Molecular investigation of tick-borne pathogens in cattle, horses and sheep in Xinjiang Uygur Autonomous Region, China

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中国新疆ウイグル自治区におけるウシ、ウマ、ヒツジのマダニ媒介病原体の分子疫学調査

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Contents

Abbreviations and unit abbreviations

Abbreviations

B	Blastn	Basic local alignment search tool
D	DNA	Deoxyribonucleic acid
	dNTP	Deoxyribonucleotide triphosphate
E	EMA-1	Merozoite antigen-1
H	htpB	Heat shock protein antigenic polypeptide
M	MSP4	Major surface protein 4
	MPSP	Major piroplasm surface protein
N	No.	Number
	N	Number
	n	Number
O	ompA	Outer membrane protein A
P	PCR	Polymerase chain reaction
R	Rap-1	Rhoptry associated protein-1
	RNA	Ribonucleic acid
S	spp.	Species
	SBP-4	Spherical body protein-4
U	UV	Ultraviolet

Unit abbreviations

bp	base pair
µl	microliter
ml	milliliter
µg	microgram
µM	micromolar
mM	millimolar
U	unit
%	percentage
°C	degree Celsius

General introduction

1. Ticks

Ticks are arachnids which obsessively feed on blood, and are listed as the second most common human disease vectors below mosquitoes, but rather the primary vectors of animal pathogens for domestic and wild animals (Fuente et al., 2008). Ticks carry and transmit a wide range of pathogens (bacteria, viruses and protozoa) which are of importance to both humans and animals globally (Fuente et al., 2008). Studies have continuously reported new pathogens identified from ticks (Qin et al., 2014; Mansfield et al., 2017), emphasizing the importance of ticks as vectors. The *Ixodes* genus consists of 241 species and 442 species in other genera including *Dermacentor*, *Haemaphysalis*, *Hyalomma*, and *Rhipicephalus* (Horak et al., 2002). In the temperate zone, ixodid ticks are usually the most important vectors of pathogens (Jongejan and Uilenberg, 2004). China has approximately 9.6 million square kilometers of land area with more than 117 tick species under seven genera and more than 30 identified emerging tick-borne pathogens (TBPs) (Chen et al., 2014; Fang et al., 2015; Zhuang et al., 2018).

2. Tick-borne pathogens

The female engorged ticks lay a large amount of eggs, and then, the eggs hatch into larvae that can attach to a host. After a few days, engorged larvae detach from the host and molt into nymphs. Nymphs attach and feed on larger hosts and then become engorged. Finally, engorged nymphs detach from the host and molt into adult

ticks (Hoogstraal and Aeschlimann, 1982). *Ixodes* ticks concentrate the ingested blood and re-injects the derived substances (excess water, hemolytic substance, pathogenic toxins and pathogens) into the host when sucking the blood. The process of transmission of pathogens from infected ticks varies with the type of pathogen. For example, *Anaplasma* spp. and some arboviruses can migrate from the intestine to the salivary glands among tick molting (Hodzic et al., 1998). Some pathogens, such as *Babesia* spp. and *Rickettsia* spp. invade ovaries and salivary glands of ticks through the hemolymph (Chauvin et al., 2009; Socolovschi et al., 2012a). After a fresh blood-sucking stimulation, *Borrelia* spp. can stay in the midgut among molting and invade the salivary glands (Silva and Fikrig, 1995). Once the pathogen invades the tick, it must cross the barrier (intestine, saliva, hemolymph, ovary) and invade a variety of different cell types in order to proliferate. Pathogens such as *Anaplasma marginale* can initially proliferate in membrane-bound vacuoles (Scoles et al., 2005).

3. Babesiosis of livestock

Babesiosis is an important tick-borne disease (TBD) of livestock (cattle, horses, sheep, goats), caused by the protozoa *Babesia* including *Babesia bigemina*, *B. bovis*, *B. divergens*, *B. ovata*, *B. major*, *B. occultans*, *B. jakimovi*, *B. caballi*, *B. motasi* and *B. ovis*. Different species have varying clinical symptoms, modes of transmission, therapies and geographical distribution (Yin et al., 1997; Schnittger et al., 2012; Rojas-Martínez et al., 2018). Clinical signs of babesiosis in livestock include fever, anemia, hemoglobinuria and can lead to death (Yin et al., 1997; Bartolomé Del Pino et al., 2016).

Babesiosis is widespread in tropical and subtropical regions including Africa,

Asia (Liyanagunawardena et al., 2016), Europe (Ribeiro et al., 2013; Bartolomé Del Pino et al., 2016), Australia (Dehhaghi et al., 2019), and North America (Barashi et al., 2019). *B. bovis* and *B. bigemina*, the common agents of bovine babesiosis, can be transmitted by *Rhipicephalus* ticks (Rojas-Martínez et al., 2018), while *B. caballi*, the causative agent of equine babesiosis, is transmitted by ticks from the genera *Hyalomma*, *Rhipicephalus*, *Ixodes* and *Dermacentor* (Bhoora et al., 2018; Ybañez et al., 2018). On the other hand, *B. motasi* and *B. ovis*, main agents of ovine babesiosis, are transmitted by *Rhipicephalus* spp. and *Haemaphysalis* spp. ticks (Schnittger et al., 2012).

4. Theileriosis of livestock

Cattle, horses, sheep and goats infected with the genus *Theileria* show different clinical signs, and morbidity and mortality rates. Naive livestock acquire some *Theileria* spp. Infections, such as *T. parva*, *T. annulata*, *T. equi*, *T. lestoquardi*, *T. uilenbergi*, and *T. luwenshuni.* In such cases, clinical diseases present with severe signs and mortality. Theileriosis in livestock is characterized by fever, anemia, anorexia, weight loss, jaundice or hemoglobinuria (Steinman et al., 2012; Hamidhi et al., 2016; Yu et al., 2017).

T. parva, the causative agent of East Coast fever, is mainly distributed in Africa (Ringo et al., 2018), while *T. annulata*, the etiological agent of tropical theileriosis, is distributed in Europe (Garcia et al., 2012), the Middle East (Hamidhi et al., 2016) and parts of Asia (Yu et al., 2017). *T. parva* and *T. annulata* are transmitted by ixodid ticks, such as *Hyalomma* spp. and *Rhipicephalus* spp.. However, *T. equi*, one of the agents of equine piroplasmosis, is distributed in Europe (Ribeiro et al., 2013), Asia

(Steinman et al., 2012), Africa (Mahmoud et al., 2016) and North America (Díaz-Sánchez et al., 2018). The pathogen is carried by the tick genera *Rhipicephalus* and *Haemaphysalis.* In small ruminants, *T. lestoquardi* presents as malignant theileriosis and can be transmitted by *Hyalomma* spp. and *Rhipicephalus* spp. in Sudan and the Middle East (Hamidhi et al., 2016). *T. uilenbergi* and *T. luwenshuni* were first reported in China and identified in tick species *Haemaphysalis* (Li et al., 2009). Other *Theileria* spp. known to infect bovine (*T. velifera*, *T. taurotragi*, *T. mutans* and *T. buffeli*/ *orientalis* group) and small ruminants (*T. separata*, *T. ovis*, *T. recondita*, *Theileria* sp.) are non-pathogenic with no significant signs and symptoms nor mortality (Berggoetz et al., 2014; Gebrekidan et al., 2014; Zhou et al., 2017).

5. Anaplasmosis of livestock

Anaplasma is a genus of obligate intraerythrocytic bacteria of cattle, horses, sheep and goats. They are the causative agents of anaplasmosis and can be transmitted by ticks or mechanically by biting flies and blood-contaminated fomites. Clinical signs of livestock anaplasmosis include fever, anaemia, icterus, and a decreased number of red blood cells (Rar and Golovljova, 2011).

Species under the genus *Anaplasma* are distributed in North America (Qurollo, 2019), Africa (Ben Said et al., 2015), Asia (Von Fricken et al., 2018), Portugal (Ribeiro et al., 2013) and Australia (Dehhaghi et al., 2019). These include *A. marginale*, *A. centrale*, *A. ovis*, *A. phagocytophilum*, *A. bovis* and *A. platys*. *A. bovis* in sheep and goats in several countries was reported by previous studies (Iqbal et al., 2019; Ben Said et al., 2015), indicating the reservoir competence of these animals for *A. bovis.* A number of *Anaplasma* species including *A. ovis*, *A. bovis* and *A.*

phagocytophilum have been documented to cause anaplasmosis in ruminants (Rar and Golovljova, 2011). *A. bovis*, *A. centrale*, *A. phagocytophilum* and *A. marginale* were identified in ruminants or ticks in Russia (Livanova et al., 2018), Philippines (Galon et al., 2019) and south India (Nimisha et al., 2019). In China, six species of *Anaplasma*, namely *A. marginale*, *A. ovis*, *A. phagocytophilum*, *A. centrale*, *A. capra*, and *A. platys*, were detected in animals and from 20 tick species (Shi et al., 2020).

6. Q fever and rickettsioses of livestock

Coxiella burnetii, a small Gram-negative bacterium, is the causative agent of Query (Q) fever and can infect cattle, sheep, goats and humans. Except in New Zealand, Q fever has occurred worldwide (González-Barrio and Ruiz-Fons, 2019), such as China (El-Mahallawy et al., 2016a), Slovenia (Knap et al., 2019) and Netherlands (Schimmer et al., 2014). Humans are infected with *C. burnetii* by breathing in dust contaminated by urine, feces, birth products and milk of infected animals. Acute case of Q fever ordinarily presents as a self-limiting febrile illness, hepatitis, or pneumonia, and only seldom infections become chronic cases (Sun et al., 2016). Recently, Q fever has received attention worldwide. Because *C. burnetii* is mainly transmitted through tick bites, the pathogen has also been reported to be transmitted by aerosol inhalation in livestock (Pan et al., 2013; Tozer et al., 2014). According to Wu et al. (2013), *C. burnetii* was detected in more than 40 tick species, including the genera *Ixodes*, *Rhipicephalus*, *Amblyomma* and *Dermacentor*. Nineteen tick species were reported to carry *C. burnetii* in China (Du, 2016).

Spotted fever rickettsioses, are important zoonotic diseases widely distributed around the world and are caused by several spotted fever group *Rickettsia* spp. (SFG

Rickettsia) (Berri et al., 2009; Han et al., 2018; Tshokey et al., 2019). The seriousness of these diseases is not only closely related to the economic losses in animal production including horses, but also pose health risks to humans (Socolovschi et al., 2012a; Pan et al., 2013; Han et al., 2018; Li et al., 2018; Von Fricken et al., 2018). According to Cicculli et al. (2019), *Rickettsia* species were the most common pathogens identified in *Rhipicephalus bursa* and *Ixodes ricinus* ticks from cattle and sheep in Corsica, France. The vectors of *R. massiliae* and *R. raoultii* (Olivieri et al., 2018) were confirmed to be *R. sanguineus* (s.l.) and *Dermacentor reticulatus*. SFG rickettsiae like *R. heilongjiangensis*, *R. raoultii*, *R. slovaca*, *R. aeschlimannii*, *R. sibirica* and *R. massiliae* have been reported in China (Han et al., 2018), and the disease has been recognized as an emerging zoonosis.

7. Diagnosis of livestock tick-borne pathogens

A lot of diagnostic methods are developed for the detection of TBPs, including Giemsa-stained blood smears, serological tests and molecular detection tools. Blood smears can be used to identify the acute stages of babesiosis and theileriosis characterized by high parasitemia. This method is simple, cheap and applicable in detection, but this method also has some disadvantages, such as low sensitivity of detecting carrier animals and sub-clinically infected animals with low parasitemia. Blood smear is also difficult for differentiating the same species of *Babesia* and *Theileria* because of some similarity in morphology. Although this method can detect babesiosis and theileriosis in the acute stage, this method requires a lot of experienced and trained people.

Serological tests have been developed for detecting TBPs, including indirect

fluorescent antibody test (IFAT), enzyme-linked immunosorbent assays (ELISA), complement fixation test (CFT) and immunochromatographic test (ICT). IFAT is usually used to diagnose and detect carrier animals for pathogens such as *A. phagocytophilum* (Chahan et al., 2005). Meanwhile, ELISA are used widely as serological diagnostic methods for the monitoring of various diseases. For example, ELISA was used in the detection of antibodies for *T. equi*, *B. caballi* (Mujica et al. 2011) and *C. burnetii* (Filioussis et al., 2017) in livestock. Other serological tests, such as CFT, have been used to detect antibody against *B. bovis* (Terkawi et al., 2013), *T. equi*, *B. caballi* (Kouam et al., 2010) and *A. phagocytophilum* (Chahan et al., 2005). ICT has been also developed to detect antibodies for *T. equi* and *B. caballi* (Ybañez et al. 2018), *T. uilenbergi* and *T. luwenshuni* (Lu et al., 2015). Although some serodiagnostic tests can be used to extensively evaluate TBP infections in the chronic stage, but an infected animal is detected with lower sensitivity in the acute stage and detection results are misinterpreted when livestock recover and in cases of earlier vaccinations.

Polymerase chain reaction (PCR) assays can identify both carriers and low parasitemic clinical infections. PCR is also an available option for identification and detection of diseases such as *B. occultans*, *T. separata* (Sun et al., 2019), *A. marginale* (Zhou et al., 2018), *C. burnetii* (Fournier and Raoult, 2003) and *Rickettsia* spp. (Han et al., 2018). Fluorescence resonance energy transfer-based real time PCR (FRET-qPCR), a more sensitive and quantitative detection method, was developed for *C. burnetii*, *B. bovis*, *B. divergens*, *B. gibsoni*, *B. canis*, *B. vogeli* and *B. microti* (Li et al., 2015b; El-Mahallawy et al., 2016b). Furthermore, loop mediated isothermal amplification (LAMP), another molecular detection method, has been established for some TBPs, such as *T. equi* and *B. caballi* (Alhassan et al., 2007).

Although time consuming and expensive equipment are some of the limitations of PCR, PCR tests are useful and suitable for confirmatory and regulatory testing because they are highly sensitive and specific, which can discriminate various TBPs in carrier infections.

8. Aim of the present study

Xinjiang Uygur Autonomous Region (XUAR) occupies one-sixth of China's land area and borders eight countries including Afghanistan, India, Kazakhstan, Kyrgyzstan, Mongolia, Pakistan, Russia and Tajikistan. XUAR is surrounded by multiple–land forms such as Gobi Desert, valleys, mountains, grasslands and plates. XUAR also has a complex climate, including temperate continental arid, semi-arid, and warm temperate continental arid. Furthermore, international livestock trade is thriving in XUAR. The abundant vegetation, various tick species, free grazing and complicated conditions in XUAR provide favorable conditions for the survival of ticks and therefore, propagation of TBPs. Six tick genera including *Dermacentor*, *Hyalomma*, *Rhipicephalus*, *Haemaphysalis*, *Ixodes*, *Argas*, and 45 tick species were identified in XUAR, China. Although there have been studies focusing on TBPs of ticks in XUAR, systematic analysis of TBPs detection in livestock remains unclear in XUAR, China.

The objective of the present study is to investigate tick-borne pathogens of cattle, horses and sheep in XUAR, China.

Chapter 1

Molecular investigation of tick-borne pathogens in cattle from Xinjiang Uygur Autonomous Region, China

1-1. Introduction

Tick-borne pathogens (TBPs) are transmitted by ticks and generally include protozoan parasites *Babesia* and *Theileria* and bacteria *Anaplasma* and *Coxiella*. Among *Babesia* species, *B. bovis* and *B. bigemina* are the most common species infecting bovines and field animals are often co-infected with both species (Niu et al., 2015). Clinical signs of infected cattle (*B. bovis* and *B. bigemina*) include fever, anemia, hemoglobinuria and even death. Both species can be transmitted by *Rhipicephalus* (*Boophilus*) *microplus* (Rojas-Martínez et al., 2018). *B. bigemina*, *B. bovis*, *B. ovata*, *B. major* and *B. orientalis* are the causative agents of bovine babesiosis that are reported in China (Luo et al., 2005a; Luo et al., 2005b).

In China, Q fever was first reported in Beijing in 1950 (Zhang et al., 1951). During the last two decades, a variety of serosurveys in humans and animals have been reported (Wu et al., 2013; Cong et al., 2015; El-Mahallawy et al., 2016b). However, molecular detection of *C. burnetii* has not been done in ruminants in Xinjiang Uygur Autonomous Region (XUAR). In addition, *A. bovis* was reported in tick vectors and in domestic and wild animals in several areas in China, including Inner Mongolia (Li et al., 2014), Guizhou , Shanxi, Yunnan, Henan (Cui et al., 2017),

Hubei, Chongqing (Zhou et al., 2018) and Gansu (Zhang et al., 2016; Lu et al., 2017; Han et al., 2018; Yang et al., 2018).

The XUAR covers one-sixth of China's land, borders eight countries. In 2010, there were more than 3 million cattle in XUAR (Statistic Bureau of Xinjiang Uygur Autonomous Region, 2011), China. *Babesia* and *Anaplasma* infections were reported in XUAR, however, *B. bovis* and *B. bigemina* were identified only in ticks but not yet in animals (Yu et al., 2015).

1-2. Materials and methods

Ethical statement

The permission and approval of all blood samples were acquired from selected farm owners. The ethical guidelines of all the procedures for treating animal samples permitted by Obihiro University of Agriculture and Veterinary Medicine (Approval ID: 18-40).

Blood collection and DNA extraction

Cattle blood samples (n = 195) were collected in Bole (n = 30), Kizilsu Kirghiz (n =30) and Altay (n =135) in XUAR, China in June 2018 (Fig. 1-1). Sampled animals were one and a half year old and above. After collection, blood samples were transported to the laboratory in cool boxes and kept at 4°C. QIAamp DNA Blood Mini Kit (Qiagen, Germany) were used for DNA extraction with manufacturer's protocol, and DNA stored at -30°C.

Detection of TBPs

All samples were screened with species specific primers for *B. bigemina Rhoptry associated protein-1a* (*Rap1a*) (Terkawi et al., 2013), *B. bovis Spherical body*

protein-4 (*SBP*-4) (Terkawi et al., 2011), *A. bovis* 16S rRNA (Reye et al., 2012) and *C. burnetii heat shock protein antigenic polypeptide* (*htpB*) (Yu et al., 2017) by nested PCR (nPCR) (Table 1-1). The PCR reaction mixture was composed of the following: 0.1 µl Taq polymerase (0.5 U; Takara, Japan), 0.25 µl of forward and reverse primers (10 µM), 0.25 µl of dNTP mix (2.5 mM), 1.25 µl of 10x buffer (Takara, Japan) and 9.4 µl distilled water. The PCR products were checked by electrophoresis in 1.5% agarose gels (Qiagen, Netherlands), stained by ethidium bromide, and then observed under UV light (Atto, Japan).

Sequencing and phylogenetic analysis

Five positive samples were randomly selected from the three sampling areas, and at least 3 clones were sequenced. When the sequences obtained from different amplicons were identical, only one amplicon was retained for sequence analysis. Representative amplicons were cloned in pGEM-T Easy Vector (Promega, USA). In brief, after extracting the amplicons from the agarose using QIAquick Gel Extraction Kit (Qiagen, Germany), purified DNA was ligated into the pGEM-T Easy Vector (Promega, USA). The plasmid construct was transformed into *Escherichia coli* DH5α and then extracted using NucleoSpin® Plasmid QuickPure (Machery-Nagel, Germany). Bigdye Terminator Cycle Sequencing Kit (Applied Biosystems, USA) and 3100 Genetic Analyzer (Applied Biosystems, USA) were used for sequencing.

The nucleotide sequence identities were determined by performing GenBank BLASTn analysis on the NCBI database. NCBI database (GenBank BLASTn) were used to analyze nucleotide sequence identities. Phylogenetic trees were then constructed based on the Kimura 2-parameter model using the Maximum Likelihood (ML) method in the MEGA version 7.0 program (Kumar et al., 2016).

1-3. Results

Among the 195 samples, the nPCR assays revealed that 141 (72.3%) cattle were infected with one to three pathogens (Table 1-2). The most detected pathogens were *B. bigemina* occurring in 34.4% of the samples, followed by *C. burnetii* (20.5%), *B. bovis* (12.3%) and *A. bovis* (5.1%) .

A total of 26 cattle (13.3%) were coinfected with two or three pathogens (double infection, n=24; triple infection, n=2). The most common coinfection was *B. bigemina* + *C. burnetii* (12/26), followed by *B. bigemina* + *B. bovis* (7/26). Meanwhile, only two cattle were infected with three TBP species, *B. bigemina* + *B. bovis* + *C. burnetii* (Table 1-3).

All *B. bigemina*, *B. bovis*, *C. burnetii* and *A. bovis* sequences in this study were of the expected sizes of 412, 503, 325 and 551 bp, respectively. The *Rap1a* gene sequences of *B. bigemina* have been deposited in GenBank under accession numbers MK345483 and MK345484. The accession numbers for *SBP*-4 gene sequences of *B. bovis* and *htpB* gene of *C. burnetii* are MK345486, MK345487 and MK345477, MK345478, respectively. The partial 16S rRNA gene sequence of *A. bovis* was assigned accession number MK345480, MK345481. The identity between MK345483 and MK345484 was 99.8%. The identity between MK345486 and MK345487 and between MK345477 and MK345478 were 99.7% and 99.8% respectively.

Phylogenetic analysis indicated that the *Rap1a* sequences of *B. bigemina* from XUAR belong to the same cluster with a sequence from Indonesia (KY484520) and Philippines (JN974300) (Fig. 1-2). The *B. bovis SBP*-4 sequences appeared to form one cluster and illustrated a close relationship with the sequences from Thailand (AB594814) and Syria (AB617641) (Fig. 1-3). Interestingly, the two *C. burnetii htpB* sequences formed a new clade (Fig. 1-4) with Nigeria isolate (JQ346187). Analysis of

the two *A. bovis* 16S rRNA sequences obtained from this study revealed a close relationship with previous XUAR strain (KJ782395) (Fig. 1-5).

1-4. Discussion

TBPs represent a serious and economic threat to global veterinary and public health. In XUAR, 45 species, 6 genera of ticks including *Hyalomma*, *Dermacentor*, *Haemaphysalis* and *Rhipicephalus* are reported (Zhang et al., 2017). In addition, *T. annulata*, *Borrelia burgdorferi* sensu stricto, *R. massiliae* and *A. bovis* were identified in *Hyalomma asiaticum* ticks (Yu et al., 2017; Wang et al., 2015). However, information on the occurrence and genetic diversities of TBPs are lacking in some areas in China, particularly in XUAR. In this study, *B. bigemina*, *B. bovis*, *C. burnetii* and *A. bovis* were identified in cattle in part of areas of XUAR, China.

B. bigemina and *B. bovis* are highly pathogenic for ruminants like cattle and buffalo. Previous studies on *B. bigemina* and *B. bovis* infection in cattle have been conducted in a forested area of northwestern China (Liu et al., 2014). Although Niu et al. (2015) reported *Babesia* infections in 14 provinces in China, no positive case for *Babesia* species was identified in cattle in XUAR. The average prevalence of *B. bigemina* from 2008 to 2013 in Chongqing, Qinghai, Gansu, Inner Mongolia and Jilin was 4.5% (14/309) (Niu et al., 2015). Interestingly, the present study revealed that infection rate of *B. bigemina* (Table 1-2) was higher than that in the previous studies (Niu et al., 2015). Furthermore, type-specific PCR assays confirmed the presence of *B. bovis* in Mongolia (Liyanagunawardena et al., 2016), while *Babesia* spp. closely related to *B. bigemina* were detected from 29 ticks species in Russsia (Rar et al., 2014). Mongolia and Russia are countries having animal trade with Altay where

bovine *Babesia* species were identified in this study. The transportation of tick-infested ruminants from one country to another may have played a role in the dissemination the TBPs within the region. Interestingly, the infection rate of *B. bigemina* and *B. bovis* in Kizilsu Kirghiz (Table 1-2) are in agreement with data from India (Kolte et al., 2017). According to Bhat et al. (2017), *R. microplus* can transmit *B. bigemina.* Local husbandry practices and business might be a reason for the spread of *Babesia* species in the studied areas. In the phylogenetic analyses, the gene sequences of *B. bigemina Rap1a* were closed with previous strains (Indonesia, Philippines). The *SBP*-4 gene sequences of *B. bovis* from my study were also closed with sequences from Benin and Mongolia (Fig. 1-2 and 1-3).

Zhou et al. (2014), revealed that *Rhipicephalus sanguineus* was distributed in 20 provinces in China and is a vector of *Coxiella*, *Rickettsia*, *Ehrlichia*, *Babesia*, and *Hepatozoon*. Furthermore, in a previous study in Dzungarian Gate of XUAR, the *C. burnetii* infection rate in *Hyaloma asiaticum asiaticum* was 22.65% (41/181) while *R. sanguineus*, *Dermacentor marginatus*, *Haemaphysalis erinacei* were negative (Luo et al., 2016). However, the molecular information available in China is still insufficient (Yin et al., 2015a). This study reports first molecular detection of *C. burnetii* in cattle in XUAR. The *htpB* gene has previously been used for the detection of *Coxiella* organisms (Fournier et al., 2003). A *C. burnetii* seroprevalence of 24.9% in cattle has previously been reported in Inner Mongolia, China (Cong et al., 2015). In addition, Tokarevich et al. (2006), reported cattle to be the main source of *C. burnetii* infection in humans in Russia. The present study identified only Bole as free of *C. burnetti* infection. The reasons for absence of *C. burnetii* infections may be tick vector distribution or the low number of exotic breeds or cross breed animals in this geographical area. The *C. burnetii* infection in Kizilsu Kirghiz and Altay may be due

to the presence of infected humans or ruminants acting as reservoirs in those areas. Interestingly, phylogenetic tree of the *htpB* sequences of *C. burnetii* illustrated high similarity to isolates documented in Nigeria (JQ346187) in Fig. 1-4.

Anaplasmosis caused by *A. bovis* has a wide host range and is known to be pathogenic to domestic ruminants including cattle, sheep, goats and other animals such as dogs, cats and deer (Yang et al., 2015). High prevalence of *A. bovis* in sheep and goats in several countries were reported by previous studies, indicating the reservoir competence of these animals for *A. bovis* (Liu et al., 2012; Ben Said et al., 2015). Additionally, it was reported to be transmitted by *Hyalomma* spp., and was detected in cattle in XUAR, recently (Yu et al., 2017). A infection rate of 5.1% for *A. bovis* in cattle in this study which was in accordance with the report of Qiu et al. (2016). Although there is a report about *Hyalomma* species occurrence in XUAR, Altay is the only sampling area for which detailed information on common tick species was available (Wang et al. 2015). *A. centrale*, *A. bovis* and *A. phagocytophilum* were identified in *Haemaphysalis longicornis* in Liaoning, China (Dong et al., 2014). In Mongolia, 8.7% infection rate of *A. marginale* by nPCR and antibody to *Anaplasma* spp. were observed in cattle herds (Ybañez et al., 2013). Based on the phylogenetic analyses, *A. bovis* 16S rRNA sequences of this study were in a single clade (Fig. 1-5), which suggests that the 16S rRNA gene of this pathogen is conserved in different locations.

Mixed infections involving 2-3 pathogens were observed in the present study. Most of co-infections were caused by *B. bigemina* + *C. burnetii* and *B. bigemina* + *B. bovis*, respectively. In addition, *B. bigemina* was in co-infection with almost all the other pathogens. Although clinical cases were not observed, it is possible that the cattle infected with multiple pathogens may have more pronounced clinical signs or

hematological abnormalities than those infected with single pathogens (Ochirkhuu et al., 2015). It is suggested that *B. bovis*, *B. bigemina*, *C. burnetii* are potential pathogens that cause mixed infection in XUAR.

This study revealed the existence and genetic diversity of *B. bigemina, B. bovis, C. burnetii* and *A. bovis* in Bole, Kizilsu Kirghiz and Altay, XUAR, China. The current data determined the infection rates of detected pathogens in cattle in that region and suggest the possible emergence of tick-borne diseases in animals in northwest China.

1-5. Summary

TBDs cause significant losses to livestock production in tropical and subtropical regions. However, information of TBPs in cattle is still insufficient in XUAR, northwestern China. *Babesia bovis*, *B. bigemina*, *Coxiella burnetii* and *Anaplasma bovis* infections were detect and analyze by nPCR assays and sequencing in XUAR. Out of 195 samples tested, 24 (12.3%), 67 (34.4%), 40 (20.5%) and 10 (5.1%) were positive for *B. bovis*, *B. bigemina*, *C. burnetii* and *A. bovis*, respectively. Sequencing analysis indicated that *B. bovis SBP-4*, *B. bigemina Rap1a*, *C. burnetii htpB* and *A. bovis* 16S rRNA genes from XUAR showed 99%-100% identity with documented strains from other countries. Phylogenetic analyses revealed that *B. bovis SBP-4*, *B. bigemina Rap1a*, *C. burnetii htpB* and *A. bovis* 16S rRNA gene sequences clustered in the same branches with isolates from other countries. To the best of my knowledge, *B. bovis*, *B. bigemina* and *C. burnetii* infections were first time reported in cattle in XUAR. This study provides considerable data for understanding the distribution of TBPs, and is expected to improve the approach for control of TBDs in XUAR, China.

Table 1-1. List of primers used in the assays.

TBP Target gene	Assay	Primer (5'- 3')	AT (°C)	EPS (bp)	References
		Forward			
B. bovis SBP-4	1st PCR	AGTTGTTGGAGGAGGCTAAT TCCTTCTCGGCGTCCTTTTC	55	907	Terkawi et al., 2011
	Nested PCR	GAAATCCCTGTTCCAGAG TCGTTGATAACACTGCAA	55	503	
B. bigemina Rap1α	1st PCR	GAGTCTGCCAAATCCTTAC TCCTCTACAGCTGCTTCG	55	879	Terkawi et al., 2013
	Nested PCR	AGCTTGCTTTCACAACTCGCC TTGGTGCTTTGACCGACGACAT	55	412	
C. burnetii htpB	1st PCR	GCGGGTGATGGTACCACAACA GGCAATCACCAATAAGGGCCG	56	501	Yu et al., 2017
	Nested PCR	TTGCTGGAATGAACCCCA TCAAGCTCCGCACTCATG	52	325	
A. bovis 16S rRNA	1st PCR	TCCTGGCTCAGAACGAACGCTGGCGGC AGTCACTGACCCAACCTTAAATGGCTG	55	1433	Reye et al., 2012
	Nested PCR	CTCGTAGCTTGCTATGAGAAC TCTCCCGGACTCCAGTCTG	55	551	

Note: AT= Annealing temperature; EPS= Expected product size.

Table 1-2. Detection of *B. bigemina*, *B. bovis*, *C. burnetii*, *A. bovis* in cattle from XUAR, China.

Pathogen	District / No. positive (%)			Overall (N=195)
	Bole (N=30)	Kizilsu Kirghiz (N=30)	Altay (N=135)	
B. bigemina	15 (50.0)	11 (36.7)	41 (30.4)	67 (34.4)
B. bovis	0	3 (10.0)	21 (15.6)	24 (12.3)
C. burnetii	0	8 (26.7)	32 (23.7)	40 (20.5)
A. bovis	7 (23.3)	0	3 (2.2)	10 (5.1)

Table 1-3. Tick-borne pathogens detected in cattle from XUAR, China.

Parameter	Pathogen	No. positive (%)
Single infection	*B. bigemina*	46 (23.6)
	B. bovis	13 (6.7)
	C. burnetii	21 (10.8)
	A. bovis	7 (3.6)
Co - infection	*B. bigemina* + *B. bovis*	7 (3.6)
	B. bigemina + *C. burnetii*	12 (6.2)
	B. bovis + *C. burnetii*	2 (1.0)
	B. bigemina + *A. bovis*	0
	B. bovis + *A. bovis*	0
	C. burnetii + *A. bovis*	3 (1.5)
	B. bigemina + *B. bovis* + *C. burnetii*	2 (1.0)
	B. bigemina + *B. bovis* + *A. bovis*	0

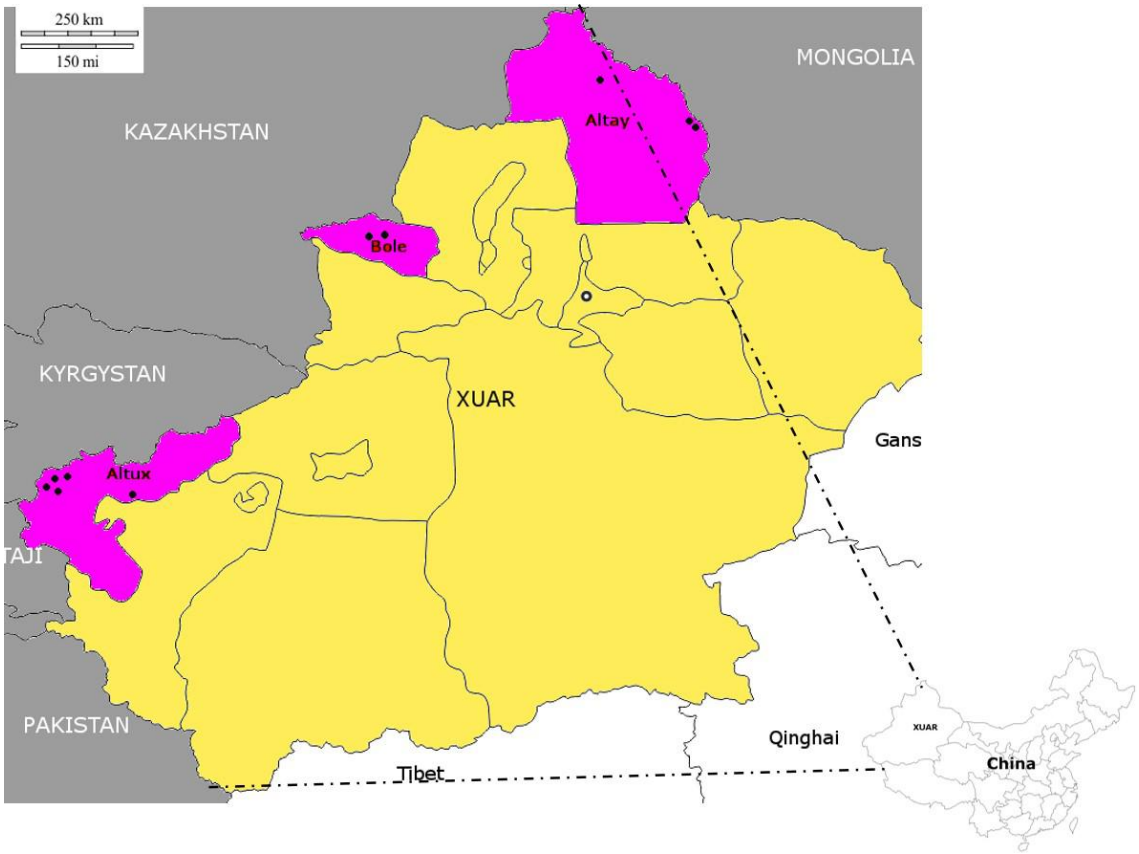


Fig. 1-1. Map of XUAR, China. The black dots indicate localities where samples were collected.

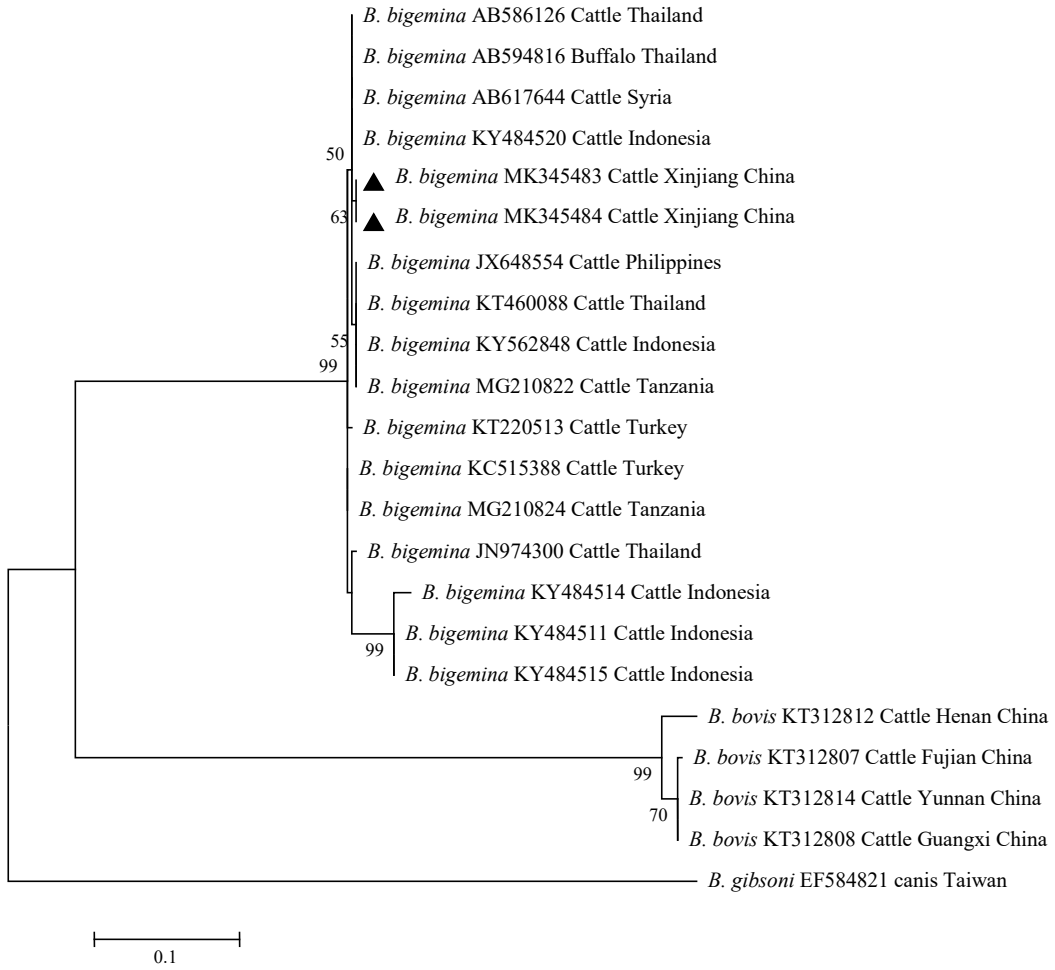


Fig. 1-2. Phylogenetic analysis of *Babesia bigemina Rap1a* gene sequences. Numbers at nodes represent percentage occurrence in 1000 bootstrap replication. Sequences from this study are marked with triangle. The values lower than 50% are hidden.

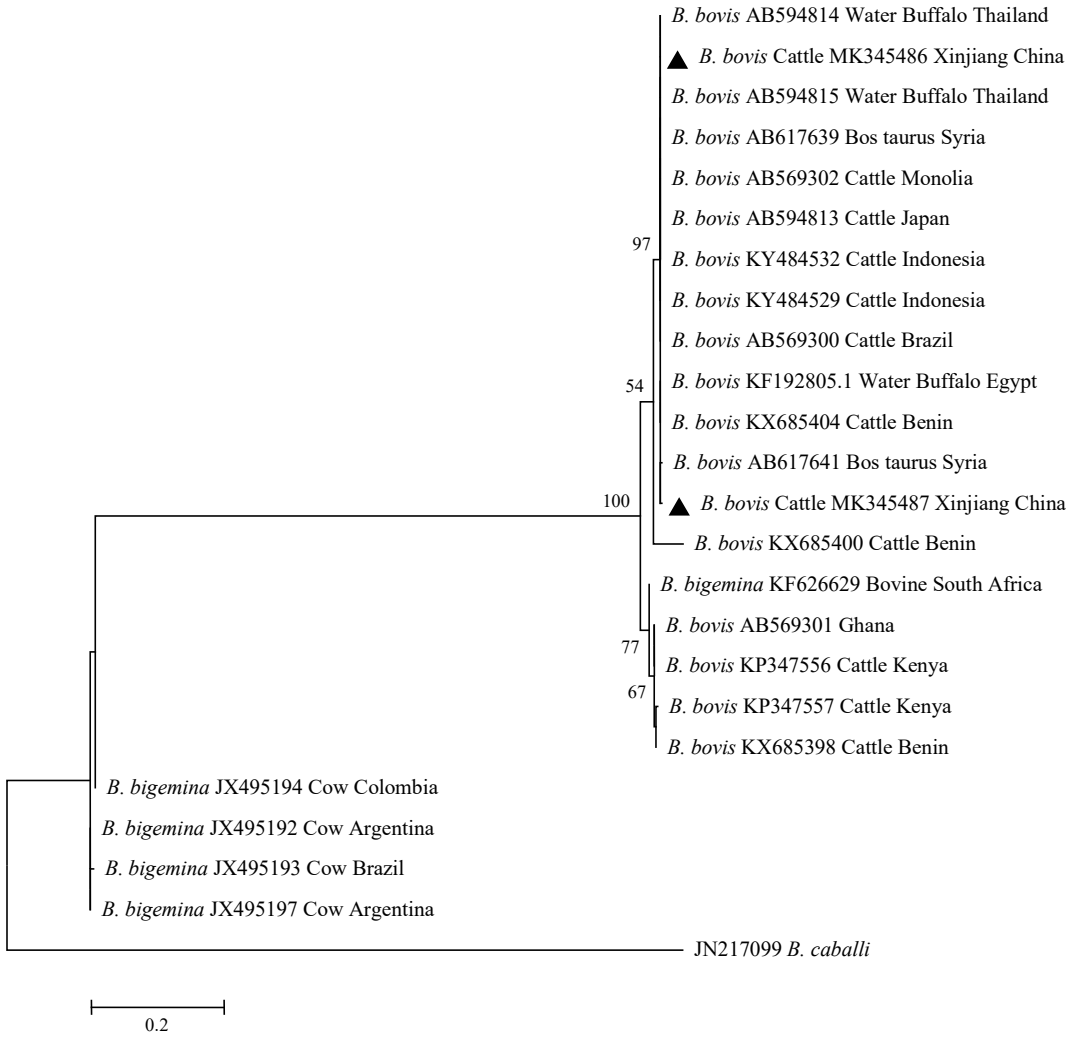


Fig. 1-3. Phylogenetic analysis of *Babesia bovis SBP-4* gene sequences. Numbers at nodes represent percentage occurrence in 1000 bootstrap replication. Sequences from this study are marked with triangle. The values lower than 50% are hidden.

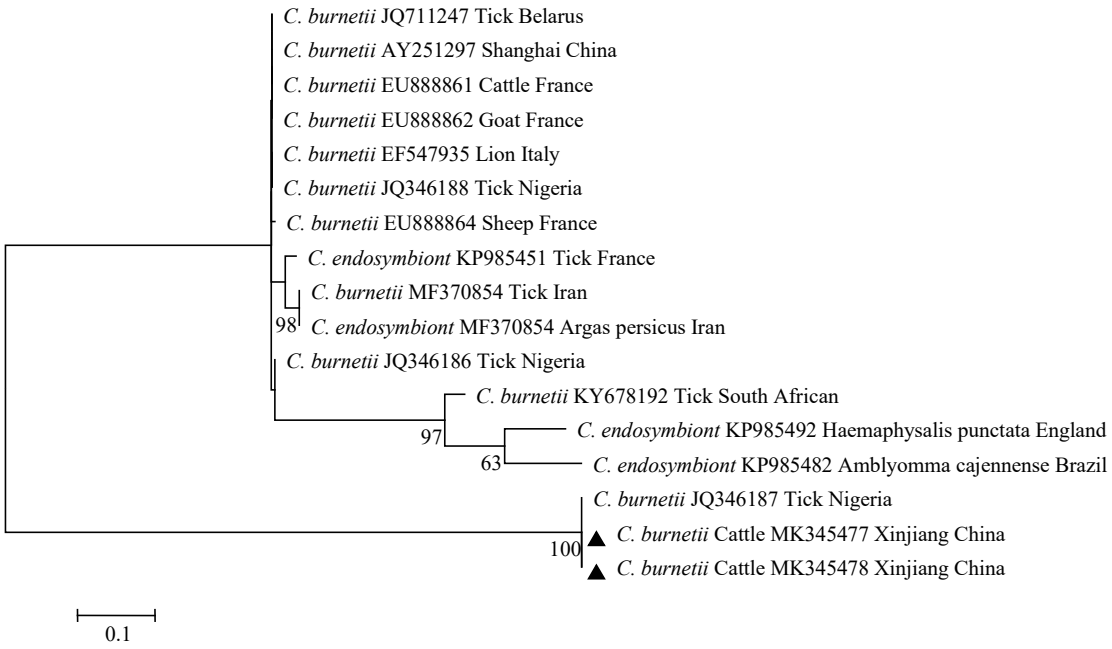


Fig. 1-4. Phylogenetic analysis of *Coxiella burnetii htpB* gene sequences. Numbers at nodes represent percentage occurrence in 1000 bootstrap replication. Sequences from this study are marked with triangle. The value lower than 50% are hidden.

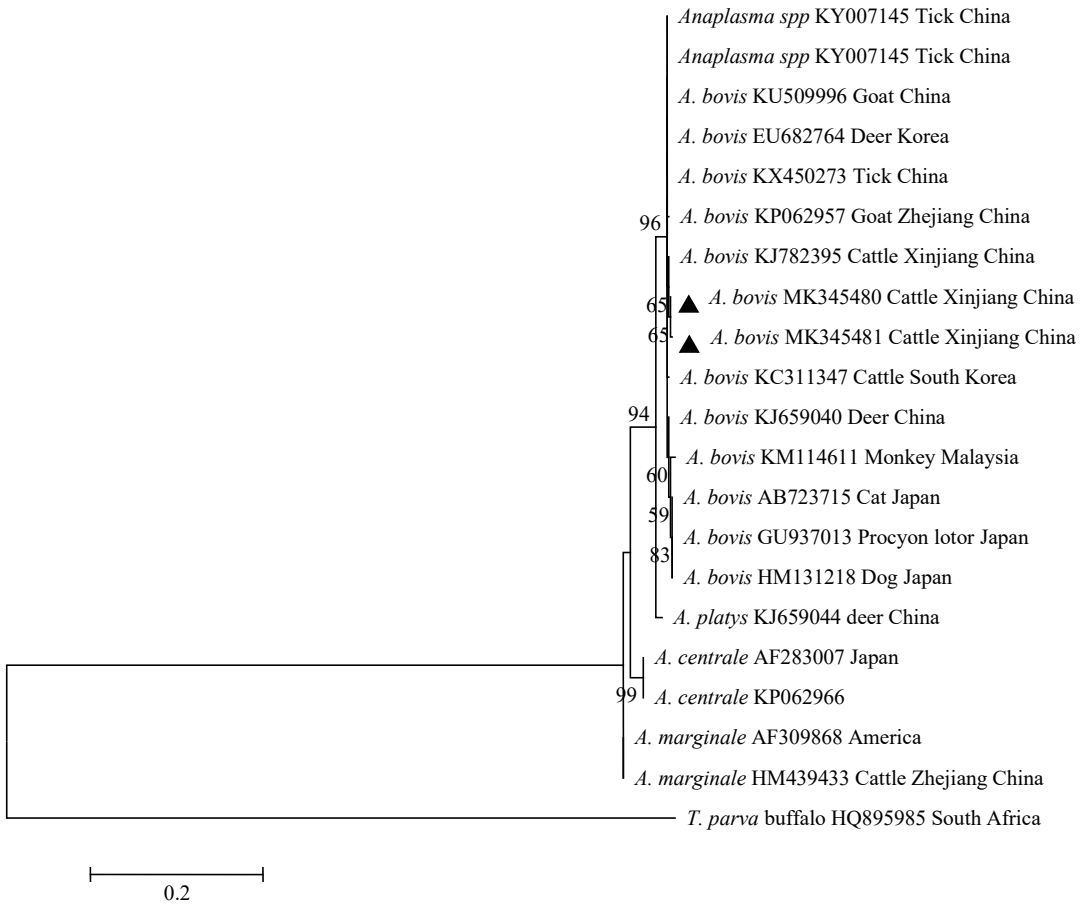


Fig. 1-5. Phylogenetic analysis of *Anaplasma bovis* 16S rRNA gene sequences. Numbers at nodes represent percentage occurrence in 1000 bootstrap replication. Sequences from this study are marked with triangle. The value lower than 50% are hidden.

Chapter 2

Molecular detection of zoonotic pathogens, *Coxiella burnetii* and *Rickettsia* spp. and piroplasma in horses in Xinjiang Uygur Autonomous Region, China

2-1. Introduction

Equine tick-borne diseases (Equine TBDs), caused by tick-borne pathogens (TBPs), are considered to be a huge risk in horses as these diseases result to severe morbidity and mortality, and also, are potential source of zoonotic pathogens which can infect humans (Veronesi et al., 2014). Q fever, spotted fever rickettsioses and Equine piroplasmosis (EP), are some of the most serious Equine TBDs caused by *Coxiella burnetii*, *Rickettsia* spp., *Babesia caballi* and/or *Theileria equi*, and they have been reported to cause economic devastation in horse industries (Socolovschi et al., 2012b; Ribeiro et al., 2013; Ebani et al., 2015; Díaz-Sánchez et al., 2018).

Rickettsioses are one of the longest worldwide known infectious diseases in arthropod vectors, animals and humans, including Xinjiang Uygur Autonomous Region (XUAR), China (Kong et al., 1982; Fan et al., 1987). EP is caused by two erythrocyte-targeting parasites, *B. caballi* and *T. equi*, which are transmitted by ticks from the genera *Hyalomma*, *Rhipicephalus*, *Ixodes* and *Dermacentor* (Bhoora et al., 2018; Ybañez et al., 2018). EP is endemic in tropical and temperate areas and typical

clinical signs are fever, depression, anaemia, icterus, oedema and anorexia (Bartolomé Del Pino et al., 2016). This disease is one of the most serious problem in horse farming industry (Díaz-Sánchez et al., 2018), specifically in China (Xu et al., 2003; Chahan et al., 2006; Zhang et al., 2017). In addition, control of EP is crucial for the international trade of horses (Bhoora et al., 2018).

XUAR, the largest province in China, is located in the most northwest area. The horse grazing is one of the main industries of XUAR, especially in Ili Kazakh Autonomous Prefecture (Ili). There were more than 900 thousands horses at 2010 in XUAR, China (Statistic Bureau of Xinjiang Uygur Autonomous Region, 2011). Although several studies focusing on EP in XUAR have been reported, prevalence and molecular identifications of these TBPs are limited in horses (Chahan et al., 2005; Chahan et al., 2006; Zhang et al., 2017). This study was initiated to determine TBPs in horses in XUAR, China and to molecularly identified the pathogens.

2-2. Materials and methods

Ethical statement

The permission and approval of all blood samples were acquired from selected farm owners. The ethical guidelines of all the procedures for treating animal samples permitted by Obihiro University of Agriculture and Veterinary Medicine (Approval ID: 18-40).

Sample collection and DNA extraction

Two hundred blood samples were randomly collected from apparently healthy female Ili horses from 10 horse ranches in 3 counties in XUAR, China from May 2018 to June 2018 (Fig. 2-1). The samples were obtained in containing Zhaosu county

(4 horse ranches, n = 80), Tekesi county (3 horse ranches, n = 60) and Nileke county (3 horse ranches, n = 60). Information on age of each animal was also recorded. DNA was extracted using the QIAamp DNA Blood Mini Kit and procedure was followed with chapter 1 materials and methods 1-2.

TBPs detection

TBPs were screened as previously described in Table 2-1. In brief, partial sequence of *Anaplasma phagocytophilum* 16S rRNA (Lee et al. 2016), *B. caballi rhoptry-associated protein 1* (*Rap-1*) (Alhassan et al., 2005), *Borrelia burgdorferi* sensu lato (*B. bsl.*) 23S rRNA (Chang et al., 2000), *C. burnetii heat shock protein antigenic polypeptide* (*htpB*) (Reye et al., 2012), *T. equi merozoite antigen 1* (*EMA-1*) (Alhassan et al., 2005) and *Rickettsia* spp. *outer membrane protein A* (*ompA*) (Kidd et al., 2008) genes were amplified by PCR (Table 2-1). DNA positive control for each pathogen were used including *B. caballi* (horse), *T. equi* (horse), *A. phagocytophilum* (cattle), *C. burnetii* (cattle) and *Rickettsia* spp. (cattle) from National Research Center for Protozoan Diseases, Obihiro University. No positive control was available for *B. bsl.*. Negative control was used by double-distilled water. In addition, some positive samples were selected for molecular identification for *B. caballi* and *T. equi* based on the 18S rRNA as previously described by Alhassan et al (2005).

Sequencing and phylogenetic analysis

The procedure of sequencing and phylogenetic analyse was followed with chapter 1 materials and methods 1-2 (Kumar et al., 2016).

2-3. Results

Among 200 horse blood samples, 163 (81.5%) were infected with at least one pathogen (Table 2-3). Of these samples, *Rickettsia* spp. was the most prevalent

pathogen (n = 114, 57.0%), followed by *C. burnetii* (n = 79, 39.5%), *T. equi* (n = 79, 39.5%) and *B. caballi* (n = 49, 24.5%) (Table 2-2).

Mixed infections were apparent in 100 samples (50.0%) with 11 different types (Table 2-3). Among these mixed-infected samples, 11 (5.5%) samples were infected with the 4 TBPs. In addition, the results showed that 36 (18.0%) samples had triple infections and 53 (26.5%) samples presented with dual infections.

The results of evaluation of the effects of age group on infection with *C. burnetii*, *Rickettsia* spp., *B. caballi* and *T. equi*, revealed that both *T. equi* and *B. caballi* infection were detected in horses for all age groups. In detail, the highest prevalence of *B. caballi* was detected in 0-4 year horse group, while the highest *T. equi* prevalence was determined in 5-8 year horse group. However, for *C. burnetii* and *Rickettsia* spp., all horses had high infection rates while 9-15 year horses were the most frequently infected (Table 2-4). The prevalence was significantly different between age group for *T. equi* and *C. burnetii*, but there was not statistically significant difference for *B. caballi* and *Rickettsia* spp..

For *C. burnetii*, *htpB* sequences grouped in the same clade as China, Nigeria and France isolates (Fig. 2-2). *Rickettsia* spp. (*ompA*) of phylogenetic analysis illustrated that isolates in this study grouped in the *R. slovaca* and *R. raoultii* clades with *Dermacentor* spp. isolate from China (Fig. 2-3). In addition, *B. caballi*, *Bc48* isolates grouped in the same cluster with isolates from other country but were in different clades (Fig. 2-4), whereas for 18S rRNA obtained isolates grouped in the same clade with isolates from China, Jordan, South Africa and Spain (Fig. 2-6). Analysis of *T. equi EMA-1* grouped horse isolates in the same clade as Brazil isolate (Fig. 2-5). However, *T. equi* 18S rRNA isolates in this study clustered with isolates recovered

from horses in China, Iran, South Korea and Switzerland, but were distant from horse isolates from South Africa, USA and Brazil (Fig. 2-6).

2-4. Discussion

The presence of *C. burnetii* in China including XUAR has previously been reported in different species, including goats, sheep, cattle, yaks, sika deer, pigs and humans (Cong et al., 2015; Yin et al., 2015a; Yin et al., 2015b; El-Mahallawy et al., 2016a; El-Mahallawy et al., 2016b; Sun et al., 2016). However, the role of horses as a reservoir in China has not been determined yet. In this study, horses were found to be infected with *C. burnetii* in XUAR, China. On the other hand, *C. burnetii* is well-known to infect animal species ranging from domestic and wild mammals, birds and reptiles, as well as arthropods such as ticks (Honarmand et al., 2012). Therefore this could also be extrapolated to an increased risk not only for horses but several mammals in the area.

Sequencing analysis of *Rickettsia ompA* gene showed that *R. slovaca* and *R. raoultii* were pathogens responsible for *Rickettsia* infection in sampled horses. This result is consistent with *R. slovaca* and *R. raoultii* detection in arthropod vector, *D. silvarum* in XUAR (Tian et al., 2012). In addition, previous studies showed the detection of *R. slovaca* and *R. raoultii* in *Dermacentor* spp. but not in other tick species in northwestern China including XUAR (Guo et al., 2016; Han et al., 2018). Therefore, this may suggest that *Dermacentor* tick species is mainly responsible for the occurrence of *R. slovaca* and *R. raoultii* in horses in XUAR. However, future study is needed to provide evidence to the suspected importance of *Dermacentor* spp. in transmitting these pathogens to horses in the study areas. In China, although *R.*

slovaca and *R. raoultii* infection were reported in humans and domestic animals (Liang et al., 2012; Li et al., 2015a), this study reports the first detection of *Rickettsia* infection in horses.

Clinically, acute EP has obvious symptoms with a serious health threat for horses, however chronic infection usually presents as asymptomatic. Therefore, epidemiological investigation is necessary and effective for prevention and control of the disease. In this study, a prevalence of 24.5% and 39.5% for *B. caballi* and *T. equi* were detected in horses from XUAR (Table 2-2). Previously, exposure of horses to these parasites have been reported by ELISA in XUAR (Xuan et al., 2002; Wang et al., 2014). On the other hand, results in this study are consistent with previous reports which detected 40.8% infection rate of *T. equi* in XUAR horses (Zhang et al., 2017).

The genotype distribution of *C. burnetii htpB* and *Rickettsia* spp. *ompA* gene sequences observed in this study. Importantly, for *Bc48* and *EMA-1* genes have been widely used as key markers to differentiate *B. caballi* and *T. equi*, but 18S rRNA gene is also important for epidemiological investigation and genotype analysis of these pathogens (Alhassan et al., 2005; Bhoora et al., 2018; Ybañez et al., 2018). However, there are a few previous reports on molecular survey and characterization of *B. caballi* and *T. equi* based on these genes in China. In this study, the genotype analysis indicated that *B. caballi Bc48* (Fig. 2-4), *B. caballi* 18S rRNA (Fig. 2-6), *T. equi EMA-1* (Fig. 2-5) and *T. equi* 18S rRNA (Fig. 2-6) genes sequences from XUAR were variable as compared with the isolates from other countries. These results provide more molecular evidence and information to the importance of those pathogens prevalence in horses in XUAR.

The high rate of mixed infections among horse ranches, may be explained by the presence of a wide range of tick-vectors (Tian et al., 2012; Guo et al., 2016). However,

all samples were negative for *A. phagocytophilum* and *B. bsl*., suggesting they may not be some of the most important TBPs in horses in the region. Although the infected horses did not show clinical symptoms, the possibility of TBDs outbreaks cannot be ruled out in the future. However, factors such as sampling regions and sampling time might have influenced of present study. The current findings are expected to provide a basis for better tick-borne disease control in the region. These results also suggest that the persons associated with horses in the region should pay attention for preventing zoonotic tick-borne pathogens from horses.

2-5. Summary

Q fever, spotted fever rickettsioses and equine piroplasmosis, are some of the most serious equine TBDs caused by *C. burnetii*, *Rickettsia* spp., *B. caballi* and/or *T. equi*. This study surveyed and molecularly identified these pathogens infecting horses in ten ranches from XUAR, China. Among 200 horse blood samples, the infection rates of 4 pathogens was 163 (81.5%). The most common pathogen was *Rickettsia* spp. (n = 114, 57.0%), followed by *C. burnetii* (n = 79, 39.5%), *T. equi* (n = 79, 39.5%) and *B. caballi* (n = 49, 24.5%). Co-infections were observed in 61.3% of positive samples. Statistically significant differences were observed between the sampling regions for *C. burnetii*, *B. caballi* and *T. equi*, and also in different age group for *C. burnetii* and *T. equi*. The genotype analysis indicated that *C. burnetii htpB*, *Rickettsia* spp. *ompA*, *B. caballi rap-1*, *B. caballi* 18S rRNA, *T. equi EMA-1* and *T. equi* 18S rRNA gene sequences from horses in XUAR were variable. To the best of my knowledge, *C. burnetii*, *Rickettsia* spp., and co-infected with piroplasma in horses were first time reported in China.

Table 2-1. TBPs primers used in this experiment.

Pathogen	Target gene	Primers (5'→ 3')	Fragment (bp)	Annealing temperature (◦C)	Note	Reference
Anapalsma phagocytophilum	16S rRNA	TCCTGGCTCAGAACGAACGCTGGC GGCAGTCACTGACCCAACCTTAAATGGCTG	1433	55	1st PCR	Lee et al., 2016
		GCTGAATGTGGGGATAATTTAATG GCTGCTTCCTTTCGGTTA	641	55	Nested PCR	
Babesia caballi	*Rap-1*	GGCTCCCAGCGACTCTGTGG CTTAAGTGCCCTCTTGATGC	610	63		Alhassan et al., 2005
	18S rRNA	TCGAAGACGATCAGATACCGTCG CTCGTTCATGATTTAGAATTGCT	585	60.5		
Borrelia burgdorferi sensu lato	23S rRNA	AGAAGTGCTGGAGTCGA TAGTGCTCTACCTCTATTAA	261	39		Chang et al., 2000
Coxiella burnetii	*htpB*	GCGGGTGATGGTACCACAACA GGCAATCACCAATAAGGGCCG	501	57	1st PCR	Reye et al., 2012
		TTGCTGGAATGAACCCCA TCAAGCTCCGCACTCATG	324	52	Nested PCR	
Rickettsia spp.	*ompA*	GCTTTATTCACCACCTCAAC TR(g/a)ATCACCACCGTAAGTAAAT	212/209	55		Kidd et al., 2008
Theileria equi	*EMA-1*	GCATCCATTGCCATTTCGAG TGCGCCATAGACGGAGAAGC	744	63		Alhassan et al., 2005
	18S rRNA	TCGAAGACGATCAGATACCGTCG TGCCTTAAACTTCCTTGCGAT	435	60.5		

Table 2-2. The prevalence of *C. burnetii*, *Rickettsia* spp., *B. caballi* and *T. equi*, in horse in XUAR, China.

Areas	Farm Number	No. tested	No. positive/(%)			
			Coxiella burnetii	*Rickettsia* spp.	*Babesia caballi*	*Theileria equi*
Zhaosu	1	20	13 (65.0)	17 (85.0)	2 (10.0)	1 (5.0)
	2	20	3 (15.0)	12 (60.0)	5 (25.0)	3 (15.0)
	3	20	2 (10.0)	7 (35.0)	4 (20.0)	3 (10.0)
	4	20	19 (95.0)	9 (45.0)	6 (30.0)	10 (50.0)
Tekesi	5	20	7 (25.0)	16 (80.0)	5 (20.0)	0
	6	20	10 (50.0)	6 (30.0)	7 (35.0)	0
	7	20	6 (30.0)	10 (50.0)	9 (45.0)	11 (50.0)
Nileke	8	20	3 (15.0)	13 (65.0)	3 (15.0)	19 (95.0)
	9	20	7 (40.0)	11 (55.0)	4 (20.0)	16 (80.0)
	10	20	9 (45.0)	13 (65.0)	4 (20.0)	16 (80.0)
	Total	200	79 (39.5)	114 (57.0)	49 (24.5)	79 (39.5)

Table 2-3. Co-infections of TBPs in horse in XUAR (n = 200).

No. positive/(%)	Pathogens	No. positive samples	(%)
Single infection (n = 63/31.5%)	*C. burnetii*	15	7.5
	Rickettsia spp.	25	12.5
	B. caballi	7	3.5
	T. equi	16	8.0
Mixed infection (n = 100/50.0%)	*C. burnetii* + *Rickettsia* spp.	17	8.5
	C. burnetii + *B. caballi*	3	1.5
	C. burnetii + *T. equi*	2	1.0
	Rickettsia spp +.*B. caballi*	6	3.0
	Rickettsia spp +.*T. equi*	22	11.0
	B. caballi + *T. equi*	3	1.5
	C. burnetii + *Rickettsia* spp.+ *B. caballi*	11	5.5
	C. burnetii + *Rickettsia* spp.+ *T. equi*	17	8.5
	C. burnetii + *B. caballi* + *T. equi*	3	1.5
	Rickettsia spp + *B. caballi* + *T. equi.*	5	2.5
	C. burnetii + *Rickettsia* spp.+ *B. caballi* + *T. equi*	11	5.5
Total		163	81.5

Table 2-4. The prevalence of *C. burnetii*, *Rickettsia* spp., *B. caballi* and *T. equi* in different sampling areas and horses age (n = 200) in XUAR.

Variable		No. tested	*C. burnetii*	*Rickettsia* spp.	*B. caballi*	*T. equi*
			No. positive (%)	No. positive (%)	No. positive (%)	No. positive (%)
Area	Zhaosu	80	37 (46.3)	45 (56.3)	17 (21.3)	17 (21.3)
	Tekesi	60	23 (38.3)	32 (53.3)	21 (35.0)	11 (18.3)
	Nileke	60	19 (31.7)	37 (61.7)	11 (18.3)	51 (85.0)
Age	0-4	26	11 (42.3)	15 (57.7)	8 (30.8)	7 (26.9)
	5-8	115	36 (31.3)	62 (53.9)	25 (21.7)	50 (43.5)
	9-15	56	31 (55.4)	34 (60.7)	15 (26.8)	22 (39.3)
	Unavailable	3	1 (33.3)	3 (100)	1 (33.3)	0
	Total	200	79 (39.5)	114 (57.0)	49 (24.5)	79 (39.5)

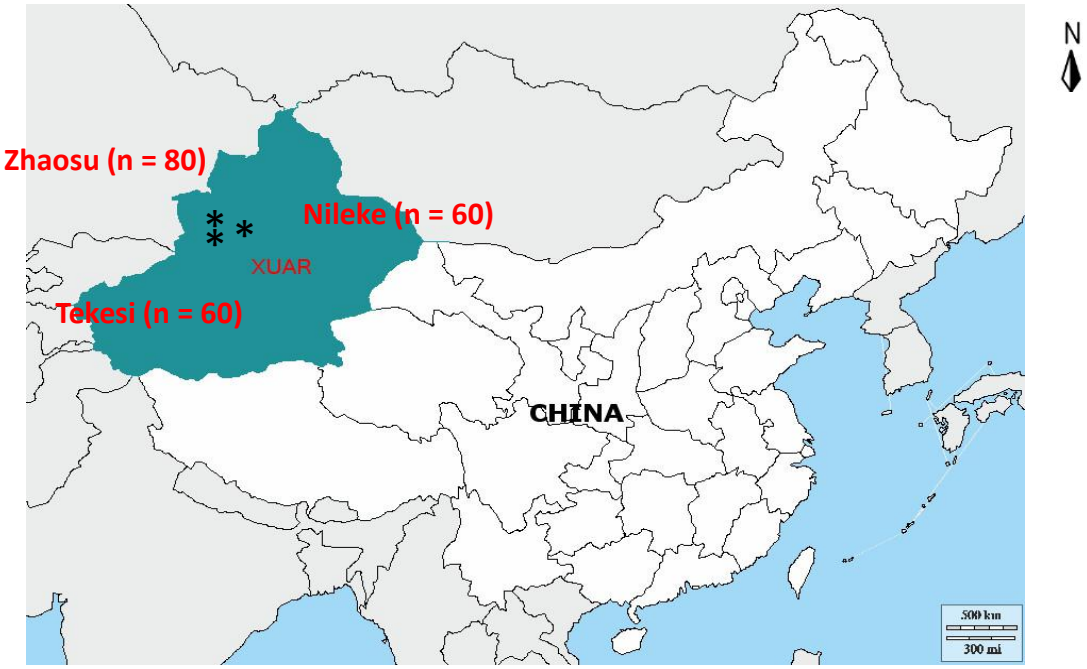


Fig. 2-1. Map of XUAR, China. The asterisk indicate samples were collected in areas. The sampling sites and sample numbers were shown in red.

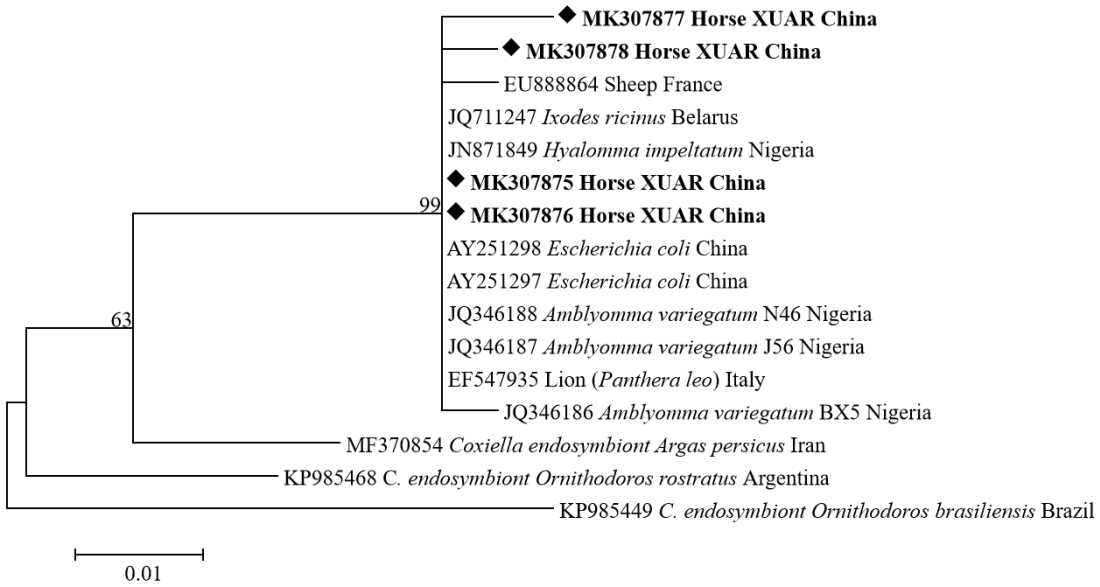


Fig. 2-2. Phylogenetic analysis of *C. burnetii htpB* gene sequence. Bootstrap replications (1000) of data are observed and numbers mean the occurrence of percentages. Black diamond marks means sequences in this study were tested. *C. endosymbiont* (MF370854, KP985468 and KP985449) was used as outgroup. The value lower than 50% are hidden.

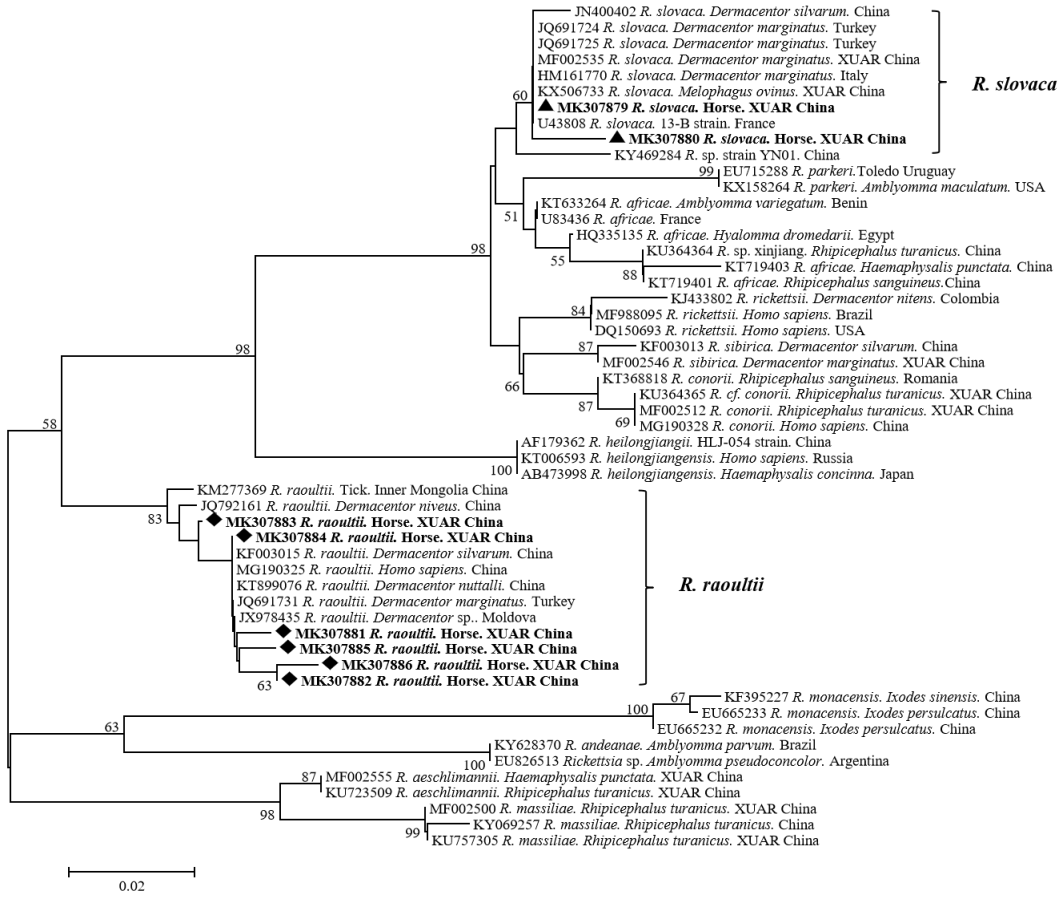


Fig. 2-3. Phylogenetic analysis of *Rickettsia* spp. *ompA* gene sequence. Bootstrap replications (1000) of data are observed and numbers mean the occurrence of percentages. Black diamond marks means sequences in this study were tested. The value lower than 50% are hidden.

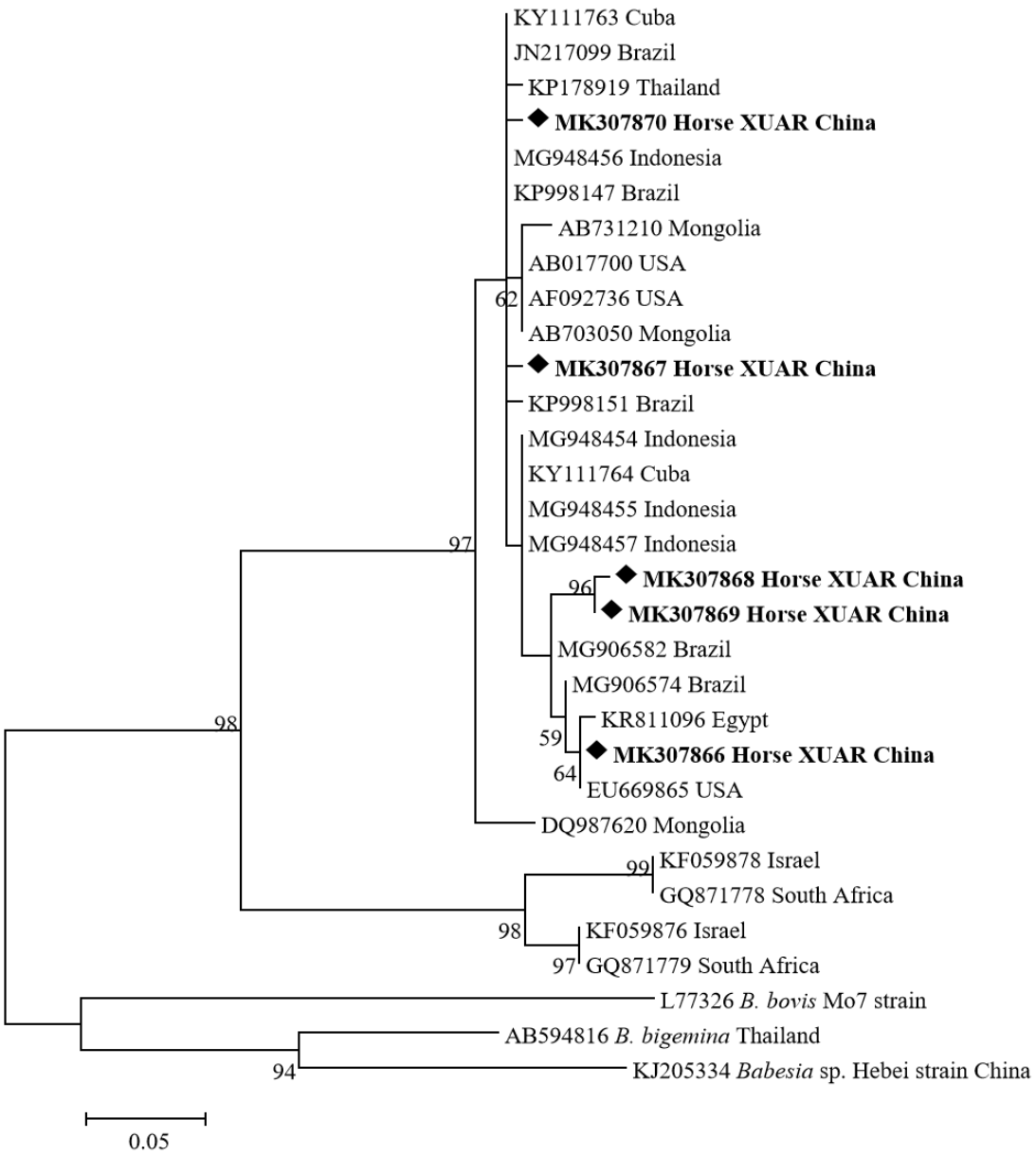


Fig. 2-4. Phylogenetic analysis of *B. caballi* 48-kDa antigen sequences. Bootstrap replications (1000) of data are observed and numbers mean the occurrence of percentages. Black diamond marks means sequences in this study were tested. *B. ovis* (L77326), *B. bigemina* (AB594816) and *Babesia* sp. Hebei (KJ205334) were used as outgroup. The value lower than 50% are hidden.

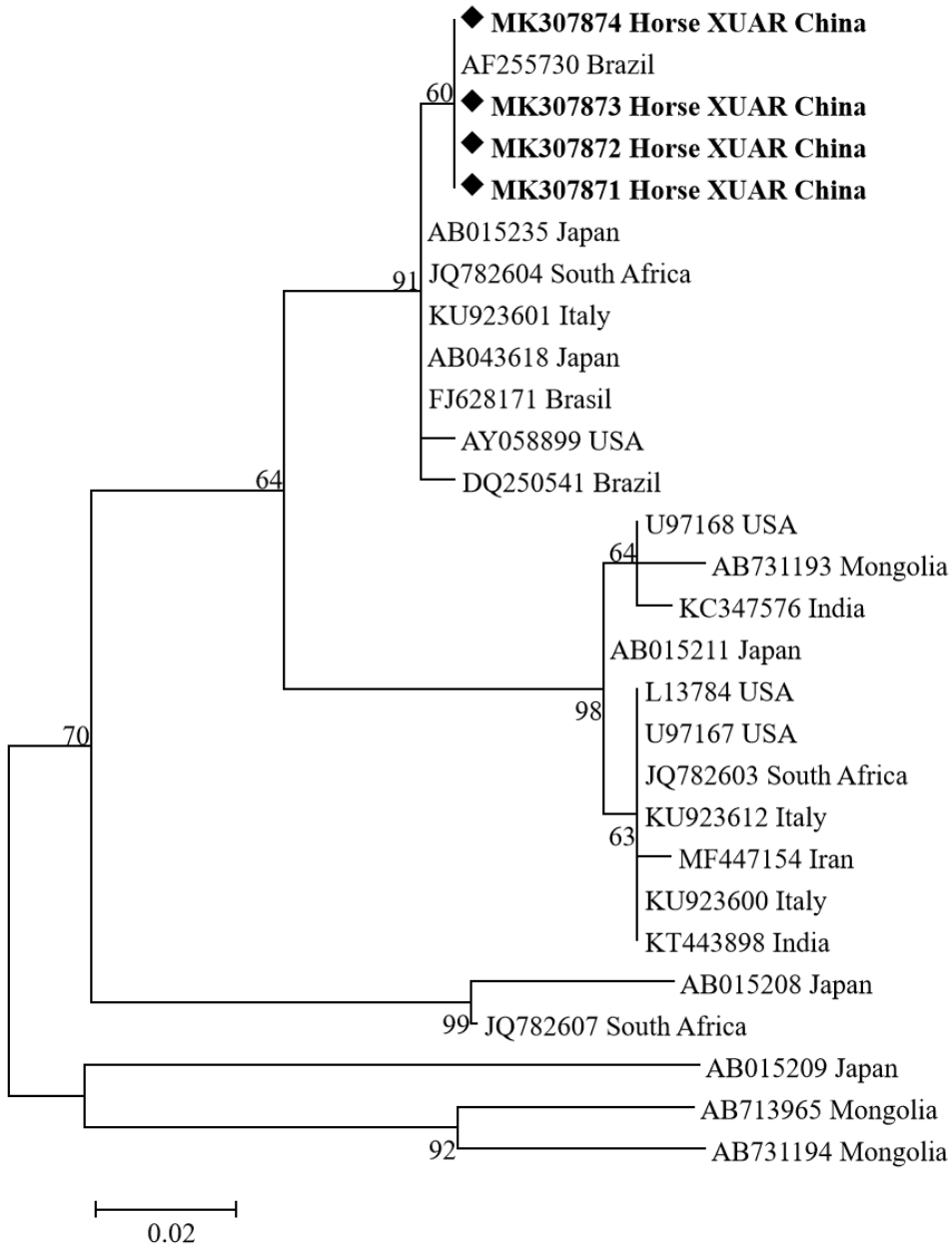


Fig. 2-5. Phylogenetic analysis of *T. equi* EMA-1 sequences. Bootstrap replications (1000) of data are observed and numbers mean the occurrence of percentages. Black diamond marks means sequences in this study were tested. The value lower than 50% are hidden.

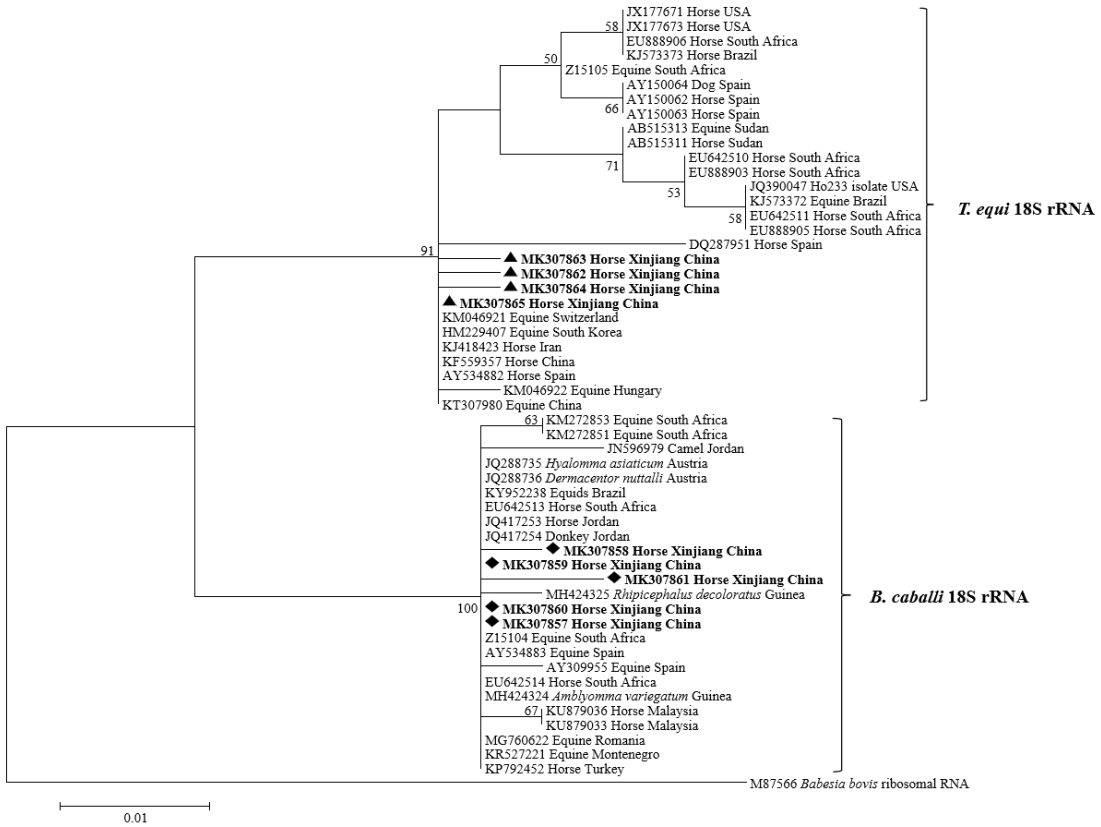


Fig. 2-6. Phylogenetic analysis of *B. caballi* and *T. equi* 18S rRNA partial sequences. Bootstrap replications (1000) of data are observed and numbers mean the occurrence of percentages. Black diamond marks means sequences in this study were tested. *B. ovis* (M87566) was used as outgroup. The value lower than 50% are hidden.

Chapter 3

Molecular detection and identification of *Babesia* spp., *Theileria* spp. and *Anaplasma* spp. in sheep from Xinjiang Uygur Autonomous Region, China

3-1. Introduction

Babesiosis, theileriosis and anaplasmosis are important diseases caused by pathogens transmitted by ticks (Zhou et al., 2014). Two generally species of *Babesia* species (*Babesia motasi* and *B. ovis*) (Yin et al., 1997; Schnittger et al., 2012) and several species of *Theileria* (*Theileria ovis*, *T. lestoquardi*, *T. sespecies*, *T. luwenshuni* and *T. uilenbergi*) of ovine babesiosis and theileriosis are distributed in Asia, Africa, Europe and the Far East (Berggoetz et al., 2014; Gebrekidan et al., 2014; Zhou et al., 2017). Furthermore, ovine anaplasmosis is mainly caused by *A. ovis* and *A. marginale.* (Splitter et al., 1955; Ciani et al., 2013). In China, *B. motasi*, *B. ovis* (Wang et al., 2019; Li et al., 2009), *T. ovis*, *T. uilenbergi*, *T. luwenshuni* (Berggoetz et al., 2014; Gebrekidan et al., 2014; Zhou et al., 2017), *A. phagocytophilum*, *A. marginale*, *A. ovis*, *A. bovis*, *A. capra* and *A. platys* (Yang et al., 2015; Wang et al., 2019) are the causative agents of ovine tick-borne disease transmitted by ticks or small ruminant that have been reported.

According to Song et al. (2018), in XUAR, five *Babesia* species, two *Theileria* speices and *A. ovis* were identified in *Haemaphysalis punctata*, *Hyalomma asiaticum*, *Dermacentor nuttalli*, *D. marginatus*, *Rhipicephalus turanicus*. In 2010, there were more than 3 million sheep and goats in XUAR (Statistic Bureau of Xinjiang Uygur Autonomous Region, 2011), but information about ovine tick-borne pathogens (TBPs) is still lacking in border regions, northwest China. Subsequently, this study was conducted to help in full filling the information gap regarding the occurrence and genetic diversity of *Babesia* spp., *Theileria* spp. and *Anaplama* spp. in sheep from northern and southern XUAR.

3-2. Materials and methods

Ethical statement

The permission and approval of all blood samples were acquired from selected farm owners. The ethical guidelines of all the procedures for treating animal samples permitted by Obihiro University of Agriculture and Veterinary Medicine (Approval ID: 18-40).

Blood collection and DNA extraction

Sheep blood samples (n=323) were collected from Fuhai (n=29), Qinghe (n=81), Jimunai (n=22), Bole (n=34), Tashikurgan (n=76) and Yecheng (n=81) between June 2018 and June 2019 in the southern and northern XUAR (Fig. 3-1).

Detection of TBPs

All samples were detected with species-specific primers for *Theileria* spp., *Babesia* spp. and *Anaplama* spp. by PCR or nested PCR (nPCR)(Table 3-1). Based on fragments of *major surface protein 4* (*MSP4*) gene of *A. ovis* (Torina et al., 2012) and

A. marginale (Torina et al., 2012), *epank1* gene of *A. phagocytophilum* (Walls et al., 2000), 16S rRNA gene of *A. bovis* (Reye et al., 2012) and *A. capra* (Yang et al., 2015), 18 ssu rRNA gene of *B. ovis* (Aktaş et al., 2005) and *T. ovis* (Aktaş et al., 2006), *Rhoptry-associated protein* (*Rap*)1-b gene of *B. motasi*-like (Niu et al., 2016), *Rap1-a* gene *of B. motasi*-like Xinjiang (Niu et al., 2014), 18S rRNA of *T. luwenshuni* (Yin et al., 2008) and *T. uilenbergi* (Yin et al., 2008) and *major piroplasm surface protein* (*MPSP*) of *T. orientalis* (Ota et al., 2009).

Sequencing and phylogenetic analyse

The methods are similar with chapter 1 materials and methods 1-2 procedure.

3-3. Results

Among the 323 samples, the PCR assays revealed that 225 (69.7%) sheep were infected with *Babesia* spp., *Theileria* spp. and *Anaplasma* spp.. The overall infection rates of sheep are presented in Table 3-2. The most frequent pathogens were *A. ovis* detected in 63.8% of the samples, followed by *B. motasi*-like (18.6%), *A. bovis* (16.7%), *T. uilenbergi* (15.8%), *A. phagocytophilum* (9.9%), *T. luwenshuni* (5.9%) and *B. motasi*-like Xinjiang (5.0%) (Table 3-2). The most common coinfection was *A. ovis* and *B. motasi*-like with an infection rate of 17.0% (55/323), followed by *A. ovis* and *T. uilenbergi* with 12.4% (40/323). Meanwhile, only 1 sheep was dual infected with *A. phagocytophilum* and *T. luwenshuni.* For triple coinfection, the infection rate of *A. ovis* and *B. motasi*-like and *T. uilenbergi* was 5.0% (16/323) (Table 3-3).

The expected sizes of *A. ovis*, *A. bovis*, *A. phagocytophilum*, *B. motasi*-like, *B. motasi*-like Xinjiang, *T. luwenshuni* and *T. uilenbergi* sequences in present study were 347, 551, 444, 536, 507, 389 and 388 bp, respectively. The *MSP4* gene sequences of *A.*

ovis (GenBank accession numbers MN946542) were 97.9% identical to other and to an isolate from Sudan (KU497712). *A. bovis* 16S rRNA gene sequences (GenBank accession nos. MN947620) was 99% identical to an isolate obtained from cattle in China (MK345480). *A. phagocytophilum epank1* gene sequences (GenBank accession nos. MN946539) was 98% identical to an isolate obtained from sheep in Germany (GU236795). **S**equences of *B. motasi*-like *Rap1-b* sequences (GenBank Accession nos. MN946540) showed 99% identity to a previous isolate from XUAR sheep (KU510048). The sequence identity of *B. motasi*-like Xinjiang *Rap1-a* (GenBank Accession nos. MN946541) with an isolate from *Haemaphysalis longicornis* in Gansu, China (KX708614) was 99%. The accession numbers for 18S rRNA of *T. luwenshuni* and *T. uilenbergi* are MN944535 and MN944557, respectively.

A. ovis MSP4 sequences of phylogenetic analysis from XUAR indicated that the sequences belong to the same cluster with a sequence from Sudan (KU497696) and Mongolia (LC412088) (Fig. 3-2). The *A. bovis* SBP-4 sequences appeared to form one cluster and illustrated an intimate nexus with the sequences between goats (KP062957) and tick (KX450273) in China (Fig. 3-3). In addition, phylogenetic analysis of *A. phagocytophilum* epank1 belong to Germany (AY282378) and Switzerland (FJ515309) (Fig. 3-4). Interestingly, the *B. motasi* Xinjiang *RAP1a* and *B. motasi RAP1b* sequences formed clade (Fig. 3-5) with China isolate (KX664714) and (KU510052). Analysis of *T. luwenshuni* 18S rRNA sequences obtained from this study revealed a close relationship with India strain KJ782395 (Fig. 3-6) and China stain KU554728, MN944557 (Fig. 3-7).

3-4. Discussion

TBPs of ovines represent a severe threat to global veterinary and public health (Yin et al., 2007; Ben et al., 2018; Sadeddine et al., 2019). In this study, three species of *Anaplasma*, two species of *Babesia* and *Theileria* were molecularly detected from sheep. The result also revealed that the total pathogen infection rates of samples were 69.7% (Table 3-2). Ordinarily, the relatively high incidence of TBPs could be attributed the abundance of vectors in the study area. In XUAR, 45 species from 6 genera of ticks were identified, and *Rhipicephalus turanicus*, *Dermacentor niveus*, *Hyalomma asiaticum*, *D. marginatus* are the most frequent tick species in domestic animals from thirty-five counties (cities) in XUAR during 2011-2017 (Sheng et al., 2019).

In this study, infections of *A. ovis* (63.8%), *A. bovis* (16.7%) and *A. phagocytophilum* (9.9%) were identified. Previously, Yang et al. (2015) found *A. ovis* and *A. phagocytophilum* in sheep in XUAR, while *A. ovis* was identified in ticks in border regions (Song et al., 2018). In addition, *A. ovis* and *A. bovis* were identified in Fuhai and Qinghe while *A. phagocytophilum* was detected in Fuhai, which both border Mongolia. *A. ovis* was identified in sheep and *D. nuttalli* tick (Enkhtaivan et al., 2019) and *A. phagocytophilum* in *I. persulcatus* in areas surrounding Mongolia (Jiang et al., 2011). *A. ovis*, *A. bovis* and *A. phagocytophilum* were also detected in Bole and *A. ovis* and *A. phagocytophilum* in Jimunai which both areas are adjoined to Kazakhstan (Shpynov et al., 2004). According to Shpynov et al. (2004), *A. phagocytophilum* DNA was identified in *I. persulcatus* ticks in the Altai and Primorye territories, which is also borders Kazakhstan. Furthermore, *A. ovis* (79.0%), *A. bovis* (24.7%) and *A. phagocytophilum* (28.4%) in Yecheng sheep in current study (Table 3-2), while *A. ovis* (1.5%), *A. marginale* (5.7%), *A. centrale* (2.7%) were identified in

ticks from Pakistan, the country that Yecheng borders (Rehman et al., 2019). Notably, *A. capra*, a newly discovered emerging zoonotic species of *Anaplasma*, was not identified in the current study, although it has been reported in both XUAR (only 4 positive samples) and Gansu of China (Peng et al., 2018; Yang et al., 2018). Such discrepant attribute to the difference in the district where the sample were collected in my study and previous one in XUAR (Peng et al., 2018).

Several *Babesia* species (*B. motasi*-like and *B. ovis*) are widely spread among sheep and goat in China, specifically in Gansu and Qinghai (Niu et al., 2014; Li et al., 2019), coherent with the distribution data of the ticks (Wang et al., 2013). The total infection rates were 0.4% for *B. motasi* in sheep from Gansu according to Sun et al. (2019). Song et al. (2018) reported that in XUAR, five *Babesia* were identified in *H. asiaticum*, *H. punctata*, *D. nuttalli* and *R. Turanicus* ticks which were collected from sheep, including *B. occultans*, *B. motasi*-like, *B. major.* Meanwhile, none of the samples was positive for *B. motasi*-like in ticks which were collected from Fuhai, Jimunai, Qinghe, Yecheng (Song et al., 2018). However, except in Yecheng, *B. motasi*-like infection was identified in sheep in Fuhai (31.0%), Jimunai (18.2) and Qinghe (25.9%). According to Niu et al. (2016), the recorded infection rate for *B. motasi*-like was 21.87% while Song et al. (2018) also reveal that *B. motasi*-like was identified in *H. punctata* ticks in Yili district. In countries nearby XUAR, such as Pakistan, *B. ovis* (16%) and *T. ovis* (23%) were also detected from sheep (Shahzad et al., 2013).

The first infection by *Theileria* spp. in small ruminants imported into Sichuan province of China was reported in 1958 (Yang et al., 1958). Infection with other *Theileria* species, such as *T. uilenbergi*, *T. luwenshuni* and *T. ovis*, infective to small ruminants, has been documented since the first report (Sun et al., 2019). Furthermore,

T. luwenshuni and *T. uilenbergi* were identified in Gansu and Sichuan, Heilongjiang, Qinghai, Hubei and Hainan, China (Yin et al., 2007; Li et al., 2019; Wang et al., 2019). Pathogenic *T. luwenshuni* was detected in sheep from five counties (Fuhai, Bole, Qinghe, Yecheng, Tashikurgan) in present study, with detection rates of 24.1%, 8.8%, 6.2%, 3.7% and 1.3% (Table 3-2), respectively. Although *T. ovis* (7.25%) was previously detected in *R. turanicus* ticks infesting sheep in Yecheng (Song et al., 2018), and *T. luwenshuni* and *T. uilenbergi* only were identified in sheep from Yecheng in this study. Asymptomatic sheep and goats was also found infecting *T. lestoquardi* from two districts of Pakistan, which was near Yecheng county (Saeed et al., 2015). In addition, *T. luwenshuni* infection in ticks from dogs and cattle were identified in DNA samples (Sun et al., 2019). Aktaş et al. (2019) revealed the presence of *T. orientalis* and *T. annulata* from cattle in Kyrgyzstan, which is near Bole. In south India, which is near Yecheng and Tashikurgan, researchers also identified *T. orientalis* in ticks (Peng et al., 2018). In previous studies, *T. ovis* were found in ticks (Li et al., 2011) and goats (Yang et al., 2014) from XUAR, but in present study, all sheep DNA samples showed negative result for *T. ovis* and *T. orientalis.* The reasonable explanation for such obtained finding might be attributed to the difference between collection sample areas and tick species with previous study in XUAR.

Although this study screened several TBPs in sheep reared in the southern and northern part of XUAR, China, while this survey did not include ticks from this area. Therefore, other future studies are required to detected those pathogens in ticks collected from different parts XUAR. Subsequently, more studies are warranted in the future defect the risk factors correlated with TBPs in XUAR.

3-5. Summary

Babesia, *Theileria* and *Anaplasma* are important causative agents of tick-borne diseases that severely affect sheep. However, there is paucity in the occurrence of the infections of tick-borne diseases in sheep in border areas of XUAR, China. Nested polymerase chain reaction (nPCR) assays and gene sequencing were used to identify tick-borne *Babesia* spp., *Theileria* spp. and *Anaplasma* spp. infections in XUAR. Out of 323 samples tested in this study, 225 (69.7%) sheep were infected with *Babesia* spp., *Theileria* spp. and *Anaplasma* spp.. Two hundred six (63.8%), 60 (18.6%), 54 (16.7%), 51 (15.8%), 32 (9.9%), 19 (5.9%), 16 (5.0%) were positive for *A. ovis*, *B. motasi*-like, *A. bovis*, *T. uilenbergi*, *A. phagocytophilum*, *T. luwenshuni* and *B. motasi*-like Xinjiang, respectively. The most common dual infection was with *A. ovis* and *B. motasi-like* while the most frequent triple coinfection was *A. ovis, B. motasi*-like and *T. uilenbergi* with coinfection rates of 17.0% (55/323) and 5.0% (16/323), respectively. Sequencing analysis indicated that *A. ovis MSP4*, *A. phagocytophilum epank1*, *A. bovis* 16S rRNA, *B. motasi*-like *Rap1-b*, *B. motasi*-like Xinjiang *Rap1-a*, *T. luwenshuni* and *T. uilenbergi* 18S rRNA from XUAR, China, showed 99%-100% identity with documented isolates from other countries. To the best of my knowledge, this is the first report of *T. uilenbergi* and *T. luwenshuni* infections of sheep in XUAR. Furthermore, these findings provide important data for understanding the distribution of *Babesia*, *Theileria* and *Anaplasma* in sheep in XUAR, China.

Table 3-1. Primers of tick-borne pathogens.

Pathogen	Target gene	Species-specific primers (5' → 3')	Fragment (bp)	Annealing temperature (◦C)	Note	Reference
A. bovis	16S rRNA	TCCTGGCTCAGAACGAACGCTGGCG GCRCAGTCACTGACCCAACCTTAAATGGCTG	1433	55	1st PCR	Reye et al., 2012
		CTCGTAGCTTGCTATGAGAAC TCTCCCGGACTCCAGTCTG	551	55	Nested PCR	
A. capra	16S rRNA	GCAAGTCGAACGGACCAAATCTGT CCACGATTACTAGCGATTCCGACTTC	1261	58		Yang et al., 2015
A. marginale	*MSP4*	CTGAAGGGGGAGTAATGGG GGTAATAGCTGCCAGAGATTCC	344	60		Torina et al., 2012
A. ovis	*MSP4*	TGAAGGGAGCGGGGTCATGGG GAGTAATTGCAGCCAGGCACTCT	347	62		Torina et al., 2012
A. phagocytophilum	*epank1*	GAGAGATGCTTATGGTAAGAC CGTTCAGCCATCATTGTGAC	444	54		Walls et al., 2000
B. motasi-like	*Rap1-b*	TGCGCCTTCGAGTTGTACAAGAG GACGGGTTGCRTAGGCTGAC	765	58	1st PCR	Niu et al., 2016
B. motasi-like xinjiang	*Rap1-a*	CGGTAGTGTGTTGATATCCTAGAG CACAAGCGTTACATCGTAGAGCCG	1400	57	1st PCR	Niu et al., 2014
		AATGACCGCCCGCGAAATCAGCCACGAC GTGGGTGGCGAAGGACAATTTGTTG	507	63	Nested PCR	
B. ovis	ssu rRNA	TGGGCAGGACCTTGGTTCTTCT CCGCGTAGCGCCGGCTAAATA	549	62		Aktaş et al., 2005
T. luwenshuni	18S rRNA	GGTAGGGTATTGGCCTACTGA TCATCCGGATAATACAAG	389	57		Yin et al., 2008
T. orientalis	*MPSP*	CTTTGCCTAGGATACTTCCT ACGGCAAGTGGTGAGAACT	776	58		Ota et al., 2009
T. ovis	ssu rRNA	TCGAGACCTTCGGGT TCCGGACATTGTAAAACAAA	520	60		Aktaş et al., 2006
T. uilenbergi	18S rRNA	GGTAGGGTATTGGCCTACCGG ACACTCGGAAAATGCAAGCA	388	55		Yin et al., 2008

Table 3-2. Detection of *Anaplasma* spp., *Babesia* spp. and *Theileria* spp. in sheep from XUAR, China.

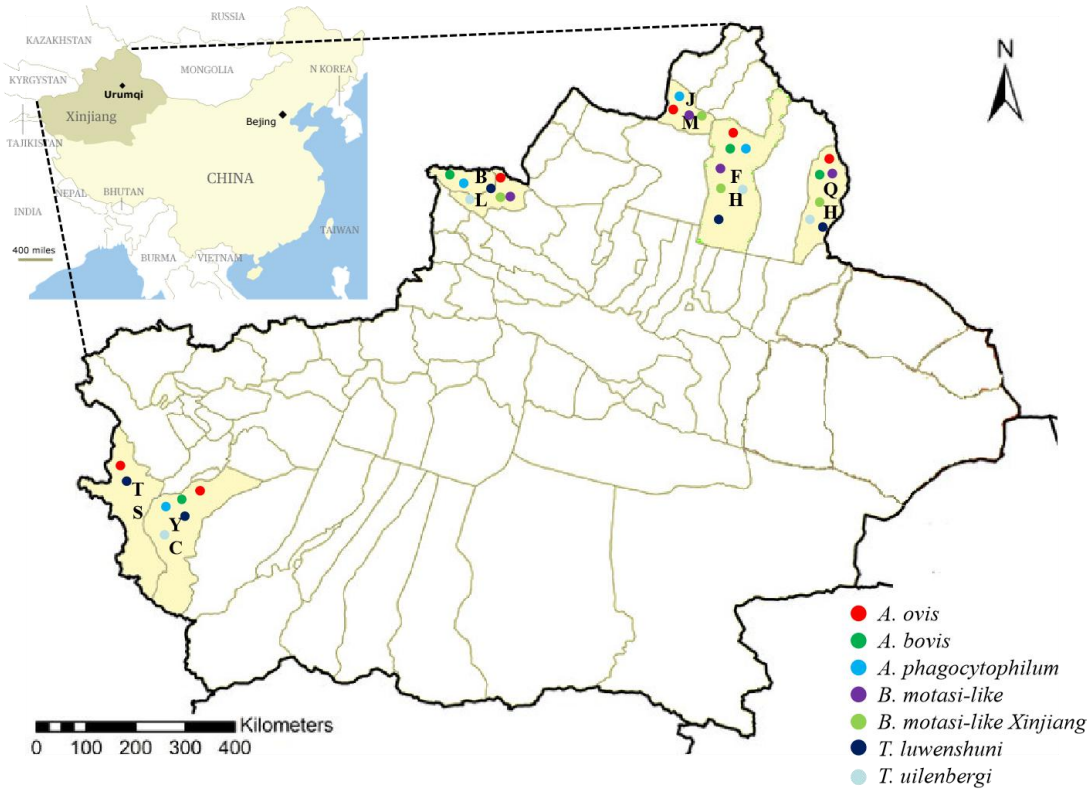
County	No. tested	No. Infected/(%)						
		A. ovis	*A. bovis*	*A. phago*	*B. m-like*	*B.m Xinjiang*	*T. luwenshuni*	*T. uilenbergi*
Tashikuergan	76	1/1.3	-	-	-	-	1/1.3	-
Bole	34	32/94.1	8/23.5	7/20.6	26/76.5	12/35.3	3/8.8	17/50.0
Fuhai	29	26/89.7	11/37.9	1/3.4	9/31.0	3/10.3	7/24.1	11/37.9
Jimunai	22	18/81.8	-	1/4.5	4/18.2	1/4.5	-	-
Yecheng	81	64/79.0	20/24.7	23/28.4	-	-	3/3.7	10/12.4
Qinghe	81	65/80.2	15/18.5	-	21/25.9	10/12.3	5/6.2	14/17.3
Total	323	206/63.8	54/16.7	32/9.9	60/18.6	16/5.0	19/5.9	51/15.8

"-" means not detected.

Table 3-3. Coinfections of *Anaplasma* spp., *Babesia* spp. and *Theileria* spp.

Type of Pathogen	No. Infected/(%)	Sampling area, No. Infected/(%)					
		Tashikuergan	Bole	Fuhai	Jimunai	Yecheng	Qinghe
A.ovis+A.bovis	40/12.4	-	6/17.6	10/34.5	-	11/13.6	13/16.0
A.ovis+A.ph	22/6.8	-	6/17.6	1/3.4	1/4.5	14/17.3	-
A.ovis+B.m	55/17.0	-	25/73.5	8/27.6	4/18.2	-	18/22.2
A.ovis+B.m XJ	19/5.9	-	12/35.3	3/10.3	1/4.5	-	3/3.7
A.ovis+T.lu	14/4.3	-	3/8.8	6/20.7	-	2/2.5	3/3.7
A.ovis+T.u	38/11.8	-	16/47.1	1/3.4	-	7/8.6	14/17.3
A.bovis+B.m	12/3.7	-	5/14.7	4/13.8	-	-	3/3.6
A.bovis+B.m XJ	7/2.2	-	4/11.8	2/6.8	-	-	1/1.2
A.bovis+T.u	11/3.4	-	3/8.8	4/13.8	-	2/2.5	2/2.4
A.ph+*B.m*	8/2.5	-	7/20.6	1/3.4	-	-	-
A.ph+T.u	9/2.8	-	6/17.6	1/3.4	-	2/2.5	-
T.u+B.m	17/5.3	-	13/38.2	2/3.4	-	-	2/2.5
A.ovis+B.m+T.u	16/5.0	-	14/41.2	2/29	-	-	2/2.5
A.ovis+A.bovis+B.m	11/3.4	-	4/11.8	5/17.2	-	-	2/2.5
A.ovis+A.bovis+T.u	8/2.5	-	2/5.9	3/10.3	-	1/1.2	2/2.5
A.bovis+B.m+T.u	5/1.5	-	3/8.8	2/6.9	-	-	-
A.ovis+A.bovis+A.ph	3/0.9	-	1/2.9	-	-	2/2.5	-

"-" means not detected.



FH: Fuhai (29); **QH:** Qinghe (81); **JM:** Jimunai (22); **BL:** Bole (34); **TS:** Tashikurgan (76); **YC:** Yecheng (81)

Fig. 3-1. Map of XUAR, China. Dots indicate localities which pathogens were detected.

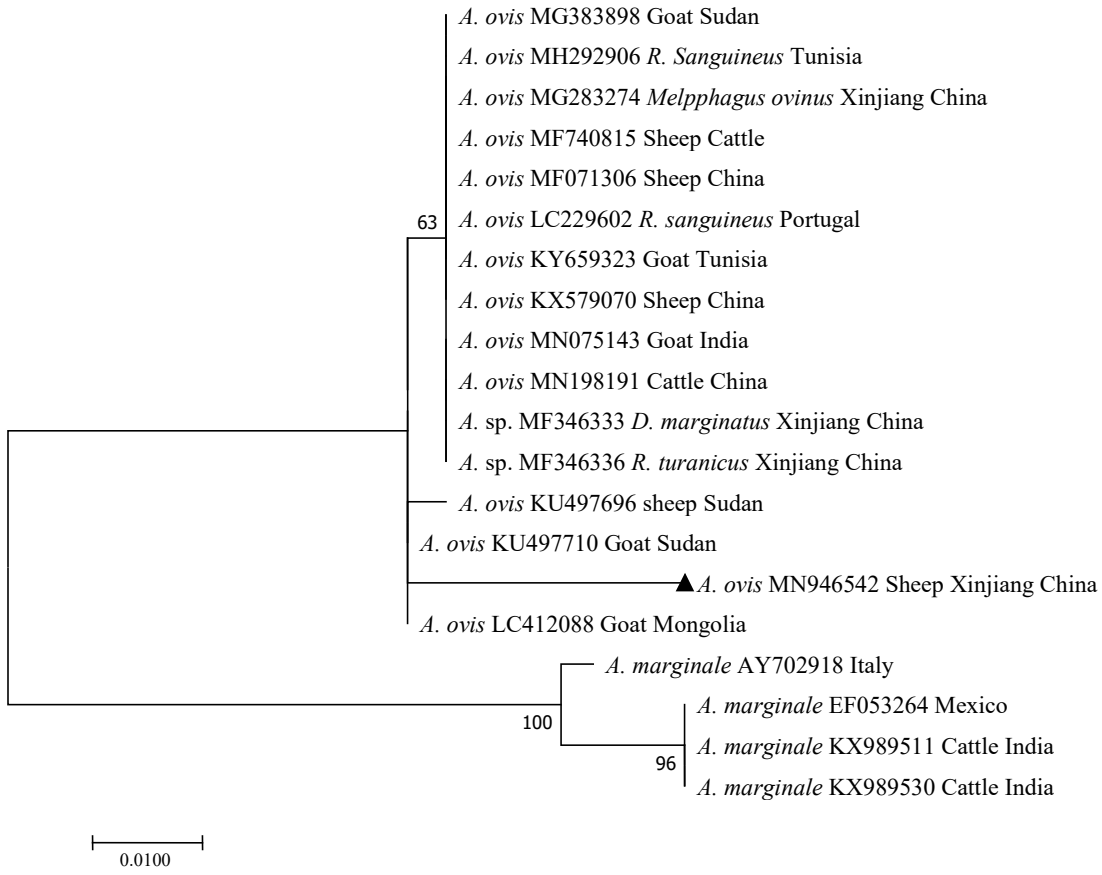


Fig. 3-2. Phylogenetic analysis of *Anaplasma ovis MSP4* gene sequences. Numbers at nodes represent percentage occurrence in 1000 bootstrap replication. Sequences from this study are marked with triangle. The value lower than 50% are hidden.

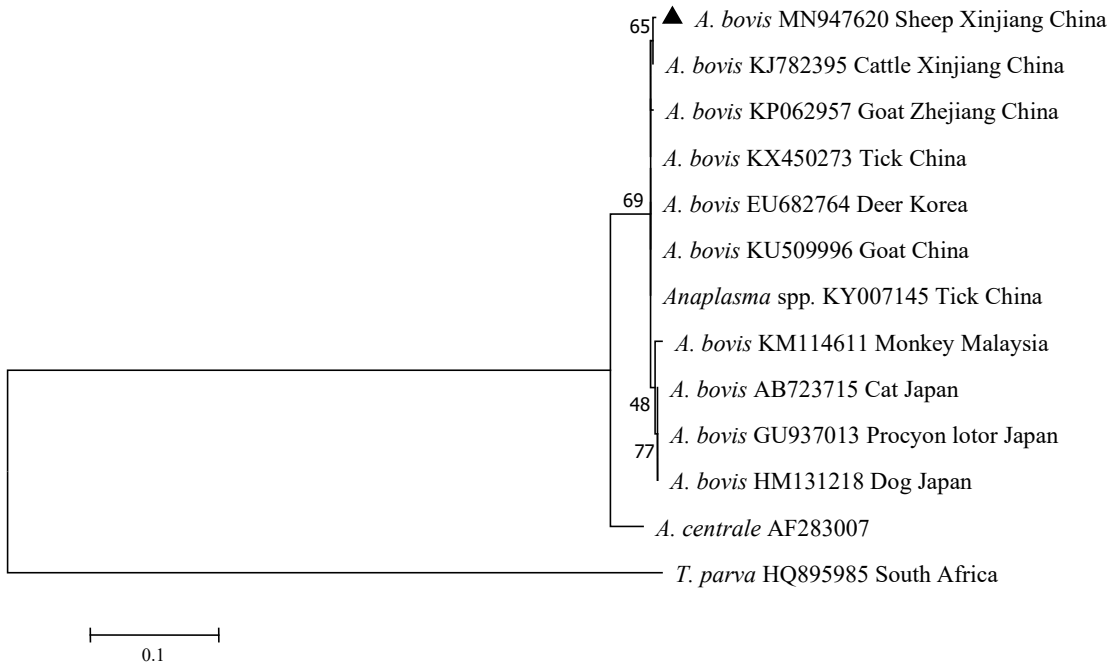


Fig. 3-3. Phylogenetic analysis of *Anaplasma bovis* 16S rRNA gene sequences. Numbers at nodes represent percentage occurrence in 1000 bootstrap replication. Sequences from this study are marked with triangle. The value lower than 50% are hidden.

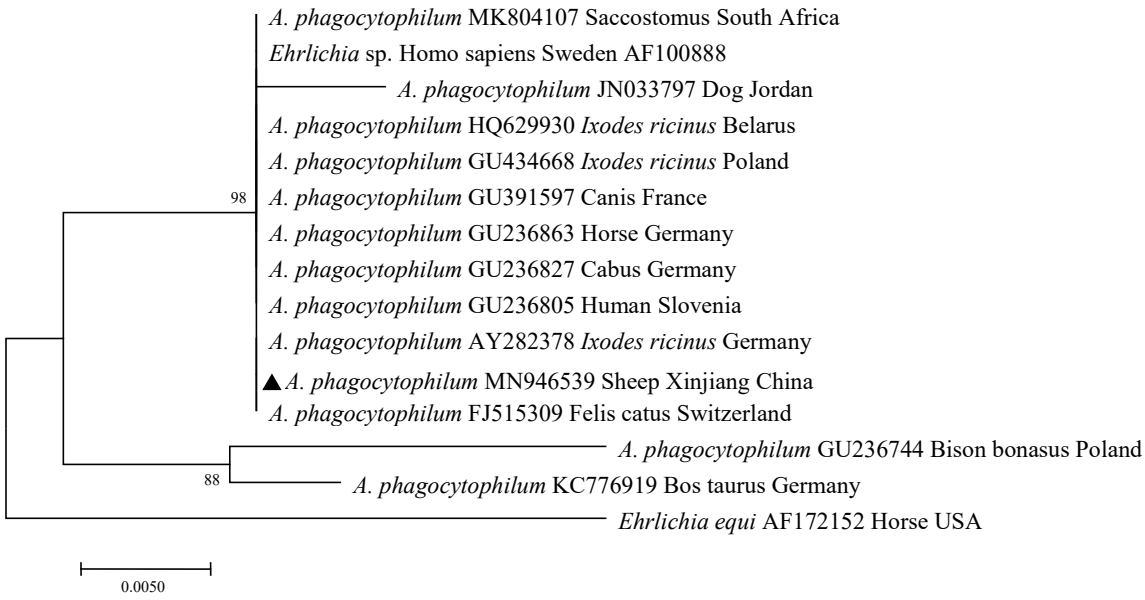


Fig. 3-4. Phylogenetic analysis of *Anaplasma phagocytophilum epank1* gene sequences. Numbers at nodes represent percentage occurrence in 1000 bootstrap replication. Sequences from this study are marked with triangle. The value lower than 50% are hidden.

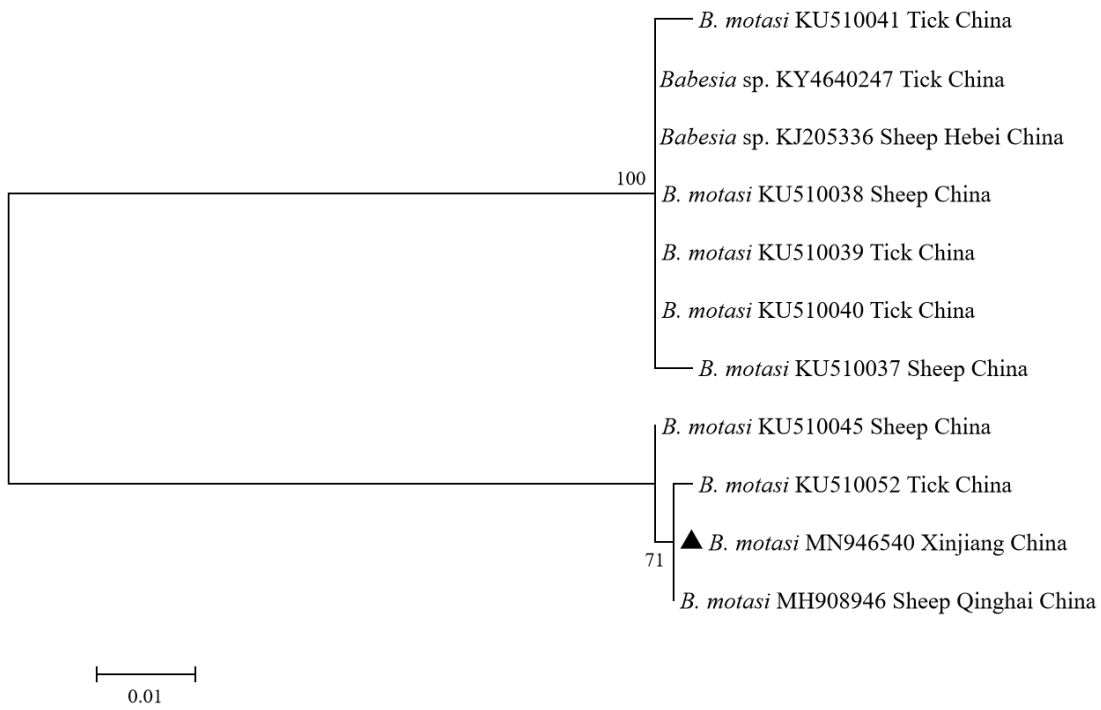


Fig. 3-5. Phylogenetic analysis of *B. motasi-like Rap1-b* gene sequences. Numbers at nodes represent percentage occurrence in 1000 bootstrap replication. Sequences from this study are marked with triangle. The value lower than 50% are hidden.

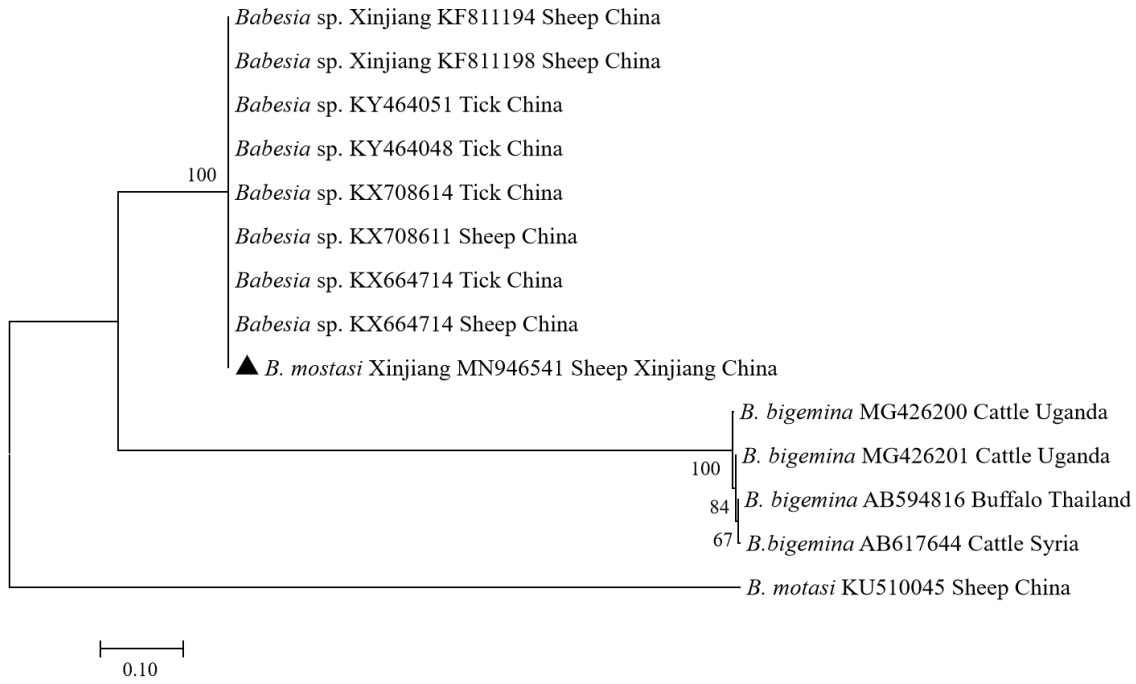


Fig. 3-6. Phylogenetic analysis of *B. motasi-like* Xinjiang *Rap1-a* gene sequences. Numbers at nodes represent percentage occurrence in 1000 bootstrap replication. Sequences from this study are marked with triangle. The value lower than 50% are hidden.

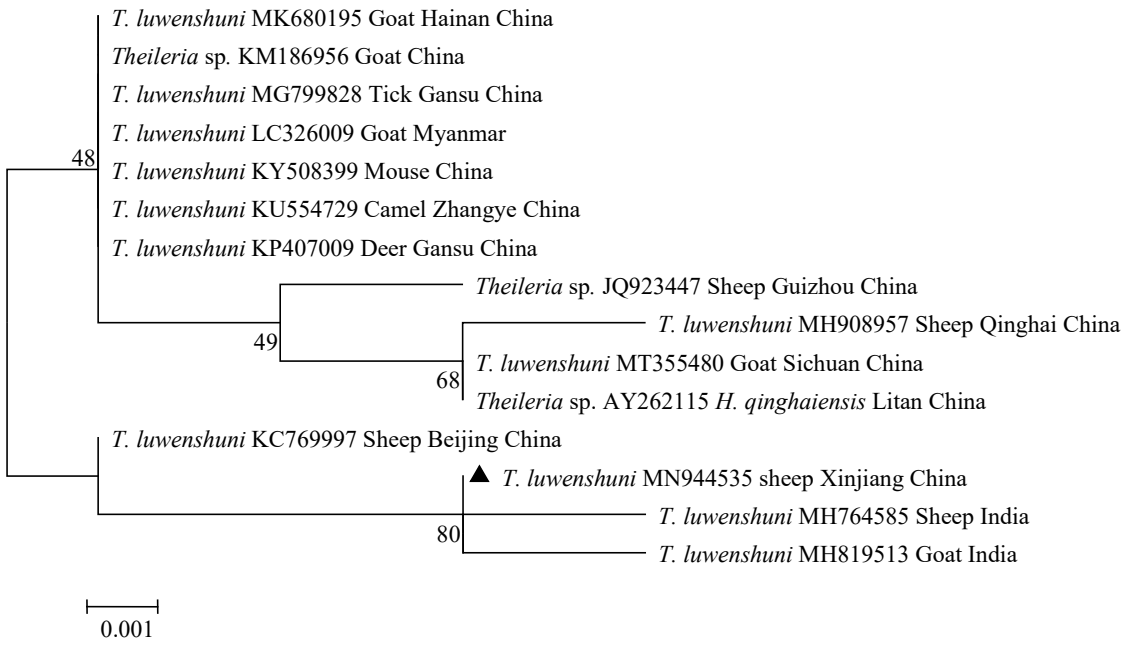


Fig. 3-7. Phylogenetic analysis of *T. luwenshuni* 18S rRNA gene sequences. Numbers at nodes represent percentage occurrence in 1000 bootstrap replication. Sequences from this study are marked with triangle. The value lower than 50% are hidden.

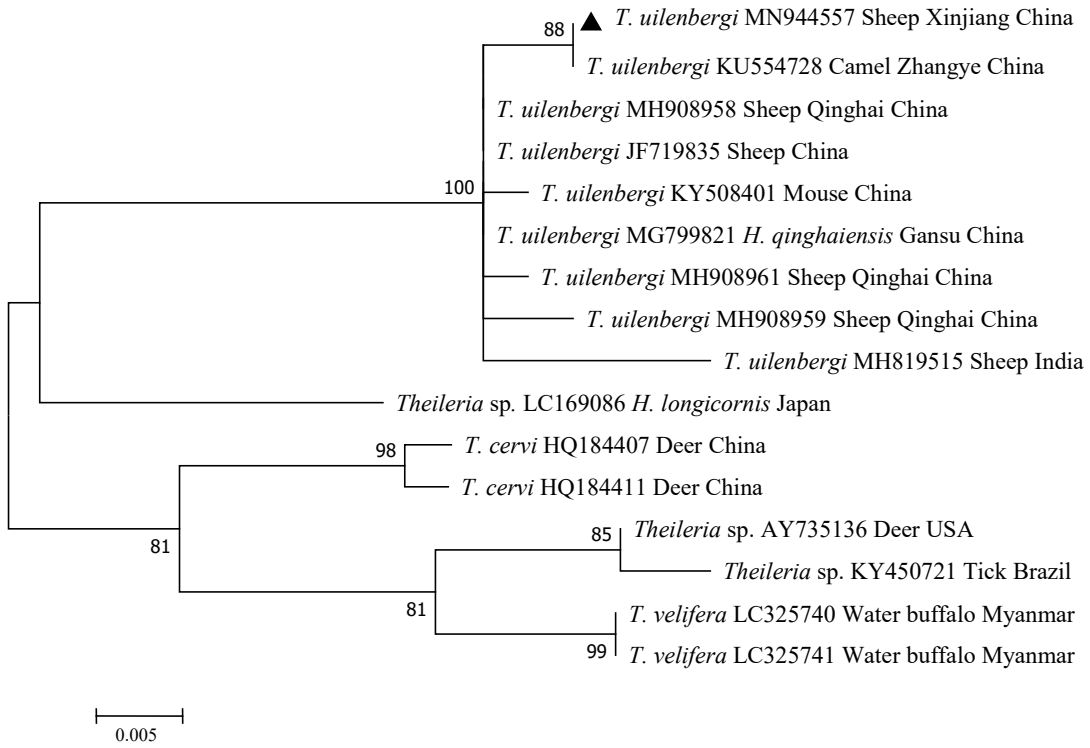


Fig. 3-8. Phylogenetic analysis of *T. uilenbergi* 18S rRNA gene sequences. Numbers at nodes represent percentage occurrence in 1000 bootstrap replication. Sequences from this study are marked with triangle. The value lower than 50% are hidden.

General discussion

Tick-borne pathogens represent a serious and economic threat to veterinary and public health worldwide. Six genera and 45 species of ticks are reported in Xinjiang Uygur Autonomous Region (XUAR), China, including *Hyalomma*, *Dermacentor*, *Haemaphysalis* and *Rhipicephalus* (Zhang et al., 2017). In the present study, 5 *Babesia* spp. (*B. bovis*, *B. bigemina*, *B. caballi*, *B. motasi*-like, *B. motasi*-like Xinjiang), 3 *Theileria* spp. (*T. equi*, *T. uilenbergi*, *T. luwenshuni*), 3 *Anaplasma* spp. (*A. ovis*, *A. bovis*, *A. phagocytophilum*), *Rickettsia* sp. and *Coxiella burnetii* were identified in cattle, horses and sheep in XUAR, China.

The first description of *A. ovis* in China was in 1985 (Ding et al., 1985), and a variant of *A. ovis* was confirmed to be a potential zoonotic pathogen (Chochalakis et al., 2010). Although a previous study (Song et al., 2018) has tested *D. nuttalli* and *D. marginatus* ticks from 11 counties of XUAR for *A. ovis*, all the samples were negative. In addition, *A. bovis* was not only identified in cattle, but also in sheep in this study. Previously, Yang et al. (2015) found *A. ovis* and *A. phagocytophilum* in sheep in XUAR, while *A. ovis* was identified in *D. nuttalli* ticks in Qinghai province which borders XUAR (Han et al., 2018).

C. burnetii was identified in cattle and horses in this study, which may be attributed to the same transmission vectors. This result suggests that *C. burnetii* circulates in horses like in other animals in China and that horse owners in XUAR need to pay more attention when handling cattle and horses as there is a substantial risk of contracting *C. burnetii* infection. However, further studies are required on the epidemiology of *C. burnetii* in cattle, horses and sheep in the wider Chinese region, and there is a need to evaluate the role of infected livestock as a source of human

infection.

B. bovis and *B. bigemina* have higher infection rates in cattle, which may be attributed to cattle grazing together with horses, sheep and goats in XUAR. Mixed grazing increases tick questing and therefore, tick infestation between animals. The identification of *B. caballi* and *T. equi* demonstrate that there is a high infection rate of these pathogens in horses in XUAR. This study also reports the first detection of *T. uilenbergi* and *T. luwenshuni* in sheep in XUAR, but there were reports of these sheep infections in Gansu and Qinghai, which are borders of XUAR (Yin et al., 2007). This suggests that surveys of *Babesia* spp. and *Theileiria* spp. in livestock are needed to clarify the risk level and in the development of effective measures.

The distribution and diversity of pathogens in cattle, horses and sheep may be attributed to the difference in macroclimate, tick species, tick-dwelling habitat and landscape between districts. Particularly, questing ticks can be found in habitats like herb layer and vegetation (Randolph and Storey, 1999; Ehrmann et al., 2018). In XUAR, cattle, horses and sheep often co-feed in the same district, and there are also many small mammals in areas with high vegetation (Kollmann and Buschor, 2002; Ehrmann et al., 2018). These expand the possibility of ticks finding an animal as its host, but reduce the possibility of ticks encountering the same animal. (Handa et al., 2014; Ehrmann et al., 2018). Furthermore, the district containing alpine and meadow climates have humid soils rich in organic matter resulting in abundant vegetation which favors the tick population (Schwarz et al., 2009; Ehrmann et al., 2018). Those aspects might explain why a lot of TBPs and different tick species were identified in XUAR. Therefore, this study was conducted to help to fill the information gap regarding the occurrence and genetic diversity of TBPs in cattle, horses and sheep in XUAR, China.

General summary

Ticks carry and transmit a wide range of pathogens (bacteria, viruses and protozoa) which are of importance to humans and animals globally. Xinjiang Uygur Autonomous Region (XUAR) is located in the northwest part of China, which occupies one-sixth of the country's land area and borders eight countries including Russia, Mongolia, Kazakhstan, Kyrgyzstan, Tajikistan, Afghanistan, Pakistan and India. Livestock production is known as a main industry in XUAR. Additionally, this region is located in halfway along the old Silk Road between eastern Asia and Europe, therefore, the international livestock trade is frequent. Although several studies focusing on tick-borne pathogens (TBPs) of ticks in XUAR have been reported, prevalence and molecular identification of these TBPs in livestock are still limited. Therefore, the present study aimed to systematically investigate tick-borne pathogens in cattle, horses and sheep in XUAR, China.

In chapter 1, TBPs were identified in cattle in XUAR. Nested polymerase chain reaction (nPCR) assays and gene sequencing were used to detect *Babesia bovis*, *B. bigemina*, *Coxiella burnetii* and *Anaplasma bovis* infections in cattle in XUAR. Out of 195 samples tested, 67 (34.4%), 40 (20.5%), 24 (12.3%) and 10 (5.1%) were positive for *B. bigemina*, *C. burnetii*, *B. bovis* and *A. bovis*, respectively. Mixed infections involving 2-3 pathogens were observed in the present study. The most common dual infections were *B. bigemina* + *C. burnetii* while the most frequent triple infections were *B. bigemina* + *B. bovis* + *C. burnetii* with co-infection rates of 6.2% (12/195) and 1.0% (2/195), respectively. Although clinical cases were not observed, it is possible that cattle infected with multiple pathogens may have more pronounced clinical signs or hematological abnormalities than those infected with single

pathogens. It suggests that *B. bovis*, *B. bigemina* and *C. burnetii* are potential pathogens that cause mixed infections in XUAR. This study revealed the existence and genetic diversity of *B. bigemina*, *B. bovis*, *C. burnetii* and *A. bovis* in XUAR. The current data determined the infection rates of detected pathogens in cattle in that region and suggest the possible emergence of tick-borne diseases in animals in XUAR.

In chapter 2, TBPs were investigated in horses in XUAR. Molecular survey of *C. burnetii*, *Rickettsia* spp., *B. caballi* and *T. equi* were investigated in horses in XUAR by using nPCR assays and gene sequencing. Out of 200 samples tested, 114 (57.0%), 79 (39.5%), 79 (39.5%) and 49 (24.5%) were positive for *Rickettsia* spp., *C. burnetii*, *T. equi*, and *B. caballi.* Mixed infections involving 2-4 pathogens were observed in the present study. The most common dual infections were with *Rickettsia* spp. + *T. equi* while the most frequent triple infections were *C. burnetii* + *Rickettsia* spp. + *T. equi* with co-infection rates of 11.0% (22/200) and 8.5% (17/200), respectively. In addition, 11 (5.5%) samples were infected with the 4 TBPs (*Rickettsia* spp.+ *C. burnetii* + *T. equi* + *B. caballi*) among these co-infection. The current findings are expected to provide a basis for better TBPs (*B. caballi*, *T. equi*, *C. burnetii* and *Rickettsia* spp.) control in the region. These results also suggest that the persons associated with horses in the region should pay attention for preventing zoonotic tick-borne pathogens from horses.

In chapter 3, TBPs were identified in sheep in XUAR. Nested PCR assays and gene sequencing were used to detect *Babesia* spp., *Theileria* spp. and *Anaplasma* spp. in sheep from the bordering area in XUAR. Out of 323 samples tested in this study, 206 (63.8%), 60 (18.6%), 54 (16.7%), 51 (15.8%), 32 (9.9%), 19 (5.9%), 16 (5.0%) were positive for *A. ovis*, *B. motasi*-like, *A. bovis*, *T. uilenbergi*, *A. phagocytophilum*,

T. luwenshuni and *B. motasi*-like Xinjiang, respectively. Mixed infections involving 2-3 pathogens were observed in the present study. The most common dual infections were *A. ovis* + *B. motasi*-like while the most frequent triple infections were *A. ovis* + *B. motasi*-like + *T. uilenbergi* with co-infection rates of 17.0% (55/323) and 5.0% (16/323), respectively. Meanwhile, only 1 sheep was dually infected with *A. phagocytophilum* + *T. luwenshuni*. These findings provide important data for understanding the distribution of *Babesia*, *Theileria* and *Anaplasma* in sheep from the bordering area in XUAR.

Overall, TBPs in livestock (cattle, horses, sheep) were investigated. These results provide important data for understanding the distribution of TBPs, and is expected to improve the approach for control of tick-borne diseases in XUAR, China.

和文要約

マダニは地球上に広く分布し、ヒトや動物に多くの病原体（ウイルス、細菌、原虫など）を媒介する。新疆ウイグル自治区（以下新疆）は、中国の西北部に位置し、全国土面積の６分の一を占めており、また、ロシアなど８ヶ国と国境を接している。家畜生産は新疆における主要産業である。また、新疆はアジアとヨーロッパを結ぶシルクロードの中継地としても知られ、家畜貿易が盛んである。これまでに、新疆におけるマダニが保有している病原体についてはいくつかの報告があるが、家畜におけるマダニ媒介病原体に関する詳細な報告はない。そこで、本研究では新疆におけるウシ、ウマ、ヒツジのマダニ媒介病原体の詳細な分子疫学調査を行った。

第１章では、新疆におけるウシのマダニ媒介病原体の調査を行った。PCR法と遺伝子塩基配列解析法を用いて、バベシア属、コクシエラ属およびアナプラズマ属の検出と解析を行った。195 頭の血液サンプル中 *B. bigemina*、*C. burnetii*、*B. bovis*、および *A. bovis* の陽性率は、それぞれ 34.4%、20.5%、12.3%、および 5.1%であった。複数の病原体が同時に検出される混合感染例も多数認められたが、ほとんどは *B. bigemina*＋*C. burnetii*（6.2%）または *B. bigemina*＋*B. bovis*＋*C. burnetii*（1.0%）であった。臨床症状を示す症例は観察されなかったが、通常混合感染は単一感染よりウシ健康へのリスクは大きいとされる。これらの結果より、バベシア属、コクシエラ属、アナプラズマ属は新疆におけるウシのマダニ媒介感染症を引き起こす主要病原体であることが示唆された。

第２章では、新疆におけるウマのマダニ媒介病原体の調査を行った。ヒトのＱ熱の病原体を含むコクシエラ属、ヒトの紅斑熱の病原体を含むリケッチア属、ならびにウマピロプラズマ症の病原体であるバベシア属とタイレリア属などが検出された。200 頭から採集した血液サンプル中リケッチア属、*C. burnetii*、*T. equi*、および *B. cabalii* の陽性率はそれぞれ 57.0%、39.5%、39.5%、および 24.5%であった。また、多数の混合感染例が認められた。その多くは *Rickettsia* 属＋*T. equi* (11.0%)または *C. burnetii*＋*Rickettsia* 属＋*T. equi* (8.5%) であった。これらの結果より、コクシエラ属、リケッチア属、バベシア属、およびタイレリア属が新疆におけるウマのマダニ媒介感染症を引き起こし主要病原体であり、その一部は、人獣共通感染症の病原体として公衆衛生上の注意喚起が必要であることが示唆された。

第３章では、新疆の国境地帯におけるヒツジのマダニ媒介病原体の調査を行った。323 頭から採集した血液サンプル中の各病原体の陽性率は、*A. ovis* で 63.8%、*B. motasi*-like で 18.6%、*A. bovis* では 16.7%、*T. uilenbergi* では 15.8%、*A. phagocytophilum* では 9.9%、*T. luwenshuni* では 5.9%、および *B. motasi*-like Xinjiang では 5.0%であった。また、多数の混合感染例が認められ、その多くは *A. ovis*＋*B. motasi*-like (17.0%) または *A. ovis*＋*B. motasi*-like＋*T. uilenbergi* (5.0%) であった。これらの結果より、バベシア属、およびタイレリア属、アナプラズマ属が新疆の国境地帯におけるヒツジの主要マダニ媒介感染症を引き起こし、これらの感染症の制御は隣国においても重要であることが示唆された。

以上のように、本研究では新疆における家畜（ウシ、ウマ、ヒツジ）のマダニ媒介病原体の分子疫学調査を行った。得られた結果は、中国新疆における家畜のマダニ媒介感染症の流行に関する重要な情報を提供し、そして、その制御対策の構築に貢献できると考えられる。

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