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# ***Bartonella* Infections in Sweden: Clinical Investigations and Molecular Epidemiology**

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#### **Abstract**

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Characteristically, in infections that are caused by the zoonotic pathogen *Bartonella* naturally infected reservoir hosts are asymptomatic, where infected incidental, non-natural, hosts develop symptomatic disease. Cat-scratch disease (CSD) is a well known example. *Bartonella* infections in humans may be self-limiting or fulminant and affect different organ systems.

The objectives of the present thesis were to (1) identify and characterise *Bartonella* infection cases in Sweden, (2) to investigate certain human populations regarding *Bartonella* infections, and (3) compare natural populations of different *Bartonella* species.

Cases with typical and atypical CSD were recognised by using a combination of PCR and serology. Gene sequence comparisons of different genes in *B. henselae* isolates from the United States and Europe showed that *ftsZ* gene variation is a useful tool for *Bartonella* genotyping.

Myocarditis was a common finding among Swedish elite orienteers succumbing to sudden unexpected cardiac death (SUCD). The natural cycle of *Bartonella* spp., the life style of orienteers, elevated antibody titres to *Bartonella* antigens, *Bartonella* DNA amplified from myocardium and the lack of another feasible explanation make *Bartonella* a plausible aetiological factor.

The first reported case of *Bartonella* endocarditis (*B. quintana*) was identified in an immunocompromised patient who underwent heart valve replacement. The patient had been body louse-infested during his childhood. It is hypothesised that a chronic *B. quintana* infection was activated by the immunosuppression.

There was no evidence of an ongoing trench fever (TF) epidemic in a Swedish homeless population, although an increased risk for exposure to *Bartonella* antigens was demonstrated. The lack of louse infestation might explain the absence of *B. quintana* bacteremia and low *B. quintana* antibody titres.

Comparisons of genetic loci and the whole genomes of environmental *B. grahamii* isolates from the Uppsala region, Sweden displayed variants that were not related to specific host species but to geographic locality. Natural boundaries seemed to restrict gene flow.

*Keywords:* Bartonella, clinical studies, molecular epidemiology

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”Katten sade: Jam,  
Jag är snäll och tam,  
Jag är snäll och inte stygg,  
Du får åka på min rygg...  
Och så sade dom adjö,  
Katten sprang så han blev rö.”

Ivar Arosenius, Kattresan

To Anna, Gustav, Ingrid and Sofia



## List of papers

The thesis is based on the following papers, which are referred to in the text by their roman numerals:

- I Ehrenborg, C., L. Wesslen, A. Jakobson, G. Friman, and M. Holmberg. 2000. Sequence variation in the *ftsZ* gene of *Bartonella henselae* isolates and clinical samples. *J Clin Microbiol* 38:682-7.
- II Wesslen, L., C. Ehrenborg, M. Holmberg, S. McGill, E. Hjelm, O. Lindquist, E. Henriksen, C. Rolf, E. Larsson, and G. Friman. 2001. Subacute *Bartonella* infection in Swedish orienteers succumbing to sudden unexpected cardiac death or having malignant arrhythmias. *Scand J Infect Dis* 33:429-38.
- III Berglund, E., C. Ehrenborg, O. Vinnere, M. Holmberg, and S.G.E. Andersson. 2007. Evolutionary Genomics of Geographically Close *Bartonella grahamii* Populations Infecting Woodland Rodents. Manuscript.
- IV Ehrenborg, C., R. Byström, E. Hjelm, G. Friman, and M. Holmberg. 2007. High *Bartonella* spp. seroprevalence in a Swedish homeless population but no evidence of trench fever. Submitted.
- V Ehrenborg, C., S. Hagberg, S. Mäkitalo, G. Myrdal, E. Larsson, E. Hjelm and G. Friman. 2007. Blood culture-negative *Bartonella quintana* endocarditis requiring heart valve replacement: First reported case of *Bartonella* endocarditis in Sweden. Manuscript.

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## Abbreviations

ARVC	Arrhythmogenic right ventricular cardiomyopathy
B	<i>Bartonella</i>
BA	Bacillary angiomatosis
BadA	<i>Bartonella</i> adhesion A
Be	<i>Bartonella elizabethaea</i>
Beps	<i>Bartonella</i> -translocated effector proteins
Bh	<i>Bartonella henselae</i>
Bq	<i>Bartonella quintana</i>
CGH	Comparative genome hybridisation
CSD	Cat scratch disease
DNA	Deoxyribonucleic acid
ECs	Endothelial cells
<i>ftsZ</i>	Cell division protein gene
FUO	Fever of unknown origin
<i>gltA</i>	Citrate synthase gene
HIV	Human immunodeficiency virus
IFA	Indirect fluorescent-antibody assay
Ig	Immunoglobulin
IVD	Intravenous drug
MLST	Multilocus sequence typing
MRT	Magnetic resonance tomography
MST	Multispacer typing
OR	Odds ratio
PCR	Polymerase chain reaction
PFGE	Pulse field gel electrophoresis
POS	Parinaud's oculoglandular syndrome
QPCR	Quantitative PCR
RFPL	Restriction fragment length polymorphism
<i>rrs</i>	16S rRNA gene
ST	Sequence type
SUCD	Sudden unexpected cardiac death
Spp	Species
TF	Trench fever
TFSS	Type four secretion system
Vomps	Variably expressed outer membrane proteins
VEGF	Vascular endothelial growth factor



# Introduction

## The history of *Bartonella*

At the end of the 19th century, the young Peruvian medical student Daniel Alcides Carrión inoculated himself with the blood of a Verruga Peruana patient to try to prove that there was not a common origin of Verruga Peruana and an anaemic febrile disease called Oroya fever. After having kept record of the following signs and symptoms, he died 21 days later in a disease identical to Oroya fever thereby having proved his thesis was faulty. Ever since, the first known human bartonellosis is called Carrión's disease. Verruga Peruana is the eruptive phase of Oroya fever which presents – if the patient survives the first phase – as dermal angioproliferative lesions. The causative agent of this disease is *Bartonella bacilliformis*. It is only known to occur in endemic regions in the South American Andean valleys between 500 and 3200 m above sea level in Peru, Colombia and Ecuador [1]. The bacterium was first described in Peru in 1909 by Alberto Barton [2].

Until the early 1990s, *Bartonella bacilliformis* was the only member of the genus *Bartonella* but because of advances in genetics-based bacterial classification and taxonomy, the former genera *Grahamella* [3] and *Rochalimaea* [4] were soon merged into an expanded genus named *Bartonella*. Since its reclassification, the genus *Bartonella* is constantly expanding and by now comprises approximately 25 species and subspecies, many of which are reported pathogenic to humans. Although most diseases we are aware of today as related to *Bartonella* infections have been known for a long time, their etiology was established just recently. In the 1980s, during the years before effective anti-retroviral medication, the AIDS epidemic gave rise to a multi-fold of opportunistic infections, including bacillary angiomatosis (BA) that is caused by *B. henselae* or *B. quintana*. Attempts to isolate the causative agents were successful to a much higher extent than before or ever since because of the immunocompromised state of the host. The AIDS epidemic in combination with the rapidly evolving molecular techniques created the necessary conditions for the onset and fast expansion of today's *Bartonella* research. Thus, bartonelloses are still referred to as emerging infections.

## Bacteriology

Members of the genus *Bartonella* are gram-negative rods, oxidase- and catalase-negative, and typically difficult to isolate. The bacteria can grow both on a free medium or intracellularly. The bacterium is haemin dependent and grows better with increased carbon dioxide pressure [5]. The optimal growing temperature is 35-37°C for all *Bartonella* spp. except *B. bacilliformis*, which prefers 28°C. The latter also expresses a flagella-like *Bartonella schoenbuchensis* and *B. clarridgeiae* but in contrast to other known *Bartonella*. The bacterium grows slowly. Primary isolation from infected humans may be very difficult and the first colony might be visible only after a prolonged incubation of 45 days. When subculturing, the generation time usually decreases successively to 4-5 days. Further, the appearance of the colonies changes after several passages from rough, adherent to smooth and less adherent colonies. This phenomenon is called phase variation and the mechanisms behind it are not fully known. It is likely caused by a variable expression of outer membrane proteins as a strategy for the bacteria to evade the host's immune response and permit adaptive interaction with different host structures [6]. *Bartonella* spp. are facultative intracellular bacteria. *Bartonella* may invade and persist in red blood cells and endothelial cells (ECs). Thus, it evades the host's immune response and increases its transmittability by blood-sucking arthropods [7].

## Molecular biology

The genus *Bartonella* is a member of the alpha sub-division of the phylum Proteobacteria. There are around 6000 species of  $\alpha$ -proteobacteria that are grouped in seven orders among which the genus *Bartonella* is a part of the order Rhizobiales (fig. 1).

The complete genomes of *B. henselae* H-1 and *B. quintana* (strain Toulouse) were recently published [9]. Both genomes contain a single circular chromosome and display a high degree of overall similarity. The size of the *B. henselae* genome was estimated to 1.93 Mb and *B. quintana* to 1.58 Mb. The backbone of the two genomes is homologous with the exception of one 55 kb prophage region and three genomic islands present in the *B. henselae* genome but absent in the *B. quintana* genome. These observations suggest that *B. quintana* is a reduced genomic derivative of *B. henselae*. This might be a consequence of the differences in vector-host ecology between the two species. *B. quintana* is more specialised in solely infecting humans with the body louse as a vector, whereas *B. henselae* is more of a generalist using vectors of broader host ranges. The utilisation of host-restricted vectors is associated with accelerated rates of genome degradation [9]. The close

genetic relationship between *B. henselae* and *B. quintana* is illustrated in a phylogenetic tree within the genus (fig. 2).

## Epidemiology

*Bartonella* infections are mainly regarded as zoonotic involving different modes of transmission, either directly from animal to human (e.g., through a cat scratch) or with arthropod vectors (Table 1). The natural cycle of *Bartonella* infections is outlined in figure 3. Naturally infected reservoir hosts are generally asymptomatic or display minor symptoms of their infection. In contrast, incidentally infected hosts are normally symptomatic. Bacteremia with different *Bartonella* spp. in seemingly healthy animals has been reported in elk [11], cattle [11-13], deer [11], roe deer [13,14] rabbits [15], squirrels [16,17], cats [18], dogs [19,20], coyotes [21] and rodent populations [22-24]. Humans are thought to be the natural hosts of *B. bacilliformis* and *B. quintana*. Asymptomatic bacteremia in humans with both *Bartonella* spp. has been encountered. However, as already mentioned, Carrion's disease may be fulminant with a high mortality rate if untreated.

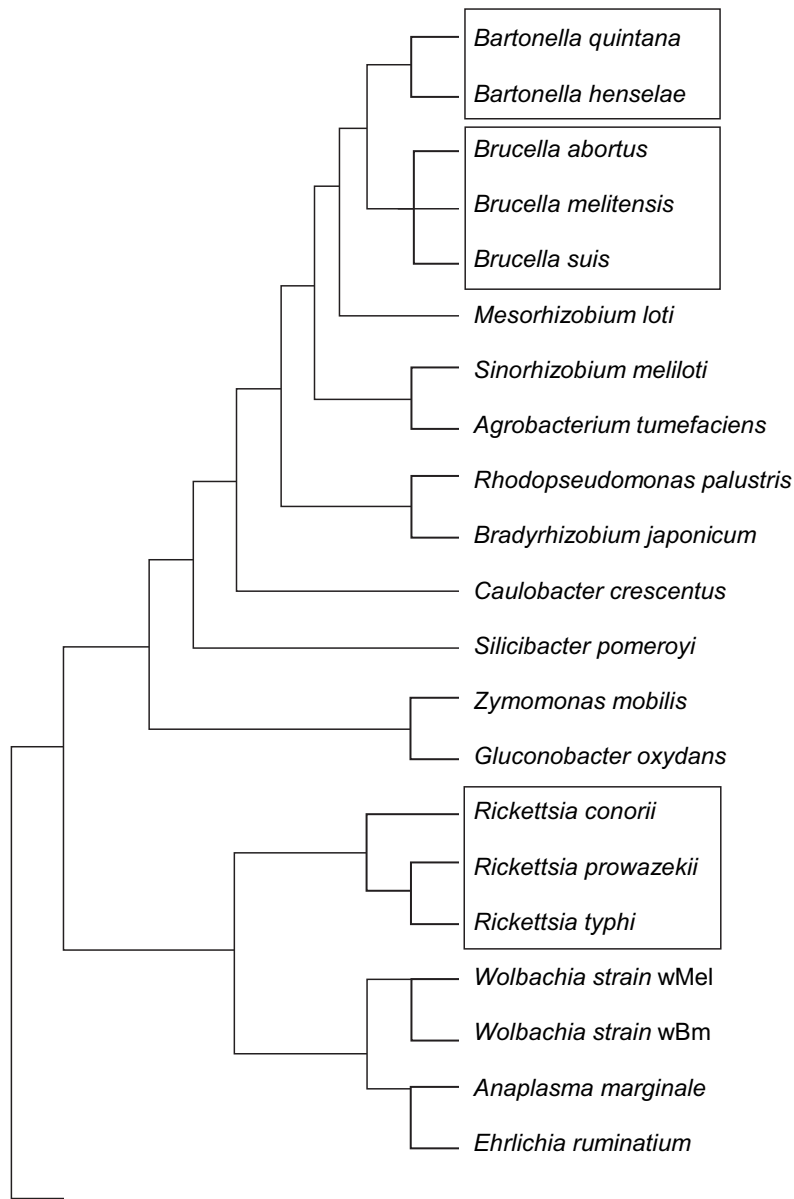


Figure 1. Adopted from reference [8]. Phylogenetic tree reconstruction inferred using the maximum likelihood method for  $\alpha$ -proteobacterial species of which the complete genome sequence is known. Only topology is shown. *Bartonella*, *Brucella* and *Rickettsia* genera are highlighted.

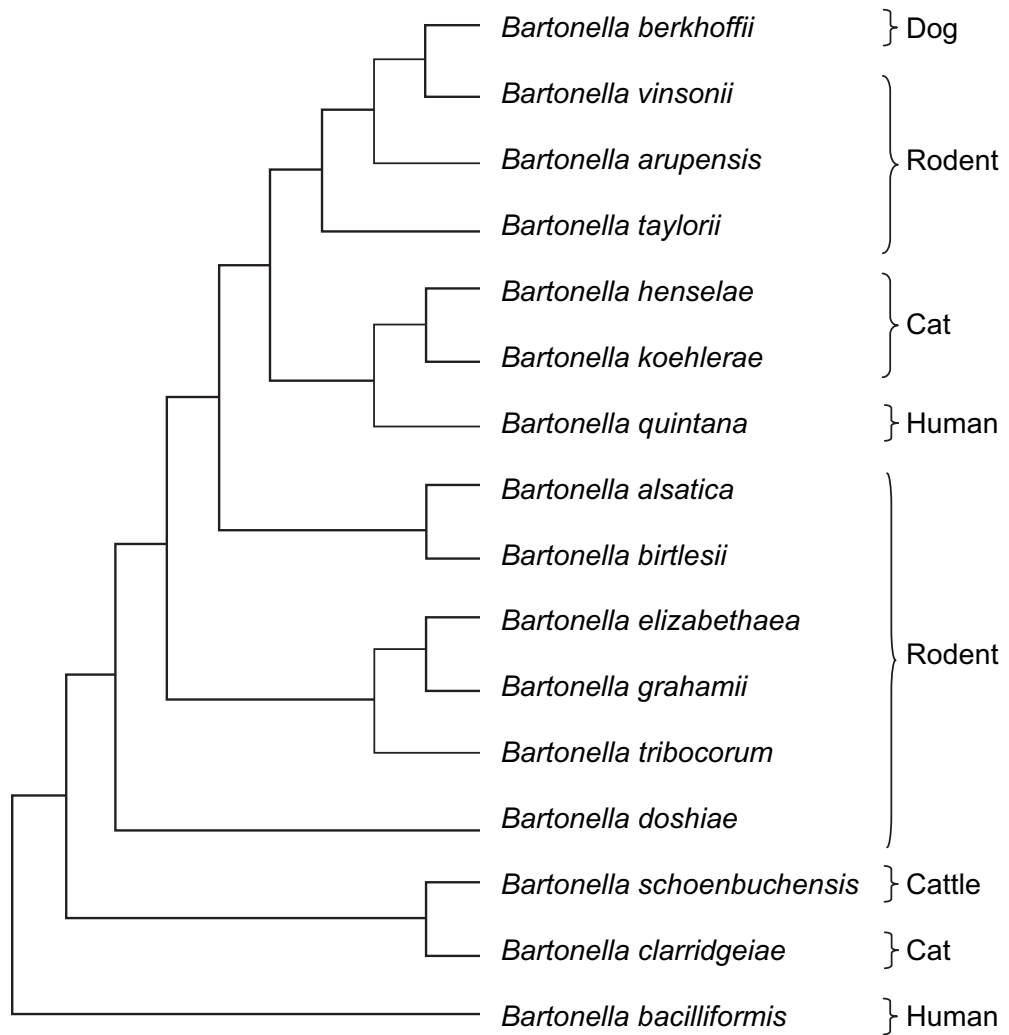


Figure 2. Adopted from reference [10]. Unrooted neighbour-joining tree resulting from comparison of *Bartonella gltA* sequences. Only topology is shown. The natural hosts for different *Bartonella* spp. are shown.

## Clinical manifestations of human bartonelloses

### Cat scratch disease

Cat scratch disease (CSD) was first described by Debré et al. in 1950 [25] but its etiology could not be established until the early 1990s. CSD is mainly caused by *Bartonella henselae* and is the most frequent clinical

manifestation of *Bartonella* infections in immunocompetent patients [26]. *Bartonella henselae* is inoculated after a cat scratch or bite and the clinical manifestations include a papular lesion of the skin and tender, enlarged lymph glands in the region of the scratch. Symptoms occur 1-7 weeks after infection [27] and usually resolve spontaneously in immunocompetent individuals. Healthy cats bacteremic with *B. henselae* constitute the infection's reservoir [18]. The cat flea (*Ctenocephalides felis*) is a proven vector among cats [28]. Traditionally, the diagnosis is based on a set of criteria from a combination of clinical (papular lesion with regional lymphadenopathy), epidemiological (cat scratch), serological (IFA), bacteriological (PCR) and histological data. The typical CSD histology shows a granuloma with central necrosis, multinucleated giant cells and microabscesses.

However, a positive *Bartonella* PCR result from an enlarged lymph node is sufficient for the CSD diagnosis even though the other criteria would not be fulfilled. If PCR would be negative, the diagnosis of CSD might be retained if two of the following criteria are fulfilled: Positive serology, cat contact and elimination of any other cause of lymph node enlargement [27].

### Atypical cat scratch disease

Although CSD is the most common presentation, there is a broad spectrum of clinical manifestations associated with *B. henselae* infection. These non-CSD *B. henselae* manifestations are called atypical and constitute approximately 15% of all *B. henselae* infections [29]. Among the atypical manifestations are bacillary angiomatosis (BA)[30], encephalopathy [31], neuroretinitis [32], Parinaud's oculoglandular syndrome (POS) [33], liver peliosis [30], endocarditis [34], myocarditis [35], fever of unknown origin (FUO) [36] and osteomyelitis [37,38].

Diagnostic criteria for atypical CSD are not fully established. However, specific pathological changes in different organs (e.g., neuroretinitis, myocarditis or endocarditis) and a positive *Bartonella* PCR from that tissue and the elimination of any other aetiological cause would indicate that *Bartonella* is the causative agent of the pathological changes. In cases of negative PCR the diagnosis of atypical CSD might still be valid if serology is positive, epidemiological information (cat contact, louse infestation etc.) is coherent with *Bartonella* infection and other causes are eliminated. Since atypical CSD is less common than classical CSD, it would be plausible to argue that the aetiological evidence needs to be stronger for atypical than for classical CSD. Thus, a positive PCR and /or a positive *Bartonella* culture would be mandatory for the atypical CSD diagnosis.

The diseases of neovascularisation (BA and liver peliosis) are confined to immunocompromised individuals. Encephalopathy including encephalitis and meningitis is the most frequent neurological manifestation of CSD and carries a good long-term prognosis[31]. There is a sudden onset of



symptoms of which convulsion is the presenting sign in 50% [39]. Antibodies to *B. henselae* can be detected in the spinal fluid suggesting a direct infection of the central nervous system (CNS) [40].

Patients with CSD neuroretinitis present with fever, malaise and blurred vision following cat exposure. Doxycycline and rifampin appear to shorten the course of disease and hasten visual recovery. Long-term prognosis is good, but some individuals may acquire a mild postinfectious optic neuropathy [41].

POS presents with conjunctivitis and parotid area swelling that are due to lymphadenitis. Symptoms spontaneously resolve within 2-4 months [42].

Bacillary angiomatosis is a proliferative vascular disease recognised predominantly in immunodeficient patients with *B. quintana* and *B. henselae* as aetiologic agents [43]. The vascular proliferations occur in various organs but above all in the skin [44] and respond promptly to macrolide antibiotic treatment because of its antiangiogenic effect, rather than to its antimicrobial effect. The time of treatment should be three months [45].

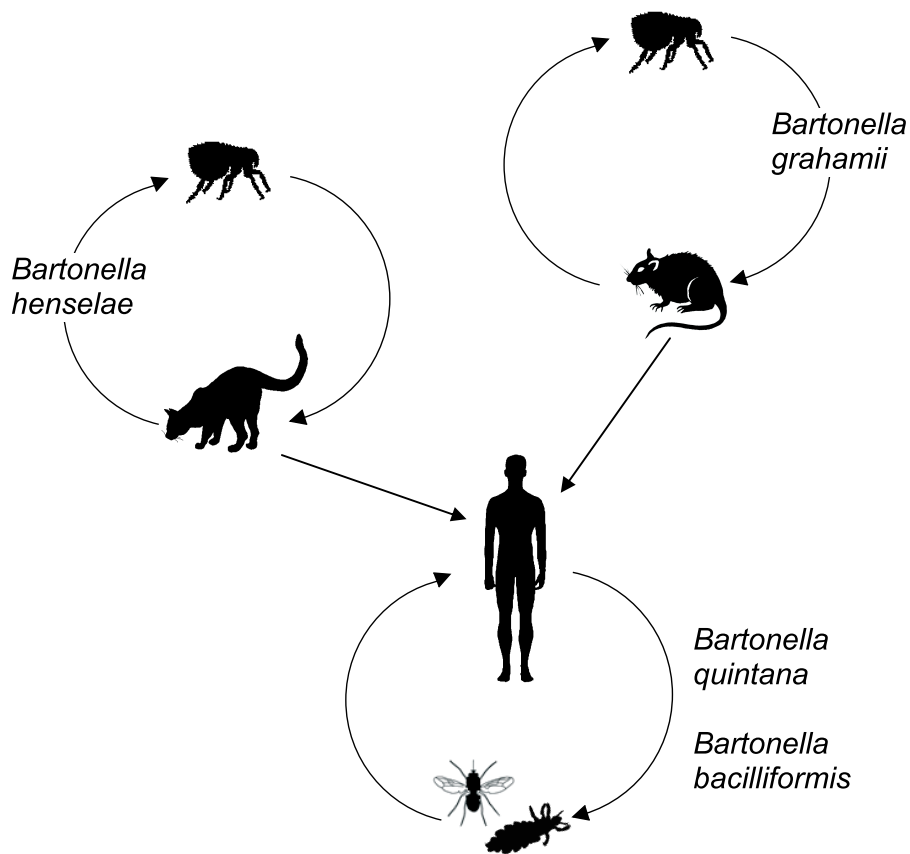


Figure 3. The natural cycle of *Bartonella* infections.

Table 1. Examples of natural hosts and human diseases in vector-born *Bartonella* infections.

Bartonella species	Vertebrate host	Arthropod vector	Disease in humans
<i>B. quintana</i>	Humans	Body louse ( <i>Pediculosis humanus corporis</i> )	Trench fever Endocarditis Chronic bacteremia
<i>B. henselae</i>	Cats	Cat flea ( <i>Ctenocephalides felis</i> )	Cat-scratch disease Osteomyelitis Bacillary angiomatosis.
<i>B. bacilliformis</i>	Humans	Sand fly ( <i>Lutzomyia verrucarum</i> )	Carrion's disease: Oroya fever (hemolytic anemia) and Verruga peruana (peruvian warts)
<i>B. grahamii</i>	Wild rodents, e.g. yellow-necked field mouse	Rodent flea ( <i>Ctenophthalmus nobilis</i> and others.)	Neuroretinitis

### Fever of unknown origin and osteomyelitis

Fever of unknown origin in children is defined as a documented daily temperature of  $\geq 38^{\circ}$  C for at least 14 days without diagnostic signs or symptoms. In a prospective evaluation of 146 children with FUO bartonellosis was an established diagnosis in 5% [36]. There are several case reports presenting data in which bartonellosis is a cause of FUO and osteomyelitis is a part of the clinical syndrome [46,47].

Osteomyelitis associated with CSD occurs both in children [37,38,48] and adults [49] and can affect any bone. It is a rare manifestation and is a part of CSD-related syndromes in 0.3 % of the cases [29]. Presenting symptoms include fever and local pain and diagnosis is based on the epidemiology, X-ray scanning (preferably MRT), inability to culture other bacteria from biopsy specimens, typical histological finding with suppurative granulomas with central necrosis [50], serology and PCR on biopsy material.. Antibody titres to *Bartonella* antigens may be low or not present but are usually elevated [51]. CSD osteomyelitis is almost invariably treated with antibiotics, although it is not fully established whether antibiotics contribute to the recovery.

### Myocarditis

Histopathology is the cornerstone of the diagnosis of myocarditis and, according to the consensus statement in the Dallas criteria of 1987, it is defined as necrosis or degeneration of the myocytes (or both) and an adjacent inflammatory infiltrate [52]. A classification based on the clinical

presentation has been suggested dividing myocarditis into fulminant, subacute, chronic active, and chronic persistent subtypes [53]. Further histopathological classification separates active myocarditis characterised by an inflammatory cellular infiltrate with evidence of myocyte necrosis from borderline myocarditis with inflammatory cellular infiltrate without evidence of myocyte injury. The inflammatory infiltrate may be lymphocytic, eosinophilic, neutrophilic or granulomatous [54]. Aetiologic agents include viral, bacterial, fungal and protozoal agents, as well as toxic or hypersensitivity reactions to drugs and autoimmune disorders [55].

Case reports as well as experimental infections have provided evidence indicating that *Bartonella* spp may be involved in the aetiology of myocarditis [35,56]. Experimentally, a majority of specific-pathogen-free cats (SPF) inoculated with *Bartonella henselae*- and/or *Bartonella clarridgeiae*-infected cat blood developed a lymphoplasmacytic myocarditis with no or mild signs of clinical disease. *Bartonella* DNA was amplified from the heart biopsies in all cases [57]. Myocarditis was suspected in a case of aggressive *B. henselae* endocarditis in an immunocompetent adult because of severely reduced left ventricular function although a heart biopsy specimen was not obtained [58]. Another case of histologically verified *B. henselae* myocarditis was suspected in a case progressing to heart failure and heart transplantation. However, immunohistochemical stainings and PCR for *Bartonella* were negative in myocardial biopsy specimens. It was hypothesised that the myocarditis in this case was caused by immune-mediated mechanisms secondary to CSD [56].

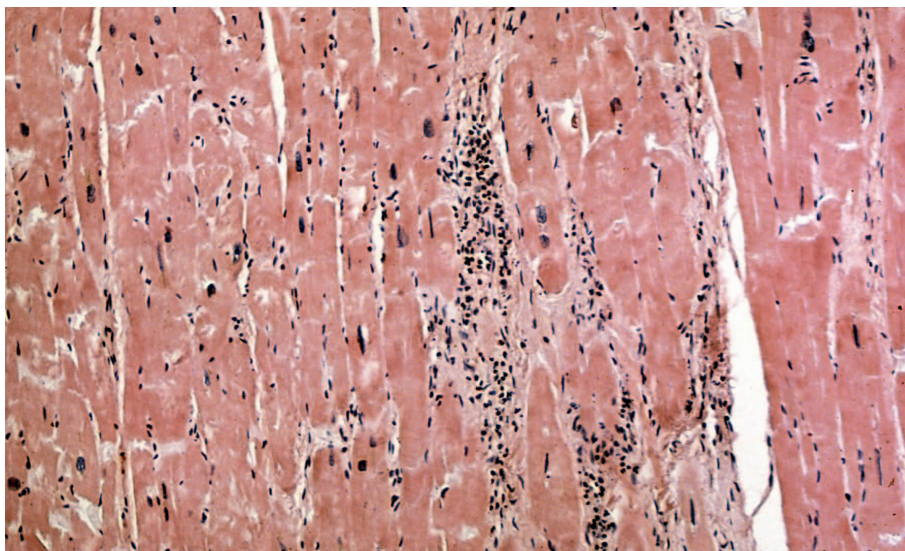


Figure 4. Myocarditis in a Swedish elite orienteer deceased in sudden cardiac death.

## ARVC

Arrhythmogenic right ventricular cardiomyopathy/dysplasia (ARVC/D) is characterised biologically by progressive fibrofatty replacement of the right ventricular myocardium and clinically by life-threatening ventricular arrhythmias [59]. Fibrofatty substitution also of the left ventricle is common in advanced disease and inflammatory infiltrates may be present [60]. There are both sporadic and familial cases and specific chromosomal loci and five disease-causing genes have been identified in the latter group. These genes encode components of the desmosome which are important for providing tissues with mechanical strength [60]. Impaired functioning of cell adhesion junctions may lead to myocyte detachment and death, accompanied by inflammation and fibrofatty repair. If infective mechanisms also have a role in the pathogenesis of ARVC and/or in the onset of malignant arrhythmias is under debate. However, myocarditis is a frequent finding in ARVC specimens and it has been postulated that infections contribute to the onset and the progression of the disease [59].

## Endocarditis

There are several reports on *Bartonella* endocarditis in human subjects, both in native and prosthetic valves and involving different *Bartonella* spp. [34,61-63]. *B. quintana* has been reported to occur more frequently than *B. henselae* in cases of endocarditis [64]. *B. quintana* endocarditis has been epidemiologically associated with immunodeficiency, chronic alcoholism, homelessness and body louse infestation and *B. henselae* endocarditis to a pre-existing valvular disease and cat contact [65]. The lack of a history of predisposing valvular heart disease in *B. quintana* endocarditis may reflect a lack of attention to medical care in homeless patients and alcoholics. Endocarditis that is caused by *B. henselae* was first reported in 1993 [34] and *Bartonella elizabethae* [62], *Bartonella koehlerae* [66], and *Bartonella alsatica* [67] have been reported in single cases of endocarditides. The incidence of *Bartonella* endocarditis cases has an uneven geographical distribution and is believed to follow a south-to-north gradient with *Bartonella* endocarditis being more frequently encountered in northern Africa than in Scandinavia. In France, for instance, *Bartonella* endocarditis constitutes 5% of all endocarditis cases [68]. In contrast, there are only two reported cases of *Bartonella* endocarditis in Scandinavia [69,70]. The reasons for these differences are nation-specific epidemiological features. In Sweden, homeless are seldom infested with lice and a Swedish homeless population had no evidence of trench fever (TF) [71]. Homeless in France are often louse infested and *B. quintana* bacteremia is common in that population [72-74]. Moreover, *B. henselae* is less prevalent in Swedish cats,

which is the agent's natural reservoir as compared with cats from warmer climates [75].

90% of the *Bartonella* endocarditis patients are febrile, have a vegetation observed on echocardiography and require valvular surgery [65]. Typically, *Bartonella* antibody titres are strongly elevated. A cut-off titre of 1:800 has been suggested [76]. For treatment of *Bartonella* endocarditis, doxycycline orally for 6 weeks should be combined with an aminoglycoside for 14 days. Aminoglycoside treatment is considered important because patients receiving an aminoglycoside for >14 days are more likely to fully recover than those treated for a shorter duration (p=0.02). If an aminoglycoside cannot be given, it should be replaced if possible with rifampin [77]. The mortality, despite medical and surgical treatment, is estimated to be 12% [65].

### *Bartonella quintana*-related syndromes

*B. quintana*'s natural reservoir is humans and the main vector is the human body louse (*Pediculus humanus corporis*). The human head louse (*Pediculus humanus capitis*) as a vector has been shown to occur [78] and the bacterium has been isolated from cat fleas [79] and detected in cat dental pulp [80], indicating a less strict host-vector ecology than was previously accepted. *B. quintana* is involved in a wide range of clinical entities similar to what is the case in *B. henselae* infections. The main manifestations are TF [81-84], endocarditis [61], bacillary angiomatosis [43] and chronic bacteremia [74,83]. The definite criteria for *B. quintana* infections are not fully established. However, pathological changes (e.g., endocarditis and bacillary angiomatosis) and a positive PCR are sufficient to define a case, whereas serology and epidemiological risk factors alone are not.

### Trench fever

TF was recognised and described during World War I by different researchers [81,82]. Their reports included natural cases and experimental infections in volunteering soldiers in whom transfused blood from typical cases reproduced natural infection in the volunteers. The putative causative agent was described as *Rickettsia*-like and was present in lice, their excreta, and their guts when they were collected from TF patients [85]. They reported on different clinical presentations ranging from a mild to a more severe course. No fatalities were recorded. The incubation time was estimated to be 15-25 days in natural infection and somewhat shorter in experimental infection. The most common presentation included an acute onset of fever (39–40°C) lasting 1-3 days followed by relapsing but decreasing episodes of fever every 5 days. Fever was accompanied by shin pain, headaches and dizziness. In 1949, Kostrzewski [83] reported on an epidemic spread in a

laboratory setting among 90 louse-feeders and described three courses: The classic relapsing form as described above; a typhoidal form characterised by a prolonged fever, splenomegaly and rash; and an abortive form, characterised by a brief, less intense course. A slightly furred tongue, conjunctivitis and a slow pulse relative to the severity of the fever were also reported [81,82]. *B. quintana* was first cultivated on axenic media by Vinson in 1966 [86].

Epidemics among homeless populations in France [87,88], the United States [89], Japan [90] and Russia [91] have recently been reported. Thus, these epidemics have been referred to as urban TF. The epidemiological risk factors for TF include chronic alcoholism, poor living conditions and louse infestation [92]. The bacteria are excreted in the faeces of the louse and transmitted when the louse-infested individual scratches an itchy wound. An epidemic of TF was reported in refugee camps in Burundi in 1997 [93]. Some TF patients develop chronic bacteremia [74,82,83] which is asymptomatic and can last for several years. Chronic bacteremia and *B. quintana* endocarditis are likely associated, although this has not been proven.

## Pathogenesis

A main feature in the pathogenesis of *Bartonella* is its propensity to invade mature erythrocytes and endothelial cells of its animal or human hosts. *B. tribocorum* experimental infections in rats have demonstrated persistent intra-erythrocytic bacteremia without causing haemolysis [94]. A type IV secretion system (TFSS) is crucial for invading the erythrocytes and for establishing the infection in an as yet elusive primary niche. From this niche, which is by some postulated to consist of endothelial cells [95] and by others by bone marrow-stemmed erythrocyte progenitors (e.g., the erythroblast) [7], the bacteria are seeded every 5 days into the bloodstream, which enables new rounds of erythrocyte invasion [94]. The five-day seeding interval coincides with the symptom interval of TF. However, in immunocompetent hosts, the anti-*Bartonella* antibody response gradually prevents seeded bacteria to invade the erythrocytes thereby subsequently clearing the bacteremia [96]. There is epidemiological evidence that this mechanism of antibody-mediated cessation of hemotropic infection also applies to the natural course of *B. quintana* infections in humans [73]. Another hallmark of *Bartonella* pathogenesis is its ability to induce angiogenesis that is seen in bacillary angiomatosis, verruga peruana and liver peliosis. In vitro studies have demonstrated the important role of the *Bartonella* VirB/VirD4 TFSS also in the interaction with ECs. The TFSS of *Bartonella* exports *Bartonella*-translocated effector proteins (Beps) into the ECs leading to cytoskeleton rearrangement and the engulfment of bacterial aggregates (invasome

formation), proinflammatory activation leading to the recruitment of circulating neutrophils and the thereby release of proangiogenic factors (such as VEGF) and enhanced cell survival that is due to an anti-apoptotic effect [97,98]. These effects promote the vasculoproliferative lesions. However, the exact mechanisms behind the processes are far from fully established.

*Bartonella* adhesion A (BadA) of *B. henselae* has recently been identified as an important pathogenicity factor [99]. BadA is essential for the bacterium's binding to ECs, the activation of a key transcription factor in angiogenesis (hypoxia-inducible factor 1) and the induction VEGF. In *B. quintana* the BadA homologues are called variably expressed outer membrane proteins (Vomps) and they have been shown to mutate at a high frequency. There is evidence that BadA is an important host-interaction gene for *Bartonella* and, furthermore, the bacterium is able to modulate the badA gene expression by as yet unknown mechanisms.

## Diagnosis

There have been major advances in *Bartonella* diagnostics over the past 20 years. Before today's methods were available, five diagnostic criteria for CSD were used: epidemiological data involving cat contact or cat scratches, the presence of a cutaneous inoculation site, regional lymphadenopathy, a granuloma present on histological examination of the lymph node biopsy or a positive skin test. Today, the skin test is completely outdated and not in practice any more. In general, serologic testing is the most widely used method to diagnose *Bartonella* infection and indirect immunofluorescence is the reference method. Drawbacks include the lack of a *Bartonella* antibody response early in the course of the infection, the occurrence of variable antibody titres when different methods of antigen preparation are used and cross-reactions with antigens of distantly related genera such as *Coxiella burnetii* [100] and *Chlamydia pneumoniae* [101]. Western blot and cross-adsorption can be used to avoid the problem with cross-reactions and to differentiate between different *Bartonella* spp. Typically, *Bartonella* endocarditis gives rise to high antibody titres.

*Bartonella* isolates from clinical samples are notoriously difficult to obtain. However, positive *Bartonella* cultures have become more common with improved techniques. The subculture of blood culture broth into shell vials or onto agar plates has been proven more efficient than the direct blood plating of infected blood. The blood sample should be frozen before the isolation attempt in order induce haemolysis and to release any intra-cellular bacteria [102].

Polymerase chain reaction (PCR) is, beside serology, the most important diagnostic tool for detecting *Bartonella* infections and has successfully been employed for various tissues. Many different target genes have been

evaluated for diagnostic PCR [38,103], although the amplification of the house-keeping gene citrate-synthase (*gltA*) has been most widely used. *gltA* has the asset of providing the highest amount of nucleotide sequence data for *Bartonella* strain comparisons in public data bases. Furthermore, a species definition for the *Bartonella* genus has been defined on the basis of the *gltA* gene [104], which reinforces its usefulness. Instead of diagnostic conventional PCR, real-time PCR or quantitative PCR (QPCR) is preferable because of decreased risk of carryover contamination, higher speed, simplicity, reproducibility and quantitative capability [105].

*B. spp* may be detected in tissues by immunohistochemistry. Reported examples are valvular tissue in patients with *Bartonella* endocarditis [106] and in skin biopsies of patients with BA [107]. The intracellular presence of *Bartonella spp.* (ECs or erythrocytes) has been visualised with staining with mouse monoclonal antibodies and using confocal microscopy [108].

## Molecular epidemiology

Different genotyping methods have been used for assessing the genetic relationships between *Bartonella* isolates. The purpose has been to investigate whether different strains possess different pathogenic potentials in humans and to acquire knowledge about the geographic distribution of different genotypes. Several nucleotide sequence polymorphisms have been discovered in various genes among *B. henselae* isolates of different geographical and host origins [38,109]. Among the most useful known polymorphisms is the variation in the *rrs* gene (16S rRNA) of *B. henselae* isolates [110]. The two *rrs* genotypes are correlated to two serotypes in humans (Houston-1 and Marseilles serotypes) [111]. Other discovered gene polymorphisms include sequence variations in *ftsZ*, *gltA* and *groEL*. By combining polymorphisms in nine genes, an allele pattern for each isolate is employed to define a sequence type (ST) in the multilocus sequence typing (MLST) assay. The MLST technique revealed seven STs resolved into three lineages among 37 human and feline *B. henselae* strains. Because a limited number of STs were associated with disease in humans, it was hypothesised that some strains might be more pathogenic to humans than other STs [103]. However, other investigators have not found such a relation between *B. henselae* genotype and disease in humans [112]. The conflicting results on the possible differences between strains in pathogenicity for humans depending on genotype might be explained by small sample numbers, that the isolates are from geographically isolated regions and that the MLST technique does not provide any data on potential virulence genes.

Multispacer typing (MST) is similar to MLST in that it employs allele patterns to define genotypes. The difference is that, in MST, polymorphisms in non-coding regions are studied, whereas MLST explores the



polymorphisms of genes. A necessary precondition for the use of MST is extensive sequence information, preferably about the entire genome, for the identification of the most variable spacers. For *Bartonella*, MST has been employed for *B. quintana* [113] and for *B. henselae* [114] as complete genome data are available for these species. For the human specialist *B. quintana*, 34 spacer loci were investigated in 71 isolates with a world-wide origin revealing only 5 genotypes [113]. Thus, the low degree of genomic heterogeneity in the human specialist *B. quintana* reflects its restricted growth environment and a limited exposure to mobile genetic elements. In contrast, MST applied to 126 *B. henselae* cat isolates from various areas of Europe, Asia and the United States identified 39 MST genotypes which were resolved into four clusters. MST showed that European and Asian isolates were different, but failed to identify pandemic strains. Because only cat isolates were studied, any information about the relationship between genotype and rate of occurrence in human infections was not obtainable [114].

Comparative genome hybridisation (CGH) to microarrays is another method for genetic comparisons between closely related strains. The method is expensive, labour intensive and detailed genetic information about a reference strain is a precondition to be able to design the microarray probes. CGH gives information about an increase or a decrease in signal intensity, which corresponds to the amount of fluorescent-labelled DNA that has hybridised to the probes of the reference strains. An increase in signal might mean a higher copy number of that gene in the test strain. Decreased hybridisation might be because of absent genes, sequence divergence or lower copy number of that gene or a combination of these factors in the test strain. Genes in the test strain that are not present in the test strain are not possible to detect. In combination with CGH, PFGE-RFLP, which provides information on the genome structure and genome size, may be used. CGH and PFGE-RFLP applied 38 *Bartonella henselae* strains isolated from cats and humans revealed genomic variation and extensive rearrangements in the prophage and in the genomic islands. Any differences in the genotypes (ST, gene content or genome structure) between feline and human strains were not observed, demonstrating that human infections with different genotypes occur [112].

## Treatment

There are several difficulties in defining treatment recommendations for *Bartonella* infections. *Bartonella* infections display a wide range of clinical manifestations. The bacterium has both extra- and intracellular niches in the infected host and the course of the disease can be acute, relapsing or chronic depending on which *Bartonella* sp is involved and the host's immune status.

There are some reported studies on susceptibilities to different antibiotic compounds but only two published randomised clinical trials for the treatment of *Bartonella* infections. Azithromycin has been evaluated in such a study design for the treatment of CSD [115]. Gentamicin and doxycycline were tested in a randomised open trial for the eradication of *Bartonella quintana* in patients with chronic bacteremia [116]. Unfortunately, few prospective multi-centre randomised clinical trials that would be necessary to better define the treatment regimens have been carried out because of the comparatively infrequent occurrence of *Bartonella* infections. The current treatment recommendations for *Bartonella* infections are listed in Table 2.

In vitro, *Bartonella* spp. are susceptible to a wide range of agents including penicillins, cephalosporins, aminoglycosides, chloramphenicol, tetracyclines, macrolides, rifampin, fluoroquinolones and cotrimoxazole [117]. Only aminoglycosides and rifampin have a reported bactericidal effect [118]. The clinician deciding on the treatment for a patient with a suspected *Bartonella* infection has to consider many factors, as always, among which maybe the patient's immune status and the bacterium's both intra- and extracellular niches are the most important.

Table 2. Adopted from reference [152]. Recommendations for the treatment of infections caused by *Bartonella* species.

Disease	Adults	Children	Strength of recommendation	Reference
CSD	Azithromycin 500 mg day 1. Days 2-5 250 mg/day.	See adults, but 10 mg/kg.	BI	[115]
TF/Chronic <i>B. quintana</i> bacteremia	Doxycycline 200 mg x 1 x 4 weeks, combined with Gentamicin 3 mg/kg x 1 x 2 weeks.	Unknown.	AI	[116]
Endocarditis, suspected	Ceftriaxone 2 g x 1 x 6 weeks, combined with doxycycline 100 mg x 2 x 6 weeks, combined with gentamicin 3 mg/kg x 1 x 2 weeks.	Unknown.	BII	[77]
			BII	[77]
			AII	[77]
Endocarditis, verified	Doxycycline 100 mg x 2 x 6 weeks, combined with gentamicin 3 mg/kg x 1 x 2 weeks. Replace gentamicin with rifampin 300 mg x 2 if necessary.	Unknown.	BII	[77]
			BII	[77]
Retinitis	Doxycycline 100 mg x 2 x 6 weeks, combined with rifampin 300 mg x 2 x 6 weeks	Unknown.	AII	[41]
BA	Erythromycin 500 mg x 4 x 3 months, or Doxycycline 100 mg x 2 x 3 months	Erythromycin 40 mg/kg (maximum 2 g/day) x 4 x 3 months.	AII	[153]

Strength of recommendation designation: A = Good evidence to support a recommendation for use. B = Moderate evidence. I = Randomized, controlled trial. II = Well-designed clinical trial.

## Aims of the study

There were four objectives of this study:

1. Develop and assess tools for molecular epidemiology
2. Conduct molecular epidemiological studies
3. Study and improve diagnostic methods
4. Identify and characterise clinical cases of and risk populations for *Bartonella* infections in Sweden

## Materials and methods

For paper I, the *ftsZ* gene was used as a target to investigate gene sequence variation among *B. henselae* isolates. The *ftsZ* alleles were compared with the *rrs* alleles of the same isolates. Because we discovered different *ftsZ* alleles in the *B. henselae* isolates, we applied the *ftsZ* genotyping to clinical samples from Swedish patients.

For paper II, cases of sudden unexpected cardiac death (SUCD) among Swedish orienteers were studied. PCR and serology for diagnosing *Bartonella* were used because other aetiological investigations had been uneventful.

For paper III, 27 *B. grahamii* isolates from a previous Swedish study were genetically compared with different methods.

For paper IV, a Swedish homeless population was studied regarding *Bartonella* infections. Because international studies previously have demonstrated urban TF among homeless populations, we wanted to investigate if a Swedish homeless population shared these risk factors and if we could find any signs of TF. We studied the *Bartonella* seroprevalence and attempted PCR and culturing on blood for *Bartonella*.

For paper V, we investigated a Swedish patient who originated from Finland with culture-negative endocarditis. We sampled the patient's blood for serum extraction and antibody detection to *Bartonella* antigens and QPCR. Histopathological investigations as well as PCR and culturing attempts for *Bartonella* were undertaken on heart valve specimens.

### Bacterial strains and growth conditions (I, III)

15 *B. henselae* strains (Table 3) were kindly provided by R. Regnery, Centers for Disease Control and Prevention, Atlanta, GA, USA. and used for paper I.

27 *B. grahamii* isolates (Table 4) were yielded from bloods of sylvatic animals captured in rural habitats near Uppsala, Sweden, as described previously [119] and used for paper III. All bacterial strains were grown for 2 weeks on Columbia blood agar containing 5% whole horse blood at 35°C in 5% CO<sub>2</sub>.

## Clinical samples (I, II, IV, V)

Tissue, blood and serum samples from Swedish patients (I, V), Swedish orienteers (II) and homeless patients from Stockholm, Sweden (IV) were obtained (Table 5).

Sera were available for all patients except from two missing samples among the homeless. For cases 6 and 7 (II), endomyocardial biopsies were only available for histological examination but not for DNA preparation. Cases 1 and 2 in paper I are identical to cases 5 and 7 in paper II.

Table 3. *B. henselae* isolates studied and their sequence types.

Strain	Source (host)	Geographical origin	<i>rrs</i> (16S rRNA) type	<i>B. henselae</i> <i>ftsZ</i> variant
Houston-1	Human	United States	I	1
SA-2	Human	United States	I	1
CA-1	Human	United States	I	1
Houston-2	Human	United States	I	1
3883	Human	United States	I	1
3884	Human	United States	I	1
4271	Cat	United States	I	1
4272	Cat	United States	I	1
Tiger-2	Cat	United States	II	1
5249	?	United States	II	2
Marseille, URLLY8	Human	France	II	2
FR97/K7ITS	Cat	Germany	I	1
FR96/BK77	Cat	Germany	II	3
FR96/BK3	Cat	Germany	II	3
FR96/BK78	Cat	Germany	II	3

## Controls (II, IV)

Controls used for paper II and IV are displayed in Table 5.

## Cultures (IV, V)

Methods were adopted from the results of studies by Brenner et al. [120] and La Scola et al. [102]. Patient whole blood was kept frozen for 2 weeks at -20°C and then thawed at room temperature. Aerobic BactAlert PF pediatric blood culture bottles (BioMerieux inc., Lyon, France) were inoculated with approximately 2 ml patient blood and then incubated in a BactAlert 3D instrument for 7 days. 1 ml of inoculated blood culture broth was removed

from the bottle and plated on a Columbia 5% defibrinated horse blood agar. Plates were examined for signs of bacterial colonies every 2 weeks during the period of incubation and incubated for up to 3 months at 35°C in 5% CO<sub>2</sub>. Prolonged incubation plates were kept in plastic bags to avoid drying out.

## DNA isolation from bacterial strains and clinical samples (I-V)

Total genomic DNA was extracted from the *B. henselae* strains (I) and from the blood and tissue samples (II, IV and V) with the Qiagen QIAamp Tissue Kit (QIAGEN, Inc., Chatsworth, CA, USA) and from the *B. grahamii* strains (III) with an AquaPure Genomic DNA Tissue Kit (Bio-RAD, Hercules, CA, USA) according to the manufacturer's instructions. Between 10 and 25 mg of tissue and 100 and 200 µl of whole blood were used.

## PCR and DNA sequencing (I-III)

PCR was applied to DNA extracted from clinical tissue specimens (I, II) and from *Bartonella* isolates (I, III). PCR amplifications for 16S rRNA (*rrs*) (I), *ftsZ* (I, III) and *gltA* (I-III) were performed with a PCR Master kit from Boehringer Mannheim Scandinavia AB (Bromma, Sweden). The 50-µl reaction mixture consisted of the template, forward and reverse primers (10 pmol/primer), 25 µl of the PCR master mixture and distilled water of PCR grade up to 50 µl. A Perkin-Elmer GeneAmp 9600 thermocycler was used for all amplifications. PCR reactions for the other loci (III) were performed as described previously [112]. Detailed protocols are described and primers are listed in each paper.

PCR products were electrophoresed through a 1% agarose gel containing ethidium bromide in Tris-borate buffer. The DNA was detected on a UV transilluminator and photographed. Single PCR products of the expected size as compared with a DNA size marker were purified by using the QIAquick purification kit from QIAGEN, Inc., Chatsworth, CA, USA following the manufacturer's instructions (I, II), or as described previously (III) [112].

Nucleotide sequences were obtained with a model ABI 310 Genetic Analyzer (Perkin-Elmer Corp., Norwalk, CN, USA). Sequencing reactions were performed using a DNA Sequencing Kit with AmpliTaq® DNA Polymerase, FS, for the BigDye™ Terminator Cycle Sequencing Ready Reaction protocol (Perkin-Elmer, Applied Biosystems, Warrington, GB.) (I, II), or as described previously (III) [112].

Table 4. *B. grahamii* strains studied and their sequence types.

Strain	Source (rodent host)	Geographical origin (Sweden)	Sequence type (ST)
mm3up	<i>M. musculus</i>	Håtunaholm	1
as4aup <sup>a</sup>	<i>A. sylvaticus</i>	Håtunaholm	1
as4bup	<i>A. sylvaticus</i>	Håtunaholm	1
af9up	<i>A. flavicollis</i>	Håtunaholm	1
af30up	<i>A. flavicollis</i>	Håtunaholm	1
af43up	<i>A. flavicollis</i>	Håtunaholm	1
af47up	<i>A. flavicollis</i>	Håtunaholm	1
af50up	<i>A. flavicollis</i>	Håtunaholm	1
cg60up	<i>C. glareolus</i>	Håtunaholm	1
cg64up	<i>C. glareolus</i>	Håtunaholm	1
af66up	<i>A. flavicollis</i>	Håtunaholm	1
af68up	<i>A. flavicollis</i>	Håtunaholm	1
cg90up	<i>C. glareolus</i>	Håtunaholm	1
af115up	<i>A. flavicollis</i>	Kumla	1
cg120up	<i>C. glareolus</i>	Kumla	2
as134up	<i>A. sylvaticus</i>	Kumla	3
af140up	<i>A. flavicollis</i>	Kumla	2
af144up	<i>A. flavicollis</i>	Kumla	2
cg147up	<i>C. glareolus</i>	Kumla	1
af156up	<i>A. flavicollis</i>	Håtunaholm	1
af163up	<i>A. flavicollis</i>	Kumla	2
af164up	<i>A. flavicollis</i>	Kumla	2
af165up	<i>A. flavicollis</i>	Kumla	2
af206up	<i>A. flavicollis</i>	Ålbo	3
as211up	<i>A. sylvaticus</i>	Ålbo	2
as224up	<i>A. sylvaticus</i>	Ålbo	2
af233up	<i>A. flavicollis</i>	Ålbo	3

<sup>a</sup> as4aup and as4bup are different isolates from the same host.



Table 5. Cases and controls studied.

Clinical data	Number of patients	Paper
ARVC, Myocarditis and Malignant arrhythmia	7	(I), (II)
Osteomyelitis	2	(I)
CSD	2	(I)
POS	1	(I)
Endocarditis	1	(V)
Homeless patients	50	(IV)
Controls: Six young males who died because of trauma.	6	(II)
Controls: Age- and sex-matched blood donors	61	(IV)

## Real-time PCR (IV, V)

A genus-specific (except *B. bacilliformis*) real-time QPCR for *Bartonella* was developed and *gltA* gene was chosen as target gene.

PCR amplifications were performed with a Taqman Universal Mastermix from Applied Biosystems (Foster City, CA, USA). Each 25- $\mu$ l reaction mixture consisted of 1  $\mu$ l template, primer BhCS781.p at a final concentration of 200 nM, primer BCS924.n at a final concentration of 600 nM, probe PBCS884.n at a final concentration of 60 nM, 12.5  $\mu$ l of the Taqman Universal Mastermix and distilled water of PCR grade up to 25  $\mu$ l. A Rotor-Gene 2000™ Real-Time Cycler (Corbett Research) was used for all amplifications and data were analysed by Rotor-Gene™ Real-Time Analysis Software 6.0.22. The thermal cycling conditions are described in paper IV.

## Analysis of sequence data and construction of phylogenetic trees (I, II, III, V)

*rrs*, *gltA* and *ftsZ* sequences were analysed with ABI Prism™ DNA Sequencing Analysis Software Version 3.0 (PE Applied Biosystems, Foster City, CA, USA) and Sequencher™ (Gene Codes Corporation, Ann Arbor, MI, USA). Other nucleotide sequences were assembled and edited with Phred, Phrap and Consed [121-123].

For phylogenetic analysis in paper I, partial primary *ftsZ* sequences of the three variants, Bh *ftsZ* 1, 2 and 3, were aligned with each other and with the corresponding *ftsZ* sequences of *B. bacilliformis* and *B. quintana* published previously by using version W of the CLUSTAL multisequence alignment programme [124]. A total of 500 bootstrap samples were produced by using the Phylo Win package [125]. A matrix of evolutionary distances was

derived from each bootstrap alignment by using the assumptions of Jukes and Cantor.

### Microarray comparative genome hybridisation (III)

The probes were spotted on Ultra-GAPS-coated slides (Corning, Inc.) in three replicates. Slides were cross-linked and prehybridised. Hybridisation of labelled DNA [126] from test and reference strain was performed at 42°C overnight.

### Analysis of CGH data (III)

Scanning and image analysis, normalisation and the construction of phylogenetic trees were performed as described previously [112].

### RFLP (III)

Bacterial cultures grown on chocolate agar plates for 10 days were suspended in TNE buffer (10mM TRIS pH 8.0, 150 mM NaCl and 1mM EDTA) and centrifuged; washes were repeated twice. DNA was isolated in agarose plugs. One mm thick slices of DNA-containing plugs were separately digested with 10 U of NotI (New England Biolabs) restriction endonuclease overnight. The DNA fragments were separated in 0.9% PFGE-grade agarose (SeaKem® Gold; Cambrex Bio Science) in 0.5x TBE buffer in GenNavigator™ System apparatus (Amersham Biosciences) at 14°C and 5.6 V/cm for a total of 65 h. The total run was separated in six phases; switch times ramped from 5 to 150 s. The sizes of the fragments were estimated using PFGE  $\lambda$ -ladder and Yeast Chromosome PFG marker (New England Biolabs).

### IFA (I, II, IV, V)

Serum samples were analysed by IFA for immunoglobulin G reactivity against crude antigens of *B. henselae* Houston-1 (ATCC 49882), *B. quintana* OK 90-268 and *B. elizabethaea* R2798 as described previously [119]. Aliquots of crude antigen were applied to 10-well Teflon-coated microscope slides (Novakemi AB, Uppsala, Sweden), air-dried, fixed in acetone and stored at 70°C until used. Serum samples and controls were diluted in phosphate-buffered saline (PBS) with 5% skim milk and applied to the slides in 30- $\mu$ l aliquots of serial dilutions, ranging from 1:32 to 1:2,048. The slides

were incubated at 35°C for 30 min and then washed in PBS, air dried and coated with a commercial fluorescein isothiocyanate-conjugated rabbit anti-human IgG (Dakopatts, Glostrup, Denmark). Following an additional 30-min incubation, the slides were washed and dried as before and mounted in buffered glycerol (Vector, Burlingame, CA, USA). Specific immunofluorescence was subjectively scored on a scale from 0 to 3+ by using a Nikon fluorescence microscope under  $\times 400$  magnification. A rating of 2+ at a 1:64 dilution was considered indicative of seropositivity for all three *Bartonella* antigens. IFA *Bartonella* titres were reported as the reciprocal of serum endpoint dilutions.

### Statistics (III, IV)

The Fisher exact test was employed to test the hypothesis that geographical location and genetic variant were mutually independent. Kumla and Ålbo were regarded as one localisation and Håtunaholm as the other (III).

*Bartonella* serology status among patients and controls was tested by using the Chi-square and Fisher's exact tests. A p-value of  $\leq 0.05$  was considered significant (IV).

### Histopathology (II, V)

Heart tissue specimens were histopathologically examined as described previously [127]. In short, slides were made from paraffin-embedded tissue blocks and stained with haematoxylin/eosin, van Gieson, elastin, periodic acid-Schiff (PAS), iron, alcian blue-PAS for visualisation of mast cells and picro-Sirius for collagen. Established criteria for myocarditis (the Dallas criteria)[52], hypertrophic cardiomyopathy [128] and ARVC [129] were applied.

# Results

## Patients and controls (I, II, IV, V)

### Atypical CSD (I, V)

Age on admission or death, gender, clinical presentation, cat contact, specimen source, diagnostic test results, treatment and outcome data for seven Swedish patients presented in paper I are summarised in Table 6. The patients were diagnosed with cardiomyopathy ( $n=2$ ), osteomyelitis ( $n=2$ ), lymphadenopathy ( $n=2$ ) and POS ( $n=1$ ).

The cardiomyopathy cases in paper I are identical to cases 5 and 7 in paper II. However, in paper I a diagnostic PCR was employed for the amplification of the *B. henselae* *gltA* and *ftsZ* genes. In paper II only the *gltA* gene was targeted in cases 1-5. This double report is motivated by different objectives: Paper I deals with the *ftsZ* gene diagnostic PCR assay for clinical samples and the possibilities to detect different *B. henselae* variants. Paper II mainly analyses the aetiological factors involved in the increased death rate for this population of orienteers. The positive PCR findings in the *ftsZ* gene amplification (but not *gltA* gene) of the second case with ARVC reported in paper I (case 7 of paper II) are not presented in paper II because only whole blood was available for DNA preparation and not myocardial tissue at that time. Histology of the heart in case 5 (II) revealed ARVC-like disease, which denotes fibrofatty replacement of myocardium in both ventricles, and in case 7 (II) ARVC.

### Orienteers (II)

The outcome of diagnostic tests and histological investigations of the cases in paper II are summarised in Table 7.

*Bartonella* spp. DNA was amplified from heart tissue in 4 of the SUCD cases and from the lung of a fifth SUCD case. Cases 1, 2, 3 and 5 displayed myocarditis and/or ARVC-like pathology on histological examination. Case 4 showed fibrosis, pericarditis and atherosclerosis. All cases, except case 5, had elevated levels of anti-*Bartonella* antibodies.

Six previously healthy young male controls deceased in traumatic accidents displayed no signs of *Bartonella* antibodies and PCR was unable to amplify *Bartonella* DNA from heart tissue.

Table 6. The outcome of diagnostic tests in *Bartonella* infection cases (I).

Age (yr), gender	Clinical diagnosis or signs	Origin of sample	<i>gltA</i> sequence	Bh <i>ftsZ</i> variant	Date of serum sampling	IgG antibodies		
						Be	Bh	Bq
37, M	SUCD, ARVC	Myocardium	Bh	1	Jan 1999	<64	<64	<64
32, M	Malignant arrhythmia, ARVC	Whole blood	Neg	2	Jan 1998	256	256	<64
8, M	Osteomyelitis	Bone (left sternoclavicular joint)	Neg	1	March 1998	<64	256	<64
12, F	Osteomyelitis	Bone (right sternoclavicular joint)	Bh	2	Feb 1999	<64	<64	<64
14, M	CSD	Enlarged axillary lymph node	Bh	1	Nov 1998	<64	<64	<64
17, F	CSD	Enlarged abdominal lymph node	Neg	2	Aug 1998	<64	<64	<64
46, F	POS	Patient's cat's blood	B.h.	1	Jan 1999	<64	<64	-

### Homeless (IV)

All 50 homeless patients were men and all patients were included with informed consent and no patients denied participation. The mean age was 49.9 years and the median age 50 (range 34-71) years. Four patients (8%) died during the study. There were no signs of ongoing louse infestation in any patient, although there were reports of earlier louse infestation in two (4%). Three patients (6%) were HIV positive. A great majority of the homeless patients had an ongoing i.v. drug addiction and/or alcohol abuse. Thus, 22 (44%) patients were abusing alcohol and 23 (46%) patients were addicted to intravenous drugs (IVDs). In total, 43 (86%) patients were misusing either alcohol or IVDs, or both. Two samples were lost during transportation between Stockholm and Uppsala.

The mean age of the 61 blood donor controls was 51 years and the median age 53 (range 34-70) years.

Table 7. The outcome of diagnostic tests and histological investigations in orienteer cases and controls (II).

Case (age at death) and controls	Date of tissue sampling at autopsy or biopsy	Histology of the heart at autopsy or biopsy	Origin of sample	<i>gltA</i> sequence	Date of serum sampling	IgG antibodies		
						Be	Bh	Bq
1 (26)	June 1992 (SUCD)	Myocarditis and streaks of fat	Heart (Small sample available)	Neg	Jan. 1992	512	512	64
			Lung	Bq	May 1992	256	<64	<64
					June 1992	512	<64	<64
2 (24)	Nov. 1992 (SUCD)	Myocarditis ; ARVC-like	Heart Thyroid gland	Bh Bh	Nov. 1992	128	<64	64
3 (59)	April 1994 (SUCD)	Myocarditis	Heart	Bq	April 1994	2048	<64	128
4 (55)	May 1993 (SUCD)	Fibrosis, pericarditis, atherosclerosis	Heart	Bh	Sep. 1992	128	128	<64
					May 1993	512	<64	<64
5 (37)	Jan. 1999 (SUCD)	ARVC-like	Heart	Bh	June 1994	<64	<64	<64
			Spleen	Bh	Jan. 1999	<64	<64	<64
			Media-stinal lymph gland	Bh				
6 (Alive)	1992 (Endo-myocardial biopsy)	Normal	- (Tissue sample not available)	-	Sep. 1992	64	<64	<64
					June 1998	64	<64	<64
7 (Alive)	1997 (Endo-myocardial biopsy)	ARVC	Blood	Bh	1993	256	256	<64
					Jan. 1998	256	256	<64
					June 1998	128	<64	<64
					Feb. 1999	<64	<64	<64
Controls (n=6)		Normal	Heart	Neg		<64	<64	<64

Homeless patients were significantly more often seropositive to *Bartonella* spp. than controls (OR 7.58 (3.30-17.39),  $p < 0.05$ ). Reactivity to the *B. elizabethaea* antigen was dominating, although the difference between patients and controls was most salient in seroreactivity to the *B. henselae* antigen. Cross-reactivity between different *Bartonella* antigens was frequently occurring and was recorded in 14 (46.7%) of the seropositive samples.

Total and specific reactivity to four *Bartonella* antigens in homeless patients ( $n=48$ ) and in age- and gender-matched controls ( $n=61$ ) is displayed in Table 8.

No *Bartonella* DNA was detected nor were any *Bartonella* spp. isolated from the clinical samples. One patient was bacteremic with an *Acinetobacter* species, which was not further characterised.

There was no evidence of an ongoing *B. quintana* epidemic.

### *B. quintana* endocarditis (V)

A 72-year-old man was suspected to have a systemic inflammatory disease with an immune-complex mediated glomerulonephritis. He received immunosuppressive therapy. In December 2006 he was admitted to hospital because of fever, chest discomfort and palpitations. Echocardiography revealed tricuspidal and aortic vegetations but blood cultures were normal. IgG antibody titres to *Bartonella* spp. were 1:1024 for *B. henselae*, 1:1024 for *B. quintana* and 1:512 for *B. elizabethaea*. He had no antibodies to *Brucella* or *Coxiella burnettii*. Despite optimal antibiotic treatment heart valve replacement surgery became inevitable. Histological examination of the heart valves was congruent with infective endocarditis fulfilling the Duke criteria (fig. 5) [130]. Real-time PCR amplification and sequencing of *gltA* and *rrs* fragments from DNA extracted from heart valve tissue specimens revealed 100% nucleotide sequence homology to *B. quintana*.

Apart from the louse infestation as a child, obvious risk factors for a *B. quintana* infection were lacking.

DNA extracted from the patient's myocardium tested positive, although weakly, by QPCR and the sequenced product was identical to that from the heart valves. The histopathological examination of the myocardium did not reveal an ongoing myocarditis, but because the coronary arteries were normal and because no peroperative signs of aortic calcification were evident, the revealed myocardial fibrosis might be a sign of a healed myocarditis. The clinical course of the infection was classical in relation to the previously reported cases in the literature.

Table 8. Total and specific reactivity to four *Bartonella* antigens in homeless patients ( $n=48$ ) and controls ( $n=61$ ) is displayed.

Antigen	Patients, $n=48$			Controls, $n=61$			Reaction to antigen only (%)	Odds ratio <sup>1</sup> (CI <sup>2</sup> )
	Reaction to antigen total <sup>3</sup> (%)	GMT <sup>4</sup>	Range	Reaction to antigen only (%)	Reaction to antigen total (%)	GMT		
Any <i>Bartonella</i>	30 (62.5)	-	-	-	11 (18.0)	-	-	7.58* (3.30-17.39)
Be $\geq 1:64$	25 (52.1)	99.8	64-256	16 (33.3)	9 (14.8)	263	64-512	6.28* (2.54-15.54)
Be $\geq 1:128$	10 (20.8)	153.6	128-256	3 (6.3)	8 (13.1)	288	128-512	1.74 (0.63-4.83) ns
Bh	14 (29.2)	123.4	64-256	4 (8.3)	1 (1.6)	128	128-128	24.7* (3.11-196.2)
Bq	2 (4.2)	64	64-64	0	1 (1.6)	256	256-256	2.61 (0.23-29.66) ns
Bg	1 (2.1)	64	64-64	0	3 (4.9)	149	64-256	0.41 (0.04-4.09) ns
Be+Bh	9 (18.8)	-	-	-	1 (1.6)	-	-	13.85* (2.58-74.47)
Bh+Bq	2 (4.2)	-	-	-	1 (1.6)	-	-	ns
Be+Bh+Bq	1 (2.1)	-	-	-	1 (1.6)	-	-	ns
Be+Bh+Bq+Bg	1 (2.1)	-	-	-	0	-	-	ns

Be: *Bartonella elizabethaea*; Bh: *B. henselae* Houston-1; Bq: *B. quintana*; Bg: *B. grahamii*.

\* Significant values ( $p \leq 0.05$ ).

ns=not significant.

<sup>1</sup> Comparisons are made between the total serum reactivity of the patients and controls.

<sup>2</sup> CI= 95% confidence interval.

<sup>3</sup> Seropositivity to at least 1 antigen. A titre of  $\geq 64$  was considered seropositive for all antigens.

<sup>4</sup> GMT = geometric mean titre, calculated only for seropositive samples.



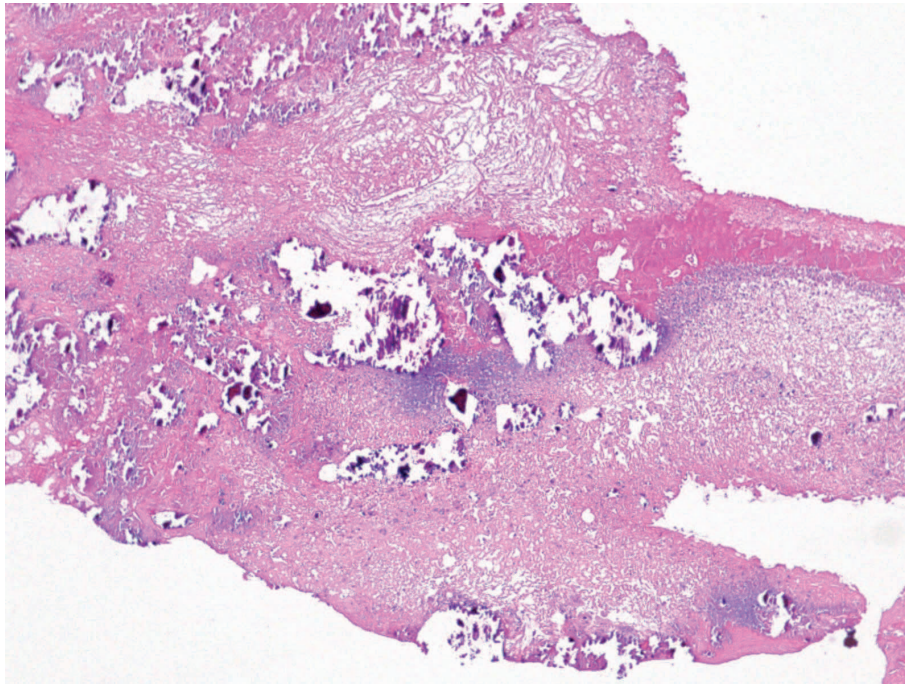


Figure 5. *B. quintana* endocarditis. The histopathological examination of the tricuspid valve displayed changes coherent with bacterial endocarditis, with necrosis, inflammatory cells and severe calcification.

## *Bartonella* isolates (I, III)

### *B. henselae* (I)

#### 16S rRNA (*rrs*) and *ftsZ* nucleotide sequence comparisons

The results of 16S rRNA (*rrs*) and *ftsZ* nucleotide sequence comparisons are displayed in Table 3.

The *ftsZ* sequence variants that were discovered were designated variants Bh *ftsZ* 1, 2 and 3. There was no *ftsZ* gene variation in the strains of 16S rRNA type I, all of which were Bh *ftsZ* 1. The type II strains constituted two groups, with nucleotide sequence variation in the *ftsZ* gene resulting in amino acid substitutions at three positions, one of which was shared by the two groups. Nucleotide sequences differed at position 1185 (A [Bh *ftsZ* 1] T [Bh *ftsZ* 2 and 3]), position 1404 (G [Bh *ftsZ* 1] A [Bh *ftsZ* 2]), position 1467 (G [Bh *ftsZ* 1] T [Bh *ftsZ* 2 and 3]) and position 1537 (C [Bh *ftsZ* 1] T [Bh *ftsZ* 3]). The correlation of the *rrs* types and *ftsZ* variants was not entirely congruent for all isolates. Strain Tiger-2 (*rrs* type II) isolate had an *ftsZ* gene sequence identical to those of the type I strains (Table 3).

A phylogenetic tree was inferred from alignments of Bh *ftsZ* 1, 2 and 3 and from the corresponding *ftsZ* sequences previously reported of *B. quintana* and *B. bacilliformis* using the neighbour-joining and maximum

parsimony methods (see paper I). The neighbour-joining and maximum parsimony methods gave identical topologies and high bootstrap support in intra-*B. henselae* phylogeny (82 and 90% of 500 bootstrap samples for the neighbour-joining and maximum parsimony methods, respectively). Within the genus, three well-supported branches were identified for *B. henselae*, *B. quintana* and *B. bacilliformis*.

### *B. grahamii* (III)

#### **Multilocus sequence typing (MLST)**

Nucleotide sequence polymorphisms were revealed in 4 (*ftsZ*, *ftsK* and two spacers) of the 17 regions examined with three observed STs. All 14 strains from Håtunaholm belonged to a single genotype, ST1, as did also two strains from Kumla. Another genotype, ST2, contained six isolates from Kumla and two from Ålbo. The third sequence type, ST3, comprised two strains from Ålbo and one from Kumla. All sequence types contained isolates from at least two hosts (Table 4).

#### **Comparative genome hybridisations (CGH)**

The number of absent loci ranged from no reported absences in all of the 6 isolates from Håtunaholm to 106 absent loci in strain af144up from Kumla. The total number of loci that were absent in any strain was 163. Lower hybridisation signals in strains of ST2 and ST3 were concentrated to loci in certain regions.

#### **RFLP**

PFGE-RFLP analysis was performed with NotI restriction enzyme. The digest resulted in four bands in all strains. It is notable that all ST1 strains have the same NotI restriction pattern, whereas the PFGE patterns for the other strains are more difficult to correlate directly with the MLST/MST or the CGH results.

#### **Correlations between ST, NotI profile, gene content, host and geographic origin**

A maximum parsimony tree based on the CGH data showed that all strains of ST1 and NotI type I grouped together in a well-supported clade that included all of the examined Håtunaholm strains and two Kumla isolates (IV). Another strongly supported group consisted of three isolates from Ålbo (af206up, as211up and af233up), which showed a NotI type IV restriction pattern and belonged to either ST2 or ST3.

**Statistics**

Statistical analysis suggests that both ST and NotI type were significantly correlated with geographic origin ( $p < 10^{-5}$  and  $p < 10^{-6}$ , respectively), but not with the rodent host. The Håtunaholm strains differed significantly from the Ålbo and Kumla strains regarding both ST ( $p < 10^{-5}$  and  $p = 0.0002$ , respectively) and NotI profile ( $p < 10^{-6}$  and  $p = 5.5 \times 10^{-4}$ , respectively), whereas there was no or only a weakly significant difference between the Ålbo and the Kumla strains ( $p = 0.062$  for ST and  $p = 0.0018$  for NotI profile).

# Discussion

## Clinical investigations (I, II, IV, V)

### Atypical CSD and *B. quintana* endocarditis (I, V)

The studies on human *Bartonella* infections in Sweden to date are listed in Table 9. The search for an infectious cause to the SUCD cases among Swedish orienteers became the starting point for these investigations and coincided with an international recognition of the importance of *Bartonella* infections. Since 1999, when the first Swedish study was published, single cases with *Bartonella* infections have been reported and certain populations studied. Seroprevalence surveys have been carried out among populations of homeless (paper IV), blood donors [131], intravenous drug addicts [132], infective endocarditises [133] and elite orienteers [134]. In summary, these investigations show an increased seroprevalence of antibodies to *Bartonella* antigens among homeless and intravenous drug addicts in Stockholm, and among elite orienteers, but not among cases with infective endocarditis from western Sweden. The cases reported include one *B. quintana* endocarditis, six myocarditis cases, two cases of osteomyelitis, three cases of lymphadenopathy and one case each of granulomatous hepatitis, lymphadenopathy and Wegener's granulomatosis. *Bartonella* as aetiologic agent in these cases was diagnosed by a combination of PCR and serology. No clinical *Bartonella* isolates were obtained. In short, the reports listed in Table 9 demonstrated an increased risk for exposure to *Bartonella* antigens in certain populations (homeless, orienteers and IVD users) and verified cases with *Bartonella* infections in Sweden.

Obviously, *Bartonella* infections in Sweden are rare. However, the true number of human *Bartonella* infections in Sweden is likely underestimated. This assumption seems plausible when considering the current diagnostic procedures. The bacterium is notoriously fastidious and it is most likely that isolation attempts fail. Serology remains the most important diagnostic means, but at a cut-off titre of 64, the sensitivity of the IFA has been estimated to be 86.8% and the specificity 74.1% in immunocompetent individuals with CSD [135]. Several of the reported *Bartonella* cases in Sweden had low or absent titres. PCR appears to be the most sensitive means for diagnosing a *Bartonella* infection with the precondition that an appropriate tissue sample is available for the DNA extraction. An

appropriate biopsy specimen is not always easy to obtain. Because *Bartonella* infections are rare, there is also the problem of a missed diagnosis because the *Bartonella* infection was never even considered.

Further, many questions remain unanswered. One of the most important is the issue of the elevated reactivity to *B. elizabethaea* seen in all *Bartonella* seroprevalence surveys in Sweden. We do not know what that increase in *B. elizabethaea* antigen seroreactivity stands for. Because intra-genus cross-reactivity is known to occur in the IFA testing (e.g., between *B. henselae* and *B. quintana*) [131], the current understanding is that the increased reactivity to *B. elizabethaea* antigen is because of cross-reactivity with an as yet unidentified *Bartonella* species. *B. elizabethaea* was responsible for one human case of *Bartonella* endocarditis in the USA in 1993 [62] and has since been isolated from rats [136]. It seems unlikely that the increase in *B. elizabethaea* antigen reactivity is caused by true exposure to this antigen. Epidemiologically, seroreactivity to *B. elizabethaea* was associated to working outdoors, hunting moose, cat contact, being out in the wild a minimum of once a week and travelling to Eastern Europe [131]. Swedish cats have been shown to have a higher seroprevalence to *B. elizabethaea* than to *Bartonella henselae* or *B. quintana* [75]. Because cats are the natural reservoir for *B. henselae*, this finding was particularly noteworthy. This exemplifies that the epidemiological features for *Bartonella* infections are influenced by geographical factors [68,137,138].

Table 9. Studies on human *Bartonella* infections in Sweden.

Study (Paper)	Patient(s)	Controls	% positive. Patients/controls.				PCR method. Analyzed material.	Main findings
			All anti-gens; Method (Cut-off)	Be	Bh	Bq		
Ehrenborg -07 (IV)	50 homeless patients. 100% healthy males.	61 age- and sex-matched healthy blood donor controls.	62* /18 IFA (1:64)	52* /15	29* /16	4 /1.6	QPCR ( <i>gltA</i> ); EDTA blood: 0. /NT	The overall <i>Bartonella</i> , <i>B. elizabethaea</i> and <i>B. henselae</i> seropositivity rates were significantly higher among patients (p<0,001) than among controls. No evidence of louse infestation or trench fever.
Ehrenborg -07 (V)	72 year-old male.	-	Titers at diagnosis IFA (1:64)	512	1024	1024	QPCR ( <i>gltA</i> ) and conventional PCR (16S rRNA) EDTA blood: 0. Heart valves and sera +.	First reported case of <i>Bartonella</i> endocarditis ( <i>B. quintana</i> ) in Sweden. The patient was louse infested during his childhood but other epidemiological risk factors were lacking.
McGill -05	498 blood donors; 63% males, 37% females.	NT	16.1 IFA (1:64)	14	1.2	0.2	NT	<i>B. elizabethaea</i> seropositivity associated to: working outdoors, hunting moose, cat contact, being out in the wild a minimum of once a week, and travelling to eastern Europe.
McGill -03.	59 dead intravenous drug addicts. 85% males.	44 forensic autopsy cases. 84% males.	39* /21 IFA (1:64)	39* /21	14* /2	3 /2	NT	The overall <i>Bartonella</i> , <i>B. elizabethaea</i> and <i>B. henselae</i> seropositivity rates were significantly higher among patients than among controls.
Werner -03	329 patients with infective endocarditis.	NT	- MIF (1:800)	NT	0	0	NT	Serologically verified <i>Bartonella</i> endocarditis was not prevalent in western Sweden.

\* Significant finding.

Study (Paper)	Patient(s)	Controls	% positive. Patients/controls.				PCR method. Analyzed material.	Main findings
			All anti-agens; Method (Cut-off)	Be	Bh	Bq		
McGill -01	1136 elite orienteers.	322 blood donors.	31* /6.8 IFA (1:64)	31* /6.8	3 /1.6	1.4 /0.3	NT	The <i>B.</i> seropositivity rate in the elite orienteers was significantly higher (p<0,001) than among controls.
Wesslén -01 (II)	Elite orienteers: 7 non-ischemic SUCD and malignant arrhythmia cases.	6 traumatic deaths	IFA (1:64) Elevated in most cases /0.	-	-	-	Semi-nested PCR ( <i>gltA</i> ); Myocardium and lung +. /0.	<i>B. quintana</i> or <i>B. henselae</i> was amplified from heart tissues of elite orienteers that had died in SUCD and had myocarditis, heart fibrosis or ARVC-like disease (fibrofatty replacement of myocardium in both ventricles).
Ehrenborg -00 (I)	7 cases with atypical CSD.	-	IFA (1:64) Elevated in most cases	-	-	-	Semi-nested PCR ( <i>gltA</i> and <i>ftsZ</i> ); Myocardium, bone, lymph nodes, EDTA blood +.	The etiological role of <i>B. henselae</i> in patients with chronic multifocal osteomyelitis, cardiomyopathy and lymphadenopathy was supported by positive <i>Bartonella</i> antibody titers and/or amplification and sequencing of <i>B. henselae gltA</i> and <i>ftsZ</i> . Patients with similar clinical entities displayed either Bh <i>ftsZ</i> variant 1 or 2.
Holmberg -99	126 assorted patient sera that had been sent for <i>Bartonella</i> testing.	100 blood donors.	8.3* /4 IFA (1:64)	-	-	-	Semi-nested PCR ( <i>gltA</i> ); Myocardium, liver +.	Overall <i>B.</i> seropositivity rate significantly higher (p<0,02) than among controls. Cases with granulomatous hepatitis, lymphadenopathy, Wegener's granulomatosis, had elevated <i>Bartonella</i> titers/and or <i>B. quintana</i> DNA was amplified.

## Orienteers (II)

During the period 1979-92, a 10- to 100-fold increase in the incidence of SUCD was observed in Swedish male orienteers < 35 years of age and competing at the elite level of orienteering [127,139]. An infectious aetiology to explain this accumulation of SUCD cases was suspected in that there were no signs of drug abuse. *Chlamydia pneumoniae* DNA was

detected in heart and lung specimens of 1 case and elevated IgG antibody titres to *C. pneumoniae* was detected in all 5 cases where serum was available [140]. However, seroprevalence studies displayed that Swedish orienteers did not show a higher prevalence of antibodies to *C. pneumoniae* than healthy blood donors [141]. Why, then, would only elite orienteers suffer from an increased rate of SUCD and not other endurance athletes if *C. pneumoniae* was the causing infectious agent? Preventive measures were taken to avoid new SUCD cases, including a discontinuation of all competition in the elite classes for a 6-month period starting in November 1992, advice to avoid competition if feeling unwell and prescription of macrolides and tetracycline on wide indications to this population in 1992-93. The measures seem to have been effective because the SUCD rate then returned to the normally expected level.

The incidence of myocarditis in previous studies of SUCD in people < 35 years of age was ranging between 0 and 30%. A review of the orienteering SUCD cases revealed a prevalence of myocarditis in 75% of the cases (12/16) and ARVC-like pathology in combination with myocarditis in 4 cases. The overall seropositivity rate to *Bartonella* spp. in a group of ranked orienteers was 31% as compared with 7% in a control group of healthy blood donors [134].

The natural cycle of *Bartonella* spp. with natural reservoirs and incidental hosts infected by arthropod vectors, the life style of the orienteers, seroprevalence data, myocarditis in the majority of cases and PCR findings in combination with the lack of another feasible explanation warrant further investigations. Larger study samples should be collected to elucidate the role of B as an aetiological factor in arrhythmogenic myocarditis.

#### Homeless (IV)

A Swedish homeless population was sampled for the purpose of investigating the prevalence of *Bartonella* infection. Swedish homeless males had a significantly higher risk of being seropositive to *Bartonella* antigens than a matched control group. Reactivity to *B. elizabethae* was dominating in seropositive samples, but the difference between patients and controls was most conspicuous in total reactivity to *B. henselae*. Reactivity to *B. quintana* and *B. grahamii* was infrequent.

The 62.5% overall reactivity to *Bartonella* antigens was higher than those previously reported in IVD users [132,142] or Swedish orienteers [134]. There was no evidence of an ongoing *B. quintana* epidemic. This might be explained by the lack of louse infestation, which also explains the absence of *B. quintana* bacteremia and the low reactivity rate to *B. quintana*. Previous investigations have demonstrated that bacteremia is associated with louse infestation and with homelessness for a shorter period of time and that presence of antibodies to *B. quintana* is correlated to homelessness for a



longer period of time [73,88]. This finding implies the development of protecting antibodies leading to the clearance of bacteremia.

We were unable to detect *Bartonella* DNA in the blood from our homeless male patients by a newly developed QPCR assay. However, we isolated *Acinetobacter* sp. from the blood of one patient. *Acinetobacter* spp. have recently been isolated from lice, as well as from blood collected from homeless individuals [72,143,144] and in experimental infections lice excreted living *Acinetobacter* species in their faeces [145]. In our study the patient with *Acinetobacter* bacteremia was an alcoholic.

## Molecular epidemiology (I, III)

### *B. henselae* (I)

To study the epidemiology of *B. henselae* infections in humans and cats regarding whether different genotypes display varying pathogenic potentials and whether epidemic or endemic strains with geographical differences occur various methods have been used for typing *Bartonella* isolates [38,103,146,147]. For whole-genome comparisons IRS-PCR and microarray comparative genome hybridisations have been employed for *B. henselae* [112,146]. Other techniques use PCR amplification of different genes and intergenic regions from clinical samples and subsequent sequencing for comparison. Those methods are especially valuable when isolates are hard to obtain, which is often the case when working with fastidious slow-growing organisms such as *Bartonella*. The publication of the complete genomes of *B. henselae* and *B. quintana* has facilitated the selection of conserved but still variable genes and intergenic spacers and enabled whole genome comparisons.

The concept of correlating alleles of different genes in order to yield a strain-specific ST has developed into the MLST for the genotyping of *B. henselae*. In the same manner, but only including the polymorphisms of two genes instead of nine genes as for the current MLST, the *rrs* genotypes I and II and *ftsZ* gene variants Bh *ftsZ* 1, 2 and 3 were correlated in 15 *B. henselae* isolates from the United States and Europe in paper I. Based on these results, *rrs* and *ftsZ* are included in the MLST for *B. henselae*.

### *B. grahamii* (III)

MLST and CGH data were combined to explore the genetic relationship of *B. grahamii* isolates derived from woodland rodent populations in three geographic localities in the vicinity of Uppsala in central Sweden. No bacterial host specificity was detected as reported by others [22,24,148]. Kosoy et al. provided data that implied a complex picture where host

bacterial specificity exists in some rodent populations but that other populations harbour genetically diverse *Bartonella* [23]. However, experimental infections in different rodent species provided evidence that bacterial host specificity can occur, suggesting bacterial-host co-speciation [149]. These data were also supported by a study by Bown et al. where grey squirrels introduced into Britain continued to be solely infected by the *Bartonella* associated with grey squirrels native to the United States [16]. Likewise, Ellis et al presented evidence of an Old World origin for *Bartonella* species recovered from Rattus species introduced into the Americas [136]. Another study by Kosoy et al. has demonstrated vertical transmission of *Bartonella* species among natural rodent hosts [150]. This way of transmission could be one possible explanation for host bacterial co-speciation that these studies suggest.

All strains from Håtunaholm were of the same ST and NotI profile. Geographic rather than host species boundaries account for the genetic diversity between *B. grahamii* isolated from animals in the different geographic localities. Except from two animals caught in Kumla all animals that harboured ST 1 were captured in Håtunaholm. All animals that harboured ST 2 or 3 were captured either in Ålbo or in Kumla. Håtunaholm is separated from Ålbo and Kumla by a bay of the lake Mälaren. Thus, it appears that geographic rather than host-species boundaries account for the genetic diversity between *B. grahamii* isolated from animals in the different geographic localities. *Apodemus* sp. and *Clethrionomys glareolus* have been reported to move at most 1500 m in a heterogeneous landscape [151].

In conclusion, nucleotide sequencing of multiple genetic loci in combination with pan-genomic sampling provide excellent tools to relate genotypic differences with epidemiological data. Future investigations with larger sample sizes, different mammalian populations and more knowledge about the ranges of different vectors may shed more light on the issue of bacterial-host interactions and specificity and the implications for public health.

## Concluding summary and future prospects

*Bartonella* infections, including cat scratch disease, osteomyelitis and endocarditis among Swedish patients have been documented. Myocarditis was a common finding among Swedish elite orienteers succumbing to SUCD. *Bartonella* as a plausible aetiological factor was supported by elevated antibody titres to *Bartonella* antigens and *Bartonella* DNA amplified from heart and lung tissues. There was no evidence of an ongoing TF epidemic in a Swedish homeless population, although an increased risk for exposure to *Bartonella* antigens was demonstrated. *ftsZ* gene variation is a useful tool for diagnostic and epidemiological purposes and has become a part of a recognised MLST for *Bartonella* isolates. MLST and CGH data of *B. grahamii* isolates from woodland sylvatic animals in the Uppsala region displayed variants that were not related to specific host species but to geographic locality.

Future studies should consider the following issues:

1. Clinicians need to take into account *Bartonella* infections more often. *Bartonella* infections are likely underdiagnosed.
2. More extensive field studies should be conducted to obtain *Bartonella* isolates from natural reservoirs such as moose and other rodent populations. Perhaps we can find an answer to the question of why *Bartonella* species are responsible for the elevated *B. elizabethae* seroprevalence in some populations. *Bartonella* are fairly easily isolated from its natural reservoir hosts.
3. Our hypotheses on the *Bartonella* aetiology in cases of sudden cardiac death among elite orienteers to be verified. These conclusions remain controversial as long as they do not gain support from other studies. A national (or international) multi-centre study should be designed for the inclusion of all non-ischaemic cardiac deaths with signs of myocarditis for *Bartonella* testing.
4. Learn more about the pathogenesis in *Bartonella* infections. An animal model might provide insights into the bacterium's ability to control its host-interaction genes.

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