DISSERTATION

LABORATORY MOUSE MODELS FOR BARTONELLA BACTERIAL INFECTION:

BACTEREMIA, HOST SPECIFICITY, AND PATHOLOGY

Submitted by

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ABSTRACT

LABORATORY MOUSE MODELS FOR BARTONELLA BACTERIAL INFECTION:

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Bartonella bacterial species are globally distributed in a diverse variety of mammalian reservoir hosts. Natural host infections are generally characterized by persistent bacteremias of long duration, seemingly without adverse host effect, whereas non-natural host infections can produce mild, self-limiting illnesses or more severe disease such as endocarditis. Incidental host infections seem to most closely resemble natural host infections when the taxonomic distance between the two hosts is small. The greater the taxonomic distance between the host of origin and the incidental host, the more likely it seems that the incidental host will either clear the bacteria or develop pathology following exposure. This level of bacterial host specificity has been demonstrated consistently and presents an enormous obstacle to the development of animal models, particularly murine models that reproduce characteristics of natural host infection or pathology consistent with human incidental infections.

In this dissertation laboratory mouse models for bartonella infection are described following the introduction and literature review (Chapter 1). Chapter 2 reports infection of mice with bartonella strains from wild *Mus* species, simulating a cross-species host switch for the bacteria. Infected mice exhibited characteristics consistent with reports of natural rodent host infection. Chapter 3 reports on a mouse infection study using four rat bartonella strains, simulating a cross-genus host switch for the bacteria. Only one of the strains infected mice and alterations in bacteremia duration and magnitude were observed relative to those reported for natural host infections. Mice also displayed organ pathology following bacteremia resolution. Chapter 4 presents a mouse infection study using an Asian house shrew *Bartonella elizabethae* strain inoculated into three different laboratory mouse stocks. Mice of all three stocks developed bacteremia following bacterial exposures, a demonstration of cross-order host switching by the bacteria. No obvious differences in infection response were observed among the mice despite differences in their genetic backgrounds. Chapter 5 describes inoculation of aged mice with either a mouse bartonella strain or human *Bartonella tamiae* strains. Mice infected with the mouse strain developed bacteremia, whereas mice infected with *B. tamiae* did not, consistent with the idea that taxonomic distance between host of origin and incidental host can be a predictor of infection outcome.

Chapter 6 details results of a study where aged mice were exposed to three different *B. tamiae* strains. The mice developed disease consistent with reports of human illness symptomatology. In summary (Chapter 7), these laboratory mouse models are presented as defined, scientific resources for research on *Bartonella* species host ecology, bacteria: host interactions, and transmission dynamics.

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CHAPTER 1

BACKGROUND AND LITERATURE REVIEW

THE GENUS BARTONELLA

Bacteria in the family Bartonellaceae are grouped in the class *Alphaproteobacteria*, phylum Proteobacteria, order Rhizobiales according to their 16S rRNA gene sequences. Today, species in the genus *Bartonella* include bacteria formerly known as *Rochalimaea* and *Grahamella* as well as the only original named species of *Bartonella*, *B. bacilliformis*. Until 1993 the genera *Bartonella* and *Rochalimaea* were classified in different families in the order Rickettsiales (Bartonellaceae and Rickettsiaceae) [1]. However, a thorough molecular investigation into the taxonomic relationships of these bacteria demonstrated a very low level of relatedness (0-2%) between them based on DNA hybridization assays of related sequences [1]. *B. bacilliformis* and *Rochalimaea* species were more closely related to one another than to the rickettsiae and ultimately proved more closely related to *Brucella, Agrobacterium*, and *Rhizobium* than to *Rickettsia*. Additional evidence for the new taxonomic placement of the species included differences among 16S rRNA sequences, the guanine: cytosine nucleic acid content of the genomes, and biochemical metabolic profiles [1]. In 1995 it was proposed that

Grahamella species also be reclassified under the genus *Bartonella* based on the same criteria [2]. The genus was therefore once again emended and two *Grahamella* species, *G. talpae* and *G. peromysci*, were added to the list of *Bartonella* species as *B. talpae* comb. nov. and *B. peromysci* comb. nov. The type genus for *Bartonella* has remained throughout these changes as described by Tyzzer, Strong, and Sellards in 1915 and the type species for the genus is *B. bacilliformis*. With the reclassification of the other bacteria and the addition of several new species a total of ten *Bartonella* species were recognized in 1996.

Since the consolidation of the family Bartonellaceae, numerous new *Bartonella* species have been described. Today there are 40 named or proposed (candidate) species in the genus *Bartonella* (Table 1.1). The rapid expansion of the genus in the last decade is mainly attributable to improvements in detection methodologies. Molecular genetic detection of bacterial DNA has been improved by increasing the sensitivity and specificity of PCR assays [3-7], while new and improved techniques for growing bacteria in eukaryotic cell culture aid in the detection of novel species [8-10]. Modified insect growth media (Schneider's drosophila liquid medium, insect growth medium DS2) have also been successfully used for bartonella culture [11, 12]. The increasing rate of discovery of new *Bartonella* species is also attributable to heightened surveillance for the bacteria as agents of human illness and the discovery of novel species in wildlife (Table 1.1) [13, 14].

New species are typically identified and described based on the proportion of their sequence similarities for concatenated sequences of 5 genes— *rpo*B,

Table 1.1 According to a literature survey there are 40 recognized or candidate (proposed) *Bartonella* species and subspecies which have been isolated from a variety of animals worldwide (English language, PubMed and Web of Knowledge). The source (S) annotation indicates hosts from which novel bacteria were isolated; (R) indicates a reservoir host (R).

Species	Animal source (S) and/ or reservoir (R)	Ref.
B. alsatica	Rabbits (R)	[15]
B. australis	Eastern grey kangaroos (R)	[16]
B. bacilliformis	Human (R)	[17]
B. birtlesii	Mice (*Apodemus* spp.) (R)	[18]
B. bovis	Cattle (R)	[19]
B. capreoli	Ruminants (R)	[5]
B. chomelii	Ruminants (R)	[20]
B. clarridgeiae	Cats (R)	[21]
B. coopersplainensis	Rats (R)	[22]
B. doshiae	Voles (*Microtus* spp.) (R)	[2]
B. elizabethae	Human (S), rodents (R)	[1, 23]
B. grahamii	Voles (*Myodes* spp., formerly *Clethrionomys* spp.) (R)	[2]
B. henselae	Cats (felines) (R)	[24]
B. japonica	Small Japanese field mouse (*Apodemus argenteus*) (R)	[25]
B. koehlerae	Cats (R)	[26]
B. peromysci	Mice (*Peromyscus* spp.) (R)	[2, 17]
B. phoceencis	Rats (R)	[27]

Table 1.1 continued.

Species	Animal source (S) and/ or reservoir (R)	Ref.
B. queenslandensis	Rat (R)	[22]
B. quintana	Human (R)	[1]
B. rattaustraliani	Rat (R)	[22]
B. rattimassiliensis	Rat (R)	[27]
B. rochalimae	Human (S),	[28]
B. schoenbuchensis	Roe deer	[29]
B. silvatica	Large Japanese field mouse (*Apodemus speciosus*) (R)	[25]
B. talpae	Shrew mole (R)	[2, 17]
B. tamiae	Human (S), Trombiculid mites (S), (R) unknown	[30]
B. taylorii	Mice (*Apodemus* spp.) (R)	[2]
B. thailandensis	Rat (*Rattus surifer*) (R)	[31]
B. tribocorum	Rat (*Rattus norvegicus*) (R)	[32]
B. vinsonii subsp. *arupensis*	Mice (*Peromyscus* spp.) (R)	[33]
B. vinsonii subsp. *berkhoffii*	Dogs (canines) (R)	[34]
B. vinsonii subsp. *vinsonii*	Voles (R)	[36, 37]
B. washoensis	Human (S); Ground squirrel (*Spermophilus beecheyi*) (R)	[38, 39]
B. washoensis subsp. *cynomysii*	Prairie dogs (R) (*Cynomys ludovicianus*)	[35]

Table 1.1 continued.

Species	Animal source (S) and/ or reservoir (R)	Ref.
Candidatus *B. antechini*	Fleas (*Acanthopsylla jordani*) (S) Ticks *(Ixodes antechini)* (S) *Antechinus flavipes* (Mardos or Yellow-footed antechinus) (R?)]	[40]
Candidatus *B. durdeni*	Flea (*Orchopeas howardi*) (S) Eastern US gray squirrels (*Sciurus carolinensis*) (R?)	[41, 42]
Candidatus *B. mayotimonensis*	Human (S), Unknown (R)	[43]
Candidatus *B. melophagi*	Sheep (S, R?), Sheep keds (S)	[44]
Candidatus *B. monaxi*	Groundhog (*Marmota monax*) (R?)	[41, 42]
Candidatus *B. volans*	Southern flying squirrel (*Glaucomys volans*) (R?)	[41, 42]

*glt*A, *fts*Z, *gro*EL, *rib*C, and the ITS (RNA polymerase subunit B, citrate synthase, filamenting temperature-sensitive mutant Z, 60-kDa heat shock protein (GroEL), riboflavin synthase, and intergenic transcribed spacer region, respectively) [45]. Identification of a new species is based on the isolate meeting two criteria. First, a 327 bp sequence of its *glt*A cannot share more than 96.0% identity with currently recognized species and second, an 825 bp sequence of its *rpo*B cannot share more than 95.4% sequence similarity to recognized species [45]. Recognition of new *Bartonella* species has been complicated by the inability of some investigators to culture type strains for new species they have discovered and characterized using bacterial DNA sequences [40, 43].

BACTERIOLOGY

Bartonella bacteria are small, <3μm in diameter, Gram negative staining coccobacilli [17]. They are aerobic and can be grown on microbiological plates but must be incubated in a 5% CO_2 atmosphere. Agar media for growth of these fastidious bacteria must be protein rich (e.g. brain heart infusion, heart infusion, or tryptic soy) and is often supplemented with 5—10% blood, usually from sheep or rabbits, to satisfy their high hemin requirements [17]. Bartonellae grow at temperatures ranging from 22—37°C which mirrors their ability to survive and grow in ectothermic arthropod vectors as well as in endothermic mammalian hosts [8, 46-48].

Bartonellae as facultative intracellular microparasites are able to infect a number of different mammalian cell types. Eukaryotic cell cultures derived from primate, cat, and mouse tissues are commonly used to grow bacteria for diagnostic or research purposes as they can easily infect these cells [8, 49-51]. Infection assays have also demonstrated that *B. henselae* can invade embryonic tick cell lines derived from *Amblyomma americanum*, *Ixodes scapularis* and *Rhipicephalus sanguineus*, respectively [52]. Nine other bartonella strains are capable of infecting the *A. americanum* cell line [52]. This promiscuity for a variety of cell types *in vitro* reflects on the capacity of many species to infect different host taxa.

Bartonella bacteria have circular chromosomes ranging from 1.5—2.6 Mb in size. Five species have been sequenced: *B. bacilliformis* (GenBank accession no. CP000525), *B. henselae*, *B. quintana*, *B. tribocorum*, and *B. grahamii* [53, 54]. The relatively small genome size and low gene coding

content of these species indicates they have undergone reductive genome evolution, a common finding for alphaproteobacteria that live an intracellular lifestyle [55]. Following radial speciation from an ancestral lineage modern species show evidence of genome expansions beyond the shared ancestral gene array. Some *Bartonella* species carry plasmids such as the cryptic plasmids pBGR1, pBGR2, and pBGR3 found in *B. grahamii* which can be shared among Gram negative bacteria by conjugation [56]. Integrated phage sequences have also been found in some species and can act in the lateral transfer of genetic material between bacteria [57]. These integrated pro-phages can also duplicate portions of the bacterial host chromosome during run-off replication [53], a potent mechanism for increasing genetic diversity.

Interactions with eukaryotic host cells

Relatively few pathogenesis factors have been identified for bartonella bacteria, the most important of which are reviewed below. Research in this area has overwhelmingly been aimed at elucidating the mechanisms for bacterial entry into host erythrocytes and/or endothelial cells and for inducing vasoproliferation. Most of these experiments have been conducted *in vitro* using eukaryotic cell lines such as HUVEC or Ea. hy926 (human umbilical vein endothelial cells), HeLa229 (human epitheloid cervix carcinoma), HMEC-1 (human microvascular endothelial cells) or J774 (murine macrophage cell line) [50, 58-61]. A number of studies have been done *in vivo* in a rat (*Rattus norvegicus*) model using *B. tribocorum* [62-64].

Bartonella bacteria contain structurally modified lipopolysaccharide (LPS) in their cell walls. Bacterial LPS commonly serves as an endotoxin during

infection and large amounts of circulating LPS in infected patients can trigger septic shock [65]. Bartonella LPS appears not to exert this effect as natural reservoir hosts for many strains maintain long term bacteremias without deleterious effect to the host [66, 67]. The LPS of *B. henselae* has some structural modifications that it shares with LPS of other intracellular bacteria such as *Legionella* and *Rhizobium* species [66]. Penta-acylation of lipid A is the major structural variation in the LPS associated with decreased endotoxic potential [66].

Bartonella bacilliformis, *B. clarridgeiae*, *B. schoenbuchensis*, *B. chomelii*, *B. capreoli*, and *B. rochalimae* have flagella [19-21, 28, 29, 68]. Flagella are cell surface structures that confer motility to bacteria [69]. The flagella of *B. bacilliformis* have been shown to play a role in erythrocyte binding and invasion though they are not the only determinants of host cell entry [68, 70-72]. On the other hand functional flagella do not appear necessary for *B. clarridgeiae* to invade HMECs *in vitro* [73]. Two non-motile *B. clarridgeiae* mutants were created by site specific mutagenesis of the flagellin gene, *fla*A, and the flagellar motor genes, *mot*BC. The mutants displayed a reduction in binding, or enhanced invasion, respectively, but no absolute loss of cell entry was observed during *in vitro* assays [73]. The role of flagella has not been investigated for the other four species, and no *in vivo* model exists for evaluating it.

Other strategies for erythrocyte or endothelial cell entry have been investigated and detailed. For *B. bacilliformis* this includes the use of the bacterial effector protein deformin to manipulate host cell membrane

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permeability and the use of invasins which are encoded by ialA-B (invasion associated locus) [74, 75]. A role for filamentous hemagglutinin in host cell adherence and entry has been hypothesized for bartonellae based on the presence of genetic homologs for these structures in *B. henselae* [76, 77]. In Gram negative bacteria adhesins (FhaB and FhaC/HecB) interact to form a periplasmic protein structure that spans the outer membrane and projects from the bacterial surface [76, 78]. During *in vitro* infection studies with *B. henselae* in HEC designed to monitor the activity of the BatR/BatS regulon, the genes encoding the filamentous hemagglutinin were upregulated along with other genes implicated in host cell invasion [79]. Research to confirm the putative role of FhaB and FhaC/HecB in host cell entry is needed.

Two type 4 secretion systems (T4SS), Trw and VirB-D4-Bep (Bep: Bartonella-translocated effector proteins) have been described in *Bartonella* species [57, 64, 80-82]. The T4SSs evolved from bacterial conjugation machinery and consist of up to 11 proteins which when assembled form a pilus and pore complex spanning both bacterial and host cell membranes [80, 83]. Bacterial effector proteins can be transported through these structures into the host cell cytoplasm [80, 83].

The specific function of the VirB-D4-Bep T4SS has been investigated *in vitro* for *B. henselae* [84], and in a rat model for *B. tribocorum* [85]. *Bartonella henselae* VirB4/D4 mutants showed significant impairments in their ability to mediate and/or abrogate host cell responses during HUVEC infection [84]. Expression of virB4/D4 is necessary for bacterial cell entry via the invasome, a unique structure resulting from bacterial rearrangement of

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the host cell's actin cytoskeleton [49, 86]. VirB4/D4 *B. tribocorum* mutants lost the ability to establish erythrocyte infection in rats compared to wild type bacteria [64].

The role of the Trw T4SS has been most extensively investigated in the *B. tribocorum*-rat model [63, 81]. It appears that the primary function of this T4SS is to mediate binding to host erythrocytes by constructing a pore and pilus complex, and not to transport bacterial effector proteins. In fact, the Trw T4SS has no VirD4 homolog, a protein which is required for the transport of Bep. TrwE deletion mutants could not establish erythrocyte infection in rats compared to wild type bacteria [81]. Because of its demonstrated role in erythrocyte binding the TrW is also believed responsible for the differential infectivity, i.e. host specificity, of different *Bartonella* species for different animal hosts [63, 81].

Phylogenetic relationships between different *Bartonella* species Trw systems have been investigated to identify influences on the evolution of host specificity [54]. In *B. henselae* gene sequences for the proteins that make up the Trw T4SS have been duplicated with as many as 7—8 copies of a single gene represented [87, 88]. An even greater number of copies exists in *B. tribocorum* and *B. grahamii*. These gene duplications have produced a high level of variability in the Trw pilus proteins [88]. Since the Trw pilus is involved in erythrocyte adherence and invasion, it is hypothesized that this variability is responsible for the erythrocyte host ranges of these bacteria [80, 88]. The variation in erythrocyte surface proteins exhibited by

mammalian hosts may have been an evolutionary driver for diversifying selection of the Trw [88].

Bartonella species are the only bacteria known to induce vasoproliferation during human infections. Bartonella bacilliformis stimulates the growth of vasoproliferative lesions known as verruga peruana, and B. quintana and B. henselae can induce bacillary angiomatosis and hepatic peliosis in humans, usually but not always in immunocompromised patients [89]. The mechanisms and effector proteins involved in these syndromes remain largely unknown and understudied.

Bartonella adhesin A (BadA) is a trimeric autotransporter adhesin, a member of a class of virulence factors common among Gram negative bacteria [90]. The protein constituents of this nonfimbrial adhesin form a construct consisting of a 'head, neck, stalk, and membrane anchor' that can transport effector proteins into the host cell cytoplasm [90, 91]. Expression of BadA is necessary for adherence of B. henselae to collagen covered glass coverslips and the head of BadA is required for binding of B. henselae to HUVECs [91]. Bad A also plays a role in activation of HIF-1 (hypoxia-inducible factor-1) during angiogenesis [90-92]. A homologue to BadA is present in B. quintana, the VompA-D (variably expressed outer-membrane proteins A-D). Though it has not been as extensively investigated as BadA in B. henselae, VompA-D appears to function in much the same way [60, 93, 94].

The upregulation of HIF-1 in B. henselae infected HeLa cells has been investigated. Increased expression of HIF-1 results in a subsequent increase

in transcription and secretion of VEGF (Vascular Epithelial Growth Factor) *in vivo* [92]. Secretion of VEGF leads to vasoproliferation [51, 92]. This pattern of angiogenesis has been previously observed in response to cellular hypoxia in tissues, such as cardiac ischemias, and also in tumor-related tissue remodeling [92]. Understanding the pathways for induction of vascular tissue growth has implications for therapeutic applications if the nature and role of bacterial effector proteins in this process can be discovered. Attempts have been made to develop an *in vivo* model for the study of this type of vasoproliferative lesion but have so far proven unsuccessful (Table 1.3).

Genetic and geographic diversity of bartonella strains

Except for *B. bacilliformis*, the sole survivor of an ancient bacterial lineage, all bartonellae appear to be derived from the same modern lineage by radial speciation [54]. These modern bacterial species commonly exist in nature as circulating genotypic variants most often associated with one or more predominant reservoir hosts [80]. The geographical and evolutionary relatedness of diverse bacterial strains has been investigated and it appears that strain diversification is associated with strain introductions into new locations with or without concomitant host shifts (or host switches) [95-97].

The rodent-associated bacterium *B. grahamii* is globally distributed and commonly isolated from a variety of rodent genera such as *Apodemus*, *Arvicola*, *Dryomus*, *Microtus*, *Mus*, *Myodes*, and *Rattus* spp., indicating a broad host range for this species [98-102]. Because of these characteristics and because it has been sequenced (2.3Mb), it is highly suitable for investigating the geographical and evolutionary origins of bartonella bacteria

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[53]. Most of the inferences made about mechanisms underlying the genetic and geographic diversification of rodent-associated bartonellae comes from genomic analyses of *B. grahamii* strains.

Genetic diversification in *B. grahamii* is quite common. Genes believed to be involved in host adaptation are located within a region of the genome containing phage sequences that experiences run-off replication (or escape replication) [53]. Sequences duplicated through this process can be selected upon or can mutate without affecting the integrity of the chromosome as a whole, so bacteria can diversify and adapt to new ecological niches easily. Strains of this species have high recombination rates and evidence for lateral gene transfers shows genetic exchanges occurring among different species that share hosts in rodent communities [53, 103]. This has been inferred among *B. grahamii* strains and between *B. grahamii* and *B. taylorii*, another species that commonly infects some of the same rodent hosts as *B. grahamii* [97, 103]. Recombination events within even a single species can explain genotypic variance among strains. Whether this variance plays a part in defining a strain's host range is unknown, but likely [96].

Geographic diversification of bartonellae can be easily conceptualized on a framework of large geographic distances and the biogeographic diversification and dispersal of rodents. However, genetical analyses of *B. grahamii* isolates from three locations within 30km of one another showed diversification even at such a small geographic scale [96]. The 20 strains analyzed had low sequence diversity, a low number of single nucleotide polymorphisms, and highly similar gene content, yet their genome structure

showed a level of diversity that could be correlated with their geographic origins [96]. This demonstrates that evolution and selection of differing genotypes/genomotypes can occur within very small areas and be driven by differences in rodent hosts, local ecology, and founder effects.

An intriguing last note on bartonella strain diversification is the suggestion that interactions between bartonella strains or between different bacterial species within a vector might contribute to strain diversity [104]. Horizontal gene transfers can potentially occur between bacteria inhabiting arthropod vectors in the gut or elsewhere. The environment within the vector would be vastly different than within a mammalian host and bacterial strains could potentially diversify to exploit that niche.

ECOLOGY OF ZOONOTIC BARTONELLAE

Bartonella bacteria can be broadly grouped into two categories: species that have man as their natural reservoir host and species that naturally infect mammalian hosts other than man. The human pathogens *B. bacilliformis* and *B. quintana* are arthropod-borne bacteria with the sandfly *Lutzomyia verrucarum* and the human body *Pediculus humanus* (Linnaeus, 1758) as their primary vectors, respectively [105]. All other known bartonella species are known or believed to be maintained in transmission cycles in non-human animal reservoirs [106-108].

Bartonellae have been isolated from rodents and other wildlife from six of the seven continents. Mammalian reservoirs encompass ungulates, rodents, insectivores, lagomorphs, carnivores, marsupials, and chiropterans (Table 1.1). Detection of the bacteria in such a wide range of mammals is made

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possible by the presence of a persistent bacteremia in natural reservoir hosts which is thought to facilitate bacterial acquisition by arthropod vectors [109]. The ability of bartonella bacteria to induce a persistent, non-pathogenic bacteremia is a unique parasitic strategy among bacteria and infections can last a year or more [110, 111]. This phenomenon is believed to be a result of a high level of adaptation of the bacteria for its natural hosts, as they appear able to maintain long term bacteremias without apparent adverse effects [108, 109, 111, 112]. Though this presumption has not been extensively investigated there is indirect evidence to support this hypothesis, several examples of which are noted below. No angiogenic lesions were found in tissues of over 100 naturally infected small woodland rodents examined histopathologically [109]. Bartonella infected fat sand rats (*Psammomys obesus*) sampled over a three year field study in Tunisia showed no effect on body mass or onset of sexual maturity relative to uninfected rats [113]. Observations from an experimental infection study of *B. tribocorum* in its natural host, *Rattus norvegicus*, also support the presumption that bartonellae do not commonly exert detrimental effects in their natural hosts [62]. In that study *B. tribocorum* parasitized erythrocytes in infected rats showed no alteration in either lifespan or functionality, though as many as 10^7 bacteria were present in 1ml of blood [62].

Natural history of bartonellae infections in rodents

The natural history of bartonellae has been investigated in a few wildlife reservoir hosts but it is still not well understood. Some host species seem to commonly harbor multiple bacterial strains or species, whereas other harbor

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only a few. Rodents in particular are reservoirs for numerous strains representing a large proportion of the diversity reported for the bacteria [108]. Recently published surveys on bartonellae in bats reveals strain diversity rivaling that observed in rodents [13, 14, 114]. Many other mammals harbor bartonella species but with less strain diversity (Table 1.1).

Bacterial host specificity seems to be a powerful determinant for the distribution of bartonella strains infecting small mammal populations. This phenomenon seems common in wildlife populations and indirect evidence for host specificity can be found in many rodent populations by simply comparing the relative prevalences of infection with different bartonella strains among different rodent taxa within an ecosystem. Specific associations between rodent taxa and circulating strains are also often apparent.

In some ecosystems the most abundant rodent species may be infected with a host specific dominant bartonella strain. This was seen with three cotton rat strains of bartonella circulating in cotton rats, *Sigmodon hispidus*, a species of New World rodent, in Georgia (species A, B, and C), and in the western U.S. in deer mice, another New World rodent, which are commonly infected with four different strain variants (D1—4) [115-117]. The dominant strains were infrequently detected in other rodent community species despite a presumptive high degree of exposure of naive rodents to infected hosts. Although the nature and frequency of inter-specific contacts between many sympatric rodent species in shared habitats have not been defined, it is likely

that contacts occur continuously since rodents will share or compete for many resources [118-120].

Studies conducted in the U.K. initially refuted the influence of host specificity on the distribution of bartonella strains among examined rodent communities, but later more in-depth studies of infection prevalence, seasonal dynamics, and host densities revealed that these strains were host specific [121-124]. Assuming that bacterial host specificity commonly exists, it explains why bartonella infection prevalences in 'non-natural' host populations surveyed in these studies never approaches the natural host population infection prevalence.

An experimental infection study using the bartonellae isolated from cotton rats further defined their host specificity among different rodent taxa, and confirmed bacterial host specificity as a determinant for the distribution of infections among rodents [125]. Three different cotton rat bartonella strains failed to infect Wistar rats (*Rattus norvegicus*), white-footed mice (*Peromyscus leucopus*), and BALB/c mice (*Mus musculus*) though bacterial doses were as high as 10^7 cfu [125]. Experimental infection studies of mice with different *Bartonella* species also reveals high levels of bacterial host specificity (Table 1.3).

Though bacterial host specificity seems to operate within, among, and between most mammal taxa there are at least two small mammal hosts inhabiting different ecosystems that appear permissive to infection by diverse strains. The North American grasshopper mouse (*Onychomys leucogaster*) commonly has a high bartonella infection prevalence (25—90%) and is

frequently infected with a variety of genotypes. Four of 10 genotypes

detected in grasshopper mice were identical to genotypes found in other

rodent genera [126]. The presence of variants in grasshopper mice

correlated with the abundance and population infection prevalence of those

variant's primary host [126]. When these indices were high, grasshopper

mice were more likely to be infected with these other variants.

In Asia the Oriental house shrew *Suncus murinus* appears to be

permissive for infection with bartonella strains that most commonly circulate

in *Rattus* and *Apodemus* spp. [127-129]. Both shrews and rats inhabit

peridomestic environments in Asia and share a common ectoparasite fauna

[130-132]. Contacts between rats and shrews and/or transfer of fleas

between hosts is a likely explanation for this finding [133, 134].

The demographic features of many bartonella infected host populations

have been investigated [108]. Small mammal populations tend to maintain

fairly high bartonella infection prevalences. A multiplicity of studies has been

done around the world to define circulating strains in reservoir hosts and to

understand the distribution and dynamics of infection in these populations

[108]. Rodent community composition and habitat appear to influence these

parameters. A few examples of such studies and their findings are

summarized below and a literature review of bartonella infection prevalences

in wildlife populations was recently published [108].

Deer mice and cotton rats in the western and southern United States

exhibit >80% and >90% population prevalences of bartonellae infection

[111, 112, 117]. In northwest England wood mice and bank voles infected

with five different *Bartonella* species had infection prevalences ranging from 0.6—38.7% depending on the host-bacteria combination [124]. Woodland and grassland rodents were sampled in Poland: 46.3% of yellow-necked mice and 29.1% of bank voles in the forest were infected with bartonellae, and 36.9% of common voles and 13.7% of root voles in grasslands were infected [135]. Numerous small mammal surveys have been done in various parts of Asia revealing an enormous array of circulating bartonella strains in tested animals [108]. Of the limited rodent genera that do not harbor bartonellae, interestingly it is *Mus* species that are most often not infected, though admittedly the capture rates for these rodents are not high [127, 129, 136-138].

Bartonella infection prevalence appears to be influenced by species diversity within rodent communities in the southwestern United States and northern Mexico [117]. Results from rodent trapping and blood sample testing of 24 rodent communities suggested that bartonellae transmission within these ecosystems was frequency-dependent [117]. Frequency-dependent bartonella transmission is predicated on some level of demonstrated host specificity of bacterial strains for particular hosts. Otherwise the presence of additional rodents within the habitat would merely increase the size of the natural reservoir population. When bacterial strains exhibit host specificity for certain rodent species or genera then an increase in refractory host abundance within the community can result in a decrease in transmissible contacts between infectious and naïve competent hosts.

A dominant species effect was observed in 2 of 24 examined sites wherein the most abundant rodent species within the community showed relatively little or no evidence for bartonella infection [117]. These dominant populations are hypothesized to monopolize resources within the ecosystem, thereby regulating population abundance of other sympatric rodent species which could be more susceptible to bartonella infection. A similar phenomenon was observed in the United Kingdom when non-native bank voles were introduced into an ecosystem with a pre-existing bartonella susceptible wood mouse population [123]. The bank voles were resistant to infection with any of the circulating strains in the native rodent populations. Bank vole population density directly affected bartonella infection prevalence in the wood mice [123]. Increases in bank vole density correlated with decreases in wood mouse infection prevalence, an example of a dilution effect [123].

Bartonella infection prevalences in surveyed rodent communities in the United Kingdom appear to be primarily density-dependent [122-124]. Within a system of four *Bartonella* species and two rodent hosts, both direct and delayed host density played a role in infection distribution and prevalence within the rodent community. It is notable that levels of host specificity for the four bacterial species examined during these studies seem much lower than those observed in U.S. rodents [122-124].

The influence of age, sex, and reproductive status on rodent bartonella infection prevalence has been assessed in several long term studies. Relationships between these parameters and population infection prevalences

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defy generalizations. In some systems older sexually mature rodents are more likely to be infected than younger immatures [122, 139, 140], but in other communities the opposite is true [112]. Sex of the rodent does not generally appear to effect infection status but there are exceptions. Male cotton rats had significantly higher bacteremia levels than females in one study [112], and male yellow necked mice (*Apodemus flavicollis*) had both higher prevalence and abundance of infection than females [135].

Seasonality seems to influence transmission of some bacterial strains among hosts [122, 124, 140]. This could be due to correlations with rodent breeding seasons and the introduction of naïve young into the population [140]. Seasonality could also be linked to fluctuations in arthropod vector abundance and host infestation rates [122]. No strong correlations between arthropod vector abundance and infection prevalence and measures of rodent host infection prevalence have been reported. This could be due to a lack of resolution in looking at ectoparasite samples coupled with a lack of information on the dynamics of vector-borne transmission within a studied system [122]. For example, studies that sampled fleas failed to identify the samples to species and samples are commonly pooled for testing [122, 139, 141].

Indigenous bartonella strains will infect both native and introduced rodent species unless a host specific barrier exists. Strains found in non-native rodent species can be related to bacteria from geographic locations where the animals originally came from [99, 142, 143]. Furthermore, when bartonellae are introduced into new areas, rodents can become infected with

the new strains, enabling detection of new strains through surveillance programs as they appear in new geographic locations. The often specific associations between rodent hosts and their bartonellae can permit both the animal source and region of origin to be deduced.

Rodent responses to naturally acquired bartonella infections

Most information about individual rodent host responses to infection comes from a few long term field studies and a very limited number of experimental infection studies evaluating rodents and their adapted bartonella strains. The threshold dose for host infection is unknown for most rodent bartonella strains. Bacteremia duration observed for naturally infected hosts was up to 8 months in cotton rats, up to 4 months for field voles, and up to a year or more for deer mice [109, 111, 112, 115, 125].

Bacteremia levels in naturally infected hosts vary widely according to the rodent species and circulating bartonella strains present. Levels of 10^6 cfu/ml have been recorded for *Sigmodon hispidus* (cotton rats) [112], 10^5 cfu/ml for *Rattus* spp. [137], 10^4 cfu/ml for *Neotoma* spp. (woodrats) [140], and 10^4 cfu/ml for *Cynomys ludovicianus* (black-tailed prairie dogs) [144]. Some field studies report categorical measures of bacteremia such as high or low [139] and others report on the number of parasitized red blood cells observed by microscopy in a blood smear [109, 113].

Bacteremia kinetics in naturally infected rodents usually seem to fall into one of four categories. Cotton rats and deer mice repeatedly captured during field studies showed patterns of relapsing bacteremias [111, 112, 115]. Rodents displayed bacteremias at one or more sampling points, then

appeared to clear the infection only to test positive for the same bacterial strain during subsequent sampling. Cotton rats and deer mice both displayed such abacteremic intervals. In the cotton rat study, some individuals showed an initial high bacteremia level that declined continuously over time. A third pattern was characterized by an initially high bacteremia which declined over time then 'spiked' again [112]. An animal might also maintain a low bacteremia for some time then experience a large increase in bacteremia level after which the level would decline again [112]. Field voles naturally infected with *Bartonella* species also displayed two patterns of response: either an initial high bacteremia that declined steadily over time, or a low level persistent bacteremia [109]. The cotton rats were infected with three different bartonella strains which could account for some of the differences in response [112, 115, 125]. Exposure dose, individual host susceptibility, and immune status could also play a role in infection response.

Homologous host:bacteria experimental infection studies have been done with cotton rats and white-footed mice (*Peromyscus leucopus*) and results generally agree with field observations [145]. The 100% infective dose was different for each of the three different cotton rat bartonella strains evaluated, ranging from 10 cfu to 1000 cfu. Cotton rats demonstrated bacteremia levels up to 10^5 cfu/ml following infection with 3 of the 4 strains. All white-footed mice inoculated with 10^7 cfu of four different *Peromyscus* spp. strains became bacteremic (100%). The dose required to infect 50% of inoculated mice varied from 10^4 to 10^7 cfu, and some mice displayed bacteremia levels >10^6 cfu/ml [145].

Vector transmission of bartonellae among hosts

Dynamics of transmission among animals naturally infected with bartonella strains remains obscure though vector transmission of several *Bartonella* species has been demonstrated. Noguchi established mechanical tick transmission of *B. bacilliformis* as early as 1926 by removing partially fed ticks from infected monkeys and placing them on naïve monkeys which later developed infections [146]. Sandflies and the human body louse are uncontended vectors of *B. bacilliformis* and *B. quintana* among humans, respectively [105]. The cat flea, *Ctenocephalides felis*, is a demonstrated vector of *B. henselae* among cats [147], and there is *in vitro*, experimental evidence for tick transmission of *B. henselae* by *Ixodes ricinus* [148]. More recently *I. ricinus* transmitted *B. birtlesii* to mice in the laboratory [149].

Flea vector transmission of two rodent bartonella species was experimentally demonstrated. Wild-caught *B. taylorii* and *B. grahamii* infected fleas (*Ctenophthalmus nobilis nobilis*) were placed in a holding pen with their natural hosts, bank voles (*Myodes glareolus*, formerly *Clethrionomys glareolus*). The twenty wild-caught fleas were allowed access to 28 naïve, laboratory bred bank voles for 4 weeks. The fleas were sampled from a population previously demonstrated to have a 60% prevalence for *Bartonella* species infection. After 4 weeks, 21/28 (75%) of the bank voles were bacteremic: 16 were infected with *B. taylorii*, five were infected with *B. grahamii*, and one vole was infected with both species. The only source of bacteria in the study was the introduced fleas, so some mechanism of flea-

borne transmission of the bacteria was indicated. Two-hundred and seventeen fleas were collected from the arena. Randomly sampled pools of 5 fleas each as well as 10 individual fleas were tested for the presence of *Bartonella* species [150]. All 10 pools were positive for bartonella and 70% (7/10) of individual fleas were positive as well. Of those seven positive fleas four were positive for *B. taylorii*, one for B. grahamii, and two fleas were positive for the presence of both species [150]. Unfortunately, it is difficult to make inferences about the transmission efficiency of the fleas from this data.

A multiplicity of flea-borne mechanisms of transmission exists and it is possible for different mechanisms to be responsible for differing portions of natural transmission cycles. The infected fleas may have bitten the voles and thus transmitted the bacteria, the voles may have eaten the fleas, or the fleas may have excreted infectious feces that infected the voles through mucous membrane exposure or intradermal exposure following self-scratching in response to flea bites.

The potential for horizontal and/or vertical transmission was also assessed in this study [150]. Eight male: female pairs of voles were caged together without fleas. For two pairs both voles were inoculated with *B. taylorii*, for two pairs only males were inoculated, and for four pairs only females were inoculated [150]. The vole pairs were kept approximately 10 weeks, or until they had produced litters (7/8 pairs). Blood cultures confirmed that all needle inoculated voles became bacteremic, but no uninoculated adults or young were found bacteremic, i.e. no horizontal or vertical transmission of the bacteria occurred [150]. Two bank vole pups

born during the experiment were negative for bacteremia though they were suckling a bacteremic female [150].

Numerous ectoparasite surveys have identified potential vectors of bartonellae through PCR detection of bacterial DNA [151, 152]. Hematophagous arthropods are often implicated as vectors when either viable bacteria or bacterial DNA is detected in them, though vector competence for bartonellae has been established for very few arthropod species [149, 151, 152]. Experimental, ecological, and epidemiological evidence is lacking for a role in transmission of bartonellae for a majority of these arthropods [52, 152]. Recent studies are beginning to address these gaps in knowledge [148, 149, 153-155].

Undoubtedly vector transmission of bartonellae does occur, but alternate transmission mechanisms may also be responsible for some proportion of transmission events in nature. There is some limited evidence implicating additional transmission modes. Bartonellae have been isolated from cotton rat and deer mouse embryos and neonates [156]. This suggests the potential for at least some *Bartonella* species to be transmitted vertically in their natural reservoir hosts [156]. Viable bartonella bacteria were detected in urine collected by cystocentesis from one cotton rat (M. Kosoy, personal communication). If urinary excretion of viable bartonellae occurs in rodents it may serve as a mechanism for environmental transmission of the bacteria [157].

One of the continuing puzzles of bartonella ecology arises from differences between bacterial strains detected in ectoparasites compared to

strains detected in hosts from which those ectoparasites were removed [155, 158-160]. First, not all ectoparasites removed from bacteremic hosts are positive for detection of bacterial DNA matching strains present in the host [141, 158]. Second, bacterial strains can be detected in ectoparasites that are not present in their hosts [160, 161]. If obligate blood feeding ectoparasites such as fleas or ticks are common vectors of bartonella strains among hosts it seems that all or most arthropods recovered from a bacteremic host should contain bacterial DNA. This is not always the case however. There are multiple explanations for this finding that do not contradict the widely held belief that arthropods commonly transmit bartonellae among hosts. Ectoparasites recovered from a host may have recently transferred from another animal [133, 134]. The efficiency of bacterial colonization of the ectoparasite may be low. A host may have been previously infected with another bacterial strain which has colonized the midgut of the vector, rendering it refractory to further colonization with a different bacterial strain. A host may be co-infected with different bartonella strains that demonstrate different growth characteristics in the ectoparasites than in their hosts [159]. This is plausible as mammalian hosts will maintain a steady body temperature, whereas ectoparasites are ectotherms subject to larger temperature fluctuations than their hosts. Strains that outcompete other bacteria in the host may in turn be out-competed in the vector. Arthropod vectors may also be more permissive to midgut colonization by some strains than by others. In addition, sensitivity for detection of co-

infecting bacteria in arthropods may be low [159, 162]. It is possible that the incidence of co-infection is much higher than currently recognized [159].

The presence of bartonella strains in reproductive tissues of small numbers of rodent fleas of five species raises the question of whether bartonellae could be vertically transmitted by arthropod vectors [155]. Bacterial strains in fleas did not match those found in their hosts [155]. Similar results were observed in another study where rodent fleas removed from bacteremic hosts were infected with both host strains and other strains [160]. In both these studies guts and ovaries dissected from fleas were positive for bartonella bacteria, supporting the potential for vertical transmission of some bartonella strains in fleas [155, 160].

The flea *Xenopsylla ramesis* has recently been shown to acquire midgut infections with bartonella strains circulating in its natural rodent host [153]. Bacterial transmission by the flea was not assessed. Testing of eggs laid by females feeding on bacteremic hosts revealed no evidence for bacterial transmission to offspring [153]. Both eggs and larval developmental stages tested negative for the presence of bacterial DNA [153]. Though there appears to be a conflict in evidence for vertical transmission of bartonellae in fleas, different flea species were investigated in these studies so the potential for vertical transmission of bacterial strains in fleas should not be ruled out.

EPIDEMIOLOGY OF HUMAN BARTONELLA INFECTIONS

Over the last decade bartonellae have repeatedly emerged or re-emerged as a cause of human illness globally [43, 163-168]. Infections with these bacteria are generally acquired from zoonotic sources such as host reservoir

animals or from hematophagous arthropods [169]. In some cases the source of infection is unknown though presumed to be a mammalian reservoir or an arthropod vector [43, 164, 165, 170]. Either immunocompetent or immunocompromised people may become ill due to bartonella infections [171]. The two *Bartonella* species most commonly recognized as causing human illness are *B. henselae*, the agent of 'cat scratch disease', and *B. quintana* which caused numerous cases of trench fever in soldiers during World War I. *Bartonella quintana* is considered a re-emerging infectious disease as the incidence of infection with this bacterium is increasing in human populations not previously recognized at risk for disease [105]. This includes homeless people and alcoholics who have higher intrinsic risk for louse parasitism and depressed immune function, both factors that can enhance their susceptibility to infection [166, 172, 173]. In South America, the lesser known though more virulent *B. bacilliformis* causes Carrion's disease in humans [105]. Carrion's disease refers to either the acute illness caused by this bacterium, known as Oroya fever, and/or a second stage chronic syndrome known as verruga peruana. During Oroya fever, infected patients may develop a severe, life-threatening hemolytic anemia [105, 172]. Human bacteremias are commonly only observed during infection with *B. bacilliformis* and *B. quintana*, the human pathogens, and may or may not be accompanied by clinical disease [105].

With respect to the zoonotic bartonellae over 22,000 cases of human infection with *B. henselae*, the 'cat scratch disease' agent, are reported annually in the United States alone [169]. The burden of human disease from

other zoonotically acquired bartonellae infections, both in the United States and on a global scale, has yet to be fully determined. Humans infected with zoonotic bartonellae can present with long term headache, myalgia, lymphadenitis, neuroretinitis, bacillary angiomatosis, hepatic peliosis, meningitis, myocarditis, and endocarditis [106, 169, 174]. While infection of incidental hosts with zoonotic bartonellae can result in disease, these bacteria do not normally cause morbidity in their natural hosts [109, 175].

Bartonella henselae is the most studied species among the zoonotic bartonella in terms of experimental *in vivo* and *in vitro* studies [106, 169, 176]. It is maintained in a feline reservoir (domestic and wild felids) and is transmitted from cats to humans most often by a cat scratch, though some human patients with the disease deny contact with cats [176]. Patients sometimes report arthropod bites, which suggests that some 'cat scratch disease' cases may be acquired from infected arthropod vectors. Considering that flea bites constitute the primary mechanism for transmission of *B. henselae* among cats, it's probably that people can also be infected this way [147].

Zoonotic bartonella infections are considered to be emerging infectious diseases because improved surveillance and diagnostics have allowed infections to be detected in populations not previously recognized to have them [30, 165, 177-180]. Infections acquired from peridomestic or commensal small mammals seem to occur at low incidence but this may be due to the inherent difficulties in diagnosing these infections [39, 165, 167, 168, 181]. The fastidious requirements of the bacteria in conjunction with

low levels of bacteremia in incidentally infected hosts makes detection of this agent complex. Bacterial isolation from incidental host blood is uncommon and though tissues and blood are often cultured in attempts to obtain an isolate, these methods are not often successful [165, 171, 182]. Species specific PCR assays and serological tests such as immuno-fluorescent assays, with four-fold changes in titer between acute and convalescent sera, are often relied upon to diagnose infections [171, 177, 178].

As zoonotic bartonella infections become more prevalent, the need to understand the transmission dynamics of the bacteria among its natural hosts and to incidental hosts has intensified. Whenever possible human cases have been followed up with epidemiological risk assessments of behavior and environment [39, 44, 164, 165, 171]. A consistently identified risk factor is a history of direct and indirect exposure to wild and domestic animals including arthropod vectors [165, 183]. Still, specific exposure events leading to infection are not often identified [44, 168, 171] (Table 1.2).

In 2008 a woman with a recent history of disposing of dead rodents was diagnosed with meningitis caused by a *B. washoensis*-like bacteria [167]. Fleas (*Oropsylla wagneri*) collected from rodent burrows and a California ground squirrel captured on her property were tested and found to contain bacteria genetically identical to the strain infecting her [167]. Whether she acquired the infection from a flea bite or rodent contact was never definitively determined [167]. In France in the last five years, three non-related cases of *B. alsatica* infection have been reported [168, 184]. One patient developed lymphadenitis and two had endocarditis [168, 184]. All

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three patients had handled rabbits, either butchering or rearing the animals [168, 184, 185]. In another instance candidatus *B. melophagi* was isolated from two chronically ill patients [44]. The bacterium was originally isolated from sheep and sheep keds. Again, specific exposures leading to patient infection could not be determined [44]. Because patients infected with zoonotic bartonella often have complex histories of animal and arthropod contacts, it is difficult to satisfactorily assess and quantify the risk of acquiring zoonotic bartonellae infections. This is due to a fundamental lack of understanding as to whether risk occurs in the context of animal and/or ectoparasite contact, and what type or how frequent such contact must be to constitute a risk.

Human infections with zoonotic bartonellae

Case reports of human infections with zoonotic bartonellae are summarized in Table 1.2 (n = 21). Human bartonella infections with species associated with domestic companion animals are not included in the table (*B. henselae, B. clarridgeiae, B. koehlerae, B. vinsonii* subsp. *berkhoffi*). Criteria for inclusion in the table were publication in English and individually reported patient data (not summary data for groups of infected patients).

Patients infected with zoonotic bartonellae can present with a wide variety of symptoms (Table 1.2). Symptoms can be quite severe leading patients to seek medical interventions. Some patients reported illness for up to two years prior to diagnosis of infection: others reported acute onsets that could sometimes be linked to potential exposures (Table 1.2, raccoon bite, travel history). The age of the patients ranged from 12—86 years old, with 12 of

Table 1.2 Epidemiological and clinical features of cases of human zoonotic bartonella infection. [yo = years old, NR = Not reported, IFA = Immunofluorescent antibody assay, IHC = Immunohistochemistry, MIF = Micro-immunofluorescent antibody assay]

Bartonella species	Clinical syndrome	Patient age / gender	Symptomatology	Co-morbidities	Patient exposures to animals or vectors	Diagnostic tests used
B. alsatica [184]	Endocarditis	74 yo male	3 weeks of fever, hemoptoic cough, aphasia	Bioprosthetic aortic valve replacement	Butchered and cared for rabbits ≥1 year prior	Cardiac tissue: Shell vial assay positive by IFA; bacterial DNA detected by PCR
B. alsatica [186]	Lymphadenitis (axillary mass)	79 yo female	10 days lymphadenopathy	NR	Butchered rabbit ~1 month prior with scratch on finger	Lymph node PCR and IHC positive; Serum positive on Western blot
B. alsatica [168]	Endocarditis	77 yo female	Fever, systolic murmur	NR	Rabbit breeder	Serum MIF and Western blot positive
B. elizabethae [23]	Endocarditis	31 yo male	2 months fatigue, malaise, anorexia, shortness of breath, weight loss	*Strongyloides stercoralis* infection; 'infected' teeth	Patient reported no animal exposures; sustained hand lacerations 2 weeks prior to illness onset	Bacterial isolation from blood

Table 1.2 continued.

Bartonella species	Clinical syndrome	Patient age / gender	Symptomatology	Co-morbidities	Patient exposures to animals or vectors	Diagnostic tests used
B. elizabethae [187]	Leber's neuroretinitis	31 yo male	Monocular loss of vision experienced over several weeks	NR (reported previously healthy)	Raccoon bite, no cat exposure	Serum titer 1:64; convalescent titer 1:32
B. grahamii [181]	Bilateral neuroretinitis	55 yo female	1 year history of loss of visual acuity, headache, behavioral changes	Insulin dependent diabetes mellitus, hypo-thyroidism	Patient owned a dog, reported no contact with cats or small rodents	Bacterial DNA in ocular fluid detected by PCR
B. grahamii [188]	Bilateral retinal artery branch occlusions	51 yo male	Relapsing fever and chills 6 months prior to, and erythematous papular rash 5 months prior to diagnosis	NR	Patient denied contact with rodents	Serological evidence
B. rochalimae [164]	Bacteremia, fever and splenomegaly	43 yo female	Fever, insomnia, myalgia, nausea, headache and mild cough of ~3 weeks duration; diffuse macular rash	NR	Travel to Peru; arthropod bites, did not own pets; denied cat contact	Bacterial isolation from blood, >4-fold change in IFA titers (IgM and IgG)

Table 1.2 continued.

Bartonella species	Clinical syndrome	Patient age / gender	Symptomatology	Co-morbidities	Patient exposures to animals or vectors	Diagnostic tests used
B. tamiae Th239 [30]	Febrile illness	38 yo male	Fatigue, myalgia, headache, and maculopapular rash of 22 days duration; 6 days of fever	NR	History of killing/trapping rodents in home	Bacterial isolation from blood
B. tamiae Th307 [30]	Bilateral pterygium	41 yo female	Headache, myalgia	NR	History of killing/trapping rodents in home	Bacterial isolation from blood
B. tamiae Th339 [30]	Febrile illness	12 yo male	Fever, fatigue, myalgia, headache, petechial rash on arms and legs for 2 days	NR	History of killing/trapping rodents in home	Bacterial isolation from blood
B. vinsonii subsp. *arupensis* [33]	Bacteremia with fever, cardiac valvulopathy	62 yo male	Dizziness, myalgia, headache, slurred speech, fatigue for 48 hours: Acute onset of confusion, difficulty walking, facial numbness and fever	An 'ill-defined' rheumatologic syndrome with neurological manifestations	Likely occupational exposure to rodents and arthropods	Bacterial isolation from blood

Table 1.2 continued.

Bartonella species	Clinical syndrome	Patient age / gender	Symptomatology	Co-morbidities	Patient exposures to animals or vectors	Diagnostic tests used
B. vinsonii subsp. *arupensis* [189]	Endocarditis	79 yo male	Felt unwell for a month, fever	Aortic valve bioprosthetic replacement	No history of animal exposure	Serum IFA, Western blot, bacterial DNA detected by PCR assay
B. washoensis [39]	Fever and myocarditis	70 yo male	NR	NR	Peridomestic rodent exposure (Patient denied rodent contact)	Bacterial isolation from blood
B. washoensis-like [167]	Meningitis	47 yo female	1 day history of fever, chills, headache, nausea, vomiting, epigastric and abdominal pain; photophobia and joint pain for several hours	None (previously healthy)	Extensive animal exposures over some years duration; recently handled dead rodents; patient denied recent arthropod exposures	Bacterial isolation from blood
B. vinsonii subsp. *arupensis* [190]	Febrile illness	20 yo female	Fever	NR	NR	Bacterial isolation from blood

Table 1.2 continued.

Bartonella species	Clinical syndrome	Patient age / gender	Symptomatology	Co-morbidities	Patient exposures to animals or vectors	Diagnostic tests used
B. vinsonii subsp. *arupensis* [190]	Endocarditis	30 yo female	Fever	NR	NR	Bacterial isolation from blood
Candidatus *B. mayotimonensis* [43]	Endocarditis	59 yo male	Progressive shortness of breath, weight loss, fatigue, and altered mental status	Aortic aneurysm	Intermittent cat contact, peridomestic rodent exposure	Bacterial DNA detected by PCR assay in aortic valve tissue, Western blot, valve section stain and IHC
Candidatus *B. melophagi* [44]	Bacteremia relapsing episodes of illness	51 yo female	Circular red lesion on thigh, cough, fatigue, myalgia, foot pain: Symptoms for 2 years; cardiac function and neurological abnormalities	NR (previously healthy); (co-infection with *B. henselae*)	Minimal occupational animal contact for ≥15 years, arthropod exposures	Bacterial isolation from blood, confirmed by PCR analysis

Table 1.2 continued.

Bartonella species	Clinical syndrome	Patient age / gender	Symptomatology	Co-morbidities	Patient exposures to animals or vectors	Diagnostic tests used
Candidatus *B. melophagi* [44]	Pericarditis (unknown etiology)	65 yo female	6 month history of fatigue, muscle weakness in arms and legs	Co-infection with *B. henselae*	Extensive daily animal and arthropod contacts over some years duration, commonly bit and scratched	Bacterial isolation from blood, confirmed by PCR analysis
Candidatus *B. volans*-like/ *Candidatus B. durdeni*-like [41]	Long term debilitating illness (~1 year)	86 yo male	Arthritic signs, short-term memory loss, incoordination, localized edema, neurologic deficit, coronary artery disease, hypertension, atrial fibrillation, persistent anemia, fungal and viral co-infections	Co-infection with *B. vinsonii* subsp. *berkhoffii* or *B. henselae*; diabetes,	Tick bites, captured mice, rats, skunks, and groundhogs, and potential rodent flea exposures during animal capture and transport	Bacterial DNA detected from enrichment culture by PCR assay, seroconversion

those patients >50 years old. Eleven of 21 cases were reported in males (age range 12—86 years old), and 10 were reported in females (age range 20—79 years old). Most patients were treated for weeks to months with antibiotics, usually aminoglycosides, and most or all symptoms seemed to resolve following treatments [191, 192]. Cases were reported from the United States, France, United Kingdom, the Netherlands, Russia, and Thailand (Table 1.2).

Co-morbidities were reported for seven patients ranging from heart valve replacements to diabetes mellitus (Table 1.2). Thirteen patients had no co-morbidities reported, and one patient was reported as not having any co-morbidities. Some patients had high occupational rates of animal exposure over time; others denied contact with animals or reported limited exposures to animals not known to harbor the strains infecting patients (Table 1.2). Exposures to arthropods were variable. Pet ownership was inconsistently reported. Epidemiological risk reporting varied widely in these reports and it is not apparent what risk factors were assessed for several cases as only positive exposures were reported (Table 1.2).

Bacteria were isolated from 12 of the 21 patients (Table 1.2). In the other cases blood cultures were either not attempted, were negative, were probably not incubated long enough to grow *Bartonella* spp., or were obtained after patients began antibiotic treatment. In the absence of bacterial isolation from blood, cases were confirmed by PCR detection of bartonella DNA in tissue, by serum IFA or Western blot, by IHC of cardiac tissue sections, or by a combination of methods (Table 1.2).

Recently zoonotic *Bartonella* species infections were reported from a group of Thai patients with febrile illnesses [165]. *Bartonella* species DNA was identified in 14/261 screened samples (7.7%). Half the bacterial strains detected were genetically related to rodent-associated *Bartonella* species (7/14), four sequences were novel, one was *B. tamiae*, and two were *B. henselae*. Patients reported owning animals (10/14) and more than half reported exposure to rats within 2 weeks of illness onset (8/14). Symptomatology associated with their illnesses is consistent with symptomatology for febrile illness cases in Table 1.2 [165].

Seroprevalence surveys for human exposures to zoonotic bartonellae

Reports of 13 seroprevalence surveys using antigens derived from rodent-associated and other *Bartonella* species were identified (English language, PubMed and Web of Knowledge) [177, 183, 193-203]. Five surveys were conducted in Sweden, five in the United States, two in Thailand and one in the Democratic Republic of Congo (DRC). Antibody prevalences to *B. henselae*, *B. elizabethae*, and *B. quintana* were assessed in all 13 studies. Additional *Bartonella* species on the IFA assay panels represent strains circulating in animal reservoirs in areas where these surveys were conducted, and which humans might potentially come in contact with.

Patient sera from serious febrile illness cases in the Four Corners area of the U.S. were screened against 9 bartonella strains [203]. These included *B. henselae*, *B. elizabethae*, and *B. quintana*, and strains from deer mice, cotton rats, meadow voles, white-throated woodrats, rock squirrels, and Ord kangaroo rats. Nine patients had serological evidence for exposure to rodent-

associated *Bartonella* species, seemingly dominated by responses to the woodrat strain antigen, though extensive cross-reactivity between strains made interpretations of results difficult [203].

In Sweden antibody to *B. grahamii* was observed following the addition antigens from the rodent-associated species *B. grahamii* and *B. vinsonii* subsp*. vinsonii* to the IFA panels. Recent serosurveys in Thailand included *B. vinsonii* subsp. *vinsonii* antigens [177, 202], and it is expected that additional antigens will be added to future panels consistent with strains detected in febrile illness patients (discussed above, also Table 1.2) [30, 165]. Antigens from six *Bartonella* species (*B. henselae*, *B. elizabethae*, *B. quintana*, *B. vinsonii* subsp. *vinsonii*, *B. vinsonii* subsp. *arupensis*, B. *clarridgeiae*) were used for IFA assay of sera obtained from febrile patients in the DRC [201]. Patients were most reactive to *B. clarridgeiae* in that study, and no antibody to rodent-associated strains was observed [201].

Most of these 13 serosurveys were conducted in populations that might be expected to have a high risk of bartonella exposure except for two in Sweden that involved healthy blood donors [193, 195]. Populations sampled included cases of chronic fatigue syndrome [199], homeless and intravenous drug users [194, 196-198, 200], febrile illness patients [177, 201-203], and cases where bartonella exposure was suspected [183, 193]. Seroprevalence to rodent-associated bartonella strains was the dominant finding, with rates ranging from 4% to 52.1% for studies positive for this response (12/13). In 10 studies the highest antibody prevalence rates observed were to *B. elizabethae* antigen (cutoff titers 1:64). Two studies could not differentiate

between strains due to cross-reactivity [177, 203], and the DRC study reported no patient antibody responses to *B. elizabethae* antigen [201].

Results from serosurveys suggest that exposures to rodent-associated bartonellae are common in some human populations. Blood donor surveys revealed antibody to *B. elizabethae* in 4% (n = 100 samples) and 14.1% (n = 498) of healthy Swedish adults screened (cutoff titer 1:64), further suggesting that exposures occur commonly among some general human populations as well [193, 195]. The disparity between number of diagnosed human infections with zoonotic bartonellae and serosurvey results is marked (Table 1.2). This highlights several important features of these infections. Detection of infection in patients can only occur if diagnostic assays are applied with reasonable suspicion of *Bartonella* species involvement in the illness. Blood cultures must often be incubated for extensive periods of time before growth will appear [30]. PCR and IFA assays and IHC are specific for detection of these micro-organisms. If bartonellae are not suspected as a cause of illnesses samples will not be screened by these methods. In addition, though cross-reactivity among *Bartonella* spp. appears common in IFA assays, appropriate antigens should be used to assess infection and exposure rates in different populations. Successful detection of bartonella infections in humans is based on knowledge of the environment, what animal reservoirs are present in a location and which strains are circulating there. This information can often raise the index of suspicion for bartonella infections allowing them to be detected [184, 185, 204].

Bartonellae infections in humans are likely under-recognized and under-reported. The human disease burden due to infections with these bacteria may be much higher than previously believed. As surveillance efforts improve and diagnostic techniques become more sensitive and specific we can expect to better define the incidence and prevalence of bartonelloses in human populations.

LABORATORY MOUSE MODELS FOR BARTONELLA INFECTION

Bacteria in the family Bartonellaceae are aptly described as stealth pathogens [205]. They are increasingly associated with zoonotic infections worldwide, creating more awareness of them as infectious agents of emerging public health concern [169, 174]. The morbidity associated with these illnesses and the bacteria's insidious tendency to infect some of the most vulnerable and resource challenged human populations in the world is slowly drawing attention (Table 1.2) [165, 169, 174, 177, 183, 193-203]. Even in more developed countries we have evidence of an impact of the bacteria. In the modern world we have both the drive and resources to pursue understanding of infectious agents and their mechanisms of persistence and virulence. Yet our ability to investigate these pathogens is being challenged in one of the most basic research areas in modern science. Almost one hundred years after the first attempts were made to model bartonelloses in animal models there remain significant impediments to the development of disease models for these bacteria [206].

Animal models for bartonella bacteria induced diseases, especially mouse models, have proven difficult to develop. Mice and many other animals

simply fail to become infected following inoculation with most *Bartonella*

species (Table 1.3) [145, 207]. Though they may develop pathology

following exposures, it does not typically match characteristics of human

infections or pathology [208-210]. Failure of bacteria to infect mice is most

probably due to host specificity and seems to be a nearly universal feature of

these species.

Though zoonotic bartonellae that infect humans have already successfully

accomplished one host switch this does not seem to translate to host

promiscuity. A prime example of this is seen with strains of *B. henselae*

isolated from both cats and infected humans. Neither strain type will infect

mice and produce bacteremia [211].

Mouse models for human disease caused by *B. bacilliformis* and *B.

quintana* have never been developed. A series of experimental infection

studies using non-human primates in the 1920's demonstrated that *B.

bacilliformis* was the causative agent of Carrion's disease [212-215]. It was

not until the 1960's that Koch's postulates were satisfied with regards to *B.

quintana*. Bacteria were isolated on blood agar plates from clinically ill

patients and used to infect other human volunteers [216, 217]. The outcome

of the studies proved the bacterium was the agent of trench fever.

Studies investigating *Bartonella* species interactions with host cells have

been done almost exclusively *in vitro*. Such studies are useful for evaluating

the role of specific genes, immune cells, or bacterial effector proteins in host

cell invasion and manipulation [79, 84, 218, 219]. Yet they cannot substitute

for, nor duplicate whole organism responses to infectious bacteria [63, 64, 92, 211, 220, 221].

Attempts have been made to develop *in vivo* models for studying bacterial interactions and mechanisms of pathogenesis but the vast majority of outcomes were unsuccessful. Published reports of laboratory mouse models for bartonella infection are summarized in Table 1.3. Of particular note are the numerous laboratory mouse stocks/strains used in these evaluations as well as the routes of exposure employed [222-224]. Mouse models for bartonella infection would have the advantage of the widespread availability of murine immunological reagents and selective mutant strains of mice for investigating specific questions about bacteria-host cell interactions.

Models for immune response elicited against *B. henselae* dominate this group and kinetics of antibody response and aspects of cellular and humoral immunity have been determined (Table 1.3). The problem is that the mice typically clear the inoculated bacteria within hours to a few days, so these models are not suitable for investigating bacterial interactions with host cells. Mice can develop granulomatous lesions in their livers following inoculation of *B. henselae* but unlike in human 'cat scratch disease' cases these lesions are neither necrotic nor suppurative [210, 220].

Mice with all manner of genetic defects in immune response have been infected with different species and strains of bartonella in unsuccessful

Table 1.3 Numerous inbred and outbred mouse stocks have been experimentally infected with *Bartonella* species in attempts to reproduce bacteria-host cell interactions observed during natural and incidental host infections. [IP = intraperitoneal, ID = intradermal, PO = per os (oral), IV = intravenous, SQ = subcutaneous]

Bartonella species	Mouse stock/strain	Mouse age	Exposure route	Desired outcome	Actual outcome	Ref.
B. henselae (6 clinical isolates) *B. quintana* (2 clinical isolates)	CD1	NR	IP	Evaluate specificity of antibody responses	Polyclonal antibodies; Species specific antibodies, intraspecies cross reactivity	[225]
B. henselae Houston-1	BALB/c An BALB/c nude BALB/c	7-9 weeks	ID	Bacillary angiomatosis	Negative blood culture, serum IFA and pathology	[226]
B. henselae Houston-1	C57BL/6 BALB/c	10-12 weeks	IP	Necrotizing granulomas	Granulomatous inflammation	[210]
B. henselae	BALB/c	4-6 weeks	PO Intranasal IV, IP, SQ	Bacteremia, organ dissemination, immune response	Induction of humoral immunity	[208]
B. grahamii	BALB/c C57BL/6 C57BL/6 *Igh-/-* C57BL/6 *Rag1-/-*	6-8 weeks	IV	Bacteremia; immune response	Bacteremia (C57BL/6 *Rag1-/-*); immune response	[85]
B. birtlesii IBS325	BALB/c	8-16 weeks	IV	Persistent infection, effect on reproductive effect	Bacteremia 10 weeks duration, reproductive failure	[227]

Table 1.3 continued.

Bartonella species	Mouse stock/strain	Mouse age	Exposure route	Desired outcome	Actual outcome	Ref.
B. henselae Berlin-1	C57BL/6	10-13 weeks	IP	Persistent liver inflammation, antibody responses	Persistent liver inflammation (20 weeks); cell mediated immune response (Th1 predominant)	[228]
B. henselae	BALB/c An BALB/c nude BALB/c	4-7 weeks	IV, IP, ID	Liver and spleen granulomas, bacteremia, ultrastructural changes in bacteria	Liver and spleen pathology, granulomas (1 immunocompetent mouse); ultrastructural changes in bacteria	[229]
B. henselae Houston-1	BALB/c BALB/c nu/nu	5 weeks	IP	Antibody response; role of T-cells	Antibody response; role of T-cells	[230]
B. bacilliformis ATCC#35685 *B. bacilliformis* (patient isolate IMTAVH#00032)	BALB/c	10-12 weeks	IP, ID, SQ, PO, intranasal	Persistent bacteremia, vasoproliferation	No bacteremia, no granulomatous lesions; liver abscesses one inoculated mouse and one control mouse; no other histopathology	[231]
B. henselae Houston-1	BALB/c	5 weeks	IP	Analyze cytokine production in spleen	Spleen cells produced IFN-γ, IL-10, and TNF-α	[232]

Table 1.3 continued.

***Bartonella* species**	**Mouse stock/strain**	**Mouse age**	**Exposure route**	**Desired outcome**	**Actual outcome**	**Ref.**
B. henselae Fr98/K8 *B. henselae* Fr98/K8aM *B. henselae* Houston-1 *B. grahamii*	BALB/c IFNARI-/- RAG1-/-	6-12 weeks	SQ	Persistent lymphadenopathy (cat scratch disease)	Persistent lymphadenopathy in popliteal lymph node	[211]
B. birtlesii	BALB/c C57BL/6 Swiss C57BL/6 IL10-/- C57BL/6 CD4-/- C57BL/6 CD8-/- C57BL/6 CD4CD8-/-	8-20 weeks	IV, SQ, ID PO, Ocular	Persistent bacteremia	Bacteremia (up to11 weeks duration)	[233]
	BALB/c	3 & 18 months	IV			
B. birtlesii M39 *B. birtlesii* P63 *B. birtlesii* Q73 *B. doshiae* C26 *B. doshiae* MAC35 *B. doshiae* R18 *B. doshiae* 70WNH *B. grahamii* F16 *B. grahamii* R16 *B. grahamii* 33TD *B. taylorii* LL-WM9 *B. taylorii* MAC36 *B. taylorii* 8TBB	A/J SCID SCID/BEIGE	6-8 weeks	IP	Bacillary angiomatosis	*B. taylorii* infected SCID/BEIGE: extramedullary hematopoiesis; bacterial aggregates in tissue; biomatrix formation; collagen deposition	[209]

attempts to recreate human disease pathologies (Table 1.3) [www.informatics.jax.org]. Immunocompromised, bartonella infected patients are the only recognized human patient population that consistently develops bacteremias during zoonotic bartonella infections. Antibody responses during incidental host infection with accompanying bacteremia were determined in immunocompetent and immunodeficient mice using *B. grahamii,* a species originally isolated from *Apodemus* mice [85]. This provided insight into what antibody subclasses were involved in sterilizing immunity in contrast with responses lacking in immunodeficient mice.

There is intense interest in developing a mouse model for the unique vasoproliferative lesions associated with some *Bartonella* species infections [209, 219, 221, 234]. Models for endocarditis, myocarditis, neuroretinitis, and lymphadenitis would be invaluable for studying virulence mechanisms and bacterial trafficking and tissue distribution during incidental host infections. Defined *in vivo* systems would be useful in determining mechanisms of microbial pathogenesis such as the role of the T4SSs.

Mice have also been evaluated for use as models of natural host infection. *Bartonella birtlesii* can infect mice and produce bacteremia but this bacterium also adversely effected reproductive function of mice and should be viewed with caution as representing a natural host infection model [227, 235]. In addition, mice infected with this strain demonstrate bacteremias of only 8-11 weeks duration, which is short relative to duration of persistent bacteremia observed in naturally infected rodent reservoir hosts [109, 111, 112, 115, 227, 235]. A mouse model that exhibited bacteremia of long duration and

was susceptible to infection with low bacterial doses would be an excellent candidate for vector transmission studies of bartonellae. Such a model would also be suitable for answering questions about the natural history of bartonellae in reservoir animals. This could include investigating the dynamics of host co-infection and the immunological responses involved in successive infections with different bacterial strains.

The Bartonella Laboratory at the Division of Vector-Borne Diseases in Fort Collins, CO has an extensive collection of bartonella strains obtained from wildlife from around the world. A unique opportunity exists there to assay strains from diverse animals in mice and to potentially develop models that better reproduce desired characteristics of bartonella infection, whether it be recapitulating dynamics of natural host infection, or reproducing human disease states. To this end strains were selected for experimental infection studies in mice based on 1) their host of origin, 2) their relatedness to known human pathogenic species, 3) their epidemiological significance in terms of human exposure risk, and 4) their isolation from ill humans and presumptive assignment of causality for that illness. Strains were to be inoculated into both outbred and inbred mouse stocks so all possible heterogeneity in response to infection could be captured. Specific aims were to develop mouse models for persistent bacteremia, bacterial host specificity, bacterial host switching, and bartonella induced pathogenesis. If desired outcomes of bacteremia or disease were observed, the study results could be published and serve as a resource for investigators interested in pursuing research in applicable areas.

CHAPTER 2

EXPERIMENTAL INFECTION OF LABORATORY MICE WITH

BARTONELLA STRAINS FROM WILD *MUS* SPECIES: A HOMOLOGOUS

HOST-BACTERIA MODEL SYSTEM AT THE GENUS LEVEL

INTRODUCTION

The natural history of bartonella bacteria is not well understood nor has it been widely investigated. Many wildlife species act as reservoirs for diverse bartonella strains. Rodents in particular harbor numerous bartonella strains and species which appear to be exceedingly well adapted to their hosts [108]. Though longitudinal field studies provide insight into rodent population infection incidence and prevalence, the dynamics of infection and transmission in rodent-bartonellae systems remain obscure. Vector transmission of several *Bartonella* species has been experimentally demonstrated but alternate transmission mechanisms have not been investigated for rodent bartonella strains. Evidence that vertical transmission may play a part in natural maintenance cycles of cotton rat and deer mouse strains was reported when fetuses and neonates of these species were found to be bacteremic [156]. Rodent urine can also contain pathogens, suggesting

a possible mechanism for environmental transmission of bartonella bacteria [157].

No studies have been published describing experimental infection of laboratory mice with bartonellae isolated from *Mus* species in nature. To better understand bartonella infection and transmission dynamics in natural hosts it would be desirable to develop a model system that pairs a natural host with its co-adapted bartonella bacteria. As *Mus musculus* is the definitive laboratory animal model, a system comprised of the laboratory mouse and a co-adapted bartonella would have great utility for research purposes. To date, the well documented host specificity of bartonellae has been an impediment to developing such a model [145, 226, 231].

With an overall goal of obtaining insight into the natural history of rodent-bartonellae systems, we designed a study to evaluate the *in vivo* infection characteristics of two *Mus* species bartonella strains in laboratory mice. Specific goals for the study were to observe 1) whether CD1 (ICR) mice (*M. musculus*) would be susceptible to infection with *Mus* species bartonella strains, 2) the response following inoculation of a range of bacterial doses, 3) bacteremia duration and magnitude in infected mice, and 4) whether viable bacteria might be present in bacteremic mouse urine. Since these strains were originally obtained from wild-caught *Mus* species (*M. caroli* and *M. cervicolor*) we thought it likely the bacteria could successfully switch from one *Mus* species to another, i.e. to *M. musculus*. Host-specificity of rodent bartonellae has been previously demonstrated at the genus level but not at the species level [145]. A laboratory mouse model that reproduces

characteristics of naturally acquired bartonella infections would be desirable for investigating bacteria-host interactions such as persistent bacteremia and bacterial host specificity, and whether vector transmission of bartonellae among hosts is important for the maintenance of these bacteria in nature.

MATERIALS AND METHODS

Mice and bacteria

Specific pathogen free, eight week old CD1 female mice were obtained from the closed, outbred mouse colony at the Division of Vector-Borne Diseases (DVBD), Fort Collins, Colorado, USA. Mice were housed six to a cage and groups were segregated based on strain identity and dose for the study duration of 27 weeks. All work with the mice was approved by and conducted under the supervision of DVBD's Institutional Animal Care and Use Committee (IACUC) in accordance with United States Public Health Service (USPHS) standards for the humane care of laboratory animals (protocol #07-004).

Source and passage history information for the study strains is detailed in Table 2.1 and does not include growth of stock for this study. Though the two strains have identical nucleotide sequence identities for their citrate synthase genes both were included in the study as they differed in host of origin. Bacterial stocks were grown on heart infusion agar plates (HIA) supplemented with 5% rabbit blood. Plates were inoculated to produce bacterial lawns and placed in a CO_2 incubator at 35°C for 5 days at which time the bacteria were harvested in physiological saline. Stocks diluted in saline were frozen at -80°C until used for mouse inoculation. Bacterial stock

concentrations were calculated by thawing frozen stock aliquots, diluting them 10-fold and inoculating them onto plates as described above to enumerate colony forming units (cfu).

Table 2.1 The *Bartonella* strains used in the study were originally isolated from *Mus* species in Thailand.

Rodent source of strain	Label	Passage history (n = number of passages)
Mus caroli (Ryukyu mouse)	MA	blood sample→ HIA + 10% rabbit blood, n=2; BHI + 10% rabbit blood[1], n=2; Swiss Webster mouse[2] (*in vivo*), n=1; BHI + 10% rabbit blood, n=1
Mus cervicolor (fawn colored mouse)	ME	blood sample→ HIA + 10% rabbit blood, n=2; BHI + 10% rabbit blood, n=2; Swiss Webster mouse[1] (*in vivo*), n=1; BHI + 10% rabbit blood, n=1

[1] Brain and Heart Infusion agar
[2] Swiss Webster mouse subcutaneously inoculated with 10^7 cfu.

Experimental design and blood collections

Thirty-six mice were used to evaluate the two strains. Three groups of six mice were each subcutaneously inoculated with a different dose of bacteria: 10, 100, or 1000 cfu, for each of the two bacterial strains (n = 18 mice X 2 strains = 36). Bacterial doses were diluted in physiological saline and mice were inoculated in the dorsal midline between the scapulae. Blood was collected from each mouse weekly for 27 weeks. Sample volumes ranged from 80—125µl depending on mouse body mass. Blood was collected by lancet puncture of the submandibular vessel plexus while mice were anesthetized (ketamine-medetomidine cocktail [50-75 mg/kg + 1 mg/kg],

administered intraperitoneally). The cheek of a mouse was first shaved and cleansed with chlorhexidine to create an aseptic site for blood collection. The puncture site was alternated weekly. Following blood collections mice were administered the anesthetic reversal agent atipamazole (5mg/kg intraperitoneally). Blood was frozen at -80°C until thawed for microbiological testing.

We observed a high mouse mortality rate during this study [6/36 (17%)], an unprecedented number in our experience working with mice. It was later learned that the ketamine used in our anesthesia cocktail was recalled due to an unduly high number of adverse events including deaths related to its use (Teva Animal Health, 2009). Three MA10, one MA100, one ME10, and one ME100 mouse died between study weeks 4 and 15. Available bacteremia data for these mice is presented in the results section.

Urine collections

Urine was collected from mice weekly for 4 weeks (weeks 3—6) by manual expression beginning soon after mice began manifesting bacteremia. While mice were anesthetized for blood collection the bladder was gently squeezed and any urine expressed was captured in a sterile tube. Cystocentesis was not performed to preclude the possibility of lacerating blood vessels during urine collection, thus contaminating the sample with blood. Urines were frozen at -80°C until thawed for microbiological plating. Attempts were made to collect urine from all mice in the study but some mice voided during handling prior to anesthesia induction. Collection volumes were variable for mice that had urine in their bladders and ranged from 20—

80µl per mouse. On a weekly basis urine was collected from 73-100% of mice.

Testing samples for viable bartonella bacteria

Frozen whole blood was thawed and mixed (50µl + 50µl) with brain heart infusion (BHI) diluent containing 15µg/ml Fungizone™. The resultant 100µl samples were inoculated onto HIA plates supplemented with 5% rabbit blood and incubated for up to 2 weeks at 35°C in a 5% CO_2 atmosphere. Samples with colonies too numerous to count were subsequently diluted so cfu could be counted and bacteremia levels could be calculated. Colonies were confirmed as bartonella by Gram stain and colony morphology (either rough or smooth) [236].

Urine samples were thawed and 50µl of each sample was mixed with BHI diluent containing 20µg/ml Fungizone™. When sample volume was less than 50µl all available urine was used for plating. Diluted samples were inoculated onto HIA plates supplemented with 5% rabbit blood and held for up to two weeks at 35°C in a 5% CO_2 incubator.

RESULTS

Bacteremia kinetics in mice

Mice were susceptible to infection with both bartonella strains evaluated and displayed a dose dependent response to both with 56—75% of mice becoming bacteremic (12/18 MA and 11/18 ME, respectively) (Table 2.1, Figures 2.1A-E). Mice inoculated with the 1000 cfu dose of MA manifested bacteremias as early as 1—2 weeks post-inoculation, whereas a temporal lag

Table 2.2 Summarized bacteremia data for mice inoculated with low doses of two *Mus* spp. bartonella strains.

Strain group (dose in cfu)	No. mice bacteremic/ No. inoculated	Range of bacteremia levels observed (cfu/ml blood)	Average time to bacteremia onset [range in weeks]	Duration of bacteremia[1] (range in weeks)	No. mice bacteremic at study end (Week 27)
MA (10)	3/6	$1 \times 10^2 - 8.5 \times 10^6$	12 [11—14]	10—13	0
MA (100)	3/6	$1 \times 10^2 - 1.1 \times 10^7$	9.6 [3—19]	8—20	2
MA (1000)	5/6	$1 \times 10^2 - 4.2 \times 10^7$	5 [1—17]	10—25	4
ME (10)	0/6	None	None	None	0
ME (100)	4/6	$1 \times 10^2 - 2.7 \times 10^6$	12.2 [6—18]	6—16	2
ME (1000)	6/6	$1 \times 10^2 - 9.4 \times 10^6$	11 [6—18]	6—13	0

[1] eight mice were still bacteremic when the study was terminated: duration of bacteremia data is therefore incomplete for those mice

of at least 5—6 weeks occurred before any mouse manifested bacteremia with ME regardless of dose. It should be noted that mice that died apparently uninfected might have manifested late onset bacteremias had they survived (n = 4/6; the other two mice were already bacteremic). (Figures 2.1A-E, Table 2.2).

Bacteremia duration in some individual mice was months long. We defined the duration as the time from first manifestation of bacteremia till last bacteremic observation or till the end of the study. This was regardless of whether bacteremia was detected at all weeks between onset and termination. Some mice displayed abacteremic intervals where high bacteremia levels were followed by periods without detectable bacteremia after which bacteremia was once again detected (Figures 2.1A-E). Abacteremic intervals were often associated with an overall pattern of decreasing bacteremia levels (Figures 2.1A-E).

Inoculated mice in both groups showed variability in time to bacteremia onset. (Figures 2.1A-E, Table 2.2). This response was especially evident in both strain groups at lower doses (10 or 100 cfu) where temporal lags in manifestation of detectable bacteremia ranged from several weeks to months for mice within the same dose group. Some mice were still bacteremic when the study was terminated (8/30 surviving mice, Table 2.2).
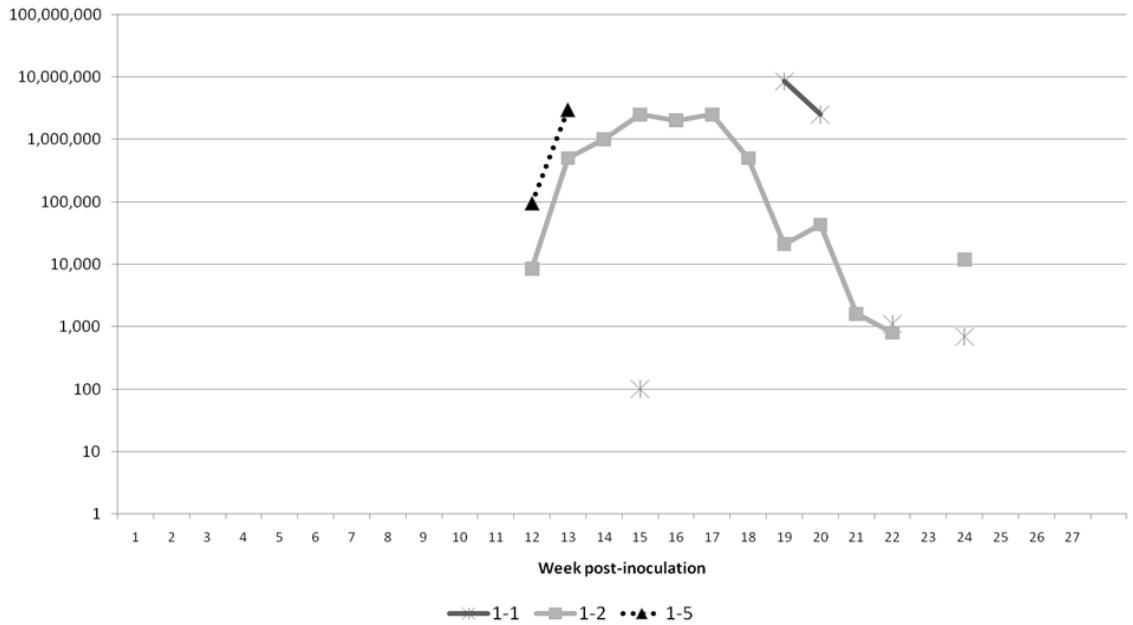
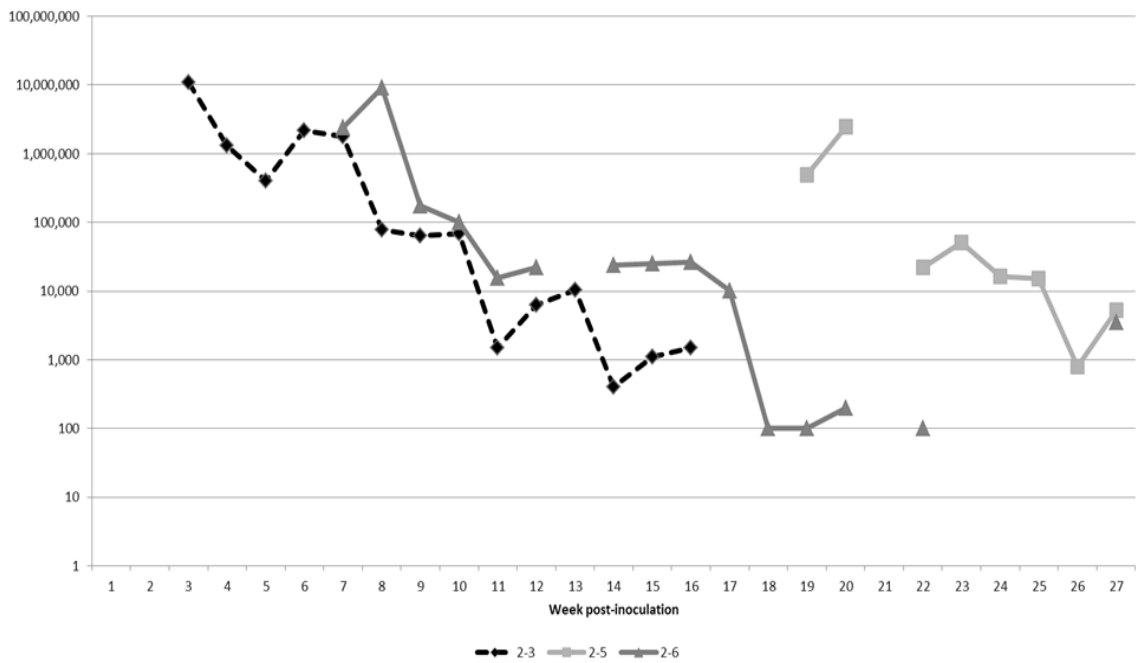Figure 2.1A. Bacteremia kinetics for CD1 mice inoculated with strain MA (10 bacteria per mouse).

Figure 2.1B. Bacteremia kinetics for CD1 mice inoculated with strain MA (100 bacteria per mouse).
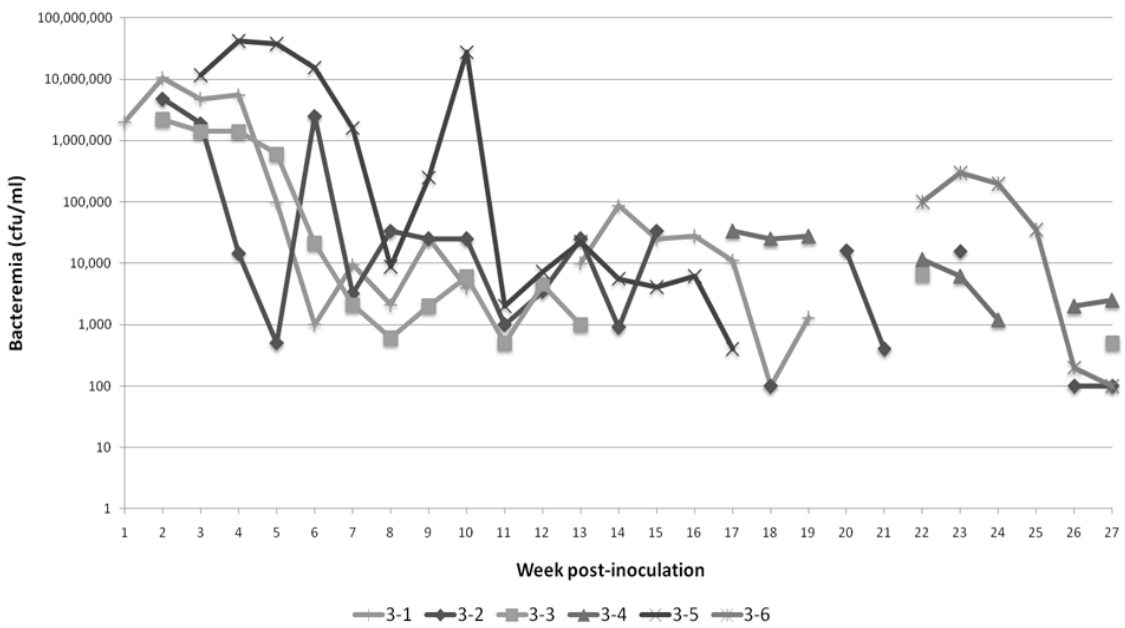
59

Figure 2.1C. Bacteremia kinetics for CD1 mice inoculated with strain MA (1000 bacteria per mouse).
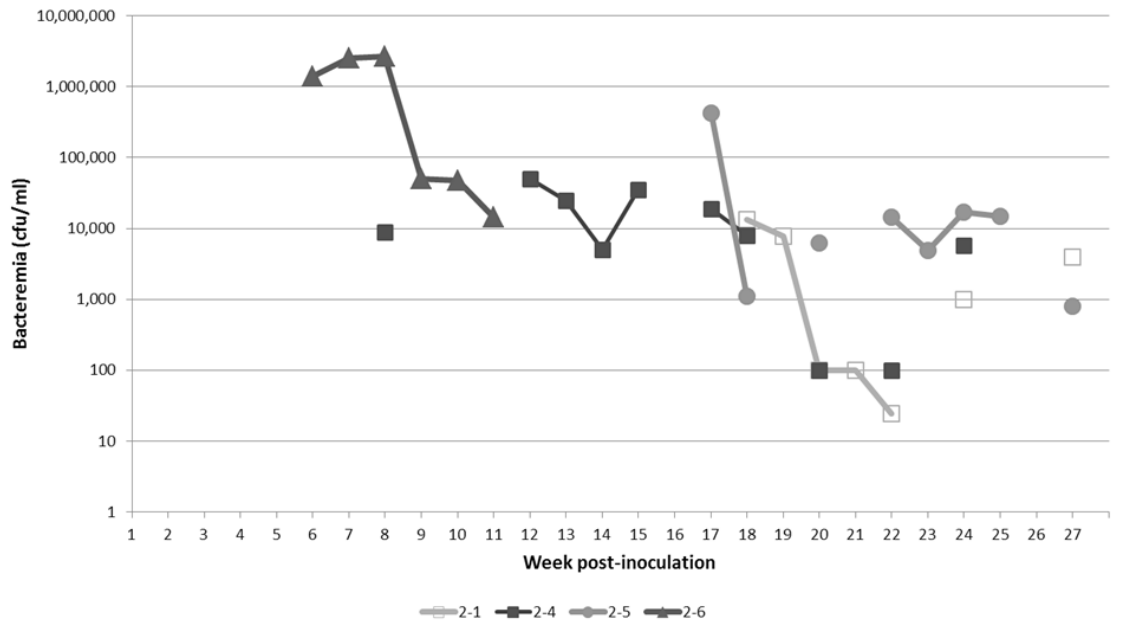

Figure 2.1D. Bacteremia kinetics for CD1 mice inoculated with strain ME (100 bacteria per mouse).
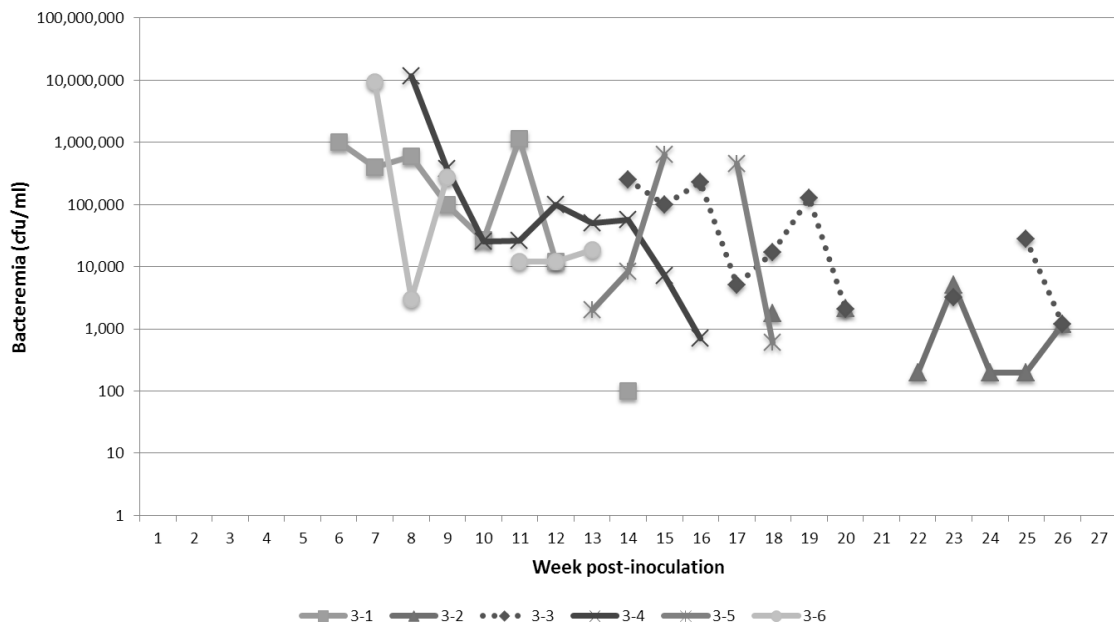
Figure 2.1E. Bacteremia kinetics for CD1 mice inoculated with strain ME (1000 bacteria per mouse).

Bacteria in urine

During the 4 week collection period urine samples were collected at least three times from all mice in both strain groups. No bartonella bacteria were detected in the mouse urines by microbiological plating and plates inoculated with urine displayed little or no contamination that might have interfered with our ability to detect bartonella bacterial growth.

DISCUSSION

This is the first report of experimental infection of laboratory mice with bartonella strains obtained from *Mus* species in nature and represents a homologous host-bacteria system at the genus level. These bacterial strains were easily able to cross-species host switch from their hosts of origin to the

laboratory mouse. Persistent bacteremia was observed following low dose exposures in a high proportion of inoculated mice. Infected laboratory mice manifested high level, long duration bacteremias with abacteremic intervals reproducing characteristics of infection reported for natural reservoirs of rodent bartonella strains [109, 111, 115].

Longitudinal field studies looking at field voles in the UK, cotton rats in Georgia, USA, and deer mice in Colorado, USA have documented bacteremias from several months to a year duration in naturally infected rodents [109, 111, 112]. Bacteremia levels reported for naturally infected rodent hosts range from 10^6 cfu/ml for bartonella strains infecting *Sigmodon hispidus* (cotton rats) [112], to levels of 10^5 cfu/ml in *Rattus* spp. [137], to 10^4 cfu/ml in *Neotoma* spp. (woodrats) [140] and *Cynomys ludovicianus* (black-tailed prairie dogs) [144].

Mice in our study exhibited high sustained levels of bacteremia following low dose exposures and represent the first immunocompetent mouse model to replicate the longevity of bartonella bacteremia well beyond 11 weeks. Two other laboratory mouse models for persistent bacteremia [85, 227, 235] have been reported. The more extensively investigated of the two used a strain of *B. birtlesii* originally isolated from *Apodemus* species [227, 235]. Laboratory mice infected with this strain manifest bacteremias up to 10 weeks duration with levels of bacteria in the blood up to 10^5 cfu/ml [227, 235]. Similarly, immunocompetent mice inoculated intravenously with 10^9 cfu of *B. grahamii* developed bacteremias up to 10^4 cfu/ml and 11 weeks duration [85]. Alterations in infection response relative to natural host

infection kinetics, such as truncated or low level bacteremias even following high dose bacterial exposures are probably due to sub-optimal levels of bacterial adaptation to an alternate host.

Temporal lags in bacteremia onset associated with different dose exposures were commonly observed during this study. The dynamics of response seemed to be primarily influenced by the dose inoculated, where lower doses generally seem to elicit a shorter duration bacteremia of somewhat lower level (Figures 2.1A-E, Table 2.2). Lags before bacteremia onset following bacterial exposure may be a common characteristic of natural infections, and may complicate attempts to define the transmission dynamics of rodent bartonella strains among their hosts. There is evidence that bartonella bacteria are transmitted by fleas [147, 150], yet analyses of data collected during longitudinal field studies of bartonella infected rodent communities have generally failed to find correlations between flea abundance, flea infection rates, and rodent host infestation rates or infection prevalence [122, 140]. Incongruences among these parameters may be explained by our study results which demonstrate that rodent hosts can have an extremely variable response in time to onset of bacteremia (Table 2.2). Differences in exposure dose and individual variation in susceptibility to infection in rodent populations can complicate attempts to find correlations among these parameters.

Results of our study provide insight into the transmission dynamics of these strains in nature in two areas. First, susceptibility to infection at low doses is a likely prerequisite for vector, specifically flea, transmission of

these strains among mice or other rodents. Therefore, either hosts must become infected at low doses of the bacteria or transmission may occur via an as yet unproven mechanism of cumulative exposures. Indeed, it may be that both these criteria need be met for bartonellae transmission to occur. Since these bacteria tend to be co-adapted to their hosts, infection at low doses is probable, and likely, in natural reservoirs. Scenarios of cumulative exposure to the bacteria leading to infection are also credible in that rodent fleas and their hosts often share a long association with fleas typically feeding upon their hosts one or more times a day. In addition, flea feces are routinely deposited in the fur of parasitized animals as well as in host burrows or nests, and could serve as a long-term exposure source to hosts as they have been shown to contain viable bartonellae.

The second area in which our observations may provide insight into the bacterial transmission dynamics is the observed dose dependent response. Mice in our experiments were susceptible to low dose inocula (10, 100, and 1000 cfu) of bacteria. In the *B. tribocorum*-rat model of bacteremia intravenous inoculation of 10^7 cfu bacterial dose resulted in bacteremia onset in five days. When cotton rats were inoculated with low and high doses of cotton rat bartonella strains, they developed bacteremia within one week as well [145]. This suggests that in natural hosts bacteremia onset is initiated by some threshold dose. In those studies the threshold was met and the response was immediate. However, in those laboratory models and this one, putative natural mechanisms of arthropod transmission were not assessed. Intradermal inoculation of the bacteria and scarification techniques for

pathogen exposure commonly serve as surrogates in the laboratory for arthropod bites or scratching which results in self-inoculation of infectious materials such as flea feces. When attempting to reconstruct natural transmission mechanisms in the laboratory, a reduction in number of bacteria ultimately colonizing the host should be considered with respect to the simulated methods. For example, if bacteria must invade the vasculature following deposition in the dermis then there is likely a reduction in the number of colonizing bacteria versus the number that were contained in the original exposure dose. Importantly, it might be expected that infection by such simulated exposure routes might take longer to develop than when viable bacteria are deposited directly into the bloodstream (intravenous route), or are placed in physiological compartments where the vasculature is more accessible to the bacteria (intraperitoneal or subcutaneous routes). Therefore intradermal inoculations or scarifications might be more likely to reveal candidates for vector transmission of the bacteria than other exposure routes. Strains evaluated in the current study would be excellent candidates for exploring whether a threshold dose for infection exists, if cumulative low dose exposures could result in infection, and whether there might be potential differences in infection response associated with different routes of bacterial exposure.

A high background of intra- and interspecific contacts exists in rodent inhabited ecosystems. Resource competition, partitioning, and sharing among rodents will occur frequently in such settings. Aggressive and amicable encounters between rodents create opportunities for flea transfer

between animals. In addition, nest or burrow sharing or opportunistic use of the same among rodents has been documented, which allows for yet more direct and indirect contacts between rodents. If susceptibility of mice to low doses of these strains reproduces susceptibility of natural rodent to bartonella infections, then it becomes more plausible that these types of contacts and/or arthropod vector exchanges between hosts can result in transmission of the bacteria.

Bartonella bacteria were not detected in the urine of infected mice during this study though mice were bacteremic at the time of sampling (weeks 3—6, Figures 2.1A-E). Since urines were only collected for 4 weeks it is possible that additional sampling might have revealed the presence of bacteria in the urine later in the study. However, in a previously published study urine collected from *B. henselae* infected, bacteremic cats was evaluated for the presence of viable bacteria or DNA without success [237]. This suggests that urinary excretion of bartonella bacteria is not common if it occurs at all. However, because the relationships between specific bartonellae and their natural hosts is still not well understood the possibility remains that this transmission mechanism could function in some natural reservoir populations.

The two *Mus* species bartonella strains evaluated in this study share 100% nucleotide sequence identity for a portion of their citrate synthase gene (*glt*A), the gene most commonly used to evaluate genetic relatedness among bartonella strains and species, yet they exhibited different infection phenotypes in the laboratory mouse. This may be due to a number of factors.

The simplest explanation is that this represents natural variability among these bacterial strains. Alternatively, clonal selection during isolation and microbiological plate passage may have resulted in expansion of a bacterial sub-population that displays differential characteristics from the parent population. This could have occurred with either or both isolates, and is always a possibility when bacteria are sampled from their natural environment and grown on microbiological media. Immune selection is also a possibility, since both of these isolates were passaged in mice. Finally, the differential infection phenotype may exist in nature and function to reduce competition for hosts between these phenotypic variants, but such an interaction would depend on a lack of cross-immunity between the strains.

Interestingly, strain MA appeared as rough colonies throughout the study when cultured from bacteremic mice, whereas cultured ME produced consistently smooth colonies with some rough colonies present at times. This phenomenon, termed phase variation, has been described from rodent bartonella isolates previously [236]. In addition, rough/smooth colony phenotypes have been investigated for *B. henselae* and the rough colony morphology was shown to be associated with the expression of pili [238]. A rough colony producing *B. henselae* strain was 100 times more invasive *in vitro* than a smooth colony producing *B. henselae* strain [238]. Increased expression of pili by strain MA relative to strain ME could also explain differences in their *in vivo* infectivity for the mice.

In summary we report on a mouse model for persistent bacteremia using bartonella strains isolated from wild caught *Mus* species. This system may be

used to evaluate the transmission dynamics of bartonella bacteria among hosts or to investigate the molecular basis for host specificity demonstrated by some bartonella strains. Susceptibility of mice to infection at low bacterial doses coupled with high bacteremia levels make this system a superior candidate for vector transmission studies. Finally, the dynamics of bacterial co-infection and host competition can be explored using both MA and ME in the mouse model, potentially providing unique insights into bartonella bacterial ecology.

CHAPTER 3

EXPERIMENTAL INFECTION OF LABORATORY MICE WITH RAT BARTONELLA STRAINS: HOST SPECIFICITY, BACTEREMIA KINETICS, DOSE DEPENDENT RESPONSE AND PATHOLOGY

INTRODUCTION

Many bartonella strains have potential to cause disease in humans (Tables 1.1, 1,2 Chapter 1). It is important to understand the epidemiological and ecological risk factors associated with human acquisition of infection [44, 106, 165, 167]. Some infected patients have histories of animal and arthropod contacts but others deny such exposures [44, 165, 171]. It is unknown what triggers some bartonella strains to perform what is essentially a host switch and infect a human incidental host. We know that some *Bartonella* species naturally cycle in a limited number of host species, whereas others may utilize animals from several genera within a taxon as natural reservoir hosts [33, 112, 122]. Perhaps bacterial host specificity could be a predictor for whether a bacterial strain might infect an incidental host.

Of all the wildlife that serve as reservoirs for bartonellae, rodents harbor the greatest strain diversity [108]. Rodents are present in peri-domestic and

agricultural settings throughout the world [239] and many rodent populations have high prevalences of bartonella infection [3, 136-138]. Infected rodents can also maintain bacteremias for long periods of time, often months, and fleas and other ectoparasites that might act as vectors are commonly found on rodents [112, 130, 239, 240]. These characteristics define a reservoir of bartonellae in nature that can pose a risk to human health.

Zoonotic disease agents that infect incidental hosts often possess an inherent capacity to infect a wide variety of mammalian hosts [241]. Evaluating the host specificity of high exposure risk bartonellae strains by simulating incidental host infections in the laboratory can provide a better understanding of a bacteria's zoonotic potential [241]. To that end we designed a study to evaluate whether rat bartonella strains could infect the common laboratory mouse, *M. musculus*. Since rats and mice are members of different genera within the subfamily Murinae (Family Muridae, Old World mice and rats), we hypothesized that exposure of mice to rat bartonella strains could provide insight into 1) whether rat bacterial strains might demonstrate host specificity, 2) what proportion of incidental hosts might become infected following exposures at different bacterial doses, and 3) whether there might be pathology subsequent to infection. Importantly, bartonellae that seem able to utilize a broad host range may be more likely to successfully host switch, or 'jump' to incidental hosts [241]. The bartonella strains chosen for this study circulate in rat hosts in areas where humans often experience rodent exposures.

MATERIALS AND METHODS

Mice

The specific pathogen free, outbred Swiss Webster (SW) mice used in the experiments were obtained either from Taconic Farms (Germantown, NY), or from a SW mouse colony maintained at DVBD, Fort Collins, Colorado, USA. The SW mouse colony at DVBD was originally established with SW mice from Taconic Farms. Female mice 5—8 weeks of age were used for all experiments and were housed seven mice to a cage except control mice which were three to a cage. All work involving mice was conducted as outlined in our approved animal use protocol (#07-004), under the supervision of DVBD's IACUC and in accordance with the USPHS standards for the humane care of laboratory animals.

Bacteria

The low passage bacterial isolates used in this study were originally obtained from *Rattus* spp. in Asia (Figure 3.1, Table 3.1) [136, 137]. The four strains differ from one another either phylogenetically and/or with respect to their hosts of origin (Figure 3.1, Table 3.1). Strains Rl5132th, Rn1532yn, and Rf1563yn share ≥92.6% similarity by *glt*A (citrate synthase gene) sequence distance analysis [ClustalW algorithm, MegAlign, DNASTAR® Lasergene software]. In comparison, Rn1691yn shares only 84.0—86.9% sequence
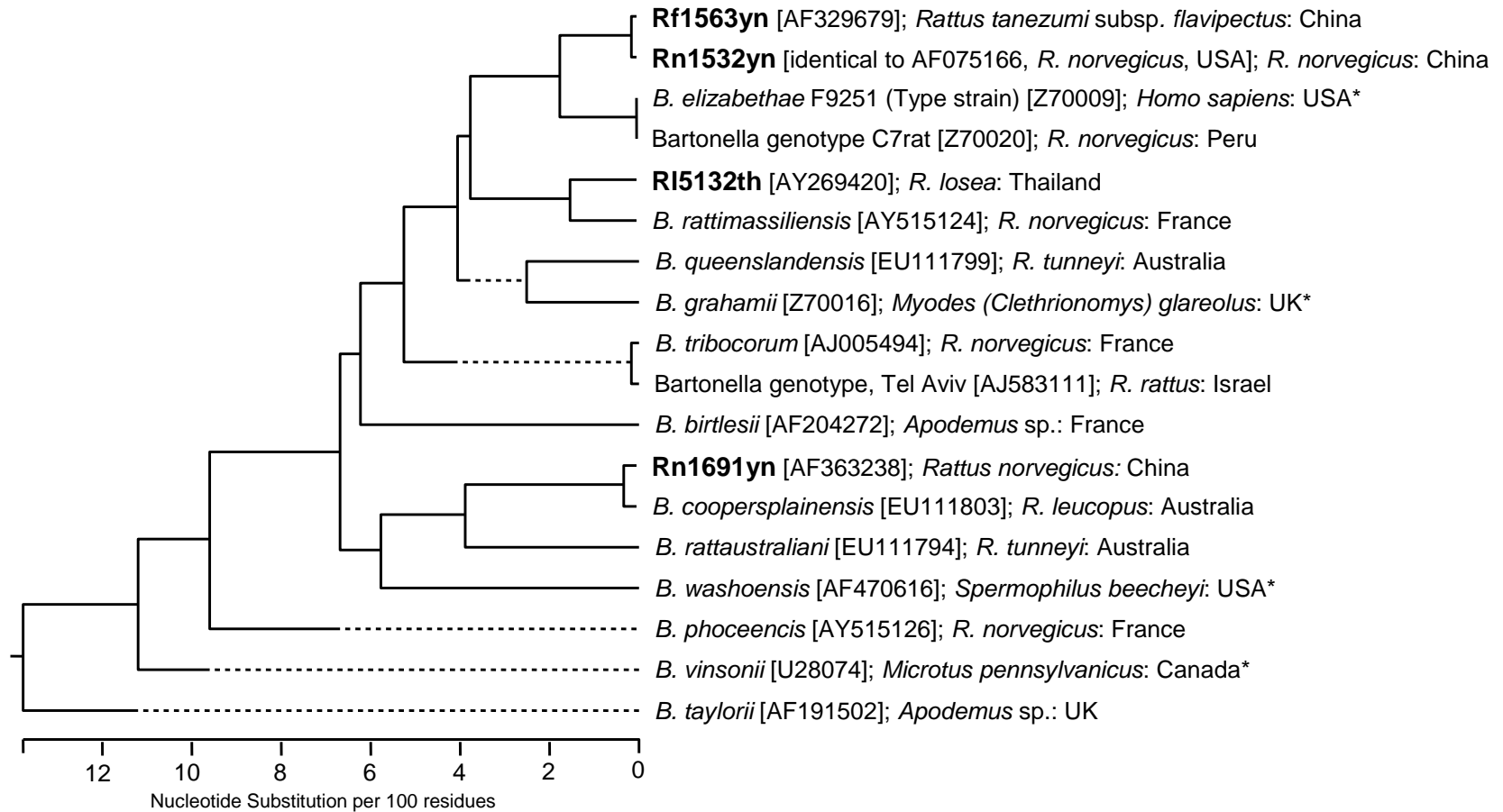
Figure 3.1 This phylogram, based on a portion of the citrate synthase gene (*gltA*), illustrates the relatedness of the four bartonella strains used in this study to one another and to other bartonellae isolated from *Rattus* spp. and other rodents worldwide. For each strain/species included above we provide the GenBank accession number for the *gltA* sequence used, and indicate the host and geographic location from which that strain was isolated. Four of the rodent associated *Bartonella* spp. included in the tree are known to have caused human disease (*).

identity with the other three isolates, though it, like Rn1532yn, was obtained

from a *R. norvegicus* in China (Figure 3.1). While the strains used in this

study were obtained from rodents in Thailand and China, a BLAST nucleotide

sequence query revealed that strains similar to these have been isolated

from rodents worldwide [BLAST = Basic Local Alignment Search Tool,

National Center for Biotechnology Information (NCBI), United States National

Library of Medicine; *glt*A sequence; also Figure 3.1]. To construct the

phylogram *gltA* sequences obtained from GenBank (NCBI) were edited to

between 334—342 bp in length, aligned with MegAlign's ClustalW, and the

phylogenetic tree was generated [DNASTAR® Lasergene software, version

8.1.2; all default parameters].

   Bacterial stocks for this study were prepared as described in Chapter 2.

Stocks were held frozen at -80°C until used for mouse inoculations and the

bacterial concentration of each stock suspension was determined as

previously described (Chapter 2). Following mouse inoculations aliquots of

each inoculum were inoculated onto HIA plates and bacterial concentrations

were confirmed by colony growth and enumeration of colony forming units

(cfu).

Inoculation of mice and experimental design for the study

   Seven groups of six mice were used to evaluate each of the four bacterial

strains (Table 3.2), hereinafter referred to as 'strain groups'. Each group

within a strain group was inoculated with a different dose of bacteria ranging

from

10^1—10^7 cfu per mouse. A seventh mouse, or sentinel, was placed in each

Table 3.1 The four bartonella strains used in this study were originally isolated from *Rattus* spp. in Asia. The bacterial passage history follows the original isolation of the strains from blood and does not include growth of stock for this study.

Strain	Rodent source	Strain origin	Passage history (n = number of passages)
Rn1691yn	*Rattus norvegicus* (Norway or brown rat)	Yunnan Province, China	BHI agar + 10% rabbit blood[1], n = 2; BALB/c *in vivo*[2], n=1; BHI agar + 10% rabbit blood, n = 1
Rl5132th	*R. losea* (Lesser rice field rat)	Chiang Rai Province, Thailand	BHI agar + 10% rabbit blood, n = 4; BALB/c *in vivo*[2], n=1; BHI agar + 10% rabbit blood, n = 1
Rn1532yn	*R. norvegicus* (Norway or brown rat)	Yunnan Province, China	BHI agar + 10% rabbit blood, n = 4; BALB/c *in vivo*[2], n = 1; BHI agar + 10% rabbit blood, n = 1
Rf1563yn	*R. tanezumi* subsp. *flavipectus* (Asian house rat)	Yunnan Province, China	BHI agar + 10% rabbit blood, n = 2; BALB/c *in vivo*[2], n = 1; BHI agar + 10% rabbit blood, n = 1

[1] BHI = Brain and Heart Infusion
[2] Mice were inoculated with a divided dose of 10^7 cfu intraperitoneally and subcutaneously.

cage of six experimentally inoculated mice to evaluate for environmental transmission of bacteria within the cage. Environmental transmission was defined as infection of a sentinel mouse resulting from contact with experimentally inoculated cage mates and/or their excreta. Sentinel mice were not inoculated, but shared cages with experimentally inoculated cage mates for the study duration. All experimental mice in the study were subcutaneously inoculated once along the dorsal midline between the scapulae with bacteria diluted in physiological saline. Groups of three mice

each were subcutaneously inoculated with physiologic saline to serve as negative controls for each strain group (n = 4 negative control groups, three mice each). Negative control groups were inoculated and sampled on the same days as their experimental counterparts and were held separately from experimental mice for the study duration.

Table 3.2 The experimental design for this study consisted of four strain groups. Each strain group contained experimentally inoculated mice, sentinel mice, and saline inoculated control mice.

Bartonella inoculated mice	Sentinel mice	Saline inoculated control mice	No. mice in a strain group
6 mice / dose group x 7 dose groups	1 mouse / dose group x 7 dose groups	3 mice / strain group	
42	7	3	52
Total number of mice in the study for four strain groups:			**208**

Blood collections

Blood was collected weekly from all mice in the study. Mice were anesthetized for all blood collections with a solution injected intraperitoneally (100 mg/kg ketamine + 50 mg/kg xylazine according to mouse body mass). Blood was collected in the manner described in Chapter 2, once a week for at least 13 weeks. Samples were stored at -80°C until tested for bacteremia. At the study endpoint(s) blood samples were collected from all mice by cardiocentesis and tested for bacteremia as described below.

Testing for bacteremia

To evaluate mice for bacteremia frozen whole blood was thawed and 50µl was added to 150µl of diluent consisting of brain heart infusion supplemented with 15 µg/ml Fungizone™. Samples (100µl) were inoculated onto plates and incubated as described previously. Bacteremic samples were subsequently diluted (from 1:5 to 1:2500) to permit cfu counts so the bacterial concentration of the blood could be calculated. Colonies were confirmed as bartonella bacteria by morphology and Gram stain.

Histopathology

Tissues were collected during necropsy 14 weeks post-inoculation from a subset of Rn1691yn infected mice. Heart, kidney, bladder, spleen, thymus, lung, liver, and lymph nodes (axillary, brachial, and inguinal) were harvested from surviving mice in the 10^6 and 10^7 cfu dose groups [n = 5 and n = 6, respectively], and fixed in 10% neutral buffered formalin. Matching tissues and organs were sampled from the three saline inoculated control mice. Following fixation all mouse tissues were subjected to standard processing and embedded in paraffin. Sections (5µm) were then stained with hematoxylin and eosin for evaluation by light microscopy (Colorado Histo-Prep, Fort Collins, Colorado, USA). Photomicrographs were taken to illustrate immunopathological findings in experimental mice with reference to healthy, saline inoculated controls, and are representative of lesions in all affected mice (AxioCam, MRc, Göttingen, Germany).

RESULTS

Bacteremia in inoculated mice

Of the four bartonella strains evaluated in mice in this study, only one, Rn1691yn, produced bacteremia in mice (Figure 3.2A-D). For mice inoculated with 10^7 cfu of Rn1691yn, 6/6 (100%) became bacteremic with onset between 1 and 2 weeks post-inoculation (Figure 3.2A). Mouse #6 in this group had the greatest observed bacteremia level for any mouse in the study: 3 X 10^5 cfu/ml blood at week 2 post-inoculation, which was also when bacteremia was first observed. The other five mice in this dose group also manifested their greatest bacteremia levels at week 2 but only reached 10^4 cfu/ml blood. Duration of detectable bacteremia for individual mice ranged from 4—8 weeks.

Five of six (86%) mice inoculated with 10^6 cfu of the Rn1691yn bacterial strain became bacteremic with onset between 1 and 2 weeks post-inoculation (Figure 3.2B). Mouse #5 manifested bacteremia at week 2 with 10^3 cfu/ml blood but then died of unknown causes the following week. Bacteremias for surviving mice ranged in duration from 5—7 weeks. The highest bacteremia observed in a mouse in this group was 10^4 cfu/ml blood. All surviving bacteremic mice (4/5) in the group also achieved this level by week 2 post-inoculation. Mouse #3 had an abrupt drop in bacteremia at week 5 to below our level of detection; bacteremia was detectable again at week 6 and then ceased or dropped below our level of detection again. Six of six (100%) mice

in the 10^5 cfu dose group became bacteremic after inoculation (Figure 3.2C).

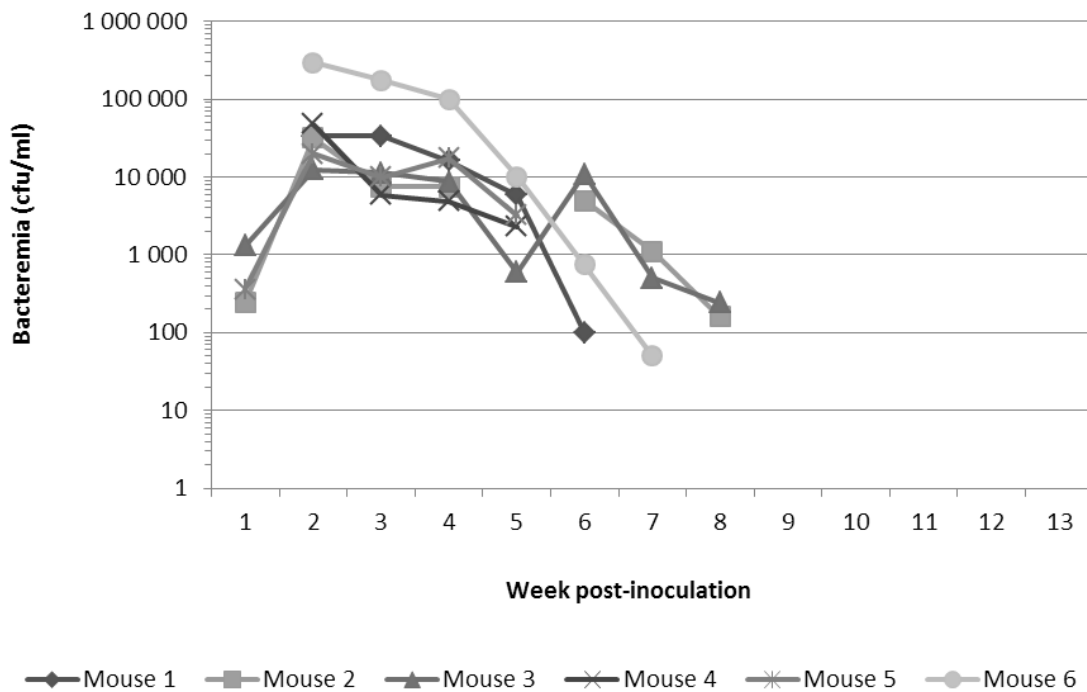Five of those six mice had bacteremia onset between 1—2 weeks post-

Figure 3.2A. All six mice inoculated with 10^7 cfu of Rn1691yn developed bacteremia within 1—2 weeks post-inoculation.

inoculation. Bacteremia duration in all six mice ranged from 5—7 weeks.

Mouse #2 in this group achieved the second highest bacteremia level overall,

2×10^5 cfu/ml blood. Mouse #1 in this group had bacteremia onset 8 weeks

post-inoculation which was also the week of highest bacteremia for this

mouse, 2.4×10^4 cfu/ml.

In the 10^4 cfu dose group, 3/6 (50%) mice became bacteremic following

inoculation (Figure 3.2D). Mice became bacteremic at week 1, week 2, and

week 10 (Mouse #5). Bacteremia duration in the mice ranged from 3—7

weeks. Mice manifesting bacteremia earlier had levels of 10^4 cfu/ml of blood,
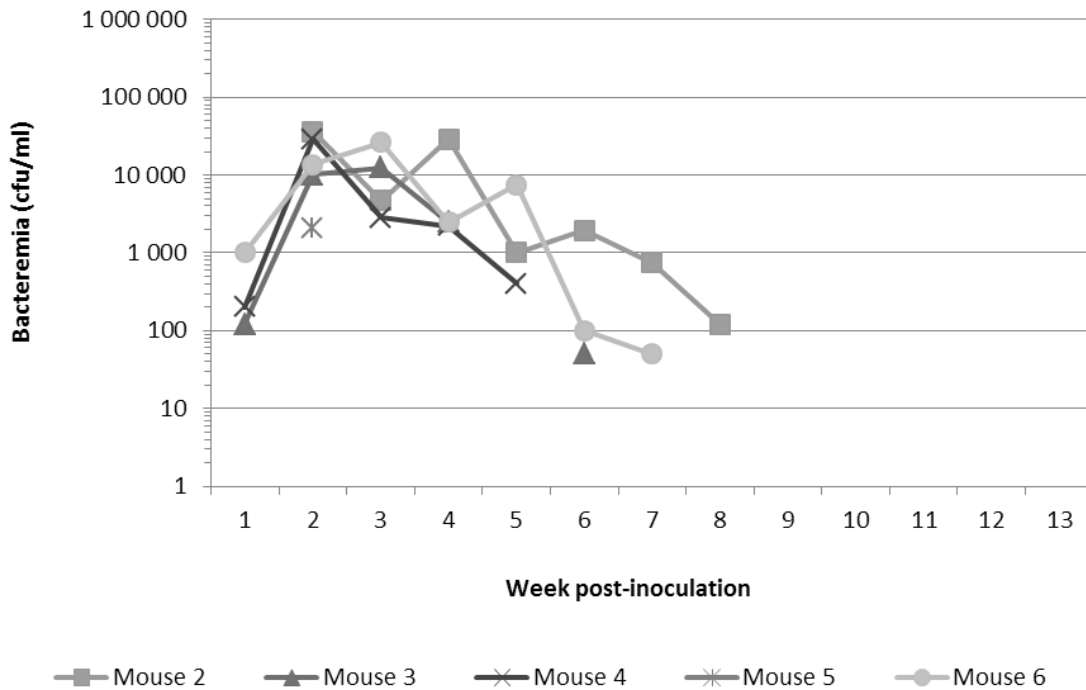
3.2B. Five of six mice inoculated with 10^6 cfu of Rn1691yn developed
bacteremia. Mouse #5 died during week 3 so no additional data are available
for her.

whereas the highest bacteremia level for Mouse #5 was 7.8 X 10^3 cfu/ml.

Bacteremia lasted only 3 weeks for Mouse #5, the shortest duration among

mice that were monitored weekly.

  The experiments evaluating infection of mice with strains Rl5132th,

Rn1532yn, and Rf1563yn were terminated after 13 weeks as no mice in

those groups had developed bacteremia. Mice inoculated with Rn1691yn

were subject to two different outcomes. Rn1691yn mice in dose groups 10^4 –

10^7 cfu were euthanatized 14 weeks post-inoculation. Mice in dose groups

10^1—10^3 cfu were held an additional 16 weeks for observation for delayed
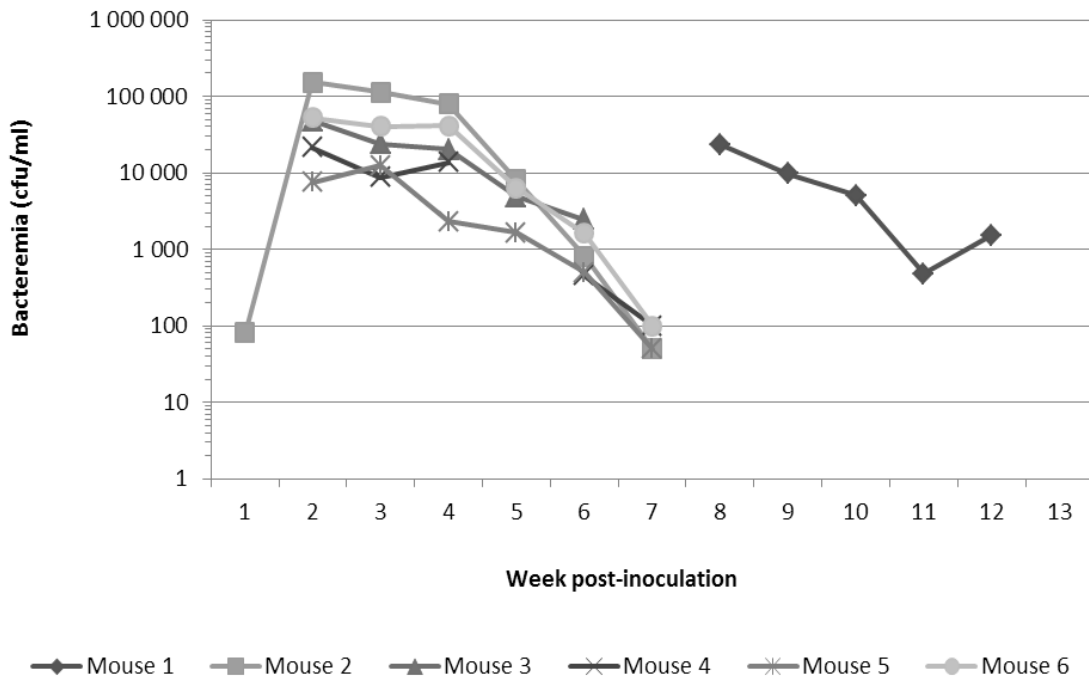

Figure 3.2C. All six mice inoculated with 10^5 cfu of Rn1691yn developed bacteremia. Mouse #1 had no bacteremia at our level of detection until 8 weeks post-inoculation.

 bacteremia onset. Those mice had blood collected every 4 weeks for an

additional four time points. Subsequently one mouse in the 10^3 cfu dose

group was found to have a 7 X 10^2 cfu/ml bacteremia at week 17. Since

blood was being collected monthly at this point bacteremia kinetics for this

mouse are unknown excepting this single time point.

   Mice in the other three strain groups had blood collected and tested

weekly for 13 weeks. No bacteremia was detected in any of those

experimentally inoculated mice at any inoculation dose. No sentinel (un-

inoculated) mice in any of the four experimentally inoculated strain groups
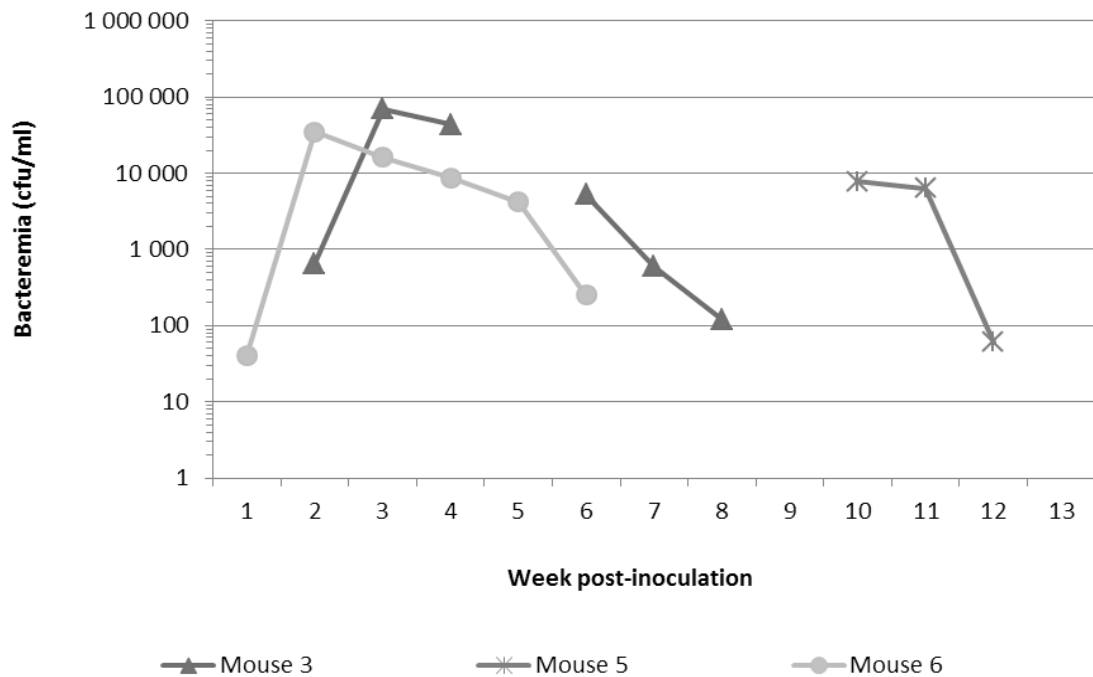
Figure 3.2D. Three of six mice inoculated with 10^4 cfu of Rn1691yn became infected and developed bacteremias following inoculation.

developed bacteremia from environmental (contact) transmission of the bacteria. In addition all blood samples collected from saline inoculated control mice were negative for bartonellae.

Histopathological observations of mice inoculated with Rn1691yn

Multifocal granulomas with associated hepatic necrosis were observed in the livers of experimentally infected mice (5/5 mice, 10^6 dose group; 5/6 mice, 10^7 dose group). These granulomatous nodules were usually seen in areas adjacent to or surrounding central veins [Figure 3.3A-B (Mouse #1, 10^7 dose group)]. Single to multiple cell hepatocellular necrosis was also seen adjacent to inflammatory nodules [Figure 3.3C, arrow (Mouse #3, 10^7 dose

group)]. The livers of control mice were consistently unaffected [Figure 3.3D (Control mouse #2)]. Large granulomas were observed in the kidneys of experimentally infected mice (1/5 mice, 10^6 dose group; 3/6 mice, 10^7 dose group). These granulomas were only seen in the cortex, perivascularly, causing displacement of glomeruli and surrounding proximal tubules [Figure 3.4A-B (Mouse #5, 10^7 dose group)]. In some areas degeneration of the
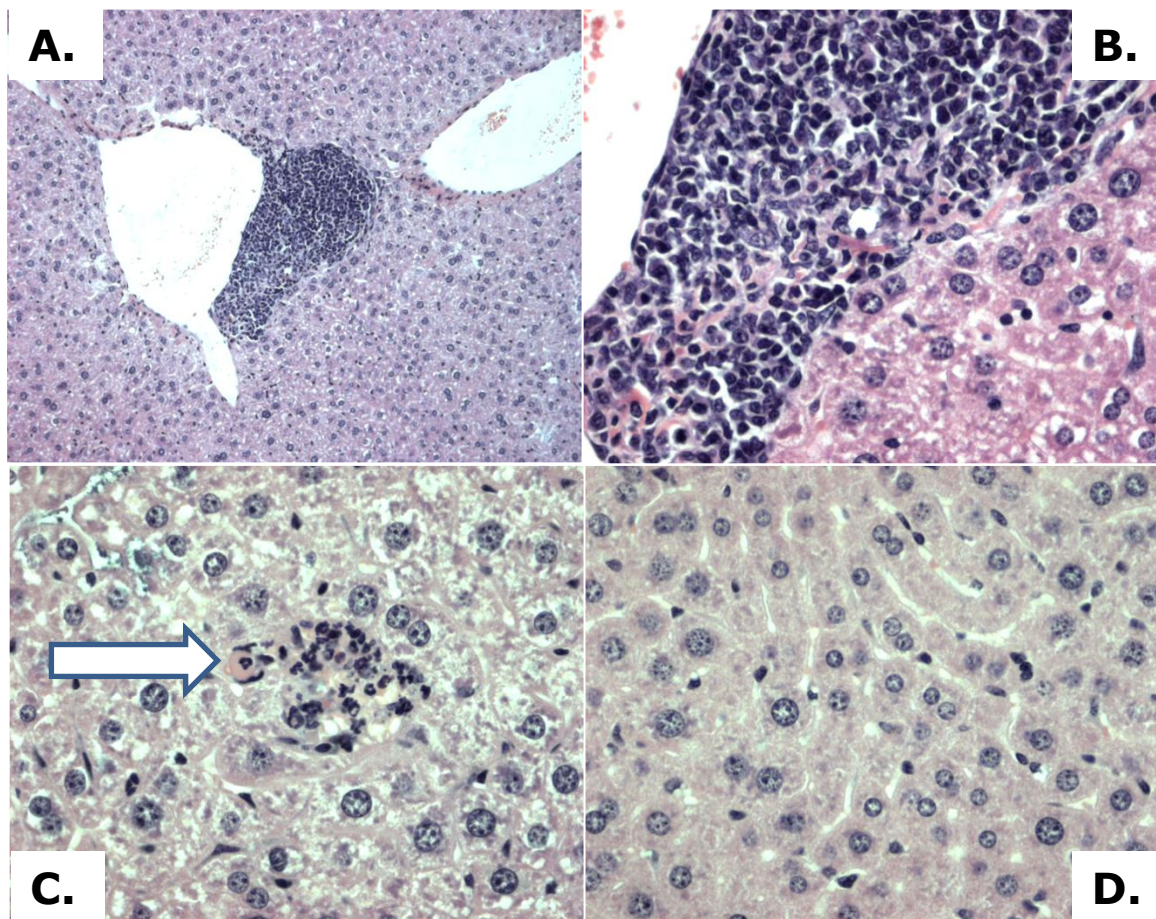
Figure 3.3 Hematoxylin and eosin stained sections show granulomatous lesions in the livers of mice experimentally infected with Rn1691yn [A (10X); B (40X)]. In C the arrow indicates a necrotic hepatocyte (40X). Livers of saline inoculated control mice were unaffected [D (40X)].

proximal tubular epithelium was noted. The kidneys of saline inoculated

control mice did not demonstrate any areas of inflammation [Figure 3.4C-D

(Control mouse #3)]
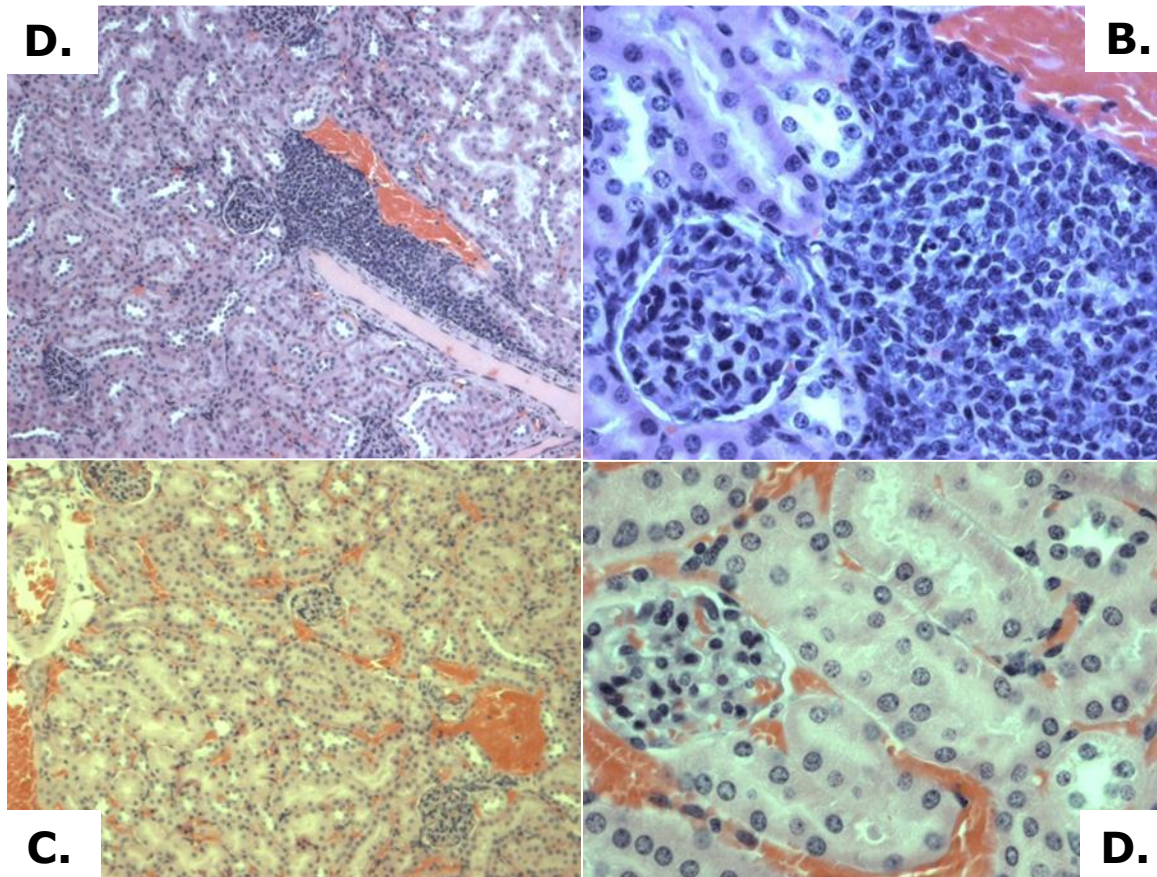
Figure 3.4 Hematoxylin and eosin stained sections show granulomatous lesions in the kidneys of mice infected with high doses of Rn1691yn [A (10X); B (40X)]. Kidneys of saline inoculated control mice did not display any lesions [C (10X); D (40X)].

Inflammatory or granulomatous lesions were not observed in other

organs examined (heart, bladder, spleen, thymus, lung, and lymph nodes)

from experimentally infected mice. Matching organs from saline inoculated

control mice did not demonstrate lesions.

DISCUSSION

Of four rat bartonella strains evaluated in this experimental infection study, only one, Rn1691yn, demonstrated an ability to infect mice. This represents a cross-genera host switch for this bartonella strain, a somewhat uncommon observation in the context of bartonella infection studies in mice [145, 210, 226, 231]. Rn1691yn was originally isolated from *R. norvegicus* in Yunnan Province, China [136], and appears to be a strain of *B. coopersplainensis,* a species later isolated and genetically characterized from *R. leucopus* in Australia [22] (Figure 3.1). Compared to the other three strains evaluated strains similar to Rn1691yn have been reported from a greater number of rodent genera worldwide [BLAST nucleotide sequence query, NCBI; Rn1691yn *glt*A sequence; ≥96% maximum identity]. This suggests that the infection phenotype observed in the laboratory is representative of the natural broad host range of *B. coopersplainensis* strains observed during field studies.

Mice responded to experimental infection in a dose dependent manner in two ways. First, a greater number of mice became infected at high bacterial doses than lower. Second, a temporal response was noted in response to the doses inoculated. Mice exposed to higher doses usually became bacteremic soon after infection (within a few weeks), whereas mice inoculated at lower doses demonstrated greater variability in time to bacteremia onset. Three mice inoculated at lower bacterial doses did not develop bacteremia until 2—4 months post-inoculation. The highest bacteremia level observed in infected mice was 10^5 cfu/ml of blood (2/21 mice, 10^3—10^7 cfu dose groups). Higher

levels and longer duration of bacteremia have been reported in natural hosts [112, 240] versus incidental hosts, and alterations in bacteremia kinetics may be a fitness trade-off for the bacteria when it infects a host to which it is not adapted. Lower bacteremia levels of shorter duration in incidentally infected rodent hosts also suggests that bacterial acquisition from these hosts by arthropod vectors may be compromised so the contribution of incidental host infections to natural transmission cycles of the bacteria may be negligible.

The failure of the other three rat bartonella strains to infect mice suggests those strains are host specific at the genus level. Bacterial host specificity appears quite common in rodent populations and has been observed in numerous field studies and in experimental infection studies using different *Bartonella* species in different host taxa (Chapter 1). By comparison Rn1691yn readily infected mice, a cross-genera host switch for this bacterium from its host of origin. Thus Rn1691yn seems inherently more capable of utilizing a mouse alternative host than the other three rat bartonella strains evaluated. This suggests that not all rat associated bartonellae have the same zoonotic potential and that human infection risk can vary according to which rodents and circulating strains are present in a particular location. The low, detected incidence of human cases of zoonotic bartonella infection, not including *B. henselae* infections, may be in part due to high levels of bacterial host specificity. Incidental human infections also likely require some measure of individual host susceptibility, as observed in this study. Furthermore if humans who acquire zoonotic bartonella infections

experience temporal lags before illness manifests this could make it more difficult to pinpoint specific exposure events leading to their infections [165, 171]. It should be noted that most mice that became bacteremic were inoculated with high doses of Rn1691yn (10^4—10^7 cfu), and the dose required to infect a susceptible human or natural reservoir host for *B. coopersplainensis* is unknown.

Temporal lags in manifestation of bacteremia following exposure of rodent hosts to bartonellae circulating in their communities may be common in nature. Though we know little about which hematophagous arthropods may transmit bartonellae and what their transmission efficiency may be [47, 148, 150], we can postulate that vector transmission would likely occur in the context of a low dose exposure. Thus variability in the time of exposure coupled with potential lags in host development of bacteremia could explain incongruities between indices of vector population abundance and infection prevalence, and host infection prevalence observed in field studies [122, 140]. It should also be noted that little is known about the rate or dynamics of bacterial acquisition by potential arthropod vectors, and differences among vectors in bacterial midgut colonization kinetics could also confound attempts to find correlations between vector and host infection prevalences. Finally, since most wild rodent populations are genetically heterogeneous (excluding founder effects), the variability observed in our outbred mice following infection can provide some insight into the variability of responses expected for incidental hosts infected through natural transmission routes.

No sentinel mice became infected and developed bacteremia including those co-housed with Rn1691yn bacteremic mice. Considering the high exposure rate of sentinel mice to infected cage mates this observation seems to rule out contact with cage mates or their feces or urine as a transmission mode, at least for this strain. However, this lack of transmission may be an experimental artifact. Since mice in our study had relatively low bacteremia levels of short duration compared to natural hosts this may have influenced the outcome of this assessment [112, 137, 240]. Still, more likely modes of bartonella transmission among rodents exist such as the bites of hematophagous arthropods [148, 150].

Persistent bacteremias in infected rodent hosts are usually considered an indicator for adaptation of a bartonella strain to that host, and the paradigm for this type of co-adaptation is that the bacteria causes no deleterious effect to the host [109]. However, in our study bacteremic mice developed organ lesions following inoculation of high bacterial doses. Similar findings have been described in two other murine infection studies using bartonella human pathogens, although those mice did not develop bacteremias [210, 242]. Mice inoculated with *B. henselae* developed liver granulomas which resolved after 3 months, and no significant pathology was observed in other organs examined [210]. Liver lesions in those mice were not associated with hepatocellular necrosis unlike the present study [210]. Rn1691yn inoculated mice examined 14 weeks post-infection had multiple large granulomas in their livers and kidneys. Whether these lesions would have resolved given more time is unknown. Differences between these models may be due to a

number of factors. The inoculation route differed between the studies and may have influenced the outcome [224, 235, 243]. Also, mice infected with Rn1691yn developed bacteremias unlike *B. henselae* inoculated mice. When we compare the current study with results reported for *B. tamiae* inoculated mice we find similar lesions in both the liver and kidneys [242]. However, Rn1691yn did not induce myocarditis or lymph node lesions as did *B. tamiae*, though it should be noted that aged mice were used in that study and young mice in the current study [242].

In summary we found that only one of four rat bartonella strains evaluated was able to infect mice [122, 140], indicating some level of host specificity for the other three strains. Our findings may contribute epidemiological and ecological insights into bartonella infection responses in natural and incidental hosts. The observation of pathology following bacteremia resolution in mice, coupled with the ability of Rn1691yn to cross-genera host switch suggests a greater zoonotic potential for *B. coopersplainensis* strains than some other rat-associated bartonellae. The incidental host infection model we present here, weeks long bacteremia in a non-natural host with concomitant pathology seems to fit somewhere between two possible extremes of bartonella infection outcomes, i.e. no deleterious effect versus debilitating disease. Though much work remains to be done in terms of defining human exposure risk to bartonellae, our study provides some insight into the likelihood of bacterial host-switching by some rat bartonella strains, and establishes a basis for estimating the probability, context, and outcome of incidental host infections by rat bartonella strains.

CHAPTER 4

EXPERIMENTAL INFECTION OF THREE LABORATORY MOUSE STOCKS WITH A SHREW ORIGIN *BARTONELLA ELIZABETHAE* STRAIN: HOST SPECIFICITY AND ZOONOTIC POTENTIAL

INTRODUCTION

In peri-domestic and peri-agricultural environments in many areas of the world, human contacts with rodents, lagomorphs and insectivores can pose heightened risk for acquiring zoonotic bartonellae infections [127, 165]. In Asia there are several species of rodents and an insectivore species, *Suncus murinus*, the Asian house shrew, that adapt easily to peridomestic environments and are frequently found infected with diverse bartonellae at high population prevalence [127, 129, 161]. These commensal small mammals often share a common ectoparasite fauna which may constitute a vector transmission risk to humans for bartonellae infections [130].

Rodents are often the focus of interest as reservoir hosts for bartonellae, but shrews have also been found to harbor both unique (shrew only) and rat-associated bartonellae [127, 128, 138]. High sequence similarities between shrew isolates from geographically distinct areas in Asia support the assertion that shrews are natural hosts for their own circulating strains of the

bacteria, but they are also found infected with *B. elizabethae*-like strains [127, 128]. Infection of shrews with bartonellae closely related to rat strains likely represents spillover events. The proportion of sequence similarity between strains from different hosts in the same geographic location can indicate how recently the bacterial strains may have switched hosts. For example, a bartonella isolate from an Asian house shrew in Vietnam shares 100% sequence identity with *B. elizabethae* by analysis of a portion of the citrate synthase gene (*gltA*). *B. elizabethae* was also isolated from two natural reservoir hosts, *Rattus norvegicus* and *R. exulans*, in that area as well. This finding points to an ongoing dynamic background of spillovers from rat populations into sympatric shrews. It also demonstrates the adaptability of a *B. elizabethae* strain in switching hosts, from rats to shrews, and suggests that this bacterium may have an inherent capacity to exploit new hosts.

Bartonella elizabethae and closely related strains have been implicated as agents of human disease [23, 165], and exposure to the bacteria seems high in some human populations, as revealed by serological surveys [196, 198, 200]. *Bartonella elizabethae*-like strains are most commonly isolated from rats and rats unquestionably serve as zoonotic sources of human infection for many viral, bacterial, and parasitic diseases [143]. The likelihood that incidental hosts can be infected by shrew bartonella strains is unknown, but may be predicted based on the host-specificity of strains obtained from these animals [241]. Some bartonellae have been shown to infect a variety of hosts [124], while others display a high level of host specificity [145].

Shrews are mammals in the Order Soricomorpha, and infection of laboratory mice (Order Rodentia) with a shrew bartonella strain could demonstrate the host switching capacity of this bacterium. Therefore, we designed a study to evaluate the ability of our Asian house shrew *B. elizabethae* strain to switch from its host of origin to the laboratory mouse, *Mus musculus*. Three laboratory mouse stocks were inoculated with a range of bacterial doses, and susceptibility to infection and bacteremia kinetics of infected mice were observed. In this study we used mice with different genetic backgrounds, thus allowing for a broad range of observations and interpretation of response to infection [244]. Recognizing the importance of individual host immune status in susceptibility to infection, we chose inbred BALB/c mice, which tend toward a TH2 type immune response, and inbred C57BL/6 mice, which display a relatively robust TH1 response for the study [244]. We also included genetically heterogeneous, outbred Swiss Webster mice in our design to assess possible variability in individual host susceptibility, with respect to the other two mouse stocks.

MATERIALS AND METHODS

Mice and bacteria

Four to 6 week old Swiss Webster (SW) female mice were obtained from an outbred closed colony at DVBD, Fort Collins, Colorado, USA. Age matched BALB/c and C57BL/6 female mice were ordered from Jackson Laboratories (Sacramento, California, USA). Mice were group caged according to stock identity and bacterial dose for the study duration. All work on the mice was approved by and conducted under the supervision of DVBD's IACUC (protocol

#07-004), in accordance with USPHS standards for the humane care of laboratory animals.

Passage history and other information about the *B. elizabethae* strain used in this study is detailed in Table 4.1 and does not include growth of bacteria for this study. Bacterial stock for mouse inoculation was prepared as described in Chapter 2. Bacterial stock concentration was determined as described previously (Chapter 2).

Experimental design

Groups of 36 mice of each mouse stock were used in the study for a total of 118 experimental mice. Six mice were inoculated at each dose. Mice were inoculated only once, subcutaneously in the in the dorsal midline between the scapulae with bacterial doses ranging from 10^1 to 10^6 cfu, diluted in saline to 350µl. Beginning 1 week post-inoculation through week 13 blood was collected weekly from mice as described in Chapter 3. Volumes of 75 -125µl of blood were collected from each mouse weekly. Samples were stored at -80°C until tested for bacteremia.

Testing for bacteremia

Frozen whole blood samples were thawed, and diluted (50µl blood : 150µl brain heart infusion with 20µg/ml Fungizone™ added). Heart infusion agar plates enriched with 5% rabbit blood were inoculated with 100ul of the diluted samples, and placed in a CO_2 incubator at 35˚C for 10 – 21 days to permit bacterial growth. Bacteremic blood samples were subsequently diluted 1:100 and 1:1000 to allow for cfu enumeration. Positive samples were

Table 4.1 The low passage *Bartonella elizabethae* strain used in this study was originally isolated from the Asian house shrew, *Suncus murinus*.

Asian house shrew *Bartonella elizabethae* strain	Sm6145vi
Place of origin	Dong Nai Province, Vietnam (2003)
Primary isolation	Whole blood inoculated onto BHI[1] supplemented with 5% rabbit blood
Subsequent passage history (n = number of passages)	BHI supplemented with 5% rabbit blood, n = 3; BALB/c *in vivo*[2], n = 1; BHI supplemented with 5% rabbit blood, n = 2
Nucleotide sequence [GenBank accession number]	*gltA* [JF523414]

confirmed as bartonella by colony morphology and microscopic appearance

as small, Gram negative bacilli following Gram staining.

RESULTS

Infection of Swiss Webster mice

Following inoculation with *B. elizabethae* Sm6145vi, 2 of 36 SW mice

became infected and developed bacteremia, 1 of 6 at the 10^6 cfu dose and 1

of 6 at the 10^5 cfu dose (Figure 4.1). Bacteremia lasted 7 weeks in the 10^6

cfu dose SW mouse, and 6 weeks in the 10^5 cfu dose mouse. Onset of

bacteremia occurred 3 weeks post-inoculation for both mice, but level at

onset for the 10^6 cfu dose mouse was 2.1 X 10^4 cfu/ml blood, whereas level

at onset for the 10^5 cfu dose mouse was 60 cfu/ml blood. The 10^6 cfu dose

SW mouse attained the highest level bacteremia of any mouse in the study

(3.1 X 10^4 cfu/ml blood). Swiss Webster mice inoculated with 10^4 cfu or less of Sm6145vi failed to develop bacteremias.
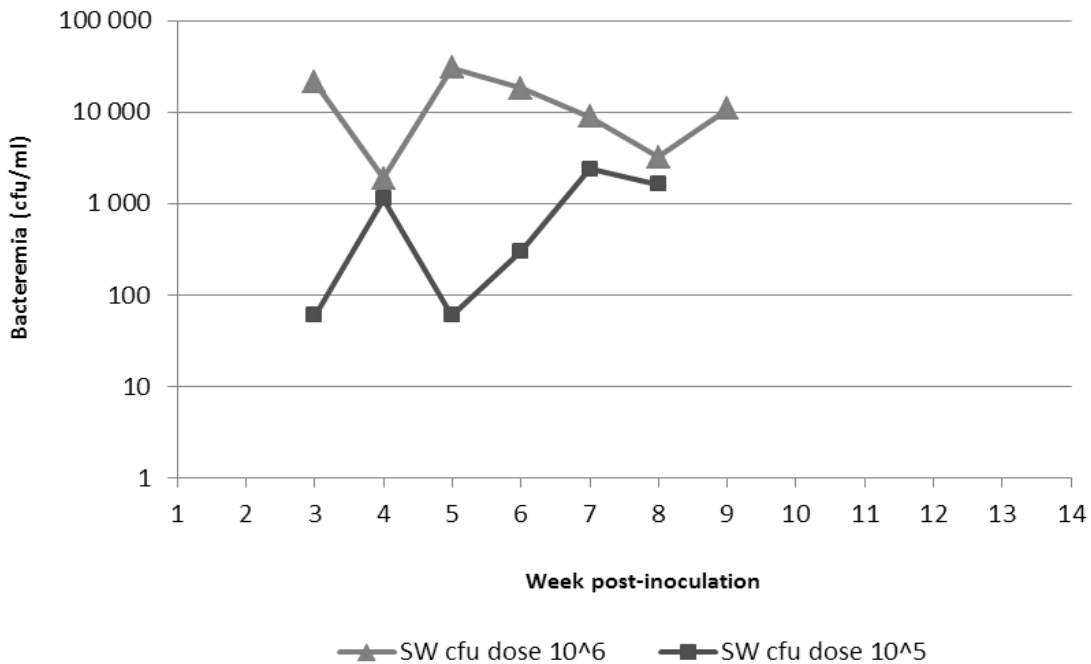
Figure 4.1. Bacteremia kinetics of Swiss Webster mice infected with *B. elizabethae* Sm6145vi.

Infection of BALB/c mice

One of six BALB/c mice became infected following inoculation with a 10^6 cfu dose of the bacteria. The duration of bacteremia for this mouse was 7 weeks, with onset at week 1 post-inoculation (Figure 4.2). The level of bacteremia at onset was 1.5 X 10^4 cfu/ml blood, which was also the highest level of bacteremia observed in this mouse over the course of infection. This mouse had the earliest onset of bacteremia (week 1) of any bacteremic mouse in the study. BALB/c mice inoculated with 10^5 cfu or less of *B. elizabethae* Sm6145vi failed to develop bacteremia.

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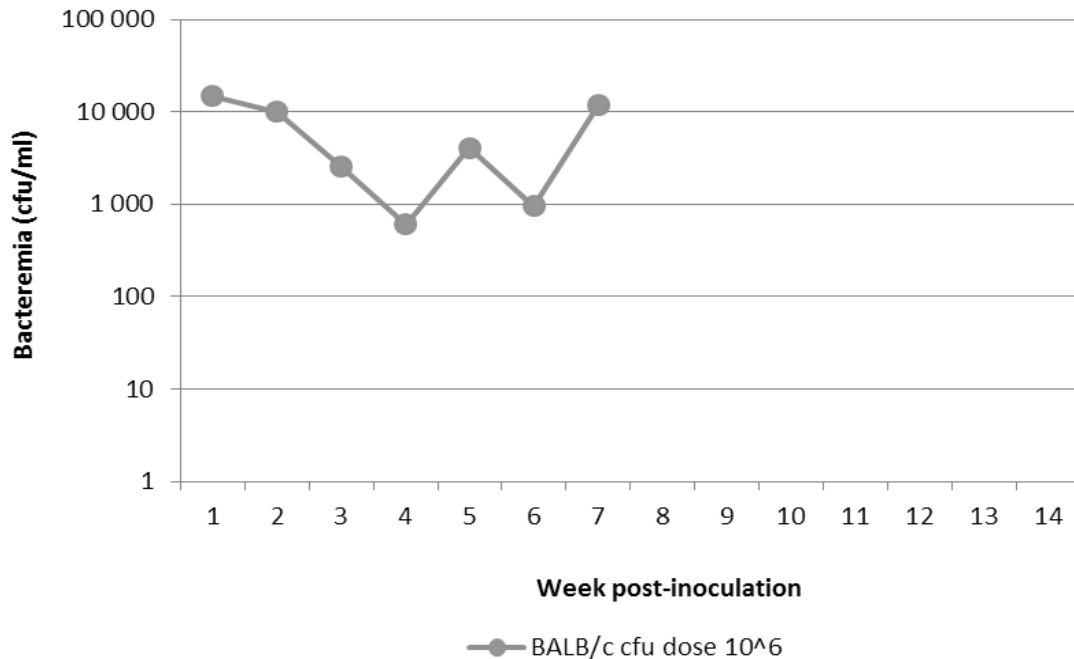
Figure 4.2 Bacteremia kinetics of a BALB/c mouse infected with
B. elizabethae Sm6145vi.

Infection of C57BL/6 mice

Two of six C57BL/6 mice became infected following inoculation of 10^6 cfu

of Sm6145vi and developed bacteremias lasting 7 and 8 weeks in length

(Figure 4.3). These two mice developed the quantitatively lowest levels of

bacteremia observed in the study. Onset of bacteremia was observed at

weeks 2 and 3 for the mice, with levels at onset of 615 and 35 cfu/ml blood,

respectively. Bacteremia peaks observed for the mice were 2.9 X 10^3 and

755 cfu/ml blood, again respectively. C57BL/6 mice inoculated with 10^4 cfu

or less of *B. elizabethae* Sm6145vi failed to develop bacteremias. Bacteremic

mice of all three stocks in the study showed fluctuations in their levels of

bacteremia which could exceed an order of magnitude from 1 week to the
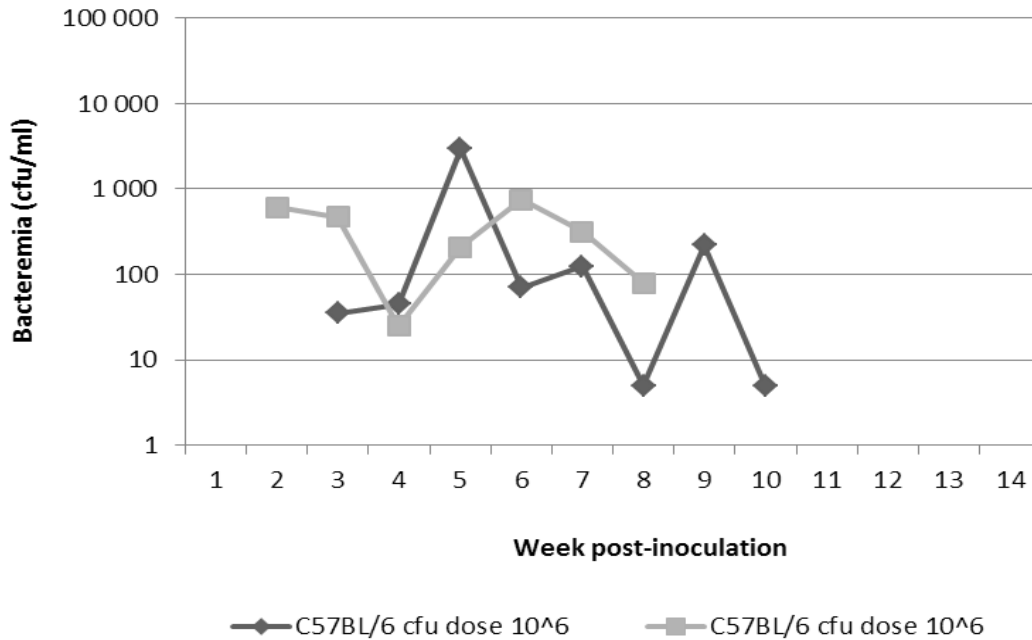
Figure 4.3 Bacteremia kinetics of C57BL/6 mice infected with
B. elizabethae Sm6145vi.

next. Finally, no control mice tested positive for bartonella bacteria at any
time during the study period.

DISCUSSION

In this study an Asian house shrew *B. elizabethae* strain (Sm6145vi)
infected laboratory mice, a demonstration of cross-order host switching by
the bacteria (from Order Soricomorpha to Order Rodentia). This is to some
extent an unusual finding as bartonella bacteria generally only infect and
produce bacteremias in hosts taxonomically close to their natural reservoir
hosts [112, 124, 145]. The ability of this strain to host switch across
mammalian orders defines a characteristic of the bacteria that can make it
more likely to undergo zoonotic transmission to humans [241]. Though this

B. elizabethae strain was isolated from a shrew its close phylogenetic relatedness to rat isolates of *B. elizabethae* in the same location means it is likely a spillover from rats to sympatric shrews in Vietnam. Therefore, unlike some other bartonellae this strain seems to have an inherent capacity to infect diverse hosts [145]. It should be noted however, that bacteremias occurred only in those mice inoculated with high doses of *B. elizabethae* Sm6145vi (10^5 and 10^6 cfu), and it remains uncertain how many bartonella bacteria are required to establish host infection under natural conditions.

Bartonella elizabethae has been reported as the causative agent of several cases of human illness [23, 165]. Still, the zoonotic potential of this bacterium is not well understood. Since *B. elizabethae* and strains phylogenetically close to it have been found in numerous commensal small mammal populations in Asia [127, 245], an understanding of the risk to humans for acquiring infections from these hosts is desirable. Given that the level of host specificity of an infectious agent is generally considered a predictor for the likelihood that the agent can switch hosts, and potentially cause illness in those hosts, our findings help define the zoonotic potential of this bacteria [241].

Previously *B. elizabethae* isolated from a human endocarditis patient was evaluated for its ability to infect several different rodent species, among them *R. norvegicus*, a natural reservoir host for the bacteria [23, 145]. In that study, the bacteria failed to infect Wistar rats (*R. norvegicus*), cotton rats (*Sigmodon hispidus*), BALB/c (*M. musculus*), and white-footed deer mice (*Peromyscus leucopus*), though inoculated doses were as high as 10^7

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bacteria [145]. It remains unknown whether the bacteria had undergone adaptation to the human host resulting in a high level of host specificity, or whether the isolate's passage history might have influenced the outcome [246]. An additional unanswered question was whether related isolates from natural reservoirs would also display a narrow host range, or limited host specificity phenotype.

In contrast to those findings we observed susceptibility of three stocks of *M. musculus* (SW, BALB/c, and C57BL/6) to infection with *B. elizabethae* Sm6145vi at doses of 10^5–10^6 bacteria. Mice developed bacteremia levels potentially high enough to infect ectoparasites, such as fleas, feeding on a host during the course of bacteremia [147]. The bacteremia levels observed in our incidental host mouse model may be sufficient to promote some secondary infections of susceptible hosts, especially if high enough levels of bacterial exposure exist, in terms of transmissible contacts, between animals.

Bacteremia of several months duration or more are commonly observed in reservoir hosts infected with their co-adapted bartonellae [109, 111, 112]. The bacteremia duration of mice in our study is shorter than that observed during such natural host infections, but is consistent with bacteremia kinetics of laboratory mice experimentally infected with non-homologous host source bartonellae [85, 235]. Truncations in bacteremia duration are likely due to the bacteria not being optimally adapted to laboratory mice, and are probably characteristic of non-natural host infections [85, 235].

Similar, small proportions of mice of each stock used in this study were susceptible to infection and developed bacteremias following inoculation of *B.*

elizabethae strain Sm6145vi. The differing genetic backgrounds among the three stocks did not appear to affect susceptibility of mice to infection, at least not with the dose range assayed. It is possible that inoculation of larger group sizes would reveal more apparent differences between stocks. We did not attempt to assess for differential response to infection for the three mouse stocks at different exposure doses, as the number of bacteremic mice in each dose group was low. It would be difficult to assign a biologically significant interpretation to such slight differences in infection rates, and level and duration of bacteremia, without knowing that the observed differences are relevant to the transmission dynamics of the bacteria. Bacteremia duration in infected hosts almost undoubtedly affects the transmission likelihood of the bacteria. Simply put, long bacteremias increase the size of the temporal window for potentially transmissible contacts to occur between infected hosts and susceptibles, or for arthropod vectors to acquire the agent [241]. Likewise, high bacteremia levels can influence the probability that contacts with infected hosts result in bacterial transmission, or arthropod vectors become infected following ingestion of infectious blood [241]. However, to reasonably extrapolate our laboratory based findings to the natural transmission cycle of *B. elizabethae* additional studies need to be done to define the transmission dynamics of the bacteria in its normal hosts.

Further evaluations of *B. elizabethae* Sm6145vi could yield more information about this bacterium's host specificity, adaptive capacity, and zoonotic potential. Additional *in vivo* passage(s) of the bacteria in laboratory mice followed by another experimental infection study might reveal

differences in the ability of a mouse adapted clone to infect the different mouse stocks. Such an experiment could also provide insight into *B. elizabethae's* rate of adaptation to a new host. Alternatively, experimental infection studies could be done to evaluate the ability of this bacterium to infect *R. norvegicus* and *R. rattus*, natural reservoir hosts of *B. elizabethae* [99, 143], to determine if adaptation to other hosts has altered its capacity to infect its natural hosts [145]. Knowledge gained about the zoonotic potential of *B. elizabethae* strains can aid us in implementing measures to reduce human infection risk in areas of the world where these strains circulate in small mammal populations.

CHAPTER 5

EXPERIMENTAL INFECTION OF AGED LABORATORY MICE WITH A MOUSE ORIGIN BARTONELLA STRAIN AND THREE HUMAN ORIGIN *BARTONELLA TAMIAE* STRAINS: TAXONOMIC DISTANCE AS A PREDICTOR OF INFECTION OUTCOME

INTRODUCTION

In a world where almost every living creature seems to be infected with bartonella bacteria how do we determine which of the strains circulating in wildlife have zoonotic potential? Are all bartonella strains equally able to infect animals other than their natural hosts, or do some strains have a greater ability to do so? From an epidemiological perspective are all hosts created equal, or do some alternate hosts exhibit characteristics that make it more likely that they can become infected? Knowing the answers to these questions and other related questions is critical to putting together a picture of how, when, and where zoonotic bartonellae may emerge to infect incidental hosts [247-249]. Equally critical is an understanding of host responses to bacterial exposures [250].

It appears that bartonella infection outcomes in immunocompetent hosts are primarily influenced by the taxonomic distance between a strain's host of

origin and the incidental host it infects. The closer the taxonomic distance between the host of origin and the incidental host, the more likely it will be to become infected and become bacteremic. Conversely, it appears that the greater the taxonomic distance between the host of origin and the incidental host, the more likely the incidental host is to either clear the bacteria or to develop pathology subsequent to bacterial exposure. Still, strict partitioning of potential host responses according to only a single criterion, host of origin for a bartonella strain, is unlikely to be warranted. In Chapter 3 laboratory mice exposed to a rat bartonella strain (Rn1691yn) developed kidney and liver pathology. Not only was a dose response observed for those groups but within a group individual mice displayed differential responses. The observation that only 1 of 5 mice inoculated at the 10^6 cfu dose of Rn1691yn developed kidney lesions versus 5/6 at the 10^7 cfu dose suggests a dose effect for bacterial infection and pathological outcome. Along with dose effect, individual host susceptibility also appears to play a role in infection outcome.

When animals are exposed to bartonella strains originating from hosts of different species or genera, they may or may not develop bacteremias (Chapters 2-4). It often seems that the duration and magnitude of bacteremia in non-natural host animals is truncated and of a lower level relative to bacteremia kinetics reported for the natural host [85, 227, 235]. This is probably because that bacterium is not adapted to that alternate host. We do not presently know how many humans are exposed to bartonella bacteria, we only see that proportion of exposed humans who become ill

from infection. Serological surveys conducted to assess exposure of high risk human populations to rodent-associated bartonella strains consistently demonstrate exposures to these bacteria [183, 193-197, 199-203, 251]. When humans become infected with zoonotic bartonellae the outcome is unknown except in cases where clinical disease develops, medical care is sought by patients and a diagnosis is made. The mouse infection studies described in Chapters 2—4 suggest that dose, bacterial strain, and individual host susceptibility can all play a role in infection outcome.

The role of individual host susceptibility to infection must be accounted for when considering all potential outcomes following bacterial exposure . Some mice did not become bacteremic following exposures to *Mus* species bartonella strains (Chapter 2), whereas a few mice became bacteremic following exposure to a shrew source bartonella strain (Chapter 4). Whether taxonomic distance is a better predictor of outcome than individual host susceptibility is arguable.

The use of outbred mouse strains in all of the studies reported in this dissertation has revealed variation in susceptibility and response to exposure likely due to the genetic heterogeneity of the mice. Though few studies have been conducted documenting heterogeneity of outbred mice strains, several report measures of allelic diversity in outbred mouse strains commensurate with the diversity in wild type *M. musculus* [252-254]. Thus, the variability in response observed can likely be considered representative of a natural mouse (rodent) population. There is no clear evidence that either inbred or outbred strains of mice or mice with defined genetic mutations in their

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immune systems can better reproduce disease states associated with bartonella infections in humans (Chapter 1, Table 1.3). However, the majority of human patients identified as having zoonotically acquired bartonella infections or experiencing adverse outcomes associated with bartonella infections are approaching middle age (Chapter 1, Table 1.2). This suggests that age related changes in immune function may affect the outcome of some bartonella infections by affecting individual host susceptibility to infection [250]. Adding an age variable to an experimental infection study that evaluates the 'taxonomic distance as predictor of infection outcome' can potentially increase the sensitivity of the system to separate outcomes related to individual host susceptibility from outcomes that are inevitable due to the intrinsic characteristics of the bartonella strains used.

We previously assessed whether exposure of mouse stocks with well documented differences in their immune response would respond differentially to exposure to a *B. elizabethae* strain and no apparent differences were observed (Chapter 4). Immunological senescence represents changes in immune response related to aging, and is not commensurate with specific defects in immune function or response such as those observed in genetically modified laboratory mouse strains [www.informatics.jax.org] [255]. Immune senescence may produce impairments or attenuation of immune response but is not typically characterized by a complete lack of function of any component of the system [256, 257].

To explore whether age could influence infection outcome we experimentally infected groups of aged mice with either a mouse bartonella strain or with strains of a human origin *Bartonella* species. The strains evaluated represent opposite ends of the spectrum for host of origin: mouse versus human. A previous study showed that outbred mice were susceptible to infection with the mouse bartonella strain and would develop bacteremias (Chapter 2). The natural reservoir host(s) of *B. tamiae* are unknown but these strains were isolated from humans so for our purposes the *B. tamiae* strains used in this experiment are pathogens of human origin.

If we accept taxonomic distance as a predictor for infection outcome then strains of *B.tamiae* should not produce bacteremias, even in aged mice. If experimental results deviate from the expected outcome it would suggest that individual host susceptibility may be a more powerful predictor of bartonella infection outcome than previously considered. In that scenario, human risk for acquiring bartonella infections should be evaluated in the context of potential host susceptibility to infection along with information about which circulating bartonella strains human populations may be exposed to through animal or arthropod contacts.

MATERIALS AND METHODS

Mice and bacteria

Four groups of six CD1 female mice aged 10 months were each subcutaneously inoculated with 10^6 cfu of a mouse bartonella strain (MA) or with *B. tamiae* strains Th239, Th307, Th339 (n = 24 mice total) as described in Chapter 2. Mice were group caged according to strain inoculated and were

kept separate from one another for the duration of the study. The mouse

bartonella strain used in this experiment is the strain designated MA in

Chapter 2. The *B. tamiae* strains used in this experiment, Th239, Th307, and

Th339, were originally isolated from infected human patients in Thailand

(Table 1.2, Chapter 1) and their passage history is detailed here in Table 5.1

Table 5.1 The *Bartonella tamiae* isolates used in the study have different *in vitro* bacterial passage histories.

B. tamiae strain	Primary isolation from human blood samples [30]	Subsequent passage history (n = number of passages)
Th239	Blood clot from Thai patient inoculated onto Vero E6 cells	HIA supplemented with 5% rabbit blood, n = 6
Th307	Blood clot from Thai patient inoculated into BAPGM	HIA supplemented with 5% rabbit blood, n = 2
Th339	Blood clot from Thai patient inoculated onto Vero E6 cells	BAPGM, n = 1; HIA supplemented with 5% rabbit blood, n = 2

HIA = Heart Infusion Agar plates
BAPGM = *Bartonella*/alpha-*Proteobacteria* growth medium [9]

Blood collections and bacteremia testing

   Blood was collected from each mouse in the study weekly for 6 weeks as

previously described (Chapter 2). Blood was frozen at -20°C until

microbiological testing. To test for bacteremia blood was thawed and 50µl

was added to 50µl of brain heart infusion diluent with 8% amphotericin B.

Samples were inoculated onto plates and incubated as described in Chapter

2. Colonies were counted on positive plates and samples with colonies too

numerous to count were diluted 1:1,000 and 1:10,000 to allow for

enumeration of bacteria. Colonies were counted 7—10 days after growth first

appeared.

RESULTS

All six mice inoculated with the mouse bartonella strain (MA) became

bacteremic in the first week post-inoculation (Figure 5.1). Bacteremias were

maintained over the 6 week study duration and reached levels >10^6 cfu/ml in

2 of the 6 mice (Figure 5.1). No mouse inoculated with a *B. tamiae* strain

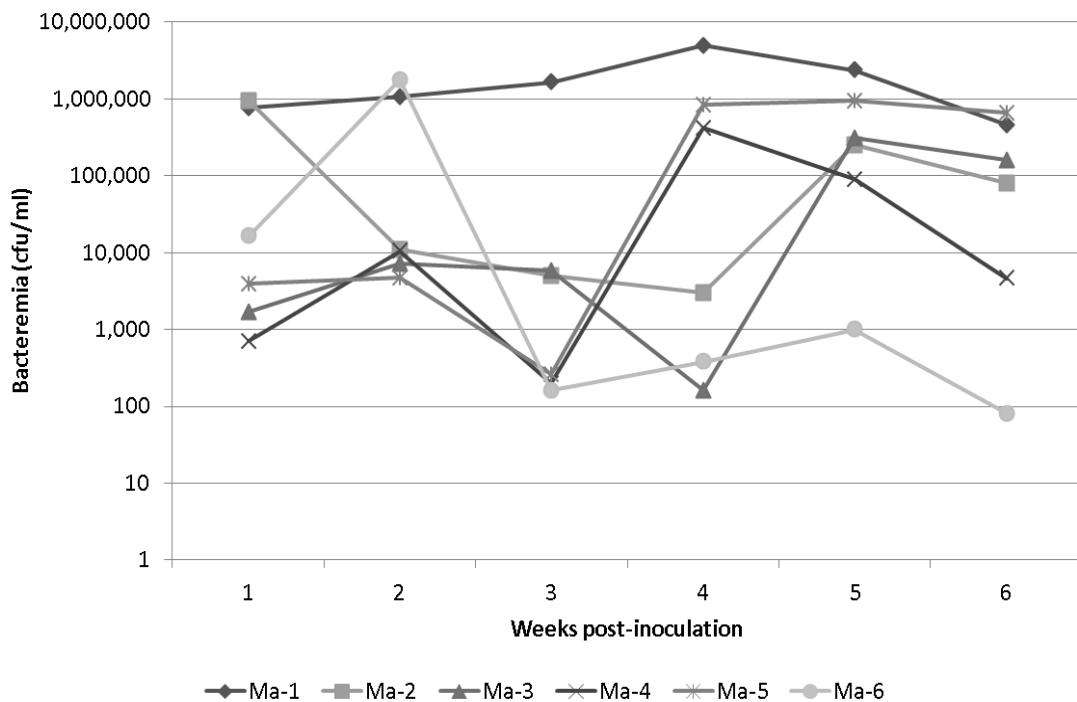became bacteremic at our level of detection during the course of the study.

Figure 5.1 Bacteremia kinetics for aged SW female mice experimentally
infected with a high dose of MA.

DISCUSSION

In this study aged female CD1 mice infected with a *Mus* species bartonella strain developed bacteremias following inoculation commensurate with the response observed in younger (8 weeks old) female CD1 mice (Chapter 2). This simple comparison between outcomes across different age groups suggests that, at least for strain MA, age alone might not be a determinant of successful infection (i.e. bacteremia manifestation). Mice inoculated with *B. tamiae* strains, however, did not become bacteremic. In this experimental system taxonomic distance was a good predictor of infection outcome. Since all six mice infected with the mouse bartonella strain became bacteremic it does not appear that mouse age affected susceptibility to infection. However, unlike the young mice that were inoculated with low doses (10, 100, and 1000 cfu), older mice were inoculated with 10^6 bacteria/mouse and might have displayed a different response to lower dose inocula.

Mice in this study were only observed for 6 weeks so their duration of bacteremia is unknown. Bacteremia duration in younger mice was months long and based on similarities in the initial pattern of response it is likely that the older mice would have maintained bacteremias for extended periods as well. Aged mice did not attain bacteremias at levels of 10^7 cfu/ml blood as did younger mice. This might be due to age-related differences in physiology or immune response in older mice as compared to younger [255-257]. However, no data were collected to evaluate antibody response to infection in either this study or the study reported in Chapter 2. Another possible

explanation is that the dynamics of response were affected by the bacterial dose. The research reported in this dissertation suggests that bacterial dose can influence the magnitude and duration of bacteremias (Chapters 2—4).

Mice inoculated with *B. tamiae* strains failed to develop bacteremias at our level of detection, not surprising as these strains were obtained from ill human patients. Though these strains have presumably already successfully accomplished a host switch by infecting humans this does not seem to have made them promiscuous for infection of another host. Adaptive changes likely occurred in the bacterial population during the course of infection. For a bacterium an infection would represent many generations upon which the selective pressures imposed by a new host could act.

Bartonella strains obtained from humans have been consistently unable to produce bacteremias in laboratory mice. This has been demonstrated for isolates of *B. bacilliformis*, *B. quintana*, *B. henselae* and *B. elizabethae* [145, 209, 210, 231]. Though *B. tamiae* inoculated mice did not manifest bacteremias, it is possible they might develop pathology following exposure as do mice infected with *B. henselae*. Future investigations into whether aged mice develop disease following exposure may be warranted, especially since older humans seem more susceptible to pathological outcomes from bartonella infections (Chapter 1, Table 1.2).

CHAPTER 6

HUMAN ISOLATES OF *BARTONELLA TAMIAE* INDUCE PATHOLOGY IN EXPERIMENTALLY INOCULATED, AGED, IMMUNOCOMPETENT SWISS WEBSTER MICE

INTRODUCTION

Numerous bartonella strains are isolated from rodents and other wildlife each year (Bartonella Laboratory, CDC, Fort Collins). Strains are also infrequently cultured from clinical samples obtained from bartonella infected human patients (Chapter 1, Table 1.2). During isolation information is collected about strain growth characteristics in eukaryotic cell culture and on microbiological media. Isolates are genetically characterized and nucleic acid sequences are deposited in GenBank (NCBI) to allow public access to the information. However, other biological information about the strains is often lacking such as whether they could infect laboratory mice. This could be an indicator of their host specificity and by implication their zoonotic potential. When bartonella strains are inoculated into mice observations can be made about the outcome of these exposures. Screening strains in this way can provide clues about whether a suspect isolate might be the actual cause of a disease.

Bartonella tamiae was originally isolated from hospitalized human patients in Thailand. That fact coupled with the absence of detection of other pathogens in the patients, as well as clinical syndromes compatible with bartonellosis led to the presumption that the bacterium was the cause of the patients' illnesses. However, an association with illness is not the same as causality. Infectious disease research is ruled by the fundamental concepts embodied in Koch's postulates. Since it is not ethically feasible to inoculate humans to see whether illness and disease could be reproduced, and since non-human primates are difficult to work with as well as extremely expensive to use as animal models, laboratory mice are often assayed in their stead.

If mice exposed to a suspected human pathogen become ill and display disease similar to that observed in ill people, and if the pathogen can be recovered from the mice then a major step toward satisfying the question of causality could be realized. For us, this would mean that the suspected role of *B. tamiae* as the causative agent of the Thai patients' illnesses could potentially be strengthened or confirmed.

In a previous study, aged mice inoculated with *B. tamiae* failed to develop bacteremias (Chapter 5). Though it seemed unlikely that Koch's postulates could be satisfied due to our inability to recover viable bacteria from exposed mice, there was still a possibility that the disease states observed in *B. tamiae* infected humans could be reproduced (Chapter 1, Table 1.2) [258]. No histological analysis of tissues had been done to investigate whether the bacterium might have induced pathology in the mice. We hypothesized that if *B. tamiae* was the cause of illness in the infected human patients then we

might observe pathology in mice experimentally infected with the bacteria. Therefore we inoculated groups of aged mice with the three available strains of *B. tamiae*. If lesions were observed in their organs consistent with disease symptomatology observed in human patients from whom the bacteria were isolated then our observations would establish a murine model for *B. tamiae* induced disease, and serve to implicate *B. tamiae* as a causative agent of human illness in Thailand.

MATERIALS AND METHODS

Mice and bacteria

Specific pathogen free, outbred Swiss Webster female mice aged 15-18 months were used for this study. Mice were obtained from a colony of Swiss Webster mice maintained at DVBD. Work with the mice was approved by, and conducted under the supervision of the DVBD IACUC [protocol # 07-012] in compliance with the standards for humane care of laboratory animals published by the USPHS.

Bacteria for the mouse inoculations were grown on heart infusion agar plates supplemented with 10% rabbit blood, in a 5% CO_2 incubator at 35°C. Stocks were prepared for mouse inoculations as described previously (Chapter 2). The *in vitro* bacterial passage history for each of the three *B. tamiae* isolates is detailed in Table 5.1 in Chapter 5 and does not include preparation of the stocks described above.

Experimental design and inoculation of mice

All experimental mice were inoculated subcutaneously along the dorsal midline between the scapulae with between 10^6 and 10^7 colony forming units

of one of three *B. tamiae* strains (Th239, Th307, or Th339) [258]. Three groups of four mice were chosen from the available pool of age matched female mice for this experiment. Each group was randomly assigned to be inoculated with one of the three available *B. tamiae* strains, such that four mice were inoculated for each of the three strains [n = 12 experimental mice]. Two age-matched female mice were subcutaneously inoculated with saline to serve as controls.

Mice were group housed by strain for the duration of the study and control mice were held separately from experimental mice. One mouse in each group was sacrificed at 3, 4, 5, and 6 weeks post-inoculation (n = 1 mouse per time point for each isolate). All of the following tissues were sampled from each mouse to include both experimental and control mice: blood, spleen, liver, lymph node(s), and kidney. Lymph nodes collected included axillary, brachial, inguinal, popliteal, and cervical nodes, generally in pairs. Lymph nodes were pooled for testing by PCR. Hearts were collected from mice during weeks 4 and 5 post-inoculation (n = 6 hearts total; 4 experimental and 2 control mice). Control mice were sacrificed at 4 and 5 weeks post-inoculation.

Gross observations

Mice were examined by visual inspection and manual palpation in the weeks following inoculation. Lymph node enlargement was noted, as were the location and number of skin lesions (Figure 6.1). Enlargement of axillary and inguinal lymph nodes was monitored by palpation, with reference to healthy, saline inoculated control mice.

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Polymerase chain reaction (PCR) detection of bartonella DNA

Genomic DNA was extracted from tissues and blood sampled from Swiss Webster mice sacrificed at 3, 4, 5, and 6 weeks after inoculation with three *B.tamiae* strains, and from saline inoculated control mice. A 200ul sample of blood and 10—30mg each of various tissues (lymph nodes, liver, spleen, kidney, and subcutaneous masses) from each mouse was subjected to DNA extraction using the manufacturer's blood or tissue protocol as outlined in the QIAamp DNA Mini kit handbook (Qiagen, Valencia, CA). Extracted DNA was stored at -20° C until tested.

Bartonella tamiae DNA in samples was detected by PCR using bartonella specific primers targeting the *gltA* (citrate synthase gene) [259], and 16S-23S rRNA intergenic transcribed spacer (ITS) region [260] (Table 6.1). Polymerase chain reaction was performed in 50 µl reaction volumes containing extracted template DNA, 10µl of 5X Green GoTaq reaction buffer, 200µM of each dNTP, 0.5µM of each forward and reverse primer and 1.25 U of Taq DNA polymerase (Promega, Madison, WI), in an Eppendorf Mastercycler (Eppendorf, Westbury, NY). The conditions for the *gltA* reactions were 2 min at 95°C, 35 cycles at 95°C for 30 s, 50°C for 45 s, 72°C for 30 s, and a 7 min extension at 72°C. The ITS PCR conditions were 2 min at 95°C followed by 40 cycles of 95°C for 30 s, 66°C for 1 min, 72°C for 1 min and a 7 min extension at 72°C. PCR products were visualized on a 1.5 % agarose gel, and amplicons matching the target length were sequenced on a 3130 Genetic Analyzer (Applied Biosystems, Foster City, CA) to confirm their identity.

Table 6.1 *Bartonella tamiae* DNA in samples was detected by PCR assay using *Bartonella* specific primers.

Primer name	Primer sequence 5'--3'	Amplicon size (bp)	Reference
gltA793F	CATGGTGGAGCTAATGAAG	344	This article
BhCS1137n	AATGCAAAAAGAACAGTAAACA		[259]
ITS325F	CTTCAGATGATGATCCCAAGCCTTCTGGCG	~300bp	
ITS1100R	GAACCGACGACCCCCTGCTTGCAAAGCA		[260]

Tissue preparation for histopathological analysis

Tissue samples were fixed in 10% neutral buffered formalin (Fisher Scientific, Kalamazoo, MI), subjected to standard processing, and embedded in paraffin. Sections of 5μm were then prepared and stained with hematoxylin and eosin for evaluation by light microscopy (Colorado Histo-Prep, Fort Collins, Colorado, USA), and with Warthin-Starry stain (Atlanta, CDC). Age-matched, saline inoculated control mouse tissues were treated in similar fashion, and all sections were read without prior knowledge of the experimental groups.

RESULTS

Examinations of inoculated mice—gross observations

Within the first week following inoculation all mice exhibited thickened, tough skin at the inoculation site in the scruff of the neck. This resolved in surviving mice by week 4. The scruff skin of saline inoculated control mice remained thin and supple throughout the course of the study. All mice inoculated with *B. tamiae* developed enlarged lymph nodes on the lateral

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thorax from 2 weeks post-inoculation (Figure 6.1A, B), and some of these

were still present at the conclusion of the study (6 weeks). Inguinal lymph

node

enlargement was easily detectable by palpation in mice during this same
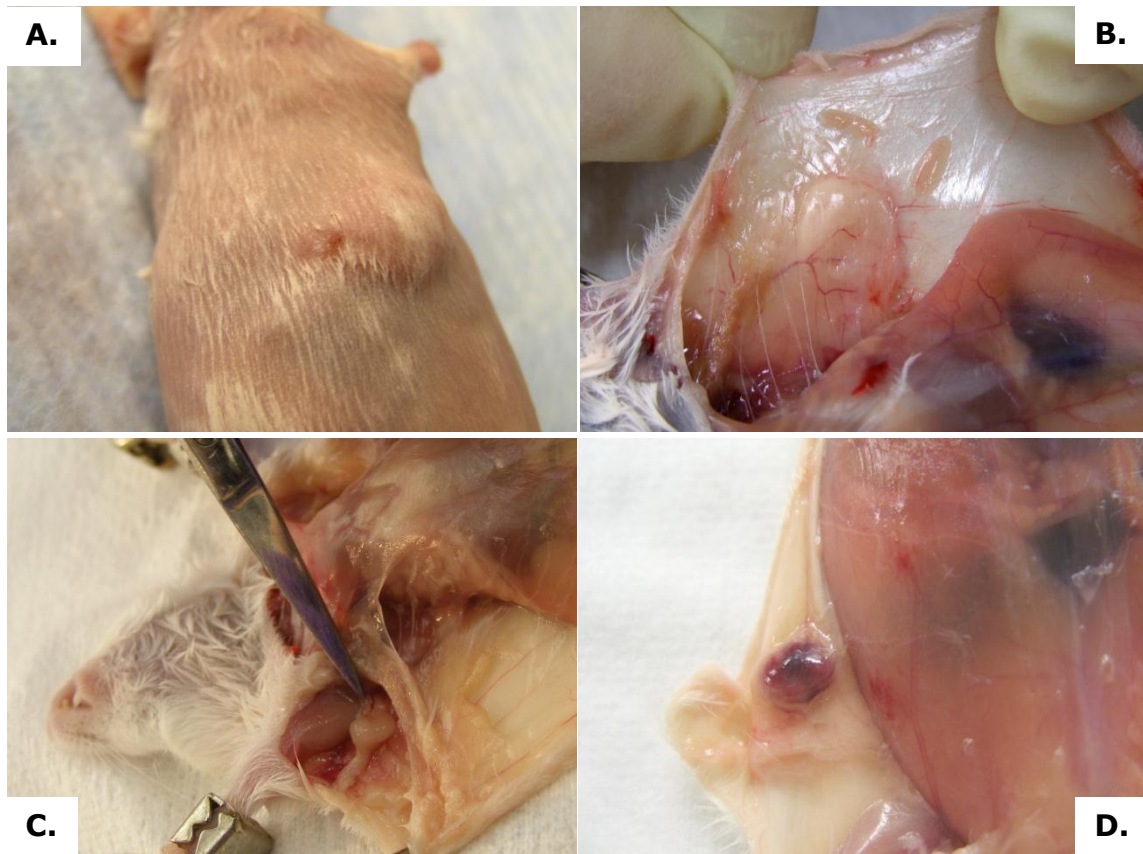
Figure 6.1 Enlarged lymph node, lateral thorax, with overlying superficial dermal lesion in a mouse inoculated with Th307 3 weeks post-inoculation (A, B). Axillary and inguinal lymph nodes of mice inoculated with three strains of *B. tamiae* were enlarged and were evident during physical exams (C, D).

time period, and in some mice axillary lymph nodes felt enlarged as well

(Figure 6.1C, D). Between weeks 2 and 3 mice inoculated with isolates Th307

and Th339 developed superficial ulcerations above several of the enlarged

lymph nodes (Figure 6.1A). Mice inoculated with Th239 did not develop skin ulcerations above enlarged lymph nodes. The lymph nodes did not appear painful upon palpation at any time during the course of the study, i.e. mice did not display aversion to handling or palpation of lymph nodes at any time.

PCR detection of bartonella DNA

Sequencing of PCR positive samples confirmed the presence of *B. tamiae* DNA in two types of mouse tissues (Table 6.2). *Bartonella tamiae* DNA was detected 3 weeks post-inoculation in a thoracic lymph node and the liver of a mouse inoculated with Th339. DNA was also detected in two different lymph nodes from another mouse inoculated with Th339, 5 weeks post-inoculation. One mouse inoculated with Th307 was also found to have a *B. tamiae* DNA positive thoracic lymph node at week 3. No blood or tissue samples collected from mice inoculated with Th239 contained detectable *B. tamiae* DNA. Samples collected from the two saline inoculated control mice were not found

Table 6.2 *Bartonella tamiae* DNA was detected in tissues of inoculated mice by two specific PCR assays

Week post-inoculation	*B. tamiae* isolates	Tissue sample	*gltA*	ITS
3	Th339[a]	Thoracic lymph node	+	+
3	Th339[a]	Liver	-	+
3	Th307	Thoracic lymph node	+	+
5	Th339[b]	Thoracic lymph node	-	+
5	Th339[b]	Lymph node (axillary or inguinal)	+	+

[a] the same mouse

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to contain *B. tamiae* DNA by PCR analysis. Nucleotide sequence analysis

indicated that the detected DNA was identical to the inoculated strains.

Histopathological observations

No bacteria were observed in tissue sections stained with Warthin-Starry

stain. Observations below are described from hematoxylin and eosin stained

tissue sections. Differences in pathogenicity among *B. tamiae* isolates were

noted (Table 6.3). Th307 appeared less pathogenic than Th239 or Th339

when inoculated into mice, as only a deep dermatitis was seen at week 3 and

a multifocal granulomatous nephritis was noted at weeks 5 and 6 after

inoculation. This contrasts with Th239 and Th339 where granulomatous

lesions were noted within the heart, kidney, liver and spleen of inoculated

mice (Table 6.3). Lesions in the dermis occurred early after inoculation with

Th307 and Th339 (3 weeks), while lesions of internal organs induced by all

three strains were noted at week 4 and persisted through week 6 post-

inoculation (the duration of the study). At week 5 a diffuse myocarditis was

noted in the hearts of mice inoculated with Th239 and Th339. Inflammation

consisted primarily of mononuclear cells (lymphocytes and plasma cells)

admixed with neutrophils occurring between myocytes (Figure 6.2-A). As

noted in Figure 6.2-B, pyknotic nuclei and necrosis of adjacent myocardial

muscle

cells was seen (Figure 6.2-B, arrow). Also, granulomas were seen within both

the right and left atria with necrosis of adjacent muscle cells (Figure 6.2-C).

Table 6.3. Histopathological observations in aged, immunocompetent mice inoculated with three different *Bartonella tamiae* strains. [NAF = No Abnormal Finding]

Bartonella tamiae isolates			
Week post-inoculation	Th239	Th307	Th339
3 Skin	NAF	Dermatitis, deep, not oriented	Necrotizing dermatitis
Spleen	NAF	NAF	NAF
Liver	NAF	NAF	NAF
4 Skin	NAF	NAF	Necrotizing dermatitis
Spleen	NAF	NAF	NAF
Liver	Granulomatous hepatitis with necrosis	NAF	Diffuse inflammatory response in sinusoids
Kidney	Granulomatous nephritis	NAF	NAF
5 Spleen	NAF		Hemosiderosis in the cortex
Liver	NAF	NAF	NAF
Kidney	Perivascular granulomatous nephritis	Granulomas in the cortex and medulla	NAF
Lymph nodes	Pyogranulomatous lymphadenitis	NAF	NAF
Heart	Myocarditis, granulomas right & left atria, infiltrates	No sample	Myocarditis, granulomas right & left atria, infiltrates
6 Skin	No sample	Deep dermatitis	No sample
Spleen	Pyogranulomatous nodules in the red and white pulp	NAF	NAF
Liver	Hepatitis, granulomas with necrosis	NAF	Perivascular granulomas, hepatocellular necrosis
Kidney	NAF	Large perivascular granulomas	NAF
Lymph nodes	Lymphadenitis	NAF	Lymphadenitis, cortical granulomas, necrotizing vasculitis

In the liver, multifocal pyogranulomatous infiltrates were noted adjacent to central veins (Figure 6.3-A) in association with hepatocellular necrosis (Figure 6.3-B, arrow). In the kidneys a perivascular granulomatous nephritis with associated degeneration and necrosis of glomeruli was noted in both kidneys (Figure 6.4-A, black arrows). These granulomas appeared to be associated with degeneration of proximal tubules (Figure 6.4-A, white arrow), and inflammation in kidneys was seen primarily within the renal cortex.
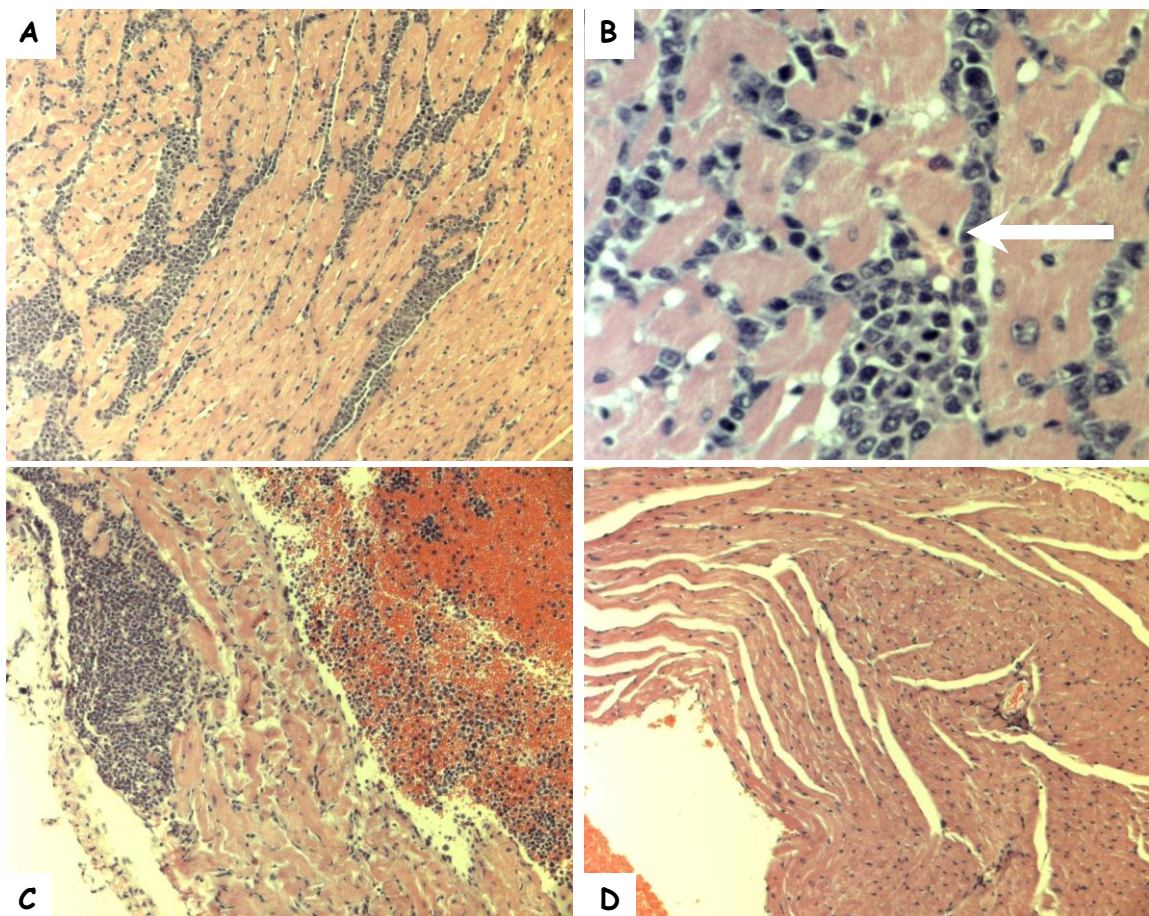

Figure 6.2 Photomicrographs of hematoxylin and eosin stained heart sections of mice sampled during the study. Mononuclear cell infiltration of the ventricle (A, 10x; B, 40x) and atrium (C, 10x) of a Swiss Webster mouse 5 weeks post-inoculation with *B. tamiae* Th339. The white arrow indicates a

myocyte with a pyknotic nucleus. (D) Ventricle of a Swiss Webster mouse inoculated with saline (10x).

Lesions within internal organs appeared to be perivascular, and, as noted in

Figure 4-B, a necrotizing vasculitis occurred in prominent arteries of the

cortex of the lymph nodes draining associated skin lesions. In the spleen a

necrotizing splenitis was seen with a mixed inflammatory response occurring

within both the red and white pulp (not shown). In the case of a mouse

inoculated with Th339 a diffuse and prominent hemosiderosis was also seen

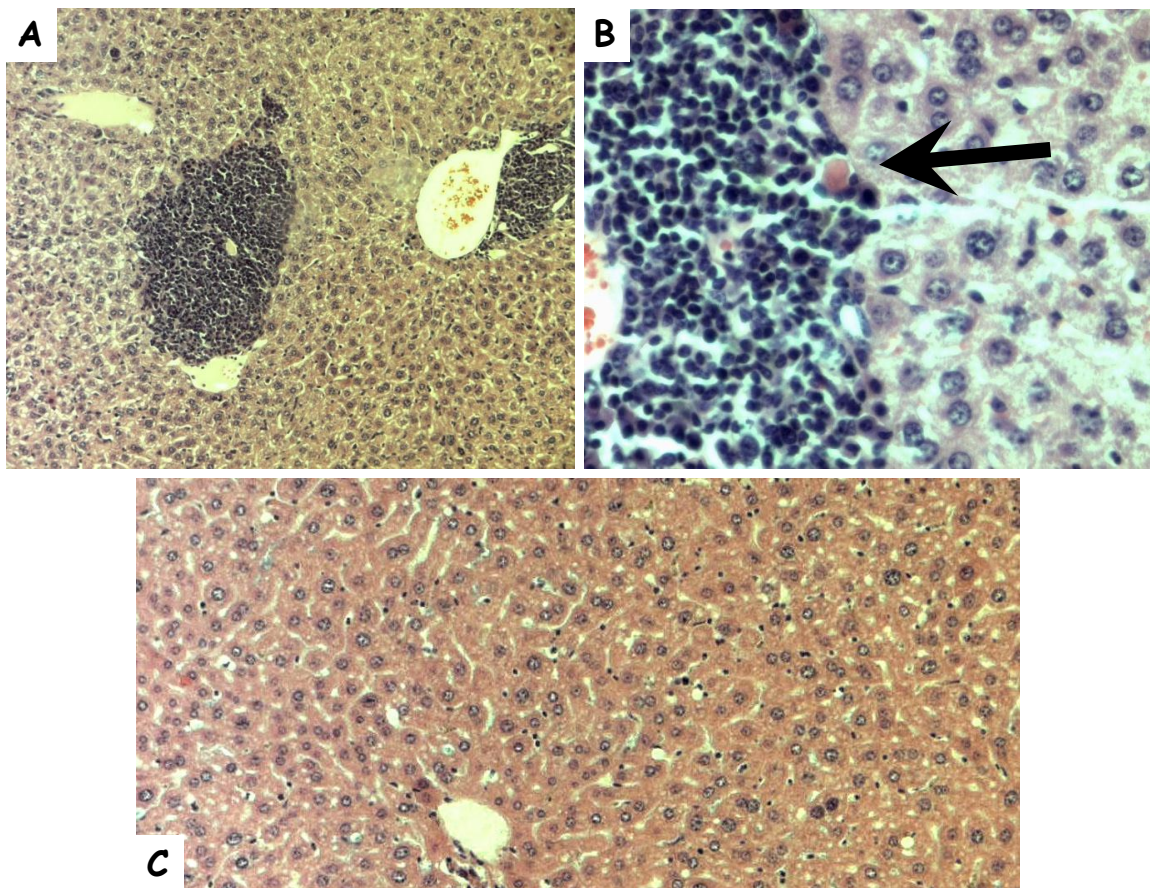within the white pulp of the spleen.

Figure 6.3 Photomicrographs of hematoxylin and eosin stained liver sections from mice sampled during the study. Granulomatous cell infiltration in liver tissue (A, 10x; B, 40x) of a Swiss Webster mouse inoculated with *B. tamiae* Th239. The black arrow indicates a necrotic hepatocyte. (C) Section of liver from a Swiss Webster mouse inoculated with saline (20x).
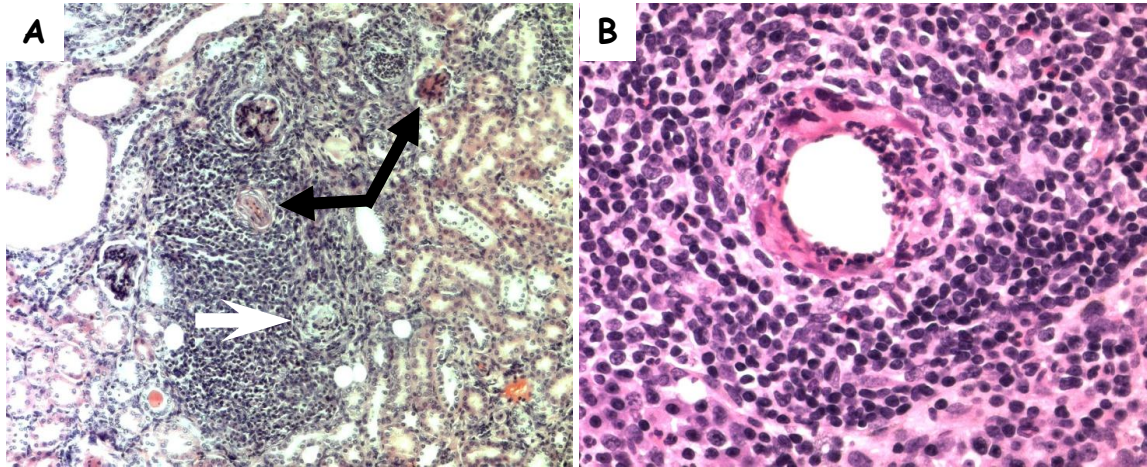
Figure 6.4 Photomicrographs of hematoxylin and eosin stained kidney and lymph node sections from mice sampled during the study.

Granulomatous cell infiltration in the kidney (A, 10x) of a Swiss Webster mouse inoculated with *B. tamiae* Th239. Black arrows indicate degenerative glomeruli; the white arrow indicates a degenerating proximal tubule. (B) Necrotizing vasculitis in an axillary lymph node recovered from a Swiss Webster mouse 6 weeks after inoculation with *B. tamiae* Th339 (40x).

In the skin, caudal and lateral to the *B. tamiae* inoculation site, a necrotizing, ulcerative dermatitis developed dorsal to the subcutaneous masses early (3 weeks after inoculation of the mice with Th339, Table 6.3). The dermatitis persisted throughout the six week study period in some mice. Grossly, the lesions induced by Th339 appeared as ulcerative nodules, while microscopically, a severe, mixed inflammatory response was noted within the deeper layers of the dermis and subcutaneous tissues with associated necrosis of adjacent structures, such as hair follicles and sebaceous glands. Inflammatory infiltrates appeared similar to what was noted in the heart, liver, and kidney lesions, consisting of neutrophils admixed with mononuclear

cells, predominantly plasma cells and mature lymphocytes. None of the above described lesions were seen in saline-inoculated control mice.

DISCUSSION

Inoculation of Swiss Webster mice aged 15-18 months with human isolates of *B. tamiae* induced disease processes consistent with clinical manifestations of disease observed in human patients [30]. Since mice experience age-related changes in immune function, which include alterations in T-cell responsiveness to antigens, this may have affected the outcome of our study [256, 261]. Future studies will include younger mice to evaluate for age differences in response to this pathogen.

Two of the evaluated isolates of *B. tamiae*, Th239 and Th339, appear more pathogenic than the third, Th307 (Table 6.3). This corresponds to the presentation of the human patients from whom these isolates were obtained [30]. Patients 239 and 339 had rashes, and were febrile for 6 and 1 day(s) respectively, whereas Patient 307 was afebrile, and presented with pterygium in both eyes: all three patients had anemia [30]. In addition, the report of liver function abnormalities found in the human patients [30] would be consistent with hepatocellular disease, also seen in mice in the present study (Table 6.3, Figure 6.2). Immunopathological changes in the liver are not uncommon following human and cat infection with *B. henselae* or *B. clarridgeiae* [176, 262-264]. *Bartonella tamiae* DNA was found in the liver of one mouse infected with Th339 three weeks after inoculation (Table 6.2). Although *B. tamiae* DNA was detected, the question remains whether persistent bacteria in tissues or an inflammatory response directed toward

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bacterial antigen(s) was responsible for the perivascular granulomas and hepatocellular necrosis seen at week 6 in our mice (Table 6.3). It remains unclear whether any of the lesions observed were induced, and persisted or progressed in the presence or absence of viable bacteria.

It is unknown whether the three human patients infected with *B. tamiae* had cardiac disease [30]. No clinical tests were reported to have been conducted to evaluate for cardiac function abnormalities [30]. In the present study *B. tamiae* Th239 and Th339 produced myocarditis in mice, with a diffuse inflammatory response associated with myocardial cell necrosis within both ventricles (Figure 6.2). Moreover, granulomatous lesions were observed in both atria 5 weeks post-inoculation (Table 6.3, Figure 6.2). Myocarditis in humans and animals has been associated with several *Bartonella* species [263, 265-268]. Advances in diagnostic techniques have implicated *B. quintana*, *B. henselae*, and *B. elizabethae* in the majority of *Bartonella*-associated human myocarditis cases [251, 269, 270], while *B. vinsonii* subspp. *berkhoffii* seems to be the main cause of *Bartonella* induced myocarditis in dogs . Histopathological findings in experimentally infected cats and in naturally acquired human and dog cases of *Bartonella* myocarditis are consistent with our observations in mice .[263, 265, 271]. Shared characteristics of infection of heart tissue among these cases and our murine model include mixed inflammatory infiltrates , and myocyte necrosis with random inflammatory foci throughout heart tissue (Figure 6.2) [263, 265, 271].

To our knowledge this is the first report of myocarditis associated with the inoculation of *B. tamiae*. Though mice had a diffuse myocarditis and no endocarditis was found in hearts sampled during our study (n = 4; hearts sampled from experimentally inoculated mice at weeks 4 and 5), it is intriguing to note that a high rate of culture negative infective endocarditis exists in Khon Kaen, Thailand [272]. This is the same area where the patients reside from whom *B. tamiae* was isolated [30]. In the human endocarditis cases the causative agent(s) is unknown but the possible involvement of bartonellae has not been stringently evaluated [272]. In Thai patients with infective endocarditis the mean period of time from symptoms to diagnosis was 5.7 weeks [272] and our present study only lasted to 6 weeks with mouse hearts sampled at weeks 4 and 5 only. Further evaluation of our mouse model for *B. tamiae* induced disease may reveal more extensive cardiac involvement, especially in the context of a longer term study. Additional studies are also needed to determine the immunopathogenesis of these lesions in this murine model.

Lymphadenitis with and without vasculitis was seen in mice inoculated with *B. tamiae* Th239 and Th339 and sacrificed at weeks 5 and 6, and week 6 respectively (Table 4). This is consistent with presentations of bartonella infections in human patients as lymphadenitis is a common symptom in immunocompetent humans infected with *B. henselae* [273]. It has also been observed in humans during infection with *B. quintana* [274] and *B. alsatica* [186], and in dogs [275] and cats infected with *B. henselae* [237]. Though lymphadenitis was observed microscopically no bartonella DNA was detected

in those three lymph node samples. A recently published *B. henselae* 'cat scratch disease' mouse model also reported persistent lymphadenopathy in mice without detection of bacteria in the lymph nodes [211]. Conversely, in this study at week 5, *B. tamiae* DNA was detected by PCR in the lymph node of a mouse inoculated with Th339 (Table 6.2), but lymphadenitis was not observed in those tissues under microscopic examination. It appears that the presence of bartonella DNA is not necessarily a predictor of pathology in the lymph nodes. Interestingly, of the four mice in our study inoculated with *B. tamiae* Th307, none displayed lymphadenitis which supports our conclusion that this isolate differs in pathogenicity compared to strains Th239 and Th339.

As little is known of the natural history of *B. tamiae* in Thailand it is difficult to assess and quantitate human risk for acquiring infection with this suspected human pathogen [30]. Although a specific animal reservoir for the bacteria has not been identified, the epidemiological profile of the three patients shows some shared exposures congruent with *Bartonella* bacterial infections manifesting most often as zoonoses [30]. All three patients had a history of exposure to rats and two had noted the appearance of rats in their homes in the weeks prior to the onset of their illness [30]. In recent years a number of rodent associated *Bartonella* species have been isolated from patients exhibiting a wide variety of clinical illnesses [61]. These cases include *B. elizabethae*: endocarditis [61], *B. grahamii*: neuroretinitis [61], *B. washoensis*: myocarditis [61] and meningitis [4], and *B. vinsonii* subspp. *arupensis*: bacteremia with fever [61] and endocarditis [189]. To date it

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remains unclear how these infections as well as the human infections with *B. tamiae* [30], were acquired, whether by direct contact with an animal reservoir or exposure to a hematophagous arthropod. Recently, *B. tamiae* DNA was detected by PCR assay in chigger mites and ticks collected from a variety of rodents in Thailand [276]. This suggests the involvement of chigger mites, ticks, and/or rodents in the transmission of *B. tamiae* to humans in Thailand.

In summary, we explored the capacity of three *B. tamiae* strains to induce disease in aged immunocompetent mice. Our observations are consistent with the classification of *B. tamiae* as a human pathogen as inoculation with the bacteria produced necrotizing dermatitis, lymphadenitis, granulomatous nephritis and hepatitis, and myocarditis in mice. Disease characteristics observed in our mouse model correlate with what has been observed in other animal models and in human bartonelloses [263, 265, 271]. This murine model lends itself to the study of the immunopathogenesis of bartonellosis caused by *B. tamiae* as it reproduces clinical symptomatology found in human patients in Thailand [30]. Future studies in mice will evaluate the role age may play in the manifestation of disease. Finally, though the natural host and transmission dynamics of *B. tamiae* are unknown at this time, several lines of evidence suggest that rodents and/or ectoparasites can present some risk to humans for acquiring infection with this bacterium.

CHAPTER 7

SUMMARY

LABORATORY MOUSE MODELS REPRODUCE HOST-BACTERIA INTERACTIONS

Research reported in this dissertation was aimed at developing mouse models for bartonella bacteria research. In doing so we explored the concept that taxonomic distance between host of origin and incidental host could be a predictor for bartonella infection outcome. Bartonella strains originating from diverse mammalian hosts were evaluated in a common system, the laboratory mouse, *M. musculus*. Mouse models were developed that span the continuum from seemingly benign infections to disease. The use of the commonly accepted laboratory mouse model in all our experiments in essence ignores the very real differences between human and mouse physiology. Certainly, a direct translation of effect and response of mice to infection would be inappropriate, but laboratory mice are accepted surrogates for humans in infectious disease studies, and little research in this area could be conducted without the use of such surrogates. In evaluating the taxonomic distance concept, we treated mice as equivalent to humans in our taxonomic distance categories, and we do recognize that this is in fact not a valid equivalency, but was done so that we could explore the concept in

controlled experiments. The validity of using the mouse as a surrogate for humans in our experiments still needs to be shown, once more information about human infections with zoonotic bartonellae is collected.

As predicted by the taxonomic distance concept, mice inoculated with *Mus* spp. origin bartonella strains displayed a response to infection that matches what we know about naturally acquired infection kinetics in rodents. As we advanced along the continuum of infection outcomes we found that three of four rat bartonella strains failed to infect mice, an example of bacterial host specificity at the genus level. These three strains seem to circulate almost exclusively in *Rattus* spp. in nature. The fourth rat bartonella strain successfully infected mice, not surprising as this strain infects a much broader host range in nature than the other isolates. Dose dependent responses following exposures including months long delays in bacteremia onset in infected mice have important implications for how we think about the transmission dynamics of these bacteria in natural maintenance cycles.

A shrew origin *B. elizabethae* strain infected three different laboratory mouse stocks. Since this strain is closely related to rat bartonella strains we might predict only a 25% probability (1:3 odds) of it infecting mice, based on the outcome described in Chapter 3—but against these odds it did. This suggests the intriguing possibility that when bartonella strains in nature jump from their natural hosts to non-natural hosts, i.e. sympatric mammals, they may undergo adaptive changes that allow the bacteria to infect a broader host range. Host switches in nature may precede the emergence of strains infecting human populations [241, 249, 277].

Though taxonomic distance between host of origin and incidental host appears to be a robust predictor of infection outcome, differences in individual host susceptibility in these dose controlled experiments were impossible to ignore. A review of the epidemiological features associated with human infections reinforced the idea that host factors may also be a powerful predictor for infection outcome (Chapter 1, Table 1.2). The demographic trend among patients with moderate to severe bartonella-associated illnesses showed 30% had co-morbidities and almost all were middle aged or older (Chapter 1, Table 1.2). In addition, a review of published literature indicated that no outstanding candidates for bartonella infection susceptibility existed among mouse strains evaluated (Chapter 1, Table 1.3). Therefore, in an attempt to add more sensitivity to our ability to induce bartonella-associated disease states we began using aged immunocompetent mice in our experiments. Aged mice responded as predicted to infection with a *Mus* spp. origin bartonella strain by producing high level bacteremias, whereas mice inoculated with *B. tamiae*, a presumptive human pathogen, were abacteremic. This indicated that aged mice would not have a universal bacteremic response to inoculation of any bartonella strain. Subsequently we showed that aged mice displayed organ pathology following inoculation with *B. tamiae.* The multi-organ pathology observed in the mice was consistent with symptomatology of human illness presumptively caused by this bacterium.

The four mouse models reported in this dissertation constitute scientific resources of great utility and wide-ranging scope. Mice are genetically similar

to humans, they have a short generation time, are easy to handle and inexpensive to maintain relative to other laboratory animals, and are amenable to genetic manipulations. They are unmatched in their usefulness and convenience in infectious disease research.

Much is currently unknown about bartonella bacteria: their natural history and ecology, their interactions with hosts and potential arthropod vectors, and their mechanisms for bacterial persistence and pathogenesis. Research interest in these areas has never been lacking, what has been lacking are suitable 'tools in the toolbox.' We present these four mouse models as new 'tools' to advance understanding of bartonella bacterial interactions with natural and incidental hosts. The potential exists for these models to be used to investigate numerous aspects of bartonella ecology, vector transmission, and disease.

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