Epidemiological Study of Tick-borne Diseases in Xinjiang Uygur Autonomous Region, China

（中国新疆ウイグル自治区における
マダニ媒介性疾患の疫学的研究）

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Contents

Abbreviations

B Bc48: *Babesia caballi* recombinant P48 protein

bp: base pair

C CFT: complement fixation test

E EMA-2t: truncated *Babesia equi* merozoite antigen 2

ELISA: enzyme-linked immunosorbent assay

EMA-1: *Babesia equi* merozoite antigen 1

G GST: glutathione *S*-transferase

gltA: citrate synthase gene

H Ha: hectare

H: hour(s)

I IPTG: isopropyl-β-D-thiogalactopyranoside

IFAT: indirect fluorescent antibody test

IgG: immunoglobulin G

K kDa: kilodalton

M mM: millimol

M: mol

mg: milligram

mL: milliliter

N nmol: nanomol

P PCR: polymerase chain reaction

PBS: phosphate-buffered saline

S Sq km: square kilometers

SDS-PAGE: sodium dodecyl sulfate-polyacrylamide gel electrophoresis

T TAE: tris-acetic acid-EDTA

TBD: tick-borne diseases

TBS: tris-HCI buffared saline

U µg: microgram

µl: microliter

General introduction

1. Xinjiang Uygur Autonomous Region and major livestock

The Xinjiang Uygur Autonomous Region also referred to as the Xinjiang was anciently known as the "XI YU" (the west-land), the hinterland of Eurasia. The region is located in the northwest of China having an area of 1,664,900 square kilometers (sq km). This region is one sixth of the Chinese territory and is famous for the Middle Asian civilization known as "the Silk Road". The Xinjiang region is surrounded, along its borders, by eight countries, which include Mongolia to the northeast, Russia, Kazakhstan, Kirghizstan, and Tadzhikistan to the northwest, as well as Afghanistan, Pakistan, and India to the south (Fig. 1). The topography of Xinjiang is very special in that there are high snow mountains, river, lakes, large-scale desert and vast grassland. The climate is predominantly dry and continental, with warm summers and cold winters having an average annual temperature of 7.3°C.

There are 47 nationalities living in Xinjiang and the major groups include Uygur, Kazak, Hui, Mongolian, Kirgiz, Xibe, Tajik, Ozbek, Tatar and the Russian people. Xinjiang has population of 20,951,900 people and is one of the five minority autonomous regions in China. Xinjiang is the largest province in China, having a beautiful natural environment, with vast grasslands, and abundant grassland resources. Thus, the region is suitable for the breeding and raising domestic animals with cattle and sheep being the main production animals. The main livestock kept in Xinjiang include sheep, cattle, goats, horses, donkeys, mules, camels, pigs, chickens, ducks and the domesticated wild animals, deer, mink amongst others. These animals provide meat, milk, eggs and other nutritious food for the people of Xinjiang.

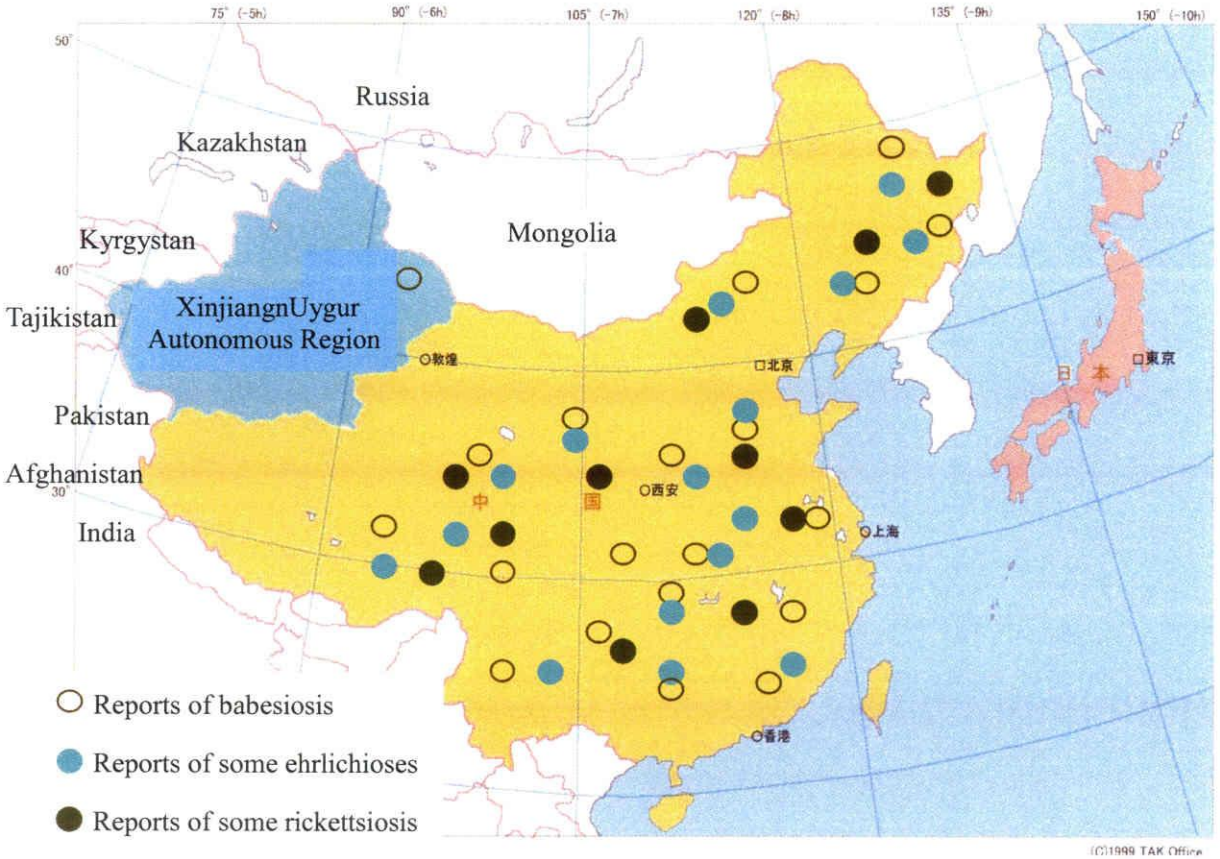


Fig.1. Reports of some tick-born diseases in domestic animals in China

According to statistics of the year 2006, the population of livestock was 53807000 in Xinjiang, consisting of 37252400 sheep, 6542600 goats, 5128600 cattle, 1249400 donkey, 920900 horse, and 2438800 pigs. Therefore, it is ranked the first and the second in China with regard to the economical value of sheep industry and that of cattle industry, respectively. Furthermore, the region is also known as the main center for the horse and donkey industries in China. The Agricultural system is mainly semi-agriculture, semi-grazing or simple grazing. The grassland area is 570000 sq km with cultivation area of 30600 sq km and a forest cover of 1870000 hectare (Ha). Because of the vast grassland resources, animal husbandry system is based mainly on

the natural pasture, needed for grazing various domestic animals that include sheep, cattle, goats, horses, donkeys, camel and other grazing livestock. Because the minority Uygur people extensively use donkeys for transportation, these animals regarded as important animals in Xinjiang. Therefore, to improve socioeconomic well-being of the people of Xinjiang, there is need to develop the breeding industry of sheep, cattle, horses, donkeys and other domestic animals.

2. The Major tick-borne diseases of Xinjiang

The hemoparasitic diseases and pathogens transmited by ticks are distributed worldwide, infect almost all domestic animals, and are a public health problem in Xinjiang Uygur Autonomous Region (Yu et al., 1996; Wang et al., 1997). Ticks are specialized group of obligate, bloodsucking, non-permanent ectoparasitic arthropods that feed on mammals, bird and reptiles in many regions worldwide (Sonenshine, 1993). Tick-vectors are of veterinary and medical importance becuase they transmit diseases to humans, domestic animals and wildlife. The superfamily Ixodoidea includes two major families: the "hard tick" and the "soft tick" (Cirad and Maisons, 1995). Both hard and soft ticks are present in Xinjiang, and the fauna distributions of the ticks constitute two-thirds of Ixodid ticks certified in China; thus making the vegetation of Xinjiang heavly tick infested. The major genera of Ixodes-ticks present in Xinjiang are as follows; *Dermacentor* (consisting of 8 species, namely, *D. silvarum*, *D. nuttalli*, *D. sinicus, D. niveus*, *D. montanus*, *D. pavlovskyi*, *D. reticulates* and *D. marginatus)*, *Hyalomma* (having 7 species, namely, *H. detritum*, *H. rufipes, H. asiaticum, H. dromedarii, H. anatolicum anatolicum*, *H. asiaticum kozlovi* and *H. scupense*) and *Boophilus* (including 1 species, *Boophilus microplus*). Other ticks species infesting the region are

Rhipicephalus (including 6 species, *R. bursa, R. schulzei, R. sanguineus, R. turanicus, R. rossicus* and *R. pumilio*), *Haemaphysalis* (including 5 species, *H. punctata, H danieli, H. erinacei turanica, H. sulcata* and *H. concinna*) and *Ixodes* (including 7 species, *I. crenulatus, I. arboricola, I. berlesei, I. redikorzevi, I. persulcatus, I. hyatti* and *I. kazakstani*) (Kong, 1983; Yu et al., 1997).

When ticks parasitize mammalian hosts, they cause direct and indirect harmful effects such as tick paralysis, various tick toxicoses, irritation, tick bite allergies, immune responses and to blood loss leading to economic losses (Sonenshine, 1991). Ticks are second only to mosquitoes as vectors of disease causing agents in humans and wild animals. They transmit a variety of pathogens including protozoa, rikettsiae, viruses, bacteria, and even fungi to human, livestock and wild animals (Balashov, 1972; Hoogstraal, 1985; Uilenberg, 2006). Heart water and zoonotic infectious diseases are also important tick-borne diseases. Many studies have focused on the co-infections transmitted by ticks in the *Ixodes ricinus* complex, including *Bebesia microti, Anaplasma phagocytophila, Ehrlichia chaffeensi* and *Rickettsia* among the others (Leviene, 1971; Bram, 1983; Wei and Xue, 1991; Liu et al., 1992; Levin and Fish, 2000; Belongia, 2002).

Tick-borne babesiosis and theileriosis. Babesiosis and theileriosis are tick-borne diseases, which cause major economic losses, and affect many domestic animals, mainly horse, cattle, donkey and sheep, in tropical and subtropical regions (Uilenberg, 1995). The *Ixodid* ticks are the vectors, which transmit these diseases. In addition to the information on the prevalence of these diseases in ruminants, the diagnosis of these diseases in animals, and the identification of these parasites in vector ticks are also important in understanding the epidemiology of the diseases. The

distribution of babesiosis and theileriosis parallels the distribution of its vector ticks, because cattle, horses, sheep, goats and donkeys graze on natural pasture heavily infested by ticks. When the ticks are active from March to September, every year, they feed on the hosts and transmit the diseases leading to large-scale prevalence of the tick-borne diseases (Xia, 1983; Lu et al., 1997; Wang et al., 1997).

Based on the numbers and distribution of the *Babesia* species, babesioses are considered one of the most ubiquitous and widespread blood parasite infections in the world animals (Leveine 1985). In China, equine babesiosis was first reported in 1943 in Heilongjiang province while bovine babesiosis was described five years later. Since then, these diseases still affect animal health (Higuchi et al., 1991; Lu et al., 1992; Yin et al., 1997). From the animal health and the economic point of views, equine and bovine babesioses are considered the most important diseases in Xinjiang.

Equine piroplasmosis is an economically important hemoparasitic tick-borne disease of horses, donkeys, mules, and zebras.The infection is caused by *B. equi* and *B. caballi*, and is characterized by fever, anemia, and icterus. Equine piroplasmosis is mostly prevalent in the tropical and the subtropical areas as well as in the temperate climatic zones (Neitz, 1956; Salabarria et al., 1981; Schein 1988; Bruning 1996). Because of the worldwide distribution of the potential tick vectors, equines, which are imported to non-endemic areas or countries must be confirmed to be negative for the babesiosis through serological tests (Tenter and Friedhoff, 1986; Schein, 1988; Bruning, 1996). Apart from the widespread infections by *B. equi* and *B. caballi* in Xinjiang, these parasites have been discovered in 11 provinces in China (Kong, 1983; Yu et al., 1997). Additionally, equine babesioses have been reported in 15 prefectures, but the enzootic areas are mainly confined to the Zhunger Basin, Takilimakn Basin and grassland of

Tianshan (Yu et al., 1996; Wang et al., 1997). *B. equi* is transmitted by *Dermacentor* (*D. niveus*, *D. montanus*, *D. reticulates*, *D. marginatus)* and *Hyalomma* (*H. detritum, H. asiaticum)* and *Rhipicephalus* species (*R. bursa, R. turanicus*) (Abramove et al., 1978) while *B. caballi* is transmitted by *Dermacentor* species (*D. silvarum, D. nuttalli D. marginatus, D. niveus*), *Hyalomma marginatum* (Salayev, 1956).

B. bigemina and *B. bovis* cause bovine babesiosis, and *Boophilus microplus* as well as some species of *Rhipicephalus* and *Ixodes ticks* transmit both parasites with exception of *B. ovata* and others *Babesia* parasites that infect animals in Xinjiang province of China (Bai et al., 1987; Lu at al., 1990; Xiang, 2005; Yuan et al., 2008). *B. bigemina* and *B. bovis* are the most important pathogens of babesioses in cattle and yak.

Ovine babesiosis is the most important hemoparasitic tick-borne disease of small ruminants and is caused by *B. ovis*, *B. motasi* and *B. crassa*, which is characterized by fever, anemia, icterus and hemoglobinuria (Kong, 1983). In China, ovine babesiosis was first reported in 1982 (Chen, 1982), where it is responsible for considerable economic losses in sheep production (Xia et al., 1983; Zhao et al., 1986; Wang et al., 1997; Yin et al., 1997, 2000).

Theileria annulata is the major form of bovine protozoan disease in Xinjiang and is transmitted by a number of *Hyalomma* ticks. *H. detritum*, *H. rufipes*, *H. asiaticum*, *H. dromedarii*, *H. anatolicum anatolicum,* and *Haemaphysalis qinghaiensis* are the main vectors in the field (Estrada et al., 2004; Yin et al., 2004). The distribution of the tick vectors, found in south northern Xinjiang, showed that the mortality rate of theileriosis (*T. annulata*) in cattle population varied between 10% and 70% with a morbidity rate of up to 65-100% (Xia et al., 1983; Guo et al., 1997; Zhang, 1997; Wang et al., 2003; Yin et al 2004; Ica et al., 2007). However, those of other domestic animals, such as dogs,

camels and pigs are by no means negligible (Fairwell et al., 1982; Xia et al., 1983; Susan et al., 2000).

Tick-borne ehrlichioses. *Anaplasma* spp. and *Ehrlichia* spp. are pathogens of medical and veterinary importance. *Anaplasma* is a tick-borne and an obligate intracellular parasite of mammals. The genus *Anaplasma* is the causative agent of anaplasmosis in ruminants and the acute phase of the disease is characterized by severe anemia, weight loss, fever, abortion, lower milk production and often death (Inokuma, 2005, 2007). The genus *Ehrlichieae* is gram negatve minute cocci, which is obligate intracellular parasite like *Anaplasma* and belongs to the family rickettsiaceae. Which are intracellular microorganisms residing within the cytoplasmic vacuoles of monocytes, granulocytes, or platelets of humans and animals (*Ehrlichia* spp. infects dogs, ruminants, and horses). *Ehrlichia* spp. elicit illnesses characterized by fever, headache, leukopenia, and thrombocytopenia (Wen et al., 2003; Inokuma, 2007). Both *Anaplasma* spp. and *Ehrlichia* spp. are transmitted by arthropod ticks and are distributed worldwide (Dumler and Bakken, 1995, 1998). The genus *Anaplasma* includes *A. marginale, A. centrale, A. ovis, A. platys*, *A. phagocytephilum* and some unidentified species closely related to these pathogens. Of these *Anaplasma* species, *A. ovis* was first found in sheep in Xinjiang Uygur Autonomous Region in China (Ma et al., 1982) and in goats in Liaoning Province of China (Ding et al., 1985). Lu et al.(1989) found that ovine anaplasmosis was widespread among sheep and goats in 13 districts and in 44 counties of Gansu Province in the Northwest of China. *A. marginale* was first isolated from cattle in Lushi county of Henan Province (Bai et al., 1987). Later Bai et al. (1992) identified that *B. microplus* and *H. longicornis* cannot transovarially transmit the parasite. The genus *Ehrlichia* includes *E. canis, E. ewingii, E. chaffeensis, E. muris, E.*

ruminantium, and some additional new *Ehrlichia* species. *A. phagocyteophilum* and *E. chaffeensis* are two major zoonotic pathogens mainly reported in the United States and European countries (Foley et al., 2004; Parola, 2004; Santos et al., 2006). *A. phagocytephilum* can cause prevalent diseases in humans, ruminants and horses while *E. chaffeensis* can cause disease in both humans and dogs. Recently, both agents have also been reported in China, Korea and other Asian countries (Cao et al., 2000a, 2003; Kim et al., 2003; Liu et al., 2005; Inokuma et al., 2007; Zhan et al., 2008). In China, *E. chaffeensis* has been detected in *Amblyomma testudinarium*, *Haemaphysalis concinna*, *Ixodes ovatus*, *Ixodes persulcatus* and *Dermacentor silvarum* ticks amongst others. Additionally, *A. phagocytophilum* has only been found in *I. persulcatus* ticks from northeastern China where Lyme disease is endemic (Cao, et al., 2003; Zhang et al., 2007; Zhang et al., 2006, 2008). Thus, this parasite is a potential threat to the livestock and human in Xinjiang, where the ixodid ticks are widely distributed.

Tick-borne rickettsiosis. The genus *Rickettsia* is a motile, Gram-negative, non-sporeforming and highly pleomorphic bacteria, which exist as cocci, rods or thread-like organisms. *Rickettsia* spp., which is an obligate intracellular parasites, depend on the entry, growth, and replication within the cytoplasm of eukaryotic host cells (typically endothelial cells) (Parola, 2004). The majority of *Rickettsia* bacteria are susceptible to antibiotics of the tetracycline group. *Rickettsia* species parasitizes many ticks, fleas, and lice, and cause diseases such as typhus, rickettsial-pox, Boutonneuse fever, African Tick Bite Fever, Rocky Mountain spotted fever, Australian Tick Typhus, Flinders Island Spotted Fever and Queensland Tick Typhus amongst others (Raoult and Roux, 1997). In China, several tick-borne *Rickettsia* species have been isolated over the last decade (Zhang et al., 2000a, 2000b; Fournier et al., 2003; Zhang et al., 2006).Three

of these *Rickettsiae*, *R. sibirica, R. heilongjiangii,* and *R. mongolotimonae* are known to be human pathogens. Recently several other new rickettsial species such as *Rickettsia aeschlimannii* (Shpynov et al., 2003), "*Candidatus Rickettsia tarasevichiae*" (Shpynov et al., 2004), *Rickettsia helvetica* (Fournier et al., 2002), and others have been reported in countries neighboring China. Although there have been few reports on the epidemiology of *Ricketsia* infection in Xinjiang, *Rickettsia sibilica* is the only known rickettsial pathogen that causes spotted fever in humans in Xinjiang (Fan et al., 1987; Zhang et al., 2000a, 2000b; Zhang et al., 2006). Ticks, fleas and lice are adapted to the grassland environment in Xinjiang and heavly infest the grassland as well as animal bodies, but it is still unknown which kinds of rickettsial diseases the vectors transmit.

Currently the only widely available practical method to control ticks is the use of chemical based acaricides. This approach is associated with serious limitation such as environmental and food chain contamination by acaricides (Shang et al., 1983; Tellam et al., 1992; Rjput et al., 2006). The treatment of these tick-borne diseases rely on the use of chemotherapeutic drugs, which can easily develop resistance; moreover, no vaccines have been developed for most of the tick-borne diseases.

3. Currently available diagnositc tests of tick-borne diseases in Xinjiang

The geographical location of Xinjiang Uygur Autonomous Region is unique, and the area has diverse parasitic fauna. Furthermore, infestation by ticks is widespread in China covering about two-third of the whole country. The above factors contribute to the transmittion of each of these tick-borne diseases, especially when the tick-vectors are active from February to September every year. Because Xinjiang geographic location is remote, information flow is restricted somehow, and except in a special

research institute, the laboratory equipments in use are generally of a lower technological specifications. Therefore, it is still difficult to conduct adequate research on some of the tick-borne diseases to facilitate the control and prevention of the diseases in this province. To date, effective methods to inactivate ticks that infest the body of animals have not been found indicating that there might still be no means of interrupting the transmition of the diseases in Xinjiang Uygur Autonomous Region.

In general, the diagnosis of tick-borne diseases is performed by using microscopy, DNA or RNA based techniques, pathological lesions, and immunodiagnosis. It is not common to use DNA or RNA based techniques such as polymerase chain reaction (PCR) to diagnose the tick-borne diseases prevalent in Xinjiang. This is because such nucleic acid based procedures require highly trained personnel as well as costly equipments, which are only available in most commercial laboratories. Serological diagnostic methods, such as complement fixation test (CFT), indirect fluorescent antibodies test (IFAT), and enzyme-linked immunosorbent assay (ELISA) are widely used for the serological diagnosis of tick-borne diseases. However, some of the serological tests have some drawbacks such as limited supply of antigen and poor specificity, as well as the cross reactions between antibodies against genetically related equine piroplasmosis (Schein, 1988; Bruning, 1996). Furthermore, cross-reactions occur between closely related *Ehrlichiae* and *Rickettsiae*, leading to misinterpretation of results and misdiagnosis (Inokuma, 2005, 2008; Zhang et al., 2006; Cao et al., 2008). Depending on the *Babesia*, *Ehrlichia*, *Anaplasma*, and *Rickettsia* species, involved, direct microscopic observation of stained blood smears is currently used to diagnose tick-borne diseases, and generally suspected to be based on clinical and routine laboratory findings in Xinjiang Uygur Autonomous Region.

The recent application of advanced techniques, in the immunological diagnosis and molecular biology has improved the diagnosis of diseases and has provided new tools for the assessment of treatment (Molad, et al., 2006). Therefore, if improved molecular diagnostic and serological tests could be developed to the detect the tick-borne pathogens and their antibodies, respectively, then numerous questions about the detection of parasitic tick-borne diseases of horses, donkeys, cattle, sheep and goats infections will be answered ultimately.

4. The aims of this study

As described above, tick-borne diseases are widely distributed in Xinjiang Uygur Autonomous Region where they cause major public health problem in hyperendemic areas. Thus, these diseases pose a great threat to livestock and human health, and impact negatively on the development of livestock husbandry (Ai et al., 1979; Lu et al., 1992; Yu et al., 1996; Wang et al 1997, Wang, 2003; Fan, 2004; Xiang et al., 2005). Therefore, there is need for scientific understanding of the major tick-borne pathogens such as *Babesia*, *Erhlichia*, *Anaplasma* and *Rickettsia.* Additionally, scientific understanding of the tick-vectors as well as knowing their distribution is important. The effective methods for the control of the diseases in Xinjiang Uygur Autonomous Region and the whole of China should be developed to faciliate the epidemiological studies of the diseases along the Silk Road of central Asian countries.

To control the tick-borne diseases, it is very important to develop specific and sensitive diagnostic methods. In most countries, particularly in Xinjiang province parasite detection is still based on morphological diagnosis using examination of stained blood smears. This method is reliable for the detection of acute cases but has limited

value for chronic or carrier cases during which only a low number of parasites exist. Sometimes, experience is required to differentiate between the different species of parasites within an infected host and vector tick.

Recently serological and molecular diagnostic tools have been developed and contribute to the improvement of detection and differentiation of the pathogenic organisms. Therefore, I used serological and molecular diagnostic tests to investigate prevalence of tick-borne diseases in horses, donkeys, cattle, sheep and goats in the Xinjiang Uygur Autonomous Region, China.

For immunodiagnosis, the sensitivity, the specificity, and the cost mainly depend on the antigens. Merozoite surface antigens play important roles in parasite recognition and attachment to as well as penetration into host erythrocytes (Jack and Ward, 1981).Equi merozoite antigen-1 (EMA-1), equi merozoite antigen-2 (EMA-2) and recombinant Bc48 are major antigens of equine *Babesia*. Therefore, these antigens are good candidates for a diagnostic reagent for the detection of antibody against the equine babesiosis (Tanaka et al., 1999; Xuan et al., 2001b; Hirata et al., 2002; Huang et al., 2003; Tamaki et al., 2004). The application of these specific antigens greatly has improved the specificity of these kinds of tests. These tests are particularly useful for identification of chronically infected horses and donkeys with significantly low parasitemia.

Anaplasma and *Ehrlichia* species were not reported in China until 1980s. Moreover, the means by which many of these species spread among livestock and wild animals is still unknown (Fan, 2004). To understand the means of spread of the pathogenic organisms among livestock and wild animals, there is need evalute and improve the serological assays for the diagnosis of the tick-borne diseases.

Diagnostic methods of emerging *Rickettsia* infection include isolation of the organism, serodiagnosis and molecular diagnosis. Isolation is the "gold standard" for diagnosis; however, this method is time-consuming and expensive. Although serodiagnosis is the most frequently used method for diagnosis, serological cross-reactions occur between closely related *Ehrlichiae*, leading to misinterpretation and misdiagnosis (Zhang et al., 2006, 2007; Cao et al., 2008). With the recent development of molecular biodiagnostic methods, specific and sensitive assays such as PCR and sequencing are now used for the detection of rickettsial disease.

The aim of the present study can be summarized as follows: To clarify the prevalence of the tick-borne diseases including Babesiosis, Erlichioses, Anaplasmosis and Rickettsiosis, and in particular to detect *B. equi*, *B. cabalii*, *A. phagocytophilum*, *E. chaffeensis* and some *Rickettsia* in domestic animals in Xinjiang Uygur Autonomous Region, China, by using serological and molecular methods.

Chapter 1

Serodiagnosis of equine babesioses in horses in Xinjiang

1-1. Introduction

Equine piroplasmosis is caused by two tick-borne haemoprotozoan parasites, *Babesia equi* and *Babesia caballi*. The diseases are endemic in most tropical and subtropical areas of the world as well as in some temperate climatic zones (Schein, 1988; Bruning, 1996). Due to the almost worldwide distribution of the various tick vectors, the introduction of carriers into non-endemic areas or countries must be prevented (Schein, 1988). Prior to importation to non-endemic areas or countries, horses must be shown to be negative for piroplasmosis through serological testing (Tenter and Friedhoff, 1986; Schein, 1998; Bruning, 1996). In endemic countries, the control of equine piroplasmosis is necessary to maintain the international market open to the horse industry. The complement fixation test (CFT) and the indirect fluorescent antibody test (IFAT) are commonly used for detecting *B. equi* infection. However, these serological tests are generally restricted by antibody detection limits and cross reactivity (Bruning, 19986; Schein, 1998). Recently, a research group has developed enzyme-linked immunosorbent assays (ELISAs) using recombinant antigens, and demonstrated that the ELISAs can be used as an alternative to CFT and IFAT for diagnosis of *B. equi* and *B. caballi* infections, respectively (Ikadai et al., 1999, 2000; Xuan et al., 2001a). In this Chapter, I investigated the prevalence of equine piroplasmosis in horses in Xinjiang Uygur Autonomous Region, China by the ELISAs using recombinant antigens.

1-2. Materials and methods

Region, animals and samples. As described above (General introduction), Xinjiang Uygur Autonomous Region, the largest province in China, lies in the middle of the Eurasian continent. Xinjiang shares borders with eight countries (see Fig.1), and Xinjiang is one of the main localities for the horse industry in China. Ili area located in west of Xinjiang, its geographical position is at 82°16′–87°21′E longitude and 43°25′–47°15′N latitude, at 2,000–3,000 m a.s.l. The climate is predominantly dry and continental, with warm summers and cold winters. The average temperature ranges from 20 to 25 °C in summer (July) and is −9.5°C in winter (January).

A total of 70 serum samples were taken from horses pastured on three farms （A,B,C） in Ili area, Xinjiang in July 2001. No apparent clinical signs were observed on all the sampled horses by macroscopic examination.

Recombinant *Babesia equi* merozoite antigen 1 (EMA-1). The *B. equi* U.S Department of Agriculture [USDA] strain was cultured in equine erythrocytes as described previously (Avarazed et al., 1997; 1998), the DNA was extracted from *B. equi*-infected erythrocytes with phenol-chloroform and precipitated with ethanol and used as a template DNA for PCR. Two oligonucleotide primers (5′-ACGGATCCCAAGATGATTTCC-3′ and 5′-ACGGATCCGTCACTTAGTAAA-3′) were used to amplify the *equi* merozoite antigen 1(EMA-1) gene by PCR. The gene encoding the entire *B. equi* EMA-1 was inserted into a baculovirus transfer vector, constructed the recombinant baculovirus AcEMA-1(Sf9 cells were infected at 10 PFU/cell with a recombinant baculovirus carrying the EMA-1 gene), and a recombinant virus expressing EMA-1 was isolated. The expressed EMA-1 was transported to the surface of infected insect cells, the expressed EMA-1 had an apparent molecular mass

of 34 kDa that was identical to that of native EMA-1(Xuan et al., 2001b). EMA-1 was purified from recombinant baculovirus AcEMA-1-infected Sf9 cell culture medium, and used as an ELISA antigen for detecting antibodies to *B. equi* in horses.

Recombinant Bc48 protein. *B. caballi* U.S. Department of Agriculture [USDA] strain was grown in horse erythrocytes in continuous microaerophilous stationary-phase cultures as described by Avarzed et al. (1997). A cDNA expression library prepared from *B. caballi* merozoite mRNA was screened with a monoclonal antibody BC11D against the rhoptry protein of *B. caballi* merozoite. A cDNA encoding a 48-kDa protein of *B. caballi* was cloned and designated Bc48. The recombinant protein expressed by the vaccinia virus vector in horse cells had an apparent molecular mass of 48 kDa, which was the same as that of the native *B. caballi* 48-kDa protein. Moreover, recombinant proteins expressed by the pGEX-4T expression vector in *E. coli* as glutathione *S*-transferase fusion proteins (Ikadai et al, 1999, 2000), and used as an ELISA antigen for the detection of antibodies to *B. caballi* in donkeys.

ELISAs. The ELISA using recombinant EMA-1expressed in insect cells by baculovirus for diagnosis of *B. equi* infection in horses was performed as described previously (Xuan et al., 2001b). The ELISA using recombinant Bc48 protein expressed in *E. coli* by pGEX-4T for diagnosis of *B. caballi* infection in donkeys was carried out as described (Ikadai et al, 1999, 2000).

Ninety-six-well microtitration plates (Nunc-Immuno Plate; Nunc, Roskilde, Denmark) were coated overnight at 4°C with 50 μl (0.1 μg/μl) of recombinant antigens (EMA-1 and BC48 protein or GST protein as the control). These antigens were diluted in a 0.05 M carbonate-bicarbonate buffer (pH 9.6). To reduce the nonspecific binding, plates were blocked for 1 H at 37°C with PBS containing 3 % skim milk. The microtiter

plates were then incubated with individual horse serum diluted 1: 80 in PBS containing 3 % skim milk for 1 H at 37°C. After six washes times with PBS containing 0.05% Tween 20, the peroxidase-conjugated goat anti-horse IgG (Cappel, Durham, N.C.) antibody diluted 1:4, 000 in PBS containing 3% skim milk was added to each well in 50 μl and incubated for 1 H at 37°C. The plates were washed as described above, and then substrate solution (0.1 M citric acid, 0.2 M sodium phosphate, 0.003% H_2O_2, and 0.3 mg/ml 2,2'-azide-bis [3-ethylbenzthiazoline-6-sulfonic acid]) (Sigma, St. Louis, Mo.USA) was added to each well in 100μl aliquots. The absorbance at 415 nm was read after 1 H of incubation at room temperature by using an ELISA reader (Corona Microplate Reader MTP-120; Corona, Japan).

On the ELISA for detection antibodies to EMA-1 the titer was expressed as the reciprocal of the maximum dilution that showed an optical density value at 415 nm equal to or greater than 0.1, which is the difference in absorbance between values for the EMA-1 antigen and control antigen. On the ELISA for detection antibodies to Bc48 the titer was expressed as the reciprocal of the maximum dilution that showed an optical density value at 415 nm equal to or greater than 0.2, which is the difference in absorbance between values for the Bc48 antigen and control antigen.

Statistical analysis. All data were presented as the mean ± S.E. The significance of differences between the 3 farms samples and different age groups were determined with Student's t-test.

1-3. Results

As shown in Table 1, of the 70 samples tested, 28 (40.0%) and 17 (24.3%) samples were positive for antibodies against *B. equi* and *B. caballi* by the ELISAs, respectively.

The ELISA antibody titers ranged from 1:80 to 1:10240 (data not shown). In addition, 11 (15.7%) samples were positive for both *B. equi* and *B. caballi* (Table 2). There were no statistically (by $\chi 2$- test, $P>0.05$) significant differences observed on the three farms sampled (Table 1). The positive rates for *B. equi* ranged from 28.6 to 45.2 % on the three farms. On the other hand, the positive rates for *B. caballi* ranged from 19.4 to 33.3 % on the three farms. As shown in Table 3, both *B. equi* and *B. caballi* were detected in horses aged 1 to over 10 years. There were no statistically (by $\chi 2$- test, $P>0.05$) significant differences among different age groups. These results indicate that equine piroplasmosis is widespread in western Xinjiang, China.

1-4. Discussion

There are only a few previous reports on the prevalence of equine piroplasmosis in China (Yin et al., 1997). To my knowledge, this study is the first report on the survey for equine piroplasmosis in Xinjiang Uygur Autonomous Region, China. My data may be considered as important information that would contribute to understanding the prevalence of equine piroplasmosis not only in China, but also to countries neighboring Xinjiang province.

Several reports on the prevalence of equine piroplasmosis in Mongolia which shares a border with Xinjiang province have been published (Avarzed et al., 1997; Ikadai et al., 1999, 2000; Xuan et al. 1998, 2001a, 2001b). These reports demonstrated that both *B. equi* and *B. caballi* infections in horses are widespread in Mongolia. Battsetseg et al. (2001) reported that both *B. equi* and *B. caballi* can be transmitted by *Dermacentor nuttalli* in horses in Mongolia. The tick vectors for equine piroplasmosis in Xinjiang Uygur Autonomous Region have not yet investigated, therefore there is a need to study

the potential tick vectors involved in the transmission of *B. equi* and *B. caballi* in horses in Xinjiang Uygur Autonomous Region, China.

In the present study, the recombinant antigens, EMA-1 and Bc48, were produced using the genes cloned from *B. equi* USDA strain and *B. caballi* USDA strain, both isolated in the USA. There may be some geographic diversity and an associated loss or gain of epitopes on the EMA-1 or Bc48 between the USDA strains and Xinjiang isolates. Therefore, there is a need to compare the EMA-1 and Bc48 genes of the USDA strains to Xinjiang isolates in the future.

1-5. Summary

The prevalence of equine piroplasmosis in the Xinjiang Uygur Autonomous Region was examined by enzyme-linked immunosorbent assays (ELISAs). A total of 70 serum samples were taken from horses pastured on three farms in Western Xinjiang, and examined for antibodies against *B. equi* and *B. caballi* by ELISAs using recombinant *B.equi* merozoite antigen 1 (EMA-1) and *B.caballi* recombinant Bc48 antigen, respectively. Of the 70 samples, 28.6 (40.0%) and 17 (24.3%) samples were positive for antibodies against *B. equi* and *B. caballi*, respectively. In addition, 11 (15.7%) samples were positive for both *B. equi* and *B. caballi*. These results indicate that equine piroplasmosis is widespread and therefore a cause for serious concern in Western Xinjiang.

Table 1. Prevalence of equine piroplasmosis in three farms in Western Xinjiang

Farm	No. of examined	No.of positive (%)	
		B. equi[a]	*B. caballi*[b]
A	31	14 (45.2)	6 (19.4)
B	21	6 (28.6)	7 (33.3)
C	18	8 (44.4)	4 (22.2)
Total	70	28 (40.0)	17 (24.3)

[a] Antibodies to *Babesia equi* were detected by ELISA using recombinant EMA-1 expressed in insect cells. The ELISA was considered positive when an optical density at 415 nm equal to or greater than 0.1 was observed at dilutions of 1:80 and above.

[b] Antibodies to *B. caballi* were detected by ELISA using the recombinant Bc48 expressed in *Escherichia Coli.* The ELISA was considered positive when an optical density at 415 nm equal to or greater than 0.2 was observed at dilutions of 1:80 and above.

Table 2. Mixed infection of *Babesia equi* and *Babesia caballi* in horses in Western Xinjiang

	B. equi +	*B. equi* -	Total
B.caballi +	11 (15.7%)[a]	6 (8.6%)	17 (24.3%)
B.caballi -	17 (24.3%)	36 (51.4%)	53 (75.7%)
Total	28 (40.0%)	42 (60.0%)	70 (100%)

Table 3. Prevalence of equine piroplasmosis in horses of different age groups

Age(years)	No. of examined	No. of positive (%)	
		Babesia equi	*Babesia caballi*
1-5	24	7 (29.2)[a]	7 (29.2)
6-10	38	16 (42.1)	8 (21.1)
>10	8	5 (62.5)	2 (25.0)

[a] Values in parenthesis are in percentage.

Chapter 2

Serodiagnosis of equine babesioses in donkeys in Xinjiang

2-1. Introduction

Equine babesiosis is tick-borne disease of equines, including horses, donkeys, mules, and zebras; the disease is caused by 2 haemoprotozoan parasites, *Babesia equi* and *B. caballi* (Bruning, 1996). The mortality rate depends upon the general immune status of the affected animals and the virulence of the pathogenic organisms. A high mortality rate occurs during the initial infection of horses from *Babesia*-free areas introduced into endemic regions. In endemic regions, the severe clinical cases of babesiosis are relatively rare. Equine babesiosis is endemic in many parts of Europe, Africa, Arabia, and Asia (except Japan). Due to the almost worldwide distribution of the potential tick vectors, the introduction of carriers into non-endemic areas or countries must be prevented (Schein, 1988). Prior to importation to non-endemic areas or countries, equines must be shown to be negative for babesiosis through serological tests (Tenter and Friedhoff, 1986; Schein, 1988; Bruning, 1996). Currently, the complement fixation test (CFT) and the indirect fluorescent antibody test (IFAT) are commonly used for detecting the presence of antibodies to *B. equi* and *B. caballi* parasites. However, these serological tests are hindered by a limited antigen supply and poor specificity (Schein, 1988; Bruning, 1996). Recently, a research group has developed enzyme-linked immunosorbent assays (ELISAs) using recombinant antigens, and demonstrated that the ELISAs can be used as an alternative to the CFT and the IFAT for diagnosis of *B. equi* and *B. caballi* infections, respectively (Ikadai et al., 1999, 2000; Xuan et al., 2001b;

Hirata et al,.2002, 2003, 2005; Huang et al., 2003; Tamaki et al., 2004). In this Chapter, I investigated the prevalence of equine babesiosis in donkeys in Xinjiang Uygur Autonomous Region, China by ELISAs using recombinant antigens.

2-2. Materials and methods

Region, animals and samples. As described above (General introduction), Xinjiang Uygur Autonomous Region, the largest province in China, is located in the northwestern part of China. It shares borders with 8 countries, i.e., Mongolia, Russia, Kazakhstan, Kirghizstan, Tadzhikistan, Afghanistan, Pakistan, and India. The Kashgar and Ili areas, located in the western part of Xinjiang, were selected to investigate the prevalence of equine babesiosis in donkeys. Kashi and Ili share borders with Kirghizstan and Kazakhstan, respectively (see Fig.1 in Chapter 3). The climate is predominantly dry and continental, with warm summers and cold winters. The average temperature is 25.7 °C (Kashgar) or 22.7°C (Ili) in summer (July) and −6.0°C (Kashgar) or −9.5°C (Ili) in winter (January). Both the Kashgar and Ili areas are located at the middle point of the Silk Road that used to extend from Rome to Xi'an, China, and are known as the main centers for the horse and donkey industries in China. Donkeys are important animals in both areas, where they are extensively used as transportation by the minority Uygur people. To date, there are no reports regarding prevalence of equine babesiosis in donkeys in Xinjaing, although prevalence of the disease in horses has been reported recently in Xinjiang (Chapter 1).

Blood samples were randomly collected from 93 donkeys in Kashgar (n=50) and Ili (n=43) areas of Xinjiang Uygur Autonomous Region, China in 2004(April to August). Blood samples were centrifuged at 1000 × *g* for 10 min, and serum was obtained and

stored at −20 °C until analysis. Age of the animals was recorded, and no apparent clinical signs were observed for any of the sampled donkeys.

Equi merozoite antigen 2 (EMA -2). U.S. Department of Agriculture strain of *B. equi* was cultured in equine erythrocytes as described previously (Avarazed et al., 1997, 1998). The DNA was extracted from *B. equi*-infected erythrocytes with phenol-chloroform and precipitated with ethanol and used as a template DNA for PCR. The EMA-2t gene was amplified by using a sense primer, 5'-ACGAATTCTAAAATGTTGAGCAAG-3', which was located at the 23rd codon (the first codon behind the cleavage site of signal sequence), and the antisense primer, 5'-ACGAATTCTTATTGGGTCTTGTAG-3', which was located at the 251st codon (the last codon before the hydropheotic C-terminal sequence) (Huang et al., 2003). The amplified DNA was inserted into the *Eco*RI site of pGEX-4T and transformed into the DH5α strain of *Escherichia Coli.* The resulting recombinant plasmid was cloned and designated pGEX-4T/EMA-2 trunacated（EMA-2t）, and expressed in *E. coli,* and the recombinant EMA-2t fusion protein with GST was extracted with TNE (50 mM Tris-HCl at pH 7.5, 100 mM NaCl, and 2 mM EDTA) containing lysozyme (100 μg/ml) and 1% Triton X-100 combined with sonication, and purified from the soluble fraction with glutathione-Sepharose 4B (Amersham Pharmacia Biotech, Uppsala, Sweden), and used as an ELISA antigen for the detection of antibodies to *B. equi* in donkeys.

Bc48 protein. The ELISA using recombinant Bc48 protein expressed in *E. coli* by pGEX-4T for diagnosis of *B. caballi* infection in donkeys was carried out as described (Ikadai et al, 1999, 2000). Recombinant Bc48 fusion protein was purified using glutathione sepharose 4B beads, and used as an ELISA antigen for the detection of antibodies to *B. caballi* in donkeys.

ELISAs. An ELISA, using recombinant EMA-2t expressed in *E. coli* by pGEX-4T vector for diagnosis of *B. equi* infection in donkeys, was performed as described previously (Huang et al 2003). The ELISA using recombinant Bc48 protein expressed in *E. coli* by pGEX-4T for diagnosis of *B. caballi* infection in donkeys was carried out as described (Ikadai et al, 1999, 2000).

ELISA was performed in 96-well microplates (Nunc, Roskilde, Denmark). The plates were coated with the diluted antigen (5 μg /ml) at 4°C overnight, the plates were blocked with 3% skim milk in PBS (blocking solution) at 37°C for 1 h. Then, the blocking solution was discarded, and 50 μl of serum sample diluted in blocking solution was added to each well. After 1 h of incubation at 37°C, the wells were washed six times with a wash solution (PBS containing 0.05% Tween 20) and then incubated with horseradish peroxidase-conjugated goat anti-horse immunoglobulin G (ICN Biochemicals) diluted in the blocking solution at 37°C for 1 h (50 μl per well). After six washing, the substrate [0.1 M citric acid, 0.2 M sodium phosphate, 0.003 % H_2O_2, 0.3 mg of 2, 2'-azino-di-(3-ethylbenzthiazoline sulfonate) per ml] was added (100 μl per well). The absorbance at 415 nm was read within 1 h by means of an MTP-120 ELISA reader (Corona Electric, Ibaraki, Japan). GST was used as a control antigen for EMA-2t. The ELISA result was determined for each sample by taking the mean optical density value of two readings with the EMA-2t or Bc48 protein and subtracting the mean value of two readings with GST protein.

The ELISA for detection antibodies to EMA-2t, the titer was expressed as the reciprocal of the maximum dilution that showed an optical density value at 415 nm equal to or greater than 0.1, which is the difference in absorbance between values for

the EMA-2t antigen and control GST antigen. The ELISA for detection antibodies to Bc48, the titer was expressed as the reciprocal of the maximum dilution that showed an optical density value at 415 nm equal to or greater than 0.2, which is the difference in absorbance between values for the Bc48 antigen and control GST antigen.

In the both ELISAs, the secondary goat anti-donkey IgG antibody (Rockland Immunochemicals, 1: 4,000) was used. The IFAT using *B. equi* parasite or *B. caballi* parasite as antigens was performed as described previously (Avarzed, et al., 1997), except that the secondary rabbit anti-donkey IgG antibody 1: 400 (Bethyl Laboratories) was used.

Statistical analysis. All data were presented as the mean ± S.E. The significance of differences between the different areas samples were determined with Student's t-test.

2-3. Results

As shown in Table 4, of the 93 donkeys tested, 9 (9.7%) and 36 (38.7%) samples were positive for antibodies against *B. equi* and *B. caballi* by the ELISAs, respectively. All ELISA-positive samples were confirmed as positive for both *B. equi* and *B. caballi* by IFATs with parasites as antigens (data not shown). However, there were also 5 and 2 ELISA-negative samples confirmed as positive for *B. equi* and *B. caballi* by IFATs, respectively. Further study is needed to clarify the discrepancy between the two methods. The distributions of the ELISA antibody titers to *B. equi* were 1:100 (2 sera), 1:200 (2 sera), 1:400 (1 serum), 1:800 (1 serum), 1:1600 (2 sera), and 1:3200 (1 serum). The distributions of the ELISA antibody titers to *B. caballi* were 1:100 (6 sera), 1:200 (8 sera), 1:400 (6 sera), 1:800 (4 sera), 1:1600 (5 sera), and 1:3200 (5 sera), and 1:6400 (2

sera). In addition, 2 (2.2%) samples were positive for both *B. equi* and *B. caballi* (Table 5). There were no statistically significant differences (by $\chi 2$- test, $P>0.05$) observed on the areas sampled (Table 1). Both *B. equi* and *B. caballi* were detected in donkeys aged 1 to 10 year (data not shown). There were no statistically significant differences among different age groups (by $\chi 2$- test, $P>0.05$).These results indicate that equine babesiosis in donkeys is widespread in western Xinjiang, China.

2-4. Discussion

There are only a few previous reports on the prevalence of equine babesiosis in horses in China (Yin et al., 1997; Xu et al., 2003). In Chapter 1, I have reported that equine babesiosis in horses is widespread and, therefore, a cause for serious concern in Xinjiang Uygur Autonomous Region, China. To my knowledge, this is the first report describing a survey on equine babesiosis in donkeys in China.

Several reports on the prevalence of equine babesiosis in Mongolia, which shares a border with the Xinjiang, have been published (Avarzed et al., 1997; Xuan et al., 2001a; Boldbater et al., 2005). These studies demonstrated that both *B. equi* and *B. caballi* infections in horses are widespread in Mongolia. Battsetseg et al. (2001) reported that *Dermacentor nuttalli* could transmit both species of *Babesia* in Mongolia. Controlling the possible tick vectors is thought to be an effective way to reduce the infection and to improve the quality of horse and donkey populations in endemic areas. The tick vectors for equine babesiosis in the Xinjiang not very clearly at the present time and, therefore, there is an urgent need to identify the potential vectors involved in the transmission of both *B. equi* and *B. caballi* in Xinjiang.

In general, *B. equi* infection is more prevalent than *B. caballi* infection in horses

(Schein, 1988). However, in the present study, the positive rate of *B. equi* infection (9.7 %) was lower than that of *B. caballi* infection (38.7 %) in donkeys in Western Xinjiang. At this moment, the reason is remained unknown. The large-scaled epidemiological studies will be needed to clarify the question.

In this study, all 93 donkeys examined were raised locally, suggesting that the equine babesiosis is endemic among the local donkey populations. However, more formally designed epidemiological studies would be needed to define the population dynamics of the infection and the role of possible vectors. In addition, all sero-positive donkeys did not show any significant clinical signs. Since these donkeys are constantly under exposure of *B. equi* and *B. caballi* infections, they may acquire comparatively high immunity.

2-5. Summary

The prevalence of *B. equi* and *B. caballi* in donkeys in Xinjiang Uygur Autonomous Region was investigated. In total, 93 serum samples were randomly collected from donkeys in the Kashgar and Ili areas, and examined for antibodies against *B. equi* and *B. caballi* by enzyme-linked immunosorbent assays using recombinant antigens of EMA-2t and Bc48. Of the 93 samples, 9 (9.7 %) and 36 (38.7 %) samples were positive for antibodies against *B. equi* and *B. caballi*, respectively. In addition, 2 (2.2 %) samples were positive for both *B. equi* and *B. caballi*. These results indicate that equine babesiosis might be extensively prevalent in donkeys in Western Xinjiang.

Table 4. Prevalence of equine babesiosis in donkeys in Western Xinjiang

Area	No. of examined	No. of positive (%)	
		B. equi [a)]	*B. caballi* [b)]
Kashi	50	7 (14.0)	16 (32.0)
Ili	43	2 (4.7)	20 (46.5)
Total	93	9 (9.7)	36 (38.7)

[a)] Antibodies to *Babesia equi* were detected by the ELISA using recombinant EMA-2t expressed in *Escherichia Coli.* The ELISA was considered positive when an optical density at 415 nm equal to or greater than 0.1 was observed at dilutions of 1:100 and above.

[b)] Antibodies to *B. caballi* were detected by ELISA using the recombinant Bc48 expressed in *E. coli.* The ELISA was considered positive when an optical density at 415 nm equal to or greater than 0.2 was observed at dilutions of 1:100 and above.

Table 5. Mixed infection of *Babesia equi* and *Babesia caballi* in donkeys in Western Xinjiang

	B. equi +	*B. equi* −	Total
B. caballi +	2 (2.2 %)[a]	34 (36.5 %)	36 (38.7 %)
B. caballni −	7 (7.5 %)	50 (53.8 %)	57(61.3 %)
Total	9 (9.7 %)	84 (90.3 %)	93 (100 %)

[a] Values in parenthesis are in percentage

Chapter 3

Serodiagnosis of *Anaplasmoses* and *Ehrlichioses* in domestic animals in Xinjiang

3-1. Introduction

Ehrlichioses are important vector-borne diseases in both humans and animals. Both *Anaplasma* and *Ehrlichia* spp. are known to be transmitted by ticks and are distributed worldwide (Dumler and Bakken, 1998). The genus *Anaplasma* includes *A. marginale, A. centrale, A. ovis, A. platys, A. phagocytophilum* and some unidentified species closely related to those pathogens. The genus *Ehrlichia* includes *E. canis, E. ewingii, E. chaffeensis, E. muris, E. ruminantium,* and some additional new *Ehrlichia* species. *A. phagocytophilum* and *E. chaffeensis* are two major zoonosis pathogens mainly reported in the United States and European countries (Foley et al., 2004; Parola, 2004). *A. phagocytophilum* can cause prevalent diseases in humans, ruminants and horses, and *E. chaffeensis* in both humans and dogs. Recently, both agents have also been reported in eastern Asia, including China and Korea (Cao et al., 2000a, 2003; Kim et al., 2003; Liu et al., 2005; Zhan et al., 2008). In China, DNA of *A. phagocytophilum* has been detected in *Ixodes persulcatus* ticks in Heilongjiang Province in northeastern China (Cao et al., 2003). *E. chaffeensis* DNA was also detected by PCR from *Haemaphysalis yeni* and *Amblyomma testudinarium* in southern China (Cao et al., 2000b). However, there is a little information available on ehrlichiosis in the western part of China. Xinjiang Uygur Autonomous Region is located in the Western most area in China. The region has a cold and dry climate with high mountains and wide deserts. The animal grazing of ruminants

on pastureland is one of the main industries of Xinjiang. Horses and donkeys are also important animals for use in transportation in this area. *Rickettsia sibilica* is the only known rickettsial pathogen that causes spotted fever in humans in Xinjiang (Ai et al., 1979; Fan et al., 1987), but it is not clear whether other tick-borne rickettsial diseases exist. The aim of this Chapter was to determine whether pathogens of *Anaplasma* and *Ehrlichia* distribute in Xinjiang Uygur Autonomous Region. Thus, the sero-prevalence of antibodies against *Anaplasma* and *Ehrlichia* in domestic animals, including cattle, sheep, goats, horses and donkeys in this area were screened by using indirect fluorescent antibody test (IFAT) for *A. phagocytophilum* and *E. chaffeensis.*

3-2. Materials and methods

Region, animals and samples. Three areas, Altai, Ili and Kashgar, were selected for the survey (Fig.3). Altai is situated in the northern part of Xinjiang, and is bounded by Russia and the People's Republic of Mongolia. It is just south-west of the Altai Mountains. Ili is situated at the north-west border of Xinjiang, and is bounded by the Kazakhstan Republic, Russia. It is also north of the Tianshan Mountains. Kashgar is at the west end of Xinjiang, bordering the Taklamakan desert in the east and the Kunlun Range in the south. It is also the eastern neighbor of Kyrgys and Tajikistan.

Sera were collected from 146 cattle, 134 sheep, 133 goats, 85 horses and 100 donkeys in Xinjiang Uygur Autonomous Region from April to August in 2004. These numbers of sera from each area and type of animal are shown in Table 6. Samples were stored at –20°C until examined. Histories and clinical symptoms of each animal were not recorded.

IFAT. Antigens for IFAT were kindly given by Dr. P. Brouqui (Unité des

Rickettsies, Université de la Méditerranée, Marseille, France). *A. phagocytophilum* (HGE agent Webster strain, originally supplied by Dr. J.S. Dumler, The Johns Hopkins University School of Medicine, Baltimore, MD, USA) and *E. chaffeensis* (Arkansas strain, originally supplied by Dr. J.E. Dawson, Center for Diseases Control and Prevention, Atlanta, GA, USA) were used as antigens in the IFAT as previously described (Brouqui et al., 1994). Sera from mice that were experimentally infected with *A. phagocytophilum* and *E. chaffeensis* were used as positive controls. Sera from healthy animals kept in Japan were used as negative controls. Sera were screened at a 1:20 dilution in phosphate-buffered saline (pH 7.4), Tween 0.5 % (PBST) and an optimized dilution (1:160 to 1:200) of fluorescein isothiocyanate-labelled anti-IgG conjugate (anti-cattle IgG; ICN Pharmaceuticals Inc., USA, anti-sheep IgG; ICN Pharmaceuticals Inc., USA, Capple, anti-goat IgG; ICN Pharmaceuticals Inc., USA, anti-horse IgG, MP Biomedicals, Inc., USA, or anti-donkey IgG; Santa Cruz Biotechnology, USA) in PBST was used as the second antibody. The positive reactions were then detected using a fluorescence microscope. Antibody levels of test samples were determined by comparison with the appropriate positive and negative controls. Those samples that reacted with any of the antigens at the screening dilution were then titrated using serial twofold dilutions to determine end titers.

3-3. Results

The results are summarized in Table 7. A total of 7 cattle serum samples of 47 (14.9 %) in Altai, 6 of 50 (12.0 %) in Ili, and 2 of 49 (4.1 %) in Kashgar reacted with at least one of the antigens at a dilution of 1:40 or more. Dual positivity was occasionally seen, but most samples reacted more strongly with one of the two antigens(Fig.2). In

Altai, all of the 7 positive cattle reacted with *E. chaffeensis* with titers ranged between 1:40 to 1:320, and showed weak reaction with *A. phagocytophilum* with titers of 1:20 or less. Five cattle serum samples in Ili showed higher titers against *E. chaffeensis* (1:40 to 1:160), while 1 showed a higher titer against *A. phagocytophilum* (1:80). In contrast, the 2 positive cattle in Kashgar showed higher titers against *A. phagocytophilum* (1:40 and 1:160) than those against *E. chaffeensis*. A total of 6 sheep serum samples among 37 (16.2 %) in Altai, 11 among 50 (22.0 %) in Ili and 8 among 47 (14.9 %) in Kashgar, reacted with *A. phagocytophilum* or *E. chaffeensis* at a dilution of 1:40 or more. In Altai, 3 of the 6 sheep sera showed higher titers against *A. phagocytophilum* (with titers of 1:40 to 1:160) than those against *E. chaffeensis*, while the other 3 samples showed the same titers (of 1:40 or 1:80) against *A. phagocytophilum* and *E. chaffeensis*. In Ili, 4 sheep samples showed higher titers against *E. chaffeensis* (1:80 to 1:160), and 1 against *A. phagocytophilum* (1:40), while the other 6 showed the same titers against both antigens. In Kashgar, 3 positive sheep sera showed higher titers against *A. phagocytophilum* (1:40, 1:80 and 1:320), 3 showed higher titers against *E. chaffeensis*, and the other 2 showed equal titers (of 1:40 and 1:80) against both antigens. None of the goat sera in Altai showed any positive reaction, while a total of 3 goat serum samples among 50 (6.0 %) in Kashgar and 1 among 33 (3.0 %) in Ili reacted with *A. phagocytophilum* or *E. chaffeensis* at a dilution of 1:40 or more. In Ili, the only positive sample showed a higher titer against *E. chaffeensis,* with titer of 1:40. In Kashgar, 2 positive goat sera showed higher titers against *A. phagocytophilum* (1:40 and 1:80) and 1 showed a higher titer against *E. chaffeensis* (1:40).

3-4. Discussion

In the present study, antibodies that reacted with *A. phagocytophilum* and *E. chaffeensis* were detected in ruminants in Xinjiang. However, the relationship between pathogenesis and antibodies against these agents was not analyzed, because the histories and clinical symptoms were not recorded in this study. In Altai, cattle showed high titers against *E. chaffeensis*, while sheep showed higher titers against *A. phagocytophilum.* This may reflect the differences of location of where the examined animals were kept. It was impossible to examine the existence of *A. phagocytophilum* and *E. chaffeensis* in these areas, because cross reaction of antibodies is commonly seen for antigens among the same genus. The positive reaction might have resulted from infection of species closely related to *A. phagocytophilum* and *E. chaffeensis*. Higher titers may be associated with multiple exposure to individual animals or recent exposure, although some younger animals also showed high titers. In Xinjiang, most ruminants are kept on pastureland, and are usually infested with many ticks from spring to autumn. Ticks may transmit the ehrlichial pathogens to animals.

All the horse serum samples were obtained in Altai, and none of there sera reacted with any of the antigens. The only positive serum sample of donkey that obtained from an animal in Kashgar. The titer against *A. phagocytophilum* was 1:40. Most of the horses and donkeys examined in this study were not kept on pastureland, but lived near the farm houses and were used for transportation. Thus, tick infestation of horses and donkeys is less likely than that of ruminants.

Recently, several new ehrlichial species were detected by molecular methods or isolated around China. *E. muris* and a new *Ehrlichia* species closely related to *E. chaffeensis* were isolated in Japan (Wen et al., 1995; Shibata et al., 2000). *E. muris* DNA

has also been detected from ticks in central Russia near Xinjiang (Shpynov et al., 2004). Another novel *Ehrlichia* DNA closely related to *E. ewingii* was detected in Tibet, Myanmar and Japan (Wen et al., 2002; Parola et al., 2003; Inokuma et al., 2004). It is possible that domestic animals in Xinjiang have been infected with some new ehrlichial pathogens and showed positive antibodies against *A. phagocytophilum* and *E. chaffeensis*. Isolation and characterization of the pathogens will be required for the next step of this study.

3-5. Summary

Serological methods were utilized to detect *Anaplasma* and *Ehrlichia* infection in domestic animals in Xinjiang Uygur Autonomous Region. Serum samples were collected from cattle, sheep, goats, horses and donkeys in Altai, Ili, and Kashgar areas. These samples were analyzed by using an indirect immunofluorescence assay to screen for antibodies against *A. phagocytophilum* and *E. chaffeensis*, which infect ruminants. Antibodies screened by IFAT showed 5.5% percent of the samples were positive for *A. phagopcytolphilum* antibodies in cattle while 17% were seropositive for the antibodies in sheep. Moreover, 8.9 % of samples were positive for the *E. chaffeensis* antibodies in cattle while 15.7% percent were positive for the pathogen antibodies in sheep. In contrast, goats, donkeys and horses examined in Altai erea were all negative for the antibodies against the pathogens. These results indicated that cattle and sheep could be infected with some the species of *Anaplasma* and *Ehrlichia* in Xinjiang Uygur Autonomous Region, China.

Table 6. Information of sera examined from each area

Area	Animals	Numbers of animals examined	Age (range, years old)
Altai	Cattle	47	5-9
	Sheep	37	3-5
	Goat	50	3-5
	Horse	85	2-20
	Donkey	50	unknown
Kashgar	Cattle	49	0-4
	Sheep	47	0-6
	Goat	50	0-6
	Donkey	50	unknown
Ili	Cattle	50	1-9
	Sheep	50	0-2
	Goat	33	0-4

Table 7. Detection for antibodies against *A. phagocytophilum* and *E. chaffeensis* in domestic animals in Altai, Ili and Kashgar area of Xinjiang Uygur Autonomous Region

Animals	Area	No.	Age (years old)	Titers	
				A.phagocytophilum	*E. chaffeensis*
Cattle	Altai	12	5	20	40
		13	6	<20	80
		26	6	<20	160
		28	6	<20	80
		29	6	<20	80
		30	5	<20	80
		31	6	<20	320
	Ili	4	5	20	40
		6	3	<20	80
		11	2	80	160
		22	4	20	80
		24	2	40	80
		36	2	80	40
	Kashgar	34	2	160	<20
		42	2	40	<20
Sheep	Altai	10	3–5[a]	80	80
		11	3–5[a]	80	80
		12	3–5[a]	160	80
		23	3–5[a]	40	<20
		44	3–5[a]	40	40
		49	3–5[a]	40	20
	Ili	6	0	40	40
		13	2	40	160
		14	2	40	20
		19	0	40	80
		26	0	40	40
		28	0	160	160
		32	2	40	80
		33	2	<20	80
		38	1	160	160
		42	2	80	80
		45	0	80	80
	Kashgar	8	0	320	80
		12	2	40	80
		13	2	20	40
		17	2	80	40
		18	2	80	80
		24	0	40	80
		33	2	40	40
		44	4	40	20
Goats	Ili	27	3	20	40
	Kashgar	8	2	80	20
		22	0	40	20
		39	2	20	40
Donkeys	Kashgar	50	8	40	20

[a] Age of the individual sheep in Altai was not recorded

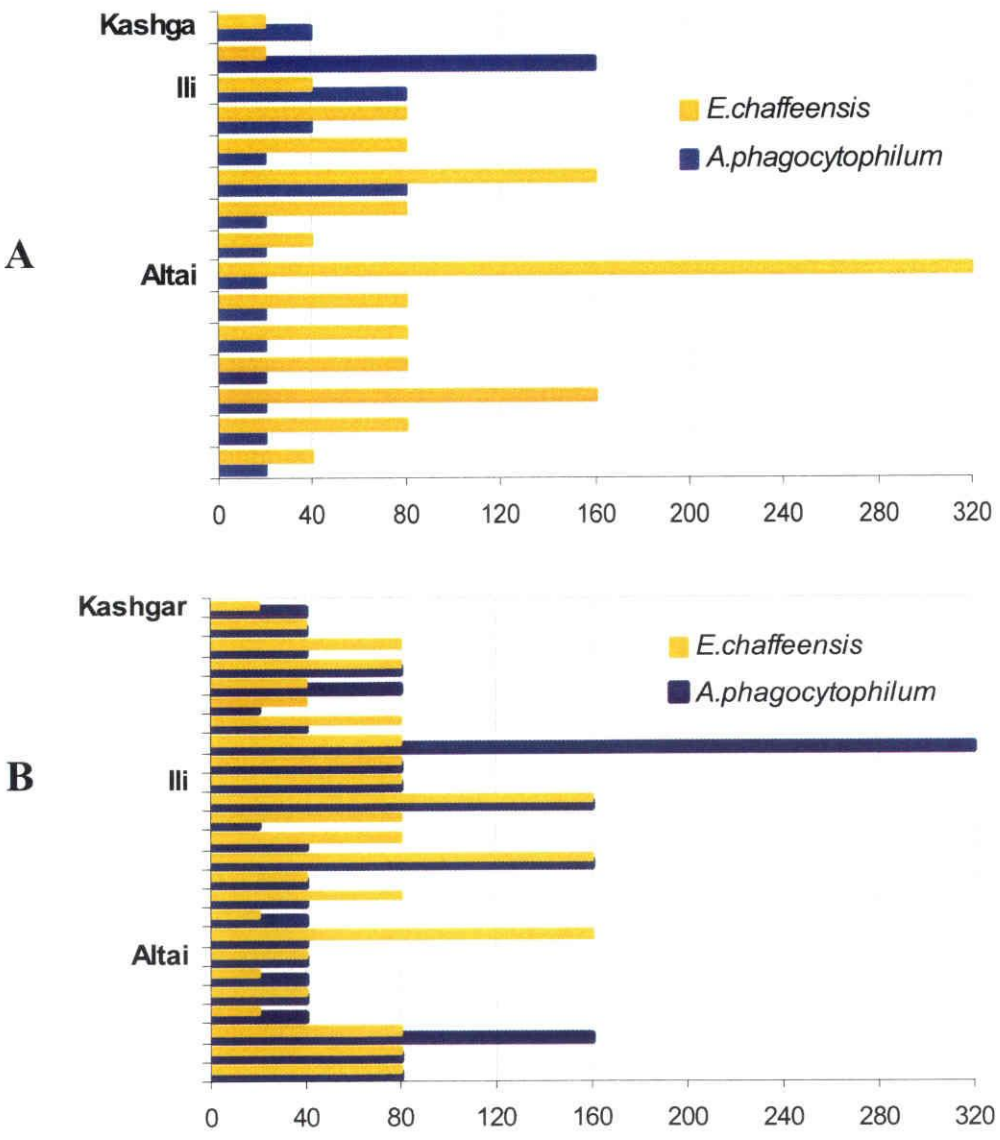


Fig.2. Antibody titers against *Anaplasma phagocytophilum* and *Ehrlichia chaffeensis* of domestic animals that showed titers of 1:40 or more against any of the agents. A: Positive sample in cattle. B: Positive sample in sheep.

Fig.3. A map of Xinjiang Uygur Autonomous Region (Urmuqui is the capital city of Xinjiag). The three study sites, Altai, Ili and Kashgar, are indicated in the figure.

Chapter 4

Molecular dignosis of *Rickettsia* infection in cattle in Xinjiang

4-1. Introduction

In China, several tick-borne *Rickettsia* species have been isolated over the last decade, including *R. sibirica* (Zhang et al., 2000b), *R. heilongjiangii* (Zhang et al., 2000a), *R. mongolotimonae* (Fournier et al., 2003) and *R. hulinensis* (Zhang et al., 2000a), which are known to be human pathogens. Recently other new rickettsial species have been reported in countries neighboring China; for example, *R. aeschlimannii* was detected in ticks in Russia and Kazakhstan (Shpynov et al., 2003), "*Candidatus Rickettsia tarasevichiae*" was detected from *Ixodes persulcatus* in Russia (Shpynov et al., 2004) and *R. helvetica* from *I. persulcatus* in Japan (Fournier et al., 2002; 2003). Xinjiang Uygur Autonomous Region Area is located north-west of China, and neighbors of several countries, including Russia, Kazakhstan, Kyrgyz, Tajikistan, Pakistan and Mongolia. Although the first Chinese spotted fever group *Rickettsia* was isolated from a patient in this area (Fan et al., 1987), there have been few reports on the epidemiology of *Ricketsia* infection in Xinjiang. Thus, in the present Chapter, the detection and analysis of *Rickettsia* species from ticks recovered from cattle in Xinjiang Uygur Autonomous Region Area were attempted using molecular methods including PCR screening and sequence analysis of the citrate synthase gene of *Rickettsia.*

4-2. Materials and methods

Region, animals and samples. Turpan area is located in the eastern part of

Xinjiang Uygur Autonomous Region, and one of the important cattle breeding industry count. A total of 28 ticks were recovered from 5 cattle kept on pastureland in Turpan, Xinjiang province, in July 2005. These 5 cattle were randomly selected from a herd which contained approximately 100 cattle. The cattle were usually infested with many ticks from spring to autumn. The ticks were stored in 70% ethanol for morphological identification. Histories and clinical symptoms of each animal were not recorded. DNA was successfully extracted from the ticks using a QIAamp DNA Mini Kit (QIAGEN GmbH, Hilden, Germany) with a method described previously (Inokuma et al., 2003).

PCR and gene analysis. PCR was used to detect rickettsial citrate synthase gene (*gltA*) fragments from the ticks. PCR amplification was performed in a 25μl reaction mixture containing 5μl of each DNA template with a screening primer set, RpCS.877p and RpCS.1273r, for most species of the genus *Rickettsia* (Roux et al., 1997). PCR was carried out under the following conditions: 35 cycles of denaturation (94 °C, 60 s), annealing (54°C, 60 s) and extension (72°C, 90 s) (Hiraoka et al., 2005). The PCR product was electrophoresed at 100 V in a 2% agarose gel (Wako Chemicals Ind) for 30 min, stained with ethidium bromide, and visualized by UV illumination. An approximately 500 bp PCR product was purified using the QIA PCR purification kit (QIAGEN) for direct sequence analysis with a Perkin-Elmer ABI Prism 3100 automated DNA sequencer at the National Research Center for Protozoan Diseases, Obihiro University of Agriculture and Veterinary Medicine. The sequence data of the PCR products were analyzed using the BLAST 2.0 program (National Center for Biotechnology Information) for homology search. The determined sequences were then analyzed for phylogenetic relationships with other sequences registered in GenBank. Multiple alignment analysis, the determination of pair-wise percent identities of the

sequences, distance matrix calculations and the construction of phylogenetic trees were all performed with the ClustalW program version 1.8 in the DNA data bank of Japan. Tree figures were generated using the Tree View program version 1.6.6. The GenBank accession

numbers of the gltA gene sequences of other species used to analyze the data are as follows: *R. prowazekii*, M17149; *R. japonica*, U59724; *R. akari,* U41752; *R. felis*, U33922; *R. slovaca*, U59725; *R. conorii*, U59730; *R. cadada*, U59713; *R. honei*, AF022817; *R. helvetica*,U59723; *R. australis*, U59718; *R. montana*, U74756; *R. massiliae*, U59719; *Rickettsia africaegi*, U59733; *R. hulinensis*, AF172943; *R. mongolotimonae*, U59731; *R. sibirica*, U59725; *Rickettsia parkeri*, U59732; *Rickettsia amblyommii*, AF031496; *R. heilongjiangii*,AF178034; *R. aeschlimannii*, U59722; *Rickettsia rhipicephali*, U59721; '*Candidatus Rickettsia tarasevichiae*',AF503167; '*Candidatus Rickettsia* prinicipis', AY578114. The nucleotide sequences of the four isolates of rickettsial *gltA* obtained from ticks in this study have been deposited in the GenBank database under the accession numbers DQ836217–DQ836220.

4-3. Results

Most of the 28 ticks removed from cattle were semiengorged ticks, and were morphologically identified as 13 *Dermacentor marginatus* (all females) (Fig.4), 13 *Haemaphysalis danieli* (5 males and 8 females) (Fig. 5) and 2 *Hyalomma asiaticum* (both females) (Fig. 6). These tick species were all typical species of cattle in this area (Yu et al., 1997).

Among the 28 samples of DNA extracted from the ticks, 4 *H. danieli* (Hd22, 23, 24 and 26) were positive for amplification of an approximately 500 bp PCR product. All

these positive samples were DNA samples extracted from *H. danieli* that were recovered from one cow. The nucleotide sequences of the 456 bp fragments gltA amplified for Hd 22, 23 and 24 were identical with each other, and that for Hd26 was similar to the other three, with 2 nucleotide differences among the 456 bp (identity level: 99.56%). These sequences belong to the same cluster in the phylogenetic tree of "*Candidatus Rickettsia* principis" (Fig. 7).

4-4. Discussion

The sequences of these four samples showed the highest levels of similarity (99.12–99.56%) with the registered sequence of "*Candidatus Rickettsia* principis", a new *Rickettsia* spp. detected from *Haemaphysalis japonica douglasi* in Russia (AY578114). Detailed information about this new *Rickettsia* spp. has not yet been published; however, it represents the first detection of "*C. R.* principis" from *H. danieli* in China. Because other semi-engorged tick samples from the same cow did not show any positive reaction in the PCR screening, *H. danieli* might be a potential vector of "*C. R.* principis." The rickettsial sequences detected in this study also showed high similarity with the sequences of other spotted fever group *Rickettsia* detected in China and neighboring countries, including *R. japonica* (99.09–99.54%), *R. heilongjiangii* (98.89–99.33%), *R. sibirica* (98.86–99.32%), *R. mongolotimonae* (98.86–99.32%), *R. aeschlimannii* (98.41–98.86%) and *R. hulinensis* (98.41–98.90%). The first four of these rickettsial species (*R. japonica, R.heilongjiangii, R. sibirica and R. mongolotimonae*) are known to be human pathogens.

Although the pathogenesis of the new "*C. R.* principis" has yet been clarified, attention should be paid to the possibility of human infections in this area. In the present

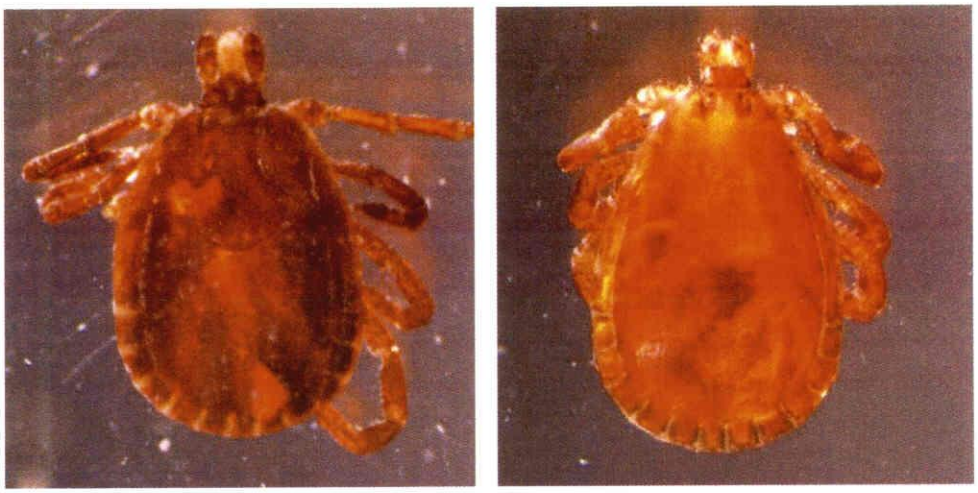
study, the cattle were not examined for rickettsial infection and, thus the role of cattle as host or reservoir animals of this new *Rickettsia* spp. is not clear at present. Further epidemiological studies will be required to clarify the relationship between the *Rickettsia* spp. and pathogenesis in both humans and animals. Reservoirs and host animals of the agent should also be clarified in further studies.

4-5. Summary

Ticks which were recovered from cattle kept on pastureland in Xinjiang Uygur Autonomous Region were examined for *Rickettsia* infections. A total of 28 ticks were collected from 5 cattle. Then, these tick samples were examined for *Rickettsia* infection by using citrate synthase gene-based PCR and nucleotide sequencing. A specific band with an approximately 500 bp was detected from the four *Haemaphysalis danieli* tick samples. The DNA sequences from these 4 samples shared high nucleotide similarity (99.12–99.56%) with the recently detected *"Candidatus* Rickettsia principis", which is a novel *Rickettsia* species found in Heilongjiang province of China. Therefore, the data obtained from this study could be used to provide future estimates of the likelihood of infections by *Rickettsia* organisms in Xinjiang Uygur Autonomous Region, China.

D. marginatus ♂

Fig. 4. *Dermacentor marginatus* collected from a pasturing cattle in Xinjiang, China.



Hae. danieli ♀ *Hae. danieli* ♂

Fig. 5. *Haemaphysalis danieli* collected from a pasturing cattle in Xinjiang, China.

Hy. asiaticum ♀ *Hy. asiaticum* ♂

Fig. 6. *Hyalomma asiaticum* collected from a pasturing cattle in Xinjiang, China.

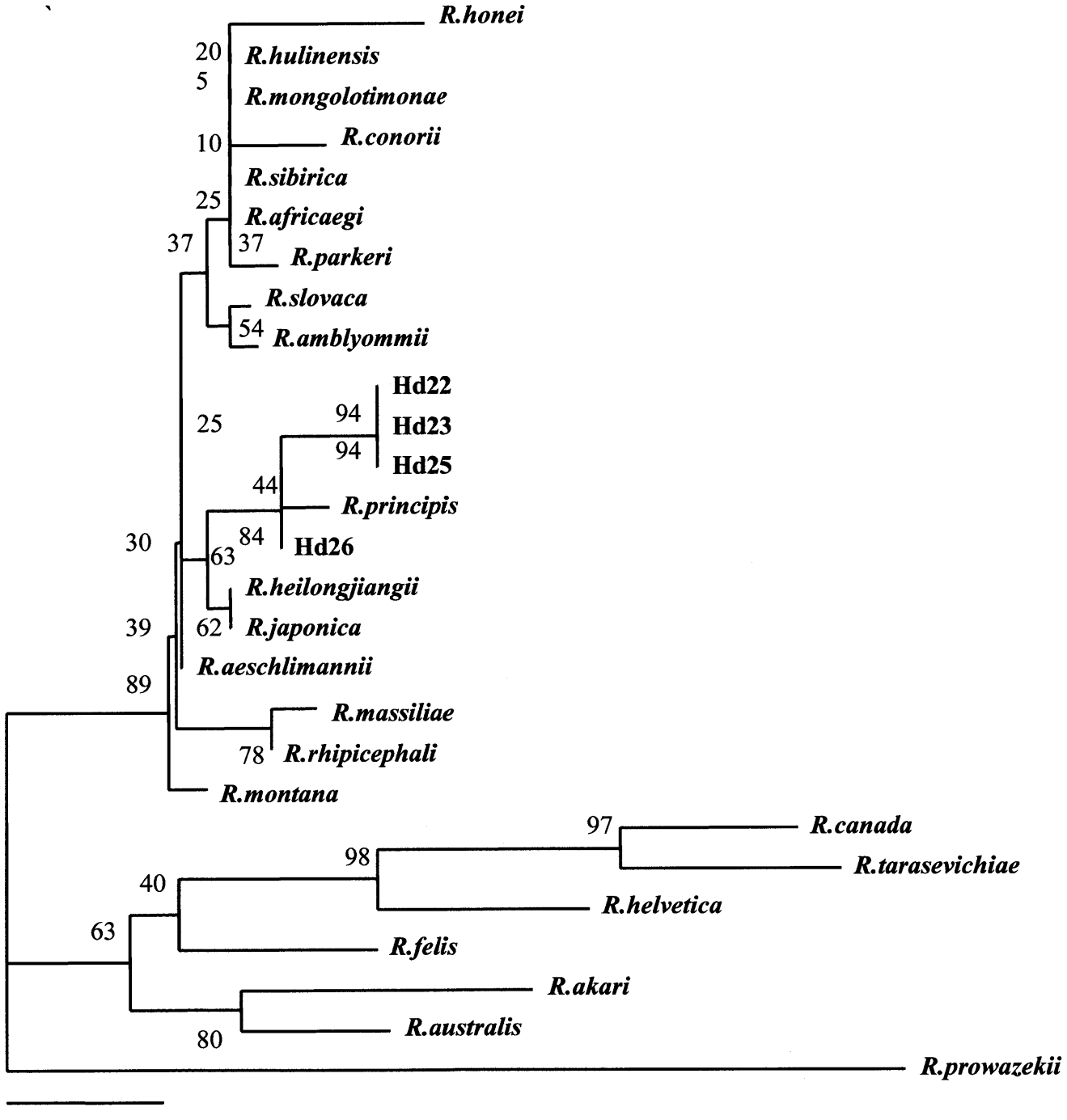


Fig.7. Phylogenetic relationship of various *Rickettsia* spp. based on the nucleotide sequences of the citrate synthase gene. The numbers at nodes are the proportions of 100 bootstrap resamplings that support the topology shown. The scale bar represents 1% divergence. The bacteria detected in this work are highlighted.

General discussion

The Xinjiang Uygur Autonomous Region is one of the important regions and is the largest province in China constituting one-sixth of the whole country. The population of major livestock is approximately 53807000 in Xinjiang, thus making livestock farming one of the major economic activities. The region is geographically located in the center of the Eurasian and is neighbored by Mongolia, Russia, India, Pakistan and other Central Asian countries.

Ticks cause great economic losses to livestock, and adversely affect livestock hosts in several ways, including "tick worry", and loss of blood, when the ticks bite. Additionally, ticks reduce the quality of hides, cause tick-bite paralysis as well as tick toxicosis and play a major influence on the kinetics of transmission of tick-borne diseases (Leviene 1971; Sonenshine, 1991, 1993). The economically most important ixodid ticks of livestock in Xinjiang region belong to the genera *Dermacentor.*, *Hyalomma.*, *Boophilus.*, *Rhipicephalus* ., *Haemaphysalis.*, and *Ixodes*. The harmful effects of ticks are most common as from late winter until autumn when the adult ticks are active, but it can occur at any time if the weather is warm and humid (Kong, 1983; Lu et al., 1997; Yu et al., 1997; Wang et al., 1997). However, major losses caused by the ticks are due to their ability to transmit the various species of *Babesia, Theileria, Ehrlichia, Anaplasma,* and *Rickettsia*, to livestock. (Ai et al., 1979; Bai et al.,1987; Lu et al., 1992; Yu et al., 1996; Wang et al., 1997; Cao et al., 2003; Xiang et al., 2005; Zhang et al., 2006). Livestock are of great economic importance in Xinjinag Uygur Autonomous Region. There are quite a few methods for controlling ticks, but every method has certain shortcomings (Tellam et al., 1992; George, 2000; Rajput et al.,

2006).

In this study, novel advanced diagnostics technology was used to investigate the epidemiology of tick-borne disease, such as equine babesiosis by the ELISA method based on recombination Bc48 protein, recombinant EMA-1 and EMA-t-2. Furthermore, I have used the IFATfor detection of antibodies against *Anaplasma* and *Ehrlichia* in domestic animals. Additionally, molecular based PCR method was used in this study to detect the DNA of R*ickettsia* in cattle infected by the parasite by specific primers to ampliy specific sequences of certain genes.

In Chapters 1 and 2, I used the recombinant EMA-1 and EMA2-t and recombinant Bc48 protein based ELISAs to investigate the prevalence of equine babesiosis in horses and donkeys in western Xinjiang of China. To investigate the prevalence of *B. equi* and *B. caballi* infections, sera were collected from horses and donkeys in Kashgar, Ili of Xinjing Uygur Autonomous Region in China. The tests used for the diagnosis of equine piroplasmosis should be sensitive enough to detect early acute infection and latent infections. Moreover, the test should be specific enough to differentiate between the two parasite species, and economical with regards to materials and time (Bruning, 1996). So far, no immunological assays using the native antigens have met the standards due to the nonspecific reaction and difficulty in preparing the antigen as well as the limitations of the available serological tests such as CFT and IFAT (McGuire et al., 1971; Knowles et al., 1991; Bruning, 1996). Recently, rEMA-1, EMA-2 and Bc48 proteins, expressed in *E. coli* and a baculovirus-insect cell system, have been used to improve the sensitivity and specificity of ELISA (Weiland, 1986; Tanaka et al; 1999; Xuan et al., 2001b). Several recombinant parasite proteins are already available and can be used for the establishment of ELISA (Ikadai et al., 1999; Xuan et al., 2001a, 2001b; Hirata et al.,

2002, 2005; Huang et al., 2003, 2004, 2006) for detection of antibodies to *Babesia* parasites in horses.

There are only a few previous reports on the prevalence of equine babesiosis in horses in China (Yin et al., 1997). Several reports on the prevalence of equine babesiosis in Mongolia, have been published (Dash, 1967; Bose et al., 1994; Avarzed et al., 1997, 1998; Xuan et al., 1998; Ikadai et al., 2000; Boldbaater et al., 2005; Battseteg et al., 2001, 2007). These studies demonstrated that both *B. equi* and *B. caballi* infections in horses are widespread in Mongolia. Recently, Xu et al (2003) have reported high prevalence of equine piroplasmosis in Jilin province northeast of China.

I have demonstrated for the first time in Chapter 2, that seropositive rate of *B. equi* infection (9.7%) is lower than that of *B. caballi* infection (38.7%) in donkeys in western Xinjiang Uygur Autonomous Region. Usually *B. equi* infection is more prevalent than *B. caballi* infection in horses (Schein, 1988). However, the results of Chapter 2 contradict the other previous reports, and the reason remains unknown.

All the 70 horses and 93 donkeys examined, in Chapter 1 and Chapter 2, were raised locally, suggesting that the equine babesiosis is endemic among the local horse and donkey populations. However, more formally designed epidemiological studies are required to define dynamics of the infections and the role played by possible vectors. In addition, no sero-positive horses and donkeys showed any significant clinical signs. Since these donkeys and horses are constantly exposured to *B. equi* and *B. caballi* infections, the animals might harbor the pathogens resulting in comparatively high-acquired immune protection. Additionally, the rEMA-1, rEMA-2 and rBc48 antigens, were produced using the genes cloned from *B. equi* USDA strain and *B. caballi* USDA strain, both isolated in the USA. It is not clear whether the recombinant

antigens reacted with anti-EMA-1, anti-EMA-2 and anti- Bc48 antibodies raised in a horse and donkeys in Xinjiang Uygur Autonomous Region. There may be some geographical diversity together with an associated loss or gain of epitopes on the EMA-1/ EMA-2 or Bc48 between the USDA strains and Xinjiang isolates. Therefore, there is a need to compare the EMA-1/ EMA-2 and Bc48 genes of the USDA strains with the Xinjiang isolates in future.

In Chapter 3, I determined the prevalence of *Anaplasma* and *Ehrlichia* infections in domestic animals in Xinjiang of China. Blood samples were collected from Altai, Ili and Kashgar including different areas of Xinjiang province, and then used to detect antibodies against *Anaplasma* and *Ehrlichia* organisms by using IFAT. *A. phagocytophilum* and *E. chaffeensis* were detected mainly in ruminants and the antibody titer was 1: 320. High antibody titers against *A. phagocytophilum* and *E. chaffeensis* were observed in cattle from Altai and sheep, respectively. This may reflect the differences of location where the examined animals were kept. The only positive serum of donkey was obtained from an animal in Kashgar and the titer against *A. phagocytophilum* was 1:40. Thus, tick infestation of horses and donkeys is less likely than in ruminants. The results obtained constitute the first serological evidence of infections by both *A. phagocytophilum* and *E. chaffeensis* in domestic animals in Xinjiang Uygur Autonomous Region.

The detection of any of the *Anaplasma* and *Ehrlichia* infections in domestic animals has not been reported in this area previously. It is well known that *A. phagocytophilum* and *E. chaffeensis* infect domestic animals and human (Inokuma et al., 2001; Zhang et al., 2008), and that the two organisms are transmitted by *I. persulcatus*, *D. silvarum*, *H. concina*, *A. testudinarium*, *B. microplus* and *D. nutalli* ticks in China (Cao et al., 2003,

2008 ; Liu et al., 2005). *A. phagocytophilum* has been found in *H. longicornis* ticks from Korea (Kim et al., 2003) and the mainland of Japan (Kawahara et al., 2006; Naitou et al., 2006). *A. phagocytophilum* is more closely related to *A. bovis* than to *A. marginale* or *A. centrale* (Lew et al., 2003; Ooshiro et al., 2008). Moreover, other two possible infectious agents of cattle, *A. bovis* and *A. phagocytophilum* have recently been detected from Ixodid ticks and sika deer in the mainland and in Hokkaido, which is the northern island of Japan (Kawahara et al., 2006; Jilintai et al., 2008). Recently, new ehrlichia species including the *E. muris* (Wen et al., 1995; Roux et al 1997; Shibata et al., 2000; Shpynov et al., 2004) and the *E. ewingii* (Wen et al., 2002; Parola et al., 2003; Inokuma et al., 2004) have been detected in China by using molecular methods. It is possible that domestic animals in Xinjiang are infected with some new *Ehrlichia* pathogens and thus, showed positive antibodies against *A. phagocytophilum* and *E. chaffeensis*.

In Chapter 4, the DNA samples of *D. marginatus*, *H. danieli*, and *H. asiaticum* from cattle in Xinjiang Uygur Autonomous Region Area of China, were examined for *Rickettsia* infection by citrate synthase gene-based PCR and sequencing. The rickettsial sequences showed high similarity with the sequences of the other *Rickettsia* spp., detected in China and neighboring countries, which include *R. japonica* (99.09–99.54%), *R. heilongjiangii* (98.89–99.33%), *R. sibirica* (98.86–99.32%), *R. mongolotimonae* (98.86–99.32%), *R. aeschlimannii* (98.41–98.86%) and *R. hulinensis* (98.41–98.90%). The first four of these rickettsial species (*R. japonica, R.heilongjiangii, R. sibirica and R. mongolotimonae*) are known to be human pathogens. Zhang et al. (2008) reported high seroprevalence rates for *Anaplasma phagocytophilum*, *Coxiella burnetii*, *Bartonella henselae*, and *Rickettsia typhi* in farm workers near Tianjin, China.

Although the pathogenesis of the new "*C. R.* principis" has not yet been clarified, the cattle were not examined for rickettsial infection and, thus the role of cattle as host or reservoir animals of this new *Rickettsia* spp. is not clear presently. Therefore, attention should be paid to the possibility of human infections in this area. According to my knowledge and with the exception of a few reports based on clinical evidence (Lu et al., 1992; Wang et al., 1997), this is the first finding, published in international journal of equine babesiosis in horses in Xinjaing, about the prevalence of equine babesiosis in donkeys in Xinjaing of China. Additionally, I report the prevalence of *A. phagocytophilum* as well as *E. chaffeensis* in domestic animals and the detection of "*C. R.* principis" from *H. danieli* in Xinjiang of China.

Xinjiang Uygur Autonomous Region is geographically remote and a big area with a large number of livestock being kept in the vast grassland. It is difficult to know clearly the prevalence of each tick-borne disease in this area, which is usually infested with many ticks from spring to autumn. The ticks may transmit the *Babesia*, *Rickettsia* and *Ehrlichial* pathogens to animals at any time during these seasons. However, the tick vectors of major tick-borne desease in Xinjiang Uygur Autonomous Region are still not well known to date. Additionally, there are widely distributed strains of pathogens that have not been isolated and characterized. Therefore, there is need for more epidemiological studies to clarify the prevalence of the major tick-borne disease in Xinjiang Uygur Autonomous Region. Moreover, the isolation and characterization of the pathogens will be required in the next step of this study.

As previously stated in the introduction, development of improved diagnostic methods is very important for the detection and the prevention of tick-borne disease in domestic animals. To prevent the infections by the tick-borne pathogens, it is impotant

to control the tick vectors and identify previous pathogens in order to improve the quality of domestic animals populations in endemic areas. Molecular Immunological techniques will be applied to study the endemic tick-borne diseases, and to develop reliable, more sensitive and more specific diagnostic methods so that antibodies against the major tick-borne diseases could be detected in livestock kept in Xinjiang Uygur Autonomous Region.

General summary

Xinjiang Uygur Autonomous Region is the largest province in China having vast and abundant natural grassland environment. This environment is good for breeding and, thus, many domestic animals are kept here including sheep, cattle, goats, horses, donkeys, camel and other grazing livestock. The animal husbandry system mainly relies on natural pasture for grazing the animals. The vegetation of the Xinjiang Uygur Autonomous Region is heavily infested by ticks, which transmit pathogens such as protozoa and *Rickettsia* to livestock, resulting in heavy economic losses in this region. Currently, the pathogenic organisms causing the tick-borne diseases in the Xinjiang Uygur Autonomous Region have been identified mainly by their morphological features in blood cells. However, these tick-borne diseases, which are prevalent in this area, have never been identified by using molecular biological methods. Furthermore, there is no data on the epidemiological studies of the tick-borne disease in some of the epidemic regions of the Xinjiang Uygur Autonomous Region. The aims of this study is to clarify the prevalence of the tick-borne diseases including Babesiosis, Erlichioses, Anaplasmosis and Rickettsiosis, and in particular to detect *B. equi*, *B. cabalii*, *A. phagocytophilum*, *E. chaffeensis* and some *Rickettsia* in domestic animals in Xinjiang Uygur Autonomous Region, China, by using serological and molecular methods.

In Chapter 1, the prevalence of equine piroplasmosis in the Xinjiang Uygur Autonomous Region was examined by enzyme-linked immunosorbent assays (ELISAs). A total of 70 serum samples were collected from horses pastured on three farms in Western Xinjiang, and examined for antibodies against *B. equi* and *B. caballi* by using ELISA based on *B. equi* merozoite antigen 1and *B.caballi* Bc48 recombinant antigens,

respectively. Of the 70 samples, 28 (40.0%) were seropositive for by *B. equi* while 17 (24.3%) were positive for *B. caballi.* In addition, 11 (15.7%) samples were positive for both *B. equi* and *B. caballi.* These results indicate that equine piroplasmosis is widespread, and therefore is a cause for serious concern in Western Xinjiang.

In Chapter 2, the prevalence of equine babesioses in donkeys in Xinjiang Uygur Autonomous Region was investigated. In total, 93 serum samples were randomly collected from donkeys in the Kashgar and Ili areas, and examined for antibodies against *B. equi* and *B. caballi* by ELISAs which is using recombinant antigens of *B. equi* merozoite antigen 2 truancate and *B.caballi* Bc48 recombinant antigens. Out of the 93 samples, 9 (9.7%) were positive for *B. equi* while 36 (38.7%) samples were positive for *B. caballi.* In addition, 2 (2.2%) samples were positive for both *B. equi* and *B. caballi.* These results indicate that equine babesiosis might be extensively prevalent in donkeys in Western Xinjiang.

In Chapter 3, serological methods were utilized to detect *Anaplasma* and *Ehrlichia* infection in domestic animals in Xinjiang Uygur Autonomous Region. Serum samples were collected from cattle, sheep, goats, horses and donkeys in Altai, Ili, and Kashgar areas. These samples were analyzed by using an indirect immunofluorescence assay to screen for antibodies against *A. phagocytophilum* and *E. chaffeensis*, which infect ruminants. Antibodies screened by IFAT showed 5.5% percent of the samples were positive for *A. phagopcytolphilum* antibodies in cattle while 17% were seropositive for the antibodies in sheep. Moreover, 8.9 % of samples were positive for the *E. chaffeensis* antibodies in cattle while 15.7% percent were positive for the pathogen antibodies in sheep. In contrast, goats, donkeys and horses examined in Altai erea were all negative for the antibodies against the pathogens. These results indicated that cattle and sheep

could be infected with some the species of *Anaplasma* and *Ehrlichia* in Xinjiang Uygur Autonomous Region, China.

In Chapter 4, ticks which were recovered from cattle kept on pastureland in Xinjiang Uygur Autonomous Region were examined for *Rickettsia* infections. A total of 28 ticks were collected from five cattle. Then, these tick samples were examined for *Rickettsia* infection by using citrate synthase gene-based PCR and nucleotide sequencing. A specific band with an approximately 500 bp was detected from the four *Haemaphysalis danieli* tick samples. The DNA sequences from these 4 samples shared high nucleotide identity (99.12–99.56%) with the recently detected "*Candidatus* Rickettsia principis", which is a novel *Rickettsia* species found in Heilongjiang province of China. Therefore, the data obtained from this study could be used to provide future estimates of the likelihood of infections by *Rickettsia* organisms in Xinjiang Uygur Autonomous Region, China.

The serological and molecular diagnostic methods used in this study could contribute to the understanding of the epidemiology of the major tick-borne disease in domestic animals. Furthermore, the findings indicate that equine babesiosis might be extensively prevalent in donkeys and horses in Western Xinjiang. Cattle and sheep are also infected with some the species of *Anaplasma* and *Ehrlichia*; moreover, "*Candidatus* Rickettsia principis" was detected in the ticks recovered from cattle grazed on pastureland in Xinjiang Uygur Autonomous Region. The results of this study have suggested that *B. caballi*, *B. equi*, *A. phagocytophilum*, *E. chaffeensis* and some *Rickettsia* infections in Xinjiang Uygur Autonomous Region are maintained in domestic animals, with ticks serving as biological and mechanical vectors of the pathogens, respectively. Infections with these pathogens in donkeys, horses, cattle, sheep and goats

are most likely transmitted by several tick species with unknown role as reservoir hosts in other wild and domesticated mammals. The present results confirm the utility of the molecular and the serological assays as valuable epidemiological tools. This study provides the first report on epidemiology of tick-borne diseases in domestic animals, and provides additional information on the prevalence of the major tick-borne diseases in Xinjiang Uygur Autonomous Region, China, thus, it will assist in developing strategies for controlling the diseases.

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