Bartonella henselae Infection and Host Response in the Zebrafish Embryo Model

Amorce Lima
University of South Florida, alima@health.usf.edu

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Bartonella henselae Infection and Host Response in the Zebrafish Embryo Model

by

Amorce Lima, B.S.

A dissertation submitted in partial fulfillment of the requirements for the degree of Doctor of Philosophy With a concentration in Signature Interdisciplinary Program in Allergy, Immunology and Infectious Diseases Department of Molecular Medicine Morsani College of Medicine University of South Florida

Major Professor: Burt Anderson, Ph.D. Robert Deschenes, Ph.D. Andreas Seyfang, Ph.D. Jahanshah Amin, Ph.D. Thomas Klein, Ph.D. My Lien Dao, Ph.D.

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Keywords: pathogenesis, cat scratch disease, bacillary angiomatosis, angiogenesis

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Dedication

I would like to thank God for giving me the wisdom and the strength to go through this doctoral training. I thank my wonderful wife Delani Lima for her support throughout this journey and through whom God has blessed me with two beautiful children: Destiny and Makayla Lima. I thank my parents Atania Sylverin and Sacrement Luma for their unwavering supports. They did not have the privilege of making it through middle school, but they understood the importance of education and had worked tremendously hard to pay for my primary education through high school in my country. I thank all my friends for their prayers and support.

I thank Dr. Burt E. Anderson for giving me the opportunity to work in his laboratory. He has been truly a great mentor. I will forever be grateful for all of the resources and time that he has invested in me. I am grateful to all of the collaborators and the present and past members of Anderson’s lab. I would like to specially thank Dr. Byeong J. Cha for providing me with microscopy training and Dr. Jahanshah Amin for microinjection training making the success of this project possible.
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List of Abbreviations

dpi ................................................................................................................ Days post infection
Bh ........................................................................................................ Bartonella henselae
dpf ...................................................................................................... Days post fertilization
hpi ........................................................................................................ Hours post infection
VEGF .......................................................................................... Vascular endothelial growth factor
EDTA .......................................................................................... Ethylenediaminetetraacetic acid
RT ........................................................................................................ Room temperature
qPCR .......................................................................................... Quantitative polymerase chain reaction
qRT-PCR .................................................................................. Quantitative real-time polymerase chain reaction
NaOAc .......................................................................................... Sodium acetate
ETOH .............................................................................................. Ethanol
LB .......................................................................................................... Luria Bertani
PBS ............................................................................................... Phosphate buffered saline
BA ......................................................................................................... Bacillary angiomatosis
BP ......................................................................................................... Bacillary peliosis
CFU ....................................................................................................... Colony forming units
TE ........................................................................................................... Tris EDTA
MyD88 .......................................................................................... Myeloid differential 88
MAL ................................................................................................. MyD88-adaptor-like
TRAM ............................................................................................. Toll/interleukin-1(IL-1) receptor
TICAM-1 ........................................................................................ TIR-domain containing adaptor inducing INF-β
SARM ............................................................................................... TRIF-related adaptor protein
NLR ..................................................................................................... Sterile α and HEAT-Armadillo motifs
LTA ....................................................................................................... Lipoteichoic acid
Scl ........................................................................................................ Stem cell leukemia
HUVEC ........................................................................................ Human umbilical vein endothelial cell
MCP-1 ........................................................................................... Monocyte chemotactic protein-1
ICAM-1 .......................................................................................... Intercellular adhesion molecule-1
NF-kB ............................................................................................ Nuclear factor kappa-light-chain-enhancer of activated B cells
SIV ....................................................................................................... Subintestinal vessel or basket
IP .......................................................................................................... Intersection point
Abstract

The Gram-negative bacterium *Bartonella henselae* (*Bh*) is an emerging zoonotic pathogen that has been associated with a variety of human diseases including bacillary angiomatosis which is characterized by vasoproliferative tumor-like lesions on the skin and internal organs of some immunosuppressed individuals. Several virulence factors associated with *Bartonella*-induced pathogenesis have been characterized. However, the study of those virulence factors has been limited to *in vitro* cell culture systems due to the lack of a practical animal model. Therefore, we wanted to investigate whether the zebrafish embryo (*Danio rerio*) could be used to model human infection with *Bh*. We investigated if *Bh* can mount an infection in zebrafish embryos during their early stage of development. Our data showed that *Tg(fli1:egfp)y1* zebrafish embryos supported a sustained *Bh* infection for 7 days with >10-fold bacterial replication when inoculated in the yolk sac. This was evident by plating of zebrafish homogenates, quantitative PCR, and confocal microscopy analysis. We assessed the interaction of *Bh* with EC and the phagocytic cells in live embryos by microscopy. Our data showed that aggregates of *Bh* interact with the endothelium of the embryo vasculature. Evidence showed that *Bh* recruited phagocytes to the site of infection in the *Tg(mpx:GFP)uwm1* embryos. We also wanted to determine the response to infection with *Bh*. Infected embryos showed evidence of a *Bh*-induced angiogenic phenotype as well as an increase in expression of genes encoding pro-inflammatory factors and pro-angiogenic markers. A deletion mutant for the entire VirB type IV secretion system (*ΔvirB2-11*) supported bacterial
replication although to a lesser degree compared to the wild type control. However, infection of zebrafish embryos with a deletion mutant in the major adhesin (BadA) resulted in little or no bacterial replication and a diminished pro-angiogenic and pro-inflammatory host response compared to wild type Bh, providing the first evidence that BadA is critical for in vivo infection. Thus, the zebrafish embryo provides the first practical animal model of Bh infection that will facilitate efforts to identify virulence factors and define molecular mechanisms of Bh pathogenesis.
Chapter 1

Introduction

1.1. *Bartonella* species, their hosts and human diseases

*Bartonella* are small, pleomorphic Gram-negative rod-shaped bacteria which belong to the \( \alpha_2 \) subgroup of the class Proteobacteria [4]. *Bartonella* formerly known as *Rochalimaea* are closely related to the genera *Brucella* and *Agrobacterium* [5]. To date, over 30 species or subspecies of *Bartonella* have been identified and classified using molecular methods such as PCR using specific primers to *Bartonella* 16S ribosomal DNA and the tmRNA gene (*ssrA*) [5,6]. As the development of new molecular methods improves, it is expected that the number of identified *Bartonella* species will increase.

The genus *Bartonella* includes fastidious, facultative intracellular bacteria that are associated with endothelial cells (EC) and erythrocytes of a variety of hosts including rabbit, cat, rodent and human (Table 1.1) [3,7-9]. All of the identified *Bartonella* species have been associated with mammalian hosts and at least 12 of them are identified as human pathogens. *B. bacilliformis* and *B. quintana* have been isolated from humans [10,11]. *Bartonella vinsonii* subsp. *berkhoffii* has been isolated from dogs [12]. *B. henselae*, *B. clarridgeiae* and *B. koehlerae* have been found in cats [13-15]. *B. doshiae*, *B. grahamii*, and *B. vinsonii* subspecies *vinsonii* have been isolated from voles [5,16]. *B. elizabethae*, *B. taylorii*, *B. birtlesii* and *B. tribocorum* have been recovered from rats [5,16-18]. *B. vinsonii* subsp. *arupensis* was isolated from mice [19].
Table 1.1. List of *Bartonella* species (subspecies), their natural hosts, vectors, and human diseases. Over 30 species and subspecies of *Bartonella* have been identified worldwide. More than 25 of those species have been associated with mammalian hosts and at least 12 of them are identified as human pathogens causing a wide array of diseases in human. Table reproduced From Vayssier-Taussat et al. 2009 with authors’ permission [3].

<table>
<thead>
<tr>
<th>Species</th>
<th>Natural Hosts</th>
<th>Vectors *s:suspected</th>
<th>Disease in Human</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>B. alsatica</em></td>
<td>Rabbit</td>
<td>Unknown</td>
<td>Endocarditis</td>
</tr>
<tr>
<td><em>B. australis</em></td>
<td>Kangaroo</td>
<td>Unknown</td>
<td>Not described</td>
</tr>
<tr>
<td><em>B. bacilliformis</em></td>
<td>Human</td>
<td>Phlebotome, <em>Lutzomyia verrucarum</em></td>
<td>Oroya fever</td>
</tr>
<tr>
<td><em>B. birtlesii</em></td>
<td>Mice (<em>Apodemus</em>)</td>
<td>Flea*</td>
<td>Not described</td>
</tr>
<tr>
<td><em>B. bovis</em></td>
<td>Ruminant</td>
<td>Unknown</td>
<td>Not described</td>
</tr>
<tr>
<td><em>B. capreoli</em></td>
<td>Ruminant</td>
<td>Unknown</td>
<td>Not described</td>
</tr>
<tr>
<td><em>B. chomelli</em></td>
<td>Ruminant</td>
<td>Unknown</td>
<td>Not described</td>
</tr>
<tr>
<td><em>B. claridgeiae</em></td>
<td>Cats</td>
<td>Fleas (<em>Ctenocephalides felis</em>)</td>
<td>Cat scratch disease (CSD)</td>
</tr>
<tr>
<td><em>B. doshiae</em></td>
<td>Mice (<em>microtus</em>)</td>
<td>Unknown</td>
<td>Not described</td>
</tr>
<tr>
<td><em>B. elizabethae</em></td>
<td>Rat</td>
<td>Flea* (<em>Xenopsylla cheopis</em>)</td>
<td>Endocarditis</td>
</tr>
<tr>
<td><em>B. grahamii</em></td>
<td>Bank vole</td>
<td>Flea (<em>Ctenocephalides nubilis</em>)</td>
<td>Ocular manifestations</td>
</tr>
<tr>
<td><em>B. henselae</em></td>
<td>Cat</td>
<td>Fleas (<em>Ctenocephalides felis</em>), Ticks</td>
<td>CSD and complications</td>
</tr>
<tr>
<td><em>B. koehlerae</em></td>
<td>Cat</td>
<td>Unknown</td>
<td>Not described</td>
</tr>
<tr>
<td><em>B. phoceensii</em></td>
<td>Rat</td>
<td>Unknown</td>
<td>Not described</td>
</tr>
<tr>
<td><em>B. peromysci</em></td>
<td>Small mammals</td>
<td>Unknown</td>
<td>Not described</td>
</tr>
<tr>
<td><em>B. quintana</em></td>
<td>Human</td>
<td>Body Lice</td>
<td>Trench fever, endocarditis</td>
</tr>
<tr>
<td><em>B. rattimasiensis</em></td>
<td>Rat</td>
<td>Unknown</td>
<td>Not described</td>
</tr>
<tr>
<td><em>B. schoenbuchensis</em></td>
<td>Ruminant</td>
<td>Fly* (<em>Lipoptena cervi</em>)</td>
<td>Not described</td>
</tr>
<tr>
<td><em>B. telpae</em></td>
<td>Shrew mole</td>
<td>Unknown</td>
<td>Not Described</td>
</tr>
<tr>
<td><em>B. taylorii</em></td>
<td>Mice (<em>Apodemus</em>)</td>
<td>Flea (<em>Ctenocephalides nubilis</em>)</td>
<td>Not described</td>
</tr>
<tr>
<td><em>B. tribocorum</em></td>
<td>Rat</td>
<td>Unknown</td>
<td>Not described</td>
</tr>
<tr>
<td><em>B. vinsonii subsp arupensis</em></td>
<td>Mice</td>
<td>Unknown</td>
<td>Fever, bacteremia, endocarditis</td>
</tr>
<tr>
<td><em>B. vinsonii subsp berkoffii</em></td>
<td>Dog</td>
<td>Ticks*</td>
<td>Fever, bacteremia, endocarditis</td>
</tr>
<tr>
<td><em>B. vinsonii subsp vinsonii</em></td>
<td>Mice</td>
<td>Flea (<em>Trombicula microti</em>)</td>
<td>Fever, bacteremia</td>
</tr>
<tr>
<td>Candidatus species</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>B. melophagii</em></td>
<td>Sheep</td>
<td><em>Melophagus ovinus</em></td>
<td>Not described</td>
</tr>
<tr>
<td><em>B. rochalimae</em></td>
<td>Unknown</td>
<td>Unknown</td>
<td>Fever</td>
</tr>
</tbody>
</table>
Bartonella are emerging pathogens that are involved in a number of disease manifestations in humans and animals. Infections caused by this group of bacteria range from chronic bacteremia with no apparent symptoms in their reservoir hosts to self-limiting regional lymphadenitis and more complicated endocarditis in the human host [20-22]. Although mammals seem to be an important reservoir host for Bartonella, most of the human diseases are caused by only three species: B. bacilliformis, B. quintana and Bh.

Humans are the only known reservoir for B. bacilliformis, the bacterial agent of the biphasic disease bartonellosis (Carrion’s disease). The bacteria are transmitted between humans through bites of infected female sand flies Lutzomyia verrucarum [23,24]. Once the bacteria enter the human blood, they colonize almost all of the erythrocytes leading to an acute and severe hemolytic anemia causing a wide array of symptoms including myalgia, fever and headache; this phase is known as oroya fever [25,26]. The second phase of the disease is verruga peruana, which may be sequential or independent of Oroya fever, and is characterized by non-fatal tumor-like lesions seen mainly on the skin [27-29]. An outbreak of bartonellosis resulting in more than 7000 deaths was reported in 1871 in the high-altitude valleys and remote areas of South American Andes [30,31]. Mortality during the acute phase of bartonellosis ranged from 1 percent in the case of patients receiving antibiotic treatment to 88 percent in the untreated although some of the mortality might be attributed to secondary infection with bacteria and viruses [10,30,32].
Like *B. bacilliformis*, humans are the primary reservoir for *B. quintana*. The pathogen is transmitted between humans by the body louse (*Pediculus humanus corporis*) and historically known to cause trench fever, characterized by a recurrent and cycling (every 5-day) fever in the human host [33]. Approximately 1 million people in Europe were affected by trench fever during World War I [34]. Although the incidence of trench fever has substantially declined since World War I, the condition has reemerged as urban trench fever among the poverty-stricken, alcoholic and homeless individuals living in poor health and hygienic conditions [35]. Moreover, for the past two decades, *B. quintana* have been associated with other infections namely endocarditis, chronic bacteremia primarily among the poor and bacillary angiomatosis in immunosuppressed individuals [36-39].

While humans are the reservoir hosts for *B. bacilliformis* and *B. quintana*, cats are the natural host for *Bh* in which the bacteria cause an asymptomatic intraerythrocytic infection [14,40]. The organism, however, can be transmitted between cats through cat fleas (*Ctenocephalides felis*), and from infected cats to humans by bites and scratches ([Fig. 1.1](#)) [41,42]. Although infected cats show an asymptomatic bacteremia, this zoonotic pathogen can cause a wide array of infections in the human incidental host. Immunocompetent patients infected with *Bh* typically suffer from cat scratch disease (CSD) which is a benign subcutaneous lymphadenopathy associated with fever [43,44]. It is estimated that 22,000 – 24,000 cases of CSD are diagnosed yearly, mainly in children, in the United States [45,46]. Although CSD is considered to be a self-limiting infection, over 10% of those cases require hospitalization and if not treated the bacteria can disseminate to other organs such as the liver, the brain and the
heart leading to more serious complications [12,45]. Immunosuppressed individuals such as HIV patients infected with *Bh* can develop a more systemic infection characterized by vasoproliferative tumor-like lesions called bacillary angiomatosis (BA) and bacillary peliosis (BP) on the skin and liver, respectively [47]. According to the CDC, the exact incidence of BA and BP is not known; however, cases have been reported in all 50 states in the U.S., with the majority in men because of the disproportionate incidence of HIV in men.

**Figure 1.1.** *Bh* infection in cat and the human incidental host. *Bh* causes intra-erythrocytic bacteremia in cats. Infection is spread between cats through the cat flea vector. The pathogen is transmitted to the human incidental host by scratches or cat bites from an infected cat. Immunocompetent individuals develop cat scratch disease and immunosuppressed individuals develop bacillary angiomatosis. Figure copied from Dehio, 2005 with author's permission [2].
1.2. *Bh* pathogenesis and infection strategy

Upon inoculation of a mammalian host, *Bartonella* are believed to reside in what researchers call a “primary niche”, outside of the bloodstream partly to avoid elicitation of a host immune response as the bacteria are not capable of directly invading the erythrocytes until the host or the bacteria are primed to do so [48]. The nature of this primary niche in *Bartonella* infection has not been completely established yet. This is perhaps, in part because infection strategy and host-pathogen specificity differ among species, and in part because of the lack of a suitable animal model. Although the cellular composition of the primary niche is not completely clear, *in vitro* data suggest that it may involve EC, migratory cells such as lymphocytes and mononuclear phagocytes which may play a role in the transport of the bacteria into the microvascular environment [2,20].

*Bh* is thought to be transmitted to the human incidental host mainly indirectly through cat flea feces via cat scratches or cat bites [49]. Once the bacteria are inoculated, they can invade many cell types including EC [50], hematopoietic progenitor cells [51], epithelial cells [8], monocytes and macrophages [52,53]. It has been shown that *Bh* entry into EC can happen by two alternative routes: single bacteria uptake via a zipper-like mechanism or as large bacterial aggregates in a structure called the “invasome” [54,55]. Bacteria-containing vacuoles accumulate in the perinuclear space where they fail to acidify and fuse with the lysosome [48]. Consequently, the bacteria are able to replicate and persist in the host cells. *Bh* was shown to induce the secretion of IL-10 in both *in vitro* and *in vivo* studies [56,57]. Secretion of IL10, a potent anti-inflammatory cytokine which suppresses the function of immune cells including
macrophages and dendritic cells, may contribute to an asymptomatic and persistent course of infection favoring *Bh* [48].

**Cat scratch Disease**

CSD is the most commonly known disease manifestation associated with *Bh* infection in immunocompetent individuals. As reviewed by Florin et al., CSD begins after 3 to 10 days with an erythematous papule at the site of inoculation with *Bh*, then the lesions progress through vesicular and papular crusted stages, and persist for 1 to 3 weeks [21]. The swelling of the lymph nodes results from granuloma formation via recruitment and stimulation of macrophages during an interferon gamma (INF-γ)-mediated T helper 1 cell response [53]. Moreover, electron microscopy of lymph node tissues of patients with CSD show the presence of *Bh* near the vascular endothelium, with organisms seen in clumps in vessel walls [58].

*Bh*-induced angiogenesis

One of the most captivating features of infection with *Bh* is its ability to cause angioproliferative lesions. The pathological lesions seen in BA patients resemble those of Kaposi’s sarcoma associated with human herpes virus 8 infection [59]. Histological studies revealed the presence of immature capillaries that are lined with swollen endothelium in BA lesions, and the lesions are packed with bacterial aggregates and infiltrated by macrophages and neutrophils [60,61]. It is believed that live *Bh* actively trigger the vasoproliferative activity since the observed EC proliferation completely regresses after treatment with antibiotic [21]. As in tumor angiogenesis, *Bartonella*-
triggered neovascularization follows a series of steps that involve disruption of the normal pattern of the extracellular matrix and basal membrane, endothelial cell migration and proliferation at the site of angiogenic stimuli [62]. Kirby developed an in vitro model of *Bh*-induced angiogenesis and showed that in addition to endothelial proliferation, *Bh* coordinated a series of events which included matrix invasion, survival of type I collagen and endothelial tubular differentiation [63].

The molecular basis of *Bh*-induced angiogenesis has been extensively studied in cell culture models. Our current understanding suggests that the process involves at least three mechanisms that work synergistically: 1) mitogenic triggering of endothelial cell proliferation, 2) inhibition of apoptosis (contact-dependent or independent), and 3) angiogenic reprogramming of infected host cells (with pro-inflammatory activation of cytokines) [64]. Both live and cell extracts of *Bh* are capable of inducing the proliferation and migration of HUVEC [65,66]. For instance, GroEL, a heat shock protein, was shown to be secreted in *Bh* extracts and caused endothelial cell proliferation by triggering the release of intracellular calcium [67,68]. EC proliferation was also shown to be dependent on anti-apoptotic activity as *Bh* inhibits the apoptosis of HUVEC by inhibiting the activities of caspase 3 and caspase 8 [69]. *Bh* infection of EC triggers an NF-κB-dependent release of ICAM-1, E-selectin, MCP-1, and IL-8, some of which may exert a direct effect on EC in an autocrine manner leading to cell proliferation, but also may recruit monocytes/macrophages and polymorphonuclear cells to the sites of infection [1,70-72]. As demonstrated by Resto-Ruiz, the interaction of *Bh* with the human macrophage cell line THP-1 triggered the release of vascular endothelial growth factor (VEGF), a potent stimulator of angiogenesis, as well as IL-1β [1]. VEGF acted on EC
leading to further cell proliferation and ultimately angiogenesis constituting the paracrine angiogenic loop (Fig. 1.2).

**Figure 1.2. Paracrine loop model of Bh-induced angiogenesis.** Upon adherence to and invasion of macrophages (mac) and neutrophils via BadA, *B. henselae* induced the production of VEGF which functions in a paracrine manner and acts as a mitogen to endothelial cells (EC) leading to cell proliferation and angiogenesis. When EC are infected with *B. henselae* through the VirB T4SS and the expression of the Bartonella effector proteins (Beps), a cascade of pathways occurs leading to production of pro-angiogenic chemokines and cytokines, which may exert a direct effect on the EC or may recruit monocytes and neutrophils to site of inflammation leading to further EC proliferation and angiogenesis. Figure is adapted from Resto-Ruiz et al. with author’s permission [1].

### 1.3. *Bh* virulence factors

For the past decade, *Bartonella* research has been advancing at a fast pace due in part to the availability of the complete genome sequence for *Bh* and the appropriate cell culture models [73]. This has enabled the establishment of gene function and definition of important virulence factors including secreted factors as well as membrane-bound proteins associated with *Bh*-induced pathogenesis for further *in vitro* studies.
**Bartonella adhesin A**

*Bartonella* adhesin A (BadA) belongs to the class of trimeric autotransporter adhesins along with YadA from *Yersinia enterocolitica* and NadA from *Neisseria meningitidis*. These proteins represent important virulence factors of Gram-negative bacteria in the form of an extracellular filament consisting of a head, neck and repetitive stalk domains assembled on a C-terminal membrane anchor domain [74,75]. There are at least two variants of the *badA* gene with the best-characterized one, *badA* full-length (BH01510), encoding a large protein made up of more than 3000 amino acids resulting in a size of 328 kDa per polypeptide chain and a length of 240 nm [64]. *In vitro* studies of *Bh* infection have shown that BadA is critical for adhesion to host cells and extracellular matrix proteins such as fibronectin and collagens, as well as inhibition of phagocytosis and induction of angiogenesis [76]. The expression of BadA has been shown to correlate with a pro-angiogenic cell response via activation of hypoxia-inducible factor 1-alpha (HIF 1-α) and the subsequent secretion of the pro-angiogenic factor VEGF [52,76-78].

**VirB/VirD4 type 4 secretion system**

Many pathogenic bacteria utilize a type IV secretion system (T4SS) to translocate DNA and effector molecules into the host cells to influence cellular functions. The VirB/VirD4 system of the plant tumor-inducing plasmid pTiC58 of *Agrobacterium tumefaciens* is the best-characterized member of this transporter family and consists of 10 essential components: VirB2-11 plus the type IV secretion substrate
The T4SS consists of a translocation channel that spans both the inner and outer membrane of Gram-negative bacteria and merges into a surface filament mediating the initial attachment to the target cells [80]. The VirB/VirD4 T4SS has also been well-characterized in Bh and shown to transfer important Bartonella effector proteins (BepA through BepG) to host cells where they disrupt cellular functions favoring survival of the bacteria [81,82]. In vitro studies of human endothelial cells cultured with Bh showed that VirB/VirD4 T4SS along with the Beps mediate cellular changes such as a massive cytoskeletal rearrangement leading to the uptake of bacterial aggregates by a structure termed the “invasome” [54]. In addition, the VirB/VirD4 T4SS is required for NF-kB-dependent pro-inflammatory cytokine activation of IL-8 [83], expression of the cell adhesion molecules ((ICAM)-1 and E-selectin) [71], and inhibition of endothelial cell apoptosis [80,83]. Recently, the role of some of the Beps was investigated by Scheidegger et al. using a 3D in vitro angiogenesis assay of collagen gel-embedded EC spheroids [84]. BepC, BepF and BepG were shown to promote invasome-mediated uptake of large Bh aggregates [84]. While BepG by itself was shown to exhibit an anti-angiogenic activity interfering with sprout formation, BepA was shown to induce sprouting of the endothelial spheroids similarly to sprouting triggered by VEGF [84].

Trw type 4 secretion system

The Trw T4SS is the other and the less studied T4SS found in Bartonella. The VirB T4SS is required for the early infection stage and involved in establishing interaction of Bh with EC and in translocating effector proteins into the host cells [79,81],
whereas the Trw T4SS is involved in adherence and invasion of erythrocytes by surface-expressed T4SS pili and has not been shown to have the ability to transfer effectors into the host erythrocytes [82]. It has been suggested that the loss of flagella is correlated with the acquisition of Trw T4SS by the modern lineage species such as Bh, and the Trw system fulfills the erythrocyte adhesion role of the flagella among non-flagellated Bartonella spp. [82]. The trw locus has multiple tandem gene duplications of trwL and trwJ-trwH which encode small adhesin-like proteins with varying copy numbers among Bartonella species [85]. The TrwJ and TrwL are the surface-exposed components that have been shown to specifically adhere to erythrocytes with TrwJ1 and TrwJ2 conferring host specificity for the bacteria [85-87].

Lipopolysaccharide (LPS)

LPS is a major component of the outer membrane of Gram-negative bacteria, and plays an important role in the pathogenicity of the bacteria. LPS induces a signaling cascade that leads to a NF-κB-dependent inflammatory response by binding to TLR4/TLR2, which interacts with accessory proteins CD14 and myeloid differentiation 2 [88]. A pronounced immune response to LPS can lead to septic shock [89]. LPS of Bartonella has been shown to exhibit very low endotoxic activity, and this has been speculated to result in remarkable interaction and persistence of Bh in the endotoxin-sensitive EC causing an angioproliferative phenotype rather than sepsis [90]. Bh LPS is structurally different than the typical LPS from E. coli [91], and evidence shows that purified Bh LPS did not stimulate TLR2 [92]. As reviewed by Harms and Dehio,
Bartonella LPS may have an immunomodulatory effect by acting antagonistically on TLR4 [48].

Other virulence factors

Outer membrane proteins (OMPs) (28, 32, 43, 52, and 58 kDa) were described in Bh and shown to adhere to EC in vitro [93]. OMP43 was shown to bind fibronectin and human umbilical vein endothelial cells and might play a very important role in Bartonella infection [94,95]. It was demonstrated that Bh OMPs were sufficient to induce NF-kB activation and expression of adhesion molecule followed by enhanced rolling and adhesion of leukocytes [71].

1.4. Previous Bartonella infection models

For over a decade, researchers have been looking into developing an animal model to study Bartonella infection and host response, but the efforts to establish such a model have met with limited success. Cats, the natural reservoir of Bh, when infected, do not exhibit symptoms such as lymphadenopathy and vasculoproliferation as seen in infected humans [96]. A primate rhesus macaque (Macaca mulatta) model was shown to reproduce characteristics of human infection with B. quintana [97], but use of this model system for Bh has not been reported. Moreover, attempts were made to infect C57BL/6 as well as BALB/c mice by inoculating 10^7 to 10^8 Bh cfu intraperitoneally or subcutaneously. However, both strains of mice were shown to clear the bacteria within days of inoculation with no increase in bacterial burden [98,99]. The mechanism by which the bacteria were cleared was not fully investigated. For those underlying
reasons, this research project aimed at investigating whether the zebrafish (*Danio rerio*) embryo model could be used to study *Bh* pathogenesis and response to infection.

### 1.5. The zebrafish embryo model

**Overview**

Many invertebrate models including fruit fly (*Drosophila*) and the nematode worm, *Caenorhabditis elegans*, have been instrumental in helping investigate fundamental concepts in genetics and early development. Historically, mice have been the model of choice for developmental and genetic studies of vertebrates. However, there are several drawbacks: because fruit fly and *C. elegans* are invertebrates, they are evolutionarily divergent from humans and many key organs and the sophisticated immune system are absent in *C. elegans* [100]. Although mice are more closely related to humans, it is difficult to directly observe the cellular development because embryogenesis occurs in the uterus. In the early 1970's, Dr. Georges Streisinger, a researcher at the University of Oregon, identified and developed the zebrafish as a model for genetic and developmental studies of vertebrates [101]. Since then the zebrafish has become a very popular model and used as a means to understand not only genetics and development of other fish but also as a means to study development in higher vertebrates.

For decades the zebrafish embryo model has been an invaluable tool for genetic and developmental studies [102,103], but recently the use of zebrafish embryos has been extended to model pathogenesis in humans including bacterial infection and tumor angiogenesis, and to study drug discovery. The zebrafish embryo model has been used
to study infections with a number of Gram-positive as well as Gram-negative bacteria. For instance, zebrafish are susceptible to *Streptococcus pyogenes, Pseudomonas aeruginosa, Staphylococcus aureus* and *Salmonella typhimurium* [104-107]. Angiogenesis-inducing factor FGF-2 and mammalian tumor xenografts have been shown to induce neovascularization in the zebrafish embryo model [108-110]. More recently, the zebrafish embryo model has been applied to large scale drug screening and to detect off-target effects of drug candidates [111-113].

The zebrafish embryo model has increasingly attracted researchers for its utility in studying human diseases. Adult zebrafish reach sexual maturity at 3 to 4 months and a female can lay an average of 200 eggs a week for up to 4 years [114]. Zebrafish embryos are translucent during the first weeks post-fertilization making them suitable for microscopy allowing real-time analysis of bacterial infection [107]. Their *ex-utero* development allows access to embryos at all stages of development making post-fertilization genetic manipulation of zebrafish embryos much easier. However, one of the most important characteristics is that they share similarities with the human immune system, having both innate and adaptive immune systems featuring the myeloid and lymphoid cells such as macrophages, neutrophils, dendritic cells, T cells and B cells [115-117].

1.6. Zebrafish innate immune system

The innate immune system, which is composed of physical barriers, cellular and humoral components, is the first line of defense against infection. In the zebrafish
embryo, antimicrobial peptides, complement components and phagocytes are present within 24 hours post-fertilization (hpf) [115,116,118].

Cellular development and maturation

In the zebrafish, hematopoiesis begins as early as 5 hpf producing three germ layers: ectoderm, mesoderm and endoderm [119]. Immune cell development occurs in waves of primitive and definitive hematopoiesis [120,121]. The primitive hematopoiesis starts in the anterior lateral plate, in which hemangioblasts differentiate into myeloid cells, and in the posterior lateral plate mesoderm which gives rise to erythrocytes [122]. The posterior lateral plate later develops and expands into the caudal hematopoietic tissue, a transitory site for erythroid and myeloid progenitor cells, from where they migrate to reside in the thymus and the pronephros, the definitive hematopoietic organs [122-124]. The pronephros develops into kidney marrow, which is the equivalent to bone marrow in humans [115]. The final wave of definitive hematopoiesis occurs in the ventral wall of the dorsal aorta in which cells that have the ability to reconstitute all of the hematopoietic lineages are produced [125,126].

Macrophages are the first leukocytes in the zebrafish embryo to differentiate [127]. The ontogeny and behavior of early macrophages were studied by Herbomel et al. using video enhanced differential interference contrast (DIC) microscopy and in-situ hybridization for hematopoietic marker genes draculin and leukocytes-specific plastin [118]. As reported by Herbomel et al., macrophages migrate into the yolk sac as precursors cells where they differentiate just before the onset of blood circulation; many subsequently enter the blood circulation while others invade the mesenchyme of the
head, retina and epidermis [118]. At 30 hpf, macrophages are seen phagocytizing large amounts of bacteria soon after intravenous injection, and they can sense microbes injected though body cavities leading to their migration and killing of the microorganisms [118]. Although myeloperoxidase (mpx), a molecular marker for neutrophils, mRNA is detected as early as 28 hpf, the presence of mature neutrophils is not documented until 48 hpf where they are seen in the trunk and tail of the zebrafish embryo [128-130]. Other myeloid cells that have been described in the zebrafish include mast cells, eosinophils and a population of dendritic-like antigen-presenting cells [117,131,132].

Transgenic reporter lines for live imaging

Transgenic reporter lines expressing fluorescent proteins driven by leukocyte-specific promoters are available to study the behavior of immune cells using live imaging. This technology helps in studying host-pathogen interactions in the zebrafish embryo model. Precursors of myeloid cells can be visualized using the spi1- or pu.-1 GFP transgenic lines [130,133,134]. In addition to being used for vasculature system visualization, the Tg(fli1:EGFP)y1 transgenic line has been used to visualize early myeloid cells [135]. The mpx or mpo promoter is used in two transgenic lines to label neutrophil populations with bright GFP and an additional population of low GFP-expressing inflammatory macrophages [136,137]. A macrophage-specific line has also been created using the promoter sequence of the mpeg1 gene [138].
Pattern recognition receptors

Like mammals and higher vertebrates, the zebrafish embryo expresses a broad range of germline-encoded pattern recognition receptors (PRRs) and adaptors. Those receptors are found on the surface, the endosomal compartments and in the cytosol of leukocytes including dendritic cells and macrophages recognizing evolutionarily conserved markers on pathogens called pathogen-associated molecular pattern (PAMPs) [139]. Some of the PAMPs expressed by zebrafish embryos include Toll-like receptors (TLRs) [140], NOD-like receptors (NLRs) [64,141], and C-type lectin receptors such as the complement activating mannose binding lectin (MBL) [142]. Upon the recognition of a PAMP, PRRs send signals to initiate pro-inflammatory and antimicrobial responses through different signaling cascades leading to production of pro-inflammatory cytokines, chemokines and antimicrobial peptides [143].

Toll-like receptors

TLRs are type I transmembrane proteins defined by the presence of an extracellular domain containing leucine-rich repeats (LRRs) and a cytoplasmic tail that contains a conserved region called the Toll/IL-1 receptor (TIR) domain that binds adaptor proteins such as MYD88, Mal, TICAM-1, TRAM or SARM at the TIR domain to initiate signal transduction [144-148]. Following interaction with PAMPs, TLRs relay signals from the cell surface to the nucleus through adaptor molecules which share a common Toll/interleukine-1 receptor (TIR) domain with TLRs [116,145]. This process leads to MAP kinases family members’ activation, NF-kB translocation to the nucleus and ultimately the activation of inflammatory cytokines and chemokines, antimicrobial
mechanisms involving production of reactive oxygen and nitrogen species, and the triggering of the adaptive immune response [149].

The TLR family was first described in *Drosophila* and was shown to be implicated not only in immune defense but also in development [150,151]. In mammals and vertebrates, however, they are mainly involved in the innate immune system [152,153]. In the zebrafish, TLRs start to express shortly after gastrulation and over 22 putative variants of TLRs including orthologs of the human TLR families have been characterized [154,155]. In humans, TLR4 recognizes lipopolysaccharide (LPS) on the outer membrane of Gram-negative bacteria, TLR1 and TLR3 recognize lipoprotein and peptidoglycan from Gram-positive bacteria, TLR5 binds to bacterial flagella, and TLRs 3, 7, 8, and 9 recognize viral nucleic acid during viral infection [156]. Most zebrafish TLRs share the same functionality with human TLRs, but because of genome duplication and alternative splicing zebrafish often have two forms of the TLRs [154]. For instance, zebrafish has two paralogs of TLR4 (TLR4a and TLR4b). However, as opposed to human, it was shown that LPS signaled through a TLR4- and MyD88-independent manner in zebrafish, and that zebrafish TLR4a and TLR4b suppressed the MyD88-dependent NF-κB activation by sequestering the TLR adaptors [157,158].

Other pattern recognition receptors

Other notable receptors described in the zebrafish antimicrobial immunity include nucleotide-binding-oligomerization-domain-(NOD-) like receptors (NLRs), RIG-I-like receptors (RLRs), scavenger receptors and C-type lectin receptors (CLRs). NLRs and RLRs are cytosolic PRRs that recognize intracellular pathogens that escape the
surveillance of the transmembrane PRRs [159]. Members of the NLR family (NOD1, NOD2) can recognize molecules on parasites and can detect the presence of bacteria through molecules produced during peptidoglycan synthesis or breakdown [160,161]. Though the function of NLR family members has not been broadly studied in the zebrafish, typical members of the mammalian NLRs are conserved and play an antimicrobial role in zebrafish embryos [162,163]. Members of the RLRs family recognize viral RNA and when activated lead to production of type I interferon which binds to its receptor to initiate expression of IFN-stimulated genes [163]. Zebrafish homologs of some RLRs have been identified; however, analysis of their putative proteins showed that their domains distribution differed from those of human [163,164].

Scavenger receptors are, on the other hand, cell surface family receptors that are not only present on immune cells namely macrophages, mast cells and dendritic cells, but they are also expressed by certain types of epithelial cells and endothelial cells [165,166]. They bind to a wide range of PAMPs which include LTA, LPS and CpG DNA [167]. Several homologs of the mammalian scavenger receptor family have been identified in the zebrafish genome; however, their functional identity remains elusive. For instance, the zebrafish macrophage receptor with collagenous structure (MARCO) gene, a specific marker for macrophages and dendritic cells, has been shown to play the same role in mammals as in zebrafish [167]. CLR s are soluble or membrane-attached carbohydrate-binding proteins expressed by most cell types including macrophages and dendritic cells [168,169]. The mannose binding lectin (MBL) is a CLR protein that binds a wide array of sugar moieties found on fungi, protozoa, viruses and bacteria and activates the complement system [170]. MBL has also been identified in
zebrafish and was shown to be associated with complement activation and resistance to bacterial infection [171].

Secreted proteins and peptide mediators of the innate immunity

Zebrfish embryos express a variety of secreted proteins and peptide mediators such as cytokines, chemokines and antimicrobial peptides that not only control the innate immune system but also play an important role in the activation of the adaptive immune system in adult zebrafish. Homologs for many mammalian secreted proteins and peptide mediators of the innate immune response have been identified in the zebrafish embryo.

Cytokines

Cytokines are small proteins secreted by a broad range of nucleated cells including macrophages, neutrophils, B and T lymphocytes and endothelial cells, and have both autocrine and paracrine activities. The primary role of cytokines is to modulate the amplitude and direction of the immune responses [172]. There are two main groups of cytokines in inflammation: the pro-inflammatory cytokines which promote inflammatory responses and the anti-inflammatory cytokines which are involved in negative regulation of the inflammatory responses. The main pro-inflammatory cytokines produced by zebrafish phagocytes include tumor necrosis factor (TNF), IL-1, IL-6, type I and type II IFNs, IL-22 and IL-26 [173-176]. In human, IL-10 is a strong anti-inflammatory cytokine and acts antagonistically to pro-inflammatory cytokine production by macrophage and T cells [177]. Zebrafish homologs IL-10 and its receptor,
IL-10R1, are identified and characterized, and are believed to play a similar role in the zebrafish immune response [178].

The importance of TNF signaling was studied in the zebrafish embryo infection model of the facultative intracellular bacterium *Mycobacterium marinum*. It was shown that TNF played a key role in controlling granuloma formation by inhibiting mycobacterial growth within macrophages and restricting their necrotic death [179]. mRNA expression levels of *tnf* and *il1b* genes were shown to increase in zebrafish embryos exposed to *Edwardsiella tarda* by static immersion as well as in adult zebrafish by injection [173]. Both pro-and anti-inflammatory cytokines were shown to be upregulated in zebrafish embryos in response to infection with *Pseudomonas aeruginosa* and *Salmonella typhimurium* [105,180]. The zebrafish homologs of the human IFNγ, IFN-γ1 and IFN-γ2, are required for resistance to bacterial infection in zebrafish embryos. The interferons were shown to be necessary to clear *E. coli* infection as well as *Yersinia ruckeri*, a natural fish pathogen that is lethal to the zebrafish embryos at low dose [181]. IFNθ and IFNθ2 are two groups of interferons in zebrafish that are closely related to human IFNs type I and type III, and shown to provide antiviral protection in a viral challenge assay [182,183].

Chemokines

Chemokines are specialized cytokines that direct the migration of cells involved in processes such as embryonic gastrulation and organogenesis, leukocyte trafficking, and immune surveillance [184]. Because of those important biological roles, chemokines have been viewed as key players in diseases involving infection,
inflammation and tumor [185]. Interleukin-8 (IL-8) or CXCL8 is the prototypical member of the CXC chemokines. This potent pro-inflammatory chemokine signals through CXCL1 and CXCL2, two G-protein coupled receptors that are known to bind chemokines other than IL-8 [186]. In addition to its chemotactic activity for neutrophils, basophils and monocytes, CXCL8 regulates the growth of endothelial cells by stimulating VEGF expression in an NF-kB-dependent manner [187]. Macrophage-derived IL-8 signaling through CXCL2 has been shown to play a role in angiogenic-dependent disorders such as tumor growth, rheumatoid arthritis, and wound healing [188].

Zebrafish homologs of CXCL8 and its receptors have been sequenced and characterized, and their signaling pathway was shown to be conserved in the zebrafish embryo [185]. However, as opposed to its mammalian counterpart, zebrafish CXCL8 lacks the angiogenic ELR (Glu-Leu-Arg) motif that is known to play an important role in the recruitment of neutrophils in humans [189,190]. Nevertheless, the expression of zebrafish embryo IL-8 was shown to be upregulated in inflammatory conditions induced by bacterial or chemical stimuli and was shown to be crucial for normal neutrophil recruitment to the wound and normal resolution of inflammation [185,191].

Complement components

The complement system is an essential humoral system of innate immunity and links the innate immune response to the adaptive immune response. This system consists of over 35 secreted and membrane-bound proteins alerting the host of the presence of pathogens and killing those pathogens [192,193]. The complement system
can be activated through three different pathways: the classical pathway which is initiated by the binding of the C1 complex to antibodies bound to structures on the surface of the pathogen, the alternative pathway which is activated by the recognition of certain structures on the surface of the microbe in an antibody-independent manner; the lectin pathway which is triggered by the binding of polysaccharides to circulating lectins, such as mannose-binding lectin [194]. These pathways merge into a common amplification step involving C3, and continuing to the cytolytic pathway forming the membrane attack complex (MAC), which lyses the cell membrane and kills the microbes [195].

Zebrafish homologs of the fundamental complement components including C3, MBL, factor B (Fb) and factor H (Fh) have been identified [142,196-198]. The mRNA levels of c3 and bf in zebrafish embryo were shown to be significantly increased in response to LPS exposure [199]. This was further supported by evidence showing that cytosol prepared from newly fertilized eggs was able to kill E. coli [195]. The antibacterial activity of zebrafish egg cytosol was also attributed to the presence of lysozyme [200]. Moreover, many zebrafish complement components could be transferred from mother to eggs as evidence showed that immunization of adult female zebrafish with Aeromonas hydrophila resulted in an increase of C3 and Bf protein levels both in the mothers and early embryos [201].

Antimicrobial peptides

Antimicrobial peptides are small cationic peptides of less than 100 amino acids that are an important component of the innate immunity, and are found in plants and
animals [202]. They have a wide bactericidal activity that includes killing of bacteria, viruses and fungi [203-205]. Small antimicrobial peptide genes such as defensins, hepcidin and phosvitin have been sequenced and characterized in the zebrafish embryo [206-209]. While some antimicrobial peptides are constitutively expressed and synthesized by cells such as keratinocytes, monocytes, neutrophils and epithelial cells, many are induced [209]. It was demonstrated that phosvitin played an important role in zebrafish embryos not only as an antimicrobial effector capable of killing microbes but also by acting as a pattern recognition receptor recognizing PAMPs of Gram-negative and Gram-positive bacteria [208]. Hepcidin gene expression was shown to be increased in fish that developed signs of bacterial infection [206].

Antisense morpholino knockdown of innate immunity mediators

Morpholinos are the most commonly used tool to knockdown genes in the zebrafish embryo. While some morpholinos work by specifically binding near the 5’ UTR of the target RNA to block the access to the ribosomal initiation complex thus inhibiting protein translation, others work by blocking pre-mRNA splicing. In either case, the effect of morpholinos is most effective when injected at the 1- to 2- cell stage of the embryo; it results in a variable period (4 to 7 days) of transient knockdown of specific genes allowing researchers to study their role in zebrafish [210,211]. The role of MyD88, the most common adaptor for TLR signaling, in response to S. typhimurium infection in the zebrafish embryo was studied using a morpholino knockdown approach [180]. The induction levels of irak3, mmp9 and il-1b were shown to be significantly suppressed in the MyD88 morphants [180]. The Spi1/Pu.1 transcription factor is important for normal
development of the myeloid cell lineage, and morpholino knockdown of this gene results in embryos lacking macrophages and neutrophils [212]. Therefore, morpholino knockdown of this gene has often been used for infection studies in the zebrafish embryo model. These morphants showed increased susceptibility to infection with bacteria such as *P. aeruginosa, E. coli* and *S. aureus* [105,106,213,214]

1.7. Zebrafish adaptive immune system

While the zebrafish embryo has a fully mature innate immune system by 48 hpf, the adaptive immune system is not fully developed until 4- to 6- weeks post-fertilization [215]. However, B cell and T cell progenitors begin undergoing recombination activating gene (*rag*)-dependent rearrangements within the kidney and the thymus, respectively, by 4 dpf [103]. Evidence for the existence of dendritic cells (DCs) and their function as professional antigen presenting cells (APCs) have been reported in the zebrafish [117,216]. As in humans, B cell and all T cell types, CD4+ T cells, CD8+ T cells and CD4+ CD25+ T regulator cells, have been described in zebrafish although their functional studies in the zebrafish are still lacking [217]. The adaptive immune response to infection initiates with APCs presenting processed foreign peptides derived from microbes to lymphocytes [217]. It is speculated that this process occurs primarily in the spleen, the secondary lymphoid organ of the zebrafish where DCs reside [117], and can also take place in the gut, where large numbers of DCs and lymphocytes can be found [218,219].
1.8. Zebrafish vasculogenesis and angiogenesis

The zebrafish circulatory system is as complex as that of mammals. Zebrafish vascular formation is composed of two main processes: vasculogenesis and angiogenesis. Like other vertebrates, zebrafish vasculogenesis involves the differentiation of lateral mesoderm to hemangioblasts which then differentiate into angioblasts and endothelial cells [112]. Angiogenesis, on the other hand, is the process through which new blood vessels are formed from existing vessels. It occurs during normal tissue growth and repair; it involves the activation and division of endothelial cells within an existing vessel inducing enzymatic activities that cause local breakdown of the vessel and the subsequent sprouting of new vessels [112]. The expression of hemangioblast markers such as the endothelium-specific receptor tyrosine kinase VEGFR2/Flk1 and the stem cell leukemia protein (SCL/Tal-1) have been detected in the lateral mesoderm of the zebrafish embryo by 12 hours post-fertilization [220,221]. By 24 hpf, the zebrafish embryo develops a simple blood circulation loop in which the blood from the dorsal aorta and axial vein circulates through the yolk sac circulating valley prior to returning to the heart. Then by 72 hpf, a complete vascular system is formed containing the intersegment vessels (ISVs) of the trunk stemming from the dorsal aorta and the subintestinal vessels (SIVs), which are originated from the Duct of Cuvier through angiogenic processes [110].

Angiogenesis plays a critical role in tumor growth and metastasis and many genes that are involved in angiogenesis in higher vertebrates and mammals have also been identified in the zebrafish embryo. Among them are the VEGF and its tyrosine
kinase receptors (Flk-1 and Flt-1), and angiopoietin [222-224]. The vegf-A gene and its splicing isoforms, including the two dominant forms in vegf-A$_{165}$ and vegf-A$_{121}$, have been isolated and characterized in the zebrafish embryo [222,225]. The role of VEGF in vasculogenesis, angiogenesis, and hematopoiesis in the development of the zebrafish embryo has been studied. Liang et al. demonstrated that the overexpression of both isoforms resulted in early onset and increased transcript levels of endothelial cell markers (flk1, tie) and hematopoietic cell lineage markers (gata1, scl) [226]. Several studies show that overexpression of vegf leads to ectopic vasculature in the developing zebrafish embryo, which occurs via the interaction of VEGF with FLK1 and sydecan-2 receptors [226-228]. Moreover, VEGF has been shown to be upregulated in pharmacologically induced vessel sprouting as well as pathologically induced angiogenesis in the zebrafish embryo [108,110,229-231].

At least three homologs of mammalian angiopoietin genes (ang1, ang2, and angptl3), and the endothelium-specific tyrosine kinase receptors (tie1 and tie2) have been isolated in the zebrafish by Pham et al. and Lyons et al. [223,232]. Their transcripts were shown to be present in all EC of both developing and mature blood vessels. Furthermore, three angiopoietin-like proteins (angptl1, angptl2 and angptl6), homologous to human angiopoietin-like proteins, were isolated and characterized in the zebrafish embryo by Kubota et al. [233]. Contrary to angiopoietin signaling which occurs through Tie 1 or Tie 2 receptors, angiopoietin-like proteins do not interact with the Tie receptors. Another study by Kubota et al. has shown that cooperative interactions of Angptl1/2 play an important role in vascular development and angiogenesis [234]. They
were shown to bind endothelial cells displaying antiapoptotic activities via the PI3-K/Akt pathway in vitro [234].

Several vascular-specific transgenic lines of the zebrafish embryo including Tg(fli1:EGFP), VEGFR2-GFP, and Tie2-GFP have been developed. The Tg(fli1:EGFP)y1 strain, which expresses green fluorescent protein in the vasculature, has been used to model tumor angiogenesis using mammalian tumor xenografts as well as human metastatic melanoma cells [108,109]. Those reports and others showed evidence of tumor cell growth accompanied with the disruption of normal vessel pattern; sprouting of the vessels and tumor metastasis as seen in mammals [108,109,235]. The angiogenic response in the zebrafish embryo model is often quantitatively assessed by measuring the size, the length and the branching of the blood vessels [110,236]. Moreover, quantitative real-time PCR (qRT-PCR) and ELISA methods to assess changes in gene expression in response to angiogenic stimuli are sometimes used to complement phenotypic observations in the zebrafish embryo model [237,238].

1.9. Objective

*Bh* causes a wide range of infection in human, but it is known mostly for its unique ability to cause bacillary angiomatosis, a disease characterized by vasoproliferative tumor-like lesions in some immunosuppressed individuals. Over the past 15 years, *Bartonella* research has been advancing at a fast pace due in part to the availability of the complete genome sequence for *Bh* [73]. This has enabled the establishment of gene function and definition of important virulence factors associated with *Bh*-induced pathogenesis. Although much is known about *Bh* and those virulence
factors from studies using in vitro systems, their molecular mechanisms and overall contribution to Bh pathogenesis remains elusive and can only be fully understood by using an in vivo model. Therefore, there has been a great need for a practical animal model to study Bh pathogenesis and host response. To date, efforts to establish an in vivo model to study Bh pathogenesis have for the most part been unsuccessful. We proposed to use the zebrafish embryo to study Bh infection and host response. Based on our literature review and our preliminary data, we hypothesized that: the zebrafish embryo is a suitable model of transient infection with Bh requiring the action of the adhesin BadA and the type IV secretion system and is ultimately cleared by the innate immune response of the embryo.

In order to test this hypothesis, the following objectives were developed and studied:

1) Define the optimal conditions necessary to maintain the longest possible sustained infection of zebrafish embryos with Bh

2) Examine the zebrafish response to Bh infection

3) Determine if the trimeric autotransporter Bartonella adhesin A (BadA) and the VirB/VirD4 T4SS are required in Bh infection in the zebrafish embryo in vivo model
Chapter 2
Materials and Methods

2.1. Bacterial strains and growth conditions

The DsRed2 labeled kanamycin-resistant *Bh* strains were cultured on heart infusion agar (Remel, Thermal Fisher Scientific, Lenexa, KS) supplemented with 1% bovine hemoglobin (chocolate agar) (Remel, Thermo Fisher Scientific, Lenexa, KS) and 50 µg/ml of kanamycin or in Schneider’s liquid medium (Sigma-Aldrich, St Louis, MO) supplemented with 10% fetal bovine serum and 50 µg/ml of kanamycin for 3-4 days at 37°C in the presence of 5% CO₂ [239]. *Escherichia coli* (*E. coli*) strains were grown in Luria-Bertani (LB) broth or agar at 37°C with no CO₂. All manipulations of infectious agents in this project were approved by the USF Institutional Biosafety Committee.

2.2. Animal care and strains

Transgenic zebrafish embryos *Tg(mpx:GFP)uwm1* were purchased from Zebrafish International Resource Center (Eugene Oregon). Adult breeders of the transgenic line *Tg(fli1:egfp)y1* were a kind gift from Dr. Brandt Weinstein (NCI, Rockville, MD). Zebrafish breeders were maintained at 28 ± 0.5°C in a 14h:10h light/dark cycle in our breeding system designed by Aquatic Habitats (Apopka, FL) in accordance with standards established in the “Zebrafish Book” [240]. In the evening adult breeders were placed in a 1.5 ml breeding tank in a female to male ratio of 1:1 or 2:1 separated with a
divider. The next morning the divider was removed to allow mating and egg production. The eggs were collected and transferred to the laboratory where they were washed with 0.065% of bleach in zebrafish embryo water and rinsed three times with fresh embryo water. After washing, the unfertilized eggs were discarded and the fertilized eggs were kept in fresh embryo water containing 2 µM of methylene blue at 30°C overnight. Methylene blue helps inhibit growth of fungi in the water. Infected embryos were kept at 30°C for a week post-fertilization.

2.3. Ethics Statement

This study was performed in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health. Zebrafish embryos and adults were maintained in accordance with standards established in the “Zebrafish Book” [240], and by the guidelines outlined by the Institutional Animal Care and Use Committee (IACUC) at the University of South Florida. The experiments using zebrafish embryos described in this report were approved by the USF IACUC under protocol R4174.

2.4. Construction of Bh knockout mutants

Bh genomic DNA extraction

Bh genomic DNA was prepared using our standard laboratory protocol to extract genomic DNA from Bh. One quarter to half plate of 3 to 4 day growth bacteria was
harvested in 400 ml of 1x Tris EDTA (TE) buffer (pH 7.5), and the bacteria were lysed in a 1% final concentration of sarkosyl and 100 µg/ml final concentration of proteinase K for 90 minutes at 55°C. After cooling down to room temperature (RT), the sample was extracted through a series of steps of phenol/chloroform treatment and centrifugation at 16,000 x g for 3 minutes using an Eppendorf Centrifuge (USA) – the sample was treated with 1 volume of saturated phenol (pH 6.6) twice followed by chloroform (chloroform/isoamyl alcohol 24:1) treatment 2 to 4 times or until the top layer which contained the DNA was clear, which then followed by a final chloroform (chloroform/isoamyl alcohol 24:1) extraction. The DNA was then precipitated in 0.1 volume of 3 M NaOAc and 2.5 volume of absolute ethanol (EtOH) overnight at -20°C. The precipitate was spun down for 30 minutes at 16,000 x g at 4°C and the supernatant was discarded. The pellet was rinsed with cold 70% EtOH and spun at 16,000 x g for 5 minutes. The supernatant was discarded and pellet was dried in a Speed Vac SC100 (Savant Instrument, Inc., Hicksville, N.Y.) for 10 minutes. The DNA was then resuspended in 30 µl of TE/RNAse (0.2 mg/ml of RNAse). Note that the TE/RNAse solution was heated at 80°C for 20 minutes to inactivate any contaminating DNAse.

Preparation of plasmid DNA from *E. coli*

*E. coli* was inoculated in LB broth and grown overnight at 37°C with shaking at 220 RPM. Plasmid DNA extraction was prepared using the Pure Yield Plasmid Midi Prep System according to the manufacturer's instruction (Promega, Madison, WI). Briefly, overnight culture was pelleted by centrifugation at 5000 x g for 10 minutes. The pellet was resuspended in Cell Resuspension Solution and an equal volume of Cell
Lysis Solution was added. The mixture was inverted 3 to 5 times and incubated for 3 minutes at RT. Neutralizing Solution was added to neutralize the lysis solution by inverting the sample and incubating for 3 minutes to allow proteins and cellular debris precipitation. The sample was then purified using a Pure yield Cleaning Column and centrifugation at 1500 x g for 5 minutes to separate the solution containing the DNA from the debris. The sample was then added to a Pure Yield Binding Column and spun at 1500 x g for 3 minutes to bind the DNA to the column. The column was washed with Endotoxin Removal Wash Solution containing isopropanol. The sample was spun at 1500 x g for 3 minutes and the column was washed again with Column Wash Solution containing EtOH and was spun as before followed by centrifugation at 1500 x g for 10 minutes to ensure that residual EtOH was removed. To elute the DNA, the column was placed in a new tube, followed by the addition of nuclease-free water and centrifugation at 2000 x g for 5 minutes.

In-frame deletion mutants of the full length *badA* (BH01510) and the *virB* operon (*virB*2-*virB*11) were constructed in *Bh* Houston-1 using the two-step mutagenesis strategy described by Mackichan et al. with some modifications [241]. *Bh* genomic DNA was used as template for PCR to generate two fragments of the gene. The first fragment contained an upstream noncoding region and included a small segment of the 5’ part of the gene whereas the second fragment contained a downstream region and a 3’ segment of the gene. The two purified PCR products were used as templates for megaprime PCR using only the forward primer from fragment 1 and the reverse primer from fragment 2. The resulting product was purified and ligated into the “suicide” plasmid pJMO5 at the *BamH*I restriction site.
The plasmids containing the deleted gene were transformed into DH12S *E. coli* (Life Technologies) and then incorporated into *Bh* Houston-1 by transconjugation using a two-step allelic exchange strategy [241]. The pJM05 derivative integrated into the *Bh* chromosome by homologous recombination with the sequences flanking the target gene. Transconjugates were selected by plating on 5% rabbit blood agar supplemented with kanamycin (30µg/ml), nalidixic acid (20µg/ml), and cefalozin (2µg/ml). The colonies were then counter-selected on agar containing 10% sucrose to promote excision of the integrated plasmid by a second cross-over event resulting in replacement of the full length gene with the truncated version. PCR was performed on genomic DNA isolated from kanamycin-sensitive sucrose-resistant colonies to confirm the knockout genotype. The deletion mutants were verified by sequencing across the deleted region and by performing RT-PCR to ensure the absence of the mRNA from the mutants.

2.5. Construction of red fluorescent protein expressing strains of *Bh*

The plasmid vector pDsRed2 (Clonetech, Mountain View, CA) was used as a template to amplify the *Discosoma sp. dsred2* gene for cloning into pNS2T5 vector containing a kanamycin resistance gene [242]. The amplicon was digested with *BamHI* and *XbaI* (New England Biolabs) and then ligated into similarly digested pNS2T5 such that the *dsred2* gene was expressed as a 6xHis tagged fusion protein. The resulting plasmid, pNS2T5-DsRed2, was electroporated into *Bh* Houston-1 wild-type, Δ*badA*, and Δ*virB* mutants making red fluorescent protein (RFP)-expressing strains
Bh/pNS2T5DsRed2, BhΔbadA/pNS2T5DsRed2, and BhvirB/pNS2T5DsRed2, respectively.

A chromosome knock in strain of Bh Houston-1 expressing DsRed2 (Bh::DsRed2) was constructed by cloning of the T5 promoter-dsred2 into the multicloning site (MCS) of EZ-Tn5 pMOD-6<Kan-2/MCS> transposon construction vector (EPICENTRE, Madison, WI). The KanT5 DsRed transposon DNA was amplified by PCR and purified using the Wizard SV Gel and PCR Clean-up System according to the manufacturer’s instructions (Promega). The stable transposome was prepared with the transposon DNA and the transposase enzyme according to manufacturers’ protocol and electroporated into Bh Houston-1. After kanamycin selection, transposon knock in strains were identified by confocal microscopy as those expressing RFP. Further, the clones were subjected to inverse PCR and sequencing methodology to confirm that the DsRed2 transposon was not inserted into an important gene. It was determined that the knock-in strain selected for further use had the transposon introduced in the intergenic region upstream of the hypothetical protein BH14030 and downstream of BH14040. Growth curve experiments showed no difference in growth between the chromosomally inserted DsRed2 or the plasmid encoded DsRed2 Bh Houston-1 strains when compared to WT Bh (data not shown).

2.6. Zebrafish embryo staging and microinjection

Before microinjection, embryos were staged at 24-28 hours post-fertilization (hpf) and manually dechorionated using two pulled-glass needles under a dissecting scope
(Nikon). Embryos were anesthetized in 0.02% tricaine methanesulfonate (Sigma) in embryo water [240]. Microinjection needles were pulled from 1.0-mm borosilicate glass micropipette using the PC-10 vertical puller (Narishige).

*Bh* and *E. coli* cultures were pelleted by centrifugation at 3000 x g for 5 min and resuspended in 0.05% phenol red in phosphate-buffered saline (PBS; pH=7.4) to help monitor the microinjected solution. The suspension was serially diluted and plated to determine the number of colony forming units (CFUs) indicating the number of viable bacteria. All *Bh* plates were incubated at 37°C with 5% CO₂ for 10 days; *E. coli* bacteria were plated on LB agar and incubated overnight at 37°C. For microinjection, anesthetized embryos were placed into a holding groove in 3% agarose to immobilize them for microinjection. Embryos were microinjected in the yolk sac or the blood circulation using thin pulled-glass needles attached to a Kite micromanipulator (WPI, Sarasota, Florida), a PV830 pneumatic pump microinjector (WPI, Sarasota, Florida), and a SMZ 1500 dissecting microscope (Nikon). To determine the infection doses, bacterial suspensions were expelled into microcentrifuge tubes containing 1X PBS before, during and after microinjection and plated for CFU enumeration or saved for qPCR experiments. For angiogenic response experiments, positive control embryos were injected with 10 ng of recombinant zebrafish VEGF (rzfVEGF) (R&D Systems; Minneapolis, MN).

2.7. Digital imaging and microscopy of zebrafish embryos

To monitor bacterial infection and host interaction in real time, embryos from the experimental and control groups were anesthetized and suspended in a depression
slide in 0.05% agarose/embryo water. Live embryos were examined with a 20x LUC Plan FLN 0.45 N.A. objective using an Olympus FV1000 confocal laser scanning microscope (Olympus America, Center Valley, PA). EGFP-vasculature of the embryo and DsRed2-expressing bacteria were imaged by a 488 nm laser with 475-519 nm spectral emission setting and a 543 nm laser with 525-615 nm emission, respectively. For time-lapse imaging, live embryos were viewed with a 20x UPLSAPO 0.75 NA, WD 0.65mm objective using an Olympus IX81 inverted microscope equipped with the 3i Yokogawa spinning disk scanner and CDD cameras. Some pictures were viewed using the FluorView 10-ASW 1.7 and analyzed by using the NIH ImageJ software. Others were analyzed using the SlideBook 5.5 software and the SMRecorder software to make 3D surface view videos showing the interaction of the RFP-expressing bacteria and the GFP-expressing endothelial cells. Adobe Photoshop CS5.1 software was subsequently used to arrange and format the images and all micrographs were reported as projected z-series.

2.8. Bacterial enumeration from zebrafish embryos

*Bh* infection and viability in the zebrafish embryo host were determined at different time-points after infection. Three to five embryos from of the infected and control groups were washed 3 times with 1X PBS and transferred to 1.5 ml tubes. Whole embryo tissues were disrupted in 0.1% saponin/PBS solution using sterile disposable plastic pestles. The suspension was serially diluted and plated on chocolate agar plates containing 50 μg/ml of kanamycin. The plates were incubated at 37°C with
5% CO₂ for 10 days to determine number of CFUs. The means of triplicate samples were used for comparison between experimental and control groups at different time points.

2.9. Genomic DNA extraction and qPCR

Infected and control zebrafish embryos were individually stored at -80°C at different time points for genomic DNA extraction. Embryos were homogenized individually and total DNA was extracted using the Wizard Genomic DNA Purification Kit (Promega) according to the manufacturer’s instructions for extracting genomic DNA from animal tissue and Gram-negative bacteria with slight modifications. Before the last isopropanol precipitation step, 5 μg of glycogen was added to help precipitate the nucleic acid. The final DNA pellet was resuspended in 20 μl of TE. To construct a standard curve, known amounts of Bh genomic DNA purified by the same procedure were utilized as template for qPCR. The genome equivalent (GE) per microliter/template was calculated by dividing the concentration (g/μl) of the extracted DNA by the molecular weight of the Bh genome (2.08 x 10⁻¹⁵ g/copy). The bacterial nucleic acid was serially diluted to generate standards facilitating conversion of real-time PCR cycle threshold values to Bh NADH dehydrogenase gamma subunit G gene (nuoG) (BH08890) copies per embryo. Quantitative polymerase reactions (qPCR) were run in 25 μl volume reactions containing 12.5 μl of 2X iQSYBR Green Supermix, 300 nmol of each primer, and 5μl of extracted DNA using the cycling parameters as follows: 95°C for 3 min, followed by 40 cycles of 60°C for 30s and 55°C for 1 min. The primer sequences
for qPCR were as follows: nuoG forward 5’-GGCGTGATTGTTCTCGTTA-3’; nuoG reverse 5’-CACGACCACGGCTATCAAT-3’ [243]. A melting curve analysis was performed to confirm that no primer-dimers were amplified. All samples were run in triplicate, and the average GE for 5 embryos was calculated.

2.10. Antibacterial activity

In order to induce the production of antimicrobial factors, zebrafish embryos were staged at 28 hpf and inoculated in the yolk sac with *Bh*. Pools of 20 embryos were saved at -70°C at day 4 post-inoculation and homogenized with a pellet pestle. The lysate was spun down at 3500 x g for 5 minutes at 4°C to remove the debris. The supernatant was collected into a 1.5 ml tube and filtered through a pre-wetted 0.45μm filter to remove any bacterial contaminant including *Bh*. HMEC cells were also harvested to use as a control. HMEC cells were cultured without antibiotics in a flask and washed 5 times with 1X PBS to remove growth supplements. HMEC lysates were sonicated using a Sonic Dismembrator 120 (Fisher Scientific) for two minutes in 15 seconds interval at 30% setting on ice. The lysate was processed as was done for zebrafish lysate. The filtered supernatants were then transferred into YM-3 centricons (Millipore, USA) and spun at 4,000 x g for up to 40 minutes at 4°C to concentrate the protein. YM-3 centricons have a 3,000 nominal molecular weight limit (NMWL) cut-off, which allows the retention of small antimicrobial peptides. The amount of protein in the lysate was calculated by a BCA protein assay. *Bh* culture was prepared in Schneider’s medium and seeded into 3 sets of triplicate wells in a 96-well plate. Embryo lysates were added into one set of triplicate wells as the experimental group, HMEC lysates...
were added into another set of triplicate wells and 1x sterile PBS was added into the last set of triplicate wells. The 96 well-plate was sealed and incubated at 30°C for up to 5 days.

### 2.11. RNA isolation

Pools of 10 embryos were stored at -80°C for RNA extraction and purification as described by Leung et al. [244]. Briefly, frozen embryos were homogenized with Trizol Reagent (Invitrogen, Grand Island, NY) using an RNase-free Kontes pellet pestle (Fisher Scientific, Pittsburg, PA). The samples were spun at 21,000 x g for 2 minutes in a QIAshredder column (Qiagen, Valencia, CA) for further homogenization. Subsequently, total RNA was extracted twice with chloroform (chloroform/isoamyl 24:1) in Heavy Phage Lock Gel tubes (Fisher Scientific, Pittsburg, PA) to separate the aqueous phase from the organic phase. The extracted RNA was then purified using the RNeasy MinElute Cleanup kit (Qiagen, Valencia, CA) according to the manufacturer’s instructions. The RNA was eluted using diethylpyrocarbonate (DEPC)-treated water. The RNA was subsequently treated with Turbo DNase (Ambion) according to the manufacturer’s protocol. The yield and the integrity of the extracted total RNA was evaluated by measuring the ultraviolet (UV) absorbance using the ND-1000 nanodrop spectrophotometer (Thermo Scientific, Wilmington, DE) and by agarose gel electrophoresis.
2.12. Microarray

The microarray experiments and data analysis were performed in the Moffitt Cancer Center and Research Institute Molecular Genomics Core Facility. RNA from pools of \textit{Bh}-infected and control embryos was isolated as described above. The resulting RNA (100 ng) was used as a template to generate amplified complementary DNA (cDNA) via a three-step process called Ribo-SPIA™ using the Ovation® Pico WTA System V2 kit and the WT-Ovation® Exon Module according to the manufacturer’s instructions (NuGEN Technologies, San Carlos, CA) and as described by Clement-Ziza et al. [245]. The hybridization mixture was prepared to accommodate 5 µg of cDNA target. The Zebrfish Gene 1.0 ST Arrays were hybridized, washed, and stained according to the Affymetrix protocol. The arrays were scanned using a GCS3000 7G scanner (Affymetrix) and images (DAT files) were converted to CEL files using AGCC software (Affymetrix). Robust Multi-array Average (RMA) data analysis was performed using expression console V1.2 Affymetrix. Differential gene expression analysis was performed using Excel.

2.13. cDNA synthesis and qRT-PCR

The DNAse-treated RNA (1 µg) was reverse transcribed into cDNA in a 20 µl reaction using the iScript cDNA Synthesis Kit (Bio-RAD). Real-time PCR was performed using the iCycler IQ real-time PCR detection system (Bio-Rad). Each reaction was performed in a 25 µl volume containing 2 µl (100 ng) of cDNA, 12.5 µl of 2X iQSYBR Green Supermix (Bio-Rad) and 300 nmol of each primer. All reactions were performed in triplicate, and zebrafish elongation factor -1 (\textit{elf1α}) or ribosomal protein L13 (\textit{rpl13})
was used as endogenous gene for normalization. Cycling parameters were 95°C for 3 min, followed by 40 cycles of 60°C for 30s and 55°C for 1 min. Melting curve analysis was performed to confirm that no primer dimers were amplified. Results were analyzed using the comparative $C_T$ method ($2^{-\Delta\Delta C_T}$) [246].

### 2.14. Statistical analysis

The SigmaPlot 11.0 software was used to graph and statistically analyze the data. One Way Repeated Measures of Analysis of Variance (One Way Repeated ANOVA) test was used to assess the significance in the increase in bacterial burden at different time points post-infection. Student’s t-test was used to determine the significance of the angiogenic phenotype difference in the mean values of the intersection points (IPs: are the intersections where the subintestinal vessels branch out) and the number and length of the subintestinal vessel among the infected and the control groups. The Mann Whitney Rank-Sum Test was used to analyze the difference in the infectivity of $Bh$ WT compared to the $\Delta$badA mutant at the different time points. The error bars represent the standard error of the mean.
Chapter 3

Results

3.1. Zebrafish embryo–site of injection with \textit{Bh} and incubation condition

In most zebrafish infectious disease models, infection is achieved by microinjecting the microbe into the embryo. However, zebrafish infection by immersion has been achieved by some natural fish pathogens as is the case for \textit{Edwardsiella tarda} [173]. We attempted to infect dechorionated zebrafish embryos at 24 hours post-ertilization (hpf) by static immersion for 16 hours with $10^8$ CFU/ml of \textit{Bh} Houston-1 (WT) carrying a plasmid expressing red fluorescent protein (\textit{Bh/pNS2T5DsRed2}). Results from confocal microscopy and colony forming unit (CFU) counts from homogenate plating of embryos were negative for \textit{Bh} (data not shown). Therefore, we used a microinjection technique as the means of introducing the bacteria into the embryos. Since zebrafish optimal temperature is 28.5°C and \textit{Bh} are exposed to 37°C in the human host, we assessed the infection pattern in infected embryos kept at constant temperatures of 28.5°C versus those that are kept at 30°C or 33°C. No significant difference in infection pattern was observed, and the proper development of the embryos did not seem to be compromised (data not shown). For all subsequent experiments, embryos were kept at 30°C to allow for both sufficient bacterial growth and to maintain the health of the embryos.
For most bacteria studied using the zebrafish embryo model, infection is achieved by microinjection via different sites depending on the type of experiment being performed. Microbes can be injected in the yolk sac, the posterior blood island or the Duct of Cuvier (yolk sac circulating valley) to initiate systemic infection, whereas a local infection can be achieved by injecting the microbes into the hindbrain, the tail muscle, or the otic vesicle (Fig. 3.1A) [107,118,130,247,248]. In an effort to determine the optimal infection route, \( Tg(fli1:egfp)^{y1} \) transgenic embryos were staged at 28 hpf and microinjected either in the yolk sac or the blood circulation valley with approximately \( 3 \times 10^3 \) CFU of \( Bh \) Houston-1 (WT) expressing red fluorescent protein from a single transposome inserted into the chromosome (\( Bh::DsRed2 \)). Bacteria injected in the blood circulation were cleared by day 2 post-injection (dpi) as shown by the DsRed2 signal, whereas those inoculated in the yolk sac near the developing subintestinal vessels replicated and persisted throughout the duration of the experiment (3 dpi) (Fig. 3.1B).

**Figure 3.1 Zebrafish embryo injection site with Bh. A)**, Schematic representation of the zebrafish embryo at approximately 48 hpf highlighting the possible sites of injection (not drawn to scale). **B**), Representative micrographs of embryos inoculated in the yolk sac circulation valley (row 1) or the yolk sac (row 2) at 28 hpf with an average of \( 3 \times 10^3 \) CFU \( Bh::DsRed2 \). The same embryos were photographed by confocal microscopy at the indicated time points. Scale bar = 100 µm.
3.2. **Bh infection and survival in the zebrafish embryo host**

In order to assess *Bh* infection and viability in the zebrafish embryo host, *Tg(fli1:egfp)y1* transgenic embryos were staged at 28 hpf and microinjected in the yolk sac near the developing subintestinal vessels with 3 x 10^3 genomic equivalents (GEs) of *Bh::DsRed2*. Microscopy results showed an increase in bacterial burden with peak fluorescence observed on day 3 post-infection (dpi) and persistence through the entire duration of the experiment (6 dpi) (**Fig. 3.2**).

To determine the viability of the bacteria in the host, embryos were inoculated with approximately 5 x 10^3 CFUs or PBS phenol red control, and three embryos were homogenized individually and plated in triplicate on selective kanamycin chocolate agar plates (50 µg/ml) at different time points post-infection for bacterial enumeration. Results showed an increase in CFU count which peaked at 3 dpi and started decreasing at 4 dpi (**Fig. 3.3A**). An alternative qPCR method using primers specific to

**Figure 3.2. Live imaging of zebrafish embryos infected with Bh.** Confocal imaging of embryos inoculated with 3 x 10^3 GEs of *Bh::DsRed2* (row 1) or PBS phenol red control (row 2) by microinjection into the yolk sac. All images shown are representatives of the pool of embryos photographed. Scale bar = 100 µm.
the *Bh* NADH dehydrogenase subunit G gene (*nuoG*, BH08890) was used to determine GEs/embryo. Embryos microinjected at 28 hpf with approximately $3 \times 10^3$ GEs of *Bh* were homogenized; the bacterial nucleic acid was extracted and the GE/embryo was determined by qPCR. Embryos mirrored the infection pattern observed by confocal microscopy and plating with bacterial burden increased by almost 10-fold by day 3 (*p = 0.02) and persisted in the embryos for 8 days (Fig. 3.3B). The limit of detection of the assay was approximately 10 copies of *nuoG* per reaction and the sequence was not amplified from the uninfected control embryos (data not shown).

**Figure 3.3. Bh infection and survival in the zebrafish embryo host** (A) Average CFUs (± SEM) from homogenate plating of embryos inoculated with approximately $5 \times 10^3$ CFUs of *Bh*. (B) Average GE/embryo determined by qPCR in embryos inoculated at 28 hpf with approximately $3 \times 10^3$ GEs of *Bh*. Values are the means (± SEM) of triplicate wells for 5 different embryos at the indicated time points. Significant increase in bacteria burden was observed from day 0 to day 2 with **p = 0.013**, from day 0 to day 3 with *p = 0.02* and from day 0 to day 4 with ***p = 0.002*. As expected the *nuoG* sequence was not amplified from uninfected control embryos.

### 3.3. Bh interaction with zebrafish embryo vascular endothelium

Tissue samples from patients with BA lesions revealed the presence of immature capillaries that are lined with inflamed endothelium and filled with bacterial aggregates
Therefore, the location of the bacteria, or bacterial aggregates, in relationship to the vascular endothelial cells in infected Tg(fli1:egfp)y1 embryos was determined by confocal microscopy (Fig. 3.4). Microscopy evidence showed that some of the red fluorescent Bh were colocalized with the green fluorescent angioblasts, the precursor of vascular endothelial cells, at day 1 post-infection (Fig. 3.4B), and with the mature vascular endothelium at day 2 post-infection (Fig. 3.4C) as shown by the arrows in inset images S1 and S2 at the respective time points. However, subsequent analysis was necessary to ascertain whether the bacteria were located either inside or merely interacting with the host cells. The SlideBook 5.5 software was used for three-dimensional analysis of the micrographs and videos were made using the SMRecorder software. Analysis showed that aggregates of bacteria were interacting with the EC; some were inside of the cells while most were located outside or away from the endothelium (data not shown).

3.4. Recruitment and accumulation of phagocytes to the site of infection with Bh

Bh-induced angiogenic lesions in HIV patients have been shown to be infiltrated with phagocytes [60,61]. To investigate if Bh could recruit phagocytes to the site of infection, we injected Bh::DsRed2 in the yolk sac of Tg(mpx:GFP)uwm1 embryos at 50 hpf, a transgenic zebrafish line that expresses GFP under the neutrophil-specific myeloperoxidase promoter [235]. Upon microscopic examination of live, infected embryos using the Olympus IX81 inverted microscope equipped with the 3i Yokogawa spinning disk scanner and CDD cameras, we observed that the infection induced the
accumulation and colocalization of neutrophils (bright green) with the bacteria (red) at the site of infection within 2 hpi (Fig. 3.5; top panel). The number of phagocytic cells increased during the course of infection as shown by the increase in neutrophils (bright green) (Fig. 3.5; arrow; top panel) and what we presumed to be macrophages (larger, light green) (Fig. 3.5, arrowhead; top panel) at day 4 post infection in the infected embryos. Time-lapse imaging further confirmed our observation with the slow moving macrophages (data not shown). Macrophages have previously been observed by 24 hpf in zebrafish embryos based on morphology and phagocytic capacity [118,213,248]. As observed in the videos, some bacteria were engulfed by the neutrophils and macrophages; however, most of the bacteria were seen outside of the phagocytic cells. Compared to the infected embryos the sham injected control embryos did not show as many phagocytes in the yolk sac (Fig. 3.5; 2 hpi and 4 dpi; bottom panels).

Figure 3.4. Interaction of Bh with GFP-labeled endothelial cells in Tg(fli1:egfp)y1 zebrafish embryo. (A) Non-infected control embryo at day 1 post inoculation with PBS/phenol red. (B) and (C) Micrographs of embryos at day 1 and day 2 post-inoculation, respectively. Insets S1 (scale grid = 50 µm) and S2 (scale grid = 10 µm) are higher magnification images of the selected area of Bh and EC interaction. Arrows show areas of adherence and intracellular location of the RFP-labeled bacteria with GFP-labeled EC of the vessels. Images are representatives of pools of embryos micrographed. Scale bar = 50 µm.
3.5. Antibacterial activity in zebrafish embryo extract

In an effort to determine if zebrafish embryo extracts possess bactericidal activity against *Bh*, extracts from pools of embryos that were primed with *Bh* were prepared after 4 days post-inoculation. This time point was chosen due to evidence showing that the bacteria burden peaked at 3 dpi and started reducing on day 4 or 5 post-infection in the embryos. HMEC lysate was used as a control. The lysates were filtered and the amount of protein was quantified by BCA protein assay. The lysates were plated to ascertain that they were free from bacterial contamination including *Bh*. An average of

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**Figure 3.5. Infiltration and colocalization of GFP-labeled phagocytes with *Bh*.** Representative micrographs from pools of *Tg(mpx:EGFP)umw1* embryos inoculated at 50 hpf with 300 CFU of *Bh* WT (top panel) or PBS/phenol red control (bottom panel) at the indicated time points post inoculation. White arrows are DsRed2 *Bh* colocalized with GFP-labeled neutrophils (bright green; 2 hpi and 4 dpi)). Arrowheads are DsRed2 *Bh* interacting with macrophages (larger phagocytes) (4 dpi only). Compared to the infected embryos, minimal numbers of GFP-labeled phagocytes are observed in the non-infected embryos (2 hpi; 4 dpi). Scale bar = 50 µm.
7.5 x 10^2 CFU/ml of *Bh* were co-inoculated with a final concentration of 138 µg/ml of embryo protein in Schneider’s medium in a 96-well plate. The culture was plated at time points 0 hr, 3 and 5 days post-exposure to the lysates for CFU count and percent of bacteria growth inhibition. The data showed that crude embryo lysates did not inhibit the growth of *Bh in vitro*; in fact it promoted the replication of the bacteria (Fig. 3.6). Compared to the PBS control at day 3 post-exposure, the HMEC lysate and the embryo lysate increased the growth of *Bh* by 80 and 10,000 fold, respectively. At day 5 post-exposure, HMEC increased bacterial survival by 1,875 fold and embryo lysate increased the bacterial survival by 2,562 fold. This might be due to the presence of nutrients in the embryo extracts that could help sustain bacterial survival.

![Graph showing bacterial growth](image)

**Figure 3.6.** *Bh* exposed to crude zebrafish extracts increase bacterial growth. 7.5 x 10^2 CFU/ml (± SEM) of *Bh* was co-incubated with zebrafish lysate, HMEC lysate or 1 x PBS for 5 days post-incubation. Representative graph showing *Bh* exposure to zebrafish embryo lysates and HMEC lysates.
3.6. Angiogenic phenotype and inflammatory and angiogenic responses in *Bh* infected embryos

In order to evaluate the angiogenic effect induced by *Bh* infection, *Tg(fli1:egfp)y1* transgenic embryos were inoculated with an average of $3 \times 10^3$ GE of *Bh::DsRed2*. Confocal laser scanning microscopy was used to examine the embryos and evaluate blood vessel formation and morphology near the site of infection. Infected embryos were analyzed for vessel sprouting at 48 hpi (Fig. 3.7). Note that the red fluorescent overlay showing the RFP bacteria in the infected panel (ii) was omitted to help better visualize the subintestinal basket (the branching network of blood vessels within the yolk sac), so the inset image represents the same embryo showing the bacteria at the site of infection. Compared to the uninfected control group (Fig. 3.7A, i) and the rzfVEGF-injected control group (Fig. 3.7A, iii), blood vessel sprouting, shown by the white arrows, could be seen at the subintestinal basket of the infected embryos (Fig. 3.7A, ii). The length of the SIVs was measured and the evidence showed a lengthening of the SIVs in the infected samples compared to the controls (Fig. 3.7B).

Moreover, the phenotypic changes induced by *Bh* infection in the transgenic embryos infected with *Bh/pNS2T5DsRed2* from an independent experiment were assessed at day 4 post-infection (Fig. 3.8); the inset image showed the presence of the red fluorescent bacteria at the site of infection. A larger subintestinal basket was observed in the infected embryos (Fig. 3.8A, ii, white oval) compared to the non-infected control embryos (Fig. 3.8A, i) and similar to the positive control injected with
rzfVEGF (Fig. 3.8A, iii), suggesting that the bacteria triggered an angiogenic response that affected the pattern of the subintestinal vessels (SIVs: vessels located in the oval) at the site of infection. This response was quantitatively analyzed using the ImageJ software measuring the length, the number and the intersection points (IPs: are the intersections where the subintestinal vessels branch out) of SIVs from 6 different embryos from infected group and 5 different embryos from the control group. Compared to the negative control embryos, the infected and the positive control embryos had significantly more SIV (**) p < 0.001) and IPs (* p = 0.003) (Fig. 3.8B), as well as significant lengthening of the SIVs (**) p < 0.001 and * p = 0.002 for WT and positive control, respectively) (Fig. 3.8C).

To determine if the observed angiogenesis was accompanied by a pro-angiogenic response, the host response to Bh infection was assessed by qRT-PCR measuring the mRNA level of pro-inflammatory cytokines and pro-angiogenic markers in pools of 10 zebrafish embryos per group at 3 dpi. Those markers were selected due to their roles in tumor angiogenesis [230,249,250], and because they have been shown to be upregulated in Bh infection in in vitro models [1,53,77,83,251-253]. The data showed that the expression of IL-1b, IL-8 (Cxl8), zVEGF_{165} (one of the dominant spliced isoforms of VEGF in zebrafish corresponding to human VEGF_{165} [222], Flk1 and angiopoietin-2 were upregulated by 4- to 23-fold in embryos inoculated with Bh WT compared to those inoculated with PBS phenol red (Fig. 3.7C).

To determine if this angiogenic response was Bh-specific, embryos were microinjected at 28 hpf in the yolk sac with an average of 4 x 10^3 CFU of Bh pNS2T5DsRed2 or E. coli DH12S/pNS2T5DsRed2. At that particular dose E. coli
DH12S, a non-pathogenic strain, lead to a lethal infection in >90% of the embryos by 20 hpi versus no death for \( Bh \)-injected and sham-injected embryos (data not shown). Our confocal microscopy showed evidence of dying embryos infected with \( E. coli \) compared to sham-injected and \( Bh \) infected embryos (Fig. 3.9). Thus, the lethal infection caused by \( E. coli \) precludes any assessment of an angiogenic response in these embryos.

Figure 3.7. Vessel sprouting and angiogenic marker expression in response to infection with \( Bh \). (A) Subintestinal vessel morphology of embryos at 48 hpi: i) PBS/phenol red control; ii) embryos infected with approximately \( 3 \times 10^3 \) GEs of \( Bh::DsRed2 \) (the overlay showing the RFP bacteria was omitted to help better visualize the subintestinal basket); inset image is the same infected embryos showing the presence of the red fluorescent bacteria at the site of infection; white arrows show SIV sprouting in the infected embryo; iii) Embryos injected with 10 ng rzfVEGF in the yolk sac near the developing subintestinal vessel. Images are representative of the pool of infected and control embryos imaged. Scale bar = 100 µm. (B) Length of SIVs for infected and control embryos at 2 dpi (measured from 200X images) (C) qRT-PCR of pro-angiogenic markers in zebrafish embryos infected with \( Bh \). Results are expressed as the mean fold change in transcript levels of \( Bh \)-infected compared to uninfected control embryos at 3 dpi (n=2).
Figure 3.8. Vessel remodeling in zebrafish embryo infected with Bh. (A) Blood vessel morphology of Tg(fli1:egfp)Y1 zebrafish embryos analyzed at 4 days post inoculation: i) PBS/phenol red control at 4 dpi; ii) embryos infected with Bh/pNST5DsRed2 visualized at 4 dpi (the overlay showing the RFP bacteria was omitted to help better visualize the subintestinal basket); white oval indicates the area of SIVs remodeling in infected embryos; inset image is the same infected embryos showing the presence of the red fluorescent bacteria at the site of infection; iii) embryos injected with 10 ng of rzfVEGF (positive control) in the yolk sac near the developing subintestinal vessel. Images shown are representative of the pool of 6 embryos photographed from the infected group and 5 embryos photographed from the control groups. Scale bar = 100 µm. (B) Number of subintestinal vessel (SIV) and intersection points (IP) forming the subintestinal basket in the yolk sac from 6 embryos (4 dpi) from each group (mean ± SEM), (** p < 0.001; * p = 0.003). (C) Length (in relative units) of SIVs (measured from 100X images) in the yolk sac from 6 embryos (4 dpi) from infected group and 5 embryos from the control groups (mean ± SEM) versus negative control, (** p < 0.001; * p = 0.002).
Table 3.1. Primers used for qRT-PCR.

<table>
<thead>
<tr>
<th>Genes</th>
<th>Sequence (5' - 3')</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>il-1b</em></td>
<td>For: ATCAAAACCCCAATCCACAGAGT</td>
<td>[185]</td>
</tr>
<tr>
<td></td>
<td>Rev: GGCACTGAAGACACCACGTT</td>
<td></td>
</tr>
<tr>
<td><em>il-8</em></td>
<td>For: TGTTTTCCTGGCATTTCTGACC</td>
<td>[185]</td>
</tr>
<tr>
<td></td>
<td>Rev: TTTACAGTGTGGGCTTGGAGGG</td>
<td></td>
</tr>
<tr>
<td><em>Vegf</em></td>
<td>For: TGCTCCTGCAAATTCACACAA</td>
<td>[254]</td>
</tr>
<tr>
<td></td>
<td>Rev: ATCTTGGCTTTTCACATCTGCAA</td>
<td></td>
</tr>
<tr>
<td><em>flk1</em></td>
<td>For: CACAAGAAGTCCAGCGATCA</td>
<td>[237]</td>
</tr>
<tr>
<td></td>
<td>Rev: CAGGGGACCACAAAATATGG</td>
<td></td>
</tr>
<tr>
<td><em>angptl2</em></td>
<td>For: GGTCAATGGATGTTCCCTTCAC</td>
<td>[233]</td>
</tr>
<tr>
<td></td>
<td>Rev: GTCTGCTCAGTGGAAGGTG</td>
<td></td>
</tr>
<tr>
<td><em>ef1α</em></td>
<td>For: AGAAGGAAGCCGCTGAGATG</td>
<td>[105]</td>
</tr>
<tr>
<td></td>
<td>Rev: TGTCAGGGGCAATCAATAAT</td>
<td></td>
</tr>
<tr>
<td><em>rpl13a</em></td>
<td>For: TCTGGAGGACTGTAAAGAGGTATGC</td>
<td>[255]</td>
</tr>
<tr>
<td></td>
<td>Rev: AGACGCACAAATCTTGAGAGCAG</td>
<td></td>
</tr>
</tbody>
</table>

Figure 3.9. Infection of zebrafish embryo with *E. coli* versus *Bh*. Micrographs of *Tg(fli1:egfp)* embryos at 24 hpi. Infected embryos were inoculated with an average of 4 x 10^{3} CFUs of *E. coli* DH12S pNS2T5DsRed2 or *Bh/pNS2T5DsRed2* in the yolk sac at 28 hpf; control embryos were inoculated with PBS/phenol red. Images are representatives of pools of embryos micrographed. Scale bar 200 µm.
3.7. *Bh* infection induces expression of genes involved in cytoskeletal rearrangement, angiogenic and inflammatory responses

The overall response of the zebrafish embryo to *Bh* was characterized by analyzing the total mRNA levels in infected embryos compared to controls by microarray using the GeneChip Gene 1.0 ST arrays designed by Affymetrix. This array was designed based on the most recent available zebrafish genome sequence. The gene chips were scanned and data were background adjusted, normalized and summarized by a Robust Multi-array Average (RMA) algorithm. In total, over 883 genes were upregulated by 2-fold or more, and 886 genes were downregulated by at least 2-fold. While several of those genes are uncharacterized, many of them are involved in metabolic pathways, inflammatory response (*IL1b*, complement component *c3c*) and tissue remodeling (*VEGF*, IL-8). The fold change in gene expression of *Bh*-infected embryos compared to uninfected control embryos was calculated, and the candidate genes with a cut-off value of 1.9 or up were selected (Table 3.2). Some genes were also selected based on the evidence of their differential expression in *Bh* infection in cell culture models. A complete excel file of the microarray data can be accessed at [http://www.liebertpub.com/zeb](http://www.liebertpub.com/zeb). The microarray result was validated by qRT-PCR on four genes (*il8, il1b, vegf, api5*) that are involved in either inflammatory response or angiogenic response to *Bh* infection of the zebrafish embryos using the remaining RNA prepared for the microarray experiment. The qRT-PCR result confirmed the upregulation of these genes due to infection of the zebrafish embryos with *Bh* (Table 3.2).
<table>
<thead>
<tr>
<th>Genes</th>
<th>Accession number</th>
<th>Microarray fold change</th>
<th>qRT-PCR fold change</th>
<th>Description/functions</th>
</tr>
</thead>
<tbody>
<tr>
<td>mapre3b</td>
<td>NM_001002170</td>
<td>+7.07</td>
<td></td>
<td>Regulate dynamic of microtubule cytoskeleton</td>
</tr>
<tr>
<td>Rho family GTPase 3a</td>
<td>NM_199522</td>
<td>+2.29</td>
<td></td>
<td>Regulate cytoskeletal dynamic</td>
</tr>
<tr>
<td>Cdc42se1</td>
<td>NM_200060</td>
<td>+2.67</td>
<td></td>
<td>Cytoskeletal assembly</td>
</tr>
<tr>
<td>IL-8</td>
<td>XM_001342570</td>
<td>+5.54</td>
<td>+2.6</td>
<td>Facilitates migration of immune cells to inflammation site; angiogenesis</td>
</tr>
<tr>
<td>IL1b</td>
<td>NM_212844</td>
<td>+1.93</td>
<td>+21.3</td>
<td>Inflammatory response, cell proliferation and differentiation</td>
</tr>
<tr>
<td>VEGF</td>
<td>NM_001044855</td>
<td>+1.92</td>
<td>+6</td>
<td>Cell proliferation; angiogenesis</td>
</tr>
<tr>
<td>Apoptosis inhibitor 5</td>
<td>NM_199540</td>
<td>+4.23</td>
<td>+9.22</td>
<td>Prevents apoptosis after growth factor deprivation</td>
</tr>
<tr>
<td>FGF13a</td>
<td>NM_001007399</td>
<td>+6.22</td>
<td></td>
<td>Cell growth; tissue repair</td>
</tr>
<tr>
<td>E-cadherin</td>
<td>NM_131820</td>
<td>+2.01</td>
<td></td>
<td>Calcium dependent adhesion molecule</td>
</tr>
<tr>
<td>FGF receptor 1b</td>
<td>NM_001161732</td>
<td>+5.20</td>
<td></td>
<td>Regulation of cell growth, formation of blood vessels</td>
</tr>
<tr>
<td>Complement component c3c</td>
<td>NM_001037236</td>
<td>-2.46</td>
<td></td>
<td>Promotes phagocytosis during an inflammatory response against pathogens</td>
</tr>
<tr>
<td>FGF2</td>
<td>NM_212823</td>
<td>-3.78</td>
<td></td>
<td>Cell survival; angiogenesis</td>
</tr>
<tr>
<td>Interferon regulatory factor 2a</td>
<td>NM_207054</td>
<td>-2.18</td>
<td></td>
<td>Regulate transcription of interferon through the JAK-STAT signaling pathway</td>
</tr>
</tbody>
</table>

Table 3.2. Selected genes involved in response to infection with *Bh*. The description and function of these genes are in humans. Fold change indicates *Bh*-infected compared to uninfected controls. Some genes have the same function in zebrafish as in humans; however, the functions of some of these genes have not yet been fully investigated in the zebrafish model.
3.8. Infection pattern of *Bh* ΔbadA and ΔvirB mutants in the zebrafish embryo

To further characterize *Bh* infection and the importance of BadA and VirB/VirD4 T4SS in *Bh* pathogenesis and survival in the zebrafish embryo host, embryos were staged at 28 hpf and microinjected with 3 x 10^3 *Bh* Houston-1 containing in-frame deletions for full-length badA (ΔbadA/pNS2T5DsRed2) or virB operon (ΔvirB2-11/pNS2T5DsRed2). Microscopy results indicated that both the ΔvirB mutant and the ΔbadA mutant could survive in the embryos with peak fluorescence observed at 3 dpi, although the levels of red fluorescence in the mutants was not as strong as in the wild type (Fig. 3.10A).

In a subsequent experiment, zebrafish embryos were randomly grouped and inoculated with an average of 7.4 x 10^3 *Bh* pNS2T5DsRed2, 5.4 x 10^3 ΔvirB2-11 pNS2T5DsRed2, and 6.7 x 10^3 ΔbadA pNS2T5DsRed2. Bacterial infection was evaluated by qPCR at different time points. Wild-type bacteria were able to replicate, with GE increasing to over 2 x 10^5 in four days and remained detectable throughout the duration of the experiment. In contrast, the ΔvirB deletion mutant appeared to follow the same infection pattern but exhibited a reduced number of GE/embryo and a shorter duration of infection. Further, the ΔbadA deletion mutant did not appear to replicate as well compared to the WT and the ΔvirB mutant in the infected embryos (Fig. 3.10B). Although there was some variability in the number of bacteria in the initial inocula, significantly higher numbers of GEs (** p < 0.016, ** p < 0.016, * p < 0.05, and *** P < 0.008 at days 2, 3, 4 and 5, respectively) were detected in embryos infected with the WT than observed in those inoculated with ΔvirB and ΔbadA mutants.
Figure 3.10. Zebrafish embryos infected with *Bh* ΔbadA and ΔvirB mutants. (A) Confocal micrographs of *Tg(fli1:egfp)* transgenic embryos infected with 3 x 10^3 CFU *Bh*ΔbadA/pNS2T5DsRed2 compared to the WT expressing DsRed2. All strains persist in the embryos at least 5 days post-infection with a peak fluorescence observed at 3 dpi with the fluorescence intensity is at least twice as much in the WT-infected compared to the mutant-infected embryos. All images shown are representative of the pool of embryos imaged. Scale bar = 100 µm. (B) Infection pattern in zebrafish embryos inoculated at 28 hpf with 6.7 x 10^3 CFU of *Bh*ΔbadA/pNS2T5DsRed2 or 7.4 x 10^3 CFU of *Bh*/pNS2T5DsRed2 and 5.4 x 10^3 ΔvirB2-11 pNS2T5DsRed2. The means of GEs between infected groups were calculated by qPCR at different time points (mean ± SEM; * p < 0.05, ** p < 0.016 and *** P < 0.008).
3.9. The *Bh* Δ*badA* and Δ*virB* mutants exhibit an attenuation response in vivo

The response to infection in pools of embryos inoculated with the Δ*virB* mutant was investigated by qRT-PCR at day 3 post-infection. Compared to embryos inoculated with WT those inoculated with Δ*virB* mutant displayed a reduced induction of IL-8 by 1.6-fold, IL-1 by 1.5-fold, VEGF by 1.7-fold, FLK1 by 1.8 fold and angiopoietin by 10.8-fold (Fig. 3.11A). In independent experiments, the response to infection in pools of embryos inoculated with the Δ*badA* mutant was also assessed by qRT-PCR. Embryos inoculated with the Δ*badA* mutant showed a reduced induction of IL-1 by 7-fold and pro-angiogenic markers IL-8, zVEGF\textsubscript{165}, Flk1 and angiopoietin-2 by 2.5-fold, 2-fold, 3-fold, and 2-fold, respectively (Fig. 3.11B). Both mutants displayed diminished ability to induce a pro-inflammatory response as well as a pro-angiogenic response compared to the WT. However, except for angiopoietin, a greater difference in expression of those markers was observed when embryos were inoculated with Δ*badA* mutant.

**Figure 3.11. Response to infection with *Bh* Δ*badA* and Δ*virB* mutants.** (A) Differential expression in pro-inflammatory genes and pro-angiogenic factors by qRT-PCR in pools of embryos infected with Δ*virB* compared to WT. Results are expressed as the mean fold change of WT-infected or Δ*virB* mutant-infected compared to uninfected control embryos at 3 dpi (n=2). (B) Differential expression in pro-inflammatory genes and pro-angiogenic factors by qRT-PCR in pools of embryos infected with Δ*badA* compared to WT. Results are expressed as the mean fold change of WT-infected or Δ*badA* mutant-infected compared to uninfected control embryos at 3 dpi (n=2).
Chapter 4

Discussion

* Bartonella* are facultative intracellular pathogens with over thirty species described that infect a range of mammalian hosts [6,8]. *Bh, B. quintana* and *B. bacilliformis* are the species most commonly known to cause human disease. Depending on the immune status of the infected individual, *Bh* infection can span the range of self-limiting cat scratch disease (CSD) to the more serious and life-threatening bacillary angiomatosis (BA) [7]. A unique aspect of some *Bartonella* species is their ability to induce blood vessel proliferation in humans characterized by blood-filled capillaries resulting from systemic infection [47]. The angiogenic response induced by *Bh* in patients with BA is believed to be a multi-step process that involves the proliferation of endothelial cells, inhibition of endothelial cell apoptosis, and angiogenic reprogramming of infected host cells. The BadA and the VirB/VirD4 T4SS proteins of *Bh* are thought to play a leading role in eliciting an angiogenic host response during infection [52,76,81,83].

Although much is known about *Bh* and those virulence factors from studies using *in vitro* systems, their molecular mechanisms and overall contribution to *Bh* pathogenesis remains elusive and can only be fully understood by using an *in vivo* model. Therefore, there has been a great need for a practical animal model to study *Bh* pathogenesis and host response. To date, efforts to establish a suitable *in vivo* model to study *Bh* pathogenesis have been unsuccessful. The fact that zebrafish have a well-
developed immune system with many similarities to mammalian systems make them an attractive model of human diseases. The zebrafish embryo is a proven model for bacterial pathogenesis; moreover, their transparency and the availability of the Tg(fli1:egfp)y1 transgenic strain with GFP-labeled vasculature has provided a unique opportunity to study Bh infection and its distinctive ability to induce vessel proliferation.

We have determined the conditions necessary to maintain a sustained infection with Bh, including the appropriate route of infection, dose and site of inoculation, the proper stage of development of the embryos, and the means of enumerating bacteria after infection. We have found that inoculation of Bh into the yolk sac as compared to the blood circulation leads to a more persistent infection. It has been reported that yolk sac infections with fast-growing bacterial species have resulted in massive bacterial growth and early death of embryos [106,107]. The yolk sac is perhaps preferred by Bh because it is a fastidious and slow growing bacterium; the yolk sac may provide the nutrients needed and enough time to adapt to the new environment before facing a strong antimicrobial response. Furthermore, we have determined that zebrafish embryos Tg(fli1:egfp)y1 sustained a consistent infection pattern when microinjected at 24 - 28 hpf in the yolk sac with an inoculum of approximately 3 x 10^3 GEs of Bh.

One area, which proved problematic, was bacterial enumeration of Bh via plating of embryo homogenates. This assay provides direct counts of the number of viable bacteria in the embryos. However, the autoagglutination of the bacteria as well as contamination seen even on kanamycin selective media plates caused some variability in enumeration as evidenced by the error bars (Fig. 3.3A). This contamination is likely
in part due to the normal flora present in the embryos. In addition, rapidly growing water-borne bacteria and fungi that are resistant to kanamycin also proved problematic since *Bh* requires at least 5 days for colonies to appear on plates; the contaminants usually grew much faster and often prevented accurate plate counting. Therefore, we alternatively used both confocal microscopy to qualitatively visualize *Bh* in embryos and qPCR to assess the GE/infected embryo. This method is more sensitive as it uses primers specific to the *Bh* NADH dehydrogenase subunit G gene to quantify the genomic equivalents of bacteria per embryo. It is likely these primers amplify DNA from nonviable bacteria; however, there is a notable reduction in GEs from day 4 to day 6 suggesting that gDNA from dead bacteria degrades and is not amplified by qPCR (Fig. 3.3B). This reduction pattern in GEs was similar to that observed by microscopy (Fig. 3.2) and by CFU counts in previous experiments (Fig. 3.3A). Despite the possible detection of nonviable bacteria, the qPCR method proved to be far more consistent and reliable than the plating method.

Much of the understanding of the mechanisms required for angiogenesis has been derived from cancer models. Like in cancer, *Bh* infection of endothelial cells induces the activation of HIF-1α and subsequent secretion of angiogenic factors such as VEGF leading to cell proliferation and angiogenesis [76,78]. An *in vivo* model is critical since multiple cell types including endothelial cells, macrophages and epithelial cells are thought to contribute to *Bh*-mediated angiogenesis via the paracrine loop model [1,77]. We have shown that aggregates of *Bh* do interact with the EC of the embryo with some intracellular bacteria observed. However, many are distant from the EC in the yolk sac and it is difficult to accurately quantify the number of intracellular *Bh*
in infected embryos. It should be noted that in humans, histological studies of samples from bacillary angiomatosis patients have shown very few intracellular \textit{Bh} and that most of the bacteria are seen as epicellular [60].

Histological examination of bacillary angiomatosis lesions from patients with \textit{Bh} infection have shown the infiltration of polymorphonuclear cells and macrophages located in close proximity to the proliferating endothelial layer [60,61]. Our evidence shows that \textit{Bh} infection induces migration and accumulation of neutrophils and macrophages at the site of infection. This result is in agreement with what was observed in tissue samples as well as what has been shown in \textit{in vitro} studies revealing the role of phagocytic cells in \textit{Bh} pathogenesis [1]. There must be a balance between \textit{Bh} causing phagocytic cells to produce proangiogenic factors and at the same time suppress bacterial killings. Although the yolk sac may be considered as a site of relative immune privilege in the zebrafish embryo, a robust innate response against infection has been described in the yolk sac, and macrophages and neutrophils have been observed ingesting bacteria in both the blood circulation and the yolk sac [106,107,195,256]. We hypothesize that the resistance to a lethal infection by \textit{Bh} from infected embryos is very likely due to the activation of an innate immune response. While our real-time observation of \textit{Bh}-infected zebrafish embryos showed an accumulation of phagocytic cells at the site of infection, the role of these cells in killing \textit{Bh} was not clearly demonstrated. It remains to be investigated whether or not this is due to the ability of this bacterium to inhibit phagocytosis.
An angiogenic phenotype was observed in our transgenic zebrafish embryo model when infected with Bh. It was characterized by an increase in length and number of the subintestinal vessels and intersection points at the site of infection in the infected compared to controls. Moreover, disruption of the smooth and normal pattern of those vessels could also be observed. Although the extent of the phenotypic response observed is not as dramatic as what is seen in tumor angiogenesis, a similar response was observed in the zebrafish yolk membrane angiogenesis assay developed by Nicoli et al. [110,257]. The angiogenic phenotype observed in the infected embryos was further substantiated by qRT-PCR analysis showing the induction of proangiogenic factors such as IL-8 (Cxcl8), zVEGF-165, zVEGFR2 and angiopoietin-2 in the infected compared to control embryos. These potent angiogenic factors have been shown to stimulate EC proliferation and angiogenic phenotypes in response to Bh infection in vitro [77,251,253]. Our efforts to use E. coli DH12S strain as a negative control for an angiogenic response in the zebrafish embryo was unsuccessful as embryos inoculated with E. coli in the yolk sac died within 20 hours post-inoculation. The death of the embryos may be a result of septic shock due to the high endotoxicity of bacterial lipopolysaccharide (LPS) of E. coli compared to the remarkably low endotoxicity of LPS of Bh [90].

Our microarray data provide evidence that there are many genes that are involved not only in inflammatory and angiogenic responses, but also in cytoskeletal rearrangement and metabolic processes. This suggests that the bacteria have a widespread impact on host gene expression patterns. Bh entry into EC and epithelial cells can happen by two alternative routes: single bacterium uptake via a zipper-like
mechanism or in the form of large bacterial aggregates in a structure called invasome [8,54]. The zipper-like mechanism has been documented in several bacteria including Listeria monocytogenes. It has been shown that the entry of L. monocytogenes into epithelial cells requires the interaction of the internalin protein with E-cadherin on the host cell [258]. In addition to its role in cell-cell adhesion and involvement in early tissue formation in zebrafish embryo [259], E-cadherin may also play a role in Bh entry into the host cell as Bh infection induces an upregulation of the gene encoding for E-cadherin protein. Formation of the invasome structure leading to the uptake of Bh aggregates was shown to be dependent in part on the expression of small Rho family GTPases and CDC42 [260]. Rho family GTPase 3a and CDC42 small effector 1 (Cdc42se1) were upregulated in the infected embryos suggesting that they may play a role in Bh-host interactions.

Antimicrobial peptides such as β-defensins, hepcidin, and phosvitin are an important component of the innate immunity. In vitro killing of pathogens with purified antimicrobial peptides from zebrafish embryo extracts have been reported [208,209]. In vitro exposure of Bh to zebrafish embryo crude extracts promotes bacterial growth, which may be due to the nutrients found in the embryo extracts. Although we do not rule out the protective role of antimicrobial peptides in zebrafish extract, it seems like the nutrients in the extracts outweighs their bactericidal effect on the Bh survival in vitro. Moreover, Bh infection did not seem to induce their expression in vivo as evidence in the microarray data showed that their transcript levels did not vary between the infected and control groups embryos.
**Bh**-induced angiogenesis is a multi-step process that partly depends on the expression of BadA protein which induces proangiogenic cell response via activation of HIF-1 and NF-kB, and the subsequent secretion of VEGF and IL-8, respectively [76-78]. **Bh**-induced angiogenesis in the zebrafish embryo may occur through the same pathway as previously shown in cell culture studies as those genes, including HIF-α (NM_2002233) and NF-κβ (NM_203184), were seen to be upregulated in the infected embryos in the microarray data (data not shown). Some notable genes that were down-regulated in the infected embryos include FGF2, which plays an important role in cell survival and angiogenesis, and complement component c3c, which is involved in promoting phagocytosis during an inflammatory response against pathogens [261-263]. Although FGF2 expression in **Bh**-induced angiogenesis has not been fully investigated *in vitro*, it is somewhat surprising that the infection with **Bh** would suppress its expression. On the other hand, **Bh** has been shown to inhibit phagocytosis [15], so it is not surprising that the evidence shows that expression of complement component c3c is suppressed by **Bh** infection in the zebrafish embryos.

As in many pathogenic bacteria, the VirB/VirD4 T4SS along with the *Bartonella* effector proteins have been shown to play important roles in **Bh** pathogenesis *in vitro* [79,82]. The expression of the VirB T4SS and some of the effector proteins have been associated with inflammatory cytokine production and pro-angiogenic activities [83,84]. A deletion mutant for genes encoding the VirB T4SS (ΔVirB2-VirB11) supports bacterial replication in the zebrafish embryos although to a lesser extent than the WT bacteria. These data suggest that the VirB machinery and even its cognate effectors are not an absolute requirement for establishing an infection in the zebrafish embryo model.
However, an abrogated response to infection with the $\Delta virB$ mutant is observed in the embryos; this further indicates that although the VirB T4SS is not absolutely required for $Bh$ survival in the zebrafish embryo, it may play a role in the pathogenicity of $Bh$ in vivo.

The genome of $Bh$ contains at least two forms of BadA-encoding genes: the full-length version (BH01510), which was the gene deleted in the mutant used in this study, and a truncated version (BH01490). It has been reported that some $Bh$ isolates do not express a functional BadA, and due to passaging histories, defined $Bh$ strains may exhibit distinct characteristics and infection phenotypes [264]. However, our $Bh$ Houston-1 strain was early passage and displayed a rough phenotype which correlates with the presence of full-length BadA protein which was further confirmed by reactivity with antibody specific for BadA (data not shown). While a deletion mutant for the major trimeric autotransporter adhesin BadA appears to increase in fluorescence intensity within the embryos over time, qPCR data indicated that this mutant was unable to replicate and remained at a relatively constant bacterial burden as determined by the GE/embryo. It has been reported that full-length BadA is essential for $Bh$ adhesion to host cells and extracellular matrix proteins leading to a direct angiogenic response by stimulating HIF-1$\alpha$ production and VEGF secretion [76-78]. Therefore, we hypothesized that, in absence of full-length BadA, $Bh$’s ability to adhere to target cells, establish infection and initiate the induction of an angiogenic response would be hampered. Our data showed that the $Bh$ mutant for full-length BadA exhibited an abrogated angiogenic response in the zebrafish embryo compared to WT although it may be possible that the diminished ability of this mutant to replicate in the zebrafish embryos precluded the establishment of a pro-angiogenic host response. Thus, our in vivo data support a major
role for BadA in establishing infection and also possibly inducing a pro-angiogenic host response. Although the function of the truncated version of BadA (BH01490) in *Bh* has not been fully investigated, a compensatory role in inducing an angiogenic phenotype following the loss of full-length BadA can’t be eliminated in the ΔbadA mutant.

In conclusion, we have developed an animal model to study *Bh* infection and pathogenesis. We demonstrated that zebrafish embryos microinjected in the yolk sac became infected with *Bh* and exhibited evidence of an angiogenic response as well as an inflammatory response involving recruitment and accumulation of neutrophils and macrophages to the site of infection. We found that the *Bh* mutants for virB T4SS and the full-length BadA induced abrogated pro-inflammatory and pro-angiogenic responses when compared to the WT in the zebrafish embryo. Moreover, we also found that the full-length BadA mutant showed a limited ability to replicate when compared to the WT suggesting that this *in vivo* model is a useful system in which to assess virulence. The infection pattern with *Bh* was not assessed beyond day 8 post-inoculation as embryos are capable of obtaining nutrition from the yolk sac during this time period and extension of the period of infection would require supplemental food sources – possibly introducing microbial contaminants. Regardless, we believe that the use of zebrafish embryos as a model of *Bh* infection proved highly successful and provided valuable insight which can only be gathered from an *in vivo* model system.
Chapter 5

Future Studies

Although the \textit{Bh} mutant for full-length BadA exhibited an abrogated response in the zebrafish embryo, because a compensatory role of the short version of BadA following the loss of full-length BadA may be at play in the \textit{ΔbadA} mutant, future studies should focus on making a double knock out of both the full-length and the truncated versions of BadA and study their roles in \textit{Bh} infection. It has been assumed that BadA and the VirB/D4 T4SS may act synergistically in that the BadA-induced adhesion to host cells brings the bacteria and the host cells into close proximity facilitating the secretion of the effector proteins by the VirB/D4 T4SS [64]. Although the synergistic effect of those two important virulence factors in \textit{Bh} has not been elucidated \textit{in vitro}, it would be interesting to investigate the effect of a double knock out mutant of those genes on \textit{Bh} pathogenesis in the zebrafish embryo. Moreover, studies complementing the genes into the respective knock out strains will be important in confirming that the phenotypic and or genotypic effects are due to the respective genes.

\textit{Bh} infection induced migration of phagocytic cells to the site of infection. Interestingly, although the phagocytic cells were recruited to the infection site, the majority of the bacteria were not phagocytized. The role of those phagocytes in clearance of the bacteria remains to be determined. The myeloid cells can be depleted in the zebrafish embryos using morpholino knockdown of the myeloid transcription factor Pu.1, which has been shown to be required for myelopoiesis in zebrafish [212].
The infection pattern and the response to infection with *Bh* could then be studied in the macrophage- and neutrophil-depleted embryos. Moreover, since there is a dramatic decrease in bacterial burden starting at day 4 or 5 post infection, there may be a respiratory burst killing of the bacteria by the zebrafish embryo immune system. The production of reactive nitrogen and reactive oxygen species is an important effector mechanism of the innate immunity in response to infection of the zebrafish embryo. The role of respiratory burst killing in clearance of the bacteria by the embryos can be assessed by an assay measuring the oxidation of 2′,7′-dihydrodichlorofluorescein diacetate (H2DCFDA), a non-fluorescent dye, to a fluorescent product dichlorofluorescein (DCF) [265].
Chapter 6

References


Appendices

Appendix I: IACUC approval letters

The Institutional Animal Care and Use Committee (IACUC) reviewed your application requesting the use of animals in research for the above-entitled study. The IACUC requested modifications/further information in response to that review and has received the required information. The IACUC APPROVED your request to use the following animals in your protocol for a one-year period beginning 4/3/2009:

- 3700 Zebrafish

Please reference the above IACUC protocol number in all correspondence regarding this project with the IACUC, Comparative Medicine, or the Division of Research Integrity and Compliance. In addition, please take note of the following:

- IACUC approval is granted for a one-year period at the end of which, an annual renewal form must be submitted for years two (2) and three (3) of the protocol. After three years all continuing studies must be completely re-described in a new application and submitted to IACUC for review.

- All Comparative Medicine pre-performance safety and logistic meetings must occur prior to implementation of this protocol [IACUC policy V.10]. Please contact the program coordinator at compassion@research.usf.edu to schedule a pre-performance meeting.

- All changes to the IACUC-Approved Protocol must be pre-approved by the IACUC [IACUC policy III.11]. Minor changes can be submitted to the IACUC for review and approval as an amendment or procedural change, whereas major changes to the protocol require submission of a new IACUC application. Minor changes are changes considered to be within the scope of the original research hypothesis or involve the original species and are submitted to the IACUC as an Amendment or Procedural change. Any change in the IACUC-approved protocol that does not meet the latter definition is considered a major protocol change and requires the submission of a new application. More information on what constitutes a minor versus major protocol change and procedural steps necessary for IACUC review and approval are available on the Comparative Medicine web site at http://www.research.usf.edu/cm/amendments.htm.

- All costs invoiced to a grant account must be allocable to the purpose of the grant [IACUC policies IV.5 and V.10]. Costs allocable to one protocol may not be shifted to another in order to meet deficiencies caused by overruns, or for other reasons of convenience. Rotation of charges among protocols by month without establishing that the rotation schedule credibly reflects the relative benefit to each protocol is unacceptable.

For more information on IACUC policies and procedures, please visit the Comparative Medicine web site at http://www.research.usf.edu/cm/default.htm.
MEMORANDUM

TO: Burt Anderson, Ph.D.  
Dept. of Med-Micro & Immunology  
MDC_7

FROM: Jay B. Dean, Ph.D. Chairperson  
Institutional Animal Care & Use Committee  
Division of Research Integrity and Compliance

DATE: 2/13/2012

PROJECT TITLE: Regulation of Virulence Factors in Bartonella henselae: A Novel Zebrafish Embryo Model to Define Virulence Factors of Bartonella henselae

AGENCY/SOURCE OF SUPPORT: NIH  
A1038178

IACUC PROTOCOL#: R 4174

PROTOCOL STATUS: APPROVED

The Institutional Animal Care and Use Committee (IACUC) reviewed your application requesting the use of animals in research for the above-mentioned study. The IACUC requested modifications/further information in response to that review and has received the required information. The IACUC APPROVED your request to use the following animals in your protocol for a one-year period beginning 2/13/2012:

- 75 Zebrafish, adult breeders
- 2100 Zebrafish, embryos

Please reference the above IACUC protocol number in all correspondence regarding this project with the IACUC, Comparative Medicine, or the Division of Research Integrity and Compliance. In addition, please take note of the following:

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- All Comparative Medicine pre-performance safety and logistic meetings must occur prior to implementation of this protocol [IACUC policy V.10]. Please contact the program coordinator at compmed@research.usf.edu to schedule a pre-performance meeting.

- All changes to the IACUC-Approved Protocol must be pre-approved by the IACUC [IACUC policy III.11]. Minor changes can be submitted to the IACUC for review and approval as an amendment or procedural change, whereas major changes to the protocol require submission of a new IACUC application. Minor changes are changes considered to be within the scope of the original research hypothesis or involve the original species and are submitted to the IACUC as an Amendment or Procedural change. Any change in the IACUC-approved protocol that does not meet the latter definition is considered a major protocol change and requires submission of a new application. More information on what constitutes a minor versus major protocol change and procedural steps necessary for IACUC review and approval are available on the Comparative Medicine web site at http://www.research.usf.edu/cm/amendments.htm

- All costs invoiced to a grant account must be allocable to the purpose of the grant [IACUC policies IV.5 and V.10]. Costs allocable to one protocol may not be shifted to another in order to meet deficiencies caused by overruns, or for other reasons of convenience. Rotation of charges among protocols by month without establishing that the rotation schedule credibly reflects the relative benefit to each protocol is unacceptable.

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Author: Sandra I. Resto-Ruiz, Michael Schmiederer, Debra Sweeney et al.

Publication: Infection and Immunity
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About the Author

Amorce Lima was born and raised in Haiti where he graduated high school. He came to the United States as a permanent resident and later became a US citizen. Having a strong desire to be the first in his family to attend college, he decided to enroll at Palm Beach Community college where he graduated with an AA degree. Due to his academic excellence at Palm Beach Community College, Amorce Lima was awarded several honors including the President's Honor Certificate for 5 consecutive semesters. Those achievements helped Amorce Lima to be admitted to the Honors College and become a Ronald E. McNair scholar when transferred to the University of South Florida in 2004. Amorce had the opportunity to be involved in undergraduate research in the laboratory of Dr. My Lien Dao and to present his work in national conferences. It was during that time that Amorce developed a passion for research. In 2006, Amorce graduated from USF with honors with a Bachelor of Science degree in Biomedical Sciences. He married his beautiful wife and his longtime high school friend Delani Lima. They were blessed with their first child, Destiny Lima in 2007 and later with their second child Makayla Lima. In 2008, Amorce decided to pursue a graduate education after awarded the McNair Doctoral Fellowship. He enrolled in the Medical Sciences Program at USF where he joined the warmth and welcoming laboratory of Dr. Burt Anderson. Amorce worked on developing the zebrafish embryo model to study *Bartonella henselae* pathogenesis for which he received his doctoral degree. Following his graduation, Amorce will pursue a career in clinical microbiology.