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Molecular identification of *Anaplasma ovis* and *Anaplasma marginale* in Sheep using PCR-RFLP method

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Abstract Anaplasma is a protozoan blood in livestock such as cattle, goats and sheep. This pathogen leads to anaplasmosis, which is tick-borne disease, production loss and high costs due to disease prevention. Therefore, it is necessary to investigate the epidemiology of Anaplasma of livestock. The results demonstrated that the semi-nested-PCR product was approximately 120 bp. The restriction endonuclease Bst1107I only recognized the sequence (GTATAC) in semi-nested PCR product of *A. marginale* and cut the sequence at the position 68. However, the restriction endonuclease was not able to cut the semi-nested PCR product of *A. ovis* at the same position because of the different sequence (GTACGC). The prevalence of *A. ovis* and *A. marginale* infection in sheep at Sa Kaeo Province was 3.66% (3/82) and 1.22% (1/82), respectively. The overall prevalence of *Anaplasma* infection in sheep was 7.32% (6/82). From this result, the PCR-RFLP method could be used to isolate *A. ovis* and *A. marginale* using this technique with high specificity and safe time to identify both types of infection.

Keywords: 16S rRNA gene, Tick-borne disease, Semi-nested-PCR

Introduction

Blood parasites in cattle are *Anaplasma* spp., *Babesia* spp., *Theileria* spp. and *Trypanosoma* spp. The first three species reside in the red blood cells of cattle. This causes lysis of the red blood cells after the animals are infected, while *Trypanosoma* spp. occupies the bloodstream, lymphatic system or bone marrow of vertebrates. Anaplasmosis is caused by rickettsia, *Anaplasma marginale* and *A. centrale* (Restic and Watrach, 1963) generally found in cows and buffalos, and *A. ovi*s, which are often found in goats and sheep. Anaplasma appear as round to oval basophilic inclusions in ruminant erythrocytes. Organisms are often located at the margin in erythrocytes when viewed on stained blood films. They turn purple when in Giemsa stain. The primary vectors of blood parasites are ticks and biting flies which can transmit the

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disease to other animals. The pathogens are transmitted biologically by tick; *Rhipicephalus (Boophilus) microplus* and transmitted mechanically by biting flies, especially by tabanids and the stable fly. Another way of disease transmission is through blood-contaminated instruments. For instance, dehorning, castration, other unclean operations, and contaminated needles in vaccination or blood test (Taweenan, 2004). The symptoms that infected animals have include anaemia, pale and/or icteric mucus membranes, abortion, fever, dehydration, depression, and anorexia and are in part related to the number of RBCs infected. These symptoms are similar to the consequences of Babesiosis, which include fever, pale and/or icteric mucus membranes, fever and abortion. *A. centrale* is less pathogenic to cattle than *A. marginale* although both of them appear to be round, are found in red blood cells, and affect cattle, goats, sheep and other wildlife such as deer and ruminants.

In the past few years, researchers have become more interested in Anaplasma especially *A. marginale, A. ovis and A. phagocytophilum* because these bacteria lead to Anaplasmosis in livestock (Rymaszewska and Grenda, 2008); therefore, it is necessary to investigate the cause of this disease. The direct method of investigating these pathogens are the microscopic examination of Giemsa stained blood smears, Indirect Fluorescence Antibody Test (IFAT) and Polymerase Chain Reaction (PCR), the latter is an effective means and accurate due to its high specificity (Kaewhom, 2014). Additionally, the PCR technique has been developed in order to diagnose blood parasites in cattle, goats and sheep effectively such as PCR-RFLP. This technique involves amplifying DNA by using specific primer of 16S rRNA (Yousefi *et al.,* 2017) or MSP4 (Ahmadi-Hamedani *et al.,* 2009) together with restriction endonuclease. Important advantages of the PCR-RFLP technique include rapid results, inexpensiveness and lack of requirement for advanced instruments. It has been applied for the detection of intraspecies as well as interspecies variation. Anaplasmosis in sheep is generally caused by *A. ovis*. However, *A. marginale* can be also found in sheep which serve as reservoir hosts. *A. marginale* and *A. ovis* were isolated from disease prevalence, and to find out the necessary information to prevent and control the disease.

Materials and methods

Study area

This study is carried out in Sa Kaeo Province that is located in the Eastern part of Thailand. Sa Kaeo Province is a connecting border with Cambodia. Amphoe Aranyaprathet, Sa Kaeo province serves as a gateway to Cambodia

which can be connecting to international commercial transport and tourism at a hectic border crossing.

Collection of blood samples

Blood samples were collected from the Jugular veins of sheep in Sa Kaeo Province for a total of 82 samples. Then, the samples were kept in test tubes which contained EDTA to prevent blood clotting, and kept in -20 ℃ until DNA extraction was carried out.

DNA extraction and DNA amplification

DNA was extracted from the blood samples by acid phenol extraction method (Chomczynsky and Sacchi, 1987). Then, the 16S rRNA gene was amplified. P1 and P2 primers were used in the first round of PCR which is specific to *Anaplasma* spp. P1 and P5 primers were used in semi-nested PCR which is specific to *A. ovis* and *A. marginale* (Table 1)(Yousefi *et al*., 2017). The PCR was performed in 100 µl reaction mixture containing 10 µl DNA template, 200 mM Tris-HCl (pH8.4); 50 mM KCl; 0.5 pM of sense and anti-sense primer; 20 mM of each nucleotide dATP, dTTP, dCTP and dGTP (Invitrogen™); 5 U Taq DNA Polymerase (Invitrogen™,USA); and 200 mM of $MgCl_2$. The PCR reaction was carried out for 35 cycles in a MyCycler™ thermal cycler (Bio-rad). The first step is incubation at 94 ^{o}C for 5 min to denature double strand DNA. The thermal profile was used for the PCR: denaturing step at 94 ℃ for 30 second, annealing step at 58 ℃ for 30 second and extension step at 72 ℃ for 30 second. The final cycle included an extension for 5 min at 72 ℃ to ensure full extension of the products. The amplified PCR products were analyzed by electrophoresis in 1.5% (w/v) agarose gel and visualized using ethidium bromide and UV transilluminator.

Table 1. The sequence details of primers used in PCR and semi-nested PCR of 16S rRNA gene

Primer	Nucleotide sequence	Nucleotide positions	Target organism	PCR product(bp)
P1	5'-AGAGTTTGATCCTGGCTCAG-3'	1-20	*Anaplasma* spp.	781
P2	5'-AGCACTCATCGTTTACAGCG-3'	796-815		
P1	5'-AGAGTTTGATCCTGGCTCAG-3'	1-20	*A. ovis* and *A. marginale*	120
P5	5'-CTATGCATTACTCACCCGTCTG CCACTAACCATACACGCAGCAAG CTGCG-3'	71-120		

PCR-RFLP

The semi-nested PCR product from individual isolates with the size of 120 bp, using P1 and P5 primers for *A. ovis* and *A. marginale* was digested with 10 U of the restriction enzyme Bst1107I (Thermo scientific, Lithuania) for 3 h at 37 ℃. After that, the digested semi-nested PCR products were separated in 1.5% agarose gel in order to examine the length of the DNA and the results were then analyzed as shown in Table 2.

Table 2. The pattern of RFLP of semi-nested PCR product of *A. ovis* and *A. marginale* using the restriction endonuclease Bst1107I

Species	Bst1107I (bp)
A. ovis	120
A. marginale	52, 68
A. ovis and *A. Marginale*	52, 68, 120

The prevalence of A. marginale and A. ovis in sheep

All samples were screened for *A. marginale* and *A. ovis* infection using PCR-RFLP analysis. Since the presence or the absence of the restriction endonuclease recognition site results in the formation of restriction fragments of different sizes, allele identification can be done by electrophoretic resolvement of the fragments. The 16s rRNA was amplified. P1 and P5 primers were used in semi-nested PCR which is specific to *A. marginale* and *A. ovis*. The restriction endonuclease Bst1107I was able to recognize and cut the semi-nested PCR product in position 68, revealing two expected DNA fragments with 52 and 68 bp in length. This indicates that the DNA of *A. marginale* exists in the sample. If the enzyme is unable to recognize and cut the semi-nested PCR product, the sample contains the DNA of *A. ovis*, which has the size of 120 base pairs. However, if the results show DNA fragments of 52, 68 and 120 bp in length, the sample is infected with both *A. marginale* and *A. ovis*. Then, the amount of each pathogen was calculated as a percentage in order to find the prevalence of *A. ovis* or *A. marginale* in sheep in Sa Kaeo Province.

Results

The blood samples of 82 sheep in Sa Kaeo Province were tested in parallel by PCR and semi-nested PCR. All positive samples, after the 16S rRNA gene was amplified, using P1 and P2 primers, showed the expected 781 bp. Then, they were amplified by semi-nested PCR using P1 and P5 primers,

and showed the expected 120 bp PCR product in an *A. ovis* or *A. marginale* positive sample (Figure 1).

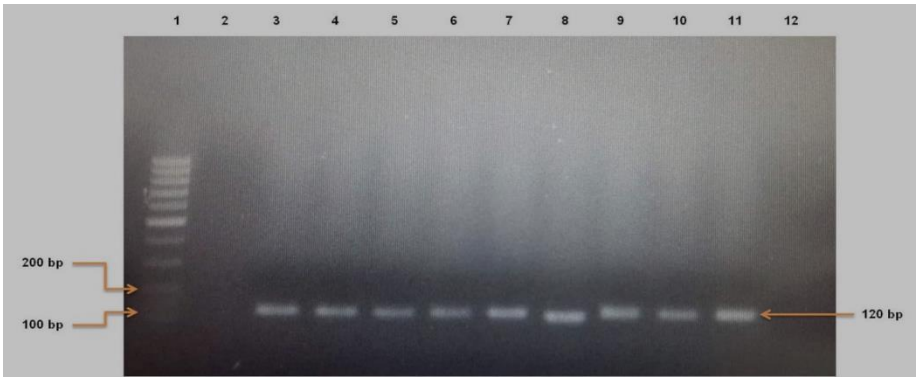

Figure 1. Analysis of PCR products of 16S rRNA gene of *Anaplasma* spp. in sheep using 1.5% agarose gel electrophoresis. A 10 µl of PCR mixture was loaded onto each lane of agarose gel. Lane 1 = DNA marker (100 bp), lane 2 = Negative control, lane 3 = *A. ovis* positive control, lane 4 = *A. marginale* positive control, lane 5 = *A. ovis* and *A. marginale* positive control, lane 6-12 = PCR product of 16S rRNA gene from sheep blood sample

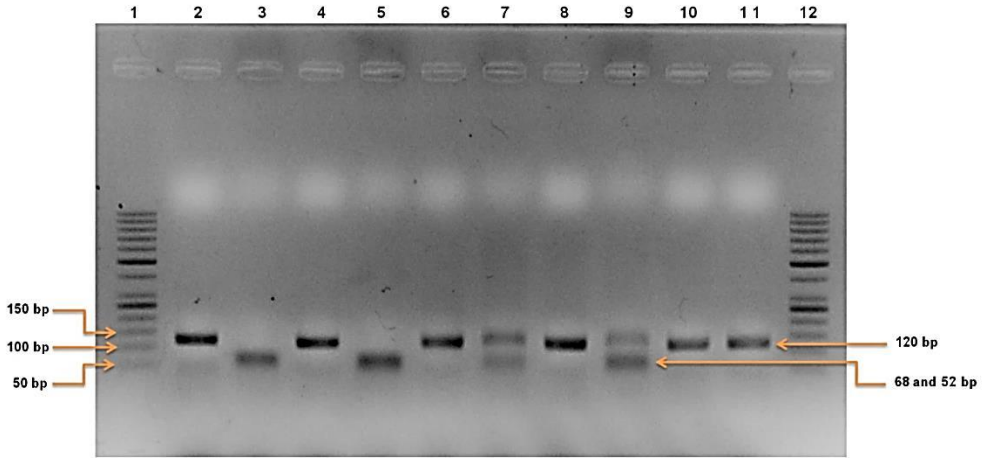

Figure 2. Restriction fragment length polymorphism by restriction endonuclease Bst1107I. A 10 µl of PCR mixture was loaded onto each lane of 1.5% agarose gel. Lane 1 and 12 = DNA marker (50 bp), lane 2 = uncut PCR product of *A. marginale*, lane 3 = digested PCR product of *A. marginale* (positive control), lane 10 = uncut PCR product of *A. ovis*, lane 11 = digested PCR product of *A. ovis* (positive control), lane 4-9 = digested PCR product of *Anaplasma* spp. from sheep

The products from semi-nested PCR were cut with the restriction endonuclease Bst 1107I, and they were analysed in 1.5% agarose gel. After cutting the semi-nested PCR product containing DNA of *A. marginale* with the restriction endonuclease, the results showed the expected 68 bp and 52 bp (Figure 2), whereas the semi-nested PCR product of *A. ovis* gave 120 bp. The restriction endonuclease Bst1107I cannot cut DNA fragment of *A. ovis* at the same position as *A. marginale* because of the different sequence at position 68, GTATAC in *A. marginale* and GTACGC in *A. ovis*.

The prevalence of *A. marginale* and *A. ovis* were then analysed, and the results were 3.66% (3/82) and 1.22% (1/82) respectively. Furthermore, the prevalence of *Anaplasma* in the sheep in Sa Kaeo province was 7.32% (6/82) as shown in Table 3.

Table 3. The prevalence of *A. marginale* and *A. ovis* in sheep in Sa Kaeo Province (n=82)

***Anaplasma* species**	**P1-P5 primer positive (n)**	**RFLP (n)**	**Prevalence (%)**
PCR			
***Anaplasma* spp.**	6		7.32 (6/82)
PCR-RFLP			
A. ovis		3	3.66 (3/82)
A. marginale		1	1.22 (1/82)
A. ovis* and *A. marginale		2	2.44 (2/82)
Total	6	6	7.32 (6/82)

Discussion

The investigation of *A. marginale* by molecular technique is one common method which uses primers from gene 16S rRNA MSP4 and MSP5 (Gisele *et al*., 2014; Shebish *et al*., 2012; Fuente *et al*., 2002) in order to increase the amount of DNA by PCR technique or real-time PCR which gives PCR products of 800-900 bp (Marvat *et al.,* 2013). Then, nested-PCR was used which gives 867 bp in the first round and approximately 344-345 bp in the nested-PCR round (Yousefi *et al*., 2017; Hamou *et al.,* 2012). Similar to *A. marginale*, primers from gene 16S rRNA MSP4 and MSP5 are commonly used in the investigation of *A. ovis*. When the nested-PCR technique is used, it gives 867 bp of PCR products in the first round, and around 347 bp in the nested-PCR round (Yousefi *et al*., 2017). However, the aforementioned techniques allow researchers to detect one organism per PCR or nested-PCR. If the PCR

technique is carried out along with other techniques such as DNA probes or, Restriction Fragment Length Polymorphism (RFLP), it is possible to detect many species of Anaplasma at the same time.

In this research, primers 16S rRNA are used to detect *A. marginale* and *A. ovis* in sheep by PCR-RFLP technique. The results showed that the band of DNA in the semi-nested PCR product was between 100 and 200 bp which meant Anaplasma existed in the blood sample, however, it was unable to identify the species. Therefore, it was then cut by the restriction endonuclease Bst1107I. The semi-nested PCR product of 16S rRNA gene of *A. marginale* will show DNA bands of 68 and 52 bp after semi-nested PCR products are cut by the restriction endonuclease Bst1107I which recognizes GTATAC. The DNA sequence (GTATAC) is commonly found in *A. marginale* strains. Therefore, the restriction endonuclease Bst1107I can cut the PCR products of 16S rRNA gene of *A. marginale*, while it cannot cut *A. centrale* and *A. ovis* because the base sequence of the same position is different. Nonetheless, the sizes of the bands were so similar that it was difficult to detect the separated bands, hence a higher percentage of agarose gel may be required. That is, 2-2.5 % agarose gel might be able to separate the bands. This corresponds with the experiment carried out by Yousefi *et al.* (2017) whose study was about using a molecular method to detect the prevalence of *A. marginale* and *A. ovis* in sheep and goats living in the highland plateau of Iran by using primers 16S rRNA and MSP4, and nested-PCR, semi-nested PCR and RFLP techniques. It was found that two species of pathogen in the same test the both in the sheep and goats by using primer 16S rRNA and it was cut by restriction endonuclease with the RFLP method. The total prevalence was 27.5 % (102/370). Besides this, another experiment was conducted in order to ensure the results. The MSP4 primer was used in nested-PCR for this as well, and the result also showed that sheep are important reservoirs of *A. marginale*. This result corresponds with the experiment of Ahmadi-Hamedani *et al.* (2009) who used a molecular method to identified pathogens that cause Anaplasmosis in goats by using PCR-RFLP with MSP4 primer and found positive results from 123 sheep out of 193 sheep which is 63.7 %. This PCR-RFLP had higher sensitivity than Giemsa stained blood smears analyzed under a microscope, which gave positive results for 22.3 %, and it could also detect *A. ovis* in goats. Therefore, the aforementioned research showed that PCR-RFLP has a high level of specificity and its sensitivity is appropriate for separating *A. marginale* and *A. ovis* in sheep. Furthermore, it can identify both species of pathogen in the same sheep by

cutting the enzyme only once, which means it consumes less time in the investigation.

There are a number of studies about the prevalence of other pathogens in sheep in many areas around the world. The researchers used molecular techniques in order to reduce the time and increase the accuracy in the detection of *Theileria ovis* (Altay *et al.*, 2005) and *Babesia ovis* (Kursat *et al.*, 2007). The prevalence of *A. ovis* and *A. marginale* in sheep in Sa Kaeo Province was 7.32 %. There were no reports about the prevalence of *A. ovis* in Sa Kaeo Province before because there were not many sheep farms in the area. Moreover, there was a study about the prevalence of this pathogen in cattle (Saetiew *et al*., 2014) and in goats (Kaewhom, 2017). The result of this study found that the prevalence of *A. marginale* in sheep in Sa Kaeo Province was very low which appeared to correspond with the experiment carried out by Kaewhom *et al.* (2017) who studied about the prevalence of *A. marginale* in goats in Watthana Nakhon District, Sa Kaeo Province. In their research, the prevalence of *A. marginale* in Watthana Nakhon District, Sa Kaeo Province was 4.32 (6/139) which was very low compared to the statistic of the prevalence of *A. marginale* in cows and buffalo which were higher (Saetiew *et al*., 2014). Therefore, this study showed that the prevalence of *A. ovis* and *A. marginale* found in Sa Kaeo Province was quite low which may be because there are not many sheep farms in the area, the location of the sheep farm was far from where goats, cows and buffalo are farmed. Besides this, the farm also played an important role in protecting and eliminating the carriers. Hence, the contagion was low and the prevalence of *A. ovis* and *A. marginale* in sheep was low. It is possible to control the outbreak of diseases effectively if there is a good plan for controlling diseases, protecting animals from being infected and treating them if they are infected.

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