รายงานวิจัยฉบับสมบูรณ์

โครงการ การพัฒนาชุดทดสอบสำหรับการตรวจวินิจฉัยโรค anaplasmosis จากการติดเชื้อพยาธิในเม็ดเลือด *Anaplasma marginale* แบบ sandwich ELISA โดยใช้โมโนโคลนัลและโพลีโคลนัลแอนติบอดีต่อ major surface proteins (MSPs)

Development of direct sandwich enzyme-linked immunosorbent assay using monoclonal and polyclonal antibodies specifically against major surface proteins (MSPs) for diagnosis of anaplasmosis caused by *Anaplasma marginale*

โดย นายปณัฐ อนุรักษ์ปรีดา (หัวหน้าโครงการวิจัยผู้รับทุน)
กลุ่มสาขาวิชาชีววิทยาศาสตร์การแพทย์ระดับโมเลกุล
สถาบันชีววิทยาศาสตร์โมเลกุล มหาวิทยาลัยมหิดล

เสร็จโครงการเมื่อ 29 พฤษภาคม 2563

สัญญาเลขที่ RSA6080071

รายงานวิจัยฉบับสมบูรณ์

โครงการ การพัฒนาชุดทดสอบสำหรับการตรวจวินิจฉัยโรค anaplasmosis จากการติดเชื้อพยาธิในเม็ดเลือด *Anaplasma marginale* แบบ sandwich ELISA โดยใช้โมโนโคลนัลและโพลีโคลนัลแอนติบอดีต่อ major surface proteins (MSPs)

Development of direct sandwich enzyme-linked immunosorbent assay using monoclonal and polyclonal antibodies specifically against major surface proteins (MSPs) for diagnosis of anaplasmosis caused by *Anaplasma marginale*

โดย นายปณัฐ อนุรักษ์ปรีดา (หัวหน้าโครงการวิจัยผู้รับทุน)
กลุ่มสาขาวิชาชีววิทยาศาสตร์การแพทย์ระดับโมเลกุล
สถาบันชีววิทยาศาสตร์โมเลกุล มหาวิทยาลัยมหิดล

สนับสนุนโดยสำนักงานกองทุนสนับสนุนการวิจัยและมหาวิทยาลัยมหิดล

ACKNOWLEDGEMENTS

My special thanks are extended to our research team in Parasitology Research Laboratory (PRL) at the Institute of Molecular Bioscience, Mahidol University, Salaya Campus, Nakhon Pathom

Likewise, I would like to acknowledge the financial support of a Research Grant from TRF Research Career Development Grant co-funded by The Thailand Research Fund and Mahidol University (grant number RSA6080071) to Panat Anuracpreeda.

.

Panat Anuracpreeda

Abstract

Project Code : RSA6080071

Project Title : Development of direct sandwich enzyme-linked immunosorbent assay using monoclonal and polyclonal antibodies specifically against major surface proteins (MSPs) for diagnosis of anaplasmosis caused by *Anaplasma marginale*

Investigator : Dr. Panat Anuracpreeda, Institute of Molecular Biosciences, Mahidol University

E-mail Address : panat.anu@mahidol.ac.th, panat.anu@mahidol.edu

Project Period : 3 years

The aim of this study was to clone and characterize recombinant major surface protein of *Anaplasma marginale* (rAmMSP) in order to produce, characterize and purify the monoclonal antibody (MoAb) and polyclonal antibody (PoAb) specific against rAmMSP and use both antibodies to study the distribution of this antigen and application in immunodiagnosis of animal anaplasmosis. The rAmMSP were expressed. Hybridoma secreting MoAb reactive against rAmMSP was obtained from fusion of rAmMSP-immunized spleen cells of BALB/C mouse with mouse myeloma cells. All clones of hybridoma that produce MoAb specific to rAmMSP, as assayed by ELISA and immunoblotting analysis, were used for cross-reactivities studies and localization of the antigen by means of indirect immunofluorescence technique. Localization of *Anaplasma* parasites by immunofluorescence showed these parasites are distributed on both the membrane and the outside of infected erythrocytes. Purified specific antibodies against rAmMSP were used to develop both sandwich ELISA and tested for MSP in sera of naturally infected ruminants. The results showed that MoAbs showed stronger reaction with rAmMSP. In addition, these MoAbs also exhibited strong reaction with native MSP of *A. marginale.* Moreover, no cross-reaction was detected in the native antigens from other parasites as compared to that from *A. marginale.* Sandwich ELISA exhibited an accuracy of 98.9%.

Keywords: *Anaplasma marginale*; *msp* gene; recombinant protein; monoclonal antibody; cross-reaction; sandwich ELISA

บทคัดย่อ

รหัสโครงการ : RSA6080071

ชื่อโครงการ : การพัฒนาชุดทดสอบสำหรับการตรวจวินิจฉัยโรค anaplasmosis จากการติดเชื้อพยาธิในเม็ดเลือด *Anaplasma marginale* แบบ sandwich ELISA โดยใช้โมโนโคลนัลและโพลีโคลนัลแอนติบอดีต่อ major surface proteins (MSPs)

ชื่อนักวิจัย : นายปณัฐ อนุรักษ์ปรีดา กลุ่มสาขาวิชาชีววิทยาศาสตร์การแพทย์ระดับโมเลกุล สถาบันชีววิทยาศาสตร์โมเลกุล มหาวิทยาลัยมหิดล

E-mail Address : panat.anu@mahidol.ac.th, panat.anu@mahidol.edu

ระยะเวลาโครงการ : 3 ปี

การศึกษาครั้งนี้มีวัตถุประสงค์เพื่อโคลนและศึกษาคุณลักษณะของ recombinant major surface protein ของเชื้อ *Anaplasma marginale* (rAmMSP) เพื่อผลิต ศึกษาคุณลักษณะ และการทำให้บริสุทธิ์ของโมโนโคลนัลแอนติบอดี (MoAb) และโพลีโคลนัลแอนติบอดี (PoAb) ที่มีความจำเพาะต่อ rAmMSP ของเชื้อ *A. marginale* และใช้ประโยชน์จากแอนติบอดีดังกล่าวในการศึกษาตำแหน่งการกระจายตัวของแอนติเจนชนิดนี้และใช้ประโยชน์ในการพัฒนาวิธีการตรวจสอบการติดเชื้อในสัตว์ที่เป็นโรค anaplasmosis จากการศึกษาพบว่าไฮบริโดมาที่ผลิตโมโนโคลนัลแอนติบอดีต่อ rAmMSP ได้มาจากการทำปฏิกิริยารวมตัวกันของเซลล์ myeloma และเซลล์จากม้ามของหนูทดลอง ซึ่งสามารถตรวจคุณสมบัติของโมโนโคลนัลแอนติบอดีดังกล่าวได้ด้วยวิธี indirect ELISA และ immunoblottin เพื่อนำมาศึกษา cross-reaction และการกระจายตัวของแอนติเจนในในตัวเชื้อศึกษาด้วยวิธี indirect immunofluorescence พบการกระจายตัวของเชื้อที่เยื่อหุ้มและนอกเซลล์เม็ดเลือดแดง แอนติบอดีที่มีความบริสุทธิ์ถูกนำมาใช้ในการใช้ในการพัฒนาวิธีการตรวจสอบการติดเชื้อในสัตว์ที่ติดโรคตามธรรมชาติด้วยวิธี sandwich ELISA ผลการศึกษาในครั้งนี้พบว่าโมโนโคลนัลแอนติบอดีที่ผลิตได้มีความจำเพาะสูงต่อ rAmMSP รวมถึงมีความจำเพาะสูงต่อ native MSP นอกจากนี้แล้วจากการศึกษาไม่พบ cross-reaction กับแอนติเจนของเชื้อชนิดอื่น วิธี sandwich ELISAค่าความแม่นยำในการตรวจสอบเท่ากับร้อยละ 98.9

คำหลัก: เชื้อพยาธิในเม็ดเลือด; *Anaplasma marginale*; ยีน*msp*; recombinant protein; โมโนโคลนัลแอนติบอดี; cross-reaction; sandwich ELISA

CONTENTS

CONTENTS (CONT.)

LIST OF TABLES

LIST OF FIGURES

LIST OF ABBREVIATIONS

BSA	=	Bovine serum albumin
°C	=	Degree Celsius
CFA	=	Complete Freund's adjuvant
cm	=	Centimeter
cm^2	=	Square centimeter
Da	=	Dalton
DMSO	=	Dimethyl sulfoxide
EDTA	=	Ethylenediamine tetraacetic acid
ELISA	=	Enzyme-linked immunosorbent assay
FCS	=	Fetal calf serum
g	=	Gram of force of gravity
h	=	hour
HAT	=	Hypoxanthine-aminopterine-thymidine
H_2O_2	=	Hydrogen peroxide
HRP	=	Horseradish peroxidase
IFA	=	Incomplete Freund's adjuvant
Ig	=	Immunoglobulin
kDa	=	Kilodalton
L	=	Liter
µg	=	Microgram
µl	=	Microliter
µm	=	Micrometer
M	=	Molar
MoAb	=	Monoclonal antibody
mg	=	Milligram
min	=	Minute
ml	=	Milliliter
mM	=	Millimolar
MW	=	Molecular weight
N	=	Normality
NaCl	=	Sodium chloride
Na_2CO_3	=	Sodium carbonate

LIST OF ABBREVIATIONS (CONT.)

$NaHCO_3$	=	Sodium bicarbonate
PAGE	=	Polyacrylamide gel electrophoresis
PBS	=	Phosphate buffered saline
PEG	=	Polyethylenegylcol
PoAb	=	Polyclonal antibody
PMSF	=	Phenylmethylsulfonyl fluoride
rpm	=	Round per minute
RPMI	=	Roswell Park Memorial Institute
sec	=	Second
SDS	=	Sodium dodecyl sulfate
TBS	=	Tris-buffer saline
TEM	=	Transmission electron microscopy
TEMED	=	N,N,N',N'-Tetramethylethylenediamine
Tris	=	Tris-(hydroxymethyl)-aminomethane
v/v	=	Volume by volume
w/v	=	Weight by volume

CHAPTER I
INTRODUCTION

Anaplasmosis is economically important diseases in ruminants caused by the rickettsia *Anaplasma* sp. (*Rickettsiales*: *Anaplasmataceae*). It results in a significant economic loss throughout the tropical and subtropical countries of the world (~40ºN to 32ºS), including South and Central America, the USA, southern Europe, Asia, Australia, and Thailand. Thailand is an agricultural country located in Southeast Asia, where the livestock industry has been hampered by the severe tick-borne haemoparasites.The current method for diagnosis of anaplasmosis in ruminants is based on direct microscopic detection of the organisms in Giemsa-stained blood smears. However, due to the low parasitemia in animals, this method is not recommended for the detection of subclinical animals or carriers. The animals response to infection has been measured by a variety of serological methods, for example, card agglutination (CA), capillary agglutination tests, complement fixation, and the indirect fluorescence antibody (IFA) techniques. Although demonstration of circulating antibodies has been used for epidemiological studies, the presence of antibodies is not the direct indicator of active infection, and cross-reactivity with other parasites is often difficult to differentiate. The detection of the circulating antigens rather than antibodies is considered to be a more reliable method for evaluating the status of infection which could be used to monitor the efficacy of treatment and the effectiveness of future candidate vaccines. The method of circulating antigen detection has been used successfully in the diagnosis of many parasitic diseases. Nevertheless, few studies have been done on the immunodiagnosis of anaplasmosis by the detection of circulating antigens.

The Initial body (IB) antigens or major surface proteins (MSPs) were found to be major circulating antigens released from *Anaplasma* sp., i.e., *Anaplasma marginale* during their course of infection. Therefore, in this project, I will focus on 1) analysis, isolation and characterization of initial body antigens or major surface protein (MSP) of *A. marginale,* 2) the production and characterization of monoclonal antibody (MoAb) and polyclonal antibody (PoAb) specifically against recombinant major surface protein of *A. marginale* (rAmMSP) and 3) use them in the direct sandwich ELISA assay, which will be developed for detecting the circulating antigens of this parasite in naturally infected animals. Hopefully, this method of detection will increase the sensitivity and specificity for the immunodiagnostic assay for anaplasmosis, especially that caused by *A. marginale.*

CHAPTER II

OBJECTIVES

Overall objective: The overall objective of this project is to devise immunodiagnostic method for anaplasmosis using monoclonal antibody (MoAb) and polyclonal antibody (PoAb) against specific major surface protein (MSP) antigens of *A. marginale.*

Specific objectives: To achieve the overall objective, there are 4 specific objectives that will be performed in steps as follows:

1. To analyze, isolate and characterize the initial body (IB) antigens or recombinant major surface protein of *A. marginale* (rAmMSP).
2. To produce and characterize MoAb and PoAb specifically against recombinant *A. marginale* major surface protein (rAmMSP).
3. To purify the MoAb and PoAb specifically against recombinant *A. marginale* major surface protein (rAmMSP).
4. To develop the direct sandwich enzyme-linked immunosorbent assay (direct sandwich ELISA) for the detection of the circulating major surface protein (MSP) antigens in serum of naturally infected animals with anaplasmosis caused by *A. marginale.*

CHAPTER III
LITERATURE REVIEW

1. Anaplasmosis

Anaplasmosis is an arthropod borne hemoparasitic disease of cattle and other ruminants including water buffalo, bison, African antelopes, and mule deer caused by the rickettsia *Anaplasma* sp. (Kuttler et al., 1984; Dumler et al, 2001). The genus *Anaplasma* (Rickettsiales: Anaplasmataceae) contains obligate intracellular organisms found exclusively within membrane-bound vacuoles in the cytoplasm of both vertebrate and invertebrate host cells (Dumler et al., 2001). The genus *Anaplasma* includes *A. marginale, A. centrale, A. bovis, and A. ovis.* Inaddition*, A. phagocytophilum* (previously recognized as *E. equi*, *E. phagocytophila*, and the human granulocytic ehrlichiosis (HGE) agent), which infects a wide range of hosts including humans, rodents, birds, dogs and cattle, as well as *A. platys* which infects dogs. *Aegyptianella*, which is infective for birds, was originally retained as a genus incertae sedis due to lack of sequence information, but was recently confirmed to be closely related to Anaplasma spp., based on 16 S rRNA and *groEL* gene sequences of *Ae. Pullorum.* However, *Aegyptianella* has yet to be formally renamed as a member of the *Anaplasma* genus (Rikihisa et al., 2003). Anaplasmosis occurs worldwide and is devastating to meat, milk, and fiber production in tropical and subtropical regions of the world and the disease causes considerable economic losses globally to both dairy and beef industries worldwide, including Thailand (National Research Council, 1982; Kocan et al., 2003). Thailand is an agricultural country located in Southeast Asia, where the livestock industry has been impeded by the severe tick-borne haemoparasites (Jittapalapong and Lieowijak, 1988). The disease results in a severe extravascular anemia associated with intraerythrocytic parasitism. After transmission by biologic or mechanical arthropod vectors (a prepatent period of 20 to 40 days), an acute phase occurs during which parasitemia increases geometrically, severe haemolytic anemia, weight loss, abortion, decreased milk production and death (Alderink and Dietrich, 1981; Wanduragala and Ristic, 1993). Cattle recovering from acute disease remain persistently infected with a low level parasitemia and serve as a reservoir for transmission of the organism (Swift and Thomas, 1983).

For animal anaplasmosis, *Anaplasma marginale* is an obligate intraerythrocytic pathogen that causes bovine anaplasmosis (Kocan et al., 2003). Biological transmission of *A. marginale* is effected by ticks and approximately 20 species of ticks have been

incriminated as vectors worldwide, while mechanical transmission occurs when infected blood is transferred to susceptible cattle by biting flies or blood-contaminated fomites (Kocan et al., 2004). Beside cattle, *A. marginale* was also found in water buffalo, American bison, Rocky Mountain elk, black tailed deer, mule deer, and white-tailed deer (Zaugg *et al*., 1996). Bovine anaplasmosis often shows clinical signs including mild to severe anemia, fever, weight loss, abortion, lethargy, icterus without hemoglobinemia and hemoglobinuria and often death in animals over 2-year old (Kocan et al., 2003, 2004). Cattle that survive acute infection develop persistent infections and serve as reservoirs of *A. marginale* providing a source of infective blood for both mechanical transmission and biological transmission by ticks (Kieser et al., 1990). One the other hand*, Anaplasma centrale* is less pathogenic for cattle than *A. marginale*, is only occasionally associated with clinical disease, and is presently used as a live vaccine in some countrys, i.e., Israel, Australia, Africa, and South America (Bock et al., 2003). In addition, *A. ovis* is a pathogen of wild and domestic sheep, but does not establish persistent infection or cause disease in cattle (Kocan et al., 2003).

In human anaplasmosis*, A. phagocytophilum* causes human granulocytic anaplasmosis (HGA), tick-borne fever of ruminants, and equine/canine granulocytic anaplasmosis. *A. phagocytophilum* is transmitted by tick species belonging to the *Ixodes persulcatus* complex, i.e., *I. scapularis* and *I. pacificus* in the United States, *I. ricinus* in Western Europe, and *I. persulcatus* in eastern Europe and Asia; however, other tick species may prove to be the vectors (Dumler et al., 2001).de la Fuente et al., 2004a). Human anaplasmosis was reported in Europe, South and North America and Asia, as well as characterized by fever, headache, myalgia, malaise, leukopenia, thrombocytopenia, and increasing levels of C-reactive protein and hepatic transaminases (Carlyon and Fikrig, 2003; Massung and Slater, 2003; Parola, 2004). Although this disease is usually self-limiting, severe complications can result in prolonged fever, shock, seizures, hemorrhage, pneumonitis, acute renal failure, rhabdomyolysis, and opportunistic infections that may result in death (Carlyon and Fikrig, 2003). Also, *A. phagocytophilum* infection occurs in white-footed mice, raccoons, gray squirrels, wild rabbits, birds, cats and white-tailed, red, and roe deer (Telford et al., 1996; Dumler et al., 2001; Daniels et al., 2002; Levin et al., 2002; Petrovec et al., 2002; Goethert and Telford, 2003; de la Fuente et al., 2004b; Lappin et al., 2004; Polin et al., 2004). Furthermore, it was currently reported that concurrent infections of *Anaplasma* sp. can occur in ruminants and ticks (Hofmann-Lehmann et al., 2004; Lin et al., 2004). The establishment of concurrent

Anaplasma spp. infections in reservoir hosts is likely to increase the risk of pathogen transmission among wildlife, domestic animals, and humans. Hence, the effective diagnostic assays are still to be taken for anaplasmosis.

2. Economic impact of bovine anaplasmosis

Presently, bovine anaplasmosis causes important economic loss in many countries, mainly due to the high morbidity and mortality in cattle flocks. The losses due to anaplasmosis are determined by several parameters including low weight gain, reduction in milk production, abortion, the cost of anaplasmosis treatments, and mortality. The annual economic loss due to anaplasmosis in beef cattle in the United States as a result of morbidity and mortality is estimated to be more than US $300 million (McCallon, 1973), whereas in Latin America those losses were measured to be US $800 million (Lonibardo, 1976). Brown (1997) reported that bovine anaplasmosis and babesiosis were responsible for causing an economic loss of US $875 millions in Latin American.

In Thailand, bovine anaplasmosis has been reported and their distributions have an impact on the economic losses of livestock production (Sithornnakoon et al., 1965; Jittapalapong et al., 1988; Chethanond et al., 1995; Chaichanapunpol et al., 1996; Phrikanahok et al., 2000; Fungfuang et al., 2006; Worasing, 2007; Yawongsa et al., 2010).

3. Transmission and development cycle of *Anaplasma marginale*

Transmission of *A. marginale* is composed of both mechanically by biting flies or blood-contaminated fomites and biologically by ticks (Dikmans, G. 1950; Kocan, K. M. 1986). Mechanical transmission frequently occurs via two ways: 1) blood-contaminated fomites, including needles, nose tongs, dehorning saws, ear-tagging equipments, tattooing instruments, and castration devices and 2) arthropods such as *Tabanus*, *Stomoxys*, and mosquitoes (Ewing, 1981; Potgieter et al., 1981; Foil, 1989). Biological transmission of *A. marginale* is effected by ticks, and approximately 20 species of ticks have been incriminated as vectors worldwide (Dikmans, G. 1950; Ewing, 1981). Tick transmission can occur from stage to stage (transstadial) or within a stage (intrastadial), while transovarial transmission from one tick generation to the next does not appear to occur (Stich et al., 1989). In addition to mechanical and biological transmission, *A. marginale* can be transmitted from cow to calf transplacentally during gestation (Norton, et al., 1983; Zaugg, 1985. Zaugg and Kuttler, 1984). Potgieter and Rensburg (1987)

revealed that prevalence rate of in utero transmission of *Anaplasma* infections in South Africa was 15.6%. Transplacental transmission of anaplasmosis may contribute to the epidemiology of this disease in some areas. In the United States, interstadial transmission of *A. marginale* has been involved with the three host ticks *Dermacentor andersoni* and *D. variabilis* (Kocan, 1986; Kocan et al., 1985). Intrastadial transmission of *A. marginale* is effected by male ticks. Kocan et al. (1992a,b) reported that male *Dermacentor* ticks may serve as reservoir and biological transmission of *A. marginale* along with persistently infected cattle (Kocan et al., 1992a,b; Ge et al., 1996; Kocan et al., 2000). In addition, transmission of *A. marginale* by male ticks could be an important mechanism of transmission of *A. marginale* by one-host ticks, including *Boophilus* sp. and *D. albipictus*. Nevertheless, the cofeeding of adult tick *Dermacentor* sp. does not influence the transmission of *A. marginale* (Kocan and de la Fuente, 2003).

The developmental cycle of *A. marginale* in ticks is complex and coordinated with the tick feeding cycle (Fig. 1). Infected erythrocytes taken into ticks (i.e., *Dermacentor* sp., *Boophilus* sp., or *Rhipicephalus* sp.) with the blood meal provide the source of *A. marginale* infection for gut cells of tick. When the ticks feed a second time, many other tick tissues (i.e., the salivary glands) become infected, from where the rickettsiae are transmitted back to vertebrates, such as cattle, during feeding. At each site of infection in ticks, *A. marginale* develops within membranebound vacuoles or colonies. There are two forms of *A. marginale* in infected tick cells. The first form seen within the colony is the reticulated (vegetative) form that divides by binary fission, forming large colonies that contain hundreds of organisms. The reticulated form then changes into the dense (infective) form, which can survive extracellularly outside the host cells. Cattle become infected with *A. marginale* when the dense form is transmitted during tick feeding via the salivary glands. The organism (initial body) was shown to enter the red blood cells by invagination of the cytoplasmic membrane with subsequent formation of a vacuole. Thereafter, the initial body multiplies by binary fission and forms an inclusion body which comprises generally of four to eight initial bodies. Inclusion bodies are numerous during the acute phase of infection. However, low-level infections persist for several years (Kocan, 1986; Ge et al., 1996; Kocan et al., 1992a,b).

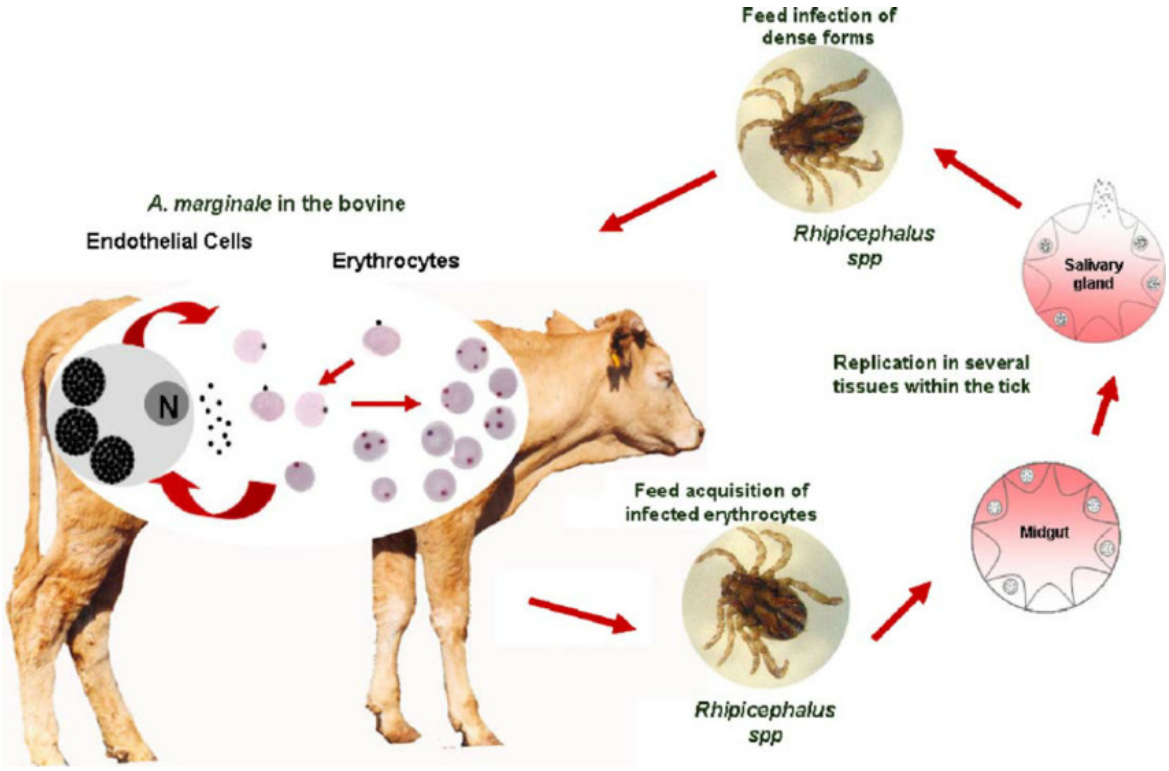


Fig. 1. Transmission and development cycle of *Anaplasma marginale* (https://www.researchgate.net/figure/Proposed-life-cycle-of-Anaplasma-marginale-The-cycle-is-modified-from-Kocan-1999-to_fig1_26853074).

4. The immunodiagnosis of anaplasmosis

A provisional diagnosis of anaplasmosis is based on the history and clinical signs of the disease. Further confirmation can be obtained by the microscopic examination of stained blood smears. However this method often results in misdiagnosis due to the low parasitemia in animals, it is not recommended for the detection of subclinical animals or carriers (Carelli *et al*., 2007). Consequently, immunological diagnosis could play the most dependable means for monitoring the infection, and can be supplemented by the standard parasitological method. It was believed that immunodiagnosis would be a better method, but up to the presence no such method had been devised. Therefore, the immunodiagnosis could be performed in two ways: 1) detection of host's antibodies against parasite' antigens such as enzyme linked immunosorbent assay (ELISA) and

immunofluorescent antibody test (IFAT), and 2) detection of parasite' antigens in the host's blood circulation such as sandwich ELISA. The method of circulating antigen detection (sandwich ELISA) has been used successfully in the diagnosis of many parasitic diseases such as *Fasciola hepatica* and *F. gigantica* infections (Moustafa et al., 1998; Anuracpreeda et al., 2009, 2013, 2016a.b). However, this sandwich ELISA has not been devised and as yet no immunodiagnosis for *Anaplasma* infection has been developed.

Up to now, immunodiagnostic antigens in anaplasmosis are the initial body (IB) antigens or major surface proteins (MSPs) of *A. marginale*. Six MSPs, MSP1a, MSP1b, MSP2, MSP3, MSP, and MSP, have been identified on erythrocyte derived organisms. MSP1a, MSP, and MSP are encoded by single genes, while MSP1b, MSP2, and MSP3 are encoded by multigene families. Some of these IB antigens or MSPs have been tested for use in serodiagnostic assays. The antigens are known to modulate the immune response of the host and thus the parasite antigens invoke the host protective immune responses which result in the death and expulsion of the parasites during the development of concomitant immunity (Palmer et al., 1985; de la Fuente et al., 2001a; Kocan et al., 2003, 2004). Shkap et al. (1990) who successfully used the ELISA and indirect fluorescent antibody (IFA) test for the detection of antibodies against *A. marginale* and *A. centrale* using initial body (IB) antigens. In addition, a competitive ELISA (cELISA) has been utilized for the detection of *Anaplasma* infection in ruminants such as cattle, ovine, and deer (Ndung'u et al., 1995; Knowles et al., 1996; de la Fuente et al., 2004b). However, this cELISA does not differentiate *Anaplasma* species in regions where co-infection with *A. phagocytophylum* and *A. marginale* or *A. centrale* occurs. The possibility of cross-infection cannot be ruled out when using this assay in these regions (de la Fuente et al., 2004a; Hofmann-Lehmann et al., 2004; Lin et al., 2004). Likewise, Morzaria et al. (1999) developed an ELISA for the detection of antibody against *A. marginale* using MSP. However, the assay has not been evaluated for cross-reactivity with other *Anaplasma* species. The MSP2-related p44 antigens of *Borrelia burgdorferi* and *A. phagocytophilum* may be used in the ELISA to detect antibodies in white-tailed deer (Magnarelli et al., 2004). In addition, MSP1a could be used to identify infections caused by *A. marginale* because it may prove to be specific for this species. A neutralization epitope, which is conserved among strains of *A. marginale,* was identified in the repeat regions of MSP1a (Palmer et al., 1987; Garcia-Garcia et al., 2004), Nowadays, few studies have been done on the immunodiagnosis of anaplasmosis by the detection of

circulating antigens. Because the detection of the parasite antigens that persist in the host's blood circulation is the more direct method for diagnosing as it indicates whether the living parasites are still in the hosts. Hence, the sandwich ELISA is one of the alternative diagnosis that provides a powerful tool for epidemiological investigations with the high accuracy of *Anaplasma* infection.

CHAPTER IV
MATERIALS AND METHODS

The materials and methods were divided into 3 sections corresponding to the four objectives as described earlier in Chapter 3.

1. Analysis, isolation and characterization of the initial body (IB) or recombinant major surface protein (rMSP) antigens of *A. marginale*

1.1 Collection of parasite specimens

Blood samples were collected from the jugular vein of infected cattle into sterile tubes containing EDTA as an anticoagulant and citrate salt to preserve the blood and stored at -20 ºC until use.

1.2 DNA extraction and PCR amplification

DNA was extracted from blood samples following the protocol of E.Z.N.A. ®Tissue DNA Kit (OMEGA bio-tek). Briefly, 25 µl of OB protease solution and 250 µl of BL buffer were added into 250 µl of blood samples, mixed and incubated at 70 ºC for 10 min. The mixture was added with 250 µl of absolute ethanol and mixed thoroughly. The samples were transferred to the HiBind® DNA Mini Column, centrifuged at maximum speed for 1 minute and discarded the filtrate. After that, 500 µl of HBC buffer was added into the column, centrifuged at maximum speed for 30 sec, and discarded the filtrate. Then, the column was washed with DNA wash buffer and eluted the DNA by elution buffer. The eluted DNA was stored at -20 ºC. The *msp* gene was amplified by polymerase chain reaction (PCR) according to Watthanadirek et al. (2018).

1.3 Construction of *msp* gene

The cloning reaction was performed by 4 µl of purified PCR products, 1 µl of salt solution and 1 µl of TOPO® vector. The reaction was mixed gently and incubated for 30 min at room temperature. For transformation, the reaction was transformed into TOP10 chemically competent *E. coli* by heat shock method. After that, the transformation was spread on a pre-warmed selective plate containing 100 µg ampicillin, and incubated at 37 ºC for overnight. The positive clones were grown in Luria Bertani (LB) broth

containing ampicillin for overnight and the plasmids were extracted by alkaline lysis and analyzed the recombinant plasmids by restriction analysis with *Hind* III and PCR.

1.4 Production and purification of the recombinant major surface protein of *Anaplasma marginale* (rAmMSP).

The recombinant plasmid was transformed into BL21 Star™ (DE3) One Shot® cells by heat shock method as per Watthanadirek et al. (2018). The transformation reaction was grown in LB broth containing 100 µg/ml ampicillin, and incubated at 37 °C for overnight with shaking. After that, The BL21/pET100-*msp* was grown in LB broth containing 100 µg/ml ampicillin at 16, 25 and 37 °C with shaking. After the culture reached 0.5 of optical density 600 (OD_{600}), the sample was induced by 0.1 mM Isopropyl β-D-1-thiogalactopyranoside (IPTG) and removed at the same cells density at every 1 h for 6 h to choose the best time for expression. The cells were harvested by centrifugation at 8,000 g at 4 °C for 10 min. The cell pellets were resuspended with 20 mM HEPES and 50 mM NaCl pH 7.4, then added 5 mg/50 µl of lysozyme and incubated at 4 °C for 16 h. Subsequently, the cells were added phenylmethane sulfonyl fluoride or phenylmethylsulfonyl fluoride (PMSF) and incubated on ice for 10 min. The cell lysate was sonicated on ice and harvested for soluble and insoluble fractions by centrifugation at 100 ×g at 4 °C for 5 min. The samples were analyzed from total, soluble and insoluble fractions of protein on 12% Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) gel and immunoblotting analysis

For rMSP protein purification was performed under the high-performance liquid chromatography (HPLC) system by using HisTrap HP (GE Healthcare Bio-sciences). The soluble fraction of rMSP was injected into the column, then washed with 20 mM HEPES, 50 mM NaCl and 25 mM of imidazole, pH 7.4. The rMSP protein was eluted by stepwise gradients of 50 mM, 60 mM, 70 mM, 80 mM, 90 mM, 100 mM, 150 mM, 150 mM and 250 mM imidazole and analyzed by SDS–PAGE.

1.5 Protein analysis by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE)

As the method of Watthanadirek et al. (2018), SDS-PAGE was perofmed. Briefly, the cell pellet was resuspended in buffer containing 20 mM HEPES and 50 mM NaCl, pH 7.4, mixed with electrophoresis sample buffer (200 mM Tris-HCl, 8% SDS, 40% Glycerol and 0.4% Bromophenol blue, pH 6.8) and boiled for 5 min. Protein samples

were separated by 12% polyacrylamide gel in electrophoresis buffer (192 mM glycine, 25 mM Tris and 0.1% SDS) at constant 80 Voltages. The gels were stained with Coomassie Brilliant Blue staining solution and destained with destain buffer (10% (v/v) of EtOH and 10% (v/v) of glacial acetic acid in distilled water).

1.6 Immunoblotting analysis

After the proteins were separated by SDS-PAGE, the gel was soaked in transfer buffer (25 mM Tris, 150 mM Glycine and 10% (v/v) ethanol). Subsequently, the proteins were transferred onto nitrocellulose membrane by wet transfer technique with constant current for 1 h. The membrane was blocked in 5% (w/v) bovine serum albumin (BSA) in phosphate buffered saline (PBS) buffer at room temperature for 45 min. Then, the membrane was incubated with Anti-His as a primary antibody at dilution of 1:3000 for 45 min. The membrane was washed with 0.1% tween 20 in PBS for 3 times. The secondary antibody (anti-mouse IgG antibody) conjugated with the alkaline phosphatase (ALP) in 5% BSA at dilution of 1:5000 was added on to the membrane and incubated for 45 min. After washing, the color reaction was developed by using ALP substrate (75 mg/ml of NBT and 50 mg/ml of BCIP) and the reaction was stopped by adding of distilled water (Watthanadirek et al., 2018).

2. Production and characterization of MoAb and PoAb specifically against recombinant *A. marginale* major surface protein (rAmMSP)

2.1 Immunization with rAmMSP antigens

Eight-week-old BALB/c mice were subcutaneously immunized with 10 μg of rAmMSP antigens in 200 μl complete Freund's adjuvant and followed by subcutaneous injection with the same dose in 200 μl incomplete Freund's adjuvant on day 21. The last immunization was performed 3 week later by intravenous injection with 20 μg of the antigens in 100 μl 0.1M PBS. Three ELISA were performed to determine the antibody titer in the immunized mouse serum. Mice that showed the highest positive ELISA titer against the antigens were sacrificed and its spleen was removed for fusion experiment to produce monoclonal antibody.

2.2 Production and screening of MoAbs

Inbred eight week-old female BALB/c mice were immunized with rAmMSP antigens according to Anuracpreeda et al. (2016a). The hybridoma clones expressing MoAb against rAmMSP antigens were produced by fusion of the splenocytes from immunized BALB/c mouse with non-secreting mouse myeloma cells (P3x63-Ag8.653), using polyethylene glycol (PEG) (Sigma-Aldrich Inc., St. Louis, MO, USA). The MoAbs produced by the hybridoma cells were screened by indirect ELISA. Briefly, 50 μl of 10 μg/ml rAmMSP antigens of *A. marginale* diluted in coating buffer, pH 9.6 were added into each well of polystyrene microtiterplate and incubated for 2 h at 37 °C. Excess antigens and unbound material were removed, and the plate was washed three times with distilled water. Each time the washing fluid is left in the wells for approximately 1 min. Consequently, the plate was tapped dry and blocked with 50 μl/well of 0.25 % bovine serum albumin (BSA), 0.05% Tween 20 (Sigma Co.) in 0.01 M PBS, pH 7.2 (blocking solution) at 37 °C for 30 min, and then washed prior to adding 50 μl of undiluted and diluted MoAbs were tested at 37 °C for 2 h. The plate was washed as above before goat anti-mouse immunoglobulin conjugated with peroxidase (Sigma Co.), at 1:6000 dilution in the blocking solution, was added to each well at 50 μl, and subsequently incubated for 1 h at 37 °C, and washed again. After excess conjugated is discarded, 50 μl of 3, 3', 5, 5'-tetramethyl benzidine (TMB) was added and the enzymatic reaction was allowed to take place for 5 min at room temperature. Finally, the enzymatic reaction was stopped by addition of 50 μl of 1N HCl. OD value at 450 nm was measured in a microplate reader. Specific MoAb class and subclass are determined by enzyme immunoassay using Mouse Typer Sub-Isotyping Kit (Bio-Rad, USA).

2.3 Cross-reactivities studies

For the cross-reactivities studies, antigens from *A. marginale* and other parasites, i.e., *Anaplasma centrale*, *Babesia bovis, B. bigemina*, *Theileria* sp. and *Trypanosoma* sp., were separated and blotted with various MoAbs by similar immunoblotting method as mentioned above. In addition, indirect ELISA was also used to confirm and quantify the degree of the cross reactions. MoAb and antigens which showed the least cross-reactions against other parasites antigens were tested against immune sera of naturally infected cattle collected in fields from various part of Thailand in order to detect the circulating antigens and/or antibodies.

2.4 Production of the PoAb against rAmMSP

Immunization was performed according to Anuracpreeda et al. (2013a, 2016a,b) as follows: each rabbit was primed by subcutaneously injection in the back region with 100 μg of rAmMSP antigens in 500 μl of PBS solution emulsified in an equal volume of complete Freund's adjuvant (Sigma-Aldrich Inc., Saint Louis, Missouri, USA). Two weeks later, each rabbit was bled and their anti-sera were tested for antibody titer against rAmMSP antigens by ELISA. The high responders were given a booster injection with 100 μg of rAmMSP antigens of *A. marginale* in PBS emulsified in incomplete Freund's adjuvant (Sigma-Aldrich Inc.) via subcutaneous route. On days 28 and 42 after boosting, rabbits were bled and anti-serum from each animal was collected and tested for the presence of antibody against the protein antigen by ELISA. Three days before blood collection, a final boosting dose of the protein was given by the same route without adjuvant. Rabbit anti-sera against rAmMSP antigens were used for characterization of these antigens and then the sera were purified by ammonium sulfate precipitation and ion-exchange chromatography for the next experiments.

2.5 Localization of *Anaplasma* parasites by immunofluorescent technique

The method of immunofluorescence detection described by Anuracpreeda et al. (2009; 2016a) was used. Briefly, the slides of blood smear were fixed in ice-cold acetone for 3 min. Thereafter, the slides were incubated with sera from rabbit immunized with rAmMSP and normal rabbit (diluted at 1:50 with PBS containing 0.05% Tween 20) at 37 ^{o}C for 2h. After washing three times with PBS containing 0.05% Tween-20, pH 7.4, for 1 min each, the slides were treated with fluorescein isothiocyanate (FITC)-conjugated goat anti-rabbit IgG (Sigma–Aldrich, Inc.), (diluted at 1:300 with PBS containing 0.05% Tween-20, pH 7.4) at 37 ^{o}C for 1h. After washing three times with the same buffer, the treated slides were air dried, examined and photographed under an EVOS FL color fluorescence microscope.

3. Purification of MoAb and PoAb specifically against recombinant *A. marginale* major surface protein (rAmMSP)

3.1 Ammonium sulfate precipitation

MoAb in culture supernatant and PoAb in serum were concentrated and purified by precipitation in saturated ammonium sulfate solution. Briefly, equal volume of ice-cold saturated solution of ammonium sulfate was added dropwise to the culture supernatant. The reaction was allowed to proceed overnight with gentle mixing. Thereafter, the mixture was centrifuged. The precipitate was collected, reconstituted to a required volume with saline, dialysed exhaustively against a large volume of saline, and finally followed by dialyzing against PBS. Protein content in dialysate was determined by measuring absorbance at 280 nm (Anuracpreeda et al., 2016a,b).

3.2 Affinity chromatography

Immunoglobulin fractions of both MoAb and PoAb were purified from by ammonium sulfate precipitation, dialyzed against an excess of PBS and applied to an affinity chromatography in a Mab trap protein G Sepharose column (for IgG fraction) or a HiTrap IgM Purification Sepharose column (for IgM fraction) (Amersham Pharmacia Biotech AB, Uppsala, Sweden). Appropriate immunoglobulin fractions were collected and pooled. The protein content was estimated from the OD values. The pooled immunoglobulin was dialyzed, lyophilized and stored at 4 $^{\circ}$C (Anuracpreeda et al., 2016a,b).

3.3 Biotin conjugation of PoAb

Chromatographically pure rabbit IgG anti-rAmMSP antigens was covalently conjugated to biotin using the method of Anuracpreeda et al. (2016a,b). Briefly, the purified immunoglobulin was dialyzed overnight, clarified by centrifugation and the protein solution was diluted to a final concentration. A biotin solution using N-hydroxysuccinimidobiotin (Sigma Co) freshly prepared in DMSO was added immediately to the immunoglobulin. The reaction mixture was allowed to take place and then exhaustively dialyzed to remove unconjugated biotin and DMSO. The supernatant was mixed with an equal volume of glycerol and stored at -20 $^{\circ}$C.

4. Development of the direct sandwich enzyme-linked immunosorbent assay (direct sandwich ELISA) for the detection of circulating MSP antigens in serum of naturally infected animals with anaplasmosis

4.1 Naturally infected cattle and buffaloes

The sera from cattle and buffaloes naturally infected with *A. marginale* and other parasites, i.e., *Anaplasma centrale*, *Babesia bovis, B. bigemina*, *Theileria* sp. and *Trypanosoma* sp., were collected from fields in many areas of Thailand, with the assistances of collaborators as mentioned in the previous section.

4.2 The lower detection limit (sensitivity) and the specificity of direct sandwich ELISA

The cut-off point was calculated as the mean optical density (OD) of negative controls plus three standard deviations (SD). The OD readings equal to or less than the cut-off point was considered negative while those readings greater than the cut-off point was considered positive. The lower detection limit (sensitivity) of sandwich ELISA was estimated by titration with the serial dilutions of rAmMSP antigens, from 0.04 pg/ml to 20 µg/ml. The end point of detection limit was considered to be the lowest amount of antigen still giving the positive OD values. To determine cross-reactivities studies, the specificity of ELISA assay was analyzed by using the antigens from other parasites, i.e., *Anaplasma centrale*, *Babesia bovis, B. bigemina*, *Theileria* sp. and *Trypanosoma* sp., which each of these antigens were prepared at various concentrations. In addition, each serum samples were tested in triplicate and expressed as an individual mean OD, and the sensitivity and specificity of were calculated and analyzed with independent-samples t-test or one-way repeated measures ANOVA using SPSS for Windows program version 19.0 (SPSS Inc., Chicago, Illinois).

4.3 Detection of circulating MSP antigens by direct sandwich ELISA

The sandwich ELISA for detection of circulating antigen was followed the method described by Anuracpreeda et al. (2016a,b). For each step, 50 µl/well was added unless otherwise mentioned. Ninety six-well plate (Nunc A/S, Roskilde, Denmark was sensitized overnight at 4 $^{\circ}$C with rabbit anti mouse IgG (Dako A/S, Glostrup, Denmark) (10 µg/ml of 0.05 M carbonate buffer, pH 9.6). The coated plate was thoroughly washed once with 0.05% Tween 20 in normal saline solution (NSST) to remove excess antibody. Each time the washing fluid was left in the wells for approximately 1 min at room

temperature. The purified MoAb diluted in 1% BSA in PBS pH 7.2 (10 µg/ml) was added and incubated for 3 h at 37 °C. After washing 3 times with the same washing buffer, unbound sites wer blocked with 150 µl/well of 5% skim milk in PBS for 1 h at 37°C. Thereafter, the plate was washed 3 times, incubated with reference antigens or samples (triplicate wells) for overnight at 4 °C. After washing, biotinylated rabbit IgG antibody against rAmMSP antigens (2 µg/ml of 1% BSA-0.05% PBST) was added. Then the plate was incubated for 90 min at 37 °C and washed with the same washing buffer. Thereafter, streptavidin-conjugated peroxidase (Zymed Laboratory Inc.) at the dilution 1:6000 in 1% BSA-0.05% PBST was added to each well. Finally, TMB substrate solution (KPL, Gaithersburg, USA) was added and incubated in a dark room for 10 min at room temperature, and then the color reaction was monitored. The reaction was stopped by adding of 1 N HCl. The OD values were read in a microplate reader (Multiskan Ascent, Labsystems, Helsinki, Finland) at 450 nm. The testing protocol is summarized in Figure 2 as follows:

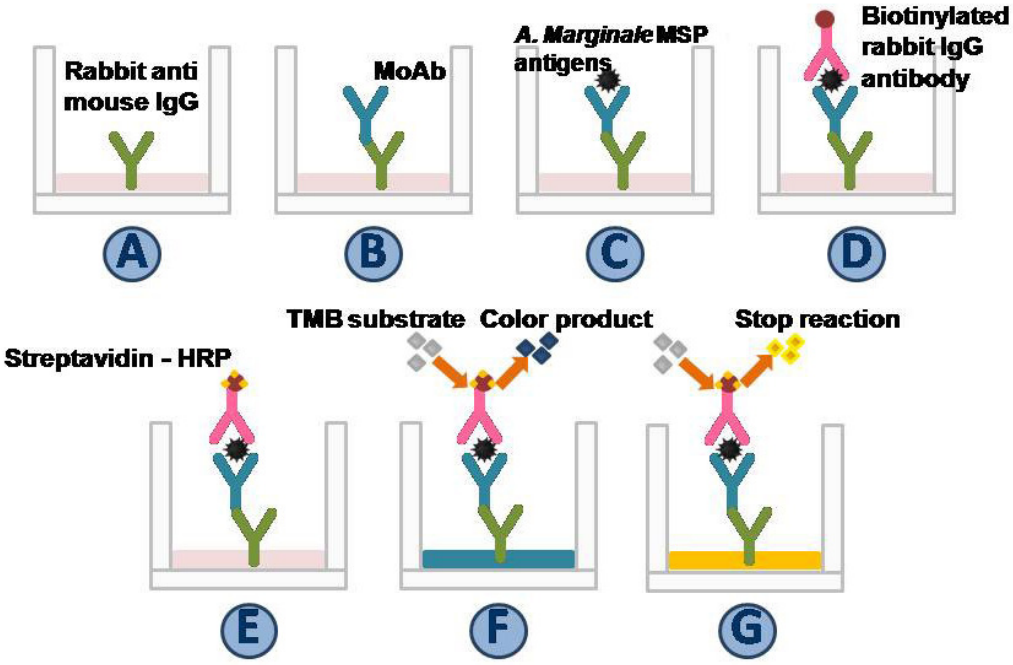


Fig. 2. Illustration of direct sandwich ELISA method.

4.4 Statistical Analysis

Each serum samples were tested in triplicate and expressed as an individual mean OD. All data from the detection of circulating antigens in serum of naturally infected animals with anaplasmosis were calculated and analyzed with independent-samples t-test or one-way repeated measures ANOVA using SPSS for Windows program version 19.0 (SPSS Inc., Chicago, Illinois). Each group was considered significant if (probabilities) *p*-value is statistically lower than 0.05 ($p < 0.05$). Standard diagnostic indices including sensitivity, specificity, accuracy and predictive values were calculated and expressed as follows: sensitivity = [no. of true positives / (no. of true positives + no. of false negatives)] x 100, specificity = [no. of true negatives / (no. of true negatives + no. of false positives)] x 100, positive predictive value = [no. of true positives / (no. of true positives + no. of false positives)] x 100, negative predictive value = [no. of true negatives / (no. of true negatives + no. of false negatives)] x 100, accuracy = [all with true positives and negatives / all test done] x 100. The primary data of the sandwich ELISA is as follows: true negative = number of control samples (other parasitosis and healthy controls) that showed negative result, true positive = number of proven *A. marginale* infection samples that showed positive result, false positive = number of control samples that showed positive result, false negative = number of proven *A. marginale* infection samples that showed negative result.

CHAPTER V

RESULTS

1. Analysis, isolation and characterization of the initial body (IB) or recombinant major surface protein (rMSP) antigens of *A. marginale*

1.1 PCR and sequencing

The DNA of *A. marginale* was isolated from infected blood and *msp* gene was amplified by PCR with specific primer. The PCR products were analyzed by 1.2% agarose gel electrophoresis and identified as the PCR products at 633 bp (Fig. 3). The purified PCR products were sent to AIT biotech Sequencing Lab for DNA sequencing and the result confirmed the sequence was similar to *A. marginale* with 99% identities.

The *msp* gene was cloned into pET100/D-TOPO® vector. The positive clones were confirmed by restriction analysis with *Hind* III

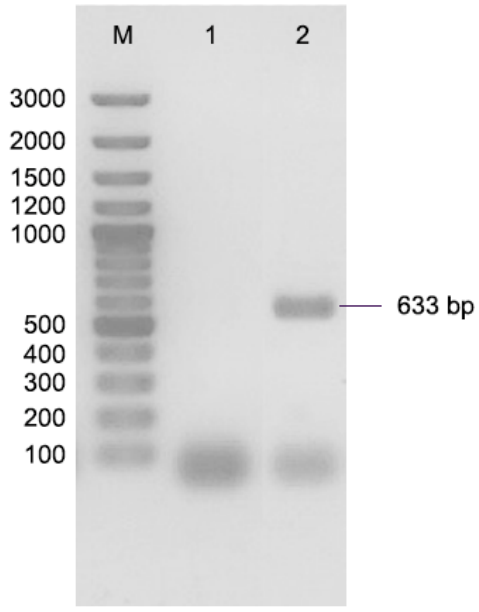


Fig. 3. PCR products of *msp* gene of *A. marginale* was showed at 633 bp: The molecular size standard is a 100 bp ladder (M), Negative control (1), Positive band of PCR products (2).

.

1.2 Expression and purification of recombinant MSP (rMSP)

The rMSP protein was analyzed by SDS-PAGE and immunoblotting. After induction with 0.1 mM IPTG at 16, 25 and 37 °C for 6 h, the results showed rMSP protein at 26 kDa on SDS-PAGE stained with Coomassie blue R-250 (Figs 4-6). However, the rMSP protein was exhibited the most evident after induction for 4 h. Moreover, rMSP protein was confirmed by immunoblotting and the results showed rMSP was reacted with commercial anti-6X His tag® antibody (Fig 7).

The soluble fraction of rMSP was purified by HisTrap HP (GE Healthcare Bio-sciences) and eluted from the column with 250 mM imidazole. The purified rMSP was analyzed by SDS-PAGE and showed the protein band at 26 kDa (Figs 8 and 9).

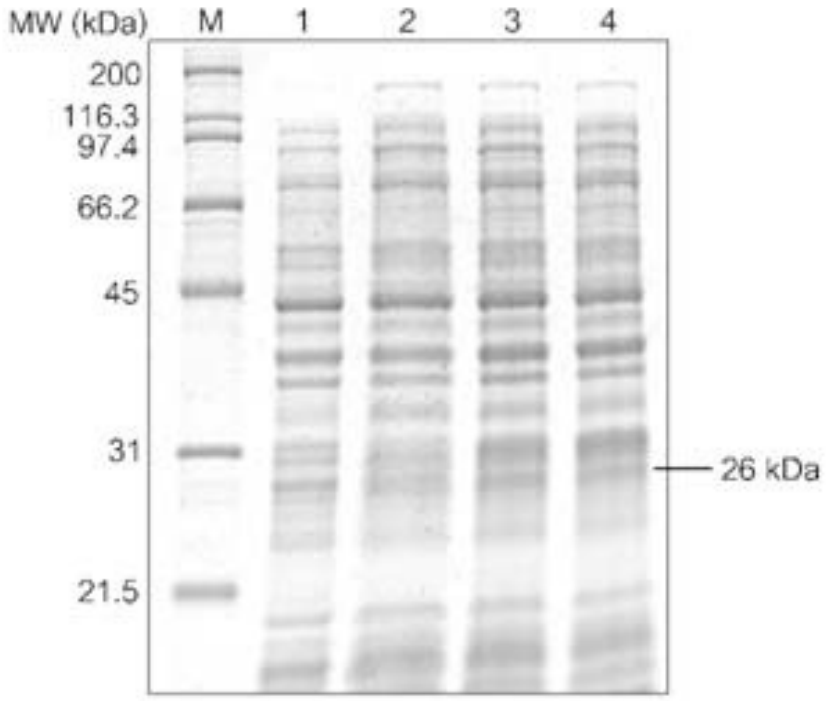


Fig. 4. SDS-PAGE stained with Coomassie Brilliant Blue showed protein expression of BL21/pET100-*msp*. The protein expression was induced by 0.1 mM IPTG at OD 0.5 - 0.8 and cultured at 16 °C. (M) Marker; (1) BL21/pET100-*msp* are not induced; (2) BL21/pET100-*msp* 2 h after induction with IPTG; (3) BL21/pET100-*msp* 4 h after induction with IPTG; (4) BL21/pET100-*msp* 6 h after induction with IPTG.

.

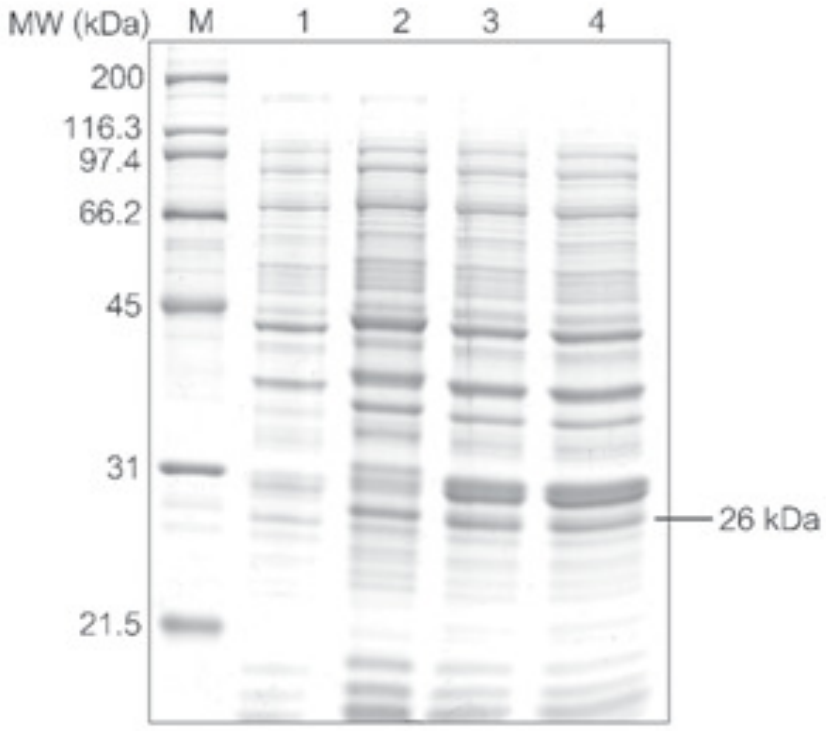


Fig. 5. SDS-PAGE stained with Coomassie Brilliant Blue showed protein expression of BL21/pET100-*msp*. The protein expression was induced by 0.1 mM IPTG at OD 0.5 - 0.8 and cultured at 25 $^{\circ}$C. (M) Marker; (1) BL21/pET100-*msp* are not induced; (2) BL21/pET100-*msp* 2 h after induction with IPTG; (3) BL21/pET100-*msp* 4 h after induction with IPTG; (4) BL21/pET100-*msp* 6 h after induction with IPTG.

.

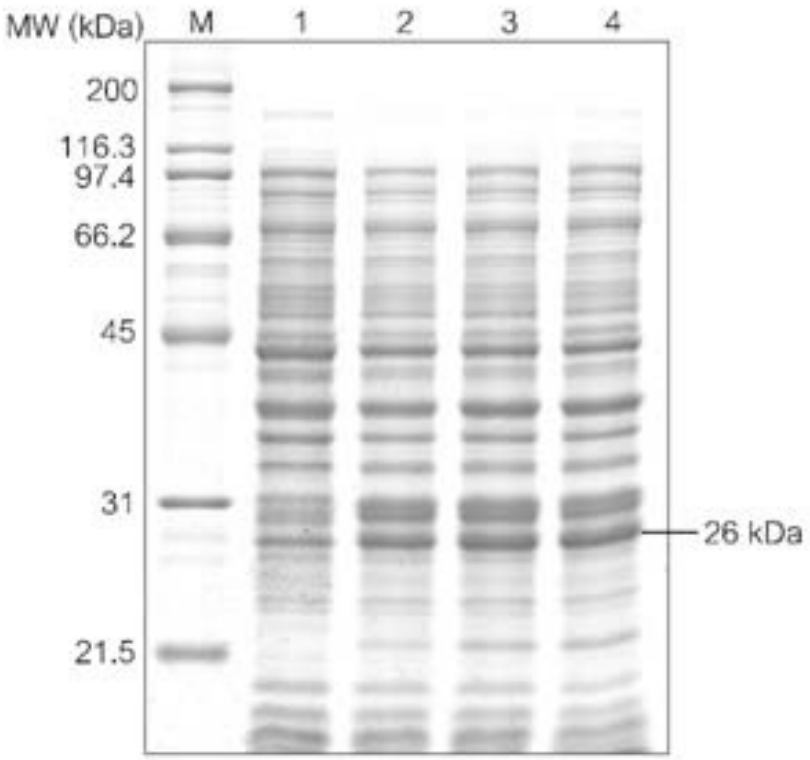


Fig. 6. SDS-PAGE stained with Coomassie Brilliant Blue showed protein expression of BL21/pET100-*msp*. The protein expression was induced by 0.1 mM IPTG at OD 0.5 - 0.8 and cultured at 37 $^{\circ}$C. (M) Marker; (1) BL21/pET100-*msp* not induced; (2) BL21/pET100-*msp* 2 h after induction with IPTG; (3) BL21/pET100-*msp* 4 h after induction with IPTG; (4) BL21/pET100-*msp* 6 h after induction with IPTG.

.

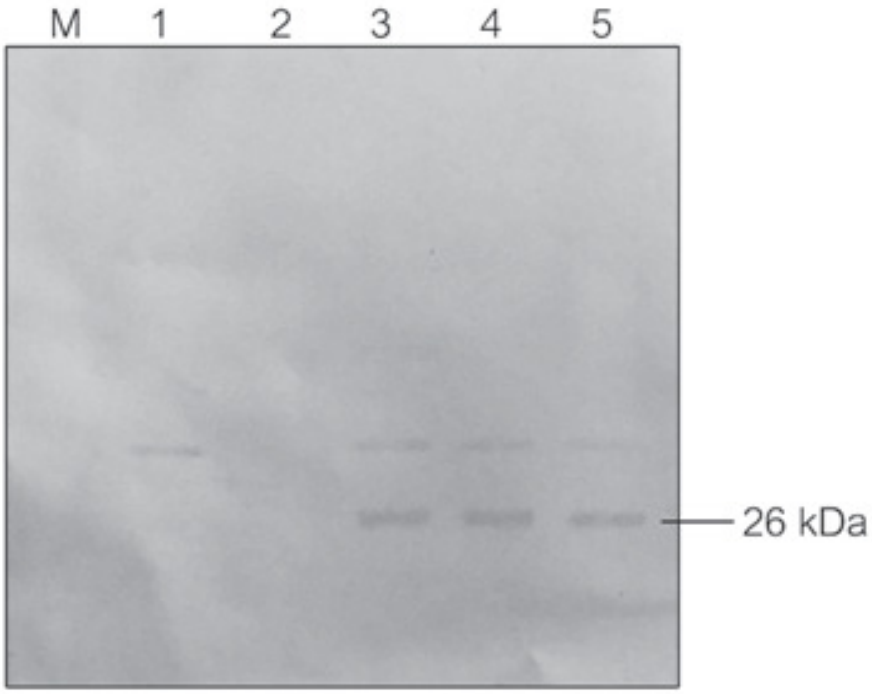


Fig. 7. Immunoblotting analysis of rMSP protein using anti-His antibodies. (M) Marker; (1) rMSP induced with IPTG (control); (2) rMSP not IPTG induced (control); (3) rMSP at 37 °C. for 2 h after induction; (4) rMSP at 37 °C. for 4 h after induction; (5) rMSP at 37 °C. for 6 h after induction.

.

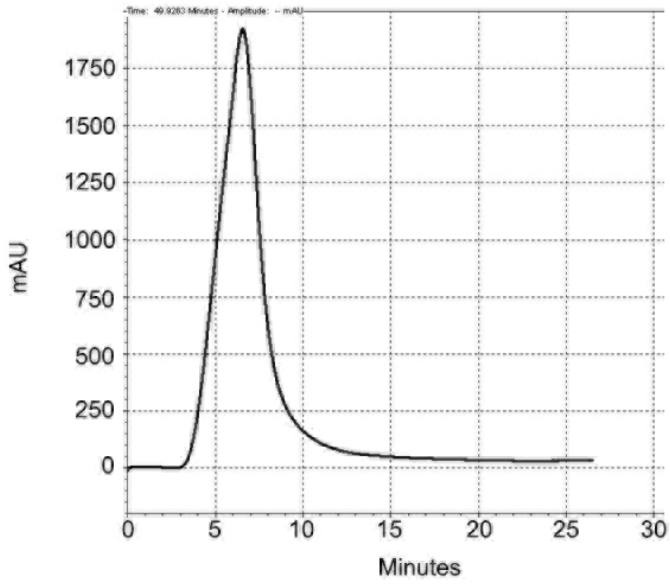


Fig. 8. The elution profile of rMSP protein was eluted with 250 mM imidazole in 20 mM HEPES and 50 mM NaCl, pH 7.4.

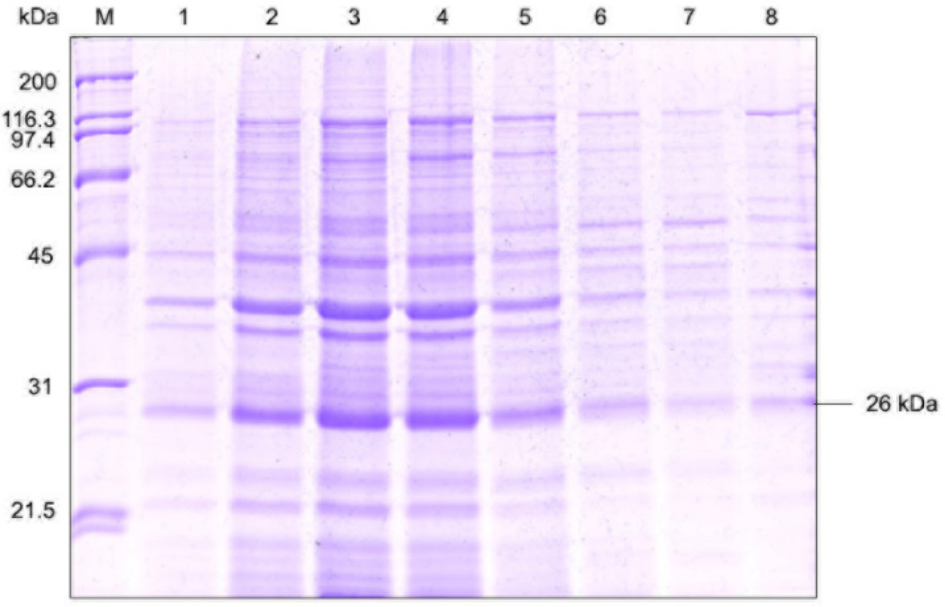


Fig. 9. The elution fractions of purified rMSP protein from HPLC were analyzed on 12% SDS-PAGE: Protein marker (M), The collected fraction of purified rMSP protein (lane 1-8).

2. Production and characterization of MoAb and PoAb specifically against recombinant *A. marginale* major surface protein (rAmMSP)

Antibody titers of mouse serum against rMSP antigen of *A. marginale* were tested by indirect ELISA method. A mouse showing OD value higher than mean value of the pre-immune serum plus 3 standard deviations (SD) is considered to be the high responder. The high responder was selected for cell fusion for production of MoAb. In addition, antibody titers of rabbit serum against rMSP antigen of *A. marginale* were tested by indirect ELISA technique. A rabbit showing OD value higher than mean value of the pre-immune serum plus 3 standard deviations (SD) is considered to be the high responder. The high responder was used for purification.

The hybridoma clones expressing MoAb against rMSP of *A. marginale* were produced by fusion of immunized spleen cells and mouse myeloma cells. The hybridoma cells that grew successfully in culture were cloned by limiting dilution methods. Only the hybridoma clones that produced high titers of antibodies against rMSP, as screened by indirect ELISA, were selected. The isotyping of monoclonal antibody was determined by indirect ELISA technique using the SBA Clonotyping^{TM} System/HRP (SouthernBiotech, USA) (Fig. 10, Table 1).

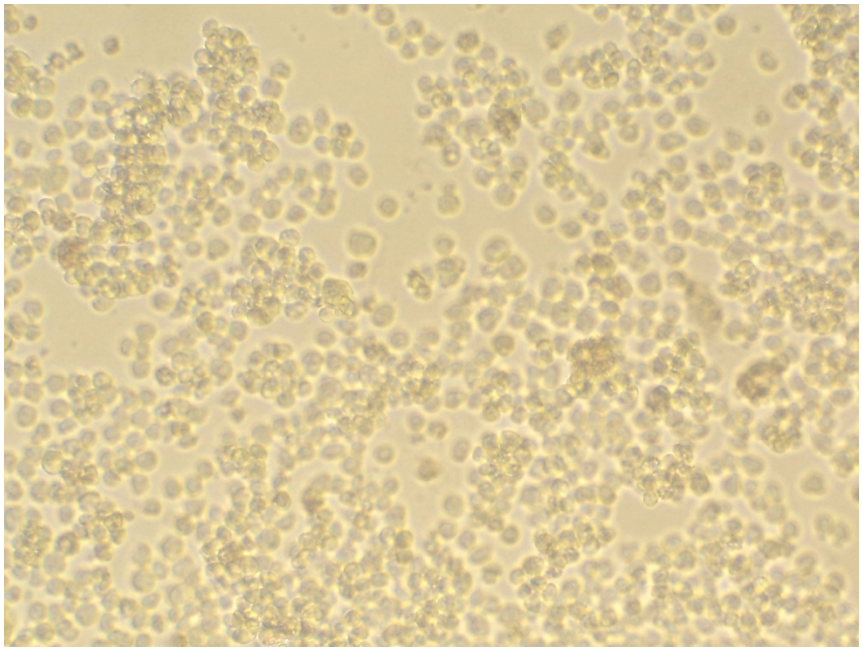
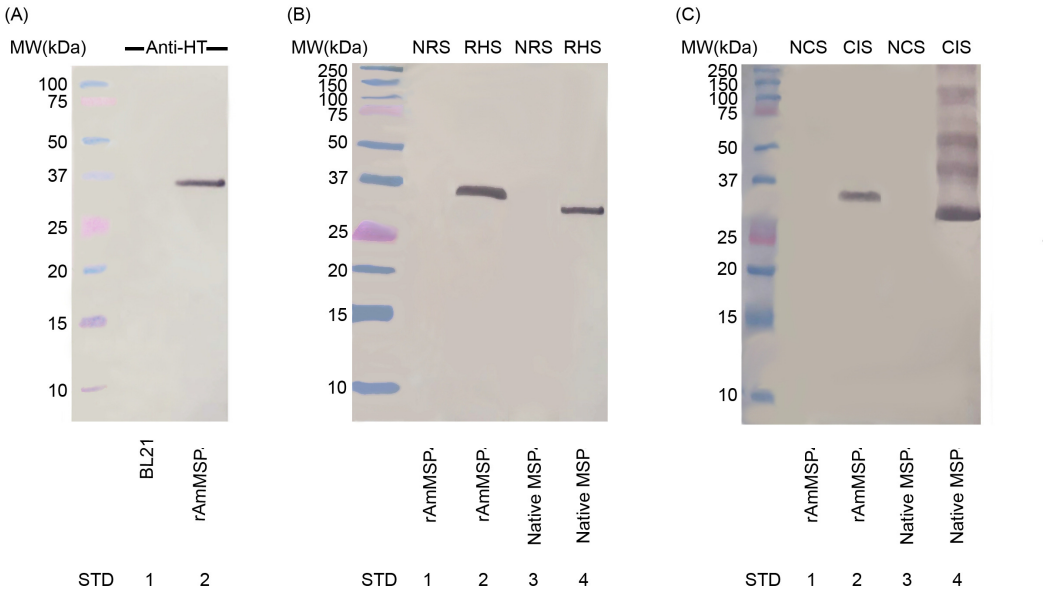


Fig. 10. The hybridoma cells that grew successfully in culture medium.

Table 1 Isotyping of MoAb specifically against rAmMSP

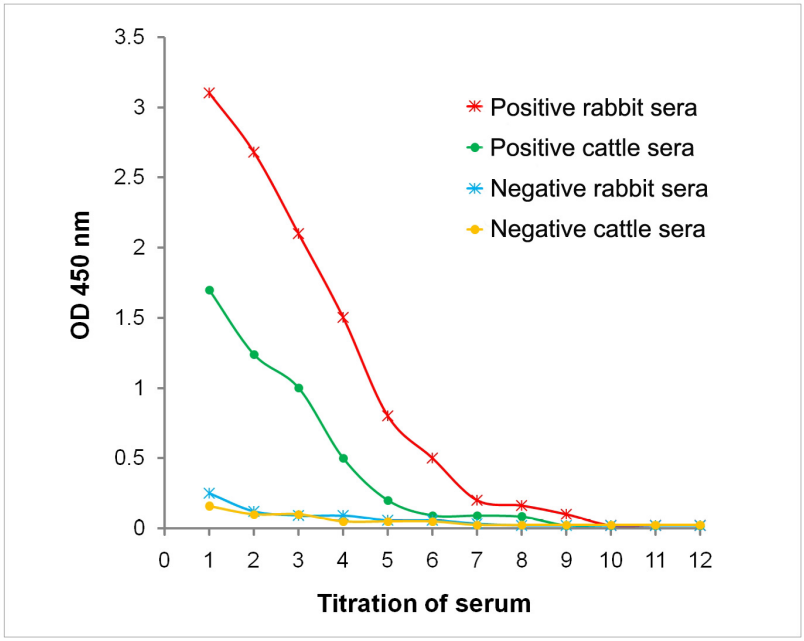
		Antibody isotyping	
Number	Clone	Heavy chain (OD 405)	Light chain (OD 405)
1	PA1	IgG_1	κ
2	PA2	IgG_1	κ
3	PA3	IgG_1	κ
4	PA4	IgG_1	κ
5	PA5	IgM	κ
6	PA6	IgG_1	κ
7	PA7	IgG_1	κ
8	PA8	IgG_1	κ
9	PA9	IgG_1	κ
10	PA10	IgM	κ

Immunobotting assay showed that all MoAbs reacted with a single band of rMSP which has a MW of 26 kDa. However, when tested against crude antigens from *A. marginale*, these MoAbs reacted with native MSP which appeared as a single band at MW 19 kDa. Taken together, similar antigenic fractions were analysed with PoAbs against native MSP, the identical single band was observed at MW 19 kDa which confirmed that proteins detected by MoAb were MSP. In the cross-reactivity study, no positive band was detected in crude antigens from other parasites, while the band was very prominent in crude antigen of *A. marginale* (Fig. 11).

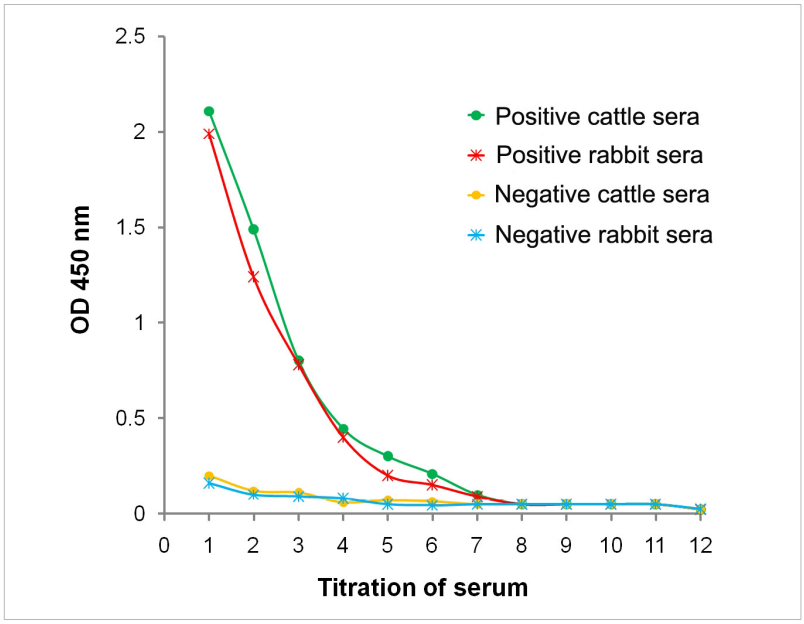


. **Fig. 11.** Immunobotting assay showed that all MoAbs and PoAbs reacted with a single band of rMSP.

Moreover, Indirect ELISA exhibited optical densities (OD) representing the levels of antigenicity of rAmMSP (A) and native MSP of *A. marginale* (B) as detected by infected cattle and hyperimmunized rabbit sera at various dilutions of 1 : 5 (number 1), 1 : 25 (number 2), 1 : 534 125 (number 3), 1 : 625 (number 4), 1 : 3,125 (number 5), 1 : 15,625 (number 6), 1 : 78,125 535 (number 7), 1 : 390,625 (number 8), 1 : 1,953,125 (number 9), 1 : 9,765,625 (number 10), 1 : 536 48,828,125 (number 11) and 1 : 244,140,625 (number 12) of pooled positive sera collected from cattle proven with *A. marginale* infection and rabbit hyperimmune sera (Fig. 12).



A



B

Fig. 12. Indirect ELISA exhibited optical densities (OD) representing the levels of antigenicity of rAmMSP (A) and native MSP of *A. marginale* (B).

The localization of *Anaplasma* parasites was detected using MoAb or PoAb in immunofluorescence technique, the parasites were found to be immunoreactive at the periphery or on the outside of the infected erythrocytes in a stained blood film (Fig. 13B and C). The negative control, using rabbit pre-immune sera, did not exhibit any positive fluorescent signal (Fig. 13A).

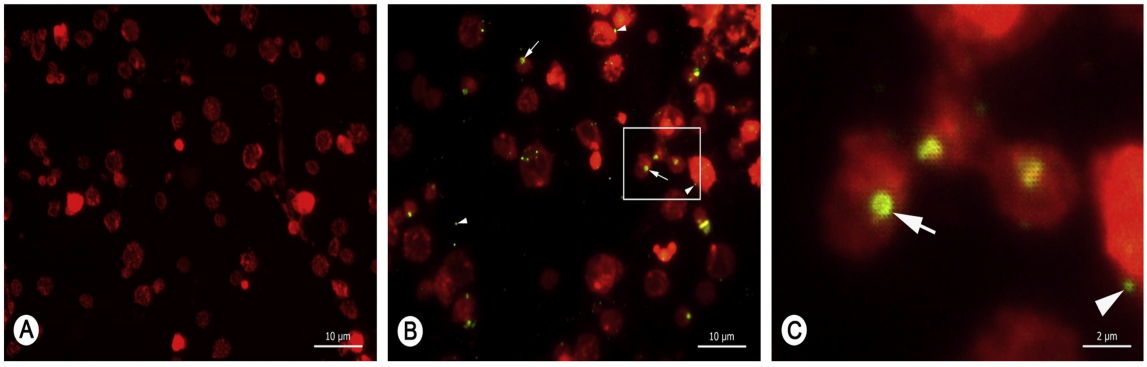


Fig. 13. Immunofluorescence staining of *Anaplasma* parasites in a stained blood film using MoAb or PoAb as probes. **(A)** The negative control did not show any positive fluorescent signal. **(B and C)** Medium and high magnification micrographs showed intense fluorescence in the parasites which occupied at the periphery (arrows) and on the outside (arrowheads) of the infected erythrocytes.

3. Purification of MoAb and PoAb specifically against recombinant *A. marginale* major surface protein (rAmMSP)

MoAb in culture supernatant and PoAb in serum specifically against rMSP have been already purified by precipitation in saturated ammonium sulfate solution. After that, MoAb and PoAb were purified by a gel-filtration and ion exchange chromatography, respectively. Appropriate immunoglobulin fractions were collected and tested for their activies. Various preparations of MoAb and PoAb obtained from the fraction were subjected to SDS-PAGE in 12.5% separating gel and 4% stacking gel (Figs 14-22).

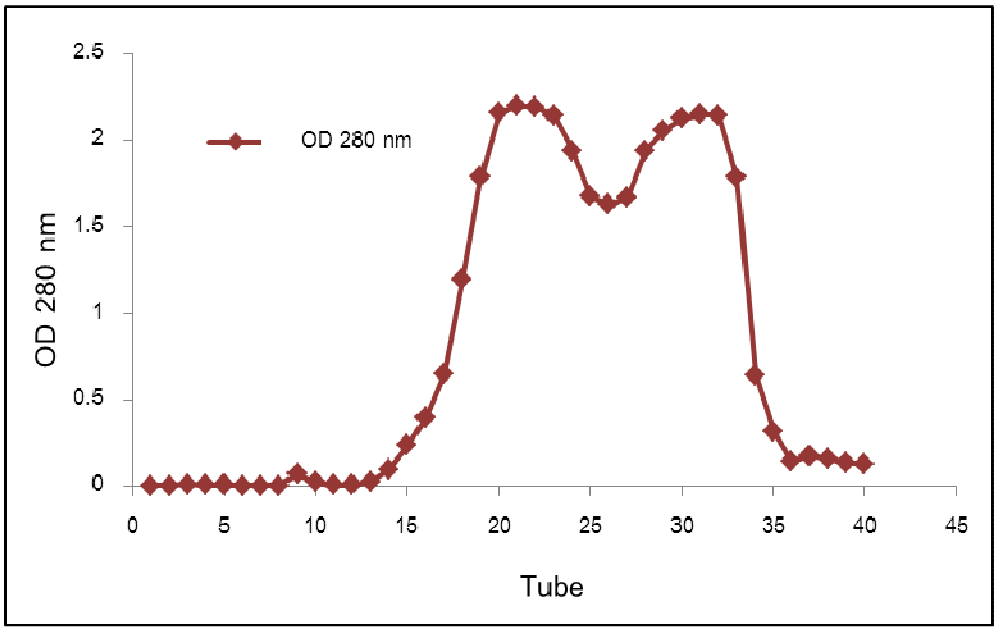


Fig. 14. The chromatographic antibody profiles of mouse MoAb (IgM) against rMSP on a Sephadex G-200 column.

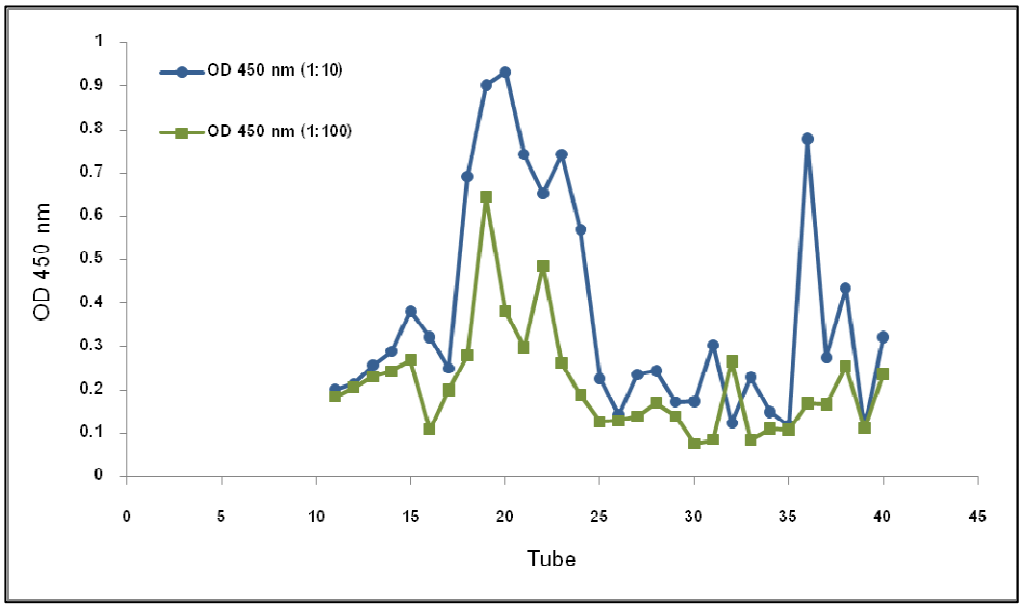


Fig. 15. Activity of MoAb (IgM) specific against against rMSP.

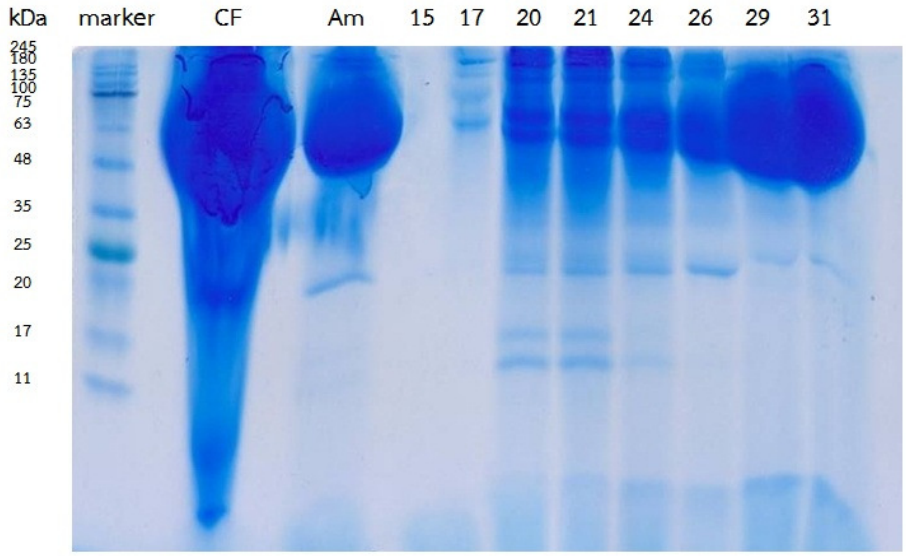


Fig. 16. SDS-PAGE of various preparations of mouse MoAb (IgM) against rMSP. Marker, Pre-stained marker; CF, Sample of hybridoma IgM; Am, Ammonium sulfate precipitated IgM; 15-31, Fraction of Sephadex G-200 purified IgM.

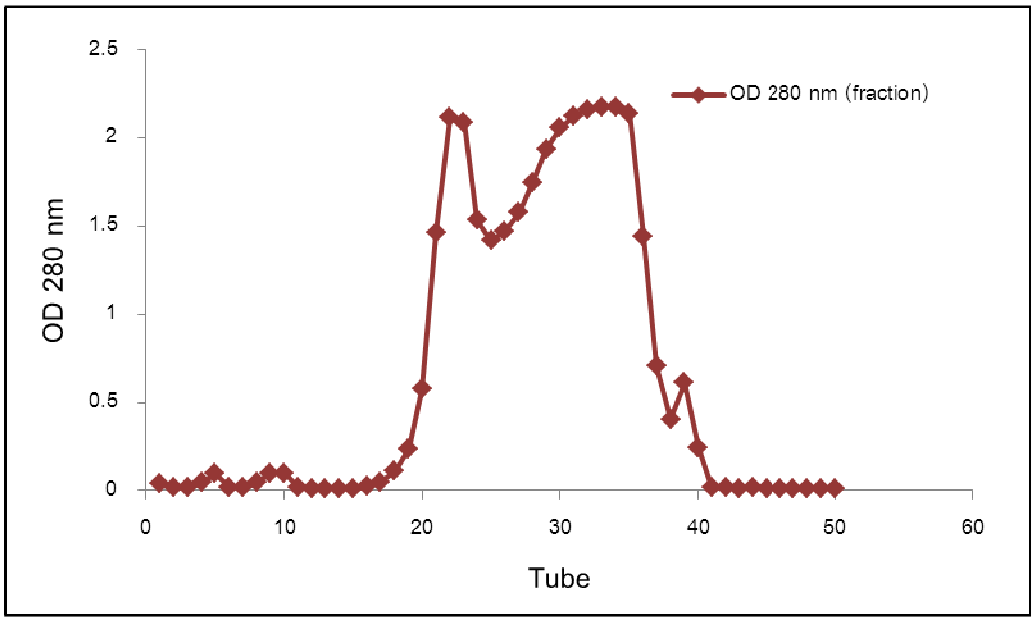


Fig. 17. The chromatographic antibody profiles of mouse MoAb (IgG_1) against rMSP on a Sephadex G-200 column.

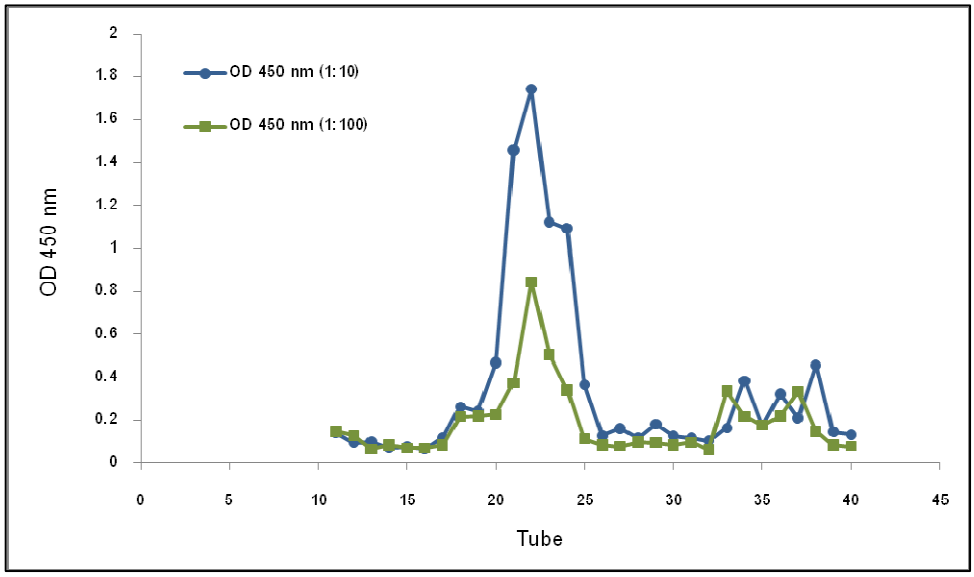


Fig. 18. Activity of MoAb (IgG_1) specific against against rMSP.

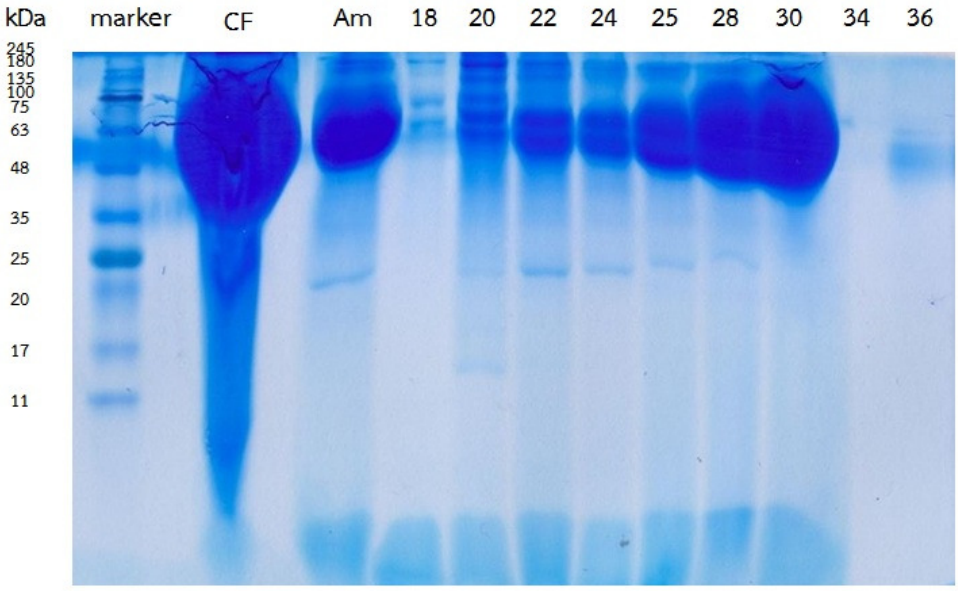


Fig. 19. SDS-PAGE of various preparations of mouse MoAb (IgG_1) against rMSP. Marker, Pre-stained marker; CF, Sample of hybridoma IgG_1; Am, Ammonium sulfate precipitated IgM; 18-36, Fraction of Sephadex G-200 purified IgG_1.

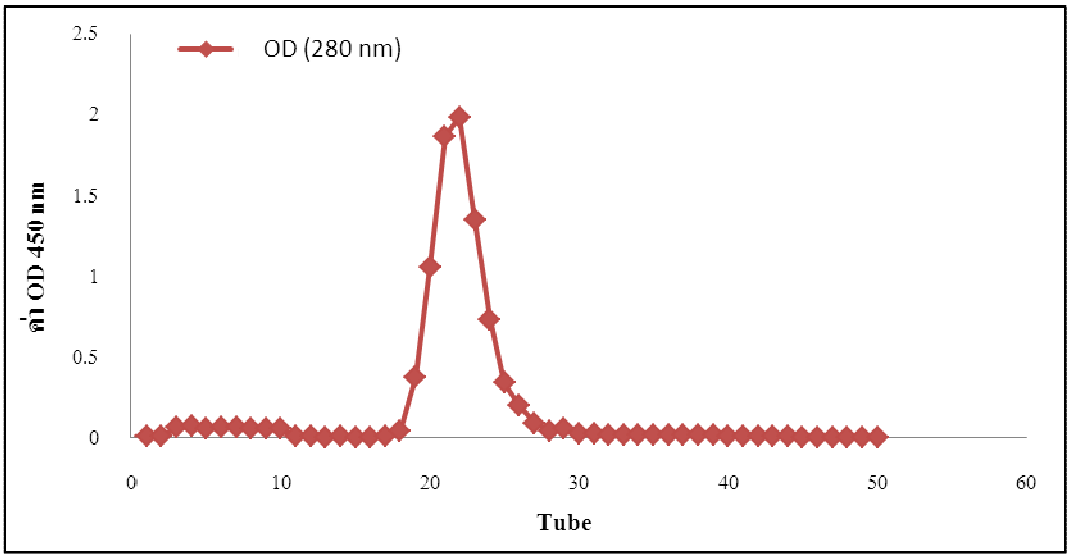


Fig. 20. The chromatographic antibody profiles of PoAb against rMSP on a DEAE column.

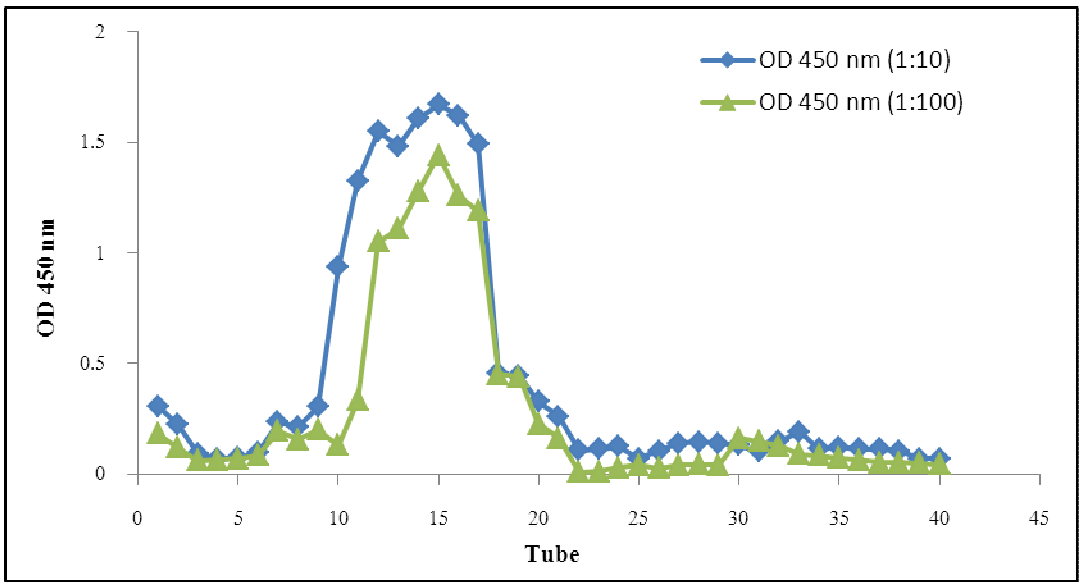


Fig. 21. Activity of PoAb specific against rMSP.

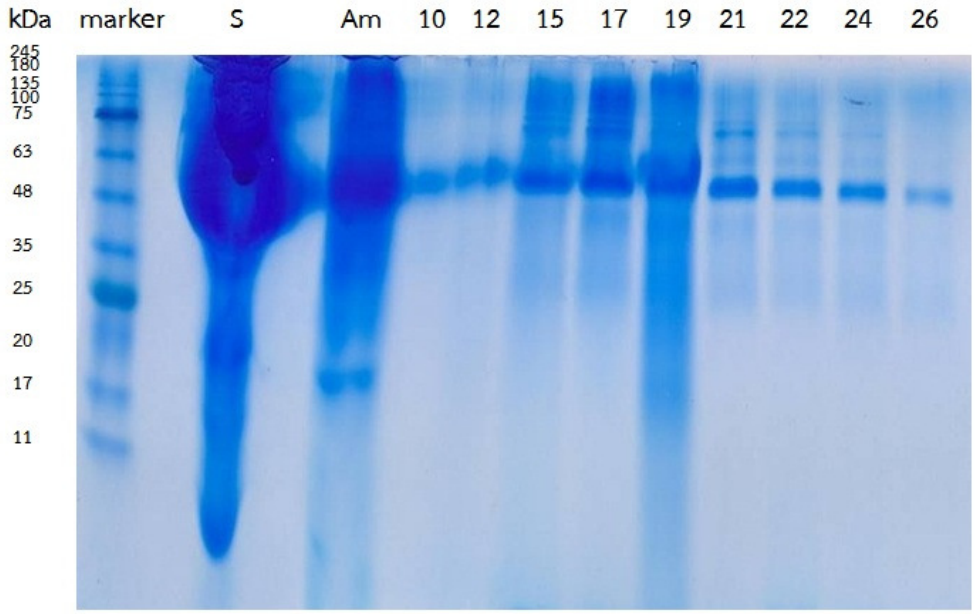


Fig. 22. SDS-PAGE of various preparations of PoAb against rMSP. Marker, Pre-stained marker; S, Sample of PoAb in serum; Am, Ammonium sulfate precipitated immunoglobulin; 10-26, Fraction of DEAE purified immunoglobulin.

4. Development of the direct sandwich enzyme-linked immunosorbent assay (direct sandwich ELISA) for the detection of circulating MSP antigens in serum of naturally animals infected with anaplasmosis

4.1 Sensitivity and specificity of the sandwich ELISA

The specificity of sandwich ELISA and the lower detectionlimitThe ELISA cut-off value was calculated as the mean opticaldensity (OD) of control non-infected sera plus 3 standard devia-tions (SD), which was at 0.109. The OD readings equal to or less than the cut-off value were considered negative while those readings greater than the cut-off value were considered positive. The lower detection limit (sensitivity) of this assay system was determined using different concentrations of rMSP and native MSP of *A. marginale.* Based on the lowest concentrations of antigen that still gave positive OD values, this assay could detect rMSP and native MSP of *A. marginale* at the lowest concentrations of 40 pg/ml (Fig. 23). Likewise, the sandwich ELISA described herein was highly specific for MSP, as no cross-reactivity was demonstrated when the assay was tested with various concentrations of other parasite antigens (Fig. 24)

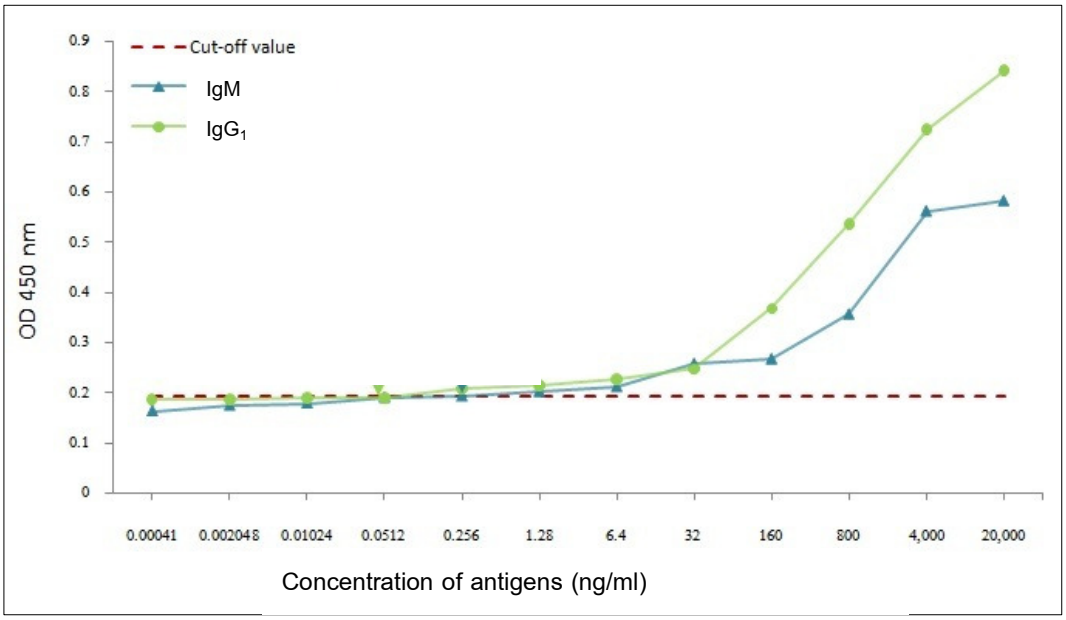


Fig. 23. Sensitivity of known quantities of *A. mrginale* antigens. Titration of rMSP and native MSP was analyzed by sandwich ELISA.

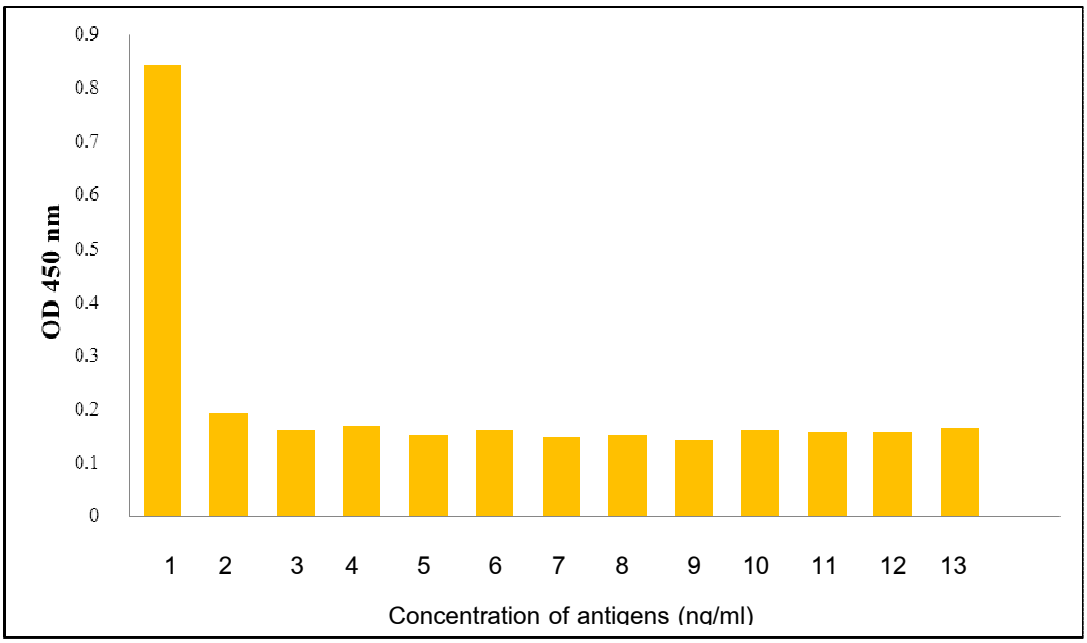


Fig. 24. Cross-reactivities study of a MoAb-based sandwich ELISA to crude preparations of various parasites. 1 = *A. marginale*, 2 = *A. centrale*, 3 = *B. bovis*, 4 = *B. bigemina*, 5 = *Theileria* sp., 6 = *Trypanosoma* sp., 7 = *Eperythrozoon* sp., 8 = *E. canis*, 9 = *H. canis*, 10 = *A. platys*, 11 = *H. felis*, 12 = *B. canis*, 13 = *B. gibsoni.*

4.2 Sandwich ELISA for the detection of circulating MSP antigen of *A. marginale*

A total of 135 serum samples together with corresponding blood smear samples from cattle were collected and examined. Sixty sera from cattle infected with anaplasmosis, 30 from cattle infected with babesiosis, 30 from cattle infected with theileriosis, 15 from cattle infected with trypanosomosis and 60 from non-infected cattle were tested. The results showed that 58 of 60 anaplasmosis sera were positive, while all 75 sera from those infected with other parasites, and all 60 sera from normal controls were negative. The ELISA cut-off value was at 0.107. Hence, the method exhibited a sensitivity and specificity of 96.7% and 100%, respectively, with a positive predictive value of 100%, a negative predictive value of 98.5%, a false positive rate of 0%, a false negative rate of 3.4%, and an accuracy of 98.9% (Table 2).

Table 2 Calculation of diagnostic values of the sandwich ELISA for MSP antigen detection in sera of cattle naturally infected with *A. marginale*.

Calculations	Natural infection (%)
1. Sensitivity	96.7
2. Specificity	100
3. Positive predictive value	100
4. Negative predictive value	98.5
5. False positive rate	0
6. False negative rate	3.4
7. Accuracy	98.9

CHAPTER VI
DISCUSSION

1. Analysis, isolation and characterization of the initial body (IB) or recombinant major surface protein (rMSP) antigens of *A. marginale*

In the present study, genomic DNA of *A. marginale* was extracted and *msp* coding sequence was sequenced. The completed sequences of *msp*coding sequence from *A. marginale* Thailand strain were reported. Close to our findings, the partial sequences of *msp* gene of *A. marginale* Thailand strain were also studied (Saetiew et al., 2015; Jirapattharasate et al., 2017). For phylogeny, we have only selected completed sequences to compare with Thailand isolates. The full length of *msp* coding sequence in Thailand strain was 633 bp, whereas the geographical variations were not observed. In addition, *msp* coding sequence of *A. marginale* was highly conserved because pairwise distance was between 0.000-0.022. Unlike previous study as obtained by Oberle et al. (1993), our *msp*coding sequence was cloned into expression vector and overexpressed in *E. coli* BL21-CodonPlus (DE3)-RIPL, and rAmMSP identity was confirmed by Mass Spectrometry. The advantage of this competent cell was having tRNA of rare codons which were mostly found in heterologous protein including rAmMSP.

Our rAmMSP was 26 kDa-protein, while AmMSP was 19 kDa-protein. The higher MW of rAmMSP was due to the addition of 38 extra amino acids which were 6xhis-tag protein, enterokinase (EK) recognition site, and EK cleavage site on pET100 vector for cloning and purification purposes (Gerlach et al., 2004). The rAmMSP was mostly found in the insoluble fraction rather than soluble fraction. Moreover, this method produced relatively high yield of rAmMSP which was used to immunize rabbits. MSP is one of the outer membrane proteins of *A. marginale* (Oberle et al., 1993; Brown et al., 1998; de la Fuente et al., 2005) and increases its hydrophobicity profile and also increases the changes of expression as inclusion bodies. In addition, the function of this protein is still unknown so far (Hope et al., 2004).

In the present study, the results of indirect ELISA revealed that the antibody titer against rAmMSP and AmMSP in the initial bodies extract was tested using the pooled positive sera of cattle infected with *A. marginale* and rabbit immunized with rAmMSP as well as negative sera of cattle uninfected with *A. marginale* and of rabbit unimmunized with rAmMSP. The rAmMSP detected the immunoglobulin G (IgG) in the sera of cattle infected with *A. marginale* and rabbit immunized with rAmMSP up to a dilution of

1:390,625 and 1:1,953,125, respectively. Moreover, the AmMSP in the initial bodies extract also detected the IgG in the sera of cattle infected with *A. marginale* and rabbit immunized with rAmMSP up to a dilution of 1:78,125. On the other hand, Oberle et al. (1993) reported that rAmMSP and AmMSP in the initial bodies extract exhibited the IgG titer at the dilution of 1:1000 in outer membrane fraction-immunized cattle sera. In this study, titer for rAmMSP is higher than the AmMSP which may be due to the impurity and the small amount of the native antigens in the initial bodies extract which lead to a decrease in the binding between antigen and antibody. In addition, these results imply that a high antibody titer is a clear reflection of the strong antigenicity of both rAmMSP and AmMSP of *A. marginale.* For immunoblotting assay, the result showed that the sera of infected cattle and hyperimmunized rabbit could react with rAmMSP at MW 26 kDa. Furthermore, they could also detect the protein band of 19 kDa AmMSP. This implied that the rAmMSP had a similar epitope with AmMSP as well as the rAmMSP which had conserved epitopes and sustained antigenicity (Brown et al., 1998; Kano et al., 2002; Oliveira et al., 2003). This result agrees with those reported by earlier study showing that MSP is a highly conserved protein encoded by a single gene (Oberle et al., 1993).

2. Production and characterization of MoAb and PoAb specifically against recombinant *A. marginale* major surface protein (rAmMSP)

In this study, we produced the specific MoAbs against rMSP of *A. marginale*. The isotype of these MoAbs are IgM and IgG_1, k light chain. These MoAbs could react with rMSP at MW of 26 kDa. However, in the initial bodies extract of *A. marginale*, the MoAb specific to rMSP could detect only a single band of 19 kDa. This was reflected by the fact that these MoAbs might cross-react with the common epitope present in initial bodies extract of *A. marginale*. In contrast to the MoAbs, PoAb could detect three bands of MSP at MW 38, 52 and 114 kDa in the initial bodies extract of *A. marginale,* which might reflect the existence of more than one isotype of these proteins in the *A. marginale* which carried different epitopes. Interestinlgy, these MoAbs did not cross reacted with antigens from other parasites due to their high quite specificity.

The localization of *Anaplasma* parasites by both MoAb and PoAb showed that these parasites are distributed not only on the membrane of infected erythrocytes, but also on the outside of erythrocytes. It is possible that these free *Anaplasma* is expected

as a result of sample preparation. From the previous study, it was believed that MSP had the signal peptide located on the erythrocytes' membrane (Oberle et al., 1993).

3. Purification of MoAb and PoAb specifically against recombinant *A. marginale* major surface protein (rAmMSP)

MoAb in culture supernatant and PoAb in serum specifically against rMSP were purified by precipitation in saturated ammonium sulfate solution. Thereafter, MoAb and PoAb were purified by a gel-filtration and ion exchange chromatography, respectively. Appropriate immunoglobulin fractions were collected and used for development of a monoclonal antibody (MoAb)-based sandwich enzyme-linked immunosorbent assay (sandwich ELISA). Hence, it is possible that this MoAb and PoAb could be used for immunodiagnosis for anaplasmosis especially in the detection of early infection.

4. Development of the direct sandwich enzyme-linked immunosorbent assay (direct sandwich ELISA) for the detection of circulating MSP antigens in serum of naturally infected animals with anaplasmosis

A provisional diagnosis of anaplasmosis is based on the history and clinical signs of the disease. Further confirmation can be obtained by the microscopic examination of stained blood smears. However this method often results in misdiagnosis due to the low parasitemia in animals, it is not recommended for the detection of subclinical animals or carriers (Carelli et al., 2007). Consequently, immunological diagnosis could play the most dependable means for monitoring the infection, and can be supplemented by the standard parasitological method. It was believed that immunodiagnosis would be a better method, but up to the presence no such method had been devised. Therefore, the immunodiagnosis could be performed in two ways: 1) detection of host's antibodies against parasite' antigens such as enzyme linked immunosorbent assay (ELISA) and immunofluorescent antibody test (IFAT), and 2) detection of parasite' antigens in the host's blood circulation such as sandwich ELISA. The method of circulating antigen detection (sandwich ELISA) has been used successfully in the diagnosis of many parasitic diseases such as *Fasciola hepatica* and *F. gigantica* infections (Moustafa et al., 1998; Anuracpreeda et al., 2009, 2013, 2016a.b). However, this sandwich ELISA has not been devised and as yet no immunodiagnosis for *Anaplasma* infection has been developed.

Up to now, immunodiagnostic antigens in anaplasmosis are the initial body (IB) antigens or major surface proteins (MSPs) of *A. marginale*. Some of these IB antigens or MSPs have been tested for use in serodiagnostic assays. The antigens are known to modulate the immune response of the host and thus the parasite antigens invoke the host protective immune responses which result in the death and expulsion of the parasites during the development of concomitant immunity (Palmer et al., 1985; de la Fuente et al., 2001a; Kocan et al., 2003, 2004). Shkap et al. (1990) who successfully used the ELISA and indirect fluorescent antibody (IFA) test for the detection of antibodies against *A. marginale* and *A. centrale* using initial body (IB) antigens. In addition, a competitive ELISA (cELISA) has been utilized for the detection of *Anaplasma* infection in ruminants such as cattle, ovine, and deer (Ndung'u et al., 1995; Knowles et al., 1996; de la Fuente et al., 2004b). However, this cELISA does not differentiate *Anaplasma* species in regions where co-infection with *A. phagocytophylum* and *A. marginale* or *A. centrale* occurs. The possibility of cross-infection cannot be ruled out when using this assay in these regions (de la Fuente et al., 2004a; Hofmann-Lehmann et al., 2004; Lin et al., 2004).

Nowadays, few studies have been done on the immunodiagnosis of anaplasmosis by the detection of circulating antigens. Because the detection of the parasite antigens that persist in the host's blood circulation is the more direct method for diagnosing as it indicates whether the living parasites are still in the hosts. Hence, the sandwich ELISA is one of the alternative diagnosis that provides a powerful tool for epidemiological investigations with the high accuracy of *Anaplasma* infection. In the present study, a MoAb-based sandwich ELISA was utilized for detection of circulating *A. narginale* MSP antigen in the sera from naturally infected cattle. The MoAbs against rMSP showed no cross-reactions with antigens of other parasites. Hence, I could use these MoAbs to detect the circulating MSP antigen in the stage of infection. The sensitivity and specificity of this sandwich ELISA were also considered very high as in sera from naturally infected cattle; they were 96.7% and 100%, respectively. Eventhough I do not know for how long animals in the field had been naturally infected, this finding indicated that this sandwich ELISA exhibited high efficiency in detecting circulating *A. narginale* MSP antigen insera of naturally infected cattle. The sera from cattle infected with *A. narginale* were collected from fields in many regions of Thailand which are endemic areas. The recurrence of infection with varying doses of the initial bodies of *A. narginale* in natural cattle could occur. Hence the paprasites release varying amount of MSP

antigen into the hosts' circulation and cattle were tested positive but with considerably different level of MSP.

In conclusion, *msp* coding sequence of *A. marginale* strain Thailand is highly conserved among *A. marginale* different strains. The 26 kDa recombinant MSP has conserved epitopes and maintains antigenicity. Hence, there is a possibility that this recombinant protein could be used for immunodiagnostic purposes. In addition, this study exhibited the efficiency of sandwich ELISA in detecting MSP antigen for the detection, which is better than the antibody detection which showed positive result very late. Hence, this MoAb-based sandwich ELISA for circulating MSP offers another promising and specific assay for anaplasmosis.

References

Alderink, F.J. and Dietrich, R. (1981). Anaplasmosis in Texas: epidemiologic and economic data from a questionnaire survey. In *Proceedings of the Seuenth National Anaplasmosis Conference.* Edited by R. J. Hidalgo and E. W. Jones. Mississippi State University

Anuracpreeda, P., Wanichanon, C., Chawengkirtikul, R., Chaithirayanon, K. and Sobhon, P. (2009). *Fasciola gigantica*: Immunodiagnosis of fasciolosis by detection of circulating 28.5 kDa tegumental antigen. *Experimental Parasitology* 123, 334-340.

Anuracpreeda, P., Chawengkirtikul, R., Tinikul, Y., Poljaroen, J., Chotwiwatthanakun, C. and Sobhon, P. (2013a). Diagnosis of *Fasciola gigantica* infection using a monoclonal antibody-based sandwich ELISA for detection of circulating cathepsin B3 protease. *Acta Tropica* 127, 38-45.

Anuracpreeda, P., Poljaroen, J., Chotwiwatthanakun, C., Tinikul, Y. and Sobhon, P. (2013b). Antigenic components, isolation and partial characterization of excretion-secretion fraction of *Paramphistomum cervi*. *Experimental Parasitology* 133, 327-333.

Anuracpreeda, P., Chawengkirttikul, R. and Sobhon, P. (2016a). Immunodiagnosis of *Fasciola gigantica* Infection using Monoclonal Antibody-based Sandwich ELISA and Immunochromatographic Assay for Detection of Circulating Cathepsin L1 Protease. *PLoS ONE* 11, 1–22. e0145650. doi:10.1371/journal.pone.0145650.

Anuracpreeda, P., Chawengkirttikul, R. and Sobhon, P. (2016b). Immunodiagnostic monoclonal antibody-based sandwich ELISA of fasciolosis by detection of *Fasciola gigantica* circulating fatty acid binding protein. *Parasitology* (In Press).

Bock, R.E., de Vos, A.J., Kingston, T.G. and Carter, P.D. (2003). Assessment of a low virulence Australian isolate of *Anaplasma marginale* for pathogenicity, immunogenicity and transmissability by Boophilus microplus. *Veterinary Parasitology* 118, 121–131.

Brown, D.C.G. (1997). Dynamic and impact of tick-borne diseases of cattle. *Tropical animal health and production* 29,1S–3S.

Brown, W.C., Shkap, V., Zhu, D., McGuire, T.C., Tuo, W., McElwain, T.F. and Palmer, G.H. (1998). CD4(+) T-lymphocyte and immunoglobulin G2 responses in calves immunized with *Anaplasma marginale* outer membranes and protected against homologous challenge. Infect. Immunol. 66, 5406–5413.

Carlyon, .JA. and Fikrig, E. (2003). Invasion and survival strategies of *Anaplasma phagocytophilum*. *Cellular Microbiology* 5, 743–754.

Carelli, G., Decaro, N., Lorusso, A., Elia, G., Lorusso, E., Mari, V., Ceci, L. and Buonavoglia, C. (2007). Detection and quantification of *Anaplasma marginale* DNA in blood samples of cattle by realtime PCR. *Veterinary Microbiology* 124, 107–104.

Chaichanapunpol, I. and Vitoorakool, P. (1996). Prevalence of parasitic infection in dairy cattle in Northern Thailand. *Proceedings of the 34th Kasetsart University Annual Conference* (Jan 30- Feb 1, 1996), 280-288.

Chethanond, U., Worasingh, R. and Srinuntapunt, S. (1995). Studies on parasitic infection in dairy cattle in the south. *Proceedings of the 33rd Kasetsart University Annual Conference* (Jan 30 – Feb 1, 1995), 398-407.

Daniels, T.J., Battaly, G.R., Liveris, D., Falco, R.C. and Schwartz, I. (2002). Avian reservoirs of the agent of human granulocytic ehrlichiosis? *Emerging Infectious Diseases* 8, 1524–1525.

de la Fuente, J., Garcia-Garcia, J.C., Blouin, E.F., Rodrı´guez, S.D., Garcı´a, M.A. and Kocan, K.M. (2001a). Evolution and function of tandem repeats in the major surface protein 1a of the ehrlichial pathogen *Anaplasma marginale*. *Animal Health Research Reviews* 2, 163–173.

de la Fuente, J., Garcia-Garcia, J.C., Blouin, E.F. and Kocan, K.M. (2001b). Differential adhesion of major surface proteins 1a and 1b of the ehrlichial cattle pathogen *Anaplasma marginale* to bovine erythrocytes and tick cells. *International Journal of Parasitology* 31, 145–153

de la Fuente, J., Vicente, J., Ho¨fle, U., Ruiz-Fons, F., Ferna´ndez de Mera, I.G., Van Den Bussche, R.A., Kocan, K.M. and Gortazar, C. (2004b). *Anaplasma* infection in free-ranging Iberian red deer in the region of Castilla-La Mancha, Spain. *Veterinary Microbiology* 100, 163–173.

de la Fuente, J., Massung, R.F., Wong, S.J., Chu, F.K., Lutz, H., Meli, M., von Loewenich, F.D., Grzeszczuk, A., Torina, A., Caracappa, S., Mangold, A.J., Naranjo,V., Stuen, S. and Kocan, K.M. (2005). Sequence analysis of the *msp4* gene of *Anaplasma phagocytophilum* strains. J. Clin. Microbiol. 43, 1309-1317.

Dikmans, G. (1950). The transmission of anaplasmosis. *American journal of veterinary research* 11, 5–16.

Dumler, J.S., Barbet, A.F., Bekker, C.P.J., Dasch, G.A., Palmer, G.H., Ray, S.C., Rikihisa, Y. and Rurangirwa, F.R. (2001). Reorganization of the genera in the families Rickettsiaceae and Anaplasmataceae in the order Rickettsiales: unification of some species of *Ehrlichia* with *Anaplasma*, *Cowdria* with *Ehrlichia* and *Ehrlichia* with Neorickettsia, descriptions of six new species combinations and designation of *Ehrlichia equi* and “HE agent” as subjective synonyms of *Ehrlichia phagocytophila*. *International Journal of Systematic Evolutionary Microbiology* 51, 2145–2165.

Ewing, S.A. (1981). Transmission of *Anaplasma marginale* by arthropods. p. 395–423. *In* R. J. Hidalgo and E. W. Jones (ed.), Proceedings of the 7th National Anaplasmosis Conference. Mississippi State University, Mississippi State.

Foil, L.D. (1989). Tabanids as vectors of disease agents. *Parasitology Today* 5, 88–96.

Fungfuang, W., Ruamthum, W., Lertchunhakiat, K., Khoomsab, K. and Kanjanarajit, S. (2006). *Anaplasma* infection in hog deers (*Cervus porcinus*) from Huay Sai wildlife captive breeding research center in Phetchaburi province. *Proceedings of 44th Kasetsart University Annual Conference* (Jan 30 – Feb 3, 2006), 557-560.

Garcia-Garcia, J.C., de la Fuente, J., Kocan, K.M., Blouin, E.F., Halbur, T., Onet, V.C. and Saliki, J.T. (2004b). Mapping of B-cell epitopes in the N-terminal repeated peptides of *Anaplasma marginale* major surface protein 1a and characterization of the humoral immune response of cattle immunized with recombinant and whole organism antigens. *Veterinary Immunology and Immunopathology* 98, 137–151.

Ge, N.L., Kocan, K.M., Blouin, E.F. and Murphy, G.L. (1996). Developmental studies of *Anaplasma marginale* (Rickettsiales: Anaplasmataceae) in male *Dermacentor andersoni* (Acari: Ixodidae) infected as adult using nonradioactive *in situ* hybridization. *Journal of medical entomology* 33, 911–920.

Gerlach, D., Schlott, B. and Schmidt, K.H. (2004). Cloning and Expression of a sialic acid-binding lectin from the snail *Cepaea hortensis*. Fems Immunol. Med. Microl. 40, 215-221.

Goethert HK and Telford III SR (2003). Enzootic transmission of *Babesia divergens* among cottontail rabbits on Nantucket Island, Massachusetts. *American Journal of Tropical Medicine and Hygiene* 69, 455–460.

Hofmann-Lehmann, R., Meli, M.L., Dreher, U.M., Go¨nczi, E., Deplazes, P., Braun, U., Engels, M., Schu¨pbach, J., Jo¨rger, K., Thoma, R., Griot, C., Sta¨rk, K., Willi, B., Schmidt, J., Kocan, K.M. and Lutz, H. (2004). Concurrent infections with vector-borne pathogens as etiology of fatal hemolytic anemia in a cattle herd from Switzerland. *Journal of Clinical Microbiology* 42, 3775–3780.

Hope, M., Riding, G., Menzies, M. and Willadsen P. (2004). A novel antigen from Anaplasma marginale: Characterization, expression and preliminary evaluation of the recombinant protein. Vaccine 22, 407-415.

http://www.ncbi.nlm.nih.gov/blast/blast.cgi

Jirapattharasate, C., Moumouni, P.F.A., Cao, S., Iguchi, A., Liu, M., Wang, G., Zhou, M., Vudriko, P., Efstratiou, A., Changbunjong, T., Sungpradit, S., Ratanakorn, P., Moonarmart, W., Sedwisai, P., Weluwanarak, T., Wongsawong, W., Suzuki, H. and Xuan, X. (2017). Molecular detection and genetic diversity of bovine *Babesia* spp., *Theileria orientalis*, and *Anaplasma marginale* in beef cattel in Thailand. Parasitol Res 116, 751-762.

Jittapalapong, S. and C. Lieowijak. (1988). Epidemiological Survey of Blood Protozoa and Rickettsia in Dairy Cow in Nongpho. *Proceedings of 26th Kasetsart University Annual Conference* (Feb 3-5) :117-122.

Kano, F.S., Vidotto, O., Pacheco, R.C. and Vidotto, M.C. (2002). Antigenic characterization of *Anaplasma marginale* isolates from different regions of Brazil. Vet. Microbiol. 87, 131-138.

Kieser, S.T., Eriks, I.E. and Palmer, G.H. (1990). Cyclic rickettsemia during persistent Anaplasma marginale infection in cattle. *Infection and Immunity* 58, 1117–1119.

Knowles, D., Torioni de Echaide S., Palmer, G., McGuire, T., Stiller, D. and McElwain, T. (1996). Antibody against an *Anaplasma marginale* MSP5 epitope common to tick and erythrocyte stages identifies persistently infected cattle. *Journal of Clinical Microbiology* 34, 2225–2230.

Kocan, K. M., Barron, S. J., Ewing, S. A. and Hair, J. A. (1985). Transmission of *Anaplasma marginale* by adult *Dermacentor andersoni* during feeding calves. *American journal of veterinary research* 46, 1565–1567.

Kocan, K. M. (1986). Development of *Anaplasma marginale* in ixodid ticks: coordinated development of a rickettsial organism and its tick host, p.472–505. *In* J. R. Sauer and J. A. Hair (ed.), Morphology, physiology and behavioral ecology of ticks. Ellis Horwood Ltd., Chichester, United Kingdom.

Kocan, K. M., Stiller, D., Goff, W. L., Claypool, P. L., Edwards, W., Ewing, S. A., McGuire, T. C., Hair, J. A., and Barron, S. J. (1992a). Development of*Anaplasma marginale* in male *Dermacentor andersoni* transferred from infected to susceptible cattle. *American journal of veterinary research* 53, 499–507.

Kocan, K. M., Goff, W. L., Stiller, D., Claypool, P. L., Edwards, W., Ewing, S. A., Hair, J. A., and Barron, S. J. (1992b). Persistence of *Anaplasma marginale* (Rickettsiales: Anaplasmataceae) in male *Dermacentor andersoni* (Acari: Ixodidae) transferred successively from infected to susceptible cattle. *Journal of medical entomology* 29, 657–668

Kocan, K. M., Blouin, E. F., and Barbet, A. F. (2000). Anaplasmosis control: past, present and future. *Annals of the New York Academy of Sciences* 916, 501–509.

Kocan, K. M., and de la Fuente, J. (2003). Co-feeding of tick infected with *Anaplasma marginale*. *Veterinary Parasitology* 112, 295–305.

Kocan, K.M., de la Fuente, J., Guglielmone, A.A. and Mele´ndez, R.D. (2003). Antigens and alternatives for control of *Anaplasma marginale* infection in cattle. *Clinical Microbiology Reviews* 16, 698–712.

Kocan, K., de la Fuente, J., Blouin, E.F. and Garcia-Garcia, J.C. (2004). *Anaplasma marginale* (Rickettsiales: Anaplasmataceae): recent advances in defining host–pathogen adaptations of a tick-borne rickettsia. *Parasitolology* 129, S285–S300.

Kuttler, K.L. (1984). *Anaplasma* infections in wild and domestic ruminants: a review. *Journal of wildlife diseases* 20, 12–20.

Laemmli, U.K. (1970). Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* 227, 680-685.

Lappin, M.R., Breitschwerdt, E.B., Jensen, W.A., Dunnigan, B., Rha, J.Y., Williams, C.R., Brewer, M. and Fall, M. (2004). Molecular and serologic evidence of Anaplasma phagocytophilum infection in cats in North America. *Journal of the American Veterinary Medical Association* 225, 893–896.

Levin, M.L., Nicholson, W.L., Massung, R.F., Sumner, J.W. and Fish, D. (2002). Comparison of the reservoir competence of medium-sized mammals and Peromyscus leucopus for Anaplasma phagocytophilum in Connecticut. *Vector Borne Zoonotic Diseases* 2, 125–136.

Lin, Q., Rikihisa, Y., Felek, S., Wang, X., Massung, R.F. and Woldehiwet, Z. (2004). *Anaplasma phagocytophilum* has a functional msp2 gene that is distinct from p44. *Infection and Immunity* 72, 3883–3889.

Lonibardo, R.A. (1976). Socioeconomic importance of the tick problem in the Americas. *PAHO Sci Publ* 316: 79.

Lowry, O.H., et al. (1951). Protein measurement with Folin phenol reagent. *The Journal of biological chemistry* 193, 265-275.

McCallon, B. R. 1973. Prevalence and economic aspects of anaplasmosis, p. 1–3. *In* E.W. Jones (ed.), *Proceedings of the 6th National Anaplasmosis Conference*. Heritage Press, Stillwater, Okla.

Magnarelli, L.A., Ijdo, J.W., Ramakrishnan, U., Henderson, D.W., Stafford, III K.C. and Fikrig, E. (2004). Use of recombinant antigens of Borrelia burgdorferi and Anaplasma phagocytophilum in enzyme-linked immunosorbent assays to detect antibodies in white-tailed deer. *Journal of Wildlife Diseases* 40, 249–258.

Massung, R.F. and Slater, K.G. (2003). Comparison of PCR assays for detection of the agent of human granulocytic ehrlichiosis, *Anaplasma phagocytophilum*. *Journal of Clinical Microbiology* 41, 717–722.

Moustafa, N.E., Hegab, M.H., and Hassan, M.M. (1998). Role of ELISA in early detection of *Fasciola* copro-antigens in experimentally infected animals. *Journal of the Egyptian Society of Parasitology* 28, 379-387.

Morzaria, S.P., Katende, J., Musoke, A., Nene, V., Skilton, R. and Bishop, R. (1999). Development of sero-diagnostic and molecular tools for the control of important tickborne pathogens of cattle in Africa. *Parasitologia* 41 (Supplement 1), 73–80.

National Research Council. (1982). Priorities in biotechnology research for international development-proceedings of a workshop. Directed by The Board on Science and Technology for International Development, Office of International Affairs. National Academy Press, Washington, DC, P. 1.

Ndung'u, L.W., Aguirre, C., Rurangirwa, F.R., McElwain, T.F., McGuire, T.C., Knowles, D.P. and Palmer, G.H. (1995). Detection of *Anaplasma ovis* infection in goats by

major surface protein 5 competitive inhibition enzyme-linked immunosorbent assay. *Journal of Clinical Microbiology* 33, 675–679.

Norton, J.H., Parker, R.J. and Forbes-Faulkner, J.C. (1983). Neonatal anaplasmosis in a calf. *Australian veterinary journal* 60, 348.

Oberle, S.M., Palmer, G.H. and Barbet, A.F. (1993). Expression and Immune recognition of the conserved MSP-4 outer membrane protein of Anaplasma marginale. Infection and Immunity 61, 5245-5251.

Oliveira, J.B., Madruga, C.R., Schenk, M.A. and Kessler, R.H. (2003). Antigenic characterization of four Brazilian isolates of *Anaplasma marginale* Rickettsiaceae: Ehlichiae. Mem. Inst. Oswaldo Cruz. 98, 395-400.

Palmer, G.H. and McGuire, T.C. (1984). Immune serum against *Anaplasma marginale* initial bodies neutralizes infectivity for cattle. *Journal of immunology* 133, 1010-1015.

Parola, P. (2004). Tick-borne rick ettsial diseases: emerging risks in Europe. *Comparative Immunology, Microbiology and Infectious Diseases* 27: 297–304.

Petrovec, M., Bidovec, A., Sumner, J.W., Nicholson, W.L., Childs, J.E. and Avsic, Z.T. (2002). Infection with *Anaplasma phagocytophila* in cervids from Slovenia: evidence of two genotypic lineages. *Wiener Klinische Wochenschrift* 114, 641–647.

Phrikanahok, N. Bunmatid, C. and Sarataphan, N. (2000). Status and prediction of infection rate of tick fever diseases among dairy cattle in some provinces of Thailand. *Kasetsart Vet* 10, 13-23.

Polin, H., Hufnagl, P., Haunschmid, R., Gruber, F. and Ladurner, G. (2004). Molecular evidence of *Anaplasma phagocytophilum* in Ixodes ricinus ticks and wild animals in Austria. *Journal of Clinical Microbiology* 42, 2285–2286.

Potgieter, F.T., Sutherland, B. and Biggs, H.C. (1981). Attempts to transmit *Anaplasma marginale* with *Hippobosca rufipes* and *Stomoxys calcitrans. The Onderstepoort journal of veterinary research* 48, 119–122.

Potgieter, F.T., and Van Rensburg, L. (1987). The persistence of colostral *Anaplasma* antibodies and incidence of *in utero* transmission of *Anaplasma* infections in calves, under laboratory conditions. *The Onderstepoort journal of veterinary research* 54, 557–560.

Saetiew, N., Simking, P., Inpankaew, T., Wongpanit, K., Kamyingkird, K., Wongnakphet, S., Stich, R.W. and Jittapalapong, S. (2015). Prevalence and genetic diversity of *Anaplasma marginale* infections in water buffaloes in Northeast Thailand. J. Trop. Med. Parasitol. 38, 9-16.

Shkap, V., Bin, H., Ungar-Waron, H. AND Pipano, E. (1990). An enzyme-linked immunosorbent assay (ELISA) for the detection of antibodies to *Anaplasma centrale* and *Anaplasma marginale*. *Veterinary Microbiology* 25, 45-53.

Sithornnakoon, T., Chaianan, P. and Dissamam, R. (1965). Study on the anaplasmosis vaccine utilization to jursey cow and controlling the occurred reaction. *Proceeding of the 4th national conference on Agricultrue and Biology Plant and Biological Science, and Animal Science section* (Jan 27-29, 1965), 479-483.

Stich, R. W., Kocan, K.M., Palmer, G.H., Ewing, S.A., Hair, J.A. and Barron, S.J. (1989). Transstadial and attempted transovarial transmission of *Anaplasma marginale* by *Dermacentor variabilis American journal of veterinary research* 50, 1377–1380.

Swift, B.L. and Thomas, *G.*M. (1983). Bovine anaplasmosis: elimi-Press. Mississippi. P. 27.

Telford III, S.R., Dawson, J.E., Katavolos, P., Warner, C.K., Kolbert, C.P. and Persing, D.H. (1996). Perpetuation of the agent of human granulocytic ehrlichiosis in a deer tick–rodent cycle. *Proceedings of the National Academy of Sciences of the United States of America* 93, 6209–6214.

Towbin, H., Staehelin, T. and Gordon, J. (1979). Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheet: procedure and some applications. *Proceeding of the National Academy of Sciences of the United States of America* 76, 4350–4354.

Worasing, R. (2007). Gastrointestinal and blood parasites in cattle in Pakpanang river basin Nakhon si thammarat province. *Thai-NIAH eJournal* 2, 138-47.

Yawongsa, A., Nunklang, G. and Rattanakunuprakarn, J. (2010). Haematological findings in Cattle infected with Trypanosomosis, Babesiosis and Anaplasmosis during 2006-2008 at Kasetsart University Veterinary Teaching Hospital in Thailand. *Kamphaengsean Academic Journal* 8, 1-6.

Zaugg, J.L. and Kuttler, K.L. (1984). Anaplasmosis: in utero transmission and the immunological significance of ingested colostral antibodies. *American journal of veterinary research* 45, 440–443.

Zaugg, J.L. (1985). Bovine anaplasmosis: transplacental transmission as it relates to stage of gestation. *American journal of veterinary research* 46, 570–572.

Zaugg, J.L., Goff, W.L., Foreyt, W. and Hunter, D.L. (1996). Susceptibility of elk (Cervuselaphus) to experimental infection with *Anaplasma marginale* and *A. ovis*. *Journal of wildlife diseases* 32, 63-66.

Output of the research

Anaplasmosis due to *A. marginale* is one of major tropical diseases that afflict ruminants, such as cattle, buffaloes and sheep, as well as quite many human cases in the tropics. In Thailand, it causes serious economic loss in the order of at least 300 million bahts per year. The discoveries of efficient immunodiagnosis are essential for the detection, monitoring, for the early treatment with drugs, and for sustainable preventive measure from the disease. The present study provides basic knowledge on the nature of target antigens that have immunodiagnostic potential. The research could also yield specific monoclonal antibodies and antigens for the development of immunodiagnostic method for field applications in large economic animals in the future.

The main output is three scientific articles published in internationally reputable journals as following below:

1. Junsiri W, Watthanadirek A, Poolsawat N, Kaewmongkol S, Jittapalapong S, Chawengkirttikul R, **Anuracpreeda P***. Molecular detection and genetic diversity of *Anaplasma marginale* based on the major surface protein genes in Thailand. Acta Trop 205. 2020, 05338. (IF = 2.787) **(*Corresponding author).**

2. Watthanadirek A**,** Chawengkirttikul R**,** Poolsawat N**,** Junsiri W**,** Boonmekam D**,** Reamtong O, **Anuracpreeda P*.** Recombinant expression and characterization of major surface protein 4 from *Anaplasma marginale.* Acta Trop. 2019; 197:105047. (IF = 2.787) **(*Corresponding author).**

3. Watthanadirek A, Doungdaolek T, Koedrith P, **Anuracpreeda P*.** Expression of recombinant major surface protein 5 of *Anaplasma marginale* (*A. marginale*) at different temperatures. Chula Med J. 2018; 62: 565-573. (IF = 2.787) **(*Corresponding author).**

Appendix

Three scientific articles published in internationally reputable journals as following below:

1. Junsiri W, Watthanadirek A, Poolsawat N, Kaewmongkol S, Jittapalapong S, Chawengkirttikul R, **Anuracpreeda P***. Molecular detection and genetic diversity of *Anaplasma marginale* based on the major surface protein genes in Thailand. Acta Trop 205. 2020, 05338. (IF = 2.787) **(*Corresponding author).**
2. Watthanadirek A**,** Chawengkirttikul R**,** Poolsawat N**,** Junsiri W**,** Boonmekam D**,** Reamtong O, **Anuracpreeda P*.** Recombinant expression and characterization of major surface protein 4 from *Anaplasma marginale* Acta Trop. 2019; 197:105047. (IF = 2.787) **(*Corresponding author).**
3. Watthanadirek A, Doungdaolek T, Koedrith P, **Anuracpreeda P*.** Expression of recombinant major surface protein 5 of *Anaplasma marginale* (*A. marginale*) at different temperatures. Chula Med J. 2018; 62: 565-573. (IF = 2.787) **(*Corresponding author).**

Acta Tropica 205 (2020) 105338

Contents lists available at ScienceDirect

Acta Tropica

journal homepage: www.elsevier.com/locate/actatropica

Molecular detection and genetic diversity of *Anaplasma marginale* based on the major surface protein genes in Thailand

Witchuta Junsiri[a,b], Amaya Watthanadirek[b], Napassorn Poolsawat[b], Sarawan Kaewmongkol[a], Sathaporn Jittapalapong[a], Runglawan Chawengkirttikul[c], Panat Anuracpreeda[b,*]

[a] Faculty of Veterinary Technology, Kasetsart University, Bangkok 10900, Thailand
[b] Institute of Molecular Biosciences, Mahidol University, Nakhon Pathom 73170, Thailand
[c] Department of Microbiology, Faculty of Science, Mahidol University, RamaVI Rd., Bangkok 10400, Thailand

ARTICLE INFO

Keywords:
Anaplasma marginale
Major surface protein genes
Genetic diversity
Phylogenetic analysis
Cattle and buffalo
Thailand

ABSTRACT

Anaplasma marginale is the rickettsial agent of anaplasmosis, a tick-borne disease, which affects cattle and other ruminants in tropical and subtropical areas of the world, and causing huge economic losses because of decreasing meat and milk production. In the present study, molecular methods have been used to determine the occurrence and genetic diversity of *A. marginale*, based on the genes encoding the major surface proteins (*msps*) genes, in blood samples from 520 cattle and 121 buffaloes in the north and northeastern regions of Thailand. The polymerase chain reaction (PCR) results based on the *msp4* gene indicated that 66 (10.30%) cattle were positive for *A. marginale*, whereas no positive result was obtained from buffaloes. The phylogenetic analysis based on the maximum likelihood method using 13, 29 and 27 nucleotide sequences from *msp2, msp4, msp5* clones, respectively, revealed that the sequences detected in this study are obviously distributed in different clusters. The sequence analysis demonstrated that *msp2* gene is genetically diverse, while *msp4* and *msp5* genes are conserved in Thailand. These findings corroborated the diversity analysis of the same sequences, which showed 13, 27 and 27 haplotypes of the *msp2, msp4* and *msp5* genes, respectively. In addition, the entropy analyses of amino acid sequences exhibited 127, 75 and 51 high entropy peaks with values ranging from 0.27119 to 2.45831, from 0.14999 to 2.17552 and from 0.15841 to 1.05453 for MSP2, MSP4 and MSP5, respectively. Therefore, the results indicate a low molecular occurrence of *A. marginale* in cattle blood samples in Thailand. From these results; however, a high degree of genetic diversity was observed in the analyzed *A. marginale* population. Hence, our finding could be used to improve the immunodiagnostics and vaccination programs for anaplasmosis.

1. Introduction

Anaplasmosis is a tick-borne disease of cattle and other ruminants caused by the rickettsial *Anaplasma marginale*, which is widely distributed throughout tropical and subtropical areas of the world such as Europe, Africa, America, Australia, Asia, including Thailand (Watthanadirek *et al.*, 2019). Transmission of *A. marginale* in cattle occurs biologically through ticks or blood-feeding flies, and mechanically through any blood contaminated equipment during ear tagging, tattooing, and dehorning (Aubry and Geale, 2011). This disease is responsible for enormous economic losses to both dairy and beef cattle industries worldwide (Kocan *et al.*, 2003), and is characterized by general weakness, weight loss, fever, severe anaemia, pale mucous membranes, abortion, lethargy, icterus, decreased milk production and often death in animals rather than two years old (Kocan *et al.*, 2015). The study of bovine anaplasmosis in foreign countries determined that the occurrence of this disease varied from 3.8% to 55.35% in Europe (Aktas and Özübek, 2017), 6.1% to 91% in Africa (Ramabu *et al.*, 2018), 15% to100% in America (Castañeda-Ortiz *et al.*, 2015), more than 50% in Caribbean (Obregón *et al.*, 2018, 2019) and 6.11% to 72.6% in Asia (Ola-Fadunsin *et al.*, 2018). The situation of bovine anaplasmosis has been reported in many regions of Thailand since 1986 (Tananyutthawongese *et al.*, 1999). The occurrence of *Anaplasma* sp. infections since from 2001 to 2017 of cattle in Thailand varied from 0.03 to 65.2% by microscopic examination and PCR assay. These provinces are Nakhon Sawan, Ayutthaya, Nan, Nakhon Pathom, Ubon Ratchathani, Surin, Buri Ram, Sakon Nakhon, and Nakorn Si Thammarat (Jirapattharasate *et al.*, 2017).

Six major surface proteins (MSPs) of *A. marginale* are well characterized as MSP1a, MSP1b, MSP2, MSP3, MSP4, and MSP5. These

* Corresponding author. Institute of Molecular Biosciences, Mahidol University, Nakhon Pathom 73170, Thailand.
E-mail addresses: panat.anu@mahidol.edu, panat.anu@mahidol.ac.th (P. Anuracpreeda).

https://doi.org/10.1016/j.actatropica.2020.105338
Received 7 October 2019; Received in revised form 9 January 2020; Accepted 9 January 2020
Available online 14 January 2020
0001-706X/

proteins are antigenically related and responsible for the production of antibodies in *A. marginale* infected animals (Tamekuni *et al.*, 2009). The *msp* genes have also been classified into six *msp* genes consisting of *msp1a, msp1b, msp2, msp3, msp4* and *msp5* (Kocan *et al.*, 2010). Although, the genetic diversity of *A. marginale* strains has been conducted using the *msp* genes such as *msp4* and *msp1a* genes (de la Fuente *et al.*, 2003; Kocan *et al.*, 2003), little is known about the genetic diversity of *A. marginale* isolates in Thailand when using *msp4* gene (Saetiew *et al.*, 2015; Jirapattharasate *et al.*, 2017; Watthanadirek *et al.*, 2019). Therefore, this study aimed to investigate the occurrence and genetic diversity of *A. marginale* in cattle from the north and northeastern of Thailand. In addition, phylogenetic relationship amino acids entropy and haplotype diversity among the isolates identified in this work and those from different countries are presented. The results are expected to provide the basic information on the genetic structure of the *A. marginale* population before further work is pursued in the development of immunodiagnostic and vaccine potential of the selected MSPs.

2. Materials and methods

2.1. Study areas and sample sizes

The present study was conducted in the north and northeastern regions of Thailand (Fig. 1). Blood samples were collected from 52 beef farms and 12 buffaloe farms in 2 provinces from Mueang Chiang Mai district in Chiang Mai province and from four districts including Kusuman, Khok Si Suphan, Mueang Sakon Nakhon and Waritchaphum in Sakon Nakhon province. The animals were separately reared with an extensive system. In total of 641, blood samples from 520 beef cattle and 121 water buffaloes were randomly collected during October 2017 to September 2018 (Table 2). The sample sizes were calculated using the formula based on the equation, $n = t^2 \times p(1\text{-}p)/m^2$, inserting the following values: the prevalence (p) of *A. marginale* infection among cattle or buffaloes in Thailand, a 95% confidence level (t) and 5% margin of error (m).

2.2. Sample collection

Ten mL of blood samples were collected directly from the jugular or caudal veins of animals. After collection, half of the blood volume was transferred to sterile tubes containing Ethylene Diamine Tetraacetic Acid (EDTA) (BD Vacutainer®, USA) as an anticoagulant and citrate salt to preserve the blood for PCR analysis. The rest was transferred to clotted blood sterile tubes (BD Vacutainer®, USA) and centrifuged at 5000 $\times g$ at room temperature for 10 min and then the serum samples were obtained. All blood and the sera were kept at -20°C for further experiments.

2.3. DNA extraction

Genomic DNA of *A. marginale* was extracted from blood samples of cattle and buffaloes using a Tissue DNA Extraction Kit (OMEGA, biotex, USA) following the manufacturer's instructions. Briefly, 250 µL of each blood sample was transferred into 1.5 mL microcentrifuge tube. Then 25 µL of OB Proteas solution and 250 µL of BL buffer were added. After incubation at 70 °C for 10 min, 250 µL of ethanol was added and the sample was transferred to HiBind® DNA Mini Column for centrifugation at maximum speed at room temperature for 1 min and then discarded the flow-through. Thereafter, 500 µL of HBC buffer was added and centrifuged at maximum speed at room temperature for

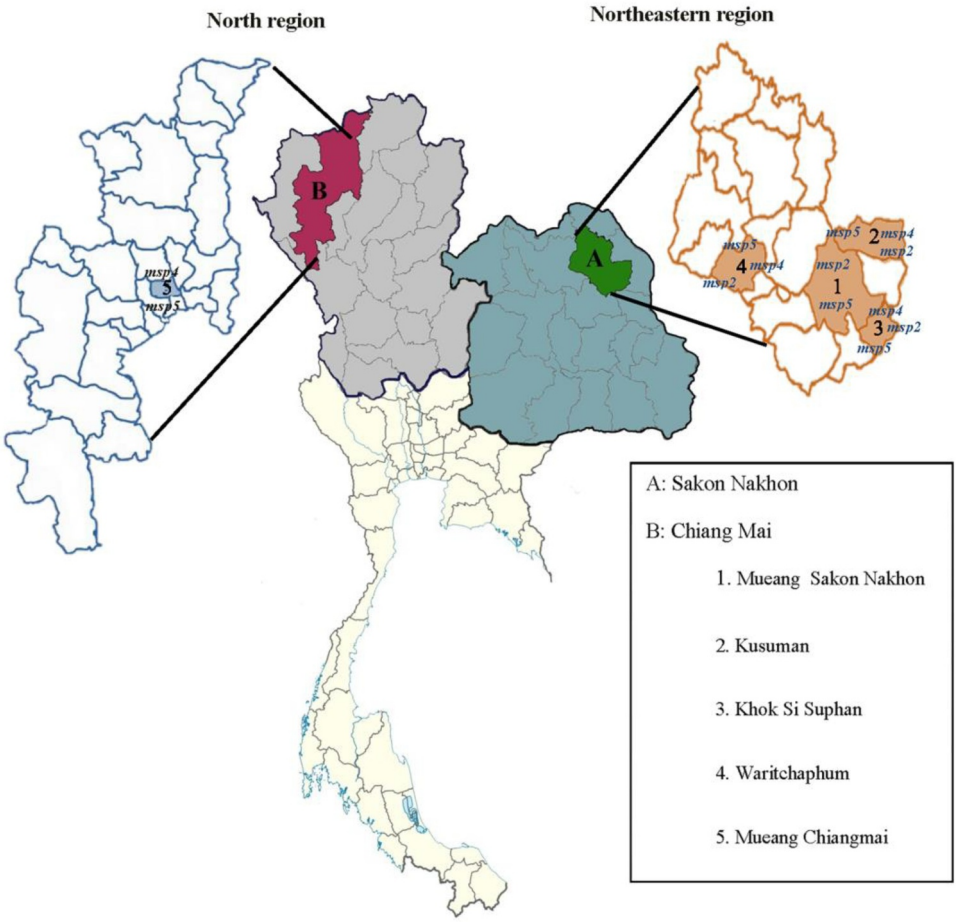


Fig. 1. Distribution of *A. marginale* Thailand strains identified in cattle from the north and northeastern regions.

30 sec and then discarded the flow-through. Subsequently, 700 µL of DNA wash buffer was added, centrifuged and discarded the flow-through. Then column was transferred into a new 1.5 mL micro-centrifuge tube. Finally, the DNA samples were eluted in 50 µl MiliQ water and stored at -20°C until used. DNA purity and concentration were determined by using NanoDrop™ 2000 Spectrophotometers (Thermo Scientific™) at the 260/280 and 260/230 ratios.

2.4. Cloning of the msp genes from A. marginale DNA

All of the specific primer pairs designed from the *A. marginale msp2, msp4* and *msp5* sequences submitted in GenBank database under accession numbers AY138958, AY127073 and M93392, respectively, namely MSP2F (5′CACCATGAGTGCTGTAAGTAATAGGAAGC3′), MSP2R (5′CTAGAAGGCAAACCTAACACCCAACTC3′), MSP43 (5′CACC ATGAATTACAGAGAATTGTTTACAG3′), MSP45 (5′TTAGCTGAACAGG AATCTTGCTCCAA3′) and MSP5F (5′CACCATGAGAATTTTCAAGATTG TGTC3′), MSP5R (5′CTAAGAATTAAGCAT3′) were used to amplify DNA fragments of the *msp2, msp4* and *msp5* genes, respectively. Four bases sequences (CACC) were added to the 5′ end of the forward primer before start codon (ATG) with the overhang sequence (GTGG) in pET100/D-TOPO® vector (Invitrogen, USA) to enable directional cloning. The designed primers were prepared by Macrogen Inc. (South Korea). The PCR reactions containing 50 ng of DNA template, 0.2 µM each of the primers, 200 µM of each deoxynucleoside triphosphate (dNTPs), 1x standard *Taq* reaction buffer, nuclease free water and 1.25 U *Taq* DNA polymerase (BioLabs®, USA), were performed in a thermal cycler (Bio-Rad, USA) with the following conditions: 95ºC for 30 sec, 35 cycles of 95ºC for 30 sec, 60ºC for 30 sec, and 68ºC for 1 min, and at 68ºC for 5 min. The PCR products were stained with FluoroStain™ DNA Fluorescent Staining Dye (SMOBIO, Taiwan) and analysed by gel electrophoresis using 1% agarose gels. The DNA sample of *A. marginale* from our previous study (Watthanadirek *et al.*, 2019) and nuclease free water was used as positive and negative controls, respectively. A 100 bp DNA Ladder M (MolBio™ HIMEDIA®, India) was used as standard for determining the molecular mass of PCR products. Positive PCR products were purified using GenepHlow™ Gel/PCR Kit (Geneaid, Taiwan) following the manufacturer's instructions for cloning. The blunt-end PCR products were quantified; 20 ng was used as an insert in the pET100/D-TOPO® vector (Invitrogen Life Technologies, USA). Chemically competent *Escherichia coli* host strain TOP10 cells (Invitrogen, USA) was then transformed with the ligation product. Then, 200 µl of transformed bacterial culture was spread on the plates containing 100 µg ampicillin and incubated at 37°C for overnight. The positive clones were selected and grown in Luria Bertani (LB) medium containing ampicillin for overnight. The plasmid extraction was performed by Presto™ Mini Plasmid Kit (Geneaid, Taiwan) following the manufacturer's instructions and analyzed for correctly sized inserts by agarose gel electrophoresis.

2.5. Sequence analysis

Purified PCR products were confirmed by Sanger sequencing. The sequences were analyzed by BLAST (The National Center for Biotechnology Information, NCBI, http://www.ncbi.nlm.nih.gov/BLAST). All DNA sequences were submitted and deposited in the GenBank database (Table 1).

2.6. Phylogenetic analysis

A. marginale msp2, msp4 and *msp5* gene sequences were used for sequence alignment and phylogenetic analysis. Multiple sequence alignment was carried out with Clustal W algorithm and then genetic inference was analyzed by neighbor-joining phylogenetic tree with a small number of gap-free positions using MEGA software version 7.0.26 (Saitou and Nei, 1987; Kumar *et al.*, 2016). Bootstrap test with 1000 repetitions was used to evaluate the confidence of the branching pattern of the trees (Felsenstein, 1985). The evolutionary distances were computed using the Kimura 2-parameter method (Kimura, 1980). The similarity was evaluated using the pairwise-distance method (Nei and Kumar, 2000).

2.7. B-cell epitopes analysis

A. marginale msp2, msp4 and *msp5* gene sequences were translated to amino acid sequences and then subjected to B-cell prediction as described by Kolaskar and Tongaonkar, (1990), using semi-empirical method (http://tools.immuneepitope.org/bcell/) for predicting antigenic determinants on the proteins.

2.8. Entropy analysis of amino acids

Entropy analysis was performed to verify the variability between the amino acid sequences. The *msp2, msp4* and *msp5* nucleotide sequences were translated into amino acids, aligned and then analyzed by the Entropy (H (x)) plot using Bioedit version 7.0.5.3 (Hall, 1999).

2.9. Haplotype diversity

The obtained alignment of the *msp2, msp4* and *msp5* gene sequences were used to calculate the nucleotide diversity (π), diversity of haplotypes (Dh), number of haplotypes, and the average number of nucleotide differences (*K*), using the DnaSP version 5.10.01 software (Librado and Rozas, 2009). In addition, these sequences were subjected to the Population Analysis with the Reticulate Trees (popART) program (Leigh and Bryant, 2015) in order to analyze the TCS Network (Clement *et al.*, 2002).

2.10. Ethics statement

Experimentation on animals was approved by the Animal Care and Use Committee (SCMU-ACUC), Faculty of Science, Mahidol University, Thailand (Protocol No. MUSC 59-009-342). The cattle sera were obtained from Department of Livestock, Ministry of Agriculture and Cooperatives, Bangkok, Thailand. Also, we have received consent to collect the blood samples from animals at the abattoir.

3. Results

3.1. Occurrence of A. marginale infection in bovine blood samples

When using the specific primers, *A. marginale* was detected and identified as the PCR products at 1230 bp (*msp2* gene), 849 bp (*msp4* gene) and 633 bp (*msp5* gene). Among the animal samples (520 cattle and 121 buffaloes), 66 (total 641, 10.3%) were positive as shown by the PCR results targeting *msp4* gene. A total positive blood samples were from cattle, but buffalo blood samples were not found to be positive for *A. marginale* infection. However, 66 (12.65%) of the 520 cattle were positive for the occurrence of *A. marginale* infection in the North and Northeastern regions of Thailand (Table 2).

3.2. Phylogenetic and similarity analysis of A. marginale msp gene sequences

The phylogenetic tree based on the alignment of the 13 sequences of the *msp2* gene obtained in this study with 10 USA sequences obtained from the GenBank was divided into 9 clusters (Fig. 2). The *A. marginale* sequences detected in this work were positioned in 7 clusters (1, 3, 5, 6, 7, 8 and 9). The sequences assigned to cluster 1, 3, 7, 8 and 9 showed phylogenetic proximity representing the genetic variability of *A. marginale msp2* sequence from four districts in Sakon Nakhon province. The remaining 5 Thailand sequences were positioned in 5th cluster and 6th

Table 1
The *A. marginale msp* nucleotide sequences amplified in Thailand strain were deposited in the GenBank database.

Provinces	Regions	Districts	Animal ID	GenBank accession numbers		
				msp2	*msp4*	*msp5*
Chiang Mai	North	Mueang	CM5		MK164538	MK164571
			CM6		MK164539	
			CM27		MK164540	MK164569
			CM28		MK164541	MK164548
			CM29		MK164542	
			CM38		MK164543	MK164570
			CM45		MK164544	MK164547
			CM74		MK164545	MK164546
Sakon Nakhon	Northeastern	Mueang	A19	MK105922		MK164566
			A26			MK164565
		Kusuman	D21	MK140720	MK140739	MK164564
		Khok Si Suphan	F21		MK140727	MK164563
			F25		MK140728	MK164562
			F26	MK140719	MK140729	MK164567
			F28		MK140730	
			F29	MK140718	MK140731	MK164561
			F31		MK140732	MK164560
			F32	MK140717	MK140733	MK164559
			F33	MK140722	MK140734	MK164558
			F36	MK140721	MK140735	MK164557
			F38		MK140736	
			F39		MK140737	MK164556
			F41	MK140716	MK140738	MK164568
			F43	MK140715	MK164535	MK164555
			F46	MK140714	MK164536	MK164554
			F47			MK164572
			F52			MK164553
			F55	MK140713	MK164537	MK164552
		Waritchaphum	H62		MK140740	MK164551
			H63	MK140712	MK140723	MK164550
			H66		MK140725	
			H74	MK140711	MK140724	MK164549
			H93		MK140726	

Table 2
Summary of *A. marginale* infection in cattle and buffaloes from the north and northeastern regions as analysed by PCR using the specific primer for *msp4* gene.

Parametors	Positive samples/ no. of tested animals (%)		Total (%)
	Beef Cattle	Water buffaloes	
Provinces			
Chiang Mai	34/58 (58.62%)	0/3 (0%)	34/61 (55.74%)
Sakon Nakhon	32/462 (6.92%)	0/118 (0%)	32/580 (5.51%)
Total	66/520 (12.69%)	0/121 (0%)	66/641 (10.30%)
Breed			
Native	5/145 (3.45%)	0/121 (0%)	5/266 (1.88%)
Mixed	61/375 (16.27%)	0	61/375 (16.27%)
Total	66/520 (12.69%)	0/121 (0%)	66/641 (10.30%)
Age			
1-3 years	57/226 (25.22%)	0/64 (0%)	57/290 (19.66%)
> 3 years	9/294 (3.06%)	0/57 (0%)	9/351(2.56%)
Total	66/520 (12.69%)	0/121 (0%)	66/641 (10.30%)
Gender			
Male	17/129 (13.18%)	0/50 (0%)	17/179 (9.49%)
Female	49/391 (12.53%)	0/71 (0%)	49/462 (10.63%)
Total	66/520 (12.69%)	0/121 (0%)	66/641 (10.30%)
Management system			
Extensive	66/520 (12.69%)	0/121 (0%)	66/641 (10.30%)
Intensive	0	0	0
Total	66/520 (12.69%)	0/121 (0%)	66/641 (10.30%)

cluster together with sequences from USA (Fig. 2). In addition, the similarity ranged between 63.9 and 88.2% for the Thailand *msp2* sequences. The similarity of the sequences within each cluster was 63-82.9% (1st cluster), 68-88.2% (3rd cluster) and 66.4-84.2% (6th cluster) (Table 3). The nucleic acid substitution rate in *msp2* sequences among *A. marginale* was estimated under the Tamura and Nei (1993) mode (Supplementary Table 1).

For phylogenetic tree of the *msp4* gene, the sequences were classified as 4 clusters in the phylogram. Twenty-nine *msp4* sequences from Chiang Mai and Sakon Nakhon provinces of Thailand were found in cluster 1 together with *msp4* sequences from Taiwan, Mexico, Argentina, Brazil, Germany, Australia, India, China and Spain. The sequences from Italy were positioned in 2nd and 3rd cluster, whereas a sequence from USA was found in 4th cluster (Fig. 3). The *msp4* gene sequences among *A. marginale* were highly conserved when compared with other strains as external groups. For the Thailand *msp4* sequences, the similarity ranged between 93.4 and 100% (Table 4). The nucleic acid substitution rate in *msp4* sequences among *A. marginale* was estimated under the Tamura and Nei (1993) mode (Supplementary Table 1).

The *msp5* gene sequences were divided into 5 clusters. The 27 Thailand *msp5* gene sequences from Chiang Mai and Sakon Nakhon provinces were classified into 4 clusters in the phylogram (1, 2, 3 and 5). Twenty-four Thailand *msp5* sequences were detected in 1st cluster together with a sequence from Germany, while one Thailand *msp5* sequence was positioned together with sequences from Japan, India, Egypt, China, Brazil and the Philippines. In 3rd cluster, only one Thailand sequence demonstrated phylogenetic proximity. In addition, one Thailand sequence (H62) was located in 5th cluster together with the sequence from Cuba (Fig. 4). The total similarity among Thailand *msp5* sequences was 91.9-98.8%, while percent similarity of the sequences within each cluster was 91.9-100% (1st cluster), 94.5-100% (2nd cluster), 96.8-100% (3rd cluster) and 100% (5th cluster) (Table 5). The nucleic acid substitution rate in *msp5* sequences among *A. marginale* was observed under the Tamura and Nei (1993) mode (Supplementary Table 1).

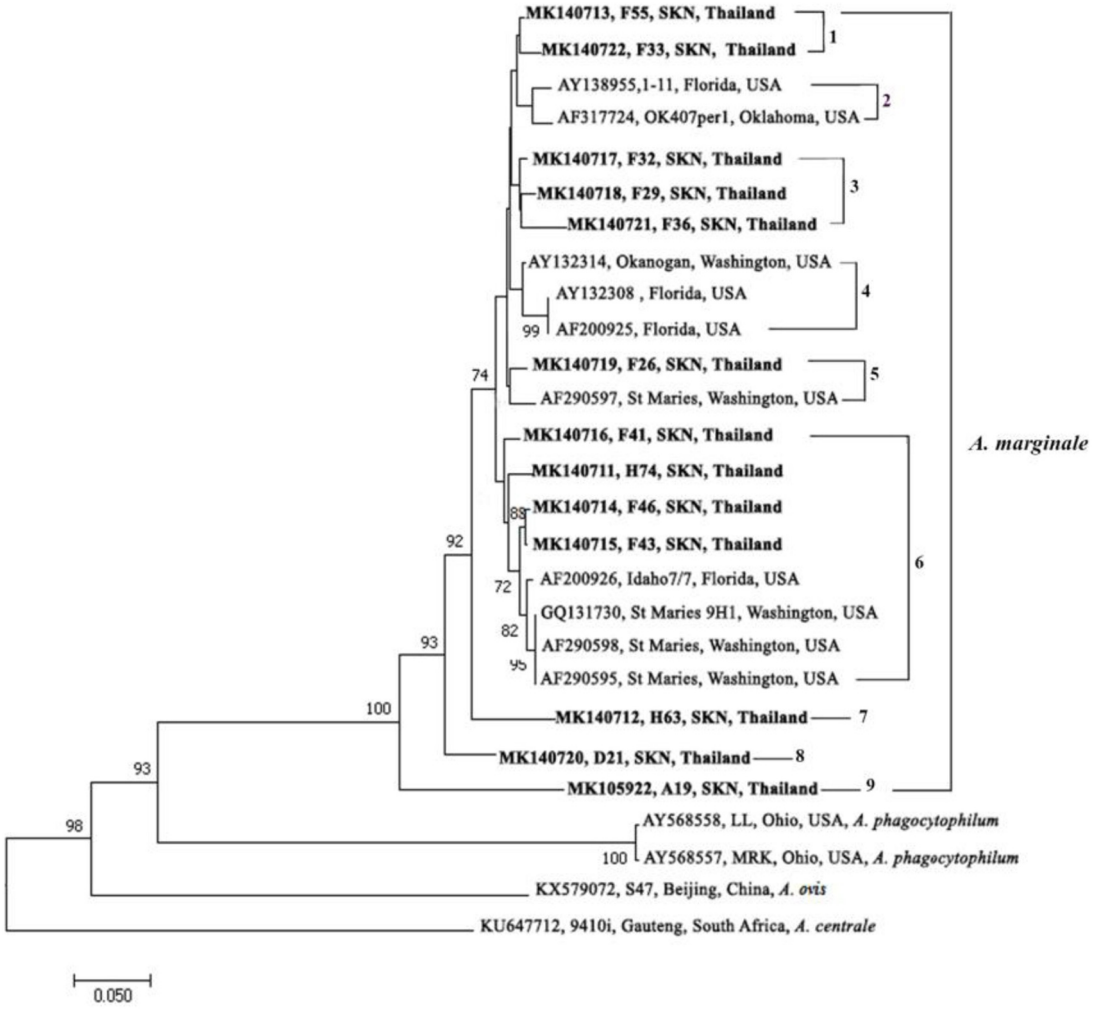


Fig. 2. Phylogenetic analysis of *A. marginale msp2* gene sequences (1156 bp) using the neighbor-joining algorithm. The numbers on each node correspond to the bootstrap analysis of 1000 replicates (only percentage greater than 60% were represented). The sequences amplified in the present study are highlighted in boldface-type letters. The GenBank accession numbers of the sequences used in the phylogenetic analysis are also shown. Four gene sequences of the other strains were used as external groups.

Table 3
Similarity of the *A. marginale msp2* gene sequences as detected in cattle sampled in Thailand.

Clade		1		5		5	6				7	8	9
Acc.No.		1	2	3	4	5	6	7	8	9	10	11	12	13
MK140713	**1**	100												
MK140722	**2**	81.5	100											
MK140717	**3**	70.7	79.7	100										
MK140718	**4**	70.5	78.2	73.9	100									
MK140721	**5**	78.6	82.6	80.4	82.6	100								
MK140719	**6**	69.2	78.2	77.7	72.2	80.8	100							
MK140716	**7**	70.4	79.5	81.3	73.4	81.6	75.3	100						
MK140711	**8**	68	76.8	68.8	74.8	81.7	65.4	70.3	100					
MK140714	**9**	74.1	82	88.2	72.8	79.8	80.8	83.9	70.9	100				
MK140715	**10**	71	79.1	76.7	70.3	79.8	70.5	78.8	68.9	80.8	100			
MK140712	**11**	70.5	79.3	79	73.8	78.6	80.8	76.8	67.3	84.2	73.5	100		
MK140720	**12**	63.9	75.6	68	73.3	87.8	67.5	69.7	72.4	68.1	66.4	69	100	
MK105922	**13**	78.3	78.6	82.9	81.2	81.5	80.4	84.1	81.6	83.6	82.6	81.6	80.5	100

Similarity (%)

3.3. B-cell epitope analysis

The B-cell epitopes predicted among Thailand MSP4 and MSP5 amino acid sequences were relatively conserved, whereas MSP2 sequences were diverse (Table 6). Nevertheless, except for three epitopes (MALVAAVVQVHSLLPAP of MSP2), (AATVCACSLLVSGAVVASP of MSP4) and (FKIVSNLLFVAAVFLGYSYV of MSP5) that were detected in the signal peptide region, all others were observed to be highly polymorphic. Interestingly, 5 of such epitopes of MSP2 were not conserved.

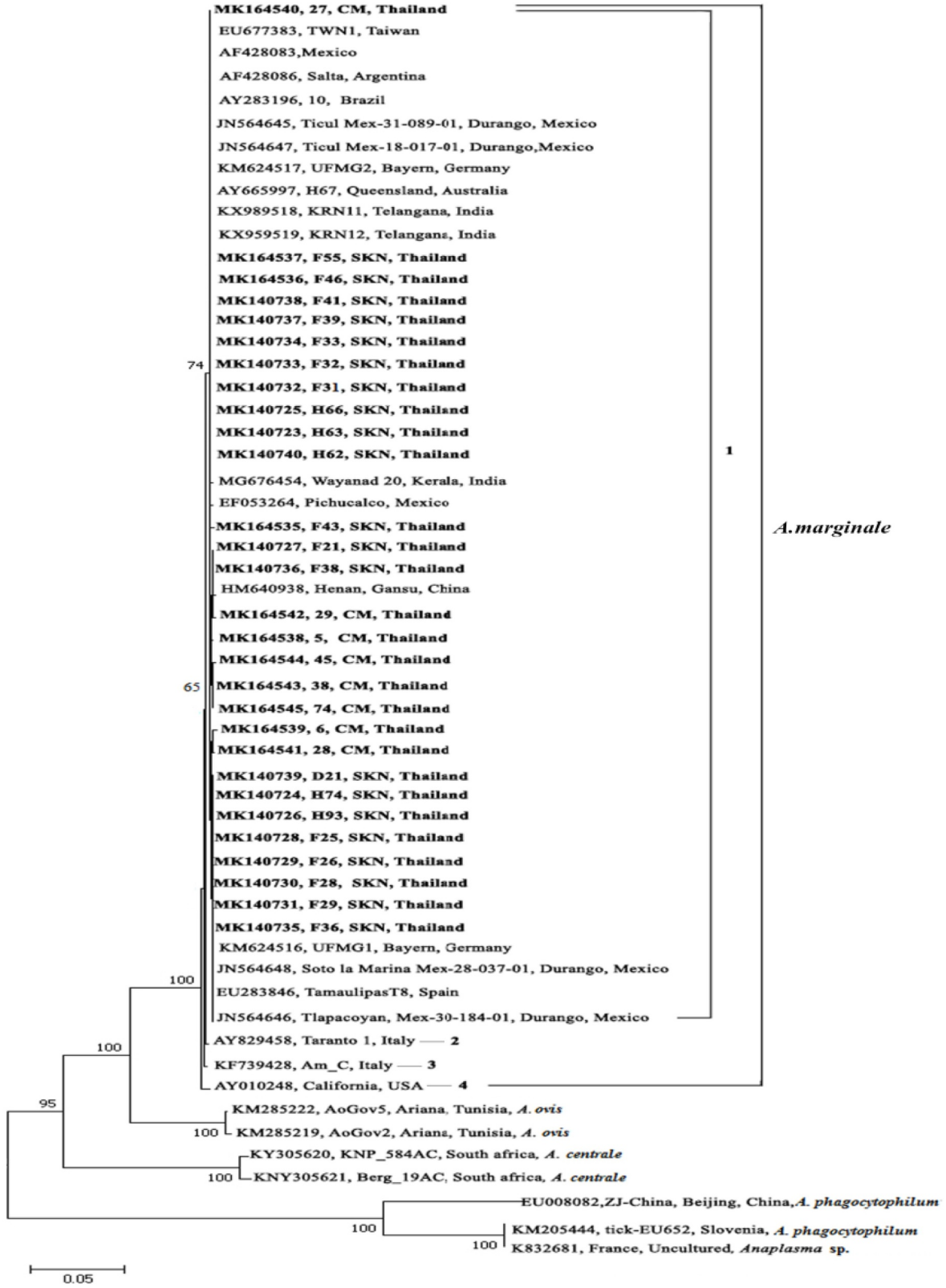


Fig. 3. Phylogenetic analysis of *A. marginale msp4* gene sequences (756 bp) using the neighbor-joining algorithm. The numbers on each node correspond to the bootstrap analysis of 1000 replicates (only percentage greater than 60% were represented). The sequences amplified in the present study are highlighted in boldface-type letters. The GenBank accession numbers of the sequences used in the phylogenetic analysis are also shown. Seven gene sequences of the other strains were used as external groups.

Table 4
Similarity of the *A. marginale msp4* gene sequences as detected in cattle sampled in Thailand.

Clade / Acc. No.		1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29
MK164540	**1**	100																												
MK164537	**2**	95.3	100																											
MK164536	**3**	95.3	98.1	100																										
MK140738	**4**	97.0	99.2	99.2	100																									
MK140737	**5**	99.4	100	100	100	100																								
MK140734	**6**	97.6	99.7	99.7	99.6	100	100																							
MK140733	**7**	99.1	99.9	99.9	99.9	99.9	99.9	100																						
MK140732	**8**	98	99.7	99.7	99.4	100	99.4	99.7	100																					
MK140725	**9**	98	99.3	99.3	99.1	100	99.6	99.7	99.0	100																				
MK140723	**10**	95.8	98.1	97.8	99.0	99.9	99.3	99.6	99.9	98.9	100																			
MK140740	**11**	97.5	99.7	99.6	99.3	99.9	99.4	99.7	100	99.0	99.9	100																		
MK164535	**12**	94.1	94.5	94.2	97.8	99.6	98.3	99.3	98.7	98.6	96.4	98.2	100																	
MK140727	**13**	99.3	99.9	99.9	99.9	99.9	99.9	99.7	99.9	99.9	99.7	99.9	99.4	100																
MK140736	**14**	99.6	99.9	99.9	99.9	99.9	99.9	99.9	99.9	99.9	99.7	99.9	99.4	100	100															
MK164542	**15**	95.8	95.0	94.9	97.5	99.4	97.9	99.3	98.3	98.0	96.2	98.1	94.5	99.6	99.9	100														
MK164538	**16**	96.4	95.1	94.9	97.6	99.4	98.0	99.3	98.6	98.1	96.5	98.2	93.6	99.3	99.6	95.1	100													
MK164544	**17**	97.9	94.4	94.3	96.7	99.3	97.3	99.0	98.0	98.1	95.6	97.2	93.8	99.1	99.4	95.7	95.9	100												
MK164543	**18**	97.9	94.6	94.6	97.1	99.4	97.7	99.1	98.2	98.3	96.1	97.6	94.1	99.3	99.6	96.4	96.3	98.5	100											
MK164545	**19**	97.8	94.3	94.1	96.8	99.4	97.4	99.1	98.2	98.3	95.7	97.4	93.9	99.3	99.6	96.2	95.4	98.9	98.7	100										
MK164539	**20**	97.7	94.0	94.0	96.7	99.3	97.3	99.0	98.0	98.1	95.6	97.2	93.4	99.1	99.4	95.5	95.5	98.8	98.5	98.8	100									
MK164541	**21**	97.7	94.1	94.1	96.7	99.4	97.3	99.1	98.0	98.1	95.6	97.2	93.9	99.3	99.6	95.6	95.2	98.8	98.4	99.5	99	100								
MK140739	**22**	95.3	95.5	95.5	98.8	99.9	99.3	99.7	99.4	99.3	97.8	99.3	93.9	99.7	99.7	94.8	94.7	95.1	95.1	94.6	95.1	94.6	100							
MK140724	**23**	97.5	99.6	99.6	99.0	99.9	99.3	99.6	99.9	98.9	99.5	99.9	98.2	99.7	99.7	98.1	98.2	97.2	97.7	97.4	97.5	97.2	99.5	100						
MK140726	**24**	98.3	99.7	99.7	99.9	99.9	99.6	99.7	99.7	99.3	99.6	99.7	99.1	99.7	99.7	98.6	98.7	98.3	98.4	98.4	98.5	98.3	99.9	99.9	100					
MK140728	**25**	97.5	99.6	99.6	99.3	99.9	99.3	99.6	99.9	98.9	99.7	99.9	98.2	99.7	99.7	98.1	98.2	97.2	97.6	97.4	97.5	97.2	99.4	100	99.9	100				
MK140729	**26**	97.9	99.9	99.9	99.7	99.9	99.6	99.7	99.6	99.1	99.4	99.6	98.7	99.7	99.7	98.1	98.4	97.9	98.0	98.0	98.1	97.9	99.9	99.7	99.9	99.7	100			
MK140730	**27**	98.8	99.9	99.9	99.9	99.9	99.9	99.7	99.7	99.6	99.6	99.7	99.1	99.7	99.7	99.0	99.0	98.7	98.8	98.8	99.0	98.8	100	99.9	100	99.9	100	100		
MK140731	**28**	97.5	99.6	99.6	99.0	99.9	99.3	99.6	99.9	98.9	99.7	99.9	98.3	99.7	99.7	98.1	98.2	97.2	97.6	97.4	97.5	97.2	99.4	100	99.9	100	99.7	99.9	100	
MK140735	**29**	97.5	99.6	99.6	99.3	99.9	99.3	99.6	99.9	98.9	99.7	99.9	98.3	99.7	99.7	98.1	98.2	97.2	97.6	97.3	97.5	97.2	99.4	100	99.9	100	99.7	99.9	100	100

Similarity (%)

Fig. 4. Phylogenetic analysis of *A. marginale msp5* gene sequences (594 bp) using the neighbor-joining algorithm. The numbers on each node correspond to the bootstrap analysis of 1000 replicates (only percentage greater than 60% were represented). The sequences amplified in the present study are highlighted in boldface-type letters. The GenBank accession numbers of the sequences used in the phylogenetic analysis are also shown. Five gene sequences of the other strains were used as external groups.

Table 5
Similarity of the *A. marginale msp5* gene sequences as detected in cattle sampled in Thailand.

Clade												1													2	3	5
Acc.No.		1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27
MK164553	**1**	100																										
MK164567	**2**	97.7	100																									
MK164558	**3**	97.9	98.1	100																								
MK164563	**4**	97.1	97.9	98.9	100																							
MK164554	**5**	97.4	97.2	96.9	96.6	100																						
MK164564	**6**	98.0	97.9	97.7	97.4	97.1	100																					
MK164556	**7**	97.2	97.1	96.8	96.9	98.0	96.9	100																				
MK164549	**8**	97.4	96.6	97.1	96.8	96.3	97.1	97.6	100																			
MK164560	**9**	97.4	96.8	96.9	96.6	96.4	96.9	96.1	96.6	100																		
MK164561	**10**	95.8	96.6	95.8	96.1	95.4	95.8	95.3	95.6	96.3	100																	
MK164557	**11**	96.7	98.2	96.8	96.6	96.9	97.2	96.4	96.1	96.6	96.8	100																
MK164562	**12**	95.8	96.4	96.1	96.1	96.4	96.3	97.6	96.3	96.9	96.1	96.6	100															
MK164552	**13**	96.9	96.7	96.8	96.4	96.4	96.6	96.3	96.4	96.6	95.5	96.4	97.2	100														
MK164550	**14**	98.1	97.2	96.8	96.4	97.2	97.1	97.2	96.9	96.8	96.1	96.7	95.9	96.4	100													
MK164559	**15**	97.2	97.1	96.9	97.6	96.6	97.1	97.6	98.1	97.1	95.6	96.4	96.1	96.9	97.4	100												
MK164565	**16**	95.4	95.2	94.9	94.9	95.4	95.6	95.6	95.4	95.1	95.2	95.4	95.7	95.4	95.6	95.7	100											
MK164555	**17**	97.2	96.9	96.9	96.4	96.7	96.9	97.0	96.7	96.5	95.9	97.0	97.5	97.0	97.4	97.0	95.9	100										
MK164546	**18**	96.1	96.4	95.7	95.6	96.2	95.4	96.1	95.6	95.9	95.4	95.7	95.7	96.6	96.2	96.2	95.9	97.3	100									
MK164569	**19**	95.8	95.9	95.1	94.9	95.1	94.9	95.3	94.9	95.6	95.6	95.7	95.0	95.8	96.1	95.9	95.7	96.7	97.9	100								
MK164572	**20**	96.1	95.9	95.4	95.1	97.6	95.1	96.7	95.6	95.9	95.9	96.1	95.9	95.8	96.6	96.1	94.9	96.9	97.1	96.3	100							
MK164568	**21**	96.6	96.6	97.1	96.7	96.4	96.9	97.2	96.7	97.1	96.1	96.7	97.7	97.2	96.7	96.7	95.4	98.0	96.4	95.9	96.3	100						
MK164570	**22**	95.9	95.7	94.7	94.7	95.9	95.4	95.9	95.1	95.9	96.0	95.9	95.6	95.6	96.5	95.7	95.4	97.0	97.2	97.7	97.5	96.4	100					
MK164547	**23**	95.4	96.4	95.3	95.0	96.6	95.9	95.8	94.3	95.9	95.3	95.9	95.3	94.8	95.3	95.1	94.1	95.9	95.4	95.4	95.3	95.8	96.1	100				
MK164548	**24**	95.1	95.7	94.9	95.0	95.1	95.1	95.1	95.0	96.1	96.2	95.4	95.6	95.5	95.8	95.1	95.3	95.9	97.5	97.7	96.3	95.9	98.5	95.8	100			
MK164571	**25**	96.5	97.0	96.3	96.0	96.7	96.2	97.0	96.3	96.4	96.8	97.0	96.5	96.5	96.8	97.2	95.5	96.9	97.7	97.7	98.0	96.8	97.8	95.5	97.5	100		
MK164566	**26**	94.9	94.8	94.5	94.0	94.8	94.8	95.0	94.5	94.5	94.3	94.9	94.8	95.3	94.5	94.4	94.1	95.2	94.8	94.0	96.1	93.6	94.6	93.2	94.3	95.7	100	
MK164551	**27**	93.0	93.3	93.3	92.9	93.0	93.3	93.0	92.5	93.3	93.2	93.6	92.9	93.5	93.3	92.5	92.0	93.4	93.6	93.0	94.5	93.8	94.6	91.9	94.0	94.5	96.8	100

Similarity (%)

Table 6
Analysis of B-cell epitopes predicted from MSPs sequences determined in this study.

MSP2		MSP4		MSP5	
Epitopes [a]	Conserved amino acids/ total amino acid	Epitopes [a]	Conserved amino acids/ total amino acid	Epitopes [a]	Conserved amino acids/ total amino acid
MALVAAVVQVHSLLPAP [b]	0/17	AATVCACSLLVSGAVVASP [b]	14/19	FKIVSNLLLFVAAVFLGYSYV [b]	18/21
EVLFSD	0/6	YVGAAYSPAFPSVT	8/14	SEVVSEG	7/7
SFYIGLDLSPAFG	0/13	TIDVSVPAN	9/9	HEGVTVS	7/7
RVEVEVG	6/7	YFVVKI	6/6	MLVIFGFSACKYT	13/13
ASVFLLGKE	5/9	CYDVLHTDLPVSPYVCAGI	19/19	ASQLLSKL	8/8
DRLATA	5/6	ASFVDIS	7/7	KLQVVFITV	9/9
SKKVCKG	0/7	EISLVAGG	8/8	KSLVENYKVYVG	12/12
KCAVGTN	0/7				
KAIVAGA	6/7				
VIEVRAI	7/7				
STSVMLNACYDLLTDGIGVVPYACAG	19/26				
FVSVVDGH	8/8				
AYRVKAGLSYALTPE	9/15				
FAGAFYHKVL	10/10				
ELPLSHIS	8/8				

[b] Conserved epitope located in the signal peptide.
[a] Amino acid residues in the epitopes predicted from the deduced amino acid sequences of MK140714 (MSP2), MK164536 (MSP4) and MK164554 (MSP5) were analyzed for their diversity among Thailand sequences.

3.4. Entropy analysis of MSPs amino acids

Amino acid entropy analysis was performed using the *msp2, msp4* and *msp5* sequence alignments. The charts exhibited 127, 75 and 51 high entropy peaks distributed along the amino acid sequences for the MSP2, MSP4 and MSP5, respectively (Fig. 5). The entropy value was observed with the range of 0.27119-2.45831 for MSP2, 0.14999-2.17552 for MSP4 and 0.15841-1.05453 for MSP5.

3.5. Haplotype diversity

Haplotype diversity based on the *msp2, msp4* and *msp5* genes of *A. marginale* sequences found in Chiang Mai and Sakon Nakhon provinces of Thailand was highly diverse when compared to worldwide sequences. The haplotype networks of *msp2, msp4* and *msp5* genes were obtained from the TCS Network tool (Fig. 6). A total of 22 different haplotypes were analyzed based on *A. marginale msp2* gene, including the 13 sequences (haplotypes #1 to #13) and 10 sequences (haplotypes #14 to #22) obtained from cattle in Sakon Nakhon province of Thailand and USA, respectively, as shown in Table 7 and Fig. 6A. The haplotype network of *A. marginale msp4* gene showed that 40 haplotypes, being haplotypes #1 to #20 were detected in cattle in Sakon Nakhon province, and haplotypes #31 to #37 were detected in cattle in Chiang Mai province. In addition, the rest of haplotypes has also been detected in other countries worldwide, as shown in Table 7 and Fig. 6B. For haplotype analysis of *msp5* gene, 25 haplotypes indicated in TCS network showed that haplotypes #1, #4 to #6, #8 to #25 were detected in cattle in Chiang Mai and Sakon Nakhon provinces. The rest of haplotypes has been observed in other countries worldwide (Table 7 and Fig. 6C).

4. Discussion

In Thailand, anaplasmosis is considered one of the major constraints to livestock improvement programs and causes serious health problems resulting in reduced animal productivity and economic losses. Up to now, some molecular methods with high sensitivity and specificity have been developed to identify *A. marginale* DNA in the infected animals (Carelli *et al.*, 2007). In the present study, the results revealed a low molecular occurrence of *A. marginale* in cattle blood samples in Thailand. The molecular detection showed that of the animals sampled, 10.30% (66/641) were positive for *A. marginale* based on the *msp4* gene (Table 2). In this work, the *msp4* gene has been used as a good marker for providing sufficient variation to create phylogeographic patterns on the large scale as well as useful for epidemiological aspects of anaplasmosis (de la Fuente *et al.*, 2005). The percent of positive animals in this work was similar to the 6.1% (42/692) reported in cattle in Sudan (Awad *et al.*, 2011), and were lower than those reported by the previous studies; for instance, 50% (25/50) in Italy (de la Fuente *et al.*, 2005), 65%-100% in South Africa (Mutshembele *et al.*, 2014), 21.3% in Egypt (El-Ashker *et al.*, 2015) and 72.6% (759/1045) in Malaysia (Ola-Fadunsin *et al.*, 2018). In Thailand, bovine anaplasmosis had been reported in different regions since 1986, for example, Ratchaburi, Nakhon Pathom, Nakhon Sawan, Ayutthaya, Nan, Ubon Ratchathani, Roi Et, Surin, Buri Ram, Sisaket, Satun and Nakhon Si Thammarat (Tananyutthawongese *et al.*, 1999). Furthermore, the occurrence of *Anaplasma* infections in beef cattle and water buffaloes during 2001 to 2017 has been shown in ranging from 0.03 to 65.2% (Saetiew *et al.*, 2015; Jirapattharasate *et al.*, 2017). Our results have revealed that the overall occurrence of *A. marginale* infection of cattle was similar to 8.8% (18/204) in water buffaloes from Sakon Nakhon (Saetiew *et al.*, 2015), 20% (11/55) in beef cattle from Chiang Rai and 3.3% (2/60) in beef cattle from Mae Hong Son (Jirapattharasate *et al.*, 2017). In addition, the results showed that the *A. marginale* infection in cattle from Sakon Nakhon and Chiang Mai were 6.93% (32/462) and 58.62% (34/58), respectively. Moreover, our investigation is the first study that demonstrated a molecular occurrence of *A. marginale* infection in cattle in Chiang Mai. From our results, it is possible that *A. marginale* infections affected in either healthy or subclinical cattle without clinical signs. Furthermore, the significant differences in occurrence between two provinces exhibited that there were some environmental factors enhancing *A. marginale* infections in cattle especially Chiang Mai located in the border area of Thailand. The migration of animals was clearly found at that province and also a risk of transboundary disease might be increased. In addition, the seasonal sampling and population density of ticks and cattle raising might be the risk factors of *A. marginale* infections. Interestingly, no positive result was obtained from buffaloes blood samples in both provinces. We could hypothesize that there is no number of buffaloes presenting *A. marginale* DNA in blood samples which might have been resulted from the innate resistance of animals to tick, e.g. *Rhipicephalus* sp., attachment to their skin.

The high genetic diversity of *msps* observed among different isolates could be involved with the immune evasion strategies of *A. marginale* (de la Fuente *et al.*, 2005). In general, the genetic diversity of *A. marginale* strains derived from bovine erythrocytes has been characterized based on the sequences of *msp* genes, and several of which have been

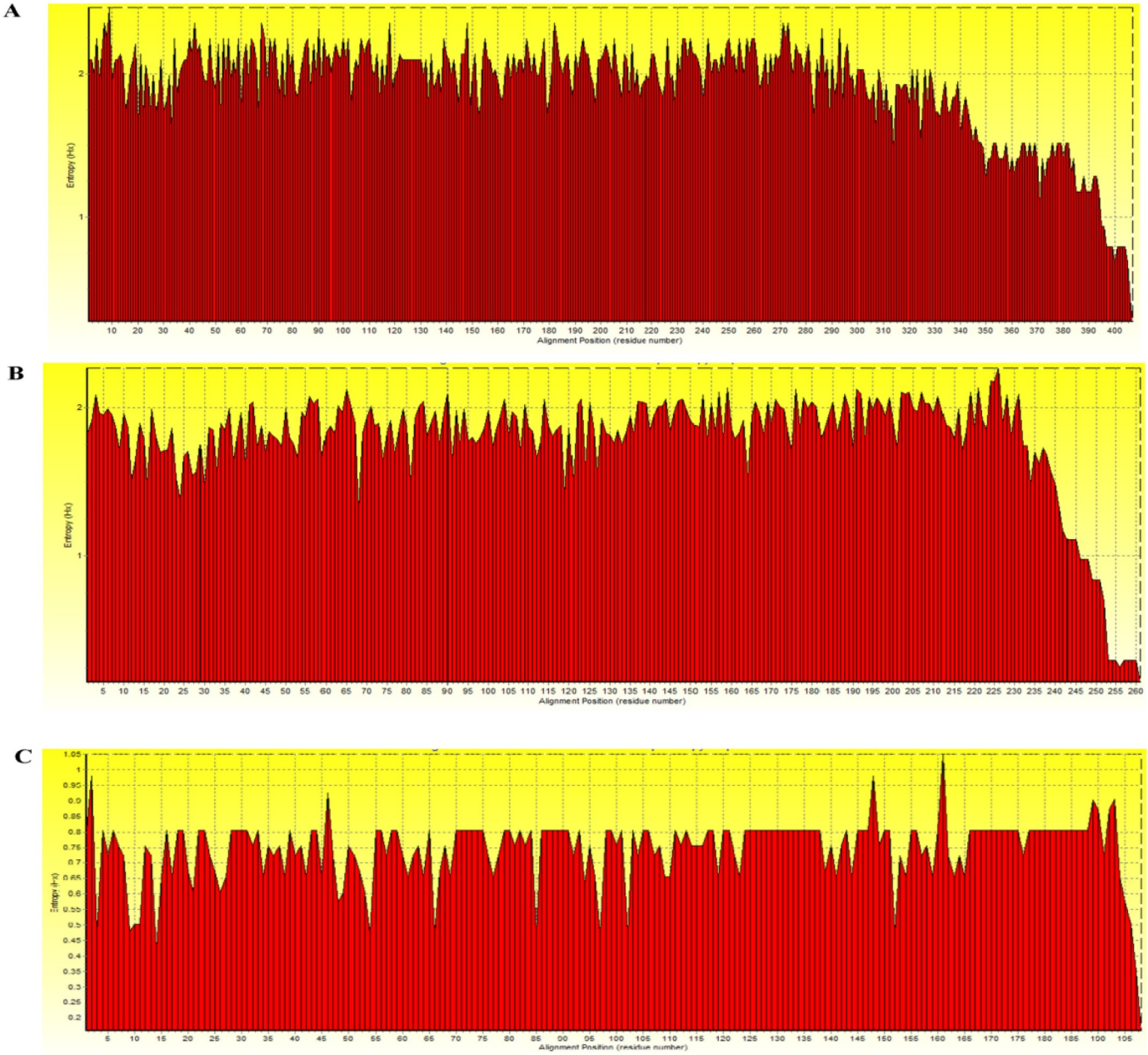


Fig. 5. Amino acid entropy plot obtained from *A. marginale msp2* (A), *msp4* (B) and *msp5* (C) gene sequences.

shown to be involved in host cell-pathogen interactions (Kocan *et al.*, 2003) such as a survival strategy of parasites (Deitsch *et al.*, 2009). The genetic diversity among the *msp* genes has been studied in several countries (de la Fuente *et al.*, 2002), almost all of these studies invariably focused on the genetic diversity of *msp1* and *msp4* genes of *A. marginale* (Löhr *et al.*, 2002). In addition, it has been reported that the *msp2* gene encoding immunodominant outer membrane protein 2 (MSP2) allowing *A. marginale* to evade the mammalian host immune system during persistent infection (Löhr *et al.*, 2002; Brown *et al.*, 2003). The genes encoding MSPs are considered as useful markers in the molecular epidemiology of *A. marginale* infection as confirmed by a number of the previous studies (Molad *et al.*, 2009). Although the genetic diversity of *A. marginale* strains has been characterized in Thailand (Saetiew *et al.*, 2015; Jirapattharasate *et al.*, 2017), the information about the genetic diversity and the phylogeny of *A. marginale* strains were still limited to the *msp4* gene analysis. In the present study, the gene fragments encoding the *msp2, msp4* and *msp5* major surface proteins in cattle population sampled in the north and northeastern of Thailand were utilized to determine the genetic diversity of *A. marginale* in these regions. The phylogenetic analysis indicated that *A. marginale* Thailand isolates could be classified into at least 7 groups by using *msp2* gene sequences. The geographical relationships among isolates were not established, because several Thailand sequences were clustered together with the sequences from Florida, Oklahoma and Washington. The phylogenetic analysis of *msp4* gene sequence revealed only 1 cluster together with the sequences from Taiwan, Mexico, Argentina, Brazil, Germany, Australia, India, China and Spain, whereas *msp5* gene sequence revealed in 4 clusters closed to the sequences from Germany, Japan, India, Egypt, China, Brazil and the Philippines. Our findings showed that the genetic diversity observed in the phylogram was further confirmed by the moderate similarity values for *msp2* gene (63.9 to 88.2%) and *msp5* gene (91.9 to 98.9%). These finding revealed that the genetic diversity among *A. marginale* populations has been varied according to the geopraphical regions with further diversification

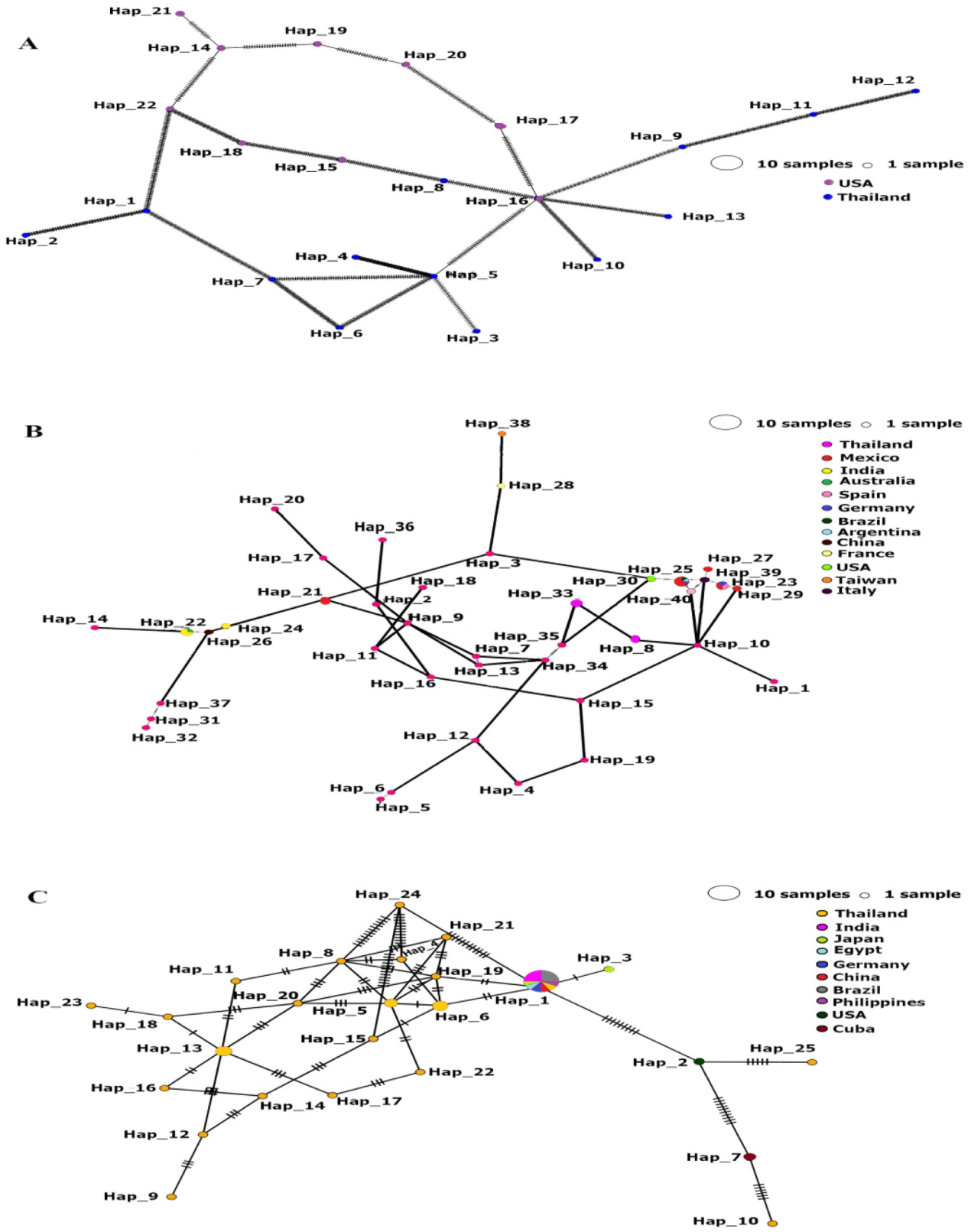


Fig. 6. TCS network of haplotypes based on the *Anaplasma marginale msp2* (A), *msp4* (B) and *msp5* (C) gene sequences detected in Thailand and worldwide (Small traits between a haplotype and another stand for mutational occurrence).

resulting from local selection pressures (de la Fuente *et al.*, 2005). Moreover, the high similarity value was detected for *msp4* gene (93.4 to 100%). This finding indicated phylogenetic proximity of the *A. marginale msp4* gene circulating in both different countries and Thailand.

The analysis of amino acid sequences of *A. marginale msp* genes approved the high polymorphism with 127, 75, and 51 entropy peaks reaching up to approximately 2.46 in *msp2*, 2.18 in *msp4* and 1.05 in *msp5* sequences, respectively. Our findings were similar to the entropy

Table 7
Polymorphism and genetic diversity of the *A. marginale msp2, msp4* and *msp5* sequences as detected in beef cattle in Thailand.

Genes	size (bp)	*N*	VS	GC%	h	Dh (mean ± SD)	π (mean ± SD)	*K*
Sequence within the Thailand								
msp2	1230	13	380	46	13	1.000 ± 0.030	0.22110 ± 0.01598	151.89774
msp4	849	29	673	49.2	27	0.995 ± 0.011	0.73639 ± 0.01054	495.59113
msp5	633	27	47	46.8	22	1.000 ± 0.010	0.01784 ± 0.00229	10.50997
Sequence worldwide								
msp2	1239	23	258	50.9	22	0.996 ± 0.014	0.34775 ± 0.02471	89.71861
msp4	859	52	673	48.7	40	0.982 ± 0.010	0.72670 ± 0.01147	489.0666
msp5	633	39	37	43	25	0.9298 ± 0.011	0.23667 ± 0.04449	8.75667

N = number of analyzed sequences; VS = number of variable sites; GC = G×C content; h = nunber of haplotypes; Dh = diversity of haplotypes; SD = standard deviation; π = nucleotide diversity (per site); *K* = average number of nucleotide differences.

peaks of *A. phagocytophilum msp2* as reported by Caspersen *et al.* (2002). Different genotype could involve being a high genetic diversity of *A. marginale* distributed in Thailand. These results implied that the variation of *msp* genes may help to the evasion of *A. marginale* from the host immunity.

In this study, the gene fragments encoding *msp* sequences in blood samples of cattle in the north and northeastern regions of Thailand were analyzed. Our results showed that the *A. marginale* population was highly diverse in Thailand, with the presence of probably more than one distinct haplotype. The genotypes of *A. marginale msp2, msp4* and *msp5* were discriminated in the haplotype networks. They were performed with the sequences detected in the present study together with other retrieved from GenBank database that found in other regions of the worlds. The result indicated that there was a high genetic diversity of the *A. marginale msp* genes observed in the different haplotype networks in Thailand and worldwide when compared to the previous study as described by Ramos *et al.* (2019) who demonstrated that *A. marginale msp4* from Brazil showed low polymorphism. In addition, sequences detected in the present study were clustered with sequences from other countries, suggesting that none of the haplotypes found in this study was unique in Thailand. Moreover, *msp2, msp4* and *msp5* sequences shared genetic traits with all sequences previously detected worldwide. These findings showed that the genetic diversity among *A. marginale* populations varied according to the geographical region. On the other hand, *A. marginale msp5* sequences were allocated in one haplotype with all sequences worldwide. This implied that the *msp5* haplotypes have a more distinct phylogenetic relationship. Furthermore, the verified genetic heterogeneity involving the antigenic variations among *A. marginale* populations may result in limiting to use of antigen or recombinant vaccines for the incomplete control of disease.

The B-cell epitope prediction analyzed in this study indicated that the polymorphisms in the epitopes might reflect the functional pertinence of the genetic diversity. Our finding exhibited that the predicted epitopes of MSPs (MSP2, MSP4 and MSP5) among Thailand isolates were highly conserved. However, five epitopes (MALVAAVVQVHSLLPAP, EVLFSD, SFYIGLDLSPAFG, SKKVCKG, and KCAVGTN) were not conserved in MSP2. Eventhough, a single epitope was highly conserved among all the MSP sequences, it is possible that this peptide might not function as an epitope, as it is located within a signal peptide. In addition, the high polymorphisms investigated among the epitopes showed that each Thailand MSP sequence might differ on its antigenic character. Unlike our study, the previous studies by Shkap, *et al.* (2002), reported that *msp2* was a protection-inducing immunogen against *A. marginale* challenged as the CD4+ T cell epitope. Moreover, this epitope is composed of conservative amino acid sequences which stimulated and recalled immune response in vaccinated cattle (Shkap *et al.*, 2002; Abbott *et al.*, 2004). MSP2 variable-region epitopes have been shown to be surface exposed, and development of variant-specific antibody is associated with variant clearance during persistent infection (French *et al.*, 1999; Oliveira *et al.* 2003; Noh, *et al.*, 2010).

In conclusion, this study indicated a low molecular occurrence of *A. marginale* in cattle blood samples in Thailand. However, we have found a high degree of genetic diversity in analyzed *A. marginale* populations. Our findings demonstrated that *A. marginale msp2* gene is genetically diverse, while *msp4* and *msp5* genes are conserved in Thailand. These findings could help to improve the understanding of phylogeny and genetic diversity among *msps* of *A. marginale* Thailand strain. Hence, there is a possibility that our finding could be used for immunodiagnosis purposes and as a vaccine candidate for anaplasmosis, which will be studied further.

CRediT authorship contribution statement

Witchuta Junsiri: Conceptualization, Formal analysis, Methodology, Validation, Investigation, Writing - original draft, Visualization, Project administration. **Amaya Watthanadirek:** Resources. **Napassorn Poolsawat:** Resources. **Sarawan Kaewmongkol:** Resources. **Sathaporn Jittapalapong:** Resources. **Runglawan Chawengkirttikul:** Resources. **Panat Anuracpreeda:** Conceptualization, Formal analysis, Methodology, Validation, Investigation, Resources, Data curation, Writing - review & editing, Visualization, Supervision, Project administration, Funding acquisition.

Declaration of Competing Interest

None

Acknowledgements

This research was financially supported by Research Grants from TRF Research Career Development Grant co-funded by The Thailand Research Fund and Mahidol University [grant number RSA6080071] and Agricultural Research Development Agency (Public Organization) or ARDA to Panat Anuracpreeda, and Royal Golden Jubilee Ph.D. (RGJ-PHD) Scholarship [grant number PHD/0035/2559] to Witchuta Junsiri.

Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.actatropica.2020.105338.

References

Abbott, J.R., Palmer, G.H., Howard, C.J., Hope, J.C., Brown, W.C., 2004. *Anaplasma marginale* major surface protein 2 CD4+-T-cell epitopes are evenly distributed in conserved and hypervariable regions (HVR), whereas linear B-cell epitopes are predominantly located in the HVR. Infect. Immun. 72, 7360–7366.

Aktas, M., Özübek, S., 2017. Outbreak of anaplasmosis associated with novel genetic variants of *Anaplasma marginale* in dairy cattle. Comp. Immunol. Microbiol. Infect. Dis. 54, 20–26.

Awad, H., Antunes, S., Galindo, R.C., do Rosário, V.E., de la Fuente, J., Domingos, A., el Husseina, A.M., 2011. Prevalence and genetic diversity of *Babesia* and *Anaplasma* species in cattle in Sudan. Vet. Parasitol 181, 146–152.

Aubry, P., Geale, D.W., 2011. A review of bovine anaplasmosis. Transbound. Emerg. Dis. 58, 1–30.

Brown, W.C., Brayton, K.A., Styer, C.M., Palmer, G.H., 2003. The hypervariable region of *Anaplasma marginale* major surface protein 2 (MSP2) contains multiple immunodominant CD4 + T lymphocyte epitopes that elicit variant-specific proliferative and IFN-gamma responses in MSP2 vaccinates. J. Immunol 170, 3790–3798.

Carelli, G., Decaro, N., Lorusso, A., Elia, G., Lorusso, E., Mari, V., Ceci, L., Buonavoglia, C., 2007. Detection and quantification of *Anaplasma marginale* DNA in blood samples of cattle by real-time PCR. Vet. Microbiol 124, 107–114.

Caspersen, K., Park, J., Patil, S., Dumler, J.S., 2002. Genetic Variability and Stability of *Anaplasma phagocytophila msp2* (p44). Infect. Immun. 70, 1230–1234.

Castañeda-Ortiz, E.J., Ueti, M.W., Camacho-Nuez, M., Mosqueda, J.J., Mousel, M.R., Johnson, W.C., Palmer, G.H., 2015. Association of *Anaplasma marginale* strain superinfection with infection prevalence within tropical regions. PLoS One 10, e0120748. https://doi.org/10.1371/journal.pone.0120748.

Clement, M., Snell, Q., Walker, P., Posada, D., Crandall, K., 2002. TCS: Estimating gene genealogies. Parallel and Distributed Processing Symposium. International Proceedings 2, 184.

Deitsch, K.W., Lukehart, S.A., Stringer, J.R., 2009. Common strategies for antigenic variation by bacterial, fungal and protozoan pathogens. Nat. Rev. Microbiol. 7, 493–503.

de la Fuente, J., Van Den Bussche, R.A., Garcia-Garcia, J.C., Rodríguez, S.D., García, M.A., Guglielmone, A.A., Mangold, A.J., Friche Passos, L.M., Barbosa Ribeiro, M.F., Blouin, E.F., Kocan, K.M., 2002. Phylogeography of New World isolates of *Anaplasma marginale* based on major surface protein sequences. Vet. Microbiol 88, 275–285.

de la Fuente, J., Van Den Bussche, R.A., Prado, T., Kocan, K.M., 2003. *Anaplasma marginale msp1*alpha genotypes evolved under positive selection pressure but are not markers for geographic isolates. J. Clin. Microbiol. 41, 1609–1616.

de la Fuente, J., Torina, A., Caracappa, S., Tumino, G., Furlá, R., Almazán, C., Kocan, K.M., 2005. Serologic and molecular characterization of *Anaplasma* species infection in farm animals and ticks from Sicily. Vet. Parasitol 133, 357–362.

El-Ashker, M., Hotzel, H., Gwida, M., El-Beskawy, M., Silaghi, C., Tomaso, H., 2015. Molecular biological identification of *Babesia, Theileria*, and *Anaplasma* species in cattle in Egypt using PCR assays, gene sequence analysis and a novel DNA microarray. Vet. Parasitol. 207, 329–334.

Felsenstein, J., 1985. Confidence limits on phylogenies: An approach using the bootstrap. Evolution 39, 783–791.

French, D.M., Brown, W.C., Palmer, G.H., 1999. Emergence of *Anaplasma marginale* antigenic variants during persistent rickettsemia. Infect. Immun. 67, 5834–5840.

Hall, T.A., 1999. BioEdit: a user-friendly biological sequence alignment editor and analysis program for Windows 95/98/NT. Nucl. Acids. Symp. Ser. 41, 95–98 http://tools.immuneepitope.org /bcell/.

Jirapattharasate, C., Moumouni, P.F.A., Cao, S., Iguchi, A., Liu, M., Wang, G., Zhou, M., Vudriko, P., Efstratiou, A., Changbunjong, T., Sungpradit, S., Ratanakorn, P., Moonarmart, W., Sedwisai, P., Weluwanarak, T., Wongsawang, W., Suzuki, H., Xuan, X., 2017. Molecular detection and genetic diversity of bovine *Babesia* spp., *Theileria orientalis*, and *Anaplasma marginale* in beef cattle in Thailand. Parasitol. Res. 116, 751–762.

Kimura, M., 1980. A simple method for estimating evolutionary rate of base substitutions through comparative studies of nucleotide sequences. J. Mol. Evol. 16, 111–120.

Kocan, K.M., Fuente, J., Guglielmone, A.A., Melendez, R.D., 2003. Antigens and alternatives for control of *Anaplasma marginale* infection in cattle. Clin. Microbiol. Rev. 16, 698–712.

Kocan, K.M., de la Fuente, J., Cabezas-Cruz, A., 2015. The genus *Anaplasma*: New challenges after reorganization. In Revue Scientifigue et technique (International Office of Epizootics) 34, 577–586.

Kolaskar, A.S., Tongaonkar, C., 1990. A semi-empirical method for prediction of antigenic determinants on protein antigens. FEBS Lett 276, 172–174.

Kumar, S., Stecher, G., Tamura, K., 2016. MEGA7: Molecular evolutionary genetics analysis version 7.0 for bigger datasets. Mol. Biol. Evol. 33, 1870–1874.

Leigh, J.W., Bryant, D., 2015. PopART: full-feature software for haplotype network construction. Methods Ecol Evol 6, 1110–1116.

Librado, P., Rozas, J., 2009. DnaSP v5: a software for comprehensive analysis of DNA polymorphism. Bioinformatics 25, 1451–1452.

Löhr, C.V., Rurangirwa, F.R., McElwain, T.F., Stiller, D., Palmer, G.H., 2002. Specific expression of *Anaplasma marginale* major surface protein 2 salivary gland variants occurs in the midgut and is an early event during tick transmission. Infect. Immun. 70, 114–120.

Molad, T., Fleidrovich, L., Mazuz, M., Fish, L., Leibovitz, B., Krigel, Y., Shkap, V., 2009. Genetic diversity of major surface protein 1a of *Anaplasma marginale* in beef cattle. Vet. Microbiol 136, 54–60.

Mutshembele, A.M., Cabezas-Cruz, A., Mtshali, M.S., Thekisoe, O.M.M., Galindo, R.C., de la Fuente, J., 2014. Epidemiology and evolution of the genetic variability of *Anaplasma marginale* in South Africa. Ticks Tick Borne Dis 5, 624–631.

Nei, M., Kumar, S., 2000. Molecular evolution and phylogenetics. Oxford University Press, New York.

Noh, S.M., Zhuang, Y., Futse, J.E., Brown, W.C., Brayton, K.A., Palmer, G.H., 2010. The immunization-induced antibody response to the *Anaplasma marginale* major surface protein 2 and its association with protective immunity. Vaccine 28, 3741–3747.

Obregón, D, Corona, BG, de la Fuente, J, Cabezas-Cruz, A, Gonçalves, RL, Antonio Matos, C, Armas, YV, Hinojosa, Y, Alfonso, P, Oliveira, MCS, Machado, RZ, 2018. Molecular evidence of the reservoir competence of water buffalo (*Bubalus bubalis*) for *Anaplasma marginale* in Cuba. Vet. Parasitol. 13, 180–187.

Obregón, D, Cabezas-Cruz, A, Armas, Y, Silva, JB, Fonseca, AH, André, MR, Alfonso, P, Oliveira, MCS, Machado, RZ, Corona-González, B, 2019. High co-infection rates of *Babesia bovis, B. bigemina* and *Anaplasma marginale* in water buffalo in Western Cuba. Parasitol. Res. 118, 955–967.

Ola-Fadunsin, S.D., Gimba, F.I., Abdullah, D.A., Sharma, R.S.K., Abdullah, F.J.F., Sani, R.A., 2018. Epidemiology and risk factors associated with *Anaplasma marginale* infection of cattle in Peninsular Malaysia. Parasitol. Int. 67, 659–665.

Oliveira, B.J., Madruga, R.C., Schenk, M., Kessler, H.R., Miguita, M., Araújo, R.F., 2003. Antigenic Characterization of Brazilian Isolates of *Anaplasma marginale*. Mem. Inst. Oswaldo. Cruz. 98, 395–400.

Ramabu, S.S., Kgwatalala, P.M., Nsoso, S.J., Gasebonwe, S., Kgosiesele, E., 2018. *Anaplasma* infection prevalence in beef and dairy cattle in the south east region of Botswana. Vet. Parasitol. 12, 4–8.

Ramos, I.A.S., Herrera, H.M., Mendes, N.S., Fernandes, S.J., Campos, J.B.V., Alves, J.V.A., Mecedo, G.C., Machado, R.Z., André, M.R., 2019. Phylogeography of *msp4*genotypes of *Anaplasma marginale* in beef cattle from the Brazilian Pantanal. Braz. J. Vet. Parasitol. https://doi.org/10.1590/s1984-29612019049.

Saetiew, N., Simking, P., Inpankaew, T., Wongpanit, K., Kamyingkird, K., Wongnakphet, S., Stich, R.W., Jittapalapong, S., 2015. Prevalence and genetic diversity of *Anaplasma marginale* infections in water buffaloes in Northeast Thailand. J. Trop. Med. Parasitol. 38, 9–16.

Saitou, N., Nei, M., 1987. The neighbor-joining method: A new method for reconstructing phylogenetic trees. Mol. Biol. Evol. 4, 406–425.

Shkap, V., Molad, T., Brayton, A.K., Brown, C.W., Palmer, G.H., 2002. Expression of Major Surface Protein 2 Variants with Conserved T-Cell Epitopes in *Anaplasma centrale* Vaccinates. Infect. Immun 70, 642–648.

Tananyutthawongese, C., Saengsombut, K., Sukhumsirichat, W., Uthaisang, W., Sarataphan, N., Chansiri, K., 1999. Detection of bovine hemoparasite infection using multiplex polymerase chain reaction. Sci. Asia. 25, 85–90.

Tamekuni, K., Kano, F.S., Atalíba, A.C., Marana, E.R.M., Venâncio, E.J., Vidotto, M.C., Garcia, J.L., Headley, S.A., Vidotto, O., 2009. Cloning, expression, and characterization of the MSP1a and MSP1b recombinant proteins from PR1 *Anaplasma marginale* strain. Brazil. Res. Vet. Sci. 86, 98–107.

Watthanadirek, A., Chawengkirttikul, R., Poolsawat, N., Junsiri, W., Boonmekam, D., Reamtong, O., Anuracpreeda, P., 2019. Recombinant expression and characterization of major surface protein 4 from *Anaplasma marginale*. Acta Trop 197, 105047. https://doi.org/10.1016/j.actatropica.2019.105047.

Chula Med J Vol. 62 No. 3 May - June 2018

Expression of recombinant major surface protein 5 of *Anaplasma marginale* (*A. marginale*) at different temperatures

Amaya Watthanadirek* Thunyaporn Doungdaolek**
Preeyaporn Koedrith* Panat Anuracpreeda***

Watthanadirek A, Doungdaolek T, Koedrith P, Anuracpreeda P. Expression of recombinant major surface protein 5 of *Anaplasma marginale (A. marginale*) at different temperatures. Chula Med J 2018 May – Jun;62(3):

Background : *Bovine anaplasmosis is an important tick-borne disease caused by Anaplasma marginale (A. marginale) and infected in ruminants, mostly in cattle. This disease occurs in tropical and subtropical regions including Thailand and causes a major problem to livestock productions. The major surface protein 5 (MSP5) is one of outer membrane protein of A. marginale which as an immunodominant protein encoded by a single gene and also highly conserved gene.*

Objective : *The aim of this study was to optimize the conditions for the expression of recombinant major surface protein 5 (rMSP5) of A. marginale.*

Methods : *The msp5 gene of A. marginale was cloned into the pET100/D-TOPO® vector to produce an pET100-msp5-6xHis fusion gene construct. The recombinant proteins were expressed by the plasmids in Escherichia coli host strain BL21 star™ (DE3) at different temperatures (16, 25 and 37 ^{o}C) for 6 h. The proteins were analyzed by SDS-PAGE and confirmed the target protein by Western blotting using antisera against His.*

* Faculty of Environment and Resource Studies, Mahidol University
** Faculty of Medical Science, Naresuan University
***Institute of Molecular Biosciences, Mahidol University

Results : *After induction with 0.1 mM of Isopropyl β-D-1-thiogalactopyranoside (IPTG) at different temperatures for protein expression, the protein was not produced at 6 h for 6 ^{o}C. On the other hand, the rMSP5 protein was produced at 25 and 37 ^{o}C for 2-6 h but the expressive protein at 25 ^{o}C showed lower yield than that at 37 ^{o}C.*

Conclusion : *In this study, the best condition for rMSP5 protein expression was cultured at 37 ^{o}C for 4 h. The protein was identified as the rMSP5 at the molecular weight of 26 kDa.*

Keywords : *Bovine anaplasmosis, Anaplasma marginale, MSP5, recombinant protein, protein expression.*

Correspondence to: Anuracpreeda P. Institute of Molecular Biosciences, Mahidol University, Salaya, Nakhon Pathom 73170, Thailand.
E-mail: Panat1@yahoo.com, panat.anu@mahidol.ac.th

Received for publication: February 15, 2018.

อมาญาส์ วัฒนดิเรก, ธันยาภรณ์ ดวงดาวเล็ก, ปรียาพร เกิดฤทธิ์, ปณัฐ อนุรักษ์ปรีดา. การแสดงออกของ Recombinant major surface protein 5 ของ *Anaplasma marginale* (*A. marginale*) ในสภาวะอุณหภูมิที่แตกต่างกัน. จุฬาลงกรณ์เวชสาร 2562 พ.ค. – มิ.ย.; 62(3):

เหตุผลของการทำวิจัย : *Anaplasma marginale (A. marginale) เป็นสาเหตุที่ทำให้เกิดโรคพยาธิในเม็ดเลือดโคและกระบือ (bovine anaplasmosis) ซึ่งก่อให้เกิดปัญหาสำคัญในอุตสาหกรรมการผลิตสัตว์ และส่งผลกระทบอย่างมากทางเศรษฐกิจ Major Surface Protein 5 (MSP5) เป็น immunodominant protein ที่อยู่บนเยื่อหุ้มเซลล์ของเชื้อ A. marginale จึงมีบทบาทสำคัญในการนำมาใช้พัฒนาการตรวจวินิจฉัยโรค bovine anaplasmosis ให้มีความแม่นยำและรวดเร็วมากขึ้น ดังนั้นการศึกษาการผลิต recombinant major surface protein 5 (rMSP5) จึงมีความจำเป็นเพื่อใช้ในการศึกษาและพัฒนาการตรวจวินิจฉัยทางภูมิคุ้มกันให้มีความจำเพาะต่อโรค ซึ่งจะเป็นประโยชน์ในการรักษาต่อไป*

วัตถุประสงค์ : *เพื่อศึกษาการแสดงออกของ rMSP5 ของเชื้อ A. marginale ในสภาวะอุณหภูมิที่แตกต่างกัน*

วิธีการทำวิจัย : *ในการศึกษานี้ยีน msp5 ได้ถูกเชื่อมต่อเข้ากับ pET100/D TOPO vector และทำการกระตุ้นให้เกิดการแสดงออกของโปรตีนใน E.coli BL21 star™ (DE3) โดยใช้ Isopropyl β-D-1-thiogalactopyranoside (IPTG) ที่อุณหภูมิ 16, 25 และ 37 องศาเซลเซียส เป็นเวลา 6 ชั่วโมง และทำการวิเคราะห์การผลิต rMSP5 ด้วยวิธี SDS-PAGE และ Western blot*

ผลการศึกษา : *การแสดงออกของ rMSP5 ในอุณหภูมิที่แตกต่างกันนั้น ไม่พบการแสดงออกของ rMSP5 ที่ 16 องศาเซลเซียส ในเวลา 6 ชั่วโมง ในขณะที่ 25 และ 37 องศาเซลเซียส พบการแสดงออกของ rMSP5 ในเวลา 2 - 6 ชั่วโมง แต่อย่างไรก็ตามที่ 25 องศาเซลเซียสพบการแสดงออกของ rMSP5 ต่ำกว่าที่ 37 องศาเซลเซียส*

สรุป : *หลังจากถูกระตุ้นให้มีการแสดงออกของโปรตีนด้วย IPTG นั้น สภาวะอุณหภูมิที่เหมาะสมในการแสดงออกของโปรตีน rMSP5 คือที่ 37 องศาเซลเซียส เป็นเวลา 4 ชั่วโมง โดยโปรตีน rMSP5 มีขนาดประมาณ 26 kDa*

คำสำคัญ : *โรคพยาธิในเม็ดเลือดโคและกระบือ, Anaplasma marginale, MSP5, recombinant protein, การแสดงออกของโปรตีน.*

Anaplasma marginale, the cause of bovine anaplasmosis in ruminants mostly cattle is endemic in the tropical and subtropical areas.[1] The transmission of *A. marginale* is affected by tick and blood contaminated fomites such as needles and ear-tagging devices. [2] The clinical symptoms of the disease include fever, severe anemia, jaundice, weight loss, abortion, decreased milk production and often death in animals over two years old. Hence, the anaplasmosis causes significant economic loss to livestock productions in many countries worldwide including Thailand. [3] The conventional method for diagnosis of anaplasmosis is based on giemsa-stained blood smears. However, this method is not reliable for detecting *A. marginale* in pre-symptomatic stage of the animals and PCR assay should be an alternative method for confirmation of the strain identity. [4]

Presently, the six major surface proteins (MSPs) of *A. marginale* have been identified from bovine erythrocyte, designated as MSP1a, MSP1b, MSP2, MSP3, MSP4 and MSP5. As for the MSP1a, MSP1b and MSP2 there have been reports about the function of these proteins which are adhesin for bovine erythrocytes. In addition, the function of MSP1a was also shown to be adhesin for tick gut cells [5], while the function of MSP3, MSP4 and MSP5 have not yet been reported.

The MSP5 is an immunodominant protein encoded by a single gene. [6] The *msp*5 gene is present in the bovine erythrocytes in all stages of *A. Marginale.* [7] The MSP5 protein is a potentially useful as a diagnostic antigen in a competitive enzyme-linked immunosorbent assay (cELISA) for diagnosis of anaplasmosis. [8] As for rMSP5, it is important to have information regarding its structure and function in the future. In this study, we have optimized the conditions for expression of rMSP5 of *A. marginale* in order to provide information for development of diagnostic technique of anaplasmosis in the future.

Materials and Methods

Construction of msp5 gene

The TOPO® cloning reaction was performed by adding of the PCR products of *msp*5 gene into the pET100/D-TOPO® vector (Invitrogen Life Technologies). The plasmid DNA was transformed into *Escherichia coli* by using the heat-shock method. Briefly, the 10 ng of plasmid DNA was added into competent *Escherichia coli* host strain BL21 star™ (DE3) (Invitrogen). The cells were incubated on ice for 30 min and transferred to heat-shock for 1 min at 42 ^{o}C. Then the cells were placed back on ice immediately. The Super Optimal broth with Catabolite repression (S.O.C.) medium (Invitrogen) was added into the transformed cells and incubated for 30 min at 37 ^{o}C. Then, the transformation reaction was added into 10 ml of Luria-Bertani (LB) broth containing 100 μg/ml of ampicillin and incubated with shaking at 37 ^{o}C for overnight.

Protein expression of the msp5 gene

The protein expression was carried out by inoculating the cells in LB broth and incubated with shaking at 37 ^{o}C to an optical density of 600 nm (OD_{600}) was 0.5 - 0.8 (mid-log phase). For induction, Isopropyl β-D-1-thiogalactopyranoside (IPTG) was added in the culture to a final concentration of 0.1 mM and incubated with shaking at difference temperature (16, 25 and 37 ^{o}C) for 6 h. The cell culture was removed

at every 2 h until 6 h to choose the best time for expression. The cells were centrifuged at 8,000 xg for 10 min at 4 ^{o}C as well as collected and frozen at -20 ^{o}C.

Protein analysis by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE)

The cell pellet was resuspended in buffer containing 20 mM HEPES and 50 mM NaCl, pH 7.4, mixed with electrophoresis sample buffer (200 mM Tris-HCl, 8% SDS, 40% Glycerol and 0.4% Bromophenol blue, pH 6.8) and boiled for 5 min. Protein samples were separated by 12% polyacrylamide gel in electrophoresis buffer (192 mM glycine, 25 mM Tris and 0.1% SDS) at constant 80 Voltages. The gels were stained with Coomassie Brilliant Blue staining solution and destained with destain buffer (10% (v/v) of EtOH and 10% (v/v) of glacial acetic acid in distilled water).

Western Blot analysis

After the proteins were separated by SDS-PAGE, the gel was soaked in transfer buffer (25 mM Tris, 150 mM Glycine and 10% (v/v) ethanol). Subsequently, the proteins were transferred onto nitrocellulose membrane by wet transfer technique with constant current for 1 h. The membrane was blocked in 5% (w/v) bovine serum albumin (BSA) in phosphate buffered saline (PBS) buffer at room temperature for 45 min. Then, the membrane was incubated with Anti-His as a primary antibody at dilution of 1:3000 for 45 min. The membrane was washed with 0.1% tween 20 in PBS for 3 times. The secondary antibody (anti-mouse IgG antibody) conjugated with the alkaline phosphatase (ALP) in 5% BSA at dilution of 1:5000 was added on to the membrane and incubated for 45 min. After washing, the color reaction was developed by using ALP substrate (75 mg/ml of NBT and 50 mg/ml of BCIP) and the reaction was stopped by adding of distilled water.

Results

Expression of recombinant MSP5 in Escherichia coli BL21 star^{TM} (DE3)

The recombinant plasmid pET100-*msp*5 was transformed into the *E. coli* BL21 star^{TM} (DE3) expression host and cultured in LB broth at different temperatures (16, 25 and 37 ^{o}C). After induction with 0.1 mM IPTG for 6 h, the induced culture at 16, 25 and 37 ^{o}C were analyzed by SDS-PAGE and compared with uninduced culture. The rMSP5 protein expressed at 16 ^{o}C did not show the target band on the gel (Figure 1). As for protein expression at 25 ^{o}C, the rMSP5 protein was shown on estimated molecular weight of 26 kDa at after induction 4-6 h (Figure 2), and the induced culture at 37 ^{o}C exhibited the rMSP5 protein on the target band on the size of 26 kDa at after induction 4 - 6 h (Figure 3).

Western blot analysis

The target band of rMSP5 protein at 37 ^{o}C was confirmed by Western blot using commercial anti-His. The results showed clearly band of expressed protein at 37 ºC for 2 - 6 h (Figure 4).

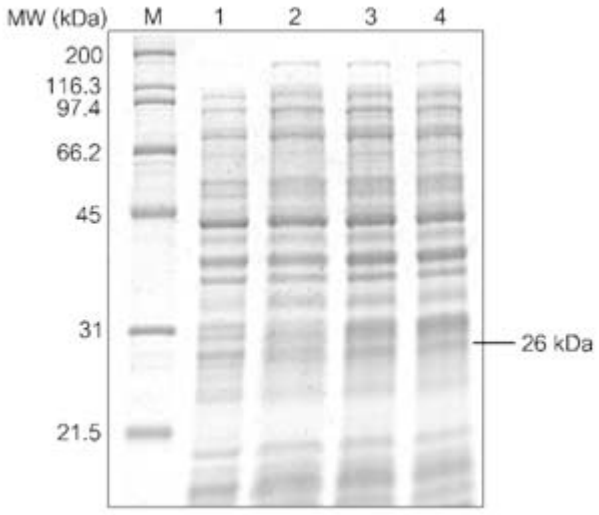


Figure 1. SDS-PAGE stained with Coomassie Brilliant Blue showed protein expression of BL21/pET100-*msp*5. The protein expression was induced by 0.1 mM IPTG at OD 0.5 - 0.8 and cultured at 16 ^{o}C. (M) Marker; (1) BL21/pET100-*msp*5 are not induced; (2) BL21/pET100-*msp*5 2 h after induction with IPTG; (3) BL21/pET100-*msp*5 4 h after induction with IPTG; (4) BL21/pET100-*msp*5 6 h after induction with IPTG.

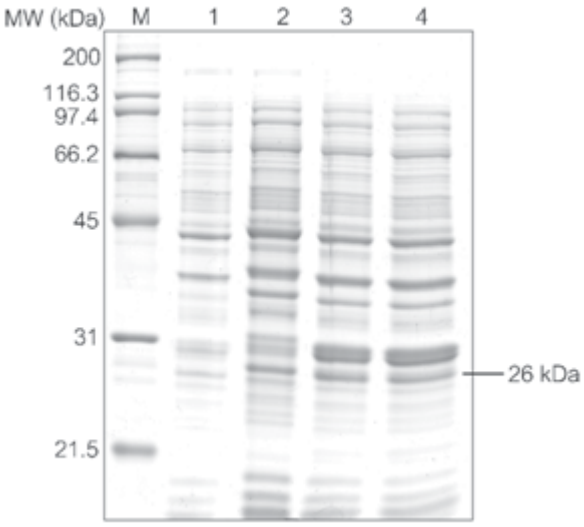


Figure 2. SDS-PAGE stained with Coomassie Brilliant Blue showed protein expression of BL21/pET100-*msp*5. The protein expression was induced by 0.1 mM IPTG at OD 0.5 - 0.8 and cultured at 25 ^{o}C. (M) Marker; (1) BL21/pET100-*msp*5 are not induced; (2) BL21/pET100-*msp*5 2 h after induction with IPTG; (3) BL21/pET100-*msp*5 4 h after induction with IPTG; (4) BL21/pET100-*msp*5 6 h after induction with IPTG.

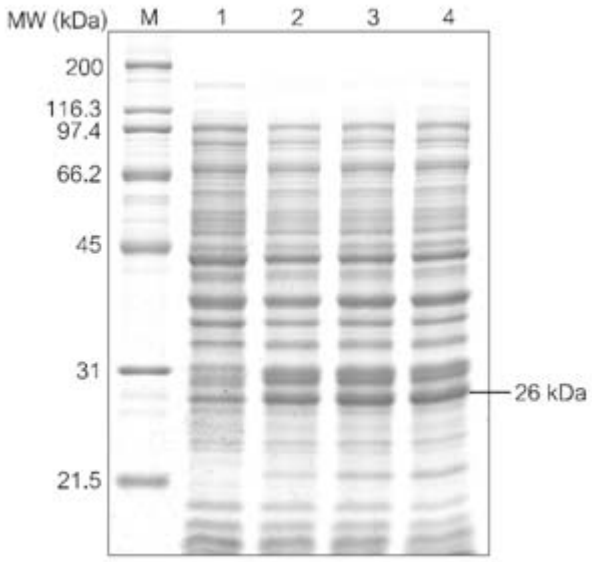


Figure 3. SDS-PAGE stained with Coomassie Brilliant Blue showed protein expression of BL21/pET100-*msp*5. The protein expression was induced by 0.1 mM IPTG at OD 0.5 - 0.8 and cultured at 37 ^{o}C. (M) Marker; (1) BL21/pET100-*msp*5 not induced; (2) BL21/pET100-*msp*5 2 h after induction with IPTG; (3) BL21/pET100-*msp*5 4 h after induction with IPTG; (4) BL21/pET100-*msp*5 6 h after induction with IPTG.

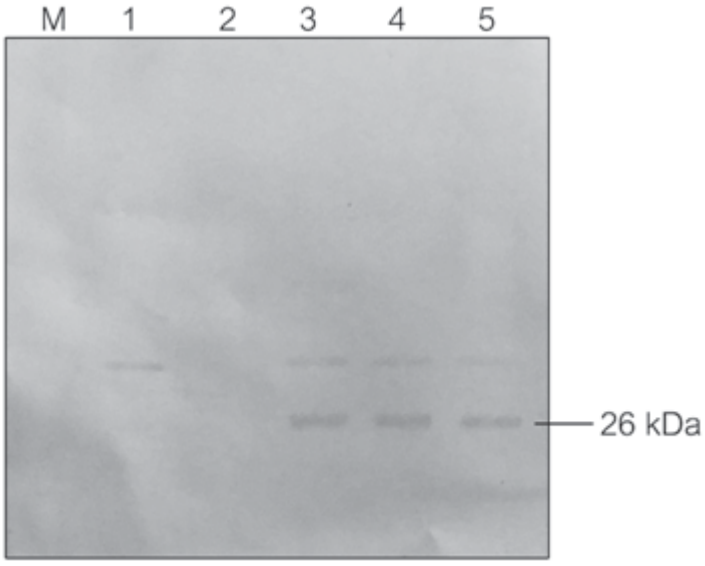


Figure 4. Western blot analysis of rMSP5 protein using anti-His antibodies. (M) Marker; (1) rMSP4 induced with IPTG (control); (2) rMSP5 not IPTG induced (control); (3) rMSP5 at 37^{o}C for 2 h after induction; (4) rMSP5 at 37 ^{o}C for 4 h after induction; (5) rMSP5 at 37 ๐C for 6 h after induction.

Discussion

This study, the *msp*5 gene isolated in Thailand and showed the molecular weight of rMSP5 protein at 26 kDa, being higher than other studied isolates. In the Florida isolate has been reported the molecular weight of rMSP5 is 19 kDa, which is constructed *msp*5 gene by using lambda ZAP and expressed protein in *E. coli* Y1090. [9] The rMSP5 from Havana isolate reported at 22 kDa. The gene of *msp*5 was cloned into pRSETB vector and expressed in *E. coli* BL21(DE3) pLysS strain. [10] This different size is occurred from different expression system. In the present study, we have used the pET100/D-TOPO® vector with histidine hexapeptide in N-terminal region for production of the recombinant fusion protein. The histidine tag residues fused to protein allow to increase the size of the recombinant protein.

The temperature of induction is one of the crucial factor affecting protein expression. In this study, the effect of different temperatures (16, 25 and 37 ^{o}C) on the rMSP5 expression levels showed the highest protein yields at 37 ^{o}C (2-6 h) compared with 25 ^{o}C. At 16 ^{o}C, rMSP5 has not been produced for 6 h because of the swift period of expression. In addition, the temperature at 25 ^{o}C showed the target band for 2 - 6 h after induction but the protein yields were lower than those at 37 ^{o}C. The duration time of expression at 37 ^{o}C for 4 h were sufficient to produce significant quantities of active protein because this time is an optimized condition for high yield of protein production. However, the higher temperature can cause a higher possibility of plasmid loss because of rapid growth of E. coli. Beside the study of temperature, for the further work, we will also concentrate on the duration time of protein expression for selecting the quality and activity of rMSP5.

Acknowledgements

This research was financially supported by a TRF Research Career Development Grant (RSA6080071) to Panat Anuracpreeda (co-funded by the Thailand Research Fund and Mahidol University).

References

1. Kocan KM, de la Fuente J, Blouin EF, Coetzee JF, Ewing S. The natural history of *Anaplasma marginale*. Vet Parasitol 2010;167:95-107.
2. Ewing SA. Transmission of *Anaplasma marginale* by arthropods. In Proceedings of the 7th National Anaplasmosis Conference Mississippi State University, Starkville, Mississippi; October 21-23, 1981. p. 395-423.
3. Atif FA. *Anaplasma marginale* and *Anaplasma phagocytophilum*: Rickettsiales pathogens of veterinary and public health significance. Parasitol Res 2015;114:3941-57.
4. Torioni dE, Knowles DP, McGuire TC, Palmer GH, Suarez CE, McElwain TF. Detection of cattle naturally infected with *Anaplasma marginale* in a region of endemicity by nested PCR and a competitive enzyme-linked immuno-sorbent assay using recombinant major surface protein 5. J Clin Microbiol 1998;36: 777-82.
5. McGAREY DJ, Allred DR. Characterization of hemagglutinating components on the *Anaplasma marginale* initial body surface and identification of possible adhesins. Infect Immun 1994;62:4587-93.
6. Ndung'u LW, Aguirre C, Rurangirwa FR, McElwain TF, McGuire TC, Knowles DP, et al. Detection of *Anaplasma ovis* infection in goats by major

surface protein 5 competitive inhibition enzyme-linked immunosorbent assay. J Clin Microbiol 1995;33:675-9.

7. Barbet A, Blentlinger R, Yi J, Lundgren A, Blouin E, Kocan K. Comparison of surface proteins of *Anaplasma marginale* grown in tick cell culture, tick salivary glands, and cattle. Infect Immun 1999;67:102-7.
8. Kocan KM, De la Fuente J, Guglielmone AA, Meléndez RD. Antigens and alternatives for control of *Anaplasma marginale* infection in cattle. Clin Microbiol Rev 2003;16:698-712.
9. Visser E, McGuire T, Palmer G, Davis W, Shkap V, Pipano E, et al. The *Anaplasma marginale* msp5 gene encodes a 19-kilodalton protein conserved in all recognized *Anaplasma* species. Infect Immun 1992;60:5139-44.
10. Corona B, Machado H, Rodriguez M, Martinez S. Characterization of recombinant MSP5 *Anaplasma marginale* Havana isolate. Braz J Microbiol 2009;40:972-9.
11. San-Miguel T, Pérez-Bermúdez P, Gavidia I. Production of soluble eukaryotic recombinant proteins in *E. coli* is favoured in early log-phase cultures induced at low temperature. SpringerPlus 2013;2.

Acta Tropica 197 (2019) 105047

Contents lists available at ScienceDirect

Acta Tropica

journal homepage: www.elsevier.com/locate/actatropica

Recombinant expression and characterization of major surface protein 4 from *Anaplasma marginale*

Amaya Watthanadirek[a], Runglawan Chawengkirttikul[b], Napassorn Poolsawat[a], Witchuta Junsiri[c,a], Dusit Boonmekam[a], Onrapak Reamtong[d], Panat Anuracpreeda[a,*]

[a] Institute of Molecular Biosciences, Mahidol University, Salaya, Nakhon Pathom, 73170, Thailand
[b] Department of Microbiology, Faculty of Science, Mahidol University, Rama VI Rd., Bangkok, 10400, Thailand
[c] Faculty of Veterinary Technology, Kasetsart University, Bangkok, 10900, Thailand
[d] Department of Molecular Tropical Medicine and Genetics, Faculty of Tropical Medicine, Mahidol University, Bangkok, 10400, Thailand

ARTICLE INFO

Keywords:
Anaplasma marginale
Major surface protein 4
msp4 coding sequence
Phylogenetic analysis
Immunolocalization

ABSTRACT

Anaplasma marginale is the rickettsia which causes the bovine anaplasmosis. The distribution of *A. marginale* is both tropical and subtropical regions of the world. The major surface protein 4 (MSP4) of this parasite was identified as an immunodominant protein. In this study, the full length of DNA encoding *A. marginale* MSP4 (AmMSP4) was cloned from the parasites. The open reading frame of *msp4* coding sequence of Thailand strain is 849 bp. Phylogenetic analysis revealed that the *msp4* coding sequence of *A. marginale* was highly conserved when compared with *Anaplasma phagocytophilum*. The recombinant plasmid was further transformed into the BL21-CodonPlus (DE3)-RIPL competent cells for over-expression of the recombinant major surface protein 4 of *A. marginale* (rAmMSP4). Sera from rabbit immunized with rAmMSP4 and from cattle infected with *A. marginale* were used to study the antigenicity of rAmMSP4 (35 kDa) and AmMSP4 (31 kDa). Both rAmMSP4 and AmMSP4 were recognized by these sera showing that recombinant and native AmMSP4 have conserved epitopes. Localization of *Anaplasma* parasites by immunofluorescence showed these parasites are distributed on both the membrane and the outside of infected erythrocytes. Regarding antigenicity, recombinant MSP4 could be used for immunodiagnostic purposes and as a possible vaccine candidate against anaplasmosis.

1. Introduction

Anaplasma marginale is an obligate intracellular rickettsia found exclusively within membrane-bound vacuoles in the erythrocytes cytoplasm of ruminants. *A. marginale* causes bovine anaplasmosis in domestic and wild animals (Dumler et al., 2001; Rymaszewska and Grenda, 2008; Kocan et al., 2010). It has been reported that *A. marginale* is the most prevalent tick-borne parasite of cattle worldwide (Kocan et al., 2010). Besides tick transmission, the transmission of *A. marginale* can occur by the use of any blood contaminated equipment during tattooing, dehorning, and ear tagging (Kocan and de la Fuente, 2003; Aubry and Geale, 2011). Bovine anaplasmosis occurs in tropical and subtropical regions thoughout the world including Thailand (Saetiew et al., 2015; Jirapattharasate et al., 2017; Sumrandee et al., 2016). Clinical signs of the disease include anemia, icterus without hemoglobinemia and hemoglobinuria, fever, anorexia, suppression of rumination, dryness of the muzzle, weight loss, lethargy and often death in animals older than two years. Abortions due to acute anaplasmosis can occur during late gestation (Correa et al., 1978; Kocan et al., 2010). The infection in animals causes the economic loss with high morbidity and mortality in most countries (Goodger et al., 1979; Kocan and de la Fuente, 2003).

A. marginale possesses several outer membrane proteins. Some are classified as major surface proteins (MSPs) including MSP1a, MSP1b, MSP2, MSP3, MSP4 and MSP5 (Barbet et al., 1987; Barbet and Allred, 1991; Visser et al., 1992; Oberle et al., 1993; Allerman and Barbet, 1996). MSP4 is an immunodominant protein and is a potential antigen for the control of anaplasmosis (Oberle et al., 1993; de la Fuente et al., 2002, 2005; Molad et al., 2004). It is therefore important to possess such information about immunogenicity, conservation among geographical strains and conservation of epitopes responsible for induction of protective immune responses by MSP4. Hence, our study aimed to clone and characterize a *msp4* coding sequence as well as overexpress and antigenically characterize MSP4 from an *A. marginale* isolate from Thailand in order to provide the basic information before further work is pursued in further studies of the immunodiagnostic and vaccine

* Corresponding author.
E-mail addresses: panat.anu@mahidol.edu, panat.anu@mahidol.ac.th (P. Anuracpreeda).

https://doi.org/10.1016/j.actatropica.2019.105047
Received 2 December 2018; Received in revised form 28 April 2019; Accepted 30 May 2019
Available online 31 May 2019
0001-706X/

potential of this protein.

2. Materials and methods

2.1. Parasite specimens

Blood samples of infected cattle were collected from abattoirs in Nakhon Ratchasima Province, Thailand. Genomic DNA of *A. marginale* was extracted from blood samples using a Blood/Cell DNA mini-kit (Geneaid, Taiwan).

2.2. Preparation of parasite antigen

The erythrocytes were isolated from the 40 ml of anticoagulant blood samples, and were washed seven times with phosphate-buffered saline (PBS), pH 7.2 by centrifugation at 9000 g at 4 °C for 20 min (Shkap et al., 1990). The pellet was resuspended in PBS and sonicated at 100 W for 3 min. The pellet was washed three times with the same buffer and centrifuged at 3000 g for 10 min. Thereafter, the pellet was resuspended in 0.4 ml of 50 mM Tris, pH 8.0 containing 5 mM EDTA and 1 mM PMSF. The concentration of the initial bodies extract was determined by Bradford's method (Bradford, 1976) and kept at – 20 °C until used.

2.3. Cloning of the msp4 coding sequence from Amarginale DNA

The *msp4* coding sequence of *A. marginale* DNA was amplified using a pair of primers constructed according to sequences in GenBank (L01987): a forward primer (5′-CACCATGAATTACAGAGAATTG-3′) and a reverse primer (5′-GCTGAACAGGA ATCTTGCTCC-3′). Four bases sequences (CACC) were added to the 5′ end of the forward primer to clone amplicon into pET100/D-TOPO® vector (Invitrogen, USA). The PCR reactions, containing 50 ng of DNA template, 1 μM each of the primers, 200 μM of each dNTPs, 1X Pfx Phusion HF buffer, nuclease free water and 0.5 U Phusion DNA polymerase (Invitrogen), were carried out in a thermal cycler (Bio-Rad, USA) with the following conditions: 94 °C for 5 min, 30 cycles of 94 °C for 1 min, 55 °C for 1 min, and 68 °C for 1 min, and at 68 °C for 7 min. The PCR products were stained with SYBRgreen (Smobio, USA) and analyzed by gel electrophoresis using 1.0% agarose gels.

The blunt-end PCR products were quantified; 20 ng was used as an insert in the pET100/D-TOPO® vector (Invitrogen Life Technologies). Chemically competent *Escherichia coli* host strain TOP10 cells (Invitrogen) was then transformed with the ligation product. Then, 200 μl of transformed bacterial culture was spread on the plates containing 100 μg ampicillin and incubated at 37 °C, overnight. The positive clones were selected and grown in Luria Bertani (LB) medium containing ampicillin, overnight. The plasmid extraction was performed by midi spin column plasmid kit (Geneaid) and analyzed for correctly-sized inserts by agarose gel electrophoresis.

2.4. Sequences and phylogenetic analysis

The presence of *msp4* inserts was confirmed by Sanger sequencing. The sequences were analyzed by BLAST (The National Center for Biotechnology Information, NCBI, http://www.ncbi.nlm.nih.gov/BLAST/). The nucleotide and amino acid sequences analysis were carried out with the computer programs MEGA 7.0 (Kumar et al., 2016) and Jalview (Clamp et al., 2004). The sequences of *msp4* were aligned by ClustalW and the phylogenetic neighbor-joining algorithm constructed with bootstrap resampling using 1000 repetitions (Kimura, 1980; Saitou and Nei, 1987)

2.5. Expression and purification of recombinant A. marginale MSP4 (rAmMSP4)

The recombinant plasmid (pET100-*msp4*) was introduced into the BL21-CodonPlus (DE3)-RIPL competent cells by heat shock transformation. Transformants were grown in 400 ml of LB medium with ampicillin and chloramphenicol at 37 °C until an optical density (OD) at 600 nm reached 0.5. Then, isopropyl-1-β-D- thiogalactopyranoside (IPTG) (Invitrogen) was added to the final concentration of 0.1 mM and the incubation was continued for 3 h at 37 °C. The cells were collected by centrifugation and the expression of soluble and insoluble fractions was analyzed on 10% sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE).

After centrifugation, the cell pellets were washed with PBS, pH 7.4 and collected by centrifugation at 8000 g for 10 min at 4 °C. Thereafter, the pellet was sonicated on ice using six 10-second bursts at high intensity with a 10-second cooling period between each burst. The lysate was centrifuged at 12,000 g for 15 min at 4 °C and the supernatant was discarded. The pellet was washed with 20 mM Tris − HCl and spun down. The washed pellet was resuspended in 20 mM Tris − HCl, pH 8.4 with 8 M Urea. Then (9/1, v/v) ice-cold absolute ethanol was added and incubated at − 20 °C. Next, the sample was spun down at 4 °C. The pellet was washed again with 90% ethanol and resuspended in PBS, pH 7.4 containing 0.1% SDS. The sample was purified with Histrap Column (GE Healthcare, USA) following the manufacturer's protocol. The protein content of the purified rMSP4 was eluted at 500 mM imidazole and measured using the Bradford's method (Bradford, 1976) and analyzed on 10% SDS-PAGE and immunoblotting. The rAmMSP4 was dialyzed in PBS, pH 7.4, and concentrated using Amicon Ultra-15 centrifugal filter units (Millipore, Billerica, MA) and kept at − 20 °C until used.

2.6. Protein identification

The rAmMSP4 band was cut and subjected to in-gel tryptic digestion. Tryptic peptide sample was analyzed for amino acid sequences using maXis IITM ESI–QTOF (Bruker Daltonics) coupled with an UltiMate 3000 nano-LC system (Dionex, Surrey, UK). Data from Mass Spectrometry analysis were searched against the non-redundant NCBI Genbank database (www.ncbi.nlm.nih.gov/) and Bacteria (Eubacteria) database using version 2.5.1.2 of the MASCOT search engine (Matrix Science, London, UK, http://www.matrixscience.com). The search parameters were composed of trypsin digest, monoisotopic mass, and allowing a maximum of one missed cleavage. Peptide and fragment mass tolerance were set as 1.2 Da and 0.6 Da, respectively. Variable modifications were set to carbamidomethylation of cysteine and oxidation of methionine. The instrument type was specified as ESI-QUAD-TOF. Proteins matched with scores over 65 were considered to be significant ($p < 0.05$). Proteins were verified with protein scores, peptide matches, and percentage of sequence coverage.

2.7. Production of rabbit hyperimmune sera against rAmMSP4

Four New Zealand White rabbits were immunized with rAmMSP4 to produce hyperimmune sera against rAmMSP4 according to the method described by Anuracpreeda et al. (2016a, 2016b). Briefly, 500 μg of rAmMSP4 was mixed with an equal volume of Titermax adjuvant (Sigma-Aldrich Inc., Germany) and injected subcutaneously into the animals. Two boosters followed at 3-week intervals with 250 μg of rAmMSP4 in PBS via the same route. The rabbits were bled, and the antibody titers in the anti-sera were tested by indirect enzyme-linked immunosorbent assay (indirect ELISA).

2.8. Indirect ELISA

Indirect ELISA method described by Anuracpreeda et al. (2017a, 2017b) was used for assessing the antigenicity of rAmMSP4 and

AmMSP4 in the initial bodies extract from erythrocytes (as described in 2.2). Briefly, 100 µl of 1 µg/mL of rAmMSP4 and the initial bodies extract diluted in coating buffer (15 mM Na_2CO_3, 35 mM $NaHCO_3$, pH 9.6) were added into each well of a flat bottom F96 microplate (Nunc A/S, Roskilde, Denmark) and incubated at 4 °C overnight. After washing three times with 0.05% Tween 20 in PBS, the plate was blocked by adding 100 µL/well of a blocking solution (0.25% bovine serum albumin (BSA), 0.05% Tween 20 in 0.01 M PBS, pH 7.2) at 37 °C for 1 h. Thereafter, the plate was similarly washed, and 100 µl of serially diluted pooled sera (from 1:5 to 1:244,140,625) from cattle infected with *A. marginale* or rabbit immunized with rAmMSP4 was added and incubated at 37 °C for 2 h. After washing, the plate was incubated with 100 µL/well of horse radish peroxidase (HRP)-conjugated goat anti-bovine antibody or HRP-conjugated goat anti-rabbit antibody (Sigma-Aldrich Inc.) at 1:6000 dilution in blocking solution at 37 °C for 1 h. Then, the plate was washed again, the color development was generated by adding 100 µL/well of 3, 3′, 5, 5′-tetramethyl benzidine (TMB) substrate (KPL, Gaitherburg, USA). The enzymatic reaction was allowed to take place at room temperature for 10 min. Finally, the enzymatic reaction was stopped by the addition of 100 µl 1 N HCl. The optical density (OD) value at 450 nm was measured using a microplate reader (Multiskan Ascent, Labsystems, Helsinki, Finland).

2.9. Immunoblotting assay

The purified proteins and the initial bodies extract were separated by 10% SDS-PAGE. The gels were either stained with Comassie blue or were used for immunoblotting. For immunoblotting, the proteins were transferred onto nitrocellulose (NC) membranes (GE Healthcare, USA). Each NC membrane was blocked with a blocking solution (5% skimmed milk in PBS, pH 7.4 containing 0.05% Tween 20) for 2 h at room temperature. The NC membranes were incubated with mouse anti-6xhistidine (Invitrogen) (diluted at 1:5000) or rabbit anti-rAmMSP4 sera (diluted at 1:100) or *A. marginale*-infected cattle sera (dilute at 1:50) for 2 h at room temperature. After washing with PBS-T (PBS + 0.05% Tween 20), membranes were incubated with AP-conjugated goat anti-mouse/rabbit/bovine IgG (Sigma-Aldrich Inc.) (diluted at 1:5000) for 1 h at room temperature. After washing, the color reaction was visualized by further incubation in a specific substrate nitro-blue-tetrazolium chloride (NBT) and 5-bromo-4-chloro-3-indolylphosphate (BCIP) (Sigma-Aldrich Inc.) for 5–10 min at room temperature. Finally, the reaction was stopped by adding distilled water.

2.10. Localization of Anaplasma parasites by immunofluorescent technique

The method of immunofluorescence detection described by Anuracpreeda et al. (2009; 2016a) was used. Briefly, the slides of blood smear were fixed in ice-cold acetone for 3 min. Thereafter, the slides were incubated with sera from rabbit immunized with rAmMSP4 and normal rabbit (diluted at 1:50 with PBS containing 0.05% Tween 20) at 37 °C for 2 h. After washing three times with PBS containing 0.05% Tween-20, pH 7.4, for 1 min each, the slides were treated with fluorescein isothiocyanate (FITC)-conjugated goat anti-rabbit IgG (Sigma–Aldrich, Inc.), (diluted at 1:300 with PBS containing 0.05% Tween-20, pH 7.4) at 37 °C for 1 h. After washing three times with the same buffer, the treated slides were air dried, examined and photographed under an EVOS FL color fluorescence microscope.

2.11. Ethics statement

Experimentation on animals was approved by the Animal Care and Use Committee (SCMU-ACUC), Faculty of Science, Mahidol University, Thailand. The cattle sera were obtained from Department of Livestock, Ministry of Agriculture and Co-operatives, Bangkok, Thailand. Also, we have received consent to collect the blood samples from animals at the abattoir.

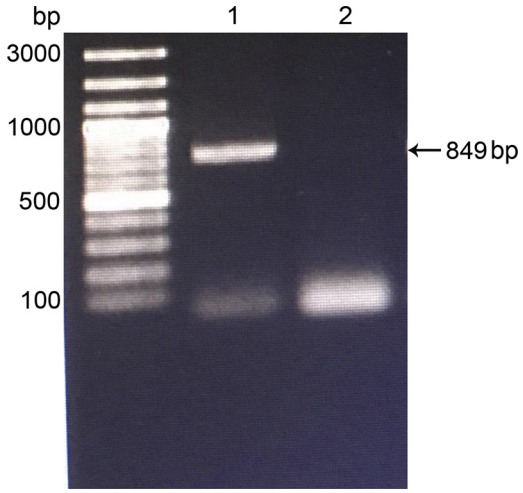


Fig. 1. PCR product of *A. marginale msp4* coding sequence of Thailand strain. Lane 1: 100 bp DNA ladder. Lane 2: The size of *msp4* coding sequence. Lane 3: negative control.

3. Results

3.1. Cloning and sequencing of msp4 coding sequence

The *msp4* coding sequence was successfully cloned into pET100/D-TOPO® vector and transformed into *E. coli* TOP10. The transformed colonies were selected by colony PCR and sequencing analysis. The open reading frame of *msp4* coding sequence amplicon of Thailand strains was 849 bp (Fig. 1). There was no genetic variation of two sequences of *msp4* coding sequence from Thailand (MH939155 and MH939156), while *msp4* coding sequence of *A. marginale* Thailand strain had the difference from other strains with pairwise distances (Supplementary Table 1). Although, the sizes of *msp4* from all strains were the same, genetic variations of *msp4* among different strains were observed. The site showing most variation was position 425 from G to A (Fig. 2). The nucleic acid substitution rate in *msp4* sequences among *A. marginale* was estimated under the Tamura and Nei (1993) mode (Supplementary Table 2). In addition, alignment of the MSP4 amino acid sequence of *A. marginale* Thailand strain and other strains was evaluated and shown in Fig. 3.

3.2. Phylogenetic relationships

In an attempt to understand the evolutionary relationship among *msp4* coding sequence of *A. marginale* in Thailand strain and other strains from different countries, a phylogenetic tree was constructed using a bootstrap-neighbor joining method. This analysis revealed that the *msp4* coding sequence among *A. marginale* was highly conserved when compared with *A. phagocytophilum* as an outer group (Fig. 4). Australian strain (AY665998) was most relevant to Thailand strain, with only 1 bp difference. The geographical clade was not observed in this tree. Not only was nucleotide variation observed but the variations in amino acid (aa) sequences were also observed. Twenty-two aa were found the genetic variation among *A. marginale* strains. The highest variation aa positions among 21 sequences were aa 99 (from N to T) (Supplementary Table 3).

3.3. Expression and purification of rAmMSP4

The recombinant plasmid pET100-*msp4* was transformed into the *E. coli* BL21-CodonPlus (DE3)-RIPL expression host, a bacterial strain designed for gene expression regulated by the T7 promoter. The

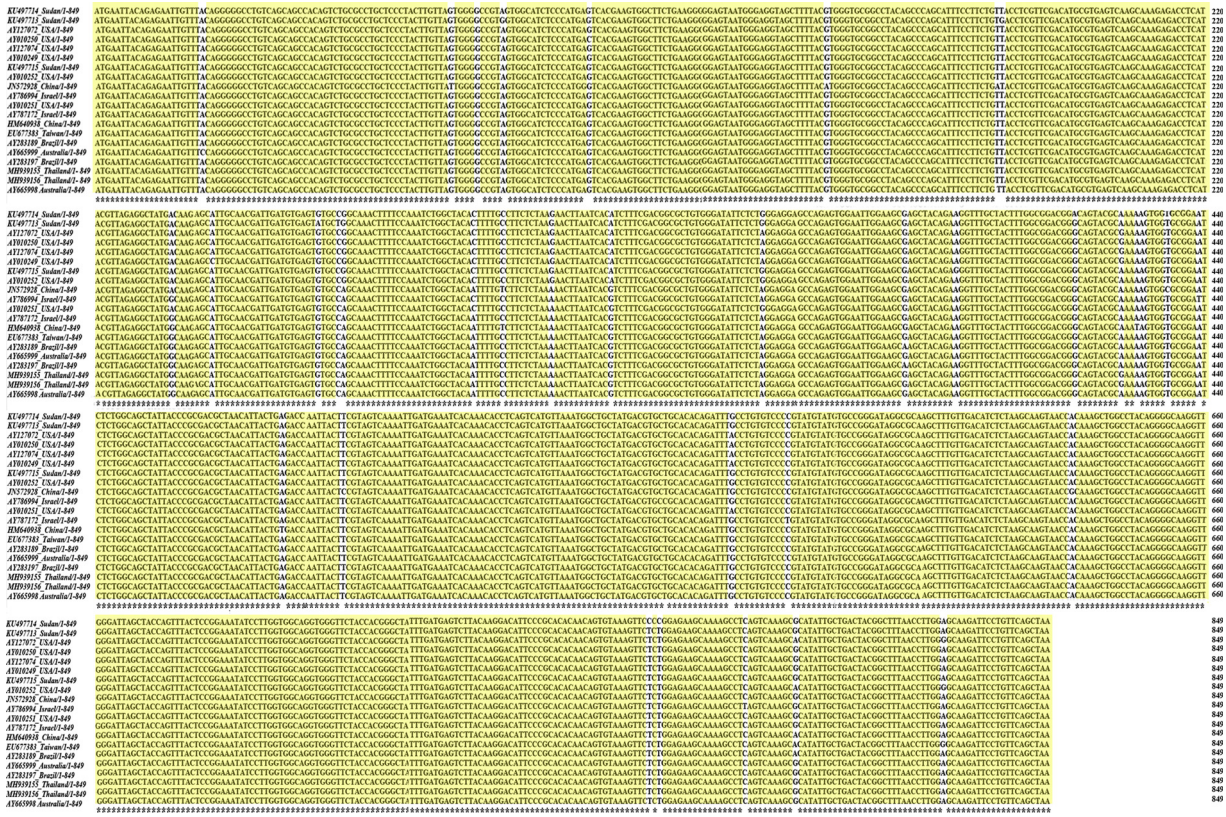


Fig. 2. Multiple sequence alignment of full length *msp4* coding sequence among *A. marginale* different strains (visualized with JalView). The symbol, (*) indicates identical consensus.

recombinant MSP4 (rMSP4) protein fusion with 6x-histidine tag and Xpress™ epitope was produced in bacterial system following the induction with 1 mM ITPG at 37 °C. The rMSP4 was resolved as a single band with an apparent molecular weight (MW) of 35 kDa on SDS-PAGE (Fig. 5), which corresponds to MW estimated for the native protein (31 kDa) fused to the 6x-histidine tag and XpressTM epitope. After the sonication, the rMSP4 band was found in the insoluble part. However, rMSP4 was recovered from the inclusion body and purified with the Histrap purification system (Fig. 5).

3.4. Protein identification of rAmMSP4

The rAmMSP4 band was identified by Mass Spectrometry. The results exhibited that those protein bands were major surface protein 4 (MSP4) of *A. marginale* (Supplementary Table 4).

3.5. Antigenicity of rAmMSP4 and AmMSP4

The antigenicity of rAmMSP4 and AmMSP4 in the initial bodies extract was evaluated using the indirect ELISA. The reaction of the sera with rAmMSP4 continued up to the dilution of 1:390,625 (*A. marginale*-infected cattle sera) and 1:1,953,125 (rAmMSP4-immunized rabbit sera) (Fig. 6A). In addition, the reaction of both *A. marginale*-infected cattle and rAmMSP4-immunized rabbit sera with AmMSP4 in the initial bodies extract continued up to the dilution of 1:78,125 (Fig. 6B). Hence, the rAmMSP4 and AmMSP4 revealed strong antigenicity.

3.6. Immunoblotting assay

The immunoblotting experiment showed that the anti-histidine tag antibody and rabbit hyperimmune sera reacted with a single band of rAmMSP4 which has a MW of 35 kDa (Fig. 7A). However, when tested against AmMSP4 with the sera of cattle infected with *A. marginale* or rabbit immunized with rAmMSP4, these antibodies reacted intensely with AmMSP4 which appeared as a single band at MW of 31 kDa (Fig. 7B). The negative control using rabbit pre-immune and uninfected cattle sera, did not show any positive bands.

3.7. Localization of Anaplasma parasites

The localization of *Anaplasma* parasites was detected using rabbit hyperimmune sera in immunofluorescence technique, the parasites were found to be immunoreactive at the periphery or on the outside of the infected erythrocytes in a stained blood film (Fig. 8B and C). The negative control, using rabbit pre-immune sera, did not exhibit any positive fluorescent signal (Fig. 8A).

4. Discussion

In the present study, genomic DNA of *A. marginale* was extracted and *msp4* coding sequence was sequenced. The completed sequences of *msp4* coding sequence from *A. marginale* Thailand strain were reported. Close to our findings, the partial sequences of *msp4* gene of *A. marginale* Thailand strain were also studied (Saetiew et al., 2015; Jirapattharasate et al., 2017). For phylogeny, we have only selected completed sequences to compare with Thailand isolates. The full length of *msp4* coding sequence in Thailand strain was 849 bp, whereas the geographical variations were not observed. In addition, *msp4* coding sequence of *A. marginale* was highly conserved because pairwise distance was between 0.000-0.022.

Unlike previous study as obtained by Oberle et al. (1993), our *msp4* coding sequence was cloned into expression vector and overexpressed in *E. coli* BL21-CodonPlus (DE3)-RIPL, and rAmMSP4 identity was confirmed by Mass Spectrometry. The advantage of this competent cell was having tRNA of rare codons which were mostly found in heterologous protein including rAmMSP4. Our rAmMSP4 was 35 kDa-protein,

Fig. 3. Alignment of MSP4 amino acid sequence from *A. marginale* Thailand strain and other strains. The signal peptide amino acid sequence of MH939155 and MH939156 has been aligned from the cleavage site. Cleavage site (arrow) take place between residue -1, amino acid position 29 which is C-terminal residue of signal peptide and residue +1, amino acid position 29 which is N-terminal residue of mature proteins. The negative numbers indicate those of the signal peptide from amino acid position 1-29. The symbol, (*) indicates identical residues, (:) indicates highly conserved residues, (.) indicates moderately conserved residues.

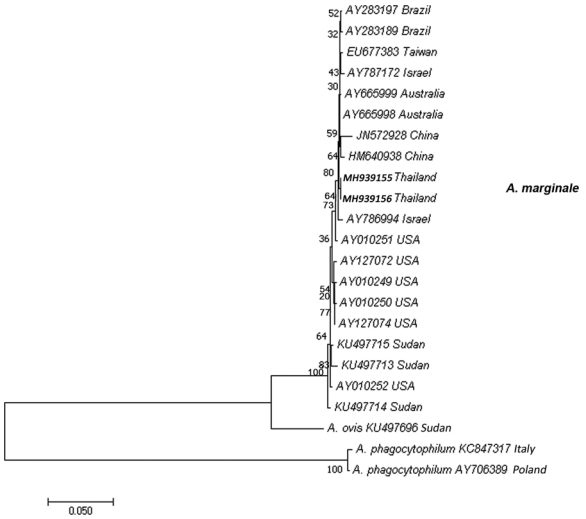


Fig. 4. The phylogenetic tree of completed *A. marginale msp4* sequences was constructed by neighbor-joining algorithm with Poisson correction and bootstrap analysis of 1000 replicates. The GenBank accession numbers of the sequences used in the phylogenetic analysis are also shown. Numbers on the branches indicated percent support for each clade.

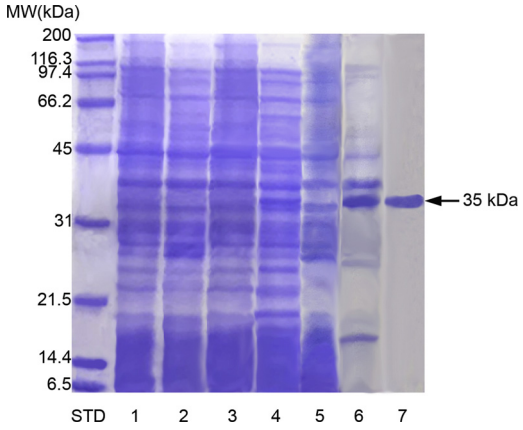


Fig. 5. SDS-PAGE exhibited the expression and purification of rAmMSP4 after Coomassie blue staining. Lane 1, *E. coli* BL21-CodonPlus (DE3)-RIPL without induction; lane 2, *E. coli* BL21-CodonPlus (DE3)-RIPL containing pET100-*msp4* without induction for 3 h; lane 3, *E. coli* BL21-CodonPlus (DE3)-RIPL with induction; lane 4, *E. coli* BL21-CodonPlus (DE3)-RIPL containing pET100-*msp4*, with induction for 3 h.; lane 5, soluble fraction; lane 6, insoluble fraction; lane 7, purified rAmMSP4. Molecular weight markers are shown in lane marked STD on the left side.

while AmMSP4 was 31 kDa-protein. The higher MW of rAmMSP4 was due to the addition of 38 extra amino acids which were 6xhis-tag protein, enterokinase (EK) recognition site, and EK cleavage site on pET100 vector for cloning and purification purposes (Gerlach et al., 2004). The rAmMSP4 was mostly found in the insoluble fraction rather than soluble fraction. Moreover, this method produced relatively high yield of rAmMSP4 which was used to immunize rabbits. MSP4 is one of the outer membrane proteins of *A. marginale* (Tebele et al., 1991; Oberle et al., 1993; Brown et al., 1998; de la Fuente et al., 2002, 2005;

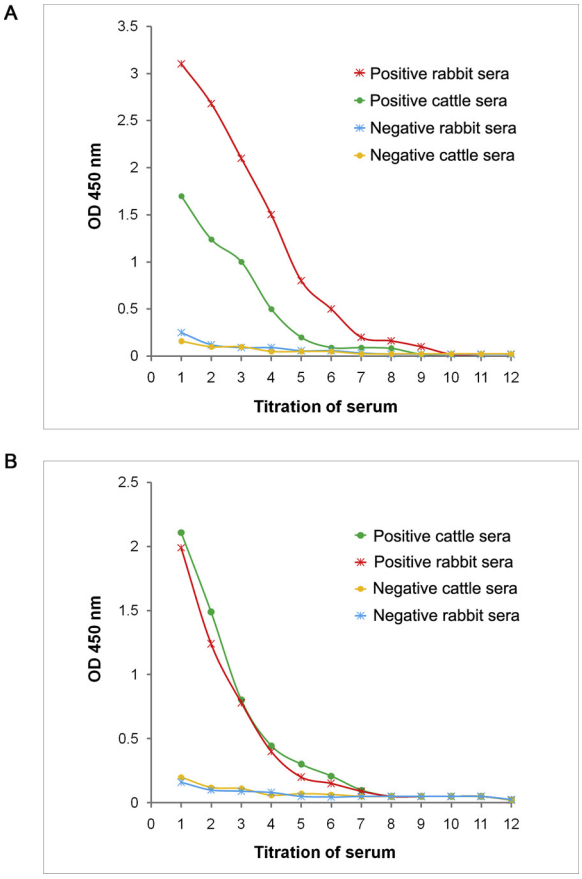


Fig. 6. Indirect ELISA exhibited optical densities (OD) representing the levels of antigenicity of rAmMSP4 **(A)** and AmMSP4 **(B)** as detected by the various dilutions of 1 : 5 (number 1), 1 : 25 (number 2), 1 : 125 (number 3), 1 : 625 (number 4), 1 : 3125 (number 5), 1 : 15,625 (number 6), 1 : 78,125 (number 7), 1 : 390,625 (number 8), 1 : 1,953,125 (number 9), 1 : 9,765,625 (number 10), 1 : 48,828,125 (number 11) and 1 : 244,140,625 (number 12) of pooled positive sera collected from cattle proven with *A. marginale* infection and from rabbit immunized with rAmMSP4.

Molad et al., 2004) and increases its hydrophobicity profile and also increases the changes of expression as inclusion bodies. In addition, the function of this protein is still unknown so far (Hope et al., 2004).

In the present study, the results of indirect ELISA revealed that the antibody titer against rAmMSP4 and AmMSP4 in the initial bodies extract was tested using the pooled positive sera of cattle infected with *A. marginale* and rabbit immunized with rAmMSP4 as well as negative sera of cattle uninfected with *A. marginale* and of rabbit unimmunized with rAmMSP4. The rAmMSP4 detected the immunoglobulin G (IgG) in the sera of cattle infected with *A. marginale* and rabbit immunized with rAmMSP4 up to a dilution of 1:390,625 and 1:1,953,125, respectively. Moreover, the AmMSP4 in the initial bodies extract also detected the IgG in the sera of cattle infected with *A. marginale* and rabbit immunized with rAmMSP4 up to a dilution of 1:78,125. On the other hand, Oberle et al. (1993) reported that rAmMSP4 and AmMSP4 in the initial bodies extract exhibited the IgG titer at the dilution of 1:1000 in outer membrane fraction-immunized cattle sera. In this study, titer for rAmMSP4 is higher than the AmMSP4 which may be due to the impurity and the small amount of the native antigens in the initial bodies extract which lead to a decrease in the binding between antigen and antibody. In addition, these results imply that a high antibody titer is a clear reflection of the strong antigenicity of both rAmMSP4 and AmMSP4 of *A. marginale.* For immunoblotting assay, the result showed that the sera of infected cattle and hyperimmunized rabbit could react with rAmMSP4 at MW 35 kDa. Furthermore, they could also detect the protein band of 31 kDa AmMSP4. This implied that the rAmMSP4 had a similar epitope with AmMSP4 as well as the rAmMSP4 which had conserved epitopes and sustained antigenicity (Brown et al., 1998; Kano et al., 2002; Oliveira et al., 2003). This result agrees with those reported by earlier study showing that MSP4 is a highly conserved protein encoded by a single gene (Oberle et al., 1993), whereas MSP1b, MSP2 and MSP3 are encoded by a multigene family (Palmer et al., 1994; Allerman et al., 1997; Brayton et al., 2002; Shkap et al., 2002).

The localization of *Anaplasma* parasites by the rabbit hyperimmune sera showed that these parasites are distributed not only on the membrane of infected erythrocytes, but also on the outside of erythrocytes. It is possible that these free *Anaplasma* is expected as a result of sample preparation. From the previous study, it was believed that MSP4 had the signal peptide located on the erythrocytes' membrane (Oberle et al., 1993). In our study, Signal IP (SignalP 4.1 Server) predicted that the signal peptide occurred at amino acid position 1 to 29 without transmembrane segment (VVA-SP D = 0.815, D-cutoff = 0.570) and the cleavage site is between amino acid position 29 and 30. The mature proteins begin with amino acid position 30 (Supplementary Fig. 1). For amino acid sequence, MSP4 of *A. marginale* (MH939155 and MH939156) showed 58.51% identity to MSP4 of *A. phagocytophilum*

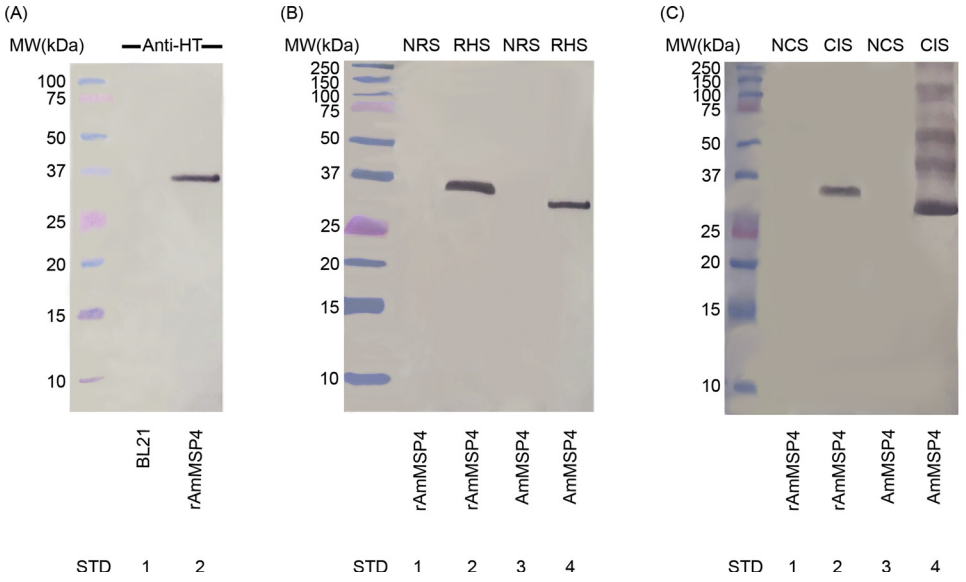


Fig. 7. Immunoblot analysis of recombinant *A. marginale* MSP4 (rAmMSP4). **(A)** Immunoblot patterns of *E. coli* BL21-CodonPlus (DE3)-RIPL (BL21, lane 1) and rAmMSP4 (lane 2) reacted with anti-histidine tag antibody (Anti-HT). **(B)** Immunoblot patterns of rAmMSP4 reacted with normal rabbit sera (NRS, lane 1) and rabbit hyperimmune sera (RHS, lane 2) as well as the AmMSP4 reacted with normal rabbit sera (NRS, lane 3) and rabbit hyperimmune sera (RHS, lane 4). **(C)** Immunoblot patterns of rAmMSP4 reacted with normal cattle sera (NCS, lane 1) and cattle infected sera (CIS, lane 2) as well as the AmMSP4 reacted with normal cattle sera (NCS, lane 3) and cattle infected sera (CIS, lane 4).
Molecular weight markers are shown in lane marked STD on the left side.

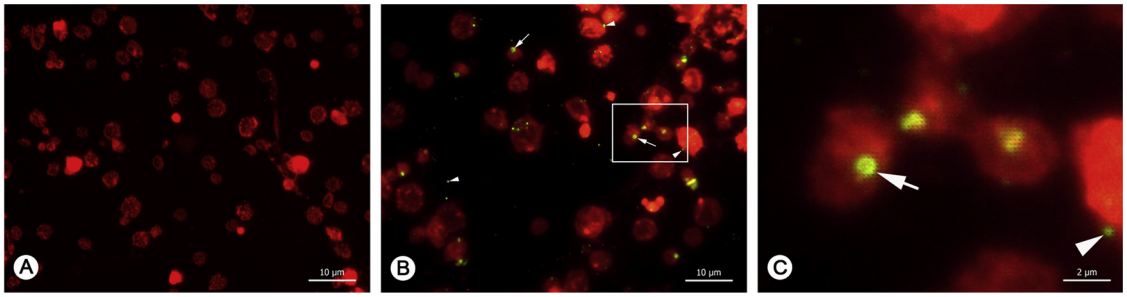


Fig. 8. Immunofluorescence staining of *Anaplasma* parasites in a stained blood film using the sera from rabbit immunized with rAmMSP4 as a probe. **(A)** The negative control stained with rabbit pre-immune sera did not show any positive fluorescent signal. **(B and C)** Medium and high magnification micrographs showed intense fluorescence in the parasites which occupied at the periphery (arrows) and on the outside (arrowheads) of the infected erythrocytes.

(KC847317) (Supplementary Fig. 2). Although the real function of MSP4 of *A. marginale* was still unknown, our result from InterPro sever predicted that MSP4 of *A. marginale* (MH939155 and MH939156) might have the similar function to that of MSP4 of *A. phagocytophilum* (KC847317) as a result of the similar homologous superfamily match to MSP4 at amino acid position 43–281, signal peptide protein amino acid position 1–29, and mild different at non cytoplasmic domain. Hence, it is likely that MSP4 of *A. marginale* might involve the interaction between host and parasite similar to MSP4 of *A. phagocytophilum* (de la Fuente et al., 2005; Contreras et al., 2017).

5. Conclusions

In conclusion, *msp4* coding sequence of *A. marginale* strain Thailand is highly conserved among *A. marginale* different strains. The 35 kDa recombinant MSP4 has conserved epitopes and maintains antigenicity. Hence, there is a possibility that this recombinant protein could be used for immunodiagnostic purposes and as a vaccine candidate for anaplasmosis, which will be studied further.

Conflict of interest

We declare there are no competing interests.

Acknowledgements

This research was financially supported by Research Grants from a Postdoctoral Fellowship of Mahidol University to Dusit Boonmekam, and TRF Research Career Development Grant co-funded by The Thailand Research Fund and Mahidol University (RSA6080071) to Panat Anuracpreeda. We would like to thank Prof. Dr.Peter Quantick, Research Director, RedKnight Consultancy Ltd, UK for assistance in correcting the manuscript.

Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.actatropica.2019.105047.

References

Allerman, A.R., Barbet, A.F., 1996. Evaluation of *Anaplasma marginale* major surface protein 3 (MSP3) as a diagnostic test antigen. J. Cli. Microbiol. 34, 270–276.

Allerman, A.R., Palmer, G.H., Mcguire, T.C., McElwin, T.F., Perryman, L.E., Barbet, A.F., 1997. *Anaplasma marginale* major surface protein 3 is encoded by a polymorphic, multigene family. Infect. Immun. 65, 156–163.

Anuracpreeda, P., Wanichanon, C., Chaithirayanon, K., Preyavichyapugdee, N., Sobhon, P., 2009. Distribution of 28.5 kDa antigen in tegument of adult *Fasciola gigantica*. Acta Trop. 100, 31–40.

Anuracpreeda, P., Chawengkirtikul, R., Sobhon, P., 2016a. Immunodiagnosis of *Fasciola gigantica* infection using monoclonal antibody-based sandwich ELISA and immunochomatographic assay for detection of circulating cathepsis L1 protease. PLoS One 11 (1), e0145650. https://doi.org/10.1371/journal.Pone.0145650.

Anuracpreeda, P., Chawenkrittikul, R., Sobhon, P., 2016b. Immunodiagnostic monoclonal antibody-based sandwich ELISA of fasciolosis by detection of *Fasciola gigantica* circulating fatty acid binding protein. Parasitology 143, 1369–1381.

Anuracpreeda, P., Watthanadirek, A., Chawengkirttikul, R., Sobhon, P., 2017a. Production and characterization of a monoclonal antibody specific to 16 kDa antigen of Paramphistomum gracile. Parasitol. Res. 116, 167–175.

Anuracpreeda, P., Tepsupornkul, K., Chawengkirttikul, R., 2017b. Immunodiagnosis of paramphistomosis using monoclonal antibody-based sandwich ELISA for detection of *Paramphistomum gracile* circulating 16 kDa antigen. Parasitology 144, 899–903.

Aubry, P., Geale, D.W., 2011. A review of bovine anaplasmosis. Transbound. Emerg. Dis. 58, 1–30.

Barbet, A.F., Allred, D.R., 1991. The msp1 multigene family of Anaplasma marginale: nucleotide sequence analysis of an express copy. Infect. Immun. 59, 971–976.

Barbet, A.F., Palmer, G.H., Myler, P.J., McGuire, T.C., 1987. Characterization of an immunoprotective protein complex of *Anaplasma marginale* by cloning and expression of the gene coding for polypeptide AM105L. Infect. Immun. 55, 2428–2435.

Bradford, M.M., 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal. Biochem. 72, 248–254.

Brayton, K.A., Palmer, G.H., Lundgren, A., Yi, J., Barbet, A.F., 2002. Antigentic variation of *Anaplasma marginale* msp2 occurs by combinatorial gene conversion. Mol. Microl. 43, 1151–1159.

Brown, W.C., Shkap, V., Zhu, D., McGuire, T.C., Tuo, W., McElwain, T.F., Palmer, G.H., 1998. CD4(+) T-lymphocyte and immunoglobulin G2 responses in calves immunized with *Anaplasma marginale* outer membranes and protected against homologous challenge. J. Infect. Dis. Immunol. Tech. 66, 5406–5413.

Clamp, M., Cuff, J., Searle, S.M., Barton, G.J., 2004. The Jalview Java alignment editor. Bioinformatics 20, 426–427.

Contreras, M., Alberdi, P., Mateos-Hernández, L., Fernández de Mera, I.G., García-Pérez, A.L., Vancová, M., Villar, M., Ayllón, N., Cabezas-Cruz, A., Valdés, J.J., Stuen, S., Gortazar, C., de la Fuente, J., 2017. *Anaplasma phagocytophilum* MSP4 and HSP70 proteins are involved in interactions with host cells during pathogen infection. Front. Cell. Infect. Microbiol. 7, 307.

Correa, W.M., Correa, C.N.M., Gottschalk, A.F., 1978. Bovine Abortion associate with Anaplasma marginale. Can. J. Com. Med. 42, 2277–2278.

de la Fuente, J., Van Den Bussche, R.A., Garcia-Garcia, J.C., Rodrı´guez, S.D., Garcı´a, M.A., Guglielmone, A.A., Mangold, A.J., Passos, L.M., Blouin, E.F., Kocan, K.M., 2002. Phylogeography of New World isolates of *Anaplasma marginale* (Rickettsiaceae: ehrlichieae) based on major surface protein sequences. Vet. Microbiol. 88, 275–285.

de la Fuente, J., Massung, R.F., Wong, S.J., Chu, F.K., Lutz, H., Meli, M., von Loewenich, F.D., Grzeszczuk, A., Torina, A., Caracappa, S., Mangold, A.J., Naranjo, V., Stuen, S., Kocan, K.M., 2005. Sequence analysis of the *msp4* gene of *Anaplasma phagocytophilum* strains. J. Clin. Microbiol. 43, 1309–1317.

Dumler, J.S., Barbet, A.F., Bekker, C.P.J., Dasch, G.A., Palmer, G.H., Ray, S.C., Rikihisa, Y., Rurangirwa, F.R., 2001. Reorganization of genera in the families Rickettsiaceae and Anaplasmataceae in the order Rickettsiales: unification of some species of Ehrlichia with Anaplasma, Cowdria with Ehrlichia and Ehrlichia with Neorickettsia, descriptions of six new species combinations and designation of Ehrlichia equi and 'HGE agent' as subjective synonyms of Ehrlichia phagocytophila. Int. J. Syst. Evol. Microbiol. 51, 2145–2165.

Gerlach, D., Schlott, B., Schmidt, K.H., 2004. Cloning and Expression of a sialic acid-binding lectin from the snail Cepaea hortensis. Fems Immunol. Med. Microl. 40, 215–221.

Goodger, W.J., Carpenter, T., Riemann, H., 1979. Estimation of economic loss associated with anaplasmosis in California beef cattle. J. Am. Vet. Med. Assoc. 174, 1333–1336.

Hope, M., Riding, G., Menzies, M., Willadsen, P., 2004. A novel antigen from Anaplasma marginale: characterization, expression and preliminary evaluation of the recombinant protein. Vaccine 22, 407–415.

Jirapattharasate, C., Moumouni, P.F.A., Cao, S., Iguchi, A., Liu, M., Wang, G., Zhou, M., Vudriko, P., Efstratiou, A., Changbunjong, T., Sungpradit, S., Ratanakorn, P., Moonarmart, W., Sedwisai, P., Weluwanarak, T., Wongsawong, W., Suzuki, H., Xuan, X., 2017. Molecular detection and genetic diversity of bovine *Babesia* spp., *Theileria orientalis*, and *Anaplasma marginale* in beef cattel in Thailand. Parasitol. Res. 116, 751–762.

Kano, F.S., Vidotto, O., Pacheco, R.C., Vidotto, M.C., 2002. Antigenic characterization of

Anaplasma marginale isolates from different regions of Brazil. Vet. Microbiol. 87, 131–138.
Kimura, M., 1980. A simple method for estimating evolutionary rate of base substitutions though comparative studies of nucleotide sequences. J. Mol. Evol. 16, 111–120.
Kocan, K.M., de la Fuente, J., 2003. Co-feeding of tick infected with Anaplasma marginale. Vet. Parasitol. 112, 295–305.
Kocan, K.M., de la Fuente, J., Blouin, E.F., Coetzee, J.F., Ewing, S.A., 2010. The natural history of Anaplama marginale. Vet. Parasitol. 167, 95–107.
Kumar, S., Stecher, G., Tamura, K., 2016. MEGA7: molecular evolutionary genetics analysis version 7.0 for bigger datasets mol. Sex. Dev. 34, 1870–1874.
Molad, T., Brayton, K.A., Palmer, G.H., Michaeli, S., Shkap, V., 2004. Molecular conservation of MSP4 and MSP5 in *Anaplasma marginale* and *A. Centrale* vaccine strain. Vet. Microbiol. 100, 55–64.
Oberle, S.M., Palmer, G.H., Barbet, A.F., 1993. Expression and Immune recognition of the conserved MSP-4 outer membrane protein of Anaplasma marginale. Infect. Immun. 61, 5245–5251.
Oliveira, J.B., Madruga, C.R., Schenk, M.A., Kessler, R.H., 2003. Antigenic characterization of four Brazilian isolates of *Anaplasma marginale* Rickettsiaceae: ehlichiae. Mem. Inst. Oswaldo Cruz 98, 395–400.
Palmer, G.H., Eid, G., Barbet, A.F., Mcguire, T.C., McElwin, T.F., 1994. The immunoprotective *Anaplasma marginale* major surface protein 2 is encoded by a polymorphic multigene family. Infect. Immun. 62, 3808–3816.
Rymaszewska, A., Grenda, S., 2008. Bacteria of the genus *Anaplasma* – characteristics of *Anaplasma* and their vectors: a review. Vet. Med. (Praha) 53, 573–584.
Saetiew, N., Simking, P., Inpankaew, T., Wongpanit, K., Kamyingkird, K., Wongnakphet, S., Stich, R.W., Jittapalapong, S., 2015. Prevalence and genetic diversity of *Anaplasma marginale* infections in water buffaloes in Northeast Thailand. J. Trop. Med. Parasitol. 38, 9–16.
Saitou, N., Nei, M., 1987. The neighbor-joining method: a new method for reconstructing phylogenetic trees. Mol. Biol. Evol. 4, 406–425.
Shkap, V., Bin, H., Ungar-Waron, H., Pipano, E., 1990. An enzyme-linked immunosorbent assay (ELISA) for the detection of antibodies to *Anaplasma centrale* and *Anaplasma marginale*. Vet. Microbiol. 25, 45–53.
Shkap, V., Molad, T., Fish, L., Palmer, G.H., 2002. Detection of the *Anaplasma centrale* vaccine strain and specific differentiation from *Anaplasma marginale* in vaccinated and infected cattle. Parastiol Re. 88, 546–552.
Sumrandee, C., Baimai, V., Trinachartvanit, W., Ahantarig, A., 2016. Molecular detection of Rickettsia, *Anaplasma, Coxiella* and *Francisella* bacteria in ticks collected from Artiodactyla in Thailand. Ticks Tick. Dis. 7, 678–689.
Tamura, K., Nei, M., 1993. Estimation of the number of nucleotide substitutions in the control region of mitochondrial DNA in humans and chimpanzees. Mol. Biol. Evol. 10, 512–526.
Tebele, N., McGuire, T.C., Palmer, G.H., 1991. Induction of protective immunity by using *Anaplasma marginale* initial body membranes. Infect. Immun. 59, 3199–3204.
Visser, F.S., McGuire, T.C., Palmer, G.H., Davis, W.C., Shkap, V., Pipano, E., Knowles, D.P., 1992. The *Anaplasma marginale* MSP-5 gene encodes a 19 kilodalton protein conserved in all recognized *Anaplasma* species. Infect. Immun. 60, 5139–5144.