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Evaluation of Human Seroreactivity to *Bartonella* Species in Sweden

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Among the species that compose the expanding genus *Bartonella*, thus far only *B. henselae* and *B. quintana* have reportedly been isolated from humans in Europe. To evaluate the prevalence of *Bartonella* infection in Sweden, we conducted a retrospective serological examination of 126 human serum samples. These samples were analyzed for antibodies to *B. henselae*, *B. quintana*, and *B. elizabethae*. Serum samples from 100 blood donors, who spanned the ages of 20 to 60 and had no apparent clinical signs of illness, were also studied as a control group. An immunoglobulin G indirect fluorescence antibody assay revealed 4 and 8.3% *Bartonella* positivity rates for the blood donor and patient group, respectively, when a cutoff titer of ≥64 was chosen. Among the blood donors, four were seropositive to *B. elizabethae*; one of these also had concordant positive titer to *B. henselae*. In the patient group, 14 serum samples were positive against *Bartonella* spp. These serum specimens represented nine patients. In three of these seropositive patients, paired serum samples displayed a fourfold increase in antibody titer to at least one of the three antigens. These three patients are discussed. In this report we also present a case study of a 60-year-old Swedish male with fatal myocarditis. Postmortem serological analysis revealed a high titer against *B. elizabethae*. PCR and nucleotide sequencing of the myocardial tissue from this patient, and of liver tissue from one of the other three patients, showed sequences similar to *B. quintana*. The age, geographical origin, animal contacts, and serological response pattern to the different *Bartonella* antigens differed among the four patients. This study substantiates the presence of *Bartonella* spp. in Sweden, documents the seroreactivity to three *Bartonella* antigens in Swedish patients, and reports the first two cases of *B. quintana*-like infections in Sweden.

Four *Bartonella* species have been isolated from human patients. Two of these have been encountered in Europe, *B. quintana* and *B. henselae. B. quintana*, the etiological agent of trench fever during the two world wars, has emerged as a cause of endocarditis in alcoholics and homeless men (4, 5, 12) and of bacillary angiomatosis in immunocompromised patients. *B. henselae* was recently characterized as the agent of cat scratch disease (9, 10) but can also give rise to endocarditis and, in immunocompromised patients, to bacillary angiomatosis and bacillary peliosis (11). *B. bacilliformis* is the cause of Carrión's disease, which is endemic to regions of the Andes. *B. elizabethae* has only been found in one human case of endocarditis in the United States (3).

The epidemiology of *Bartonella* infections is poorly understood; most *B. henselae* infections are probably acquired from infected cats, but no animal reservoir has been implicated for *B. quintana*. However, this infection can be transmitted by the human body louse (7). In Scandinavia, a few cases of seropositivity to *Bartonella* have been reported from Denmark (1) and one case of endocarditis caused by *B. quintana* was reported in Finland (5). We are not aware of any reports of *Bartonella* infections in Sweden.

With the aims of studying the occurrence of *Bartonella* infections in Sweden and identifying *Bartonella* species and strains, we have evaluated the seroreactivity to *Bartonella* antigens of selected patients and blood donors. Some Swedish *Bartonella*-infected patients with unusual clinical presentation were found and characterized.

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MATERIALS AND METHODS

Samples and patients. One hundred serum samples from healthy Swedish blood donors were used as controls. The specimens were obtained from the local blood bank at Uppsala University Hospital, representing donors from the region around Uppsala, and were collected during 1992. The serum samples were divided into four age groups: 20 to 29 year olds, 30 to 39 year olds, 40 to 49 year olds, and 50 to 59 year olds; 25% of the serum samples were in each group.

One hundred twenty-six serum samples, sent to our clinical microbiology laboratory to be tested for *Bartonella* antibodies, were serologically evaluated. The specimens had been obtained from 1994 through 1997 from 109 patients living in different parts of Sweden. The majority of the patients were adults (75% were >18 years of age) with a median age of 34 years (range, 1 to 82 years); cases were equally distributed between the sexes (female: male = 1:1.2). For 14 patients, more than one serum sample was available.

For one additional patient, who suddenly died in April 1994 with fatal myocarditis, tissue samples and a serum sample were obtained at autopsy. DNA was extracted from the heart tissue samples by using the QiaAmp Tissue Kit (Qiagen Inc., Stanford, Calif.), with an additional final ethanol precipitation. Heart tissue samples from six patients with no known heart disease were used as negative controls and were treated in the same way as the heart tissue samples from the myocarditis patient.

Cultivation of *Bartonella* spp. Bacterial strains were cultivated on 5% defibrinated rabbit blood heart infusion agar (BBL Microbiology Systems, Cockeysville, Md.) at 34°C in the presence of CO_2. The strains used were *B. henselae* Houston-1 isolate, (ATCC 49882), *B. elizabethae* F9251 (ATCC 49927), and *B. quintana* (OK 90-268). Cultures were incubated for a period of 3 to 4 days. Cocultivation of Vero cells with *Bartonella* spp. was subsequently performed in accordance with previously established standards (10). All organisms were inactivated by gamma irradiation (500,000 rads) and stored at −70°C prior to further use.

IFA. Serum samples were analyzed by an indirect fluorescence antibody assay (IFA) for immunoglobulin G (IgG) reactivity against the aforementioned three *Bartonella* strains. The IFA assay was adapted from a previously described protocol (10) with slight modifications. Briefly, aliquots of crude antigen were applied to 10-well Teflon-coated microscope slides (Novakemi AB, Uppsala,

TABLE 1. Oligonucleotides complementary to *Bartonella GltA* that were used in the PCR assays

Name	Sequence	Target organism	Target gene	Nucleotide start position (direction)
BHCS212.p	GTT ATC CTA TTG ACC AA	*Bartonella* spp.	*GltA*	212(→)
BHCS613.n	TAT TCT TCA CAA GGA AC	*Bartonella* spp.	*GltA*	613(←)
BHCS510.p	AAC TCT TGC CGC TAT GG	*Bartonella* spp.	*GltA*	510(→)
BHCS897.n	CCA AAA CCC ATA AGG CG	*Bartonella* spp.	*GltA*	897(←)

Sweden), air-dried, fixed in acetone, and stored at −70°C until being used. Serum samples, including appropriate controls, were diluted in phosphate-buffered saline (PBS) with 5% skim milk and applied to the slides in 30-μl aliquots of serial dilutions, ranging from 1:32 to 1:2,048. Following incubation at 35°C for 30 min, slides were washed in PBS, air dried, and coated with a 1:120 working dilution of commercial fluorescein isothiocyanate-conjugated rabbit anti-human IgG (Dakopatts, Glostrup, Denmark). The slides were then incubated for an additional 30 min, washed and dried as before, and mounted in buffered glycerol (Vector, Burlingame, Calif.). Using a Nikon fluorescence microscope under ×40 magnification, we subjectively scored specific immunofluorescence on a scale of 0 to 3+; a rating of 2+ at a 1:64 dilution was considered indicative of seropositivity for all three *Bartonella* antigens. IFA *Bartonella* titers were reported as the reciprocal of serum endpoint dilutions.

PCR and nucleotide sequencing. PCR assays targeting the *gltA* gene were performed. A set of oligonucleotide primers amplifying 685 bp of the gene was constructed with the assistance of Oligo version 4.0 for Macintosh (National Biosciences, Inc., Plymouth, Minn.). The primers were chosen to match the three different species, *B. henselae*, *B. quintana*, and *B. elizabethae* (Table 1). A seminested amplification protocol was employed. The first PCR amplification used primers BHCS212.p and BHCS897.n in the following protocol: 95°C for 20 s, 1°C decrease in annealing temperature each cycle starting at 50°C for 1 min, 72°C for 1.5 min for 10 cycles. Subsequently, 40 cycles of 94°C for 20 s, 40°C for 1 min, and 72°C for 1.5 min were performed. This was followed by a second amplification at 94°C for 1 min, 49°C for 1 min, and 72°C for 1 min for 40 cycles with 1 μl from the first reaction as template in a total reaction volume of 50 μl. Two separate seminested PCRs were performed, with either the primers BHCS212.p and BHCS613.n or the primers BHCS510.p and BHCS897.n. All reactions included positive and negative controls. Measures were taken to prevent carryover contamination by using different rooms for template handling of the first and second reaction, amplification, and analysis on gels.

The four oligonucleotides were used to generate sequencing products. DNA sequencing was performed by using a DNA sequencing kit (Dye terminator cycle sequencing kit; Applied Biosystems Inc., Foster City, Calif.) to generate sequencing products. The sequence analysis was performed with a 310 automated sequencer (Applied Biosystems).

RESULTS

Seroreactivity to *Bartonella* spp. in Swedish blood donors. Of the 100 blood donor serum samples, 4 were positive for *B. elizabethae* at a titer of 64 or higher. One of these also reacted positively to *B. henselae*, but none reacted positively to *B. quintana*. The overall positivity rate was thus 4%. For titers and age distribution, see Table 2.

Seroreactivity to *Bartonella* spp. in Swedish patients. Fourteen of the 126 serum samples (11%) were considered positive with a titer of ≥64. These 14 samples represented 9 patients of a total of 109 patients (8.3%). The frequency of seropositivity in this cohort was significantly higher than in the blood donors ($P < 0.02$, calculated by using the χ^2 test). For five of the seropositive patients, paired serum samples were available. Three of these displayed a fourfold or greater increase in titer from the acute to the convalescent phase to at least one of the *Bartonella* species. Of the nine seropositive patients, three had a granulomatous hepatitis, two had lymphadenopathy, two had Wegener's granulomatosis, and one had a paralysis in her arm after a cat scratch. In the remaining seropositive patient, we did not know the clinical history. Of the seronegative patients with specified clinical histories, 26 had lymphadenopathy, 17 had fever of unknown origin, 7 had hepatitis, 3 had Wegener's granulomatosis, 5 had symptoms affecting the central nervous system, and 3 had heart involvement. In 33 patients there was no specified clinical history (30%).

TABLE 2. Seropositivity to *Bartonella* antigens among 100 Swedish blood donors

Age group (yr)	No. positive	Titer to:	
		B. elizabethae	*B. henselae*
20–29	1	128	<64
30–39	1	64	128
40–49	0		
50–60	2	128	<64
		64	<64

Three patients with fourfold increases in *Bartonella* titers in consecutive serum samples. Patient 1 was an 18-year-old woman with chronic suppurative submandibular lymph nodes. A biopsy of the lymph node showed granulomatous inflammation with multinucleated giant cells but no sulfur granules. Standard bacterial aerobic and anaerobic cultures were negative as were fungal and mycobacterial cultures. Furthermore, serology for *Toxoplasma* and tularemia was negative but was weakly positive for *Chlamydia pneumoniae* (IFA titer of 64). She was treated with several antibiotics (penicillin V, trimethoprim-sulfamethoxazole, clindamycin, and metronidazole) during prolonged periods, with no permanent effect. The patient developed a positive anti-neutrophil cytoplasmic antibody test (cANCA) and was diagnosed as having Wegener's granulomatosis. She was found to have classical alpha-1-antitrypsin deficiency (Pi type ZZ). Immunosuppressive treatment was initiated and her clinical condition improved. The patient owned a cat. Serum specimens from a 3-year period were assayed for *Bartonella* antibodies. The patient had an initial increase in titer to all three *Bartonella* species. After 2 years a low titer to *B. elizabethae* persisted, and after 3 years the patient was seronegative (Table 3).

Patient 2 was a 17-year-old woman with a 2-year history of relapsing ulcerating submandibular lymph nodes. Curettage of the ulceration showed unspecific granulomatous tissue with no giant cells or acid-fast bacilli. Bacterial and fungal cultures showed only normal dermal bacteria, and mycobacterial cultures were negative. *Toxoplasma* and tularemia serology was negative, but serology was weakly positive against *C. pneumoniae* with a titer of 64. Acute-phase reactants in serum were within normal limits, but the patient had antinuclear antibodies (1/100). Computerized tomography of the mandible showed a dental radix cyst or granuloma that had fistulated and was subsequently treated by a dental surgeon. After *Bartonella* serology was found to be positive with a fourfold titer rise between two serum samples, clindamycin was prescribed. The mandibular ulcer healed. The patient had been in frequent contact with cats.

Patient 3 was a 73-year-old man with history of relapsing pain in the right costal region accompanied by night sweat and fever since December 1996. A computerized tomography (CT) scan in May 1997 showed a 5-cm-diameter tumor-like attenu-

TABLE 3. Four patients with significant change in titers and/or PCR positivity to *Bartonella*

Patient no.	Age (yr)	Diagnosis	Date of serum sample	Titer to:			Animal contact
				B. henselae	*B. elizabethae*	*B. quintana*	
1	18	Wegener's granuloma	03/10/95	128	128	256	Cat
			03/28/95	128	512	256	
			05/17/95	<64	128	64	
			01/09/97	<64	64	<64	
			05/12/97	<64	<64	<64	
			05/18/98	<64	<64	<64	
2	17	Submandibular ulcer	05/05/97	128	<64	64	Cat
			06/16/97	128	128	128	
3	73	Hepatic granuloma	06/18/97	<64	<64	<64	Mice, rats
			10/09/97	<64	128	<64	
			05/10/98	<64	512	<64	
4	59	Myocarditis	04/03/94	<64	2,048	128	

ation in the right liver lobe. At laparotomy, a firm fibrosis-like alteration was found, and a liver biopsy showed chronic granulomatous inflammation and necrosis but no malignant cells. A PCR test for tubercle bacilli was negative, as was serology for Q fever, ehrlichiosis, tularemia, and brucellosis. There were no IgM antibodies to *Toxoplasma gondi*, and a cANCA test for Wegener's granulomatosis was negative. However, the patient had a stationary IgG titer of 1/512 to *C. pneumoniae*. The symptoms gradually disappeared without any antibiotic treatment, but a relapse occurred 6 months later. A new CT scan showed that the tumor had increased in size to 7.5 cm in diameter. A fourfold titer rise of antibodies to *B. elizabethae* was then found. A needle biopsy of the liver showed chronic inflammation with no specific features. PCR of the liver tissue was positive by using *Bartonella*-specific citrate synthase primers, and sequencing from the PCR product was performed. Sequence alignment showed a 99.7% (683 of 685 nucleotide positions) similarity to *B. quintana* Fuller strain, and 85.0% (582 of 685 nucleotide positions) similarity to the *B. elizabethae* type strain. Treatment with clarithromycin and amoxycillin was then instituted. After 3 weeks of antimicrobial chemotherapy a new CT scan showed that the liver tumor had decreased in size to 2 cm in diameter. The patient was not aware of any cat contact but worked as a janitor and had frequently cleaned up basements of old houses. He was also an ardent rat and mouse catcher, setting traps every fall and winter.

From a fourth patient with a fatal myocarditis of unknown origin, several tissues were tested with PCR assays by using *Bartonella*-specific oligonucleotide primers. Products from tissues derived from the right ventricle and left atrium were positive with bands of the expected size. Sequencing of the PCR product showed two point mutations in a 685-bp fragment of the citrate synthase gene compared to the *B. quintana* reference strain. The sequence derived from this patient was identical to that derived from patient 3. The patient had no recent cat contacts.

For serology results from all four patients, see Table 3.

DISCUSSION

Nine of 109 Swedish patients and 4 of 100 healthy Swedish blood donors had antibodies to *Bartonella* spp. in an IFA. The patients, most of whom were adults, lived in different parts of the country. A fourfold or greater increase in titer to *Bartonella* antigens was demonstrated in three of the patients, indicating recent infections. Two patients were positive for *Bartonella* by a PCR assay. None of these patients with significant rises in antibody titer or PCR positivity had visited other countries recently, indicating that transmission of *Bartonella* occurred in Sweden. The clinical signs and symptoms, the epidemiology, and the serological response differed among the four patients described. Two were young women, had cat contacts, were from the northern part of the country, and reacted strongly to all three *Bartonella* antigens tested. The other two were older men from central and southern Sweden with no known cat contacts. They reacted strongly to *B. elizabethae* antigens (and in one case also weakly to *B. quintana*) but were demonstrated to harbor DNA from a *B. quintana*-like organism. Our findings thus suggest that both *B. henselae* and *B. quintana* are transmitted in Sweden.

It is well-known that *Bartonella* spp. cross-react within the genus, as well as to some other species, such as *Coxiella* (6) and *Chlamydia* (8). Our patients were negative in serological tests for Q fever and did not have detectable levels of antibodies against tularemia or *Brucella*. Three of the patients were weakly (64) and one patient was strongly (512) positive for *C. pneumoniae*. However, the high *C. pneumoniae* titer in this patient was stationary during the time when the *Bartonella* antibody titer increased. We conclude that *C. pneumoniae* was not likely to be related to that patient's illness, but represented an earlier exposure to *C. pneumoniae*.

In the blood donors, as well as in the patients, antibodies to *B. elizabethae* were the most frequent finding. This pattern has previously been observed in a study of urban intravenous drug users (2). However, our two patients with PCR-verified *B. quintana* infections both had high titers against *B. elizabethae* but not (or only weakly) against *B. quintana*. An isolated *B. elizabethae* titer in the IFA may thus not always indicate a specific response to that species but may represent a cross-reaction between *Bartonella* species. We speculate that an antigenic variant of *B. quintana* might exist in Sweden, sharing antigenic epitopes with the *B. elizabethae* type strain used in the IFA.

Including *B. elizabethae* antigens in the IFA allowed us to identify several patients with positive *Bartonella* serology and signs of recent infection who would otherwise have been overlooked. Using antigens from more than one *Bartonella* species in the IFA can thus increase the sensitivity of this assay. Further studies of both human and animal populations are clearly needed to isolate and characterize *Bartonella* species and strains in Sweden.

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