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HOST MICROBE INTERACTIONS

Dynamics of Co-Infection with *Bartonella henselae* Genotypes I and II in Naturally Infected Cats: Implications for Feline Vaccine Development

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Abstract *Bartonella henselae* is an emerging bacterial pathogen causing cat-scratch disease and potentially fatal bacillary angiomatosis in humans. Bacteremic cats constitute a large reservoir for human infection. Although feline vaccination is a potential strategy to prevent human infection, selection of appropriate *B. henselae* strains is critical for successful vaccine development. Two distinct genotypes of *B. henselae* (type I, type II) have been identified and are known to co-infect the feline host, but very little is known about the interaction of these two genotypes during co-infection in vivo. To study the in vivo dynamics of type I and type II co-infection, we evaluated three kittens that were naturally flea-infected with both *B. henselae* type I and type II. Fifty individual bloodstream isolates from each of the cats over multiple time points were molecularly typed (by 16S rRNA gene sequencing), to determine the prevalence of the two genotypes over 2 years of persistent infection. We found that both *B. henselae* genotypes were transmitted simultaneously to each cat via natural flea infestation, resulting in mixed infection with both genotypes. Although the initial infection was predominately type I, after the first 2 months, the isolated genotype shifted to exclusively type II, which then persisted with a relapsing pattern. Understanding the parameters of protection against both genotypes of *B. henselae*, and the competitive dynamics in vivo between the two genotypes, will be critical in the development of a successful feline vaccine that can ultimately prevent *B. henselae* transmission to human contacts.

Keywords *Bartonella henselae* · Genotypes · Feline bacteremia · Vaccine · *hbpA*

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Introduction

Bartonella henselae is a fastidious, gram-negative, bacterial pathogen that causes cat-scratch disease (CSD) and bacillary angiomatosis (BA). Nearly 13,000 new cases of CSD are reported in the USA each year. Five hundred of these patients require hospitalization, making CSD one of the most common zoonotic, non-foodborne infectious diseases [1]. In immunocompetent individuals, CSD usually is characterized by self-limiting lymphadenopathy following traumatic contact from a cat [2]. In immunocompromised individuals, *B. henselae* infection is associated with serious clinical manifestations such as potentially fatal BA, peliosis hepatis, and endocarditis [3–6].

Domestic cats are the major reservoir for *B. henselae*, and the cat flea (*Ctenocephalides felis*) is the principal vector of transmission among cats [7]. After transmission, *B. henselae* achieves a high density in the bloodstream of its feline host (10^4 to 10^6 colony-forming units [CFU]/ml) and can persist, causing relapsing bacteremia for months and sometimes years [8, 9]. In some regions, the prevalence of *B. henselae*

bacteremia in the cat population is 41% [10], with *B. henselae* seroprevalence of up to 81% [7]. Despite high numbers of *B. henselae* bacteria in the bloodstream, most infected cats remain asymptomatic. Humans usually are infected when flea feces containing *B. henselae* are inoculated during a scratch from a contaminated cat claw [11].

Two genotypes of *B. henselae* have been identified, based on 16S rRNA gene (ribosomal DNA (rDNA)) sequence differences: type I (*B. henselae* Houston-1, ATCC type strain) and type II (Marseille strain) [12, 13]. The genetically distinct types I and II correspond to two unique *B. henselae* serotypes [13, 14], and there also are phenotypic differences between type I and II bloodstream isolates: Type I colonies are usually smooth and faster-growing, but type II colonies appear dry and pit the agar surface [15].

Although the *B. henselae* type I and II genotypes are very closely related, they interact differently with feline and human hosts during infection. *B. henselae* type I is more frequently detected in humans with CSD, even when cats in the environment are more prevalently infected with type II [12, 16–18]. *B. henselae* type I also appears to be more virulent in humans than type II [18–20], and type I has a unique tropism for invasion of human liver and spleen [19]. The few cases of feline endocarditis caused by *B. henselae* belong to type I [21]. In cats, either type I or type II *B. henselae* can cause bacteremia, but infection with one genotype is not fully protective against infection with the heterologous genotype [9]. Prior infection with *B. henselae* type I was shown to be protective against challenge with the same type (type I) and was sometimes protective against challenge with type II [9]. In contrast, prior infection with *B. henselae* type II protects only against challenge with type II (unidirectional cross protection).

Simultaneous, naturally occurring co-infection of cats with both type I and type II genotypes has been documented [22], but it is not known whether transmission of *B. henselae* type I and type II to these cats occurred simultaneously or as distinct events and whether one of the genotypes has a competitive advantage during prolonged feline bloodstream infection. Nothing is known about the interactions of these two genotypes during simultaneous infection in the cat reservoir or about the basis for the differences in bacterial virulence and host immune response.

Controlling *B. henselae* infection in the feline reservoir is essential to preventing *B. henselae* infection in humans, thus reducing morbidity and mortality. One obstacle to the successful eradication of *B. henselae* in feline populations is the difficulty in identifying asymptomatically infected cats [10, 23–25]. Additionally, although antibiotic treatment of infected cats is associated with a reduction in *B. henselae* CFU/ml in the bloodstream, complete and durable clearance of *B. henselae* from the feline bloodstream usually is not achieved [10, 24, 25]. However, because cats that naturally clear *B. henselae* infection are protected against re-infection with the same genotype [23, 25–27], immunization of cats against both type I and type II *B. henselae* infections likely would be most effective in limiting human exposure to *B. henselae*. No successful feline vaccine against *B. henselae* has been developed to date, despite several attempts. It is evident that selection of appropriate *B. henselae* strains and antigens is critical for the development of a successful feline vaccine.

Studies have been conducted in cats experimentally infected with *B. henselae* by intradermal inoculation [28, 29]. The course of *B. henselae* bacteremia in these experimentally infected cats, especially when using *B. henselae* type I (Houston-1), is different from that in naturally, flea-infected cats: Experimental infection is of significantly shorter duration, often with spontaneous resolution within 3 months post-inoculation [9]. Longitudinal bacteremia studies of naturally infected cats have been of limited value because the time of inoculation is unknown [7, 22, 30–32]. Thus, studies of cats naturally infected at a known time point are crucial to understanding interactions between *B. henselae* and the feline host and also between the two *B. henselae* genotypes during bloodstream infection and persistence. Such studies will provide important information about *B. henselae* pathogenesis and inform strategies for vaccine development.

We sought to better understand the interactions of *B. henselae* with its mammalian feline host, as well as the interactions between the two *B. henselae* genotypes during prolonged bloodstream infection after natural inoculation at a documented time. To accomplish this, we studied *B. henselae* isolates from the blood of flea-infected cats cultured over a 2-year period [11]. At the time of original cultures, the existence of two genotypes was not known, but after the subsequent demonstration that the *B. henselae* genus is comprised of two genotypes [12, 13], we reexamined the archived primary cultures from these three kittens, to determine if both *B. henselae* type I and type II genotypes could be detected in the bloodstream of each cat over time and if the prevalence changed temporally. We determined the prevalence of *B. henselae* type I and type II by PCR amplification of the 16S rDNA, in 50 individually selected colonies from the cryopreserved population at different time points, for each cat. To understand the dynamics of outer membrane protein (OMP) expression at the host-pathogen interface over time, we analyzed total OMP (TOMP) extracted from a population of early and late bloodstream isolates from each cat. Changes in the TOMP pattern during prolonged infection were identified by comparative 2D gel electrophoresis, and differential spots were characterized to better understand genotype-specific differences in *B. henselae*. The data from this study emphasize the relevance of both *B. henselae* genotypes in vaccine design and suggest elements that influence bacterial persistence and infection in vivo.

Materials and Methods

B. henselae Strains

JK33 and JK9R were isolated from the BA lesions of HIV-infected patients with *B. henselae* infection [33]. Subtype analysis of these strains demonstrated that they are *B. henselae* type I and type II, respectively, and they were thus chosen as positive controls for this study.

Experimental Transmission of *B. henselae* to SPF Kittens via Fleas Collected on *B. henselae* Bacteremic Cats Specific pathogen-free (SPF) kittens (3–5 months old) were infected via flea infestation as previously described [11]. Fleas were collected from 7 cattery cats that lived in a single household of 47 cats. The donor cats were heavily infested with fleas (five or more detectable fleas per animal), and all were actively infected with *B. henselae*, as confirmed by blood culture and PCR analysis. At time of culture and PCR, subspeciation of *B. henselae* was not performed. Fourteen to 15 fleas were collected, and 4–5 fleas were deposited on each of the bodies of three SPF kittens (cats #94552, #95019, and #94602). An additional 14 fleas were collected to evaluate for the presence of *B. henselae* DNA by PCR. *B. henselae* DNA was detected in 45% of the fleas collected [11]. Prior to infestation, the kittens were confirmed to be negative for *Bartonella* exposure by serum indirect immunofluorescence assay and blood culture. Each week, the infested kittens were clinically examined and blood was drawn for culture, serology, and complete blood count. After the fleas were placed on the kittens, they immediately burrowed into the feline fur, and subsequent examination of the kittens did not identify fleas in the course of the experiment. The kittens were housed in facilities free from arthropod pests and in an environment not conducive to flea reproduction. The kittens were cared for according to the Animal Welfare and Protection Rules, under a protocol approved by the University of California at Davis.

Isolation of *B. henselae* from Three Naturally Infected Kittens Approximately 1.5 ml of blood was drawn weekly, then every other week for a 2-year period into pediatric lysis centrifugation tubes (Wampole, Cranbury, NJ). The tubes were centrifuged at 1700×*g* for 70 min at room temperature, and the pellet was spread onto heart infusion agar plates supplemented with 5% fresh defibrinated rabbit blood (HIAR). The plates were incubated in candle extinction jars at 36 °C for 3 weeks. Bacterial isolates from blood cultures were confirmed to be *B. henselae* by citrate synthase PCR restriction fragment length polymorphism analysis [34]. Individual colonies, as well as a population of isolates, were harvested and cryopreserved at −80 °C in M199 medium (Cellgro, Mediatech, Herndon, VA) supplemented with 20% (*v*/*v*) heat-inactivated fetal bovine serum (FBS; Thermo Scientific, Fremont, CA) and 10% (*v*/*v*) dimethyl sulfoxide (DMSO; Fisher Scientific, Fair Lawn, NJ) until used in the current experiments.

16S rDNA PCR Genotyping of Feline *B. henselae* Isolates PCR amplification of the 16S rDNA was performed to genotype *B. henselae* isolates as type I or type II. Time points chosen were determined by availability of archived samples. From each available time point, the cryopreserved population of blood isolates was grown as individual colonies on chocolate agar plates, and 50 single colonies were randomly selected and clonally expanded to obtain sufficient biomass for subsequent PCR-based typing analysis. The clonally derived bacteria were then harvested and resuspended in 50 μl sterile H_2O, boiled for 10 min, and centrifuged at 16,000×*g* for 10 min. A 1:10 dilution of the supernatant was used as the DNA template for each PCR reaction. Two reactions were performed for each colony, using either the BH1/16SF or BH2/16SF primer sets [12] and goTaq DNA polymerase (Promega, Madison, Wisconsin). Thermal cycling conditions were followed as previously described [12]. The expected size of 16S rDNA fragments resulting from PCR with these sets of primers was approximately 185 bp. The amplified products were separated by gel electrophoresis on a 1.5% agarose gel, then stained with ethidium bromide and visualized on a UV transilluminator.

Subcellular Fractionation of *B. henselae* for Total Outer Membrane Protein Preparations Archived specimens were available for cat #94602 at 2 and 108 weeks post-infestation (p.i.), representing the longest duration of bacteremia. A population of *B. henselae* colonies isolated from the bloodstream were harvested into cold phosphate-buffered saline (PBS), centrifuged, and stored as pellets at −80 °C. Sarkosyl-based fractionation was performed as described [35]. The TOMP fraction was solubilized in 10 mM HEPES pH 8.0, with 0.1% sodium dodecyl sulfate (SDS). Protein concentration of the TOMP fraction was measured with the MicroBCA Protein Assay Kit (Pierce, Rockford, IL).

2D SDS-Polyacrylamide Gel Electrophoresis (PAGE) Analysis of *B. henselae* TOMP from Sequential Isolates After Natural Feline Infection 2D SDS-PAGE was performed as described [36]. Isoelectric focusing was performed in glass tubes with an inner diameter of 2 mm using 2% ampholines, pH 4 to 8 (BDH, Hoefer Scientific Instruments, San Francisco, CA) for 9600 V h. After equilibration for 10 min in buffer O (10% glycerol, 50 mM dithiothreitol, 2.3% SDS, and 0.0625 M Tris, pH 6.8), tube gels were laid on top of 10% acrylamide slab gels (0.75 mm thick) and SDS slab gel electrophoresis was carried out for 4 h at 12.5 mA/gel (Kendrick Labs Inc., Madison, WI). Proteins were visualized with silver stain as previously described [37].

N-Terminal Peptide Sequencing Individual protein spots of interest from the 2D SDS-PAGE separation of TOMP were excised from silver-stained gels. Three protein spots detected in the TOMP preparation of isolates from early and late time points were selected and transferred to a polyvinylidene difluoride membrane, and the N-terminal sequence of each was determined (Kendrick Labs, Inc., Madison, WI).

Sequencing of *hbpA* (840 bp) from Multiple, Temporally Distinct *B. henselae* Isolates Ten individual colonies were selected from cryopreserved populations of *B. henselae* isolated from cat #94602 at weeks 2 and 108. These 20 colonies were clonally expanded, harvested, and resuspended in 50 μl sterile H_2O. This material was then boiled for 10 min and centrifuged for 10 min at 16,000×*g*. Eight microliters of this clonal colony suspension was used as template in 100 μl PCR reactions to amplify the *hbpA* gene. Amplification was carried out with the following primer pairs: 5′-CGGG TACGGATTGGTTTTGCTGCTGAG-3′ HbpCAB3F and 5′-GCATTAGCTTTTTTAAGGGAATC-3′ HbpCAB3R, and 5′-GAATTTTTCGAGTAAGGTTGAAATAAC-3′ HbpCAB4F and 5′-GTCACAAAAAAATAGAGGATTTG-3′ HbpCAB4R. Reaction mixtures contained 0.8 μM final concentration of each primer, 200 μM of each deoxyribonucleoside triphosphate, and 1.5 mM $MgCl_2$, in a volume of 100 μl. Amplification was carried out as follows: 5 min denaturation at 95 °C; 30 cycles of: 60 s at 95 °C, 60 s at 45 °C, and 90 s at 72 °C; followed by a final extension at 72 °C for 10 min. Amplicons were analyzed on a 1% agarose gel stained with ethidium bromide and visualized on a UV transilluminator. All PCR products were purified using the QIAquick PCR product purification kit. DNA sequencing was performed using the HbpCAB3F/HbpCAB3R and HbpCAB4F/HbpCAB4R primer pairs. Both strands of DNA were sequenced with a BigDye Terminator Cycle Sequencing Ready Reaction Kit (Applied Biosystems) and an automated DNA sequencer (ABI, model 377), compiled, and analyzed with SerialCloner 2.1 software. Amino acid sequences were generated using the ExPASy translate tool (http://ca.expasy.org/tools/dna.html), and theoretical isoelectric point (pI) values and molecular weights were calculated using the Compute pI/MW tool (http://www.expasy.ch/tools/pi_tool.html). Alignments were performed with Clustal (http://www.ebi.ac.uk/Tools/msa/clustalo/).

Results

***B. henselae* Type I and Type II Were Co-Transmitted to the Feline Reservoir Host by the Flea Arthropod Vector, During Natural Infection** Within 2 weeks of infestation with fleas collected from highly bacteremic cattery cats, three SPF kittens (cats #94552, 95019, 94602) developed *B. henselae* bacteremia [11]. In the flea-infected kittens #94552, 95019, 94602, bacterial CFU/ml peaked for each animal at 3, 4, and 6 weeks p.i., respectively (Fig. 1). Maximum levels of bacteremia peaked at 4.2–7.3 × 10^6 CFU/ml in the kittens. Blood samples from these cats were cultured for 115 weeks (two cats) and 145 weeks (one cat). Over the course of infection, relapsing bacteremia was observed in all cats, with intermittent periods of negative blood cultures (Fig. 1). Infection eventually resolved spontaneously in the cats (defined by five or more successive negative blood cultures) after 106, 95, and 136 weeks p.i., respectively [11].

Of interest, we observed mixed colony morphologies on the blood culture plates from several time points after the kittens were infected, but at that time, existence of two genotypes was not known. After the subsequent demonstration that the *B. henselae* genus is comprised of two genotypes [12, 13], we reexamined the archived primary cultures from these three kittens, to determine if both *B. henselae* type I and type II genotypes could be detected in the bloodstream of each cat over time and if the prevalence changed temporally. Fifty single colonies were randomly picked from the population of cryopreserved blood isolates cultured at multiple time points for each cat (Fig. 1, arrows with dates), and differential 16S rDNA PCR was performed to distinguish type I from type II *B. henselae* (Fig. 2). Typing of isolates recovered from the first positive blood cultures 2 weeks p.i. revealed co-infection with both *B. henselae* type I and type II in two of the three kittens (#95019 and #94602; Table 1); the prevalence of type I at this time point was significantly greater than type II in both. For the third cat (#94552), all isolates typed from the first positive blood culture were *B. henselae* type I. However, at the subsequent time point for #94552, at 11 weeks p.i., a dramatic shift in genotype dominance was observed: All 50 typed isolates were identified as *B. henselae* type II, suggesting an undetected initial co-infection with both type I and type II genotypes.

Although *B. henselae* Type I Predominated in the Blood Early After Flea Infection, Type II Outcompeted Type I Over Time and Was the Only Genotype Isolated from the Blood of All Three Cats at Late Time Points 16S rDNA genotyping revealed a predominance of type I isolates recovered from all three cats early after infestation. Eighty two to 100% of colonies picked from the earliest bacterial isolates (2 weeks p.i.) were identified as *B. henselae* type I (Table 1). Isolates from cat #95019 at 2 and 10 weeks p.i. were mostly or all type I, but isolates after 51 weeks were all type II. For cat #94552, all isolates at 2 weeks were type I, but all subsequent isolates were type II. Genotyping of late isolates for the three kittens indicated a shift in type I genotype dominance until 100% of isolates were identified as *B. henselae* type II: #95019 (at 51, 60, and 81 weeks p.i.), #94552 (at 11 and 96 weeks p.i.), and #94602 (at 108 weeks p.i.) (Table 1).

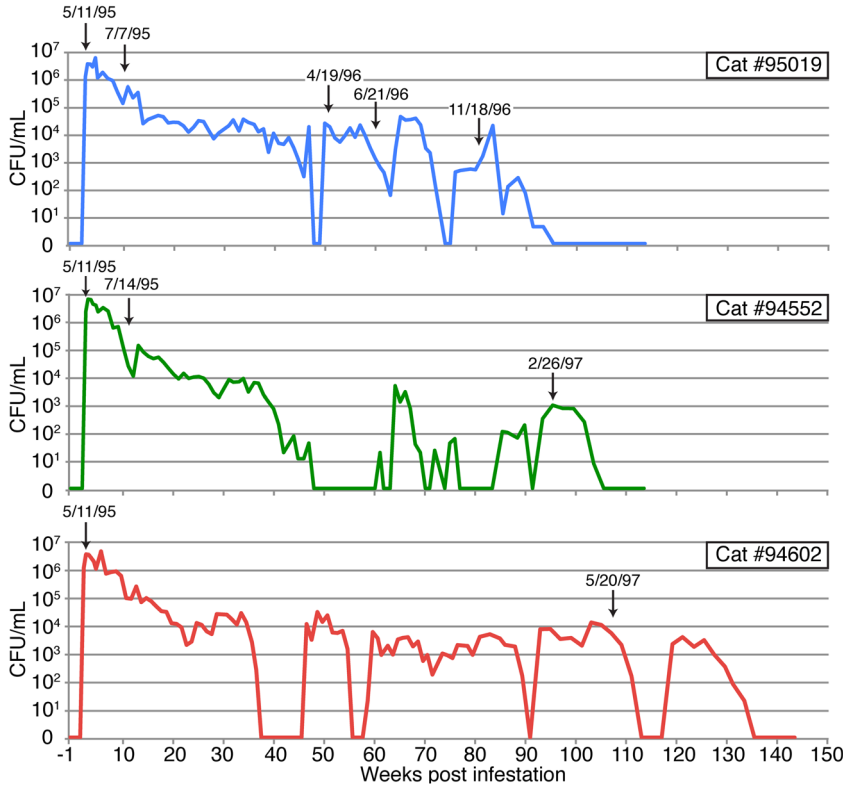

Fig. 1 Infestation of SPF kittens with fleas fed on *B. henselae* bacteremic cats establishes prolonged and relapsing bloodstream infection. Cats were naturally infected by fleas taken from bacteremic cattery cats; infestation of SPF kittens occurred at time 0. The number of *B. henselae* in the bloodstream was determined (CFU/ml; *y*-axis) at time points post-infestation (weeks post-infestation; *x*-axis). *Arrows* indicate time points for which isolates were typed by 16S rDNA PCR to determine the type I or type II genotype of the infecting *B. henselae* bacteria

2D SDS-PAGE of TOMP Confirmed a Shift in *B. henselae* Genotype Predominance from Type I to Type II During Persistent, In Vivo Feline Infection but Did Not Detect Antigenic or Phase Variation, at the Protein Level We sought to identify changes in the TOMP profile during prolonged bloodstream infection, as was done for the related species, *B. quintana* [38]. We performed 2D SDS-PAGE separation of TOMP fractions from *B. henselae* isolated from each of the three cats at different time points; the pattern of protein spots from the TOMP of early and late isolates for all three cats showed the same distinct difference between early and late time points. Figure 3 shows the 2D SDS-PAGE TOMP profile for cat #94602 at 2 and 108 weeks p.i. A comparison of all TOMP spots from early isolates for each cat with those from late isolates revealed several changes in protein spots: There was a prominent group of acidic protein isoforms at ~28–35 kDa in the TOMP from 2 weeks p.i. (Fig. 3a, spots 1, 2) that was not present in the TOMP from the later time point, 108 weeks p.i. (Fig. 3b). In addition, a new protein spot with a similar mass, but at a more basic pI, appeared at the later time point (Fig. 3b, spot 3). Individual protein spots 1–3 (Fig. 3) were each identified as the important virulence factor, hemin-binding protein A (HbpA), by N-terminal sequencing. The first 16–20 amino acids of the N terminus of the mature protein were determined unequivocally and were identical in all three proteins (ADVIVPHEVAPTVISAPAFS).

Sequencing of the TOMP Virulence Gene, *hbpA*, from Individual *B. henselae* Colonies Identified a Localized Region with Nucleotide Differences Between Type I and Type II *hbpA* but Did Not Provide Evidence of Antigenic Variation Over Time Among Type II *hbpA*, at the Genetic Level To further investigate the striking differences in the 2D SDS-PAGE between the pI and pattern of HbpA from the two genotypes, we sequenced the *hbpA* genes from individual isolates of 9 type I and 1 type II from the early time point (May 11, 1995) and 10 type II from the latest time point (May 20, 1997) of cat #94602 (Supplemental Fig. 1). The *hbpA* gene sequence was conserved among all type I cat isolates (9 colonies of early time point); the *hbpA* sequences of all type II isolates (1 colony of early and 10 colonies of late time points) were identical to each other and differed from the type I *hbpA* sequences. In addition, the feline type I *hbpA* sequences were 100% identical to *B. henselae* type I strains from two human patients: JK33 [33] and the *B. henselae* ATCC type strain, Houston-1 [39]. Similarly, our *B. henselae* type II cat sequences for *hbpA* were identical to a human type II strain isolate, JK9R (Supplemental Fig. 1) [33].

The *hbpA* type I and type II sequences were 96% identical at the nucleotide level and 92% identical at the amino acid level (Table 2). This divergence in amino acid sequence between the two genotypes resulted in a shift of predicted pI from 5.22 in type I to 6.24 in type II (although the shift we

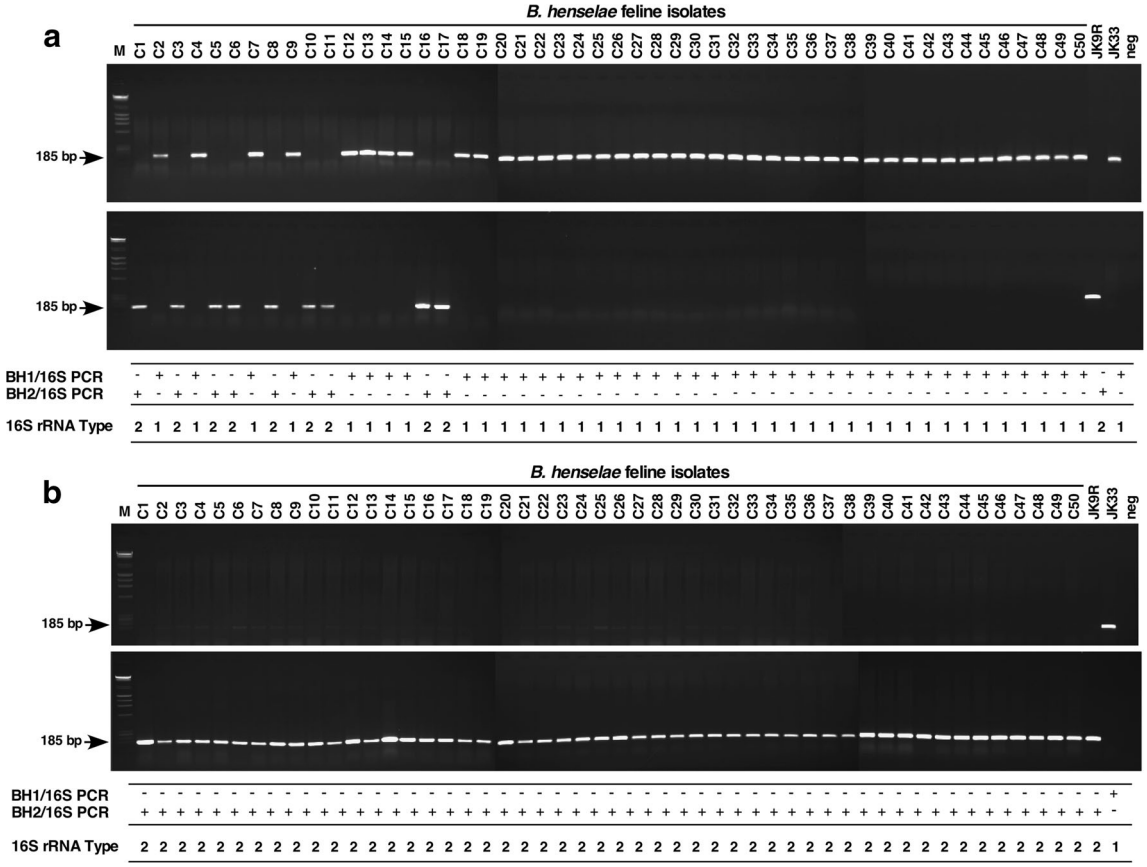

Fig. 2 16S rDNA PCR analysis of individual *B. henselae* isolates from cat #95019 demonstrates **a**) initial mixed infection with *B. henselae* type I and type II and **b**) in vivo selection for *B. henselae* type II during prolonged bloodstream infection. Fifty colonies were randomly picked from a population of bacteria isolated at **a**) 2 weeks post-infestation (p.i.) and **b**) 51 weeks p.i. Individual colonies were analyzed by PCR using two distinct 16S rDNA primer sets, allowing identification of *B. henselae* type I or type II genotype. Human isolate strains JK33 (*B. henselae* type I) and JK9R (*B. henselae* type II) were used as positive PCR controls. Water instead of DNA template was used for negative PCR controls

actually observed for *B. henselae* type I vs. type II HbpA in the 2D gel was greater, from 5.2 to 6.4, than was predicted by the amino acid sequence). The location of the divergent amino acids between type I and type II is shown in Supplemental Fig. 2 (adapted from Minnick, et al. [40]). *hbpA* gene sequences of type I and type II feline strains and of human isolates JK9R and JK33 were deposited in the NCBI GenBank database: isolate JK9R, JX431936; isolate JK33, JX431937; strain 94602 (May 20, 1997), JX431935; strain 94602 (May 11, 1995), JX431934.

Table 1 16S rDNA typing of *B. henselae* recovered from the blood of three naturally infected kittens at different time points after flea infestation demonstrates co-infection with type I and type II early after infection and the early predominance of type I, compared with late predominance of type II, during prolonged infection

Cat #	Culture date	Approximate weeks post-infestation	Total colonies screened	No. of type 1 colonies	No. of type 2 colonies
Cat #95019	May 11, 1995	2	50	41	9
	July 7, 1995	10	50	50	0
	April 19, 1996	51	50	0	50
	June 21, 1996	60	50	0	50
	November 18, 1996	81	50	0	50
Cat #94552	May 11, 1995	2	50	50	0
	July 14, 1995	11	50	0	50
	February 26, 1997	96	50	0	50
Cat #94602	May 11, 1995	2	50	49	1
	May 20, 1997	108	50	0	50

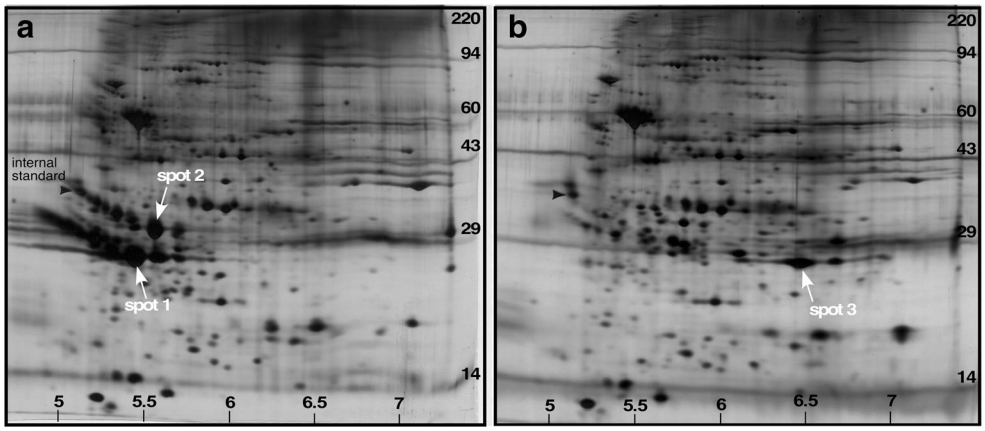

Fig. 3 2D SDS-PAGE of total outer membrane proteins (TOMPs) reveals distinct differences in protein patterns between *B. henselae* type I and *B. henselae* type II isolates from cat #94602 over 2 years; the major differences observed are attributable to hemin-binding protein A (HbpA). TOMP fractions were prepared from isolates from the bloodstream of cat #94602, drawn **a**) 2 weeks (type I) and **b**) 108 weeks (type II) post-infestation (p.i.). Proteins were separated by isoelectric point (*x*-axis, pI) and molecular mass (*y*-axis, kDa) and were visualized by silver staining. Spots 1, 2, and 3 were identified as HbpA by N-terminal sequencing. The *black arrowhead* indicates an internal standard, tropomyosin, which runs as a doublet with a molecular mass of 33 kDa and a pI of 5.2 for the lower spot

Discussion

B. henselae is an important zoonotic pathogen whose high prevalence in the bloodstream of domestic cats provides a major source of human infection. Understanding the diversity, dynamics, and survival mechanisms of *B. henselae* genotypes during persistent infection of the feline host is critical to developing strategies to prevent human infection. To better understand these dynamics, we evaluated *B. henselae* blood isolates from three cats infected "naturally" by fleas, drawn from the time of infection via *B. henselae*-infected fleas, to the time of spontaneous resolution of bacteremia ~2 years later. Our goal was to identify in vivo dynamics between the two *B. henselae* genotypes, type I and type 2, and to identify any adaptive gene and protein changes in *B. henselae* during the 2 years of prolonged bloodstream infection. Our study had the advantage of observing the course of a natural feline *B. henselae* infection, which more closely recapitulates the bacteremia pattern than intradermal experimental inoculation, but with a defined source and time of infection (not possible in cats naturally infected in an uncontrolled setting).

Early after flea infestation, two of the cats (and likely the third) were bacteremic with both type I and type II concomitantly, indicating that the fleas were able to infect naïve cats with both *B. henselae* genotypes. It is likely that individual fleas were simultaneously infected with, and transmitted, both genotypes to the kittens, although we cannot rule out separate infection events from individual fleas, one infected with *B. henselae* type I and another with type II. Forty-five percent of the infesting fleas had detectable *B. henselae* DNA by PCR, but genotyping was not performed [11]. However, co-infection of fleas with several *Bartonella* species has been demonstrated [41].

Early in infection, *B. henselae* type I predominated among the 50 individual colonies chosen randomly for genotyping from each cat (Table 1). As the duration of infection increased, *B. henselae* type II was isolated with increasing frequency, and at the final time point, all 50 individual colonies evaluated for each of the three cats belonged to type II genotype. For one cat (#94552), all 50 isolates genotyped 2 weeks p.i. were type I, but all 50 isolates at 11 weeks p.i., and subsequently, were type II. This suggests that *B. henselae* type II was present in the

Table 2 Divergence of the predicted HbpA amino acid sequences from *B. henselae* type I (2 weeks p.i.) compared with type II (108 weeks p.i.) results in the MW and pI changes observed in the 2D SDS-PAGE (Fig. 3) and confirms the shift of genotype in the bloodstream of cat #94602 during persistent infection

	Cat #94602 HbpA		Human HbpA		
	Week 2	Week 108	JK33 (type I)	JK9R (type II)	Houston (type I)
Molecular mass (kDa)	29.92	30.21	29.92	30.21	29.92
Predicted pI (mature protein)	5.22	6.24	5.22	6.24	5.22
Percent amino acid identity to type strain Houston-1	100	92	100	92	100
Percent nucleotide identity to type strain Houston-1	100	96	100	96	100

bloodstream of this cat at the initial time point, but in numbers too small to be detected with a sampling of 50 random colonies. The notable shift in *B. henselae* genotype prevalence in all three cats could indicate a competitive interaction between the two genotypes in vivo or an intrinsic difference between the ability of the two genotypes to persist. The underlying mechanism also could be a combination of these two factors. Interestingly, a recent study conducted in shelter cats in the San Francisco Bay area, California, showed that *B. henselae* type I was more frequently isolated from kittens and type II was more commonly isolated from young adult cats [42], indicating that this type I to type II shift could occur in nature.

It has been shown that experimental inoculation of cats with the human isolate Houston-1 (type I) leads to a shorter bacteremia with the absence of relapses [9, 25, 29], compared to cats experimentally infected with feline strains of *B. henselae* type I [32] or type II [9]. In studies of cats experimentally infected with feline-derived type I *B. henselae*, the average duration of bacteremia was 11 weeks [29], and 8 weeks in another study [43]. Cats experimentally infected with *B. henselae* type II were bacteremic for 26 weeks in one study and 41 weeks in another [26]. In studies of cats naturally infected with *B. henselae*, the duration of bacteremia is significantly longer than with experimental inoculation. In one study of cats naturally infected (at an unknown time) with *B. henselae* type I, bacteremia persisted for 70–133 weeks after initial examination [32]. In another study, natural infection with type II was documented for at least 96 weeks [28]. In our study, the relatively short duration (~8 weeks) of detectable *B. henselae* type I in the bloodstream of cats naturally co-infected with both genotypes could have resulted from an interaction between the genotypes, with a competitive advantage of type II over type I in vivo over time.

The *B. henselae* bloodstream infection lasted approximately 2 years in all three cats, with recurrent peaks of cultivable *B. henselae*, alternating with time points of undetectable bloodstream infection. All isolates from cats #95019 and #94552 during this relapsing phase were *B. henselae* type II (Fig. 1). A relapsing pattern of bacteremia has been documented previously in cats infected with both *B. henselae* type I [24, 26, 29, 32, 44] and type II [17]. It has been suggested that this relapsing pattern of *B. henselae* infection is related to antigenic or phase variation [45, 46]. Indeed, in *B. quintana*, a closely related *Bartonella* species also causing prolonged and relapsing bloodstream infection (in humans), phase variation was observed in sequential bloodstream isolates by 2D SDS-PAGE, in genes encoding the variably expressed outer membrane protein (Vomp) family of virulence factors [35, 38]. For *B. henselae*, potential evidence of antigenic variation in *B. henselae* sequential feline bloodstream isolates was identified previously using restriction enzyme fragment length polymorphism (RFLP) analysis [45, 46], but this has not been documented in specific genes.

To identify any phase variation, as well as differences between the closely related *B. henselae* genotypes type I and type II that could contribute to the in vivo phenotypic differences in duration we observed, we compared the TOMP profile of *B. henselae* type I bloodstream isolates (2 weeks p.i.) and type II (108 weeks p.i.) from cat #94602, using 2D SDS-PAGE. Two distinct protein profiles were evident for *B. henselae* types I and II (Fig. 3). The same 2D TOMP pattern differences also were identified between type I and type II isolates for cat #95019 (data not shown). For *B. henselae* type I, a similar pattern of protein spots at ~28–35 kDa was reported previously in TOMP analyzed by 2D electrophoresis [47]. N-terminal sequencing in our study (Fig. 3a, spots 1 and 2) and mass spectrometry performed by others [47] confirmed that these protein spots at ~28–35 kDa from *B. henselae* type I represent isoforms of HbpA, a virulence OMP found in *Bartonella* species pathogenic for humans [48, 49].

For *B. henselae* type II, however, this acidic group of highly visible spots was absent (Fig. 3b). Instead, we identified a spot of similar molecular mass, but at a higher pI of 6.4 (Fig. 3b, spot 3) that was not observed in the type I TOMP. N-terminal sequencing of this *B. henselae* type II spot 3 identified it as HbpA. Interestingly, differences at the amino acid level between HbpA from genotype types I and II were found primarily in the predicted surface-exposed loops between transmembrane regions 3 and 4 (Supplemental Fig. 2, adapted from Minnick et al. [40]). Two previous proteomic analyses of a *B. henselae* type II strain did not detect the presence and pI shift of HbpA in *B. henselae* type II [50, 51]; thus, this important difference between *B. henselae* type I and type II HbpA has not been reported previously.

The numerous isoforms of the OMP HbpA observed in *B. henselae* type I, and the dramatic difference in the pI of type I compared with type II HbpA, raised the possibility that the *hbpA* gene might display different types of plasticity, e.g., genetic variation during prolonged bloodstream infection, as we had identified in the *B. quintana* VOMP. To explore this, DNA was extracted from early and late time point isolates, and the *hbpA* genes sequenced. We found that all type I *hbpA* gene sequences were identical, and the type II *hbpA* gene sequences were different from type I, but identical to each other. Thus, our sequence analysis of the *hbpA* genes did not show evidence for phase or antigenic variation in the type II *hbpA* gene over a 2-year time period.

It is unknown what is the nature of the modification(s) resulting in the *B. henselae* type I HbpA isoforms or why *B. henselae* type II HbpA lacks isoforms. The type I isoforms with molecular mass and pI shifts could represent post-translational modifications that contribute to *B. henselae* survival in vivo. Advantageous adaptations in *B. henselae* could include strategies to better exploit host substrates or evade host immune detection. Interestingly, *B. henselae* type I uniquely targets liver, spleen, and bone marrow, but type II

does not; modifications in the type I HbpA could expand access to hemin, the only source of iron for *B. henselae*. Changes in the OMP, such as those observed in HbpA, also could contribute to the difference in duration of bacteremia and the relapsing phenotype of *B. henselae* type I compared with type II. Interestingly, studies demonstrate that other OMP, e.g., *Neisseria* AniA, are likely protected from immune recognition by glycosylation [52]; additionally, upon glycosylation, Ag43 of *Escherichia coli* exhibits enhanced binding to human cells [53]. Further characterization of HbpA in both *B. henselae* type I and type II will likely provide insight into the mechanisms and differences of persistence in vivo of the two *B. henselae* genotypes and data relevant to feline vaccine development.

To develop an effective feline vaccine against infection with both *B. henselae* type I and type II genotypes, it is critical to understand the interactions between *B. henselae* type I and type II, as well as the protective efficacy of each of the genotypes against the heterologous genotype. In one study, infection with a *B. henselae* type I strain was protective against homologous challenge with another type I strain [9]. However, challenge of *B. henselae* type I-infected cats with type II revealed only partial protection by the initial type I infection against type II infection. Finally, in cats initially infected with *B. henselae* type II, there was no protection against challenge with type I in 100% of the cats [9].

Several attempts have been made to develop a vaccine with type I genotype to prevent *B. henselae* infection in cats, without success. A live attenuated *B. henselae* type I vaccine resulted in no protective advantage against a type I challenge [54]. A subunit vaccine using the highly immunogenic *B. henselae* OMP P26 [55, 56] did not provide protection against subsequent *B. henselae* infection. Two studies tested a killed whole cell, adjuvanted *B. henselae* type I strain vaccine: the human-derived Houston-1 type I strain, patented by Regnery et al. [57], and a feline-derived type I strain [58]. Protection against reinfection with the homologous *B. henselae* type I genotype was demonstrated, but vaccine protection from infection with the heterologous type II genotype was never assessed. To date, no experimental inoculation of cats with a *B. henselae* type II or a combined type I/type II vaccine candidate has been performed.

The ideal *B. henselae* strain and vaccine component(s) for prevention of feline infection have not yet been identified. An in vitro analysis of sera from naturally infected cats [27] identified 13 differentially seroreactive antigens between genotypes I and II. This variation in the antigenic properties between the two strains could contribute to the lack of cross protection in heterologous human and feline infections. Based on findings from our and others' studies, a feline vaccine will require inclusion of a protective antigen that is conserved in either both *B. henselae* type I and type II or a cocktail of protective proteins from both genotypes.

In this study, we documented the experimental co-transmission of two *B. henselae* genotypes, type I and type II, to SPF kittens by the natural vector, the cat flea. Co-transmission of both type I and type II occurred in all three kittens and appeared to result in in vivo competition between the two genotypes, with a selective advantage of type II over type I during the 2 years of persistent bloodstream infection. A caveat of this study is its small sample size. However, despite only three experimental subjects, the consistent and unequivocal results observed across each individual animal suggest that our findings are valid and applicable to infection in a natural setting. Another limitation of this study is the lack of subspeciation of *Bartonella* in the fleas collected from the cattery cats; unfortunately, the existence of two genotypes was not known at the time PCR typing was performed on the fleas. In conclusion, understanding the behavior of the two *B. henselae* genotypes in vivo is critical to the development of an effective feline vaccine that can disrupt the infection cycle, thus limiting the occurrence of CSD and preventing potentially fatal bacillary angiomatosis and heart valve infection in humans. These data also contribute to the understanding of bacterial persistence in the mammalian reservoir host and provide insight into the pathogenesis of *B. henselae*.

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Compliance with Ethical Standards

Sequence Data The nucleotide sequence data reported are available in the GenBank database under the following accession numbers: JX431936, JX431937, JX431935, and JX431934.

Conflict of Interest The authors declare that they have no conflict of interest.

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