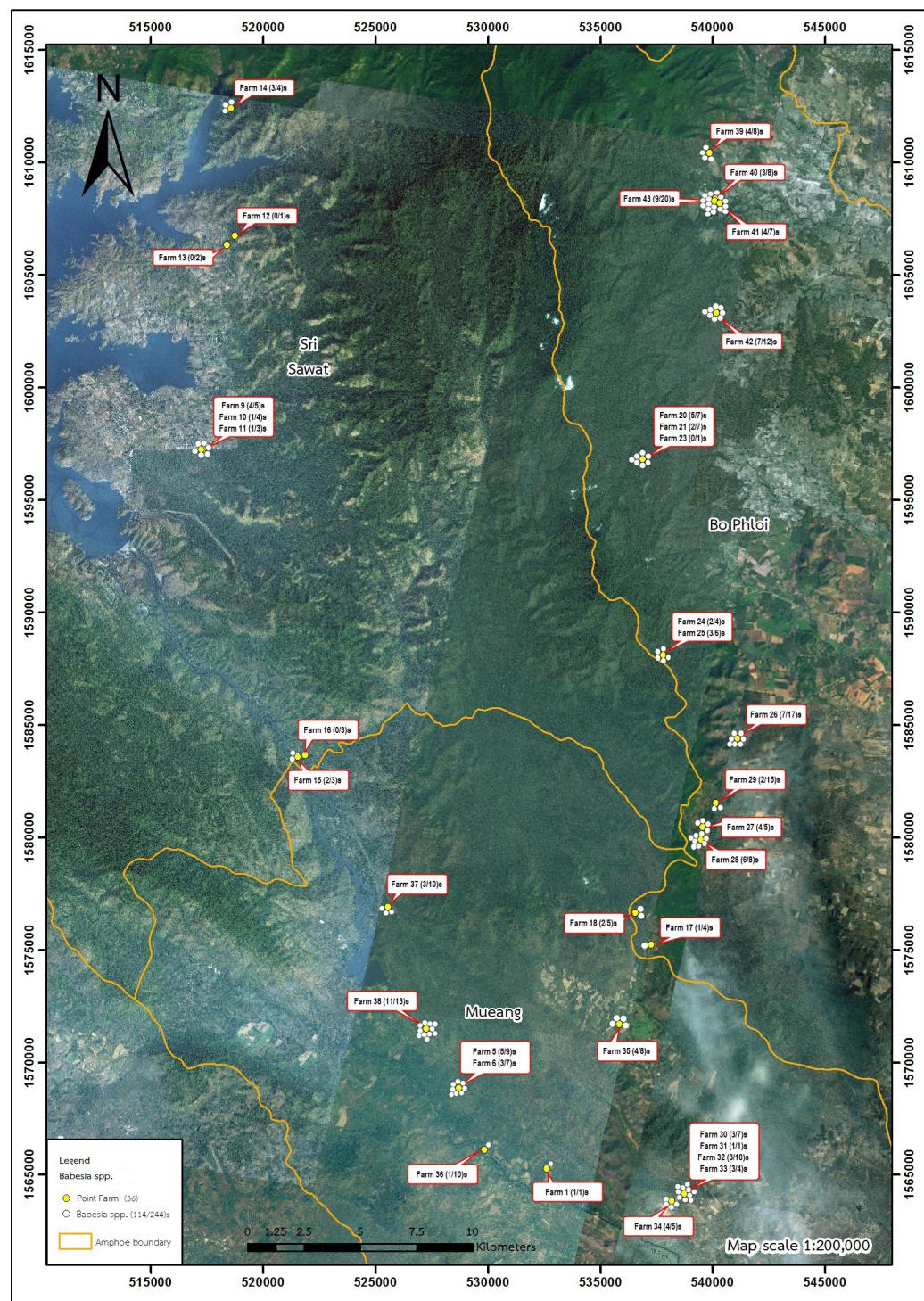


Fig 2 GIS map demonstrating the distribution of *Babesia* infection of cattle in the Salakpra Wildlife Sanctuary areas

Table 1 Univariate analysis for the risk factors associated with *Babesia* spp. infection

Variables	No.	No. Positive (%)	χ^2 values	df	p-value	<i>B. bovis</i>	<i>B. bigemina</i>	Mixed infection
Areas			0.13	2	0.93			
1. Mueang	94	45 (47.9%)				8 (8.5%)	33 (35.1%)	4 (4.2%)
2. Sri Sawat	25	11 (44%)				4 (16%)	5 (20%)	2 (8.0%)
3. Bo Phloi	125	58 (46.4%)				1 (0.8%)	57 (45.6%)	0
Herd size			16.37	2	0.0002*			
1. Small (1 – 40 cattle)	60	25 (41.7%)				6 (10%)	18 (30%)	1 (1.7%)
2. Medium (40 – 80 cattle)	105	64 (61.0%)				5 (4.8%)	54 (51.4%)	5 (4.8%)
3. Large (over 80 cattle)	79	25 (31.6%)				2 (2.5%)	23 (29.1%)	0
Age group			6.01	2	0.049*			
1. 0 – 1 year	37	23 (62.2%)				5 (13.5%)	14 (37.8%)	4 (10.8%)
2. 1 – 5 years	129	52 (40.3%)				7 (5.4%)	43 (33.3%)	2 (1.6%)
3. > 5 years	78	39 (50.0%)				1 (1.3%)	38 (48.7%)	0
Sex			1.63	1	0.20			
1. Male	40	15 (37.5%)				1 (2.5%)	14 (35.0%)	0
2. Female	204	99 (20.4%)				12 (5.9%)	81 (39.7%)	6 (2.9%)
Dry season management			1.21	1	0.27			
1. Free roaming	231	106 (45.9%)				13 (5.6%)	87 (37.7%)	6 (2.6%)
2. in Barn	13	8 (61.5%)				0	8 (61.5%)	0
Insect Density			2.70	2	0.25			
1. Low (0 – 10/cattle)	99	40 (40.4%)				6 (6.1%)	34 (34.3%)	0
2. Moderate (11 – 20/cattle)	50	25 (50%)				2 (4%)	22 (44%)	1 (2%)
3. High (>20/cattle)	95	49 (51.6%)				5 (5.3%)	39 (41.1%)	5 (5.3%)
Forage Density			7.94	2	0.018*			
1. Low	84	38 (45.2%)				8 (9.5%)	27 (32.1%)	3 (3.6%)
2. Moderate	147	65 (44.2%)				3 (2.0%)	60 (40.8%)	2 (1.4%)
3. High	13	11 (13.6%)				2 (15.4%)	8 (61.5%)	1 (7.7%)
Deworming			0.85	1	0.35			
1. Yes	195	94 (48.2%)				12 (6.2%)	76 (40.0%)	6 (3.1%)
2. No	49	20 (40.8%)				1 (2.0%)	19 (38.8%)	0
Pet in the farm			2.28	1	0.13			
1. Yes	208	93 (44.7%)				11 (5.3%)	79 (37.9%)	3 (1.4%)
2. No	36	21 (58.3%)				2 (5.6%)	16 (44.4%)	3 (8.3%)
Tick infestation on the animal			0.08	1	0.77			
1. Yes	219	103 (47.0%)				13 (5.9%)	84 (38.4%)	6 (2.73%)
2. No	25	11 (44.0%)				0	11 (44.0%)	0
Tick existence in barn			0.14	1	0.70			
1. Yes	80	36 (45.0%)				6 (7.5%)	28 (35%)	2 (2.5%)
2. No	164	78 (47.6%)				7 (4.3%)	67 (40.9%)	4 (2.4%)
Grazing areas			1.55	1	0.21			
1. Selected area	157	78 (49.7%)				10 (6.4%)	66 (42.0%)	2 (1.3%)
2. Public area	87	36 (41.4%)				3 (3.4%)	29 (33.3%)	4 (4.6%)
Total	244	114 (46.7%)				13 (5.3%)	95 (38.9%)	6 (2.4%)

Table 2 Results of the multivariate analysis of risk factors associated with *Babesia* infection in SWS areas.

Risk factors	β	SE β	Adjusted OR ^a (95%CI) ^b	p-value ^c
Age group				
1. 0 – 1 year	-	-	1	
2. 1 – 5 years	-1.138	0.049	0.32 (0.14 – 0.72)	0.005
3. > 5 years	-5.01	0.425	0.60 (0.26 – 1.39)	0.23
Herd size				
1. Small (1 – 40 cattle)	-	-	1	
2. Medium (40 – 80 cattle)	0.856	0.402	2.35 (1.07 – 5.18)	0.033
3. Large (over 80 cattle)	-0.360	0.409	0.69 (0.31 – 1.55)	0.37

^a OR, odds ratio^b CI, confidence interval^c Wald testmulti

technique since different genes primers or tests might yield the different prevalence [9].

This protective forestry location (SWS) has 1,277 domestic cattle resided in the 859 km² [17]. In this study, the result revealed that the medium sized herd (40–80 animals/herd) had more significantly risk effect (Odds ratio = 2.35, CI = 1.07 – 5.18, $p = 0.33$) to the infection than the large (>80 animals/herd) and small herds (1–40 cattle). This effect might be due to the overcrowded cattle in the herd and the poor management. During the raining season, all of the cattle were roamed in the grazing areas that can be highly contaminated since the temperature and humidity were the essential factors to support the tick survival and population. The annual precipitation of Kanchanaburi was over 1,000 mm (data from Thai Meteorological Department) while the annual precipitation above 700 mm is suitable for enzootic stability [30]. The high humidity (>50%) has influenced the tick biological cycle, leading to the stability of the pathogen in the areas. However, there were no significant differences by seasonal factors in this study. In addition, *Babesia* cannot be transmitted by the other vectors such as stable flies or tabanus. Therefore, the insect density might not have the effect on *Babesia* infection in this area.

The other factors such as sex, farm management,

and grazing location were not significantly related to the *Babesia* infection in this study. Evidently, sharing of the grazing location for rearing the cattle might increase the spreading of the cattle ticks, leading to the great risks of transmitted babesiosis [31]. Cattle in SWS areas roamed around the public pasture. Frequently, these animals had been intentionally or unintentionally grazed in the SWS areas. Naturally, this protected forestry area is the habitat of the wildlife such as guar, sambar deer, bantang and elephant [17]. This trespassing cattle combined with available ticks might create the high risk environment for the wildlife. Moreover, the numbers of trespassing cattle in the protected forestry areas is currently increasing. Therefore, the situation was menacing the wildlife's health. SWS is one of the protected forestry areas that encounter the problem of the cattle trespassing [17]. Most cattle wandered in or nearby the forestry areas since there was no real boundary to limit the trespassing [32]. Some cattle diseases can be transmitted by the invaded cattle to the wildlife or vice versa. Both infected wildlife and cattle can become a carrier with high potential of disease transmission [1,17]. Up to date there are no evidence of the disease transmission between the farms and the wildlife. However, there were a few reports of ticks in animals and environment so that ticks are potential to transmit pathogen from

both animals and wildlife. To confirm a route of transmission or carrier, further investigation would be required.

CONCLUSION

In this study, the high infection of *Babesia* (46.7%) might have been concerned as a threat on the wildlife's health. These results also confirmed that Kanchanaburi was the endemic area of *Babesia* infection (38.9% *B. bigemina* and 5.3% *B. bovis*) compared to the previous report (10.8% *B. bigemina* and 1.6% *B. bovis*) in Salakpra Wildlife Sanctuary. However, one of existing is the trespassing of animals from the neighboring community.

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