Tickborne Rickettsial Diseases: Epidemiological studies in China

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Chapter 1

General introduction

Tickborne rickettsial diseases: pathogens, vectors, animal hosts and the diseases

Rickettsial diseases are vector-borne zoonoses caused by obligate intracellular bacteria within the order *Rickettsiales*, which was previously described as short, Gram-negative rod bacteria that retained basic fuchsin when stained by the method of Gimenez. As development in molecular technologies, the taxonomy of the fastidious bacterial species in the order *Rickettsiales* has been modified (Dumler *et al*. 2001), and certain agents such as *Coxiella burnetii*, the pathogen of Q fever have recently been removed from this order (Raoult & Roux 1997). Although specialists in the field of rickettsiology frequently disagree over species definitions, the taxa as well as names of species or subspecies based on polyphasic taxonomic studies by integrating phenotypic and phylogenetic data (Fournier *et al*. 2003) are currently accepted and used in this thesis. Three groups of diseases are usually classified as rickettsial diseases. These include (i) rickettsioses caused by the spotted fever group (SFG) and the typhus group rickettsiae of the genus *Rickettsia* within the family Rickettsiaceae, (ii) ehrlichioses and anaplasmoses due to microorganisms within the family Anaplasmataceae, and (iii) scrub typhus caused by *Orientia tsutsugamushi* (Raoult & Roux 1997; Dumler *et al*. 2001; Hechemy *et al*. 2003; Watt & Parola 2003). Among these rickettsial diseases, scrub typhus is transmitted by trombiculid mites (Watt & Parola 2003), and cat flea typhus (also called flea-borne spotted fever) due to *Rickettsia felis* is transmitted by flea (Adams *et al*. 1990; Higgins *et al*. 1996). Tickborne rickettsial diseases are caused by two groups of intracellular bacteria belonging to the order *Rickettsiales*, *i.e.* the SFG of the genus *Rickettsia* within the family Rickettsiaceae and several genera of *Anaplasma* and *Ehrlichia* groups within the family Anaplasmataceae. These pathogens infect and proliferate in the organs of ticks, particularly in the salivary glands, and can be transmitted to animal hosts during feeding (Parola & Raoult 2001). Because each tick species favours particular optimal environmental conditions and biotopes, the geographic distribution of the ticks is usually restricted to a specific area (small or large) and tickborne rickettsial diseases are natural focus diseases. This is particularly true for most of the spotted fever rickettsiae, which are maintained in ticks through transstadial (from larvae to nymphs to adults) and transovarial (from one generation to the next through ovaries) transmissions (Raoult & Roux 1997).

Ticks are not insects but Arachnids, a class of Arthropods, which also includes mites, spiders and scorpions. They are divided into two groups – hard bodied and soft bodied – both of which are capable of transmitting diseases. Many ticks have four

stages in their life cycle (Figure 1), *i.e.* egg, the 6-legged larva, and 8-legged nymph and adult (male or female). After the eggs hatch, each stage of tick must digest a blood meal to develop into the next stage. Each of the three active stages usually feed on a different individual host animal, taking a single blood meal. Larvae generally feed on small animals, drop to the ground and molt to nymphs. The nymphs often feed on small to media-sized hosts, engorge and molt to adults. The adult ticks usually feed on larger animals. A replete or engorged female tick will produce a large batch of eggs and then die. Depending upon the species of tick, egg mass deposited range roughly from 1,000 to 18,000 eggs. Some ticks may have one-host (all stages staying and feeding on only one animal host before the female drops off) or other multi-host lifecycles. Soft ticks have a multi-host life cycle with multiple nymphal stages; and adults take multiple small blood meals, laying small egg batches after each feeding (Stanford III 2007).

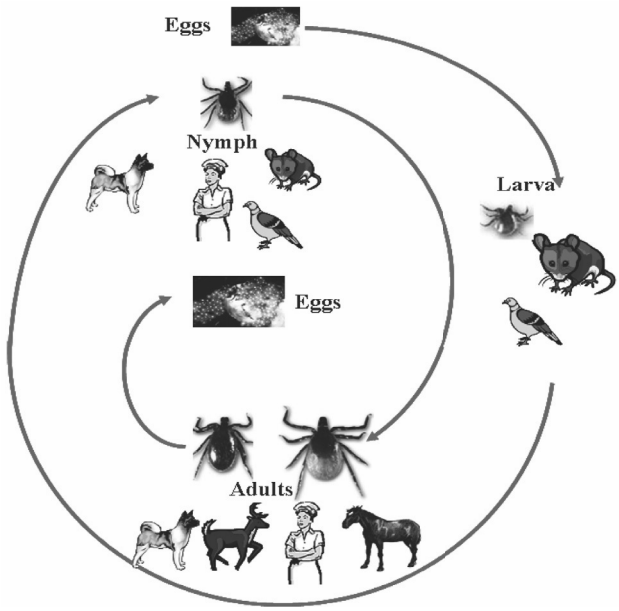

Figure 1. Tick life cycle

Ticks, like many mite species, are obligate blood-feeders, requiring a host animal for food and development. Ticks are parasites that feed by latching on to an animal host, imbedding their mouthparts into the host's skin and sucking its blood. This method of

feeding makes ticks the perfect vectors (organisms that harbor and transmit disease) for a variety of pathogenic agents. Ticks are able to maintain agents in natural cycles involving ticks and animal reservoirs. Ticks become infected while feeding on blood from an animal infected with a *Rickettsia*. While feeding, they can transmit infections to humans and animals (Parola & Raoult 2001). After the tick develops into the next stage, the infection may be transmitted to humans or other animals during the feeding process (Figure 1). Both male and female ticks may bite humans but it is the females that are responsible for most transmission.

Hard ticks were first considered as vectors of SFG rickettsioses in 1906, when the Rocky Mountain wood tick (*Dermacentor andersoni*) was discovered to transmit *Rickettsia rickettsii*, the agent of Rocky Mountain spotted fever in the USA (Ricketts 1906; Wolbach 1919). The public health significance of ticks was recognized with the identification of *Borrelia burgdorferi* as the pathogen of Lyme disease in the 1980s (Hengge *et al*. 2003). However, ticks are known to be vectors of various zoonoses caused by bacteria (rickettsial diseases, recurrent fever borreliosis, tularemia and maybe Q fever) (Parola & Raoult 2001), virus (Dumpis *et al*. 1999), protozoa (Homer *et al*. 2000; Herwaldt 2003), as well as toxaemic diseases (Greenstein 2002).

Tickborne rickettsial diseases are a set of distinct diseases with similar signs and symptoms that are transmitted to humans by tick bites. *R. rickettsii* had been considered as the only agent of tick-borne rickettsioses in America throughout the 20th century. Although other SFG rickettsiae were detected from ticks there, they were considered to be "non-pathogenic" (Raoult & Roux 1997; Raoult 2002). In other continents, various tick-borne *Rickettsia* species were found to be pathogenic to humans, including *R. conorii* in Europe and Africa, *R. sibirica* in the former Soviet Union and China, and *R. australis* in Australia (Raoult & Roux 1997). In the past two decades, nine novel species or subspecies of tick-borne SFG rickettsiae were recognized as emerging pathogens throughout the world (Raoult & Roux 1997; Raoult 2002).

The causative agents of ehrlichioses and anaplasmoses are maintained through enzootic cycles between ticks and animals, and have long been considered to be only of veterinary importance (Rikihisa 1991). However, in recent years, three species have been identified as emerging tick-borne pathogens in humans: (i) human monocytic ehrlichiosis (HME) due to *Ehrlichia chaffeensis*, (ii) human granulocytic anaplasmosis (HGA) (formerly human granulocytic ehrlichiosis) due to *Anaplasma phagocytophilum*, and (iii) granulocytotropic ehrlichiosis due to *E. ewingii* (Dumler *et al*. 2001). The first HME case was diagnosed in 1987 in the USA and initially assumed to be caused by *E. canis*, the agent of canine monocytic ehrlichiosis (Maeda 1987). The causative agent

of HME was isolated in 1991 from a patient residing in Chaffeen town and nominated as *E. chaffeensis* (Dawson *et al*. 1991). In 1994, human granulocytic ehrlichiosis was first reported in the USA (Chen *et al*. 1994). The causative agent, first known as the "HGE agent", had been found to be closely related to *E. equi* and *E. phagocytophila* (pathogens of horses and ruminants, respectively). Systematic phylogenic analyses revealed that differences among the three agents were insufficient to support separate species designations, therefore these agents are grouped as a single species, i.e. *A. phagocytophilum* (Dumler *et al*. 2001), and the disease has now been renamed as HGA. Finally, *E. ewingii*, the agent of canine granulocytic ehrlichiosis, was found in 1999 to cause disease in humans (Buller *et al*. 1999), and reported primarily in immunocompromised patients (Chapman *et al.* 2006).

Epidemiology and public health significances in China

In China, five species of tick-transmitted SFG rickettsiae were previously reported, three of which have been known to cause human diseases (reviewed in Fan *et al*. 1999). *R. sibirica,* the pathogen of Siberian tick typhus, transmitted by *Dermacentor spp*. is frequently encountered especially in the northern China. Another SFG *Rickettsia* was first identified from *Hyalomma asiaticum* in Inner Mongolia, and isolated from the blood and skin of a patient in Marseille, France. Therefore, the agent was named as *R. mongolotimona* (Raoult *et al.* 1996). "*R. heilongjiangiensis*" initially isolated from *D. silvarum* in Heilongjiang Province was then found in patients from Jilin and Heilongjiang provinces (Lou *et al*. 1989, Zhang *et al*. 2000a). In addition to the 3 human pathogens, some rickettsiae of unknown pathogenicity were recognized from ticks, including "*R. hulinii*" from *Haemaphysalis concinna* (Zhang *et al*. 2000a) and BJ-90 strain from *D. sinicus* (Zhang *et al*. 2000b).

HME due to *E. chaffeensis*, HGA due to *A. phagocytophilum*, and granulocytotropic ehrlichiosis due to *E. ewingii* (Dumler *et al*. 2001) are emerging tick-transmitted rickettsial and ehrlichial infections that were first recognized in the United States (Walker *et al*. 2008). Serological and molecular evidence suggests that human infection exists in Asia such as Korea, Japan, and China (Li *et al*. 1993; Heo *et al*. 2002; Chae *et al*. 2003; Park *et al*. 2003; Wen *et al*. 2003; Ohashi *et al*. 2005).

Tick-borne rickettsial diseases have been relatively neglected by health care and infectious disease physicians, all of which are remarkable for their uniform susceptibility to doxycycline but are clinically difficult to distinguish from viral infections

and each other (Walker *et al*. 2008). Therefore, only a limited number of laboratory-confirmed cases have been reported, mostly from countries in Europe and North America, although these tick-borne pathogens are globally distributed (Dumler 2005). In China, only sporadic Siberian tick typhus cases caused by *R. sibirica* (Fan *et al*. 1999) and a cluster of 8 HGA cases occurring in a hospital (Zhang *et al.* 2008) have been diagnosed. However, tens of thousands of patients with unexplained febrile illnesses are observed during the peak period of tick activity. The data on the clinical characteristics, exposure history to ticks and presumptive therapy strongly suggests that at least some of the patients are infected by tickborne rickettsial pathogens. For the time being, the presence and geographical distribution of tickborne rickettsial agents, especially the emerging ehrlichiae and anaplasmae, have not been investigated. Lack of such knowledge has inhibited us to understand the ecology, epidemiology and potential threats of the pathogens to human health. Furthermore, given the existence of rickettsial pathogens in mainland China, it is unclear if the agents are genetically similar to the strains or variants found in other countries.

Background and research questions

Ticks, which maintain the microorganisms in a natural cycle through transovarial and transstadial passage, act as both reservoirs and vectors of tickborne rickettsial pathogens. While feeding, they can transmit rickettsial infections to humans and animals (Parola and Raoult 2001). The different agents are usually associated with specific tick species in certain endemic areas. Small wild animals, the common hosts of immature stages of ticks, are implicated as natural reservoirs for many tickborne agents in various continents (Telford *et al*. 1996; Bown *et al*. 2003). Moreover, a single tick bite could transmit more than one agent and lead to the development of a single tickborne disease or a coinfection (Parola *et al*. 2005). Patients coinfected with two tickborne pathogens usually show more severe clinical manifestations and experience longer duration. Coinfection often leads to variations in clinical manifestations in humans and animals as a consequence of tick bites.

The specific research questions of the thesis are as follows:

1. Do *E. chaffeensis* and *A. phagocytophila*, the emerging human pathogens of public health significance, exist in mainland China?
2. What are the prevalences of tickborne ricketsial infections in mainland China?
3. Which species of ticks and rodents are naturally infected by rickettsial agents?

4. What are the genetic characteristics of the tickborne agents detected in mainland China?
5. What are the co-infection rates of tickborne agents in ticks and animal hosts?

Structure of the thesis

To determine the presence of tick-borne rickettsial infections in mainland China, epidemiological surveys on various rickettsial agents in ticks were carried out in different geographical locations. **Chapter 2** reports the molecular detection of spotted fever group Rickettsia in D. silvarum, in an area where Siberian tick typhus is endemic. In the survey, a Rickettsia variant (JL-02) was discovered. **Chapter 3** provides the evidence of *A. phagocytophilum* infection in *I. persulcatus* from a forest area of northeastern China, where Lyme disease is known to be endemic. **Chapter 4** presents the identification of E. chaffeensis, the causative agent of HME, by nested PCR in adult and nymphal ticks collected from three provinces in southern China during the period of 1996 to 1998.

In this thesis, findings of tickborne rickettsial infections in animal hosts are presented. **Chapter 5** gives findings of a field survey on the natural infection of *A. phagocytophilum* in both ticks and rodents from a forest area of Jilin Province. **Chapter 6** reports the identification of a novel *A. phagocytophilum* variant in rodents from southeastern China. **Chapter 7** records the isolation of A. phagocytophilum from rodents and sheep in the same area through propagation in Balb/c mice followed by cell culture.

In addition to the infection of a single rickettsial agent, co-infections with two or more tickborne microorganisms in either ticks or animals are investigated. Co-infection with both *A. phagocytophila* and *Borrelia burgdorferi* were observed in *I. persulcatus* ticks from northeastern China (**Chapter 8**). In a comprehensive survey, *A. phagocytophilum*, *B. burgdorferi*, SFG *Rickettsia* and *Francisella tularensis* were detected in diverse species of rodents collected from different areas of China, and 10 rodents species were found to be positive for 2 or 3 agents (**Chapter 9**).

In the general discussion (**Chapter 10**), concise answers are provided to the questions posed above. Topics remaining to be investigated in the future are indicated in the end of each answer. Finally, conclusions and recommendations are made.

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Chapter 2

Molecular Detection of Spotted Fever Group *Rickettsia* in *Dermacentor silvarum* from a Forest Area of Northeastern China

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Molecular detection of spotted fever group *Rickettsia* in *Dermacentor silvarum* from a forest area of northeastern China.

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Abstract

In total, 676 *Dermacentor silvarum Olenev (Acari: Ixodidae)* from a forest area of Jilin Province in northeastern China were examined by PCR for the presence of spotted fever group (SFG) *Rickettsia*. The overall positive rate was 10.7% with the 95% *CI* from 8.3% to 13.0%. The SFG *Rickettsia* infection was more prevalent in adults than in nymphs ($\chi^2 = 9.17$, $P = 0.002$), and in fed ticks obtained from domestic animals than in those collected on vegetation ($\chi^2 = 12.86$, $P < 0.001$). Sequence analysis of the partial *ompA* gene confirmed the existence of *R. sibirica* and discovered a novel rickettsial agent in this area, the sequence of which was identical to that of DnS14 genotype *Rickettsia* previously reported in the former Soviet Union.

Introduction

Spotted fever group (SFG) rickettsiae are obligately intracellular gram-negative bacteria associated with arthropods. Ticks, which maintain the microorganisms in a natural cycle through transovarial and transstadial passage, act as both reservoirs and vectors. While feeding, they can transmit SFG infections to humans and animals (Parola and Raoult 2001). In China, five species of tick-transmitted SFG rickettsiae were previously reported, three of which have been known to cause human diseases (reviewed in Fan *et al*. 1999). *R. sibirica,* the pathogen of Siberian tick typhus, transmitted by *Dermacentor spp*. is frequently encountered especially in the northern China. Another SFG *Rickettsia* was first identified from *Hyalomma asiaticum* in Inner Mongolia, and isolated from the blood and skin of a patient in Marseille, France. Therefore, the agent was named as *R. mongolotimona* (Raoult *et al*. 1996). "*R. heilongjiangiensis*" initially isolated from *D. silvarum* in Heilongjiang Province was then found in patients from Jilin and Heilongjiang provinces (Lou *et al*. 1989, Zhang *et al.* 2000a). In addition to the 3 human pathogens, some rickettsiae of unknown pathogenicity were recognized from ticks, including "*R. hulinii*" from *Haemaphysalis concinna* (Zhang *et al*. 2000a) and BJ-90 strain from *D. sinicus* (Zhang *et al*. 2000b).

Detection and identification of the tick-borne SFG rickettsiae before 1990s mainly depended on culture and epitope recognition techniques, such as immunoflourescence and agglutination tests as well as serotyping with monoclonal antibodies. Recently, new molecular techniques have enabled the development of useful, sensitive, and rapid tools to detect agents in ticks (Sparagano *et al*. 1999). Sequence comparison of the gene encoding outer membrane protein A (*omp*A) (Fournier *et al*. 1998) is a currently accepted method for recognizing rickettsiae (Billings *et al*. 1998, Marquez *et al*. 1998, Weller *et al*. 1998, Rydkina *et al*. 1999, Ammerman *et al*. 2004). The molecular-sequence-based identification techniques facilitate genetic characterization without need for bacterial culture. In the present study, polymerase chain reaction (PCR) and sequence analysis of amplified products were carried out to identify rickettsiae in *D. silvarum* from a forest area of Jilin Province in northeastern China.

Materials and Methods

Tick collection

D. silvarum were collected in 2005 in the hinterland of Changbai Mountains situated at 42° – 43° north latitude and 126° – 128° east longitude within Jingyu county, Jilin province. The terrain consists of forested rolling hills with an average elevation of 775 m. The annual precipitation is about 800 mm, and the relative humidity is around 70%. The temperature ranges from -41.4°C to 33.6°C, with the average of 4.0°C. Questing ticks were collected by dragging a standard 1 m^2 flannel flag over vegetation. Fed ticks were collected from domestic animals including cattle, sheep and dogs. Ticks were identified by an entomologist to the species level and the development stage, and were kept alive in a refrigerator until DNA extraction was performed.

DNA extraction

The ticks to be tested were soaked in 70% ethanol for 15 min, and then rinsed three times in sterile water. DNA was extracted by a method previously described (Cao *et al*. 2000). Briefly, the ticks were individually placed into micro-tubes and mechanically crushed with sterile scissors in 50 µl DNA extraction buffer (10 mM Tris pH 8.0, 2 mM EDTA, 0.1% sodium dodecyl sulfate, 500 µg of proteinase K per ml). The samples were incubated for 2 h at 56°C, and then boiled at 100°C for 10 min to inactivate proteinase K. After centrifugation, the supernatant was transferred to fresh sterile micro-tubes and purified by extracting twice with an equal volume of phenol-chloroform. The DNA was precipitated by adding 3 volume of ice-cold absolute ethanol and 100 µl of 3 M sodium acetate to the samples and placing them at -20°C for 24 h. The DNA was pelleted at 10,000 g for 15 min at 4°C in a microcentrifuge tube and washed twice with ice-cold 70% ethanol. After drying, the DNA was resuspended in 50 µl of DNase-free water, and used as template for PCR amplification. The blank micro-tubes with only 50 µl DNA extraction buffer were assessed in parallel with the tick samples during DNA extraction.

Polymerase chain reaction (PCR) amplification

PCR was performed using primers Rr190.70p (Regnery *et al*. 1991) and 190-701 (Roux *et al*. 1996), which amplifies a 629 or 632-bp fragment of the gene encoding a 190-kD *omp*A specific for the SFG rickettsiae. The PCR amplifications were performed in a 30-µl reaction mixture containing of 3.0 µl purified DNA, 200 µM each deoxynucleoside triphosphate (dATP, dCTP, dGTP and dTTP), 0.8 µM of *Taq* polymerase and 0.5 µM each of forward and reverse primers. In parallel with each amplification of tick specimens,

negative (sterile water) and positive controls (DNA from *R. conorii*) were included. The reaction was carried out in a PCR thermal cycler (AB Geneamp, PCR System 2700) with the following step-wise procedure: initial denaturation at 94°C for 6 min, followed by 35 cycles of 94°C for 15 s, 54°C for 15 s, 70°C for 30 s, and a final extension at 70°C for 5 min. The PCR products were visualized under UV light after electrophoresis on a 1.2% agarose gel stained with ethidium bromide. To avoid contamination, DNA extraction, the reagent setup, amplification and electrophoresis were conducted in separate rooms.

DNA sequencing and data analysis

The amplicons of positive ticks were then sequenced directly by a dideoxynucleotide cycle sequencing method with an automated DNA sequencer (ABI PRISM 377, Perkin-Elmer, Inc.). Two sequencing reactions of each PCR product were performed to limit errors in sequencing. In case of different sequences obtained, the further sequencing reactions were carried out to generate a consensus sequence and determine the potential presence of another co-infected *Ricketsia*. The sequences of either 629 or 632-bp fragments obtained at present study were compared with the corresponding sequences of other rickettsiae deposited in GenBank by means of BLAST.

SFG *Rickettsia* infection rate with its 95% confidence interval (*CI*) of the ticks was estimated on the basis of binomial distribution. χ^2 test was used to compare infection rates across the strata of selected characteristics. A *P*-value < 0.05 was considered statistically significant.

Results

In total, 676 *D. silvarum* were examined by PCR for presence of SFG *Rickettsia*. The distribution and infection rates of ticks in light of sex and stage, origin and month of collection are shown in Table 1. The overall positive rate for SFG *Rickettsia* in *D. silvarum* was 10.7% with the 95% *CI* from 8.3% to 13.0%. The variation in prevalence of rickettsial infection among male, female and nymph ticks was statistically significant (χ^2 = 12.09, degree of freedom [df] = 2, *P* = 0.002). Although no difference in infection rate was observed between male and female ticks (χ^2 = 2.63, df = 1, *P* = 0.105), infection with *Rickettsia* was more frequent in adults than that in nymphs (χ^2 = 9.17, df = 1, *P* = 0.002). The infection rate in fed ticks obtained from domestic animals was significantly higher than that in questing ticks collected on vegetation (χ^2 = 12.86, df = 1, *P* < 0.001). There was no significant diversification in positive rates among ticks in different months (χ^2 = 1.29, df = 2, *P* = 0.525).

Table 1. Prevalences of SGF *Rickettsia* infection in *D. silvarum* ticks from Jilin province of northeastern China

Characteristics	No. of ticks tested	Prevalence (95% CI)	P value
Sex / Stage			0.002
Male	239	14.6 (10.2 -19.1)	
Female	352	10.2 (7.1 – 13.4)	
Nymph	85	1.2 (0 – 3.5)	
Origin			<0.001
Questing ticks from vegetation	518	8.3 (5.9 – 10.7)	
Fed ticks from animals	158	18.4 (12.3 – 24.4)	
Month of collection			0.265
May	213	8.9 (5.1 – 12.7)	
June	346	11.0 (7.7 – 14.3)	
July	117	12.8 (6.8 – 18.9)	

All the amplicons of the 72 positive ticks were directly sequenced. The partial nucleotide sequences of *ompA* gene obtained using primers Rr190.70p / 190.701 from 47 ticks (accounting for 65.3% of the total positive samples) were identical to those of *R. sibirica* genotype (GenBank accession number U43807). While an unreported agent in China (named JL-02, GenBank accession No. AY093696) was discovered from the other 25 ticks, the sequences of which were 100% homology with that of DnS14 genotype previously recognized in the former Soviet Union (Rydkina *et al*. 1999). The sequence was closely related to DnS28 and RpA4 genotypes in the former Soviet Union and *R. montanensis* in the United States, but different from *R. sibirica*, *R. mongolotimonae*, *R. heilongjiangii* and *R. hulinii* previously reported in China.

Discussion

The prevalence of SGF *Rickettsia* infection in *D. silvarum* was investigated in a forest area of Jilin province in northeastern China. As a result, 10.7% ticks were found to be infected, which is comparable to the infection rate in *D. nuttallii* from the former Soviet Union (Rydkina *et al*. 1999), *Ixodes ricinus* from Italy (Beninati *et al*. 2002), but is lower than that in *Amblyomma variegatum* from Caribbean (Kelly *et al*. 2003). The phenomena of wide range of *Rickettsia* infection rate in various ticks has been observed in an annual survey in United States (Azad and Beard 1998). The finding that

the infection rates of male and female ticks were not significantly different is consistent with that of a previous study (Ammerman *et al*. 2004). However, adult ticks seem more likely to be infected with *Rickettsia* than nymphs. This may be because the ticks at adult stage have fed on more animal hosts with rickettsemias. *Rickettsia* infection was found more common in fed ticks from animals than that in ticks on vegetation. The possibility is that the feeding activation of *Rickettsia* spp., in which the agent infecting a tick can multiply in response to feeding, increases the probability of detecting the *Rickettsia* using PCR techniques. *D. silvarum* is one of the most abundant tick species in northeastern China, and often parasitizes large domestic and wild animals such as cattle, horse, sheep and deer, and also readily feeds on humans as alternate hosts. The significances of the tick species in both veterinary medicine and public health deserve further investigations.

The findings of sequence analysis confirmed the existence of *R. sibirica* in Jilin province, where Siberian tick typhus is endemic. In addition, a *Rickettsia* variant (JL-02) was found in *D. silvarum* from this region. The partial nucleotide sequences of *ompA* gene of the agent is identical to the corresponding part of the *Rickettsia* recognized in *D. nutallii* from former Soviet Union (Rydkina *et al*. 1999), and it is genetically distinct from all the rickettsiae previously reported in China. Unfortunately, we did not sequence two separate PCR products or two clones from one PCR reaction, dual infection of the *Rickettsia* variant and *R. sibirica* in a single tick remains to be investigated. In the United States, various tickborne rickettsiae exist in areas endemic for *R. rickettsii,* the agent of Rocky Mountain spotted fever (Niebylski *et al*. 1997). Similarly, several recently described rickettsiae were found in ticks of the *Rh. sanguineus* complex in the regions endemic for Mediterranean spotted fever caused by *R. conorii* (Marquez *et al*. 1998). Although coinfections of *Rickettsia* could be detected in ticks, the transmission to the egg was for only one species. It is believed that only a single species of *Rickettsia* is transmitted transovarially. The clinical importance of the phenomenon is that “non-pathogenic” *Rickettsia* can exclude tick infection with “pathogenic” strains, altering the incidence and prevalence of rickettsial disease in animals and humans in one area (Burgdorfer *et al*. 1981).

The sequence analysis of *ompA* gene fragment alone is not entirely sufficient to identify the agent as a new *Rickettsia*. However, it is a useful tool for preliminary classification, and has been widely used to recognize tickborne *Rickettsia* in field surveys (Billings *et al*. 1998, Marquez *et al*. 1998, Weller *et al*. 1998, Rydkina *et al*. 1999, Ammerman *et al*. 2004). A recently published paper discussed an approach to genetic classification of *Rickettsia* species, in which 4–5 gene targets were used for sequencing. As an example,

the approach was applied for confirming the classification of *R. heilongjangiensis* (Fournier *et al*. 2003). Further studies are required to classify the JL-02 strain and to understand its public health significance.

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Chapter 3

Identification of *Ehrlichia chaffeensis* by Nested PCR in Ticks from Southern China

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Identification of *Ehrlichia chaffeensis* by Nested PCR in Ticks from Southern China.
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Abstract

A total of 717 ticks collected from southern China were examined by nested PCR for the presence of *Ehrlichia chaffeensis*. Sixteen (55.2%) of 29 adult *Amblyomma testudinarium*, 28 (11.7%) of 240 adult and at least 4.2% of 215 nymphal (pooled specimens) *Haemaphysalis yeni* tested positive. Four other species of ticks were negative. Selected positive amplicons were confirmed by DNA sequencing.

Introduction

Human monocytic ehrlichiosis (HME) is an acute febrile illness characterized by nonspecific clinical manifestations mainly including fever, headache, myalgia, chills, malaise, anorexia, and vomiting, sometimes with leukopenia, thrombocytopenia and elevated hepatic aminotransferase levels. It is usually moderate to severe and sometimes fatal(Walker and Dumler 1996). Since first reported in 1987 (Maeda *et al*. 1987), the disease has been diagnosed in more than 30 states of the United States(Walker and Dumler 1996), as well as in Europe (Morais *et al*. 1991) and Africa (Uhaa *et al*. 1992). The etiologic agent, *Ehrlichia chaffeensis* has been associated with ticks including *Amblyomma americanum, Dermacentor variabilis* (Anderson *et al*. 1993; Lockhart *et al*. 1997a; Roland *et al*. 1998) and *Ixodes pacificus* (Kramer *et al*. 1999), which may serve as vectors. Wild white-tailed deer, *Odocoileus virginianus* are believed to be the natural reservoirs of *E*. *chaffeensis* (Lockhart *et al*. 1997a; Lockhart *et al*. 1997b). Although HME has never been reported in Asia, there is immunoserologic evidence of exposure to *E. chaffeensis* among some individuals from Thailand (Heppner *et al*. 1997) and southern China (Li *et al*. 1993). The main purpose of this study was to determine the presence of *E*. *chaffeensis* in ticks from China.

Materials and Methods

Tick collection

Adult and nymphal ticks were collected from three provinces in southern China during the period of 1996 to 1998. Ticks were collected from domestic and wild animals including cattle, dog, southern China hare (*Caprologus sinensis*), goat-like deer (*Muntiacus reevesi)*, short-eared rabbit (*Lepus sinensis)* and white-abdomened grant rat (*Rattus edwardsi*) (Table 1). In the laboratory, ticks were examined morphologically and sorted by species, developmental stage and collection site. Tick specimens were then stored at -20°C until DNA extraction was performed.

DNA extraction

DNA was extracted from ticks by a modification of a previously described method (Magnarelli et al. 1995). Briefly, the ticks were placed into micro-tubes and mechanically crushed with sterile scissors in 50 µl DNA extraction buffer (10 mM Tris pH 8.0, 2 mM EDTA, 0.1% sodium dodecyl sulfate, 500 µg of proteinase K per ml). The samples were

incubated for 2 h at 56°C, and then boiled at 100°C for 10 min to inactivate proteinase K. After centrifugation, the supernatant was used directly for PCR or purified by extracting twice with an equal volume of phenol-chloroform before using.

Table 1. Results of nested PCR for the identification of E. chaffeensis in ticks collected in southern China

Tick species	Collection site (province)	Animal host(s)	No. tested	No. positive (%)
A. testudinarium	Yunnan	Cattle	29	16 (55.2)
H. yeni	Fujian	Muntiacus reevesi and Lepus sinensis	185	22 (11.9)
H. yeni	Fujian	Cattle	37	2 (5.4)
H. yeni	Fujian	Caprologus sinensis	18	4 (22.2)
H. yeni (nymph)	Fujian	Muntiacus reevesi and Lepus sinensis	215	9 (4.2*)
H. hystricis	Fujian	Muntiacus reevesi and Lepus sinensis	54	0
I. granulatus	Fujian	Rattus Edwardsi and dog	50	0
I. sinensis	Fujian	Muntiacus reevesi	9	0
H. longicornis	Zhejiang	Rattus edwardsi and cattle	120	0

* Minimum infection rate

Nested PCR

Nested PCR was performed using primers derived from the 16S rRNA gene of *E. chaffeensis*. For the initial amplification, 3 ml of each template sample was amplified in a 30-ml reaction mixture containing the primers HE1 (5'-CAATTGCTTATTACCTTTTGGTTATAAAT-3') (Anderson *et al*. 1992) and PER2 (5'-CTCTACACTAGGAATTCCGCTAT-3')(Goodman *et al*. 1996). For the nested amplification, 1 ml of the primary PCR product was used as the template in a second 30-ml reaction mixture with specific primers HE1 and HE3 (5'-TATAGGTACCGTCATTATCTTCCCTAT-3') (Anderson *et al*. 1992) The PCR amplifications were performed in a Perkin-Elmer model 480 thermal cycler, using the following protocol: preheating at 95°C for 3 min, followed by 35 cycles of 94°C for 1 min, 56°C for 75 sec, and 72°C for 1 min, then a final extension at 72°C for 7 min. In each set of amplifications, both a negative control (distilled water) and a positive control (plasmid containing the *E. chaffeensis* 16S rRNA gene) were included. PCR products were separated by agarose gel electrophoresis, stained with ethidium bromide, and visualized under UV light.

PCR product cloning and DNA sequencing

PCR products were purified and then ligated into the plasmid Vector pGEM-T (Promega Corp.) according to the manufacturer's instructions. The ligation products were transformed into *Escherichia coli* XL1-Blue, and white colonies were selected after growth on Luria-Bertani agar with IPTG (isopropyl-ß-D-thiogalactopyranoside), X-Gal (5-bromo-4-chloro-3-indolyl-ß-D-galactopyranoside) and ampicillin. Recombinant plasmids were extracted and purified from overnight cultures using Qiagen plasmid Kit (QIAGEN GmbH). The nucleotide sequence of the plasmid insert was determined by a dideoxynucleotide cycle sequencing method with an automated fluorescent ABI. PRISM™ 377 DNA sequencer (Perkin-Elmer, Inc.).

Results

The sensitivity of the nested PCR was assessed by a spiking experiment with dilution of a plasmid containing the *E. chaffeensis* 16S rRNA gene sequence. The linearized plasmid DNA was diluted with DNA extracted from uninfected adult ticks. Serial dilutions of the quantified plasmid DNA were tested by the nested PCR assay. To control matrix effects, the same amount of uninfected tick background DNA was included in each initial amplification. Under these conditions, 4 copies of the double-stranded DNA could be identified (figure 1). The result was the same when the original tick DNA preparations purified from different uninfected (PCR-negative) tick species (*Haemaphysalis yeni*, *H. longicornis*, *I. persulcatus*) were used as a nonspecific competitor (data not shown). Inhibitory effects did not appear when 10% of the background tick DNA amount was used. To assess the specificity of the assay, DNA templates extracted from various ehrlichial species (including *E. canis, E. platys, E. ewingii, E. equi* and *E. risticii*) and possible tick infectious agents (including *Rickettsia rickettsii, R. conorii, R. sibirica, R. japonica, Borrelia burgdorferi* senso stricto, *B. garinii* and *B. afzelii*) were tested by the nested PCR, and no products were amplified.

A total of 717 ticks were tested for the presence of ehrlichial DNA. The species and origin of ticks are shown in table 1. After the first round of amplification with initial primers HE1 and PER2, 12 specimens including 2 *A. testudinarium* and 10 *H. yeni* adults generated characteristic 587 bp products. The nested PCR detected ehrlichial DNA in *A. testudinarium*, adult and nymphal *H. yeni* ticks, evidenced by the presence of a 389-bp band (table 1 and figure 2). Of 29 *A. testudinarium* from Monla county of Yunnan province, 16 tested positive. Among the ticks collected from Wuyishan city

and Ninghua county of Fujian province, the positive rate of *H. yeni* adults was 11.7% (28/240) on average and varied from 5.4% to 22.2% with host origin. 215 *H. yeni* nymphs from the same area were examined in pools (each containing 5 ticks), and 9 pools were positive at a minimum frequency of 4.3%. PCR tests were negative for all of 54 *H. hystricis*, 50 *I. granulatus* and 9 *I. sinensis* ticks from Wuyishan city of Fujian province, and 120 *H. longicornis* from Longyan county of Zhejiang province (Table 1).

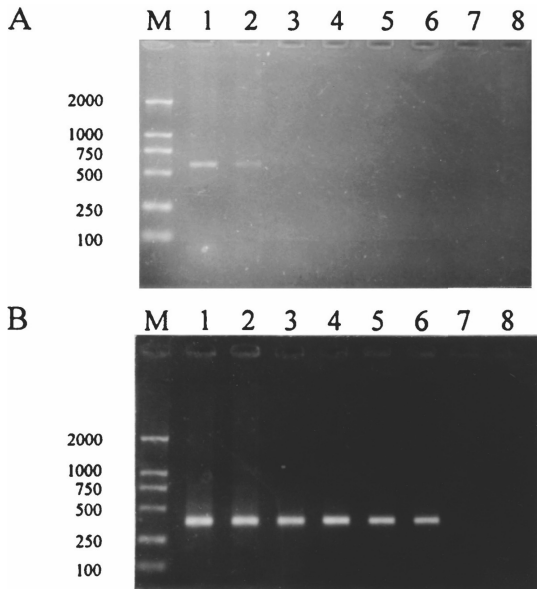

Figure 1. Analytical sensitivity of nested PCR for detection of *E. chaffeensis* 16S rRNA genes in Chinese ticks. Lanes labeled M represent molecular standards, sizes (in base pairs) are indicated on the left. (**A**) Products of the primary amplification using serial dilutions of plasmids containing *E. chaffeensis* 16S rRNA gene as templates. Lanes 1 through 7 correspond to template copy numbers of 8×10^4, 8×10^3, 8×10^2, 80, 8, 4, 2 respectively. Lane 8 is the negative (water) control. The expected size (587 bp) of the primarily amplified product is indicated on the right. (**B**) Products of the nested PCR using 1 µl of the corresponding primary product as the template, with expected size (389 bp) indicated.

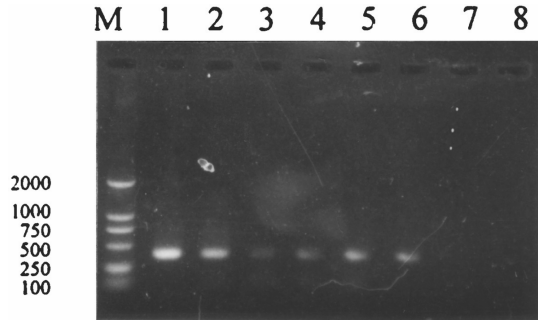

Figure 2. Nested PCR products amplified from representative tick samples. Lane M, DNA marker, sizes (in base pairs) are indicated on the left. Lane 1, positive control (plasmid containing the *E. chaffeensis* 16S rRNA gene); Lane 2, *A. testudinarium* adult from cattle; Lane 3, *H. yeni* adult from *Muntiacus reevesi*; Lane 4, *H. Yeni* adult from cattle; Lane 5, *H. yeni* adult from *Caprologus sinensis*; lane 6, *H. yeni* nymph from *Lepus sinensis*; Lane 7, water (as negative control). The expected size (389 bp) indicated.

The nucleotide sequences determined for the 587-bp PCR products from 2 *A. testudinarium* and 2 *H. yeni* positive ticks after initial amplification were identical to each other and to the corresponding part of the 16S rRNA gene sequence of the *E. chaffeensis* agent previously described by Anderson et al. (GenBank accession number M73222) (Anderson *et al*. 1991). Furthermore, the sequences of the 389 bp nested PCR amplicons from representative positive samples were all identified as partial sequence of the *E. chaffeensis* 16S rRNA gene.

Discussion

The results of the current study demonstrate the presence of *E. chaffeensis* in *A. testudinarium* and *H. yeni*. To our knowledge, this is the first evidence of *E. chaffeensis* in ticks from China and the first finding of ehrlichial infection in *Haemaphysalis* species in the world. *E. chaffeensis* has been detected in a variety of ticks including *A. americanum* (Anderson et al. 1993; Lockhart et al. 1997a; Roland et al. 1998), *D. variabilis* (Lockhart *et al*. 1997a; Roland *et al*. 1998) and *I. pacificus* (Kramer *et al*. 1999). Our findings, together with the evidence previously accumulated, suggest that *E. chaffeensis* is probably widespread and a variety of tick species may be involved in

transmission of the infectious agent. *A. testudinarium* is commonly seen in farmland and mountainous areas of southern and southwestern China. *H. yeni* is a dominant species in Fujian province, which accounts for more than 80% of adult and 85% of immature ticks collected from host animals such as *Muntiacus reevesi* and *Lepus sinesis* (Xu and Luo 1998). Further studies are needed to determine the competence of *A. testudinarium* and *H. yeni* as vectors of *E. chaffeensis*.

Nested PCR may enhance sensitivity of detection of target nucleotide sequences (Haff 1994). This technique has been shown to be sensitive for direct identification of ehrlichiae in ticks (Roland *et al*. 1998; Kramer *et al*. 1999; Barlough *et al*. 1997). In this study, the ability of the assay to detect ehrlichial DNA in ticks was assessed by using a plasmid containing *E. chaffeensis* 16S rRNA gene, and the sensitivity was 4 copies. This method may be minimally sufficient to identify ehrlichiae in individual ticks, and could be useful for field surveys. The specificity of the nested PCR was also evaluated, and no products were amplified from various ehrlichial species other than *E. chaffeensis* and other possible tick-harbored organisms, demonstrating the high specificity of the assay. The specificity of the assay was also confirmed by sequencing the PCR amplicons. All the resulting sequences of selected positive specimens were identified as partial sequence of the *E. chaffeensis* 16S rRNA gene.

This study provides primary data regarding the prevalence of *E. chaffeensis* in ticks from southern China. Of *A. testudinarium*, 55.2% (16/29) were infected, higher than the infection rate of *A. americanum*, a closely related species found in North America (Roland *et al*. 1998). In addition, 11.7% adult and at least 4.3% nymphal *H. yeni* ticks were positive for *E. chaffeensis*. Attempts to detect the agent in other tick species were unsuccessful. This study is not intended as a comprehensive survey of the *ehrlichia* distribution in ticks; rather it was designed to investigate the presence of *E. chaffeensis* in China. Because the number of ticks examined was limited, the infection rates of the present study could be biased. A randomized sampling scheme and further collection of the ticks should be made to obtain a reliable estimate.

It is known that large domestic and wild animals such as cattle, horse, sheep and deer are hosts for adult *A. testudinarium*. *H. yeni* often parasitizes variety of animals as listed in Table 1. However, it is not so clear to what extent the two tick species feed on humans as alternate hosts. It remains to be determined whether the agent found in ticks in this study cause human disease. Isolation and identification of causative agents from patients will eventually provide direct evidence for human infection. However, ehrlichiosis should be considered, when a patient has an unexplained fever with thrombocytopenia, leukopenia and elevated hepatic aminotransferase levels and

recent history of tick bite especially in the areas where *A. testudinarium* or *H. yeni* are abundant.

The accession number of nucleotide sequence

The nucleotide sequence reported in this paper has been deposited in GenBank under the accession no. AF147752.

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Chapter 4

Granulocytic Ehrlichia in *Ixodes persulcatus* Ticks from an Area in China where Lyme Disease is Endemic

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Granulocytic *Ehrlichiae* in *Ixodes persulcatus* ticks from an area in China where Lyme disease is endemic.

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Abstract

A total of 372 adult *Ixodes persulcatus* ticks were collected from vegetation in a forest area of Heilongjiang province in northeastern China, where Lyme disease is known to be endemic. The ticks were examined for the presence of granulocytic ehrlichiae by hemi-nested PCR using primers derived from 16S rRNA gene. Of 310 ticks obtained from Dahe forestry farm, 2 pools (each containing 5 ticks) were found positive, with a minimum infection rate of 0.6%. Ehrlichial DNA was also detected in one female (1.6%) of 62 ticks collected from Yulin forestry farm. The overall minimum infection rate of the 372 *Ixodes persulcatus* adults was 0.8%. The nucleotide sequences of 919-bp PCR products from the three positive tick specimens were identical to each other, and very closely related to the members in *Ehrlichia phagocytophila* genogroup. This is the first identification of granulocytic *ehrlichia* in ticks in Asia, and first report of infection in *I. persulcatus* anywhere.

Introduction

Human granulocytic ehrlichiosis (HGE) was initially described in the United States in 1994 (Chen *et al*. 1994), and presents clinically as an acute febrile illness characterized by fever, headache, chills, myalgia, lethargy, and arthralgia (Walker and Dumler 1996). Laboratory findings suggestive of the disease mainly include leukopenia, anemia, thrombocytopenia and elevated hepatic aminotransferase levels (Walker and Dumler 1996; Bakken *et al*. 1996). The causative agent of HGE has not yet been fully defined, but 16S rRNA gene sequence analysis demonstrates that it is closely related to *Ehrlichia equi*, the agent of the world wide equine granulocytic ehrlichiosis, and *E. phagocytophila*, the well recognized pathogen of tick-borne fever of ruminants in Europe (Chen *et al*. 1994; Walker and Dumler 1996). In addition, the HGE agent also can cause a form of granulocytic ehrlichiosis in horses (Barlough *et al*. 1995; Madigan *et al*. 1996; Madigan *et al*. 1995) and dogs (Greig *et al*. 1996). It is suggested that the HGE agent, *E. equi*, and *E. phagocytophila* may constitute variants of a single species now called *E. phagocytophila* genogroup.

The granulocytic ehrlichiae have been associated with ixodid ticks that may act as vector, including *Ixodes scapularis* (Magnarelli *et al*. 1995; Pancholi *et al*. 1995) and *I. pacificus* (Barlough *et al*. 1997a; Kramer *et al*. 1999; Schwartz *et al*. 1997) in the United States, and *I. ricinus* (Guy *et al*. 1998; Pusterla *et al*. 1999; von Stedingk *et al*. 1997) in Europe. These ticks are known to transmit *Borrelia burgdorferi*, the pathogen of Lyme disease, and recent studies indicated that they were could be coinfected with *B. burgdorferi* and granulocytic ehrlichiae (Schwartz *et al*. 1997; Baumgarten *et al*. 1999; Cinco *et al*. 1997; Leutenegger *et al*. 1999). It is suggested that the natural cycle of granulocytic ehrlichiae is probably similar to that of *B. burgdorferi* (Walker and Dumler 1996). *I. persulcatus* is the vector of Lyme borreliosis (Ai *et al*. 1994) and tick-borne encephalitis in northeastern China, but the occurrence of *Ehrlichia* in ticks has not been established for this same region. The objectives of this study were to determine whether or not ehrlichial DNA is present in *I. persulcatus* ticks in an area where Lyme disease is endemic, and to provide initial data regarding the presence of granulocytic *ehrlichia* in China.

Materials and Methods

Tick collection

Adult *I. persulcatus* ticks were collected from a forest area of Heilongjiang province in northeastern China in 1997. The collection site is near Mudanjiag city located at 50° north latitude and 128° east longitude, which is a highland (elevation from 500 to 600 m above sea level) belonging to Small Xing-An Mountains. In the area investigated, *I. persulcatus* is abundant, and Lyme borreliosis is known to be endemic, as indicated by the report of human infection (Ai *et al*. 1994) and isolations of *B. burgdorferi* from ticks (Takada *et al*. 1998). In this study, ticks were collected by dragging a standard 1 m^2 flannel flag over vegetation, and stored alive in the refrigerator until use.

Processing of tick specimens

Ticks were processed individually or in pools (each containing five ticks). DNA extraction was performed by a modification of a method previously described(Magnarelli *et al*. 1995). Briefly, the ticks were placed into micro-tubes and mechanically crushed with sterile scissors in 50 µl DNA extraction buffer (10 mM Tris pH 8.0, 2 mM EDTA, 0.1% sodium dodecyl sulfate, 500 µg of proteinase K per ml). The samples were incubated for 2 h at 56°C, and then boiled at 100°C for 10 min to inactivate proteinase K. After centrifugation, the supernatant was transferred to fresh sterile micro-tubes and purified by extracting twice with an equal volume of phenol-chloroform. The DNA was precipitated by adding 3 volume of ice-cold absolute ethanol and 100 ?l of 3 M sodium acetate to the samples and placing them at -20°C for 24 h. The DNA was pelleted at 10,000 g for 15 min at 4°C in a microcentrifuge and washed twice with ice-cold 70% ethanol. After drying, the DNA was resuspended in 50 µl of DNase-free water, and used as template for PCR amplification.

Hemi-nested PCR amplification

Hemi-nested PCR amplifications were performed with primers designed to amplify the 16S rRNA gene of *E. phagocytophila* genogroup. Primers GE9f (5'-AACGGATTATTCTTTATAGCTTGCT-3') and GE10r (5'-TTCCGTTAAGAAGG-ATCTAATCTCC-3') previously described by Chen *et al*. (Chen *et al*. 1994) were applied for the initial amplification. Two primer pairs were used in the hemi-nested PCR amplification. The primer pair GE9f and GE2 (5'-GGCAGTATTAAAAGCAGCTCCAGG-3') (Massung *et al*. 1998) can specifically produce a 546-bp fragment, and the primer pair Ehr521 (5'-TGTAGGCGGTTCGGTAAGTTAAAG-3') (Pancholi *et al*. 1995) and

GE10r yields a 441-bp product. Primary reaction used 3 µl of purified DNA as the template in a total volume of 30 µl. The hemi-nested PCR was performed using 1 µl of the primary PCR product as the template in a volume of 30 µl. For either initial or nested amplification, reaction mixture contained 200 µM of each deoxynucleoside triphosphate (dATP, dCTP, dGTP, and dTTP), 0.8U of Taq polymerase and 0.5 µM each primer. The PCR amplification was performed in a Perkin-Elmer 480 thermal cycler. The cycling conditions were identical for primary and nested amplifications, which involved the following step-wise procedure: preheating at 95°C for 2 min; 35 cycles of 94°C for 1 min, 55°C for 75 s, and 72°C for 1 min; and a final extension at 72°C for 7 min. Reaction products were then analyzed by agarose gel electrophoresis or purified for DNA sequencing. A negative control (distilled water) and a positive control (a plasmid containing the 16S rRNA gene of HGE agent, GenBank accession number U02521) were included with each set of amplifications. To minimize contamination, DNA extraction, the reagent setup, amplification and agarose gel electrophoresis were performed in separate rooms.

Cloning of PCR products and DNA sequencing

PCR products after nested amplification were purified and then ligated into the plasmid Vector pGEM-T (Promega Corp.) according to the manufacturer's instructions. The ligation products were transformed into *Escherichia coli* XL1-Blue, and white colonies were selected after growth on Luria-Bertani agar with IPTG (isopropyl-β-D-thiogalactopyranoside), X-Gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside) and ampicillin. For sequence analyses, recombinant plasmids were extracted and purified from overnight cultures using QIA prep Spin Miniprep Kit (QIAGEN). The nucleotide sequence of the plasmid insert was determined by a dideoxynucleotide cycle sequencing method with an automated fluorescent ABI PRISM™ 377 DNA sequencer (Perkin-Elmer, Inc.).

Nucleotide sequencing accession number

The nucleotide sequence reported in this study has been deposited in GenBank under the accession no. AF205140.

Results

A total of 372 adult *I. persulcatus* ticks were examined for the presence of granulocytic ehrlichiae by hemi-nested PCR with initial primer pair GE9f and GE10r and subsequent primer pair GE9f and GE2. The prevalence of PCR-positive ticks from the different sources and sex are shown in table 1. The results are expressed as the positive rate or the minimum positive rate, obtained by the calculation that the positive number of specimens was divided by the total number of ticks examined. The calculation is based on the assumption that each PCR-positive pool contains at least one tick with detectable ehrlichiae. Ticks collected from Dahe forestry farm were examined in pools, each containing five ticks. Two of 62 pools (310 ticks) were found positive, and the minimum infection rate was 0.6%. Of 62 ticks from Yulin forestry farm, ehrlichial DNA was detected in one female, with a positive rate of 1.6%. Overall, the minimum positive rate of the 372 ticks was estimated 0.8%. When the tick samples were examined using initial primer set of GE9f and GE10r and subsequent primer set of Ehr521 and GE10r, exactly the same results were obtained.

Table 1. Results of hemi-nested PCR for the identification of granulocytic *ehrlichia* in *I. persulcatus* ticks using initial primer pair GE9f and GE10r and subsequent primer pair GE9f and GE2

Origin	Sex	No. ticks tested	No. positive	Positive rate (%)
Dahe	Male	120 (24 pools)	1 pool	0.8*
	Female	190 (38 pools)	1 pool	0.5*
Yulin	Male	23	0	0
	Female	39	1	2.6
Total		372	3	0.8*

* Minimum positive rate.

The primary PCR product (amplified with primer GE9f and GE10r) of each positive specimen was respectively reamplified with subsequent primer sets GE9f-GE2 and Ehr521-GE10r. For all nested PCR amplicons, both DNA strands were sequenced twice. As a result, a specific nucleotide sequence of 919-bp long was obtained for each tick specimen. The ehrlichial 16S rDNA sequences determined from the three positive tick samples were identical to each other, and differed from the corresponding sequences of HGE agent, *E. phagocytophila* and *E. equi* all by 4 bases respectively, but at different positions (table 2). A variable region is discovered near the 5' end of 16S rRNA gene at the position from 76 to 84 (according to HGE agent, GenBank accession

no. U02521). It is remarkable that the G at position 77 or 80 was unique to the *Ehrlichia* variant in *I. persulcatus*. In contrast, there is the A at both the positions for all known granulocytic *ehrlichia* variants(data not shown).

Table 2. Nucleotide differences among the 919-bp partial 16S rRNA gene sequences of *Ehrlichia* variant in *I. persulcatus* and members in the *E. phagocytophila* genogroup

Organism	Nucleotide difference at position[a]					GenBank accession no.
	76	77	80	84	886[b]	
HGE agent	A	A	A	G	G	U02521
E. equi	A	A	A	A	–	M73223
E. phagocytophila	A	A	A	A	–	M73220
Ehrlichia variant in *I. persulcatus*	G	G	G	A	G	AF205140

[a] The position of nucleotide relative to the sequence of HGE agent reported by Chen *et al.* (8).

[b]. – indicating no nucleotide corresponds to HGE agent; a gap was required at this position to align the adjacent sequences.

Discussion

Granulocytic *Ehrlichia* DNA was amplified from *I. persulcatus* collected in a forest area of Heilongjiang province in northeastern China, where Lyme disease is known to be high endemic. To our knowledge, this is the first detection of granulocytic *ehrlichia* in ticks in Asia, and the first report of infection in *I. persulcatus* anywhere. *I. scapularis*. and *I. pacificus* have been identified as potential vectors of HGE agent and *E. equi* in the United States (Kramer *et al*. 1999; Barlough *et al*. 1996; Barlough *et al*. 1997b; Richter *et al*. 1996). *E. phagocytophila* genogroup was found in *I. ricinus* ticks from many European countries (Guy *et al*. 1998; Pusterla *et al*. 1999; von Stedingk *et al*. 1997). The findings of this study add to the evidence that the *E. phagocytophila* genogroup is specifically associated with the *Ixodes persulcatus* complex. *I. persulcatus* ticks are distributed over an extensive area from Russia to eastern Asia, where about one-fifth human population of the world resides. The presence of granulocytic *Ehrlichia* in northeastern China suggests a potential health threat to both humans and animals in the area, where *I. persulcatus* are abundant. Detailed epidemiological studies are required to investigate the distribution of ticks infected with ehrlichiae, to determine the animal reservoirs of the ehrlichial agents, and especially to detect ehrlichiae in patients with acute febrile illnesses following tick bite in Lyme disease endemic areas.

The overall minimum infection rate of granulocytic *Ehrlichia* in *I. persulcatus* adults in this study was 0.8%, which is comparable to that in adult *I. pacificus* from California (Barlough *et al*. 1997a) and in free-living adult *I. ricinus* from tick-borne fever endemic areas in Switzerland (Pusterla *et al*. 1998). The percentage reported in this study might have been falsely reduced, because ticks were examined in pools, and some pools might contain more than one positive tick. However, even if every tick in the positive pools had harbored detectable ehrlichiae, the overall prevalence would have only gone up to 3.0%. Higher prevalence of *E. phagocytophila* genogroup was reported in *I. scapularis* in the United States (Magnarelli *et al*. 1995; Pancholi *et al*. 1995; Schwartz *et al*. 1997) and *I. ricinus* in some European countries (Guy *et al*. 1998; von Stedingk *et a*l. 1997; Cinco *et al*. 1997; Schouls *et al*. 1999). This discrepancy in positive rate could be due to differences in sampling approaches, tick species, and ehrlichial life cycle, to geographic and seasonal variations of infected ticks, or to limits of PCR sensitivity.

Sequence analysis of PCR products from tick samples revealed a granulocytic *Ehrlichia* variant that slightly differs from members of the *E. phagocytophila* genogroup (table 2). The sequence variants of granulocytic *ehrlichia* 16S rRNA gene have previously been detected in ticks in many places (Baumgarten *et al*. 1999; Massung *et al*. 1998; Parola *et al*. 1998). It is unlikely that the 16S rDNA variants represent different *Ehrlichia* species. The specific sequence polymorphism of the *Ehrlichia* variant identified in this study has not been reported before, and whether the variant can cause disease in humans and animals remains to be determined.

I. persulcatus is abundant in northeastern China, and is known as the vector of *B. burgdorferi*, the agent of Lyme disease (Ai *et al*. 1994). Studies elsewhere demonstrate that ixodid ticks are coinfected with *B. burgdorferi* and the HGE agent (Schwartz *et al*. 1997; Baumgarten *et al*. 1999; Cinco *et al*. 1997; Leutenegger *et al*. 1999), and that simultaneous human infection with the two agents is reported (Weber *et al*. 1998; Nadelman *et al*. 1997; Duffy *et al*. 1997). Coinfection may explain variations in clinical manifestations in human and animals as a consequence of tick bites. The identification of granulocytic *Ehrlichia* in *I. persulcatus* suggests the probability of coinfection and cotransmission of the two agents in the area. Further studies are needed to investigate this phenomenon, and the possible occurrence of ehrlichiosis should be considered in the differential diagnosis of febrile patients with a history of tick bite in northeastern China, particularly when clinical symptoms and signs are atypical for Lyme disease.

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Chapter 5

Natural *Anaplasma Phagocytophilum* Infection of Ticks and Rodents from a Forest Area of Jilin Province, China

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Natural Anaplasma phagocytophilum infection of ticks and rodents from a forest area of Jilin Province, China

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Abstract

PCR integrated with sequence analysis was carried out to investigate the natural infection of *Anaplasma phagocytophilum* in ticks and rodents from Jilin Province, China. Four (4.0%) of 100 *Ixodes persulcatus* and 2 (0.7%) of 286 *Dermacentor silvarum* ticks collected by flagging vegetation were positive. Nine (8.8%) of 102 rodents as well as 2 (2.8%) of 71 *I. persulcatus* parasitized on 25 rodents were infected. The nucleotide sequences of 919-bp partial 16S rRNA gene amplified from rodents and ticks were identical to each other and to that (GenBank accession no. AF205140) previously reported in Heilongjiang Province of China, but different from those of other *A. phagocytophilum* variants. The sequence analysis of a 357-bp fragment of citrate synthase gene revealed that all the amplification products had 100% homology, and varied from known *A. phagocytophilum* agents.

Introduction

Anaplasma phagocytophilum is a Gram-negative obligate intracellular bacterium, which encompasses former *Ehrlichia phagocytophila*, *E. equi*, and the human granulocytic ehrlichiosis (HGE) agent based on phylogenetic analyses (Dumler *et al.* 2001). This bacterium has long been recognized as a veterinary agent. Since HGE (now called human granulocytic anaplasmosis), the human infection, was first reported in the United States in 1994 (Bakken *et al*. 1994; Chen *et al*. 1994), *A. phagocytophilum* has been considered as an emerging pathogen of public health importance (Walker and Dumler 1996). The disease usually presents as an acute febrile illness characterized by headache, chill, myalgias, arthralgia, malaise and hematological abnormalities, such as thrombocytopenia, leukopenia and elevated hepatic aminotransferase levels (Bakken *et al.* 1996).

A. phagocytophilum is thought to be naturally maintained in a tick-rodent cycle, with humans being involved only as incidental "dead-end" hosts (Bown *et al*. 2003; Liz *et al*. 2000; Pancholi *et al.* 1995; Polin *et al*. 2004; Telford *et al*. 1996). The agent is usually associated with genus *Ixodes* ticks, including *I. scapularis*, *I. pacificus*, and *I. spinipalpis* in the United States (Pancholi *et al*. 1995; Burkot *et al*. 2001; Richter *et al.* 1996), *I. trianguliceps* in the United Kingdom(Bown et al. 2003; Ogden et al. 1998), *I. ricinus* in mainland Europe (Liz *et al*. 2000; Polin *et al*. 2004; Cinco *et al*. 1997; Guy *et al.* 1998; Oteo *et al*. 2001; Pusterla *et al*. 1999; Schouls *et al*. 1999) and Africa (Sarih *et al*. 2005), and *I. persulcatus* in eastern Europe (Alekseev *et al*. 2001) and Asia (Cao *et al.* 2000; Cao *et al*. 2003). Wild rodents have been implicated as natural reservoirs for *A. phagocytophilum* in the United States, the United Kingdom, and mainland Europe (Bown *et al*. 2003; Liz *et al*. 2000; Polin *et al*. 2004; Telford *et al*. 1996).

A. phagocytophilum was only documented in *I. persulcatus* ticks from northeastern China where Lyme disease is endemic (Cao *et al*. 2000; Cao *et al*. 2003). However, the existence of the agent has not been established in Jilin Province. Furthermore, no information is available concerning animal reservoirs of *A. phagocytophilum* in Asia. Lack of such knowledge has inhibited us to understand the ecology, epidemiology and potential threats of the pathogens to human health. The objectives of this study were to investigate the presence of *A. phagocytophilum* in Jilin Province of China, and to determine if rodents, the common hosts of immature stages of ticks, are naturally infected by the agent.

Materials and Methods

Study site

Rodents and actively questing ticks were collected in May 2005 in the hinterland of Changbai Mountains situated at 42°45'north latitude and 130°35' east longitude within Hunchun County, Jilin Province. The terrain consists of forested rolling hills with an average elevation of 825 m. The annual precipitation is about 800 mm, and the relative humidity is around 70%. The temperature ranges from -41.4°C to 33.6°C, with the average of 4.0°C.

Sample collection

Host-seeking ticks were collected by flagging vegetation. Ticks were identified to the species level and the developmental stage, and were stored alive until use. Rodents were captured using box traps with peanuts as baits. Attached feeding ticks were removed by using sterile forceps, identified by species and life stage, and stored in 1.5-ml microcentrifuge tubes in the refrigerator until DNA extraction. After identification of species and sex, the spleen was removed from each rodent and stored at -20°C until tested.

DNA extraction

DNA extraction from ticks was performed as previously described. Briefly, each tick was placed into a microtube and mechanically disrupted with sterile scissors in 50 µ?l DNA extraction buffer (10 mM Tris pH 8.0, 2 mM EDTA, 0.1% sodium dodecyl sulfate, and 500 µg of proteinase K per ml). The sample was incubated for 2 h at 56°C, and then boiled at 100°C for 10 min to inactivate proteinase K. After centrifugation, the supernatant was transferred to fresh sterile microtube and purified by extracting twice with an equal volume of phenol-chloroform before use.

A small piece of spleen (about 500 mg) from individual rodent was used for DNA extraction. Briefly, each spleen specimen was crushed with Trizol (Invitrogen, Carlsband, CA, USA) to separate DNA from RNA after centrifugation. The precipitated DNA were obtained after washing twice in a solution containing 0.1 M sodium citrate in 10% ethanol, then the DNA pellet was suspend in 75% ethanol and kept at room temperature for 10-20 min. After centrifuging at 2000 g at 2-8°C for 5 min, the DNA was dissolved in 8 mM NaOH and stored at 4°C overnight. Then TE (0.01 M, pH 8.0) was added to dissolve DNA for PCR use.

PCR amplification

A nested PCR was performed with primers designed to amplify the partial 16S rRNA gene of *A. phagocytophilum* (Cao *et a*l. 2000; Cao *et al*. 2003). Primers GE9f and GE10r, previously described by Chen *et al*. (1994), were applied for the primary amplification. In nested PCR, Ehr521 (Pancholi *et al*. 1995) and GE10r were used as primers, and can yield a 441-bp product. Both primary and nested PCR amplifications were performed in a volume of 30 µl in a Perkin-Elmer model 2400 thermal cycler. Cycling conditions involved an initial 3-min denaturation at 95°C followed by 35 cycles of 94°C for 15 s, 55°C for 20 s, and 72°C for 15 s, and a final extension at 72°C for 5 min. In parallel with each amplification, a positive control (a plasmid containing the 16S rRNA gene of HGE agent, kindly provided by Dr. J. Stephen Dumler at Department of Pathology, The Johns Hopkins Medical Institutions) and a negative control (distilled water) were included.

To further identify the agent in the samples positive for the *A. phagocytophilum* 16S rRNA gene, another nested PCR was performed to amplify partial sequence of the citrate synthase gene (*gltA*) of *A. phagocytophilum* (Inokuma *et al*. 2001). The primers W1 (5'-TGTTTTGGAGTGTGGAGAC-3') and W2 (5'-GGTGAACCAATCTCAGCAA-3') for the initial amplifications, and the primers N1 (5'-ATATAGAAAATCTGATCGG-3') and N2 (5'-CTCTAAGTTTGCGTCAGC-3) for the nested reactions were designed, and expected to produce a 357-bp fragment. PCR amplifications were conducted in a volume of 30 µl in a Perkin-Elmer model 2400 thermal cycler. The cycling conditions were identical for primary and nested amplifications, which involved 3-min denaturation at 95°C, followed by 35 cycles of 94°C for 15 s, 50°C for 20 s, and 70°C for 20 s, and a final extension at 70°C for 5 min.

All the products from the nested PCRs were separated by agarose gel electrophoresis, stained with ethidium bromide, and visualized under UV light. To minimize contamination, DNA extraction, the reagent setup, amplification and agarose gel electrophoresis were performed in separate rooms.

Sequence analysis

The PCR products were collected and purified. The nucleotide sequences of positive samples were determined by a dideoxynucleotide cycle sequencing method with an automated DNA sequencer (ABI PRISM 377, Perkin-Elmer, Inc.). The sequences obtained at present study were compared with the previously published sequences deposited in GenBank using BLAST program from the National Center for Biotechnology Information website.

Statistical analysis

χ^2 test or Fisher's exact test was used to compare the proportions. A *P*-value < 0.05 was considered statistically significant.

Nucleotide sequencing accession number

The nucleotide sequences of partial 16S rRNA gene and *gltA* obtained in this study were deposited in GenBank under the accession nos. DQ160227 and DQ160228 respectively.

Results

A. phagocytophilum was first detected by a nested PCR specifically targeting the 16S rRNA gene. A total of 386 unfed, host-seeking ticks, including 100 adult *I. persulcatus* and 286 *Dermacentor silvarum*, were collected on vegetation and individually examined. Infection rates of ticks in light of species, sex, and stage are shown in Table 1. The positive rate of *A. phagocytophilum* in *I. persulcatus* (4.0%) was significantly higher than that in *D. silvarum* (0.7%) (Fisher's exact test, *P* = 0.041). The male *I. persulcatus* ticks had significant higher infection rate than females did (Fisher's exact test, *P* = 0.011). There was no significant difference in positive rates among male, female and nymphal *D. silvarum* ticks (χ^2 = 3.18, degree of freedom [df] = 2, *P* = 0.204).

Table 1. Results of nested PCR for the detection of *A. phagocytophilum* in ticks collected by flagging vegetation from Jilin, China

Tick species	Sex and stage	No. tested	No. (%) positive
I. persulcatus	Male	34	4 (11.8)
	Female	66	0 (0.0)
D. silvarum	Male	137	0 (0.0
	Female	111	2 (1.8)
	Nymph	38	0 (0.0)
Total		386	6 (1.6)

A total of 102 rodents from 7 species were captured and detected for the presence of *A. phagocytophilum*. Nine rodents from 3 species, including Japanese field mouse (*Apodemus peninsulae*), black-striped field mouse (*A. agrarius*) and Siberian chipmunk

(*Tamias sibiricu*), were found naturally infected, with an overall positive rate of 8.8% (Table 2). The difference in infection rates among species was not statistically significant (χ^2 = 9.82, df = 6, P = 0.132).

Only *I. persulcatus* ticks were found to be parasitized on the captured rodents. Among the 102 rodents, 25 (24.5%) hosted 71 *I. persulcatus* ticks (Table 2), including 56 larvae and 15 nymphs. Only 2 nymphs were positive for *A. phagocytophilum* with an overall infection rate of 2.8%. The 2 infected nymphs were respectively from a PCR-positive *A. peninsulae* and a PCR-negative *A. agrarius* (Table 2).

Table 2. Results of nested PCR for the detection of *A. phagocytophilum* in rodents and their parasitizing ticks collected from Jilin, China

Rodent species	No. of rodents tested	No. of rodents positive (%)	No. of rodents parasitized with ticks	No. of parasitizing ticks tested	No.of ticks positive (%)
Japanese field mouse (*Apodemus peninsulae*)	43	3 (6.98)	12	40	1 (2.5)
Black-striped field mouse (*Apodemus agrarius*)	24	5 (20.8)	5	7	1 (14.3)
Grey red-backed vole (*Clethrionomys rufocanus*)	23	0	7	15	0 (0.0)
Great long-tailed hamster (*Tscherskia triton*)	4	0	1	9	0 (0.0)
Siberian chipmunk (*Tamias sibiricu*)	3	1 (33.3)	0	0	0 (0.0)
Small home rat (*Mus musculus*)	4	0	0	0	0 (0.0)
Brown home rat (*Rattus norvegicus*)	1	0	0	0	0 (0.0)
Total	102	9 (8.8)	25	71	2 (2.8)

All the nested-PCR positive specimens, including 6 questing ticks, 9 rodents and 2 parasitizing *I. Persulcatus,* were re-amplified with the initial primer set (GE9f and GE10r). A 919-bp product obtained from each specimen was sequenced and compared with the previously published sequences deposited in GenBank. The nucleotide sequences from the above positive specimens were identical to each other and to the *A. phagocytophilum* sequences (GenBank accession no. AF205140) previously reported in Heilongjiang Province of China (Cao *et al*. 2000; Cao *et al*. 2003), but different from the corresponding sequences of *A. phagocytophilum* variants in other countries.

The samples positive for the *A. phagocytophilum* 16S rRNA gene were further tested by another nested-PCR assay targeting *gltA*, and all showed positive results. The sequence analysis of partial *gltA* (357-bp) revealed the nucleotide sequences amplified from any rodents and ticks had 100% homology, and varied from all the known *A. phagocytophilum* sequences deposited in GenBank. The agent identified in the study appeared to be most closely related to *A. phagocytophilum* detected in *I. persulcatus* tick from Russia (GenBank accession no. AY339602), with only 2-bp differences (99.4% similarity). The homology of the sequence with other *A. phagocytophilum* variants ranged from 87%–93%. Furthermore, the deduced amino acid sequence of the agent found in this study only differed at 1 position, with 99.1% identity to the Russian strain mentioned above.

Discussion

The findings of the current study demonstrate the presence of *A. phagocytophilum* in a mountain forest area of Jilin Province, China. To our knowledge, this is the first identification of *A. phagocytophilum* in this area. The infection rate of *A. phagocytophilum* in *I. persulcatus* ticks was 4.0%, which is in agreement with the prevalence of 4.6% in the same tick species previously collected in northeastern China (Cao *et al*. 2003), but obviously higher than that in our initial study (Cao *et al*. 2000). The infection rate in male ticks seems higher than that in the female. These diversities could be attributable to geographical and seasonal variations of infected ticks or to different sampling approaches. Further studies using a greater number of tick samples in different seasons, locations, and habitats are required to understand the infection levels. Discrepant infection rates of *A. phagocytophilum* in ticks have been observed around the world. The prevalence of *A. phagocytophilum* infection could be < 1% in adult *I. pacificus* from California (Barlough *et al*. 1997), and in *I. ricinus* from the United Kingdom and Switzerland (Guy *et al*. 1998; Pusterla *et al*. 1999). Higher prevalences were reported in *I. scapularis* in the United States (Pancholi *et al*. 1995; Varde *et al.* 1998) and *I. ricinus* in Europe (Schouls *et al*. 1999; von Stedingk *et al*. 1997). *I. persulcatus* is a three-host tick known to bite various wild and domestic animals as well as humans in China. Therefore, the presence of *A. phagocytophilum* in the tick poses a potential health threat to humans and animals.

In the study, *A. phagocytophilum* was first detected in *D. silvarum* in the world, although the infection rate in the tick (0.7%) was significantly lower than that in *I.*

persulcatus. *A. phagocytophilum* is well documented to be associated with *Ixodes* ticks that may act as vectors, however, the agent has also been reported in *Dermacentor* ticks such as *D. reticulates* in Austria (Sixl *et al*. 2003) and *D. variabilis* in California (Holden *et al.* 2003). The presence in alternate ticks may be due to the existence of secondary maintenance cycles, in which *A. phagocytophilum* circulates between relatively host-specific, usually nonhuman-biting ticks and their hosts. The additional cycles would possibly buffer the agent from local extinction and assist to re-establish the primary cycles (Goethert and Telford 2003). *D. silvarum* is an three-host tick well adapted to a broad range of habitats and infests a variety of domestic animals such as scalper, sheep, goat and horse, but has seldom been found to feed on humans in China. The finding of this study provides further evidence to the above. The competency of *D. silvarum* as a vector for *A. phagocytophilum* and its significance in veterinary medicine has yet to be demonstrated.

Among the 7 rodent species trapped in the study, *A. peninsulae, A. agrarius* and *T. sibiricu* were found positive for *A. phagocytophilum.* The overall infection rate of 102 rodents was 8.8%. As far as we know, this is the first observation of *A. phagocytophilum* infection in wild animals from China. In the forest areas of northeastern China, *Apodemus* mice and *Clethrionomys* voles are dominant rodents and important hosts for larval and nymphal *I. persulcatus* ticks. This was also the case in this study. It is unknown if *A. phagocytophilum,* as some other tick-borne agents, is maintained through transovarial transmission in ticks (Telford *et al*. 1996). A 2-year longitudinal study carried out in a woodland area in northwest England indicates a seasonal variation in rodent infections, which appears to be associated with seasonal increases in the abundance of *I. trianguliceps* nymphs and adults, but not larvae (Bown *et al.* 2003). This finding further testifies the transstadial, but not transovarial, maintenance of *A. phagocytophilum* by ixodid ticks. Although the *A. phagocytophilum* infection in rodents could be low because they would receive few bites from infected ticks, a rodent-tick cycle of infection was verified. In the United States, molecular and serological studies indicate that the white-footed mouse (*P. leucopus*) is the main host for immature *I. scapularis* ticks, and is apparently the reservoir for *A. phagocytophilum* (Telford *et al.* 1996; Walls *et al*. 1997; Yeh *et al.* 1997). Antibodies against *A. phagocytophilum* were also detected in *Tamias striatus*, *Clethrionomys gapperi* (Walls *et al.* 1997), *Neotoma* spp. and other *Peromyscus* spp (Nicholson *et al*. 1998).

About a quarter of rodents (24.5%) in the study were parasitized with ticks, all of which were *I. persulcatus*. Most of the ticks collected on rodents were larvae (78.9%), and the others were nymphs. Two nymphs were found positive, but no larva was

infected. The roles of rodents in transmission and maintenance of *A. phagocytophilum* in China require further investigations.

In the study, spleen specimens were used to identify the natural infection of *A. phagocytophilum* in rodents with reference to previously reported results. Experimental studies on monkeys infected with *A. phagocytophilum* showed that the spleen harbors the organism for the longest period of time and is the best source for the diagnosis of carrier state by PCR (Foley et al. 1999). In mice that were experimentally infected with the HGE agent, splenic infection was obvious and persistent (Bunnell et al. 1999). A field investigation indicates that spleens from wild small animals are most often infected over other samples such as blood, livers and ears (Liz *et al.* 2000).

The nucleotide sequences of partial 16S rRNA gene amplified from rodents as well as questing and parasitizing ticks were identical to each other and to those previously published in China (Cao *et al*. 2000; Cao *et al*. 2003). To further classify and determine the agents, DNA of *gltA* (357-bp) was amplified and sequenced. The *gltA* sequence analyses indicate that the agent detected in the study is closely related to *A. phagocytophilum* identified in Russian *I. persulcatus* (GenBank accession no. AY339602), with 2-bp difference (99.4% similarity) at nucleotide level and 1-position difference (99.1% similarity) at amino acid level. The nucleotide sequence of the agent has only 87%–93% homology with other *A. phagocytophilum* strains. Sequences of *gltA* exhibit higher variation than the 16S rRNA gene, therefore allowing better discrimination among *Rickettsia* species (Inokuma *et al*. 2001). The agent discovered in this study is unique and worthy of studying its public health and veterinary significances.

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Chapter 6

A Newly Discovered *Anaplasma phagocytophilum* Variant in Rodents from Southeastern China

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A Newly discovered *Anaplasma phagocytophilum* variant in rodents from southeastern China

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Abstract

Anaplasma phagocytophilum was detected by polymerase chain reaction in 13 (14.1%) of 92 rodents captured from a mountainous area of Zhejiang Province in southeastern China. The nucleotide sequences of 1442-bp, nearly entire 16S rRNA gene amplified from these rodents, had 100% identity, but varied from all known corresponding sequences of *A. phagocytophilum* deposited in GenBank. To further identify and classify the variant, fragments of 357-bp partial citrate synthase gene (*gltA*), 849-bp major surface protein 4 gene (*msp4*), and 443-bp groESL heat shock operon gene, were amplified and analyzed. The nucleotide sequences of the partial *gltA* gene amplified from the rodents were identical to each other, but distinct from previously reported *A. phagocytophilum* sequences, as were msp4and groESL. These findings indicate that the newly discovered agent represents a novel *A. phago-cytophilumvariant*.

Introduction

Anaplasma phagocytophilum, which encompasses the former *Ehrlichia phagocytophila*, *E. equi*, and the human granulocytic ehrlichiosis (HGE) agent (Dumler *et al.* 2001), has long been recognized as a veterinary agent and is considered to be an emerging human pathogen of public health significance. Human disease caused by *A. phagocytophilum* (now called human granulocytic anaplasmos is [HGA]) was initially described in the United States in 1994 (Bakken *et al.* 1994, Chen *et al*. 1994), and usually presents as an acute, undifferentiated febrile illness characterized by headache, myalgia, malaise, and hematological abnormalities, such as thrombocytopenia and leukopenia as well as elevated levels of hepatic aminotransferases (Bakken *et al.* 1996). Although confirmed cases of HGA have been rarely reported in other parts of the world, serological and molecular investigations reveal that *A. phagocy-tophilumis* widely distributed across Europe, the Middle East, and Asia (Blanco *et al*. 2002,Cao *et al*. 2000, 2003, 2006, Chae *et al*. 2003,Kawahara *et al*. 2006, Keysary *et al*. 1999, Kim *et al*. 2006, Ogden *et al*. 1998, Ohashi *et al*. 2005, Oteo *et al*. 2001).

A. phagocytophilum can be maintained in various animal reservoirs. Although human infection could be transmitted by direct contact with infected animal blood (Bakken *et al*. 1996), tic bite is considered the most common transmission route. *A. phagocytophilum* has been detected in the following tick species: *Ixodes scapularis, I. pacificus*, and *I. spinipalpis* in the United States (Burkot *et al*. 2001, Daniels *et al*. 1997, Holden *et al*. 2006, Lane *et al*. 2004), *I. ricinusin* continental Europe (Fingerle *et al.* 1999, Oteo *et al*. 2001, Pusterla *et al.* 1999), *I. trianguliceps* in the United Kingdom (Bown *et al*. 2003), and *I. Persulcatus* in eastern Europe (Alekseev *et al.* 2001) and Asia (Cao *et al.* 2000, 2006, Chae *et al.* 2003, Kawahara *et al.* 2006, Ohashi *et al.* 2005). Serologic and molecular evidence has confirmed that wild rodents are naturally infected by *A. phagocytophilum* in the United States (Dumler *et al.* 2005, Yeh *et al*. 1997), Europe (Blanco *et al*. 2002, Bown *et al.* 2003, Ogden *et al*. 1998) and Asia (Cao *et al*. 2006, Kim *et al*. 2006).

In China, *A. phagocytophilum* was detected in *I. persulcatus* and *Dermacentor silvarumticks* and rodents from northeastern provinces where Lyme disease is endemic (Cao *et al.* 2000, 2003, 2006). However, the presence of the agent has not been investigated in southern parts of China. Considering the vast territory of China, limited knowledge about the geographic distribution of *A. phagocytophilum* has prevented diagnosis and study of the potential public health problems of this emerging infectious disease. Furthermore, given the existence of *A. phagocytophilum* in southern China, it

is unclear if the agent is genetically similar to those strains or types found in northern China. The objectives of the current study were to investigate the prevalence of *A. phagocytophilum* in rodents from Zhejiang Province of southeastern China, and to characterize a novel variant of the agent identified by molecular techniques.

Methods

Collection of specimens

In December, 2004, small animals in a mountainous area of Tiantai County in Zhejiang Province of southeastern China were captured using box traps with peanuts as bait (figure 1). After identification of rodent species and sex the spleen was removed from each rodent and stored at 20C°until DNA was extracted.

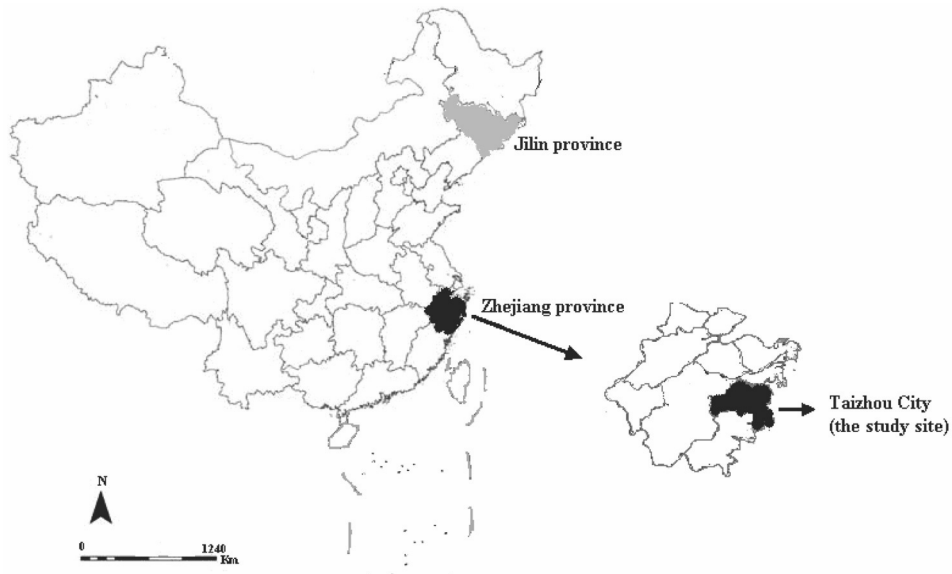

Figure 1. Study site (black shade) where rodents were collected. The gray shade indicates the areas where *Anaplasma phagocytophilumwas* previously detected in rodents and ticks.

DNA extraction

DNA was extracted from spleens using Trizolagent (Invitrogen, Carlsband, CA) following the instructions of the manufacturer. Briefly, about 300 mg of spleen from individual rodents was crushed in Trizol to separate DNA from RNA after centrifugation.

Precipitated DNA was obtained after washing twice in a solution containing 0.1 M sodium citrate in 10% ethanol; then the DNA pellet was suspended in 75% ethanol and kept at room temperature for 10-20 min. After centrifuging at 2000 x *g*, 2-8°C for 5 min, the DNA was dissolved in 8 mM NaOH and centrifuged to remove insoluble material. The supernatant containing the DNA was transferred to a new tube, adjusted to pH 7-8 with HEPES buffer, and stored at 4C° for polymerase chain reaction (PCR).

Polymerase chain reaction amplification

Nested PCR was performed with primers designed to amplify a part of rrs (16S rRNA gene) of *A. phagocytophilum*, as previously described (Cao *et al*. 2006). Both primary and nested PCR amplifications were performed in a volume of 30 µL using a Perkin-Elmer model 2400 thermal cycler. Cycling conditions involved an initial 3-min denaturation at 95°C followed by 35 cycles of at 94°C for 15 sec, 55°C for 20 sec, and72°C for 15 sec, followed by a final extension at 72°C for 5 min. In parallel with each amplification, a positive control (a plasmid containing rrs of *A. phagocytophilum* from the Department of Pathology, The Johns Hopkins University School of Medicine) and a negative control (distilled water) were included.

To further identify the agent in the samples positive for the *A. phagocytophilum* rrs, partial sequences of the *gltA* (citrate synthase gene), *msp4* (major surface protein 4 gene), and *groESL* (heat shock gene) were amplified as previously described (Cao *et al.* 2006, de la Fuente *et al.* 2005, 2007, Inokuma *et al.* 2001, Sumner *et al.* 1997). The primers and cycling conditions used for gltA were described in our previous report (Cao *et al.* 2006), and expected to produce a 357-bp fragment. An expected 849-bp *msp4* gene was amplified directly by MSP4-AP3 and MSP4-AP5 primers (de la Fuente et al. 2005), and an expected 443-bp partial *groESL* was amplified using HS1 and HS6 as outer primers and HS43 and HS45 as inner primers (Sumner *et al*. 1997). PCR amplifications were conducted in a volume of 30 µL using a Perkin-Elmer model 2700 thermalcycler. In parallel with each amplification, a negative control (distilled water) was also included.

All PCR products were separated by agarosegel electrophoresis, stained with ethidium bromide, and visualized under ultraviolet (UV) light. To minimize contamination, DNA extraction, the reagent setup, amplification, andagarose gel electrophoresis were performed in separate rooms.

Sequencing and phylogenetic analyses

All samples positive for *rrs* were re-amplified to obtain a 1442-bp nearly entire rrs, a partial 357-bp *gltA*, 849-bp *msp4*, and 443-bp *groESL*. PCR amplified products were purified by TIANgel Mini Purification Kit (Tiangen Biotech Co., Ltd, Beijing, China) and sequenced directly on both strands by automated dideoxynucleotide cycle sequencing (ABIPRISM 377, Perkin-Elmer, Inc.). The sequences obtained were compared with previously published sequences deposited in GenBank using BLAST and the non redundant nucleotide(nr/nt) database at the National Center for Biotechnology Information website.

Phylogenetic analyses were performed using Mega 3.0 software (Kumar *et al.* 2004). Phylogenetic trees were constructed using the Neighbor-joining (NJ) algorithm method with the Kimura two-parameter model or Poisson-correction method for nucleotide sequence or deduced mino acid sequence analysis, respectively. To examine the effect of the method of analysis on the resulting phylogeny, Maximum parsimony (MP) analyses were also conducted. The stability of the trees obtained was estimated by boot-strap analysis with 1,000 replications.

Statistical analysis

x^2 test or Fisher's exact test (whenever necessary) was used to compare proportions. A *P*-value of <0.05 was considered statistically significant.

Nucleotide sequence accession numbers

The nucleotide sequences of *A. phagocytophilum* amplified from the rodents in thisstudy were deposited in GenBank under the accession numbers DQ458805, DQ458807, and DQ458808 (for 1442-bp rrs), DQ458809, DQ458810, and DQ458811 (for 357-bp *gltA*), and EU008082 and EU008083 (for 849-bp *msp4* and 443-bp *groESL*).

Results

A total of 92 small mammals comprising seven species were collected and tested by nested PCR targeting *A. phagocytophilum rrs*. Thirteen (11 male and 2 female) rodents were positive, with an overall infection rate of 14.1%.There was no significant difference in infection rates between male and female rodents (Fisher's exact test, $P = 0.728$). Five rodent species were found to be infected with *A. phagocytophilum*. The highest positive rate (33.3%) was observed in brown house rats (*Rattus norvegicus*), followed

by 12.5% for Chinese white-bellied rats (*Niviventer confucianus*) and 10.0% for wood rats (*Apodemus sylvaticus*). In addition, one of two lesser rice-field rats (R. losea), and one of four white-bellied giant rats (*N. coxingi*) were also positive (Table 1). Although the prevalence varied from species to species, the difference in infection rates among species was not statistically significant (Fisher's exact test, $P = 0.134$).

Table 1. Results of nested PCR for detection of *A. phagocytophilum* in rodents from southeastern China

Rodent species	No. tested	*A. phagocytophilum*	
		No. positive	Positive rate (%)
Chinese white-bellied rat (*Niviventer confucianus*)	48 (40)†	6 (5)	12.5
Black-striped field mouse (*Apodemus agrarius*)	4 (4)	0	0
Wood rat (*Apodemus sylvaticus*)	20 (18)	2 (2)	10.0
Brown house rat (*Rattus norvegicus*)	9 (7)	3 (2)	33.3
Lesser ricefield rat *(Rattus losea*)	2 (1)	1(1)	50
White-bellied giant rat (*Niviventer coxingi*)	4 (3)	1 (1)	25
Black hamster (*Cricetulus spp.*)	5 (3)	0	0
TOTAL	92 (76)	13 (11)	14.1

† The number in parentheses is the number of male animals

Samples positive for partial *rrs* were re-amplified to obtain a 1442-bp nearly complete *A. phagocytophilum rrs*. After amplicons sequencing, the nucleotides amplified from these rodents were 100% identical and varied from all known *A. phagocytophilum* sequences in Gen-Bank. The newly detected *A. phagocytophilum* sequences were most similar to the sequences amplified from the strain detected in rodents and ticks from Jilin Province of northeastern China (GenBank accession no. DQ342324 and DQ449948; Figuur 1), possessing only a 5-bp difference. The nucleotide sequence of the south China rodent *A. phagocytophilum* differed from sequences most commonly associated with the HGA agent in the USA (GenBank accession no.U02521) by 8 bp, from Japanese *I. ovatustick* isolates (GenBank accession no. AY969010-15) by 11-15 bp, and from the Japanese wild deer ES34 variant (GenBank accession no. AB196720,

1338-bp) by 20 bp. The phylogenetic tree based on the alignments of rrs sequences showed that the agent detected in this study was distant from the previously reported A.phagocytophilum, although they were in the same clade (Figure 2).

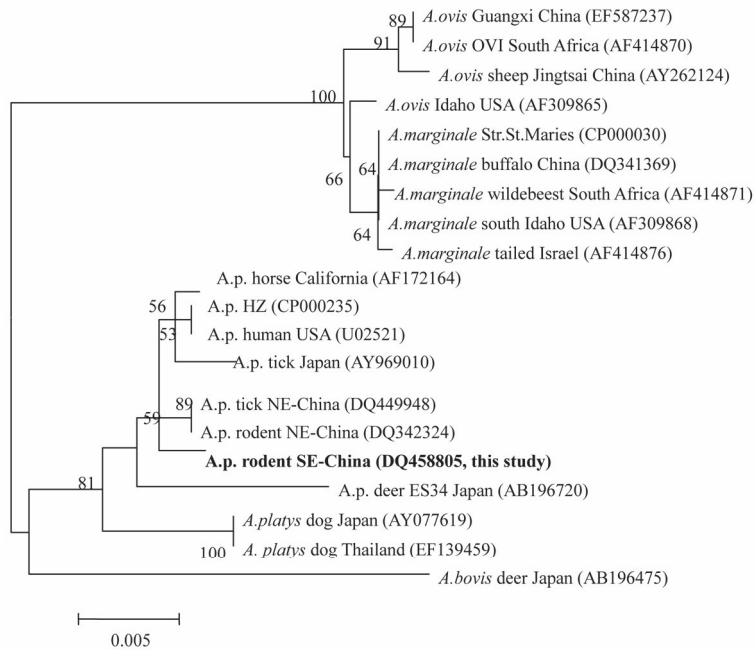

Figure 2. Phylogenetic tree based on 16S rRNA gene (1338-bp) sequences obtained using Neighbor-Joining methodwith Kimura two-parameter analysis and bootstrap analysis of 1,000 replicates. Numbers on the branches indicatethe percent of replicates that reproduced the topology for each clade. The bar represents the number of nucleotidesubstitutions per 1,000 bp. The numbers in parentheses are the GenBank numbers of the sequences used in the phy-logenetic analysis. A.p., *Anaplasma phagocytophilum*; NE-China, Northeast China; SE-China, Southeast China.

To further identify and classify the *A. phagocytophilumvariant* detected in this study, partial sequences of *gltA* (357-bp), *msp4* (849-bp), and *groESL* (443-bp) were also analyzed. The nucleotide sequences of *gltA* fragments amplified from rodents were identical to each other, and showed 85.2%-86.6% identity with previously reported *A. phagocytophilum* strains, and the similarity of deduced amino acid sequences ranged from 81.4% to 83.9%. The topology of phylogenetic trees constructed using NJ andMP

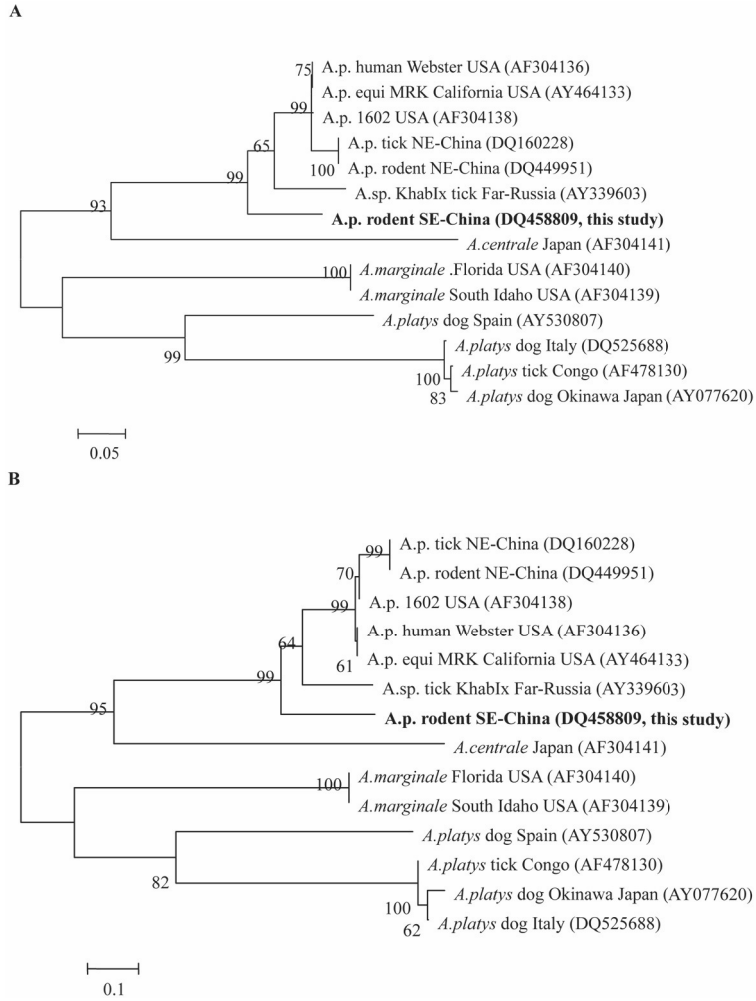

Figure 3. Phylogenetic tree based on *gltA* gene (357-bp; A)and amino acid (B) sequences obtained using Neighbor-Joining method with Kimura two-parameter analysis or Poisson corrections and bootstrap analysis of 1,000 replicates. Numbers on the branches indicate the percent of replicates that reproduced the topology for each clade. The bar represents the number of nucleotide or amino acid substitutions per 1,000 bp or residues. The numbers in parentheses are the GenBank numbers of the sequences used in the phylogenetic analysis. A.p.,*Anaplasma phagocytophilum*; NE-China, Northeast China; SE-China, Southeast China.

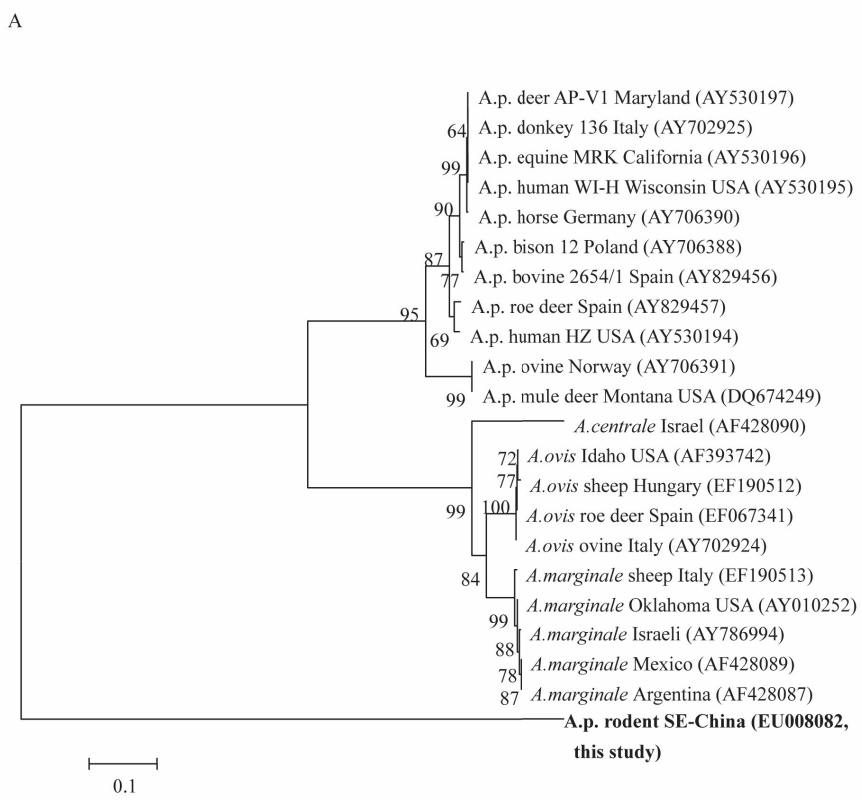

Figure 4. Phylogenetic tree based on *msp4* nucleotide (A)and amino acid (B) sequences using the Neighbor-Joining method with Kimura two-parameter analysis or Poisson corrections and bootstrap analysis of 1,000 replicates. Numbers on the branches indicate percent of replicates that reproduced the topology for each clade. The bar represents the number of nucleotide or amino acid substitutions per 1,000 bp or residues. The numbers in parentheses are the GenBank numbers of the sequences used in the phylogenetic analysis. A.p., *Anaplasma phagocytophilum*; SE-China,Southeast China.

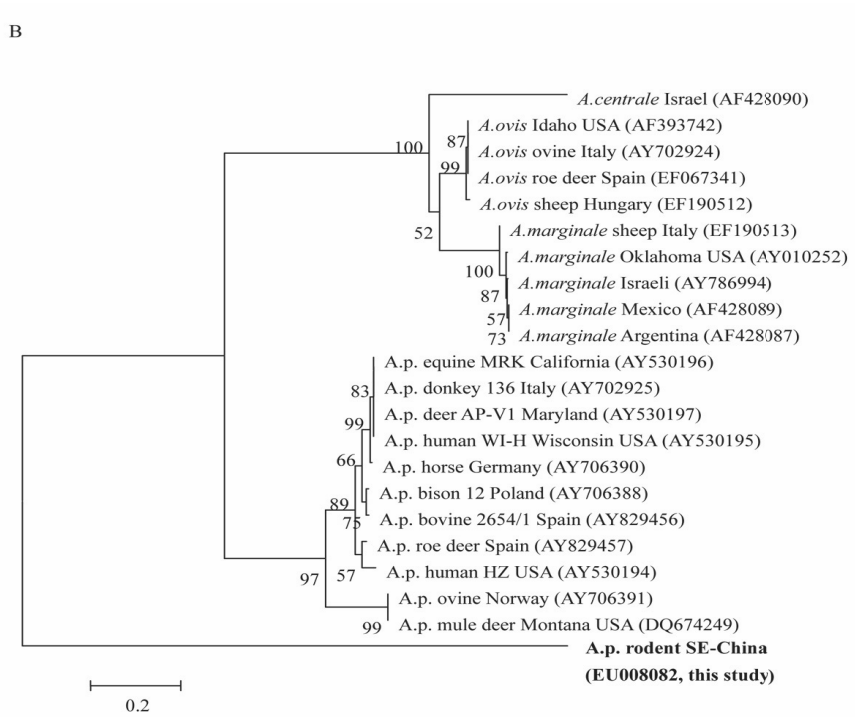

Figure 4. *(Continued).*

methods were similar (data not show), and indicated that the variant was most closely related to the KhabIx *A. phagocytophilum* sequences amplified from the Russian Far East (GenBank accession no. AY339603), distinct from previously reported variants in north-eastern China (GenBank accession no.DQ160228) and in other places (Figure 3). The sequences of 849-bp *msp4* fragments amplified from different rodent species were 100% identical and had 84%-88% identity in nucleotide sequences and 88%-91% identity in deduced amino acid sequences with *A. phagocytophilum* strains available in GenBank. The identities in all corresponding nucleotide sequences with *A. marginale* were 78% and deduced amino acidsequences varied from 59% to 60%, and with *A. ovis*, the identities in nucleotide sequences varied from 66% to 69% and the deduced amino acid sequences changed from 58% to 60%. The msp4-based phylogenetic tree (Figure 4) showed more distance than the tree derived from analyses of other genes. The agent identified in the study was genetically far away from the previously reported

A. phagocytophilum, distinct from other *Anaplasma* species. The 443-bp *groESL* partial gene sequences from positive samples were also compared with known *A. phagocytophilum* sequences in Gen-Bank, and the identity varied from 93% to 95%. In addition, the deduced 124 amino acid residues were 100% identical to those from known *A. phagocytophilum* sequences in Gen-Bank. Phylogenetic analysis of *groESL* showed that the *A. phagocytophilum* identified in this study is placed on a separate branch with the strain detected in Swiss tick, which was distant from those reported in other places (figure 5).

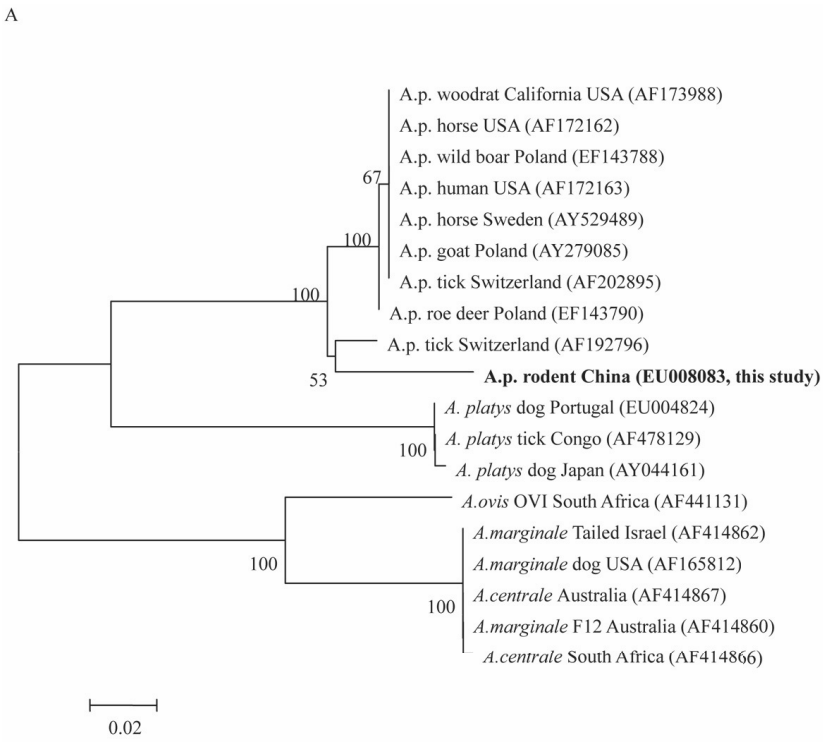

Figure 5. Phylogenetic tree based on *groES* nucleotide, (373-bp, (A) and amino acid (B) sequences using the Neighbor-Joining method with Kimura two-parameter analysis or Poisson corrections and bootstrap analysis of 1,000 replicates. Numbers on the branches indicate percent of replicates that reproduced the topology for each clade. The ba represents the number of nucleotide or amino acid substitutions per 1,000 bp or residues. The numbers in parentheses are the GenBank numbers of the sequences used in the phylogenetic analysis. A.p., *Anaplasma phagocytophilum*; SE-China, Southeast China.

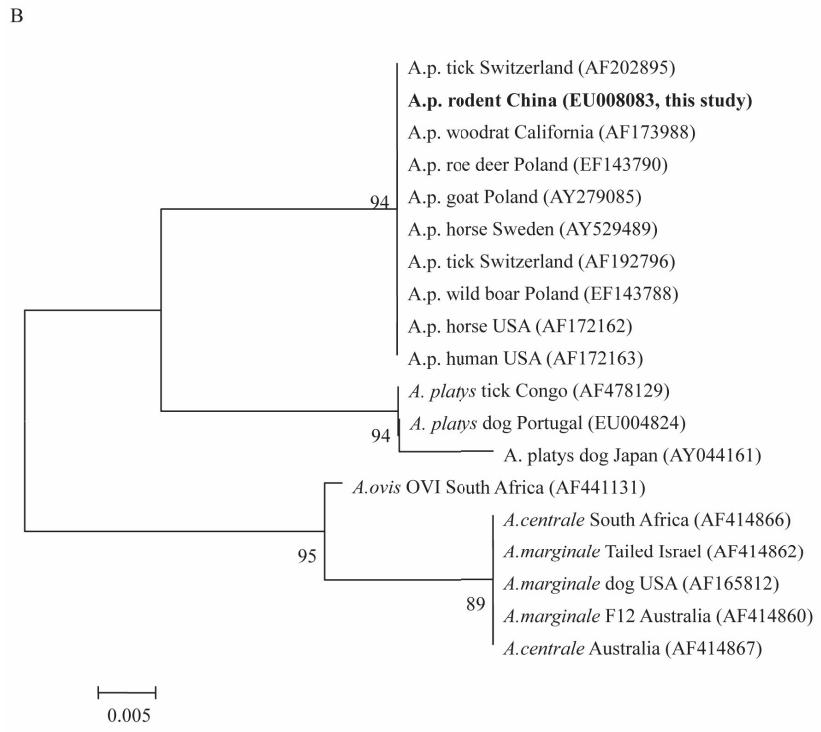

Figure 5. *(Continued).*

Discussion

The current study constitutes part of a larger effort to investigate the distribution of *A. phagocytophilum* in various regions of mainland China. The agent of *A. phagocytophilum* was detected in five rodent species, including *R. norvegicus*, *N. confucianus*, *A. sylvaticus*, *R. losea*, and *N. coxingi* captured from Tiantai, Zhejiang Province (figure 1), with an overall infection rate of 14.1%. These findings provide evidence tha *A. phagocytophilum* is present in wild animals from southeastern China, and imply that rodents may play a role in the enzootic maintenance of *A. phagocytophilum* in the region. Previously, molecular and serological studies demonstrated natural infection of *A. phagocytophilum* in the white-footed mouse (*Peromyscus leucopus*), Neotoma spp., gray squirrels (*Scrius carolinensis*) (Levin *et al*. 2002), other Peromyscus spp. in North America (Levin *et al*. 2002, Stafford *et al*. 1999, Walls *et al*. 1997), Apodemus mice in Europe (Liz *et al*. 2000, Ogden *et al*. 1998), and Japanese field mice (*A.*

peninsulae), Siberian chipmunk (*T. sibiricu*),and Black-striped field mice (*A. agrarius*) in northeastern China (Cao *et al.* 2006) and Korea (Kim *et al*. 2006). *A. phagocytophilum* seems competent to infect a variety of rodent species. Moreover, experimental infection of rodents through tick-bite is also well established (Blas-Machado *et al*. 2007). Even though the role of rodents in transmission and maintenance of *A. phagocytophilum* in the region still remains to be determined, the presence of *A. phagocytophilum* in wild animals suggests a potential health threat to domestic animals and humans, and the public health as well as veterinary importance of these findings should be further investigated.

The nucleotide sequences of the nearly entire *A. phagocytophilum rrs* (1442-bp) amplified from rodents in this study were identical to each other but distinct from all known *A. phagocytophilum rrs* sequences previously reported. The newly characterized *A. phagocytophilum* sequences were most closely related to those from northeastern China (Cao *et al.* 2003, 2006) according to *rrs* sequence analyses. This geographic correlation implies that *A. phagocytophilum* detected in this study will likely reveal high degrees of sequence identity with other Asian strains. In fact, its *rrs* sequence varies from those detected in nearby Japan (Kawahara et al. 2006), and is more similar to sequences in North American isolates. Such a geographic divergence phenomenon has been previously described (de la Fuente *et al*. 2007, Kawahara *et al*. 2006), but remains to be investigated.

The 16S rRNA gene is the standard for species identification and will remain so. To further characterize and classify the novel *A. phagocytophilum* detected in this study, partial *gltA*, *msp4*, and *groESL* were amplified and sequenced. These genes are more variable and are therefore useful for the differentiation of closely related strains of *A. phagocytophilum* (de la Fuente *et al*. 2005, 2007, Inokuma *et al*. 2001, Sumner *et al.* 1997). Sequence analysis of the 357 bp partial *gltA* revealed 100% identity among nucleotide sequences amplified from each positive rodent but greater variation when compared to *A. phagocytophilum* sequences in GenBank. The *gltA*-based phylogenetic tree (Figure 3) showed that the agent identified in the study appeared to be most closely related to sequences amplified from *A. phagocytophilum* KhabIx in the Russian Far East (GenBank ac-cession no. AY339603), but obviously differed from *A. phagocytophilum* detected in northeastern China (GenBank accession no. DQ160228). These phylogenetic analyses indicate that the southeastern China *A. phagocytophilumis* genetically distinct from *A. phagocytophilum* reported in the geographically distinct regions such as North America (Figure 3). Similarly, both

phylogenetic trees based on *msp4* and *groESL* sequences show that southeastern China *A. phagocytophilum* studied here occupy a distinct clade from all known *A. phagocytophilum* not only in ticks, but also infecting ruminants, other small mammals, and humans in the United States and Europe (Figures 4 and 5). The phylogenetic trees based on predicted amino acid sequences provide further support for then ucleotide sequence results. Interestingly, when compared with amino acid sequences de-posited in GenBank, the deduced amino acid sequence of south China *A. phagocytophilum groESL* was 100% identical to most other *A.phagocytophilum* sequences (124 amino acids were compared). Thus, all or most of the nucleotide changes represent silent mutations underscoring the distinct but related genetic history, yet unaltered biological function.

It was recently reported that at least one different *A. phagocytophilum* variant (Ap-variant 1) isolated from deer could have special host tropisms. White-tailed deer (*Odocoileus virginianus*) serves as a reservoir for "Ap-variant 1 strains" in nature, but human patients infected by this agent have yet to be identified. The "Ap-variant 1" has 2 bp different in *rrs* compared to "Ap-ha strains" from human patients, and are unable to establish infection in white-footed mice or SCID mice, but could infect goats by experimental inoculation (Massung *et al*. 2003, 2006, 2006). Sheep infected by genetic variantsof *A. phagocytophilum* have different clinical manifestations, hematological abnormalities, and serological responses (Stuen *et al.* 2003). Obvious gene variation exists in *A. phagocytophilumin* rodents from southeastern China. Further study is required to determine the pathogenicity potential and host range of these new "genotypes" of *A. phagocytophilum*.

In summary, all *A. phagocytophilumgene* sequences obtained from rodents in our study showed diversity that distinguishes them from *A. phagocytophilumin* other ticks, animals, and human patients not only in the United States, but also in Europe. Although more study is needed to fully understand the characteristics of this agent, the data here support the hypothesis that *A. phagocytophilum* identified inrodents from southern China is distinct fromother characterized isolates, strains, or sequences, and represents a novel variant.

Acknowledgments

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Chapter 7

Anaplasma phagocytophilum from Rodents and Sheep, China

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Anaplasma phagocytophilum from rodents and sheep, China.
Emerg Infect Dis, 2010;16(5):764-8.

Abstract

To characterize the strains of *Anaplasma phagocytophilum* in wild and domestic animals in China, we isolated the organism from rodents and sheep in northeastern China. We isolated 3 strains (2 from rodents and 1 from sick sheep) through propagation in BALB/c mice and then cell culture in HL60 cells. The 3 isolates were identified by right-Giemsa staining, immunofluorescence, and electronic microscopy and were characterized by sequence analyses of the 16S rRNA gene, partial citrate synthase gene, major surface protein 4 gene, and heat shock protein gene. The multiple sequences of the 3 isolates were identical to each other but different from all known strains from other countries. The public health and veterinary relevance of the isolates deserves further investigation.

Introduction

Anaplasma phagocytophilum has long been recognized as a veterinary agent and has been proved to be an emerging human pathogen of public health significance. Approximately ≈3000 human granulocytic anaplasmosis (HGA) cases caused by *A. phagocytophilum* were diagnosed in the United States from 1994 to 2005 (Dumler *et al.* 2007) , and sporadic and clustered cases were reported in Europe and China in the past a few years (Stuen 2007; Beltrame *et al*. 2006; Remy *et al*. 2003; Zhang *et al*. 2008). Human are usually infected by exposure to tick bites, although perinatal transmission or contact with infected animal blood were reported in some occasions (Dumler *et al*. 2005). A broad range of animal species are known to carry *A. phagocytophilum,* and humans are involved only as incidental "dead-end" hosts (Dumler *et al*. 2001).

Various *A. phagocytophilum* strains have been isolated from patients (Dumler *et al*. 2001), domestic and wild animals, and ticks in the United States and Europe (Dumler *et al*. 2001; Dumler *et al*. 2005; Foley *et al*. 2008; Kawahara *et al*. 2006; Massung *et al*. 2006a) However, no *A. phagocytophilum* isolate has been obtained so far in Asia, though serological and molecular evidence suggested that ticks, rodents as well as humans were infected by the agent in many Asian countries including China, Japan and Korea (Zhang *et al*. 2008; Cao *et al*. 2003; Cao *et al*. 2006; Kawahara *et al*. 2006; Ohashi *et al*. 2005; Park *et al*. 2003; Chae *et al*. 2003; Zhan *et al*. 2008). Failure to establish the propagation of *A. phagocytophilum* in Asia has inhibited us to understand the biology, pathogenesis, epidemiology, and potential threats of the pathogen to human health. The objectives of this study were to obtain isolates of *A. phagocytophilum in vitro* using HL60 cell line, and to characterize the Chinese strains originated from wild and domestic animals.

Materials and Methods

Collection and preparation of specimens

In May 2009, live rodents were captured in wire mesh traps in the hinterland of the Changbai Mountains (42°45'N, 130°35'E) in Jilin Province, China, where natural infections with A. phagocytophilum in ticks and rodents have been reported (Cao et al. 2006). After their species and sex were identified, trapped rodents were euthanized and anatomized. The spleen was removed from each rodent and ground with sterile normal

saline. Four dying sheep were found at the same site at the same time; blood samples were aseptically collected into tubes containing EDTA-K^{2+}.

Propagation of *A. phagocytophilum* in Balb/c mice

For isolation of *A. phagocytophilum*, the spleen suspensions of the rodents were pooled into 12 groups according to species, and 0.3 mL of spleen suspension was intraperitoneally injected into 48 BALB/c mice (4 in each group). Blood samples from the 4 sheep were also pooled and injected into a group of BALB/c mice by the same means. After 7–14 days, blood samples were collected from each inoculated mouse and evaluated for infection by real-time PCR. All animal experiments were performed according to the approved Institutional Animal Care and Use Committee guidelines.

Isolation of *A. phagocytophilum* in HL60 cells

The HL60 leukemia cell line was used to cultivate *A. phagocytophilum* as described (Goodman *et al*. 1996). A volume of 100–300 µL blood (in EDTA-K^{2+}) from infected BALB/c mice was inoculated into HL60 cells at densities of 2×10^5 to 6×10^5 cells/mL (Goodman *et al*. 1996).

Wright-Giemsa staining and immunofluorescence microscopy

Slides of peripheral blood or the cultured cells were stained with Wright-Giemsa (BaSO DIAGNOSTICS, INC, Zhuhai, China). An indirect immunofluorescence assay was performed after the slides of culture cells were fixed for 10 minutes in a 1:1 solution of methanol and acetone as described (Goodman *et al*. 1996). Horse anti-*A. phagocytophilum* serum (kindly provided by Jenet E. Foley, University of California, Davis, CA, USA) and fluorescein isothiocyanate-conjugated goat antihorse immunoglobulin G (Zhongshan Biotechnique, Inc., Beijing, China) were used for the assay. Serum samples from healthy horses were used as negative controls.

Electronic microscopy

Infected HL60 cells were processed as previously described (Goodman *et al*. 1996). Electron microscopic examination was conducted by using a Tecnai 10 electron microscope (Philips, Amsterdam, the Netherlands).

PCR and sequence analysis

Real-time PCR selective for the major surface protein 2 gene (*msp2*) was used as described by Drazenovich *et al*. (2006). To characterize the *A. phagocytophilum* strains

isolated in the study, we amplifi ed, purifi ed, sequenced, and compared the 16S rRNA gene (*rrs*), partial sequences of the citrate synthase gene (*gltA*), major surface protein 4 gene (*msp4*), and heat shock protein gene (*groEL*) as described (Cao *et al*. 2006; de la Fuente *et al*. 2005; Zhan *et al*. 2008). Phylogenetic analyses were performed and phylogenetic trees were constructed by using Mega 3.0 software (Kumar *et al.* 2004; Zhan *et al.* 2008).

Nucleotide sequence accession numbers

The nucleotide sequences of *A. phagocytophilum* in this study were deposited in GenBank. Accession numbers are GQ412337–GQ412339 for 1431-bp *rrs*, GQ412340–GQ412342 for 342-bp to 350-bp *gltA*, GQ412343–GQ412345 for 428-bp *groEL*, and GQ412346–GQ412348 for 743-bp *msp4*.

Results

A. phagocytophilum in Balb/c mice

A total of 47–20 black-striped field mouse (*Apodemus agrarius*) and 27 great long-tailed hamster (*Tscherskia triton*) – were captured. When tested 7-14 days post inoculation, every mouse in 5 of the 12 groups of inoculated Balb/c mice were positive for *A. phagocytophilum* according to real-time PCR selective for the *msp2* gene; 3 groups came *A. agrarius* mice and 2 were *T. triton* hamsters. Two Balb/c mice were inoculated with anticoagulated blood samples of 4 sick sheep were PCR positive for *A. phagocytophilum* according to PCR. Typical morulae were observed in granulocytes of experimentally infected Balb/c mice (Figure 1, panel A).

A. phagocytophilum in HL60 cells

Three *A. phagocytophilum* strains were propagated in HL60 cells: 1 from *A. agrarius* mice was named China-C-Aa, 1 from *T. triton* hamsters was named China-C-Tt, and 1 from sheep was named China-C-Y. *A. phagocyto-philum* was first observed in Wright-Giemsa stain preparations 5 days after preparation of cultures (Figure 1, panel B). Morulae were found in ≈70% of HL60 cells at 10 days postinoculation. PCR showed all 3 agents cultured to be *A. phagocytophilum*. Blank control cultures (HL60 cells only) and cultures inoculated with blood of uninfected BALB/c mice showed no evidence of infection by Wright-Giemsa stain or PCR. Immunofluorescence microscopy demonstrated specific staining of *A. phagocytophilum* in infected cells (Figure 1, panel C).

Such staining was not observed in uninfected cells or in cells incubated with control serum. Electron microscopy showed cytoplasmic inclusions in infected HL60 cells. The size of individual bacteria varied, and double-layered membranes were clearly observed surrounding electron-lucent and electron-dense forms (Figure 1, panel D).

A. phagocytophilum isolate Sequences

The 1431-bp nearly entire *rrs* sequences of the 3 *A. phagocytophilum* isolates from cultured cells were identical to each other and to the sequences amplified from infected mice as well as from field-collected rodents and sheep. The tested rrs sequences was also identical to the sequences amplified from ticks and rodents captured 3 years ago (GenBank accession no. DQ342324 and DQ449948) in the same area (Cao *et al*. 2006), but different from all known *A. phagocytophilum* sequences depositted in GenBank.

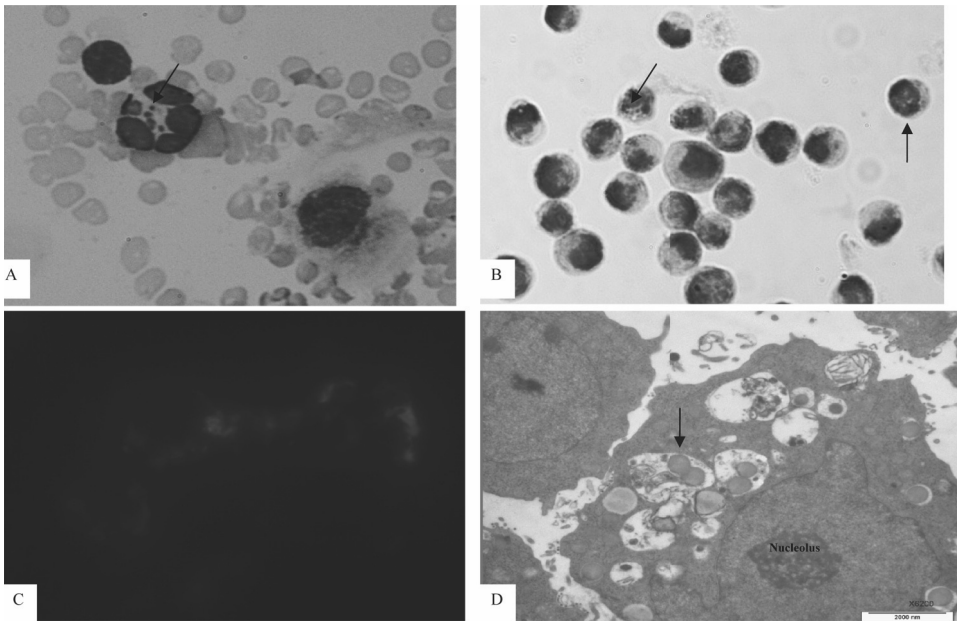

Figure 1. Photomicrographs of cells infected with *Anaplasma phagocytophilum*. A) Wright-Giemsa–stained granulocytic cell of a BALB/c mouse. B) Wright-Giemsa-stained HL60 cells. C) Immunofluorescent-stained infected HL60 cells. D) Electron photomicrographs of an HL60 cell. Original magnifications ×1,500 (A–B), ×1,000 (C) and ×6,200 (D).

To further characterize the *A. phagocytophilum* strains isolated in this study, partial sequences of *gltA* (342-bp), *msp4* (743-bp) and *groESL* (428-bp) were analyzed. The nucleotide sequences of *gltA* fragments amplified from the three isolates were identical to each other, and showed 84% to 99% identity with previously reported *A. phagocytophilum* strains with 3~52 bp difference and 83%-99% similarity of deduced amino acid sequences. Three clades were structured on a phylogenetic tree based on 342-bp nt of the *gltA* gene – including, a clade of strains from the United States, the Russian Far East, and this study; a clade comprising strain from rodent from southeastern China; and a clade of other *Anaplasma* spp., such as *A. centrale, A. marginale and A. platys* (Figure 2).

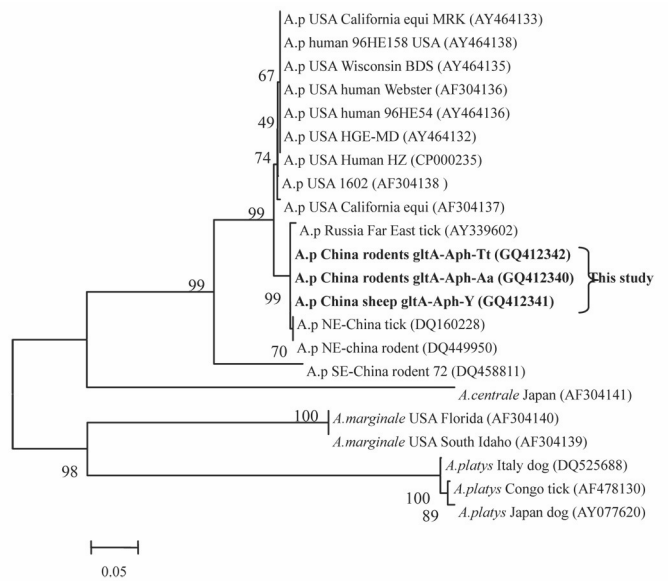

Figure 2. Phylogenetic tree based on partial (348-bp) gltA sequences of *Anaplasma* spp., obtained by using neighbor-joining method with Kimura 2-parameter analysis and bootstrap analysis of 1,000 replicates. Numbers on the branches indicate percentage of replicates that reproduced the topology for each clade. Parentheses enclose GenBank numbers of the sequences used in the phylogenetic analysis. Boldface indicates sequences obtained from rodents and sheep from northeastern China, May 2009. Scale bar indicates number of nucleotides per 1,000 bp. *phago, phagocytophilum*.

The sequences of 779-bp *msp4* fragments amplif ed from the 3 isolates were also 100% identical and had 98%–87% nt sequence identity and 99%–88% deduced 268-*A.*

phagocytophilum strains available in GenBank. When compared with the sequences from rodents in southeastern China (GenBank accession no. EU008082), nucleotide identity was only 87% with a 95-bp difference, and induced amino acid identity was 88% with a 31-aa difference. Phylogenetic analysis placed the *A. phagocytophilum* isolates in this study on a separate branch and in the same clade as the strains from the United States and Europe but far from the strains from sheep in Norway (GenBank accession no. AY706391), mule deer in Montana (DQ674249), and rodents in southeastern China (EU008082) (Figure 3). The other *Anaplasma* spp. were in a separate clade.

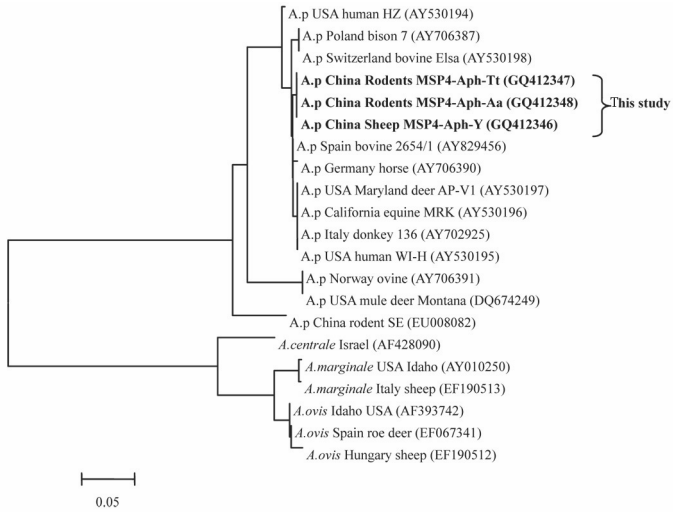

Figure 3. Phylogenetic tree based on partial (779-bp) *msp4* nt sequences of *Anaplasma* spp., obtained by using the neighbor-joining method with Kimura 2-parameter analysis and bootstrap analysis of 1,000 replicates. Numbers on branches indicate percent of replicates that reproduced the topology for each clade. Parentheses enclose GenBank numbers of the sequences used in the phylogenetic analysis. **Boldface** indicates sequences obtained from rodents and sheep from northeastern China, May 2009. Scale bar indicates number of nucleotides per 1,000 bp. *phago, phagocytophilum*.

When the 428-bp groEL sequences of the 3 isolates were compared with known *A. phagocytophilum* sequences in GenBank, the identity varied from 93% to 99%. The phylogenetic tree of *groEL* showed the *A. phagocytophilum* isolates in this study on a separate branch. The strains from hu-mans in China and the United States, horses in United States, dogs in Slovenia, roe deer in Poland and Austria, and ticks in Germany

were in another clade (Figure 4); however, their deduced amino acid sequences were identical to those from patients and rodents in southeastern China.

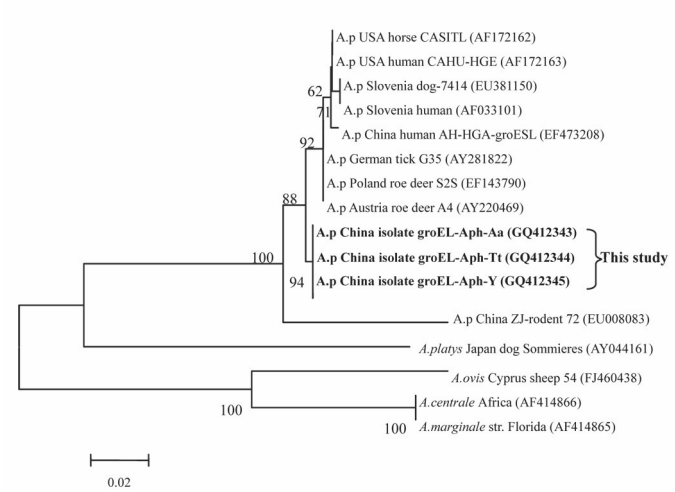

Figure 4. Phylogenetic tree based on partial (428-bp) *groEL* nt sequences of *Anaplasma* spp., obtained by using the neighbor-joining method with Kimura 2-parameter analysis and bootstrap analysis of 1,000 replicates. Numbers on branches indicate percent of replicates that reproduced the topology for each clade. Parentheses enclose GenBank numbers of the sequences used in the phylogenetic analysis. **Boldface** indicates sequences obtained from rodents and sheep from northeastern China, May 2009. Scale bar indicates number of nucleotides per 1,000 bp. *phago, phagocytophilum*.

Discussion

We isolated 3 strains of *A. phagocytophilum* from black-striped f eld mice, great long-tailed hamsters, and sheep in northeastern China. The availability of the isolates in a cell line will permit studies on the genetic, proteomic, and pathogenic characteristics of this agent.

A. phagocytophilum is reportedly maintained in vari-ous animal reservoirs, such as white-footed mice (Ravyn *et al.* 2001), woodrats (Foley *et al*. 2008), goats, sheep (Woldehiwet 2006), and horses (Scorpio et al. 2008) Our isolation of 3 *A. phagocytophilum* strains from *A. agrarius* and *T. triton* rodents and from sheep indicates that both small wild animals and domestic animals may act as competent reservoirs of *A. phagocytophilum* in northeastern China. Although we found cultivation of this organism

from ex-perimentally infected mice to be reliable, the sensitivity of cultivation from wild and domestic animals is uncertain. In addition, the specimens used for isolation were pooled. Consequently, we were unable to ascertain the exact prevalence of infection in the rodents collected for this study. In a previous survey, we found a natural infection rate of 8.8% for *A. phagocytophilum* in rodents in the same area (Cao *et al.* 2006) To determine the level of infectivity in rodents as well as domestic animals, further studies are needed. The nucleotide sequences of the 3 strains in this study were identical to each other in corresponding genes. The 1,431-bp nearly entire *rrs* sequences were most closely re-lated to those detected in rodents from southeastern China (Cao *et al*. 2003; 2006), but they differed from other known strains. The sequence divergences and the phylogenetic analyses of partial *gltA, msp4*, and *groESL* genes indicated that a novel strain of *A. phagocytophilum* might be prevalent in north-eastern China.

Different *A. phagocytophilum* strains seem to have special host tropisms (Foley *et al.* 2008). Strains from sciurids and white-footed mice infect various laboratory animals and perhaps humans as well. *A. phagocytophilum* – variant 1 and the strains from woodrats are found in association with wild-life only; human infections with these strains have yet to be identified. *A. phagocytophilum* – variant 1 has been unable to infect white-footed mice or SCID (severe combined immunodeficiency) mice but could infect goats by experimental inoculation (Massung *et al*. 2003). Holden *et al*. have documented that the pathogenicity of an A. phagocytophilum strain causing human disease waned with mouse passage in C3H mice but could be resurrected by passage in SCID mice (Holden *et al.* 2005). In our study, *A. phagocytophilum* strains with the same molecular characteristics were isolated not only from wild rodents but also from domestic sheep. Furthermore, they could propa-gate in BALB/c mice in the laboratory. The host tropisms and pathogenicity of the isolates remain to be clarified, and the relevance of these findings to public health and veterinary medicine deserves further investigation.

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Chapter 8

Prevalence of *Anaplasma phagocytophila* and *Borrelia burgdorferi* in *Ixodes persulcatus* Ticks from Northeastern China

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Wu XM, Wen BH, Zhang XT, Habbema JDF

Prevalence of *Anaplasma phagocytophila* and *Borrelia burgdorferi* in *Ixodes persulcatus* ticks from northeastern China.

Am J Trop Med Hyg, 2003;68(5):547-550

Abstract

A total of 1345 *Ixodes persulcatus* ticks collected from northeastern China were investigated for the presence of *Anaplasma phagocytophila* and *Borrella burgdorferi* by nested polymerase chain reaction (PCR). Sixty-two (4.6%) ticks were positive for *A. phagocytophila*, and 454 (33.8%) were positive for *B. burgdorferi*. Seven (0.5%) were coinfected with both agents. Sequence analysis of 919-bp PCR amplicons revealed 3 types of *A. phagocytophila*. Type 1 was identical to the published sequences of *A. phagocytophila*sis (HGE). The other two variants differed from HGE agent sequence at one and four positions, respectively. These findings imply that *A. phagocytophila* poses a potential health threat to both humans and animals in northeastern China, and ehrlichiosis should be considered in the differential diagnosis of febrile patients with a history of tick bite, particularly when clinical manifestations are atypical for Lyme disease.

Introduction

Human granulocytic ehrlichiosis (HGE) is an emerging tick-borne disease, first recognized in the upper midwestern United States in 1994 (Bakken *et al*. 1994). Patients with HGE often present relatively nonspecific symptoms that include fever, myalgia, headache, chills, lethargy, and arthralgia and sometimes with leukopenia, thrombocytopenia and a mild elevation of the transaminases (Bakken *et al*. 1996; Walker and Dumler 1996). The etiological agent of HGE is closely related to *Ehrlichia phagocytophila* and *Ehrlichia equi* (Chen *et al*. 1994). However, these species are now considered as one species, and they were named *Anaplasma phagocytophila* in a reclassification (Dumler *et al*. 2001).

A. phagocytophila is transmitted by genus *Ixodes* ticks, including *I. scapularis* (Magnarelli *et al*. 1995; Pancholi *et al*. 1995), and *I. pacificus* (Barlough *et al*. 1997a; Kramer *et al*. 1999) in the United States, *I. ricinus* in Europe (Gug E *et al*. 1998.; Pusterla *et al*. 1999), and *I. persulcatus* in Asia (Cao *et al*. 2000a). These ticks also serve as the vectors of *Borrelia burgdorferi*, the causative agent of Lyme disease. Coinfection of ticks with *A. phagocytophila* and *B. burgdorferi* has been increasingly reported in recent years (Baumgarten *et al*. 1999; Christova *et al*. 2001; Leutenegger *et al*. 1999; Schouls *et al*. 1999). Northeastern China is highly endemic with Lyme disease (Ai *et al*. 1994), where the infection rate of *B. Burgdorferi* in adult *I. persulcatus* ticks was up to 44.1% (Takada *et al*. 1998). Recently, the presence of *A. phagocytophila* in ticks from the region has been reported (Cao *et al*. 2000a). Therefore, an extensive study was carried out to confirm the previous findings, and to investigate coinfection of *I. persulcatus* ticks with *A. phagocytophila* and *B. Burgdorferi*.

Materials and Methods

Collection of tics

I. persulcatus ticks were collected by flagging vegetation from four areas in Inner Mongolia Autonomous Region and Heilongjiang Province in the summers of 1999 and 2001, respectively. All the collection sites are forested highlands belonging to Great Xing-An Mountains and Small Xing-An Mountains. Ticks were kept alive in refrigerator until tested.

Extraction of DNA

After identification, ticks were soaked in 70% ethanol for a few minutes, and then rinsed three times in sterile water. Extraction of DNA was performed as previously described (Cao *et al.* 2000b). Briefly, the ticks were placed into microtubes and mechanically disrupted with sterile scissors in 50 µl DNA extraction buffer (10 mM Tris pH 8.0, 2 mM EDTA, 0.1% sodium dodecyl sulfate, 500 µg of proteinase K per ml). The samples were incubated for 2 h at 56°C, and then boiled at 100°C for 10 min to inactivate proteinase K. After centrifugation, the supernatant was transferred to fresh sterile microtubes and purified by extracting twice with an equal volume of phenol-chloroform before use.

Amplification by a polymerase chain reaction (PCR)

A nested PCR was performed with primers designed to amplify the 16S rRNA gene of *A. phagocytophila* (Cao *et al*. 2000a). Primers GE9f and GE10r, previously described by Chen and others (Chen *et al*. 1994), were used for the primary amplification. The PCR amplifications were performed in a volume of 30 µl in a Perkin-Elmer model 2400 thermal cycler. An initial three-minutes denaturation at 95°C was followed by 35 cycles of 94°C for 15 s, 55°C for 20 s, and 72°C for 15 s, and a final extension at 72°C for 5 min. In nested PCR, the components and conditions were similar to those for the primary amplification, except that primers GE9f and GE2 were used(Massung et al. 1998), and 1 µl of the primary PCR product was used as the template. A positive control (a plasmid containing the 16S rRNA gene of HGE agent, kindly provided by Dr. J. Stephen Dumler at Department of Pathology, The Johns Hopkins Medical Institutions) and a negative control (distilled water) were included with each set of amplifications.

For amplification of *B. burgdorferi* DNA, a nested PCR was carried out with primers derived from *B. burgdorferi* outer surface protein A *(ospA)* gene (Vasiliu *et al*. 1998). For the primary amplification, 3 µl of each template sample was amplified in a 30-µl reaction mixture containing the primers OA1 and OA4. One microliter of the primary PCR product was then used as the template in a second 30-µl reaction mixture with primers BSL and OA4. The PCR amplifications were performed in a Perkin-Elmer model 2400 thermal cycler, using the following protocol: preheating at 95°C for 3 min, followed by 40 cycles of 94°C for 20 s, 50°C for 20 s, and 72°C for 30 s, then a final extension at 72°C for 7 min. In parallel with each amplification of tick specimens, DNA from a *B. burgdorferi* isolate was used as positive control and distilled water was used as a negative control.

Reaction products were separated by agarose gel electrophoresis, stained with ethidium bromide, and visualized under UV light. To minimize contamination, DNA

extraction, the reagent setup, amplification and agarose gel electrophoresis were performed in separate rooms.

Cloning of PCR products and DNA sequencing

The products of nested amplification were collected, and then the purified DNA fragments were cloned with the plasmid vector pGEM-T (Promega Corp., Madison, WI) and transformed into competent cells (*Escherichia coli* XL1-Blue) according to the manufacturer's instructions. The recombinant plasmids were extracted and purified from overnight cultures using QIA prep Spin Miniprep Kit (Qiagen, Valencia, CA). The nucleotide sequences of the plasmid inserts were determined by a dideoxynucleotide cycle sequencing method with an automated DNA sequencer (ABI PRISM 377, Perkin-Elmer).

Results

A total of 1345 *I. persulcatus* ticks were examined for the presence of *A. phagocytophila* and *B. burgdorferi* sensu lato. The distribution of ticks according to origin, sex and stage, and infection status is shown in table 1. *A. phagocytophila* were detected in 4.6% of theticks. Of 643 *I. persulcatus* ticks collected from Wuerqihan and Moerdaoga forestry farms in the Great Xingan Mountains of Inner Mongolia, 40 (6.2%) contained *A. phagocytophila.* The difference in infection rates among male, female and nymphal ticks was not significant (χ^2 = 1.69, degree of freedom [df] = 2, P = 0.43). The positive rate of ticks from Weihe forestry farm in the Small Xingan Mountains of Heilongjiang was 3.1% (22 of 702 ticks), which was significantly lower than that at Inner Mongolia (χ^2 = 6.59, df = 1, P = 0.01). Although *A. phagocytophila* DNA was not detected in nymphal ticks in this area, the frequency of positive ticks was not statistically different regardless of sex or stage (χ^2 = 3.71, df = 2, P = 0.16).

The prevalence of *B. burgdorferi* in ticks from the Great Xingan Mountains was 27.7% (178 of 643 ticks). The infection rate (47.9%) in adults was significantly higher than that (8.0%) in nymphs (χ^2 = 29.28, df = 1, P < 0.001). In contrast to *A. phagocytophila* infection, *B. burgdorferi* infection was more prevalent in ticks at the Small Xingan Mountains (39.3%) than that at the Great Xingan Mountains (χ^2 = 19.80, df = 1, P < 0.001). There was a significant difference in *B. burgdorferi* infection between adult and nymphal ticks from the Small Xingan Mountains (χ^2 = 8.95, df = 1, P = 0.003). Among 62 *A. phagocytophila*-positive *I. persulcatus* ticks, seven were coinfected with

B. burgdorferi. The overall coinfection rate was 0.5%. No difference in proportions of coinfected ticks was demonstrated between the two studied areas.

Table 1. Results of nested PCR for the identification of *A. phagocytophila* and *B. burgdorferi* in *I. persulcatus* ticks from northeastern China

Origin	Stage and sex	No. of ticks	No. (%) of ticks infected with:		
			A. phagocytophila	***B. burgdorferi***	***A. phagocytophila* and *B. burgdorferi***
The Great Xingan Mountains	Male	323	18 (5.6)	106 (32.8)	3 (0.9)
	Female	199	16 (8.0)	63 (31.7)	0
	Nymph	121	6 (5.0)	9 (7.4)	1 (0.8)
The Small Xingan Mountains	Male	283	12 (4.2)	108 (38.2)	1 (0.4)
	Female	341	10 (2.9)	150 (44.0)	2 (0.6)
	Nymph	78	0	18 (23.1)	0
Total		1345	62 (4.6)	454 (33.8)	7 (0.5)

Ten ticks positive for *A. phagocytophila* by the nested PCR were randomly selected for sequence analysis. Of the 10 positive specimens, six (3 males, 2 females and 1 nymph) was from the Great Xingan Mountains, and 4 (2 males and 2 females) were from the Small Xingan Mountains. A 919-bp nucleotide sequence amplified with primer pair GE9f and GE10r (Chen *et al*. 1994) was obtained from each tick specimen. Sequence analysis of the 10 PCR amplicons revealed 3 variants of *A. phagocytophila* (Table 2). The nucleotide sequences from 2 male and 1 female ticks of the Great Xingan Mountains and 1 male tick from the Small Xingan Mountains, were identical to the published sequences of HGE agent. In addition, two sequence variants were detected. Variant 1, isolated from a male, a female and a nymphal tick of the Great Xingan Mountains had a T instead of an A at position 81 according to the corresponding sequence of HGE agent (GenBank accession no. U02521). Variant 2, isolated from a male and two female ticks of the Small Xingan Mountains differed from HGE agent sequence by 4 bases at position 76, 77, 80 and 84 (Table 2).

Table 2. Comparison of partial 16S rRNA gene sequences of *A. phagocytophila* in *I. persulcatus* from northeastern China with published sequences of *A. phagocytophila* with different origins

Geographic origin	Biological origin	Nucleotide difference at position[a]						GenBank accession no.	Reference
		76	77	80	81	84	886		
USA	Human	A	A	A	A	G	G	U02521	4
USA	Horse	A	A	A	A	A	-[b]	M73223	31
USA	Goat	A	A	A	A	A	-[b]	M73220	31
USA	Roe deer	G	A	A	A	A	G	AF384213	20
China	*I. persulcatus*	A	A	A	T	G	G	AY079425	This study
China	*I. persulcatus*	G	G	G	A	A	G	AF205140	This study
Sweden	*I. ricinus*	A	A	A	A	G	G	AJ242785	23
Sweden	*I. ricinus*	G	A	A	A	G	G	AJ242783	23
Sweden	*I. ricinus*	G	A	A	A	A	G	AJ242784	23
Canada	*I. scapularis*	G	A	A	A	A	G	AF311343	30

[a] The position of nucleotide relative to the sequence of HGE agent reported by Chen et al[4].

[b]. – Indicating no nucleotide corresponds to HGE agent; a gap was required at this position to align the adjacent sequences.

Discussion

The prevalence of *A. phagocytophila* infection in *I. persulcatus* ticks was investigated in the forest areas of northeastern China where Lyme disease and the tick-borne encephalitis are endemic. As a result, 4.6% ticks were found to be infected, which further confirmed the existence of *A. phagocytophila* in the region. However, the overall infection rate of ticks determined in the present study is remarkably higher than that in our previous investigation (Cao *et al*. 2000a). A significant difference in positive rate was also demonstrated between ticks from the Great Xingan Mountains and ticks from the Small Xingan Mountains. Recently, discrepant infection rates of *A. phagocytophila* in ticks from different areas around the world have been reported. The prevalence of *A. phagocytophila* infection was 0.8% in adult *I. pacificus* from California(Pancholi *et al*. 1995), and in free-living adult *I. ricinus* from tick-borne fever endemic areas in Switzerland (Pusterla *et al*. 1999), which are comparable to our previous findings (Cao *et al*. 2000a). Higher prevalences were reported in *I. scapularis* and *I. pacificus* in the United States (Barlough *et al.* 1997a; Barlough *et al*. 1997b; Dumler *et al*. 2001; Kramer *et al.* 1999; Magnarelli *et al*. 1995), and *I. ricinus* in Europe (Christova et al. 2001; Gug

et al. 1998.; Pusterla *et al*. 1999; Schouls *et al*. 1999; von Stedingk *et al*. 1997). This discrepancy in positive rate could be attributable to differences in sampling approaches, tick species, to geographic and seasonal variations of infected ticks, or to limits of PCR sensitivity. A study carried out in southern Norway showed that tick samples taken from different locations and at different time points might have different infection rates of infection with *A. phagocytophila* (Jenkins *et al*. 2001). All these findings imply that estimates based on spot investigations may have only local and temporary applicability, and have limited value in forming public health policy *I. persulcatus* ticks are distributed over an extensive area from Russia to eastern Asia, where approximately one-fifth human population of the world resides. The results obtained in the present survey demonstrate that *A. phagocytophila* infection poses a potential health threat to both humans and animals where *I. persulcatus* is abundant, and should be useful to alert public health officials and clinicians of the presence of ehrlichiosis in northeastern China. In contrast to our study, an investigation conducted in the Baltic region of Russia failed to demonstrate *A. phagocytophila* infection in *I. persulcatus* (Alekseev *et al*. 2001). In southern Germany, *ehrlichia*-positive ticks were only found in 1 of 5 surveyed regions, arguing that the distribution of *A. phagocytophila* seems to be focal (Fingerle *et al*. 1999). Further epidemiological studies are required to clarify the diversity, to identify the natural foci, and especially to define potential human and animal infection risks following tick bites in areas infested with *I. persulcatus*-infested.

In forest areas of northeastern China, *I. persulcatus* is the most abundant tick species and responsible for the majority of tick bites in humans. This tick species infests multiple animal hosts, and therefore, may acquire more than one pathogen from different reservoirs. Coinfection of ixodid ticks with *A. phagocytophila* and *B. burgdorferi* has been reported in the United States and many European countries with various prevalences (G Christova *et al*. 2001; Gug E *et al*. 1998; Kramer *et al*. 1999; Jenkins *et al*. 2001 ; Schouls *et al.* 1999;). In our study, seven of 62 ehrlichia-positive ticks were found to harbor both *A. phagocytophila* and *B. burgdorferi*. Coexistence of the two pathogens in *I. persulcatus* ticks from Asia has not been previously reported. The finding suggests that humans may become coinfected with the two pathogens as a consequence of a single tick-bite. In fact, simultaneous human infection with the two agents has already been reported (Duffy *et al*. 1997), and it may lead to variations in clinical symptoms and signs(Nadelman *et al*. 1997; Weber *et al*. 1998). If one considers that *A. phagocytophila* can cause immunosuppression in its mammal hosts, coinfection with two or more tick-borne agents may aggravate the clinical pictures of Lyme disease and tick-borne encephalitis. The identification of *A. phagocytophila* in *I. persulcatus*,

and the finding of coinfection with *B. burgdorferi* in the current study imply that the possible occurrence of ehrlichiosis should be considered in the differential diagnosis of febrile patients with a history of tick bite in the forest areas of northeastern China, particularly when clinical manifestations are atypical for Lyme disease. Unfortunately, *B. burgdorferi* genospecies was not determined in the present investigation. A previous study reported 64.4% *B. garinii* and 35.6% *B. afzelii* based on a polymerase chain reaction-restriction fragment length polymorphism analysis of 45 *B. burgdorferi* isolates from this area (Takada *et al.* 1998). Whether the coinfection was associated with *B. burgdorferi* genospecies remains to be determined.

The nucleotide sequences of PCR products from tick samples were all identified as part of *A. phagocytophila* 16S rRNA gene, and showed high level of identity (99.6% to 100%) with published sequences of the HGE agent (Table 2). Three types of *A. phagocytophila* sequences were found: one with identical sequence of the HGE agent, and two variants with nucleotide differences from the HGE agent by 1 and 4 base pairs, respectively. A variable region was found near the 5' end of 16S rRNA gene at the position from 76 to 84 (according to HGE agent [GenBank accession no. U02521]). This result is consistent with findings of previous studies in other places, in which nucleotide differences were also identified in this variable region but at different positions (Anderson et al. 1991; Baumgarten *et al*. 1999; Drebot *et al*. 2001; Massung *et al*. 1998; von Stedingk *et al*. 1997). It remains to be determined whether each molecular variant of *A. phagocytophila* can cause a disease in humans or animals.

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Chapter 9

Tick-borne Agents in Rodents, China, 2004-2006

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Tick-borne agents in rodents, China, 2004-2006

Emerg Infect Dis, 2009;15(12):1904-1908

Abstract

A total of 705 rodents from 6 provinces and autonomous regions of mainland People's Republic of China were tested by PCR's for the tick-borne agents (*Anaplasma phagocytophilum*, *Borrelia burgdorferi* sensu lato, spotted fever group rickettsiae and *Francisella tularensis)*. Infection rates were 5.5%, 6.7%, 9.1% and 5.0%, respectively. Eighteen (2.6%) rodents of 10 species were positive for 2 or 3 agents. Sequence analysis of PCR products confirmed the presence and genotypes of the detected agents. These findings demonstrate that the tick-borne agents co-circulate and that a variety of rodent species may be involved in their enzootic maintenance.

Introduction

Anaplasma phagocytophilum, Borrelia burgdorferi sensu lato, spotted fever group (SFG) rickettsiae and *Francisella tularensis* are the causative agents of human granulocytic anaplasmasis, Lyme disease, spotted fever and tularemia, respectively. These agents are naturally maintained in animal reservoirs and considered as emerging or reemerging pathogens with serious public health implications. Although these agents could infect human through various routes, ticks play a significant role in the transmission from animal hosts to humans.

Coinfection of these agents have been found in many tick species including *Ixodes scapularis* in northeastern United States, *I. pacificus* and *I. spinipalpis* in western United States, *I. ricinus* in Europe, and *I. persulcatus* in Asia (Swanson *et al.* 2006). Patients coinfected with two tick-borne pathogens usually experience more sever clinical signs of longer duration (Swanson *et al.* 2006). Experimental concurrent infections with *A. phagocytophilum* and *B. burgdorferi* could suppress interleukin-2 (IL-2) and interferon-γ production, promote IL-4 response, increase pathogen load, and intensify Lyme arthritis (Nyarko et al. 2006; Thomas *et al*. 2001; Zeidner et al. 2000;). Natural infection and co-infection of these four agents were reported in China in various tick species (Cao et al. 2003; Z Huang et al. 2006; Zhang et al. 2008) such as *I. persulcatus, Dermacentor silvarum*, *Haemaphysalis concinna, H. longicornis* and *H. warburconi,* which are known to feed on small mammals as well as humans. We hypothesized that multiple agents might be present in rodents of tick-infested areas. The purpose of this study was to identify presence and coexistence of *A. phagocytophilum, B. burgdorferi,* SFGR and *F. tularensis* in rodents from mainland China and to better understand the public health role of these emerging and reemerging pathogens.

Materials and Methods

Sample Collection

During 2004-2006, the rodents were collected at 6 study sites in Heilongjiang Province, Inner Mongolia Autonomous Region, Jilin Province, Zhejiang Province, Guizhou Province and Xinjiang Autonomous Region (Figure 1) at various times accoding to peak seasons of tick species. The first 3 sites were forested highlands in northeastern China, belonging to the Small Xing'an Mountains and the Great Xing'an Mountains, where local residents worked and exposed to rodents and ticks. The study sites in

Zhejiang and Guizhou provinces were forested rolling hills with typical scenery of temperate zone vegetation, these regions attracted hundreds of thousands of tourists per year. The study site in Xinjiang autonomous region was a forest with a rural resident population. Rodents were trapped by using peanut butter as bait. After captured rodent species were identified,the spleen specimens were collected and stored at -20°C until DNA was extracted.

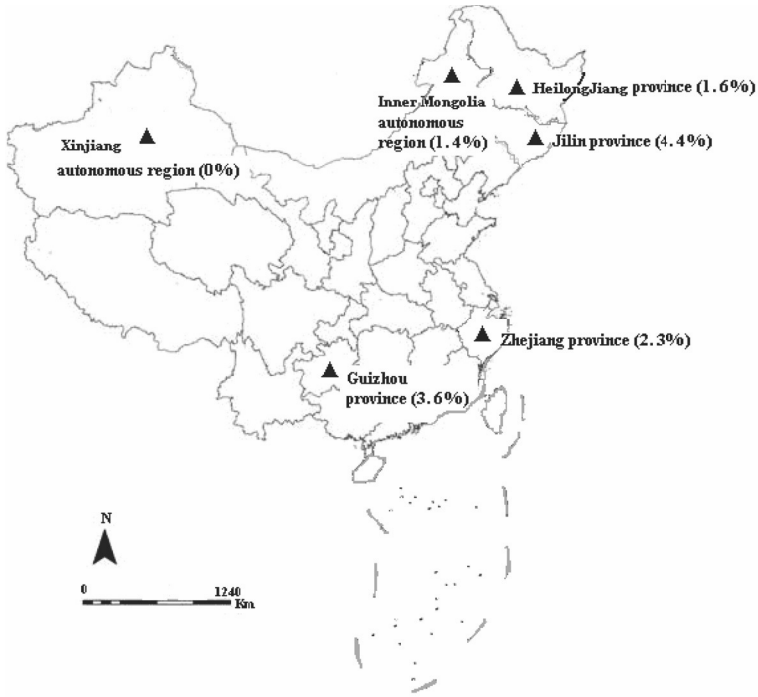

Figure 1. Study sites (black triangles) where rodents were collected , 2004-2006. The numbers in the parentheses are the coinfection rates of rodents with 2 or 3 agents.

Extraction of DNA

Total DNA was extracted from spleen samples using Trizol agent (Invitrogen, Carlsband, CA, USA) following the instructions of the manufacturer. Briefly, approximately 300 mg of spleen tissue from each rodent was crushed in Trizol reagent, and DNA was separated from RNA by centrifugation. DNA was precipitated after washing twice in a solution containing 0.1 M sodium citrate in 10% ethanol, then the DNA pellet was washed in 75% ethanol and kept at room temperature for 10 – 20 min. After centrifugation at 2000

g at 2 – 8°C for 5 min, DNA was dissolved in 8 mM NaOH and centrifuged to remove insoluble material. The supernatant containing the DNA was transferred to a new tube, adjusted with HEPES buffer to pH 7-8.

Polymerase Chain Reaction (PCR)

Nested PCR was conducted with primers design to amplify a part of 16S rRNA gene of *A. phagocytophilum*, as previously described (Cao *et al*. 2000). For amplification of *B. burgdorferi* DNA, a nested PCR was carried out with primers derived from the *B. burgdorferi* 5S-23S rRNA intergenic spacer (Chu *et al*. 2008a). PCR was performed using primers Rr 190.70p and Rr 190-701n to amplify a fragment of the gene encoding a 190-kDa outer membrane protein A *(ompA)* specific for SFGR (Parola *et al*. 2005). Samples were tested for *F. tularensis* by a nested PCR targeting the *fopA* gene, as previously described (Fulop *et al*. 1996). All the PCRs were conducted using a Perkin-Elmer model 2700 thermal cycler. The PCR products were separated by agarose gel electrophoresis, stained with ethidium bromide, and visualized under UV light. In order to avoid contamination, DNA extraction, the reagent setup, amplification, and agarose gel electrophoresis were performed in separate rooms, and negative controls (distilled water) were included in each of amplification.

DNA sequencing and analysis

PCR products of positive samples were sequenced directly by a dideoxynucleotide cycle sequencing method with an automated DNA sequencer (ABI PRISM 377, Perkin-Elmer, Inc.). Two sequencing reactions of each PCR product were performed to limit errors in sequencing. In case of different sequences obtained, the further sequencing reactions were carried out to generate a consensus sequence. The sequences obtained in the present study were compared with the corresponding sequences deposited in GenBank using the BLAST program from the National Center for Biotechnology Information website (http://blast.ncbi.nlm.nih.gov/Blast.cgi).

Statistical analysis

Chi-square tests or Fisher's exact tests (whenever necessary) were used to compare proportions. A P-value < 0.05 was considered statistically significant.

Results

A total of 705 rodents were captured. The number of rodents tested and the positive rate of each agent varied in different survey sites (Table 1). *A. phagocytophilum* was detected only in eastern regions of China (Heilongjiang, Jilin and Zhejiang Provinces) (Figure 1). *B. burgdorferi* was detected in rodents captured at all the 6 survey sites. SFG rickettsiae were detected in rodents captured at all sites except Jilin Province. *F. tularensis* was detected in rodents captured only in northern China (Heilongjiang and Jilin Provinces), and in the Inner Mongolia and Xinjiang Autonomous Regions (Figure 1).

Table 1. Infection rates of *A. phagocytophilum, B. burgdorferi,* SFGR and *F. tularensis* in rodents, China

Study sites	No. of rodents tested	No. positive (%)				p Value
		A.p	B.b	SFGR	F.t	
Heilongjiang province	64	3 (4.7)	3 (4.7)	1 (1.6)	5 (7.8)	0.424
Jilin province	205	20 (9.8)	17 (8.3)	0 (0)	26 (12.7)	0.329
IMAR	148	0 (0)	8 (5.4)	32 (21.6)	2 (1.4)	0.000
XJAR	44	0 (0)	1 (2.3)	4 (9.1)	2 (4.5)	0.348
Zhejiang province	216	16 (7.4)	16 (7.4)	21 (9.7)	0 (0)	0.598
Guizhou province	28	0 (0)	2 (7.1)	6 (21.4)	0 (0)	0.252
Total	705	39 (5.5)	47 (6.7)	64 (9.1)	35 (5.0)	

IMAR: Inner Mongolia autonomous region; XJAR: Xinjiang autonomous region; A.p: *Anaplasma phagocytophilum;* B.b: *Borrelia burgdorferi* sensu lato; SFGR: spotted fever group rickettsiae; F.t: *Francisella tularensis*.

In Heilongjiang province, all 4 agents were detected in rodents at similar frequences (χ^2 = 2.80, degree of freedom [df] = 3, p = 0.424). No SFG-rickettsiae were detected in rodents from Jilin Province. The positive rates of the other 3 agents in Jilin Province did not significantly differ (χ^2 = 2.23, df = 2, p = 0.328). Infectivity rates for SFRS-rickettsiae were was significantly higher than those for *B. burgdorferi* and *F. tularensis* in rodents from Inner Mongolia Autonomous Region did not differ significantly (χ^2 = 39.76, df = 2, p < 0.001). Infectivity rates for the 3 agents in Xinjiang Autonomous Region (χ^2 = 5.01, df = 2, p = 0.082). Except *F. tularensis*, the other 3 agents presented in Zhejiang Province with similar positive rates (χ^2 = 1.30, df = 2, p = 0.523). Only *B.*

burgdorferi and SFG-rickettsiae were found in Guizhou Province, and the difference in their infectivity rates was not significant (Fisher's exact test, p = 0.525).

A total of 18 (2.6%, 95% confidence interval 1.4% – 3.8%) rodents from 5 survey sites except for Xinjiang Autonomous Region were positive for 2 or 3 agents, among which 15 were positive for 2 agents. A *Clethrionomys rufocanus* from Heilongjiang Province was positive for *A. phagocytophilum*, *B. burgdorferi* and SFG-rickettsiae, and 2 rodents *A. agrarius* and *Tamias sibiricu*) from Jilin Province were positive for *A. phagocytophilum*, *B. burgdorferi* and *F. tularensis* (Appendix Table 1, available from www.cdc.gov/EID/content/15/12/1904-appT1.htm).

Overall except for 6 unclassified rodents, 23 species of rodents captured at the 6 survey sites were identified. Rodent species composition varied greatly at different sites (Appendix Table 2, available from www.cdc.gov/EID/content/15/12/1904-appT2.htm). *Rattus norvegicus* were found at all survey sites except Xinjiang Autonomous Region. *A. agrarius, A. peninsulae, Clethrionomys rufocanus, Mus musculus* and *T. sibiricu* rodents were found in northeastern China; *A. sylvaticus, N. confucianus*, and *R. losea* were mainly found in southern China; and *Meriones unguieulataus* and *Mus musculus* were found mainly in western China. The dominant rodent species was different at various study sites. *Clethrionomys rufocanus* (57.8%) was dominant in Heilongjiang Province, *A. agrarius* (36.2%) and *A. peninsulae* (27.1%) in Jilin Province, *A. agrarius* (29.7%) and *Microtus maximowiczii* (23.7%) in Inner Mongolia Autonomous Region, *Mus musculus* (50.0%) and *Meriones Unguieulataus* (34.1%) in Xinjiang Autonomous Region, *N. confucianus* (53,0%) in Zhejiang Province and *R. norvegicus* (32.14%) and *Mus musculus* (28.6%) in Guizhou Province (Appendix Table 2).

To confirm the presence and determine genotypes of detected microorgnisms, PCR products were sequenced and analysed. A 919-bp partial 16S rRNA gene of *A. phagocytophilum* was obtained from each positive specimen (Cao *et al*. 2000). The *A. phagocytophilum* sequences detected in rodents from Heilongjiang and Jilin Provinces (GenBank accession no. DQ342324) were identical and differed from that of Zhejiang Province (GenBank accession no. DQ458808) by 2 bp, from those from ticks in United Kingdom and Sweden (GenBank accession no. AY082656 and AJ242784.1, respectively) by 2 bp, and from other known *A. phagocytophilum* sequences by more than 3 bp.

Sequence analysis of the *B. burgdorferi* 5S–23S rRNA intergenic spacer showed that the agents in rodents from Heilongjiang province, Inner Mongolia autonomous region, Jilin Province and Xinjiang Autonomous Region belonged to *B. garinii* genospeciese similar to that detected in ticks (GenBank accession no. DQ150540) in northern China.

Appendix Table 1. Coexistence of *A. phagocytophilum, B. burgdorferi,* SFGR and F. tularensis in rodents, China

Study sites	No. of rodents tested	No. positive (%)												Total (%)
		A.p & B.b	A.p & SFGR	A.p & F.t	B.b & SFGR	B.b & F.t	SFGR & F.t	Dual infection	A.p & B.b & F.t	A.p & B.b & SFGR	A.p & SFGR & F.t	B.b & SFGR & F.t	Triple infection	
Heilongjiang province	64	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	1 (1.6)	0 (0)	0 (0)	1 (1.6)	1 (1.6)
Jilin province	205	0 (0)	0 (0)	2 (1.0)	0 (0)	5 (2.4)	0 (0)	7 (3.4)	2 (1.0)	0 (0)	0 (0)	0 (0)	2 (1.0)	9 (4.4)
IMAR	148	0 (0)	0 (0)	0 (0)	2 (1.4)	0 (0)	0 (0)	2 (1.4)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	2 (1.4)
XJAR	44	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)
Zhejiang province	216	1 (0.5)	1 (0.5)	0 (0)	3 (1.4)	0 (0)	0 (0)	5 (2.3)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	5 (2.3)
Guizhou province	28	0 (0)	0 (0)	0 (0)	1 (3.6)	0 (0)	0 (0)	1 (3.6)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	1 (3.6)
Total	705	1 (0.1)	1 (0.1)	2 (0.3)	6 (0.9)	5 (0.7)	0 (0)	15 (2.1)	2 (0.3)	1 (0.1)	0 (0)	0 (0)	3 (0.4)	18 (2.6)

IMAR: Inner Mongolia autonomous region; XJAR: Xinjiang autonomous region; A.p: *Anaplasma phagocytophilum;* B.b: *Borrelia burgdorferi* sensu lato; SFGR: spotted fever group rickettsiae; F.t: *Francisella tularensis.*

Appendix Table 2. Species compositions and PCR results for tick-borne agents in rodents, China

Species of rodents	No. of rodents tested *	Study sites*						No. positive (%)				No. (%) Coinfection
		Heilongjiang province	Jilin province	IMAR	XJAR	Zhejiang province	Guizhou province	A.p	B.b	SFGR	F.t	
Apodemus agrarius	142 (20.1)	13 (20.3)	75 (36.2)	44 (29.6)	0 (0)	10 (4.6)	0 (0)	14 (9.9)	4 (2.8)	27 (19.0)	14 (9.9)	4 (2.8)
Apodemus peninsulae	74 (10.4)	13 (20.3)	56 (27.1)	5 (3.4)	0 (0)	0 (0)	0 (0)	4 (5.4)	9 (12.2)	1 (1.4)	7 (9.5)	3 (4.1)
Apodemus sylvaticus	21 (3.0)	0 (0)	0 (0)	0 (0)	0 (0)	21 (9.7)	0 (0)	2 (9.5)	3 (14.3)	7 (33.3)	0 (0)	3 (14.3)
Cricetulus barabensis	11 (1.6)	0 (0)	0 (0)	10 (6.8)	0 (0)	1 (0.5)	0 (0)	0 (0)	1 (9.1)	0 (0)	0 (0)	0 (0)
Cricetulus migratourius	2 (0.3)	0 (0)	1 (0.5)	0 (0)	0 (0)	1 (0.5)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)
Cricetulus triton	5 (0.7)	0 (0)	5 (2.4)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	1 (20.0)	0 (0)	1 (20.0)	1 (20.0)
Cricetulus sp.	39 (5.5)	0 (0)	17 (8.3)	0 (0)	5 (11.3)	17 (7.9)	0 (0)	2 (5.1)	0 (0)	0 (0)	2 (5.1)	0 (0)
Clethrionomys rufocanus	65 (9.2)	37 (57.8)	23 (11.1)	5 (3.4)	0 (0)	0 (0)	0 (0)	3 (4.6)	6 (9.2)	1 (1.5)	2 (3.1)	1 (1.5)
Clethrionomys rutilus	13 (1.8)	0 (0)	0 (0)	13 (8.8)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	1 (7.7)	0 (0)	0 (0)
Meriones unguieulataus	15 (2.1)	0 (0)	0 (0)	0 (0)	15 (34.1)	0 (0)	0 (0)	0 (0)	0 (0)	1 (6.7)	1 (6.7)	0 (0)
Microtus maximowiczii	35 (4.9)	0 (0)	0 (0)	35 (23.7)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	1 (2.9)	0 (0)
Mus pahari	7 (1.0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	7 (25.0)	0 (0)	0 (0)	1 (14.28)	0 (0)	0 (0)
Mus musculus	46 (6.5)	0 (0)	4 (1.9)	12 (8.1)	22 (50.0)	0 (0)	8 (28.6)	0 (0)	2 (4.7)	8 (18.6)	0 (0)	1 (1.3)

Appendix Table 2. *Continued*

Species of rodents	No. of rodents tested *	Study sites*						No. positive (%)				No. (%) Coinfection
		Hei-longjiang province	Jilin province	IMAR	XJAR	Zhejiang province	Guizhou province	A.p	B.b	SFGR	F.t	
Niviventer confucianus	115 (16.2)	0 (0)	0 (0)	0 (0)	0 (0)	115 (53.2)	0 (0)	6 (5.2)	10 (8.7)	8 (7.0)	0	2 (1.7)
Niviventer coxingi	4 (0.6)	0 (0)	0 (0)	0 (0)	0 (0)	4 (1.8)	0 (0)	1 (25)	1 (25)	0 (0)	0 (0)	0 (0)
Niviventer fulvescens	2 (2.8)	0 (0)	0 (0)	0 (0)	0 (0)	2 (0.9)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)
Rattus losea	32 (0.5)	0 (0)	0 (0)	0 (0)	0 (0)	30 (13.9)	2 (7.1)	1 (3.1)	3 (9.4)	6 (18.8)	0 (0)	1 (3.1)
Rattus norvegicus	47 (6.6)	1 (1.6)	13 (6.2)	12 (8.1)	0 (0)	12 (5.6)	9 (32.2)	4 (8.5)	3 (6.4)	2 (4.3)	2 (4.3)	1(2.1)
Tamias sibiricu	18 (2.5)	0 (0)	11 (5.3)	7 (4.7)	0 (0)	0 (0)	0 (0)	1 (5.6)	2 (11.2)	0 (0)	5 (27.8)	1 (5.6)
NI	6 (0.9)	0 (0)	0 (0)	5 (3.4)	0 (0)	1 (0.5)	0 (0)	0 (0)	2 (20)	0 (0)	0 (0)	0 (0)
Others	6 (0.9)	0 (0)	0 (0)	0 (0)	2 (4.6)	2 (0.9)	2 (7.1)	1 (10.0)	0 (0)	0 (0)	0 (0)	0 (0)
Total	705 (100.0)	64 (100.0)	205 (100.0)	148 (100.0)	44 (100.0)	216 (100.0)	28 (100.0)	39 (5.5)	47 (6.7)	63 (8.9)	35 (5.0)	18 (2.6)

IMAR: Inner Mongolia autonomous region; XJAR: Xinjiang autonomous region; A.p: *Anaplasma phagocytophilum;* B.b: *Borrelia burgdorferi* sensu lato; SFGR: spotted fever group rickettsiae; F.t: *Francisella tularensis.*

* The numbers in the parentheses are the proportions of rodent species.

Twelve of the 16 *B. burgdorferi* detected in Zhejiang Provice belonged to *B. garinii* genospeciese, and the other 4 were clasified as *B. valaisiana*-related group (GenBank accession no. EU160458, EU160459). The 2 strains found in Guizhou Province were also *B. valaisiana*-related group (GenBank accession no. EU247840).

For identification of SFG-rickettsiae, the partial nucleotide sequences of *ompA* gene were obtained from the positive specimens in Heilongjiang Province and Inner Mongolia Autonomous Region, and all identical to those of *R. sibirica* genotype (GenBank accession U43807). The nucleotide sequences of .35 specimens positive for *F. tularensis* were identical to each other and to the published sequences of *F. tularensis* subsp. *Holarctica* strain (GenBank accession no. AF247642.2).

Discussion

We detected *A. phagocytophilum*, *B. burgdorferi*, SFG-rickettsiae, and *F. tularensis* in diverse species of rodents from different areas of China. Our findings and previous evidence previously accumulated (Chu *et al*. 2008a; Chu *et al*. 2008b; Zhang *et al.* 2006; Zhang *et al*. 2008; Zhan et al. 2008) suggest that several tick-borne agents co-circulated in mainland China, and a variety of rodent species may be involved in the enzootic maintenance of these agents.

This study is not intended as a comprehensive survey on the active infections of *A. phagocytophilum*, *B. burgdorferi*, SFG-rickettsiae and *F. tularensis.* Rather, it was designed to investigate the presence and extent of these agents in China. If one considers that human infections of *A. phagocytophilum*, *B. burgdorferi*, SFGR and *F. tularensis* have been reported in various regions of mainland China (Ai *et al*. 1987; Fan *et al*. 1999; Li *et al.* 1985; Zhang *et al*. 2008*)*, the presence of these agents in rodents in the study areas suggests a potential threat to humans, and the public health role of these findings should be further investigated.

Although infectivity rates varied at different survey sites (Table 1 and Appendix Table 1), we could not make a conclusion on the geographical diversity of these agents in rodents. The number of rodents examined was limited; therefore, infectivity rates in current study could be biased. In addition, the intensity of circulation of any vector-borne agent fluctuates dramatically both throughout the season and from year to year even at the same location (Bown *et al*. 2003; Wielinga *et al*. 2006), we could not justify comparing infectivity rates between different sites based on unsynchronized one-time collections over a 3-year period. A randomized sampling scheme and further collection of rodent are required to clarify this issue. Unfortunately, we did not collect the ticks from

the captured rodents for testing the tick-transmitted agents. This limitation prevents us from understanding vector potential.

In this study, *A. phagocytophilum* was only discovered in eastern regions of China (Table 1, Figure 1), where it coexisted with the other 3 agents (Appendix Table 1). The *A. phagocytophilum* detected in Heilongjiang, Jilin and Zhejiang provinces were closely related to each other, but distant from other known strains in other countries *B. burgdorferi* presented in all the 6 survey sites. As observed in the previous study (Chu *et al*. 2008a), *B. garinii* was the dominant genospeciese in mainland China, and *B. valaisiana*-related group was present in southern China.

SFG-rickettsiae, including about 20 species of rickettsiae, can be transmitted to animals and humans not only by ticks but also by other arthropod such as infected lice, fleas, and mites (Parola *et al*. 2005). In this study, we amplified *ompA* gene, which is present in most of SFG-rickettsiae was amplified (Parola *et al*. 2005). The overall positive rate of SFG-rickettsiae was highest (9.1%) among the 4 tested agents (Appendix Table 1). Through sequence analysis, the *Rickettsia* identified in Heilongjiang Province and Inner Mongolia Autonomous Region was *R. sibirica* which is known to cause Siberian tick typhus (Fan *et al*. 1999). However, we did not sequenced the PCR products amplified from the rodents at other study sites due to the limited amount of samples. Although the sequence analysis of *ompA* gene fragment alone is not sufficient to identify the agent (Mitsuhiro Ishikura *et al*. 2003), it is commonly used to recognize tick-borne *Rickettsia* in field surveys (Ammerman *et al*. 2004).

F. tularensis was found only in northern China, which verifies our belief that *F. tularensis* is present only in north of 30°N latitude. In many endemic areas, ticks are known to play an important role in transmitting *F. tularensis* from animal hosts to humans, although other arthropods such as deer flies, fleas, mites, mosquitoes are known to carry the bacterium. Sequence analysis showed that all *F. tularensis* in this study belong to *holarctica* subspecies.

Interference of infections among *A. phagocytophilum*, *B. burgdorferi*, SFG-rickettsiae and *F. tularensis* in rodent hosts are not clear. Our findings indicates that the infection of *A. phagocytophilum* dose not intensify for transmission of the other three agents, and vice versa. *B. burgdorferi* in rodent appears increases risk for infection with *F. tularensis*, but does not increase the possibility of infection with SFG-rickettsiae or *A. phagocytophilum*. Further investigations are needed to prove positive or negative interactions of the pathogens and to establish whether this interference is associated with the animal species.

Out of 705 rodents tested in this study, 15 were infected with 2 agents and 3 were infected with 3 agents. These findings indicate that mixed natural-foci of tick-borne agents are present at in the study sites. Because *A. phagocytophilum*, *B. burgdorferi*, SFG-rickettsiae and *F. tularensis* were found in ticks collected in the study areas (Cao *et al*. 2000; Cao *et al*. 2006; Chu *et al*. 2008a; Chu *et al*. 2008b; Huang *et al*. 2006; Zhan *et al*. 2008; Zhang *et al.* 2008), it is not surprising that multiple agents were detected in rodents. Coexistence of multiple agents might be caused by a single bite of a tick infected with several agents or multiple bites of ticks infected with at least one agent. The presence of the 4 pathogens in the study areas demonstrates the risk for multiple infections in humans, which may lead to variations and aggravation of clinical manifestations (Swanson *et al*. 2006). Therefore, the differential diagnosis should be made for febrile patients with a history of tick bite in these areas, particularly when clinical manifestations are atypical for one disease or a related disease.

Among 23 rodent species trapped in this study, 21 were infected with at least one agent (Appendix Table 2). Only 2 species (*Cricetulus migratourius* and *N. fulvescens*), each of which had 2 samples, were negative for all 4 agents. Which species is the main host of each agent remains unknown, because none of the agents is predominantly associated with one or a few related rodent species, regardless of their geographic origin. However, *A. phagocytophilum, B. burgdorferi,* SFG-rickettsiae*,* and *F. tularensis* in various rodent species illustrates their potential roles in maintaining these tick-borne agents. Systematic epidemiologic studies that investigate characteristics of natural foci and the role of small wild animals in transmission of these agents to humans are needed.

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Chapter 10

General Discussion

Answering the research questions

In the general introdution of this thesis, five research questions were formulated. These questions have been addressed in different chapters of the thesis. The answers to the specific questions are as follows.

1. Do *E. chaffeensis* and *A. phagocytophila*, the emerging human pathogens of public health significance, exist in mainland China?

E. chaffeensis and *A. phagocytophila*, the pathogens of HME and HGA, have long been recognized as veterinary agents and are considered to be emerging human pathogens of public health significance (Dumler *et al*. 2001; Rikihisa 1991). They are maintained through enzootic cycles between ticks and animals.

An investigation was carried out in southern China (**Chapter 3**) and found the presence of the 16S rRNA gene DNA of *E. chaffeensis* in *A. testudinarium* and *H. yeni*. This is the first detection of *E. chaffeensis* in ticks from China and the first report of infection in *Haemaphysalis* species in the world. The findings of the study provide preliminary evidence for the presence of *E. chaffeensis* in China, and suggest that a variety of tick species may carry the agent.

It is suggested that the natural cycle of *A. phagocytophila* is probably similar to that of *B. burgdorferi* (Walker and Dumler 1996). *I. persulcatus* is the vector of Lyme borreliosis (Ai *et al*. 1994) in northeastern China, but the occurrence of *A. phagocytophila* in ticks has not been established for this same region. A study was carried out to determine whether or not *A. phagocytophila* is present in *I. persulcatus* ticks in an area where Lyme disease is endemic, and to provide initial data regarding the presence of the agent in China (**Chapter 4**). This is the first detection of *A. phagocytophila* from ticks in Asia, and the first report of infection in *I. persulcatus* anywhere. Then natural infection of *A. phagocytophilum* was confirmed in both ticks and rodents from a forest area of Jilin Province (**Chapter 5**). In southeastern China, a novel *A. phagocytophilum* variant was identified in rodents (**Chapter 6**). These findings indicate that the natural foci of *A. phagocytophilum* extensively exist in mainland China.Three srains of *A. phagocytophilum* were isolated from rodents and sheep in the same area through propagation in Balb/c mice followed by cell culture (**Chapter 7**) (Zhan *et al*. 2009), which represents the first report of isolation of *A. phagocytophilum* in Asia.

2. What are the prevalences of tickborne ricketsial infections in mainland China?

The prevalence of SGF *Rickettsia* infection in *D. silvarum* ticks was 10.7% (**Chapter 2**), which is comparable to the infection rate in *D. nuttallii* from the former Soviet Union (Rydkina *et al*. 1999), *I. ricinus* from Italy (Beninati *et al*. 2002), but is lower than that in *Amblyomma variegatum* from the Caribbean (Kelly *et al*. 2003). The phenomenon of the wide range of *Rickettsia* infection rate in various ticks has been observed in an annual survey in United States (Azad and Beard 1998). The finding that the infection rates of male and female ticks were not significantly different is consistent with that of a previous study (Ammerman *et al*. 2004). However, adult ticks seem more likely to be infected with *Rickettsia* than nymphs. This may be because the ticks at adult stage have fed on more animal hosts with rickettsemias. *Rickettsia* infection was found more common in fed ticks from animals than that in ticks on vegetation. The possibility is that the feeding activation of *Rickettsia s*pp., in which the agent infecting a tick can multiply in response to feeding, increases the probability of detecting the *Rickettsia* using PCR techniques.

Our primary data regarding the prevalence of *E. chaffeensis* in ticks from southern China is shown in **Chapter 3**. Of *A. testudinarium*, 55.2% (16/29) were infected, higher than the infection rate of *A. americanum*, a closely related species found in North America (Roland *et al*. 1998). In addition, 11.7% adult and at least 4.3% nymphal *H. yeni* ticks were positive for *E. chaffeensis*. Attempts to detect the agent in other tick species were unsuccessful. This study is not intended as a comprehensive survey of the ehrlichia distribution in ticks; rather it was designed to investigate the presence of *E. chaffeensis* in China. Because the number of ticks examined was limited, the infection rates of the present study could be biased. A randomized sampling scheme and further collection of the ticks should be made to obtain a reliable estimate.

The infection rates of of *A. phagocytophilum* in ticks from different study sites vary remarkably (**Chapter 4, 5, 8**). Discrepant infection rates of *A. phagocytophila* in ticks from different areas around the world have been reported. The prevalence of *A. phagocytophila* infection was 0.8% in adult *I. pacificus* from California (Pancholi *et al*. 1995), and in free-living adult *I. ricinus* from tick-borne fever endemic areas in Switzerland (Pusterla *et al*. 1999), which are comparable to our previous findings (Cao *et al*. 2000b). Higher prevalences were reported in *I. scapularis* and *I. pacificus* in the United States (Magnarelli *et al*. 1995; Barlough *et al*. 1997; Kramer *et al*. 1999; Barlough *et al*. 1997), and *I. ricinus* in Europe (Gug *et al*. 1998; Schouls *et al*. 1999; Christova *et al*. 2001; Von Stedingk *et al*. 1997). This discrepancy in positive rate could

be attributable to differences in sampling approaches, tick species, to geographic and seasonal variations of infected ticks, or to limits of PCR sensitivity. A study carried out in southern Norway showed that tick samples taken from different locations and at different time points might have different *A. phagocytophila* infection rates (Jenkins *et al*. 2001). All these findings imply that estimates based on spot investigations may have only local and temporary applicability, and have limited value in forming public health policy.

3. Which species of ticks and rodents are naturally infected by rickettsial agents?

D. silvarum ticks are found to be positive for SGF *Rickettsia* (**Chapter 2**). *D. silvarum* is one of the most abundant tick species in northeastern China, and often parasitizes large domestic and wild animals such as cattle, horse, sheep and deer, and also readily feeds on humans as alternate hosts. The significances of the tick species in both veterinary medicine and public health deserve further investigations.

A. *testudinarium* and *H*. *yeni* ticks are found to be infected by *E*. *chaffeensis* in the current study (**Chapter 3**). *A*. *testudinarium* is commonly seen in farmland and mountainous areas of southern and southwestern China. *H*. *yeni* is a dominant species in Fujian Province, which accounts for more than 80% of adult and 85% of immature ticks collected from host animals such as *Muntiacus reevesi* and *Lepus sinesis* (Xu *et al*. 1998).

I. persulcatus and *D. silvarum* ticks are found to be infected by *A. phagocytophilum* (**Chapter 4, 5, 8**) (Cao *et al*. 2000b, 2003, 2006). *A. phagocytophilum* is well documented to be associated with *Ixodes* ticks that may act as vectors, however, the agent has also been reported in *Dermacentor* ticks such as *D. reticulates* in Austria (Sixl *et al*. 2003) and *D. variabilis* in California (Holden *et al*. 2003). The presence in alternate ticks may be due to the existence of secondary maintenance cycles, in which *A. phagocytophilum* circulates between relatively host-specific, usually nonhuman-biting ticks and their hosts (Bown *et al*. 2003; Goethert *et al*. 2003). The additional cycles would possibly buffer the agent from local extinction and assist to re-establish the primary cycles (Goethert *et al*. 2003). *D. silvarum* is an three-host tick well adapted to a broad range of habitats and infests a variety of domestic animals such as scalper, sheep, goat and horse, but has seldom been found to feed on humans in China. The finding of this study provides further evidence to the above.

Among over 20 rodent species trapped in this study, nearly all the species were found positive for at least one agent (**Chapter 5, 6, 9**). Only 2 species (*Cricetulus*

migratourius and *N. fulvescens*), each of which had 2 samples, were negative for all the agents. Which species is the main host of each agent remains unknown, because none of the agents is predominantly associated with one or a few related rodent species, regardless of their geographic origin. However, the presence and coexistence of *A. phagocytophilum, B. burgdorferi* and SFG *Rickettsia* in various rodent species illustrates their potential roles in maintaining these tick-borne agents.

4. What are the genetic characteristics of the tickborne agents detected in mainland China?

Detection and identification of the tick-borne rickettsiae before 1990s mainly depended on culture and epitope recognition techniques, such as immunoflourescence and agglutination tests as well as serotyping with monoclonal antibodies. Recently, new molecular techniques have enabled the development of useful, sensitive, and rapid tools to detect pathogens in ticks (Sparagano *et al*. 1999) and animal hosts. The molecular-sequence-based identification techniques facilitate genetic characterization without need for bacterial culturing ((Billings *et al*. 1998).

The DNA sequences determined for the 587 bp products from the 2 tick species were identical (**Chapter 3**) to the corresponding part of the 16S rRNA gene sequence of the *E. chaffeensis* agent isolated from patients in the United States (GenBank accession number M73222) (Anderson *et al*. 1991).

Obvious gene variation exists in *A. phagocytophilum* in rodents from southeastern China. The nucleotide sequences of partial 16S rRNA gene amplified from rodents in northeastern China were identical to each other (**Chapter 4, 5, 7**). To further classify and determine the agents, DNA of *gltA* (357-bp) was amplified and sequenced. The *gltA* sequence analyses indicate that the agent detected in the study is closely related to *A. phagocytophilum* identified in Russian *I. persulcatus* (GenBank accession no. AY339602), with 2-bp difference (99.4% similarity) at nucleotide level and 1-position difference (99.1% similarity) at amino acid level. The nucleotide sequence of the agent has only 87% – 93% homology with other *A. phagocytophilum* strains. Sequences of *gltA* exhibit higher variation than the 16S rRNA gene, therefore allowing better discrimination among *Rickettsia* species. The agent discovered in this study is unique and worthy of studying its public health and veterinary significances. In southerneastern China, a novel *A. phagocytophilum* variant were identified in rodents (**Chapter 6**). Although more study is needed to fully understand the characteristics of this agent, the data here support the hypothesis that *A. phagocytophilum* identified in rodents from

southern China is distinct from other characterized isolates, strains, or sequences, and represents a novel variant.

5. What are the co-infection rates of tickborne agents in ticks and animal hosts?

Coexistence of the two pathogens in *I. persulcatus* ticks from Asia has not been reported before. The finding suggests that humans may become coinfected with the two pathogens as a consequence of a single tick-bite (**Chapter 8**). In fact, simultaneous human infection with the two agents has already been reported (Duffy *et al*. 1997), and it may lead to variations in clinical symptoms and signs (Nadelman *et al*. 1997; Weber *et al*. 1998). Considering the fact that *A. phagocytophila* can cause immunosuppression in its mammal hosts, coinfection with two or more tick-borne agents may aggravate the clinical pictures of Lyme disease and tick-borne encephalitis. The identification of *A. phagocytophila* in *I. persulcatus*, and the finding of coinfection with *B. burgdorferi* in the current study imply that the possible occurrence of ehrlichiosis should be considered in the differential diagnosis of febrile patients with a history of tick bite in the forest areas of northeastern China, particularly when clinical manifestations are atypical for Lyme disease.

Out of 705 rodents tested in this study, fifteen and 3 were found to be positive for 2 and 3 agents, respectively. These findings indicate that mixed natural-foci of tick-borne agents exist in the study sites. Since *A. phagocytophilum*, *B. burgdorferi*, SFG *Rickettsia* and *F. tularensis* could been found in ticks collected in these areas, it is not surprising that multiple agents were detected in rodents (**Chapter 9**). The coexistence of multiple agents might be caused by a single bite of a tick infected with several agents or multiple bites of ticks infected with at least one agent. Therefore, the differential diagnosis should be made for febrile patients with a history of tick bite in these areas, particularly when clinical manifestations are atypical for one or another related disease.

Lessons learned about the public health significance of the tickborne rickettsial diseases

The epidemiological studies in this thesis are not intended as comprehensive surveys on the active infections of tickborne rickettsial agents, rather designed to investigate the presence and extent of them in mainland China. The findings indicates that *E. chaffeensis*, *A. phagocytophilum* and SFG-rickettsiae exist in extensive areas, and

often co-circulate with other microorganisms such as *B. burgdorferi* and *F. tularensis*. A variety of ticks and rodent species may be involved in the enzootic maintenance of these tickborne agents. In fact, the tickborne rickettsial diseases have been relatively neglected and not been included as notifiable infectious diseases as in other edemic countries. However, human infections of *A. phagocytophilum* and SFG *Rickettsia* have been diagnosed in various regions (Fan *et al*. 1999; Zhang *et al*. 2008*)*. The wide distribution and extensive tick vectors and animal hosts of these agents suggests a potential threat to humans, and the public health significance of these findings should be taken into account.

The infection rates of these tickborne agents varied in ticks or animal hosts from different survey sites. It is not justifiable to compare the positive rates between different sites based on unsynchronized one-time collections over years. Because the intensity of circulation of any vector-borne agent fluctuates dramatically both throughout the season and from year to year, even in the same location (Bown *et al*. 2003; Wielinga *et al*. 2006). A randomized sampling scheme and further collection of samples are required to clarify this question. In mainland China, tens of thousands of patients with febrile diseases are observed each year in tick-infested seasons in different areas. From a public health point of view, we should emphasize the awareness among clinicians, pediatricians, emergency room physicians, and other primary care workers of the widespread presence and possible mixed infections, and its rapid response to doxycycline that characterize these diseases in mainland China, thus to effectively defend against these tick-transmitted infections. The patients who never had a confirmed diagnosis or accurate public health reporting should be empirically treated with doxycycline as early as possible. Accurate and rapid laboratory diagnoses would assist public health strategic planning and sharpen medical knowledge of these diseases.

Conclusions and recommendations

Conclusions

- The emerging rickettsial agents including *E. chaffeensis* and *A. phagocytophilum* are present in a variety of ticks and rodents, which may be involved in the enzootic maintenance of these tickborne agents. The universality of vectors and animal hosts indicates an obvious threat to humans and livestock in mainland China.

- *E. chaffeensis* is only found in southern China. *A. phagocytophilum* and SFG *Rickettsia* distribute in extensive areas, where the tick vectors and animal hosts seem different.
- The *A. phagocytophilum* strains discovered in mainland China are genetically different from those in other continents, and worthy of studying its public health and veterinary significances.
- The existence of *R. sibirica* in China is confirmed, and a SFG *Rickettsia* variant similar to a strain from the former Soviet Union is identified. Spotted fever remains a public health problem in mainland China..
- *A. phagocytophilum* and SFG *Rickettsia* often co-circulate with other micro-organisms such as *B. burgdorferi* and *F. tularensis* in the same natural foci. The fact demonstrates the risk for multiple infections in humans and domestic animals, which may lead to variations and aggravation of clinical manifestations.
- The epidemiological surveys only concentrate on the presence and prevalence of tickborne rickettsial agents in ticks and animal hosts, and have not investigated human infections. Therefore, the public health significances of these tickborne rickettsial pathogens can not be directly appraised according to the findings in the thesis.

Recommendations

- Systematic epidemiological studies are required to estimate the infectivity rates of each agent in ticks and animal hosts from different areas of mainland China, to determine their roles in transmission and maintenance of these agents, and to clarify their public health significance.
- It remains to be determined whether *E. chaffeensis* found in this study cause human disease. The agent should be detected in patients with acute febrile illnesses following tick bites.
- Further studies are required to fully understand the characteristics of the *A. phagocytophilum* strains isolated from animals in northeastern China to understand the potential for human pathogenicity and the public health significance.
- Coexistence of the tickborne agents in the studied areas demonstrates the risk for multiple infections in humans, which may lead to variations and aggravation of clinical manifestations. Therefore, the differential diagnosis should be made for febrile patients with a history of tick bite, particularly when clinical manifestations are atypical for one or another related disease.

- Further investigations are needed to prove positive or negative interactions of these tickborne microorganisms and to establish whether this interference is associated with the animal species.
- Seroepidemiological investigations on the infections of tickborne rickettsial agents should be carried out in human populations especially those living in the tick-infested areas, to obtain the evidence for evaluating their public health significance. In addition, isolation and identification of causative agents from patients will eventually provide direct evidence for human infections.

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Summary

Tickborne rickettsial diseases are a set of distinct diseases with similar clinical manifestations and caused by two groups of intracellular bacteria belonging to the order *Rickettsiales*, *i.e*. the spotted fever group (SFG) of the genus *Rickettsia* within the family Rickettsiaceae and several genera of *Anaplasma* and *Ehrlichia* groups within the family Anaplasmataceae. Various tickborne SFG *Rickettsia* species were found to be pathogenic to humans, including *R. rickettsii* in America, *R. conorii* in Europe and Africa, *R. sibirica* in the former Soviet Union and China, and *R. australis* in Australia (Raoult & Roux 1997). In the past two decades, sveral novel species or subspecies of tickborne SFG rickettsiae were recognized as emerging pathogens throughout the world (Raoult & Roux 1997; Raoult 2002). *Ehrlichia* and *Anaplasma* have long been considered to be only of veterinary importance (Rikihisa 1991). However, in recent years, three species have been identified as emerging tickborne pathogens in humans, *i.e. E. chaffeensis* causing human monocytic ehrlichiosis (HME), *A. phagocytophilum* causing human granulocytic anaplasmosis (HGA), and *E. ewingii* causing granulocytotropic ehrlichiosis (Dumler *et al*. 2001).

These rickettsial pathogens infect and proliferate in the organs of ticks, which act as both reservoirs and vectors of tickborne agents. While feeding, they can transmit rickettsial infections to humans and animals (Parola and Raoult 2001). Because each tick species favours particular optimal environmental conditions and biotopes, the geographic distribution of the ticks is usually restricted to a specific area (small or large) and tickborne rickettsial diseases are natural focus diseases. The different agents are usually associated with specific tick species in certain endemic areas. Small wild animals, the common hosts of immature stages of ticks, are implicated as natural reservoirs for many tickborne agents in various contenent (Telford *et al*. 1996; Bown *et al*. 2003). Moreover, a single tick bite could transmit more than one agents and lead to the development of a single tickborne disease or a coinfection (Parola *et al*. 2005). Patients coinfected with two tickborne pathogens usually showed more sever clinical manifestations and experienced longer duration. Coinfection often leads to variations in clinical manifestations in human and animals as a consequence of tick bites.

In China, only sporadic Siberian tick typhus cases caused by *R. sibirica* (Fan *et al*. 1999) and a cluster of 8 HGA cases occurred in a hospital (Zhang *et al.* 2008) have been diagnosed. However, tens of thousands of patients with unexplained febrile illnesses are observed during the peak period of tick activity. The data on the clinical characteristics, exposure history to ticks and presumptive therapy strongly suggests that at least some of the patients are infected by tickborne reckettsial pathogens. For the time being, the presence and geographical distribution of tick-borne reckettsial agents,

especially the emerging ehrlichiae and anaplasmae, have not been investigated. Lack of such knowledge has inhibited us to understand the ecology, epidemiology and potential threats of the pathogens to human health. Furthermore, given the existence of rickettsial pathogens in mainland China, it is unclear if the agents are genetically similar to the strains or variants found in other countries. To determine the presence of tickborne rickettsial infections in mainland China, epidemiological surveys on various rickettsial agents in ticks were carried out in different geographical locations.

Spotted fever group *Rickettsia* infection in ticks

A total of 676 *Dermacentor silvarum* from a forest area of Jilin Province in northeastern China were examined by PCR for the presence of SFG *Rickettsia*. The overall positive rate was 10.7% with the 95% *CI* from 8.3% to 13.0%. The SFG *Rickettsia* infection was more prevalent in adults than in nymphs ($\chi^2 = 9.17$, $P = 0.002$), and in fed ticks obtained from domestic animals than in those collected on vegetation ($\chi^2 = 12.86$, $P < 0.001$). Sequence analysis of partial *ompA* gene confirmed the existence of *R. sibirica* and discovered a novel rickettsial agent in this area, sequence of which was identical to that of DnS14 genotype *Rickettsia* previously reported in the former Soviet Union (**Chapter 2**).

E. chaffeensis infection in ticks

A total of 717 ticks collected from 3 provinces in southern China during the period of 1996 to 1998 were examined by nested PCR for the presence of *E. chaffeensis*. Sixteen (55.2%) of 29 adult *Amblyomma testudinarium*, 28 (11.7%) of 240 adult and at least 4.2% of 215 nymphal (pooled specimens) *Haemaphysalis yeni* tested positive. Selected positive amplicons were confirmed by DNA sequencing (**Chapter 3**). This is the first detection of *E. chaffeensis* in ticks from China and the first report of infection in *Haemaphysalis* species in the world. The findings of the study provide preliminary evidence for the presence of *E. chaffeensis* in China, and suggest that a variety of tick species may carry the agent.

A. phagocytophilum infections in ticks and animal hosts

A total of 372 adult *Ixodes persulcatus* ticks were collected from vegetation in a forest area of Heilongjiang province in northeastern China, where Lyme disease is known to be endemic. The ticks were examined for the presence of *A. phagocytophilum* by hemi-nested PCR using primers derived from 16S rRNA gene. Of 310 ticks obtained from Dahe forestry farm, 2 pools (each containing 5 ticks) were found positive, with a

minimum infection rate of 0.6%. *A. phagocytophilum* DNA was also detected in one female (1.6%) of 62 ticks collected from Yulin forestry farm. The overall minimum infection rate of the 372 *Ixodes persulcatus* adults was 0.8%. The nucleotide sequences of 919-bp PCR products from the three positive tick specimens were identical to each other, and very closely related to the members in *A. phagocytophilum* genogroup (**Chapter 4**). This is the first identification of granulocytic *ehrlichia* in ticks in Asia, and first report of infection in *I. persulcatus* anywhere.

In another survey conducted in Jilin Provice, 4 (4.0%) of 100 *Ixodes persulcatus* and 2 (0.7%) of 286 *Dermacentor silvarum* ticks collected by flagging vegetation were positive. Nine (8.8%) of 102 rodents were infected, as well as 2 (2.8%) of 71 *I. persulcatus* parasitizing on 25 rodents. The nucleotide sequences of 1442-bp *A. phagocytophilum* 16S rRNA gene amplified from rodents and ticks were identical to each other and to that previously reported in Heilongjiang Province of China (GenBank accession no. AF205140), but different from those of other countries. The sequences of 357-bp partial citrate synthase gene from the above specimens were homologic, and varied from known *A. phagocytophilum* agents (**Chapter 5**). These findings add new information on the ecologic features of *A. phagocytophilum* and indicate the threat of anaplasmosis in the area.

A. phagocytophilum was detected in 13 (14.1%) of 92 rodents captured from a mountainous area of Zhejiang Province in southeastern China. The nucleotide sequences of 1442-bp nearly entire 16S rRNA gene amplified from these rodents had 100% identity, but varied from all known corresponding sequences of *A. phagocytophilum* deposited in GenBank. To further identify and classify the variant, fragments of 357-bp partial citrate synthase gene (*gltA*), 849-bp major surface protein 4 gene (*msp4*) and 443-bp *groESL* heat shock operon gene, were amplified and analyzed. The nucleotide sequences of the partial *gltA* gene amplified from the rodents were identical to each other, but distinct from previously reported *A. phagocytophilum* sequences, as were *msp4* and *groESL* (**Chapter 6**). These findings indicate that the newly discovered agent represents a novel *A. phagocytophilum* variant.

Three srains of *A. phagocytophilum* were isolated from northeastern China through propagation in Balb/c mice followed by cell culture. Two isolates from wild rodents and one from sick sheep were achieved in HL60 cells, and identifed by Wright-Giemsa staining, immunofluorescence and electronic microscopy. Then the 3 isolates were characterized by sequens analyses of the 16S rRNA gene, partial citrate synthase gene, major surface protein 4 gene, and heat shock gene. The multiple sequences of the 3 isolates were identity to each other but different from all know isolation strains

from other contries (**Chapter 7**). This is the first time *Anaplasma phagocytophilum* was isolated in Asia. Successfully isolating *A. phagocytophilum* from both wild rodents and domestic animals confirmed the existence of focus of *Anaplasma phagocytophilum* in the Northeastern China. The public health and veterinary significance of the isolates deserve further investigation.

Coinfection of tickborne agents in ticks and rodents

A total of 1345 *I. persulcatus* ticks collected from northeastern China were investigated for the presence of *A. phagocytophila* and *B. burgdorferi* by nested PCR. Sixty-two (4.6%) ticks were positive for *A. phagocytophila*, and 454 (33.8%) were positive for *B. burgdorferi*. Seven (0.5%) were coinfected with both the agents. Sequence analysis of 919-bp PCR amplicons revealed 3 types of *A. phagocytophila*. Type 1 was identical to the published sequences of human *A. phagocytophila*sis. The other two variants differed from the agent sequence by 1 and 4 positions, respectively (**Chapter 8**). These findings imply that *A. phagocytophila* infection poses a potential health threat to both humans and animals in northeastern China, and anaplasmosis should be considered in the differential diagnosis of febrile patients with a history of tick bite, particularly when clinical manifestations are atypical for Lyme disease.

A total of 705 rodents from 6 provinces and autonomous regions of mainland China were tested by polymerase chain reaction (PCR) assays for the tick-borne agents including *A. phagocytophilum*, *B. burgdorferi* sensu lato, SFG *Rickettsia* and *Francisella tularensis*. Their overall positive rates were 5.5%, 6.7%, 9.1% and 5.0%, respectively. Eighteen (2.6%) rodents of 10 species were found to be positive for 2 or 3 agents. Sequence analysis of PCR products confirmed the presence and genotypes of the detected agents (**Chapter 9**). These findings demonstrate that the tickborne agents co-circulate, and a variety of rodent species may be involved in their enzootic maintenance.

Conclusions and recommendations

The existence of *R. sibirica* in China is confirmed, and a SFG *Rickettsia* variant similar to a strain from the former Soviet Union is identified. The emerging rickettsial agents including *E. chaffeensis* and *A. phagocytophilum* are present in a variety of ticks and rodents, which may be involved in the enzootic maintenance of these tickborne agents. The universality of vectors and animal hosts indicates an obvious threat to humans

and livestock in mainland China. *E. chaffeensis* is only found in southern China. *A. phagocytophilum* and SFG *Rickettsia* distribute in extensive areas, where the tick vectors and animal hosts seem different. The *A. phagocytophilum* strains discovered in mainland China are genetically different from those in other continents, and worthy of studying its public health and veterinary significances. *A. phagocytophilum* and SFG *Rickettsia* often co-circulate with other microorganisms such as *B. burgdorferi* and *F. tularensis* in the same natural foci. The fact demonstrates the risk for multiple infections in humans and domestic animals, which may lead to variations and aggravation of clinical manifestations. The epidemiological surveys only concentrate on the presence and prevalence of tickborne rickettsial agents in ticks and animal hosts, and have not investagated human infections. Therefore, the public health significances of these tickborne rickettsial pathogens can not be directly appraised according to the findings in the thesis.

Systematic epidemiological studies are required to estimate the infectivity rates of each agent in ticks and animal hosts from different areas of mainland China, to determine their roles in transmission and maintenance of these agents, and to clarify their public health significance. It remains to be determined whether *E. chaffeensis* found in this study cause human disease. The agent should be detected in patients with acute febrile illnesses following tick bites. Further studies are required to fully understand the characteristics of the *A. phagocytophilum* strains isolated from animals in northeastern China to understand the potential for human pathogenicity and the publich health significance. Coexistence of the tickborne agents in the studied areas demonstrates the risk for multiple infections in humans, which may lead to variations and aggravation of clinical manifestations. Therefore, the differential diagnosis should be made for febrile patients with a history of tick bite, particularly when clinical manifestations are atypical for one or another related disease. Further investigations are needed to prove positive or negative interactions of these tickborne microorganisms and to establish whether this interference is associated with the animal species. Seroepidemiological investigations on the infections of tickborne rickettsial agents should be carried out in human population especially those living in the tick-infested areas to obtain the evidence for evaluating their public health significance. In addition, isolation and identification of causative agents from patients will eventually provide direct evidence for human infections.

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Curriculum Vitae

Wuchun Cao was born on August 10, 1963 in Shandong Province, People' Republic of China. He received a medical degree from Shandong University in 1985. After graduation, he worked on the control of tropical diseases for several years. After getting a WHO fellowship in 1990, he was trained in Epidemiology of Infectious Diseases at the Faculty of Tropical Medicine, Mahidol University, Bangkok, Thailand. Then he enrolled the Master programme in Health Economics at Chulalongkorn University, Thailand. He transferred to Erasmus University in Rotterdam, where he received his Master and Doctor of Science degrees in Health Services Research at the Netherlands Institute of Health Sciences. Since returning to China in 1996, he has been working at Beijing Institute of Microbiology and Epidemiology (BIME), first as a postdoctoral fellow and subsequently as an associate professor and a professor. As a visiting scholar, he has worked in Institute of Public Health, Cambridge University, UK and Karolinska Institute, Stockholm, Sweden. He is currently the general director of BIME, and the director of the State Key Laboratory of Pathogen and Biosecurity. His research activities are molecular epidemiology, epidemiology and control of infectious diseases, especially emerging infectious diseases, and the application of spatial information technologies (such as GIS and RS) in public health.

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PhD Portfolio Summary

Summary of PhD training and teaching activities

Name PhD candidate: Wu-Chun Cao Erasmus MC Department: Public Health Research School: NIHES	PhD period: 2006-2010 Promotors: Prof.dr. ir. J.D.F. Habbema and Prof.dr. J.H. Richardus

1. PhD training

	Year	Workload
Research skills and in-depth courses		
▪ Epidemiology of Infectious Diseases (Mahidol University, Thailand)	1993-1994	50 hours
▪ Health Economics (Chulalongkorn University, Thailand)	1994 1994-1995	170 hours 600 hours
▪ MSc Health Service Research (NIHES, The Netherlands)	1995-1996	60 hours
▪ DSc Health Service Research (NIHES, The Netherlands)		
Presentations		
▪ First Isolation and Characterization of *Anaplasma phagocytophilum* from Rodents and Sheep in China (Wuhan, China)	2010	0.5 hour
▪ Application of Spatial Information Technology in Infectious Disease Control (Beijing, China)	2010	1 hour
▪ Global Status of Infectious Disease (Beijing, China)	2009	1 hour
▪ Microbial Threats to Health: Challenges and Responses (Munich, Germany)	2007	1 hour
▪ Epidemiological Studies on SARS (Madrid, Spain)	2006	1 hour
International conferences		
▪ International Forum for Surveillance and Control of Mosquitoes and Mosquito-borne Diseases (Beijing, China)	2009	4 days
▪ Tularemia Wet-Lab Exercise (Umea, Sweden)	2009	3 days
▪ The 3rd International Symposium on Emerging Infectious Diseases (Wuhan, China)	2008	3 days
▪ 11th Biological Medical Defense Conference (Munich, Germany)	2007	2 days
▪ 3rd Annual Meeting of the EPISARS Project (Madrid, Spain)	2006	4 days

2. Teaching activities		
	Year	**Workload**
Lecturing		
▪ Infectious Disease Epidemiology for MSc Students of Beijing Institute of Microbiology and Epidemiology (BIME)	2006-2010	12 h annually
▪ Epidemiology on Biodefense for MSc Students of BIME	2006-2010	4 h annually
▪ Survey of Vector-borne Diseases for MSc Students of BIME	2006-2010	8 h annually
▪ Emerging Infectious Disease Control for PhD Students of BIME	2006-2010	20 h annually
Supervising Master's theses		
▪ Supervision of 15 masters and 12 doctoral students	2003-2010	150 h annually