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RESEARCH

Prevalence and genetic diversity of *Anaplasma marginale* infections in water buffaloes in Northeast Thailand

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Abstract

Bovine anaplasmosis caused by *Anaplasma* spp. is normally found in tropical and subtropical regions. Anaplasmosis poses important problems for animal health in Thailand since this major tick-borne disease is prevalent and causes animal health problems and production losses in the cattle industry. However, there is limited information on anaplasmosis in water buffaloes. The objective of this study was to determine the prevalence and genetic diversity of *A. marginale* infections of water buffaloes in Northeast, Thailand. In total, 625 blood samples of water buffaloes from 6 provinces consisting of Ubon Ratchathani, Roi Et, Buri Ram, Sakon Nakhon, Surin, and Sisaket were examined for *A. marginale* infection using a major surface protein4 (*msp4*) gene-PCR assay. The factors associated with *Anaplasma* spp. infections were compared on the basis of age, gender, geographic region, and herd. A total of 8% (50/625) of the buffaloes was positive for *A. marginale* infections. The only potential factor associated with *Anaplasma* infection was herd prevalence ($p<0.05$). The PCR products of *A. marginale* were sequenced to identify strains for phylogenetic studies and 4 strains were subsequently recognized. The most abundant *A. marginale* Thai strain 1 (AmT1) was found in Ubon Ratchathani, Buri Ram, Sakon Nakhon, Surin, and Sisaket, while the AmT2 strain existed only in Surin province. The AmT3 strain was found in Buri Ram and Sisaket while AmT4 was found in Ubon Ratchathani and Sakon Nakhon. To our knowledge, this report was the first molecular characterization to classify *A. marginale* strains using the *msp4* gene in Thai buffaloes.

Keywords: Water buffaloes, *Anaplasma marginale*, *msp4* gene, Northeast Thailand

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Introduction

Anaplasmosis is one of the major tick-borne diseases of cattle and other ruminants caused by *Anaplasma* (Rickettsiales: Anaplasmataceae) [1]. Bovine anaplasmosis is characterized by haemolytic anemia, high fever, weight loss, abortion, decreased milk production, and death [2]. Transmission of *A. marginale* in cattle occurs biologically and mechanically through ticks, blood-feeding flies, and blood contamination. *A. marginale* has been reported in water buffalo (*Bubalus bubalis*), American bison (*Bison bison*), white-tailed deer (*Odocoileus virginianus*), mule deer (*Odocoileus hemionus*), black tailed deer (*Odorcoileus hemionus columbianus*) and Rocky Mountain elk (*Cervus elaphus nelsoni*) [3]. In Thailand, water buffaloes are important animals in the Northeast region since they provide meat, horns, milk products, leather, land plowing, and transportation of people and crops. However, like cattle, buffaloes might be infected by many bovine pathogens; in contrast to cattle, buffaloes may show less apparent damages due to its breed resistance. However, there is limited information on *A. marginale* infection in buffaloes.

The conventional method for detecting *Anaplasma* infection is based on a microscopic examination of stained blood smears. However, due to the low parasitemia in animals, this method is not recommended for the detection of subclinical animals or carriers [4]. According to its specificity, polymerase chain reaction (PCR) is one of the alternative diagnosis tools for epidemiological investigations based on selected specific genes such as *msp* (major surface protein) [5,6]. PCR has a considerable advantage in decreasing the occurrence of false negatives when testing animals are as subclinical or carriers. *Msp* genes of *A. marginale* have been characterized and classified into 6 *msp* genes consisting of *msp1a*, *msp1b*, *msp2*, *msp3*, *msp4*, and *msp5* [7]. However, only *msp1a*, *msp4*, and *msp5* have been extensively used for the molecular characterization of *A. marginale*. In addition, *msp4*, encoded by a single gene, is highly conserved among geographic strains [1,8] and is an immune-dominant outer membrane protein with orthologs in all *Anaplasma* spp. examined so far [5]. The *msp4* gene has been also used for phylogenetic studies providing information on the biogeography and the evolution of *Anaplasma* spp. [1,5]. The objectives of this study were (1) to detect *A. marginale* infections of buffaloes and (2) to identify the variation of *A. marginale* strains existing among buffaloes in Northeast Thailand.

MATERIALS AND METHODS

1. Study areas

Water buffaloes located in six provinces of Northeast Thailand, Ubon Ratchathani, Roi Et, Buri Ram, Sakon Nakhon, Surin, and Sisaket, were randomly selected based on the census of buffalo populations reported by DLD.

2. Animals

Six hundred and twenty five buffaloes (535 females and 90 males) were recruited; 138 were collected from Ubon Ratchathani, 81 from Roi Et, 73 from Surin, 70 from Buri Ram, 204 from Sakon Nakhon, and 59 from Sisaket provinces. They were classified by age group (i) < 1 year (n=37), (ii) between 1 and 5 years (n=334), and (iii) > 5 years (n=254). Blood samples from each animal were collected by random sampling between June 2009 and May 2010. Ten ml of blood samples were collected from the jugular vein and placed into sterile tubes containing EDTA as an anticoagulant and citrate salt to preserve the blood for PCR analysis. Blood samples were kept on ice during transportation to the Department of Parasitology, Faculty of Veterinary Medicine, Kasetsart University, Bangkok, Thailand and stored at -20°C until use.

3. DNA extraction

The DNA was extracted from each sample following the protocol of Sambrook [9] and stored at -20°C prior to use.

4. Polymerase chain reaction (PCR)

Primers MSP43 (5'CCGGATCCTTAGC GAACAGGAATCTTGC-3') and MSP45 (5'GGAGCTCCTATGAATTACAGAGAATTGT

TAC-3') were used to amplify an approximately 849 bp fragment of the *msp4* gene [5]. Negative control (ddH_2O) were used every 10 samples to ensure the absence of contamination. The first PCR-positive sample, which was confirmed with sequence analysis, was subsequently used as the positive control. *Msp4* was amplified from 2 µl (approximately 100 ng) of total DNA by PCR. The PCR condition for the *msp4* gene were identical, and consisted of a PCR reagent mixture of 20 µl, 1 pmol/µl of each primer, 0.2 mM deoxynucleotide triphosphates, 1.0 mM $MgCl_2$, 0.5 unit of Taq DNA polymerase, 1x PCR buffer (200 mM Tris-HCl (pH 8.4), 500 mM KCl) (Invitrogen™, Brazil) in a 0.2 ml PCR tube which was used in the T1 Thermocycler (Biometra, Germany). Reactions were performed in the automated DNA thermal cycler for 40 cycles. After an initial denaturing step of 2 min at 95°C, each cycle consisted of a denaturing step of 30 sec at 95°C, an annealing step of 30 sec at 60°C, and an extension step of 1 min at 72°C. PCR products were electrophoresed on 1% agarose gel to confirm the size of amplified fragments.

5. PCR product purification

PCR products were electrophoresed on 1% agarose gels to confirm the size of amplified fragments by comparing with the DNA molecular weight marker. *Msp4* amplicons were silica purified (UltraClean®15 DNA Purification Kit; USA). Briefly, after electrophoresis, the selected DNA bands were cut from the agarose gel and melted irreversibly in a chaotropic salt solution. The DNA was then bound to Ultra Bind® silica particles in the presence of Ultra Salt®. The DNA/silica complex was pelleted in a microcentrifuge, and the melted gel was discarded. The pellet was washed once and the concentrated DNA was maintained in water.

6. Sequence analysis

Amplified fragments were used directly for sequencing these strands by double-stranded, dye-termination cycle sequencing. The *msp4* gene was completely sequenced and these sequences were used to evaluate the genetic distance using the *p* distance method contained in the MEGA program.

7. Statistical analysis

Positive numbers of *A. marginale* infections were analyzed using the factors including gender, age group, geographic region, and herd prevalence. Chi-square test and the Number Cruncher Statistical System (NCSS) ver 2000 (Kaysville, UT) program were used to assess differences in the prevalence and intensity of infection. Comparisons were conducted

(i) between gender (male, female),

(ii) between age groups (< 1 year, between 1 and 5 years, > 5 year)

(iii) between geographic regions, and

(iv) between herds within each region.

Results

1. PCR results

Using the specific primer, *A. marginale* was detected and identified as the PCR product at 849 bp (Fig. 1). The PCR result showed a total of 8% (50/625) positive samples for *A. marginale* (Table 1). Herd prevalence was 13.6% (39/287) with statistically significant differences ($p<0.05$). The prevalence of water buffaloes aged < 1 year and between 1 and 5 years was 8.1% (3/37) and 8.1% (27/334), respectively, while 7.9% (20/254) was reported in animals aged > 5 years. Males were slightly more infected (10%, 9/90) than females (7.7%, 41/535), and Ubon Ratchathani had the highest prevalence of 11.6% (16/138).

2. Molecular characterization of the *A. marginale* strains

The amplified sequences from *A. marginale*-infected buffaloes were sequenced and divided into 4 different nucleotides and amino acid sequences (Table 2). The strains were classified as *A. marginale* Thai (AmT) consisting of AmT1, AmT2, AmT3, and AmT4. Of the *A. marginale* strains existing in buffaloes from Northeast Thailand (Fig 2), the most abundant, AmT1, was found in Ubon Ratchathani, Buri Ram, Sakon Nakhon, Surin and Sisaket, while AmT2 was existed only in Surin province. AmT3 was found in Buri Ram and Sisaket and AmT4 was found in Ubon Ratchathani and Sakon Nakhon. The average genetic distances calculated

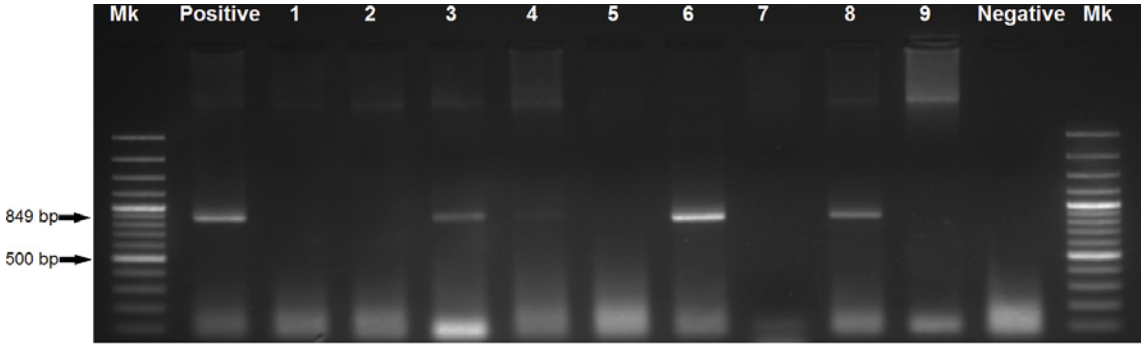

Fig. 1 PCR assay of 849 bp fragment of *msp4* gene of *A. marginale*
(Lanes 3, 4, 6 and 8 show positive band from field sample. The molecular size standard is a 100-bp ladder)

Table 1 Prevalence and strains of *A. marginale* infection in water buffaloes of Northeast, Thailand

Parameter		Prevalence	Statistical significance	Strain
Individual Prevalence	**Province**			
	Ubon Ratchathani	16/138(11.6%)		AmT1, AmT4
	Roi Et	0/81(0%)		-
	Surin	6/73(8.2%)		AmT1, AmT2
	Buri Ram	5/70(7.1%)		AmT1, AmT3
	Sakon Nakhon	18/204(8.8%)		AmT1, AmT4
	Sisaket	5/59(8.5%)		AmT1, AmT3
	Total	**50/625(8%)**	df=5, χ^2=9.75, P=0.08	
Herd Prevalence	**Province**			
	Ubon Ratchathani	13/59(22%)		
	Roi Et	0/58 (0%)		
	Surin	5/28(17.9%)		
	Buri Ram	3/22(13.6%)		
	Sakon Nakhon	16/98(16.3%)		
	Sisaket	2/22(9.1%)		
	Total	**39/287(13.6%)**	df=5, χ^2=17.86, P<0.05	
Age (year)				
	<1	3/37(8.1%)		
	1-5	27/334(8.1%)		
	>5	20/254(7.9%)		
	Total	**50/625(8%)**	df=2, χ^2=0.009, P=0.99	
Sex				
	Male	9/90(10%)		
	Female	41/535(7.7%)		
	Total	**50/625(8%)**	df=1, χ^2=0.57, P=0.45	

Abbreviations: AmT1=*A. marginale* Thai strain 1, AmT2=*A. marginale* Thai strain 2, AmT3=*A. marginale* Thai strain 3 and AmT4=*A. marginale* Thai strain 4

Table 2 Differences in the *msp4* nucleotide and amino acid sequences among *A. marginale* strains, Thailand

Msp4 strain	msp4 Nucleotide position						
	29	236	296	354	423	564	797
Oklahoma; AY127073.1	G, *G*	A, *D*	C, *T*	G, *L*	A, *A*	A, *L*	C, *A*
AmT1	**A, *D***	*	*	A	G	*	*
AmT2	*	*	*	A	G	*	*
AmT3	**A, *D***	*	*	A	G	*	**A, *E***
AmT4	*	**G, *G***	**A, *N***	A	*	G	*

Only variable nucleotide positions are shown with nucleotides identical to the reference Wetumka (Oklahoma) isolate sequence marked with asterisks. Adenine in the translation initiation codon = position 1. Encoded amino acids are shown in italics for the sequence of the Wetumka isolate. Nucleotide changes that result in amino acid changes are shown in bold letters with the encoded amino acid shown in bold italics.

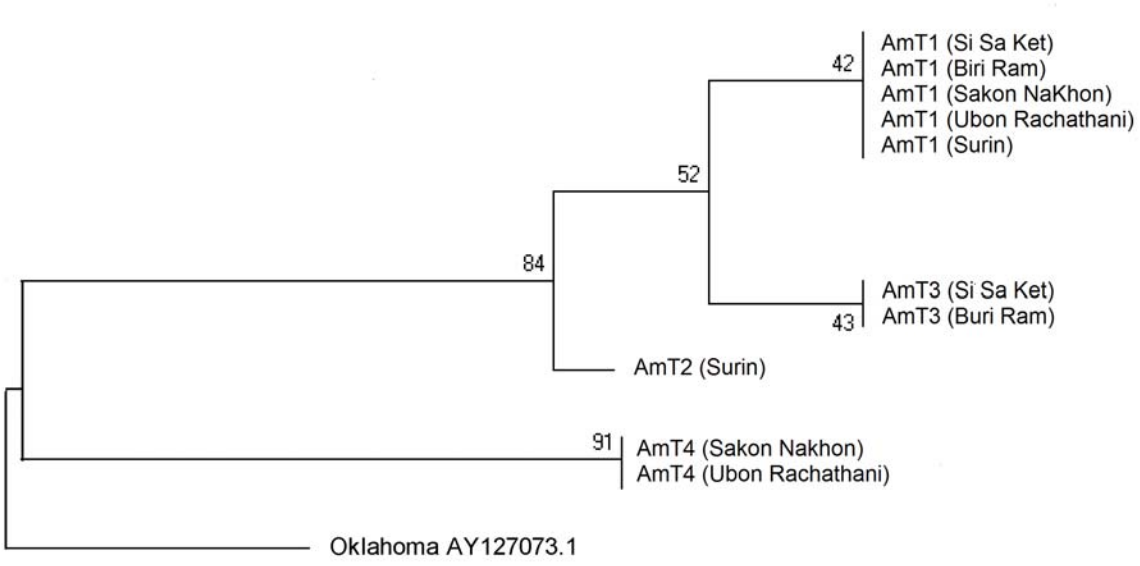

Fig. 2 Phylogenetic analysis of *A. marginale* strains based on MP analysis of *msp4* sequence data and bootstrap analysis with 1000 iterations
(Numbers on branches indicate >40% support for each clade. Strains of *A. marginale* are described in Tables 1 and 2.)

using MEGA highlight the low degree of genetic variation within groups in Thailand (0.001- 0.007). Among groups, calculations of averages revealed greater genetic distances, especially between the 4 genotypes of the Thai strains identified and the Oklahoma strain (Table 3).

Discussion

In Thailand, anaplasmosis is normally detected using microscopic examination (ME) with some limitations. ME is the gold standard test but it can only detect 106 infected erythrocytes per ml [11]. Currently, some molecular techniques

Table 3 Genetic distance (lower diagonal) and standard deviation values (upper diagonal) obtained from *msp4* gene sequence analysis

Strain	AmT2	AmT1	AmT3	AmT4	Oklahoma_AY127073.1
AmT2	-	0.0011	0.0019	0.0025	0.0043
AmT1	0.0012	-	0.0016	0.0026	0.0043
AmT3	0.0035	0.0024	-	0.0026	0.0043
AmT4	0.0059	0.0071	0.0071	-	0.0033
Oklahoma_AY127073.1	0.0153	0.0165	0.0165	0.0094	-

with high sensitivity and specificity have been developed to identify *A. marginale* DNA in the infected animals [4]. To improve the sensitivity of PCR, the *msp4* gene was used and this gene provided sufficient variation to create phylogeographic patterns on the large scale and was also useful for epidemiological studies of anaplasmosis. Previous epidemiological studies of *Anaplasma* spp. infections of cattle in Thailand varied from 0.03 to 74.2% by different methods and were associated with various factors including age, sex, breed, tick density, season and management [12-18]. Our result have shown that the overall prevalence of *A. marginale* infection of water buffaloes in the Northeast was 8% by PCR, compared to 6.3% by ME in 1995 [14]. This result clearly indicated that *A. marginale* infections existed in healthy or subclinical water buffaloes without clinical signs. Moreover, the significant differences in prevalence among 6 provinces demonstrated that there were some factors enhancing *A. marginale* infections in buffaloes particularly in Ubon Ratchathani which is located in the border areas of Thailand. Migration of animal was evidently found at that province; therefore, it might increase a risk of transboundary diseases. Northeastern part of Thailand is the major buffalo production area of the country, since this region houses 70% of buffaloes and more than 35% of cattle husbandry of the country [19]. Anaplasmosis affects animal health and their productivity ranging from draft animals for paddy rice and to food animals for meat and skin. *A. marginale* infections are mostly transmitted among buffaloes through tropical cattle ticks (*Rhipicephalus microplus*) that are widely distributed in Thailand [20]. To minimize the loss, a strategy of vector control should be considered.

The *msp4* primer employed in the PCR was specific for *A. marginale* and sequenced amplicons confirmed the presence of *A. marginale* infections in buffaloes. The sequence of *msp4* is highly conserved among *A. marginale* strains and between *A. marginale* and other *Anaplasma* spp. [5,6]. However, the differences at the nucleotide level of the *msp4* gene allow the differentiation of *Anaplasma* spp. and recently, phylogenetic analysis of *Anaplasma* species was created using the *msp4* data for *A. marginale* [5,6,21]. The results of this study reported herein demonstrated that *msp4* strains of *A. marginale* might vary among geographic regions due to the movement of infected hosts. AmT1 is the most common strain in the Northeast Thailand (Fig 3). Phylogenetic analysis of *msp4* sequences by MP and NJ resulted in trees with similar topology (Fig. 2). Weak support (>52%) was found for the clade containing AmT1, AmT3, AmT2 and AmT4 of Thai *A. marginale* strains (Fig. 2). The AmT1 and AmT4 strains were found in both Ubon Ratchathani and Sakhon Nakhon which were located near the border line between Laos, Cambodia, and Thailand. Genetic diversity of *A. marginale* strains has been described within cattle herds and geographic locations, suggesting that multiple introductions of genetically diverse strains of the pathogen occurred in most geographic areas

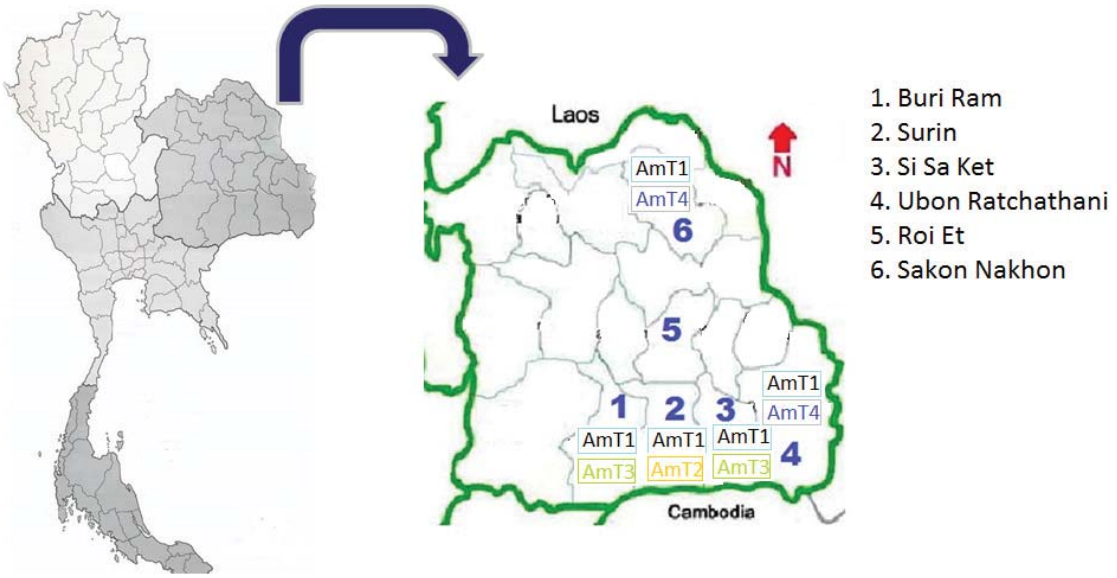

Fig. 3 Distribution of *A. marginale* strains identified in buffaloes form Northeast Thailand

(Abbreviations: AmT1=*A. marginale* Thai strain 1, AmT2=*A. marginale* Thai strain 2, AmT3=*A. marginale* Thai strain 3 and AmT4=*A. marginale* Thai strain 4)

with further diversification resulting from animal movement [5].

The results reported herein demonstrated that *A. marginale msp4* strains might vary among geographic regions due to the unrestricted movement of infected hosts. Different *A. marginale* strains exist within herds in endemic areas due to available vectors such as ticks and blood sucking flies [22]. Therefore, if infected buffaloes have moved into the non-endemic areas, they might introduce a new *A. marginale* strains by mechanical or biological transmission to susceptible hosts. The diversity of *A. marginale* strains and the correlation of infection prevalence in buffaloes have provided information to design control strategies such as a vaccine or tick control based on the presence of genetically heterogeneous populations of *A. marginale* during disease outbreaks.

Acknowledgments

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