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ORIGINAL INVESTIGATION

Bartonella quintana and *Bartonella vinsonii* subsp. *vinsonii* bloodstream co-infection in a girl from North Carolina, USA

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Abstract
The genus *Bartonella* consists of globally distributed and highly diverse alpha-proteobacteria that infect a wide-range of mammals. Medically, *Bartonella* spp. constitute emerging, vector-borne, zoonotic, intravascular organisms that induce long-lasting bacteremia in reservoir-adapted (passive carrier of a microorganism) hosts. At times, these bacteria are accidentally transmitted by animal scratches, bites, needles sticks or vectors to animal or human hosts. We report the first documented human case of blood stream infection with *Bartonella vinsonii* subsp. *vinsonii* in a girl from North Carolina, USA, who was co-infected with *Bartonella quintana*. Limitations of *Bartonella* spp. serology and the challenges of microbiological culture and molecular diagnostic confirmation of co-infection with more than one *Bartonella* spp. are discussed. When and where these infections were acquired is unknown; however, exposure to rodents, fleas and cats in the peri-equestrian environment was a suspected source for transmission of both organisms.

Keywords *Bartonella quintana* · *Bartonella vinsonii* subsp. *vinsonii* · Bacteria · Vector · Equestrian

Introduction

During the past two decades, there has been increasing scientific interest in the genus *Bartonella*, in conjunction with an increasing medical emphasis on the seemingly diverse spectrum of cardiovascular, neurological and rheumatologic disease manifestations that can result from persistent bloodstream infection with these bacteria in animals and humans [1–3]. Historically, *Bartonella quintana*, the cause of Trench Fever in World War I, has been more recently referred to as Urban Trench Fever, most often infecting homeless individuals with human body louse exposure [2]. *Bartonella vinsonii* subsp. *vinsonii* was isolated from voles during the 1940s by James Baker, Veterinary Corps, U.S. Army, while stationed at the War Disease Control Station at Grosse Isle, Quebec Province, Canada [4]. The organism was named in honor of J. William Vinson, who along with Henry S. Fuller first determined that *Bartonella* spp. could occasionally be isolated on blood agar plates. Subsequently, *B. vinsonii* subsp. *berkhoffii* [5], *B. vinsonii* subsp. *arupensis* [6], and *B. vinsonii* subsp. *yucatanensis* [7] were described in 1996, 1998, and 2016, respectively. Infection with *B. vinsonii* subsp. *berkhoffii* and *B. vinsonii* subsp. *arupensis* have been previously reported in association with human illnesses [8, 9], but to our knowledge, infection with *B. vinsonii* subsp. *vinsonii* has not been reported in a human being.

Materials and methods

Case report

In September 2017, the mother of a 14-year-old Caucasian female contacted our research group requesting that her daughter be tested for evidence of *Bartonella* spp. bloodstream infection. With signed parental permission, the child was entered into North Carolina State University Institutional Review Board (IRB) Protocol #1960, Detection of *Bartonella* species in the blood of healthy and sick people with animal contact or arthropod exposures. Based upon responses to our IRB research questionnaire, the girl's historical duration of illness was 5 years, during which time she had experienced intermittent fever, ocular pain,

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balance problems, headaches, irritability, blurred vision, mental confusion, disorientation, hallucinations, memory loss, insomnia, tremors, anxiety and panic attacks. Other listed symptoms included fatigue, joint pain involving the elbows, shoulders, hips, knees and ankles, muscle weakness and pain, tachycardia and diarrhea. During the course of illness, the severity, frequency and number of symptoms progressively increased. Historically, the girl was an avid equestrian (riding nearly every day of the week), had dog and horse exposure for the past 5 years, and had traveled throughout the United States and to Europe. Vector exposure was reported for mosquitoes and ticks. There was no historical exposure to pet rodent species.

Prior diagnostic testing had been performed by her pediatrician, ear, nose and throat specialist, and allergist. Previous diagnoses listed by the parents on the research questionnaire had included ADHD (Attention Deficit Hyperactivity Disorder), migraines, allergies to dairy products, gluten, and oak tree pollen, auditory processing disorder, learning disability (reading), *Clostridium difficile* colitis and "neuroimmune disorder" (myalgic encephalomyelitis). Repeated physical examinations were unremarkable. White blood cell counts, ranging from 3.3 to 5.5 × 103/μL, were often low despite a concurrent fever. Serial biochemical and urinalyses results were normal. IgA, IgM, IgE and IgG immunoglobulin subclass values were age appropriate. Extensive viral, protozoal, and bacterial (including *Borrelia burgdorferi*) testing was either unremarkable or diagnostically inconclusive. *Mycoplasma pneumoniae* IgG and Coxsackie A&B subtype antibody titers were at times increased.

Beginning in September 2016, osteopathic treatments consisted of manipulation, neuromuscular reeducation, vitamin C, MgCl supplementation, iodine, melatonin, and herbal dietary supplements. Due to ongoing symptoms, doxycycline and cefuroxime were administered in October 2016. In January 2017, a commercial diagnostic laboratory (Galaxy Diagnostics Inc. Research Triangle Park, NC) reported a *B. quintana* serum indirect fluorescent antibody (IFA) titer of 1:64, after which her physician administered doxycycline, rifampin, and clarithromycin for 2 months. Again, antibiotic administration resulted in only minimal symptomatic improvement.

Bartonella spp. serology and enrichment blood culture/PCR testing

Upon entry into our research study in September 2017, blood and serum specimens were obtained aseptically on September 21st and 22nd for *Bartonella* spp. indirect fluorescent antibody serology and BAPGM (*Bartonella* alpha proteobacteria growth medium) enrichment blood culture/PCR testing. Previously described indirect fluorescent antibody serology assays [10, 11, 15] were used to test the girl's serum sample. In addition, *B. quintana* (NCSU 11-MO-01, Monkey origin) organisms were passed from 5% sheep blood agar into cell lines to obtain antigens for IFA testing as described previously [12]. For all six IFA assays, heavily infected cell cultures were spotted onto 30-well Teflon coated slides (Cel-Line/Thermo Scientific, Waltham, MA), air dried, acetone fixed and stored frozen until tested. Serum samples were diluted in phosphate buffered saline (PBS, pH 7.4) containing normal goat serum, Tween-20 and 0.5% of powdered nonfat dry milk to block non-specific antigen binding sites. Sera were tested at dilutions of 1:16–1:8192. Serum that was reactive to all six antigens and a non-seroreactive serum to all antigens were run concurrently with the girl's serum at two independent testing time points.

BAPGM enrichment blood culture, DNA extraction, and DNA sequencing methods were performed as previously described [10, 11]. Quantitative PCR targeting of the *Bartonella* ITS region was performed using oligonucleotides BsppITS325s: 5′ CCT CAG ATG ATG ATC CCA AGC CTT CTG GCG 3′and BsppITS543as: 5′ AAT TGG TGG GCC TGG GAG GAC TTG 3′ as forward and reverse primers, respectively, and oligonucleotides BsppITS438probe: 5′ FAM-GGT TTT CCG GTT TAT CCC GGA GGG C-BHQ1 3′ and BsppITS438Rprobe: 5′ CY5-AGG AAG GTT TTT ACC RAT TTA CTC CTG AGG- IowaBlack3′as taqman probes. Amplification was performed in a 25 μl final volume reaction containing 12.5 μl of SsoAdvanced™ Universal Probes Supermix (Bio-Rad Laboratories, USA), 0.2 μl of 100 μM of each forward primer, reverse primer, and taqman probe (Integrated DNA Technologies, INC., IDT® DNA Technology, Skokie, Illinois), 7.5 μl of molecular–grade water, and 5 μl of DNA from each sample tested. PCR negative controls were prepared using 5 μl of DNA from the blood of a healthy dog. Positive controls for PCR were prepared using 5 μl of DNA from a serial dilution (using dog blood DNA) of *B. henselae* genomic DNA equivalent to 0.1, 0.01, and 0.001 pg/ μl. Quantitative PCR was performed in an CFX96® (Bio-Rad Laboratories, USA) thermocycler under the following conditions: a single hot-start cycle at 95 °C for 3 min followed by 45 cycles of denaturing at 94 °C for 10 s, annealing at 68 °C for 10 s, and extension at 72 °C for 10 s. Amplification was completed by an additional cycle at 72 °C for 30 s. Positive amplification was assessed by analysis of detectable fluorescence by cycle values at Channel 1 and Channel 4. All qPCR positive amplicon products were sent to a commercial laboratory (Genewiz, Research Triangle Park, NC) for DNA sequencing. Bacteria species and strain were defined by comparing similarities with other sequences deposited in the GenBank database using the Basic Local Alignment Search Tool (Blast v. 2.0), and an in-house curated database.

Also, each DNA extraction was tested with *B* spp. *groEL*-20 s (5′ CGTGAAGTTGCTTCTAAAACCAATGACATT

G 3′) and *B* spp. *groEL*-590as (5′ CGAGSGTTGCCAAA GCTTCACCTTC 3′) and with *B* spp, *gltA*-850 s (5′ AGA ATTCCTGAATTYATTGCACGTGCAAAAG 3′) and *B* spp. *gltA*-1120as (5′ CCTAGAGCTTTTAATGTAATW CCAGAATAGAAATC 3′) primers, respectively, that were previously validated to amplify *Bartonella* spp. DNA in our laboratory (unpublished data).

Results

By qPCR targeting the 16S-23S ITS region, *B. quintana* DNA was PCR amplified and sequenced from the girl's blood DNA extraction and *B. vinsonii* subsp. *vinsonii* DNA was amplified and sequenced from a 14-day BAPGM enrichment blood culture. There was no agar plate bacterial growth following subculture from the liquid enrichment medium at 7, 14, or 21 days. The *B. quintana* ITS sequence was 100% similar to GenBank accession DQ648598. The *B. vinsonii* subsp. *vinsonii* DNA sequence was 100% similar to BAO16SRC. Comparative 16S-23S ITS DNA sequences for *B. quintana, B. vinsonii* subsp. *vinsonii* and other selected *Bartonella* spp. or subsp. ranging from 79 to 93% are provided in Table 1. PCR testing of all blood, serum and enrichment culture specimens targeting *groEL* and *gltA* genes failed to result in amplification. By IFA testing, her *B. quintana, B. henselae, B. vinsonii* subsp. *berkhoffii* genotypes I, II and III, and *B. koehlerae* antibody titers were 1:32, 1:32, < 1:16, < 1:16, < 1:16, and < 1:16, respectively (with a non-reactive or negative titer being < 1:64). Identical IFA titers were obtained when the serum was tested at a later time point. *Bartonella vinsonii* subsp. *vinsonii* IFA slides were not available for genotype serological testing.

Phylogenetic analyses using the short DNA sequences amplified from blood or BAPGM enrichment blood culture further supported co-infection with *B. quintana* and *B. vinsonii* subsp. *vinsonii* (Fig. 1). In an effort to further assess qPCR gene target sequence similarities among rodent isolates of the *B. vinsonii* subsp. complex, the analogous region of the *Bartonella* 16S-23S rRNA gene from isolates derived from white-throated woodrats (*Neotoma albigula*) captured in New Mexico (NA789) and Colorado (NA251), from the Pinyon mouse, *Peromyscus truei* (PT719) captured in New Mexico, from the deer mouse (*Peromyscus maniculatus*) [PM370] and brush mouse *Peromyscus boylii* (PB230) captured in Colorado were kindly provided by Dr. Michael Kosoy, Centers for Disease Control and Prevention, Fort Collins, Colorado. The *B. vinsonii* subsp. *vinsonii* qPCR DNA sequence from the girl differed from the five rodent isolates from Colorado and New Mexico, but was 100% similar to the type strain of *B. vinsonii* subsp. *vinsonii* (GenBank accession BAO16SRC), the Baker isolate type strain that originated from Grosse Isle, Quebec Province, Canada.

Consistent with Institutional Review Board requirements, serology and PCR research results were communicated to the girl's attending physician for consideration during future clinical assessments.

Discussion

Using targeted qPCR amplification and DNA sequencing, we confirmed co-infection with two *Bartonella* spp.; *B. quintana* in blood and *B. vinsonii* subsp. *vinsonii* in a 14-day enrichment blood culture, in a girl with historically unexplained symptomatology. *Bartonella* spp. co-infections have been reported infrequently, and to our knowledge only in sick individuals with high exposure risks (i.e., frequent arthropod or animal contact) [15–17]. Due to low bacterial numbers, blood culture isolation of a *Bartonella* spp. is rarely achieved in research intensive or diagnostic clinical settings [17, 18]. PCR using DNA extracted following enrichment culture in insect biochemical growth media [19, 20] or the

Table 1 Number and percentage of bases comparing the 16S-23S intergenic spacer region DNA sequence similarities for *Bartonella quintana* and *Bartonella vinsonii* subsp. *vinsonii* to other selected *Bartonella* species or subspecies

	Sample # 11,241 (blood)	Sample # 11,242 (C14 enrichment blood culture)
Bartonella quintana (DQ648598)	167/167 (100%)	133/167 (80%)
Bartonella v. vinsonii (BAO16SRC)	132/167 (79%)	167/167 (100%)
Bartonella v. arupensis (JN402327)	135/167 (81%)	155/167 (93%)
Bartonella henselae Houston-I (CP020742)	135/167 (81%)	136/167 (81%)
Bartonella koehlerae (AF312490)	135/167 (81%)	135/167 (81%)
Bartonella v. berkhoffii genotype I (DQ059762)	136/167 (81%)	149/167 (89%)
Bartonella v. berkhoffii genotype II (DQ059763)	135/167 (81%)	145/167 (87%)
Bartonella v. berkhoffii genotype III (DQ059764)	136/167 (81%)	149/167 (89%)
Bartonella v. berkhoffii genotype IV (DQ059765)	135/167 (81%)	145/167 (87%)

v. vinsonii subsp

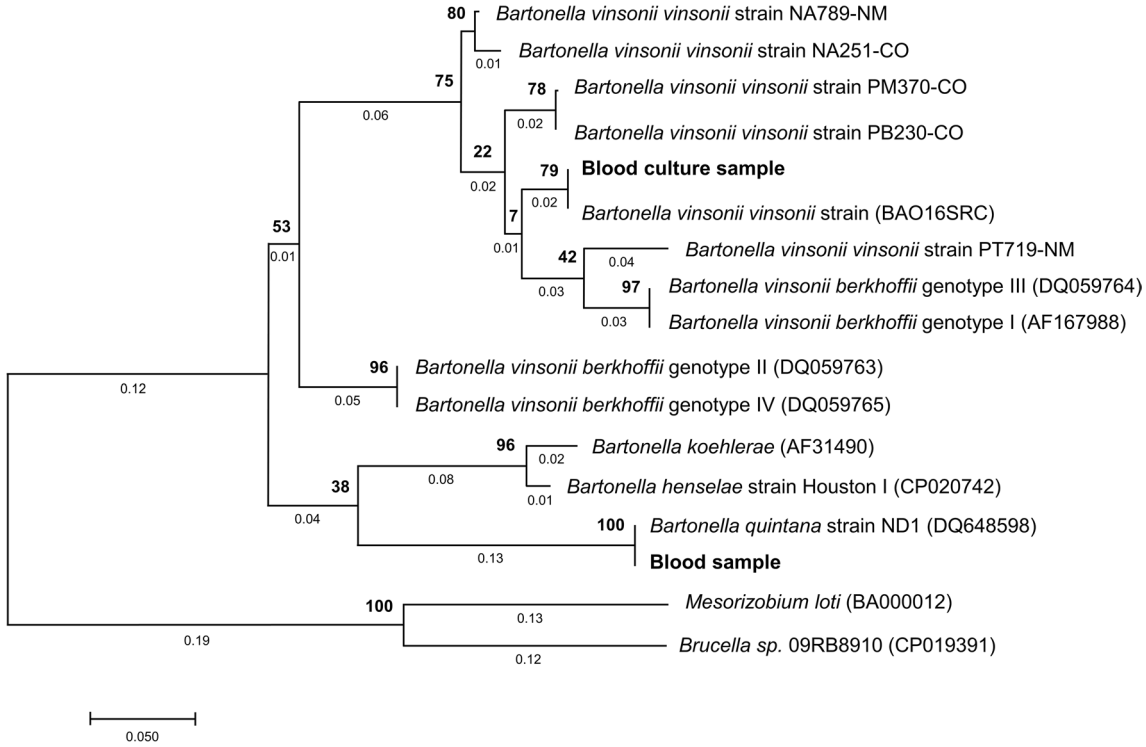

Fig. 1 Using the *B. quintana* and *B. vinsonii* subsp. *vinsonii* 16S-23S ITS sequences generated from the girl's blood or enrichment blood culture, an evolutionary history was inferred using the Maximum Likelihood method based on the Tamura-Nei model. The tree with the highest log likelihood (−742.4888) is shown. The percentage of trees in which the associated taxa clustered together is shown next to the branches. Initial tree(s) for the heuristic search were obtained automatically by applying Neighbor-Join and BioNJ algorithms to a matrix of pairwise distances estimated using the Maximum Composite Likelihood (MCL) approach, and then selecting the topology with superior log-likelihood value. The tree is drawn to scale, with branch lengths measured in the number of substitutions per site (next to the branches). The analysis involved 11 nucleotide sequences. There were a total of 194 positions in the final dataset. Evolutionary analyses were conducted in MEGA7 [13, 14]

use of cell-based isolation systems [18, 21, 22] improves *Bartonella* spp. isolation and/or PCR DNA amplification. Due to presumed relapsing bacteremia, testing serial blood specimens [23] in conjunction with prolonged enrichment culture incubation times [18, 22] are often necessary to successfully document infection. In this case *B. vinsonii* subsp. *vinsonii* DNA was only amplified and sequenced from one 14-day enrichment blood culture (7 and 21 day cultures were PCR negative). Similarly, *Bartonella quintana* DNA was only amplified and successfully sequenced from one of two blood specimens (both sera were PCR negative). In addition to testing sequential blood cultures, co-infections are more frequently detected by targeting multiple *Bartonella* spp. gene targets, and at times only using well-defined species or genotype PCR primer sets [16, 24, 25]. Consistent with PCR results in this and other studies [26, 27], primers targeting the 16S-23S ITS region are generally more sensitive than *glt*A or *groEl* PCR primers, unless testing DNA derived from *Bartonella* spp. isolates [27]. The advent of more sensitive molecular diagnostic assays and ongoing improvements in microbiological culture techniques have resulted in the association of an increasing number of *Bartonella* spp. with a seemingly diverse array of disease manifestations [1–3, 27]. Based upon this case report, *B. vinsonii* subsp. *vinsonii* should be added to the list of zoonotic *Bartonella* spp.

Why patients with documented persistent *Bartonella* spp. infections are not seroreactive to the infecting *Bartonella* spp. is most likely multifactorial. Immunological anergy, strain variation in immunogenicity, and IFA technical limitations may all be contributing factors [10, 16]. Consistent with previous reports [10, 16], this girl's sera (IFA results repeatable at a later date) were minimally reactive to six *Bartonella* spp./genotypes. Additional efforts to enhance the diagnostic sensitivity of serology, culture, and molecular-based diagnostic modalities are warranted.

Of comparative medical importance, disease (bartonellosis) occurs when one or more reservoir-adapted *Bartonella* spp. are transmitted to a non-reservoir-adapted host, such as a human [1, 27]. Due to the relatively recent recognition of long-lasting *Bartonella* spp. blood stream infections in immunocompetent humans [10, 28], including healthy blood donors [29, 30], the clinical, diagnostic and pathological

consequences of accidental, ecological spillover of one or more *Bartonella* spp. into a non-reservoir adapted human host remains unclear and incompletely investigated [1–3, 28]. However, on the basis of evolving comparative microbiological and pathological data, *Bartonella* spp. may be a historically unsuspected cause or cofactor in a spectrum of cardiovascular, neurological and rheumatological diseases in animals and human patients [3, 11, 15, 17]. Similar to the medical history reported in this girl, investigators in France have reported longstanding, non-specific symptoms in conjunction with bloodstream infection with known and previously undescribed *Bartonella* spp. in humans [17]. The girl in this report experienced symptoms most indicative of neurological (headache, irritability, blurred vision, mental confusion, disorientation, hallucinations, memory loss) and rheumatological (pain involving multiple joints) diseases. Our research group has focused primarily on testing animal health workers, who often experience frequent exposures to arthropods and infected animals [12–16, 31, 32]. We suggest that animal health workers and others with extensive arthropod and animal contact will become the sentinel populations by which the microbiological and medical relevance of bartonellosis is more thoroughly defined. Due to similarities in *Bartonella* spp. associated pathology among animals, particularly dogs and humans, we and others have proposed a comparative One Health approach to better clarify the medical importance of the genus *Bartonella* [1–3, 33].

As the isolation and PCR detection of rodent, bat and other reservoir-adapted *Bartonella* spp. from human blood lacks sensitivity, infection may occur more frequently than has been historically suspected [34, 35]. For example, studies from Thailand, Nigeria, the United States and other countries, have documented human infections with *Bartonella* spp. that are genetically similar to those found in regional rodents [35–37]. Rodents, the primary reservoir hosts for *B. vinsonii* subsp. *arupensis, B. vinsonii* subsp. *vinsonii* and *B. vinsonii* subsp. *yucatanensis* are most likely infected by fleas or other hematophagous arthropod vectors [6, 36, 38]. Recent studies from northern Mexico and the Yucatan ecosystem found up to 58% of *Peromyscus yucatanicus* (deer mice) and other *Peromyscus* spp. were infected with *B. vinsonii* subsp. *vinsonii* or *B. vinsonii* subsp. *yucatanensis* [7, 36, 37]. These and other studies illustrate the high prevalence of bacteremia found among many rodent reservoir hosts. Based upon comparative host–pathogen co-evolutionary analyses, *Bartonella* spp., unlike comparative analyses of *Leptospira* spp., have co-evolved with bats, rodents and other mammals, with minimal evolutionary host switching [37]. It is possible that exposure to peridomestic rodents or rodent fleas, potentially at an equestrian center, could have been the source of the girl's *B. vinsonii* subsp. *vinsonii* and *B. quintana* infections. Cats, dogs, horses, humans and rodents and their associated arthropod vectors are all commonly found around equestrian stables and farms. In 2007, we reported *B. quintana* blood stream infection in an equestrian instructor from North Carolina and in the blood of the feral cat that most likely infected her with *B. quintana* via a bite wound [39]. Other equestrian case-based [40] and epidemiological studies [41] have reported potential associations between *Bartonella* spp. exposures with cattle and horse farms. Whether infection with *B. quintana* and *B. vinsonii* subsp. *vinsonii* were transmitted to this girl simultaneously or sequentially, and the extent to which co-infection may have contributed to the girl's historical symptoms could not be determined.

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Compliance with ethical standards

Conflict of interest E.B.B., in conjunction with Sushama Sontakke and North Carolina State University, holds US Patent No. 7,115,385, Media and Methods for Cultivation of Microorganisms, which was issued October 3, 2006. E.B.B. is a founder, shareholder and the chief scientific officer for Galaxy Diagnostics, a company that provides diagnostic testing for the detection of *Bartonella* species and other vector borne pathogens in animals and human patients. R.G. Maggi has lead research efforts to optimize the BAPGM platform and is the scientific technical advisor for Galaxy Diagnostics.

Ethical approval All procedures performed in studies involving human participants were in accordance with the ethical standards of the institutional and/or national research committee and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards. North Carolina State University (NCSU) Institutional Review Board provided (IRB 1960) ethical approval for this study.

Informed consent Informed consent was obtained from the parents of the participant included in this study.

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