Epidemiological and Bacteriological Aspects of Spotted Fever Rickettsioses in Humans, Vectors and Mammals in Sweden

KARIN ELFVING
Rickettsiae are obligate intracellular gram-negative bacteria transmitted by arthropod vectors. Rickettsiae sometimes cause disease in humans, typically with high fever, headache and occasionally an eschar.

In Sweden, Rickettsia helvetica, belonging to the spotted fever group, is the only tick-transmitted rickettsia found free in nature. The pathogenic role of R. helvetica has not been fully investigated, but it has been implicated in aneruptive fever and cardiac disease.

This thesis describes parts of the transmission pathways of rickettsiae in Sweden. Rickettsia infection rates in ticks collected from birds were analysed, and the birds' role as disseminators and reservoirs was studied. We found that more than one in ten ticks was infected with rickettsia bacteria, predominantly R. helvetica, and that migrating birds contribute not only to long-distance dispersion of bacteria, but also to an inflow of novel and potentially pathogenic rickettsia species, in this case R. monacensis and R. sp. strain Davousti-like species, into Sweden.

Further, wild and domestic animals were found to have seroreactivity against R. helvetica, which shows that they are exposed and susceptible to rickettsia. Their role as reservoirs has not been determined, yet they may indirectly be involved in transmission of rickettsia to humans by infected ticks feeding on them.

The seroreactivity in humans was also studied. Patients investigated for suspected Borrelioses and blood donors had detectable antibodies against Rickettsia spp., with the highest prevalence detected in the suspected Borreliosis group. This shows that humans in Sweden are exposed to and develop an immune response against rickettsia. The suspicion that R. helvetica may cause severe symptoms was verified by a patient with subacute meningitis where the bacterium was shown for the first time to cause an invasive infection with CNS involvement and where the bacterium was isolated from the patient’s cerebrospinal fluid.

Growth characteristics and morphology of R. helvetica were studied to better understand invasiveness and virulence. The findings indicate that the invasiveness is comparable with other rickettsia, though R. helvetica seems to have a stable but slightly slower growth.

Rickettsia helvetica is endemic in Sweden and therefore needs to be considered when investigating disease after a tick bite.

Keywords: Rickettsia helvetica, ticks, cultivation, serology, polymerase chain reaction (PCR), DNA sequencing, western blot, electron microscopy, meningitis, seroprevalence

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List of Papers

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


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Pictures on the cover

**Upper left:** A *Rickettsia helvetica* bacterium. Picture taken with a scanning electron microscopy (SEM JEOL JSM-5200) at magnification x 20,000.

**Upper right:** *Rickettsia helvetica* bacteria grown in Vero cells, fixated on a slide and visualised with FITC using a fluorescence microscopy, x 400.

**Lower left:** Amplification curves of a dilution series containing *Rickettsia helvetica*, detected by a rickettsia specific real-time PCR.

**Lower right:** DNA fragments amplified in a rickettsia nested PCR and visualised using gel-electrophoresis.
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<tbody>
<tr>
<td>bp</td>
<td>Base pair</td>
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<tr>
<td>CNS</td>
<td>Central nervous system</td>
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<td>CSF</td>
<td>Cerebrospinal fluid</td>
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<tr>
<td>DNA</td>
<td>Deoxyribonucleic acid</td>
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<tr>
<td>EM</td>
<td>Erythema migrans</td>
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<tr>
<td>FITC</td>
<td>Fluorescein isothiocyanate</td>
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<td>IF/IFA</td>
<td>Immunofluorescence assay</td>
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<td>IFN-γ</td>
<td>Interferon gamma</td>
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<td>IgG</td>
<td>Immunoglobulin G</td>
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<tr>
<td>kDa</td>
<td>Kilo Dalton</td>
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<tr>
<td>LPS</td>
<td>Lipopolysaccharide</td>
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<tr>
<td>Mb</td>
<td>Mega base pair</td>
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<tr>
<td>Omp</td>
<td>Outer membrane protein</td>
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<tr>
<td>PBS</td>
<td>Phosphate buffered saline</td>
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<tr>
<td>PCR</td>
<td>Polymerase chain reaction</td>
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<tr>
<td>RFLP</td>
<td>Restriction fragment length polymorphism</td>
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<tr>
<td>RNA</td>
<td>Ribonucleic acid</td>
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<tr>
<td>rRNA</td>
<td>Ribosomal ribonucleic acid</td>
</tr>
<tr>
<td>rrs gene</td>
<td>16S rRNA gene</td>
</tr>
<tr>
<td>SDS-PAGE</td>
<td>Sodium dodecyl sulfate polyacrylamid gel electrophoresis</td>
</tr>
<tr>
<td>SFG</td>
<td>Spotted fever group</td>
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<tr>
<td>SMI</td>
<td>Swedish Institute for Infectious Disease Control</td>
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<tr>
<td>SVA</td>
<td>Swedish National Veterinary Institute</td>
</tr>
<tr>
<td>sp.</td>
<td>Species, singular</td>
</tr>
<tr>
<td>spp.</td>
<td>Species, plural</td>
</tr>
<tr>
<td>TBE</td>
<td>Tick-borne encephalitis</td>
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<tr>
<td>TEM</td>
<td>Transmission electron microscopy</td>
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<tr>
<td>TG</td>
<td>Typhus group</td>
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<tr>
<td>TNF-α</td>
<td>Tumour necrosis factor alpha</td>
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<tr>
<td>WHO</td>
<td>World Health Organization</td>
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Introduction

Ticks are hematophagous acarines that parasitize every class of vertebrate (including man) and have a worldwide distribution. In Sweden and Europe, the most common hard tick *Ixodes ricinus* can transmit viral as well as bacterial and protozoal infections. Ixodes play an important role as the vector of common infections such as Borreliosis, tick borne-encephalitis (TBE) and Tularemia [1]. Another example of a bacterial infection is the tick-borne rickettsioses caused by obligate intracellular bacteria belonging to the spotted fever group (SFG) within the genus *Rickettsia*. The existence of a spotted fever rickettsia in Sweden, *R. helvetica*, has been known since 1997, and it is the only established tickborne rickettsia species in Sweden [2]. The pathogenic role of *R. helvetica* is still unclear, but documented patients have presented with a mild, self-limited disease associated with fever, headaches and myalgias. *R. helvetica* has, however, previously been associated with acute perimyocarditis, sarcoidosis and unexplained febrile illness [3-5].

Rickettsia

General characteristics

The bacteria were described in 1906 by Howard Ricketts as a causative agent of Rocky Mountain spotted fever, and the genus *Rickettsia* was later named after him [6]. The bacteria belong to the genus *Rickettsia* within the Family *Rickettsiaceae* in the order *Rickettsiales*, a genetically diverse group of α-Proteobacteria. The order *Rickettsiales* is currently comprised of the genera *Anaplasma, Ehrlichia, Neorickettsia, Orientia, Rickettsia* and *Wolbachia* [7, 8] (Figure 1).

Rickettsiae were originally characterized into two main groups, the spotted fever group (SFG) and the typhus group (TG), although it has been suggested that a few species should be classified into two additional groups: the ancestral and the transitional group [9-11]. Currently, the *Rickettsia* genus contains about 25 officially validated species and several dozen as yet uncharacterized strains [7]. The spotted fever group contains the largest number of species, and the typhus group is made up of three species (Figure 1). Around 16 species are established human pathogens and another two are
suspected to cause rickettsioses, but the number is increasing as the knowledge grows [12]. Modern diagnostic tools including culture and molecular biology have contributed to the great expansion of newly identified rickettsia species, especially from human samples [12, 13].

Figure 1. Rickettsia taxonomy.

Rickettsiae are gram-negative bacteria with an exclusively intracellular replication cycle, requiring host cells in which to replicate [13]. In other words, in the laboratory, rickettsia can only be cultivated in viable eukaryotic host cells, e.g., in cell culture, embryonated eggs, or susceptible animals [14]. The bacteria replicate via binary fission where the single DNA molecule first replicates and when the cell begins to pull apart, the replicate and the original chromosome are separated [15]. The reproductive doubling time of Rickettsia spp. (prowazekii and rickettsii) is 9-12 hours, which is slightly longer than for other bacteria [16, 17].

The bacilli of rickettsiae are short rods that are poorly stained by gram but retain basic fuchsin when stained using the method of Giménez. They measure 0.8 – 2.0 µm in length and 0.3–0.5 µm in diameter [18]. Rickettsiae occur singly, in pairs, or in strands. SFG Rickettsiae differ from the bacteria in the typhus group in that they can be observed both in the cytoplasm and in the nuclei of the host cell [19].
The cell wall structure is typical of gram-negative bacteria with an inner and an outer membrane separated by a peptidoglycan layer. Rickettsia cells are surrounded by a crystalline proteic layer, S-layer, which represents 10-15% of the total protein mass [20]. The outer membrane contains lipopolysaccharide (LPS), however this layer does not seem to exert endotoxic effects, a phenomenon seen in pathogens like *Coxiella burnetti* and *Chlamydia trachomatis*. The cell wall structure also contains a major 120-kDa rickettsial outer membrane protein (OmpB), thought to mediate entry into the host cell, a 17-kDa lipoprotein and a 190-kDa major immunodominant surface exposed protein (OmpA), with a variable number of nearly identical tandem repeats. Genetic organization of these repeated regions has been compared between different spotted fever group rickettsiae, and their distinctive arrangements are responsible for encoding species-specific conformational epitopes of the protein [12, 21].

The rickettsia genome consists of a single circular chromosome and the genome of SFG rickettsiae is highly conserved, with similar synteny and content [19]. Recent studies have reported that several rickettsia species also carry plasmids, but the plasmids role in virulence and host adaptation is unknown [22]. Genome sequencing is now complete for several rickettsial species. The genome sizes of the species in the spotted fever group are small, usually between 1.2 and 1.3 Mb [18]. As for other host-associated organisms, rickettsiae have undergone dramatic genome reduction. The close association with the host has caused elimination of biosynthetic metabolic pathways, and several of the pathways have been replaced by transport systems [21]. The bacteria rely on the host for the synthesis of many amino acids and nucleotides [10].

**Phylogeny and taxonomy**

Until the 1990s, the phylogenetic studies of rickettsiae were based on morphological, antigenic and metabolic features that were unreliable. After the advent of molecular methods and the possibility of genome sequencing, the phylogenetic relationships between species could be more reliably estimated. As a consequence, several bacteria initially classified in the genus *Rickettsia* were excluded and divided into other genera, e.g., *Orientia tsutsugamushi* [7].

The guidelines established for extracellular bacteria do not accord well with the strict intracellular nature of rickettsiae. Application of the phenotypic characteristics, used for extracellular bacteria, to the order *Rickettsiales* is limited, as few are expressed by these bacteria [23]. Many novel rickettsia isolates have been characterized by genetic methods that have generated controversy regarding the appropriate taxonomy of rickettsia species [24]. For the taxonomic classification of rickettsial isolates at the genus, group and species level, gene sequence-based criteria, using five rickettsial genes,
including *rrs*, *gltA*, *ompA*, *ompB* and gene D, have been proposed [23]. Another proposed criterion for the establishment of a new species is a divergence of the *rrs* gene (16S rRNA gene) by 0.2% [24].

Vectors and reservoirs

Most rickettsial diseases are transmitted by arthropod vectors, including ticks, mites, fleas, and lice [25]. Spotted fever group rickettsiae are predominantly transmitted by ticks, while the agents of typhus group rickettsia are transmitted to humans through the faeces of lice and fleas [26, 27]. Two families of ticks are of medical importance regarding rickettsioses: *Ixodidae* (hard ticks) and *Argasidae* (soft ticks). Most ticks infected with SFG *Rickettsiae* belong to the *Ixodidae* family [26]. More than 95% of the ticks found on humans in Sweden belong to the *I. ricinus* species [28]. The *Ixodes ricinus* tick has a three-host life cycle, i.e., it ingests a blood meal in each life stage before it moults.

Ticks acquire SFG rickettsial species through transovarial transmission (adult female to egg) and transstadiol passage (egg to larva to nymph to adult) and by horizontal acquisition during feeding on a rickettsiemic host [10, 19] (Figure 2). Given that larvae, nymphs and adults may all be infective for susceptible vertebrate hosts, the ticks must be regarded as the main reservoir host of rickettsiae [19].

Almost all organs in the intervertebrate host are infected. Ixodid ticks feed once within each stage, but often for a period of several days. This blood-feeding may involve a great variety of vertebrates that occupy very diverse habitats. To sustain a successful life cycle, it is likely that wild animals act as natural reservoirs for rickettsia. Free-living vertebrates and especially small mammals like the vole, mouse, rat, rabbit, hare and squirrel are reported to be potential reservoirs for rickettsia [26, 29]. Large mammals like cervids and cattle are also suggested to be natural reservoirs for rickettsia [30-32]. Humans are only occasional hosts for ticks and play no role in the maintenance of the bacteria in nature.
Distribution

The genus *Rickettsiae* has a world-wide distribution, and the existence and geographical spread of rickettsioses is increasing (Figure 3 and 4) [13]. The distribution of SFG rickettsia corresponds to the geographical distribution of its vector *Ixodes ricinus*. The geographical distribution of *I. ricinus* in Sweden is located to the southern and south-central parts of the country and the coastal areas in the north. The northern limit corresponds to snow cover (mean duration of 150 days) and a vegetation period averaging 170 days [33, 34].

The distribution and prevalence of *I. ricinus* ticks carrying rickettsia have been investigated mostly in southern and central Sweden. An initial study detected a prevalence of rickettsia DNA in 1.7% of the collected ticks [2]. Follow-up studies analysed ticks collected at seven different localities, and rickettsiae were detected both in the inland of southern Sweden and in coastal areas in the south-east and northern Sweden. The study showed occurrence of only one rickettsia species, *R. helvetica*, with an overall prevalence of 13.7% [35]. Another study from 29 localities in southern and central Sweden showed a prevalence of 9.6% for *Rickettsia* spp. in ticks. The majority of the positive samples represented *R. helvetica*, but one tick was infected with a rickettsia species closely related to *R. sibirica* [36].

In recent years several studies have shown that environmental factors like climate, biotope and the abundance of ticks and their hosts are important to the geographic distribution of ticks and the rickettsiae they transmit. A changing climate and the impact of human behaviour on the environment affect the spread of ticks and the pathogens they carry [34, 37-39]. The range and abundance of *I. ricinus* ticks have increased markedly in Sweden during the past two decades, probably because of an increased duration of the vege-
lation period and an increase of the roe deer population [40]. This affects the tick-borne infections, including Lyme borreliosis and TBE, which also increase in incidence [34, 41]. In Sweden, the main host for adult ticks is the roe deer (*Capreolus capreolus*), but also other medium- and large-sized mammals are important mating and blood hosts for *I. ricinus* [40, 42]. Host migration events are also responsible for expanding the habitat of ticks by potentially introducing them into new geographic regions. Migrating birds, for example, can act as long-distance vectors for several microbial agents of human disease and are also incidentally reported for rickettsiae [43]. Rapidly evolving rickettsiae may adapt to different hosts and environmental conditions, and prevalence of currently minor pathogenic species may be favoured by changing environmental conditions [44].
Figure 3. Map from Parola et al. 2005 illustrating Rickettsia distribution in Europe.

Coloured symbols indicate pathogenic rickettsia. White symbols indicate rickettsia of possible pathogenicity and Rickettsia of unknown pathogenicity.

Figure 4. Map from Parola et al. 2005 illustrating Rickettsia distribution in Asia.

Coloured symbols indicate pathogenic rickettsia. White symbols indicate rickettsia of possible pathogenicity and Rickettsia of unknown pathogenicity.
Pathogenesis

Arthropods transmit rickettsia bacteria through salivary secretion (ticks) into the bite or via faeces (flea and louse), thus contaminating the skin area around the bite [45]. The strict intracellular environment entails technical difficulties in studying the organism in detail. Interaction between the rickettsiae and the host cell involves several steps including recognition, entry, phagosome escape, growth, actin-based motility, cell-to-cell spread and cell lysis, as seen in Figure 5 [12, 46].

After the rickettsial entry into the dermis, the transmitted rickettsiae are hematogenously disseminated, and the bacteria preferentially infect endothelial cells lining the small blood vessels [12, 46, 47]. Rickettsiae may also enter phagocytic cells such as macrophages (a secondary target of most rickettsiae) by antibody-mediated opsonisation as well as invade underlying tissue such as smooth muscle cells and monocytes [48, 49].

Rickettsial penetration into the host cell is considered to be mediated by parasite-induced phagocytosis [50, 51]. Once in the host cell, the bacteria lyse the phagosome membrane with a phospholipase and get into the cytoplasm [52]. In the cytosol, they acquire nutrients and components required for growth, and the bacteria start replicating. A virulence mechanism unique to SFG rickettsiae, in contrast to the typhus rickettsiae, involves the utilization of the intracellular actin-based motility system to promote direct cell-to-cell spread [12]. SFG rickettsiae are also able to move within the cell and can enter the nuclei because of the actin polymerization [19]. Mode of exit from the host cell varies depending on the species. Some exit by cell lysis and others are extruded from the cell by local projections, filopodia (filamentous actin), which associate with the bacteria and help to push them out (Figure 5). Another mode of exit is by budding through the cell membrane; in this case the bacterium remains enveloped in the host cell membrane as it infects other cells [50].

Pathogenesis is primarily due to irreversible destruction of the cells by the replicating bacteria, because rickettsiae do not produce soluble toxins [47]. Destruction of the endothelial cells, most critical in the lungs and brain, results in leakage of fluid from the bloodstream due to increased vascular permeability. The fluid is accumulated in the surrounding tissue, edema, with subsequent organ and tissue damage [46].

An aspect of the pathogenesis of rickettsial infections is the host defences. Studies of murine models of spotted fever and typhus group rickettsioses have identified mechanisms of immunity. Gamma interferon (IFN-γ) and tumour necrosis factor alpha (TNF-α) are cytokines secreted by host immune cells, and they activate the infected cells to kill intracellular rickettsiae [53, 54].
Clinical manifestation and treatment

Several species are potentially harmful to humans, for example *R. prowazekii*, the causative agent of epidemic typhus, *R. rickettsii*, which causes Rocky Mountain spotted fever, and *R. conorii*, the agent of Mediterranean spotted fever [13]. *R. prowazekii* and *R. rickettsii* are considered potential biological weapons and are therefore currently on the bioterrorism watch list [55]. *R. slovaca* is one example of a rickettsia that has long been presumed to be nonpathogenic or of undetermined pathogenicity but that is now known to cause illness in humans [24]. Infections caused by *R. slovaca* have symptoms with distinctive features, especially enlarged lymph nodes, which led to the name tick-borne lymphadenopathy (TIBOLA) [56].

Rickettsioses can present with an array of clinical signs and symptoms, varying with the rickettsial species involved [49]. Clinical features of rickettsioses in humans begin 6-10 days after a tick bite and are somewhat nonspecific ‘flu-like’ symptoms including fever, headache, myalgia, fatigue and restlessness/insomnia [25, 49]. Rash and inoculation eschar at the site of the tick bite can follow an infection with rickettsia, although spotless fever has also been reported [57]. Some rickettsioses can be harmful and even life-threatening if left untreated, owing to late diagnosis or misdiagnosis, as in the case of Rocky Mountain spotted fever [58]. Spotted fever rickettsial dis-
cases may cause CNS infection, and *R. rickettsii, R. conorii* and *R. japonica* have a documented association with meningitis [25, 59].

The earlier a diagnosis is established, the shorter the course of rickettsial illness is, after appropriate treatment with antirickettsial antibiotics. In the treatment of infections caused by *Rickettsia* spp., usually doxycycline or another tetracycline antibiotic is usually used to inhibit bacterial cell growth [25, 60].

After recovery from SFG rickettsial infection, immunity is solid and long lasting; no human reinfections have been reported. Experimental animals that have recovered from SFG Rickettsioses are solidly immune to rechallenge [61]. Antibodies against rickettsial OmpA and OmpB, but not rickettsia lipopolysacharide, are protective against reinfection [54]. However there are cases in which relapses of typhus have occurred in treated patients. *Rickettsia prowazekii* is the only pathogen rickettsial species with acknowledged capacity to remain persistent in convalescent patients [51]. The organism appears to lie dormant in endothelial cells until they are reactivated and causes another acute but milder infection, called Brill-Zinssser disease [62, 63].

**Coinfections**

Coinfections between *Rickettsia* spp. and other pathogens are common in host-feeding ticks [64]. Rickettsia together with *Borrelia* spp. and *Babesia* spp., respectively, have been detected in ticks collected in Germany [65]. Other occurring coinfections are *Rickettsia* spp., *Anaplasma* spp. and *Borrelia* spp., all three simultaneously detected in *Ixodes ricinus* ticks [66]. Mixed infections in ticks can potentially influence transmission dynamics, owing either to interactions between bacteria within the ticks or to pathogenic effects on tick behaviour or survival. A negative interaction between rickettsiae within ticks is the transovarial transmission interference of the pathogen *R. rickettsii* in ticks coinfected with the nonpathogen *R. peacockii* [64]. In ticks coinfected with the two bacteria, only the non-pathogenic species *R. peacockii* is transovarially transmitted. A positive interaction is an increased spread of *Coxiella burnetii* into tissues of Dermacentor ticks in the presence of *Rickettsia phytoseiuli* [67].

Coinfections with several infectious agents have also been demonstrated in humans. Infection with two different bacterial agents was detected serologically in forest workers in Poland [68]. Antibodies to both *Rickettsia* spp. and *A. phagocytophilum* were present in 1.6% of the studied individuals. Workers seropositive for *Rickettsia* spp. also showed antibodies to *Bartonella* spp. (9%) and *B. burgdorferi* (7%), respectively. Coinfection with viruses and rickettsia also occurs. In a patient with meningoencephalitis, both rickettsia and Herpes simplex virus 2 were detected in the CSF [69].
Diagnostic tools

Traditional diagnosis and identification methods used in bacteriology cannot be applied to rickettsiae owing to their strictly intracellular habitat [19].

Isolation

Rickettsiae are isolated most commonly from blood, skin biopsy specimens or ticks. Inoculation on cell culture systems, preferably Vero or L-929 cells, and isolation by centrifugation shell-vial technique are the most suitable methods. SFG rickettsiae have an optimal growth temperature of 32ºC [7]. Isolation is laborious and time consuming; it takes weeks to cultivate rickettsia on cells and it is also less sensitive than other methods. Several rickettsia species also require a Bio safety lab 3 for cultivation of the bacteria. Therefore it is not a diagnostic alternative in clinical settings. Detection of rickettsiae within the cells can be achieved using microscopic examination, immunodetection or PCR [70].

Serological tests

Serological tests have been the easiest and also the most widely used methods for diagnosing tick-borne rickettsial infections for several years. One disadvantage of methods based on antibodies is that interpretation of serological data can be confounded by the cross-reactivity that occurs among the spotted fever rickettsiae [70]. Rickettsiae cross-react not only within and between the groups, but also with other bacteria such as Legionella and Proteus species [14]. A combination of different serological methods can give a reliable result on the species level.

Immunofluorescence

The most commonly used serological test for diagnosis of rickettsial diseases is immunofluorescence assay (IFA) because it is easy to perform and a relatively sensitive, specific and reproducible method. IFA can detect immunoglobulin G (IgG) and IgM antibodies and has a sensitivity of 84-100% [13, 71]. Whole cell bacteria are used as the antigen, and the cells are fixated on microscopic slides. Small amounts of serum are incubated on the slides allowing antibodies specific to the antigen to bind to the antigen. Unbound antibodies are washed away. A second antibody, labelled with a fluorochrome, is directed against the human antibodies and results can be detected in a fluorescence microscopy [72]. IFA methods are not useful in diagnosis of infections in the acute phase, because there is a delay of 7-15 days between onset of infection and the appearance of detectable antibody titres. Demonstration of a four-fold rise in antibody titres between an acute phase and a convalescent phase sera provides evidence of a recent infection [73]. The method is often retrospectively used for analysis of frozen serum samples.
**Western blot**

Western blot immunoassay is a serodiagnostic tool for confirmation of serologic diagnoses obtained using conventional tests [14]. The method allows differentiation among the SFG rickettsiae, provided that acute-phase sera are used. However, humans do not produce a high level of the species-specific antibodies against outer membrane proteins (OmpA, OmpB), which causes difficulties in species differentiation [19]. Western blot requires a laboratory equipped for cultivation of rickettsia owing to the large amount of antigens needed.

**PCR**

PCR has become the established quick and preferred method for confirming rickettsiae in clinical and biological materials [6]. The PCR amplification of rickettsial DNA must be performed before initiation of antibiotic treatment and before the antibody level is detectable [19]. PCR assays are rapid, sensitive and allow for simultaneous examination of a panel of samples. One advantage of PCR is the possibility to either design species-specific methods or if needed to design a genus-group-specific method. There are small genetic differences within the SFG rickettsiae, which makes species-specific PCR assays difficult to develop. Group-specific PCR assays can be combined with sequencing to make differentiation among the group possible [6, 13].

**Immunohistochemistry**

Rickettsiae can be detected in tissue specimens by various histochemical stains, including Giemsa or Gimenez stain. Most available assays are SFG specific, but not species specific [13]. Detection of rickettsiae using immunodetection allows confirmation of infection in patients prior to their seroconversion [14]. Today, PCR assays are more common for detection of bacteria in tissues or ticks [13].

**Transmission electron microscopy (TEM)**

The technique is seldom used for diagnosis of rickettsia infection in human tissues or other specimen. On the other hand, electron microscopy is usable for ultrastructural studies of the bacteria and the infected cell, for example in the vector or in experimental studies, when rickettsia-infected cells are propagated in cell culture [45]. TEM is also used for bacterial morphology studies. Rickettsia can usually be visualized with an ordinary light microscope, but high resolution electron microscopy has greatly contributed to our knowledge of the ultrastructural features of rickettsiae and has also been successfully applied to determine the subcellular localization of bacterial proteins [74].
**Rickettsia helvetica**

In 1979, a strain of rickettsia, isolated from *Ixodes ricinus* in Switzerland, was designated as an undescribed spotted fever group rickettsia. Serological typing indicated that this strain differed from all other strains of SFG rickettsia [75]. Not until 1993 was the strain officially validated as a new species of SFG rickettsia. The growth characteristics and the results of IF serologic typing, SDS-PAGE, Western blotting (immunoblotting) with specific mice sera, and a PCR followed by RFLP analysis confirmed previously reported preliminary findings. The new rickettsia was given the name *Rickettsia helvetica* [76]. The existence of *R. helvetica* in Sweden has been known since 1997, and was until recently the only tick-transmitted rickettsia species reported in the country [2, 77]. In 2012, a tick collected from a dog in Sweden was infected with a *R. sibirica*-like species, but no other findings confirm *R. sibirica* as an established species in Sweden [36].

*R. helvetica*’s genome size is 1.397 Mb and is somewhat larger than the others in the same group [18]. *R. helvetica* has both a circular chromosome and a plasmid [22, 78]. The cell wall structure of *R. helvetica* contains the surface protein OmpB (outer membrane protein) and a 17kDa lipoprotein, but it is unclear whether or not the cell wall contains the immunodominant protein OmpA. In an attempt to analyse the corresponding gene *ompA* for all spotted fever rickettsia, the gene was not amplified for *R. helvetica* and no published sequence for *R. helvetica* *ompA* is available in GenBank [79]. The *ompA* sequences among SFG rickettsia are very divergent and, for example *R. peacockii* is unable to express the OmpA protein because the bacterium possesses an *ompA* gene that contains three premature stop codons [80].

**Vector and reservoirs for R. helvetica**

The main vector for *R. helvetica* is *I. ricinus*, but the organism has also been reported in *I. ovatus*, *I. persulcatus* *I. monospinosus* and most recently in *Dermacentor reticulates* ticks [7, 81]. *R. helvetica* occurs across a large geographical area with a distribution from north-western Europe to central Asia (Figure 3, 4) [13, 70]. The infection rate of *R. helvetica* in ticks varies between 0.6–46.45% in different parts of Europe and Asia [26]. In a vegetation-rich dune area in France, an exceptionally high prevalence of *R. helvetica* in ticks was found to be ~66% [32]. An estimated prevalence of *R. helvetica* in ticks, in Sweden, was 22% when ticks collected from dogs, cats, roe deer, moose, humans and vegetation were analysed in pools [77]. Two recent studies performed in southern and central Sweden showed a *Rickettsia helvetica* prevalence of 9.6% (individual samples) and 1.5-17.3% (pooled samples) in ticks from vegetation [35, 36].

Coinfections in ticks with *R. helvetica* and one or more agents have been reported in Europe. In Croatia, one tick coinfected with *R. slovaca* and *R.*
*R. helvetica* was found [81]. Also in Portugal there were ticks infected with *R. helvetica* and *Borrelia lusitaniae* simultaneously [82].

In ticks, *R. helvetica* are vertically transmitted through the next generation with high efficiency [32]. The transovarial transmission rate – the proportion of infected females giving rise to at least one positive egg or larva – is 100% [26]. Pathogens that benefit from efficient transovarial transmission hardly depend on vertebrate hosts as reservoir, and *Ixodes ricinus* can therefore be considered a reservoir host [32].

The role of animals, both rodents and larger mammals, in the life cycle of tick-borne rickettsiae in Sweden is still unclear, and this is a subject area that requires closer examination. In the Netherlands, whole blood from wild animals was examined to investigate the animals’ role as a reservoir for *rickettsia* spp. *Rickettsia helvetica* DNA was present in mice 43/146 (29%), roe deer 4/21 (19%) and wild boar 2/29 (7%) [32]. However a study from Poland showed that not one out of 323 examined blood samples from birds, rodents and cervids was PCR positive for *Rickettsia* spp. [83]. Animals positive for rickettsia in the Netherlands showed no clinical signs of infection, therefore they may act as reservoir hosts and could be involved in further geographical dispersion of *R. helvetica* [32]. Genetic material from *Rickettsia helvetica* was also detected in the spleen of a roe deer in central Slovakia. Spleens from 109 wild animals including deer, wild boar and mouflon were analysed [84]. Nucleotide sequences of *R. helvetica* genes were detected in peripheral blood samples of sika deer in Japan, which suggests that sika deer could be a reservoir animal for *R. helvetica* [31].

*Rickettsia helvetica* has no pathogenic effect on Swiss mice, guinea pigs or domestic rabbits. Small rodents, however, have been shown to be susceptible to *R. helvetica* infection, for example meadow voles, bank voles, European shrews and European woodmice [85].

Seasonal and habitat variation in the prevalence of *Rickettsia helvetica* were observed in *Ixodes ricinus* ticks from Denmark. The infection rate was higher in ticks collected in the spring compared with those collected in the summer and autumn. Regarding habitat, the high tick density areas in ecotones had a higher prevalence of *R. helvetica* compared to spruce or beech forests [86].

**R. helvetica in relation to clinical diseases**

*R. helvetica* has until recently been classified as a suspected human pathogen rickettsia species, but has lately been accepted as a pathogenic species and is now listed by the US Centers for Disease Control and Prevention (CDC) as a pathogen causing aneruptive fever [7, 87].

In 1999, an association between *R. helvetica* and perimyocarditis was reported. Two young men died of sudden cardiac failure during exercise and showed signs of chronic perimyocarditis. Rickettsia was detected by PCR in tissues from their hearts. In one of the cases, a seroresponse to rickettsia was
found and also verified at WHO, Rickettsial and Ehrlichial Research Laboratories, Texas [3].

Genetic material from *R. helvetica* was detected in samples obtained from autopsies of two patients with sarcoidosis. It is possible that rickettsia may contribute to a granulomatous process, as seen in sarcoidosis. Serum samples were not available and therefore seroconversion tests were not performed [4].

On the other hand, sera from 20 well-characterized sarcoidosis patients were analysed for anti-rickettsia IgG antibodies using immunofluorescence to investigate whether serological findings support the association presented by Nilsson et al. (2002). None of the investigated sera revealed serological signs of rickettsial infection, and the study does not support the association with sarcoidosis [88].

Seroconversion to *Rickettsia helvetica* has been shown for several patients in European countries. In France a man with unexplained febrile illness seroconverted to *R. helvetica* four weeks after the onset of fever, and 9.2% of forestry workers were seropositive against *R. helvetica* [57]. In Sweden, 22.9% (8.9% verified with *R. rickettsii*) of 35 recruits showed a four-fold increase in IgG titres against *R. helvetica*, reflecting a high rate of exposure [28]. Serological evidence of *R. helvetica* infection was also detected by immunofluorescence in eight patients from France, Italy and Thailand. The infection presented as a mild flu-like disease associated with fever, headache and myalgia [5]. In a recent study in Sweden, 20 out of 206 (9.7%) patients investigated for *Borrelia* were positive for *Rickettsia* spp., as shown by immunofluorescence [89].

A report from Denmark showed coinfections with *Rickettsia helvetica* and *Borrelia* sp. in Danish patients [72]. Patients with a confirmed borreliosis were tested for *R. helvetica* antibodies and 12.5% tested positive. The results are similar with findings in Sweden, where antibodies against *Rickettsia* spp., *Borrelia* spp and *Anaplasma* spp. were simultaneously detected in patients’ serum samples [89].

Rickettsial infection, in samples from three Swedish patients, was confirmed by serological tests in combination with visualization of the rickettsial organism by electron microscopy. The patients had febrile illness with myalgia and eschar and two out of three patients had documented tick-bite sites [28].

**Rickettsia felis**

*R. helvetica* was long the only SFG rickettsia detected in Sweden, but new findings have revealed another species: *Rickettsia felis*. Retrospectively analysed CSF from two patients, from Sweden, with subacute meningitis was found to be positive for rickettsia, and sequence analysis showed *R. felis* [90]. The bacterium was first detected in the USA, in 1990, in cat fleas.
(Ctenocephalides felis) [91]. Fleas serve as the primary reservoir and vector and have a central role in the transmission of human illness. Molecular characterization of R. felis designates the bacterium as a member of the spotted fever group rickettsiae [92]. The clinical manifestations of R. felis infections resemble those of a typical rickettsioses: high fever, myalgia and rash [93]. Central nervous system involvement has not only been reported in patients from Sweden, but also in patients from Mexico [94].
Aim

The general aim of the thesis was to investigate whether rickettsiae are of clinical relevance in Sweden and to survey the prevalence and distribution of the bacteria in vectors, potential reservoirs and humans.

Specific aims

- To study the prevalence of rickettsial antibodies in a human population in Sweden by comparing a tick-exposed group with a group of blood donors as controls to determine Rickettsia exposure (Paper I).

- To study rickettsia infection rates in ticks from birds migrating to and from Sweden and to characterize the infecting rickettsia. The aim was also to define birds’ role as disseminators of *Rickettsia* spp.-infected arthropod vectors and to define their role as a potential reservoir of the agents (Paper II).

- To study whether patients with various neurological symptoms and investigated for borreliosis are infected by rickettsia bacteria (Paper III).

- To study growth characteristics of *R. helvetica*, in an experimental system after inoculation of a host cell-line, as well as to study host cell interactions of the bacteria and the possible association to invasiveness and virulence of *R. helvetica*. This includes studies of morphological and ultrastructural changes in the organism and host cell (Paper IV).

- To study whether wild animals, i.e. deer and moose, or domestic animals like horse, dog or cat are exposed to rickettsial infections and therefore may be potential reservoir hosts for spotted fever rickettsia (Paper V).
Material and methods

Study material (ticks, animal and human samples)

In **Paper I**, a total of 236 Swedish patients seeking medical attention with symptoms of infectious disease after a previous tick bite were analysed for the presence of rickettsial antibodies. All patients had been exposed to ticks and most of them had been tick bitten during the past month, although the period ranged from 3 weeks to 2 years. 137 of the patients were positive for *Borrelia burgdorferi* IgG, giving a group with confirmed tick bite. Samples analysed for rickettsial antibodies were serum samples collected between 2002–2006 and stored at -20°C. 161 healthy blood donors with unknown history of tick bites were chosen as a control group.

13,260 migrating birds were trapped during spring and autumn 2001 at Ottenby bird observatory, and 1127 ticks parasitizing 437 of the birds were collected for further studies [95, 96]. This gave an infestation rate of 2.1-2.6 ticks per infested bird. Ticks were analysed to identify species and stage and then stored at -70°C until further analysis. Due to loss of sample material during earlier studies, only 957 ticks representing 407 birds were available for rickettsia analysis in **Paper II**. Most of these ticks were identified as *I. ricinus*, but 25 were partly damaged and could only be characterized as *Ixodes* spp. Four nymphs from one bird were identified as *I. lividus*.

In **Paper III**, a 56-year-old woman, included in an ongoing project searching for fastidious organisms, was investigated for presence of rickettsia species. Cerebrospinal fluid (CSF) from the woman was stored in a freezer and retrospectively analysed. The samples were taken when she was hospitalized after three weeks of illness due to worsening headache and fever. Symptoms and laboratory findings indicated sub-acute meningitis.

*Rickettsia helvetica* isolated from the hemolymph of a tick collected by random blanket-dragging from vegetation in central Sweden was used for further inoculation procedures in **Paper IV**. The *Rickettsia helvetica* strain was high passaged and was therefore considered a standard type.

Serum samples collected either from screening of healthy animals before vaccination, from animals investigated for disease or from harvested animals were stored for further analysis by the Swedish National Veterinary Institute.
(SVA). The study in Paper V comprised serum samples from wild animals, deer (n=107) and moose (n=90) and from domestic animals, horses (n=63), cats (n=90) and dogs (n=100). Moose samples represented 47 adult animals and 43 moose calves. Deer and moose samples were collected during hunting season. Horses included were mostly sick animals investigated for microbial agents. Serum samples from cats were both from screening before breading or vaccination and from sick animals. Dog samples were from screening of rabies before vaccination, thus mostly healthy dogs. The majority of the samples were collected throughout Sweden, but a few samples from horses were from Denmark and Iceland. The samples were collected during 2010-2011, with the exception of deer serum, which was sampled during the period 1990-1993.

Analysis techniques

Immunofluorescence (IF)
Indirect micro-immunofluorescence assay was used for analyses of antibodies against rickettsia. The only rickettsia species isolated in Sweden was R. helvetica, and for this reason the antigen was prepared from Vero cell-grown isolates of R. helvetica (isolated from domestic Ixodes ricinus) [2] (Paper I, III, V). There is cross-reactivity among spotted fever rickettsia when using IF, so other species than R. helvetica are also detected. A sample was positive if it showed bright green fluorescence (FITC-conjugated) to rickettsia in a fluorescence microscopy at magnification 1x400 at or above the specific cut-off.

In human samples, rickettsia IgG antibody titres at or above cut-off 1:80 were considered positive (Paper I, III). Antibodies visualized with FITC-conjugated animal specific antibodies; goat anti-deer (moose and deer), -horse, -cat and -dog at or above cut-off 1:64 were considered positive (Paper V).

All deer samples were also analysed for Anaplasma phagocytophilum with commercially available IF-glass (FOCUS Diagnostics, CA, USA), cut-off titre 1:128 (Paper V).

Western blot
Human serum samples positive in IF analysis were also analysed using Western blot to verify the existence of antibodies against Rickettsia helvetica whole cell antigen. The antigen blotting procedure ran over night followed by incubation with serum the day after. The results were visualized using HRP-conjugated IgG (Paper I).
DNA purification

DNA from ticks collected from birds were extracted earlier using Puregene DNA isolation protocol (Gentra Systems) and stored at -20°C for further analyses (Paper II). Bacterial DNA from human CSF and inoculated Vero cells were extracted using automated purification with MagNa Pure kit (Roche Diagnostics GmbH, Mannheim, Germany) (Paper III, IV).

PCR amplification

Real-time PCR

DNA extracts from ticks collected from birds (Paper II), from humans CSF (Paper III) and from inoculated Vero cells (Paper IV) were assayed with a rickettsia genus-specific real-time PCR targeting the citrate-synthase gene (gltA) [97]. The method was chosen because several rickettsia species other than R. helvetica exist outside Sweden and perhaps also inside Sweden, indicating the need for a PCR designed to determine the presence of spotted fever group rickettsia. In each reaction, 0.25 µl LC Uracil-DNA glycosylase (Roche) was included to minimize the risk of contamination.

Conventional PCR

To obtain readable sequence data and to make species differentiation possible, all gltA PCR-positive samples were rerun using two nested PCR assays amplifying parts of the 17kDa-gene and the ompB-gene [98, 99] (Paper II, III). In case of difficulties in distinguishing between the infecting rickettsia species, two additional conventional PCR assays representing the gltA-gene and the ompA-gene were used [79, 100] (Paper II). The ompA-gene is very divergent among SFG rickettsiae, but the gene is not amplified for R. helvetica, therefore it is a good alternative in species differentiation when species other than R. helvetica are suspected. In the human case, the sample was further analysed using the nested PCR assay amplifying the 16S rRNA-gene fragment instead of the ompA-PCR [2] (Paper III). Expected sizes of the PCR fragments were confirmed using gel electrophoresis (2% agarose).

DNA sequencing

All PCR-derived products (amplicons) generated from the nested PCR assays and the conventional PCR assays were analysed using direct cycle sequencing at KI gene (Department for Genetic Analysis at CMM, KI, Stockholm) (Paper II, III). The amplicons were sequenced twice, in the forward and reverse directions, and similarities to and differences from other rickettsiae in the spotted fever group were examined using the Blast function in GenBank.
Isolation
CSF from the patient was inoculated onto Vero cells for bacteria isolation as previously described [101] (Paper III). The cell culture was maintained in Eagle’s medium to allow rickettsiae to multiply. Detection of growing rickettsiae was monitored using Gimenez staining and an immunofluorescence assay. Presence of rickettsial DNA was verified by spotted-fever-specific real-time PCR [97]. Also nested PCR for 17kDa and ompB-genes and sequencing of obtained fragments of the Vero cell grown isolate were performed.

Cultivation
*R. helvetica*, isolated from a tick, was cultivated on Vero cells in three parallel series (Paper IV). The bacteria were inoculated in Eagle’s minimal essential medium, containing 10% foetal calf serum and 1% l-glutamine. After centrifugation, the cell cultures were incubated in a humid cell chamber in 5% CO₂, 32°C for 14 days. A static system, i.e. without addition or replacement of cell medium, was used. The entire content of each well was harvested each 24h interval of infection for each day. Two of the three cultivation series were frozen and later quantified using qPCR; the third series was stored in glutaraldehyde for examination using TEM and light microscopy. The harvested cells and bacteria were centrifuged, and the pellet was resuspended in 200µl PBS. DNA extraction was performed in a MagNA pure LC (Roche). To determine the number of bacterial copies in the inoculation suspension a PCR, based on the gltA gene [97], was performed in a LightCycler 2.0 (Roche).

Immunohistochemistry
For the immunohistochemistry analysis, cultivated cells were fixed on microscope slides and incubated with a specific anti-rickettsial rabbit antiserum [85]. IgG antibodies were detected by FITC-conjugated goat anti-human globulin (Dako, Glostrup, Denmark) (Paper IV).

Transmission electron microscopy (TEM)
For morphological analysis (Paper IV), using TEM, the content from every second day was processed by fixation in 2% glutaraldehyde in a 0.1 M sodium cacodylate buffer (pH 7.2), supplemented with 0.1 M sucrose, post-fixation in 1% osmium tetroxide, dehydration in ethanol and embedding in epoxy resin Agar 100 (Agar Scientific, Stansted, UK). Ultrathin sections were placed on formvar-coated copper grids, contrasted with 4% uranyl acetate and Reynolds lead citrate, and analysed in a Tecnai Bio TWIN electron microscope.
microscope (FEI, Eindhoven, the Netherlands). For immunocytochemical labelling, the Vero cells were processed according to a low-temperature protocol, as previously described [102]. These sections were initially etched in 1 N NaOH for 1 min to compensate for the epoxy resin embedding [102]. Optimal dilution of the specific anti-rickettsial rabbit antisera was 1:50 diluted in 0.05 M TBS pH 7.2. A 10-15 nm gold-conjugated goat anti-rabbit IgG (GAR-G10 or GAR-G15; Amersham International, Amersham, Bucks, UK) was used as a secondary antibody.

Controls

Serum from a patient with proven Mediterranean spotted fever (R. conorii) and an end-point IgG titre of 1:160, confirmed at SMI (Swedish Institute for Infectious Disease Control), was used as the positive control in the IF tests, when human samples were analysed (Paper I, III). Reference controls positive for SFG Rickettsia in IF were not available for each specific animal species analysed (Paper V). Instead positive samples were included in each IF run of animal samples. Phosphate-buffered saline (PBS) was included as negative control in all IF tests (Paper I, III, V).

For the Western blot analyses, a hyperimmune serum from rabbit immunized with R. helvetica was used as the positive control, and the secondary assay antibody alone served as the negative control (Paper II).

Extracted DNA from R. helvetica originally isolated from a domestic Ixodes ricinus was used as the positive control in the PCR assays (Paper II, III). Sterile water was included as the negative control in each amplification trial (Paper II, III, IV).

A standard calibration curve was made from a plasmid containing a gltA fragment of R. helvetica. The plasmid standard was a 10-fold dilution series containing 1.5-1.5 x 10^8 copies, and the standard was used in the real-time PCR for the quantification of R. helvetica organisms in each well from each day of cultivation (Paper IV).

A human serum with an end-point titre of 1/640 for R. helvetica was used as the positive control in the immunohistochemistry analysis and uninoculated cells were used as the negative control (Paper IV).

In the TEM analysis, negative controls were obtained by excluding the primary antiseraum or replacing it with nonimmune serum (Paper IV).
Results and discussion

Seroprevalence of *Rickettsia* spp. in humans

In **paper I**, a serological pilot study conducted on tick-exposed humans (n=236) and on blood donors (n=161) was performed to survey whether and to what extent humans are exposed to SFG rickettsia. We detected rickettsial IgG antibodies at an overall prevalence of 2.6% (10/397). In the tick exposed group, 137 had tested positive for *Borrelia burgdorferi*, which confirms a previous tick bite. This group also had the highest prevalence of antibodies against *Rickettsia helvetica* (4.4%) compared with the tick exposed but Borrelia-negative group (3.0%) and the blood donors (0.6%). This indicates that humans are exposed to infected ticks and are susceptible to rickettsia infection.

Whole cell bacteria from *R. helvetica* were used as antigen in **Paper I**, but cross-reactivity occurs among the spotted fever group. *R. helvetica* is the only tick-transmitted rickettsia species established in Sweden to date, making a presumption of *R. helvetica* as the agent probable.

The *R. helvetica* prevalence detected in **Paper I** is in accordance with previous findings in Europe, e.g., in Denmark where a prevalence of 12.5% was recorded in patients with confirmed borreliosis and in France where 9.2% of forestry workers were seropositive for *R. helvetica* [57, 72]. In a recently published study conducted in the eastern Alps, human seroprevalence rate of IgG antibodies against *R. helvetica* amounted to 7.7% [103].

After the publication of **Paper I**, another seroprevalence study was conducted in the southern parts of Sweden [89]. Samples from patients with erythema migrans (EM) and/or general signs of infection following known or probable tick bite were retrospectively analysed for *Rickettsia* spp., *Borrelia* spp., and *Anaplasma* spp. Out of the 365 analysed samples, 36 (10%) had IgG and/or IgM antibodies against *R. helvetica* antigen above the cut-off.

The geography of tick-borne rickettsioses is determined by the distribution of ticks [70]. In southern and central Sweden, previous studies have detected rickettsia prevalence in ticks ranging from 1.7% to maximum 36.8% when ticks collected from nature and animals were investigated [2, 35, 36, 77]. Seroprevalence in the human population often coincides with the
detection of SFG rickettsiae in ticks from the same region. The present study was conducted in a low endemic area for ticks, which makes it probable that the rickettsia prevalence in humans is higher in tick endemic areas, compared to the findings in Paper I. As in the case with 10% positive patients from the south of Sweden as well as the case with a small survey of military recruits from the highly tick endemic area of Gotland in Sweden, where 22.9% (or 8.9% if only those confirmed with R. rickettsii are counted) had antibodies against R. helvetica antigen [28, 89]. The recruits were followed during their first basic field training period and the prevalence reflects a high exposure to rickettsia. Patients in Paper I, however, had not been exposed to the same extent as the recruits and the prevalence is therefore lower.

In one serum sample in Paper I, the presence of antibodies to R. helvetica was confirmed by Western immunoblot. An antibody-specific response to the membrane proteins in the ~120 kDa region was demonstrated, but no antibodies against lipopolysaccharide (LPS) were detected. The fact that LPS antibodies are absent probably impacts the interpretation of IF results due to a weaker fluorescence signal.

The present study suggests that coinfections between Rickettsia helvetica and other pathogenic agents, in this case Borrelia, can occur in humans in Sweden. The same findings were reported from Denmark and recently also from southern Sweden [72, 89]. In the latter study in Sweden, more than every other patient seropositive for Rickettsia spp. was simultaneously seropositive for Borrelia spp., and one in four patients positive for Rickettsia spp. was also seroreactive for both Borrelia spp. and Anaplasma spp. This makes diagnosing more difficult and reliable tests that can determine whether antibodies are a reaction to recent or previous infection are needed. Paper I shows that humans are exposed and susceptible to rickettsioses and that they develop an immune response that needs to be considered when trying to diagnose an illness after tick exposure.

Rickettsia isolation from human

R. helvetica causes febrile illness, but the bacteria have also been associated with severe symptoms [3-5]. The human case with meningitis presented in Paper III was included from the outset in a larger study in which CSF samples from a total of 631 patients were retrospectively re-examined using real-time PCR to detect Rickettsia spp. The 631 patients were originally investigated based on symptoms and/or laboratory findings indicating possible central nervous system (CNS) infection. Rickettsial DNA was detected in eight of these patients, but not all cases yielded fragments of the right sizes when further examinations with nested PCR were performed. The quality of 430 of the examined samples was probably reduced because the extracted DNA had been used before and stored in a freezer for a long period of time.
One of the patients positive for rickettsia is described in Paper III. The other samples are presented in an upcoming publication.

Sequencing of the amplified fragments in the nested PCR assays showed 99-100% sequence similarity with the corresponding gene fragments of *R. helvetica* (accession no. EU407139, EU407140) and significant sequence differences from the other rickettsiae in the spotted fever group.

Findings in Paper III demonstrated that it is possible to detect *Rickettsia* spp. in CSF, but if the presence of bacteria is low, as suspected, in CSF material, then the amount of bacteria may be near the detection limit for PCR assays. Also very little is known about the extent to which rickettsia-infected patients also develop an invasive infection in the CSF, and the time span of Rickettsemia in the CSF is probably short. All these factors may impact the outcome of the PCR result in CSF and can be an explanation for the low number of patients with rickettsia-positive CSF. For early diagnosis, the first choice of diagnostic method is PCR, because a reliable immune response appears later in the infection, but PCR assays also have limitations.

Inoculation of CSF from the rickettsia-positive patient was performed on Vero cells. After six weeks of bacterial growth on the cells, many intracellular bacteria were observed inside the cells and the presence of rickettsial DNA was verified using real-time PCR. Nucleotide sequences of the 16S rRNA, *ompB*, and 17kDa genes were identical to the sequences from the CSF, and the isolate was identified as *R. helvetica*. This was the first time the bacteria were isolated from CSF, and it shows that the infection is invasive. Isolation of *Rickettsia helvetica* from a patient with suspected meningitis has only succeeded once so far, therefore it is important to conduct further studies in an attempt to understand the association between meningitis and *R. helvetica*. However, isolation of rickettsia from human samples requires patience, because of difficulties associated with these organisms due to their slow growth and obligate intracellular nature [17]. Spotted fever rickettsial diseases has been shown to cause CNS infection, and *R. rickettsii, R. conorii, R. japonica* and *R. felis* have a documented association with meningitis [25, 59, 90].

IF assays showed IgG end titres of 160 and 320 in the early phase serum when the isolates of *R. helvetica* from tick and patient, respectively, were used as antigen. One reason for this may be that the surface protein expressions differ between a rickettsia isolated from its vector and reservoir tick, in comparison with bacteria isolated from humans, but this has to be further investigated. No antibodies in the CSF were detected in Paper III, but the serological method also has not been evaluated for CSF.

Rickettsioses can easily be confused with other febrile illnesses because the symptoms are often non-specific and the disease is sometimes self-healing. This makes it difficult to distinguish rickettsioses from other febrile illnesses and agents causing meningitis.
Growth characteristics

In Paper IV, two parallel cultivation series, with R. helvetica inoculated Vero cells, were analysed separately and the mean value of bacterial count was calculated. Growth characteristics of the bacteria showed a short lag phase at day 1-3 followed by an exponential increase in bacteria with a maximum at day 6-7 before the stationary phase occurred. Similar findings for R. slovaca have demonstrated the highest point of multiplication at day 4 when using standard type bacteria and either Vero cells or L929 cells, but for R. conorii L929 cells were more optimal for growth [104-106]. The earlier maximum of multiplication seen in R. slovaca (day 4) and R. rickettsii (day 2-3) compared with R. helvetica can be explained by their high number of bacteria in the inoculum [104, 107]. Regarding R. helvetica, the number of bacteria in the inoculum is considerably lower leading to a prolonged lag phase. Higher propagation rates and earlier maxima of replication have been detected for wild type strains of bacteria compared with standard type strains [104]. In Paper IV, standard type bacteria were used, but it is likely that a wild type R. helvetica also would give a higher replication rate.

The calculated generation time for R. helvetica was 20-22h which is in accordance with that for R. slovaca (20.34h) [106]. In contrast, a shorter doubling time has been reported for R. prowazekii (10h) and R. rickettsii (9-12h) [16, 17]. Both of them are highly pathogenic species, and cultivation requires a bio safety lab 3, which reflects their pathogenicity. This might be an indication of a slower progress of infection in hosts for R. helvetica and similar species like R. slovaca compared with R. prowazekii and R. rickettsii.

No death phase was recorded for the first 14 days of R. helvetica cultivation. Concerning other species, e.g. R. slovaca (standard type) and R. rickettsii (wild type), the death phase started the same day or one day after the replication peak, with gradual degradation of DNA [104, 107]. This may indicate that the R. helvetica strain used in Paper IV is more stable than what has been reported for R. slovaca and R. rickettsii or is more adapted to the Vero cell line. Another possible reason could be that both the cells and culture medium were harvested and included in DNA analyses, thus explaining the low amount of losses in DNA copies. In electron microscopy, no bacteria with vacuoles typical for the bacteria in the stationary phase were recorded, which indicates that the death phase occurs later than the 14 days studied. This might perhaps influence the establishment of infection in hosts after a tick bite.

Analysis in microscopy demonstrated that R. helvetica bacteria invaded the host cell by phagocytosis and escaped by lysis. Four days post-infection, debris from collapsed cells was revealed. Binary fission was observed as well as sparse penetration of the nuclear membrane in the host cell. Cell-to-cell spread via filopodia filled with rickettsiae was seen in the microscopy.
All of these are characteristic features of spotted fever rickettsia, which is now also demonstrated for *R. helvetica*.

Although the symptoms of a *R. helvetica* infection are generally somewhat milder than what has been reported for Mediterranean spotted fever (*R. conorii*) and Rocky Mountain spotted fever (*R. rickettsii*), no difference in invasiveness for *R. helvetica* in the host cell was seen compared with what has been reported for other SFG rickettsiae.

Vector and reservoirs

Tick infested birds

The most important vector for SFG rickettsia is ticks. In northern Europe, the tick species *Ixodes ricinus* is the most common vector, and it is also considered the main vector implicated in human cases. The only SFG rickettsia detected in ticks in Sweden was *R. helvetica*. In Paper II, we aimed to study the prevalence of SFG rickettsia in ticks collected from birds and also determine the ecological pressure migrating birds contribute to while moving across continents.

The tick samples in Paper II had previously been used for other analyses, therefore extracted DNA from only 957 ticks representing 529 larvae, 409 nymphs and 19 undetermined was available for rickettsial DNA analyses. Most of the ticks were identified as *I. ricinus*, another 25 ticks were damaged and were classified as *Ixodes* spp. and four nymphs were identified as *I. lividus*.

Of the 957 ticks, 216 (22.8%) were collected during spring and 741 (77.4%) during autumn migration. Using the rickettsia genus-specific PCR, 108/957 (11.3%) ticks were found to be infected with a *Rickettsia* spp. The result is in accordance with findings published shortly after Paper II, where a prevalence of 7.2% in ticks collected from birds in a nearby area of the Baltic see was reported and a prevalence of 15.1% in bird-feeding ticks from north-western Russia [108, 109]. The time span of seven years between the collected samples in the three studies shows an established level of rickettsia-infected ticks in the region.

Due to the small volumes of extracted DNA in Paper II, only 81 of 108 samples that were positive in the genus-specific PCR could be further analysed with nested PCR. Sequence analysis of the amplicons from the nested PCR assays revealed *R. helvetica* in 57 of the samples (sequence similarity with *R. helvetica* in GenBank, 99-100%), while 24 samples represented other spotted fever rickettsiae. Out of the 24 samples, 9 had highest similarity with *Rickettsia monacensis* for the ompB gene and *R. monacensis* or strain IrITA3 for the 17kDa gene. The strain IrITA3 has highly similar sequences with
strain IRS3, which clusters phylogenetically with *R. monacensis*, making *R. monacensis* as the most likely infecting agent [110, 111].

In 15 samples, the ompB and 17kDa sequences showed the highest similarity (97-99%) with *Rickettsia japonica*, *marmioni*, *heilongjiangii* and *heilongjiangensis*, while the ompA sequence had 100% similarity with *Rickettsia* sp. strain Davousti. There are no registered sequences in GenBank for 17kDa and *ompB* genes for *R. sp. strain Davousti*. Phylogenetically, *R. sp. Davousti* is placed into the *R. japonica*-like group, where Davousti clusters with *R. japonica*, *R. heilongjiangensis* and another nine species [112, 113].

Because of the close relationship between SFG rickettsia species and the fact that not all genes from the suggested rickettsia species are available in GenBank, it is difficult to determine the exact species infecting 15 of the ticks. Also the limited amount of extracted DNA is a problem for species determination. It is well known that the primer set of OmpA does not detect *R. helvetica* [79], which confirms the findings of species different from *R. helvetica* owing to their positive result in OmpA PCR.

Shortly after the publication of Paper II, another two reports from similar studies were published, one conducted on an island in the Baltic Sea and one in north-western Russia [108, 109]. At both collection sites, all analysed ticks were identified as *I. ricinus* and the ticks were infected with *R. helvetica* and *R. monacensis*; in Russia also *R. japonica* was found. The studies did not present evidence that migratory birds have reservoir status, but did show that migratory birds act as vehicles in the spread of pathogenic rickettsia species over long distances, consistent with our findings.

In Paper II, four nymphs from the same bird were identified as *I. lividus*, three of these were positive for *R. sp. strain Davousti* belonging to the *R. japonica* group. Similar findings are reported from the UK where five *I. lividus* ticks were infected with a *R. sp* closely related to the *R. japonica*-like group [113].

*R. helvetica* was the only SFG rickettsia species reported in ticks from Sweden until the *R. monacensis* and *R. sp. strain Davousti*-like species was identified in Paper II. Since then another species, *R. sibirica*, has been found in a tick collected from a dog [36].

Higher rickettsia prevalence was detected in ticks from birds migrating during spring season (68/108=63%) than during autumn season (40/108=37%). Several bird species are long-distance migrants passing the Mediterranean area, where they are infested with infected ticks on their way to Sweden. In the present study, the ground-foraging birds were four times more likely to carry infected ticks (59/6174=1%) than other bird species were (18/7085=0.25%). This indicates that the former group of birds has a higher risk of exposure to ticks as a result of their feeding habits, which is the case for *Borrelia* sp. [95]. Rickettsiae were more common among nymphs (68/409=16.6%) than among larvae (36/529=6.8%). The higher
prevalence in nymphs is presumably a result of accumulation of infection during consecutive feedings.

To investigate the birds’ role as reservoir host for *Rickettsia* spp., the infestation with larvae and the prevalence of *Rickettsia* spp. in the larvae were studied. It is difficult to investigate an animal’s reservoir competence when the infecting agent is transovarially transmitted in the tick, as is the case with SFG rickettsia. When transovarial transmission of a pathogen is absent or very rare, as is the case with *Borrelia* spp., *A. phagocytophilum*, and *Babesia microti*, detection of the agent in feeding larvae is an indication of pathogen transmission from an infected reservoir host to the tick [65]. Therefore, the proportion of birds infested with multiple infected larvae and the observed counts of infected larvae on individual birds were calculated in Paper II. To investigate whether or not larvae were infected by the birds, 215 larvae-infested birds were analysed by comparing the proportion of birds with infected larvae among birds with a single larva and the proportion of birds with ≥1 infected larva among birds with ≥2 larvae. The prevalence of rickettsia infection in larvae was 9/127 (7.1%) individuals among single infested birds and 22/86 (25.6%) individuals among multiple infested birds. The higher prevalence of infection in larvae among multiple than among single infested birds could be an indication of either reservoir competence among birds or transmission between ticks via a co-feeding mechanism. To investigate which alternative is the most likely, the count of infected larvae at 7.1% prevalence (single infested) was compared with the observed prevalence of infection after the first positive larva had been identified. 22 birds were multiple infested with 102 larvae, of which 28 were positive. The expected count of positive larvae was (102-22) x 7.1% = 5.7 larvae compared to the 6 observed, which does not support the reservoir theory.

On the other hand, a blue tit, included in Paper II, was suspected of acting as a competent reservoir for *Rickettsia* spp., because the blue tit was infested with two *Rickettsia* spp.-positive tick species, *I. ricinus* and *I. lividus*, and six out of seven collected ticks from the bird were infected with the same rickettsia species. This might indicate the ability for some passerine bird species to act as rickettsial disease reservoirs. In a study from Cyprus, three percent of birds’ blood samples were positive for *Rickettsia* spp., indicating an ongoing rickettsemia [114].

Birds are reservoirs of several agents [95, 115], but even if the results in Paper II do not indicate that this applies to spotted fever rickettsia, it is shown that migratory passerine birds are an important factor for the distribution and expansion of *Rickettsia* spp.

**Mammals**

Roe deer and other medium- and large-sized mammals are known to be important blood hosts for the *I. ricinus* tick [42]. Several studies have investi-
gated the presence of *Rickettsia* spp. in their tick vector, but data on the natural life cycle of SFG rickettsia in Europe are limited. Identifying natural infection is a useful step to determining natural hosts that may constitute potential reservoirs. Natural infection is determined by identifying previous infection through antibody detection [116]. In **paper V**, we analysed serum samples from wild and domestic mammals in Sweden to see whether they are natural hosts and potential reservoirs of *Rickettsia* spp. Out of the 450 samples analysed, 104 (23.1%) contained IgG antibodies against *R. helvetica*. The detected seroreactivity is in line with other international investigations, e.g. in Germany where 23.3% of small mammals showed a positive antibody reaction to *R. helvetica* [29]. The serosurvey on humans in Sweden, presented in **paper I**, showed a prevalence of rickettsial antibodies in 2.6% of the local population. It is expected that the seroprevalence of *Rickettsia* spp. in wild and domestic animals should be higher than in humans, which is a probable reflection of the greater exposure of animals to tick vectors.

In detail, considering animal species, the numbers of sera found positive in **paper V** were: 23/107 (21.5%) among deer, 21/90 (23.3%) among moose, 23/63 (36.5%) among horses, 20/90 (22.2%) among cats and 17/100 (17%) among dogs.

The lowest value of seroreactivity was detected in the dog population (17%), representing the only analysed animal species without a free roaming habitat and therefore also the least tick exposed group. Dogs are also frequently treated with tick-repellent by their owners. Cat samples were found to have a seroreactivity of 22%. Horses, on the other hand, showed the highest value of seroreactivity (36.5%). One explanation could be that the horses were sick animals investigated for microbial infections, while the other analysed groups consisted of mostly healthy animals. The prevalences detected in **paper V** should probably have been even higher, if domestic animals were not treated with agents for debugging on a regular basis. The role of domestic animals in the ecology of *Rickettsia* spp. is of interest because of their close connection to humans and their ability to spread diseases.

**Paper V** is the first report of detection of rickettsial antibodies in serum samples from moose (*Alces alces*). A prevalence of 23.3% was found, which is similar to the prevalence in deer (21.1%). Moose and deer are often infested with a large number of ticks. In Japan, *R. helvetica* DNA was detected in the blood of Sika deer in 7.8% of the analysed animals, indicating that this species may represent a potential reservoir host of *R. helvetica* [31].

Deer samples in **paper V** were also tested for antibodies against *Anaplasma phagocytophilum* and 91/107 (85%) tested positive. This is in line with previously reported data from Denmark, where 96.6% of the examined roe deer were positive, and deer are probably the main natural host for *A. phagocytophilum* [117].

*Rickettsia* spp. are characterized by their transovarial and transstadial mode of transmission, and all tick stages are infective for mammal hosts.
Infection of mammals may result in a rickettsemia that allows non-infected ticks to become infected, enabling the life-cycle to continue. In this perspective, the tick is considered as the actual reservoir and the animal as an intermittent reservoir during the transient rickettsemia [19]. During this short time of rickettsemia, the animal may be involved in further geographical dispersion of rickettsia bacteria. This assumption is supported by a previous study, where feeding ticks collected from animals were PCR positive at a higher rate than free-living ticks in nature [77]. The seroreactivity (Paper V) against SFG rickettsia among animals in Sweden is high, which identifies the country as an endemic region for exposure to these bacteria.

As the serosurvey in Paper V only involved single sera from the animals without convalescent serum or DNA samples being available, it is not possible to determine whether the animals were currently infected with a SFG rickettsia or had been exposed in the past. Most of the analysed samples were anonymized, thus it was not possible to determine the correlation between animal sickness and seropositivity. Even though animals positive for rickettsia might not show clinical signs of infection, they may act as natural hosts and potential reservoirs and could be involved in further geographical dispersion of Rickettsia bacteria.

In addition to the samples presented in Paper V, other samples from small and medium-sized mammals have also been analysed earlier for rickettsial DNA. The study material comprised blood from wild animals (n=24), e.g. fallow deer, red deer, roe deer, moose, fox and pig. Also biopsies from the lung, kidney, bladder and heart of field mice (n=18) and deer (n=1) were included, as well as biopsies from Norwegian moose (n=44). Rickettsia genus-specific real-time PCR, targeting the gltA-gene was used. None of the samples tested had detectable DNA. The low number of samples impacts the outcome. Biopsies from field mice were primarily from internal organs, while other studies have shown that skin biopsies are more suitable for detection of rickettsia by PCR in mammals. A study in Germany, on small mammals, showed that rickettsial organisms are best detected in ear tissue [29].

Climate changes

The geographical distribution of several tick-transmitted diseases corresponds to the distributional area of its tick vector Ixodes ricinus [34].

Ticks and their hosts are affected directly or indirectly by temperature and humidity in their environment. An increase in the daily mean temperature gives both a prolonged vegetation period and a movement of the northern limit of the vegetation period. Changes in weather and climate will influence the occurrence, distribution, abundance, activity and survival of ticks, but climate changes will also impact its hosts, especially deer and other medium-
and large-sized mammals [33, 39, 40]. This, in turn, affects the occurrence and distribution of tick-borne pathogens, for example the densities of *I. ricinus* nymphs and Borrelia-infected nymphs are significantly correlated [33]. *Rickettsia* spp. may also become more abundant and expand its distribution range northwards when the climate changes, which is the case for TBE virus and *Borrelia burgdorferi* [34, 41].

The rickettsia seroprevalence detected in Paper I (2.6–4.4%) was conducted in a low endemic area for ticks, but an increasing number of ticks in those areas will most likely also affect the rickettsia prevalence in humans. Weather patterns also affect disease risk by influencing human behaviour in the short term, which means that humans spend more time outdoors and are exposed to ticks to a greater extent when the temperature is higher.

The incidence of other tick-borne diseases, like Lyme borrelioses and TBE, has increased during recent years [34, 41], and there is evidence of an increased incidence during the winter months probably due to increased tick activity resulting from global warming [118]. Because domestic Rickettsioses have not been diagnosed previously, there are no statistics on incidence in Sweden. It is likely that Rickettsioses are affected in the same way as other tick-borne pathogens. One study on patients positive for rickettsia in France revealed that increased temperature lead to an increased period of activity of Ixodid ticks as well as an increased aggressiveness to bite humans [119].

Birds are transporters of ticks and frequently disperse them large distances which contribute to an expanded distribution range. Birds are also responsible for import of novel tick-borne rickettsiae into new localities, as shown in Paper II. The establishment of novel pathogenic rickettsia species, for example *R. monacensis* and *R. japonica*-like species dispersed by migrating birds as reported in Paper II, may be a reality when climate changes are beneficial to new rickettsia species and their ability to survive in Sweden.

Vertebrates and especially larger mammals like deer and moose are often infested with a high number of ticks. Seroreactivity against *Rickettsia* spp. among wild and domestic animals, as presented in Paper V, demonstrates that mammals are exposed to ticks infected with rickettsia and that they develop an immune response. A higher tick infestation rate among animal hosts of rickettsia will be the reality when abundance and activity of ticks increases as a consequence of climate warming. The animal hosts of rickettsia are also capable of contributing to the expansion of the rickettsia distribution range.
Conclusions

- **Paper I** shows that humans are exposed and susceptible to rickettsioses and that humans develop an immune response. Patients with a confirmed tick bite (positive borreliosis) have a higher prevalence of rickettsial antibodies than blood donors. Coinfection with rickettsiae can occur in patients with acquired borreliosis, and people exposed to ticks are also exposed to rickettsiae. Spotted fever rickettsioses are likely to be endemic in Sweden and should be taken into consideration in the diagnosis of tick-transmitted infections.

- **Paper II** shows that migratory birds are important in the distribution and expansion of *Rickettsia* spp., but they do not seem to serve as reservoir hosts for these bacteria even though birds might be rickettsiemic. *R. helvetica* was the only tick-transmitted spotted fever rickettsia reported in Sweden prior to findings revealing another two species of ticks infesting birds. The inflow of novel *Rickettsia* spp. by birds into the environment provides opportunities for establishment of new endemic areas with new rickettsia species.

- **Paper III** shows that *R. helvetica* can be an invasive infection and a possible agent for suspected meningitis. For early diagnosis, the method of choice should be PCR. Isolation of *R. helvetica* bacteria from a patient’s CSF is possible. Although rickettsial isolation in culture remains the most definitive diagnostic method, this technique is typically performed only in reference laboratories. When seeking to diagnose possible agents of meningitis, the relevance of the broader clinical spectrum of acute febrile illness caused by *R. helvetica* should be considered.

- **Paper IV** shows that *R. helvetica* possesses features typical for the rickettsia bacteria, like binary fission, penetration of the cell nuclei and formation of philopodia. Furthermore, the bacterial growth was characterized by a lag-, exponential and stationary phase, but with a slower generation time than for other rickettsia species. Invasiveness of *R. helvetica* in host cells seems to be the same as for other SFG rickettsia reported.
Paper V shows that both wild and domestic animals in Sweden are exposed to *Rickettsia* spp. to a great extent and that the bacteria are causing an immune response in their hosts. The reservoir competence of the animals has not been fully investigated, but although the animals may not develop clinical symptoms of infection, they may act as potential reservoir hosts and be involved in further geographical dispersion of rickettsia bacteria.

Avhandlingen beskriver delar av smittkedjan för SFG rickettsia i Sverige. Bakteriernas förekomst i fästingar plockade från fåglar har studerats, likaså det ekologiska tryck som flyttfåglars bärarskap av infekterade fästingar bidrar med när de korsar olika världsdelar. Mer än var tionde fästing var infekterad med rickettsiabakterier, i huvudsak *R. helvetica*. Det visade sig att flyttfåglar bidrar inte bara till långväga spridning av bakterier utan även till införsel av nya potentiellt patogena rickettsiaarter. I detta fall identifierades *R. monacensis* och en *R. sp* strain Davousti liknande art.

Vidare analyserades seroreaktivitet mot *Rickettsia helvetica* hos både tamdjur och vilda djur, vilket visade på antikroppsutveckling, som uttryck för smittexposition, i mer än vart femte djur. Djurens roll som reservoar för bakterien är inte klarlagd, men oavsett är djuren indirekt involverade i spridningen av bakterien till människa via infekterade fästingar som suger blod.

Seroreaktivitet hos människa har också studerats. Patienter, provtagna på grund av misstanke om borreliainfektion, samt blodgivare hade detekterbara antikroppar mot rickettsia, med högst prevalens i gruppen med misstänkt borreliainfektion. Fynden visar att människor i Sverige är exponerade för och utvecklar en immunreaktion mot rickettsia. Att *R. helvetica* skulle kunna ge allvarlig sjukdom verifiersas av ett patientfall med subakut meningit där bakterien för första gången visats ge invasiv infektion med påverkan på nervsystemet (CNS engagemang) och där bakterien isolerats från patientens ryggmärgsvätska.

Morfologi och tillväxtegenskaper för *R. helvetica* undersöktes för att bättre förstå bakteriens invasivitet och virulens. Fynden indikerar att invasiviteten är jämförbar med andra rickettsiaarter men *R. helvetica* verkar ha en stabil men något långsammare tillväxt.

*Rickettsia helvetica* är endemisk i Sverige och måste tas i beaktande vid sjukdomsutredning efter ett fästingbett.
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