PREVALENCE AND GENETIC ANALYSIS OF ANAPLASMA PHAGOCYTOPHILUM AND SPOTTED FEVER GROUP RICKETTSIAE IN THE TICK IXODES RICINUS IN URBAN AND PERIURBAN SITES IN SOUTHERN GERMANY

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INAUGURAL-DISSERTATION
zur Erlangung der tiermedizinischen
Doktorwürde (Dr. med vet.)
der Tierärztlichen Fakultät der
Ludwig-Maximilians-Universität München

édition scientifique
VVB LAUFERSWEILER VERLAG

1. Auflage 2008

1st Edition 2008

VVB LAUFERSWEILER VERLAG
édition scientifique

STAUFENBERGRING 15, D-35396 GIESSEN
Tel: 0641-5599888 Fax: 0641-5599890
email: redaktion@doktorverlag.de

www.doktorverlag.de

Aus dem Institut für Vergleichende Tropenmedizin und Parasitologie
der Tierärztlichen Fakultät der Ludwig Maximilians-Universität München
Vorstand: Univ.-Prof. Dr. Kurt Pfister

Arbeit angefertigt unter Leitung von Dr. Jérémie Gilles

Prevalence and genetic analysis of *Anaplasma phagocytophilum* and spotted fever group rickettsiae in the tick *Ixodes ricinus* in urban and periurban sites in Southern Germany

Inaugural -Dissertation zur Erlangung
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von

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geb. Semmler

aus München

München 2008

Gedruckt mit Genehmigung der Tierärztlichen Fakultät
der Ludwig-Maximilians-Universität München

Dekan:	Univ.-Prof. Dr. Braun
Berichterstatter:	Univ.-Prof. Dr. Pfister
1. Korreferent:	Univ.-Prof. Dr. Förster
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3. Korreferent:	Univ.-Prof. Dr. Klee
4. Korreferent:	Univ.-Prof. Dr. DDr. hc. Stolle

Tag der Promotion: 18. Juli 2008

Die vorliegende Arbeit wurde nach § 6 Abs. 2 der Promotionsordnung für die Tierärztliche Fakultät der Ludwig-Maximilians-Universität München als kumulative Dissertation gestaltet.

Für Petre

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1. Introduction

Ixodes ricinus is the most abundant hard tick of the family *Ixodidae* in central Europe and has long been known as a reservoir and vector of pathogens (MacLeod, 1932; Parola et al., 2005a).

In 1982, the spirochete *Borrelia burgdorferi* was identified as the causing agent of Lyme disease in *I. scapularis* ticks in the USA (Burgdorfer et al., 1982). Since then, the worldwide medical interest in tick-borne diseases has continued to grow due to newly found pathogens causing so-called emerging diseases and the growing number of clinical cases (Parola et al., 2005b). In Europe, *B. burgdorferi*, the tick-borne encephalitis virus (TBEV), *Anaplasma phagocytophilum* and some other rickettsial species are transmitted by *I. ricinus*. *A. phagocytophilum* causes tick-borne fever of ruminants and canine, equine and human granulocytic anaplasmosis and is widespread in tick populations across Europe, North America and Asia (Rikihisa, 1991; Parola et al., 2005a; Cao et al., 2006). Studies in Germany have shown that *A. phagocytophilum* is present among the *I. ricinus* population with an uneven distribution and an average prevalence ranging from 1.0% to 4.5% (Baumgarten et al., 1999; Fingerle et al., 1999; Hildebrandt et al., 2002; Hartelt et al., 2004; Leonhard, 2005). The 'English Garden', a large recreational park situated in the centre of Munich, State of Bavaria, has been suggested to be a focal point for *A. phagocytophilum* with prevalence up to 8.7% (Fingerle et al., 1999; Leonhard, 2005).

Rickettsial diseases include both some of the oldest and the most recently discovered infectious diseases. Nearly half of the currently recognized rickettsioses have been discovered within the last two decades (Raoult and Roux, 1997). *Rickettsia helvetica*, a member of the spotted fever group (SFG) rickettsiae was first detected in Swiss *I. ricinus* ticks in 1979 and since then from *I. ricinus* in many European countries (Burgdorfer et al., 1979; Parola et al., 1998; Nilsson et al., 1999a; Beninati et al., 2002; Christova et al., 2003; Prosenc et al., 2003; Fernández-Soto et al., 2004; Nielsen et al., 2004; Sréter-Lancz et al., 2005; Bertolotti et al., 2006; Skarphédinsson et al., 2007). In 1999, *R. helvetica* was associated with chronic perimyocarditis in the sudden cardiac death of two young male patients (Nilsson et al., 1999b). Only recently, members of the genus *Rickettsia* have been detected in ticks in Germany, like *R. helvetica* and *R. monacensis* strain IrR/Munich in *I. ricinus* and *Rickettsia* strain RpA4 in *Dermacentor reticulatus* (Simser et al., 2002; Hartelt et al., 2004; Dautel et al.,

2006; Pichon et al., 2006; Wölfel et al., 2006). In 2007, *R. monacensis* has been associated with the febrile disease of two human patients in Spain (Jado et al., 2007).
When evaluating the risk for tick-borne infections in a given geographical area, the first step is the detection of the pathogen in the vector under natural conditions. The highly seasonal character of the tick-borne encephalitis virus and its close relationship to the activity and activation of its tick vector are well known (Korenberg, 2000). This has lead to the discussion whether other tick-borne disease agents follow similar patterns.

The present study focused on three aspects:

(i) The prevalence of *A. phagocytophilum* and SFG rickettsiae in *I. ricinus* in urban and periurban areas of Munich and comparative sites in Southern Germany, determined by molecular methods;

(ii) The analysis of possible seasonal or geographical effects and the effect of the stage and gender of the tick on prevalence rates;

(iii) The genetic diversity of rickettsial species based on sequencing.

As there is only limited data available on the epidemiology and genetic characterization of rickettsial bacteria in *I. ricinus* in Southern Germany, this work will enhance the knowledge and understanding of the endemic cycles and the genetic make-up of these tick-borne bacteria. Thereby, it will contribute to determining risks for the health of people and animals alike.

2. Literature review

2.1. *Ixodes ricinus*

2.1.1. Taxonomy

Ticks belong to the subclass *Acari* within the class *Arachnida* in the phylum *Arthropoda* (Figure 1). They are subdivided into three families: '*Ixodidae*', the hard ticks, '*Argasidae*', the soft ticks, and '*Nuttalliellidae*' (Sonenshine, 1991: Eckert et al., 2005). They are obligate hematophagous arthropods, found as ectoparasites on mammals, birds and reptiles in almost all terrestrial regions on earth. One hundred of the more than 800 known species serve as vectors of important infectious organisms of humans and animals (Hillyard, 1996; Jongejan and Uilenberg, 2004). The most abundant tick species in Germany is *I. ricinus* with a high affinity to biting people (Parola et al., 2005a).

Taxonomy of ticks	
Phylum: Arthropoda	
Subphylum: Amandibulata	
Class: Arachnida	
Subclass: Acari	
Parasitiformes	
Metastigmata (Ixodida)	
Ixodidae	*Amblyomma, Anocentor, Boophilus, Dermacentor, Haemaphysalis, Hyalomma, Ixodes, Rhipicephalus*
Argasidae	*Argas, Ornithodorus, Otobius*
Nuttalliellidae	*Nuttalliella*

Figure 1: Taxonomy of ticks (modified from Eckert et al., 2005)

2.1.2. Distribution and habitat

The habitat of *I. ricinus* stretches from the Iberian Peninsula to the Caspian Sea, from the Southern part of Scandinavia to North Africa in altitudes ranging from 0 to 2000m above sea level (Sonenshine, 1993; Eckert et al., 2005). The favored habitats are forest with more than 75% relative humidity, meadows and pastures (Sonenshine, 1993). Three different climatic

forms are important for survival: *(i)* the macroclimate, above the vegetative layer, *(ii)* the mesoclimate, within the vegetative layer, and *(iii)* the microclimate, within the soil and dense layer of leaf and mould. In the latter, ticks live whilst developing into a new instar or in-between periods of questing activity, as heat loss is reduced and relative humidity is increased. They ascend to the mesoclimate for active questing. The distribution of ticks is also determined by the macroclimate of a geographic region and the availability of suitable hosts (Sonenshine, 1993).

2.1.3. Life cycle

The life cycle of *I. ricinus* includes four stages, the embryonated egg and three active instars, the larva, the nymph and the adult. Each of the active instars needs to take a bloodmeal before continuing development. However, the adult male *I. ricinus* does not need to feed. The reproductive strategy is based on a single gonotrophic cycle. The female finds a suitable sheltered microhabitat, lays several thousand eggs within one to four weeks, and dies (Sonenshine, 1991). The entire life cycle of *I. ricinus*, depending on climatic conditions and the availability of hosts, lasts between two and six years (Sonenshine, 1993).

2.1.4. Seasonal activity

The three active life stages undergo a bimodal seasonal pattern. During the active period, the tick engages in host questing (see 2.1.6.) which is controlled by the photoperiod, solar energy and temperature changes. The developmental cycle of *I. ricinus* consists of spring and autumn feeding populations which are independent of each other (Sonenshine, 1993). Adult tick activity starts when the average daytime temperature reaches 7°C or more and 50% of a given tick population reach the active stage within 10 to 20 days (Korenberg, 2000). Activity quickly reaches a peak in May or early June, and declines over the summer to reach a second smaller peak in autumn (Sonenshine, 1993; Eckert et al., 2005). The exact time of this peak is dependent on the habitat of the questing tick. Ticks living in exposed habitats reach the peak earlier than ticks in sheltered, dense habitats. In its northernmost distribution range, only a single peak is reached (Korenberg, 2000). In general, nymphal and larval peaks follow the same pattern. The colder the climate, the less probable is that the next tick stage will become active in the same year (Sonenshine, 1993; Eckert et al., 2005).

2.1.5. Diapause

Diapause is a period of reduced metabolism and locomotory activity and enables ticks to synchronize the population growth within optimal environmental conditions. Its start and end are regulated by neurohormones reacting to climatic conditions. There are two types of diapause, behavioral and morphogenetic. In the first, the tick stops questing, even when offered suitable hosts. The latter describes a condition where a stage of tick does not develop into the next, thus interrupting its development. All stages of *I. ricinus* can enter morphogenetic diapause (Belozerov, 1982; Sonenshine, 1993).

2.1.6. Host questing

I. ricinus has an exophilic questing behavior of the ambush type, a passive host-finding strategy with the tick waiting on an exposed leafy stem to find a host by direct contact (Sonenshine, 1993; Eckert et al., 2005). Ticks have a highly efficient sensory system for host detection. The forelegs contain the Haller's organ, which can detect CO_2, NH_3, lactic acid and other odors, body temperature and vibration. Until a suitable host is found, *I. ricinus* can stay in the active stage of questing for days or weeks. Changes in the climate can force it to find a more favorable microhabitat. When the climate becomes favorable again, the tick restarts questing. This can be repeated many times (Sonenshine, 1993). *I. ricinus* is questing at a height depending on the host it is targeting. It is not host specific and has three different hosts during its lifetime (Parola et al., 2005a). Usually larvae and nymphs are found lower to the ground (smaller hosts), whereas adults are found at levels of up to more than one meter (larger hosts) (Eckert et al., 2005).

2.1.7. Feeding behavior

I. ricinus takes only one blood meal per stage. Up to 5ml of blood are taken in one meal, remarkably more than in other blood-sucking arthropods (Sonenshine, 1993).

Without directly piercing a blood vessel, the tick cuts through the dermis of the host with its mouthparts and creates a feeding lesion with the aid of salivary secretion. The lesion is massively infiltrated with neutrophilic granulocytes (Herron et al., 2005). Feeding can be divided into two phases: the first phase lasts between two and ten days. Cells and tissue liquid

are taken from the feeding pool and are immediately digested. During this phase, the tick gains about ten times of its original weight. During the second phase, lasting 12 to 24 hours, blood starts to enter the feeding pool (Eckert et al., 2005). The tick gains 70 to 120 times of its original weight. At the end of the feeding period, the tick drops of the host (Sonenshine, 1993).

2.1.8. *Ixodes ricinus* as a vector

Ticks are the most important vectors of diseases to domestic animals worldwide. They transmit an unsurpassed variety of pathogens including viruses, rickettsiae, bacteria, fungi, protozoa and helminthes (Hillyard, 1996; Eckert et al., 2005). *I. ricinus* is known to transmit a variety of diseases in Europe (Table 1).

Table 1: Pathogens and associated diseases transmitted by *Ixodes ricinus*

Pathogen	Disease	Hosts
	Viral diseases	
TBE-Virus	Tick-borne encephalitis	Mammals, humans
Louping-ill virus	Louping-ill	Sheep, humans
	Rickettsioses	
Anaplasma phagocytophilum	Granulocytic anaplasmosis	Humans, horses, dogs, cattle
Rickettsia helvetica	Myocarditis	Humans
Coxiella burnetii	Q-Fever	Sheep, humans
	Bacterial diseases	
Borrelia spp.	Lyme disease	Humans
Staphylococcus aureus	Tick pyraemia of lambs	Lambs
Francisella tularensis	Tularemia	Humans, cats, sheep, rabbits, rodents
	Protozoan diseases	
Babesia divergens	Redwater fever	Cattle
Babesia microti	Human babesiosis	Humans (very rare)

(modified from Eckert et al., 2005 and Hillyard, 1996)

Different routes of transmission of pathogens are known within ticks: (a) transstadial; the tick is infected and the pathogen is transmitted to the next stage. Thus, the pathogen persists in at least two stages; (b) transovarial or vertical; an infected female transmits the infection to embryonated eggs; (c) simultaneous; infection of a non-infected tick by an infected tick by feeding on the same host, probably through neighbouring pools (Eckert et al., 2005); and (d)

sexual transmission from males to females (Hayes et al., 1980). Possibilities to release the pathogen from the tick are excrements, coxal fluid and saliva (Řeháček, 1989).
When the agent is transmitted transovarially and transstadially, the tick becomes a competent reservoir, and serves both as a vector and the main reservoir. The distribution of the pathogen is then equal to the distribution of the tick. If the pathogen is transmitted transstadially only, the tick acts as a competent vector. The reservoir must then be a vertebrate host, on which the distribution of the pathogen is dependent (Parola and Raoult, 2001a).

2.2. The order *Rickettsiales*

Prior to the availability of modern molecular genetic tools for the classification of bacteria, the taxonomic structure of the order was based on features such as morphology, the type of infected cell or serological cross-reactivities (Rikihisa, 1991). With molecular phylogenetic tools, many species were moved within the order, or removed completely. It became clear that there was great disarray within the order, for example, species appeared in two families at the same time. In 2001, Dumler et al. proposed the reorganization of the order *Rickettsiales* on the basis of homology in the nucleotide sequences of the *16S rRNA* gene.
With this reorganization, the order *Rickettsiales* comprises now the families *Rickettsiaceae* and *Anaplasmataceae*. All tribes in the order have been emended, because the affinities of the species are better recognized at family than tribe level. The family *Rickettsiaceae* is composed of the closely related genera *Rickettsia* and *Orientia* and the family *Anaplasmataceae* of the genera *Wolbachia, Ehrlichia, Cowdria, Neorickettsia, Aegyptianella* and *Anaplasma*.

2.3. *Anaplasma phagocytophilum*

2.3.1. Systematics and morphology

The genus *Anaplasma* contains the species *A.* (*Ehrlichia) bovis*, *A.* (*Ehrlichia) platys* and *A. phagocytophilum* (the former *Ehrlichia phagocytophila* group) (Dumler et al., 2001). The *E. phagocytophila* group contained the human granulocytic ehrlichiosis (HGE) Agent, *E. equi* and *E. phagocytophila* (Rikihisa, 1991). Due to great similarity and insufficient differences of the *16S rRNA* gene nucleotide sequences between *E. equi* and the HGE agent, Chen et al. (1994) and Bakken et al. (1994) proposed that these species should be reclassified into one

single species, *E. phagocytophilum*. This finding was further accentuated by Johannson et al. (1995), when ehrlichial *16S rRNA* gene sequences derived from blood samples from dogs and horses suffering from granulocytic ehrlichiosis were identical to the previously deposited sequences derived from a human patient. With the reorganization of Dumler et al. (2001) the name *A. phagocytophilum* was proposed. Uilenberg et al. (2004) strongly criticized the reclassification based on small portions of the genome, as it did not take into account phenotypical characteristics.

According to the emended description of the new species *A. phagocytophilum*, it is a small (0.5 – 1.5 µm), pleomorphic, gram-negative, obligate intracellular organism (Dumler et al., 2001). It infects granulocytes of mammals, where it is found in cytoplasmatic, cell-membrane derived vacuoles. The life cycle of *A. phagocytophilum* begins with the "elementary bodies". These are basically only a nucleus penetrating the cell where they become surrounded by a membrane and reproduce by binary fission. The result is the "initial body" which lies in a cytoplasmatic vacuole. The nuclei keep replicating until after a few days the full membrane surrounded vacuole (morula) ruptures with the host cell, releasing infecting elementary bodies and the cycle starts again (Liebisch et al., 2006).

2.3.2. Genetic diversity

A. phagocytophilum has a single circular chromosome, with numerous repeats in the genome (Dunning Hotopp et al., 2006). Genetic variants show differences concerning vectors, host tropism, DNA sequence, pathogenicity and geographical distribution and have been found in both mammals and ticks (Massung et al., 2002; Stuen et al., 2002; Stuen et al., 2003; De la Fuente et al., 2005).

The polymorphic multigene family *p44* encoding the major surface proteins (*msp*) is likely to be important in the pathogenesis in the mammalian host (Lin et al., 2004). *Msp* of *A. phagocytophilum* are heterogenic from different geographic origins and could play a role in pathogenicity and persistence (Caspersen et al., 2002; De la Fuente et al., 2005). An analysis of different loci on the *p44* multigene family showed a great diversity in US strains, and differences to European strains (Lin et al., 2004).

In general, American and European lineages show differences and are heterogenic amongst themselves. For example, in Slovenia, genetic diversity of the *16S rRNA* gene and the *groESL* heat shock operon was detected in sequences derived from roe and red deer. All red deer

sequences clustered with those from humans, whereas the roe deer sequences clustered separately. In Sweden, two lineages with respect to the *16S rRNA*, *groESL* and *ank* genes of equine origin were detected. None of these had been previously obtained from human patients (Bjoersdorff et al., 2002; Petrovec et al., 2002). Two genetic lineages of *groESL* were also found in Austria, one variant associated with anaplasmosis in humans (Polin et al., 2004). Von Loewenich et al. (2003a) found seven different *16S rRNA* gene sequence types in Germany. Further sequencing of the *groESL* and *ankA* genes revealed even greater gene diversity and brought up the question whether there may be differences in European and American strains concerning pathogenicity. This hypothesis was supported by a further *msp5* characterization of *A. phagocytophilum* strains which revealed great heterogenicity of European isolates and differences to the US isolates (Strik et al., 2007).

All data speak in favor of two distinct lineages in Europe, which are different from the lineage in the United States. However, *groESL* sequences in Sardinia were distinct from the two lineages described in Europe, but closely related to the lineage from the USA (Alberti et al., 2005).

In Japan, deer were found infected with an *A. phagocytophilum* strain whose *16S rRNA* gene was different from those detected previously in mammals in the USA and Europe as well as different from those detected in ticks in Asia (Kawahara et al., 2006).

2.3.3. Vectors and transmission

Hard ticks of the genus *Ixodes* are vectors of *A. phagocytophilum*. The main vector in Europe is *I. ricinus*. In North America the main vector on the East Coast is *I. scapularis* and on the West Coast *I. pacificus* (Parola et al., 2005a). There have been reports on the detection of *A. phagocytophilum* in *I. spinipalpis* and *I. dentatus* in the USA (Zeidner et al., 2000; Goethert and Telford, 2003). In Asia, *A. phagocytophilum* has been detected in the hard ticks *I. persulcatus* and *I. ovatus* (Cao et al., 2000; Ohashi et al., 2005). Apart from the genus *Ixodes*, *A. phagocytophilum* has also been detected in *D. variabilis* in California and *D. silvarum* in China (Holden et al., 2003; Cao et al., 2006).

Even though not yet determined as a vector, *A. phagocytophilum* has also been identified in *Neotrombicula autumnalis* mites and *Syringophilidae* quill mites (Fernández-Soto et al., 2001; Skoracki et al., 2006).

In *I. scapularis*, *A. phagocytophilum* induces the expression of a specific salivary gland protein which is required for the pathogen in order to persist within the vector (Sukumaran et al., 2006). Transovarial transmission seems to be inefficient, and there is still discussion on the efficiency of the transstadial transmission (Ogden et al., 1998; 2002). Therefore, a susceptible vertebrate host is necessary for the maintenance of *A. phagocytophilum* in nature. The transmission from the infected tick to the host occurs during the first two days after attachment (Herron et al., 2005). However, the dynamics of transmission to mammals have not yet been fully elucidated (Parola et al., 2005a).

A reciprocal cross-transmission experiment was carried out in a study on the transmissibility of different strains of *A. phagocytophilum* within different tick vector species. The transmissibility of an East Coast and West Coast North American *A. phagocytophilum* strain was tested in the West and East Coast North American tick species, *I. pacificus* and *I. scapularis*. *I. pacificus* showed higher vector competency and the East Coast isolate higher transmissibility. When these data were compared with epidemiological data (lower prevalence in *I. pacificus* and fewer cases at the Pacific Coast), the results indicated that variation in host susceptibility and transmissibility of *A. phagocytophilum* may play a more important role than the vector competency of the tick (Teglas and Foley, 2006). Adult sheep in the UK were found infected with *A. phagocytophilum* at a rate of 38%, but the prevalence declined significantly with the sheep's age and varied significantly with the number of ticks infesting them. Furthermore, with a rising number of adult ticks feeding on the sheep, the transmission efficiency from sheep to immature ticks rose as well (Ogden et al., 2002).

2.3.4. Hosts and reservoirs

A. phagocytophilum is thought to be maintained in nature in a tick-ruminant-rodent-cycle, with humans only being dead-end hosts (Blanco and Oteo, 2002; Woldehiwet, 2006). Antigenetic variation of the major surface proteins have been suggested to influence the persistence in mammals, which is in turn vital for subsequent transmission to the tick and the distribution between different areas (Brayton et al., 2001; Stuen, 2007). In Europe, large wild mammals have been suggested as reservoir hosts, amongst them mainly roe deer (*Capreolus capreolus*) and red deer (*Cervus elaphus*), but also, to a lower extent, chamois (*Rupicapra rupicapra*), wild boars (*Sus scrofa*), and foxes (*Vulpes vulpes*) (Pusterla et al., 1999a; Schouls et al., 1999; Liz et al., 2002, Petrovec et al., 2002; Hulínská et al., 2004; Polin et al., 2004; De

la Fuente et al., 2005; Skarphédinsson et al., 2005; Beninati et al., 2006; Naranjo et al., 2006; Smetanová et al., 2006; Adamska and Skotarczak, 2007; De la Fuente et al., 2007). Roe deer is considered to possibly be the main reservoir in Europe (Skarphédinsson et al., 2005), but Halos et al. (2006) could not support this hypothesis as their findings indicated a host from pasture sites. Polin et al. (2004) found a peak in infected roe deer in late summer and autumn. This corresponds to a significant seasonal variation in Denmark, where roe deer were infected more than double during summer compared to autumn (Skarphédinsson et al., 2005).

Small mammals, such as bank voles (*Clethrionomys glareolus*), wood mice (*Apodemus sylvaticus*), yellow-necked mice (*A. flavicollis*) and shrews (S*orex araneus* and *Crocidura russula*) may also act as reservoirs (Liz et al., 2000; Bown et al., 2003; Hulínská et al., 2004; Barandika et al., 2007; Marumoto et al., 2007). In Switzerland, the infection rate found in voles was significantly higher than in mice (Liz et al., 2000). More recently, the root vole (*Microtus oeconomus*) has been added to the list of potential reservoirs whereas most birds may not serve as reservoirs, and *I. ricinus* may not be a competent vector for transmission to birds (Grzeszczuk et al., 2006a; Skotarczak et al., 2006).

In the USA, the main reservoirs discussed are the white-tailed deer (*Odocoileus virginianus*) and the white-footed mouse (*Peromyscus leucopus*) (Levin et al., 1999; Munderloh et al., 2003; Michalski et al., 2006). However, white-tailed deer seems to be a reservoir for a variant strain (Ap-V1) not associated with human infection, but not for the strain causing human disease (Ap-ha) (Massung et al., 2005).

In recent years, alternate rodent–tick driven cycles possibly providing an efficient niche reservoir for *A. phagocytophilum* have been detected. *I. trianguliceps* has an endemic cycle with field voles (*Microtus agrestus*), the most abundant rodent in the UK (Bown et al., 2003). These rodents host large numbers of nymphal and larval *I. trianguliceps* and *I. ricinus* ticks which have both been found infected with *A. phagocytophilum*. In the USA, the cottontail rabbit (genus *Sylvilagus*), which has an enzootic cycle with *I. dentatus*, has been found infected with *A. phagocytophilum* (Goethert and Telford, 2003). From these endemic cycles, the anthropophilic ticks *I. ricinus* or *I. scapularis* could acquire the infection. However, *A. phagocytophilum* infection in rodents might be short-lived and ticks rather than rodents might carry the infection over the winter (Bown et al., 2003; 2006).

2.3.5. Geographical distribution of *Anaplasma phagocytophilum*

2.3.5.1. Questing ticks in Europe

A. phagocytophilum is spread circumglobal in the northern hemisphere (Teglas and Foley, 2006). It has been detected by PCR in ticks and mammals in almost all countries in Europe (Strle, 2004).

In Germany, the overall prevalence ranges from 1.0% to 4.5% (Table 2). The overall prevalence in Europe ranges from 0.25% to 57.14% (Alberdi et al., 1998; Mantelli et al., 2006).

Table 2: Prevalence of *Anaplasma phagocytophilum* in *Ixodes ricinus* in Germany

Year	Location in Germany	No. of ticks	Average prevalence	Reference
1998	South	287	2.2%	Baumgarten et al., 1999
1999	South	492	1.6%	Fingerle et al., 1999
1998-2001	South and North	1,022	4.1%	Von Loewenich et al., 2003a
1999-2001	South	5,424	1.0%	Hartelt et al., 2004
2002	South	9,189	2.6 – 3.1%	Oehme et al., 2002
2003	Middle	305	2.3%	Hildebrandt et al., 2002
2003	North	127	3.9%	Pichon et al., 2006
2003-2004	South	625	4.5%	Leonhard, 2005

Great variation in prevalence is found in every European country. Ranges are from 0.4% to 15% in France (Ferquel et al., 2006; Halos et al., 2006), from 2.99% to 57.14% in Italy (Cinco et al., 1997; Mantelli et al., 2006; Piccolin et al., 2006), from 0.25% to 2.0% in Scotland (Alberdi et al., 1998), 3% in Estonia (Mäkinen et al., 2003), from 5.1% to 8.7% in Austria (Sixl et al., 2003, Polin at al., 2004), from 0.5% to 2.2% in Switzerland (Leutenegger et al., 1999; Pusterla et al., 1999b; Liz et al., 2000), up to 40.5% in Denmark (Skarphédinsson et al., 2007) and up to 14% in various Eastern European countries (Petrovec at al., 1999; Derdáková et al., 2003; Hulínská et al., 2004; Stańczak et al., 2004; Koči et al., 2007). *I. ricinus* removed

from human patients in Poland had a very high prevalence of 36.8% in females and 16.6% in males and the prevalence in questing ticks in Southeastern Europe was equally high (Grzeszczuk and Stańczak 2006c; Christova et al., 2001, 2003).

The infection rate in adults is often higher than in nymphs. In Thuringia (Germany), for example, adults have been significantly more often infected (6.5%) than nymphs (1.2%; Hildebrandt et al., 2002). Similar results were found in other studies (Wicki et al., 2000; Grzeszczuk and Stańczak, 2006c; Wielinga et al., 2006). In a study in Poland, adult females were significantly more often infected than males and nymphs (Chmielewska-Badora et al., 2007). However, *I. ricinus* nymphs in a forest area of Berlin and in Italy had a high proportion of infection, in Italy with significant spatial variation (Mantelli et al., 2006; Pichon et al., 2006). Significant geographic variation was also detected in Poland (Chmielewska-Badora et al., 2007). In the UK and Portugal, the prevalence in nymphs compared to adults was higher (Walker et al., 2001; Santos et al., 2004).

A. phagocytophilum may have a focal distribution and differences between different vegetation zones have been detected. Fingerle et al. (1999) conducted a study on five different sites in Southern Germany. The overall prevalence of adult ticks was 1.6%, but all infected ticks were found at one site, the 'English Garden' in Munich, increasing the infection rate at this site to 2.6%. No nymphs were found to be infected. Even though the difference was statistically not significant, it did suggest a focal distribution of granulocytic *Ehrlichiae*. Another epidemiological study investigating three sites in Bavaria, including the 'English Garden', detected an overall prevalence of 4.5% in 625 ticks. However, in the 'English Garden' alone the prevalence was 8.7% (Leonhard, 2005). On the other hand, a study on different vegetation zones in the Netherlands has revealed a higher infection rate of ticks from natural forest than from city parks (Wielinga et al., 2006). In France, double as many ticks collected from pastures than ticks from woods carried *A. phagocytophilum* DNA and in the U.K., a significantly higher prevalence was found in upland than in woodland ticks (Ogden et al., 1998; Halos et al., 2006).

Results indicating a seasonal variation were found in Norway and Austria, where the prevalence was highest in spring (Jenkins et al., 2001; Polin et al., 2004). Two epidemiological studies in Bulgaria found strong seasonal variation, but no pattern was identified (Christova et al., 2001, 2003). Substantial year to year variation is frequently detected in yearly follow-up studies (Grzeszczuk and Stańczak, 2006b; Wielinga et al., 2006).

Coinfection with different tick-borne pathogens in the same vector tick has been known for a long time. For example, coinfection of *A. phagocytophilum* with *B. burgdorferi* or rickettsial

agents has been discovered in various European countries (Cinco et al., 1997; Baumgarten et al., 1999; Fingerle et al., 1999; Leutenegger et al., 1999; Hartelt et al., 2004; Mantelli et al., 2006; Piccolin et al., 2006; Wielinga et al., 2006).

2.3.5.2. Questing ticks in North America and Asia

Regional variations have been found in the United States, where ticks on the East Coast tend to have a higher infection rate than on the West Coast. In Eastern States like Connecticut, Maine, Rhode Island, Pennsylvania, Indiana, New Jersey, New York State and Wisconsin, infection rates from 1.9% to up to 50% have been detected in *I. scapularis* (Levin et al., 1999; Massung et al., 2002; Courtney et al., 2003; Adelson et al., 2004; Holman et al., 2004; Michalski et al., 2006; Moreno et al., 2006; Steiner et al., 2006). Studies carried out on *I. pacificus* in California have revealed prevalences between 0.8% and 8.3% (Barlough et al., 1997; Kramer et al., 1999; Holden et al., 2003; 2006). The difference in infection rates between the East and the West Coast could be due to factors such as the climate, vector or reservoirs (see 2.3.3.). As in European countries, there seems to be a focal distribution and year to year variation (Courtney et al., 2003; Holman et al., 2004). Significant differences between infection rates of adults and nymphs were found by Levin et al. (1999). Adult infection was higher, furthermore the infection rate increased in two consecutive years.
In China and Western Siberia, *A. phagocytophilum* has been detected in *I. persulcatus* ticks with prevalence between 0.6% and 4.6% (Cao et al., 2000; 2003; 2006; Rar et al., 2005). A significant difference has been found in the prevalence between two investigated sites in China (Cao et al., 2003). Spatial variations have also been found in *I. persulcatus* and *I. ovatus* in Japan with prevalences up to 12% (Ohashi et al., 2005).

2.3.6 Seroprevalence

2.3.6.1. Seroprevalence in humans

Confirmed clinical human cases with *A. phagocytophilum* have been reported in the USA (Chen et al., 1994; Demma et al., 2005) and in Europe (Petrovec et al., 1997; Arnež et al., 2001; Walder et al., 2003). So far, there have been no confirmed *A. phagocytophilum*

infections of humans in Germany, despite the fact that it has been detected in *I. ricinus* in various regions in Germany and risk populations show high serological antibody titers against the agent.

In general, the proportion of persons seropositive to granulocytic *Ehrlichiae* increases with the age of the person and is higher in tick-exposed populations (Strle, 2004). Forestry workers, febrile patients after tick exposure, and male soldiers exercising in the outdoors have been seropositive between 11.4% and 14.9% in different European countries (Fingerle et al., 1997; Woessner et al., 2001; Lillini et al., 2006). The seroprevalence of Lyme borreliosis patients compared to control groups can be significantly higher (Fingerle et al., 1997; Hunfeld and Brade, 1999; Zwolinski et al., 2004; Kowalski et al., 2006). In Germany, the seroprevalence detected ranges from 1.9% to 16%, depending on factors such as patient history, tick exposure and age (Fingerle et al., 1997; Woessner et al., 2001; Oehme et al., 2002; Von Loewenich et al., 2003b; Kowalski et al., 2006). Similar results were found in other European countries where seroprevalence ranges from 1.4% to 21% (Cinco et al., 1998; Oteo et al., 2001; Skarphédinsson et al., 2001; Walder et al., 2003).

2.3.6.2. Seroprevalence in animals

Studies on potential reservoir hosts revealed seroprevalences of up to 95.6% and PCR positivity of 42.6% of *A. phagocytophilum* roe deer (Petrovec et al., 2002; Skarphédinsson et al., 2005; De la Fuente et al., 2007). Serological evidence in sheep flocks points to up to 14.5% seroprevalence (Lillini et al., 2006; Torina et al., 2007). Cattle in Italy was PCR- and seropositive in 16.67% and 16.07% of cases, respectively (Torina et al., 2007) and in Switzerland, an increase in seroprevalence from 16% prior to the pasture period to a maximum of 63% in September in a herd of cattle (n=70) has been detected (Pusterla et al., 1998).

Seroprevalence studies in dogs have been mainly carried out on animals under suspicion of the disease due to clinical symptoms. In Germany and Switzerland, positivity in dog sera recently tested with IFAT (immune fluorescence assay test) ranges from 17.6% to 50.1% (Barutzki et al., 2006; Liebisch et al., 2006; Jensen et al., 2007; Krupka et al., 2007; Schaarschmidt-Kiener and Müller, 2007). Seroreactivity in a control group showed no significant difference, even though dogs with a history of high tick infestation were significantly more often seroreactive (Jensen et al., 2007). Regional variations were detected

in one study where the prevalence was highest in the State of Bavaria (Krupka et al., 2007). Between 1.87% and 44.83% of investigated dog sera have been positive in Italy (Lillini et al., 2006; Torina and Caracappa, 2006; Torina et al., 2007). Even though 44.83% of the 87 dogs were positive, none of them provided amplification in PCR (Torina et al., 2007). In the USA, Hinrichsen et al. (2001) showed a positive correlation between abundance of *I. scapularis* and dogs being seropositive to the HGE agent.

In Spain and in the USA, 1.8% (n=168), 4.3% (n=460) and 4.6% (n=122) of cats were positive to *A. phagocytophilum*, respectively. The infections could not be confirmed by PCR (Aguirre et al., 2004; Solano-Gallego et al., 2006; Billeter et al., 2007).

Only 0.3% (n=563) of horses have been seropositive in Italy in 1997, even though clinical cases had been reported since 1996 (Scarpulla et al., 2003). Seroprevalence had increased to 7.79% in an extensive study covering blood samples from the years 2003 to 2005, but PCR prevalence remained 0% (Torina et al., 2007). On the other hand, 6 out of 61 horses with fever of unknown origin in the Netherlands were PCR positive (Butler et al., 2008). The seroprevalence in donkeys in Italy was 18.92% (Torina et al., 2007).

2.3.7. Diagnostic and phylogenetic tools

Bloodsmear examination

A. phagocytophilum morulae can be visualized in bloodsmears stained with Giemsa. However, the infection rate in peripheral blood neutrophils may vary from 0.5% to 73% (Rikihisa, 1991) and even though the detection of morulae is evidence for an *Ehrlichia* or *Anaplasma* infection, the species involved can not be specified. Furthermore, the detection of morulae depends on the experience of the microscopist and the duration and stage of the illness. Usually they can be seen during the acute fever period of the disease, but false-negative results are possible (Blanco and Oteo, 2002).

Serology (IFAT)

Fluorescent detection of antibodies is the most common diagnostic technique. No standardized assay exists and false-positive results are possible, due to possible cross-reactivity with *E. chaffeensis*, and cross-reaction with other rickettsial agents (Blanco and Oteo, 2002).

PCR

A rapid and sensitive tool, PCR is useful for detection, identification and phylogenetic analysis of *A. phagocytophilum* from blood, skin biopsy specimen and ticks (Blanco and Oteo, 2002). *16S rRNA* gene analysis has become the gold standard for the phylogenetic classification of bacteria. The comparison of more variable genes of *A. phagocytophilum* is of value when very closely related strains of genetic variants need to be further specified. Candidates are the *groESL* heat shock operon, the *ankA* gene, which encodes a 160-kDa cytoplasmatic protein antigen and genes encoding major surface proteins (Bjöersdorff et al., 2002; Von Loewenich et al., 2003a).

2.3.8. Granulocytic anaplasmosis

A. phagocytophilum infects granulocytes, a unique niche among bacteria, as they offer a harsh environment (Bakken and Dumler, 2006). However, it has been shown that they are unlikely maintaining the infection in the early phase of disease. *A. phagocytophilum* is generally transmitted during the first 24 hours of attachment, a time when blood vessels remain intact at the site of the tick bite, and, therefore, neutrophils can not return from the tick bite lesion and spread the pathogen. Infection of the microvascular endothelium may be involved (Herron et al., 2005). The incubation time of seven to ten days equals the time from infection to first rupture of the host cells and the freeing of elementary bodies (Parola et al., 2005a; see 2.3.1). Symptoms include fever, anorexia, malaise, lethargy and depression. Laboratory findings typically include thrombocytopenia, leukopenia, and sometimes anemia (Dumler, 2005; Parola et al., 2005a).

Human granulocytic anaplasmosis (HGA) typically occurs in spring or summer (Parola et al., 2005a). In the USA, HGA is one of the most important tick-borne infections with the highest number of cases being reported in the north eastern and upper Midwestern regions (Dumler et al., 2005; Parola et al., 2005a). In 2005, 700 cases were reported, altogether more than 2,500 since 1994 and the number continues to rise (Dumler et al., 2007). American strains seem to be associated with higher mortality rates and more severe cases of HGA than European ones (Blanco and Oteo, 2002). In Europe, up to March 2003, 65 patients, amongst them one child, were confirmed with HGA (Petrovec et al., 1997; Arnež et al., 2001; Strle, 2004).

Clinical manifestations range from mild and self-limiting to serious disease, especially in elderly patients, with up to 50% of patients requiring hospitalization and 7 % intensive care.

Complications involve toxic and septic shock-like syndromes, coagulopathy, acute renal failure and heart failure. The fatality rate reaches approximately 0.7% (Dumler et al., 2007).
In canine granulocytic anaplasmosis, lymphadenopathy, lameness, central nervous system signs, splenomegaly and hepatosplenomegaly can occur as additional symptoms (Greig et al., 1996; Dumler et al., 2001; Liebisch et al., 2006; Kohn et al., submitted). Feline granulocytic anaplasmosis further includes neutrophilia as a laboratory finding and vomiting and polyarthritis as symptoms (Billeter et al., 2007).
Equine granulocytic anaplasmosis is known in the USA since 1969; reports in Europe started in the 1990s (Gribble, 1969; Bermann et al., 2002; Bjoersdorff et al., 2002; Scarpulla et al., 2003; Von Loewenich et al., 2003b; Butler et al., 2008). Additional clinical signs include a lower limb edema, petechia, icterus, and ataxia (Rikihisa, 1991; Dumler, 2005).
A. phagocytophilum infection of ruminants cause an acute febrile disease, resulting in reduced milk production, hemorrhage and abortion in cattle, sheep, sometimes goats, also in red and roe deer. In Norway, moose was found to be infected as well (Pfister et al., 1987; Rikihisa, 1991; Liz, 1994; Dumler, 2005; Woldehiwet, 2006).

2.4. Spotted fever group rickettsiae

2.4.1. Systematics and morphology

Historically, there were three groups within the genus *Rickettsia*, based on phenotypic and clinical criteria: the spotted fever group (SFG), the typhus group (TG) and the scrub typhus group (STG) (Roux and Raoult, 2000; Blanco and Oteo 2006). However, applying those traditional classification criteria to rickettsiae is not always possible due to their strictly intracellular nature (Raoult and Roux, 1997).
In recent years, phylogenetic research has shown that these groupings were not consistent with species relationships based on molecular genetic data (Weisburg et al., 1991; Roux et al., 1997; Fournier et al., 1998; Roux and Raoult, 2000; Sekeyová et al., 2001). Currently, the genus is divided into the heterogenic and large SFG rickettsiae and the TG rickettsiae with *R. prowazekii* and *R. typhi*. The STG was excluded from the genus *Rickettsia* and reclassified as the new genus *Orientia* (Roux and Raoult, 2000). However, this division of rickettsiae into two groups is not supported by genomic data and continues to be discussed (Roux and Raoult, 2000; Sekeyová et al., 2001).

All members of the family *Rickettsiaceae* are described as small, rod-shaped, gram-negative, obligate intracellular bacteriae growing freely in the cytoplasm of the eukaryotic host cells and retaining basic fuschin when stained by the method of Gimenez (Dumler et al., 2001; Raoult et al., 2005). After invasion of the tick's digestive tract, rickettsiae cause a generalized infection in the hemocoel. They undergo a developmental cycle in the organisms of the tick, and are found in saliva, faeces or coxal fluids. SFG rickettsiae can also be found in the nuclei of the host cells, whereas TG rickettsiae are found only in the cytoplasm (reviewed in Raoult and Roux, 1997). The genome of rickettsiae is highly conserved. Reductive evolution has lead to a small genome relying on the host cell for many biosynthetic functions (Walker, 2007).

2.4.2. Vector, transmission and reservoir

Vectors of rickettsiae can be ticks, mites, lice, fleas and the ladybird beetle (Raoult and Roux, 1997; Blanco and Oteo, 2006). The TG rickettsiae are transmitted by lice or fleas and *O. tsutsugamushi* by mite larvae (Nilsson et al., 1997; Roux and Raoult, 1997; Urakami et al., 1994).

Hard ticks were first suggested as vectors of the SFG rickettsiae in 1906, when the Rocky Mountain wood tick (*D. andersoni*) became known in the USA as the vector for the agent of the Rocky Mountain spotted fever (Ricketts, 1906). SFG rickettsiae cycle on the one hand between ixodid ticks and vertebrate hosts, but are also maintained in tick populations through efficient transstadial and transovarial transmission (Raoult and Roux, 1997). Due to transovarial transmission, which has been shown for a large part of the SFG rickettsiae, ticks are considered to be the main reservoirs (Raoult and Roux, 1997; Parola et al., 2005a). Consequently, the distribution of rickettsial diseases is dependent on the geographic distribution of its arthropod host (Parola and Raoult, 2001a; b). Furthermore, an association exists in evolution between rickettsiae and arthropods, leaving the assumption that mammals are only subordinate in the evolutionary strategy (Roux and Raoult, 1995). Rickettsiae seem not to be strictly bound to one tick species and appear to be able to switch between different tick hosts (Ishikura et al., 2002). It has been suggested that rickettsiae are primarily symbionts of invertebrates and have only a secondary role as pathogens of vertebrates (Perlman et al., 2006). Electron microscopy research has shown that *I. ricinus* testicular tissue harbored masses of *R. helvetica* and it could therefore be possible that sexual transmission occurs (Hayes et al., 1980).

The role of vertebrates as reservoirs of rickettsiae is still under discussion. To be an efficient reservoir, they need to be susceptible hosts developing a relatively long rickettsiemia (Raoult and Roux, 1997). All small mammals, most of them wood mice (*A. sylvaticus*), captured during a study in Spain were PCR negative for SFG rickettsiae (Barandika et al., 2007) and roe deer from Denmark were also negative (Nielsen et al., 2004; Skarphédinsson et al., 2005). All 73 dogs investigated in a study in Grenada were both seronegative and PCR negative for *Rickettsia* spp. (Yabsley et al., 2008). On the other hand, rickettsial infections (*R. aeschlimannii, R. helvetica* and *R. massiliae*) were detected in ticks from wild birds in Portugal and therefore, birds could play a role in the maintenance and dissemination of ticks and rickettsial infection (Santos-Silva et al., 2006). In Japan, *R. helvetica* was detected in blood samples of Sika deer (*C. nippon yesoensis*), which suggests a possible reservoir host (Inokuma et al., 2008).

2.4.3. Geographical distribution and prevalence in *Ixodes ricinus* and other Ixodid ticks

Rickettsiae have been detected in *I. ricinus* in many European countries, with prevalence ranging from 1.6% to 38.5% (Bertolotti et al., 2006; Piccolin et al., 2006). *R. helvetica* was first detected in Swiss *I. ricinus* ticks in 1979, at the time called the "Swiss agent", a rickettsial strain of unknown pathogenicity (Burgdorfer et al., 1979). In 1993, it was confirmed to be a new member of the SFG rickettsiae and was named *R. helvetica* (Beati et al., 1993).

In Germany, *I. ricinus* ticks from the States of Bavaria and Baden-Württemberg contained *R. helvetica* with prevalence ranging between an average of 8.9% and 12% (Hartelt et al.; 2004; Wölfel et al., 2006). *I. ricinus* nymphs from a forest in the urban areas of Berlin showed an infection rate of 14.2% with *R. helvetica* (Pichon et al., 2006). Another species of the SFG rickettsiae has been detected in *I. ricinus* in the 'English Garden' in Munich: *R. monacensis* sp. nov., type strain IrR/ Munich, closely related to the strain IRS 4 (Simser et al., 2002). From 135 *D. reticulatus* collected from deer, 23% were positive for *Rickettsia* spp. and sequencing showed that they had 100% identity with strain RpA4, isolated first from *Rhipicephalus sanguineus* in Russia. The pathogenicity of this strain remains unknown (Dautel et al., 2006; Ibarra et al., 2006).

R. helvetica has been detected in *I. ricinus* in Switzerland (Beati et al., 1994), Italy (Beninati et al., 2002; Bertolotti et al., 2006; Piccolin et al., 2006), France (Parola et al., 1998; Halos et al., 2006), Spain (Fernández-Soto et al., 2004), Denmark (Nielsen et al., 2004; Skarphédinsson et al., 2005; 2007), Sweden (Nilsson et al., 1997; 1999a), Slovenia (Prosenc et al., 2003), Hungary (Sréter-Lancz et al., 2005; 2006), Poland (Stańczak, 2006; Stańczak et al., 2008) and the Netherlands (Nijhof et al., 2007). *R. helvetica* has further been found in *I. ventalloi* in Portugal and *I. hexagonus* in the Netherlands (Santos-Silva et al., 2006; Nijhof et al., 2007). Coinfection of *R. helvetica* with *B. burgdorferi* or *A. phagocytophilum* has also been detected (Piccolin et al., 2006; Pichon et al., 2006).

R. monacensis and all its strains form a separate cluster within the SFG rickettsiae based on phylogenetic analysis (Sréter-Lancz et al., 2005). The strains *Rickettsia* IRS3 and IRS4 were first detected in Slovakia, and later in Hungary (Sekeyová et al., 2000; Prosenc et al., 2003; Sréter-Lancz et al., 2005; 2006). In Italy, strains detected were IrR/Munich, IRS4, IRS3 and *ompA* strains IrITA2, IrITA3 (Beninati et al., 2002; Bertolotti et al., 2006). In Albania, strain IRS3 has also been detected in *Hyalomma plumbeum* and *R. bursa* (Christova et al., 2003). *I. ricinus* collected from human patients in Spain harbored strains IRS3 and IRS4, but also *R. massiliae*/Bar29, genotype RpA4 and *R. aeschlimannii* (Fernández-Soto et al., 2004). In Bulgaria, 67% of adult *I. ricinus* were infected with SFG *rickettsiae*, 59% of which were *R. helvetica* and 58% strain IRS3. Coinfection with two rickettsial species occurred in 17% of adults and 53% of nymphs (Christova et al. 2003).

SFG rickettsiae have also been detected in ixodid ticks in Japan. Strains in questing or engorged *I. persulcatus*, *I. monospinosus* and *I. ovatus* were identical or closely related to *R. helvetica* with prevalences of up to 10% (Ishikura et al., 2002; 2003; Yano et al., 2004; Hiraoka et al., 2005; Inokuma et al., 2007). *R. helvetica* was first thought to be a European strain, but might well be distributed upon the Eurasian land mass. It has also been detected in *Haemaphysalis flava* and *R. sanguineus* (Ishikura et al., 2003; Hiraoka et al., 2005). Another strain, detected in *I. nipponensis*, was genetically close to the strains IRS3, IRS4 and Ir/R Munich (Fournier et al., 2002; Ishikura et al., 2003).

2.4.4. Seroprevalence

Seroprevalence in the human population often coincides with the detection of SFG rickettsiae in ticks from the same region (Parola et al., 1998). In Sweden and France, 22% of 35 recruits and 9.2% of forestry workers, respectively, were seropositive for *R. helvetica* (Nilsson et al., 2005; Fournier et al., 2000a). In another study, a total of 2.6% seropositive individuals were made up of a *Borrelia* positive group (4.4%) and a control group (0.6%; Elfving et al., 2007). A survey amongst Danish patients (seropositive for borreliosis) revealed a seroprevalence of 12.5% (Nielsen at al., 2004). These findings add evidence to the hypothesis that *R. helvetica* may cause a threat to exposed human populations.

2.4.5. Diagnostic and phylogenetic tools

Haemolymph Test

To examine a living tick, a distal portion of a leg is severed, a drop of heamolymph collected, stained on a slide by the Giménez' method and microscopically examined for the presence of bacteriae (Burgdorfer, 1970).

Serology

Serologic typing with mouse sera was developed in the late 1970s and was long the method of reference for identifying new SFG rickettsiae. The antigenetic determinants were the outer membrane proteins rompA and rompB (Raoult and Roux, 1997).

Isolation

Different methods have been used: *(i)* inoculation in guinea pigs, rats and voles; *(ii)* inoculation in embryonated eggs; and *(iii)* the currently most widely used system for primary isolation is *in-vitro* cultivation in tick or mammalian cell lines (Raoult and Roux, 1997).

PCR

PCR is the most widely used method for detection and analysis. Molecular phylogenetic classification began with *16S rRNA* gene analysis, where all SFG rickettsiae were grouped in the same cluster. However, the differences were not enough for a precise phylogenetic analysis (Roux and Raoult, 1995; Fournier et al., 1998).

The *gltA* gene, a highly conserved gene encoding the citrate synthase, is very suitable for initial screening, as it is shared by all rickettsiae. However, phylogenetics can only be carried out for rickettsiae which diverged early in evolution (Roux et al., 1997; Fournier et al., 1998; Roux and Raoult, 2000).

The *ompA* gene encodes an antigenetic membrane protein of high molecular mass and is a very good candidate for phylogenetic analysis of SFG rickettsiae, as it is specific for SFG rickettsiae and differences amongst species are higher than with *gltA* (Fournier et al., 1998; Walker, 2007). The gene encoding the outer membrane protein B (*ompB*), also a protein of high molecular mass is present in almost all rickettsiae (Roux and Raoult, 2000). The outer membrane proteins and the protein encoding gene D are reliable tools in phylogenetic classification (Sekeyová et al., 2001). Genetic guidelines have been proposed for the classification of new rickettsial isolates at genus, group and species level based on homology levels with existing rickettsial agents of the above mentioned genes (Fournier et al., 2003; Raoult et al., 2005).

2.4.6. Tick-borne rickettsioses in Europe

Until recently, Mediterranean spotted fever, caused by *R. conorii* and some genetic variants, was thought to be the only autochthonous tick-borne rickettsiosis in Europe. During the last decade, new rickettsioses have been discovered in Europe. These have been associated with *R. sibirica mongolotimonae* (1996), *R. slovaca* (1997) and *R. helvetica* (1999) (Raoult et al., 1997; Nilsson et al., 1999b; Fournier et al., 2000b; Blanco and Oteo, 2006). These species were classified to be non-pathogenic or of unknown pathogenicity upon their discovery. New species of rickettsiae continue to be isolated from ticks all over the world. In most cases their pathogenicity remains to be determined (Fernández-Soto et al., 2004).

In general, clinical symptoms of SFG rickettsioses begin six to ten days after the tick bite and typically include fever, headache, muscle pain, a rash, local lymphadenopathy and a characteristic inoculation eschar (“tache noire”) at the site of the tick bite (Parola et al., 2005a).

Mediterranean spotted fever, endemic in Southern Europe, is most probably transmitted by *R. sanguineus* (Parola and Raoult, 2001a; Blanco and Oteo, 2006). Most cases occur during summer (Blanco and Oteo, 2006).

Tick-borne lymphadenopathy (TIBOLA) and *Dermacentor*-borne-necrosis-erythema-lymphadenopathy (DEBONEL) are caused by *R. slovaca*; the vectors are *D. marginatus* and *D. reticulatus*. Recent research has shown that other strains such as RpA4, DnS14 and DnS28 may be involved in the etiology (Ibarra et al., 2006). Most cases occur during winter (Parola and Raoult, 2001a; Blanco and Oteo, 2006).

Lymphangitis-associated rickettsiosis (LAR) is caused by *R. mongolotimonae* (originally found in *Hyalomma asiaticum* ticks in Mongolia). Most European cases occurred in France in spring and the individuals concerned had no travel record; therefore, the vector in Europe remains to be identified (Fournier et al., 2000b; Parola and Raoult, 2001a; Blanco and Oteo, 2006). *R. aeschlimannii* has also been associated with human disease (Raoult et al., 2002). This rickettsial species has been identified in ixodid ticks (Blanco and Oteo, 2006).

In 1999, a case of fatal perimyocarditis was associated with *R. helvetica* infection in Sweden and further cases of rickettsial infection have followed (Nilsson et al., 1999b; 2005). A suggested connection with sarcoidosis was not confirmed (Planck et al., 2004). *R. helvetica* has also been associated with febrile illnesses in France, Italy and Thailand. In most cases there was no evidence of a cutaneous rash (Fournier et al., 2000a; 2004; Ciceroni et al., 2006). *R. monacensis* was associated with an acute tick-borne rickettsiosis in two human patients in Spain (Jado et al., 2007).

2.5. Polymerase Chain Reaction

2.5.1. Principle of Polymerase Chain Reaction

The polymerase chain reaction is a method to amplify a defined DNA sequence with the help of two 15-25 bp oligonucleotides (primers) flanking the target sequence and a heat resistant DNA polymerase. The process is carried out in a thermocycler and contains usually 30 to 50 amplification cycles. Each amplification cycle consists of three steps:

1. Denaturation. The double-stranded DNA is separated by heating to 94 – 95°C.

2. Annealing. The temperature is lowered and the primers hybridize with the complementary part of the single-stranded target DNA.

3. Elongation. With the help of a DNA polymerase the DNA sequence is filled with free nucleotides to form a new double-stranded DNA.

The target sequence is amplified identically. The primers anneal also with the new double-stranded DNA fragments after denaturation during the following cycles. This leads, under ideal conditions, to an exponential increase of the target sequence (Löffler and Petrides, 1998). The PCR products are made visible during a final step with agarose gel electrophoresis.

2.5.2. Nested PCR

A part of a PCR product of a first amplification is amplified during a second PCR with a second set of primers. These are chosen to amplify within the target sequence of the first PCR. Thereby, the sensitivity is increased.

2.5.3. Real-time PCR

Amplification and detection are combined in a single step. The PCR products are made visible during the amplification process by adding either unspecific DNA-binding fluorescent dyes or special fluorescent hybridization probes. The amplification is first detected when fluorescence intensity is greater than the background intensity. The intensity and the onset cycle of fluorescence correlates with the product concentration in the initial sample. Real-time PCR requires no post-amplification handling which greatly reduces the risk of contamination (Wong and Medrano, 2005; Dorak, 2006).

The fluorescent dye SYBR Green I binds to double-stranded DNA and is widely used due to its low cost and applicability in different PCR protocols. Bound dye fluoresces stronger than free dye and increase in intensity is proportional to the product concentration. A limitation is that it binds to any double-stranded DNA, therefore unspecific PCR products and primer dimers are also made visible and give false-positive results (Bell and Ramford-Cartwright, 2002; Wong and Medrano, 2005). For this reason, real-time PCR protocols often include sequence-specific fluorescent probes. These are oligonucleotids marked with a fluorophore complementary to the target sequence. After hybridization with the target gene, a fluorescent signal is created and measured. The quench of a reporter fluorescent is either diminished or there is an increase in a fluorescent resonance energy transfer (FRET) from a donor to an acceptor fluorophore (Wong and Medrano, 2005).

These are the most common types of probes:

Hybridization probes. They bind specifically to the sequence between the primers and are marked with a reporter fluorophore at the 5'- end and an acceptor fluorophore at the 3'-end. The reporter dye absorbs light of a specific wavelength and transmits the energy to the acceptor dye (FRET). The acceptor emits the transferred energy as a fluorescent signal. When the distance between acceptor and reporter is diminished, the signal of the acceptor becomes stronger (Wong and Medrano, 2005).

TaqMan® probes. These are half-moon-shaped hydrolysis probes with a reporter dye at the 5'-end and a quencher dye at the 3'-end. In the intact probe, the quencher reduces the intensity of the reporter dye. Once the probe anneals to the target sequence, it is hydrolyzed by the 5'-3' exonuclease activity during the elongation step. Thus, the reporter and the quencher are separated and there is an increase in fluorescence by diminishing the quench (Wong and Medrano, 2005).

Molecular beacons. They consist of a target sequence-specific "loop"-region flanked by two complementary "stems". When the probe is free in solution, the terminal ends attach to each other and the fluorescence is quenched as each end of the molecule contains either a reporter or quencher dye. By binding to the target sequence, the reporter and quencher are separated resulting in emission of the fluorescence (Wong and Medrano, 2005).

The following are primer-probe combinations. With them, a gel must be run to ensure the presence of a single PCR product, as priming and detection are not independent in these systems (Wong and Medrano, 2005).

Scorpions are a combination of detection probes and PCR primers. At the 5'-end there is a fluorophore, at the 3'-end a PCR primer, a DNA polymerase blocker and a quencher dye and in-between a stem-loop structure with a specific sequence (Wong and Medrano, 2005).

Sunrise primers. Primer and detection mechanism are also combined with a self-complementary sequences at the 5'-end, marked with reporter and quencher. The 3'-end contains the PCR primer. After annealing and subsequent extension, the hairpin structure of the 5'-end is separated far enough for the reporter to emit its signal. (Wong and Medrano, 2005).

Light upon extension (LUX) primers are another variation, which do not contain a quencher fluorophore, but emission is reduced through the secondary structure of the 3'-end, making them less expensive than other probes (Wong and Medrano, 2005).

3. Materials and methods

3.1. Tick collection

3.1.1. Sampling method

The tick collection was designed as a two-phased sampling model. Phase 1 consisted of collecting actively questing ticks at eight different locations from the study area in Munich (see 3.1.2.), from May to September 2006. The location of these sites is shown in Figure 1 of publication 1 (see 4.4.1., p. 53) and Figure 1 in publication 2 (see 4.4.2., p. 81). Ticks were taken directly from the vegetation using the flagging method. The aim was to gain an overview on the occurrence of *I. ricinus* at the different sites by collecting all stages until an adult tick count of 200 was reached. In phase 2, nymphs and adults (as available 30 females, 30 males and 30 nymphs per months per site), were randomly chosen from the phase 1 sample and included in laboratory investigation. Collected larvae were not included in laboratory investigaton.

3.1.2. Study area

3.1.2.1. City parks

Sites A1, A2 and A3 (Figures 2, 3 and 4) are situated in the ‘English Garden’, a large and heavily frequented park in the centre of Munich. A1 and A2 are located in the southern part of the park (‘Südteil’) which is enclosed to three sides by roads and houses, and separated from the northern part (‘Nordteil’) by a busy 4-laned road.

The vegetation at sites A1 and A2 consists of well-groomed lawn, small bushes and deciduous trees which are maintained by gardening activities. Site A3 is located in the northern part of the ‘English Garden’ which has no borders to the North and transitions into unkept areas. Vegetation at site A3 is also maintained by gardening, and includes areas for horse-back riding.

Figure 2: Site A1
(altitude 510m, 48°09'21.51"N, 11°35'25.92"E)

Figure 3: Site A2
(altitude 509m, 48°09'03.65"N, 11°35'26.02"E)

Figure 4: Site A3
(altitude 503m, 48°10'54.78"N, 11°37'11.06"E)

Figure 5: Site B
(altitude 529m, 48°06'15.20"N, 11°33'25.27"E)

Site B (Figure 5) is situated in a smaller city park in the southern parts of the city. This park borders to the banks of the river Isar to the western side and to the city to the eastern side. It is also a landscaped park, even though grass is less frequently cut than in the ‘English Garden’.

3.1.2.2. Riparian forests

Sites C, D, E1 and E2 are situated along walkways in natural riparian and deciduous forests along the river Isar. All have relatively thick and leafy undergrowth and are not maintained by gardening.
Site C (Figure 6) is situated in the southern part of the city, on the western bank of the river Isar and Site D (Figure 7) in the northern part. Both are visited by people for hiking and biking. Sites E1 and E2 (Figures 8 and 9) are situated in the north outside of the city boundaries in the riparian forest ‘Isarauen’. The area is used for horse-back riding.

Figure 6: Site C
(altitude 532m, 48°05'20.72"N, 11°32'32.17"E)

Figure 7: Site D
(altitude 492m, 48°11'55.58"N, 11°37'48.06"E)

Figure 8: Site E1
(altitude 493m, 48°13'08.23"N, 11°38'52.86"E)

Figure 9: Site E2
(altitude 488m, 248°14'13.20" N, 11°39'53.31" E)

3.1.2.3. Sites outside of Munich

For comparison, three sites were chosen outside of Munich. Their location is shown in Figure 1 in publication 1 (see 4.4.1., p. 53) and Figure 1 in publication 2 (see 4.4.2., p. 81).

Site W: Bad Wörishofen is located in the district Unterallgäu. It is a spa town with surrounding landscapes of high recreational value. Its altitude is 641 m (48°01’51.75” N, 10°36’34.51”E) in a prealpine area with mixed forest and thick undergrowth.
Site L: The Lechstaustufe 23, along a barrier of the river Lech, close to the city of Mehring in the district Augsburg, (altitude 518m, 48°17’29.21”N, 10°56’55.93”E) is a recreational area with mixed forest and thick undergrowth.
Site K: Kreßbronn am Bodensee, district Bodenseekreis (altitude 669m, 47°35’36.80”N, 9°37’22.45”E) is a holiday town in the State of Baden-Württemberg situated directly at the Lake Constance. The site is situated in a pine forest with moderate undergrowth.
None of the comparative sites is maintained by gardening.

3.2. Identification of ticks and DNA-Extraction

All collected adult ticks were identified to species level by standard taxonomic keys (Hillyard, 1996). For every collection, the site and day of sampling were noted and the ticks, separated by sex and stage, were frozen individually at -26°C until further use.
Nymphs chosen for the DNA extraction were identified to species level and each tick (adults and nymphs) was mechanically crushed in an individual 1.5ml tube, using a metal spatula prior to extraction. High pure PCR Template Preparation Kit (Roche Diagnostics GmbH, Mannheim, Germany) was used for the isolation of DNA from ticks according to manufacturer's instruction, with modifications. A volume of 200µl sterile water was added to each tube before tissue lysis, and the materials were kept over night in a 55°C water bath to allow dissolving of the tissues. The elution volume was 200µl. At the beginning and end of each extraction line, a tube which contained no DNA was added as quality control to ensure that no contamination had occurred during the extraction process.

3.3. Quality control of extraction and quantization of DNA

To verify the quantity and quality of DNA extraction, samples were measured in a full-spectrum (220-750nm) spectrophotometer (NanoDrop®ND-1000, PeqLab, Erlangen, Germany) according to manufacturer's instructions (NanoDrop® User Manual, 2004).

3.4. Polymerase Chain Reaction for detection of *Anaplasma phagocytophilum* DNA

3.4.1. Real-time PCR for detection of the *msp2* gene of *Anaplasma phagocytophilum*

A real-time PCR (modified for the diagnostic laboratory) from Courtney et al. (2004) was chosen for an initial screening. The target sequence lies on the major surface protein 2 (*msp2*) gene encoding a 44 kDa immunodominant surface protein, supposed to be unique to *Anaplasma* species (Courtney et al., 2004). It is a multicopy gene (>10), with a highly variable central region flanked by highly conserved regions (Brayton et al., 2001). Major

surface proteins are involved in host-pathogen interactions and changes in *msp2* expression may be related to antigenetic variability (Dunning Hotopp et al., 2006). The primers ApMsp2f and ApMsp2r and the TaqMan® probe ApMsp2p-Hex hybridize with the conserved region of the gene and generate a 77-bp fragment (Table 3). By targeting a multicopy gene, the sensitivity is comparable to that of a traditional nested PCR (Massung et al., 1998). Genomic DNA from *A. marginale*, *E. canis*, *E. chaffeensis*, *Neorickettsia sennetsu*, *R. rickettsii*, *R. prowazekii*, *E. coli* and *Bartonella henselae* is not amplified (Courtney et al., 2004). The reaction was carried out in a BioRad iCycler IQ (Bio-Rad, Munich, Germany).

Tables 4 and 5 show the reaction and cycling conditions used. Negative controls (DNA free extraction quality controls or sterile water) were always included. The unmodified protocol by Courtney et al., (2004), originally tested for *I. scapularis* ticks, has also been used to retest a part of the samples which did not give clear amplification curves. Cycling conditions in the original protocol were initial activation of the Taq Polymerase for 10 minutes at 95°C, denaturation for 15 seconds at 95°C and annealing-extension for 1 minute at 60°C, for 40 cycles.

Table 3: Primers for real-time PCR detection of the *msp2* gene of *Anaplasma phagocytophilum*

Primer	Oligonucleotide sequence	Reference
ApMSP2f	5'-ATG GAA GGT AGT GTT GGT TAT GGT ATT-3'	Courtney et al., 2004
ApMSP2r	5'-TTG GTC TTG AAG CGC TCG TA-3'	
ApMSP2p-HEX	5'-TGG TGC CAG GGT TGA GCT TGA GAT TG-3' labeled 5'-HEX, 3'-TAMRA	

Table 4: Reaction conditions for real-time PCR detection of the *msp2* gene of *Anaplasma phagocytophilum*

Reagent	Volume	
Buffer 10x	2.5	µl
$MgCl_2$ (25mM)	4.5	µl
dNTPs (10mM each)	0.5	µl
ApMSP2f (100µM)	0.225	µl
ApMSP2r (100µM)	0.225	µl
ApMSP2p-HEX (100µM)	0.063	µl
Taq Polymerase (5U/µl)	0.25	µl
Pure H_2O	11.737	µl
Template DNA	5.0	µl
Total volume	**25.0**	**µl**

Table 5: Cycling conditions for real-time PCR detection of the *msp2* gene of *Anaplasma phagocytophilum*

Cycle	Step	Temperature	Duration
Cycle 1:	initial activation	95.0°C	15 min
1x			
Cycle 2:	denaturation	94.0°C	15 sec
50x	annealing-extension	60.0°C	60 sec

(Modified from Courtney et al., 2004)

3.4.2. Nested PCR for detection of the *16S rRNA* gene of *Anaplasma phagocytophilum*

The sensitivity and specificity of the *msp2* PCR is very high, but the sequences obtained can not be used for differentiation of closely related strains (Courtney et al., 2004). Massung et al. (1998) developed a nested PCR with a sensitivity of two copies of the *16S rRNA* gene of *A. phagocytophilum*. *E. chaffeensis, E. canis, E. sennetsu, E. risticii, B. henselae, R. rickettsii* and others are not amplified. As it was intended to gain an overview on the genetic variants present in the study area, this protocol was applied to 30% of the samples showing a positive *msp2* result and products were subsequently sequenced.

The primer pair ge3a/ge10r generates a 932-bp fragment, the primer pair ge9f/ge2 a 546-bp fragment (Table 6). A Thermocycler Gene Amp PCR System 2700 (Applied Biosystems, Weiterstadt, Germany) was used for the amplifications with the reaction mix and cycling conditions as shown in Tables 7 and 8.

Table 6: Primers for nested PCR detection of the *16S rRNA* gene of *Anaplasma phagocytophilum*

Primer	Oligonucleotide sequence	Reference
Primary amplification		
ge3a	5'-CAC ATG CAA GTC GAA CGG ATT ATT C-3'	Massung et
ge10r	5'-TTC CGT TAA GAA GGA TCT AAT CTC C-3'	al., 1998
Second amplification		
ge9f	5'-AAC GGA TTA TTC TTT ATA GCT TGC T-3'	Massung et
ge2	5'-GGC AGT ATT AAA AGC AGC TCC AGG-3'	al., 1998

Table 7: Reaction conditions for nested PCR detection of the *16SrRNA* gene of *Anaplasma phagocytophilum*

Reagent	Volume	
Buffer 10x	5.0	µl
$MgCl_2$ (25mM)	3.0	µl
dNTP (10mM each)	1.0	µl
Primer r (100µM)	0.25	µl
Primer p (100µM)	0.25	µl
Taq Polymerase (5U/µl)	0.5	µl
Pure H_2O	35.0	µl
Template DNA	5.0	µl
Total volume	**50.0**	**µl**

Table 8: Cycling conditions for nested PCR detection of the *16S rRNA* gene of *Anaplasma phagocytophilum*

Cycle	Step	Temperature	Duration
Primary amplification			
Cycle 1:	Initial denaturation	95.0°C	2 min
1x			
Cycle 2:	Denaturation	94.0°C	30 sec
40x	Annealing	55.0°C	30 sec
	Extension	72.0°C	1 min
Cycle 3:	Final extension	72.0°C	5 min
1x			
Second nested amplification			
Cycle 1:	Initial denaturation	95.0°C	2 min
1x			
Cycle 2:	Denaturation	94.0°C	30 sec
30x	Annealing	55.0°C	30 sec
	Extension	72.0°C	1 min
Cycle 3:	Final extension	72.0°C	5 min
1x			

3.5. Polymerase Chain Reaction for detection of *Rickettsia* spp. DNA

3.5.1. PCR for the detection of the *gltA* and *ompA* genes

Detection of *Rickettsia* spp. was performed with PCRs targeting the *gltA* and *ompA* genes. The primer pair RpCS.877p and RpCS.1258n (Table 9) was derived from the citrate synthase gene of *R. prowazekii* which is shared by all rickettsiae (Regnery et al., 1991). It is therefore suitable for an initial screening. Primer pair Rr190.70p and Rr190.602n (Table 9) was derived from the 190-kDa SFG antigen of the outer membrane protein A of *R. rickettsii* (R strain) (Regnery et al. 1991). The *ompA* PCR was used on all samples that had tested positive in the screening for *gltA*, as it is suitable for differentiation of closely related SFG rickettsiae.

Table 9: Primers for the detection of the *gltA*, *ompA*, *ompB* and *16S rRNA* genes of *Rickettsia* spp.

gene	primer	oligonucleotide sequence	Reference
gltA	RpCS.877p RpCS.1258n	5'-GGG GGC CTG CTC ACG GCG G-3' 5'-ATT GCA AAA AGT ACA GTG AAC A-3'	Regnery et al., 1991
ompA	Rr190.70p Rr190.602n	5'-ATG GCG AAT ATT TCT CCA AAA-3' 5'-AGT GCA GCA TTC GCT CCC CCT-3'	Regnery et al., 1991
16S rRNA	fD1 Rc16S.452n	5'-AGA GTT TGA TCC TGG CTC AG-3' 5'-AAC GTC ATT ATC TTC CTT GC-3'	Márquez et al., 1998
ompB	120-2788 120-3599	5'-AAA CAA TAA TCA AGG TAC TGT-3' 5'-TAC TTC CGG TTA CAG CAA AGT-3'	Roux and Raoult, 2000

PCR reaction conditions and cycling protocols are shown in Tables 10 and 11. Negative controls were the same as in the *A. phagocytophilum* PCR. As positive controls, the first positive samples, confirmed by sequencing from this study, were subsequently used.

Table 10: Reaction conditions for PCR detection (*gltA*, *ompA*, *ompB* and *16S rRNA* genes) of *Rickettsia* spp.

Reagent	Volume	
Buffer 5x	10.0	µl
$MgCl_2$ (25mM)	3.0	µl
dNTP (10mM each)	1.0	µl
Forward primer (100µM)	0.25	µl
Reverse Primer (100µM)	0.25	µl
Taq Polymerase (5U/µl)	0.5	µl
Pure H_2O	30.0	µl
Template DNA	5.0	µl
Total volume	**50.0**	**µl**

Table 11: Cycling conditions for the detection of the *gltA* and *ompA* genes of *Rickettsia* spp.

Cycle	Step	Temperature	Duration
gltA			
Cycle 1: 1x	Initial denaturation	94.0°C	3 min
Cycle 2: 35x	Denaturation	95.0°C	20 sec
	Annealing	48.0°C	30 sec
	Extension	60.0°C	120 sec
Cycle 3: 1x	Final extension	72.0°C	7 min
ompA			
Cycle 1: 1x	Initial denaturation	94.0°C	3 min
Cycle 2: 35x	Denaturation	95.0°C	45 sec
	Annealing	55.0°C	30 sec
	Extension	72.0°C	90 sec
Cycle 3: 1x	Final extension	72.0°C	7 min

(Bertolotti et al., 2006)

3.5.2. PCR for the detection of the *16S rRNA* and *ompB* genes of *Rickettsia* spp.

To clarify results, a part of the *gltA* positive samples whose sequences gave no sufficient sequence similarity to known rickettsial species in sequence analysis, were further investigated for the presence of a SFG rickettsiae specific 426-bp portion of the *16S rRNA* gene with the primer pair fD1 and Rc16S.452n (Márquez et al., 1998) and of a 765-bp portion

of the o*mpB* gene with the primer pair 120-2788 and 120-3599, which was present in all rickettsiae studied by Roux and Raoult (2000) (Table 9). Tables 10 and 12 show the reaction mix and the cycling conditions used.

3.6. Agarose gel electrophoresis

Conventional PCR products were visualized under UV light after 1.5% agarose gel electrophoresis (1.5g Agarose/100 ml TAE Buffer) and subsequent staining with ethidiumbromide solution. For comparison a standardized DNA-Ladder was added to each electrophoresis.

Table 12: Cycling conditions for the detection of the *16S rRNA* and *ompB* genes of *Rickettsia* spp.

Cycle	Step	Temperature	Duration
16S rRNA			
Cycle 1:	Initial denaturation	94.0°C	3 min
1x			
Cycle 2:	Denaturation	95.0°C	20 sec
40x	Annealing	59.0°C	30 sec
	Extension	72.0°C	45 sec
Cycle 3:	Final extension	72.0°C	7 min
1x			
ompB			
Cycle 1:	Initial denaturation	95.0°C	3 min
1x			
Cycle 2:	Denaturation	95.0°C	30 sec
40x	Annealing	50.0°C	30 sec
	Extension	68.0°C	90 sec
Cycle 3:	Final extension	68.0°C	7 min
1x			

3.7. DNA Purification

The purification of PCR products was carried out with the QIAquick PCR Purification Kit (Qiagen, Hilden, Germany) according to manufacturer's instruction.

3.8. Sequencing and Sequence Analysis

After purification, all rickettsial PCR products and the *16S rRNA* products of *A. phagocytophilum* were sent off for sequencing (MWG Biotech, Martinsried, Germany). After evaluating the specificity of results with Chromas©Lite (www.technelysium.com.au/chromas_lite.html), sequence similarity searches were made, without the flanking primers, by BLASTn analysis (www.ncbi.nlm.nih.gov.library.vu.edu.au/BLAST/). The obtained sequences were further analyzed with each other and with GenBank sequences by multiple alignments (www.ebi.ac.uk/clustalw/index.html). Multiple alignment files were transformed into boxshade files with Boxshade 3.21 (www.ch.embnet.org/software/BOX_form.html).

3.9. Statistical Analysis

The statistical analysis of this work was carried out in close cooperation with the StabLab (Statistical Consulting Unit) of the Department of Statistics, Ludwig-Maximilians-University, Munich. To investigate the effect of monthly variation, geographic location, tick developmental stage and gender of ticks on the probability of infection with *A. phagocytophilum,* a statistical analysis based on logistic regression was performed. Wald-Tests and an analysis of deviance were used for this model to study the effect of the above covariates and possible interactions. Values of $p<0.05$ were regarded as significant. For *A. phagocytophilum,* given the low prevalence in the investigations the resulting odds-ratios of the logistic regressions were interpretable as relative risks (RR). Calculation of the monthly prevalence was performed by a weighted analysis taking into account the two-phase sampling design for stratification. Phase 1 corresponded to a simple random sample, which could be stratified by gender. In phase 2, a fixed number of samples per month was drawn at random within each gender stratum, thus month was an additional stratum. Estimates were based on the Horvitz-Thompson estimator and corresponding 95% confidence intervals were computed by a parametric bootstrap conditioning on the Phase 1 sample sizes (Särndal et al., 1992). All computations for *A. phagocytophilum* were performed using R version 2.5.0 and for *Rickettsia* spp. using R version 2.6.2. (R Development Core Team, 2007).

4. Results

4.1. Tick collection

In the first phase of sampling, a total of 9,469 actively questing ticks of all stages (4,932 adults, 3,573 nymphs and 964 larvae) were collected during all flagging sessions at the study sites. 1 nymph and 36 larvae were collected in July at another site close to Site W, but were not included in the statistical analysis. The number of females, males and nymphs collected at different sites and months during this phase varied (Table 13, Figure 10). Species identification of adult ticks revealed no other tick species than *I. ricinus*.

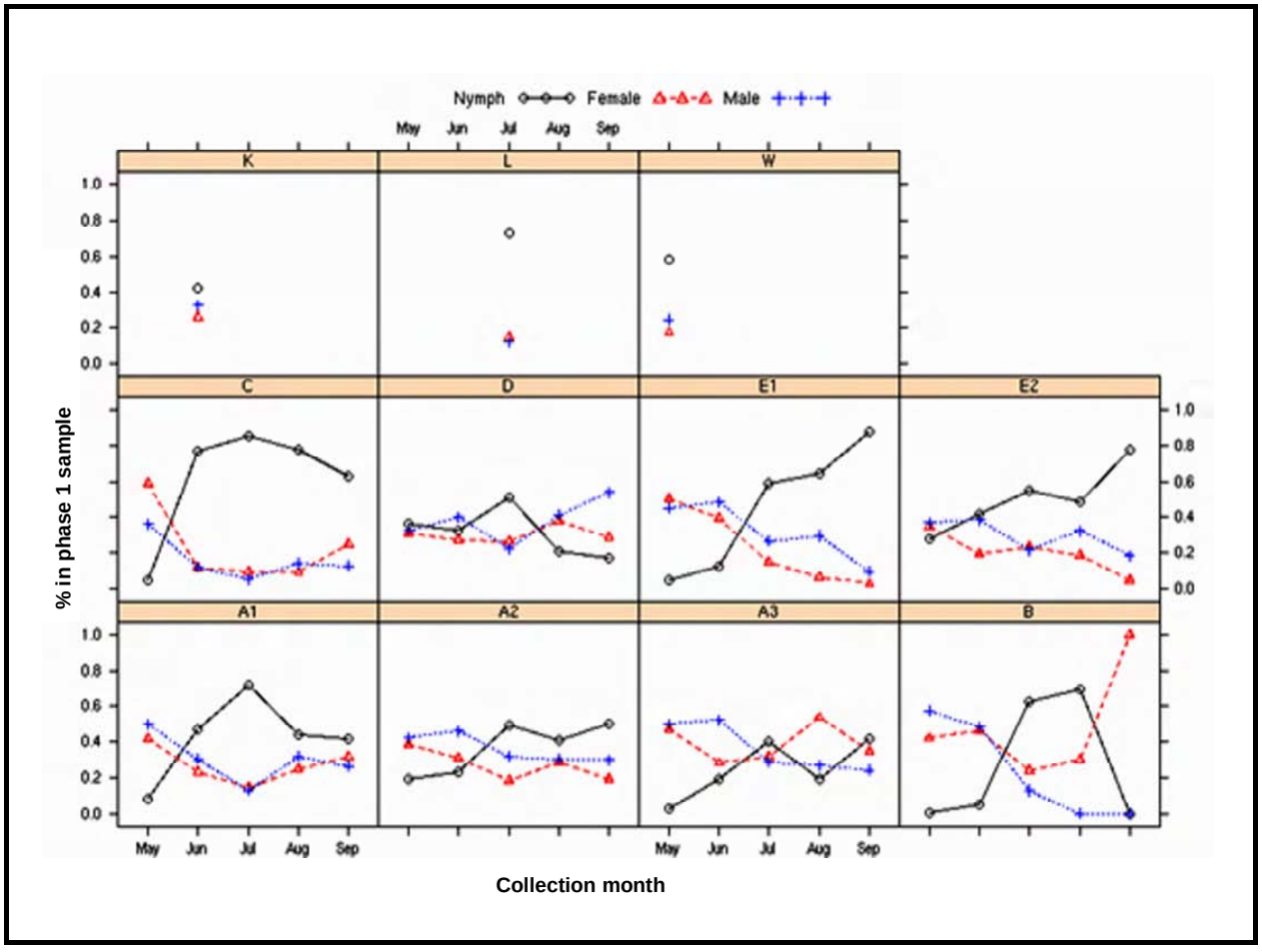

Figure 10: Proportion of females, males and nymphs collected each month at each collection site during phase 1.
[a] For number of ticks collected compare table 13.

Table 13: Numbers of all ticks collected at each collection site during phase 1.

Month	Stage[a]	Collection site[b]										
		A1	A2	A3	B	C	D	E1	E2	W	K	L
May	M	101	102	105	124	8	105	147	109	189	-	-
	F	84	93	98	91	13	99	164	104	137	-	-
	N	17	47	6	1	1	117	14	83	457	-	-
	L	0	0	0	0	0	0	0	0	248	-	-
Juni	M	90	117	126	66	53	116	118	138	-	89	-
	F	68	78	68	63	52	79	95	70	-	70	-
	N	139	58	47	7	351	94	29	151	-	114	-
	L	52	0	0	0	16	6	0	1	-	1	-
July	M	8	93	71	6	10	74	52	88	-	-	54
	F	9	53	78	11	18	88	29	94	-	-	66
	N	44	145	99	29	161	170	115	226	-	-	325
	L	97	122	0	52	22	20	14	19	-	-	5
Aug.	M	13	31	7	0	11	61	5	70	-	-	-
	F	10	29	14	3	7	56	1	41	-	-	-
	N	18	42	5	7	61	31	11	106	-	-	-
	L	76	56	0	0	0	0	0	4	-	-	-
Sept.	M	5	48	7	0	1	69	7	12	-	-	-
	F	6	31	10	1	2	37	2	3	-	-	-
	N	8	81	12	0	5	22	66	51	-	-	-
	L	10	139	2	0	0	2	0	1	-	-	-

[a]M, male; F, female; N, nymph; L, larva
[b]for descriptions of study sites see 3.1.2.

4.2. NanoDrop

The number of DNA extracts obtained for each stage from each collection site and month are shown in the appendix table in publication 1 (4.4.1., p. 56). The total number of ticks investigated in the laboratory equals the total number of DNA extracts. For SFG rickettsiae, one male tick less from site D, month of May, was investigated. The average amount of DNA extracted measured with NanoDrop® was 16.67ng/µl in female ticks, 9.16ng/µl in male ticks and 9.86ng/µl in nymphs.

4.3. Real-Time PCR

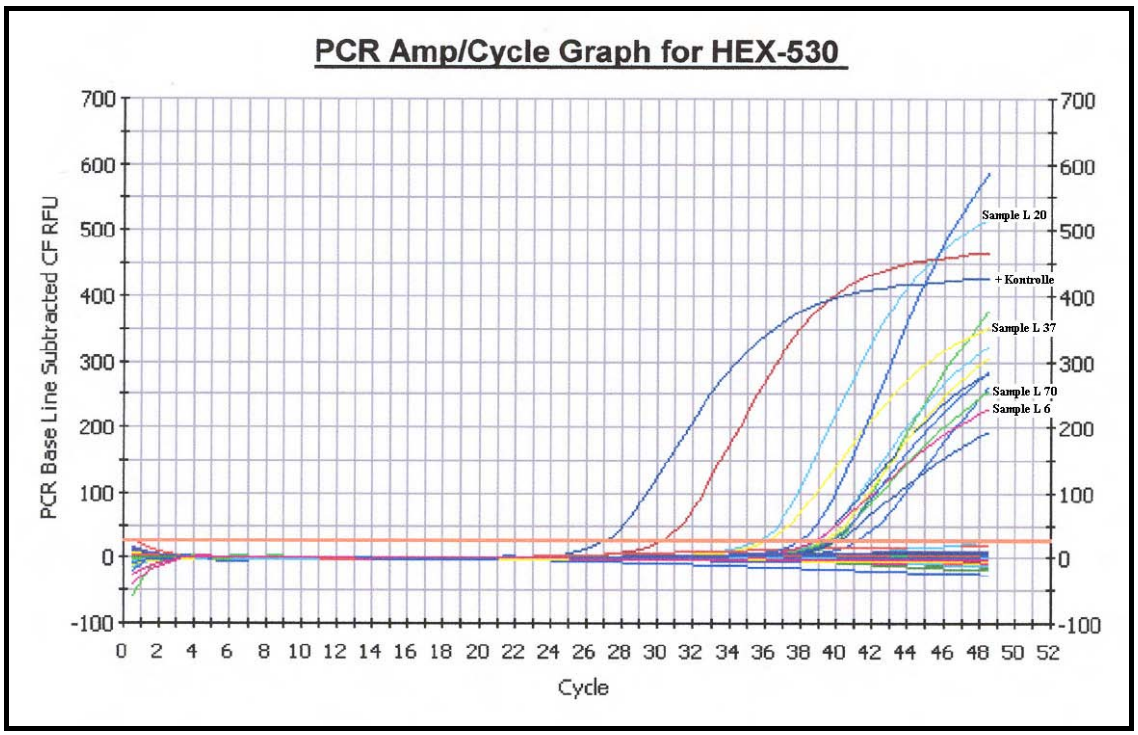

Figure 11: *Msp2* real-time PCR results with the modified protocol with 50 cycles.
(L 70: green curve)

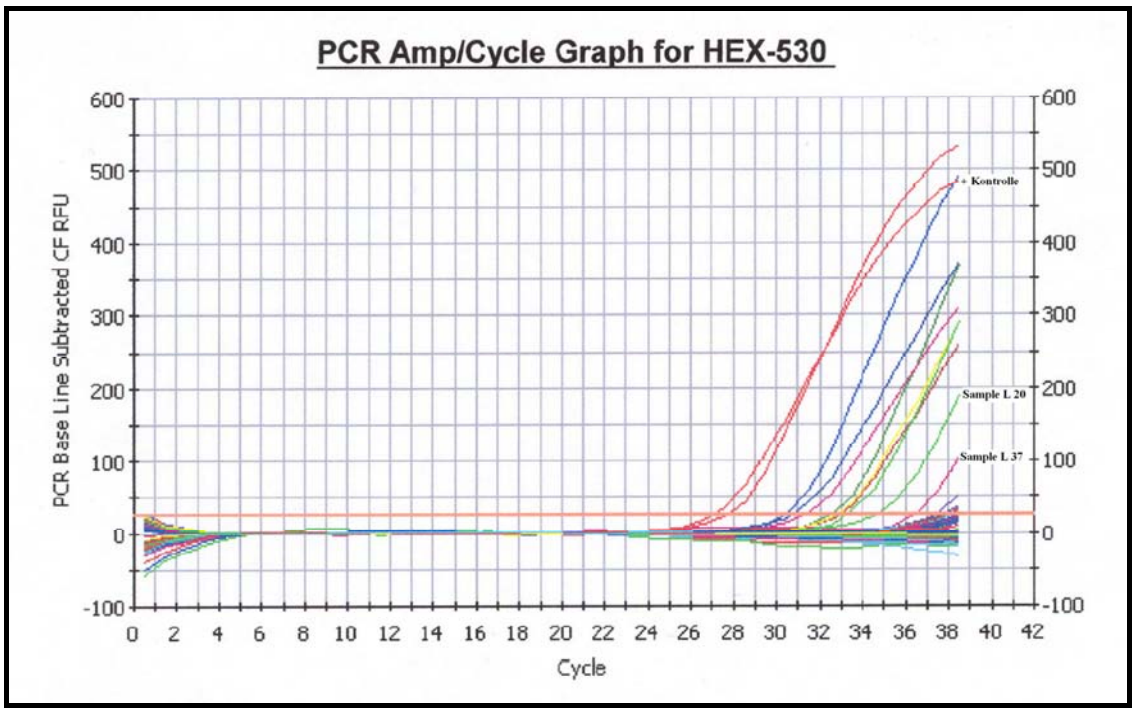

Figure 12: *Msp2* real-time PCR results with the original protocol with 40 cycles.

During amplification in the real-time PCR for detection of the *msp2* of *A. phagocytophilum*, amplification later than cycle 40 was considered negative, however, some samples remained doubtful with the modified protocol due to late amplification between cycles 38 and 40 (see 3.4.1.). Sample L 70 and L 6 have been chosen as examples (Figure 11). These samples could be verified and most of them excluded as negative with the original protocol from Courtney et al. (2004). Exemplary samples L 70 and L 6 did not amplify in the real-time PCR with 40 cycles (Figure 12).

4.4. Publications

The PCR results, the statistical analysis of the prevalence and the sequencing results were organized in two publications and submitted to peer-reviewed journals.

4.4.1. Publication 1

Anaplasma phagocytophilum infection in *Ixodes ricinus*, Bavaria, Germany

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Emerging Infectious Diseases (2008), Vol. 14 (6), 972-974

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PDF file available from:

http://www.cdc.gov/eid/content/14/6/972.htm

Anaplasma phagocytophilum Infection in *Ixodes ricinus*, Bavaria, Germany

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Abstract

Anaplasma phagocytophilum DNA was detected by real-time PCR, which targeted the *msp2* gene, in 2.9% of questing *Ixodes ricinus* ticks (adults and nymphs; n = 2,862), collected systematically from selected locations in Bavaria, Germany, in 2006. Prevalence was significantly higher in urban public parks in Munich than in natural forests.

Anaplasma phagocytophilum, an obligate intracellular bacterium, causes a febrile disease in ruminants and granulocytic anaplasmosis in dogs, horses, and humans (1). A reorganization of the order Anaplasmataceae reclassified *Ehrlichia equi*, *E. phagocytophila*, and the human granulocytic ehrlichiosis (HGE) agent to the single species *A. phagocytophilum* (2), which in Europe is transmitted by the sheep tick, *Ixodes ricinus* (3). The agent is found among the *I. ricinus* population in Germany; average prevalence rates are 1% to 4.5% (4,5). The English Garden, a large (3.7km2) public park in Munich (state of Bavaria, Germany), has been suggested in 2 previous studies as a focal point for *A. phagocytophilum* (5,6). We investigated *A. phagocytophilum* in questing ticks in urban areas of Munich and focused on seasonal and geographic effects on the prevalence.

The Study

The sampling consisted of 2 phases. First, to gain an overview on the occurrence of *I. ricinus*, were collected questing ticks by the flagging method at 8 locations (labeled A1, A2, A3, B, C,

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D, E1, E2) close to the Isar River in the Munich area from May through September 2006 (Figure 1). Sites A1 and A2 were located in the city center part of the English Garden, which is enclosed by roads and houses. The vegetation of this heavily frequented area consists of groomed lawns, bushes, and deciduous trees.

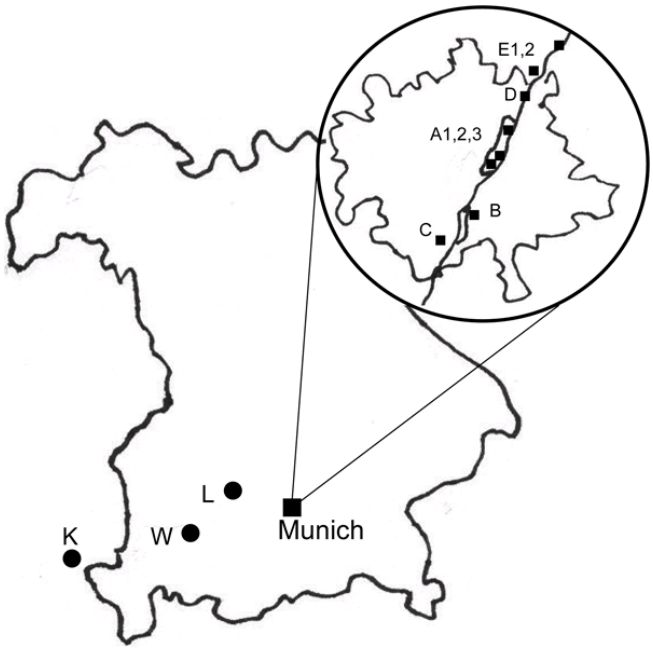

Figure 1. Location of collection sites. Large map, Bavaria, Germany; circled inset, city of Munich (with the Isar River). Sites in Munich area: A1, 2, 3, English Garden park; B, city park; C, D, E1, 2, riparian and deciduous forest; K, L, W, mixed forest areas outside of Munich.

Site A3 was located in the northern part of the Garden, where vegetation was maintained by gardening, but bushes and trees were denser and grassland less frequently cut. The site was also used for horseback riding. Site B was a landscaped public green in the southern part of the city with groomed lawns and deciduous trees. Sites C, D, E1, and E2 were periurban riparian and deciduous forests. Three natural mixed forest sites (K, L, W) outside of Munich were sampled once (Figure 1). Ticks were registered and frozen individually at –26°C; adults were identified to species level by standard taxonomic keys (7). In the second phase, DNA was extracted from randomly chosen ticks (as available, 30 females, males, and nymphs, respectively, per month per site) with the High Pure PCR Template Preparation Kit (Roche, Mannheim, Germany) according to manufacturer's instructions with modifications. In individual 1.5-mL tubes, each tick was crushed mechanically with a metal spatula; sterile water (200 µL) was added, and the sample was kept overnight in a 55°C water bath for complete tissue lysis. At the beginning and end of each extraction line, a negative control was added. Quality and quantity of extracted DNA were tested with a spectrophotometer (NanoDrop ND-1000, PeqLab, Erlangen, Germany). A real-time PCR targeting the *msp2* gene of *A. phagocytophilum* (8) was performed with modifications in a Bio-Rad iCycler iQ (Bio-Rad, Munich, Germany). In a reaction volume of 25 µL, the HotStarTaq Buffer Set was

used with 1.25 U HotStarTaq Polymerase (both QIAGEN, Hilden, Germany), 6 mmol/L MgCl2, 200 µmol/L each dNTP, 900 nmol/L each primer (ApMSP2f / ApMSP2r [8]), 125 nmol/L probe ApMSP2p-HEX (8), and 5.0 µL template DNA. Cycling conditions were as follows: initial activation (95°C, 15 min), 50 cycles denaturation (94°C, 15 s), and annealing–extension (60°C, 60 s). The original protocol was also used for part of the samples (8). Thirty-one DNA extracts, positive in real-time PCR, were amplified in a Thermocycler GeneAmp PCR System 2700 (Applied Biosystems, Weiterstadt, Germany) with a nested PCR (9) targeting the 16S rRNA gene, amplification of which is necessary to differentiate closely related strains (8). Negative and known positive controls were always included. After the final products were analyzed by 1.5% agarose gel electrophoresis and purified with the QIAquick PCR Purification Kit (QIAGEN) according to manufacturer's instruction, the 497-bp fragments, without flanking primers, were sent for sequencing to MWG, Martinsried, Germany. The results were evaluated with ChromasLite (www.technelysium.com.au/chromas_lite.html), sequence homology searches made by BLASTn analysis of GenBank sequences (www.ncbi.nlm.nih.gov/BLAST), and multiple alignments (www.ebi.ac.uk/clustalw/index.html). The effects of month, location, stage, and sex of ticks on probability of infection were investigated with logistic regressions by using R version 2.5.0 (10); $p<0.05$ was regarded as significant. Due to low prevalence of *A. phagocytophilum*, odds ratios were interpretable as relative risks (RR). We calculated monthly prevalence with a weighted analysis, taking into account the sampling design: phase 1, a random sample, is stratified by sex, and in phase 2, a fixed number was drawn monthly at random within each sex stratum. Estimates were based on the Horvitz-Thompson estimator and corresponding 95% confidence intervals (CIs) computed by parametric bootstrap conditioning on phase 1 sample sizes (11). A total of 9,507 ticks (4,932 adults, 3,573 nymphs, and 1,001 larvae) were collected, and adults were identified as *I. ricinus*. Real-time PCR was performed for 2,862 ticks (Table; online Appendix Table, available from www.cdc.gov/EID/content/14/6/972-appT.htm). With the modified protocol, atypical amplification occurred in ~10% of samples, whereas with the original protocol, which had been tested on *I. scapularis* ticks, no amplification occurred. This difference suggests unspecific reactions in the modified protocol. *A. phagocytophilum* was detected in 5.67% of females, in 4.00% of males, and in 1.14% of nymphs (Table). The overall prevalence was 2.9% (95% CI 2.3%–3.5%). Significantly more females and males were infected than nymphs (RR = 4.906 for females, RR = 3.439 for males; $p<0.001$).

Table. Total *Anaplasma phagocytophilum*–infected *Ixodes ricinus* ticks per site, southern Germany, 2006*

	N° infected ticks/N° total ticks (%)		
Study site	Females	Males	Nymphs
A1	11/87 (12.64)	5/88 (5.68)	3/104 (2.88)
A2	10/149 (6.71)	12/153 (7.84)	3/150 (2.00)
A3	7/114 (6.14)	4/105 (3.81)	1/83 (1.20)
B	8/80 (10.00)	5/65 (7.69)	0/42 (0)
C	1/68 (1.47)	1/60 (1.67)	0/96 (0)
D	5/150 (3.33)	5/152 (3.29)	2/142 (1.41)
E1	3/92 (3.26)	1/101 (0.99)	2/114 (1.75)
E2	5/122 (4.10)	1/134 (0.75)	0/140 (0)
K	1/30 (3.33)	1/31 (3.23)	0/30 (0)
L	1/30 (3.33)	2/30 (6.67)	0/30 (0)
W	2/30 (6.67)	1/30 (3.33)	0/30 (0)
Total	54/952 (5.67)	38/949 (4.00)	11/961 (1.14)

*A1, A2, A3, English Garden in Munich; B, other park in Munich; C, D, E1, E2, periurban forest areas of Munich; W, K, L, forests outside of Munich (compare Figure 1)

Prevalence was significantly higher in the city parks (A1, A2, A3, B) than in natural forest areas (C, D, E1, E2, K, L, W; RR = 0.368, $p<0.001$). Prevalence was significantly lower in the riparian forest, Isarauen (E1, E2) in the north of Munich, than in the English Garden (A1, A2, A3) (RR = 0.314, $p<0.001$). Variations among the collection months, ranging from 0% to 20% for females and males and from 0 to 9.1% for nymphs (online Appendix Table), were not significant ($p = 0.40$).

Alignment of the partial 16S rRNA gene sequences showed that 30 sequences were 100% identical (Gen-Bank accession no. EU490522); 1 sequence differed in 2 nucleotide positions (accession no. EU490523). The 30 homologous sequences were 100% identical to *Ehrlichia* sp. Frankonia 2 when compared with GenBank sequences (Figure 2) of *Ehrlichia* sp. Frankonia 2, *A. phagocytophilum* isolate X7, *A. phagocytophilum* isolate P80, and the prototype sequence of the HGE agent (GenBank accession nos. AF136712, AY281805, AY281794, and U02521, respectively). For Frankonia 2 and *A. phagocytophilum* isolate X7, the remaining sequence differed in 1 nt position. All differed in 1 nt position from the prototype HGE agent and *A. phagocytophilum* isolate P80 and in 2 more nt positions from P80.

Appendix Table. *Anaplasma phagocytophilum* prevalence in *Ixodes ricinus*, Munich, Germany, 2006*†

N° infected ticks/N° total ticks

Study Site	May			June			July			August			September		
	F	M	N	F	M	N	F	M	N	F	M	N	F	M	N
A1	6/31	1/32	1/17	2/30	2/30	0/30	1/10	1/8	2/31	2/10	0/13	0/18	0/6	1/5	0/8
A2	2/30	1/30	0/30	3/30	2/32	1/30	1/30	3/30	0/30	3/29	5/31	2/30	1/30	1/30	0/30
A3	4/30	2/31	0/6	1/30	0/30	0/30	1/30	2/30	0/30	0/14	0/7	0/5	1/10	0/7	1/12
B	4/35	3/30	0/1	4/30	2/30	0/6	0/11	0/5	0/29	0/3	0/0	0/6	0/1	0/0	0/0
C	1/12	0/8	0/1	0/30	0/30	0/30	0/17	0/10	0/30	0/7	1/11	0/30	0/2	0/1	0/5
D	2/30	0/31	0/30	0/30	0/31	0/30	0/30	2/30	1/30	1/30	2/30	0/30	2/30	1/30	1/22
E1	1/30	0/30	1/14	1/30	1/30	0/29	1/29	0/29	0/30	0/1	0/5	1/11	0/2	0/7	0/30
E2	1/30	0/31	0/20	2/30	0/31	0/30	2/30	0/30	0/30	0/30	1/30	0/30	0/2	0/12	0/30
Total	**21/228**	**7/223**	**2/119**	**13/240**	**7/244**	**1/215**	**6/187**	**8/172**	**3/240**	**6/124**	**9/127**	**3/160**	**4/83**	**3/92**	**2/137**

*F, females; M, males; N, nymphs; A1, A2, A3, English Garden in Munich; B, other park in Munich; C, D, E1, E2, periurban forest areas of Munich. †Monthly prevalence in all sites (includes sites K, L, W, [forests outside Munich] and stages calculated), considering the 2-phased sampling design. Monthly prevalence for May, 4.0% (95% confidence interval [CI] 2.6%–5.3%); June, 2.3% (95% CI 1.3%–3.2%); July, 2.1% (95% CI 1.0%–3.0%); August, 3.6% (95% CI 1.8%–5.2%); September, 2.3% (95% CI 0.7%–3.8%).

GenBank accession no.	Nucleotide at position 7	11	16	97	303
EU490522	A	G	T	C	**A**
EU490523	–	–	**A**	**T**	**A**
AF136712	A	G	T	C	**A**
AY281805	A	G	T	C	**A**
AY281794	**G**	**A**	T	C	G
U02521	A	G	T	C	G

Figure 2. Comparison of the 497-bp sequences of *Anaplasma phagocytophilum* obtained from *Ixodes ricinus* ticks, Bavaria, Germany, 2006, in relation to selected GenBank sequences.

Conclusions

Our results indicate that city parks of Munich may be focal points for *A. phagocytophilum*. Focal distribution depends mainly on mammalian reservoir hosts because of lack of transovarial transmission in ticks (12). Wood mice, yellow-necked mice, voles, roe, and red deer have been suggested as reservoirs in Europe (13,14). In the parks, a different reservoir host might be present. Large numbers of people and their domestic dogs pass through the parks, and the possibility of dogs acting as reservoirs for *A. phagocytophilum* should be investigated in further studies. *Ehrlichia* sp. Frankonia 2 was first detected in adult ticks collected from domestic dogs in central Germany (15) and was later found in questing adults in Munich (5). However, neither *Ehrlichia* sp. Frankonia 2 nor the closely related *A. phagocytophilum* isolate X7 has been detected in humans or animals; thus, they can be regarded as strains of unknown pathogenicity. Future studies should aim at characterization of this strain and its possible role as a human or veterinary pathogen, as well as the identification of potential reservoir hosts in the city parks.

Acknowledgments

We thank Cecilia Hizo-Teufel and Sarah Leonhard for instruction on working with tick DNA, Ingrid Pradel for excellent technical advice, Sarah Pfalzer and Sophie Neuvecelle for help with DNA extraction, Helmut Küchenhoff for valuable discussions on the statistical analysis of the data, and Lygia Friche Passos for critically reviewing the manuscript.

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4.4.2. Publication 2

Prevalence of Spotted Fever Group Rickettsiae in *Ixodes ricinus* (Acari: Ixodidae) in Southern Germany

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Journal of Medical Entomology

Submitted: February 14th 2008
Accepted for publication: May 23rd 2008

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Silaghi et al.: *Rickettsia* spp. in Ticks, Southern Germany

Journal of Medical Entomology
Vector-borne Diseases

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Prevalence of Spotted Fever Group Rickettsiae in *Ixodes ricinus* (Acari: Ixodidae) in Southern Germany

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Abstract

Host-seeking *Ixodes ricinus* (L.) ticks were collected systematically, from May to September 2006, at selected sites in Southern Germany, including a large city park in Munich. Polymerase Chain Reactions for amplification of genes of the rickettsial citrate synthase (*gltA*), the outer membrane proteins A and B (*ompA* and *ompB*), and the 16S rDNA were used to investigate 2,861 specimen (adults and nymphs). G*ltA* sequences of spotted fever group rickettsiae were detected in 151 of all samples (5.3%; 95% CI 4.3% to 6.2%). Sequencing revealed *Rickettsia helvetica* in 91.4% of them and *R. monacensis in* 8.6%. Amplification of *ompA* was not possible for *R. helvetica*, but in all except one of the *R. monacensis*. The results were analyzed statistically to test the effects of season, location, developmental stage and gender of the tick on prevalence of *Rickettsia* spp. Although rickettsial DNA was detected in all investigated sites, sites in natural forest areas had significantly higher prevalences than sites in landscaped city parks ($p<0.001$). Adult female and male ticks had a similar prevalence and were significantly more often infected than nymphs ($p<0.001$). Monthly differences were not statistically significant. These results indicate that *R. helvetica* might lead to a public health threat to humans, especially after tick exposure in areas of high prevalence.

Keywords

Rickettsia helvetica, Rickettsia monacensis, Ixodes ricinus, epidemiology, Germany

Various members of the genus *Rickettsia* have been detected in recent years in ticks in Germany (Pichon et al. 2006, Dautel et al. 2006). Historically, the genus *Rickettsia* consisted of three groups, based on phenotypic criteria: (*i*) the spotted fever group (SFG), (*ii*) the typhus group (TG) and (*iii*) the scrub typhus group (STG) (Blanco and Oteo 2006). However, more recent phylogenetic studies have shown that these groupings are not consistent with interspecies relationships (Sekeyová et al. 2001). It is currently accepted that the heterogenic SFG rickettsiae comprise seven lineages, TG rickettsiae only *R. prowazekii* and *R. typhi,* and STG rickettsiae were excluded from the genus and reclassified as the new genus *Orientia* with one species, *O. tsutsugamushi*. Nevertheless, this classification is still not definite, and a reorganization may be necessary (Roux and Raoult 2000).

SFG rickettsiae are transmitted to vertebrate hosts via saliva during bloodfeeding of ixodid ticks, and within the ticks transstadially and transovarially (Parola and Raoult 2001). Therefore, rickettsial infections can be maintained in nature without the presence of a vertebrate host and ticks act both as a vector and a reservoir (Parola et al. 2005). Consequently, distribution of rickettsial diseases is dependent on the geographic distribution of the arthropod host (Parola and Raoult 2001).

New SFG rickettsiae and associated emerging diseases have been discovered all over Europe in the past decades. *R. helvetica* was first detected in Swiss *Ixodes ricinus* (L.) ticks in 1979, as a rickettsial strain of unknown pathogenicity (Burgdorfer et al. 1979), and was confirmed to be a new member of the SFG rickettsiae in 1993 (Beati et al. 1993). It has since been isolated from *I. ricinus* in many European countries (Blanco and Oteo 2006), with prevalence ranging from 2.5% to 59% (Parola et al. 1998, Christova et al. 2003). *R. helvetica*, previously only known to exist in European countries, has also been detected in *I. persulcatus*, *I. ovatus* and *I. monospinosus* in different parts of Japan where evidence is accumulating for a wide distribution across the islands (Inokuma et al. 2007).

In 1999, *R. helvetica* was linked to chronic perimyocarditis (Nilsson et al. 1999). There have since been several reports of serologic association with disease in Europe and Asia (Fournier et al. 2000, Fournier et al. 2004, Nilsson et al. 2005).

Amongst the SFG rickettsiae present in *I. ricinus* in Germany are *R. helvetica* with prevalence ranging from 8.9% to 14.2% and *R. monacensis* strain IrR/Munich (Simser et al. 2002, Hartelt et al. 2004, Pichon et al. 2006, Wölfel et al. 2006). Based on phylogenetic analysis, *R. monacensis* and all its strains comprise a separate cluster of SFG rickettsiae (Sréter-Lancz et al. 2005). Recently, *R. monacensis* has been associated with a febrile disease in man in northern Spain (Jado et al. 2007).

To provide further information on the distribution of SFG rickettsiae in Germany, a large number of ticks was tested by molecular methods for detection of rickettsial DNA. The results were analyzed statistically to evaluate seasonal, geographical and stage developmental effects on the prevalence and to determine areas of risk. Sequence analysis of all amplified DNA was conducted to obtain additional information on genetic differences in rickettsial species in Germany.

Material and Methods

Study Area and Tick Collection. The study area stretches over 20 km along the river Isar in the city of Munich (State of Bavaria, Germany) and includes eight sites in the vicinity of the river in the city area with elevation from 488 to 532 m a.s.l. (Fig. 1). The sites cover an area of about 0.25 km^2 each. Sites A1, A2 and A3 are located in the 'English Garden', one of the largest landscaped city parks in the world (more than 400 hectare). Sites A1 and A2 are in the Southern part of the park, enclosed to all sides by roads and houses and separated from site A3, which is in the Northern part of the park, by a four-lane city road. The Northern part transitions gradually into the natural riparian forest to the north. The landscaped vegetation in the parks consists of singular or small groups of large deciduous trees, in the majority beech (*Fagus sylvaticus* L.) and ash (*Fraxinus excelsior* L.), further yew (*Taxus baccata* L.) and frequently cut lawns. Foliage is removed in autumn. In the Northern part of the park, the grass is cut less frequently, trees and scrubs are denser and sheep are used in some areas for landscaping purposes. A part of Site A3 is also used for horse riding activities. Site B is in a landscaped public green in the South of Munich. Access to the 'English Garden' and the other park is free and therefore not controlled. It is estimated that during the summer month hundreds of thousands of people visit especially the Southern part of the 'English Garden'. Trespassing of lawns is allowed in all parks. Sites C, D, E1 and E2 are in natural riparian and deciduous forests. Whereas Sites C and D are located within the city boundaries, Sites E1 and E2 (the forest 'Isarauen') are north of the main city in periurban landscapes. The main tree species are *F. excelsior* and alder (*Alnus glutinosa* L.). Undergrowth is generally thick and includes blackberry bushes (*Rubus fruticosus* L. s.l.).

Three comparative sites (K, L, W) were chosen to cover additional vegetation types in areas of high recreational value (Fig. 1). Site K (altitude 669 m a.s.l.) is located in a natural mixed forest near the Lake Constance close to the town of Kreßbronn, and Site W (641 m a.s.l.) is located in a natural mixed forest near the prealpine spa town of Bad Wörishofen. The main coniferous tree species is the Norway spruce (*Picea abies* L.). Site L (528 m a.s.l.) is located close to a barrage of the river Lech near the city of Augsburg. The main tree species

are *A. glutinosa* and *P. abies*; *R. fruticosus* make up a large part of the thick undergrowth. Silviculture is practiced in all forest areas.

Regular tick samples were taken from the eight sites in the Munich area over a period of five months from May to September 2006. The aim was to collect all stages of ticks randomly from the vegetation by the flagging method until at least 200 adult ticks per month and site were obtained. In the parks, ticks were collected from along walkways and lawns, in forest areas from undergrowth of the natural riparian forest and along the river banks. The three sites outside of Munich were sampled once during the months May, June and July, respectively.

Roe deer (*Caproleus caproleus* L.) is abundant in almost all forest areas in Southern Germany, whereas red deer (*Cervus elaphus* L.) is less frequent. Small to medium sized mammals are common in the city parks: e. g., hedgehogs (*Erinaceus europaeus* L.), rabbits (*Oryctolagus cuniculus* L.*)*, hares (*Lepus europaeus* L.) and red foxes (*Vulpes vulpes* L.), but inhabit also the forest areas. Small rodents and birds are present in all areas. Generally speaking, city climates can differ greatly from rural climates as such that in many large cities the wind speed may be reduced and the temperature increased.

During the collection phase, all adult ticks were identified to species level by standard taxonomic keys (Hillyard 1996), classified according to date of collection, separated by sex and developmental stage, and frozen individually at -26°C.

DNA Extraction. From this first large random sample, a fixed number of ticks (30 females, 30 males and 30 nymphs per month and site, according to availability) were randomly chosen for further laboratory investigations. The chosen nymphs were identified to species level. Each of the chosen tick specimen was crushed with a sterile metal spatula prior to DNA extraction, which was carried out with the High pure PCR Template Preparation Kit (Roche Diagnostics GmbH, Mannheim, Germany), following the manufacturer's instructions. Additionally, 200µl sterile water were added to each tube before tissue lysis, and the materials were kept overnight in a 55°C water bath for better tissue lysis. A negative control was added at the beginning and at the end of each extraction line. Quality and quantity of extracted DNA were evaluated in a spectrophotometer (NanoDrop® ND-1000, PeqLab, Erlangen, Germany).

Polymerase Chain Reaction for detection of *Rickettsia* spp. and Sequencing. The PCRs were performed under conditions previously published (Regnery et al. 1991, Bertolotti et al. 2006) and targeted a 380-bp portion of the *gltA* gene using the primer pair RpCS.877p / RpCS.1258n (Regnery et al 1991), and a 530-bp portion of the *ompA* gene using the primer pair Rr190.70p / Rr190.602n (Regnery et al. 1991). All amplifications were performed in a

Thermocycler Gene Amp PCR System 2700 (Applied Biosystems, Weiterstadt, Germany) and were carried out in a 50µl reaction mix with 5µl of DNA template, 10µl PCR Buffer 5x, 3µl $MgCl_2$ (25mM), 1µl dNTP Mix (10mM), 0.25µl of each primer (100pM), and 0.5µl Taq (5U) with the Expand High Fidelity Plus PCR System (Roche Applied Science, Mannheim, Germany). Sterile water or DNA-free extraction controls served as negative controls and the first positive samples, confirmed by sequencing, were subsequently used as positive controls. The final PCR products were analyzed by 1.5% agarose gel-electrophoresis. Initially, all individual DNA extracts were screened for the presence of the *gltA* gene, encoding for the citrate synthase, a highly conserved enzyme present in all rickettsiae (Roux and Raoult 2000). Samples positive in *gltA* PCR were evaluated for amplification of the *ompA* gene, encoding for an autotransporter present only in the SFG rickettsiae (Fournier et al. 1998). To clarify samples which gave no clear results from the *gltA* and *ompA* PCR, further investigation for the presence of a 426-bp portion of the 16S rRNA gene with the primer pair fD1 / Rc16S.452n (Márquez et al. 1998) and of a 765-bp portion of the *rOmpB* gene with the primer pair 120-2788 / 120-3599 (Roux and Raoult 2000), were carried out as described above. Cycling conditions for the 16S rDNA PCR started with 94°C for 3 min, followed by 40 cycles (95°C 20 sec, 59°C 30 sec, 72°C 45 sec), and ended with a final extension for 7 min at 72°C. The amplification of *ompB* began with 3 min at 95°C, followed by 40 cycles (95°C 30 sec, 50°C 30 sec, 68°C 90 sec) and a final extension step at 68°C for 7 min. The PCRs were established using positive controls and subsequent sequencing. All PCR products were sequenced after purification with the QIAquick PCR Purification Kit (Qiagen, Hilden, Germany). After evaluation of the sequences with the software Chromas©Lite (www.technelysium.com.au/chromas_lite.html), sequence homology searches, without the flanking primers, were made by BLASTn analysis of GenBank (www.ncbi.nlm.nih.gov.library.vu.edu.au/BLAST/). The partial sequences obtained were aligned with sequences deposited in the GenBank database using the multiple alignment software ClustalW (www.ebi.ac.uk/clustalw/index.html).

Statistical Analysis. A statistical analysis based on logistic regression was performed to evaluate the effect of month, site, developmental stage and gender of the ticks on the probability of infection with *Rickettsia* spp. All significance tests in the logistic model were performed by Wald tests and $p<0.05$ was considered to indicate a statistically significant difference. The overall goodness of fit test was performed using the unweighted sum of squares test described in Hosmer et al. (1997). For the calculation of monthly and overall (i.e. May to September) prevalence, the two-phase sampling design of the study has to be taken

into account. Phase 1 consists of all ticks collected for each site and month, whereas phase 2 consists of the gender stratified subsets (30 for each gender, as available) investigated for disease status. For a specific site and month the prevalence can thus be computed by a weighted sum of the prevalence for each gender. Weights are the gender's proportion of the population, which can be estimated for each site and month from the phase 1 sample. Altogether the computed prevalence estimates correspond to the Horwitz-Thompson estimator for a two-phase sample design for stratification (Särndal et al. 1992). 95% confidence intervals were computed by a parametric bootstrap conditioning on the phase 1 sample size. Monthly overall prevalences were computed as follows: using all available site prevalences for that month, a weighted mean according to the site's phase 1 sample size was computed to partly take the varying population size into account. A similar procedure was performed to get an overall prevalence estimate. Exact Clopper-Pearson confidence intervals were computed for binomial proportions. All statistical computations described above were performed with R version 2.6.2 using the add-on packages 'Design', 'boot' and 'binom'.

Results

Tick collection. A total of 4,932 adults and 3,573 nymphs were collected at all sites from May to September 2006. Species identification of adult ticks revealed no other tick species than *I. ricinus*. For the investigation a subsample of 2,861 ticks was taken.

Prevalence of SFG Rickettsiae in *I. ricinus.* A total of 151 out of the investigated 2,861 samples contained DNA of the SFG rickettsiae, of which 91.4% were *R. helvetica* and 8.6% belonged to the *R. monacensis* cluster. This corresponds to a weighted average May-to-September prevalence of 5.3% (95% CI 4.3% to 6.2%). Weighted prevalences for the individual months under investigation are 6.7% (95% CI 4.4-8.0%) for May, 5.8% (95% CI 4.1-7.5%) for June, 3.2% (95% CI 1.8-4.4%) for July, 5.9% (95% CI 3.5-8.0%) for August and 3.7% (95% CI 1.4-5.7%) for September. With regard to developmental stage and gender, (unweighted) prevalences of 7.5% (95% CI: 5.9-9.3%) for females (71 out of 952), 7.2% (95% CI 5.6-9.0%) for males (68 out of 948) and 1.2% (95% CI 0.6-2.2%) for nymphs (12 out of 961) were found based on the binomial distribution.

PCR prevalences for the individual gender/stage in the different investigated months and sites indicate a higher prevalence during the summer months and in the natural forests (Tables 1 and 2). For ease of exposition the number of samples and infected ticks were here simply summed over all months and sites for each gender in Tables 1 and 2, respectively. Calculations are thus performed as simple binomial proportions without weighting. Taking

into account each factor (stage or gender of the tick, site and month), raw prevalences ranging from 0.0% to 40.0% were observed (data not shown).

In the statistic modeling, all interactions of stage, gender, site and month were investigated, but a stagewise elimination showed that only the main effects of site ($p<0.001$) and gender ($p<0.001$) were significant. An overall goodness of fit test for this main effects logistic model resulted in a p-value of 0.90. Generally, significantly more ticks were infected in sites from natural forest (C, D, E1, E2, K, L, and W together) compared to sites from the city parks (A1, A2, A3, and B together; $p<0.001$). Significant differences between single sites were also observed with site W having the highest prevalence in adult ticks (see also Table 2). The prevalence in males and females was similar and significantly higher than the prevalence in nymphs ($p<0.001$), whereas the monthly variation was not significant. DNA of *R. helvetica* was detected at every site, *R. monacensis* at six out of the eleven studied sites. Ten out of thirteen *R. monacensis* were detected in natural forest. No tendency of a species being present more often in a certain stage or gender of *I. ricinus* was observed. The infection ratios of *R. monacensis* to *R. helvetica* were always comparable at roughly averaging 1 to 12 regarding gender/stage and vegetation type.

***GltA* gene.** Out of the 2,861 samples, 171 yielded bands of the correct nucleotide size in the gel electrophoresis. Sequencing and similarity search with Blastn revealed that 138 samples showed 100% similarity to *R. helvetica*, 13 samples showed >99% to 100% similarity to the *R. monacensis* cluster, eight samples showed 72% to 73% similarity to *Coxiella burnetii*, seven samples showed 74% to 97% similarity to small parts of the citrate synthase gene of various *Bartonella* species, and five samples showed 88% to 97% similarity to other previously described rickettsial species. All 138 *R. helvetica* partial *gltA* sequences were aligned to each other and were 100% identical (consensus sequence submitted to GenBank, accession no. EU596563; Fig. 2), whereas alignment of the 13 *R. monacensis* partial *gltA* sequences revealed that 12 were 100% identical and one sample (D-2) differed in one nucleotide position (submitted to GenBank, accession nos. EU596564 and EU596562, respectively; Fig. 2).

***16S rRNA* Gene.** Sequencing of 20 *gltA* positive samples could attribute them to neither *R. helvetica* nor *R. monacensis* and was giving uncertain results. These samples were tested for amplification of the 16S rRNA gene. Three yielded bands of the correct size. Their *gltA* sequence had been related to rickettsial species. Sequencing and similarity search with Blastn revealed that two were ≥ 99% homologue to *Rickettsia* endosymbionts and one ≥99% to *R. bellii*.

***OmpA* gene.** All *gltA* positive samples were tested. As expected, amplification was not achieved for *R. helvetica*, but for 12 samples belonging to the *R. monacensis* cluster. In one sample (E1-12), amplification of *ompA* was not achieved. None of the samples with *gltA* results related to *C. burnetti*, *Bartonella* spp. or other rickettsial species amplified *ompA*. Alignment of the 12 *ompA* sequences of *R. monacensis* revealed that 11 were 100% identical to each other, and sample D-2 differed in four nucleotide positions (submitted to GenBank, accession nos. EU596565 and EU596561, respectively; Fig. 3).

***OmpB* Gene.** Thirteen samples attributed to *R. monacensis* and 20 not clearly attributed to a specific species were tested. All 13 *R. monacensis* and two of five samples related to rickettsial species yielded bands of the correct size. Homology search of the latter revealed 73% similarity to *R. slovaca* and 91% to previously described, but not further specified rickettsial species. Alignment of the thirteen partial *R. monacensis ompB* sequences of the showed that twelve were 100% identical, sample D-2 differed in one nucleotide position (submitted to GenBank, accession nos. EU330639 and EU330640; respectively; Fig. 2). None of the samples showing a relationship to *C. burnetii* or *Bartonella* spp. in *gltA* amplified any other gene.

Multiple Sequence Alignment. Sequences obtained have been aligned for similarity search with sequences previously deposited at GenBank (Figs. 2 and 3). The *gltA* sequence of *R. helvetica* obtained in this study differed in one nucleotide position from the prototype sequence of *R. helvetica* (accession no. U59723). The *R. monacensis gltA* sequence of sample D-2 was 100% homologous to *Rickettsia* sp. IRS3 and sp. PoTiR1dt, while the other 12 samples were identical to *R. monacensis* (IrR/ Munich strain) and *Rickettsia* sp. IRS4. The *R. monacensis ompA* sequence of sample D-2 was 100% homologous to *Rickettsia* sp. PoTiR1dt. The others were 100% homologous to *Rickettsia* sp. IrR/Munich and sp. IrITA2. All twelve had two nucleotide differences to *R. monacensis* Rp-Sp2 from a human patient, *Rickettsia* sp.IRS3 and sp.IRS4. The *ompB* sequence of D-2 had one nucleotide difference to sp. Ir/Munich.

Discussion

We detected an overall May to September prevalence of 5.3% which might appear low compared to previous reports from Germany (Hartelt et al. 2004, Pichon et al. 2006). Wölfel et al. (2006) found an average prevalence of 12% in questing ticks and ticks collected of small rodents in Bavaria including Munich. Hartelt et al. (2004) detected an average prevalence of 8.9%. However, the range between three different sites was 5.6 to 13.3%, indicating variations occur between different geographic locations. The lower overall prevalence in the

present study may be attributed to two factors. First, a large cross-section of vegetation zones was examined, with landscaped parks having low prevalence. Second, only 1.25% of nymphs were infected whereas in previous studies in Germany, up to 14.2% were infected with *R. helvetica* (Pichon et al. 2006, Wölfel et al. 2006). Due to transovarial transmission, an infected female tick gives the infection to the entire next generation. High nymphal prevalence could therefore be a result of a high proportion of infection either in the parent tick generation or in the mammalian host. Conduction of yearly follow-up studies of tick populations in a given area could clarify if high prevalence in adults leads to high prevalence in the next generation of nymphs.

Prevalence in city parks was significantly lower than in natural forests in the present study and causes could lie in host-pathogen-tick relationships and geographic or climatic circumstances. The role of mammals as potential reservoirs for SFG rickettsiae has not yet been established. Small mammals studied in Spain, the majority wood mice (*Apodemus sylvaticus* L.), were all PCR negative for SFG rickettsiae (Barandika et al. 2007). On the other hand, rodent host DNA and *R. helvetica* DNA correlated in *I. ricinus* nymphs from Germany (Pichon et al. 2006). In Japan, *R. helvetica* was detected in blood samples of Sika deer (*Cervus nippon* (Temminck) sp. *yesoensis*), which suggests a possible reservoir host (Inokuma et al. 2008). However, the evolution of ticks and rickettsiae was closely linked together, leaving the assumption that mammals may be only subordinate in the evolutionary strategy (Roux and Raoult 1995). The potential role of mammals, especially red, but also roe deer, needs nonetheless further investigations. The forest areas with high prevalence of SFG rickettsiae in the present study were also areas with large numbers of roe deer.

Tick populations in forests undergo stable endemic cycles with their natural hosts. In city parks, questing areas are altered and the ticks themselves removed during gardening activities like grooming and mowing or due to occasional infestations on passing hosts, e.g. domestic animals. Every infected female tick removed results in lower prevalence in the next generation and could be an explanation for the lower prevalence in the city parks. Studies on other city parks should be conducted to reveal if this is a phenomenon also occurring elsewhere.

Nine adults (eight females, one male) were coinfected with *A. phagocytophilum* (strain *Ehrlichia* sp. ‘Frankonia 2’) and *Rickettsia* spp. (Silaghi et al. accepted for publication). Eight ticks were coinfected with *R. helvetica*, one with *R. monacensis* and coinfection was not clustered to a specific area. Previous systematic studies from Germany detected *R. helvetica* only, but coinfection with *A. phagocytophilum* has been revealed (Hartelt et al. 2004, Pichon

et al. 2006, Wölfel et al. 2006) and has also been detected in Bulgaria and Spain (Christova et al. 2003, Fernández-Soto et al. 2004). No tick was infected with more than one rickettsial species, but in Bulgaria, 67% of adult *I. ricinus* were infected with SFG *rickettsiae*, from which 59% were *R. helvetica* and 58% strain IRS3. Coinfection with two rickettsial species occurred in 17% of adults and 53% of nymphs (Christova et al. 2003).

R. helvetica was associated with human disease in 1999 and has been serologically associated with patients in Europe and Asia (Nilsson et al. 1999, Fournier et al. 2000, Nilsson et al. 2005). Seropositivity in humans (borreliosis patients, forestry workers) in Europe has been detected at 9.25% and 12.5% (Fournier et al. 2000, Nielsen at al. 2004). We detected up to 20% of active adult questing ticks infected in recreationally used areas. These results indicate that the potential human pathogen *R. helvetica* may be widespread in Europe, at least in tick-exposed areas.

One of the *R. monacensis* strains from the present study had greatest similarity to one previously detected in Portugal, and the other is 100% similar to *R. monacensis* sp. Ir/Munich which was first isolated from an *I. ricinus* in a city park in Munich and later found also in Italy (Simser et al. 2002, Bertolotti et al. 2006). Our results suggest that *R. monacensis* may be more frequent in forest areas than in city parks. Prevalence of *R. monacensis* in the present study was very low and the ratio of *R. monacensis* to *R. helvetica* was roughly 1 to 12. Other studies in Europe found ratios of up to almost 20 to 1 (Bertolotti et al. 2006, Fernández-Soto et al. 2004, Prosenc et al. 2003, Christova et al. 2003). *R. monacensis* has recently been etiologically associated with human disease, but the partial *ompA* sequences from the present study differed in two nucleotides (Jado et al. 2007). Larger genome studies are needed and future research should address the possible influence of gene diversity on the pathogenicity of strains of this heterogenic cluster. This could also clarify why one of our *R. monacensis* did not amplify *ompA*.

Five ticks contained *gltA* sequences with a high percentage of similarity to previously described rickettsial species. Comparison of PCR results with genetic guidelines indicated that two might not belong to the genus *Rickettsia* and the other three were most probably not SFG rickettsiae (Fournier et al. 2003). It is most likely that these sequences belonged to rickettsial or closely related endosymbionts which are very frequent in ixodid ticks (Noda et al. 1997, Moreno et al. 2006). The products showing similarity to *C. burnetii* or *Bartonella* spp. may have been detected due to unspecific amplification, possibly because of the low annealing temperature of the *gltA* PCR.

In conclusion, the data obtained in this study demonstrates a wide, but uneven distribution of SFG rickettsiae previously associated with human disease in Southern Germany. Thus, further studies should be aimed at investigating the seroprevalence in patients and control groups in Germany to gain knowledge on the clinical situation. Even though no clinical case has yet been reported in Germany, clinicians should be aware of a potential risk of tick-borne rickettsial infection when confronted with cases of unexplained febrile disease after tick exposure.

Acknowledgments

We thank Mrs C. Hizo-Teufel, Miss S. Neuvecelle, and Miss S. Pfalzer for very helpful technical assistance, Dr. S. Leonhard for help with the tick collection, and Prof. L. Friche Passos for reviewing this manuscript. The third author was financially supported by the Munich Center of Health Sciences, Germany.

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Tables:

Table 1: Prevalence of Spotted Fever Group rickettsiae in questing *Ixodes ricinus* ticks collected in Southern Germany in the investigated months 2006.

Month	Females			Males			Nymphs		
	No. infected/ No. total	%	95% CI	No. infected/ No. total	%	95% CI	No. infected/ No. total	%	95% CI
May	15/258	5.8	3.3-9.4	21/252	8.3	5.2-12.5	2/149	1.3	0.2-4.8
June	28/270	10.4	7.0-14.6	21/275	7.6	4.8-11.4	1/245	0.4	0.0-2.3
July	15/217	6.9	3.9-11.1	6/202	3.0	1.1-6.4	5/270	1.9	0.6-4.3
Aug.	11/124	8.9	4.5-15.3	12/127	9.4	5.0-15.9	2/160	1.2	0.2-4.4
Sept.	2/83	2.4	0.3-8.4	8/92	8.7	3.8-16.4	2/137	1.5	0.2-5.2

Table 2: Prevalence of Spotted Fever Group rickettsiae in questing *Ixodes ricinus* ticks from selected sites in Southern Germany in 2006.

Site	Females			Males			Nymphs		
	No. infected/ No. total	%	95% CI	No. infected/ No. total	%	95% C	No. infected/ No. total	%	95% C
A1	6/87	6.9	2.6-14.4	1/88	1.1	0.0-6.2	1/104	1.0	0.0-5.2
A2	5/149	3.4	1.1-7.7	5/153	3.3	1.1-7.5	0/150	0.0	0.0-2.4
A3	7/114	6.1	2.5-12.2	7/105	6.7	2.7-13.3	0/83	0.0	0.0-4.3
B	5/80	6.2	2.1-14.0	7/65	10.8	4.4-20.9	0/42	0.0	0.0-8.4
C	5/68	7.4	2.4-16.3	2/60	3.3	0.4-11.5	3/96	3.1	0.6-8.9
D	11/150	7.3	3.7-12.7	15/151	9.9	5.7-15.9	3/142	2.1	0.4-6.0
E1	6/92	6.5	2.4-13.7	12/101	11.9	6.3-19.8	3/114	2.6	0.5-7.5
E2	17/122	13.9	8.3-21.4	8/134	6.0	2.6-11.4	1/140	0.7	0.0-3.9
K	3/30	10.0	2.1-26.5	4/31	12.9	3.6-29.8	0/30	0.0	0.0-11.6
L	0/30	0.0	0.0-11.6	1/30	3.3	0.1-17.2	0/30	0.0	0.0-11.6
W	6/30	20.0	7.7-38.6	6/30	20.0	7.7-38.6	1/30	3.3	0.1-17.2

[a] for location of study sites see Fig 1.

Figure legends

Fig. 1. Study area: ■ Natural forest sites outside of Munich; K- Kressbronn at the Lake Constance (State of Baden-Württemberg); L – near Augsburg at the river Lech, W – near the Spa town Bad Wörishofen (both State of Bavaria). ● Munich - City Parks: English Garden (A1, A2, A3); Park in the south of the city (B), Natural Forest sites: southern part of the city (C); northern part of the city (D, E1, E2).

Fig. 2. Sequence comparison of *Rickettsia helvetica* (*gltA*) and *Rickettsia monacensis* strains (*gltA* and *ompB*) from Southern Germany with sequences from GenBank (-, gap in the sequence). [a] Position of nucleotides relative to the position of the 342 nucleotide sequences obtained in this study; [b] Position of nucleotides relative to the position of the 770 nucleotide sequence of strain A3-264; #, sequences obtained in this study.

Fig. 3. Sequence comparison (*ompA*) of *Rickettsia monacensis* strains from Southern Germany with sequences from GenBank (- , gap in the sequence; n.a., not available).
[a] Position of nucleotides relative to the position of the 488 nucleotide sequences obtained in this study; #, sequences obtained in this study.

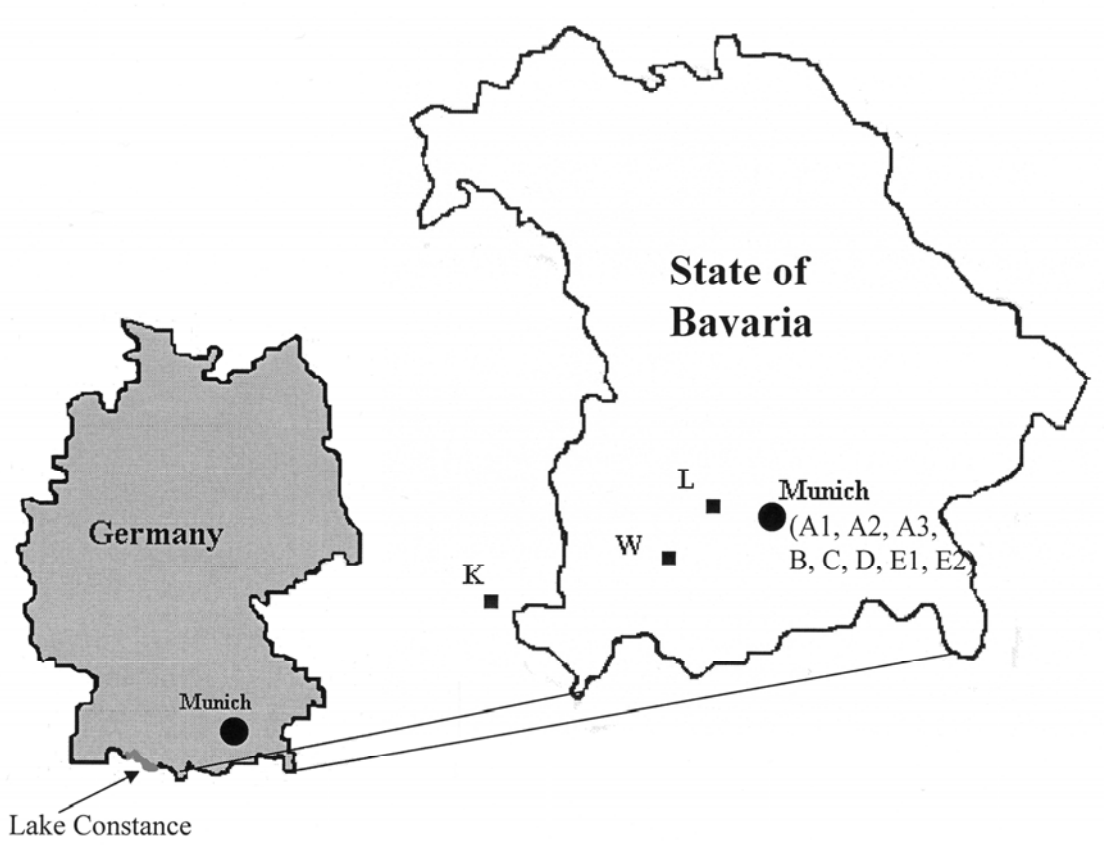
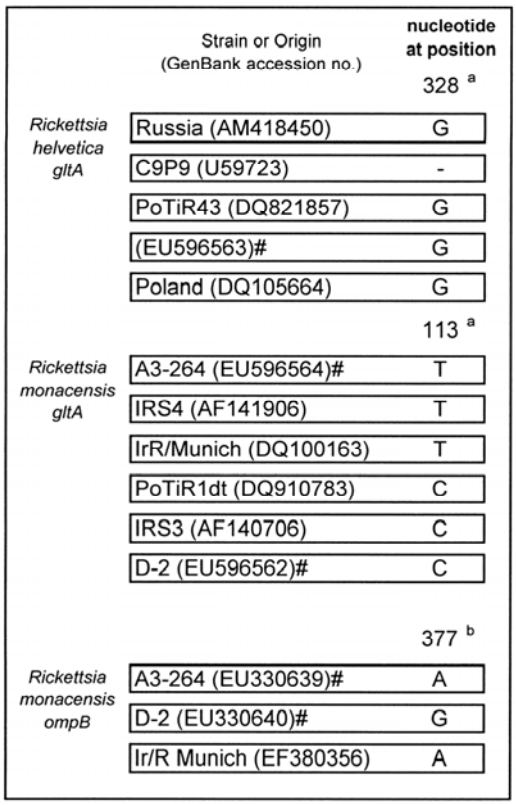

State of
Bavaria
Germany
Munich
L
Munich
(A1, A2, A3,
B, C, D, E1, E2)
W
K
Lake Constance

Fig. 1



	Strain or Origin (GenBank accession no.)	nucleotide at position
		328 [a]
Rickettsia helvetica gltA	Russia (AM418450)	G
	C9P9 (U59723)	-
	PoTiR43 (DQ821857)	G
	(EU596563)#	G
	Poland (DQ105664)	G
		113 [a]
Rickettsia monacensis gltA	A3-264 (EU596564)#	T
	IRS4 (AF141906)	T
	IrR/Munich (DQ100163)	T
	PoTiR1dt (DQ910783)	C
	IRS3 (AF140706)	C
	D-2 (EU596562)#	C
		377 [b]
Rickettsia monacensis ompB	A3-264 (EU330639)#	A
	D-2 (EU330640)#	G
	Ir/R Munich (EF380356)	A

Fig. 2

Rickettsia monacensis ompA Strain (GenBank accession no.)	nucleotide at position [a] 16	17	202	262	321	362	447	469
PoTiR1dt (DQ910781)	A	T	G	G	A	G	G	G
D-2 (EU596561)#	A	T	G	G	A	G	G	G
IRS3 (AF141909)	C	C	G	G	A	G	G	G
IRS4 (AF141911)	C	C	A	A	G	A	G	G
RpSp1 (DQ157778)	n.a.	n.a.	A	A	G	A	-	A
IrR/Munich (AF201329)	A	T	A	A	G	A	G	G
A3-264 (EU596565)#	A	T	A	A	G	A	G	G
ITA2 (AJ427884)	A	T	A	A	G	A	G	G
IrR/Munich (DQ100169)	A	T	A	A	G	A	G	G

Fig.3

5. Discussion

The prevalence rates and distribution of *A. phagocytophilum* and SFG rickettsiae in the anthropophilic tick *I. ricinus* were investigated in urban public parks and natural forests in Munich and Southern Bavaria, with focus on the effects of geographic location, season and tick developmental stage on prevalence variations. For this purpose, a large number of *I. ricinus* was collected regularly over five months by the flagging method and tested in the laboratory by molecular methods.

The calculated weighted prevalence of *A. phagocytophilum* and SFG rickettsiae in this study was 2.9% and 5.3%, respectively. This result is comparable to that of other studies on *A. phagocytophilum* in Germany, but previously detected prevalence rates of SFG rickettsiae were higher (Baumgarten et al., 1999; Hildebrandt et al., 2002; Hartelt et al., 2004; Wölfel et al., 2006). Compared to other studies in Europe, the results might appear rather low. For example, a total of 14% of *I. ricinus* were infected with *A. phagocytophilum* in Poland, the range was from 0 – 27.6% and statistically significant between the different sites under investigation (Stańczak et al., 2004). However, the present study included a large cross-section of vegetation zones with wide variation amongst the sites. Statistical analyis weighted the PCR prevalence detetected in the laboratory according to the phase 1 sample size which consisted of actively questing ticks from vegetation. Therefor the overall prevalence calculated according to month and gender is a realistic estimate of the true prevalence in questing ticks in the Munich area.

The significant differences observed between collection sites in the present study substantiate the suggested focal distribution of *A. phagocytophilum*, especially in the big city parks of Munich (Fingerle et al., 1999; Leonhard, 2005). The results show that ticks collected in these parks in Munich were significantly more often infected with *A. phagocytophilum* than in natural forest areas. Contrary, in a previous study comparing city parks with forest and heather areas in the Netherlands, the lowest infection rate with *A. phagocytophilum* was found in the city parks (Wielinga et al., 2006). In a study in Finland, ticks could not be found at all in a city park (Mäkinen et al., 2003). This indicates that ticks from city parks are not always highly infected and the special circumstances of the ‘English Garden’ in Munich need further investigation. For SFG rickettsiae, the results were vice versa and ticks were significantly higher infected in natural forest than in city parks. A previous study from Bavaria showed

regional variation, but no clustering to a specific area. However, in this study, city parks were not investigated (Wölfel et al., 2006).

Variation in the prevalence rate of a tick-borne bacterial agent could be attributed to differences between individual tick species, susceptibility of tick populations to the agent and variations in bacterial strains (Řeháček, 1989), but also to differences in the enzootic cycle, e.g. different reservoir host species or differences in the susceptibility of the same reservoir host species, the molecular genetic make-up of the local tick population, or differences in the microclimate.

Only 0.31% of all infected ticks in the present study were co-infected with *A. phagocytophilum* and SFG rickettsiae. The fact that *Rickettsia* spp. prevalence was significantly higher in the forest areas and *A. phagocytophilum* prevalence significantly higher in the city parks indicate that different factors influence the enzootic cycles in the same vector species.

SFG rickettsiae are transmitted in the ticks both transstadially and transovarially, whereas transovarial transmission seems inefficient for *A. phagocytophilum* (Ogden et al., 1998; 2002; Parola et al., 2005a). *I. ricinus* can therefore be regarded as the main reservoir in nature for SFG rickettsiae. The role of mammals as potential reservoirs for SFG rickettsiae has not been established (Parola et al. 2005a), even though *R. helvetica* has been detected in Sika deer *(C. nippon yesoensis)* in Hokkaido, Japan (Inokuma et al., 2008). However, it seems more likely that the reasons for the differences in prevalence lie in either the tick population itself or in the microclimatic circumstances of the city parks. The tick population, especially of the Southern part of the ‘English Garden’ is separated from tick populations of, for example, the ‘Isarauen’ in the North of Munich. The reason for the lower prevalence could therefore lie in the development of a lower susceptibility to SFG rickettsiae.

Ticks in forests are living in stable endemic cycles with their natural hosts, whereas in city parks, ticks are constantly removed by the gardening activities. Passing hosts, e.g. domestic animals, also take ticks away through occasional infestation. As a tick once infected with SFG rickettsiae remains so for the rest of its life and gives infection to almost the entire next generation, this may be an explanation for the lower infection of ticks in the city parks. If the possibility was considered that deer could provide a potential reservoir (Inokuma et al., 2008), this still explains the higher prevalence in the forest areas, as large wild mammals are very rare in the park areas in the city centers.

A. phagocytophilum, on the other hand, is dependent on a vertebrate host to maintain stable endemic cycles in nature. Roe deer play a central role in the life cycle of *I. ricinus* by feeding

large numbers of this arthropod. The probability of roe deer being rickettsiaemic can therefore change through season. However, in the central urban parts of the 'English Garden', where the highest infection rate with *A. phagocytophilum* was found, deer do not normally live. The significantly higher infection rate leads to the conclusion that either a different reservoir host is frequenting these areas or that special circumstances of the tick population give them higher susceptibility. Especially the Southern part of the 'English Garden' could be regarded as an isolated area where hardly any exchange of animal populations with other areas takes place. Thus, separate endemic cycles could develop. In the United States, *A. phagocytophilum* has the sylvan zoonotic cycle, but is also present in urban areas where domestic animals like cats and dogs, but also urban rodents like Norway rats (*Rattus norvegicus*) and house mice (*Mus musculus*) could serve as amplifying hosts and therefore help maintain transmission cycles in urban areas (Comer et al., 2001).

The requirement for a natural vertebrate reservoir is that infection results in a lasting bacteriaemia. This enables a new generation of uninfected ticks to become infected, thus creating a natural cycle (Parola, 2005a). It is known that the 'English Garden' is heavily frequented by rabbits and foxes. Of 1,550 red foxes in Switzerland, 2.8% were seropositive for *A. phagocytophilum* (Pusterla et al., 1999a) and in the Czech Republic 1 of 25 foxes was PCR positive for *A. phagocytophilum* (Hulínská et al., 2004). The possible role of domestic animals as a reservoir for *A. phagocytophilum* needs investigation. Cats as reservoir hosts have been examined. The seroprevalence detected has been low, and no PCR confirmation of infection could be obtained. However, PCR confirmation of a granulocytic ehrlichiosis has been obtained in a feline patient in Sweden. Nonetheless, it is unlikely that the cat is a reservoir host (Bjoersdorff et al., 1999; Billeter et al., 2007). Large numbers of dogs pass through the 'English Garden' every day. High seroprevalences or naturally infected dogs were found in Germany and even though only 2.9% of 209 ticks removed from dogs in Poland harbored *A. phagocytophilum* DNA, future studies should focus first of all on this animal (Jensen et al., 2007; Zygner et al., 2008; Kohn et al., submitted).

Female and male adult ticks were significantly more often infected with both pathogens than nymphs, but differed not significantly among each other. As *A. phagocytophilum* is only transmitted transstadially, this result is not surprising. For SFG rickettsiae, one could expect higher prevalence in nymphs, as infected female ticks give infection to almost the entire next generation.

In the present study monthly fluctuations for both pathogens were not significant. It has been suggested previously that due to either seasonal effects or random fluctuations over time, spot

check samples may not reflect the true prevalence in an area (Jenkins et al., 2001). Our results could not establish seasonal effects. However, numbers of collected and therefore of investigated ticks declined over the summer. Some sites had only very few ticks in the late summer months and the number may have been too low for statistical comparison.

The 16S rDNA sequence of *Ehrlichia* sp. 'Frankonia' 2 was first detected in adult ticks collected from domestic dogs in Central Germany (Baumgarten et al., 1999). It has also been found in *I. ricinus* ticks from Estonia (Mäkinen et al., 2003). So far, *Ehrlichia* sp. 'Frankonia 2' has not been detected in infected humans or animals, and can therefore, at this moment, be considered as a strain of unknown pathogenicity.

In Europe, the discrepancy between seroprevalence of *A. phagocytophilum* in humans and animals, PCR prevalence in ticks and the low number of proven clinical cases is high (Strle, 2004). Disease in Europe could be either self-limiting, undetected by clinicians not aware of the disease, or another explanation could be the presence of non-pathogenic strains. The non-pathogenic variant ApV1 has been detected in Spain (Portillo et al., 2005). Stuen et al. (2002) detected more than one *A. phagocytophilum* variant in one single sheep. An experimental infection of sheep with two variants of *A. phagocytophilum* brought out a marked difference in the clinical manifestation, the amount of neutropenia, the antibody response and the cross protection (Stuen et al., 2003).

Therefore, prevalence rates of *A. phagocytophilum* have to be regarded with care, as the calculated risk of encountering an infected tick does not equal the risk of encountering a truly human pathogenic strain (Zeman et al., 2007). Sequencing and further characterization of the pathogenic role of detected *A. phagocytophilum* strains will be a most important research topic of future studies. This has also been shown in the USA, where the pathogenic variant Ap-ha and the non-pathogenic ApV1 are frequently detected alongside (Courtney et al., 2003). Routine sequencing of the 16S rDNA is therefore of utmost importance (Michalski et al., 2006).

R. helvetica strains were all 100% identical and differed in one nucleotide position from the prototype sequence. *R. helvetica* has been associated with human disease, but there have been no clinical reports in Germany (Nilsson et al., 1999b). The point prevalence of up to 20% in one study side leads to the question whether this nucleotide difference from the prototype could indicate different types concerning pathogenicity. More genes need to be sequenced and their function concerning pathogenicity analyzed in order to gain knowledge on this. *R. helvetica* is special amongst SFG rickettsiae, as its genome is longer than that of most rickettsiae and the *ompA* and large parts of the *ompB* gene cannot be amplified (Eremeeva et

al., 1994; Roux and Raoult, 2000). On the other hand, *R. helvetica* could cause an undiagnosed febrile disease, not noticed by practicing physicians, who need to be aware of the disease in case of unexplained febrile illness after tick bites. Dogs investigated for *Rickettsia* spp. have been seronegative and PCR negative, however, it has to be noted that the primers used amplified the *ompA* gene (Yabsley et al., 2008). *R. helvetica* presence could therefore have been overlooked and its possible role in animal disease should be addressed in future studies.

Two different strains of the heterogenic *R. monacensis* cluster were detected, one of them 100% homologue to a strain that has been detected previously in the 'English Garden' in Munich (Simser et al., 2002). Even though *R. monacensis* had first been detected in a city park the current results indicate that it may be more frequent in forest areas than in city parks. *R. monacensis* has recently been associated with a spotted fever rickettsiosis in two patients in northern Spain (Jado et al., 2007). The strains detected in the present study differed in two nucleotide positions in the *ompA* partial sequence from the one described by Jado et al. (2007). Future studies need to address the possible influence of differences in the nucleotide sequences on the pathogenicity of the different strains of this heterogenic cluster.

The flagging method is an established way of collecting questing Ixodid ticks. From personal experience, a drawback of this method is that it functions only well in fairly good weather, as both strong wind and wet weather conditions limit the outcome. It has also been reported that different collectors obtain different results with this method (Ginsberg and Ewing, 1989). The aim at the beginning of the study was to collect until at least 200 adults per month and site were obtained for comparative figures. In most sites, the number of ticks declined so much over the summer months that it was impossible to achieve these figures with only a single collector.

The prevalence of both pathogens investigated was very low in nymphs. All DNA from nymphs was extracted individually. Even though the average DNA amount extracted as measured with NanoDrop® was similar to male ticks, the range for nymphs was very wide from 0.1ng/µl to 64.4ng/µl. This low amount of extracted DNA in some nymphs could be one explanation for the low positivity, as the limit of sensitivity of our PCR method could have been reached.

The *msp2* PCR for detection of *A. phagocytophilum* was modified for the diagnostic laboratory of the institute with 50 amplification cycles. When dealing with arthropod DNA, background amplification can become too high and it can be difficult to decide whether a sample is truly positive (Pradel, personal communication). Therefore, the original PCR

protocol with 40 amplification cycles, established for *I. scapularis* ticks, was used for a number of samples not giving clear results in the first PCR.

The annealing temperature of 48°C for the *gltA* PCR was very low. This can lead to unspecific amplifications (Pradel, personal communication). These false positive samples could be excluded from the analysis by sequencing.

6. Conclusion

The results confirmed a wide distribution of tick-borne rickettsial bacteria in *I. ricinus* in the urban areas of Munich and contributed to the genetic characterization of *A. phagocytophilum, R. helvetica* and *R. monacensis*. High prevalences of *A. phagocytophilum* in the city parks and of SFG rickettsiae in prealpine forest areas were observed in *I. ricinus*.

Further studies are urgently needed to understand the differences between prevalences in the city parks and in the natural forests with special focus on identification of potential reservoir hosts of *A. phagocytophilum* (e.g. rodents, deer, foxes, domestic dogs). It will also be interesting to conduct investigation in other city parks to find out if this phenomenon occurs elsewhere.

As strains of unknown pathogenicity of *A. phagocytophilum* and two rickettsial agents previously associated with human disease have been detected, it will be necessary to focus research on studies to unravel the clinical situation in Southern Germany, especially after tick exposure. Host-pathogen and tick-pathogen interactions should be addressed in gene expression studies to identify the potential pathogenic role and the mechanisms involved of the detected strains.

7. Summary

In recent years, *Anaplasma phagocytophilum* and *Rickettsia* spp. have been detected in *Ixodes ricinus* in Germany and a focal distribution has been suggested for *A. phagocytophilum*. In the present study the prevalence of *A. phagocytophilum* and spotted fever group (SFG) rickettsiae was investigated in *I. ricinus*. DNA-extracts were taken from 2,862 unfed *I. ricinus* ticks (adults and nymphs) from eight sites in Munich, sampled over a five-month period. Single samples from three comparative sites outside of Munich were also included. A real-time PCR targeting the *msp2* gene of *A. phagocytophilum* was used for screening and a nested PCR targeting the *16S rRNA* gene for sequencing of 30% of positives. Screening for *Rickettsia* spp. was performed with a PCR targeting the citrate synthase gene (*gltA*), followed by PCRs detecting the *ompA* gene for all *gltA* positives, and the *ompB* and *16S rRNA* genes for clarifying results of some samples. The overall prevalence was 2.90% (95% CI 2.27 to 3.48%) for *A. phagocytophilum* and 5.28% (95% CI 4.31 to 6.17%) for SFG rickettsiae. Only 0.31% of the ticks investigated were coinfected. Statistical analysis revealed that prevalence of *A. phagocytophilum* in ticks from city parks in Munich was significantly higher than in ticks from natural forest, whereas the prevalence of *Rickettsia* spp. was the opposite. For both, the prevalence in adults was significantly higher than in nymphs. Although wide ranges of prevalence were observed monthly, the variations were not significant along the observational period. Sequence analysis of *16S rRNA* PCR products (n=31) revealed 100% homology to *Ehrlichia* sp. “Frankonia 2”, only one differed in one nucleotide position. All differed in one nucleotide position from the HGA agent detected in human patients. All rickettsial PCR products were also sequenced. All *gltA* sequences of *R. helvetica* (n=138) were 100% identical to each other and differed in one nucleotide position from the prototype sequence. Two different *R. monacensis* strains (n=13) were detected, which differed in up to 4 nucleotide positions in *gltA*, *ompA* and *ompB*. Further rickettsial strains (n=3) most probably belonging to rickettsial endosymbionts were also found. These results show, by molecular methods, a wide distribution of *A. phagocytophilum* and SFG rickettsiae in *I. ricinus* ticks in Southern Germany. SFG rickettsiae which are thought to be involved in human disease (*R. helvetica* and *R. monacensis*) had a significantly higher prevalence in natural forest areas. Prevalence of *A. phagocytophilum* was significantly higher in city parks; the genetic strain has not yet been associated with human infection.

8. Zusammenfassung

In den letzten Jahren wurden *Anaplasma phagocytophilum* und Rickettsien der Spotted Fever Group (SFG) in *Ixodes ricinus* in Deutschland entdeckt und eine fokale Verteilung von *A. phagocytophilum* vermutet. In der hier vorliegenden Studie wurde die Prävalenz von *A. phagocytophilum* und SFG Rickettsien in *I. ricinus* untersucht. Dazu wurden Zecken über fünf Monate an acht Orten in München und einmal an drei Vergleichsorten außerhalb Münchens gesammelt. Von 2,862 ungesaugten *I. ricinus* (Adulte und Nymphen) wurde DNA extrahiert. Eine Real-Time PCR mit der Zielsequenz auf dem *msp2* Gen von *A. phagocytophilum* wurde für ein Screening ausgewählt und zum Sequenzieren von 30% der positiven Proben eine nested PCR mit der Zielsequenz auf dem *16S rRNA* Gen. Ein Screening aller Proben auf *Rickettsia* spp. wurde mit einer auf das Citrat Synthase (*gltA*) Gen abzielenden PCR durchgeführt, gefolgt von PCRs zur Detektion des *ompA* Gens bei allen *gltA* positiven Proben, und der *ompB* und *16S rRNA* Gene zur Abklärung der unklaren Proben. Die Gesamtprävalenz lag bei 2,90% (Konfidenzintervall 95%: 2,27%-3,48%) für *A. phagocytophilum* und bei 5,28% (Konfidenzintervall 95%: 4,31%-6,17%) für SFG Rickettsien. Nur 0,31% der Proben waren coinfiziert. Die statistische Analyse ergab, dass die Prävalenz von *A. phagocytophilum* im „Englischen Garten“ in München signifikant höher war als in naturbelassenen Waldstücken, wohingegen sich die Prävalenz von *Rickettsia* spp. gegenteilig verhielt. Die Prävalenz in adulten Zecken war in beiden Fällen signifikant höher als in Nymphen. Obwohl in den untersuchten Monaten große Spannweiten in der Prävalenz beider Erreger gefunden wurden, waren diese statistisch nicht signifikant. Dreißig *A. phagocytophilum 16S rRNA* PCR Produkte (n=31) waren 100% identisch mit *Ehrlichia* sp.”Frankonia 2“ und eine unterschied sich in einer Nukleotidposition. Alle unterschieden sich in einer Nukleotidposition von dem HGA Agens, das in Humanpatienten gefunden wurde. Die rickettsialen PCR Produkte wurden alle sequenziert. Alle *R. helvetica* (n=138) waren 100% identisch miteinander und unterschieden sich in einer Nukleotidposition vom Prototyp von *R. helvetica*. Zwei verschiedene genetische Varianten von *R. monacensis* (n=13) wurden gefunden, welche sich in bis zu vier Nukleotidpositionen in den *gltA*, *ompA* und *ompB* Sequenzen unterschieden. Weitere Rickettsiensequenzen (n=3) gehörten höchstwahrscheinlich zu rickettsialen Endosymbionten. Die Ergebnisse dieser molekularbiologischen Untersuchungen bestätigen eine weite Verbreitung von *A. phagocytophilum* und SFG Rickettsien in *I. ricinus* in Süddeutschland. SFG Rickettsien,

welche mit humanen Erkrankungsfällen in Verbindung gebracht wurden (*R. helvetica* und *R. monacensis*) hatten eine signifikant höhere Prävalenz in den naturbelassenen Waldstücken. Die Prävalenz von *A. phagocytophilum* war in den Stadtparks signifikant höher, die genetische Variante wurde noch nicht mit humanen Erkrankungsfällen in Verbindung gebracht.

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10. Abbreviations

bp	base pair
°C	degree Celsius
CO_2	carbon Dioxide
dATP	2’-deoxyadenosine-5’-triphosphate
dCTP	2’-deoxycytidine-5’-triphosphate
dGTP	2’-deoxyguanosine-5’-triphosphate
DNA	deoxyribonucleic acid
dNTP	deoxynucleotide triphosphate
dTTP	2’-deoxythymidine-5’-triphophate
EDTA	diaminoethanetetraacetic acid
E. coli	*Escherichia coli*
FRET	fluorescent resonance energy transfer
g	gram
gltA	citrate synthase gene
H_2O	water
HEX	hexa chlorine fluorescine
HGA	Human granulocytic anaplasmosis
HGE	Human granulocytic ehrlichiosis
IFAT	Immun Fluorescence Assay Test
kDa	kiloDalton
mg	milligram
$MgCl_2$	magnesium chloride
ml	milliliter
mM	millimolar
msp	membranous surface protein
µg	microgram
µl	microliter
µm	micrometer
µM	micromolar
ng	nanogram
NH_3	hydrogen nitride

ompA	outer membrane protein A
ompB	outer membrane protein B
PCR	polymerase chain reaction
pM	pikomolar
rRNA	ribosomal ribonucleic acid
SFG	spotted fever group
STG	scrub typhus group
TAE	tris-acetate-EDTA
TBEV	tick-borne encephalitis virus
TAMRA	6-carboxytetramethylrhodamin
Taq	*Thermus aquaticus*
TG	typhus group
Tris	trishydroxymethylaminomethane
U	Unit

11. Figures

12. Tables

13. Annex

13.1. Materials

13.1.1. Kits, oligonucleotids and chemicals

High Pure PCR Template Preparation Kit	(Roche Applied Science, Mannheim, Germany)
Expand High Fidelity Plus PCR System, dNTPack	(Roche Applied Science, Mannheim, Germany)
Magnesium chloride (25mM)	(Roche Applied Science, Mannheim, Germany)
PCR grade nucleotide mix	(Roche Applied Science, Mannheim, Germany)
dNTP mix, PCR grade	(Qiagen, Hilden, Germany)
ApMSP2f, ApMSP2r	(MWG Biotech, Ebersberg, Germany)
ApMSP2p (probe with HEX-TAMRA)	(MWG Biotech, Ebersberg, Germany)
AP-ge3a, AP-ge10r	(MWG Biotech, Ebersberg, Germany)
AP-ge9f, AP-ge2	(MWG Biotech, Ebersberg, Germany)
RpCS.877p, RpCS.1258n	(MWG Biotech, Ebersberg, Germany)
Rr190.70p, Rr190.602n	(MWG Biotech, Ebersberg, Germany)
fD1-16SrDNA, Rc16S-452n	(MWG Biotech, Ebersberg, Germany)
120-2788, 120-3599	(MWG Biotech, Ebersberg, Germany)
Loading buffer 6x Loading Dye	(Fermentas Life Science, Leonrot, Germany)
Gene Ruler™ DNA Ladder Mix	(Fermentas Life Science, Leonrot, Germany)
QIAquick PCR purification Kit	(Qiagen, Hilden, Germany)
Ethanol > 99.8%	(Roth, Karlsruhe, Germany)
Tris (14g/mol) (Tris–hydroxymethyl–aminomethane)	(Roth, Karlsruhe, Germany)
Sodium acetate	(Roth, Karlsruhe, Germany)
Ethidiumbromide (10mg/ml)	(Roth, Karlsruhe, Germany)
Agarose, electrophoresis grade	(invitrogen, Paisley, UK)

13.1.2. Enzymes

HotStarTaq DNA polymerase	(Qiagen, Hilden, Germany)
Expand High Fidelity Plus PCR System, Thermostable DNA polymerase mixture	(Roche Applied Science, Mannheim, Germany)

13.1.3. Buffer and solution for the agarose gel electrophoresis

50x TAE buffer:	50 mM Tris
	20 mM sodium acetate
	1 mM EDTA
	With concentrated acetic acid adjusted to pH=8.3

Ethidiumbromide staining solution: diluted to 5µl/ml

13.2. Sequencing data

13.2.1. *GltA* sequence comparison of *Rickettsia helvetica*

AM418450: *R. helvetica* from Russian *I. persulcatus*
U59723: prototype sequence of *R. helvetica*
DQ821857: *R. helvetica* PoTiR43
DQ105664: *R. helvetica* from Polish *I. ricinus*
A1-12: consensus sequence obtained in this study and submitted to GenBank (accession no. EU596563)

```
AM418450      1 GGGCTAATGAAGCGGTAATAAATATGCTTAAAGAAATTGGTAGTTCTGAGAATATCCCTA
U59723        1 GGGCTAATGAAGCGGTAATAAATATGCTTAAAGAAATTGGTAGTTCTGAGAATATCCCTA
DQ821857      1 GGGCTAATGAAGCGGTAATAAATATGCTTAAAGAAATTGGTAGTTCTGAGAATATCCCTA
A1-12         1 GGGCTAATGAAGCGGTAATAAATATGCTTAAAGAAATTGGTAGTTCTGAGAATATCCCTA
DQ105664      1 -GGCTAATGAAGCGGTAATAAATATGCTTAAAGAAATTGGTAGTTCTGAGAATATCCCTA

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DQ821857     61 AATATATAGCTAAAGCTAAGGATAAAAATGATCCGTTTAGGTTAATAGGCTTCGGTCATC
A1-12        61 AATATATAGCTAAAGCTAAGGATAAAAATGATCCGTTTAGGTTAATAGGCTTCGGTCATC
DQ105664     60 AATATATAGCTAAAGCTAAGGATAAAAATGATCCGTTTAGGTTAATAGGCTTCGGTCATC

AM418450    121 GTGTATATAAAAACTACGATCCACGTGCCGCAGTACTTAAAGAAACTTGTAAGGAAGTAT
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DQ821857    121 GTGTATATAAAAACTACGATCCACGTGCCGCAGTACTTAAAGAAACTTGTAAGGAAGTAT
A1-12       121 GTGTATATAAAAACTACGATCCACGTGCCGCAGTACTTAAAGAAACTTGTAAGGAAGTAT
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DQ821857    181 TAAAGGAACTCGGACAGCTAGAAAACAATCCGCTCTTACAAATAGCAATAGAACTTGAAG
A1-12       181 TAAAGGAACTCGGACAGCTAGAAAACAATCCGCTCTTACAAATAGCAATAGAACTTGAAG
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DQ821857    301 ATTCGGGTATTATCTATAAAGCTATGGGTATACCGTCGCAAA
A1-12       301 ATTCGGGTATTATCTATAAAGCTATGGGTATACCGTCGCAAA
DQ105664    300 ATTCGGGTATTATCTATAAAGCTATGGGTATACCGTCGCAAA
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13.2.2. *GltA* sequence comparison of the *Rickettsia monacensis* cluster.

AF141906: *Rickettsia* sp. IRS4
DQ100163: *R. monacensis* strain IrR/Munich
DQ910783: *Rickettsia* sp. PoTiR1dt
AF140706: *Rickettsia* sp. IRS3
A3-264: submitted to GenBank as consensus sequence for the 12 identical ones (accession no: EU596564)
D-2: submitted to GenBank (accession no. EU596562)

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W-28          1 -GGCTAATGAAGCGGTAATAAATATGCTTAAAGAAATCGGTAGTTCTGAGAATATCCCTA
W-82          1 GGGCTAATGAAGCGGTAATAAATATGCTTAAAGAAATCGGTAGTTCTGAGAATATCCCTA
E1-297        1 GGGCTAATGAAGCGGTAATAAATATGCTTAAAGAAATCGGTAGTTCTGAGAATATCCCTA
E2-250        1 GGGCTAATGAAGCGGTAATAAATATGCTTAAAGAAATCGGTAGTTCTGAGAATATCCCTA
D-249         1 GGGCTAATGAAGCGGTAATAAATATGCTTAAAGAAATCGGTAGTTCTGAGAATATCCCTA
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D-2       301 ATTCAGGTATTATCTATAAAGCTATGGGTATACCGTCGCAAA
```

13.2.3. *OmpA* sequence comparison of the *Rickettsia monacensis* cluster

DQ100169: *R. monacensis* strain IrR/Munich
AF201329: *R. monacensis* sp. IrR/Munich
AJ427884: *R. monacensis* sp. IrITA2
AF141911: *Rickettsia* sp. IRS4;
DQ910781: *R. monacensis* sp. PoTiR1dt
AF141909: *Rickettsia* sp. IRS3;
DQ157778: *Rickettsia monacensis* strain Rp-Sp1
A3-264: submitted to GenBank as consensus sequence for the 12 identical ones (accession no: EU596565)
D-2: submitted to GenBank (accession no. EU596561)

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AF141911    1 TTATTTCAAAAGGCACCTCAAAAAGGTCTTAAAACCGCTTTATTCACCACCTCAACCGCA
DQ157778    1 ------------------------------------------------------------
W-28        1 ----------------------------TTAAAACCGCTTTATTCACCACCTCAACCGCA
AF201329    1 TTATTTCAAAAGGCAATTCAAAAAGGTCTTAAAACCGCTTTATTCACCACCTCAACCGCA
A3-264      1 TTATTTCAAAAGGCAATTCAAAAAGGTCTTAAAACCGCTTTATTCACCACCTCAACCGCA
E2-250      1 TTATTTCAAAAGGCAATTCAAAAAGGTCTTAAAACCGCTTTATTCACCACCTCAACCGCA
W-87        1 TTATTTCAAAAGGCAATTCAAAAAGGTCTTAAAACCGCTTTATTCACCACCTCAACCGCA
AJ427884    1 TTATTTCAAAAGGCAATTCAAAAAGGTCTTAAAACCGCTTTATTCACCACCTCAACCGCA
D-249       1 TTATTTCAAAAGGCAATTCAAAAAGGTCTTAAAACCGCTTTATTCACCACCTCAACCGCA
W-82        1 TTATTTCAAAAGGCAATTCAAAAAGGTCTTAAAACCGCTTTATTCACCACCTCAACCGCA
E1-297      1 TTATTTCAAAAGGCAATTCAAAAAGGTCTTAAAACCGCTTTATTCACCACCTCAACCGCA
E1-196      1 TTATTTCAAAAGGCAATTCAAAAAGGTCTTAAAACCGCTTTATTCACCACCTCAACCGCA
A3-163      1 TTATTTCAAAAGGCAATTCAAAAAGGTCTTAAAACCGCTTTATTCACCACCTCAACCGCA
DQ100169    1 TTATTTCAAAAGGCAATTCAAAAAGGTCTTAAAACCGCTTTATTCACCACCTCAACCGCA
B-4         1 TTATTTCAAAAGGCAATTCAAAAAGGTCTTAAAACCGCTTTATTCACCACCTCAACCGCA
E2-167      1 TTATTTCAAAAGGCAATTCAAAAAGGTCTTAAAACCGCTTTATTCACCACCTCAACCGCA
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DQ910781   61 GCATTAATGCTGAGTAGTAGCGGGGCGTTGGGTGTTGCTGCGGGTGTTATTTCTATTAAT
D-2        61 GCATTAATGCTGAGTAGTAGCGGGGCGTTGGGTGTTGCTGCGGGTGTTATTTCTATTAAT
AF141909   61 GCATTAATGCTGAGTAGTAGCGGGGCGTTGGGTGTTGCTGCGGGTGTTATTTCTATTAAT
AF141911   61 GCATTAATGCTGAGTAGTAGCGGGGCGTTGGGTGTTGCTGCGGGTGTTATTTCTATTAAT
DQ157778    1 ------------------------GCGTTGGGTGTTGCTGCGGGTGTTATTTCTATTAAT
W-28       33 GCATTAATGCTGAGTAGTAGCGGGGCGTTGGGTGTTGCTGCGGGTGTTATTTCTATTAAT
AF201329   61 GCATTAATGCTGAGTAGTAGCGGGGCGTTGGGTGTTGCTGCGGGTGTTATTTCTATTAAT
A3-264     61 GCATTAATGCTGAGTAGTAGCGGGGCGTTGGGTGTTGCTGCGGGTGTTATTTCTATTAAT
E2-250     61 GCATTAATGCTGAGTAGTAGCGGGGCGTTGGGTGTTGCTGCGGGTGTTATTTCTATTAAT
W-87       61 GCATTAATGCTGAGTAGTAGCGGGGCGTTGGGTGTTGCTGCGGGTGTTATTTCTATTAAT
AJ427884   61 GCATTAATGCTGAGTAGTAGCGGGGCGTTGGGTGTTGCTGCGGGTGTTATTTCTATTAAT
D-249      61 GCATTAATGCTGAGTAGTAGCGGGGCGTTGGGTGTTGCTGCGGGTGTTATTTCTATTAAT
W-82       61 GCATTAATGCTGAGTAGTAGCGGGGCGTTGGGTGTTGCTGCGGGTGTTATTTCTATTAAT
E1-297     61 GCATTAATGCTGAGTAGTAGCGGGGCGTTGGGTGTTGCTGCGGGTGTTATTTCTATTAAT
E1-196     61 GCATTAATGCTGAGTAGTAGCGGGGCGTTGGGTGTTGCTGCGGGTGTTATTTCTATTAAT
A3-163     61 GCATTAATGCTGAGTAGTAGCGGGGCGTTGGGTGTTGCTGCGGGTGTTATTTCTATTAAT
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DQ100169    61 GCATTAATGCTGAGTAGTAGCGGGGCGTTGGGTGTTGCTGCGGGTGTTATTTCTATTAAT
B-4         61 GCATTAATGCTGAGTAGTAGCGGGGCGTTGGGTGTTGCTGCGGGTGTTATTTCTATTAAT
E2-167      61 GCATTAATGCTGAGTAGTAGCGGGGCGTTGGGTGTTGCTGCGGGTGTTATTTCTATTAAT

DQ910781   121 GATGCAGCATTTAGTGATCTTGCTGCTGCCGGTAATTGGAATAAGATAACGGCTGGAGGA
D-2        121 GATGCAGCATTTAGTGATCTTGCTGCTGCCGGTAATTGGAATAAGATAACGGCTGGAGGA
AF141909   121 GATGCAGCATTTAGTGATCTTGCTGCTGCCGGTAATTGGAATAAGATAACGGCTGGAGGA
AF141911   121 GATGCAGCATTTAGTGATCTTGCTGCTGCCGGTAATTGGAATAAGATAACGGCTGGAGGA
DQ157778    37 GATGCAGCATTTAGTGATCTTGCTGCTGCCGGTAATTGGAATAAGATAACGGCTGGAGGA
W-28         93 GATGCAGCATTTAGTGATCTTGCTGCTGCCGGTAATTGGAATAAGATAACGGCTGGAGGA
AF201329   121 GATGCAGCATTTAGTGATCTTGCTGCTGCCGGTAATTGGAATAAGATAACGGCTGGAGGA
A3-264     121 GATGCAGCATTTAGTGATCTTGCTGCTGCCGGTAATTGGAATAAGATAACGGCTGGAGGA
E2-250     121 GATGCAGCATTTAGTGATCTTGCTGCTGCCGGTAATTGGAATAAGATAACGGCTGGAGGA
W-87       121 GATGCAGCATTTAGTGATCTTGCTGCTGCCGGTAATTGGAATAAGATAACGGCTGGAGGA
AJ427884   121 GATGCAGCATTTAGTGATCTTGCTGCTGCCGGTAATTGGAATAAGATAACGGCTGGAGGA
D-249      121 GATGCAGCATTTAGTGATCTTGCTGCTGCCGGTAATTGGAATAAGATAACGGCTGGAGGA
W-82       121 GATGCAGCATTTAGTGATCTTGCTGCTGCCGGTAATTGGAATAAGATAACGGCTGGAGGA
E1-297     121 GATGCAGCATTTAGTGATCTTGCTGCTGCCGGTAATTGGAATAAGATAACGGCTGGAGGA
E1-196     121 GATGCAGCATTTAGTGATCTTGCTGCTGCCGGTAATTGGAATAAGATAACGGCTGGAGGA
A3-163     121 GATGCAGCATTTAGTGATCTTGCTGCTGCCGGTAATTGGAATAAGATAACGGCTGGAGGA
DQ100169   121 GATGCAGCATTTAGTGATCTTGCTGCTGCCGGTAATTGGAATAAGATAACGGCTGGAGGA
B-4        121 GATGCAGCATTTAGTGATCTTGCTGCTGCCGGTAATTGGAATAAGATAACGGCTGGAGGA
E2-167     121 GATGCAGCATTTAGTGATCTTGCTGCTGCCGGTAATTGGAATAAGATAACGGCTGGAGGA

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D-2        181 GTAGCTAATGGTACTTCTGTTGACGGTCCTCAAGACAATAAGGCATTTACTTACGGTGGT
AF141909   181 GTAGCTAATGGTACTTCTGTTGACGGTCCTCAAGACAATAAGGCATTTACTTACGGTGGT
AF141911   181 GTAGCTAATGGTACTTCTGTTAACGGTCCTCAAGACAATAAGGCATTTACTTACGGTGGT
DQ157778    97 GTAGCTAATGGTACTTCTGTTAACGGTCCTCAAGACAATAAGGCATTTACTTACGGTGGT
W-28        153 GTAGCTAATGGTACTTCTGTTAACGGTCCTCAAGACAATAAGGCATTTACTTACGGTGGT
AF201329   181 GTAGCTAATGGTACTTCTGTTAACGGTCCTCAAGACAATAAGGCATTTACTTACGGTGGT
A3-264     181 GTAGCTAATGGTACTTCTGTTAACGGTCCTCAAGACAATAAGGCATTTACTTACGGTGGT
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W-87       181 GTAGCTAATGGTACTTCTGTTAACGGTCCTCAAGACAATAAGGCATTTACTTACGGTGGT
AJ427884   181 GTAGCTAATGGTACTTCTGTTAACGGTCCTCAAGACAATAAGGCATTTACTTACGGTGGT
D-249      181 GTAGCTAATGGTACTTCTGTTAACGGTCCTCAAGACAATAAGGCATTTACTTACGGTGGT
W-82       181 GTAGCTAATGGTACTTCTGTTAACGGTCCTCAAGACAATAAGGCATTTACTTACGGTGGT
E1-297     181 GTAGCTAATGGTACTTCTGTTAACGGTCCTCAAGACAATAAGGCATTTACTTACGGTGGT
E1-196     181 GTAGCTAATGGTACTTCTGTTAACGGTCCTCAAGACAATAAGGCATTTACTTACGGTGGT
A3-163     181 GTAGCTAATGGTACTTCTGTTAACGGTCCTCAAGACAATAAGGCATTTACTTACGGTGGT
DQ100169   181 GTAGCTAATGGTACTTCTGTTAACGGTCCTCAAGACAATAAGGCATTTACTTACGGTGGT
B-4        181 GTAGCTAATGGTACTTCTGTTAACGGTCCTCAAGACAATAAGGCATTTACTTACGGTGGT
E2-167     181 GTAGCTAATGGTACTTCTGTTAACGGTCCTCAAGACAATAAGGCATTTACTTACGGTGGT

DQ910781   241 CGTCATATTATCACTGCAGATGAAGTCGGTCGTATTATTACGGCTATAAATGTTGCGGCT
D-2        241 CGTCATATTATCACTGCAGATGAAGTCGGTCGTATTATTACGGCTATAAATGTTGCGGCT
AF141909   241 CGTCATATTATCACTGCAGATGAAGTCGGTCGTATTATTACGGCTATAAATGTTGCGGCT
AF141911   241 CGTCATATTATCACTGCAGATAAAGTCGGTCGTATTATTACGGCTATAAATGTTGCGGCT
DQ157778   157 CGTCATATTATCACTGCAGATAAAGTCGGTCGTATTATTACGGCTATAAATGTTGCGGCT
W-28        213 CGTCATATTATCACTGCAGATAAAGTCGGTCGTATTATTACGGCTATAAATGTTGCGGCT
AF201329   241 CGTCATATTATCACTGCAGATAAAGTCGGTCGTATTATTACGGCTATAAATGTTGCGGCT
A3-264     241 CGTCATATTATCACTGCAGATAAAGTCGGTCGTATTATTACGGCTATAAATGTTGCGGCT
E2-250     241 CGTCATATTATCACTGCAGATAAAGTCGGTCGTATTATTACGGCTATAAATGTTGCGGCT
W-87       241 CGTCATATTATCACTGCAGATAAAGTCGGTCGTATTATTACGGCTATAAATGTTGCGGCT
AJ427884   241 CGTCATATTATCACTGCAGATAAAGTCGGTCGTATTATTACGGCTATAAATGTTGCGGCT
D-249      241 CGTCATATTATCACTGCAGATAAAGTCGGTCGTATTATTACGGCTATAAATGTTGCGGCT
W-82       241 CGTCATATTATCACTGCAGATAAAGTCGGTCGTATTATTACGGCTATAAATGTTGCGGCT
E1-297     241 CGTCATATTATCACTGCAGATAAAGTCGGTCGTATTATTACGGCTATAAATGTTGCGGCT
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E1-196     241 CGTCATATTATCACTGCAGATAAAGTCGGTCGTATTATTACGGCTATAAATGTTGCGGCT
A3-163     241 CGTCATATTATCACTGCAGATAAAGTCGGTCGTATTATTACGGCTATAAATGTTGCGGCT
DQ100169   241 CGTCATATTATCACTGCAGATAAAGTCGGTCGTATTATTACGGCTATAAATGTTGCGGCT
B-4        241 CGTCATATTATCACTGCAGATAAAGTCGGTCGTATTATTACGGCTATAAATGTTGCGGCT
E2-167     241 CGTCATATTATCACTGCAGATAAAGTCGGTCGTATTATTACGGCTATAAATGTTGCGGCT

DQ910781   301 ACTAATCCTATAGGCCTAAAAATTGCTGAAAATACCAGCGTCGGTTCTATTGTTACAGAT
D-2        301 ACTAATCCTATAGGCCTAAAAATTGCTGAAAATACCAGCGTCGGTTCTATTGTTACAGAT
AF141909   301 ACTAATCCTATAGGCCTAAAAATTGCTGAAAATACCAGCGTCGGTTCTATTGTTACAGAT
AF141911   301 ACTAATCCTATAGGCCTAAAGATTGCTGAAAATACCAGCGTCGGTTCTATTGTTACAGAT
DQ157778   217 ACTAATCCTATAGGCCTAAAGATTGCTGAAAATACCAGCGTCGGTTCTATTGTTACAGAT
W-28       273 ACTAATCCTATAGGCCTAAAGATTGCTGAAAATACCAGCGTCGGTTCTATTGTTACAGAT
AF201329   301 ACTAATCCTATAGGCCTAAAGATTGCTGAAAATACCAGCGTCGGTTCTATTGTTACAGAT
A3-264     301 ACTAATCCTATAGGCCTAAAGATTGCTGAAAATACCAGCGTCGGTTCTATTGTTACAGAT
E2-250     301 ACTAATCCTATAGGCCTAAAGATTGCTGAAAATACCAGCGTCGGTTCTATTGTTACAGAT
W-87       301 ACTAATCCTATAGGCCTAAAGATTGCTGAAAATACCAGCGTCGGTTCTATTGTTACAGAT
AJ427884   301 ACTAATCCTATAGGCCTAAAGATTGCTGAAAATACCAGCGTCGGTTCTATTGTTACAGAT
D-249      301 ACTAATCCTATAGGCCTAAAGATTGCTGAAAATACCAGCGTCGGTTCTATTGTTACAGAT
W-82       301 ACTAATCCTATAGGCCTAAAGATTGCTGAAAATACCAGCGTCGGTTCTATTGTTACAGAT
E1-297     301 ACTAATCCTATAGGCCTAAAGATTGCTGAAAATACCAGCGTCGGTTCTATTGTTACAGAT
E1-196     301 ACTAATCCTATAGGCCTAAAGATTGCTGAAAATACCAGCGTCGGTTCTATTGTTACAGAT
A3-163     301 ACTAATCCTATAGGCCTAAAGATTGCTGAAAATACCAGCGTCGGTTCTATTGTTACAGAT
DQ100169   301 ACTAATCCTATAGGCCTAAAGATTGCTGAAAATACCAGCGTCGGTTCTATTGTTACAGAT
B-4        301 ACTAATCCTATAGGCCTAAAGATTGCTGAAAATACCAGCGTCGGTTCTATTGTTACAGAT
E2-167     301 ACTAATCCTATAGGCCTAAAGATTGCTGAAAATACCAGCGTCGGTTCTATTGTTACAGAT

DQ910781   361 CGTAACTTGTTGCCTGTTAATATTACTGCCGGCAAAAGTTTAACTTTAACCGGTACTGCT
D-2        361 CGTAACTTGTTGCCTGTTAATATTACTGCCGGCAAAAGTTTAACTTTAACCGGTACTGCT
AF141909   361 CGTAACTTGTTGCCTGTTAATATTACTGCCGGCAAAAGTTTAACTTTAACCGGTACTGCT
AF141911   361 CATAACTTGTTGCCTGTTAATATTACTGCCGGCAAAAGTTTAACTTTAACCGGTACTGCT
DQ157778   277 CATAACTTGTTGCCTGTTAATATTACTGCCGGCAAAAGTTTAACTTTAACCGGTACTGCT
W-28       333 CATAACTTGTTGCCTGTTAATATTACTGCCGGCAAAAGTTTAACTTTAACCGGTACTGCT
AF201329   361 CATAACTTGTTGCCTGTTAATATTACTGCCGGCAAAAGTTTAACTTTAACCGGTACTGCT
A3-264     361 CATAACTTGTTGCCTGTTAATATTACTGCCGGCAAAAGTTTAACTTTAACCGGTACTGCT
E2-250     361 CATAACTTGTTGCCTGTTAATATTACTGCCGGCAAAAGTTTAACTTTAACCGGTACTGCT
W-87       361 CATAACTTGTTGCCTGTTAATATTACTGCCGGCAAAAGTTTAACTTTAACCGGTACTGCT
AJ427884   361 CATAACTTGTTGCCTGTTAATATTACTGCCGGCAAAAGTTTAACTTTAACCGGTACTGCT
D-249      361 CATAACTTGTTGCCTGTTAATATTACTGCCGGCAAAAGTTTAACTTTAACCGGTACTGCT
W-82       361 CATAACTTGTTGCCTGTTAATATTACTGCCGGCAAAAGTTTAACTTTAACCGGTACTGCT
E1-297     361 CATAACTTGTTGCCTGTTAATATTACTGCCGGCAAAAGTTTAACTTTAACCGGTACTGCT
E1-196     361 CATAACTTGTTGCCTGTTAATATTACTGCCGGCAAAAGTTTAACTTTAACCGGTACTGCT
A3-163     361 CATAACTTGTTGCCTGTTAATATTACTGCCGGCAAAAGTTTAACTTTAACCGGTACTGCT
DQ100169   361 CATAACTTGTTGCCTGTTAATATTACTGCCGGCAAAAGTTTAACTTTAACCGGTACTGCT
B-4        361 CATAACTTGTTGCCTGTTAATATTACTGCCGGCAAAAGTTTAACTTTAACCGGTACTGCT
E2-167     361 CATAACTTGT----------------------------------------------------

DQ910781   421 GCGTTTGTTCCACGTCATGGTGTTGGTGCTTTTGCTGATACTTATACGGGTTTAGGAAAT
D-2        421 GCGTTTGTTCCACGTCATGGTGTTGGTGCTTTTGCTGATACTTATACGGGTTTAGGAAAT
AF141909   421 GCGTTTGTTCCACGTCATGGTGTTGGTGCTTTTGCTGATACTTATACGGGTTTAGGAAAT
AF141911   421 GCGTTTGTTCCACGTCATGGTGTTGGTGCTTTTGCTGATACTTATACGGGTTTAGGAAAT
DQ157778   337 GCGTTTGTTCCACGTCATGGTGTTG-TGCTTTTGCTGATACTTATACAGGTTTAGGAAAT
W-28       393 GCGTTTGTTCCACGTCATGGTGTTGGTGCTTTTGCTGATACTTATACGGGTTTAGGAAAT
AF201329   421 GCGTTTGTTCCACGTCATGGTGTTGGTGCTTTTGCTGATACTTATACGGGTTTAGGAAAT
A3-264     421 GCGTTTGTTCCACGTCATGGTGTTGGTGCTTTTGCTGATACTTATACGGGTTTAGGAAAT
E2-250     421 GCGTTTGTTCCACGTCATGGTGTTGGTGCTTTTGCTGATACTTATACGGGTTTAGGAAAT
W-87       421 GCGTTTGTTCCACGTCATGGTGTTGGTGCTTTTGCTGATACTTATACGGGTTTAGGAAAT
AJ427884   421 GCGTTTGTTCCACGTCATGGTGTTGGTGCTTTTGCTGATACTTATACGGGTTTAGGAAAT
D-249      421 GCGTTTGTTCCACGTCATGGTGTTGGTGCTTTTGCTGATACTTATACGGGTTTAGGAAAT
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W-82       421 GCGTTTGTTCCACGTCATGGTGTTGGTGCTTTTGCTGATACTTATACGGGTTTAGGAAAT
E1-297     421 GCGTTTGTTCCACGTCATGGTGTTGGTGCTTTTGCTGATACTTATACGGGTTTAGGAAAT
E1-196     421 GCGTTTGTTCCACGTCATGGTGTTGGTGCTTTTGCTGATACTTATACGGGTTTAGGAAAT
A3-163     421 GCGTTTGTTCCACGTCATGGTGTTGGTGCTTTTGCTGATACTTATACGGGTTTAGGAAAT
DQ100169   421 GCGTTTGTTCCACGTCATGGTGTTGGTGCTTTTGCTGATACTTATACGGGTTTAGGAAAT
B-4        421 GCGTTTGTTCCACGTCATGGTGTTGGTGCTTTTGCTGATACTTATACGGGTTTAGGAAAT
E2-167         ------------------------------------------------------------

DQ910781   481 ATAACTTT
D-2        481 ATAACTTT
AF141909   481 ATAACTTT
AF141911   481 ATAACTTT
DQ157778   396 ATA-----
W-28       453 ATAACTTT
AF201329   481 ATAACTTT
A3-264     481 ATAACTTT
E2-250     481 ATAACTTT
W-87       481 ATAACTTT
AJ427884   481 ATAACTTT
D-249      481 ATAACTTT
W-82       481 ATAACTTT
E1-297     481 ATAACTTT
E1-196     481 ATAACTTT
A3-163     481 ATAACTTT
DQ100169   481 ATAACTTT
B-4        481 ATAACTTT
E2-167         --------
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13.2.4. *OmpB* sequence comparison of the *Rickettsia monacensis* cluster

All sequences obtained in this study.
A3-264 submitted to GenBank as consensus sequence for the 12 identical ones (accession no: EU330639)
D-2 submitted to GenBank (accession no: EU330640)

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B-4          1 AATTCTTAGCGGCGGTATTCCTAATACCCCTGGTACGATTTATGGCTTAGGTATAGAGAA
D-249        1 AATTCTTAGCGGCGGTATTCCTAATACCCCTGGTACGATTTATGGCTTAGGTATAGAGAA
E1-196       1 AATTCTTAGCGGCGGTATTCCTAATACCCCTGGTACGATTTATGGCTTAGGTATAGAGAA
A3-163       1 AATTCTTAGCGGCGGTATTCCTAATACCCCTGGTACGATTTATGGCTTAGGTATAGAGAA
A3-264       1 AATTCTTAGCGGCGGTATTCCTAATACCCCTGGTACGATTTATGGCTTAGGTATAGAGAA
E1-12        1 AATTCTTAGCGGCGGTATTCCTAATACCCCTGGTACGATTTATGGCTTAGGTATAGAGAA
E1-297       1 AATTCTTAGCGGCGGTATTCCTAATACCCCTGGTACGATTTATGGCTTAGGTATAGAGAA
E2-167       1 AATTCTTAGCGGCGGTATTCCTAATACCCCTGGTACGATTTATGGCTTAGGTATAGAGAA
E2-250       1 AATTCTTAGCGGCGGTATTCCTAATACCCCTGGTACGATTTATGGCTTAGGTATAGAGAA
W-28         1 AATTCTTAGCGGCGGTATTCCTAATACCCCTGGTACGATTTATGGCTTAGGTATAGAGAA
W-82         1 AATTCTTAGCGGCGGTATTCCTAATACCCCTGGTACGATTTATGGCTTAGGTATAGAGAA
W-87         1 AATTCTTAGCGGCGGTATTCCTAATACCCCTGGTACGATTTATGGCTTAGGTATAGAGAA
D-2          1 AATTCTTAGCGGCGGTATTCCTAATACCCCTGGTACGATTTATGGCTTAGGTATAGAGAA

B-4         61 TGGTACTCTAAAGTTAAAGCAAGTAACGTTTACTACAAACTATAACAACTTAGGTAGTAT
D-249       61 TGGTACTCTAAAGTTAAAGCAAGTAACGTTTACTACAAACTATAACAACTTAGGTAGTAT
E1-196      61 TGGTACTCTAAAGTTAAAGCAAGTAACGTTTACTACAAACTATAACAACTTAGGTAGTAT
A3-163      61 TGGTACTCTAAAGTTAAAGCAAGTAACGTTTACTACAAACTATAACAACTTAGGTAGTAT
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A3-264    61 TGGTACTCTAAAGTTAAAGCAAGTAACGTTTACTACAAACTATAACAACTTAGGTAGTAT
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E1-297    61 TGGTACTCTAAAGTTAAAGCAAGTAACGTTTACTACAAACTATAACAACTTAGGTAGTAT
E2-167    61 TGGTACTCTAAAGTTAAAGCAAGTAACGTTTACTACAAACTATAACAACTTAGGTAGTAT
E2-250    61 TGGTACTCTAAAGTTAAAGCAAGTAACGTTTACTACAAACTATAACAACTTAGGTAGTAT
W-28      61 TGGTACTCTAAAGTTAAAGCAAGTAACGTTTACTACAAACTATAACAACTTAGGTAGTAT
W-82      61 TGGTACTCTAAAGTTAAAGCAAGTAACGTTTACTACAAACTATAACAACTTAGGTAGTAT
W-87      61 TGGTACTCTAAAGTTAAAGCAAGTAACGTTTACTACAAACTATAACAACTTAGGTAGTAT
D-2       61 TGGTACTCTAAAGTTAAAGCAAGTAACGTTTACTACAAACTATAACAACTTAGGTAGTAT

B-4      121 TATTGCAACTAACGCAATAATTAATGACGGTGTAACTGTTACTACAGGCGGTATAGCCGG
D-249    121 TATTGCAACTAACGCAATAATTAATGACGGTGTAACTGTTACTACAGGCGGTATAGCCGG
E1-196   121 TATTGCAACTAACGCAATAATTAATGACGGTGTAACTGTTACTACAGGCGGTATAGCCGG
A3-163   121 TATTGCAACTAACGCAATAATTAATGACGGTGTAACTGTTACTACAGGCGGTATAGCCGG
A3-264   121 TATTGCAACTAACGCAATAATTAATGACGGTGTAACTGTTACTACAGGCGGTATAGCCGG
E1-12    121 TATTGCAACTAACGCAATAATTAATGACGGTGTAACTGTTACTACAGGCGGTATAGCCGG
E1-297   121 TATTGCAACTAACGCAATAATTAATGACGGTGTAACTGTTACTACAGGCGGTATAGCCGG
E2-167   121 TATTGCAACTAACGCAATAATTAATGACGGTGTAACTGTTACTACAGGCGGTATAGCCGG
E2-250   121 TATTGCAACTAACGCAATAATTAATGACGGTGTAACTGTTACTACAGGCGGTATAGCCGG
W-28     121 TATTGCAACTAACGCAATAATTAATGACGGTGTAACTGTTACTACAGGCGGTATAGCCGG
W-82     121 TATTGCAACTAACGCAATAATTAATGACGGTGTAACTGTTACTACAGGCGGTATAGCCGG
W-87     121 TATTGCAACTAACGCAATAATTAATGACGGTGTAACTGTTACTACAGGCGGTATAGCCGG
D-2      121 TATTGCAACTAACGCAATAATTAATGACGGTGTAACTGTTACTACAGGCGGTATAGCCGG

B-4      181 AACAGATTTCGACGGTAAAATTACTCTTGGAAGTGTTAACGGTAACGCTAATGTAAGATT
D-249    181 AACAGATTTCGACGGTAAAATTACTCTTGGAAGTGTTAACGGTAACGCTAATGTAAGATT
E1-196   181 AACAGATTTCGACGGTAAAATTACTCTTGGAAGTGTTAACGGTAACGCTAATGTAAGATT
A3-163   181 AACAGATTTCGACGGTAAAATTACTCTTGGAAGTGTTAACGGTAACGCTAATGTAAGATT
A3-264   181 AACAGATTTCGACGGTAAAATTACTCTTGGAAGTGTTAACGGTAACGCTAATGTAAGATT
E1-12    181 AACAGATTTCGACGGTAAAATTACTCTTGGAAGTGTTAACGGTAACGCTAATGTAAGATT
E1-297   181 AACAGATTTCGACGGTAAAATTACTCTTGGAAGTGTTAACGGTAACGCTAATGTAAGATT
E2-167   181 AACAGATTTCGACGGTAAAATTACTCTTGGAAGTGTTAACGGTAACGCTAATGTAAGATT
E2-250   181 AACAGATTTCGACGGTAAAATTACTCTTGGAAGTGTTAACGGTAACGCTAATGTAAGATT
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W-82     181 AACAGATTTCGACGGTAAAATTACTCTTGGAAGTGTTAACGGTAACGCTAATGTAAGATT
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D-2      181 AACAGATTTCGACGGTAAAATTACTCTTGGAAGTGTTAACGGTAACGCTAATGTAAGATT

B-4      241 TGCTGACGGTACATTTTCTGATTCTACAAGTATGATTGTTACTACTAAAGCTAATAGCGG
D-249    241 TGCTGACGGTACATTTTCTGATTCTACAAGTATGATTGTTACTACTAAAGCTAATAGCGG
E1-196   241 TGCTGACGGTACATTTTCTGATTCTACAAGTATGATTGTTACTACTAAAGCTAATAGCGG
A3-163   241 TGCTGACGGTACATTTTCTGATTCTACAAGTATGATTGTTACTACTAAAGCTAATAGCGG
A3-264   241 TGCTGACGGTACATTTTCTGATTCTACAAGTATGATTGTTACTACTAAAGCTAATAGCGG
E1-12    241 TGCTGACGGTACATTTTCTGATTCTACAAGTATGATTGTTACTACTAAAGCTAATAGCGG
E1-297   241 TGCTGACGGTACATTTTCTGATTCTACAAGTATGATTGTTACTACTAAAGCTAATAGCGG
E2-167   241 TGCTGACGGTACATTTTCTGATTCTACAAGTATGATTGTTACTACTAAAGCTAATAGCGG
E2-250   241 TGCTGACGGTACATTTTCTGATTCTACAAGTATGATTGTTACTACTAAAGCTAATAGCGG
W-28     241 TGCTGACGGTACATTTTCTGATTCTACAAGTATGATTGTTACTACTAAAGCTAATAGCGG
W-82     241 TGCTGACGGTACATTTTCTGATTCTACAAGTATGATTGTTACTACTAAAGCTAATAGCGG
W-87     241 TGCTGACGGTACATTTTCTGATTCTACAAGTATGATTGTTACTACTAAAGCTAATAGCGG
D-2      241 TGCTGACGGTACATTTTCTGATTCTACAAGTATGATTGTTACTACTAAAGCTAATAGCGG

B-4      301 TACCGTAACTTATTTAGGTGATGCGTATGTTGGTAATATAGGTGCTTCAGATACTCCTGT
D-249    301 TACCGTAACTTATTTAGGTGATGCGTATGTTGGTAATATAGGTGCTTCAGATACTCCTGT
E1-196   301 TACCGTAACTTATTTAGGTGATGCGTATGTTGGTAATATAGGTGCTTCAGATACTCCTGT
A3-163   301 TACCGTAACTTATTTAGGTGATGCGTATGTTGGTAATATAGGTGCTTCAGATACTCCTGT
A3-264   301 TACCGTAACTTATTTAGGTGATGCGTATGTTGGTAATATAGGTGCTTCAGATACTCCTGT
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E1-297  301 TACCGTAACTTATTTAGGTGATGCGTATGTTGGTAATATAGGTGCTTCAGATACTCCTGT
E2-167  301 TACCGTAACTTATTTAGGTGATGCGTATGTTGGTAATATAGGTGCTTCAGATACTCCTGT
E2-250  301 TACCGTAACTTATTTAGGTGATGCGTATGTTGGTAATATAGGTGCTTCAGATACTCCTGT
W-28    301 TACCGTAACTTATTTAGGTGATGCGTATGTTGGTAATATAGGTGCTTCAGATACTCCTGT
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13.3. Statistic figures and tables

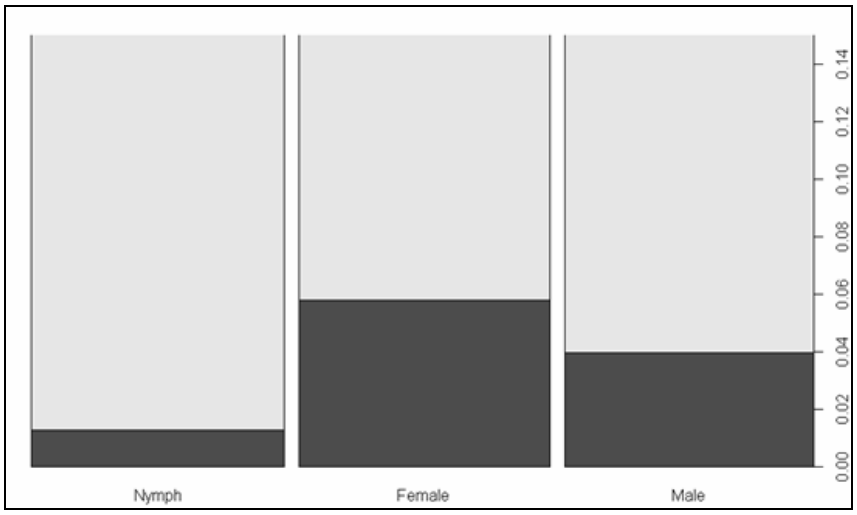

Figure 13: Overview of *Anaplasma phagocytophilum* infected ticks separated by gender and stage[a]

[a]the width of each bar is proportional to the number of ticks (Nymphs=961; Females=952; Males=949)

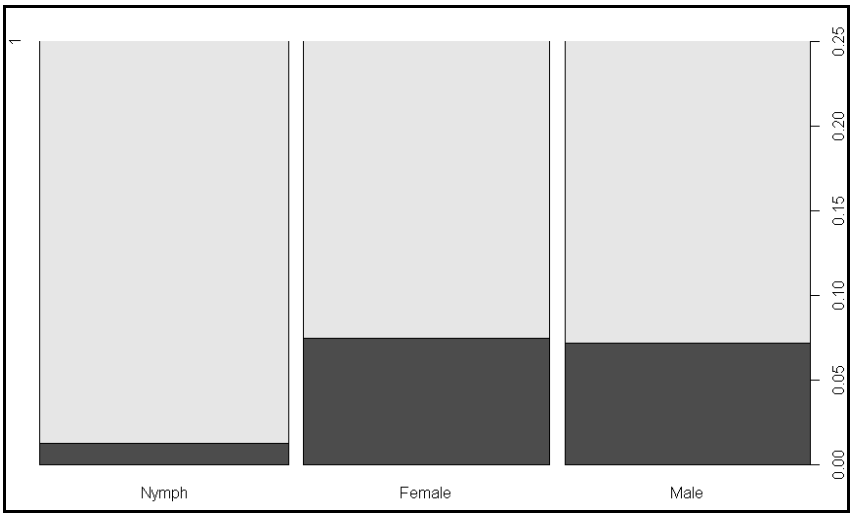

Figure 14: Overview of spotted fever group rickettsiae infected ticks separated by gender and stage[a]

[a]the width of each bar is proportional to the number of ticks (Nymphs=961; Females=952; Males=948)

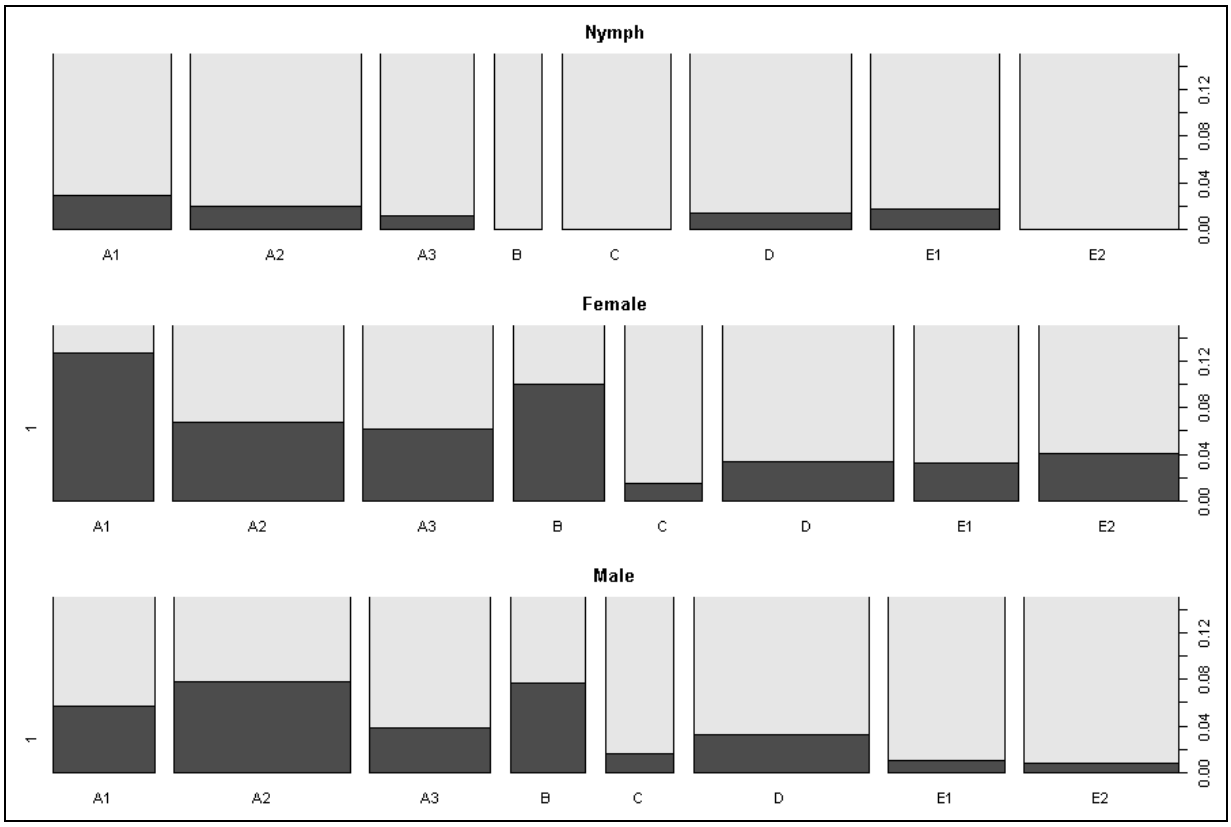

Figure 15: Overview on the proportion of *Anaplasma phagocytophilum* infected ticks
In dark: ticks groupd by gender and collection site. The width of each bar is proportional to the number of ticks

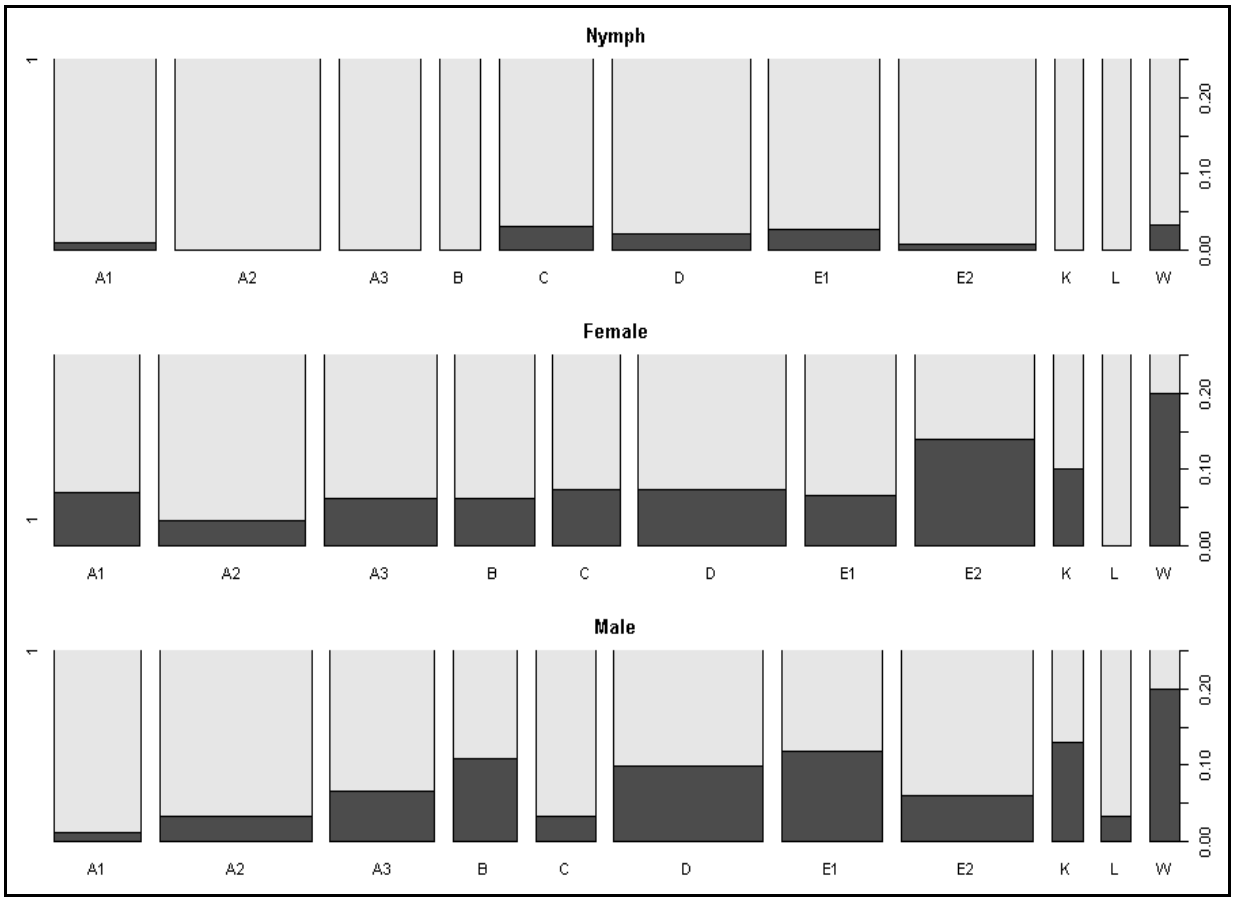

Figure 16: Overview on the proportion of spotted fever group rickettsiae infected ticks
In dark: ticks grouped by gender and collection site. The width of each bar is proportional to the number of ticks

Table 14: Number of infected ticks in all sites, months and stages investigated

		N° infected ticks/N° total ticks					
		Anaplasma phagocytophilum			SFG rickettsiae		
Site	Month	Nymph	Female	Male	Nymph	Female	Male
A1	May	1/17	6/31	1/32	0/17	3/31	1/32
	June	0/30	2/30	2/30	0/30	2/30	0/30
	July	2/31	1/10	1/8	1/31	0/10	0/8
	Aug.	0/18	2/10	0/13	0/18	1/10	0/13
	Sept.	0/8	0/6	1/5	0/8	0/6	0/5
A2	May	0/30	2/30	1/30	0/30	1/30	0/30
	June	1/30	3/30	2/32	0/30	2/30	1/32
	July	0/30	1/30	3/30	0/30	0/30	0/30
	Aug.	2/30	3/29	5/31	0/30	1/29	3/31
	Sept.	0/30	1/30	1/30	0/30	1/30	1/30
A3	May	0/6	4/30	2/31	0/6	1/30	2/31
	June	0/30	1/30	0/30	0/30	3/30	3/30
	July	0/30	1/30	2/30	0/30	3/30	0/30
	Aug.	0/5	0/14	0/7	0/5	0/14	0/7
	Sept.	1/12	1/10	0/7	0/12	0/10	2/7
B	May	0/1	4/35	3/30	0/1	1/35	4/30
	June	0/6	4/30	2/30	0/6	2/30	3/30
	July	0/29	0/11	0/5	0/29	2/11	0/5
	Aug.	0/6	0/3	0/0	0/6	0/3	0/0
	Sept.	0/0	0/1	0/0	0/0	0/1	0/0
C	May	0/1	1/12	0/8	0/1	0/12	0/8
	June	0/30	0/30	0/30	1/30	4/30	1/30
	July	0/30	0/17	0/10	1/30	1/17	0/10
	Aug.	0/30	0/7	1/11	1/30	0/7	1/11
	Sept.	0/5	0/2	0/1	0/5	0/2	0/1
D	May	0/30	2/30	0/31	1/30	2/30	1/30
	June	0/30	0/30	0/31	0/30	3/30	5/31
	July	1/30	0/30	2/30	0/30	3/30	3/30
	Aug.	0/30	1/30	2/30	1/30	2/30	3/30
	Sept.	1/22	2/30	1/30	1/22	1/30	3/30
E1	May	1/14	1/30	0/30	0/14	1/30	5/30
	June	0/29	1/30	1/30	0/29	3/30	4/30
	July	0/30	1/29	0/29	2/30	2/29	1/29
	Aug.	1/11	0/1	0/5	0/11	0/1	2/5
	Sept.	0/30	0/2	0/7	1/30	0/2	0/7
E2	May	0/20	1/30	0/31	0/20	0/30	2/31
	June	0/30	2/30	0/31	0/30	6/30	0/31
	July	0/30	2/30	0/30	1/30	4/30	1/30
	Aug.	0/30	0/30	1/30	0/30	7/30	3/30
	Sept.	0/30	0/2	0/12	0/30	0/2	3/12
K	June	0/30	1/30	1/31	0/30	3/30	4/31
L	July	0/30	0/30	1/30	0/30	0/30	1/30
W	May	0/30	2/30	1/30	0/30	6/30	6/30

Table 15: Percentage of infected ticks in all sites, months and stages investigated.

		Anaplasma phagocytophilum[a]			SFG rickettsiae[a]		
Site	Month	Nymph	Female	Male	Nymph	Female	Male
A1	May	5.9	19.4	3.1	0.0	9.7	3.1
	June	0.0	6.7	6.7	0.0	6.7	0.0
	July	6.5	10.0	12.5	3.2	0.0	0.0
	Aug.	0.0	20.0	0.0	0.0	10.0	0.0
	Sept.	0.0	0.0	20.0	0.0	0.0	0.0
A2	May	0.0	6.7	3.3	0.0	3.3	0.0
	June	3.3	10.0	6.2	0.0	6.7	3.1
	July	0.0	3.3	10.0	0.0	0.0	0.0
	Aug.	6.7	10.3	16.1	0.0	3.4	9.7
	Sept.	0.0	3.3	3.3	0.0	3.3	3.3
A3	May	0.0	13.3	6.5	0.0	3.3	6.5
	June	0.0	3.3	0.0	0.0	10.0	10.0
	July	0.0	3.3	6.7	0.0	10.0	0.0
	Aug.	0.0	0.0	0.0	0.0	0.0	0.0
	Sept.	8.3	10.0	0.0	0.0	0.0	28.6
B	May	0.0	11.4	10.0	0.0	2.9	13.3
	June	0.0	13.3	6.7	0.0	6.7	10.0
	July	0.0	0.0	0.0	0.0	18.2	0.0
	Aug.	0.0	0.0	NA	0.0	0.0	NA
	Sept.	NA[b]	0.0	NA	NA	0.0	NA
C	May	0.0	8.3	0.0	0.0	0.0	0.0
	June	0.0	0.0	0.0	3.3	13.3	3.3
	July	0.0	0.0	0.0	3.3	5.9	0.0
	Aug.	0.0	0.0	9.1	3.3	0.0	9.1
	Sept.	0.0	0.0	0.0	0.0	0.0	0.0
D	May	0.0	6.7	0.0	3.3	6.7	3.3
	June	0.0	0.0	0.0	0.0	10.0	16.1
	July	3.3	0.0	6.7	0.0	10.0	10.0
	Aug.	0.0	3.3	6.7	3.3	6.7	10.0
	Sept.	4.5	6.7	3.3	4.5	3.3	10.0
E1	May	7.1	3.3	0.0	0.0	3.3	16.7
	June	0.0	3.3	3.3	0.0	10.0	13.3
	July	0.0	3.4	0.0	6.7	6.9	3.4
	Aug.	9.1	0.0	0.0	0.0	0.0	40.0
	Sept.	0.0	0.0	0.0	3.3	0.0	0.0
E2	May	0.0	3.3	0.0	0.0	0.0	6.5
	June	0.0	6.7	0.0	0.0	20.0	0.0
	July	0.0	6.7	0.0	3.3	13.3	3.3
	Aug.	0.0	0.0	3.3	0.0	23.3	10.0
	Sept.	0.0	0.0	0.0	0.0	0.0	16.7
K	June	0.0	3.3	3.2	0.0	10.0	12.9
L	July	0.0	3.3	6.7	0.0	0.0	3.3
W	May	0.0	6.7	3.3	3.3	20.0	20.0

[a]for total number of ticks investigated see Table 14
[b](NA=not applicable)

Table 16: Full model of the logistic regression with all interaction of stage/gender, site and month

	Anaplasma phagocytophilum p	*Rickettsia* spp. p
Stage/gender	6.542e-08 *	1.796e-13 *
Site	5.893e-04 *	5.509e-05 *
Month	0.40	0.29
Stage : Site	0.80	0.11
Stage : Month	0.33	0.08
Site : Month	0.24	0.63
Stage : Site : Month	0.96	0.95

(95% Confidence interval; * p<0.05 regarded as significant)

Table 17: Reduced model of the logistic regression
Reference categories: Stage: Nymph; Site: A1

	Anaplasma phagocytophilum		*Rickettsia* spp.	
	Estimate	p	Estimate	p
Stage: Female	1.59	2.61e-06 *	1.87	3.48e-09 *
Stage: Male	1.24	0.0004 *	1.82	9.49e-09 *
Site: A2	-0.27	0.40	-0.32	0.51
A3	-0.65	0.09	0.39	0.40
B	-0.12	0.75	0.68	0.15
C	-2.01	0.008 *	0.54	0.27
D	-1.04	0.007 *	0.81	0.05 *
E1	-1.25	0.009 *	0.93	0.03 *
E2	-1.62	0.0007 *	0.86	0.04 *
K	-0.98	0.21	1.00	0.06
L	-0.68	0.32	-1.02	0.34
W	-0.96	0.14	1.74	0.00025 *

(95% Confidence interval; * p<0.05 regarded as significant)

Table 18: Logistic regression: Statistical difference between male and female ticks

	Anaplasma phagocytophilum		*Rickettsia* spp.	
	Estimate	p	Estimate	p
Female : Male	-0.35	0.11	-0.05	0.78

(95% Confidence interval; * p<0.05 regarded as significant)

Table 19: Logistic regression: comparison of investigated areas
(A1, A2, A3 together as A = English Garden; E1, E2 together as E = Isarauen; A as reference)

	Anaplasma phagocytophilum		*Rickettsia* spp.	
Vegetation type	Estimate	p	Estimate	p
B	0.15	0.64	0.65	0.06
C	-1.76	0.02 *	0.52	0.17
D	-0.74	0.02 *	0.79	0.003 *
E	-1.16	0.0003 *	0.86	0.0003 *
K	-0.94	0.20	0.98	0.03 *
L	-0.50	0.40	-1.04	0.31
W	-0.50	0.40	1.71	1.51e-0.6 *

(95% Confidence interval; * p<0.05 regarded as significant)

Table 20: Logistic regression: comparison between natural forest sites and city parks[a]

	Anaplasma phagocytophilum		*Rickettsia* spp.	
	Estimate	p	Estimate	p
Forests : Parks	-1.00	2.98e-06 *	0.69	0.0002 *

[a]Forest sites: C, D, E1,E2, K, L,W together; City parks: A1, A2, A3, B together
(95% Confidence interval; * p<0.05 regarded as significant)

Table 21: Calculated weighted monthly and overall prevalence of all stages adjusted to the sampling design

	Anaplasma phagocytophilum			*Rickettsia* spp.		
	Prevalence (%)	CI 95%[a] lower	CI 95%[a] upper	Prevalence (%)	CI 95%[a] lower	CI 95%[a] upper
May	4.04	2.54	5.35	6.69	4.33	8.74
June	2.34	1.35	3.24	5.83	4.08	7.45
July	2.11	1.09	3.02	3.16	1.77	4.36
Aug.	3.60	1.82	5.17	5.85	3.56	8.03
Sept.	2.35	0.66	3.76	3.70	1.40	5.68
Total	2.90	2.27	3.48	5.28	4.31	6.17

([a] 95% Confidence interval)

14. Acknowledgments

First of all, I would like to thank Prof. Dr. Kurt Pfister for having given me the opportunity to work on this thesis in his institute and laboratories. I am invaluably grateful for his immense support and advice concerning all the different features of this doctorate.

I wish to express my gratitude to Dr. Jérémie Gilles for the superb mentoring of this work, his great support and patient and numerous discussions.

I wish show my deep appreciation to Dr. Michael Höhle and to Prof. Dr. Helmut Küchenhoff of the Statistische Beratungslabor (STABLAB), Department of Statistics, Ludwig-Maximilians-University Munich, for their invaluable help with the statistical analysis of this work.

I am very grateful for having had the opportunity to work and learn in the laboratory of the Max-von-Pettenkofer Institute, National Reference Center for Borrelia, Ludwig-Maximilians-University, Munich, especially for the excellent technical advice of Mrs. Cecilia Hizo-Teufel.

I wish to thank Prof. Dr. Lygia Friche Passos for critically reviewing the publications and this manuscript, and for numerous encouraging discussions.

Miss Sophie Neuvecelle I thank for outstanding assistance with DNA extractions and real-time PCR and lots of chats and laughs.

Further acknowledgments go to Dr. Sarah Leonhard for her help with the tick collection and advice on working with tick DNA and to my colleagues at the Institute of Comparative Tropical Medicine and Parasitology for their help, especially to Ingrid Pradel for excellent technical advice in the laboratory, to Sarah Pfalzer for help with the laboratory work and to Heidrun Schöl for guidance in identifying tick species.

Very special thanks go to Dr. Alexandra Heim for a first review of this manuscript, for long cheering chats during the days of laboratory work, for buffering panic attacks and lots of helpful advice and to Dr. Manfred Schweigert for brightening up those long commuting hours

in the train and solving many a logistic problem. Further I wish to thank Anne Kupča and Dr. Dietmar Hamel for their critical correction of this manuscript.

My dearest friend Andrea Mares I wish to thank for giving me shelter and much beyond for the long weeks of the tick collection.

Also I wish to express my gratefulness to my family for their support throughout this work, in good times and in bad times.

At last, my deepest and warmest thank you goes to my husband for always being there for me, encouraging me and believing in me, no matter what.

édition scientifique
VVB LAUFERSWEILER VERLAG

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STAUFENBERGRING 15
D-35396 GIESSEN

Tel: 0641-5599888 Fax: -5599890
redaktion@doktorverlag.de
www.doktorverlag.de

ISBN 3-8359-5332-X