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Spotted Fever Rickettsioses in Sweden

Aspects of Epidemiology, Clinical Manifestations and Co-infections

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Abstract
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The spotted fever group rickettsiae are emerging diseases. They cause damage in their hosts by invading the endothelium in small to medium-sized blood vessels, which results in vasculitis that can cause clinical manifestations from most organs.

The present thesis describes the prevalence of *Rickettsia helvetica* in ticks, the incidence of rickettsial infection based on seroreactivity and seroconversion in humans and their symptoms, from different parts of Sweden and the Åland Islands in Finland. This was accomplished through serological analysis of both retrospective and prospective serum samples from confirmed and suspected tick-bitten individuals compared to individuals with no knowledge of tick exposure (blood donors). We found a comparable seroprevalence to *Rickettsia* spp. in different geographical areas where ticks are present; it was also comparable to the seroprevalence of *Borrelia* spp. Seroprevalence was also more common, as suspected, in the tick-exposed group compared to blood donors. In comparison with co-infections with other tick-borne infections (*Anaplasma* spp. and *Borrelia* spp.), we could conclude that co-infections do exist and that, based on clinical findings, it is difficult to distinguish which microorganism causes certain clinical manifestations. For reliable conclusions regarding the causative microorganism, the diagnosis should basically rely on diagnostic tests. In comparison with *Borrelia* spp., seroconversion to *Rickettisa* spp. was more common in the areas we investigated, indicating that rickettsiosis is a common tick-borne infection in Sweden and most likely underdiagnosed.

When investigating patients with meningitis, we found *R. felis* in cerebrospinal fluid from two patients with subacute meningitis. This was the first report in which *R. felis* was found and diagnosed in patients in Sweden. The patients recovered without sequelae and without causal treatment. To provide guidelines on when to treat *Rickettisa* spp. infections, more investigations are needed.

The present thesis shows that *Rickettsia* spp. are common in ticks and do infect humans. Rickettsial infection should be considered in both non-specific or specific symptoms after a tick bite. It was also shown in the thesis that flea-borne rickettsiosis (*R. felis*) occurs in Sweden and may cause invasive infections

Keywords: Rickettsia helvetica, Rickettsia felis, co-infection, erythema migrans, meningitis, serology, PCR, western blot

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To Kristin and our children Louise,
Sara and Frida

List of Papers

This thesis is based on the following papers, which are referred to in the text by their Roman numerals.

I Elfving, K., Lindblom A., and Nilsson K. (2008) Seroprevalence of *Rickettsia* spp. infection among tick-bitten patients and blood donors in Sweden. *Scandinavian Journal of Infectious Diseases,* 40(1): 74-7.

II Lindblom, A., Wallménius, K., Nordberg, M., Forsberg, P. Eliasson, I., Påhlson, C., Nilsson, K. (2013) Seroreactivity for spotted fever rickettsiae and co-infections with other tick-borne agents among habitants in central and southern Sweden. *Eur J Clin Microbiol Infect Dis*, 32(3): p. 317-323.

III Lindblom, A., Wallménius, K., Sjöwall, J., Fryland, L., Wilhelmsson, P., Lindgren, P-E., Forsberg, P., Nilsson, K. Prevalence of *Rickettsia* spp. in ticks and serological and clinical outcomes in tick-bitten individuals in Sweden and on the Åland Islands. *Manuscript.*

IV Lindblom, A., Severinson, K. and Nilsson, K. (2010) *Rickettsia felis* infection in Sweden: report of two cases with subacute meningitis and review of the literature. *Scandinavian Journal of Infectious Diseases*, 42(11-12): 906-909.

Contents

Abbreviations

bp	Base pair
CDC	Centers for Disease Control and Prevention
CRP	C-reactive protein
CSF	Cerebrospinal fluid
Ct	Cycle threshold
ELISA	Enzyme-linked immunosorbent assay
EM	Erythema migrans
gltA	Citrate synthase
HGT	Horizontal gene transfer
IFA	Immunofluorescence assay
IHC	Immunohistochemistry
LFCIA	Lateral flow coal immunochromatographic assay
Mb	Mega base pair
MIF	Microimmunofluorescence assay
MSF	Mediterranean spotted fever
Omp	Outer membrane protein
PCR	Polymerase chain reaction
RMSF	Rocky Mountain spotted fever
rrs	16S rRNA
Sca	Surface cell antigens
SFG	Spotted fever group
sp.	Species, singular
spp.	Species, plural
TBE	Tick-borne encephalitis
TEM	Transmission electron microscopy
TG	Typhus group

Introduction

Historical notes

Rickettsia species are old bacteria, having been around for approximately 150 million years. A development occurred about 50 million years ago that led to differentiation into most of the species we know today [1].
Some historians believe epidemic typhus caused the Athens plague during the 5th century BC [2]. Recent research has suggested that the Athens plague was instead caused by *Salmonella typhi*, the agent of typhoid fever [3]. Epidemic typhus and typhoid fever were hard to differentiate until 1739, when John Huxham distinguished epidemic typhus from the less severe typhoid fever. The two diseases were more surreally distinguished from each other by William Wood Gerard in his publication in 1837 [4]. In the 16th century, the Italians Girolamo Cardano and Fracastorius described typhus, and the latter distinguished it from plague. The disease was well known during wartime, as described by Zavorziz in 1676 in his book "The Infection of Military Camps" [5]. In 1899, Edward E Maxey first described Rocky Mountain spotted fever from an outbreak in Idaho, USA [6]. Louis B Wilson and William M Chowning concluded, in 1904, that wood ticks transmitted Rocky Mountain spotted fever [7]. In a series of studies between 1906 and 1909, Howard T Ricketts identified *Rickettsia rickettsii,* the agent of Rocky Mountain spotted fever, and showed that it circulated among ticks and mammals [8-10]. Unfortunately, he died from typhus in 1910 while investigating the disease in Mexico. In 1909, Charles Nicolle discovered that the body louse transmits typhus and thereafter received the Nobel Prize in 1928 for his discovery. Stanislaus von Prowazek studied typhus and could confirm Howard T Ricketts' discovery that the causative agent of typhus was *Rickettsia prowazekii*. While investigating typhus in a Russian prison camp in Costbus in Prussia, he contracted the disease and died in 1915. His friend and colleague Henrique da Rocha Lima named the bacteria after von Prowazek and Ricketts [6]. In 1896, Nathan Brill described a milder form of typhus. Hans Zinsser published data in 1934 suggesting that Brill's disease was a recrudescent form of typhus. The disease is now known as Brill-Zinsser disease [11]. In 1997, a rickettsia, *Rickettsia helvetica*, was for the first time detected and later isolated from ticks in Sweden [12]. Since the earliest discoveries, a number of rickettsiosis have been detected, mainly from the spotted fever group (SFG), but there are still a great many questions

concerning the pathogenesis and symptomatology of rickettsioses left to be answered.

Rickettsia

General characteristics

Rickettsiae are small obligate intracellular gram-negative rods, measuring 0.3 – 1.0 μm. They are hard to stain using conventional methods, but can be stained using the Gimenez method or acridine orange. Because it is an obligate intracellular bacteria, it has to be cultivated on embryonic eggs, experimental animals or cell culture (e.g., Vero, HEL, MRC5 or L-929 cells) [13]. The rickettsiae from SFG penetrate into the nucleus of the host cell, as opposed to the rickettsiae from the typhus group (TG), which are restricted to the cytoplasm [14]. SFG have the ability to move within the cell by actin polymerization, an ability that TG lack. This is suggested to be the mechanism SFG use to penetrate into the cell nucleus. Motility by actin polymerization also helps SFG to move directly from one host cell to another without contact with extracellular space. In contrast, TG multiply in the host cell until it lysis and thus spread in the host.

The generation time of rickettsia is about 10 hours, which is longer than for most bacteria. As a comparison, the generation time for *E. coli* is about 40 minutes [15]. SFG rickettsiae's optimal growth temperature is 32 °C, and for the TG rickettsiae it is 35 °C [16].

Like other gram-negative bacteria, rickettsiae have a cell wall with an inner and outer membrane separated by a peptidoglycan layer. The cell wall is surrounded by a crystallic layer, the S-layer (slime-layer) [17, 18]. This S-layer contains "surface cell antigens" (*Sca*). Among the *Sca* proteins are outer membrane protein A (*OmpA* or *Sca0*) and outer membrane protein B (*OmpB* or *Sca5*), which are important in the process of *Rickettsia* entrance into the host cell. *OmpB* is present in both the SFG and TG, while *OmpA* is only present in the SFG. *OmpA* and *OmpB* are immunogenic, and antibodies to these epitopes seem to protect against reinfection [19].

The rickettsial genome is very small, consisting of 1.11 – 2.1 Mb in a single circular chromosome. Most of the genomes from *Rickettsia* spp. are known today [20]. Through evolution, the genome size of rickettsia has been reduced [21]. It is believed that the free-living bacteria, from which *Rickettsia* has evolved, had a genome size four to five times larger. Genome reduction is associated with increased pathogenicity and adaption to an intracellular lifestyle [22]. As a result of selection during genome loss, the bacteria loses the ability of metabolism but keeps the basic functions, such as replication, transcription and translation, as it may benefit from the metabolic pathways of the

host cell [23]. Due to the loss of metabolism, rickettsiae are dependent on metabolites from the host cell. To come around this problem, rickettsiae benefit from other systems including autotransporters, such as *OmpA, OmpB, Sca1, Sca2* and *Sca3*, as well as type IV secretion. Type IV secretion transports proteins and DNA across bacterial and eukaryotic cell walls [24].

It was previously thought that rickettsiae lack plasmids, but recent studies have found plasmids in most *Rickettsia* spp. Some rickettsiae even carry multiple plasmids [25]. The discovery of plasmids in rickettsiae has opened up the possibility for horizontal gene transfer (HGT), and studies have shown evidence for HGT [26].

There have been reports of recombination in rickettsiae, although recombination seem to be infrequent because different genes have similar phylogenetic histories [1].

Taxonomy and phylogeny

Before molecular methods were introduced, phylogenetic studies were based on serology, geographical distribution, vectors, metabolic characters and optimal growth temperature (32 °C for SFG and 35 °C for TG). These methods were unreliable, and some *Rickettsia* spp. do not fit well into this classification. For example, SFG have been described as living in ticks, but *R. felis* and *R. akari* are transmitted by cat fleas and mites, respectively [16]. The development of molecular methods has changed the classification. From the study of the 16S rRNA gene (*rrs*), *Coxiella burnetii* have been classified as *Legionellaciae*, *Wolbachia melophagi* within *Bartonellaceae*. *Orientia tsutsugamishi* was classified into a new genus, *Orientia*. Subsequently, sequences from more divergent genes have been used, such as citrate synthase (*gltA*), the gene coding for 17-kDa protein, *OmpA*, *OmpB* and genes coding for other autotransporters [27].

Rickettsiae belong to the phylum *Proteobacteria*, the class *α-Proteobacteria*, the order *Rickettsiales*, the family *Rickettsiaceae* and the genus *Rickettsia*. When only phenotyping methods were available to classify *Rickettsia*, several families were included in *Rickettsiales* that are now divided into different families. Since the introduction of molecular methods as new taxonomic tools, the order *Rickettsiales* now consists of the families *Rickettsiaceae* and *Anaplasmataceae*. The family *Anaplasmataceae* includes the genera *Anaplasma, Erlichia, Wolbachia* and *Neorickettsia* [28].

Traditionally, *Rickettsia* has been divided in the typhus group (TG) and the spotted fever group (SFG) (Figure 1). Phylogenetic studies have suggested division into two additional groups: the ancestral group, consisting of *R. belli* and *R. canadensis,* and the transitional group, consisting of *R. australis, R. akari* and *R. felis* [29].

New species belonging to the genus *Rickettsia* are continuously being discovered, and the number of species is constantly increasing.

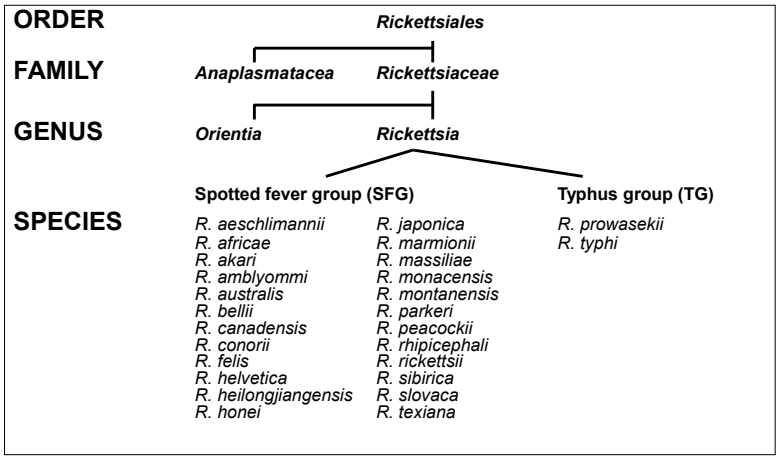

Figure 1. Rickettsia taxonomy

The vectors

Arthropods serve both as vectors and reservoirs for rickettsiae (Figure 2). Until recently, only ticks, lice, fleas and mites have been known as vectors. *Rickettsia* spp. have recently been demonstrated in the Whitefly (*Bemisia tabaci*) [30]. In a laboratory model, the malaria mosquito (*Anopheles gambiae*) has been infested with *R. felis* [31]. These discoveries suggest that rickettsiae are transmitted by several more vectors than the classic ones.

Among TG, *R. prowazekii* has lice as vectors and the vectors for *R. typhi* are fleas.

Rickettsia akari, which causes rickettsial pox, is transmitted by the house mouse mite, *Liponyssoides sanguineus*. The life cycle of the mite consists of a larval stage, which goes through a nymph stage before reaching the adult stage. The nymph and adult forms feed on blood every three or four days. In the mite, *R. akari* spreads transovarilly [32].

Rickettsia helvetica, which is normally transmitted by hard ticks, has also been found in the hedgehog flea, *Archaeopsylla erinacei,* suggesting other vectors than ticks for these bacteria [33].

R. felis was first discovered in the cat flea, *Ctenocephalides felis* [34]. The cat flea is still considered the main vector and reservoir for *R. felis,* although the bacteria has been discovered in other fleas including the dog flea, *Ctenocephalides canis,* as well as in ticks, *Haemaphysalis flava, Ixodes ovarus* and *Rhipicephalus sanguineus,* and mites [35, 36]. *R. felis* has also been demonstrated in booklice, *Liposcelis bostrychophila* [37]. Within the cat flea, *R. felis* has been discovered in the midgut, muscle cells, tracheal matrix, hypodermis, ovaries, testes and salivary glands [34, 38, 39]. Because *R. felis* have been

cultivated in mosquito cells (*Aedes albopictus*), mosquitoes have been suggested as a possible vector [40].

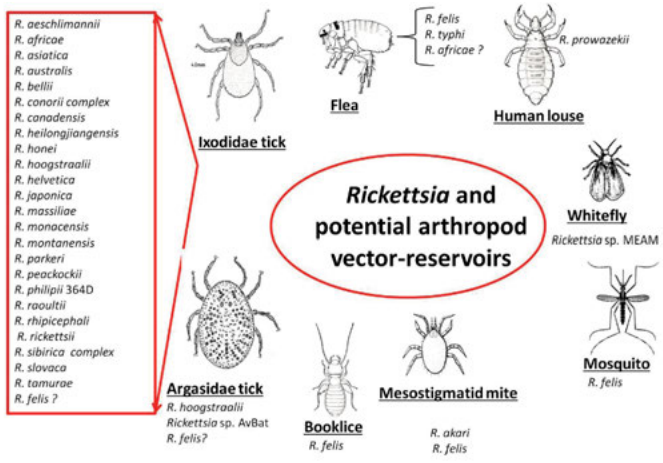

Figure 2. Rickettsia and potential arthropod vectors and reservoirs. Reprinted from Infect Genet Evol, 25C, Merhej, V., et al., Genotyping, evolution and epidemiological findings of *Rickettsia* species, p. 122-137, 2014, with permission from Elsevier.

The Tick

The main vector for the SFG rickettsiae is the tick. For the majority of SFG rickettsiae, the vector is the hard tick (*Ixodidae*), but the soft tick (*Argasidae*) can also serve as vector. A third family of ticks (*Nutalliellidae*), a monotypic genus of soft ticks, exists in southern Africa.

Ticks have three life cycle stages: larvae, nymph and adults (male and female) (Figure 3). Larvae have six legs. After moulting into the nymphal stage, they acquire eight legs. Hard ticks are more well-adapted as vectors than soft ticks are. They feed for longer periods, and their bite is usually painless. For each life stage, they have only one blood meal, and they feed on a variety of vertebrate hosts (Figure 4). Soft ticks feed for shorter periods, more frequently and usually on a single species.

When the hard tick feeds it inserts its feeding organ, the hypostome, into the host animal. Then it inserts substances produced by the salivary glands, including anaesthetics, into the host. During the feeding period, which takes 2–15 days, regurgitation also occurs. For hard ticks, only one blood meal is taken during each of the three life stages. After mating, the adult male hard tick dies. A few weeks after mating, the adult female hard tick lays thousands of eggs and then dies. Ticks are blind but have a highly developed sensory system, which includes sensitivity to chemicals (such as CO_2 and NH_3), humidity, aromatic chemicals, vibrations and the body temperature of host animals. Rickettsiae multiply in almost all organs and fluids of the tick, especially in the salivary glands, but also in the ovaries. Thus, rickettsiae may be transmitted both during feeding and transovarially. Transmission then occurs to the

next life cycle stage; this is called transstadial transmission. The tick can also be infected by cofeeding, when several ticks are feeding on the same host at the same time, allowing rickettsiae to spread from one tick to another [41]. An increase in *Rickettsia* spp. in the salivary glands of ticks during feeding has been shown [42].

Ticks can feed on any part of the human body, but the most common parts are the legs, followed by the trunk/dorsum and arms [43].

Figure 3. Life cycle stages of *Ixodes ricinus*. From left: blood-filled adult female, larvae (six legs), blood-filled larvae, adult female, adult male, nymph and blood-filled nymph. Photo by Thomas G.T. Jaenson, used by permission.

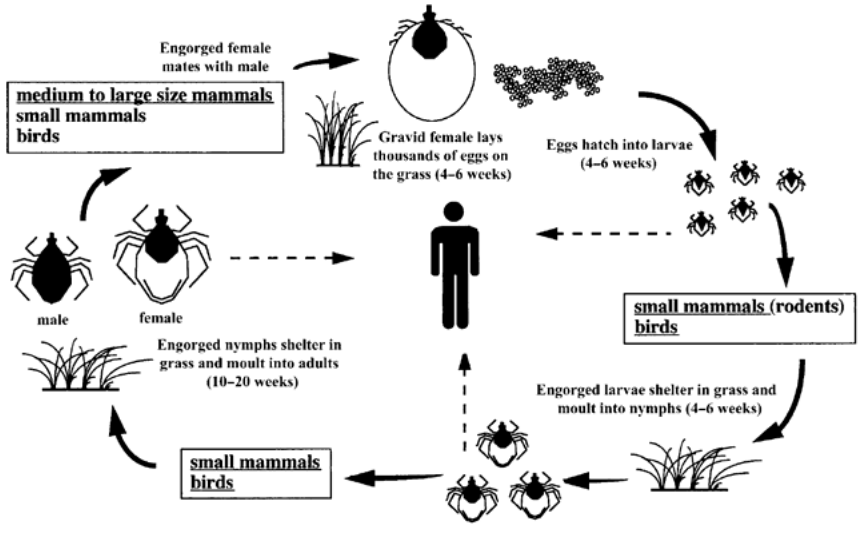

Figure 4. Life cycle of *Ixodes ricinus*. From Parola, P. and D. Raoult, Ticks and tickborne bacterial diseases in humans: an emerging infectious threat. Clin Infect Dis, 2001. 32(6): p. 897-928, by permission from Oxford University Press.

In Sweden, *Ixodes ricinus* is the most common tick, but several other ticks are permanently present. Of the ticks present in Sweden, only *Ixodes uriae, Ixodes ricinus, Ixodes hexagonus* and *Haemaphysalis punctate* feed regularly on humans [44].

Ixodes ricinus is active in the temperature range from 5°C to approximately 30°C, and they require high relative humidity. This temperature corresponds

to the vegetation period, which in Sweden begins in May and continues for eight to nine months in southern and seven months in central Sweden [45]. *Ixodes ricinus* is found in south-central Sweden and up through the Baltic Sea coast in the northern part (Figure 5). Over time, the distribution will spread northwards due to climate change.

Figure 5. Estimated range of *I. ricinus* in Sweden in the early 1990s (left map) and 2008 right map. Reprinted from Parasit Vectors, 2012. 5: p. 8, Jaenson, T.G., et al., Changes in the geographical distribution and abundance of the tick *Ixodes ricinus* during the past 30 years in Sweden. Originally published by BioMed Central.

Epidemiology

Rickettsiae are distributed all over the world (Figure 6). The distribution of rickettsiae is closely determined by the ecology of the vector. One *Rickettsia* sp. can have different vectors, and in these cases transmission is independent of the distribution of one single vector. Fleas, lice and mites are globally distributed, and therefore the rickettsiae that have these vectors, for example *R. felis, R. akari* and the TG rickettsiae, are distributed worldwide. The prevalence of *R. felis* in cat fleas from non-endemic and endemic areas in California has been estimated to 134 and 234 per 1000 fleas, respectively [46].

For SFG rickettsiae, the hard tick is the most important vector, and the distribution of SFG is closely linked to that of hard ticks. Several factors contribute to the spread of *Ixodes ricinus*, the dominant tick in Sweden. Due to climate change, the tick has spread northward in Sweden [47]. *I. ricinus* seldom walks for longer distances than 5 meters and is, therefore, dependent on host animals for dispersing longer distances [41]. The habitat and the host of *I. ricinus* also play a role in its distribution [48]. Roe deer is considered the main host for ticks in Sweden. Other large to medium-sized mammals also serves as host for ticks [47, 49]. The distribution of these animals affects the spread of tick-borne rickettsiosis.

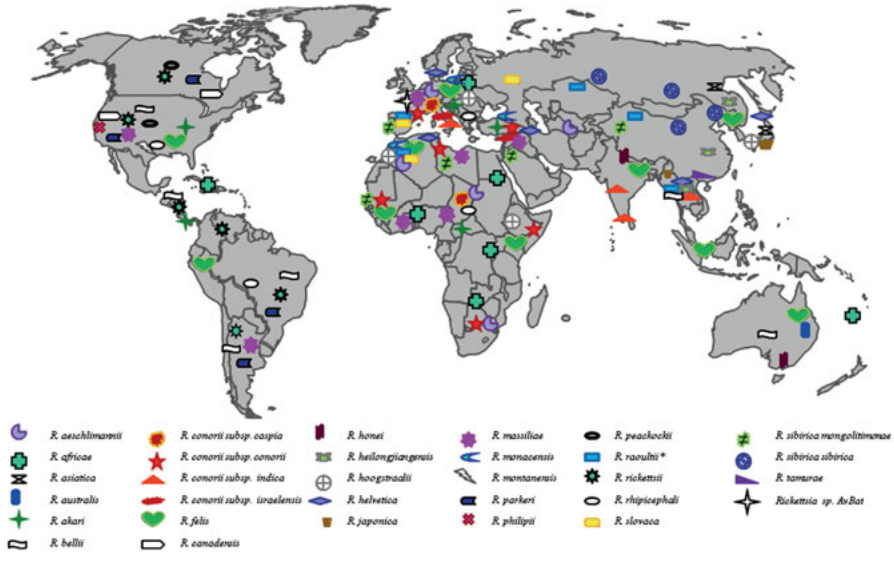

Figure 6. Geographical distribution by continent of *Rickettsia* spp. Isolated from athropods. Reprinted from Infect Genet Evol, 25C, Merhej, V., et al., Genotyping, evolution and epidemiological findings of *Rickettsia* species, p. 122-137, 2014, with permission from Elsevier.

Humans play a role in the spreading of ticks in ways other than merely being hosts. In the 18th and 19th centuries, the African tick *Amblyomma variegatum* was introduced in the West Indies by cattle imported from Senegal. As a result, *Rickettsia africae*, which causes African tick-bite fever and has *Amblyomma variegatum* as a vector, was discovered in the West Indies. *R. africae* has been demonstrated in humans as well as in cattle in Guadeloupe in the West Indies [50].

Ticks can also travel longer distances while attached to migrating birds. From ticks collected from migrating birds, rickettsiae as well as other microorganisms have been discovered [51-54]. In a study where ticks were collected from birds in Sweden, *R. helvetica* was the most common *Rickettsia* found and was present in 67% of the samples. Other rickettsiae isolated were *Rickettsia monacensis, Rickettsia japonica, Rickettsia heilongjiangensis,* and *Rickettsia spp.* strain Davousti [51]. In contrast, ticks collected from the Mediterranean area showed different *Rickettsia* spp. In a study where ticks from Capri and Antikythira were collected, *Rickettsia aeschlimannii* was the dominant species, found in 96% of the samples [52].

Until recently, *R. helvetica* was the only known *Rickettsia* affecting humans in Sweden, and thus it is the most studied *Rickettsia* sp. in Sweden. The prevalence of *Rickettsia helvetica* in ticks in Sweden has been reported to range from 1.7% to 36.8% [12, 55-57]. In Denmark, the prevalence in ticks was reported to range from 1.1 to 13.0% [58]. *Rickettsia* spp. have been found in Finland in a prevalence of 5.10% in adult ticks and 1.10% in nymphs. Of the

20 sequenced *Rickettisa* spp. 16 were identified as *R. helvetica* and three as *R. monacensis* and one sample could not be identified [59]. Prevalence studies from other parts of Europe have shown different figures. The prevalence of *R. helvetica* in ticks from Poland was reported at 5.5% [60]. From France and Italy, the prevalence has been reported at 1.4-6% and 13.1%, respectively [61-63]. In Germany and the Netherlands higher prevalence figures have been found. In a study where ticks were collected from the city of Hannover, the prevalence was 48.6%, and in a study where ticks were collected from the Hamburg area, the prevalence was 52.5% [64, 65]. In the latter study, there were prevalence differences between ticks collected in the summer compared to other seasons. The prevalence in April was 36.5%, in May 29.5% and in June between 55.0% and 64.5%. In the Netherlands, a prevalence of *R. helvetica* in ticks of 66% was reported [66]. More recently in a study where ticks were collected from different countries in Europe, the figures reported for prevalence in ticks were 14.3% in France, 10.4-14.3% in Denmark and 4.5%-11.9% in the Netherlands [67]. In these studies, *R. helvetica* is the most prevalent *Rickettsia* sp. found in *I. ricinus* ticks.

The distribution area of *R. helvetica* is from Europe to Southeast Asia, Japan and North Africa [68-75]. Thus far, *R. helvetica* has only been isolated from *I. ricinus* in Sweden, but from *Ixodes ovatus, Ixodes persulcatus*, *Ixodes monospinosus, Dermacentor taiwanensis, Haemaphysalis flava* and *Haemaphysalis japonica* in Japan, from *Dermacentor reticulatus* in Croatia and from *Dermacentor marginatus* in Algeria [72, 75, 76]. The ability of *R. helvetica* to use different ticks as vectors explains findings on the bacteria in different parts of the world, as opposed to rickettsiae which are dependent on a single tick sp.

Serological studies in humans have shown a seroprevalence of 9.2% for *R. helvetica* among forest workers in Alsace, France; among forest workers in Italy, 3.9% were seropositive for *Rickettsia conorii* and *R. helvetica* as a single or dual infection [77, 78]. In Denmark, 12.5% of patients seropositive for *Borrelia* were also seropositive for *R. helvetica* [79]. In Tyrol, Austria, 7.7% of blood donors were seropositive for *R. helvetica* [80]. A study of recruits in Sweden showed a four-fold increase in antibody titre in 22.9% (8/35) and an additional twofold increase in antibody titre in 5.7% (2/35) of the recruits. The recruits were followed for six months of their military service [81].

Pathogenesis and pathophysiology

Rickettsiae are transmitted from arthropods to humans. Tick- and mite-borne rickettsiae are transmitted from the salivary glands of the arthropods while flea- and lice-borne rickettsiae are transmitted by faeces. It is believed that the infected faeces are autoinoculated by scrubbing the pruritic bite site [82]. After inoculation, rickettsiae enter the small to medium-sized blood vessels and multiply in the endothelium. They can proliferate locally and cause an eschar

or tache noire and spread via the lymphatic vessels and bloodstream throughout the body [83, 84]. Examinations of biopsies from tache noire have shown vascular injury, intravascular and intramural infiltrations of lymphocytes and mononuclear cells and cutaneous necrosis [85]. The maculopapular rash is believed to result from increased vascular permeability and perivascular oedema [83]. Rickettsial infections can disseminate to every organ of the body. Except for endothelial cells, the underlying smooth muscle cells and perivascular cells such as monocytes and macrophages can also be infected [86]. The outer surface membranes, *OmpA* and *OmpB*, play an important role in the adhesion and invasion of host cells. *OmpA* adherence to the endothelium is dependent on the interaction with α2β1 integrins on the host cell [87]. This interaction mediates invasion of the host cells. It has been demonstrated that *OmpB* binds specifically to the protein Ku70, which is a DNA-dependent protein kinase located in the cytoplasm and on the plasma membrane as a receptor. The *OmpB*-Ku70 interaction mediates rickettsial invasion into the host cell. After induced phagocytosis, the phagosome lysis and the SFG rickettsiae escape the phagosomes and multiply in the cytoplasm and nucleus. Rickettsiae then spread to adjacent cells by actin polymerization (Figure 7) [88].

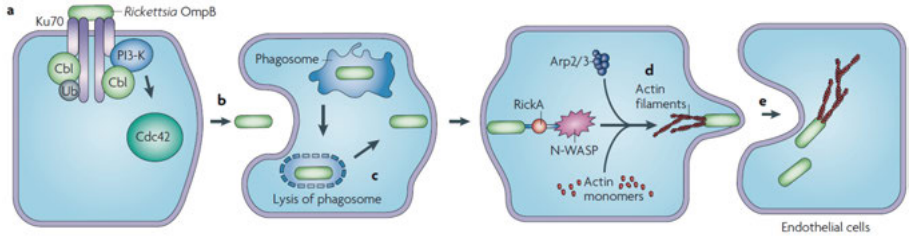

Figure 7. Host cell interactions of rickettsiae showing attachment to the Ku70 receptor by *OmpB*, phagocytosis, lysis of phagosome and movement by actin polymerization.
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The growth of rickettsiae in the endothelium stimulates oxidative stress, involving production of superoxide anions (O_2^-), hydrogen peroxide (H_2O_2) and hydroxyl radical (OH^-), causing endothelial cell damage, and thus inducing increased vascular permeability, generalized vascular inflammation and release of vasoactive mediators leading to oedema, hypovolemia and hypoperfusion. Postmortem studies have shown that thromboses are uncommon [84]. The vascular injury affects nearly all organ systems, but is most evident as interstitial pneumonia, vasculitis of the lungs, meningoencephalitis, granulomatous inflammation in the liver and perivasculitis in the kidneys and testes [19, 86, 89].

Immunological response

In the early stage of infection, natural killer cells (NK cells) inhibit growth of rickettsiae. Cytotoxic CD 8 T cells eliminate infected endothelial cells by apoptosis. Both CD 4 and CD 8 T cells contribute to protective immunity. Macrophages and lymphocytes are involved in the clearance of rickettsia from the infection foci. Antibodies (to *OmpA* and *OmpB*) are protective against re-infection. They appear after recovery from the infection and do not play a role in the acute defence mechanism against infection.

Host factors that play a role in the severity of the disease include old age, glucose-6-dehydrogenase deficiency, diabetes mellitus and male sex [90, 91].

Clinical manifestations

Typhus group

Epidemic typhus

Epidemic typhus or louse-borne typhus is caused by *R. prowazekii*. The incubation time is 10 to 14 days. The majority of patients develop fever, headache, chills, muscle tenderness, arthralgia and anorexia. Less common symptoms are nonproductive cough, dizziness, photophobia, nausea, abdominal pain, tinnitus and constipation [92]. A maculopapular or petechial rash may appear in 20-40% of cases. Laboratory findings show thrombocytopenia, elevated transaminase levels, hyperbilirubinemia and high urea concentrations. In the pre-antibiotic era, the mortality was as high as 60%, but with antibiotic treatment is currently about 4% [5].

Murine typhus

The microorganism causing murine typhus is *R. typhi.* The incubation time is 7-14 days. The most common symptoms include fever, which can last for three to six days, headache, myalgia and arthralgia. Development of rash ranges from 20-80%, and rash lasts for one to four days. Other symptoms include pulmonary infiltration, hepatosplenomegaly, nausea, abdominal pain, diarrhoea and central nervous system involvement. Laboratory findings include thrombocytopenia, elevated transaminase levels, hyperbilirubinemia, elevated erythrocyte sedimentation rate, elevated CRP levels and hypoalbuminemia. The mortality rate was 4% prior to antibiotic use and has decreased to 1% with use of proper antibiotics [93-95].

Spotted fever group

Rickettsial diseases from SFG constitute an expanding field in which new rickettsiae are being discovered and knowledge of clinical manifestations is improving. Clinical manifestations of the most studied, severe diseases and the rickettsiae discovered in Sweden thus far are described below. Many clin-

ical features are the same in different SFG diseases, though rash is more frequent with some spp. than with others. The exanthema with rickettsialpox, caused by the mite-borne bacteria *R. akari*, is slightly different because the maculopapular lesion develops a vesicle on the second to third day [14]. Other rickettsiosis associated with a vesicular rash include *R. africae* and *R. australis* [96]. Moreover, laboratory findings are similar in infections with different SFG. Most of the knowledge has been collected from Rocky Mountain spotted fever (RMSF) and Mediterranean spotted fever (MSF).

Rocky Mountain spotted fever (RMSF)

RMSF is caused by *R. rickettsii*. It is the most severe of the SFG rickettsial infections and had a case-fatality rate of 66% from 1873 to 1920 in the US [97]. The incubation time for RMSF is 2-14 days (mean seven days). Approximately 60% recall having had a tick bite. Initially, the symptoms include high fever, headache, malaise, myalgia, nausea, vomiting, anorexia, abdominal pain, diarrhoea and photophobia. At this stage of the disease, it is often misdiagnosed as a viral disease. The classical rash usually appears after three days of fever. It first appears as small papules on the wrists and ankles. Then it spreads to the arms, legs and trunk. The rash evolves into a maculopapular rash with central petechiae by the end of the first week. The classical triad of rash, headache and fever appears in 60-70% during the two weeks after a tick bite. About 10% of the patients do not develop a rash [98]. Lack of rash is most common in fatal cases, older patients and Afro-Americans. Eschar is uncommon in RMSF. Other symptoms include neurological manifestations, mucosal ulcers, jaundice, ocular manifestations and pneumonia. Severe cases often develop acute renal failure [82, 99]. The life-threatening symptoms are due to the microvascular damage and increased vascular permeability, which result in oedema, localized haemorrhage and hypoperfusion of several organ systems. Laboratory tests show thrombocytopenia, normal white blood cell count or leukopenia and hypoalbuminemia [100, 101]. Between 1999-2007, mortality in the US was 0.5% (40 of 7,738 cases of RMSF) [102]. These data are in accordance with later data from the US between 2008-2012, when the case fatality rate was 0.4% (36 of 10,356) [103]. The risk of death was higher among females, immunosuppressed, people of Hispanic ethnicity, American Indians/Alaska natives and Asian/Pacific Islanders.

Mediterranean spotted fever (MSF)

MSF is caused by *R. conorii*. The incubation time is 1-15 days (mean six days) [82, 104]. The onset is often abrupt with high fever, flue-like symptoms (headache, chills and arthromyalgias) and a black eschar (tache noire) at the tick-bite site (Figure 8). After one to seven days (mean four days), a generalized rash involving the palms and soles appears, usually sparing the face. In most cases, the patient recovers in 10 days without sequelae. Laboratory tests show

anaemia in 33%, leukopenia in 12-20%, leukocytosis in 11-28%, thrombocytopenia in 12.5%, and hyponatremia in 23%. C-reactive protein is often elevated, and elevation of transaminases is often seen [105]. Severe forms can occur with neurological symptoms and multiorgan involvement in 5-6% of cases. The mortality rate is about 2.5% [14, 82].

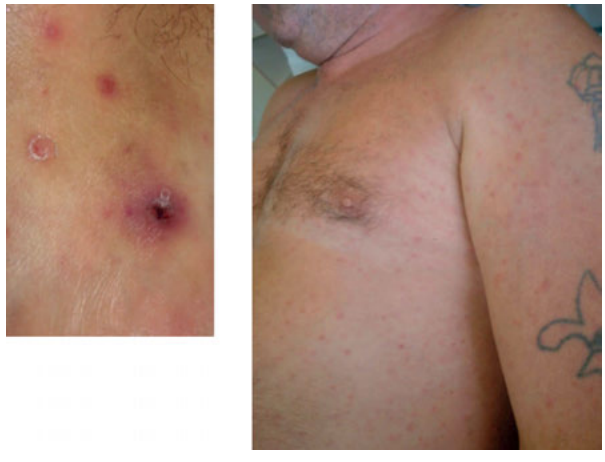

Figure 8. Inoculation eschar (left) and maculopapular rash (right) on patient with Mediterranean spotted fever.
Reprinted from Clinical Microbiology Reviews, 2005. 18(4): p. 719-56; Parola, P., C.D. Paddock, and D. Raoult, Tick-borne rickettsioses around the world: emerging diseases challenging old concepts. With permission from American Society for Microbiology.

Infection caused by Rickettsia helvetica

R. helvetica was isolated from *I. ricinus* ticks for the first time in Switzerland in 1979 [106]. It was discovered that *R. helvetica* was distinct from other rickettsiae belonging to SFG. From the outset, the newly discovered *Rickettsia* was called the "Swiss agent", and it was not until 1993 that it was confirmed to be a new sp. and named *R. helvetica* [107]. It was detected for the first time in Sweden in 1997 and was for many years the only known *Rickettsia* sp. causing infection in humans in Sweden [12]. It was long considered a non-pathogenic bacteria, but in 1999 two patients with fatal perimyocarditis in Sweden were described [108]. By using Polymerase chain reaction (PCR) and DNA sequencing, *R. helvetica* was detected in the patients' heart. One of the patients also showed a significant seroresponse in immunofluorescence to the *R. helvetica* and *R. rickettsii* that were used as antigens. Transmission electron microscopy (TEM) and immunohistochemistry examination further strengthened the diagnosis. From another study in Sweden, three patients with fever, myalgia and eschar were diagnosed with *R. helvetica* between 1999 and 2001. The diagnosis was confirmed by serological tests, microscopic visualization of the rickettsial organisms and by electron microscopy of biopsy samples

[81]. In 2000, a patient from eastern France with *R. helvetica* infection was described with prolonged fever, fatigue, myalgia and headache [77]. Laboratory tests showed elevated C-reactive protein (48 mg/L), increased fibrogen level (6.0 g/L), high erythrocyte sedimentation rate (34 mm) and elevated aminotransferases. The patient seroconverted after infection debut, and the diagnosis was confirmed with Western blot. After two weeks, the patient recovered spontaneously. In 2004, eight patients with aneruptive fever diagnosed as *R. helvetica* infection were described [69]. Of the patients, two were French, three Italian and three Thai. All patients presented mild, aneruptive fever and headache. Six patients developed myalgia, five arthralgia, two conjunctivitis and one an inoculation eschar. None of the patients developed a rash. Laboratory tests showed elevated liver enzymes and thrombocytopenia in 75% of cases. Two of the patients were treated with doxycycline and one with cefotaxime. All patients recovered without sequelae. Patients were diagnosed with microimmunofluorescence and confirmed with Western blot. Five cases with possible *R. helvetica* infection from the Thai-Myanmar border have been described. All patients had headache, chills and myalgia. Two patients had nausea and vomiting, two patients coughing and one splenomegaly. Thrombocytopenia was present in two patients and elevated liver enzymes in three. Infection was documented by MIF and Western blot. In Laos, eight patients were reported with serologic evidence of acute *R. helvetica* infection, six presented headache, four vomiting, one diarrhoea, two cough, two dyspnoea, seven myalgia and four had a palpable liver. Two patients showed rash. In two patients, the level of aminotransferases was three times over the upper limit [70]. In a patient with meningitis in Sweden, *R. helvetica* was detected in the cerebrospinal fluid using PCR [109]. The patient had slightly elevated C-reactive protein (56-128 mg/L) and thrombocytopenia. Treatment with cefuroxime had no effect, but after changing to doxycycline 100 mg twice daily the fever disappeared within two to three days. At follow-up one year later, the patient had recovered but had been asthenic for several months. Furthermore a previously healthy man was diagnosed with septicaemia caused by *R. helvetica*, and this was confirmed by PCR of blood and serology [110]. The patient presented septic fever, myalgia, arthralgia, severe headache and photophobia. One to two days after onset of illness, he developed a macular rash. Laboratory tests showed elevated C-reactive protein, elevated leukocyte count and slightly increased serum AST. The patient was treated with doxycycline 200 mg daily for 14 days. The fever disappeared three to four days after treatment was started. He recovered but muscle weakness and headache remained for months. In a recent prospective study, patients with facial palsy and sudden deafness were investigated, and 8.3% (5/60) with facial palsy and 11.9% (8/67) with deafness showed confirmed serological evidence of *Rickettsia* spp. infection [111]. An additional seven patients showed evidence of recent or current infection. Three of the patients (one with facial palsy and three with sudden deafness) were positive in liquor for *R. felis*. Because *R. helvetica* is

the most common SFG *Rickettsia* in Sweden, the antibody response could reflect *R. helvetica* infection in some of the cases. To further strengthen this assumption, *R. helvetica* has been isolated in liquor from a patient with meningitis [109].

Based on the studies performed thus far, *R. helvetica* infection seems in most cases to be a self-limited infection with fever, myalgia, headache and in some cases with a rash and eschar, but can also be a severe disease with septicaemia and neurological symptoms as well as perimyocarditis.

Infection caused by Rickettsia felis

This infection is also called California flea rickettsiosis, because it was first detected in the Los Angeles area in California where Adams et al. described the bacteria in 1990 [34]. The bacteria was first called ELB agent, named after El Labs, Soquel, California, which was one of the labs from which cat fleas containing *R. felis* were obtained. It was designated as a new species in 1996 [112]. Because the main vector for *R. felis* is the cat flea (*Ctenocephalides felis*), it has the same distribution area as the vector, i.e. worldwide. *R. felis* (then still called ELB agent) was first detected from a human in Texas in 1991, suggesting that the bacteria might be a human pathogen [113]. In 2000, three patients form Yucatán, Mexico, with fever, exanthema, headache and central nervous system involvement were diagnosed as having *R. felis* infection with PCR and seroconversion to rickettsial antigens [114]. Since then, patients with *R. felis* infections have been reported from France, Germany, Spain, Brazil, Asia and Africa. The symptoms include fever, rash, eschar, neurological signs, digestive symptoms and pneumonia [115]. Apart from these very mild cases, two patients from Mexico with severe infection due to hepatitis were described in 2009 [116]. From Sweden, three cases with neurological symptoms (one with facial palsy and three with sudden deafness) have been confirmed with PCR from liquor [111].

Prevention

No vaccines are available for rickettsial diseases, though the possibility to develop a vaccine against another *Rickettsiaceae, Orientia tsutsugamushi,* has not been ruled out [117]. To avoid tick-borne rickettsial diseases, the main strategy is to avoid tick bite and remove attached ticks. Wearing protective clothing and staying away from tick-infested habitats are ways to reduce exposure to rickettsial diseases. Lemon eucalyptus oil with the active ingredient *trans*-p-methane-3,8-diol (PMD) seems to be an effective repellent with a long-lasting effect [118]. DEET (*N,N*-diethyl-*m*-toluamide) has a shorter effect up to two to five hours. PMD seems to be a better choice than DEET as a repellent, though the Centers for Disease Control and Prevention (CDC) recommend DEET [119]. Permethrin is effective to prevent tick bites and can be used to treat clothing, but should not be applied to the skin [120]. An attached

tick should be removed using a fine-tipped forceps or tweezers, grasping the tick close to the skin and removing it without twisting [121].

Treatment

The drug of choice for treatment of all rickettsiae is doxycycline. It is also recommended for treatment of children and pregnant women, despite risk for side effects, though shorter courses are recommended. In vitro studies show effects of chloramphenicol, rifampicin and some fluoroquinolones. There are few in vivo studies of these drugs, so doxycycline is still the first-line treatment [122]. Because rickettsiae are intracellular bacteria, they are resistant to penicillins, cephalosporins, aminoglycocides and trimethoprim-sulfametoxazole, all of which have limited intracellular activity. Furthermore, erythromycin is resistant. For treatment of RMSF, CDC recommends doxycycline as first-line treatment at a dose of 100 mg twice daily for adults and 2.2 mg/kg twice daily for children [119]. The treatment should continue for at least three days after fever subsides, usually a minimum period of five to seven days. Fever usually disappears after four to five days of doxycycline therapy. Chloramphenicol is an alternative drug and is recommended for pregnant women in the third trimester to avoid grey baby syndrome. For MSF, doxycycline is also the recommended treatment. The dose is either 200 mg twice daily for one day or 200 mg daily for two to five days (or one day after fever has subsided) [123]. One-day treatment was shown to be as effective as several-day treatment in a randomized trial [124]. Josamycin (a macrolide) is an alternative and is recommended for treatment of pregnant women. The dose is 1 g every eight hours for five days. Although fluoroquinolones have an in vitro effect against MSF, in vivo studies have shown a poor or fatal outcome due to a toxin-antitoxin effect [125]. Fluoroquinolones are therefore not recommended for treatment of MSF. For other SFG rickettsiosis, doxycycline is recommended at a dose of 200 mg as a single dose daily or 100 mg twice daily for two to five days or until three days after apyrexia [122]. An in vitro study of *R. felis* showed the same induced cell apoptosis with chloramphenicol as observed with fluoroquinolone treatment of MSF [123].

Laboratory diagnostics

Serology

Immunofluorescence assay (IFA)

Immunofluorescence assay (IFA) is considered the gold standard of serology testing for rickettsial diseases and is commercially available. It detects both IgM and IgG antibodies. Microimmunofluorescence (MIF) is often used for detecting rickettsial antibodies. The advantage of this method is that it can detect antibodies to several antigens with the same drop of serum in a single

well containing multiple antigen dots [126]. The sensitivity of IFA after 14 days is estimated at 94-100% and is increased if paired sera are tested. The specificity has been estimated at 99.8-100%. For MSF, the sensitivity ranges from 46% (when serum is collected on day five to nine) to 100% (when serum is collected on day 29) [41]. Antibodies are usually detected from day 7-15 after onset of disease. For *R. africae,* antibodies are detected somewhat later from day 28 and 25 for IgG and IgM antibodies, respectively. Treatment with doxocycline within seven days after debut of disease has been shown to prevent the development of antibodies to *R. africae,* and this is probably the case for other *Rickettsia* spp. [127]. Cross-reactivity is a problem for interpreting the data and seems to be typically group specific rather than species specific, although cross-reactivity occurs between TG and SFG as well as other bacteria such as *Legionella* and *Proteus* [126]. In a study from Germany, differentiation of SFG rickettsiae when using MIF was enabled in 70.4% of cases in sera from dogs. The criterion for differentiating different *Rickettsia* spp. was at least a twofold higher titre against one *Rickettsia* sp. than the others. In the German study, antibodies to *R. helvetica, R. raoulti, R. slovatica, R. monacensis* and *R. felis* were examined [128]. False positive IgM antibodies can be seen with rheumatoid factor and in viral and parasitic infection generating unspecific lymphocyte B proliferation (cytomegalovirus, Epstein-Barr virus and malaria) [129]. IgM antibodies wane after three to four months, while IgG antibodies persists for at least eight months [130]. To confirm acute infection, a four-fold or greater increase in antibody titre should be demonstrated in paired sera taken after a two- to four-week interval, according to CDC [119]. The rickettsia laboratory in Marseille (Unité des Rickettsies) suggests IgG titres $\geq$ 128 and/or IgM titres $\geq$ 64 as indicative of infection [127].

Enzyme-linked immunosorbent assay (ELISA)

ELISA was first introduced for detection of antibodies to *R. prowazekii* and *R. typhi*. It is now used for diagnosing RMSF. It seems to be as sensitive as IFA is in detecting antibodies to RMSF and more sensitive in detecting low levels of antibodies [130]. Available ELISA tests are qualitative and cannot effectively monitor an increase or decrease in antibody titres [119].

Western blot (WB)

Western blot is more sensitive and specific than IFA is in detecting early antibodies [131]. Western blot also allows differentiation of SFG species if acute-phase sera are used. Early in the infection, homologous reactions dominate, which makes specific diagnose possible. The test is aimed at two types of antigens: a lipopolysaccharide and the outer membrane proteins *OmpA* and *OmpB*. Because these proteins are highly species specific, the test allows for differentiation of species [14].

Lateral flow immunochromatography assay (LFIA)

LFIA is not a standard procedure in detecting antibodies to rickettsiae. The test has been used to diagnose infections from different organisms. The advantages are that it is easy to use and fast. Antibodies can be visualized within minutes. Labels are usually made of colloidal gold, latex and carbon. It is a qualitative test or semiquantitative test used for detecting antibodies [132].

Polymerase chain reaction (PCR)

PCR can be performed to detect rickettsiae as well in ticks, fleas and lice as in human tissues [14]. The test is not suitable to detecting rickettsiae in blood samples, as low numbers of rickettsiae circulate in the blood, although rickettsiae have been demonstrated in blood [110, 119]. It is more suitable to detect rickettsiae in skin biopsies and especially in eschars, because an eschar contains a high number of bacteria. *Rickettsia* spp. have been detected using PCR from skin biopsies as well as lesion swabs from eschars [133, 134]. In an animal model skin biopsies taken away from the site of tick emplacement and without eschars, could provide molecular diagnose of up to 60-70% [135]. Other bodily fluids in which rickettsia have been demonstrated include cerebrospinal fluid [109, 111]. Sensitivity up to 68% and specificity up to 100% have been described [82]. Doxycycline treatment can decrease the sensitivity [119]. Primers targeting the genes encoding for 16 sRNA (*rrs*), citrate synthase (*gltA*), 17-kDa protein, *OmpA* and *OmpB* are typically used [14]. Several PCR techniques are available for diagnosis of rickettsiosis. Nested PCR increases the sensitivity but contamination is a risk. Real-time PCR (RT-PCR) offers low risk for contamination and possibility of quantitation. In a study from CDC, RT-PCR was found to be the most sensitive PCR method in detecting rickettsial DNA, followed by semi-nested PCR and conventional PCR [136]. For further differentiation, sequencing can be carried out.

Isolation

Because rickettsiae are obligate intracellular organisms, common bacterial culture procedures are not applicable. The culture has to be done on cell lines or embryonic eggs. For this purpose, Vero, L929, HEL, XTC-2 or MRC5 cells can be used [82]. SFG rickettsiae grow at a temperature of 32 °C [27]. Culturing rickettsiae is a time-consuming and labour-intensive technique that takes several weeks. For this reason, other techniques are preferable, such as PCR for diagnostic purposes. Several rickettsiae require Biosafety Level-3 for cultivation [119].

Immunohistochemistry (IHC)

This method can be done to stain formalin-fixed and paraffin-embedded biopsies or autopsy tissues. The Gimenez method or Giemsa orange can be used

for staining [82]. Using this method, infection can be detected prior to seroconversion. A specificity of 100% and sensitivity between 53% and 75% have been reported [126]. PCR is currently used more often for detection of rickettsiae in tissues and vectors.

Transmission electron microscopy (TEM)

TEM has been used in the past to describe new *Rickettsia* in new locations [12, 34]. At present, other methods are used to detect rickettsiae in tissues and vectors. TEM is useful for morphology studies and to enhance our knowledge of the ultrastructure of the bacteria [137].

Aim

The general aim was to investigate the prevalence of spotted fever rickettsiosis in Sweden and its correlation with various symptoms, both those that are expected and those that are not normally associated with rickettsiosis. An additional aim was to find rickettsioses not previously described in humans in Sweden and to examine the extent to which co-infections with other tick-borne agents occur.

Specific aims

- To examine the seroprevalence of *Rickettsia* spp. antibodies in a subgroup exposed to tick bites compared with the seroprevalence in blood donors **(Paper I)**.
- To examine the seroprevalence and seroconversion of spotted fever group *Rickettsia* spp. among habitants in an endemic area and the occurrence of co-infections with other tick-borne microorganisms **(Paper II)**.
- To examine seroconversion for *Rickettsia* spp. after a tick bite together with reported symptoms and prevalence of co-infections in a population exposed to *Borrelia* spp. **(Paper III)**.
- To investigate whether species of rickettsiae other than *Rickettsia helvetica* are present in Sweden and can cause disease in humans **(Paper IV)**.

Material and methods

Materials and subjects

In **Paper I**, 236 Swedish patients seeking medical attention for various symptoms after a previous tick bite were analysed for the presence of rickettsial antibodies. Most of the patients were exposed and had been bitten during the past month prior to collection of serum samples, but with a range from three weeks to two years before sampling. The samples were collected between 2002 and 2006 from the central part of Sweden and submitted to the Department of Microbiology, Falu County hospital. Samples were stored at -20°C. Of the 236 patients, 137 were positive for *Borrelia burgdorferi* antibodies, thus giving a group with confirmed tick bites.

In **Paper II**, two study groups were examined. In Study 1, sera were obtained from 206 patients seeking medical care from May to December 2001 for flu-like symptoms or erythema migrans (EM) after suspected or observed tick bite in southeastern Sweden. Sera were sampled on enrolment day, six to eight weeks later and a third sample six months after enrolment. The last sera were not used in this study. From the samples taken six to eight weeks after enrolment, all 206 were screened for IgG antibodies to *Rickettsia* spp. Samples with a titre ≥ 1:64 were retested for IgM. The corresponding sera from the enrolment day were then examined for antibodies to *Rickettsia* spp. Data on symptoms and laboratory tests were taken from medical records of the initial examination and follow-up interviews six to eight weeks after enrolment. Prior to this study, sera had been analysed for antibodies to *Borrelia* spp., *Anaplasma* spp. and TBE [138]. In Study 2, 112 patients who, regardless of indication, had submitted samples for analysis of LB and 47 patients analysed for *Mycoplasma pneumoniae* at Uppsala University Hospital during the period March–April 2012 were also examined for the presence of rickettsial antibodies in the same manner as in Study 1.

In **Paper III**, 218 participants from the TBD STING study, a prospective study following recently tick-bitten individuals for three months, were enrolled. The participants were included from May 2008 to September 2009. Through the local public media, persons ≥ 18 years with a recent tick bite were asked to bring the tick after detachment to their Primary Health Care (PHC) Centres. Ticks and blood samples were collected and the participants were asked to complete a questionnaire. A follow-up visit was made after three months, at which time a new questionnaire was completed and new blood

samples taken in order to obtain paired sera, and ticks from further tick bites were collected. The PHC centres were located in south-central Sweden, represented by Västra Götaland (Lidköping); Östergötland (Söderköping; Kisa; Vikbolandet) Jönköping County (Bankeryd), southernmost Sweden (Kalmar) and the Åland Islands in Finland. Prior to this study, the blood samples were examined for *Borrelia* spp. Samples were transported to the University Hospital in Linköping and frozen at -70°C within three days for later analyses. In the first questionnaire, participants were asked about number of tick bites, geographical location where the tick bites occurred and medical history of tick-borne diseases. The second questionnaire included questions about new tick bites, symptoms associated with tick-borne diseases and whether the participants had sought medical care. Symptoms in the questionnaire included fatigue, headache, loss of appetite, weight loss, nausea, fever, neck pain, vertigo myalgia/arthralgia, numbness, radiating pain and cognitive difficulties. EM was determined by examining medical records and was defined as an expanding rash of at least five cm in diameter, with or without central clearing.

From one patient who sought medical care and was hospitalized in Falu County Hospital in May 2008 with symptoms of meningitis, cerebrospinal fluid (CSF) was collected and examined regarding rickettsial disease (**Paper IV**). Samples from CSF had been previously stored at -20°C and were thawed. General cultures from blood and CSF and tests for enteroviruses, tick-borne encephalitis virus (TBE) and varicella were also taken and analysed, as were antibodies in serum and CSF against *B. burgdorferi*. From another patient showing signs of meningitis and urinary tract infection, hospitalized in Uppsala University Hospital in May 2008, CSF was collected and analysed for rickettsial disease. Culture from CSF and test for herpes and enteroviruses were also taken.

Subject controls

In **Paper I**, 161 healthy blood donors were chosen to form a control group. In the second study (Study 2) in **Paper II**, the control group consisted of 80 healthy blood donors; they were tested for rickettsial antibodies.

Methods

Laboratory methods

Immunofluorescence assay (IFA)

Indirect microimmunofluorescence assay (MIF) were used to detect antibodies to *Rickettsia* spp. Antigen was prepared from Vero cell-grown isolates of *R. helvetica* from a domestically harvested *Ixodes ricinus* tick. The antigen was applied to each well of microscope slides, air-dried, fixed in acetone for 15 minutes and incubated with serial dilutions of serum. Immunoglobulin G

(IgG) antibodies were detected by fluorescein isothiocyanate-conjugated (FITC) g-chain-specific polyclonal rabbit anti-human IgG (Ref: F0202; Dako, Denmark) **(Papers I-III)**. For detection of Immunoglobulin M (IgM), Mu chain conjugated antibodies were used (Ref: F0203; Dako, Denmark) **(Paper II, III)**. The IgM antibodies were examined after a pre-treatment procedure with rheumatoid factor adsorbent (Immunkemi, Stockholm, Sweden) to remove complex bound IgG antibodies. A sample was positive if it showed green fluorescence to *Rickettsia* in a fluorescence microscope with a magnification of 1x400 equal to or above the specific cut-off. In **Paper I**, a cut-off at 1:80 was considered positive. For **Paper II**, an IgM titre < 1:64 was considered negative and ≥1:64 recent or current infection. A probable infection was defined as a four-fold increase in IgG between sera collected at enrolment and sera collected six to eight weeks later or with a titre >1:128 as the highest titre in the second sera collected. In **Paper III**, IgG/IgM titres <1:64 were considered negative. A confirmed case was defined as a four-fold rise in titre or seroconversion between the first and second sera. A probable case was defined as a single or repeatable IgG endpoint titres of ≥ 1:256. A seroreactive case was defined as IgG and/or IgM titres ≥ 1:64 and ≤ 1:128. This was considered indicative of either a past infection or early response (IgM) to infection. Persisting IgG titres with or without IgM reactivity were considered indicative of past infection. Persisting IgM antibodies alone were interpreted as non-specific cross-reactivity due to exposure to other organisms or autoimmune responses or possibly as a sign of previous exposure.

Enzyme-linked immunosorbent assay (ELISA)

Prior to our work, ELISA was used to detect antibodies to *B. burgdorferi, Anaplasma phagocytophilum* and tick-borne encephalitis virus (TBE) **(Paper II)** [138]. *B. burgdorferi* IgG and IgM were examined using a commercial enzyme-linked immunosorbent assay (ELISA), according to the manufacturer's instructions for use and interpretation (Genzyme Virotech GmbH, Rüsselsheim, Germany). Positive or equivocal samples from ELISA were further tested by Western blot (WB). Positive tests were based on seroconversion, a significant rise in IgG titre and/or new significant bands in the WB banding pattern between the paired sera. *A. phagocytophilum* were detected using a commercial kit (Focus Technologies, Cypress, CA, USA). Patients with a titre >1:80 were considered positive, and patients with seroconversion or a four-fold rise in titres in the paired sera were considered to have a laboratory confirmed infection. A probable infection was defined as a permanently high IgG antibody titre of ≥1:640 or at least a four-fold decrease in IgG antibody titre during the investigation period. Infection with TBE virus was based on a positive IgM screen on the first collected serum [Immunozym FSME (Frühsommer-Meningoenzephalitis) IgM or Progen Biotechnik GmbH, Germany] and confirmed by the rapid fluorescent focus inhibition test (RFFIT), as previously described [138]. In Study 2, sera were analysed for IgG and IgM antibodies to

Borrelia spp. using the Euroimmun's ELISA kit [Euroimmun AG (Aktiengesellschaft), Lübeck, Germany], according to the manufacturer's instructions. In **Paper III**, sera were analysed prior to our study for both IgG anti-flagellum antibodies (IDEIATM Borrelia IgG, Oxoid, Cambridgeshire, UK) and IgM/IgG anti-C6 antibodies (C6 Lyme ELISATM kit, Immunetics, Inc., Cambridge, MA), according to the manufacturer's instructions for use and interpretation [139]. Seropositive samples were defined as samples that had optical density (OD) values above the cut-off levels in each assay from either the first sample or the second sample or both. Seroconversion was defined as samples that had OD values above the cut-off levels in the second sample and negative in the first sample or a minimum of a twofold increase in OD values between the first and second samples in at least one of the two ELISA assays. The samples were further analysed using commercial immunoblot (recomLine Borrelia IgG assay, Microgen, Neuried, Germany). Seroconversion in the immunoblot assay was defined as either a change from seronegative to seropositive, or as detection of new clear/strong bands in the second sample.

Western blot (WB)

Western blot was used to verify positive IFA **(Paper I-III)**. In **Paper I**, serum from one patient's antibodies was tested against *R. helvetica* whole-cell antigen. After electrophoresis, antigen was transferred to a membrane and the blotting process run overnight and serum was incubated the day after. The result was visualized with horseradish peroxidase (HRP)-conjugated goat anti-human IgG. In **Paper II**, sera from three patients were analysed with WB. As antigen, a 1,401 bp cloned DNA fragment of the *OmpB* gene of *R. helvetica* was used. After the blotting process, serum was incubated and visualized with HRP-conjugated goat anti-human IgG. In **Paper III**, sera were diluted to titres 1:200 and tested against *R. helvetica* whole-cell antigen using Amersham WB system (GE Healthcare) with the secondary antibody Antihuman IgG DyLight™549 (Rockland Inc. cat.no 609-142-123) in the concentration of 1:10,000 in, in accordance with the manufacturer's instructions.

Lateral flow coal immunochromatographic assay (LFCIA)

To verify the presence of SFG rickettsia IgG antibodies, in **Paper IV** lateral flow coal immunochromatographic assay (LFCIA) was used. *R. helvetica* cultured in Vero cells were used as antigens. Serum from one patient was added on a pad at one end of the strip. Coal-conjugated γ-chain-specific polyclonal rabbit anti-human IgG was added. The result was read after a few minutes. Black-colouring of the spot indicated presence of serum antibodies reactive with rickettsial antigens.

Polymerase chain reaction (PCR) on human samples

Samples of CSF from two patients were analysed using a genus-specific quantitative real-time PCR (RT-PCR) with the probe (Taqman) and primers targeting the citrate synthase (*gltA*) gene **(Paper IV)**. In each reaction, 0.25 µL LC Uracil-DNA glycosylase (Roche) was included to reduce the risk of contamination [140].

For sequencing analysis, two nested PCR were performed amplifying the 17-kDa and outer membrane B (*OmpB*) fragments [141, 142]. Expected fragment sizes were confirmed using gel electrophoreses (2% agarose).

Polymerase chain reaction (PCR) on tick samples

Prior to our study, ticks were analysed for *Borrelia* spp. [139]. Ticks were homogenized and total nucleic acid was extracted, followed by reverse-transcription **(Paper III)**. A borrelia genus-specific region of the 16S rRNA gene was used to detect and quantify the borrelia cells in a Light Upon eXtensionTM (LUX) real-time PCR assay. Positive samples were species determined by conventional PCR assays, using primers targeting regions within the 5S–23S and the 16S–23S intergenic spacers, respectively. This was followed by nucleotide sequencing.

For analysing *Rickettsia* spp., a real-time PCR targeting the citrate synthase (*gltA*) was used as previously described **(Paper III)** [52, 57]. Two to five µl cDNA was used as a template in each reaction, together with 0.25 µl LC Uracil-DNA glycosylase (UNG) (Roche Diagnostics, Mannheim, Germany) to minimize the risk of contamination. The reactions were run in a Rotor-Gene 3000 (Qiagen, Sydney, Australia) using LightCycler® TaqMan® Master (Roche Diagnostics, Mannheim, Germany).

DNA sequencing

In **Paper III**, cDNA samples from the ticks that were positive in real-time PCR were further amplified for analysis of a fragment of the genes coding for *ompB*, 17-kDa or by a semi-nested PCR targeting the *gltA* gene. PCR products considered for sequencing were cleaned using Exonuclease I and FastAPTM Thermosensitive Alkaline Phosphatase (Fermentas GmbH). Sequencing analysis of PCR products was performed at Macrogen Inc. (Macrogen Europe, Amsterdam, Netherlands). DNA Baser version 2.80.0 (HeracleSoftware, Lilienthal, Germany) and BioEdit Sequence Alignment Editor Version 7.0.5.3 (Ibis Therapeutics, Carlsbad, CA) were used for sequence alignments. For species identification, sequences were examined using the Basic Local Alignment Search Tool (BLAST).

In **Paper IV**, PCR products from the nested PCR were analysed by a cycle sequencing analysis of both strands using an automatic Hitachi 3100 Avant Plus Genetic Analyser (Applied Biosystems, Tokyo, Japan); this was done at the Centre for Genomics and Bioinformatics, KI, Stockholm (KI Seq). For one

patient it was not possible to amplify the 17 kDa gene, probably because the amount of DNA was near the detection limit and the extracted sample was limited.

Laboratory controls

For IFA as a positive control a serum from a patient with proven Mediterranean spotted fever, with an endpoint IgG titre of 1:160 confirmed at the Swedish Institute for Infectious Disease Control, was used **(Paper I, II)**. In **Paper III**, serum with proven end-point titres of 1:256 and 1:128 of IgG/IgM, respectively, was used as a positive control. Serum from a blood donor with no history of tick bite was used as a negative control **(Paper I-III)**.

For Western blot, a serum from rabbit immunized with purified *R. helvetica* was used as the positive control and the secondary antibody alone served as the negative control **(Paper I-II)**. In **Paper III** a serum from a patient with a proven rickettsial infection served as the positive control. The secondary antibody alone served as the negative control together with serum sample from a healthy blood donor.

In **Paper III**, sterile water was used as a negative control in the PCR analysis of the ticks. As a positive control, a standard plasmid constructed by cloning the PCR product into a PCR 4-TOPO vector (TOPO® TA Cloning® kit for Sequencing, Invitrogen, Carlsbad, CA, USA), and containing the cloned 74 bp fragment of the *gltA* gene included in 10-fold serial dilutions, was used.

For RT-PCR as a standard, the 74-base pair (bp) fragment of *R. helvetica*, amplified in the real-time PCR reaction was used **(Paper IV)**. Ten-fold serial dilutions of extracted plasmids were used to establish standard curves for the PCR runs. The quantification was linear over a range of 10 to 109 copies, and the detection limit was shown to be 1–10 copies per reaction. For the nested PCR, purified DNA of *R. helvetica* was used as a positive control.

Tick feeding duration

To calculate the tick feeding duration, the scutum index (the ratio of the width of the scutum to the length of the idiosoma) or the coxal index (the ratio of the width of the scutum to the distance between the basal coxae of the fourth pair of legs) was used. From these indices feeding duration was calculated using the equations described by Gray et al. [143]. This was done prior to our study [139].

Statistical methods

The difference between the tick-exposed group and the control group in **Paper I** was calculated using the Pearson´s 2-sided χ^2 test. In **Paper III**, the Fischer's exact test was used to calculate differences in symptoms between the rickettsia

seropositive groups and the seronegative group and differences in seroreactivity between the two geographical areas: southernmost and south-central Sweden and the Åland Islands in Finland. A p-value ≤ 0.05 (two-tailed) was considered statistically significant.

Ethics

Approvals were obtained from the Regional Ethics Committees in Lund (Reg. no. 218-01), Linköping (Reg. no. 546-03, Reg. no. 133-01, Reg. no. 132-06), Uppsala (Reg. no. 2007/085, Reg. no. 2013/420) and the Ethics Committee of the Åland Health Care (2008-05-23).

Results and discussion

Seroprevalence and incidence in humans

In **Paper I**, of the 236 tick-exposed persons 137 tested positive for *B. burgdorferi*. Antibodies to *R. helvetica* were detected using microimmunofluorescence (MIF) assay in nine of the 236 (3.8%) sera from the tick-exposed group when a titre of 1/80 was used as the cut-off. Six of 137 (4.4%) were positive in the *Borrelia* group and three of 99 (3.0%) were positive in the *Borrelia*-negative but tick-exposed group. One of 161 (0.6%) was positive in the control group consisting of blood donors. The findings indicate that *R. helvetica* is present in Sweden and confirm that tick-exposed persons become infected with *R. helvetica* to a greater extent than non-exposed persons do. The difference between the tick-exposed groups and the control group was statistically significant (χ^2=3.97, df=1, p=0.046). Cross-reactions do occur when using serological tests, but *R. helvetica* is the only known tick-borne *Rickettsia* affecting humans in Sweden, which is why seroconversion should reflect antibodies to *R. helvetica*.

In this study, serum from one patient was verified with antibody-specific response to the high-molecular protein antigens in the 110-150 kDa region, but no antibodies to lipopolysaccharide were found, indicating lower fluorescence signal in the IFA.

In **Paper II**, of the 206 patients in Study 1, 20 of 206 (9.7%) had IgG and/or IgM antibodies equal to or higher than the cut-off value of 1:64. Seven (3.4%) of the patients showed seroconversion or significant rise in titre, indicating recent or current infection; 13 patients had titres compatible with past infection, of which five cases were judged as probable infection. In the second study (Study 2), 16 of 159 (10.1%) patients primarily sampled for *Borrelia* or *Mycoplasma pneumoniae* were seroreactive for *Rickettsia* spp. Eleven patients were primarily tested for *Borrelia* spp., and five were primarily tested for *M. pneumonia*. In the control group of 80 healthy blood donors, one patient was interpreted as having previous exposure to *Rickettsia* spp., showing IgG 1:64 and IgM 1:64-1:128. Three of the patients had only IgM between 1:64-1:128 and were non-reactive for IgG, probably as a result of non-specific reactivity or previous exposure.

Out of the 218 participants in **Paper III**, 96 (44.0%) had IgG equal to or higher than the cut-off titre of 1:64. Forty (18.3%) seroconverted with a titre of at least 1:128 or showed a four-fold increase in IgG titre (Group 1). Four

(1.8%) showed a single titre equal to or above 1:256 (Group 2). Fifty-two (23.8%) had an IgG titre between 1:64 and 1:128 (Group 3), and 122 (56.0%) were seronegative (Group 4). In summary, 44 (20.2%) patients showed either a seroconversion with a four-fold increase in titre or a titre ≥ 1:256, indicating a recent or current infection. There were no differences in serological findings between the Swedish areas compared to the Åland Islands.

Paper I, **Paper II** and **Paper III** show that the seroprevalence for *Rickettsia* spp. differs in different areas of Sweden. The sera from patients in **Paper I** were collected from an area in Sweden with low prevalence of *Borrelia*. The investigated population in **Paper II** was from an area with higher *Borrelia* prevalence and the prevalence of *Rickettsia* spp. antibodies was, as anticipated, correspondingly higher. In **Paper III**, even more of the surveyed population showed seroreactivity and seroconversion against *Rickettsia* spp., indicating that these areas are high endemic areas for *Rickettsia* spp. The result is also supported by a previous study of recruits from the eastern coastal area of Sweden showing a seroconversion rate of 22.9% [81]. In this area of Sweden, the seroprevalence of *Borrelia* is among the highest. We could conclude that patients who had been more exposed to tick bites, measured as suspected or confirmed tick bites, or patients seeking medical care for suspected *Borrelia* infection had a higher seroprevalence for *Rickettsia* spp. Our findings are consistent with those from other studies performed in Europe. A seroprevalence study from France showed antibodies to *R. helvetica* in 9.2% of forest workers, who are also presumed to be at high risk for tick bites [77]. In Denmark, 12.5% of serum samples from patients seropositive for *Borrelia* were also found to be positive to *R. helvetica* [79]. A study from the Tyrol in Austria showed that 7.7% of blood donors had IgG at a titre of 1:128 or higher against *R. helvetica*. The prevalence was higher in the north of Tyrol (10.6%) than in the south (7.4%). In the area with a high prevalence of *Borrelia* and tick-borne encephalitis, a high seroprevalence of *R. helvetica* was also seen [80]. The serological results from IFA were confirmed with Western Blot (WB) (**Paper I-III**). In **Paper I** one IFA seropositive patient was tested positive in WB, in **Paper II** three IFA seropositive patients were tested positive in WB and in **Paper III** 16 IFA seropositive patients were tested positive in WB. All negative controls were negative and all positive controls were positive.

Prevalence of *Rickettsia* sp. in ticks and its correlation with serological response in humans

Ticks in which it was possible to determine life cycle stage and these correlation to serological response are given in Table 1 **(Paper III)**. Most of the ticks were nymphs and just a few were larvae. This is in accordance with other European findings [144].

Life cycle stage	Sero-Group 1	Sero-Group 2	Sero-Group 3	Sero-Group 4	Total
Female adult	21	0	21	48	90
Nymph	91	4	117	76	288
Larvae	7	0	6	1	14
Total	119	4	144	125	392

Table 1. *Life cycle stages of ticks examined in relation to serologic groups (Group 1 = seroconversion or fourfold IgG titre increase; Group 2 = IgG titre ≥1:256; Group 3 IgG titre ≥ 1:64 to ≤ 1:128; Group 4 seronegative).*

Of the 472 ticks collected from the different locations (Lidköping 98, Jönköping 97, Östergötland 98, Kalmar 82 and Åland Islands 97), 8.3% (range 0-20.6%) were positive for *Rickettsia* spp. in real-time PCR. Twenty-three amplicons showed a sequence that completely matched (100%) the deposited sequences in Gen Bank representing *R. helvetica*. The other 16 samples had weak signals in real-time PCR and showed no product by amplification with the other PCR assays. Of the ticks with confirmed infestation with *R. helvetica,* 11/98 (11.2%) were from Lidköping, 4/98 (4.1%) from Östergötland, 7/97 (7.2%) from Jönköping, 1/82 (1.2%) from Kalmar and 0/98 (0%) from the Åland Islands. The amplicons that were sequenced all represented *R. helvetica* sequences. These findings support earlier studies showing that *R. helvetica* is the dominant tick-borne *Rickettsia* sp. in Sweden. It is known from previous studies that the prevalence of *R. helvetica* in ticks differs in different parts of Sweden, with a range from 1.7% to 20% [12, 56]. None of the ticks collected from the Åland Islands contained *Rickettsia,* although the participants showed the same percentage of seroconversion and seroreactivity as those from the other study areas. All reported that they had acquired their tick bites on the Åland Islands. The participants from that area reported more tick bites (average 2.7) than the participants from other areas did (average 1.7). One explanation could be that even though the prevalence of *Rickettsia* in ticks on the Åland Islands is lower, the population is more exposed to tick bites and therefore has the same serological response as in other areas. Similar discrepancies between prevalence of *Rickettsia* spp. in ticks and seroprevalence have been reported from Austria [80].

Five of the 16 participants (31.3%) bitten by a rickettsia-positive tick showed seroconversion. Of the 10 ticks in whom *R. helvetica* was quantified by PCR, the mean value for Group 1 (four ticks) was 12793 copies/µl reaction

and for Group 4 (13 ticks) 35847 copies/ µl. Accordingly, there was no correlation between higher number of DNA copies/µl and seroconversion. Because most of the participants in Group 1 (60%) were bitten by a *Rickettsia*-negative tick, it is plausible that the ticks that were collected were not the ones that caused the infection. It is therefore difficult to determine the risk of infection when bitten by a rickettsia-infested tick, although the risk seems low.

Blood feeding duration was calculable in 327 ticks, of which 90 belonged to Group 1, four to Group 2, 117 to Group 3 and 116 to Group 4. The median blood feeding duration was 27 hours (h) in Group 1, 32 h in Group 2, 32 h in Group 3 and 34 h in Group 4. There was no correlation between blood feeding duration and infection rate. This was in accordance with a previous report where transmission duration for *Rickettsia* was reported to be between 10 minutes and ≥10 hours [145].

Symptoms of *Rickettsia helvetica*

In **Paper II**, all but one patient with IgG or/and IgM over the cut-off titre had medical records of the time of disease. In this study, 206 patients seeking medical care with erythema migrans (EM) or flu-like symptoms after suspected or observed tick bite in southeastern Sweden were investigated (Study 1). In the corresponding group, sampled for the analysis of *Borrelia* spp. and *M. pneumonia*, symptoms were reported by physicians (Study 2). Of the 19 patients with recent, current or past infection in Study 1, where medical records were obtained, only seven patients were solely seroreactive to *Rickettsia* spp. The others were also seroreactive to *Borrelia* spp., *Anaplasma* sp. or all three agents. Because ticks can be infected with several microorganisms and thus are able to spread them to humans, this should be kept in mind when evaluating symptoms of tick-borne diseases [57, 146, 147]. Despite this, co-infection in humans have rarely been studied [148]. Of the patients in Study 1 with seroreactivity solely to *Rickettsia* spp., two had fever, four had headaches, one muscle pain, three rash, three respiratory symptoms, and six had erythema migrans (EM). Fifteen of 20 patients presented EM. Five had fever (37.5-39°C) lasting less than one week, and four of them experienced chills for up to three days. Eight had headache lasting up to seven days. Five had muscle pain. Seven had respiratory symptoms, primarily coughing (Table 1). In this study, the patients had symptoms comparable to Lyme borreliosis (LB). The symptoms were similar and gave no guidance as to the causative agent. This could be related to the selection of the study group, 174 of whom were recruited on the basis of EM and 32 for flulike symptoms in combination with a preceding tick bite. These symptoms were therefore overrepresented, compared to when the study group was selected based on other symptoms or was symptom-free when included. For this reason, it was not surprising that many patients had EM. Note that six of seven patients who seroconverted only for *Rickettsia* spp.

had EM. This might indicate that rickettsia disease was causative, that the *Borrelia* infection did not produce antibody development or that there was an interaction between the two agents. The associations of EM and rickettsial disease have previously been described, but the causative agents have not been fully investigated given that the studies depended solely on serology [149, 150]. Other symptoms are similar to other rickettsial diseases described, thus implying that the symptoms can be caused by *Rickettsia* spp. However, the number of co-infections renders the interpretation unclear. In previous studies, co-infections have not been investigated and co-infections could be more common than has been expected thus far.

All patients had normal values for haemoglobin, white blood cell count, platelet cell count, alanine and aspartate aminotransferase, and lactate dehydrogenase in both sera. Normal laboratory values can be seen in *Rickettsia* diseases, and this does not rule out a current disease.

Three of the patients were treated with doxycycline 100 mg once a day and the rest with phenoximethylpenicillin. All patients except one (no. 7) were cured at follow-up after two months. The patient not cured showed persisting skin problems. Treatment with doxycycline can inhibit antibody production [127].

Patient no.	Age/ sex	Tick bite (no.)	Fever	Headache	Muscle pain	Rash	Respiratory symptoms	EM	*Borrelia* serology	*Anaplasma* serology	Treatment
1	76 M	ND	ND	ND	ND	ND	ND	ND	ND	ND	pc
2	70 F	Y	N	Y	N	N	N	Y	Neg	Neg	pc
3	59 F	Y	N	Y	N	Y	N	Y	Neg	Neg	pc
4	59 M	S	N	N	N	Y	N	Y	Neg	Neg	pc
5	57 F	Y	N	N	N	N	Y	Y	Neg	Neg	pc
6	57 F	Y	Y	Y	N	Y	Y	Y	Neg	Neg	pc
7	46 M	Y (>1)	Y	Y	Y	N	Y	N	Neg	Neg	pc
8	20 F	Y	N	N	N	N	N	Y	Neg	Neg	pc
9	74 M	Y	N	N	Y	Y	N	N	Pos	Pos	doxy
10	70 F	Y	N	N	N	Y	N	Y	Pos	Neg	pc
11	68 F	S	N	N	N	N	N	Y	Neg	P	pc
12	61 F	Y	N	Y	N	Y	N	Y	Pos	P	pc
13	57 F	Y	N	Y	N	N	Y	Y	Pos	Neg	pc
14	56 M	Y	N	N	N	Y	N	Y	Pos	Neg	pc
15	55 F	Y	N	N	N	N	N	Y	Pos	Neg	doxy
16	54 F	Y	N	N	N	Y	Y	Y	Pos	P	pc
17	52 M	Y (>1)	Y	Y	Y	N	N	Y	Pos	P	pc
18	51 M	Y (>1)	Y	Y	Y	N	Y	N	Pos	P	doxy
19	43 F	S	N	N	N	Y	N	Y	Pos	Neg	pc
20	26 F	Y (>1)	Y	N	Y	N	Y	N	Pos	Neg	pc

ND no data available; *Y* yes; *N* no; *S* suspected; *P* probable; *EM* erythema migrans; *doxy* doxycycline, *pc* penicillin

Table 2. *Clinical symptoms, number of tick bites, treatment, and results of serology for Borrellia spp. and Anaplasma sp. for the Rickettsia spp. seroreactive patients in Study 1.*

Three patients had IgG antibodies in their first serum, probably due to a past infection. Seventeen patients showed seroconversion or four-fold rise in IgG titre, of whom seven (no. 1, 6, 7, 9, 14, 15 and 19) had a recent or current

infection. Thirteen patients had a past infection, of whom five (no. 5, 8, 13, 17 and 18) were judged as cases of probable infection (Figure 2).

Western blot for three patients showed a specific response to the mass-specific protein antigen in the 60-kDa region for IgG showing specificity in the serological response.

The assumption is that the patients showed seroconversion to *R. helvetica* because it is the only tick-borne *Rickettsia* spp. found in humans in Sweden. Even though cross-reactions occur it is unlikely that any other *Rickettsia* spp. should cause seroconversion.

In Study 2, the symptoms of the seroreactive patients are similar to the symptoms in Study 1, which were skin manifestations, culture negative arthritis, headache and cough (Figure 3). In one case with positive serology to *Mycoplasma pneumonia,* respiratory symptoms such as coughing could be explained. In other cases, the cause was not obvious. One explanation could be pulmonary vasculitis due to rickettsial infection.

Patient no.	S1		S2	
	IgG	IgM	IgG	IgM
1	<64	128	512	64
2	<64	<64	64	<64
3	128	128	64	<64
4	64	64	64	<64
5	<64	128	128	64
6	<64	<64	1,024	64
7	<64	64	256	128
8	<64	64	128	<64
9	<64	128	256	64
10	<64	128	64	<64
11	<64	256	<64	64
12	<64	256	64	64
13	<64	128	128	64
14	<64	128	512	<64
15	<64	128	512	<64
16	<64	128	64	<64
17	<64	128	128	64
18	64	<64	128	64
19	<64	128	512	<64
20	<64	128	64	<64

Table 3. *Antibody titres of serums 1 and 2 for 20 Rickettsia spp.-reactive patients in Study 1.*

Patient	S1		*Borrelia* serology	M.p. serology	Symptoms
	IgG	IgM			
A	128	128	Neg	ND	EM
B	128	64	Neg	ND	Arthritis
C	256	1,024	Pos (IgG)	ND	EM
D	256	<64	Neg	ND	Abdominal
E	64	<64	Pos (IgG/M)	ND	Arthritis
F	<64	64	Pos (IgM)	ND	EM
H	<64	128	Neg	ND	Cough
I	512	512	Neg	ND	Cough, arthritis
J	32	256	Pos (IgG)	ND	Myalgia/tendinitis
K	64	128	Pos (IgG)	ND	Cough
L	256	<64	Neg	ND	Neuropathia
P	2,048	256	ND	Neg	Fever, cough
Q	128	64	ND	Neg	Fever, cough
R	<64	256	ND	Neg	Cough
S	64	64	ND	Pos (IgG)	Cough
T	64	128	ND	Neg	Abdominal

Table 4. *Antibody titres and symptoms in Study 2.*
M.P. Mycoplasma pneumonia; ND no data available; EM erythema migrans.

In the prospective study **(Paper III)**, where participants with an observed and recent tick bite were enrolled through public media, only a few participants sought medical care due to the symptoms and none was diagnosed with a tick-borne disease. Forty-seven (21.6%) self-reported at least one non-specific symptom at the follow-up visit. The symptoms that were more common in participants with confirmed or possible infection (combination of Group 1 and 2) compared to the seronegative group (Group 4) were nausea (p=0.006) and radiating pain (p=0.041). The participants who showed seroreactivity and were bitten by a *Rickettsia* spp.-positive tick had significantly more non-specific symptoms compared to the seronegative group (Group 4) (p=0.041). Three participants developed EM, one each from Group 1, 3 and 4. The one from Group 1 was bitten by a rickettsia-positive tick and was negative in borrelia serology. The participant from Group 3 was seroreactive to both *Borrelia* and *Rickettsia*, but did not seroconvert. The participant in Group 4 seroconverted to *Borrelia*. Few participants developed EM, and based on this study no conclusions can be drawn concerning which agent caused EM. In this study, a cohort of tick-bitten individuals was followed, and symptoms that developed were notified. This explains why we saw less EM in this study than in the study in **Paper II**, where EM was one of the inclusion criteria. This low incidence of EM should more accurately reflect the true incidence of EM after tick bites.

The findings of clinical manifestations indicate that *R. helvetica* is in most cases a subclinical disease, but that, as previously described, it can in some cases cause severe symptoms such as meningitis, septicaemia and myocarditis. Differentiating symptoms of *R. helvetica* from symptoms of other tick-borne diseases is difficult, and this increases the risk of misdiagnosis between tick-borne diseases. It is also a challenge for clinicians to know when to start

treatment, and more knowledge is needed for development of treatment guidelines.

Co-infection

Co-infection with several tick-borne agents has previously been studied in ticks. In a study from Sweden, ticks were found to be co-infected with *Anaplasma phagocytophilum* and *Rickettsia* spp. [57]. Co-infections in ticks with *Rickettsia* spp. and other agents have been reported from many countries, including Portugal, Croatia, China, Germany, Italy, Poland and France [65, 146, 147, 151-153]. In China, five species have been isolated in ticks. In ticks collected from an urban park in Rome, Italy, 30% were co-infected with two pathogens and 12% carried three pathogens. The most common double infection was infection with *Rickettsia* SFG and *Borrelia burgdorferi* sensu lato. Triple infection most commonly involved *Rickettsia* SFG, *Borrelia burgdorferi* sensu lato and *Coxiella burnetii,* among the pathogens examined [154]. In *Ixodes ricinus* ticks infected with *Borrelia* collected in Hamburg, Germany, 22.9% were co-infected with *Rickettsia* spp. [155]. Co-infection can occur either by being infected by a single tick containing multiple infectious agents or by being infected by several ticks, each containing one or more infectious agents. It is well known that persons living in areas where ticks are common get multiple tick bites each season and can in this way contract many different tick-borne agents from different ticks. Interaction of co-infections in ticks may alter replication of infectious agents in ticks. It has been shown that prior infection of tick cell cultures with *B. burgdorferi* enhanced subsequent replication of the pathogen *Ehrlichia ruminantium* [156]. Another study showed that co-infection in ticks with *R. rickettsii* and *R. peacockii* in *Dermacentor andersoni* ticks inhibits transovarial transmission of *R. rickettsii* [157]. Positive relationships between pathogens can also occur as increased spread of *Coxiella burnetii* into tissues of *Dermacentor reticulatus* in the presence of *Rickettsia phytoseiuli* [158]. Even though several infectious agents appear in ticks, co-infections in humans have rarely been studied [148]. The fact that co-infections occur in humans, including co-infections with different *Rickettsia* SFG species, has been demonstrated in case reports and small surveys [147, 159-162]. The possibility that interactions due to co-infection may change the infection and symptoms in humans cannot be excluded. In **Paper II**, co-infections among *Rickettsia* spp. seroreactive patients were examined for co-reactivity with *Anaplasma* sp., *Borrelia* spp. and tick-borne encephalitis (TBE). None of the patients showed antibodies for TBE. We were able to demonstrate that, in patients with symptoms and seroreactivity to *Rickettsia* spp., co-infection against *B. burgdorferi* and *Anaplasma* sp. was common. Thirteen out of 19 patients had seroconverted to two or more bacteria.

Of the participants in **Paper III**, seven (3.2%) seroconverted against *Borrelia* spp. Three (1.4%) of these seroconverted to both *Rickettsia* spp. and *Borrelia* spp., and four were seronegative for *Rickettsia* spp. In addition, 88 participants (40.4%) were seropositive to *Borrelia* spp., of whom 17 of 40 (42.5%) were positive in Group 1, three of four (75.0%) in Group 2, 24 of 52 (46.2%) in Group 3, and 44 of 122 (36.1%) in Group 4. In comparison, 40 (18.3%) participants seroconverted against *Rickettsia* spp. Ninety-five (43.6%) participants were either seropositive or showed seroconversion to *Borrelia* spp. compared to 96 (44.0%) for *Rickettsia* spp. The risk of seroconversion was higher for *Rickettsia* spp. than for *Borrelia* spp., but the number of participants with antibodies to both agents was equal in this study. This can be explained by the fact that antibodies remain longer for *Borrelia* spp. than for *Rickettsia* spp. *Borrelia* spp. antibodies can remain for years, while antibodies to *Rickettsia* spp. remain for at least 8-12 months [130, 163, 164].

For both rickettsial diseases and *Borrelia* infection, symptoms are quite general and do not allow us to distinguish between the agents. Even erythema migrans appeared at the same rate in relation to both infections and could not provide any clues as to what infectious agent was involved. Skin biopsies further examined by PCR or immunochemistry would be an option for gaining information on which agent corresponds to a specific symptom. In a study performed in Croatia, skin biopsies were taken and examined regarding *Borrelia* and *Rickettsia* [147]. The numbers were small, and out of 67 skin biopsies from EM, only one was *Rickettsia* positive. This sample was also shown to harbour *Borrelia afzelii*. This provides no evidence that *Rickettsia* causes EM, and larger studies must be performed to acquire more knowledge about the relationship between *Rickettsia* and EM.

Infection caused by *Rickettsia felis*

In **Paper IV**, two cases of meningitis due to *R. felis* were described. This was the first time *R. felis* was detected in Sweden. Because it is a flea-borne agent that is distributed worldwide, the possibility that *R. felis* occur in Sweden could not be excluded. Even though the cat flea (*Ctenocephalides felis*) is the most common vector, no confirmed vector has been reported in Sweden thus far. Patient 1 was a male, 47 years of age and hospitalized in May 2008. He had a week earlier started to feel ill with headache and fatigue. The headache progressed. He had no symptoms of infection other than a slight sore throat. He felt like he had a fever, but did not check his body temperature. He had noticed a tick bite eight months prior to debut of disease. He had no memory of contact with cats or dogs. Laboratory tests showed C-reactive protein (CRP) 23 mg/L, white blood cell count (WBC) 10.9 x 10^9/L, normal haemoglobin 136 g/L and normal platelet count 250 x 10^9/L. Cerebrospinal fluid (CSF) showed pleocytosis of 32 x 10^6/L mononuclear cells and elevated albumin 407

g/L, but a normal serum CSF ratio. Computed tomography of the brain was normal. Cultures from blood and CSF were negative. Tests for herpes, enteroviruses, tick-borne encephalitis (TBE) were all negative, as were tests for *Borrelia burgdorferi* in serum and CSF. He resumed for two days in hospital, then he felt better and returned home. He received no antibiotics. At a follow-up one year later, he was still healthy and had no sequelae.

Patient 2 was an 89-year-old woman hospitalized in Uppsala in May 2008 with symptoms of fever and back pain. She had a medical history of asthma and a newly discovered atrial fibrillation. At admission she had a fever of 38.5°C, was tired and a bit confused, but without focal neurology. No lymphadenopathy or cutaneous lesions were seen, but she had pain over her left kidney loge. Laboratory tests showed WBC 9.8-12.6 x 10^9/L, haemoglobin 123 g/L, platelet count 204-429 x 10^9/L and CRP 139 g/L. She was diagnosed with a urinary tract infection and following cultures, treatment with piperacillin/tazobactam was started. Urine culture showed >100,000 bacteria/mL of *Klebsiella pneumoniae*. Computed tomography of the brain was normal. CSF showed 16 x 10^6/L mononuclear, normal albumin, slightly elevated lactate (3.0 mmol/L) and lowered glucose ratio (<0.5). The antibiotic was changed after two days to cefuroxime and ampicillin i.v. in doses for meningitis. She slowly recovered and left the hospital after 10 days of treatment. Cultures from CSF and tests for herpes and enterovirus were negative. After informed consent was obtained, both patients were retrospectively included in the study, wherein both patients were found to be positive in real-time PCR. CSF was further examined with nested PCR and sequencing revealed the presence of *Rickettsia felis*. The sequences shared 99–100% similarity with the corresponding gene sequences of *R. felis* (GenBank accession numbers DQ102709.1 (17-kDa) and AF210695.1 (*ompB*)) and showed significant nucleotide differences from the other rickettsiae in the spotted fever group. The sequences obtained from Patient 1 (17-kDa, *ompB*) and Patient 2 (*OmpB*) have been deposited at GenBank; accession numbers GQ182891 and GQ182892. The PCR analysis of CSF verified the presence of *R. felis*. According to the standard curve, the Ct (cycle threshold) values indicated only 1 – 10 DNA copies/ µL in the samples. However, amplicon contamination seems very unlikely, as we have never amplified, cultured or used *R. felis* as an antigen in serological assays in our laboratory. The 17-kDa and *ompB* sequences had the same length as the amplified products from *R. helvetica,* but differed in 13 and 11 bp from *R. helvetica,* which indicates another species. All negative controls were negative, and DNA amplification used primers targeting three different genes. Microimmunofluorescence assay (MIF) showed antigen reactivity with *R. helvetica* with end titres of 1:64 in the early phase. No other sera were available, which is why it was not possible to determine seroconversion. Lateral flow coal immunochromatographic assay tested in Patient 1 confirmed the presence of specific IgG antibodies to SFG *Rickettsia*. The relative low titre in MIF is probably due to cross-reaction between *R. helvetica*, used as

antigen, and antibodies from *R. felis*. This determination is also made at the rickettsial reference laboratory in Marseille (Unité des Rickettsies), where a IgG titre between 1:64 and 1:128 is considered to indicate cross-reactivity [127].

Neurological symptoms due to *R. felis* infection have previously been reported from Yucatán, Mexico [114]. The patients were diagnosed with PCR from skin biopsies, confirmed with DNA sequencing and positive serological tests. The case reports consisted of three patients, all showing neurological symptoms: photophobia in two patients and hearing loss and signs of meningism in the other. No samples were taken from CSF in these cases, and the diagnosis meningitis was not verified by laboratory tests. All of them had cutaneous manifestations, and two of them maculopapular rash, which our patients lacked. In a summary of previously published cases, 10 out of 34 (29.4%) patients had no cutaneous manifestations, showing that rash does not always appear [165]. Because it is easier to discover rickettsial infection when rash occurs, the number of patients with infection and without rash is probably higher than what has been shown in past studies and, thus, the disease is probably underdiagnosed. One of our patients did not receive antibiotics and the disease was self-limited, implying that rickettsiae as a possible cause of meningitis may be overlooked. Although our cases were not severe, more severe forms of meningitis cannot be ruled out in all cases. Of the 34 cases of *R. felis* infection published previously, five (14.7%) had neurological signs suggesting that *R. felis* should be considered as a cause of meningitis [165]. Because *R. felis* has a worldwide distribution, neurological symptoms due to *R. felis* could be a common cause. Other clinical findings for *R. felis* resemble febrile diseases and are often mistaken for murine typhus in areas where murine typhus is common, indicating that infection with *R. felis* is probably more common than previously thought.

Meningitis has been described as a symptom of rickettsial disease [109]. In that case, PCR examination of CSF identified the agent as *R. helvetica*. The patient had symptoms of meningitis and CSF showed slight pleocytosis (28 x 10^6/L of which 18 x 10^6/L were mononuclear cells). The patient also had a pulmonary infiltrate on X-ray. Cefuroxime had no effect, but following change to doxycycline the patient recovered after three days. At the time of admission, the patient had experienced a total of three weeks of fever and headache.

Both our patients showed CRP levels that were slightly elevated, as is commonly seen in rickettsial diseases [105]. Thrombocytopenia was not present, and aminotransferases were not analysed. WBC was normal or slightly elevated. In the patients from Yucatán, both leukopenia and leukocytosis were present [114]. In a case report on one patient in France, the only abnormal values were elevated aminotransferases [165]. Laboratory test results seem to vary in *R. felis* infections, in the same way as with other rickettsial diseases,

although the number of cases published thus far is low and no certain conclusions can be drawn.

Conclusions

- **Paper I** shows that rickettsia infections occur in Sweden and that the seroprevalence differs as a result of the degree of exposure to tick bites. Patients with confirmed tick bites (borrelia seropositive patients) have a higher prevalence of seropositivity than do patients at low risk for tick bites (blood donors). There is a geographical difference in seroprevalence between low endemic and high endemic areas. Co-infection in humans occurs between *Rickettsia* spp. and *Borrelia* spp.
- **Paper II** shows the seroprevalence of *Rickettsia* spp. in southeastern Sweden among patients seeking medical care for erythema migrans or flu-like symptoms and seroprevalence in central Sweden among patients sampled for *Borrelia* spp. and *Mycoplasma pneumoniae*. It also shows that co-infection between *Rickettsia* spp., *Anaplasma* sp., *Borrelia* spp. occurs and is more common than previously assumed. Erythema migrans is common regardless of any combination of the tick-borne infections studied or infection from a single agent. Symptoms alone give no clue as to which agent is responsible for the disease. Diagnostic tests, not solely valuation of clinical manifestations, have to be performed to determine the identity of the infectious agent.
- **Paper III** shows the rate of seroconversion against *Rickettsia* spp. and *Borrelia* spp. after tick bites in south-central and southernmost Sweden as well as on the Åland Islands in Finland. Seroconversion against *Rickettsia* spp. was more common after a tick bite than seroconversion against *Borrelia* spp. in these areas. This shows the risk of seroconversion against *Rickettsia* spp. after being bitten by *Rickettsia* spp.-infested ticks. A longer blood feeding duration did not seem to affect transmission of *Rickettsia* spp. A higher number or bacteria in the tick did not seem to raise the risk of seroconversion. Seroconversion against *Rickettsia* spp. seldom gave clinical symptoms that required medical care, but non-specific symptoms after a tick bite could be a sign of infection. We examined to what extent a serological response to both *Rickettsia* spp. and *Borrelia* spp. occurs following a tick bite.
- **Paper IV** shows the first documented cases of *Rickettsia felis* infections in humans in Sweden and that *R. felis* can cause meningitis.

Future directions

Spotted fever group rickettsiae, except for Rocky Mountain spotted fever and Mediterranean spotted fever, have often been described as causing mild, flu-like diseases. Previous studies and our studies have showed that spotted fever group (SFG) rickettsiae can also cause severe diseases such as perimyocarditis, meningitis and hepatitis. Because SFG rickettsial diseases cause vasculitis, symptoms from all organs are possible and severe diseases in other organs should be more investigated with regard to rickettsiae in order to enhance our knowledge. Most of the symptoms are mild. Thus, if we are to discover whether more severe diseases are caused by *Rickettsia* spp., we must keep in mind to test for *Rickettsia* when the cause of the disease is unknown.

Co-infections of tick-borne diseases in humans are rarely studied. More investigations are needed to discover to what extent co-infections occur, which could add to the knowledge base on tick-borne diseases. Furthermore, interactions by bacteria causing co-infections in humans could be a field for further investigation.

More large-scale studies would improve our knowledge of the clinical manifestations of SFG rickettsiae. Most of the clinical manifestations from SFG rickettsiae come from case reports and small-scale studies.

For *R. felis,* no vector has been detected in Sweden thus far. Searching for possible vectors for this agent could be a future field of investigation.

Continuing to search for new *Rickettsia* spp. not previously observed in Sweden could also be a field of interest.

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Sammanfattning på svenska

Rickettsiae är obligat intracellulära bakterier som finns spridda över hela världen. Den huvudsakliga vektorn för spotted fever gruppen (SFG) av rickettsiae är fästingar. Olika rickettsiaarter har olika utbredningsområden beroende av vad dess vektor har för utbredningsområde. Arter som har löss, loppor och kvalster som vektorer är spridda över hela världen. Rickettsiae är gamla bakterier och har under årens lopp anpassat sig till ett liv i värdcellerna genom minskat genomiskt material. Bakterierna invaderar endotelcellerna och orsakar vaskulit som kan drabba flera organssystem. Symptombilden hos SFG rickettsiae skiljer sig åt mellan arter men har många gemensamma drag som utslag, eschar vid bettmärket, feber, influensalikande symptom som huvudvärk, frysningar, mag-, tarmsymptom samt muskel- och ledvärk. Allvarliga fall finns beskrivna med meningit, perimyokardit och septikemi. Standardbehandling vid kliniska symptom är Doxycyklin.

Denna avhandling visar skillnader i seroreaktivitet och serokonversion i olika delar av Sverige och på Åland i Finland. För att ta reda på detta har vi gjort serologiska undersökningar av personer med säkerställda eller misstänkta fästingbett. Vi har även jämfört med personer med okänd fästingexposition (blodgivare). Seroprevalensen skiljer sig åt i olika delar av Sverige och är högre där risken för fästingbett anses som störst. Inte heller förvånande är seroprevalensen hos blodgivare lägre än för de med kända eller misstänkta fästingbett. Serokonversion efter fästingbett har i våra studieområden visat sig vara högre än för *Borrelia*. Rickettsioser kan vara en vanligare infektion än vi hittills trott och är antagligen underdiagnostiserad.

Vid undersökning av kombinerade infektioner med *Anaplasma* spp. och *Borrelia* spp. kunde vi konstatera att dessa förekommer. Symptomen är i våra studier oftast ospecifika och milda och det är därför svår att knyta dem till en specifik fästingburen infektion. För att säkerställa diagnos behövs även analyser för mikrobiologisk diagnostik.

När vi studerat patienter med meningit har vi hittat två patienter där *R. felis* kunnat påvisas i likvor med PCR, Det är första gången som *R. felis* konstaterats i Sverige. Det är även första gången som sjukdom av *R. felis* är konstaterad i Sverige. Båda patienterna tillfrisknade utan restsymptom. Ingen av de båda patienterna fick effektiv behandling mot *Rickettsia*.

Avhandlingen visar att *Rickettsia* är vanlig i fästingar och kan infektera människa. Rickettsianfektion skall misstänkas vid ospecifika eller specifika symptom efter fästingbett. När en rickettsiainfektion skall behandlas är ännu

inte helt klarlagt och fler studier behövs för att utröna detta. Vi har även visat att förutom *R. helvetica* förekommer även *R. felis* i Sverige och kan orsaka infektion.

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