Abstract

VARANAT, MRUDULA. Potential Role of *Bartonella* sp. in Vasoproliferative Disorders in Dogs. (Under the direction of Edward B. Breitschwerdt).

Bartonella sp. are highly fastidious, vector borne zoonotic agents. *Bartonella* sp. cause persistent intraerythrocytic bacteremia in reservoir hosts whereas endothelial cells are the major target cells in incidental hosts. Three species of *Bartonella* are associated with vasoproliferative disorders including verruga peruana (*B. bacilliformis*), bacillary angiomatosis (*B. henselae, B. quintana*) and peliosis hepatis (*B. henselae*). *Bartonella* sp. induces proliferation of endothelial cells by both direct mitotic stimulation and by inhibiting endothelial cell apoptosis.

Commonly used diagnostic methods for *Bartonella* include liquid enrichment cultures, serology, polymerase chain reaction (PCR), immunohistochemistry, silver staining and fluorescent *in situ* hybridization (FISH). We developed fluorescent in situ hybridization probes specific for *Bartonella* sp., *B. henselae* and *B. vinsonii* subsp. *berkhoffii*. We tested paraffin embedded tissues with these FISH probes and compared the results with PCR, Warthin-Starry silver staining, immunohistochemistry and FISH with the universal probe Eub338. Even though the sensitivity of these probes were lower compared to the other techniques, it may be useful for the detection and localization of *Bartonella* sp. in tissues when combined with other diagnostic techniques like PCR.

Paraffin embedded tissues are readily available and highly useful for diagnosis of infectious diseases and retrospective studies. PCR is a highly sensitive and specific technique for diagnosis of bartonellosis from blood and tissues. Extreme care should be taken to avoid contamination or carryover of DNA between samples. This is especially important in case of paraffin embedded tissues, since there are many steps involved in the preparation and processing of the paraffin blocks which could be potential sources of DNA carry over. We found presence of *Bartonella* sp. DNA on different parts of the microtome, necropsy room table and in the paraffin from the tissue processor. Our results emphasize the need of extreme care and proper controls when paraffin embedded tissues are used for the diagnosis and research studies.

B. bacilliformis, *B. henselae* and *B. quintana* are known to induce vasoproliferative disorders in human beings and animals. Here we present evidence that *B. vinsonii* subsp. *berkhoffii* also has ability to induce vasoproliferation. *B. vinsonii* subsp. *berkhoffii* was isolated and/or detected from various vasoproliferative disorders from humans and animas. We also demonstrated that *B. vinsonii* subsp. *berkhoffii* induces VEGF production in HeLa 229 cells in a dose dependent manner. Taken together these results strongly support the addition of *B. vinsonii* subsp. *berkhoffii* to the list of bartonellae capable of inducing vasoproliferation.

Next, we performed a case control study to detect the molecular prevalence of *Bartonella*, *Babesia* and hemotropic *Mycoplasma* sp. in splenic biopsy tissues from dogs with splenic fibrohistiocytic nodules (FHN), hemangiosarcoma (HSA) and lymphoid nodular hyperplasia (LNH). The prevalence of *Bartonella* sp. DNA was higher in FHN and HSA when compared to LNH. Prevalence of *Bartonella* sp. was significantly higher when compared to the prevalence of *Babesia* and *Mycoplasma* sp. in both FHN and HSA whereas no significant difference was noted in the prevalence of the three genera tested in LNH group. Our studies indicate a plausible role of *Bartonella* sp. in pathogenesis of splenic FHN and HSA.

We studied the effect of *Bartonella* sp. infection in human brain vascular pericytes *in vitro*. We demonstrated invasion of pericytes by *B. henselae*. *Bartonella* decreased the proliferation of pericytes when compared to uninfected controls. *Bartonella* did not have any significant effect on the apoptosis of pericytes. *Bartonella* induced the production of VEGF production by pericytes. Our results indicate that *B. henselae* can infect pericytes and induce production of VEGF from pericytes which may act in turn help in the proliferation of the endothelial cells in a paracrine manner.

Potential Role of *Bartonella* sp. in Vasoproliferative Disorders in Dogs

by
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DEDICATION

This dissertation is dedicated to my wonderful husband Manoj Kumar Ramanunninair, without whose support it would not have been possible for me to finish this work. I would also like to dedicate this dissertation to my parents Mr. Venugopalan Puliyamkott and Mrs. Komalavally Varanat who believed in me from the beginning and gave me the best education and let me follow my dreams, and to my wonderful sisters, brother-in-laws and nieces who are always there to share my happiness and sorrows and to encourage me in each step of my career.

BIOGRAPHY

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Bartonella sp. – Pathology, Pathogenesis and Potential role in the development of vasoproliferative tumors

Pathology of *Bartonella* sp. infection in humans and animals

Introduction

The genus *Bartonella* belongs to the α-subdivision of the class Proteobacteria and is comprised of more than 24 species and subspecies, out of which 13 are associated with human diseases (Chomel *et al.,* 2009). Bartonellae are highly fastidious, intracellular, Gram negative organisms which stain positive with silver stains (argyrophilic). Arthropod vectors including biting flies, fleas, lice and potentially ticks play an important role in the transmission of these bacteria (Billeter *et al.,* 2008). Bartonellosis is also transmitted by animal scratches and bites (Chomel *et al.,* 2006) making it an important zoonotic disease. Recently, suspected cases of needle stick transmission of *Bartonella* from animals to veterinarians have been reported (Oliveira *et al.,* 2010; Lin *et al.,* 2010a). Suspected perinatal transmission of *B. henselae* and *B. vinsonii* subsp. *berkhoffii* to a child was also reported recently (Breitschwerdt *et al*., 2010b) whereas an earlier study in cats failed to detect any evidence of perinatal transmission of *B. henselae* (Guptill *et al*., 1998). Bartonellae are highly adapted pathogens which can cause persistent intravascular infections in reservoir hosts, which helps in their transmission by blood sucking arthropods. In incidental hosts, *Bartonella* cause a variety of clinical manifestations. Intraerythrocytic infection is not a characteristic in incidental hosts instead, endothelial cells are considered to be the primary target cells in these hosts (Dehio, 2004).

The disease manifestations associated with *Bartonella sp.* infection in humans include cat scratch disease (*B. henselae*, *B. clarridgeiae*) (Anderson *et al.,* 1994; Kordick *et al.,* 1997), Oroya fever and verruga peruana (*B. bacilliformis*) (Ihler, 1996), trench fever (*B. quintana*) (Ohl and Spach, 2000) endocarditis (*B. alsatica*, *B. elizabethae*, *B. henselae*, *B.*

koehlerae, *B. quintana*, *B. vinsonii* subsp. *arupensis*, *B. vinsonii* subsp. *berkhoffii*, Candidatus *B. myotimonensis*) (Daly *et al.,* 1993; Roux *et al.,* 2000; Klein *et al.,* 2002; Avidor *et al.,* 2004; Fenollar *et al.,* 2005; Raoult *et al.,* 2006; Walls *et al.,* 2006; Lin *et al.,* 2010b), musculoskeletal disorders (*B. henselae*) (Maman *et al.,* 2007), neurological disorders (*B. henselae*, *B. vinsonii* subsp. *berkhoffii*) (Breitschwerdt *et al.,* 2008; Breitschwerdt *et al.,* 2010a), neuroretinitis (*B. henselae, B. grahamii*, *B. quintana*) (Kerkhoff *et al.,* 1999; George *et al.,* 2006; Irshad and Gordon, 2009), bacillary angiomatosis (BA) (*B. henselae*, *B. quintana*) (LeBoit *et al.,* 1988; Sala *et al.,* 2005) and bacillary peliosis hepatis (BP) (*B. henselae*) (Mohle-Boetani *et al.,* 1996).

In dogs, *Bartonella* infections have been associated with endocarditis (*B. henselae, B. quintana, B. vinsonii* subsp. *berkhoffii*, *B. clarridgeiae*, *B. koehlerae, B. washoensis and B. rochalimae*) (Chomel *et al.,* 2001; Chomel *et al.,* 2003; Kelly *et al.,* 2006; Cockwill *et al.,* 2007; Henn *et al.,* 2009a; Ohad *et al.,* 2010) granulomatous hepatitis, lymphadenitis and rhinitis (*B. henselae, B. clarridgeiae* and *B. vinsonii* subsp. *berkhoffii*) (Pappalardo *et al.,* 2000; Gillespie *et al.,* 2003), bacillary angiomatosis (*B. vinsonii* subsp. *berkhoffii*) (Yager *et al.,* 2010), peliosis hepatis (*B. henselae*) (Kitchell *et al.,* 2000) and various other systemic diseases (*B. henselae* and *B. elizabethae*) (Mexas *et al.,* 2002).

Cats can be infected with 6 species of Bartonellae including *B. henselae*, *B. clarridgeiae*, *B. quintana*, *B. bovis*, *B. koehlerae* and *B. vinsonii* subsp. *berkhoffii* (Breitschwerdt, 2008; Varanat *et al.*, 2009). Cats are considered to be the reservoirs for *B. henselae* and potentially for *B. clarridgeiae*. Cat fleas act as vectors for transmission of *B. henselae* and *B. clarridgeiae* and most likely *B. koehlerae* among cats (Chomel *et al.*, 1996). Different studies have reported 4.3-41% prevalence of *B. henselae* in healthy cats worldwide based on serological, culture and molecular evidence (Jameson *et al.*, 1995; Maruyama *et al.*, 2001; Kamrani *et al.*, 2008). Following natural infection, cats generally remain outwardly healthy carriers of *B. henselae* for months to years; however self-limiting fever, transient anemia, neurologic dysfunction, lymphadenopathy and reproductive disorders have been documented in experimentally infected cats (Guptill *et al.*, 1998; Kordick *et al.*, 1999). *B.*

henselae has also been implicated as the causative agent for aortic valve endocarditis in cats from Australia and North America (Chomel *et al.,* 2003b; Chomel *et al.,* 2009; Perez *et al.,* 2010). Recently natural infection with *B. henselae* has been associated with the development of pyogranulomatous myocarditis and diaphragmatic myositis in cats housed in a shelter (Varanat *et al*., 2011*).* The stress associated with shelter conditions may have contributed to the development pathology in these cats. The virulence of the infecting strain of *B. henselae* may also be a contributing factor, as differences in pathogenicity among different strains of *B. henselae* have been reported (O'Reilly *et al*., 1999; Yamamato *et al.,* 2002). *B. vinsonii* subsp. *berkhoffii* has been isolated from blood of a cat with recurrent osteomyelitis using *Bartonella* alpha Proteobacteria growth medium (BAPGM) (Varanat *et al.,* 2009). DNA of *B. vinsonii* subsp. *berkhoffii* was subsequently amplified and sequenced from a digit amputated 18 months earlier, suggesting persistent infection with these bacteria.

Bartonella infection has also been documented in a wide variety of other animals including horses, monkeys, cattle, raccoons, coyotes, foxes, rodents and marine mammals (Heller *et al.,* 1999; O'Rourke *et al.,* 2005; Hams *et al.,* 2008; Chomel *et al.,* 2009; Henn *et al.,* 2009b; Chinnadurai *et al.,* 2010). Horses can be infected with *B. henselae* and such infection has been associated with abortion (Johnson *et al.,* 2009). Grey foxes and raccoons are considered to be natural reservoirs of *B. rochalimae* (Henn *et al.,* 2009b) whereas Coyotes are considered natural reservoirs for *B. vinsonii* subsp. *berkhoffii* (Chang *et al.,* 2000). Cattle can be infected with *B. bovis*, *B. chomelii*, and *B. henselae* (Maillard *et al.,* 2004; Cherry *et al.,* 2009) with *B. bovis* causing endocarditis in cattle (Maillard *et al.,* 2007). Rodents act as reservoir hosts for a number of *Bartonella* sp. with *B. grahamii* (neuroretinitis), *B. vinsonii* subsp. *arupensis* (endocarditis), *B. elizabethae* (endocarditis) and *B. washoensis* (endocarditis) being associated with disease in humans (Chomel *et al.,* 2004; Chomel *et al.,* 2009).

Bartonella is the only genus of bacteria known to induce vasoproliferation in both immunocompromised and immunocompetent hosts. *In vitro*, *Bartonella* is able to induce proliferation of endothelial cells and to prevent endothelial cell apoptosis (Maeno *et al.,*

1999; Kirby and Nekorchuk, 2002). Vascular Endothelial Growth Factor (VEGF) plays an important role in *Bartonella*-induced vasoproliferation and BA and BP lesions show increased expression of VEGF (Kempf *et al.,* 2001). The authors also showed that the proliferating endothelial cells promote the growth of *Bartonella* sp. Whether *Bartonella*-triggered vasoproliferation is a pathogenic strategy used by these bacteria to expand a specific host cell habitat (the endothelial cell) is currently unknown. This review will be focusing mainly on the pathology and pathogenesis of *Bartonella* sp. with special emphasis on their ability to induce vasoproliferation.

Cat scratch disease

Cat scratch disease (CSD) is caused by *B. henselae* and, potentially, by *B. clarridgeiae* (Kordick *et al.,* 1997; Chomel *et al.,* 2004). CSD bacillus is transmitted from cat to human by cat scratch or bite and is most commonly seen in children and young adults. CSD is characterized by regional lymphadenopathy and fever in immunocompetent people. The disease starts as an erythematous papule at the site of scratch or bite 7-12 days after the inoculation. Regional lymphadenopathy develops 1-3 weeks later (Carithers, 1985) and most commonly involves the axillary and epitrochlear nodes (46%), nodes in the head and neck (26%) or the groin (17.5%) (Florin *et al.,* 2008). Usually it is a self-limiting condition which resolves even without antibiotic therapy; however, about 15% of the patients with CSD develop atypical complications including endocarditis, hepatosplenic manifestations, osteomyelitis, hemolytic anemia, glomerulonephritis and neuroretinitis (Chomel *et al.,* 2004). In United States, it is estimated that about 22,000 humans develop cat scratch disease each year, out of which about 2000 cases need hospitalization (Jackson *et al.,* 1993), with an estimated annual health care cost of about 12 million dollars (Jackson *et al*., 1993). Histologically, CSD is characterized by granulomatous lymphadenitis with multiple microabcesses and the bacilli (*B. henselae*) can be demonstrated in the lesions by silver stains.

Endocarditis

Infection with *Bartonella* sp. has been increasingly recognized as the cause of blood culture negative endocarditis in humans and animals. Endocarditis due to *Bartonella* infections has been reported in humans, dogs, cats, cattle and sea otters (Table 1). In human beings, *Bartonella* endocarditis is more common in patients with preexisting heart problems and exposure to cats (Gouriet *et al.,* 2007) and accounts for about 15% of all infective endocarditis cases (Benslimani *et al.,* 2005). In dogs, studies in Northern California concluded that *Bartonella* infection is responsible for 18-28% of all endocarditis cases referred to a tertiary care facility (MacDonald *et al.,* 2004; Sykes *et al.,* 2006). Clinical and pathological findings of *Bartonella* endocarditis are similar in human beings and dogs. Pathologically it is characterized by vegetative lesions on the valves; the most commonly affected being the aortic valve (Chomel *et al.,* 2009). Calcification, fibrosis and neovascularization of the affected valve are frequent findings. Most common clinical abnormalities include cardiac murmurs, fever, hypoalbuminemia, leukocytosis, monocytosis and thrombocytopenia.

Musculoskeletal manifestations

Various musculoskeletal manifestations including myalgia, arthritis, joint pain, tendinitis and osteomyelitis have been reported as secondary complications of cat scratch disease in humans (Maman *et al.,* 2007). In their study, about 10% of the CSD patients presented with musculoskeletal manifestations. Co-infection with *B. henselae* and *B. vinsonii* subsp. *berkhoffii* has been reported in dogs with polyarthritis (Diniz *et al.,* 2009). *B. vinsonii* subsp. *berkhoffii* was also attributed to be the causative agent of recurrent osteomyelitis in a cat (Varanat *et al.,* 2009). These findings further emphasize the fact that *Bartonella* infections in humans and animals share similar clinical and pathologic characteristics.

Hepatosplenic manifestations

Hepatomegaly and splenomegaly can develop as a complication of CSD (Laham and

Kaplan, 2008). Granulomatous hepatitis has been reported in both humans and dogs following infection with *Bartonella* sp. (Liston and Koehler, 1996; Pappalardo *et al.,* 2000; Scolfaro *et al.,* 2008). Hepatosplenic manifestations are seen in both immunosuppressed and immunocompetent patients and *B. henselae*, *B. quintana* and *B. vinsonii* subsp. *berkhoffii* have been identified in humans and dogs with hepatosplenic diseases (Liston and Koehler, 1996; Pappalardo *et al.,* 2000; Durupt *et al.,* 2004) Development of granulomatous hepatitis following a liver transplant in a pediatric patient (Scolfaro *et al.,* 2008b) indicates that it is plausible that *Bartonella* could be transmitted from the donor to the recipient during organ transplantation. Rarely the necrotizing splenitis caused by *Bartonella* may lead to splenic rupture (Daybell *et al.,* 2004).

Neurological manifestations

Bartonella infection has been associated with neurological symptoms in humans and animals (Breitschwerdt *et al.,* 2010a; Barber *et al.,* 2010). Neurological complications of CSD and chronic *B. henselae* bacteremia include memory loss, disorientation, paralysis, stroke, seizures, cerebral arteritis, status epilepticus, coma and encephalitis (Gerber *et al.,* 2002; Breitschwerdt *et al.,* 2008). In addition transient neurological dysfunction was reported in cats experimentally infected with *B. henselae* (Guptill *et al.,* 1997).

Vasoproliferative diseases caused by *Bartonella* sp.

Vasoproliferative lesions induced by *Bartonella* sp. infection include bacillary angiomatosis (BA), bacillary peliosis (BP) and verruga peruana. *B. henselae* and *B. quintana* cause BA and BP, where as verruga peruana is caused by *B. bacilliformis* (Recavarren and Lumbreras, 1972; Koehler *et al.,* 1997). Recently, isolation and molecular detection of *B. vinsonii* subsp. *berkhoffii* genotype II has been reported from a dog with hemangiopericytoma and a boy with epithelioid hemangioendothelioma (Breitschwerdt *et al.,* 2009). The role of *Bartonella* in the development of these tumors needs further investigation.

A. Bacillary Angiomatosis

BA is an unusual vascular lesion caused by *Bartonella* sp. Three species of *Bartonella* have been reported to cause BA, including *B. henselae, B. quintana* and *B. vinsonii* subsp. *berkhoffii* (Koehler *et al.,* 1997; Yager *et al.,* 2010). BA has been reported in both human beings and dogs (Koehler *et al.,* 1997; Yager *et al.,* 2010) and is mainly seen in immunocompromised individuals, although BA has been reported in immunocompetent patients (Tappero *et al.,* 1993a). The disease is predominantly seen among patients in the late stages of Human Immunodeficiency Virus (HIV) infection and with CD4 lymphocyte counts of less than 100 cells per mm3 (Tappero *et al.,* 1995). BA is treated successfully with antibiotics, but if left untreated can be fatal (Cockerell *et al.,* 1987). BA lesions have been reported associated with pregnancy and in both immunocompromised and immunocompetent children (Myers *et al.,* 1992; Riley and Tuomala, 1992; Turgut *et al.,* 2004).

Historic perspective

BA was first reported in individuals infected with HIV (Stoler *et al.,* 1983). The association between Cat Scratch Disease (CSD) bacilli and BA was first demonstrated by LeBoit and co-workers in 1988. Numerous bacilli were demonstrated in BA vascular lesions using Warthin-Starry silver stain and transmission electron microscopy. The appearance of the bacilli was identical to that of the CSD bacillus (LeBoit *et al.,* 1988 and 1989), though efforts to isolate the organism were not successful until 1992. Analysis of the 16S rRNA and citrate synthase genes by Polymerase Chain Reaction (PCR) and Restriction Fragment Length Polymorphism (RFLP) showed that this agent is closely related to the trench fever agent *Rochalimaea quintana* and the organism was named as *Rochalimaea henselae* (Regnery *et al.,* 1992; Welch *et al.,* 1992). *R. henselae* was isolated from one patient and *R. quintana* from two patients with cutaneous and osseous BA lesions using the lysis centrifugation method and co-cultivation with an endothelial cell monolayer (Koehler *et al.,* 1992). The genera *Bartonella* and *Rochalimaea* were combined, the family *Bartonellaceae* removed from the order Rickettsiales in 1993, (Brenner *et al.,* 1993) and the agent of CSD

and BA was renamed *Bartonella henselae*.

The association between the development of BA and exposure to cats was demonstrated in a study by Tappero *et al.,* where BA patients were also more likely than controls to have a household kitten (Tappero *et al.,* 1993b). In another study involving 49 patients with BA, *B. henselae* infection was epidemiologically linked to cat and flea exposures, whereas infections with *B. quintana* were characterized by low income, homelessness, and exposure to lice (Koehler *et al.,* 1997). In a study conducted in Seattle, WA focused on chronic alcoholics, *B. quintana* was isolated from blood of 10 patients and 3 of them had recent cat scratches (Spach *et al*., 1995). *B. quintana* was also isolated from patients with endocarditis, CSD or BA and contact with cats (La-Scola and Raoult, 1999) indicating that exposure to cats may be a risk factor for the infection with *B. quintana*.

Use of more sensitive molecular techniques like PCR has allowed detection of new *Bartonella* sp. associated with BA. *B. vinsonii* subsp. *berkhoffii* genotype I DNA was detected in an immunocompromised dog with BA lesions by PCR (Yager *et al.,* 2010). Similar to *B. henselae, B. vinsonii* subsp. *berkhoffii* genotypes I, II and III can also induce activation of hypoxia inducible factor I and production of VEGF in a dose dependent manner by HeLa 229 cells (Kempf, Personnel communication).

Clinical presentation

BA lesions commonly involve skin. Focal lesions have also be reported in bone, brain, lymph nodes, respiratory tract, cardiac valves, gastrointestinal tract, oral mucosa, conjunctiva, scrotum, penis, cervix, vulva and bone marrow (Cockerell *et al.,* 1987; Baron *et al.,* 1990; Chan *et al.,* 1991; Slater and Min, 1992; Spach *et al.,* 1992; Lee *et al.,* 1994; Fagan *et al.,* 1995; Eden *et al.,* 1996; Long *et al.,* 1996; López de Blanc *et al.,* 2000). Cutaneous lesions of BA arise as small, erythematous vascular papules which may enlarge to form pedunculated, friable nodules surrounded by scaliness with or without erythema (Koehler *et al.,* 1992). Their number can vary from one to hundreds and these lesions bleed profusely when traumatized (Cockerell *et al.,* 1987). In dark skinned people the cutaneous BA lesion

may appear as an indurated, hyperpigmented black plaque (Koehler and Tappero, 1993). The cutaneous BA overlying osteolytic lesions may appear as cellulitic plaques (Webster *et al.,* 1992). Subcutaneous lesions of BA may arise below the skin surface as one or more nodules covered with flesh-colored or reddish skin. Enlarging soft tissue masses may erode through the skin giving rise to the cutaneous vascular lesions (Koehler *et al.,* 1992). BA may also be presented as soft tissue nodules without any cutaneous involvement (Schinella and Greco, 1990)

Bone lesions in BA usually involve the long bones like the tibia, fibula and radius (Baron *et al.,* 1990). Other locations where osseous BA has been reported include calcaneum, rib and vertebrae (Baron *et al.,* 1990; Herts *et al.,* 1991, Omarini *et al.,* 1994). Lesions may involve single or multiple sites. Radiographically, the lesions appear as well-circumscribed osteolysis, sometimes accompanied by periostitis (Baron *et al.,* 1990). BA lesions involving bone marrow and skin were reported in a patient with AIDS, and the bacilli were demonstrated by Warthin–Starry staining in the bone marrow (Fagan *et al.,* 1995).

Histologically confirmed BA lesions of the oral cavity were reported in two HIV positive patients (Lopez de Blanc *et al.,* 2000). Lesions were present in maxillary and mandibular gum line, soft palate, hard palate and the pharyngeal wall. Lesions appeared as red, elevated macular areas which bled profusely on palpation. BA lesions in lymph nodes are most commonly seen as enlarged lymph nodes (Koehler *et al.,* 1992; dos Santos *et al.,* 2007). Involvement of different lymph nodes has been reported, with the majority of cases involving the inguinal lymph node (Chan *et al.,* 1991; Kohler *et al.,* 1992).

Differential diagnosis of BA includes Kaposi's sarcoma (KS), pyogenic granuloma, angiosarcoma and verruga peruana (Kohler and Tappero, 1993). Most of the time, biopsy and histopathology are needed for the differentiation of BA from other lesions. Presence of small bacilli in the lesions that can be demonstrated by Warthin-Starry silver stain and electron microscopy differentiate BA from other vascular lesions by histopathology (Kohler and Tappero, 1993). The vascular tumors of BA can be treated successfully with antibiotics (Rudikoff *et al.,* 1989; Anderson and Newman, 1997). Concurrent occurrence of BA and KS

lesions has been reported. BA was successfully treated with antibiotics in that patient whereas KS lesions persisted (Berger *et al.,* 1989). Relapse of BA lesions can occur (Berger *et al.,* 1989; Koehler *et al.,* 1992), though this may be prevented by a longer course of antibiotic therapy (Koehler and Tappero, 1993).

Histopathology

Tissue specimen stained with routine hematoxylin and eosin, shows proliferation of small capillaries of various sizes and shapes in a characteristic lobular pattern (LeBoit *et al.,* 1988 and 1989; Yager *et al.,* 2010). The vessel walls are lined by protuberant endothelial cells and some areas may also contain clusters of plump endothelial cells without visible lumens (Juskevicius and Vnencak-Jones, 2004). Proliferating capillaries are surrounded by an edematous stroma and mixed inflammatory infiltrates. The inflammatory infiltrates are predominantly neutrophils but also contain lymphocytes and macrophages (LeBoit *et al.,* 1988), and many of the leukocytes showed degenerative changes including pyknosis and karyorrhexis of the nuclei (Yager *et al.,* 2010). BA lesions of bone, lymph nodes and brain may be less lobular and may not have prominent neutrophilic infiltrate (Koehler and Tappero, 1993). The connective tissue surrounding the proliferating capillaries shows the presence of basophilic granular material in H&E stained sections, which on Warthin–Starry staining reveal numerous bacilli in close proximity to the proliferating vessels and the inflammatory infiltrates. Although most of the bacilli appear extracellular, organisms can also be seen very close to or within the endothelial cells (Yager *et al.,* 2010). Organisms are not visualized using an acid fast stain for Mycobacterium, Gomori's methenamine silver stain for fungi or Brown-Brenn tissue gram stain (Koehler and Tappero, 1993; Juskevicius and Vnencak-Jones, 2004). Organisms can be demonstrated by electron microscopy as small bacilli with trilaminar cell wall and electron dense granular material (LeBoit *et al.,* 1988). *B. henselae* can be visualized in tissues by immunohistochemistry using monoclonal antibodies (Caponetti *et al.,* 2009). Fluorescent *in situ* hybridization assays for detection of *B. henselae* and *B. quintana* have also been reported (Hercik *et al.,* 2002; Gescher *et al.,* 2008), and these

tests may be of value in demonstration and differentiation of *B. henselae* and *B. quintana* in patients with BA.

B. Bacillary Peliosis Hepatis

Bacillary Peliosis (BP) refers to the vasoproliferative lesion caused by *Bartonella* in parenchymatous organs. It is commonly seen in liver and spleen and is characterized by blood filled spaces in these organs (Perkocha *et al.,* 1990; Slater *et al.,* 1992). Bacilli staining positively with silver stains are found associated with the proliferating endothelial cells. BP has been reported in human beings and dogs (Slater *et al.,* 1992; Ahsan *et al.,* 1998; Kitchell *et al.,* 2000). Even though peliosis hepatis is common in cats, the reservoir host of *B. henselae*, a recent study failed to detect any association between *B. henselae* and peliosis hepatis in cats (Buchmann *et al.,* 2010). The pathogenesis of BP caused by *B. henselae* is not clear, although vascular endothelial growth factor seems to play a role in the development of these unique vascular lesions (Kempf *et al.,* 2001). Peliosis hepatis, not due to *Bartonella* infection, has been well described and has been shown to occur in association with tuberculosis, anabolic and androgenic steroids, oral contraceptives, malignant tumors, and immunosuppressive agents such as azathioprine and cyclosporine (Schoenlank, 1916; Nadell and Kosek, 1977; Larrey *et al.,* 1988; Elsing *et al.,* 2007; Choi *et al.,* 2009; Kleger *et al.,* 2009). This review primarily focuses on peliosis hepatis caused by *Bartonella* sp. infection.

Historic perspective

The term ‘Peliosis hepatis’ was introduced in 1916 by Schoenlank to describe unique blood filled cystic lesions in a 33 year old woman who died of tuberculosis (Schoenlank, 1916). Peliosis hepatis associated with Acquired Immunodeficiency Syndrome (AIDS) was first reported by Czapar and co-workers in 1986. Peliosis lesions were observed in the livers of 2 AIDS patients and one patient had lesions also in the spleen and portal lymph node (Czapar *et al.,* 1986). Presence of rod-shaped bacteria in the peliotic lesions were first observed by Perkocha and associates (Perkocha *et al.,* 1990), and *B. henselae* was first

isolated from a patient with histologically confirmed BP in 1992 (Slater *et al.,* 1992). A single case of BP due to *B. henselae* in a dog was reported in 2000 (Kitchell *et al.,* 2000).

Clinical presentation

BP, like bacillary angiomatosis, is commonly found in immunocompromised patients such as people with AIDS, tuberculosis or those who undergo immunosuppressive therapy for cancer or organ transplants (Slater *et al.,* 1992; Sandrasegaran *et al.,* 2005). Patients with BP may present clinically with abdominal pain and fever (Perkocha *et al.,* 1990) and physical examination abnormalities may include hepatomegaly and splenomegaly. BA and BP lesions may exist concurrently in some patients. Mild to moderate increase in the level of hepatic enzymes may be noted (Kohler and Tappero, 1993) and alkaline phosphatase levels were more consistently elevated compared to transaminases (Perkocha *et al.,* 1990). BP has also been attributed as the cause of acute anemia in an AIDS patient (Garcia-Tsao *et al.,* 1992). Low density lesions in the hepatic parenchyma could be noted in computed tomography and ultrasonography (Sandrasegaran *et al.,* 2005). Diagnosis is confirmed by biopsy and histopathology (Kemper *et al.,* 1990; Steeper *et al.,* 1992). BP has also been reported in spleen (Perkocha *et al.,* 1990; Slater *et al.,* 1992). BP is successfully treated with antibiotics, with complete resolution of the lesions (Perkocha *et al.,* 1990; Slater *et al.,* 1992), but, if left untreated, may lead to spontaneous rupture of the vascular lesions in the liver (Choi *et al.,* 2009).

Histopathology

Histologically, BP presents as multiple blood-filled spaces in the parenchymatous organs. There can be a spectrum of changes ranging from dilated capillaries to multiple thin-walled blood-filled spaces in the parenchyma lined with endothelial cells which stain positive with Factor VIII antigen (Kitchell *et al.,* 2000). These peliotic spaces are surrounded by myxoid stroma (Perkocha *et al.,* 1990; Tsokos and Erbersdobler, 2005). Mixed inflammatory cells are seen in the stroma mixed with purple-stained granular material which on silver

staining and electron microscopy reveals clumps of bacilli (Perkocha *et al.,* 1990; Kohler and Tappero, 1993). Ultrastructural studies have demonstrated the bacilli in the hepatic sinusoidal endothelial cells (Leong *et al.,* 1992). Organisms were detected in both intracellular and extracellular locations.

C. Verruga peruana

Verruga peruana is a vasoproliferative disorder of human beings caused by the bacteria *B. bacilliformis*. The disease is known as Carrion's disease or Oroya fever. *B.bacilliformis* was the first identified member of the genus *Bartonella* and the only member in the genus until 1993, when the genus *Rochalimaea* was combined with the genus *Bartonella* (Brenner *et al.,* 1993). The disease caused by *B. bacilliformis* in humans is a biphasic disease characterized by an acute febrile illness, accompanied by a hemolytic anemia, which can be fatal and, if the individual survives, is followed by formation of wart-like vasoproliferative lesions called verruga peruana. Infection with *B. bacilliformis* has been restricted to the Andes region of South America, mainly Peru, Columbia and Ecuador (Ihler, 1996). Reports from other parts of the world have involved individuals travelling to the endemic areas (Matteelli *et al.*, 1994; Lydy *et al.,* 2008). The confined occurrence of this disease is thought to be due to the limited distribution of its vector. *B. bacilliformis* is transmitted by the sand fly *Lutzomyia verrucarum* (previously known as *Phlebotomus verrucarum*), and potentially other closely related sand flies (Alexander, 1995). Transmission of *B. bacilliformis* from infected to normal rhesus macaques by the tick *Dermacentor andersoni* was demonstrated experimentally (Noguchi, 1926), but there is no report of natural transmission of *B. bacilliformis* by ticks. To date, humans are the only known reservoir of *B. bacilliformis* and the infection has not been reported in any other animal species.

Historic Perspective

B. bacilliformis has been known to cause disease in human beings for several hundred years as evidenced by the pottery and carvings of the pre-Columbian natives in

South America (Alexander, 1995; Minnick and Battisti, 2009). The most devastating outbreak of bartonellosis occurred among the laborers working on the Central railroad of Peru during the late 19th century, causing the death of thousands due to an acute febrile disease (Karem *et al.,* 2000). Association between the acute febrile phase and chronic verruga phase was first demonstrated by Daniel Carrion in 1885. Carrion, a Peruvian medical student inoculated himself with the materials taken from a verruga lesion. Unfortunately, he succumbed to the disease and died in October 1885. In his honor, the disease was named Carrion's disease (Garcia-Caceres and Garcia, 1991). The causative agent of Carrion's disease (*B. bacilliformis*) was demonstrated within erythrocytes by Alberto Barton in 1905.

In 1913, a group of researchers from Harvard School of Tropical Medicine, conducted extensive studies and made the incorrect conclusion that Oroya fever and verruga peruana were separate disease entities (Strong *et al.,* 1915). The single volunteer they inoculated with the extract from verruga peruana survived. Thus they came to the conclusion that Oroya fever is caused by *B. bacilliformis* and verruga peruana is caused by a virus. However in 1926, Noguchi and Battistini isolated *B. bacilliformis* from the blood of a patient who died of Oroya fever and injected it into rhesus monkeys where upon the monkeys developed verruga lesions, thereby proving Daniel Carrion's observations in a reciprocal manner (Noguchi and Battistini, 1926). Noguchi also noted that the development of the disease depends on the immune status of the host and the virulence of the strain of bacteria and that different disease manifestation, ranging from mild skin lesions to severe hemolytic anemia were observed in the experimental animals inoculated with the same strain of the bacteria (Noguchi, 1927). In another study, he also reported that the bacteria from Oroya fever and verruga peruana showed complete cross immunity. This further validated that Oroya fever and verruga peruana are caused by the same organism (Noguchi, 1927b). It was Charles Townsend who first proposed that the disease is transmitted by the sand fly *Lutzomyia* (*Phlebotomus*) *verrucarum* (Strong *et al.*, 1915).

Even with the availability of antibiotics, Carrion's disease continues to be a problem in some parts of Peru, Columbia and Ecuador. Outbreaks of both acute and chronic forms of

the disease have been reported in recent years (Gray *et al.,* 1990; Cooper *et al.,* 1997; Ellis *et al.,* 1999; Kosek *et al.,* 2000; Maco *et al.,* 2004). The rate of occurrence is especially high in children less than 5 years old (Chamberlin *et al.,* 2002). The recent studies have documented a wide spectrum of disease manifestations that is significantly milder than the classic Oroya fever (Kosek *et al.,* 2000).

Clinical presentation

B. bacilliformis causes a biphasic disease. The acute form of the disease is characterized by fever and severe hemolytic anemia. Other symptoms include hepatospenomegaly, rapid pulse, malaise, weakness, myalgia, neurological complications and circulatory collapse (Ihler, 1996; Maco *et al.,* 2004). If left untreated, the mortality can be as high as 88 % (Ihler, 1996). Transient immunosuppression leading to secondary bacterial infections can contribute to the high mortality rate (Maco *et al.,* 2004). Even with proper antibiotic treatment, mortality of 9% is associated with Oroya fever (Kosek *et al.,* 2000). During the infection 100% of the red blood cells may become infected. Anemia is due to the rapid removal of the infected red blood cells by the cells of the reticuloendothelial system leading to a dramatic drop in hematocrit (Mernaugh and Ihler, 1992). Hematocrit values may drop below 20% (Maguina *et al.,* 2001). *B. bacilliformis* is the only species of *Bartonella* that can be visualized on the surface of erythrocytes with Wrights or Geimsa stain (Bentzel *et al.,* 2008). Persistent infection and bacteremia without hemolytic anemia has been reported in a 56 year old splenectomized patient. More than 90% of the erythrocytes were infected in this patient, but there was no hemolytic anemia present. This finding clearly demonstrates the role of the spleen in the pathogenesis of acute anemia in Oroya fever patients (Henríquez *et al.,* 2004). In a prospective study in the endemic areas of Peru, 0.5% of healthy individuals were asymptomatic carriers of *B. bacilliformis* (Chamberlin *et al.,* 2002).

The acute phase of the disease can last for weeks to months. If the patient survives the acute phase, it can be followed by the chronic form in some individuals. In endemic areas,

verruga peruana can also develop without any noticeable hematic phase (Kosek *et al.,* 2000). Verruga peruana is characterized by nodular vasoproliferative lesions on the skin. Other non-specific symptoms include muscular and joint pain, headache, and malaise. The nodular lesions of verruga peruana can be presented in three forms; miliary, nodular (subdermic), and mular (Maguina *et al.,* 2001). Miliary lesions are multiple small (1-4mm), erythematous lesions usually confined to the papillary dermis and are often accompanied by pruritus. Nodular lesions are medium sized and appear as subcutaneous nodules without any change to the overlying skin. Mular lesions involve all layers of the dermis and subcutaneous tissue and are usually larger lesions which bleed profusely. The most common form of these lesions is miliary (Ihler, 1996; Maguina *et al.,* 2001). Verruga peruana lesions are commonly located in the upper and lower extremities. Lesions may also be present in conjunctiva and oral and nasal mucosa. The lesions are friable and bleed profusely on trauma. Secondary bacterial infection may also be a complication (Maguina *et al.,* 2001).

Carrion's disease and verruga peruana are treatable with antibiotics. Historically, chloramphenicol, penicillin G, tetracycline and erythromycin have been used successfully to treat Oroya fever, and streptomycin and rifampicin to treat the eruptive verruga peruana (Maguina *et al.,* 2001; Rolain *et al.,* 2004). *In vitro* studies have demonstrated susceptibility of *B. bacilliformis* to many antibiotics including most β-lactams, aminoglycosides, chloramphenicol, rifampin, macrolides, tetracyclines, cotrimoxazole, and fluoroquinolones (Sobraques *et al.,* 1999); however more recent studies have demonstrated quinolone, rifampicin and erythromycin-induced resistant mutants of *B. bacilliformis,* whereas the organisms were susceptible to gentamicin and doxycycline even after 16 passages *in vitro* (Biswas *et al.,* 2007; Dell Valle *et al*., 2010).

Histopathology

Histologically, verruga peruana resembles BA, but unlike BA, the lesions of verruga peruana are restricted to the skin (Karem *et al.,* 2000). Lesions are characterized by proliferation of endothelial cells which may be seen as solid sheets or as well-formed

capillaries with patent lumen (Arias-Stella *et al.,* 1986). Phagosomes containing degenerating bacteria may be seen in the proliferating endothelial cells and these inclusion bodies seen in the cytoplasm of the endothelial cells are known as Rocha-Lima inclusions (Areas-Stella *et al.,* 1986). Mixtures of inflammatory cells, predominantly made of lymphocytes and plasma cells, are seen surrounding the proliferating vessels. Clumps of bacteria can be demonstrated by Warthin-Starry silver stain in close proximity to the proliferating endothelial cells. Bacteria can also be demonstrated by electron microscopy (Recavarren and Lumbreras, 1972).

Pathogenesis of Bartonellosis

Interaction with erythrocytes

Bartonella species are emerging zoonotic pathogens associated with a variety of clinical manifestations in both human beings and animals. *Bartonella* can cause prolonged intraerythrocytic bacteremia in the reservoir hosts, which helps in the transmission of these bacteria by blood sucking arthropods (Schulein *et al.,* 2001; Billeter *et al.,* 2008). *B. bacilliformis* and *B. quintana* typically infect human red blood cells and *B. henselae* infects feline erythrocytes (Cuadra and Takano, 1969; Kordick and Breitschwerdt, 1995; Rolain *et al.,* 2002). Recently, *B. henselae* has been demonstrated to infect human erythrocytes *in vitro* (Pitassi *et al.,* 2007). They can survive for prolonged periods (more than 35 days) in human erythrocytes stored at 4^0C (Magalhaes *et al.,* 2008), indicating potential for transmission by blood transfusion. The same authors also documented a case of death suspected to be due to bartonellosis in a patient with aplastic anemia, after receiving a blood transfusion (Magalhães *et al.,* 2009). However, intraerythrocytic infection of *B. henselae* in human erythrocytes has not been documented in naturally-occurring infections to date. Studies suggest that *B. henselae* can also invade CD34 hematopoietic progenitor cells, and infection with *Bartonella* does not affect erythroid differentiation of hematopoietic progenitor cells. Therefore, infection of CD34 progenitor cells results in intracellular presence and replication of *B. henselae* in erythroid differentiated cells (Mandle *et al.,* 2005). *Bartonella* is adapted to

survive in the mammalian erythrocytes without causing hemolysis. An exception to this is *B. bacilliformis*, which causes a fatal hemolytic anemia in humans due to the destruction of infected erythrocytes by the spleen (Ihler, 1996). *B. bacilliformis* infects up to 100% of the erythrocytes (Maguina *et al.*, 2001), whereas the percentage of red blood cells infected is very low for *B. quintana* (0.001-0.005%) (Rolain *et al*., 2002). Electron microscopic studies in cats persistently infected with *B. henselae* showed up to 6.2% of the erythrocytes containing intracellular bacteria (Kordick and Breitschwerdt, 1995)

Animal models of Intraerythrocytic parasitism

Most of the studies on intraerythrocytic infection of *Bartonella* have been performed in animal models (Boulouis *et al.*, 2001; Koesling *et al.*, 2001; Schulein *et al.*, 2001; Marignac *et al.*, 2010). In a rat model, green fluorescent protein (GFP)-expressing *B. tribocorum* was used to study the course and kinetics of intraerythrocytic infection (Figure 1) (Schulein *et al.*, 2001). After intravenous inoculation of culture-grown *B. tribocorum* (isolated from the blood of wild rats) into 6 week old Wistar rats, the bacteria were cleared from the blood within hours of inoculation and blood remained culture negative for 3-4 days. Bacteria reappeared in the blood stream 4-5 days post infection and periodic increases in the number of infected erythrocytes occurred at intervals of approximately five days. These results indicate that following infection the bacteria are readily cleared from the blood stream, subsequently colonize a 'primary niche', and multiply there with release into the blood stream after a period of 5 days. The identity of the 'primary niche' in which this replication occurs is not experimentally proven, but it is considered that endothelial cells and other cells of the reticuloendothelial system may act as the primary site (Schulein *et al.*, 2001; Dehio, 2008). After release from the primary niche, the bacteria adhere to and invade erythrocytes. Following invasion, the bacteria replicate inside the erythrocytes in membrane bound compartments. The degree of replication is limited, achieving up to 15 (average 8) bacteria per erythrocyte (Schulein *et al.*, 2001). Once this critical density is attained, the bacteria survive for the entire lifespan of the erythrocytes, and the infected cells are virtually

indistinguishable from the uninfected cells (Schulein *et al.,* 2001). In rats, approximately 0.1% of the erythrocytes get infected. The hemotropic infection in this model was self limiting, with complete clearance of the bacteria after 10 wks. Notably, in natural infection with *B. quintana,* the fever occurs at an interval of 5 days, and trench fever is also called five day fever.

Similar kinetics of infection has been noted in other rodent models of *Bartonella* infection (Boulouis *et al.,* 2001; Koesling *et al.,* 2001). In the mouse model of *B. birtlesii* infection (Boulouis *et al.,* 2001), female BALB/c mice became bacteremic on day 8 post-infection, with the peak bacteremia noted on day 14. All mice were blood culture negative by 10 weeks. Pregnant mice showed reproductive abnormalities including fetal loss, vascular lesions in the placenta, and transplacental transmission of organisms to the fetus. The clearance of infection may be due to the immune response of the host as shown by Kosoy and co-workers (Kosoy *et al.,* 1999). In their experiments with cotton rats experimentally-infected with three different *Bartonella* sp., challenging convalescent animals with the same *Bartonella* sp. did not result in reinfection, whereas the animals became bacteremic upon infection with a different *Bartonella* sp. Similar results were obtained by Yamamoto and co-workers in their studies with cats infected with four different species or strains of *Bartonella*. They noted that *B. henselae* type I is protective against *B. henselae* type II, whereas type II did not provide protection against subsequent challenge with type I. *B. clarridgeiae* did not protect the cats against secondary infection with either *B. henselae* type I or type II (Yamamoto *et al*., 2003). In another mouse model of infection with *B. grahamii*, supplementation of immune serum to B-cell deficient mice resulted in abrogation of persistent bacteremia, indicating that a specific antibody response is important in protection of mice against *B. grahamii* infection (Koesling *et al.,* 2001).

Molecular pathogenesis of intraerythrocytic infection

Different virulence determinants for the erythrocytic invasion have been identified in *Bartonella* sp. They include flagella-mediated motility, Trw type IV secretion system

(T4SS), deformin and an invasion-associated locus (*ial*) (Mernaugh and Ihler, 1992; Scherer *et al.,* 1993; Dehio, 2008). Flagella-mediated motility is important for the flagellated pathogen *B. bacilliformis*, which lacks the Trw T4SS (Minnick and Battisti, 2009). Flagellin of *B. bacilliformis* is a 42 kilodalton (kDa) protein (Scherer *et al.,* 1993) which is partially resistant to trypsin digestion. Motile bacteria adhere to and form deformations in erythrocytes. Non-motile or azide-killed bacteria could not adhere to erythrocytes or form deformations (Benson *et al.,* 1986). Treatment of bacteria with antiflagellin antibodies reduced the invasion of erythrocytes by 99.7% (Scherer *et al.,* 1993), indicating that flagellin is essential for the attachment and invasion of erythrocytes. Other species of *Bartonella,* such as *B. henselae, B. quintana* and *B. tribocorum,* are non-flagellated but can invade the erythrocytes. They possess the Trw T4SS which helps them during erythrocyte invasion.

Bartonella possess three different type IV secretion systems (VirB/VirD4, Vbh and Trw) which act as host adaptability factors (Saenz *et al.,* 2007). The VirB/VirD4 T4SS mediates the infection of endothelial cells by subverting the endothelial cell functions (Schmid *et al.,* 2004). The VirB/VirD4 system is also essential for the establishment of intraerythrocytic infection in the rat model of *B. tribocorum* infection (Schulein and Dehio, 2002). A deletion mutation in either VirB or VirD4 genes of *B. tribocorum* resulted in the loss of the ability of the bacteria to infect erythrocytes. The second T4SS, Trw, is also required for the intraerythrocytic infection in the rat model of *B. tribocorum* infection (Seubert *et al.,* 2003). Trw was first identified in *B. henselae* during a search for the promoters specifically activated during *in vitro* infection of endothelial cells (Seubert *et al.,* 2003). The Trw of *B. tribocorum* shares 80% amino acid similarity with the Trw conjugation machinery of the antibiotic resistance plasmid R388 of *E. coli* (Seubert *et al.,* 2003). It is conserved not only structurally, but also functionally, as evidenced by the fact that *trw*D and *trw*H mutations in R388 can be trans-complemented for conjugation by the homologues of *B. tribocorum*. A deletion mutation in the trwE of *B. tribocorum* resulted in the loss of infectivity to erythrocytes, indicating that the Trw T4SS is essential for erythrocytic parasitism; however, trwE mutants appeared to be able to infect the primary niche as shown

by the appearance of this mutant in the blood for a short period on the day 5 post infection (Dehio, 2004; Schroder and Dehio, 2005). Similar findings were also reported in the mouse model of *B. birtlesii*, in which bacteria carrying mutations in the *trw* locus were unable to cause intraerythrocytic infection (Marvis *et al.*, 2005). Multiple copies of *trwL* and *trwJ* encode different forms of surface-exposed pilus components (Dehio, 2008). The other duplicated genes *trwI* and *trwH* are essential for pilus elongation and anchorage to the outer membrane respectively. It is assumed that these various pili may facilitate the interaction with different receptors on the erythrocytes, thus representing a major determinant of host range or host specificity (Dehio, 2008). Cat-specific *B. henselae* and human-specific *B. quintana* expressing the Trw of rat-specific *B. tribocorum* were able to infect rat erythrocytes, demonstrating that Trw mediates host-specific erythrocyte infection (Vayssier-Taussat *et al.*, 2010). Variable, surface-located TrwL and TrwJ appear to be the T4SS components that determine host-specificity of erythrocyte parasitism. The Vbh and VirB/VirD4 T4SSs are closely related and considered to be functionally redundant. Vbh (VirB homologue) is functional only in species in which VirB/VirD4 T4SS is absent (e.g. *B. bovis*). In the presence of VirB/VirD4 system, Vbh shows accumulation of mutations or complete loss of the locus (Saenz *et al.*, 2007).

Another virulence factor associated with the intraerythrocytic parasitism is 'deformin', a secreted bacterial product that has been identified in *B. bacilliformis* and *B. henselae* (Iwaki-Egava and Ihler, 1997). Deformin was first reported to be a protein (Xu *et al.*, 1995), and later work showed that it is a small hydrophobic molecule with a high affinity for albumin (Derrick and Ihler, 2001). Deformin can cause extensive pitting and invagination of erythrocyte membranes, which are considered to be the point of entry of bacteria into erythrocytes (Benson *et al.*, 1986). Deformin–induced deformations can be reversed by the treatment with ATP inhibitors such as vanadate, or by increasing intracellular calcium ion levels with ionophores (Mernaugh and Ihler, 1992; Xu *et al.*, 1995). A hemolysin that acts independently of deformin has also been identified in *B. bacilliformis* which is considered to be responsible for the β-hemolytic phenotype observed in *B. bacilliformis* grown on blood

agar plates (Hendrix, 2000). Another cohemolysin called CAMP-like factor autotransporter (Cfa) which is an 18kDa autotransporter protein, has been identified in *B. henselae*. The α-domain of Cfa is secreted into the growth medium and it has some homology to repeat in toxin (RTX) (Litwin and Johnson, 2005). Cfa orthologues are also present in *B. quintana*. The virulence function of these hemolysins needs further clarification.

Invasion-associated locus (*ial*) of *Bartonella* is composed of two genes *ialA* and *ialB*. Introduction of *ialA* and *ialB* genes of *B. bacilliformis* conferred an erythrocyte-invasive phenotype to laboratory-adapted strains of *E. coli* (Mitchell and Minnick, 1995). The ial locus is highly conserved in the genus *Bartonella* (Minnick and Battisti, 2009). IalA is a 21kDa, dinucleoside polyphosphate hydrolase of MutT motif family thought to be involved in reducing levels of stress-induced dinucleotides during invasion, thus aiding bacterial survival (Cartwright *et al.*, 1999). IalB is an 18kDa protein located in the inner membrane of *B. bacilliformis*, whereas it was identified in the outer membrane of *B. henselae* (Mitchell and Minnick 1995; Coleman and Minnick 2001; Chenoweth *et al.,* 2004). Insertional mutagenesis of *ialB* results in approximately 50% reduction in human erythrocyte invasion of *B. bacilliformis*, compared to the wild type and trans complementation of wild type *ialB* which restored the ability to invade erythrocytes to a level comparable to the wild type (Coleman and Minnick, 2001). *ialB* expression levels vary depending on the environmental conditions. The expression is higher in arthropod like conditions (20^0C, pH 5.0) when compared to human like conditions (37^0C, pH 7.2) (Coleman and Minnick, 2003). This may be important in the priming of bacteria during its time in the sand fly, enhancing the virulence for subsequent transmission to humans. The molecular mechanisms by which the Ial A and B proteins enhance erythrocyte invasion remain unclear.

The receptors on the erythrocytes and the bacterial adhesins involved in adherence of *Bartonella* sp. to erythrocytes are not yet identified. Earlier studies demonstrated that *B. bacilliformis* adhered poorly to alpha- or beta-glucosidase-treated erythrocytes, but pronase or subtilisin treatment of erythrocytes stimulated adherence, indicating that Bartonellae probably adhere to an erythrocyte glycolipid moiety (Walker and Winkler, 1981). Recent

studies indicate that *B. bacilliformis* interacts with multiple surface exposed proteins on human erythrocytes including Glycophorins A and B (Buckles and McGinnis, 2000). Also, the inactivation of the proton motive force or respiration by chemicals significantly reduces the bacterial adherence to the erythrocytes indicating that the adhesion is energy dependent (Benson *et al.*, 1986).

Interaction of *Bartonella* with endothelial cells

Invasion of endothelial cells

The molecular mechanism underlying *Bartonella*-induced vasoproliferation is not well-defined, and most of the knowledge is gathered from the *in vitro* studies using a cell culture model of human umbilical vein endothelial cells (HUVEC) (Dehio *et al.,* 1997; Verma *et al.,* 2000; Fuhrmann *et al.,* 2001; Schmid *et al.,* 2004). *Bartonella* adheres to and enters HUVECs by an actin-dependent process within hours of infection. The uptake of *B. bacilliformis* is dependent on the small GTPases Rho, Rac and Cdc42 (Verma *et al.,* 2000; Verma and Ihler, 2002). Internalization of *B. bacilliformis* was blocked by inactivation of intracellular Rho by C3 exoenzyme (Verma *et al.,* 2000). Bacteria appear as small clusters in membrane-bound compartments localized in the perinuclear region (Brouqui and Raoult, 1996; Dehio *et al.,* 1997; Verma *et al.,* 2000). *B. henselae* can invade the endothelial cells using a second mechanism called ‘invasome-mediated uptake’ (Dehio *et al.,* 1997). Invasome-mediated uptake is a slower process which can take up to 24 hrs to complete. The process starts with establishing contact to sedimented bacteria at the leading lamella of endothelial cells and mediating bacterial aggregation by rearward transport on the cell surface. The bacterial aggregate is then engulfed and internalized by a unique host cellular structure, the invasome. The membrane protrusions entrapping the bacterial aggregate are rich in Cortical F-actin, intercellular adhesion molecule-1 (ICAM- I) and phosphotyrosine (Dehio *et al.,* 1997). The formation of invasomes was found to be inhibited by cytochalasin D but unaffected by nocodazole, colchicine or taxol, indicating that invasome-mediated invasion is an actin-dependent and microtubuli-independent process. Invasome-mediated

uptake is dependent on the VirB/VirD4 T4SS (Schmid *et al.,* 2004), and the Bartonella effector protein G (BepG) is required for the triggering of invasome formation (Rhomberg *et al.,* 2009). BepG promotes the invasome-mediated internalization by inhibiting bacterial entry by endocytosis.

Bartonella possess a group of trimeric autotransporter adhesins (TAAs) including Bartonella adhesin A (BadA) of *B. henselae*, variably expressed outer membrane proteins (Vomps) of *B. quintana* and *Bartonella* repetitive protein (Brp) of *B. vinsonii* subsp. *arupensis* (Zhang *et al.,* 2004; Gilmore *et al.,* 2005; Kaiser *et al.,* 2008). BadA is a 340kDa nonfimbrial adhesin located on the outer membrane of *B. henselae* (Riess *et al.,* 2004). This study showed that BadA mediates auto-aggregation and attachment to the endothelial cells by binding to the β1-integrins and prevents phagocytosis of *B. henselae* by macrophages. BadA also mediates binding to the extracellular matrix proteins; in this mechanism, the head of BadA binds to the collagen and the stalk is essential for binding to fibronectin (Kaiser *et al.,* 2008). BadA is also responsible for the activation of hypoxia inducible factor- Iα (HIF- Iα) and secretion of VEGF, which play an important role in *Bartonella*- induced vasoproliferation (Riess *et al.,* 2004). The Vomps of *B. quintana* are a family of four highly conserved 100kDa proteins. Two of these proteins Vomp A and Vomp C function as adhesins, Vomp C binds to collagen IV, and Vomp A mediates auto aggregation (Zhang *et al.,* 2004). The variable expression of Vomps is mediated by deletion of one or more *Vomp* genes during chronic infection. The *brp* gene family of *B. vinsonii* subsp. *arupensis* codes for 3 proteins, Brp A-C. These show significant similarity to the BadA of *B. henselae* and Vomps of *B. quintana* (Gilmore *et al.,* 2005). The biological functions of the Brps need further investigation. Other potential adhesins of *Bartonella* include the heme binding proteins (Hbps/pap31), which can bind to the extracellular matrix components such as fibronectin and heparin (Dabo *et al.,* 2006)

B. henselae avoids the normal endocytic pathway, by forming specialized non-endocytic membrane bound Bartonella containing vacuoles (BCVs) which lack endocytic marker proteins, fail to acidify and do not fuse with lysosomes (Kyme *et al.,* 2005) in the

endothelial cells, whereas their fusion with the lysosomes is delayed in macrophages and requires bacterial viability. The mechanism by which *Bartonella* inhibit the endosome maturation is unclear, but evidence suggests that *Bartonella* plays an active role in the process, as dead bacteria are processed by the normal phagolysosome formation.

Subversion of cellular functions mediated by the type IV secretion system

Bartonella can subvert host cell functions to its advantage. This is mediated by the VirB/VirD4 T4SS and its effector proteins (Schmid *et al.,* 2004) (Figure 2). T4SSs are protein assemblies that mediate the transfer of DNA and proteins from a bacterium to other cells (Christie *et al.,* 2005). They are ancestrally related to the bacterial conjugation machinery. *Bartonella* possess 3 T4SSs, namely VirB/VirD4, Trw and Vbh, of which VirB/VirD4 T4SS is required for the endothelial cell infection. VirB/VirD4 T4SS is well-conserved within the genus *Bartonella* (Dehio, 2005). T4SS consists of a substrate translocation channel spanning the bacterial membranes and a pilus-like structure extending from the bacterial surface, which is thought to establish focal contact with the host cell. The translocation process is thought to be activated by receptor docking, and the translocation channel is presumed to extend to, or even across, the target cell membrane to facilitate substrate translocation (Christie *et al.,* 2005).VirB/VirD4 T4SS is encoded by an operon of 10 genes (virB2-virB11) and the downstream virD4 gene (Dehio, 2005).

Seven Bartonella effector proteins have been identified (Bep A-G) and they are responsible for all the known Vir B-mediated endothelial cell changes (Schulein *et al.,* 2005). The amino acid sequence of these proteins resembles plasmid-encoded conjugative relaxases of the bacterial conjugation system (Pulliainen and Dehio, 2009) which mediates the transfer of plasmid DNA by cleaving and covalently attaching to the DNA strand and directing the transport of the complex into the recipient cell (Christie *et al.,* 2005). Beps contain a C-terminal Bep intracellular delivery (BID) domain followed by a short positively- charged tail sequence. These two together form the signal for translocation of these proteins into the host cells (Schulein *et al.,* 2005). Bep E, Bep F and Bep G contain more than one BID domain.

Bep A, Bep B and Bep C contain a filamentation induced by the cAMP (FIC) domain, the function of which is currently unknown in *Bartonella*, although it is involved in cellular morphogenesis in *E. coli* (Utsumi *et al.,* 1982). Bep D, Bep E and Bep F contain N-terminal tandem-repeated motifs resembling eukaryotic tyrosine – phosphorylation motifs (EPLYA) (Pulliainen and Dehio, 2009). These motifs are similar to the EPIYA motif of CagA protein which is a T4SS substrate of *Helicobacter pylori*. CagA is phosphorylated after being translocated into the gastric epithelial cells by Src-family kinases. Tyrosine-phosphorylated CagA interferes with various signaling pathways in the host cell, resulting in cytoskeletal rearrangements, cell migration and malignant transformation (Hatekeyama, 2004). It has been shown that Bep D also gets tyrosine phosphorylated after being translocated into the endothelial cells by host tyrosine kinases (Schulein *et al.,* 2005). If the other Beps are also phosphorylated in the host cell and whether they modulate the host cell signaling pathways requires further research.

Activation of proinflammatory response in endothelial cells

B. henselae can activate the NF-κB pathway resulting in the upregulation of the adhesion molecules like intracellular adhesion molecule I (ICAM-I) and E-selectin in infected HUVECs *in vitro* (Fuhrmann *et al.,* 2001). Purified bacterial outer membrane proteins (omps) activated the endothelial cells with similar efficiency as live bacteria, indicating that bacterial omps are responsible for this effect. Upregulation of the adhesion molecules was accompanied by increased rolling and adhesion of neutrophils. Neutrophil infiltration is a characteristic feature of the vasoproliferative lesions induced by *Bartonella* (LeBoit *et al.,* 1988). NF-κB activation also results in the production of IL-8 which is a powerful chemoattractant triggering trans-endothelial migration of lymphocytes (McCord *et al.,* 2006). *B. henselae* is also able to induce the production of the chemokine monocytes chemoattractant protein-1 (MCP-1) from human microvascular endothelial cells (HMEC-1) (McCord *et al.,* 2005) which triggers monocyte migration into lesions. The influx of these cells is believed to help in the *Bartonella*-induced vasoproliferation by producing angiogenic

proteins such as VEGF (Resto-Ruiz *et al.,* 2002).

Bartonella-induced vasoproliferation

Bartonella can induce proliferation of endothelial cells in 3 ways (Figure 3). *Bartonella* can adhere and invade endothelial cells, resulting in the direct mitogenic stimulation of the endothelial cells. Second, *Bartonella* can also inhibit the endothelial cell apoptosis. The third mechanism by which Bartonella induces vasoproliferation is through the activation of NF-κB which results in a proinflammatory phenotype and recruitment of macrophages. Colonization of macrophages by *Bartonella* results in production of VEGF which acts on the endothelial cells in a paracrine manner to induce proliferation (Dehio, 2005).

A. Direct stimulation of mitosis.

Three species of *Bartonella* (*B. henselae*, *B. quintana* and *B. bacilliformis*) are capable of inducing proliferation of HUVECs *in vitro* (Garcia *et al.,* 1992; Conley *et al.,* 1994; Maeno *et al.,* 1999). A soluble bacterial protein was implicated as the angiogenic factor in *B. bacilliformis* (Garcia *et al.,* 1990) which was also shown to be capable of inducing neovascularization in a surgically implanted sponge in rats. Later research showed that GroEL, which is a chaperonin protein, plays a role in the mitogenic activity of *B. bacilliformis* on HUVECs (Minnick *et al.,* 2003). Mitogenic activity is significantly reduced by the treatment with anti-GroEL and anti-GroES antibodies. It may also be possible that GroEL is acting indirectly by protecting other unknown mitogenic factors through its chaperonin activity (Minnick and Battisti, 2009). Even though *B. bacilliformis* GroEL has mitogenic activity, it does not have an anti-apoptotic effect on the HUVECs; in fact overexpression of GroEL actually leads to induction of apoptosis. Mitogenic activity of *B. bacilliformis* is much greater than the mitogenic activity of *B. henselae* (Minnick *et al.,* 2003).

B. henselae can cause proliferation and migration of HUVECs through a porous

membrane *in vitro*, and this property was attributed to a trypsin sensitive factor in the soluble fraction of bacterial lysates (Conley *et al.*, 1994). Later work showed that the stimulatory effect of *B. henselae* on the proliferation of HUVECs was mediated by soluble mitogens secreted into the medium (Maeno *et al.*, 1999). UV or heat-killed *B. henselae* did not have this stimulatory effect, indicating that a live bacterium is necessary for the stimulation of proliferation. No direct contact of the bacteria with the cells was necessary for the stimulatory effect as shown by the ability of *B. henselae* separated from the HUVECs by a filter membrane to stimulate the proliferation of HUVECs. This stimulatory effect on proliferation is specific for endothelial cells (Maeno *et al.*, 1999). A more recent study has also shown the ability of *B. henselae* to induce tube formation of collagen-embedded HUVECs (Kirby, 2004). All these studies support a direct effect of *Bartonella* on stimulation of endothelial cell proliferation.

B. Inhibition of endothelial cell apoptosis

Mammalian cells respond to extrinsic insults, including bacterial infection, by inducing apoptosis (programmed cell death) of the affected cells, and many bacterial pathogens have coevolved with their host by acquiring different means to prevent apoptosis (Hacker *et al.*, 2006). Thus, it is particularly important for intracellular pathogens such as *Rickettsia*, *Chlamydia* and *Bartonella* to keep their host cells alive. *Bartonella* sp. can inhibit the apoptosis of HUVECs *in vitro*, and inhibition of endothelial cell apoptosis is partly responsible for *Bartonella*-induced angiogenesis (Kirby and Nekorchuk, 2002). Inhibition of apoptosis was also observed with *Bartonella*-conditioned medium, indicating that direct contact of the bacteria with the cell is not necessary for this action, but anti-apoptotic activity was dependent on bacterial RNA and protein synthesis (Kirby and Nekorchuk, 2002). Anti-apoptotic property was shown by *B. henselae* and *B. quintana*, both of which are associated with vasoproliferative disorders, whereas *B. elizabethae* and *B. vinsonii* VR 152, which are not associated with vasoproliferation, did not have an inhibitory effect on endothelial cell apoptosis.

In addition to endothelial cells, *Bartonella* is also shown to be capable of inhibiting apoptosis in monocytes (Mono Mac 6 cells) *in vitro* (Kempf *et al.,* 2005). *Bartonella*-induced vasoproliferative lesions (e.g. bacillary angiomatosis) is characterized by the infiltration of macrophages and monocytes (LeBoit *et al.,* 1988), and these cells are capable of producing angioproliferative factors such as VEGF in response to *Bartonella* infection, which act in a paracrine manner on the endothelial cells, favoring proliferation (Resto-Ruiz *et al.,* 2002). Unlike the results obtained by Kirby and Nekorchuk, direct contact of the bacteria with the cell has been shown to be required for inhibition of apoptosis in these cells; however, intracellular presence of bacteria was not essential, as shown by the lack of effect of cytochalasin D (inhibitor of actin polymerization and bacterial intake) on inhibition of apoptosis by *Bartonella* (Kempf *et al.,* 2005). This discrepancy may be due to the fact that VEGF, which is a soluble factor released into the medium, has anti-apoptotic effects on endothelial cells whereas VEGF does not have this effect on monocytes (Kempf *et al.,* 2001; Kempf *et al.,* 2005). BadA expression is found to be important for the inhibition of apoptosis as Bad A^- mutants show significantly reduced ability to inhibit apoptosis. Heat and antibiotic killed bacteria also show significant inhibition of apoptosis indicating that bacterial viability is not essential.

Bartonella inhibits both early (caspase activation) and late (DNA fragmentation) events of apoptosis. *Bartonella* inhibits the activity of both caspase 3 and caspase 8 (Kirby and Nekorchuk, 2002; Kempf *et al.,* 2005). Inhibition of apoptosis of monocytes by *Bartonella* was associated with the activation of NF-κB (Kempf *et al.,* 2005) which has anti-apoptotic activity (Karin and Lin, 2002). NF-κB mediates the transcriptional activation of inhibitors of apoptosis proteins (IAPs). *Bartonella* infection can induce the production of cellular IAP-1 and 2 (cIAP1 and 2) (Kempf *et al.,* 2005).

The Vir B/Vir D4 type IV secretion system (T4SS) is essential for the anti-apoptotic activity of *B. henselae* (Schmid *et al.,* 2004). This T4SS is related to the bacterial conjugation machineries, and it helps to inject bacterial effector proteins directly into the host cell cytosol (Schulein *et al.,* 2005). The C-terminus of the effector proteins contain a 140 amino acid

domain called the Bep intracellular delivery (BID) domain and a short positively-charged tail sequence (Schulein *et al.,* 2005). The BID domain, together with the tail sequence, forms the signal for the translocation of the proteins into the host cell. Bep A is responsible for the anti-apoptotic property of *B. henselae* and *B. quintana* (Schmid *et al.,* 2006). The Bep A BID domain is sufficient for the anti-apoptotic effect. The anti-apoptotic property was limited to the Bep A orthologs of *B. henselae* and *B. quintana* (which are associated with vasoproliferative diseases), where as the Bep A of *B. tribocorum* (which is not associated with vasoproliferative disease) does not have anti-apoptotic activity. The anti-apoptotic effect of Bep A is independent of NF-κB pathway and is dependent on the cAMP-dependent CREM/CREB pathway. Increase in the intracellular cAMP level induced by the adenylate cyclase activating drug forskolin and the phosphodiesterase inhibiting drug IBMX mimicked the anti-apoptotic effect of Bep A, indicating that the anti-apoptotic effect of Bep A is due to an increase in the intracellular cAMP. The authors also demonstrated that Bep A can protect the endothelial cells against apoptosis triggered by the cytotoxic T cells (CTLs) using a co-culture system of HUVECs with human CTLs (Schmid *et al.,* 2006).

Apoptosis is controlled by the balance between the proapototic *bax* and anti-apoptotic *bcl-2* genes. *Bartonella* can induce the production of IL-8 by the host cells (Resto-Ruiz *et al.,* 2002) and IL-8, in turn, can cause overexpression of the bcl-2 protein and decreased production of the bax protein (Li *et al.,* 2002), leading to increased cell survival. Addition of anti IL-8 antibodies resulted in an increase in bax and a decrease in bcl2 levels in *Bartonella*-infected HUVECs, indicating an autocrine role of IL-8 in *Bartonella*-induced inhibition of endothelial cell apoptosis (McCord *et al.,* 2006).

Studies with *B. quintana* have shown that live bacteria induce apoptosis of the HUVECs earlier in the infection (up to 6 hrs), whereas later in the course of infection, *B. quintana* inhibits the apoptosis (Liberto *et al.,* 2004). *B. quintana* induces apoptosis in the earlier stages of infection by activating p38 MAPK and SAPK/JNK, which are activated by extracellular bacteria (Cobb, 1999; Liberto *et al.,* 2004), but after the bacteria are internalized, p38MAPK and SAPK/JNK are inhibited, leading to an inhibitory effect on

apoptosis. *B. quintana* infection also induces overexpression of anti-apoptotic protein bcl-2 (Liberto *et al.*, 2003).

C. Paracrine loop of angiogenesis

VEGF plays an important role in *Bartonella*-induced vasoproliferation (Kempf *et al.*, 2001) but endothelial cells are not a major source of VEGF following *Bartonella* infection (Maeno *et al.*, 1999; Kempf *et al.*, 2001). Vasoproliferative lesions induced by *Bartonella* are characterized by an inflammatory infiltrate composed of neutrophils, monocytes and macrophages (LeBoit *et al.*, 1988). *Bartonella* infection results in the production of MCP-1 from endothelial cells, and this protein is a potent chemoattractant for monocytes and macrophages (McCord *et al.*, 2005). Monocytes and macrophages can produce proangiogenic factors like VEGF upon activation (Ono, 2008). *Bartonella* infection can lead to production of IL-1β and VEGF from macrophage cell lines (THP-1) and the conditioned medium from infected macrophages can induce proliferation of HMECs *in vitro* (Resto-Ruiz *et al.*, 2002). These observations support a paracrine loop of angiogenesis induced by *Bartonella*, where *Bartonella* infection results in proinflammatory response leading to the recruitment of macrophages and monocytes to the site of infection. These cells produce proangiogenic cytokines which, in turn, act on endothelial cells in a paracrine manner, resulting in their proliferation.

The concept of 'primary niche'

As discussed above, in experimental infection of rats with *B. tribocorum*, the bacteria are cleared from the blood of the host after a few hours of infection and reappear in the blood stream after 4-5 days (Schulein *et al.*, 2001). This observation led to the concept of a primary niche, which supports the initial replication of *Bartonella*, and from where *Bartonella* are released into the blood stream at regular intervals. It is also proposed that infection of the primary niche is required to gain competency for erythrocytic infection (Dehio, 2004). The identity of the primary niche of *Bartonella* is not proven experimentally, but endothelial cells

are considered to be the strongest candidate (Schulein *et al.,* 2001). *Bartonella* can infect and replicate within the endothelial cells *in vitro*. In vasoproliferative lesions caused by *Bartonella* (BA, BP and verruga peruana), these bacteria are seen inside, or in close proximity to, the endothelial cells (Arias-Stella *et al.,* 1986; Koehler and Tappero, 1993). The proximity of endothelial cells to the blood stream may also be a favorable factor for the vector transmission of these intracellular pathogens.

Bartonellae are also able to invade and replicate in CD34+ hemopoietic progenitor cells *in vitro* as shown by gentamicin protective assay and confocal laser scanning microscopy (Mandle *et al.,* 2005). Infection did not affect the erythroid differentiation of the progenitor cells and *Bartonella* was subsequently detected in the differentiated erythrocytes. These findings indicate that CD34+ hemopoietic progenitor cells may act as the primary niche of *Bartonella*. This hypothesis is also supported by the studies of Rolain *et al.,* in which they found *B. quintana* in the hemopoietic precursor cells in the bone marrow of bacteremic patients (Rolain *et al.,* 2003). Further studies are warranted to find out the identity of the primary niche of *Bartonella* and whether the same niche is utilized by all *Bartonella* sp.

Potential role of *Bartonella* sp. in vascular tumor formation

Introduction

Oncogenesis is a multistep process involving multiple genetic and epigenetic events that result in disturbances in the regulation of cell growth leading to enhanced cell survival and uncontrolled proliferation (Monier, 2000). The genetic events leading to the development of cancer may involve activation of oncogenes or inactivation of tumor suppressor genes. These mutations accumulate in the cell and lead to malignant transformation and later invasion into the surrounding tissues by metastasis. Three major steps involved in carcinogenesis are initiation, promotion and progression. For example, in a chemically-induced skin carcinogenesis model in mice (Wu and Pandolfi, 2001), application of the skin carcinogen 7, 12-dimethyl-benzantracene (DMBA) leads to irreparable DNA damage and

mutation of the Ha-ras oncogene (initiation). Promotion is achieved by repeated applications of 12-*O*-tetradecanoyl-phorbol-13-acetate (TPA) which causes cyclin D1 overexpression and leads to selective clonal expansion of the initiated epidermal cells, resulting in the formation of papillomas. Progression occurs spontaneously and is characterized by high levels of genetic instability, resulting in high levels of Cyclin D1 and inactivation of the tumor suppressor gene p53. These changes may lead to the progression of benign papillomas in to malignant squamous cell carcinomas.

Infectious agents and cancer

Multiple genetic and environmental factors act as cofactors in the development of cancer. The role of infectious agents like virus, bacteria and parasites as cofactors of cancer development has been extensively studied (Mueller, 1995; Hateckayama, 2004; Mazigo *et al.,* 2010; Yang *et al.,* 2010). According to a World Health Organization report, about 20% of all cancers in human beings are caused by infectious agents (WHO, 2006). Infectious agents may induce cancer directly, by oncogenic viral or bacterial proteins, or indirectly, by inducing chronic persistent infections and inflammatory responses associated with the chronic infections (Koraitim *et al.,* 1995)

A. Viruses and cancer

Tumor viruses are classified in two general groups based on whether an RNA or DNA genome is packaged into the infectious viral particle. Besides the difference in replication and life cycle, RNA and DNA viruses differ also in their general mechanisms of inducing cellular transformation/immortalization. At least six human viruses, namely Epstein-Barr virus (EBV)/human herpes virus-4 (Burkitts's lymphoma, Hodgkin's disease, nasopharyngeal carcinoma, gastric carcinoma), Hepatitis B virus (HBV) (hepatocellular carcinoma), Hepatitis C virus (HCV) (Hepatocellular carcinoma), Human papilloma virus (HPV) (Cervical cancer), Human T-cell leukemia virus (HTLV) (leukemia) and human herpes virus-8 (Kaposi's sarcoma) are etiologic agents of human cancers (Chow *et al.,* 1990;

Cader *et al.*, 2010; McClune and Tong, 2010; Madhoun *et al.*, 2010). RNA tumor viruses (Retroviruses and Flaviviruses), in particular animal retroviruses, are usually characterized by the ability to carry and/or alter important cellular growth regulatory genes, namely oncogenes, and the cells transformed by non defective retroviruses also express a full range of viral proteins and antigens and new virions bud from their membranes (Swanton and Jones, 2001). The proteins encoded by these cellular genes are not essential for viral replication but are usually key players in cell cycle control. Retroviral oncoproteins acts as intracellular signal transducers and nuclear transcription factor. DNA tumor viruses (like Leporipox, SV40, mouse polyoma virus, adenovirus, papilloma virus, herpes virus) cause cell transformation by encoding proteins of exclusively viral origin and are essential for viral replication (Brinkman and Schulz, 2006). Transformation by DNA viruses usually occurs in cells undergoing non-productive infection where viral DNA is integrated into the cellular DNA, except in the case of papilloma viruses and herpes viruses where the viral DNA remains episomal.

B. Bacteria and cancer

Bacterial organisms have also been implicated as co-factors in the development of cancers. Unlike viral oncogenesis, the mechanism by which bacterial organisms cause cancer is not well-elucidated. The relationship between *Helicobacter pylori* and gastric carcinoma is the most studied model of bacteria induced tumorigenesis (Hateckayama, 2004). WHO has classified *H. pylori*, which selectively colonizes the gastric epithelium (Peek *et al.*, 2010), as a class I carcinogen for gastric tumors. Several host and pathogen factors determine the outcome of infection. Chronic infection with *H. pylori* in human beings leads to chronic gastritis and peptic ulcers. In a small proportion of the patients the infection can lead to the development of mucosal associated lymphoid tissue (MALT) lymphoma. MALT lymphoma is responsive to antibiotic therapy and can be reversed by the elimination of *H. pylori* infection (Chen *et al.*, 2005). In a smaller fraction of patients, *H. pylori* infection can lead to formation of gastric carcinoma. Some strains of *H. pylori* possess type IV secretion systems

(T4SS), called *cag* Pathogenicity Island, and the bacterial protein called CagA is translocated into the host cell by this T4SS. CagA acts as a bacterial oncoprotein, which can inhibit apoptosis of host cells *in vitro* and *in vivo*. Transgenic expression of CagA in mice leads to abnormal proliferation of gastric epithelium and formation of gastric carcinoma (Ohnishi *et al.,* 2008). Another virulence factor associated with disease induction is Vac A (vacuolating cytotoxin). Vac A can bind to the receptor-type protein tyrosine phosphatase (RPTP-β) that modulates cell proliferation, differentiation and adhesion (Peek *et al.,* 2010). Although the molecular mechanisms for *H. pylori* carcinogenesis have not been fully elucidated, chronic inflammation, accompanied by release of oxygen free radicals, resulting in DNA damage and transformation of gastric epithelial cells to a neoplastic phenotype, has been proposed (Naito and Yoshikawa, 2002). Other bacterial organisms that are considered to have oncogenic potential include *Salmonella typhi* (gall bladder carcinoma in humans), *Citrobacter rodentium* (colon cancer in mice), and *Campylobacter jejuni* (MALT lymphoma in humans) (Lax and Thomas, 2002). The evidence linking these organisms with cancer is mainly epidemiological and further studies are needed to elucidate the mechanisms by which these bacteria contribute to the formation of cancer.

Mycoplasmas are cell wall-deficient bacteria belonging to the class Mollicutes. They are capable of causing persistent intravascular infections in humans and animals, generally without causing clinical disease (Tsai *et al.,* 1995). Mycoplasmas are able to transform normal cells into cancer cells. *Mycoplasma*-mediated oncogenesis is characterized by a long period of latency, and it requires persistent infection (Tsai *et al.,* 1995). *M. genetalium* and *M. hyorhinis* are capable of inducing malignant transformation of human epithelial cells and are thought to play a role in the development of various cancers including prostate cancer and gastric carcinoma (Namiki *et al.,* 2009; Yang *et al.,* 2010). Mycoplasmal membrane protein p37 has the ability to induce antisenescence in mammalian cells (Liu *et al.,* 2007), and it promotes invasiveness of cancer cells and metastasis by the activation of MMP-2 (Gong *et al.,* 2008).

C. Parasites and Cancer

Schistosoma sp. have been implicated as the causative agent of human cancers, including colorectal, bladder and hepatic cancers (Salim *et al.,* 2010). *S. hematobium* infection is associated with the development of bladder carcinoma (Koraitim *et al.,* 1995), and *S. japonicum* and *S. masoni* infections have been associated with colorectal carcinoma (Salim *et al.,* 2010). Eggs of Schistosomes can be sequestered in the mucosa and submucosa of the bladder and intestine and elicit chronic inflammatory reactions and granuloma formation. This may further lead to microabcess formation and neoplastic transformation. Epidemiological studies show a strong correlation between prevalence of schistosomiasis and incidence of colorectal carcinoma in the same geographical location (Xu and Su, 1984).

Can *Bartonella* induce cancer?

Bartonella infections have been reported in dogs and human beings with cancer (Liston and Koehler, 1996). Recently, *B. vinsonii* subsp. *berkhoffii* was isolated form a dog with hemangiopericytoma and from a human patient with epithelioid hemangioendothelioma (Breitschwerdt *et al.,* 2009). A recent study by our research group reported an 18% prevalence of *Bartonella* sp. infection in Golden retrievers with lymphoma and in healthy, age and sex matched controls (Duncan *et al.,* 2008). This study was not a longitudinal study, and whether the *Bartonella* positive animals in the control group developed lymphoma later in their life was not investigated.

Bartonella can cause persistent intravascular and intraerythrocytic infections in the host and can persist without causing any overt clinical signs in reservoir and accidental hosts. *Bartonella* has the unique ability to induce pathological proliferation of endothelial cells leading to formation of vascular tumors (BA, BP and verruga peruana), especially in immunocompromised patients. Vascular tumors induced by *Bartonella* sp. are benign and disappear after successful elimination of the bacteria by antibiotic therapy (Koehler and Tappero, 1993). *Bartonella* sp. can induce the production of vascular endothelial growth factor (VEGF) from the host cells, and VEGF plays an important role in the pathogenesis of

Bartonella-induced angiogenesis, as discussed earlier. *Bartonella* can also inhibit apoptosis in the host cells, which helps the bacteria induce a chronic infection and also plays a role in the development of vascular tumors.

Inflammation, hypoxia and angiogenesis play a crucial role in the development and metastasis of cancers. In human beings, approximately 25% of all cancers in adults result from chronic inflammation (Coussens and Werb, 2002). Chronic inflammatory response promotes tumor growth, angiogenesis and metastasis by activation of the surrounding stromal cells and recruitment of inflammatory cells, especially macrophages. Macrophages can promote cell proliferation through the production of growth factors, and enhancement of angiogenesis. *Bartonella* infection can activate the NF-κB pathway, leading to the recruitment of inflammatory cells including neutrophils, lymphocytes and macrophages, to the site of infection (Dehio, 2004). Hypoxia also promotes tumor growth and metastasis by inducing angiogenesis. Hypoxia activates the hypoxia inducible factor- Iα (HIF-Iα), a transcription factor for many downstream genes, which promote angiogenesis. Bartonella infection has been shown to activate the HIF-Iα, leading to the increased expression of the growth factor VEGF (Kempf *et al.*, 2001). Action of VEGF, along with the direct mitogenic activity of *Bartonella* on endothelial cells and inhibition of apoptosis, leads to proliferation of the endothelial cells.

Bacteriophages have been detected and isolated from *B. henselae, B. bacilliformis* and *B. vinsonii* subsp. *berkhoffii* (Barbian and Minnick, 2000; Anderson *et al.*, 2004; Breitschwerdt *et al.*, 2007). Interactions between bacteriophages and their respective *Bartonella* sp. could promote recombination divergence, which is known to play a critical role in the evolution of many gram-positive and gram-negative bacteria. For other bacterial species, phages enhance bacterial survival, facilitate bacterial adaptation to new environmental conditions, assist in the adaptation to a new host species, and enhance bacterial evasion or inactivation of host defense mechanisms. In addition, phage-associated chromosomal rearrangement has been postulated to play a crucial role in host specialization of *B. quintana* (a “specialist” bacteria that generally uses humans and nonhuman primates as

reservoir hosts), which appears to be a genomic derivative of the zoonotic pathogen *B. henselae* (a "non-specialist" that infects humans and many animals) (Alsmark *et al.,* 2004). In fact, the majority of genes found in *B. henselae* that are not found in *B. quintana* are located in four genetic clusters flanked by several phage integrases. This finding further supports the role of phages as potential vehicles for *B. henselae* genetic DNA rearrangements, which could prove important in oncogenesis. Moreover, partial sequence analysis of the *B. tribocorum* genome (a rodent-adapted species) has identified more than 30 phage genomes that are found within several genetic islands within this organism (Schuster *et al.,* 2004). Collectively, these observations clearly support the concept that a high degree of complexity is involved in *Bartonella* gene assembly, that phages may play an important role as vectors for the horizontal and vertical transfer of gene clusters or pathogenicity islands, and implicates a potential role for bacteriophages as mediators of the recombination events that take place in *Bartonella* sp.

Summary

The genus *Bartonella* comprises of more than 24 species and subspecies of vector borne organisms. They are associated with a wide range of diseases in both humans and animals. Disease conditions associated with *Bartonella* infections include cat scratch disease, Carrion's disease, bacillary angiomatosis, bacillary peliosis hepatis, endocarditis, myocarditis, musculoskeletal manifestations, neurological manifestations and neuroretinitis.

Bartonella can cause persistent intraerythrocytic bacteremia in the reservoir hosts, which promote transmission via blood sucking arthropods. After primary infection, the bacteria disappear from the blood stream and infect an unidentified 'primary niche'. After a period of approximately 5 days, bacteria are released into the blood stream and invade fresh erythrocytes where they replicate and can survive the entire life span of the erythrocytes. The virulence determinants for erythrocytic infection include flagella-mediated motility, Trw type IV secretion system (T4SS), deformin and invasion-associated locus (*ial*). The receptors on the erythrocytes and the bacterial adhesins involved in adherence of *Bartonella* sp. to

erythrocytes are not yet identified.

In accidental hosts, endothelial cells are considered to be the primary target cells of this bacterium. *Bartonella* sp. are unique in their ability to induce pathological proliferation of endothelial cells leading to the formation of vascular tumors in humans and animals (bacillary angiomatosis, bacillary peliosis hepatis and verruga peruana). *Bartonella* has a type IV secretion system, which allows the bacteria to inject the Bartonella effector proteins (Beps) into the host cell cytoplasm. Beps are responsible for the subversion of host cell functions, including cytoskeleton rearrangements, inhibition of host cell apoptosis and induction of a proinflammatory phenotype. This assists in the invasion of the bacteria into the host cell and also induces proliferation of the endothelial cells.

VEGF plays an important role in *Bartonella*-induced vasoproliferation. *Bartonella* infection activates a proinflammatory phenotype, leading to the recruitment of inflammatory cells, including neutrophils, lymphocytes and macrophages. *Bartonella* can, in turn, infect macrophages, leading to the activation of HIF-Iα and increased production of VEGF by macrophages. This VEGF can act on the endothelial cells in a paracrine manner to induce proliferation of these cells.

There is increasing evidence that infectious agents like viruses, bacteria and parasites play a major role in the development of cancers. Integrated interactions among viruses, bacteria, *Mycoplasma* and parasites are infrequently studied and remain incompletely characterized. According to the World Health Organization about one fifth of all human cancers are caused by infectious agents. The role of viruses in oncogenesis is well-understood, whereas the mechanisms by which bacterial organisms induce cancer are not yet clear. Recent research has shown that inflammation, hypoxia and uncontrolled angiogenesis are important factors in the pathogenesis of tumors. *Bartonella* sp. cause persistent infection in the host and have the unique ability to induce pathologic angiogenesis. *Bartonella* can also recruit inflammatory cells, including macrophages, into sites of infection. Chronic inflammatory process can promote tumor growth and metastasis through production of growth factors and enhanced angiogenesis. Further studies are required to determine the role

of *Bartonella* sp. as a cause of, or cofactor in, the development of cancers in animals and human patients.

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Table 1. *Bartonella* sp. associated with endocarditis in humans and animals

***Bartonella* sp.**	**Host species affected**	**Reference**
B. henselae	Humans, dogs, cats	Baorto *et al.*, 1998; Chomel *et al.*, 2003b; Ohad *et al.*, 2010
B. quintana	Humans, dogs	Klein *et al.*, 2002; Kelly *et al.*, 2006
B. vinsonii subsp *berkhoffii*	Humans, dogs	Roux *et al.*, 2000; Cockwill *et al.*, 2007
B. vinsonii subsp *arupensis*	Human	Fenollar *et al.*, 2005
B. alsatica	Humans	Raoult *et al.*, 2006
B. elizabethae	Humans	Daly *et al.*, 1993
B. washoensis	Humans, Dogs	Chomel *et al.*, 2003
B. clarridgeiae	Dogs	Chomel *et al.*, 2001
B. volans like	Sea otters	Chomel *et al.*, 2009
Candidatus *B. mayotimonensis*	Humans	Lin *et al.*, 2010b
B. rochalimae	Dogs	Henn *et al.*, 2009a
B. bovis	Cattle	Maillard *et al.*, 2007
B. koehlerae	Humans, dogs	Avidor *et al.*, 2004; Ohad *et al.*, 2010

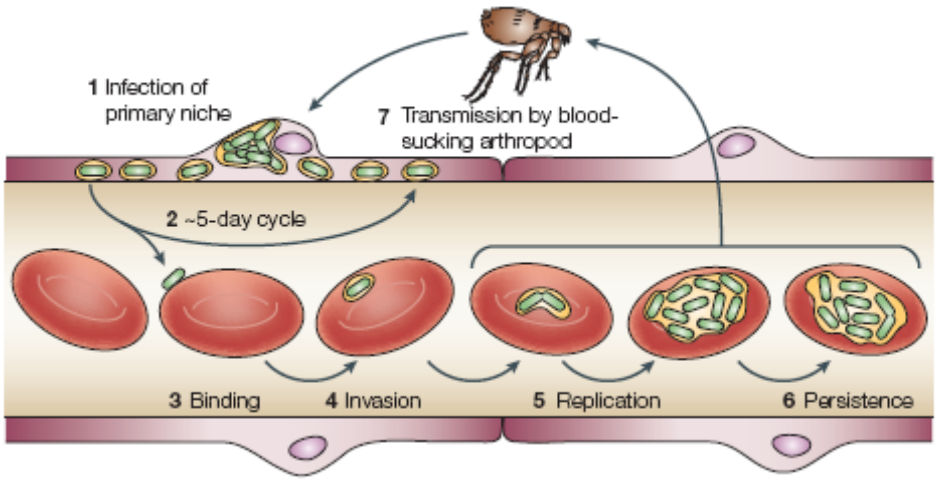

Figure 1. Model of the course of *B. tribocorum* infection in rat. (Source: Dehio, 2005)

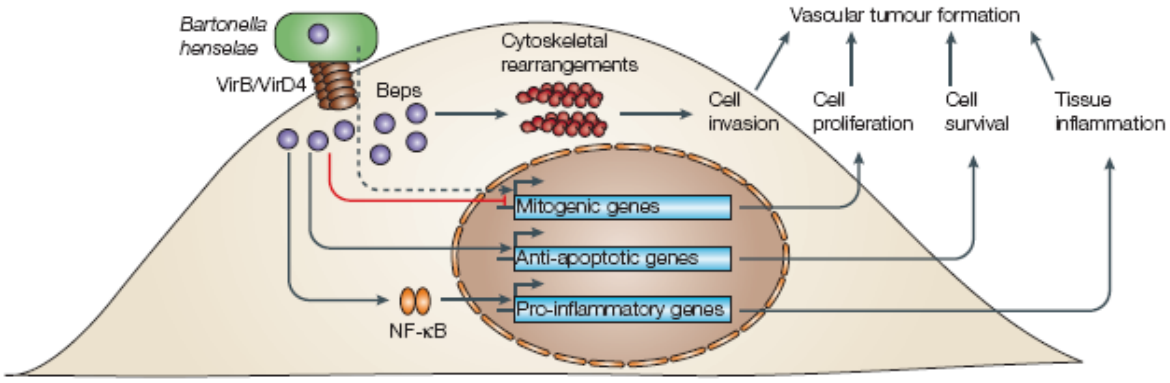

Figure 2. Subversion of host cell functions by Bartonella effector proteins translocated by the type IV secretion system (Source: Dehio, 2005)

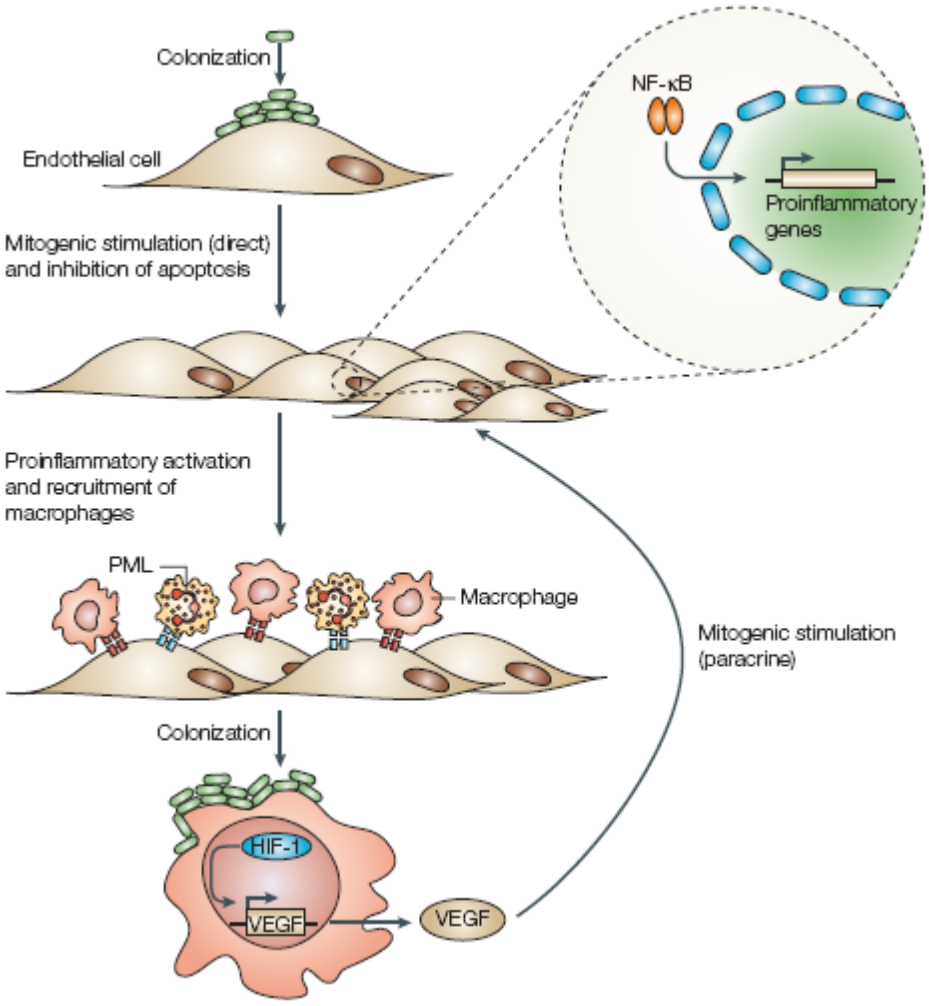

Figure 3. Model of *Bartonella* induced vascular tumor formation. *Bartonella* can induce proliferation of endothelial cells in three ways. 1) direct mitogenic stimulation 2) Inhibition of endothelial cell apoptosis 3) Paracrine loop of mitogenic stimulation by the VEGF produced by the macrophages which are recruited by the proinflammatory activation through the NF-κB activation induced *Bartonella* (Source: Dehio, 2005)

Chapter 2.

Challenges associated with diagnosis of *Bartonella* species

Part A

Routinely used methods for diagnosis of *Bartonella* species and Fluorescent *in situ* Hybridization as a tool for the visualization of *Bartonella* species in tissues

Routinely used methods for diagnosis of *Bartonella* sp. and Fluorescent *in situ* Hybridization as a tool for the visualization of *Bartonella* sp. in tissues

Introduction

Bartonella sp. are associated with a wide variety of disease manifestations in human beings and animals including cat scratch disease, bacillary angiomatosis, bacillary peliosis hepatis and endocarditis (Chomel *et al*., 2006). *B. quintana*, *B. henselae* and *B. vinsonii* subsp. *berkhoffii* are the most important species known to infect humans and dogs, although infection with other *Bartonella* sp. have been associated with clinical disease in both these species (Chomel *et al*., 2004). *Bartonella* sp. are facultative intracellular organisms with a predilection to erythrocytes and endothelial cells (Dehio, 2005). *Bartonella* can also infect other cell types, such as macrophages, monocytes, dendritic cells, CD34+ hemopoietic progenitor cells and epithelial cells *in vitro* (Kempf *et al*., 2005; Mandle *et al*., 2005; Vermi *et al*., 2006).

The spectrum of clinical disease associated with *Bartonella* sp. infection is ever increasing, and many of the symptoms associated with *Bartonella* sp. are nonspecific, including fever, lymphadenopathy, neurological and musculoskeletal manifestations, especially when infection occurs in immunocompetent people (Ridder *et al*., 2002; Maman *et al*., 2007; Breitschwerdt *et al*., 2010a). Atypical cat scratch disease may be mistaken for tuberculosis or cancers leading to unnecessary hospitalization and increased cost of patient care (Weinspach *et al*., 2010). Treatment of bartonellosis has proven to be challenging, with increased evidence to support the development of antibiotic resistance (Biswas *et al*., 2009). Furthermore prolonged treatment with one or more antibiotics may be necessary for the complete elimination of *Bartonella* (Elliot *et al*., 2004; Sykes *et al*., 2010).

Early and accurate diagnosis is important for the successful treatment of any disease

caused by bacteria. Even with the development of improved and more sensitive diagnostic methods, detection of *Bartonella* sp. remains a challenge today. *Bartonella* sp. are highly fastidious organisms that are extremely difficult to culture in the laboratory and require special enrichment medium and prolonged incubation periods for successful isolation from clinical samples (Maggi *et al*., 2005a; Riess *et al*., 2008; Lynch *et al*., 2011). The most commonly used diagnostic techniques for the detection of *Bartonella* from clinical samples include serology, molecular detection by PCR and histological detection in the affected tissues by silver staining and/ or immunohistochemistry and fluorescent *in situ* hybridization (FISH) (Maggi *et al*., 2005a; Caponetti *et al*., 2009; Vermeulen *et al*., 2010). Each of these diagnostic modalities has been found to be useful in the past, but there is no one diagnostic test for *Bartonella* which can be considered as the gold standard. This chapter discusses the advantages and disadvantages associated with different methods used for diagnosis of *Bartonella* sp., with special emphasis on the visualization of *Bartonella* sp. in tissues and also describes our efforts to develop fluorescent *in situ* hybridization probes to detect *Bartonella* sp. in formalin-fixed paraffin-embedded tissues.

Isolation of *Bartonella* sp. by culture

Isolation of *Bartonella* sp. from blood, lymph node aspirates and tissues had been attempted using various culture techniques, including direct plating onto blood agar or chocolate agar plates sometimes with lysis centrifugation or freeze-thaw techniques, enrichment culture using various liquid media and culture using various mammalian and tick cell lines (Maggi *et al*., 2005a; Billeter *et al*., 2009; Lynch *et al*., 2011). *Bartonella* sp. are highly fastidious and isolation by direct plating of blood or other clinical samples is not always successful. Lysis centrifugation and freeze-thaw techniques are used to release bacteria from the blood cells to facilitate growth on agar plates. *Bartonella* sp. require specific growth conditions (35-37^{0}C, 5% CO_2), and it may take up to 6 wks for visualization of these bacteria on blood agar following direct plating (Maggi *et al.*, 2005a). This is especially true of blood culture in the case of non-reservoir host samples since the number of

bacteria circulating in the blood may be very low.

A. Liquid enrichment culture

In order to improve the chances of isolation of *Bartonella* sp. from clinical samples, various liquid enrichment media have been developed. Our laboratory has developed a liquid enrichment medium called *Bartonella* alpha proteobacteria growth medium (BAPGM) (Maggi *et al*., 2005a), which is a chemically-modified insect cell culture medium. We routinely use this medium in combination with molecular detection using polymerase chain reaction (PCR) and isolation by plating the enrichment culture on to blood agar plates (Figure 1), for the detection and isolation of *Bartonella* sp. in clinical samples from human beings and animals (Cadenas *et al*., 2007; Duncan *et al*., 2007). This approach has significantly improved the detection and isolation of *Bartonella* sp. from clinical samples and has resulted in the identification of novel *Bartonella* sp. in human patient samples (Maggi *et al*., 2009). Co-infection with more than one species of *Bartonella* has also been reported using the BAPGM platform and blood culture samples from dogs and human patients (Diniz *et al*., 2009; Breitschwerdt *et al*., 2010a). Other genera of bacteria in addition to *Bartonella*, including *Arthrobacter, Bacillus, Dermabacter, Methylobacterium, Propionibacterium, Pseudomonas* and *Staphylococcus* have also been isolated from patient clinical samples using BAPGM (Cadenas *et al*., 2007).

Other laboratories have used various liquid enrichment media successfully to grow *Bartonella* sp. Chenoweth and co-workers developed a liquid enrichment medium, consisting of a *Brucella* broth with HEPES buffer supplemented with 0.4% histidine, and were successful in culturing *B. henselae* from the blood of experimentally-infected cats (Chenoweth *et al*., 2004). Riess *et al*. utilized Schneider's drosophila powder medium supplemented with 10% fetal calf serum to successfully grow *B. henselae*, *B. quintana* and *B. vinsonii* subsp. *berkhoffii* (Riess *et al*., 2008); however, Schneider's media has not been used to isolate *Bartonella* sp. from clinical samples. In a recent study, Lynch *et al*. compared different methods for culturing *Bartonella* (mammalian vs. insect media, presence or absence

of mammalian cells and 35^0C vs. 37^0C). Their data suggests that a combination media containing both mammalian and insect cell culture media supported the growth of *B. henselae, B. quintana, B. elizabethae* and *B. tamiae* and that co-culture with mammalian cells was not a requirement when this media is used (Lynch *et al*., 2011).

B. Cell cultures

Both mammalian and insect cell cultures have been used for isolation of *Bartonella*. Most commonly used mammalian cell lines for the culture of *Bartonella* sp. include mouse fibroblast cell lines (L-929 cell line), Vero E6 cells (kidney epithelial cells) and endothelial cells (Chmielewski *et al*., 2007; Kosoy *et al*., 2008). Candidatus *B. tamiae* was first isolated from a human patient using Vero E6 cell line (Kosoy *et al*., 2008). Due to the endotheliotrpic nature of these bacteria, endothelial cells are widely used in *Bartonella* research as an excellent in vitro model (McCord *et al*., 2007), which is especially important because a suitable animal model for *Bartonella*-induced vasoproliferation has not yet been developed. Tick cell lines used for the culture of *Bartonella* sp. include an *Ixodes scapularis* cell line (ISE6) and an *Amblyomma americanum* cell line (AAE12) (Tate *et al*., 2005; Billeter *et al*., 2009). The AAE12 cell line was shown to support the growth of *B. henselae, B. vinsonii* subsp. *berkhoffii, B. volans, B. bovis, B. durdenii, B. elizabethae* and *B. monaxi*. Cytopathic effects and cell lysis were noted with all the species except *B. bovis* (Billeter *et al*., 2009).

Serology

Detection of *Bartonella* antibodies in the serum of patients is a routine diagnostic methodology used in both human and veterinary diagnostics, and these assays include indirect immunofluorescent assay (IFA), enzyme linked immunosorbent assay (ELISA), microarrays and Western Blot (Breitschwerdt *et al*., 2003; Vermeulen *et al*., 2007; Gouriet *et al*., 2008; Wagner *et al*., 2008). Advantages of serological diagnostic tests include faster results, ease of obtaining samples and that these techniques do not need overly specialized equipment and that these assays are comparatively cheaper (Florin *et al*., 2008).

IFA is the most commonly used serological assay. IFA has been used for the

diagnosis of various *Bartonella* sp. including *B. henselae, B. vinsonii* subsp. *berkhoffii, B. quintana, B. bacilliformis* and *B. koehlerae* (Chamberlin *et al*., 2000; Balakrishnan *et al*., 2008; Breitschwerdt *et al*., 2010b). The Centers for Disease Control and Prevention (CDC) developed the first IFA test for *B. henselae* using *B. henselae* organisms grown in Vero E6 cells (Regnery *et al*., 1992). According to CDC guidelines, a titer of 1:64 or higher is defined as positive for current infection or prior exposure. Patients with CSD mount both IgM and IgG antibody responses (Anderson and Neuman, 1997). A positive IgM response is considered to be indicative of an acute infection of less than three months duration (Florin *et al*., 2008). Negative IgM results do not prove absence of acute infection, because of the short duration of the IgM antibody response. In contrast IgG responses can persist up to a year, making it difficult to distinguish between active infection and previous exposure (Florin *et al*., 2008). Sensitivity of the IgG IFA may range from 14-100% and specificity from 34-100% depending on the antigen used (Sander *et al*., 2001). In a recent study involving serum samples from humans with CSD, sensitivity of IgM IFA was found to be lower (50-62%) than that of IgG IFA (88-98%), whereas IgM IFA was more specific (Vermeulen *et al*., 2010). Different antigenic preparations, such as, agar-grown bacteria or bacteria co-cultured with mammalian cells have been used by different laboratories and the difference in antigens may influence the results of IFA testing (Dalton *et al*., 1995). Differences in the IFA results were also observed when different strains of *B. henselae* were used as antigens (Drancourt *et al*., 1996). In a study by Zangwill and co-workers the highest antibody titers were detected in patients with CSD, 0-16 weeks following the onset of lymphadenopathy and the antibody titers dropped to borderline after 25-28 weeks of infection (Zangwill *et al*., 1993).

Serological diagnosis using enzyme immune assays (EIA) have also been used by different laboratories with varying results. Sensitivity of IgG EIA was less than that of IgG IFA whereas IgM EIA was more sensitive than IgM IFA (Bergmans *et al*., 1997). Loa and coworkers developed an IgG-specific ELISA using the 17kDa protein of *B. henselae* which had a sensitivity of 71% and a specificity of 93% (Loa *et al*., 2006), when testing CSD patient sera, whereas IgM-specific ELISA developed by the same group showed a sensitivity

of 100% and specificity of 97.1 % (Hoey *et al*., 2009). When agar grown whole *B. henselae* lysate was used as antigen for IgM specific ELISA, the sensitivity was only 56% in one study (Harremans *et al.,* 2009). Other proteins such as pap31, SucB and P26 have also been suggested as potential antigens for serodiagnosis (Taye *et al*., 2005; Werner *et al*., 2008). These results indicate a wide range of sensitivity and specificity depending on the assay conditions and the antigens used.

Serological results do not always correlate with culture or molecular diagnostic results (Dalton *et al*., 1995). In a recent study conducted in our laboratory only 13% of 322 human patients tested by IFA and PCR were positive by both. 46% of the patients were IFA positive and PCR negative, and 7% were PCR positive and IFA negative (Breitschwerdt *et al*., 2010b). Other disadvantages of serological techniques include cross reactivity among different *Bartonella* sp., especially between *B. henselae* and *B. quintana* where it can be as high as 95% (Sander *et al*., 1998). Cross reactivity has also been reported between *Bartonella*, *Coxiella burnetii* and *Chlamydia* sp. (La Scola and Raoult, 1996; Maurin *et al*., 1997). There can be variation in the results due possibly to subjectivity of reading the IFA slides.

Molecular diagnosis by polymerase chain reaction (PCR)

Diagnosis of *Bartonella* sp. by PCR is widely used. Different PCR assays including conventional, nested PCR and real-time PCR has been developed for diagnosis of *Bartonella* sp. The most commonly targeted genes include 16S rRNA, 16S-23S intergenic spacer region (ITS), citrate synthase (*gltA*), phage associated protein (*pap31*), NADH dehydrogenase gamma subunit (nuoG), RNA polymerase subunit β (*rpoB*), cell division gene (*ftsZ*), riboflavin synthase (*ribC*) and the heat shock protein *GroEL* (Lindsey and Patel, 2008; Colborn *et al*., 2010; Kernif *et al*., 2010; Ohad *et al*., 2010). PCR is highly sensitive and specific and is less time consuming than culture, which can facilitate an early diagnosis of these highly fastidious bacteria. We use a conventional PCR targeting the ITS region for the routine diagnosis of *Bartonella* sp. from clinical samples. This is a genus-specific PCR with

high sensitivity and specificity, the limit of detection of this reaction can be as low as 5 bacteria (10 genome copies of ITS region) per reaction (Maggi, personal communication). PCR in combination with BAPGM enrichment culture (Figure 1) has improved the chance of successful and accurate diagnosis of *Bartonella* sp. in our laboratory from blood and other clinical samples including tissues.

Even though PCR is highly sensitive, there are a number of limitations. PCR requires specialized equipment, highly trained personnel and can be expensive to perform (Brunt *et al*., 2006). Also, extreme care must be taken to avoid amplicon contamination within the laboratory. Recently, our research group documented the potential for carryover of DNA when paraffin-embedded tissues were used for *Bartonella* sp. PCR (Varanat *et al*., 2009). Due to previous tissue processing activities with naturally exposed cat, dog and bovine samples, *Bartonella* DNA was amplified from various parts of the microtome, necropsy room and from the paraffin used in the tissue processor. To avoid any potential DNA carryover, we now shave test samples from paraffin blocks using a sterile single use scalpel blade instead of a microtome. In addition, we test biopsy specimens whenever possible to avoid possible DNA carryover in the necropsy room. A blank paraffin block is always included as a negative control. PCR results obtained from random, single paraffin-embedded tissues should be interpreted with caution and when ever possible aseptically obtained fresh or frozen tissues should be used for PCR testing and BAPGM enrichment culture. Detection of DNA by PCR does not prove the presence of viable organisms in the sample (Rovery *et al*., 2005). False negative results may be obtained due to the presence of PCR inhibitors in the samples, low or intermittent bacteremia and/or prior treatment with antibiotics (Brunt *et al*., 2006). Most PCR assays are not standardized between laboratories which can also result in varying sensitivities and specificities depending on laboratory conditions (Brunt *et al*., 2006). Furthermore, PCR from tissues cannot distinguish between the presence of *Bartonella* DNA in the tissue cells versus DNA in the blood that may be circulating through the tissue at the time of collection.

Histological diagnosis

Since diagnosis of bartonellosis is extremely difficult by culture, histological diagnosis, although requiring more invasive tissue sampling than blood collection, is traditionally used to confirm serological diagnosis especially in patients with CSD if/when a lesion is present (Florin *et al*., 2008). Lesions caused by *Bartonella*, if present, are usually granulomatous in nature (Daybell *et al*., 2004; Varanat *et al*., 2011) and CSD is characterized by granulomatous lymphadenopathy. Histologically, it is characterized by reactive follicular hyperplasia in the earlier stages which progress to formation of suppurative granulomas. Granulomas usually show a necrotic center with surrounding histiocytes, plasma cells and lymphocytes (Caponetti *et al*., 2009).

Bartonella sp. can also induce vasoproliferative lesions (bacillary angiomatosis and peliosis hepatis), especially in immunocompromised patients (Koehler and Tappero, 1993; Yager *et al*., 2010). Bacillary angiomatosis is characterized by lobular proliferation of small capillaries of various sizes and shapes, lined by protuberant endothelial cells (LeBoit *et al.*, 1988; Yager *et al.*, 2010). Some areas may also contain clusters of plump endothelial cells without visible lumens (Juskevicius and Vnencak-Jones, 2004). Proliferating capillaries are surrounded by an edematous stroma and mixed inflammatory infiltrates made predominantly of neutrophils but also contain lymphocytes and macrophages (LeBoit *et al.*, 1988). Histologically, bacillary peliosis is presented as multiple blood-filled spaces in the parenchymatous organs. This is accompanied by a spectrum of changes, ranging from dilated capillaries to multiple thin-walled blood-filled spaces in the parenchyma lined with endothelial cells which stain positive with Factor VIII antigen (Kitchell *et al.*, 2000) and surrounded by myxoid stroma with mixed inflammatory cells (Perkocha *et al.*, 1990).

Another disease condition caused by *Bartonella* sp. in which histological diagnosis is routinely used in conjunction with serological and molecular diagnosis is, blood culture negative endocarditis (Lepidi *et al*., 2000). *Bartonella* sp. endocarditis is characterized by a more fibrotic reaction with calcification and with vegetative growths that are smaller than when compared to non-*Bartonella* endocarditis vegetative growths (Lepidi *et al*., 2000).

Inflammatory infiltrates, predominantly made of lymphocytes and macrophages with fewer neutrophils, are also noted. Recently, we reported granulomatous myocarditis and diaphragmatic myositis in two cats in which *B. henselae* was detected by PCR, Warthin-Starry silver staining and *B. henselae* immunohistochemistry (Varanat *et al*., 2011). The lesions were characterized by partially- organized pyogranulomas consisting of aggregates of neutrophils and macrophages with fewer lymphocytes and plasma cells in the heart and diaphragm. The center of the lesions showed necrosis and loss of myocytes (Figure 5A).

Even though histopathological examination helps with the diagnosis, in many cases the histological appearance of lesions induced by *Bartonella* sp. are not specific. Special stains are required to demonstrate the bacteria in the lesions. *Bartonella* sp. appear as basophilic granular material in routine H&E staining (Koehler and Tappero, 1993). Different techniques including silver stains, immunohistochemistry and fluorescent *in situ* hybridization (FISH) and transmission electron microscopy (TEM) are used to demonstrate the bacilli within the lesions. Even though less sensitive, these techniques can be useful for the tissue localization of *Bartonella* sp.

A. Silver staining

Different silver impregnation techniques like Steiner and Steiner and Warthin-Starry are used to demonstrate *Bartonella* sp. in tissues (Varanat *et al.,* 2011). Silver stains are less specific, as many other Gram negative organisms can also be stained with these staining techniques (Caponetti *et al*., 2009). Sensitivity of the silver stains is also low (around 50% in CSD patient lymph nodes), but was the most sensitive test when compared to PCR and immunohistochemistry in a study where lymph node samples from patients with CSD were evaluated (Caponetti *et al*., 2009). But in our laboratory PCR is much more successful in detecting *Bartonella* sp. when compared to silver staining. Silver stains are also very tedious and expensive to perform. The outcome of silver staining may vary depending on the individual laboratory setting. In a study involving pyogranulomatous myocarditis and diaphragmatic myositis in cats caused by *B. henselae*, Steiner and Steiner silver stain failed while the Warthin-Starry silver staining technique was successful in demonstrating bacteria

in the lesions in our laboratory, whereas Steiner and Steiner technique was successfully used on the same tissue block to demonstrate positively staining bacilli in the center of the lesion in commercial laboratory (Varanat *et al*., 2011). *Bartonella* sp. appear as black, short rods of about 1-2µm length, arranged in chains or clumps (Figure 3C) (Yager *et al*., 2010; Varanat *et al*., 2011). Bacteria may be seen extracellularly or in close proximity to the endothelial cells or, in some cases, inside the endothelial cells (Yager *et al*., 2010).

B. Immunohistochemistry (IHC)

Demonstration of *Bartonella* sp. by immunohistochemical staining using specific monoclonal or polyclonal antibodies has been successfully used in endocarditis, myocarditis and bacillary angiomatosis lesions (Reed *et al*., 1992; Lepidi *et al*., 2000; Varanat *et al*., 2011) (Figure 2). IHC can be performed on both fresh frozen and paraffin-embedded tissues, making it a useful tool for retrospective studies although prolonged fixation in formalin may lead to antigen masking and false negative results. In a study by Webster and co-workers, immunoreactivity to *B. henselae* was completely lost by 9 weeks of formalin fixation (Webster *et al*., 2010). Monoclonal antibodies specific to *B. henselae* are commercially available (Abcam, Cambridge, MA), and this antibody does not cross react with *B. quintana*, *B. bacilliformis*, *B. elizabethae*, *B. grahamii*, *B. taylorii*, *B. doshiae*, *B. vinsonii* strains, spirochete, *H. pylori* or *M. tuberculosis*. Various researchers have also used monoclonal and polyclonal antibodies developed against different *Bartonella* sp. with varying results (Lin *et al*., 2006; Angelakis *et al*., 2008). Immunohistochemical staining using the patients' own serum ("Autoimmunohistochemistry") was used by Lepidi and co-workers to stain *Bartonella* sp. in cardiac valves from patients with infective endocarditis (Lepidi *et al*., 2006). In a study by Caponetti *et al*., IHC was the least sensitive compared to silver staining and PCR to detect *B. henselae* from the lymph nodes of patients with CSD (Caponetti *et al*., 2009). At this time secific monoclonal antibodies for IHC are not available commercially for any other *Bartonella* sp. except *B. henselae.*

C. **Fluorescent *In situ* Hybridization (FISH)**

Fluorescent *in situ* hybridization (FISH) is a useful method for the detection of

bacteria in tissues. FISH detects specific nucleic acid sequences of the bacteria in relation to their anatomical sites of infection. This technique has been successfully used for the detection and identification of spirochetes from digital dermatitis lesions of cattle and bacteria in patients with chronic periodontitis (Choi *et al*., 1997; Colombo *et al*., 2007). FISH utilizes oligonucleotide probes labeled with fluorescent molecules for the *in situ* detection and identification of bacteria. FISH is an important tool that helps in the detection of the distribution of bacteria and is widely used to study interactions of bacteria with their biotic and abiotic environments, especially in marine environments (Jurgens *et al*., 2000). Oligonucleotide synthesis is much cheaper than production of monoclonal antibodies, which makes FISH much more economical compared to IHC (Amann and Fuchs, 2008). FISH is also helpful in the detection of fastidious and uncultured bacteria (Moter and Gobel, 2000). False negative results may be obtained due to the low copy number of the target or due to poor permeabilization of the cells. Differences in binding affinity of fully complementary oligonucleotide have also been reported (Amann and Fuchs, 2008). Recently, nucleic acid modifications, such as, peptide nucleic acid (PNA) and locked nucleic acid (LNA), have been developed to improve the affinity of binding (Silahtaroglu *et al*., 2004; Almeida *et al*., 2010).

Development of a FISH assay for *B. henselae* and *B. vinsonii* subsp. *berkhoffii*

Bartonella sp. are highly fastidious and difficult to culture (Maggi *et al*., 2005a). Monoclonal antibodies are available commercially only for *B. henselae*. Since the sequences of many of the species in the genus *Bartonella* are now available, FISH may prove helpful in the diagnosis of *Bartonella* sp. in conjunction with PCR and serology (Hercik *et al*., 2002). Moreover FISH helps in the visualization of bacteria in the tissues and thus morphology can be used to help rule out false positive PCR results (Varanat *et al*., 2009). *B. henselae* and *B. vinsonii* subsp. *berkhoffii* are the most frequently documented species of *Bartonella* infecting immunocompetent human beings and dogs (Chomel *et al*., 2006). Our aim was to develop fluorescent probes specific to the genus *Bartonella*, to *B. henselae* and to *B. vinsonii* subsp. *berkhoffii* and to evaluate the efficiency of these probes to detect the bacteria in formalin-fixed paraffin-embedded tissues compared to PCR, Warthin-Starry silver staining and FISH using the universal probe Eub 338 (Amann *et al*., 1990).

Materials and Methods

Bacteria and cell culture

E. coli was kindly provided by Dr Paul Orndorff (NCSU). *E. coli* was grown on trypticase soy agar (TSA) with 10% sheep blood for 48 hrs. *B. quintana* was grown in TSA with 10% sheep blood for 7 days. Bacterial colonies were harvested and resuspended in SPG buffer and stored at -80^{0}C until use. 10µl of the bacterial suspension was spotted onto Teflon-coated 24 well slides. Slides were air dried and stored at -20^{0}C until use. DH82 cells were grown in RPMI with 20% fetal calf serum and infected with *B. henselae, B. koehlerae, B. volans* or *B. vinsonii* subsp. *berkhoffii* for 3-5 days. Cells were harvested and centrifuged to remove the medium and resuspended in PBS. The cells were then spotted on to Teflon-coated 24 well slides. Slides were air dried and stored at -20^{0}C until use.

Paraffin-embedded tissues

Paraffin-embedded tissue blocks from endocarditis, myocarditis and bacillary

angiomatosis cases were collected from other studies conducted in the lab and reported elsewhere (Cadenas *et al*., 2008; Yager *et al*., 2010; Varanat *et al*., 2011).

Hybridization

Teflon-coated slides were fixed in acetone for 10 minutes, washed in PBS and treated with proteinase K (100µg/ml) for 15 min at 37^0C. Sections were cut 5 µm thick from the paraffin-embedded tissues onto Plus Slides (Thermo Scientific, Hudson, NH). Slides were deparaffinised, rehydrated and washed with PBS. Tissues were treated with proteinase K (100µg/ml) for 15 min at 37^0C. Slides were washed in PBS and hybridized overnight with fluorescent probes at a concentration of 10 ng/µl in hybridization buffer (0.9M NaCl, 0.02M Tris HCl, 0.01% SDS, pH 7.2). Slides were then washed with wash buffer (0.9M NaCl, 0.02M Tris HCl, pH 7.2) at 56^0C for 30 min and mounted with Vectashield mounting medium (Vector Laboratories, Burlingame, CA), followed by examination under an epifluorescent microscope. Probes used in this study are given in Table 1. Universal probe Eub 338 was used as a reference standard and the nonsense probe nonEub 338 (Wallner *et al*., 1993) was used as a negative control for hybridization.

PCR

The primers used in this study are given in Table 1. PCR targeting the 16S-23S rRNA intergenic transcribed spacer (ITS) region was performed using primers 438s and 1100as as described previously (Maggi *et al*., 2005b). All amplicons were sequenced to establish species, strain and genotype.

Silver staining and immunohistochemistry (IHC)

5µ sections from paraffin embedded tissues were deparaffinized and rehydrated. Warthin-Starry silver staining was carried out in an automated stainer. For IHC, sections were treated with proteinase k for antigen retrieval. After blocking with normal goat serum, the sections were incubated with 1:100 dilution of mouse monoclonal antibody against

Bartonella henselae in PBS (Abcam, Cambridge, MA). After incubation with primary antibody, immunodetection was carried out using biotinylinated goat anti-mouse immunoglobulin followed by alkaline phosphatase conjugated streptavidin. Tissues sections were counterstained with Mayer's hematoxylin prior to coverslipping.

Results

To test the specificity of the probes, DH82 cells infected with *B. henselae*, *B. koehlerae*, *B. volans* or *B. vinsonii* subsp. *berkhoffii* and suspensions of *B. quintana* and *E. coli* spotted on Teflon-coated 24 well slides were used (Figure 2). All bacteria tested were positive with the Eub338 probe. With the Bspp 438 short probes (*Bartonella* genus specific probe) *B. henselae*, *B. quintana*, *B. koehlerae*, *B. volans* and *B. vinsonii* subsp. *berkhoffii* showed positive hybridization, whereas *E. coli* was negative. All the bacteria tested showed no fluorescence with the nonsense probe nonEub338. Bh371 probe showed specific hybridization with *B. henselae* whereas no hybridization was noted with *B. quintana*, *B. koehlerae*, *B. volans*, *B. vinsonii* subsp. *berkhoffii* or *E. coli*. The Bvb P1 probe hybridized specifically to *B. vinsonii* subsp. *berkhoffii*, whereas *B. henselae*, *B. quintana*, *B. koehlerae*, *B. volans* and *E. coli* were negative.

Next, we tested paraffin embedded tissues with the probes and compared the results with Warthin-Starry silver staining and immunohistochemistry (in the case of *B. henselae* only). Paraffin blocks were collected from bacillary angiomatosis, pyogranulomatous myocarditis and endocarditis cases from which *Bartonella* DNA was amplified by PCR. Results are summarized in Table 2. *B. vinsonii* subsp. *berkhoffii* genotype II was amplified and sequenced from the bacillary angiomatosis lesion (Yager *et al*., 2010). Bacilli staining positive with Warthin–Starry silver impregnation were demonstrated within the lesions closely associated with the proliferative capillaries (Figure 3C). Bacteria were also demonstrated by FISH using Eub338 probes. Not enough tissue was available for FISH with Bspp 438short, Bvb P1 or Bh 371 probes. A paraffin-embedded block from the endocarditis lesion was collected from pathology archives. This was from the dog from which *B. vinsonii*

subsp. *berkhoffii* genotype III was isolated for the first time (Cadenas *et al*., 2008). DNA was extracted from the block, and *B. vinsonii* subsp. *berkhoffii* genotype III DNA was amplified by PCR and sequenced. Bacteria were demonstrated by Warthin–Starry silver staining and by FISH with Eub 338, Bspp 438 short and Bvb PI probes (Figure 4). FISH was negative with Bh 371 and nonEub338 probes (results not shown).

Recently, we reported pyogranulomatous myocarditis and diaphragmatic myositis in cats caused by *B. henselae* (Varanat *et al*., 2011). Paraffin embedded heart tissue was tested by PCR, Warthin-Starry silver staining, *B. henselae* immunohistochemistry, and FISH using Eub 338, nonEub 338, Bspp 438 short, Bh 371 and Bvb PI probes. *B. henselae* Cal I strain DNA was amplified and sequenced from the tissue. Bacteria were demonstrated in the center of the pyogranulomas by Warthin-Starry silver staining, *B. henselae* immunohistochemistry and FISH using the Eub 338 probe (Figure 5). Bspp 488 short and Bh 371 probes failed to detect bacteria in the lesion. FISH was also negative with Bvb P1 and non Eub 338 probe as expected.

Discussion

In this study we developed FISH probes specific to the *Bartonella* genus, *B. henselae* and *B. vinsonii* subsp. *berkhoffii*. We targeted a partial sequence in the 16S-23S rRNA ITS region with fluorescently-labeled oligonucleotide probes. Our probes were able to bind specifically in the cell cultures infected with the specific *Bartonella* sp. *Bartonella* is an important emerging zoonotic pathogen implicated as causative agent of a wide variety of disease manifestations in both human beings and animals. Diagnosis of *Bartonella* sp. remains challenging even with recent developments in culture and molecular diagnostic techniques (Maggi *et al*., 2005a). *Bartonella* sp. infection does not follow Koch's postulates (Jacomo *et al*., 2002) and, because of this, proving causation is extremely difficult in *Bartonella* sp. infections. Most of the associations between *Bartonella* sp. infections and disease conditions are made based on molecular and serological detection (Angelakis *et al*., 2008; Breitschwerdt *et al*., 2010a). There are no well-defined animal models for *Bartonella*

sp. infections, especially for vasoproliferation (Chiaraviglio *et al*., 2010) and most of the pathogenesis studies are carried out using *in vitro* cell culture models (Mc Cord *et al*., 2007; Chang *et al*., 2011). Since *Bartonella* sp. is difficult to culture from clinical samples, FISH may prove to be an important tool for the diagnosis in conjunction with other diagnostic methods such as PCR and serology. FISH allows for the specific localization of the infectious agent in infected tissues, facilitating the establishment of spatial distribution of these bacteria in the tissues.

Visualization of *Bartonella* sp. in tissues is extremely difficult. Even though *Bartonella* is a Gram negative coccobacilli, Gram's stains are not usually successful in demonstrating the bacteria within the paraffin-embedded tissues. *Bartonella* sp. can be demonstrated by silver staining. Different silver staining methods like Warthin-Starry and Steiner and Steiner have been used preferentially by different laboratories. Silver stains are tedious to perform and may give varying results depending on the laboratory conditions (Caponetti *et al*., 2009; Varanat *et al*., 2011). Immunohistochemistry using specific monoclonal antibodies is another technique used for the visualization of *Bartonella* in tissues (Caponetti *et al*., 2009; Johnson *et al*., 2009); however, commercial antibodies are only available against *B. henselae* which limits the usefulness of this technique.

FISH utilizes fluorescently-labeled oligonucleotides for the detection of specific nucleotide sequences of the organism of interest in the tissues. Since sequences of many of *Bartonella* sp. are available now, and oligonucleotide synthesis is comparatively cheaper than development of monoclonal antibodies, FISH can be used as an economical diagnostic test for the visualization of *Bartonella* sp. Other researchers have developed FISH probes for the detection of *Bartonella* sp. (Hercik *et al*., 2002; Gescher *et al*., 2008). But those probes were not used on paraffin embedded tissues. To the best of our knowledge, FISH probes for *B. vinsonii* subsp. *berkhoffii* have not been reported in the literature.

Paraffin-embedded tissues are readily available and are useful for diagnostic testing and also retrospective research. FISH probes that can be used on paraffin-embedded tissues will be a valuable tool for both diagnosis and research applications. We tested our probes

using paraffin-embedded tissues from bacillary angiomatosis, endocarditis and myocarditis lesions. The Bvb P1 probe (specific to *B. vinsonii* subsp. *berkhoffii*) successfully hybridized in bacillary angiomatosis (caused by *B. vinsonii* subsp. *berkhoffii* genotype II) and in endocarditis (caused by *B. vinsonii* subsp. *berkhoffii* genotype III) lesions. The Bh371 probe, which is specific to *B. henselae*, failed to detect *B. henselae* organisms in paraffin embedded myocarditis lesions in which bacteria were demonstrated using Warthin-Starry silver staining, *B. henselae* specific immunohistochemistry and FISH using Eub338 probe. Bspp 438 short probes (specific to the *Bartonella* genus) successfully hybridized with *B. vinsonii* subsp *berkhoffii* in the endocarditis case but failed to hybridize in *B. henselae* myocarditis. Fewer numbers of bacteria were present in the myocarditis case compared to the bacillary angiomatosis and endocarditis cases, which may be a reason for the failure of the probes to hybridize. The most important limitation of FISH is the low sensitivity especially when fewer organisms are present in the lesions. Also this method requires more invasive sampling techniques, compared to serology or PCR.

Conclusions

We evaluated FISH probes specific to the *Bartonella* genus, *B. henselae* and *B. vinsonii* subsp. *berkhoffii*, for their specific hybridization and usefulness in the diagnosis of *Bartonella* in formalin-fixed paraffin-embedded tissues. Our probes were successful in demonstrating *Bartonella* in DH82 cells. We were also able to demonstrate *B. vinsonii* subsp. *berkhoffii* in bacillary angiomatosis and endocarditis lesions using Bvb P1 probe. The most important limitation was the low sensitivity of the probes, leading to failure of hybridization when the number of bacteria present in the tissues was low. Our results show that FISH can be used successfully for the diagnosis of *Bartonella sp*. in paraffin-embedded tissues.

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Table 1. Primers and probes used in this study

Primer/probe	Sequence	Reference
438s	GGTTTTCCGGTTTATCCCGGAGGGC	Maggi *et al*., 2005b
1100as	GAACCGACGACCCCCTGCTTGCAAAGCA	Maggi *et al*., 2005b
Eub338	6FAM GCTGCCTCCCGTAGGAGT	Amann *et al*., 1990
Non Eub338	6FAM ACTCCTACGGGAGGCAGC	Wallner *et al*., 1993
Bspp438short	TYE563 CGGTTTATCCCGGAGGGC	This study
Bh371	6 FAM CGTGGGCTTTGAAAAACGCT	This study
BvbP1	6 FAM AATCGGTTTAGGGGTTAGCGC	This study

Table 2. Summary of PCR, Warthin-Starry, *B. henselae* immunohistochemistry and FISH results on paraffin-embedded tissues.

Tissue	**Diagnosis**	**PCR**	**Warthin-Starry**	**Bh IHC**	**FISH (Probes)**			
					Eub338	**Bspp 438 short**	**Bh371**	**BvbP1**
Skin	Bacillary angiomatosis	Bvb II	Positive	ND	Positive	ND	ND	ND
Aortic valve	Endocarditis	Bvb III	Positive	ND	Positive	Positive	Negative	Positive
Heart	Pyogranulo-matous myocarditis	Bh CalI	Positive	Positive	Positive	Negative	Negative	Negative

Bh- *B. henselae*, Bvb- *B. vinsonii* subsp. *berkhoffii*

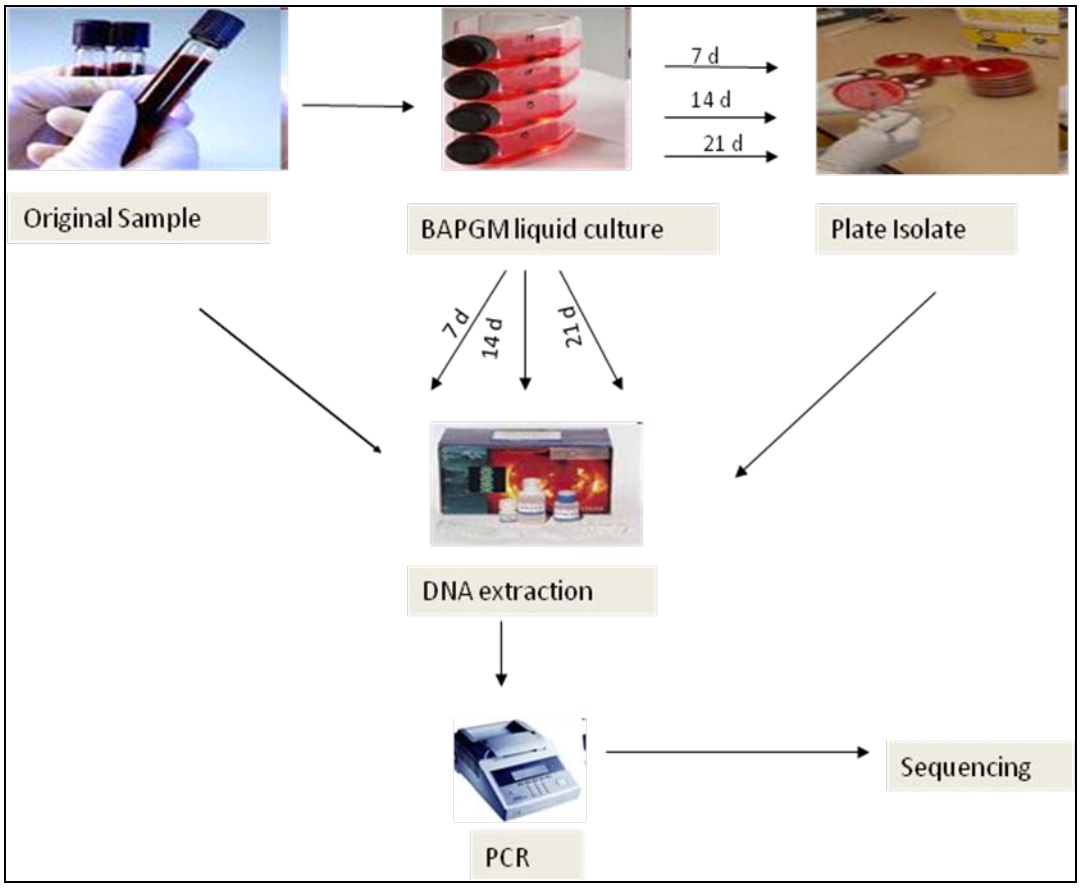

Figure 1. BAPGM platform for the diagnosis of *Bartonella* sp. from clinical samples. DNA is extracted from 200µl of the blood sample and *Bartonella* sp. DNA is amplified by PCR. 1ml of the blood sample is inoculated in to 10ml of BAPGM and incubated for 21days at 35^0C with 5% CO_2. DNA is extracted from 200µl of the culture on days 7, 14 and 21 post culture, followed by *Bartonella* sp. PCR. 1ml from the BAPGM culture is plated on to TSA agar with 10% sheep blood on days 7, 14 and 21. If an isolate is obtained, the identity is determined by PCR. Any amplicons obtained by PCR are sequenced to determine the *Bartonella* species, strain or genotype.

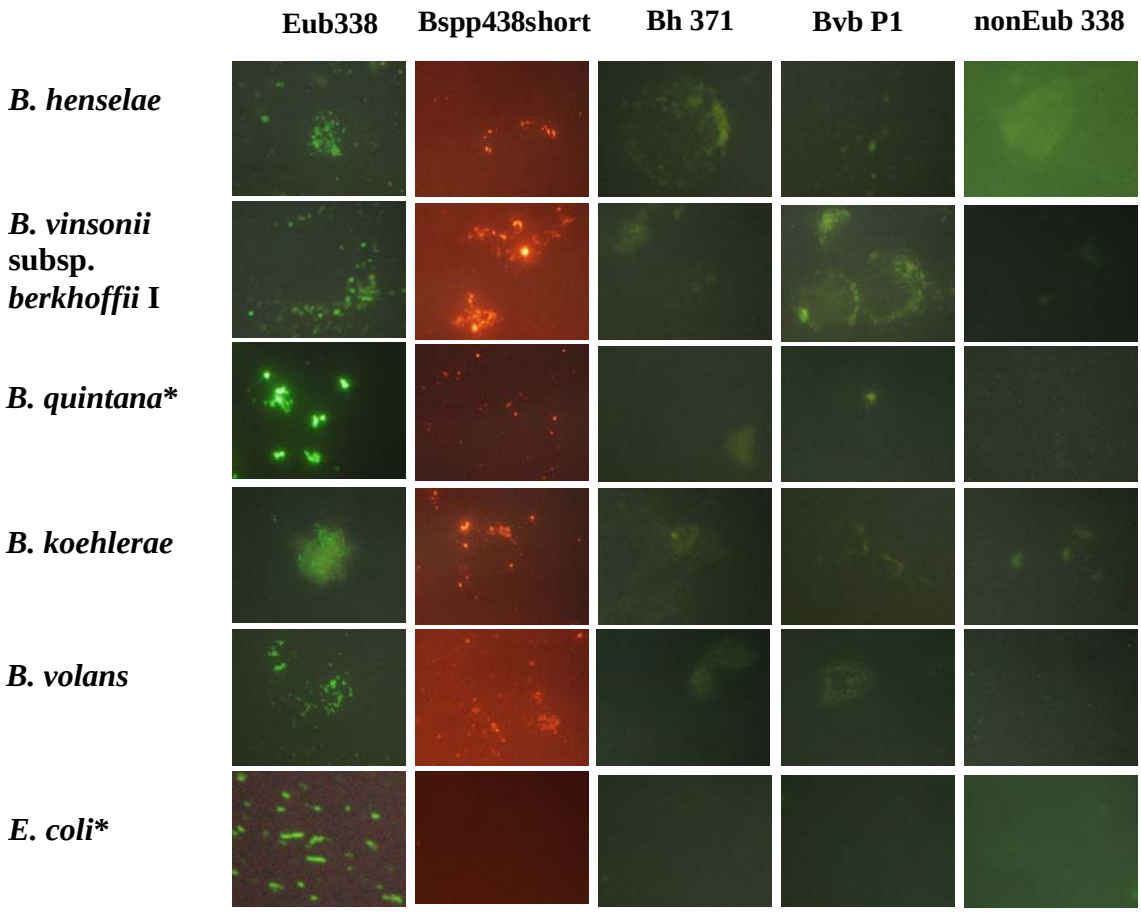

Figure 2. Fluorescent *in situ* hybridization on DH82 cells infected with *Bartonella* sp. DH82 cells were infected with *B. henselae*, *B. vinsonii* subsp. *berkhoffii*, *B. koehlerae* or *B. volans*, and spotted on to teflon coated slides. FISH was performed using the probes Eub 338, Bspp 438, Bh 371, Bvb P1 and Noneub 338. The probes hybridized specifically to the respective *Bartonella* sp. No hybridization was observed using the nonsense probe noneub 338.* Suspensions of bacteria were used in case of *B. quintana* and *E. coli*.

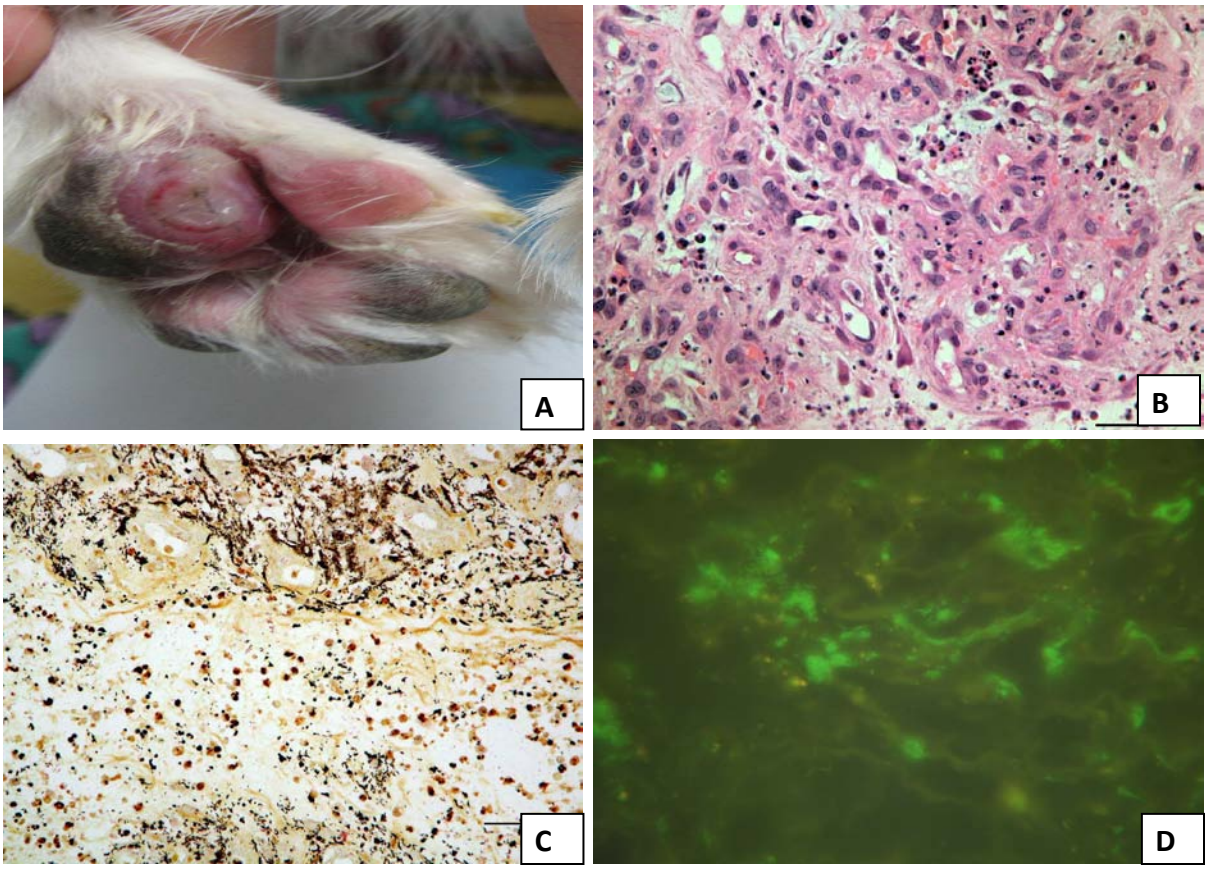

Figure 3. Bacillary Angiomatosis in a dog (Yager *et al*., 2010). A) Erythematous lesion on the foot pad. Lesion was not ulcerated, but had a depressed center. B) The proliferating capillaries lined by plump endothelial cells. The intervening edematous stroma contains pink staining strands of fibrin and degenerating neutrophils and macrophages. H&E. Bar = 30 µm. C) Positively stained bacilli are present within the lesion, more concentrated in areas of capillary proliferation. Warthin–Starry. Bar = 30 µm. D) Green fluorescent bacteria seen within the lesion. Fluorescent *in situ* hybridization with Eub 338 probe labeled with 6-FAM

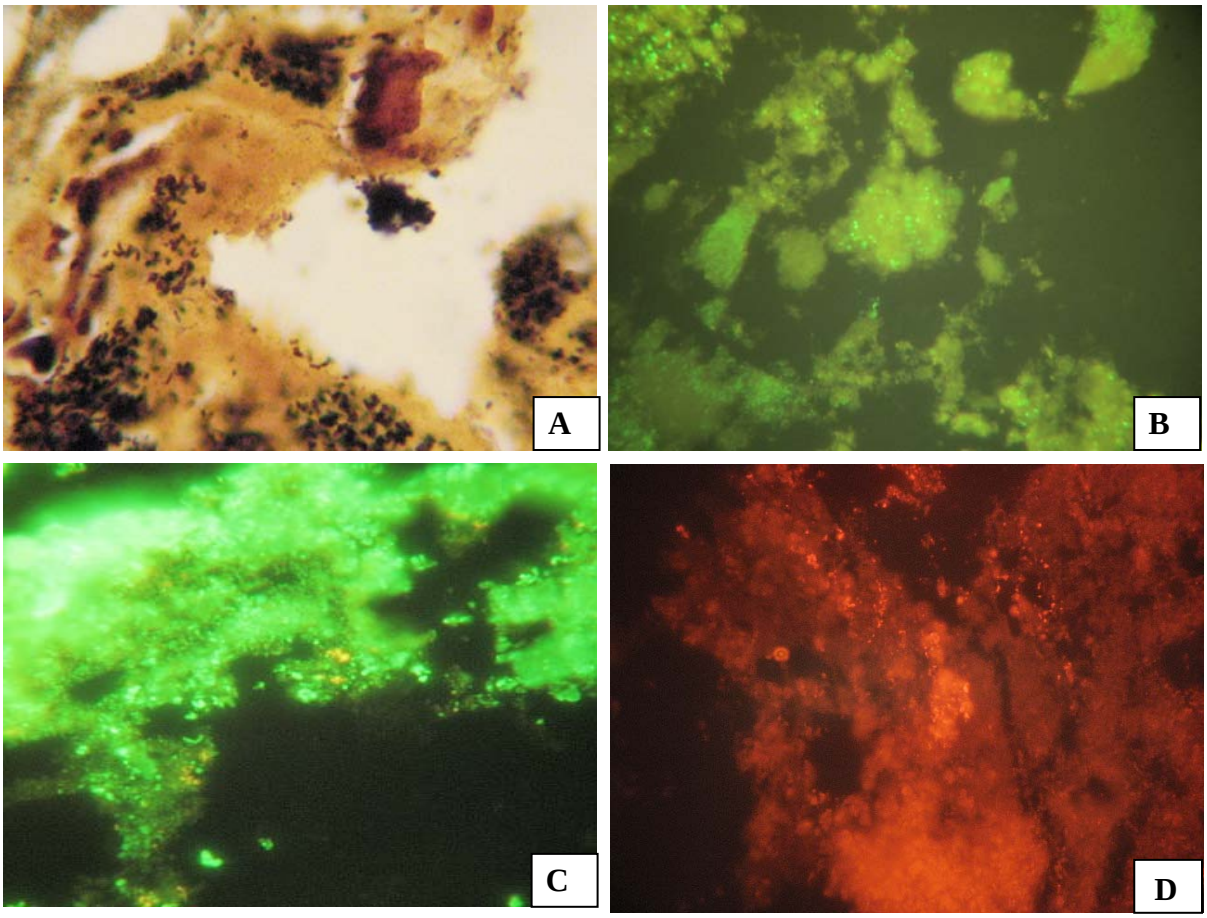

Figure 4. *B. vinsonii* subsp. *berkhoffii* endocarditis in a dog. A) Warthin-Starry silver staining B) FISH using Eub 338 probe labeled with 6-FAM. C) FISH using Bvb P1 probe labeled with 6-FAM. D) FISH using Bspp 438 probe labeled with TYE563. Note the rod shaped bacteria.

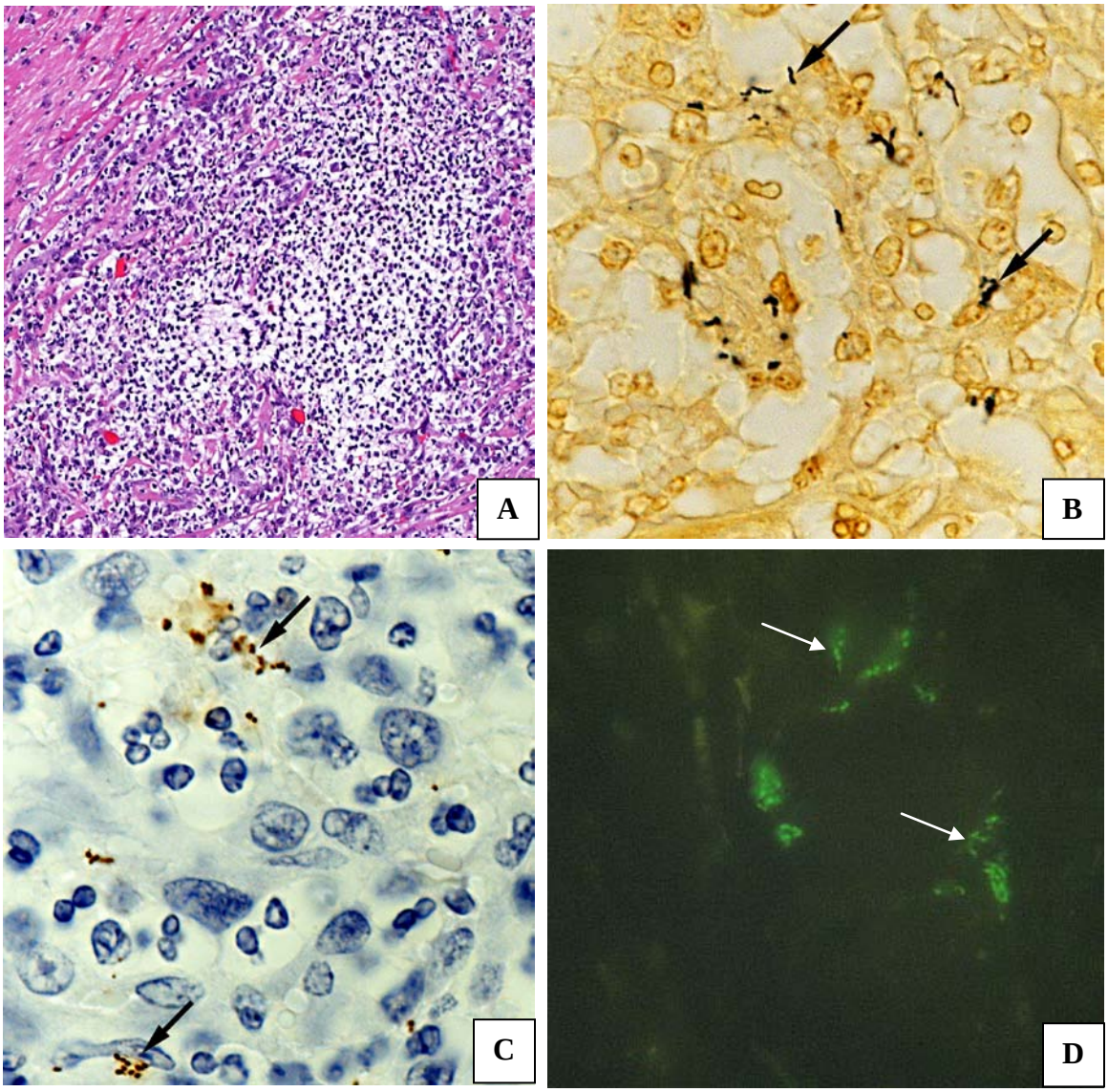

Figure 5. Pyogranulomatous myocarditis in a cat due to *B. henselae* (Varanat *et al*., 2011). A) Partially organized pyogranuloma in heart, H&E. B) Warthin-Starry silver staining C) *B. henselae* immunohistochemistry. D) FISH using Eub 338 probe labeled with 6-FAM. Note the rod shapes bacteria in the center of lesion in B, C and D.

Part B

Cross-contamination in the molecular detection of *Bartonella* from paraffin-embedded tissues

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Cross-contamination in the molecular detection of *Bartonella* from paraffin-embedded tissues

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Short Title: Cross contamination of *Bartonella* spp. DNA

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Abstract

The genus *Bartonella* is comprised of a group of gram-negative, argyrophilic, and highly fastidious bacteria. Due to diagnostic limitations associated with culture and serological testing, polymerase chain reaction (PCR) has become a powerful tool for the confirmation of *Bartonella* spp. infection in blood and tissue samples. As numerous wild and domestic animals harbor *Bartonella* spp., these somewhat ubiquitous intravascular bacteria pose special challenges for pathologists, when obtaining or processing animal tissues that might subsequently be used for *Bartonella* PCR testing. In this study, we describe evidence of *Bartonella* DNA dissemination and carry over identified in the necropsy room and during the subsequent processing of formalin-fixed paraffin-embedded tissues. *Bartonella* DNA was amplified from different parts of the microtome, different areas of the necropsy room and from the liquid paraffin in the tissue processor. Transfer of *Bartonella* DNA among tissues obtained from different animals or patients during embedding and sectioning processes could result in erroneous diagnoses or inaccurate research conclusions. Unless specific policies are established and rigidly followed, molecular diagnosis of *Bartonella* spp. infection using tissue samples obtained at necropsy or tissues processed in a multispecies diagnostic histopathology laboratory, including surgical biopsies, may not provide diagnostically valid results.

Key words: Bartonellosis, cross contamination, necropsy, paraffin-embedded tissue, PCR contamination

The genus *Bartonella* currently includes at least 22 species of gram negative, fastidious, aerobic bacilli, with many additional unnamed or candidatus species having been identified within arthropod vectors and the blood of various animal reservoir hosts, mostly on the basis of PCR amplification and DNA sequencing. From a clinical and pathological perspective, these intravascular bacteria appear to cause a wide spectrum of pathology in animals and human beings (Breitschwerdt and Kordick, 2000). In addition, various *Bartonella* spp. have evolved to facilitate efficient transmission by biting flies, fleas, lice, sand flies, and potentially ticks among a spectrum of reservoir-adapted hosts (Billeter *et al*, 2008). Improvements in culture and PCR have resulted in the detection of *Bartonella* spp. in association with an increasingly diverse spectrum of diseases (Duncan *et al*., 2007). However, certain *Bartonella* species, such as *B. henselae* in cats, *B. vinsonii* subsp. *berkhoffii* in coyotes, *B. bovis* in ruminants and numerous *Bartonella* spp. in small mammals and rodents are evolutionarily well adapted to cause persistent, non-clinical, intravascular infections in the reservoir host (Breitschwerdt and Kordick, 2000). As an example, blood samples obtained from 80 % of the beef cattle from North Carolina contained *B. bovis* DNA by PCR testing (Cherry *et al*., 2009). A similar high prevalence of *B. bovis* bacteremia has been reported from cattle in Africa, Europe and North America (Breitschwerdt *et al*, 2001; Kelly *et al*., 2005). Cross contamination of tissues with bovine blood during necropsy, which frequently contains *B. bovis* DNA, could result in DNA carry over from a cow to another necropsied animal, such as a cat. As *B. bovis* has been infrequently isolated from cat blood samples (Breitschwerdt, 2008), it would be difficult to establish if the cat was infected with *B. bovis* or if there was transfer of DNA from cow blood due to cross contamination at necropsy.

Because of historical and ongoing limitations associated with culturing these bacteria from the clinical samples, problems related to serological cross reactivity among *Bartonella* spp., and the poor correlation between serology and bacteremia in reservoir hosts, dogs and human patients, molecular detection of *Bartonella* DNA by polymerase chain reaction (PCR)

has gained favor as a reliable diagnostic aid in the clinical microbiology laboratory. Researchers have targeted different *Bartonella* genes, including the 16S rRNA gene, 16S-23S rRNA intergenic transcribed spacer (ITS) region, groEL gene, the citrate synthase gene (gltA) and others for the molecular detection of *Bartonella* infection (La Scola *et al*., 2003; Maggi and Breitschwerdt, 2005). Although PCR amplification of various target genes provides a rapid diagnostic alternative for the detection of *Bartonella* spp., there can be limitations related to poor test sensitivity and specificity. As an example, our laboratory reported non-specific PCR amplification of *Mesorhizobium* species using previously published 16S-23S intergenic spacer (ITS) primers designed to amplify *Bartonella* DNA (Maggi and Breitschwerdt, 2005). As *Mesorhizobium* DNA can be found as a contaminant in molecular grade water, false positive PCR test results were documented and the ITS primers were redesigned to ensure amplification specificity. In conjunction with efforts to enhance PCR sensitivity for the microbiological detection of *Bartonella*-specific DNA sequences, we have recently incorporated pre-enrichment culture of aseptically-obtained diagnostic samples (blood, cerebrospinal, aqueous, and joint fluids and effusions) using a liquid insect cell culture based medium (*Bartonella* alpha-Proteobacteria growth medium (BAPGM) prior to PCR testing (Duncan *et al*., 2007). BAPGM pre-enrichment culture increases *Bartonella* bacterial numbers so as to achieve a higher level of PCR detection. By combining pre-enrichment culture with PCR amplification, substantial improvement in molecular diagnostic sensitivity has been achieved, subculture agar plate isolates have been obtained for detailed microbiological studies, and human infections with novel *Bartonella* species have been reported (Maggi *et al.*, 2009). This approach is applicable to pathological specimens, assuming they are collected and transported aseptically.

Archival collections of paraffin embedded tissues provide an important and readily available resource for retrospective pathological studies of bartonellosis and other novel or emerging bacterial infections (Imrit *et al.*, 2006). Techniques used for DNA extraction from formalin-fixed paraffin-embedded tissue have been studied and as described above, various PCR assays have been validated (Sato *et al.*, 2001). In conjunction with initial efforts to

define the cellular localization of *Bartonella* spp. in paraffin embedded tissues, we repeatedly detected *Bartonella* DNA in negative control samples used during PCR testing. Initially, to investigate the source of DNA contamination, all reagents used for DNA extraction and PCR amplification were individually tested and shown to be negative for amplicon contamination. When amplicon contamination within the testing laboratory was eliminated as a source of the problem, we investigated the possibility of DNA transfer in the necropsy service area and during tissue processing and sectioning in the histopathology laboratory.

In an effort to address DNA carry over during the sample processing stages, blank paraffin blocks (negative controls) and positive paraffin blocks containing known *Bartonella* spp were cut on a microtome in an alternating fashion. Using this approach, *Bartonella* DNA could be amplified by PCR from 4/6 blank (negative control) paraffin blocks, however, samples obtained from the same blank paraffin blocks using a scalpel blade were consistently PCR negative. All samples were extracted and tested in an identical manner.

After implicating the microtome as a source of DNA carry over, other potential sources of DNA transfer were investigated within our institution's necropsy room and histopathology laboratory. In total, 5 microtomes were assessed, one each from two diagnostic laboratories in our institution and three from within a local state diagnostic laboratory. Dry, sterile cotton swabs were used to collect samples from the necropsy room (the trim board, the tissue strainer and the necropsy table tops) at the end of a business day, and after these areas had been routinely cleaned. Sterile cotton swabs were also used to collect samples from the processing area in the histopathology laboratory (the trim board, the formalin container for collecting cassettes after trimming and the water bath used for slide mounting of tissue sections). Used paraffin samples were collected directly from the tissue processor from the oldest and newest liquid paraffin holding tank. Sampling was conducted at two times, several days apart. Unused sterile swabs and new paraffin, unexposed to the processor, served as negative controls during extraction and PCR testing of the necropsy and histopathology laboratory samples.

For each of five different microtomes, located in three different diagnostic

laboratories, dry, sterile cotton swabs were individually used to obtain samples from different parts of the microtome including, the blade holder, block holder, platform, slanting platform, bench top and used microtome blades. Samples were taken at the end of the workday and after microtomes were routinely cleaned. One new sterile swab was tested as a negative control with each set of microtome collected swabs.

DNA from the swabs was extracted using the QIAamp DNA mini kit (QIAGEN Inc., Valencia, CA) as per manufacturer's instructions. Molecular grade water was used as an additional control during DNA extraction. After extraction, DNA concentration and purity were measured with an absorbance ratio of 260 to 280 nm using Nanodrop (Thermo Scientific, Wilmington, DE) and stored at -20^0C until used. The 16S-23 ITS primers used included 325S: 5' CTTCAGATGATGATCC CAAGCCTTCTGGCG 3' and 1100AS: 5' GAACCGACGACCCCTGCTTGC AAAGCA 3'. Primers were synthesized by MWG Biotech (Highpoint, NC). PCR was performed with a final reaction volume of 25 μl, containing 12.5 μl of premix Ex Taq (perfect real time) (Takara Bio USA Inc, Madison, WI), 7 μl of molecular grade water 0.25 μl each of forward and reverse primers and 10 μl of template DNA. Amplification of the ITS region was performed under the following conditions in a Mastercycler epgradient (Eppendorf, Westbury, NY) a single hot-start cycle at 95°C for 2 min followed by 55 cycles of denaturing at 94°C for 15 sec, annealing at 66°C for 15 sec, and extension at 72°C for 18 sec. Amplification was completed by an additional cycle at 72°C for 2 min. All amplification products were analyzed by 2% agarose gel electrophoresis, stained by ethidium bromide and visualized under UV light. Amplicon sizes were determined by comparison with the molecular weight marker, Hyladder 1 kbp (Denville scientific Inc., Metuchen, NJ). *Bartonella henselae* DNA (.001pg/μl) and DNA from a healthy un-infected dog were used as positive and negative controls respectively.

Bartonella ITS PCR amplification products were sequenced directly by gel extraction using QIAquick gel extraction kit (Qiagen Inc., Valencia, CA.) or by cloning into the plasmid pGEM T easy vector system (Promega, Madison, WI). After E.coli DH5α transformation,

the recombinant white colonies were selected based on the correct insert size and the plasmids were isolated using QIAprep Spin Miniprep kit (Qiagen Inc., Valencia, CA). From each transformation, at least three independent clones were selected for sequencing. Sequencing of each plasmid insert was done by MWG Biotech (MWG, High Point, NC). Sequence analysis and alignment with GenBank sequences was performed using AlignX software (Vector NTI Advance 10, Invitrogen Inc., Carlsbad, CA).

Bartonella DNA could be amplified by PCR from swabs taken from used microtome blades and from different parts of the microtome (Table 1). Three out of five microtomes sampled, after daily cleaning, were PCR positive for DNA of three different *Bartonella* spp. *Bartonella* spp. DNA could be amplified from 3 of 7 swabs taken from microtome 1, and 1of 7 swabs from microtomes 2 and 3. DNA was also amplified from 1 of 2 used paraffin samples obtained from the tissue processor and from 1 of 2 necropsy room trim board samples. All negative control sterile swabs and all extraction and PCR controls remained negative. Amplicons from the flat platform and the block holder of microtome 1 had 97% and 99.5 % sequence similarities respectively with *Bartonella henselae* (ITS strain Houston I). An amplicon, obtained from a used microtome blade, had 99.6 % similarity with *B.vinsonii* subsp. *berkhoffii* genotype II. This microtome was routinely used for preparation of dermatopathology slides and cat and dog tissues were the predominant tissue sections processed on this microtome. The amplicons from microtomes 2 and 3 had 99.2 % sequence similarity to *B. henselae* (ITS strain San Antonio 2) and 99.8% *B.vinsonii* subsp. *berkhoffii* genotype II, respectively. The amplicon from the necropsy room trim board was 85.6 % similar to Candidatus *Bartonella volans* and the amplicon from the paraffin from the tissue processor was 99.5% similar to *B. henselae* (ITS strain Houston I). Cat, dog, rat and wood chuck necropsies were carried out during the several day period prior to obtaining samples for PCR testing.

The results of this study indicate that different *Bartonella* species and strains can be amplified from necropsy and histopathology processing areas, both of which are potential sources of sample cross contamination. There are several possible mechanisms by which

cross contamination might occur during the collection and processing of necropsy tissues or surgical biopsies. Although many *Bartonella* spp. have evolved in conjunction with a preferred vector and reservoir host, it is increasingly obvious that DNA of these bacteria can be found in an array of vectors and hosts. For example, *B. henselae* is most often found in the blood of cats and dogs, but has also been isolated or amplified from blood of other animals, including dolphins, horses, porpoises, sea turtles and whales (Hams *et al.*, 2008; Jones *et al*., 2008; Maggi *et al.*, 2005). To date, *B. vinsonii* subsp. *berkhoffii* has only been detected in dogs, coyotes, gray foxes and human beings (Chang *et al*., 2000). *Bartonella vinsonii* subsp. *berkhoffii* genotype II is the most frequently detected genotype in dog and human samples in our laboratory. Although the most similar GenBank sequence for one amplicon was Candidatus *B. volans*, a novel *Bartonella* sp. isolated from southeastern ground squirrels, (GenBank sequence EU 294521, Reeves WK, Unpublished data), the low homology suggests that this sequence represents a novel *Bartonella* sp., not previously sequenced in our or other research laboratories. Somewhat surprisingly, *B. bovis* DNA was not amplified from any microtome. This may be related to the minimal numbers of beef cattle tissue samples processed on the microtomes tested in this specific study, which was based in an urban area.

Because veterinary pathologists provide necropsy and biopsy services for a wide array of animal species, many of which can serve as reservoir hosts for specific *Bartonella* species, transfer of DNA from these ubiquitous intravascular bacteria is highly problematic for both molecular diagnostic studies and for retrospective research studies. The increasing utilization of molecular-based diagnostic testing for infectious agents in postmortem tissues has created an entirely new paradigm for tissue collection and cleaning procedures used at the time of necropsy when molecular diagnosis and/or molecular research are anticipated goals. Potential contamination of equipment (knives, saws, cleavers, gloves, tables, etc) within the necropsy room could result in the transfer of *Bartonella* spp. DNA to subsequent carcasses or tissues, which might result in a false positive diagnosis when using PCR.

In this study *Bartonella* DNA was amplified from the bench top near the microtome,

from the used discarded microtome blades and directly from the microtome. False positive results have been a previously reported problem when PCR is used for the detection of infectious disease agents using formalin-fixed paraffin-embedded tissues (Schewe *et al*., 2005). Hole punch carry over contamination and environmental contamination of the laboratory work space and equipment have also been documented as potential sources of false positive results when using PCR (Bonne et al, 2008; Taranger et al., 1994).

Although our study was less than exhaustive, microtomes are an important source of DNA carry over, as small bits of paraffin and infected tissue can adhere to different parts of the microtome, which could potentially contaminate the next block that is processed. Although lacking practicality, measures like special cleaning the microtome between blocks and using a new microtome blade for each block processed could potentially minimize the chance of DNA carry over. For molecular-based diagnostic studies, blank paraffin control blocks should always be used in between the sample blocks in order to detect potential DNA carry over while processing sequential blocks. In addition, processing positive control blocks in the same laboratory and on the same microtome as patient or research sample blocks should be avoided. Using individual new scalpel blades to shave off tissue from each block processed is a means to limit microtome carry over; however, this approach does not limit the potential of paraffin contamination in automated tissue processors, as identified in this study. Prospective studies should include individual hand embedding of tissues using new reagents. Fresh frozen clinical diagnostic samples would be favored over paraffin embedded tissues samples for PCR-based testing, particularly where communal exposure to different tissues in automated processors cannot be avoided. This is especially critical for identifying novel associations between various pathologies and these highly fastidious infectious agents. In situ antibody or molecular testing, such as immunohistochemistry, in situ hybridization or in situ PCR offers additional challenges because of a need for microtome preparation of samples. While relevant morphological localization of agents within tissue lesions, the lack of an amplification step, and documentation of repeatable results can guard against processor related contamination of samples, isolated results from individual cases should be cautiously

interpreted.

In conclusion, PCR is a powerful tool for the detection of DNA from *Bartonella* spp. and other infectious agents in blood and tissue samples obtained from animals and human beings; however, caution should be exercised in interpreting the results. On the basis of this study, several steps involved in the preparation of paraffin embedded tissue blocks could serve as potential sources of DNA carry over between different tissues and different patient samples. Care should be taken in the collection, processing and sectioning of the tissue blocks, if the samples are intended for molecular diagnostic testing by PCR.

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Table 1. Results of PCR from swabs taken from the different parts of the five different microtomes.

Sample Source	PCR Results				
	M1	**M2**	**M3**	**M4**	**M5**
1) Negative control (swab)	Negative	Negative	Negative	Negative	Negative
2) Platform	Negative	Negative	Negative	Negative	Negative
3) Block holder	Positive	Positive	Negative	Negative	Negative
4) Slanting platform	Positive	Negative	Negative	Negative	Negative
5) Used blades	Positive	Negative	Negative	Negative	NP*
6) Microtome Bench top	Negative	Negative	Positive	Negative	Negative
7) Blade holder	Negative	Negative	Negative	Negative	Negative
8) Extraction control	Negative	Negative	Negative	Negative	Negative

NP* Not performed

Chapter 3.

Bartonella vinsonii subsp. *berkhoffii* as an agent of vasoproliferative disorders

Part A

Detection of *Bartonella vinsonii* subsp. *berkhoffii* from a dog with bacillary angiomatosis

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(Varanat M, was responsible for the PCR testing, DNA sequencing, silver staining and FISH assays)

Bacillary Angiomatosis in an Immunosuppressed Dog

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Running Heading: Bacillary Angiomatosis

Abstract

A dog being treated with immunosuppressive doses of prednisone and azathioprine for pancytopenia of unknown origin developed, over a 2-week period, multiple erythematous nodular lesions in the skin including footpads. Skin samples revealed lesions identical to those of human bacillary angiomatosis (BA). The nodules were composed of multifocal proliferations of capillaries, each lined by protuberant endothelial cells. The capillary clusters were separated by an oedematous connective tissue, lightly infiltrated with degenerate inflammatory cells, including neutrophils and macrophages. Tissue sections stained with Warthin–Starry silver stain revealed large numbers of positively stained bacilli in the stromal tissue, most heavily concentrated around the proliferating capillaries. Lesions of vascular degeneration and inflammation were evident. *Bartonella vinsonii* subsp. *berkhoffii* genotype 1 was independently amplified and sequenced from the blood and the skin tissue. The pathognomonic nature of the histological lesions, demonstration of compatible silver-stained bacilli in the tissue, and identification of *B. vinsonii* subsp. *berkhoffii* in the blood and tissue indicates that this is most likely the aetiologic agent responsible for the lesions. Antibiotic therapy was successful in resolving the nodules. It would appear that *B. vinsonii* subsp *berkhoffii*, like *Bartonella henselae* and *Bartonella quintana*, has the rare ability to induce angioproliferative lesions, most likely in association with immunosuppression. The demonstration of lesions identical to those of human BA in this dog is further evidence that the full range of clinical manifestations of human Bartonella infection occurs also in canines.

Introduction

The clinical manifestations of infection by bacteria of the *Bartonella* genus are protean.[1–4] Members of this genus of gram-negative bacteria are highly fastidious intracellular pathogens that infect erythrocytes, endothelial cells and macrophages.[5,6] Their persistence in erythrocytes in their reservoir hosts potentiates the chance of transmission via the various blood-sucking arthropods that act as vectors.[1,7] Within the reservoir host, the bacteria are usually so well adapted that they do not cause disease; for example, cats are

healthy carriers of *Bartonella henselae*, the main cause of cat scratch fever in humans.[1] Most, although not all, diseases caused by *Bartonella spp*. occur in accidental hosts and these organisms are being increasingly implicated in zoonotic infections.[1,2] *Bartonella* infections have been described as an emerging epidemic.

In humans, at least six and possibly up to 11 species or subspecies of *Bartonella* are responsible for a wide range of symptoms, relating to multiple organ systems.[2,3] The most commonly diagnosed is cat scratch fever, a localized granulomatous lymphadenitis caused by *B. henselae*.[3] *Bartonella quintana* is associated with trench fever, a disease of great significance during World War I, and one that is re-emerging in homeless humans.[4] *Bartonella bacilliformis*, the ancestral pathogen in this genus, is endemic to localized areas in South America, particularly Peru, Columbia and Ecuador, reflecting the range of the sand fly vector. In acute infections the organism causes haemolytic anaemia, known as Oroya fever; chronic infections result in cutaneous angioproliferative lesions known as verruga peruana.[8] Endocarditis has been associated with *B. quintana*, *B. henselae* and *Bartonella vinsonii* subsp. *berkhoffii* infections.[3,4] Other manifestations of human disease include bacteraemia with fever (*B. henselae*), neuroretinitis (*B. henselae*), encephalitis and peliosis hepatitis, an angioproliferative lesion in the liver, typically caused by *B. quintana* or *B. henselae*.[3,4]

The pathogenesis of most diseases caused by the Bartonellae is predicated upon their well-adapted status as facultative intracellular pathogens. Initial infection of endothelial cells, leads, in the reservoir hosts, to infection of erythrocytes. In the reservoir hosts, the bacteria multiply to a critical density without damaging the host erythrocyte. The exception is *B. bacilliformis*, which causes haemolytic anaemia in humans (Oroya fever).[9] This organism, geographically restricted to the Andes region of South America, is considered a 'missing-link' from which other species have evolved by radial speciation.[10,11] In the accidental hosts, intracellular infection of endothelial cells is the main mechanism whereby chronic infections are established, but the bacteria also infect macrophages, CD34+ cells and microglia.[12,13] Intra-endothelial infection explains why some infections are characterized by lesions resembling vasculitis.[14] The granulomatous nature of the inflammatory response, typical of

bartonellosis in immunocompetent individuals, is due to interferon-γ mediated T helper immune response resulting in macrophage activation.[15]

Bartonella spp. infections are being recognized in dogs with increasing frequency as diagnostic techniques have become more sophisticated.[16–18] *Bartonella vinsonii* subsp. *berkhoffii* was first recovered from a dog with endocarditis in 1993[19] and its designation was formalized in 1996.[20] This organism has been isolated from the blood of normal dogs and serological evidence suggests that the infection in dogs is worldwide.[20] In temperate climates, the seroconversion rate of dogs is low indicating that dogs,[21,22] unlike coyotes[23], are not likely to be a reservoir of infection. However, in tropical climates serologic surveys point to a much higher rate of infection of domestic and wild dogs.[24] In both situations, canine Bartonella infection is considered a sentinel for zoonoses.[2] Other Bartonella species isolated from dogs include the organism of cat scratch fever, *B. henselae* which was recovered from the blood of a dog with peliosis hepatis[25] and in another with granulomatous hepatitis.[26] There is serological evidence of widespread exposure to this species in dogs in south-eastern USA.[27] *Bartonella clarridgeiae* has been recovered from a dog with endocarditis.[28] Other species implicated in canine infections include *Bartonella elizabethae*, *Bartonella washoensis* and *B. quintana*.[2,29]

Bartonella infections in dogs, as in humans, exhibit a wide range of clinical signs, relating to the involvement of many organ systems. Lesions may affect the central nervous system,[30,31] eye,[32] nasal cavity,[33] endocardium,[19] myocardium,[34] liver,[25] lymph nodes,[33] joints,[31] skin and subcutaneous tissues.[30,31,33] Lesions in the subcutaneous tissues may occur in concert with lesions affecting other systems, particularly the nervous system.[30] Subcutaneous lesions may be single or multiple, nodular and non-ulcerated, and are characterized by granulomatous or pyogranulomatous inflammation of the panniculus.[30]

One manifestation of human *Bartonella* infection has such characteristic histologic lesions that it has been accorded a specific designation, namely bacillary angiomatosis (BA).[35] It is a unique manifestation of bacterial infection in that the lesions are angioproliferative [35,36] and occur mostly, but not exclusively, in immunosuppressed

individuals.[36,37] The lesion mimics a vascular tumour and was, in fact, first described as such, and named epithelioid angiomatosis.[36] BA is very different from the pyogranulomatous lesions that characterize the few cases of subcutaneous *Bartonella* infection recognized, to date in canines.[30,31] This report describes lesions, which are identical to those of human BA in an immunocompromised dog infected with *B. vinsonii* subsp. *berkhoffii*.

Case report

Clinical findings

In January 2008, a 3-year-old female papillon was diagnosed with pancytopenia by a veterinary internist. The dog was anaemic (normocytic, normochromic and nonregenerative), hypoproteinemic (total serum protein 40.0 g⁄L; normal range 50–74 g⁄L) and markedly leukopenic (mildly neutropenic and markedly lymphopenic) with a regenerative left shift and toxic neutrophils (see Table 1). The dog was also thrombocytopenic (Table 1). The presence of moderate numbers of giant platelets indicated bone marrow regeneration: the clinical pathologist's comment suggested a drug reaction but no drug history was evident. Six days later, a bone marrow aspirate was performed because the anaemia and leucopenia had worsened (see Table 1). The marrow was hypercellular. Approximately 70% of the haematopoietic cells were erythroid precursors and there were many bone marrow reticulocytes. Neutrophilic hyperplasia was described as mild, whereas megakarocytes were markedly increased. Maturation of all cell lines was complete and orderly. A moderate number of macrophages contained erythrocytes, nuclear remnants and hemosiderin, indicating increased haemophagocytosis. The findings suggested a recent and transient destruction of mature blood cells (hypersplenism) and⁄or suppression of bone marrow precursor cells and subsequent restoration of marrow function. Based upon the bone marrow findings, the dog was treated from January 2008 to April 2008 for pancytopenia with immunosuppressive drugs: prednisone 2.5 mg⁄kg orally twice a day and azathioprine (Imuran; Glaxo Smith Kline, Research Triangle Park, NC, USA) 1.5 mg⁄kg twice a day. At the time of presentation to the veterinary dermatologist in April 2008, despite

immunosuppressive therapy, the dog remained mildly anaemic (Table 1).

In April 2008, the dog, whilst receiving this immunosuppressive treatment, developed lameness and was referred to a veterinary dermatologist (NZ). In the preceding 5 days, multiple nodules had developed on the dorsum, thorax, flanks, caudal thighs, right elbow and right hock, lips, periocular skin and left fore and right hind footpads (Figure 1). There was no involvement of the mucous membranes or the nasal planum. The cutaneous lesions were between 5 and 13 mm in diameter, round to oval, erythematous and sometimes alopecic. The footpad lesions were centrally located on the pads. There was no ulceration but the centre of the pad lesions was depressed.

Cytologic examination of aspirates from the nodules revealed neutrophils, some of which contained intracellular cocci, and rare histiocytes. Six nodules were sampled with a 6-mm biopsy punch and submitted in 10% formalin to the authors for histologic evaluation. Standard 5-μm sections were prepared from the paraffin-embedded blocks and stained with Haematoxylin and Eosin (H&E) and Warthin–Starry silver.

Whilst awaiting the histological results, the dog was treated with clindamycin (10.4 mg⁄kg mg orally twice a day) and the immunosuppressive therapy was continued. After receiving the skin sample results, and at the time blood was collected for microbiological analysis, the clindamycin treatment was discontinued and replaced with 11.1 mg⁄kg azithromycin orally once a day for 1 week and then every other day for 5 weeks.

Within 2 days of the commencement of the azithromycin treatment, the cutaneous lesions began to resolve, despite continuation of the immunosuppressive therapy. The nodules regressed over the next week and no recurrence has been reported since cessation of the antibiotic therapy. The immunosuppressive therapy was tapered over 6 weeks. Haematologic testing in late May 2008 revealed a normal haemogram and leukogram (Table 1).

Histologic lesions

On low-power examination, the lesion was nodular and located in the subcutis

(Figure 2). Individual nodules were composed of multifocal clusters of small capillaries within an oedematous stroma, giving a pseudo-neoplastic appearance to the lesion (Figure 3). Mild fibrinoid degeneration of the vessels was apparent. Fibrin was also present in the perithelial tissues (Figure 4). The intervening connective tissue was very oedematous, sometimes mucinous, with separation of the fine collagen bundles. There was a light infiltration of the lesion by poorly preserved leukocytes, mostly neutrophils, but also by macrophages and lymphocytes. Many of the leukocytes were pyknotic or karyorrhectic (Figure 4). Swollen, mitotically active, hyperchromatic endothelial cells, protruded into the vessel lumina (Figure 5). A faint basophilic stippling of the connective tissue in the H&E-stained sections, equated, on Warthin–Starry silver-stained sections, to the presence of myriads of extracellular bacilli (Figure 6). These were most numerous in the areas of capillary proliferation, but were also scattered throughout the intervening connective tissue. Most organisms were extracellular but some appeared close to, or within, endothelial cells.

Microbiological findings

Serological testing of serum, collected after commencement of the clindamycin therapy, was negative for *Ehrlichia canis*, *B. henselae*, *B. vinsonii*, *Babesia canis*, *Anaplasma spp*. There was no evidence of heartworm infection. The paraffin-embedded tissue blocks prepared from the skin samples and a fresh blood sample were submitted to the Intracellular Pathogens Research Laboratory, College of Veterinary Medicine, North Carolina State University. The blood was cultured using a specifically designed liquid medium BAPGM (Bartonella–Alphaproteobacteria growth medium).[18] PCR amplification of the 16S–23S rRNA intergenic transcribed spacer (ITS) gene and DNA sequencing were carried out on the blood sample and subsequently, on the formalin-fixed lesion tissue as described previously.[16] *Bartonella vinsonii* subsp. *berkhoffii* genotype I DNA was amplified and sequenced from both the blood and tissue block. BAPGM culture failed to grow any bacteria, presumably because the dog was being treated with clindamycin at the time the blood culture sample was obtained. *Bartonella vinsonii* subsp. *berkhoffii* DNA was localized in the lesions by fluorescent *in situ*

hybridization (FISH) using the eubacterial probe EB 16S (FAM 5'-CCACTGCTGCCTCCCGTAGGAGTC- 3') and *B. vinsonii* subsp. *berkhoffii* specific probe Bvb490 (TYE563 5'-TKWAWKGGKRGTTTKYTTATYTAAGAGTTTTCCGGG- 3'). FISH was conducted using the protocols previously described, except that the hybridization was conducted at 56 ^{0}C.[38] No hybridization was detected using *B. henselae*-specific probe Bh 371 (FAM 5'-GTCCACCGTGGGCTTTGAAAAACGCT- 3').

Discussion

Bacillary angiomatosis, which has been described only in humans, was first identified in 1983 in a person with acquired immunodeficiency syndrome (AIDS).[39] Other reports followed, also in AIDS patients, suggesting that immunodeficiency might be a necessary requirement for the development of these lesions.[36] At that time, the infectious nature of the disease was not recognized and it was termed epithelioid angiomatosis, reflecting the vasoproliferative nature of the lesions.[36] The demonstration of silver-positive bacteria in large numbers within the lesions raised the possibility of an underlying infectious aetiology, [39] but the highly fastidious organisms could not be cultured by routine methods.[40] Progress was not made until the advent of molecular techniques,[41] whereby the use of PCR to amplify the bacterial DNA and subsequent sequencing showed that the bacteria in bacilliary angiomatosis lesions most closely resembled the agent of cat scratch fever, then known as Rochalimaea. In 1993, the genera of *Rochalimaea* and *Bartonella* were united.[42] It was recognized early on that BA was not restricted to the skin.[36] Peliosis hepatis is the hepatic form, but lesions may affect other body systems.[37]

The histological lesions in the skin samples from this dog are identical to those of human BA[35,39] with vascular proliferative pathognomonic lesions in conjunction with the presence of argyrophilic bacilli. It is typical of lesions of human BA to contain very large numbers of bacteria, as seen in this dog. In immunocompromised patients, the bacteria are largely extracellular but often grow in close association with the endothelial cells as demonstrated here. This likely is not fortuitous as it has been shown that endothelial cells

stimulated with vascular endothelial growth factor (VEGF) promote the growth of *B. henselae*.[43]

In none of the cases of putative *Bartonella*-induced dermatitis or panniculitis in dogs, published to date, has the organism been demonstrated in the skin lesions by histological, bacteriological or molecular techniques.[30,31] The demonstration of *B. vinsonii* subsp. *berkhoffii* in the blood and the lesion of this dog by PCR and FISH, the pathognomonic microscopic lesions and the presence of large numbers of silver-positive bacilli in the lesions strongly suggest that the bacterium responsible is *B. vinsonii* subsp. *berkhoffii*. Although seemingly unlikely based upon the PCR and FISH results, it cannot be absolutely ruled out, however, that another species of *Bartonella*, such as *B. henselae* or *B. quintana*, was responsible for the angioproliferative lesions in the skin of this dog. Co-infection by multiple tick-borne pathogens, including co-infection with *B. vinsonii* subsp. *berkhoffii* and *B. henselae* is documented in dogs.[44] It is perhaps of interest, in this context, that in vitro infection of human umbilical vein endothelial cells (HUVECs) with other *B. vinsonii* subspecies, although probably different strains or genotypes than detected in this dog, did not result in the protection of the endothelial cells from apoptosis. The *Bartonella spp*. that cause angioproliferative lesions in humans, namely *B. henselae, B. quintana* and *B. bacilliformis*, were able to do so.[45]

Bartonella–Alphaproteobacteria growth medium is a novel, chemically modified insect-based liquid culture medium that supports the growth of at least seven *Bartonella* species.[18] BAPGM culture of the blood from this dog failed to grow any organisms. This can occur due to the low number of the circulating bacteria in the blood or due to the lack of viable bacteria in the cultured blood sample. As this dog was receiving immunosuppressive drug therapy, which generally enhances isolation of *Bartonella spp*. from samples, concurrent administration of clindamycin was the most likely reason for the lack of bacterial growth. *Bartonella vinsonii* subsp. *berkhoffii* genotype I DNA was independently amplified and sequenced from both blood and the paraffin-embedded tissue by PCR. *Bartonella vinsonii* subsp. *berkhoffii* DNA could also be demonstrated in the paraffin-embedded tissue

by FISH using specific probes. Importantly, FISH was negative using the *B. henselae*-specific probe. This, along with the PCR detection of *B. vinsonii* subspecies *berkhoffii* genotype I from both the blood and tissues strongly suggest that *B. vinsonii* subsp. *berkhoffii* was the causative agent for the BA lesions in this dog.

The immunosuppressive treatment for the pancytopenia presumably paved the way for the development of the skin lesions in this dog. Angioproliferative lesions in *Bartonella* infection have been described only in humans. Immunosuppressed individuals, such as AIDs patients[36] and transplantation patients, are predisposed, although BA does occur infrequently in competent individuals. While this genus is very well adapted to interfere with the host immunity, both innate and specific [46,47] it would appear that additional immunosuppression is required for the bacteria to promote angiogenesis. In the immunosuppressed individual, the balance is tipped even further in favour of the bacterial pathogen, thus allowing the bacterium to effectively 'build its own shelter' by causing proliferation and protection from death of the very cells it infects. These cells, in turn, promote the growth of the organisms.

Bartonellae are unique in the bacterial kingdom in their ability to promote host angiogenesis.[48] To date, bacterially induced angiogenesis has been attributed to only three species; namely *B. henselae*, *B. bacilliformis* and *B. quintana*.[49] The pathogenesis of the distinctive and peculiar vascular proliferation in the lesions of BA is complex. In vitro studies using HUVECs infected with these three species of *Bartonella*, have identified several important and likely collaborative mechanisms.[49] These include direct mitotic stimulation of endothelial cells, induction of endothelial cell migration (both essential to angiogenesis) and inhibition of apoptosis, which also allows the bacteria to maintain the longevity of their host cell.[45,48] In addition, the intracellular infection of macrophages induces a paracrine loop of endothelial proliferation as a result of secretion of VEGF and interleukin-1β by the activated macrophages.[43] Lesions of BA in humans exhibit high levels of VEGF.[43]

These angioproliferative lesions are very different, histologically, from any previously recognized cutaneous manifestation of bartonellosis in dogs.[30,31] The few reports of skin disease in dogs describe the lesions as granulomatous or pyogranulomatous.[30] Details

are, however, sparse. In one report of three dogs presenting with subcutaneous nodules, the diagnosis of pyogranulomatous inflammation was based solely on cytology in two dogs.[30] In the third dog, the cutaneous lesions were listed as pyogranulomatous and lymphoplasmacytic in type, but no detailed description of the lesions was given. In the only report of putative *Bartonella*-induced panniculitis in which there is a more detailed description of the histopathologic lesions, nodules of inflammatory cells in the panniculus, were composed of macrophages, neutrophils and occasional giant cells with fibrosis. Histologically these lesions could not be differentiated from other forms of panniculitis in dogs. In that particular dog, it is also possible that the lesions were solely or partially caused by the demonstrated concurrent alpha-1 proteinase inhibitor deficiency.[30,31] The equivalent enzyme deficiency in humans causes lesions of panniculitis in a small proportion of patients, and it is possible that the same may occur in the dog.

In humans, BA is highly responsive to antibiotic therapy. In this dog, the cutaneous nodules disappeared rapidly and completely following treatment with azithromycin. The choice of antibiotics in human patients is based on clinical observations rather than on prospective studies.[3] A variety of antibiotics, including azithromycin, have been reported as efficacious. However, somewhat surprisingly, the efficacy of erythromycin may be due to the demonstrable (in vitro) anti-angiogenic effect of this antibiotic.[50]

Although it is most likely that the immunosuppressive therapy administered for pancytopenia allowed an existing, or subsequently acquired *Bartonella* infection to be expressed, the question arises as to whether the *Bartonella* infection could have been responsible for some or all of the initial haematological abnormalities in this dog. Thrombocytopenia is the most common haematological abnormality in humans and dogs suffering from Bartonellosis. [3,21] Immune-mediated haemolytic anaemia and less commonly, non-regenerative anaemia have been reported in dogs seroreactive to *B. vinsonii* subsp. *berkhoffii*. [21] Although not previously reported, neutropenia has been noted in chronic *B. henselae* infection in humans and in some dogs (E. Breitschwerdt, personal communication).

Pancytopenia is associated with *E. canis* infection and as discussed above, co-

infection of dogs with *E. canis* and *Bartonella spp*. is more frequent than infection with *Bartonella spp.* alone (3.6% versus 36%).22 Of most interest are the implications of the recent discovery that *B. henselae* is capable of infecting CD34+ progenitor cells in the bone marrow.[12] The haematologic pattern in the bone marrow of this dog is inconsistent with bone marrow suppression but suggests ongoing peripheral destruction of mature circulating cells (erythrocytes, neutrophils and thrombocytes), most likely by the spleen. A hyperplastic marrow, in conjunction with pancytopenia, suggests hypersplenism. However, infection of bone marrow progenitor cells could also contribute to the release and dissemination of *Bartonella*-infected cells into the systemic circulation, resulting in immunological recognition of cellular pathology and selective removal of infected cells by the spleen (hypersplenism). The presence of bone marrow erythrophagocytosis would also support activation of this protective mechanism in the bone marrow. Even more recently, *B. henselae* has been shown to infect endothelial progenitor cells in the bone marrow.[51] Bone marrow progenitor cell infection could promote persistent infection of mature endothelia which might contribute to a subtle loss of endothelial integrity with accompanied platelet utilization, overt leukocytoclastic vasculitis, as reported in association with *B. henselae* infection in humans, or BA as reported in this dog.

Finally, the fact that the immunosuppressive therapy alone failed to reverse the anaemia completely, whereas there was complete resolution once azithromycin treatment was instigated, raises the intriguing possibility that pancytopenia and hypersplenism might be attributed to *Bartonella* infection. Interestingly, prior to immunosuppressive therapy, this dog was severely lymphopenic. Although the stress of illness may have contributed to the lymphopenia, two previous studies have documented a progressive CD8 lymphopenia in dogs experimentally infected with *B. vinsonii* subsp. *berkhoffii.*[46] Although thrombocytosis can be induced by hypercortisolism, the thrombocytosis in this dog was remarkable after the initiation of immunosuppressive drugs. Previously, thrombocytosis has been reported in dogs and humans infected with *B. henselae* [52] therefore, the dramatic increase in platelet numbers may have been related to the concurrent use of prednisone in a *Bartonella*-infected dog.

This case report draws attention to this rare, but highly characteristic form of *Bartonella* infection, affecting the skin and subcutis. Given the emerging nature of *Bartonella* infections, possibly accelerated by climate change expanding vector range, and the increasing use of immunosuppression in the treatment of canine ailments, this manifestation of canine bartonellosis may be more common than currently recognized. Misdiagnosis of the lesions as vascular neoplasms, the original fate of this condition when first recognized in humans, would preclude the instigation of curative antibiotic therapy.

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Table 1. Sequential haematology before and after diagnosis of bacillary angiomatosis

TEST and Reference Range	DATE OF TEST						
	01/01/08	07/01/08	23/01/08	14/02/08	27/03/08	22/04/08[1]	27/05/08[2]
Hemoglobin g/dL 12.5-20.3	7.5 **L**	3.8 **L**	9.7 **L**	10.6 **L**	10.5 **L**	10.4 **L**	14.3
Hematocrit % 36-60	22.1 **L**	11.3 **L**	28.7 **L**	30.7 **L**	32.2 **L**	31.4 **L**	41.8
RBC 4.8-9.3 x 10^6/mL	2.9 **L**	1.5 **L**	3.8 **L**	4.0 **L**	4.1 **L**	4.1 **L**	5.8
WBC 4 – 15.5 x10^3/mL	1.6 **L**	0.8 **L**	24.0 **H**	12.0	12.8	8.1	5.3
MCV 58-79 fl	74	73	75	76	80	77	72
MCH 19-28 pg	25.3	24	25.3	26.2	25.9	25.4	24.7
MCHC 30-38 g/dL	33.9	24.7 **L**	33.8	34.5	32.6	33.1	34.2
Reticulocytes 0-1 %	0.7	0.8	0.1	3.5	NR	NR	NR
Absolute <60,000	20,790	12,320	3840	141,400	NR	NR	NR
Corrected 0-1%	0.3	0.2	0.1	2.2	NR	NR	NR
Polychromasia	MODERATE	slight	NR*	NR	NR	NR	NR
Ansisocytosis	MODERATE	NR	NR	NR	NR	NR	NR
Serum Protein 5.0-7.4g/dL	4.0 **L**	NR	6.0	5.4	6.0	6.1	6.6

Table 1 continued

TEST and Reference Range	DATE OF TEST						
	01/01/08	07/01/08	23/01/08	14/02/08	27/03/08	22/04/08[1]	27/05/08[2]
DIFFERENTIAL COUNT							
PMN Absolute & %	1440 **L** (90%)	672 **L** (84%)	20640 **H** (86%)	10440 (87%)	12032 **H** (94%)	8019 (99%)	4717 (89%)
2060 -10600 x10^3/mL							
B**ands Absolute & %**	32 (2%)	0 (0%)	0 (0%)	0 (0)%	0 (0%)	0 (0%)	0 (0%)
0-300 x10^3/mL							
Lymphocytes Absolute &%	96 **L** (6%)	72 **L** (9%)	1680 **H** (7%)	240 **L** (2%)	384 **L** (3%)	81 **L** (1%)	212 **L** (4%)
690-4500 x10^3/mL	16 (1%)	16 (1%)	1680 **H** (7%)	1320 **H** (11%)	384 (3%)	0 (0%)	318 (6%)
Monocytes Absolute &%							
0-840 x10^3/mL	16 (1%)	40 (5%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)
Eosinophils Absolute &%							
0-1200 x10^3/mL	0 (0%)	0 (0%)	0(0%)	0 (0%)	0 (0%)	0 (0%)	53 (1%)
Basophils Absolute & %							
0-150 x10^3/mL							
PLATELET Count/ Estimate	8 **L** Adequate	35 **L** Adequate	1773 **H** High	1253 **H** High	668 **H** High	393 Adequate	434 **H** High
(170-400 x x10^3/mL)							
Giant platelets/ other	moderate	moderate	clumping				

1. Patient, under treatment for pancytopenia of undetermined cause, presented with cutaneous masses. 2. After treatment for BA. NR = not reported. L = Low H= High

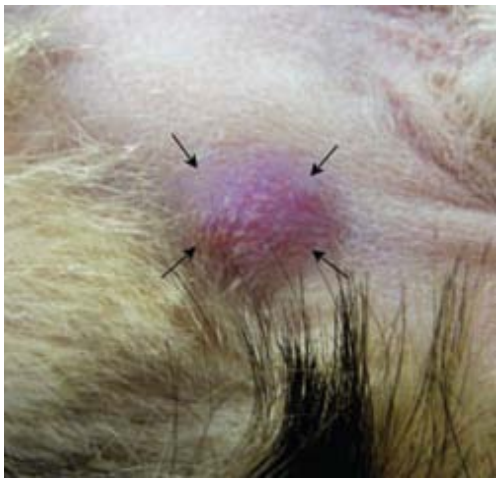

Figure 1. Erythematous nodular lesion on the thigh (arrows)

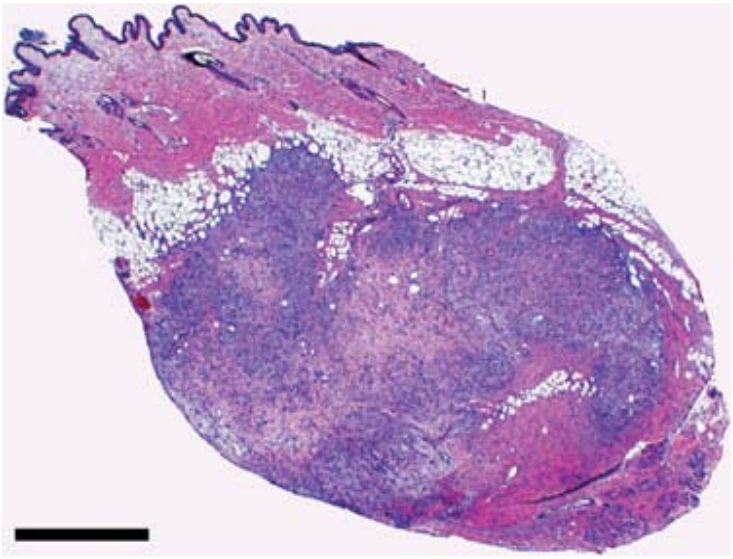

Figure 2. Low-power photomicrograph of haired skin adjacent to footpad, showing the nodular appearance of the lesion in the subcutaneous fatty tissues of the footpad area. H&E. Bar = 1 mm.

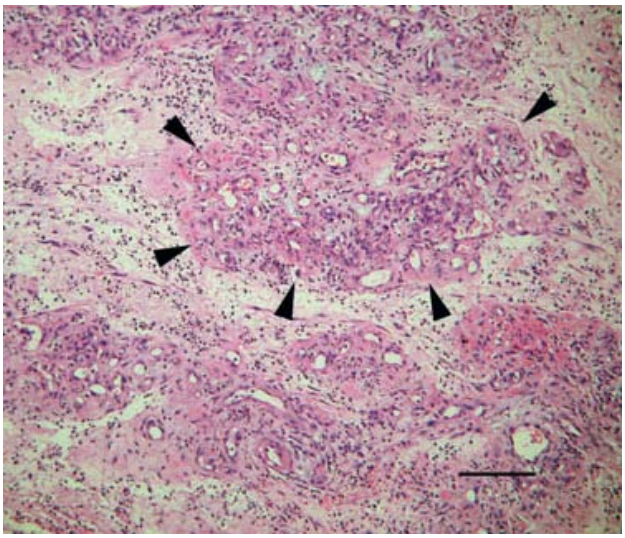

Figure 3. The nodules are composed of groups of proliferating capillaries (outlined by arrowheads) in an oedematous and inflamed connective tissue stroma. H&E. Bar = 200 μm.

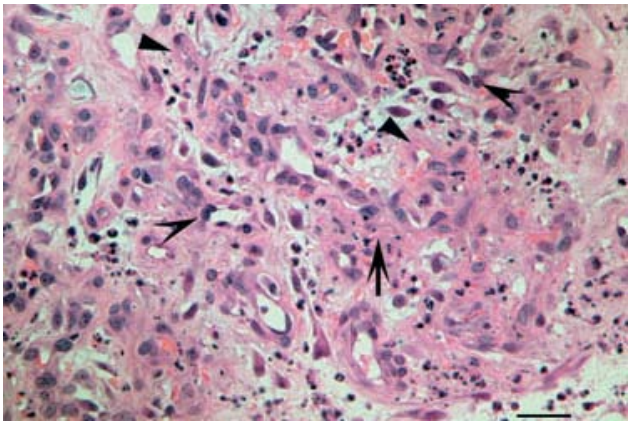

Figure 4. The capillaries are lined by plump endothelial cells (divided arrowheads). Some vessels show fibrinoid degeneration (black arrowheads). The intervening oedematous stroma contains pink staining strands of fibrin and degenerating neutrophils (black arrow) and macrophages. H&E. Bar = 30 μm.

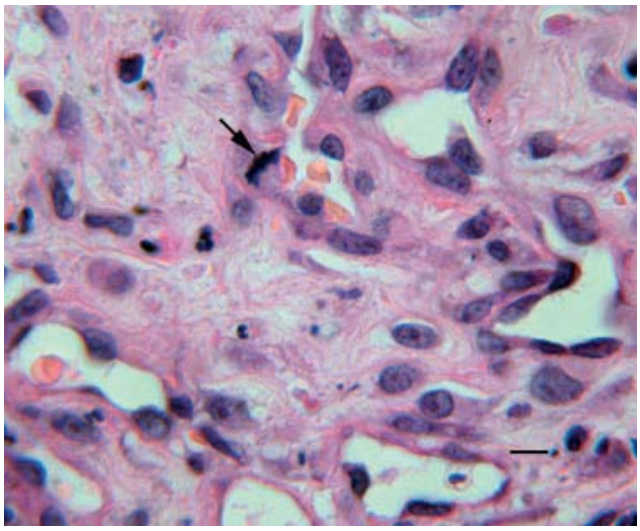

Figure 5. The endothelial cells (EC) protrude into the vessel lumen. Mitotic figures are present (arrow). Note the basophilic stippling of the ground substance. H&E. Bar = 10 μm

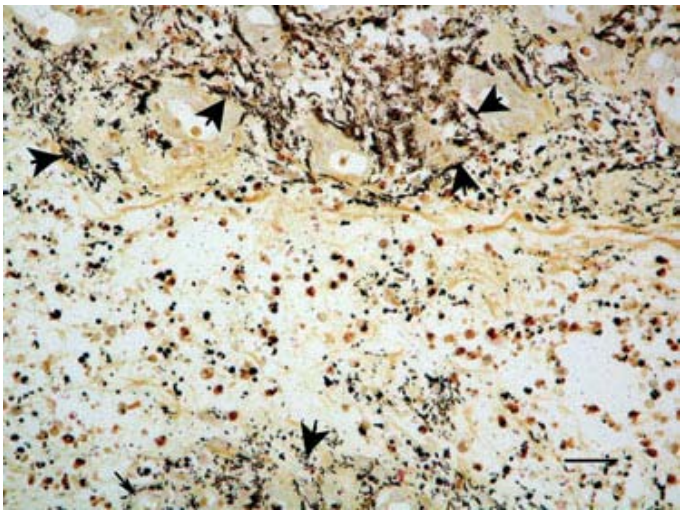

Figure 6. Myriads of positively stained bacilli are present within the lesion, more concentrated in areas of capillary proliferation Warthin– Starry. Bar = 30 μm.

Part B

Isolation of *Bartonella vinsonii* subsp. *berkhoffii* from vascular tumors of humans and dogs

Manuscript as published in Journal of Clinical microbiology:

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(Varanat M, was involved in PCR testing of the biopsy tissues and in generation of the manuscript).

Isolation of *Bartonella vinsonii* subsp. *berkhoffii* genotype II from a dog with hemangiopericytoma and from a boy with epitheloid hemangioendothelioma

Running Title: Vasoproliferative neoplasia and Bartonella infection

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Abstract:

In this report we describe isolation of *Bartonella vinsonii* subsp *berkhoffii* genotype II from a dog with hemangiopericytoma and a boy with epitheloid hemangioendothelioma. These results suggest that B. *vinsonii* subsp *berkhoffii* may cause vasoproliferative lesions in both human beings and dogs.

Keywords: Vascular tumors, VEGF, infection, cancer,

Case reports

Case 1. A 13-year-old male presented with acute onset of severe right upper quadrant pain and hepatomegaly. The patient had a several month history of malaise and fatigue, which was attributed to mononucleosis (positive monospot test), but the boy was otherwise previously healthy. A computed tomography scan identified large heterogeneously enhancing tumors throughout the liver. The patient had a mild microcytic anemia (hemoglobin level, 12.5 g/dl; mean corpuscular volume, 71.1 fl) with very low serum iron (23 μg/dl), normal iron binding capacity (292 μg/dl), and low percent iron saturation (7.9%). The patient's level of C-reactive protein was elevated at 18 mg/liter. Liver function test results were normal except for a mildly elevated gamma-glutamyl transpeptidase level of 76 IU/liter (normal levels, 11 to 63 IU/liter). A biopsy specimen of the tumor was obtained laparoscopically. Histological analysis yielded a diagnosis of epithelioid hemangioendothelioma (EHE) with tumor cells staining strongly for endothelial markers CD31 and CD34. Subsequently and at different diagnostic time points, multiple venous blood samples, drawn following sterile skin preparation, were sent to the Intracellular Pathogens Research Laboratory of North Carolina State University (NCSU-IPRL) for culture in *Bartonella* alpha Proteobacteria growth medium (BAPGM) and 16S-23S intergenic spacer (internal transcribed spacer [ITS]) PCR as previously described (3, 4, 9). In addition, scrolls cut from the formalin-preserved, paraffin-embedded block of the EHE tumor were tested for *Bartonella* sp. DNA using 16S-23S ITS PCR. *Bartonella vinsonii* subsp. *berkhoffii* genotype II was initially amplified and sequenced directly from a venous blood sample (Table 1). Subsequently, *B. vinsonii* subsp. *berkhoffii* genotype II DNA was amplified and sequenced from the tissue block; this was done again for a third time from a second BAPGM enrichment blood culture. Four distinct *B. vinsonii* subsp. *berkhoffii* genotypes have been characterized based upon defined insertion and deletion sequences within the ITS region (5, 21). Only genotype II DNA was sequenced from the blood or tissue samples from this boy. After consultation with infectious disease physicians, antibiotic treatment was instituted. The antibiotic treatment consisted of triple drug therapy for 12 weeks. Oral doxycycline and rifampin were given for 12 weeks. For the

initial 2 weeks, intravenous gentamicin was given, which was replaced by oral azithromycin for the remaining 10 weeks. This regimen was chosen to achieve sufficient intracellular concentrations of rifampin and azithromycin and serum concentrations of doxycycline and to invoke the putative bactericidal properties of aminoglycosides for the treatment of *Bartonella* infection. Constitutional symptoms improved during the period of treatment, and improvement has continued during the subsequent year. The patient grew 5 cm and gained 7.7 kg. The patient reports rare, brief episodes of right upper quadrant pain but otherwise remains asymptomatic. Serial computed tomography scans during the year following treatment have shown no change in the size or number of tumors. The patient remains mildly anemic (hemoglobin level, 12.7 g/dl). However, the C-reactive protein concentrations (8 mg/liter) and gamma-glutamyl transpeptidase (58 IU/dl) dropped to near normal and normal values, respectively; other liver function test values have remained in the normal range. For over 1 year following antibiotic administration, *B. vinsonii* subsp. *berkhoffii* was not isolated and *Bartonella* sp. DNA was not amplified from five blood culture samples (direct extraction, BAPGM enrichment liquid culture, and agar plate subcultures all negative) (Table 1). There has also been a progressive decrease in seroreactivity to *Bartonella henselae*, *B. vinsonii* subsp. *berkhoffii* genotype I, and *B. vinsonii* subsp. *berkhoffii* genotype II antigens, eventually achieving undetectable levels (Table 1).

Case 2. A 12-year-old female English sheepdog was referred to the North Carolina State University Oncology Service for evaluation of a recurrent right antebrachial mass. Three years earlier, a similar, large mass had been surgically resected by the referring veterinarian from the same anatomic site. One year later, a smaller, approximately 4.0- to 6.0-cm mass was removed after recurrence at the surgical excision site. Although no lameness was reported, the dog would frequently lick the masses, presumably due to pain or local irritation. Historically, the dog had otherwise been healthy. At the time of referral, there were three distinct right foreleg masses measuring 0.5 by 0.3 by 1.5 cm on the medial surface of the right antebrachium, 3.0 by 3.0 by 1.5 cm on the cranial right antebrachium, and 4.0 by 4.5 by

2.0 cm on the lateral right antebrachium. The masses were discrete, nontender, fixed, and located between 2.0 and 5.0 cm distal to the olecranon. There was also a firm 2.0 by 2.0 by 1.0 cm mass on the medial aspect of the right rear leg. Complete blood count and serum biochemical profile values were normal. The dog was mildly proteinuric (urine specific gravity, 1.030; 2+ urine protein; negative urine sediment). Aspiration cytology of the right antebrachial and right medial thigh masses identified sheets or clusters of fusiform cells with a high nuclear to cytoplasmic ratio, basophilic cytoplasm, and prominent nucleoli, consistent with a mesenchymal neoplasm. On thoracic radiographs, there was no evidence of pulmonary metastases. Core biopsy specimens were obtained from the antebrachial and medial thigh masses. Histopathology of the masses identified densely packed and interweaving streams and fingerprint whorls of plump, elongated spindle-shaped cells separated by a scant fibrillar stroma, which contained numerous small blood vessels. Neoplastic cells occasionally whorled around small blood vessels. The cells contained scant to moderate amounts of amphophilic, weakly fibrillar cytoplasm, oval nuclei with finely stippled chromatin, one to two nucleoli per cell, and infrequent mitoses. There was mild anisokaryosis and anisocytosis and rare multinucleated cells. The location, morphology, and recurrent nature of these tumors were most consistent with hemangiopericytoma (HPC).

The dog was referred to our teaching hospital in 1995, 2 years after the NCSU-IPRL had made the first isolate of *B.vinsonii* subsp. *berkhoffii* (ATCC type strain 93-CO-1) genotype I from a dog with endocarditis (1). Using *B. vinsonii* subsp. *berkhoffii* genotype I as the antigen source, indirect immunofluorescent- antibody titers (n = 4) were consistently 1:256 over the next 3 months, and a *Bartonella* sp. was isolated by lysis centrifugation blood culture. Retrospectively, the strain was determined to be *B. vinsonii* subsp. *berkhoffii* genotype II based on sequencing the 16S-23S intergenic spacer region in 2008, using a previously described approach (21).

Three months after the initial oncology consultation, the owners elected surgery. Mild basophilia (200 cells/µl; normal value, less than 100 basophils/µl) was the only hematological abnormality, and there were no serum biochemical abnormalities. The medial

thigh mass was totally resected, and the three antebrachial masses were partially resected. The owner declined radiation therapy for the antebrachial mass. The dog was treated with enrofloxacin for 6 weeks at which time the *B. vinsonii* subsp. *berkhoffii* antibody titer was 1:128 and a lysis centrifugation blood culture was negative. Also in 2008, *B. vinsonii* subsp. *berkhoffii* genotype II DNA was amplified and sequenced from the paraffin-embedded biopsy specimen of the antebrachial HPC; the block had been stored for 13 years.

Discussion

In this report, infection with *B. vinsonii* subsp. *berkhoffii* genotype II is described in a boy with EHE and a dog with HPC. Neoplastic recurrence at the same anatomic location over a 3-year period, in conjunction with the temporal association of *B. henselae* and *Bartonella quintana* with vasoproliferative lesions in human immunodeficiency virus-infected humans (25, 26), initiated our efforts to determine whether the dog was infected with a *Bartonella* sp. The boy was cultured after a literature search by an attending physician revealed a well-established association between *Bartonella* sp. infection, vascular endothelial growth factor (VEGF) induction and angioproliferative disease (6, 7, 13, 27). An association of increased tissue VEGF levels with the formation of hemangioendotheliomas lent credence to the hypothesis of a potential causal association between *Bartonella* infection and hemangioendothelioma (18, 30).

Dogs and humans infected with *Bartonella* spp. can develop similar disease manifestations and pathological lesions, including prototypical vasoproliferative lesions, such as bacillary angiomatosis and peliosis hepatis (2, 6). In humans, *Bartonella henselae* and *Bartonella quintana* cause cutaneous vasoproliferative lesions (bacillary angiomatosis) and parenchymal vasoproliferative lesions of the liver, spleen (bacillary peliosis), and less frequently other tissues, particularly in human immunodeficiency virus-infected patients (25, 26). The NCSU-IPRL recently cultured *B. vinsonii* subsp. *berkhoffii* genotype I from a dog with bacillary angiomatosis (J. Yager, E. B. Breitschwerdt, et al., unpublished data), and *B. henselae* DNA had previously been amplified from the liver of a dog with peliosis hepatis

(14). Long-lasting intravascular infection with *B. henselae* (for months to years) has been documented in naturally and experimentally infected cats (16, 17), and persistent intravascular bacteremia for 18 months with *B. vinsonii* subsp. *berkhoffii* genotype II was reported in a naturally infected healthy pet dog (15). More recently, persistent infection with *B. vinsonii* subsp. *berkhoffii* and *B. henselae*, as well as coinfection with both organisms, has been reported in immunocompetent people with substantial arthropod and animal contact (3, 4). The seemingly unique capability of bacteria of the genus *Bartonella* to invade and induce long-lasting intraerythrocytic and intraendothelial infections, in conjunction with the ability of at least three *Bartonella* spp. (*B. henselae*, *B. quintana*, and *Bartonella bacilliformis*) to induce VEGF-mediated vasoproliferative disease in immunocompromised or immunocompetent individuals suggests that these novel emerging bacterial pathogens might contribute to the development of other vascular tumors (7, 8, 12).

Bartonella vinsonii subsp. *berkhoffii* genotype I was isolated for the first time from a dog with epistaxis, recent-onset seizures, and endocarditis in 1993 (1). Subsequently, three additional genotypes (designated II to IV), all of which have been implicated as a cause of endocarditis in dogs, were described based upon sequence differences in the *Bartonella* 16S-23S intergenic spacer region and the Pap31 gene (5, 21). Genotypes I, II, and III have also been implicated in humans with vascular infections (3, 4, 29). Although seemingly well-adapted on an evolutionary basis to induce persistent infection in canine reservoir hosts (dogs, foxes, and coyotes), *B. vinsonii* subsp. *berkhoffii* has only rarely been isolated from pet dogs (2, 6). In pet dogs, both seroprevalence studies and blood culture isolation studies indicate infrequent exposure to or active infection with any of the four *B. vinsonii* subsp. *berkhoffii* genotypes, whereas infection is more frequent in coyotes and feral dog populations (6, 21). Although a source of infection was not determined in either case, the dog in this case report was from a rural area and had experienced recurrent ectoparasite exposures. The boy had a tick bite roughly 1 year prior to the diagnosis of EHE, spent summers in heavily wooded areas, and lived in a suburb where coyote sightings are common. Coyotes are likely a reservoir host for *B. vinsonii* subsp. *berkhoffii* genotype II, and tick transmission of this

subspecies has been proposed on the basis of epidemiological evidence (2, 6). In regard to human infections, there is one case of endocarditis and eight cases in which *B. vinsonii* subsp. *berkhoffii* was isolated or sequenced from blood cultures obtained from immunocompetent people with arthritis, fatigue, or neurological or neurocognitive abnormalities (3, 4, 29). Four of these eight individuals infected with *B. vinsonii* subsp. *berkhoffii* were coinfected with *B. henselae* and *B. vinsonii* subsp. *berkhoffii*, of which genotype II was sequenced from all but one person, who was infected with genotype I. We were unable to detect DNA evidence of *B. henselae* infection using species-specific primers in samples from the boy or the dog. Therefore, isolation of the same bacterial genotype from vascular tumors occurring in a dog and a human suggests that *B. vinsonii* subsp. *berkhoffii* may play a role in the development of some vasoproliferative tumors. Over a decade separated the efforts to isolate *B. vinsonii* subsp. *berkhoffii* from the dog and the human patient in this study. In the intervening period, there were important advances in the microbiological isolation and molecular detection of *Bartonella* spp. in blood and tissue samples from immunocompetent individuals, which continue to facilitate a redefinition of the pathogenic role of this genus in animals and humans (3, 4, 9).

In all mammals, including dogs and humans, endothelial cells appear to be an important target cell following direct or vector-borne transmission of a *Bartonella* sp. (6–8). Based upon in vitro infection of human endothelial cell lines, *B. henselae* has been shown to induce angiogenesis and endothelial cell proliferation (20). *Bartonella* spp. also subvert many functions of human endothelial cells, including the induction of mitogenic and proinflammatory genes, cytoskeletal rearrangements, and suppression of endothelial cell apoptosis (7, 8). Clinically, the resulting vascular proliferation induces tumor- like lesions (verruga peruana, bacillary angiomatosis, and peliosis hepatis), especially in immunocompromised individuals (2, 6, 7). VEGF is an important mediator of tumor angiogenesis, and its production has been specifically induced by *B. henselae* in vitro (13, 23). In addition, increased VEGF levels were found in tissues from patients with bacillary angiomatosis and peliosis hepatis (23). Recently, it has been shown that infection of human

endothelial cells by *B. henselae* resulted in interleukin-8 (IL-8) production and upregulation of IL-8 receptors CXCR2 (23). IL-8 promotes angiogenesis through enhanced endothelial cell survival and enhanced vascular proliferation (13, 19, 23). The results of this study suggest that *B. vinsonii* subsp. *berkhoffii*, a *Bartonella* species that appears to have coevolved with canines (2, 6), may also contribute to vasoproliferative lesions in dogs and human beings, potentially by enhancing cell proliferation in conjunction with inhibition of apoptosis. Whether *Bartonella*-triggered vasoproliferation is a pathogenic strategy used by these bacteria to expand a specific host cell habitat (the endothelial cell) is currently unknown (12, 24).

HPC is a vascular neoplasm thought to originate from pericytes, capillary subendothelial lining cells predominantly found in distal extremity vessels (11, 22). As described in this report, these tumors in dogs are generally subcutaneous and tend to involve the limbs (11, 22). HPCs are most often found in adult dogs, they do not occur more often in male or female dogs, local recurrence is common, and metastasis is rare (11, 22). EHE is a rare vascular neoplasm having a malignant potential between benign hemangioma and angiosarcoma. First described by Weiss and Enzinger in 1982 (30), EHE is characterized by positive immunostains for endothelial antigens CD34 and factor VIII. EHE typically presents with various combinations of hepatic, pulmonary, cutaneous, or bony disease. The natural history is extremely unpredictable, and there is substantial interpatient variability in disease progression. Intervals of rapid growth are frequently interrupted by long periods of quiescence. EHE is generally unresponsive to standard cytotoxic chemotherapy and radiation therapy. Despite anecdotal reports of success with antiangiogenics, there have been no clinical trials of this approach. Surgery is considered the treatment of choice in hepatic disease, where options include resection for localized disease or transplant for the more common presentation of multilobar, multicentric liver involvement, as would be the case for the boy in this report. In two large studies, there was a high percentage of long-term survivors following liver transplant, even in the presence of metastases (18, 28). If the proposed association of *Bartonella* spp. with EHE were confirmed, it is plausible that

eradicating the bacterial infection or interrupting *Bartonella*-induced angiogenic and proliferative cell signals could slow tumor progression and improve patient outcomes.

On a comparative medical basis, dogs and humans infected with *Bartonella* spp. can develop similar disease manifestations, including endocarditis, granulomatous lymphadenitis, granulomatous hepatitis, bacillary angiomatosis, peliosis hepatis, seizures, and arthritis (2, 6). Therefore, medical information generated in one species (dogs or human beings) can prove beneficial while attempting to characterize the role of *Bartonella* species as a pathogen in the comparable species. As is true of many other infectious diseases, a "one medicine" approach to the current clinical and research understanding of canine and human bartonellosis has proven beneficial for the health care of animals and human patients (10, 31). During the past decade, researchers have provided substantial evidence to support a role of infectious agents, including bacteria, viruses, mycoplasmas, and protozoa, as cofactors in the development of cancer in humans. Substantial epidemiologic and microbiological research is needed to test the potential causal relationship of *Bartonella* sp. infection with EHE and HPC and to determine whether members of the genus *Bartonella* will be added to the list of oncogenic infectious agents in the future.

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The dog *B. vinsonii* subsp. *berkhoffii* genotype II isolate reported in this study was obtained by Brandee Pappalardo while a Ph.D. student in the Intracellular Pathogens Research Laboratory at North Carolina State University. We thank Julie Bradley for providing *Bartonella* serological test results, Tonya Lee for editorial assistance, and the many clinicians who facilitated the care of these patients.

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Table 1. Serological, PCR and culture results for a 13-year-old male with a hepatic epitheloid hemangioendothelioma and *Bartonella vinsonii* subsp. *berkhoffii* infection.

	***Bartonella* IFA Reciprocal Titers**			**PCR/BAPGM Culture Results**		
Date/Sample	***B. henselae***	**Bvb *I***	**Bvb *II***	Direct Extraction	Enrichment Culture	Blood Agar Plate Isolate
1/2/2008/Blood	16	64	16	*Bvb* II*	-	-
1/02/2008/Liver Tissue	NA	NA	NA	*Bvb* II*	NA	NA
1/9/2008/Blood	64	16	32	-	*Bvb* II*	-
1/17/2008/Blood	128	32	64	-	-	-
2/14/2008/Blood	32	64	16	-	-	-
4/4/2008/Blood	128	16	64	-	-	-
6/17/2008 Blood	64	<16	32	-	-	-

Bvb=Bartonella vinsonii subsp. *berkhoffii*, *Denotes 16S-23S ITS DNA sequence result that defines genotypes I-IV. NA= Not applicable

Part C

Bartonella vinsonii subsp. *berkhoffii* induces production of vascular endothelial growth factor from HeLa 229 cells *in vitro*

Bartonella vinsonii subsp. *berkhoffii* induces production of vascular endothelial growth factor from HeLa 229 cells *in vitro*

Introduction

Bartonella sp. are emerging vector borne pathogens responsible for a wide spectrum of disease manifestations in humans and animals, including cat scratch disease, Oroya fever, Trench fever, endocarditis, bacillary angiomatosis, peliosis hepatis, musculoskeletal and neurological manifestations (Breitschwerdt *et al*., 2010a). A unique characteristic of *Bartonella* is its ability to induce proliferation of endothelial cells (Dehio, 2005). *B. bacilliformis*, *B. henselae* and *B. quintana* are known to induce vasoproliferative tumors in human beings and animals (Koehler and Tappero, 1993; Ihler, 1996). *B. bacilliformis*, which is a human specific *Bartonella*, causes a biphasic disease in human beings known as Carrion's disease. The acute phase of Carrion's disease is characterized by hemolytic anemia, which could be life-threatening if not treated. In the chronic phase of the disease bacteria colonizes the endothelial cells in the skin, leading to formation of vascular tumors known as verruga peruana (Ihler, 1996). *B. henselae* and *B. quintana* are the causative agents of bacillary angiomatosis (BA) and peliosis hepatis (BP) (Koehler and Tappero, 1993). BA is a vasoproliferative tumor which resembles verruga peruana in histological appearance (Karem *et al*., 2000). Lesions of verruga peruana are limited to skin, whereas BA lesions can occur in skin, subcutis, brain, lymph nodes, respiratory tract, cardiac valves, gastrointestinal tract, oral mucosa, conjunctiva, scrotum, penis, cervix, vulva and bone marrow (Cockerell *et al.*, 1987; Baron *et al.*, 1990; Chan *et al.*, 1991; Slater and Min, 1992; Spach *et al.*, 1992; Lee *et al.*, 1994; Fagan *et al.*, 1995; Eden *et al.*, 1996; Long *et al.*, 1996; López de Blanc *et al.*, 2000).

Recently BA was reported in an immunocompromised dog infected with *B. vinsonii* subsp. *berkhoffii* (Yager *et al*., 2010). *B. vinsonii* subsp. *berkhoffii* genotype I DNA was detected in the blood and in the BA lesions sampled by biopsy. *B. vinsonii* subsp. *berkhoffii* genotype II was isolated from a boy with epithelioid hemangioendothelioma (EHE) and a

dog with hemangiopericytoma (Breitschwerdt *et al*., 2009) (See parts A and B of this chapter). These reports were the first to indicate that *B. vinsonii* subsp. *berkhoffii* may also have vasoproliferative properties, like *B. bacilliformis*, *B. henselae* and *B. quintana*.

Vascular endothelial growth factor (VEGF) plays a critical role in *Bartonella*-induced vasoproliferation (Kempf *et al*., 2001). *Bartonella* can induce production of VEGF from host cells, including macrophages, which then act on the endothelial cells in a paracrine manner (Resto-Ruiz *et al*., 2002). VEGF mediated vasoproliferation is also noted in tumor angiogenesis, where tumor cells and other stromal cells such as pericytes are the major source of VEGF (Carmeliet, 2005). The aim of this study was to determine if *B. vinsonii* subsp. *berkhoffii* can induce VEGF production from mammalian cells *in vitro*.

Materials and Methods

Cell culture and Bacteria

HeLa 229 cells (human cervical carcinoma cells), were maintained in RPMI-1640 medium supplemented with 2g/L NaHCO3 (Biochrom, Berlin, Germany), 10% heat inactivated fetal calf serum (FCS; Sigma Aldrich, Taufkirchen, Germany), 1% L-glutamine (Gibco, Karlsruhe, Germany) and 10mg/mL streptomycin and 100U penicillin (Biochrom, Berlin, Germany). Cells were trypsinized with 0.05% Trypsin-EDTA (Gibco, Karlsruhe, Germany), and after trypsinization, Trypsin-EDTA was neutralized by adding Media containing FCS. The day before infection experiments, 0.5×10^6 cells/well were seeded in a 6 well plate, in cell culture media without antibiotics (to allow bacterial growth). Infection experiments were performed in cell culture media without antibiotics and without FCS to avoid non-specific hypoxia inducible factor-1α (HIF-1α) activation.

B. vinsonii subsp.*berkhoffii* genotypes I, II and III were used for the infection experiments. Bacteria were grown on Columbia Sheep Blood agar (BD, Heidelberg, Germany) at 37^0C, 5% CO_2, 96% humidity. Bacterial colonies were harvested after 3 days and washed with PBS (Gibco Karlsruhe, Germany). Bacteria were quantitated by measuring optical density at 600nm (OD of 1 = ~ 5×10^8 bacteria). Cells were inoculated with bacteria at

MOIs of 250 and 500 for 6 hrs for the Western blot experiments and 24 and 48 hrs for ELISA experiments. Desferrioxamine (DFO, 200µmol/L, Sigma Aldrich) treated cells were used as positive control and uninfected cells were used as negative control.

Detection of HIF-1α activation by immunoblotting

Protein-Extraction

For the detection of HIF-1α activation by immunoblotting, proteins from cell cultures were extracted with protein extraction buffer (7M Urea, 1% SDS, 10% Glycerol, 10mM Tris/HCl (pH 6.8), 1 tablet protease inhibitor^{TM} Cocktail (Roche) and 5mM DTT). Six hours after infection cells were washed with ice cold PBS, 250µl of extraction buffer was added and cells were resuspended with a cell scraper and transferred to an eppendorf tube (all steps were performed on ice). Cells were subsequently lysed and homogenized using an Ultra-Turrax for 5-7 sec (IKA, Staufen, Germany), heated for 10 min at 70°C, and stored at – 80 °C until further use.

SDS-Polyacrylamide-gel electrophoresis and Western-Blotting

Proteins were separated on an 8% SDS-polyacrylamide gel. Before loading the gel, samples were diluted 1:5 with 5X SDS loading buffer. Gels were run at constant amperage of 30mA per gel. Subsequently, proteins were transferred to polyvinylidene difluoride (PVDF) membranes (Millipore, Schwalbach, Germany). Blotting conditions were 72mA per blot, 120 min (Mini Trans-Blot Electrophoretic Transfer Cells, Biorad). After blotting, membranes were incubated shortly in methanol and air-dried for at least 15 min. For reversible visualization of the protein transfer, membranes were incubated for 5 min in 1% Ponceau S-rouge-solution. Staining was removed by a washing step with double distilled H_20 and membranes were incubated with 3% blocking buffer (3% low fat dry milk in washing buffer) for blocking nonspecific binding sites (1 hour, room temperature (RT), on a rocking platform). Afterwards, membranes were incubated with mouse-anti-HIF-1α primary-antibody (BD) (1:1000 in 3% blocking buffer, 4°C, overnight on a rocking platform). Membranes were washed (3 x 5 min) with washing-buffer (10mM Tris/HCl pH 7.4, 0.15M NaCl, 0.1% Tween 20 in ddH20) and than incubated with a HRP-coupled rabbit anti-mouse

IgG secondary antibody (Dako) (1:1000 in 3% blocking buffer, 1 hr, RT on a rocking-platform). Membranes were washed with washing-buffer (3 x 10 min) and HIF-1α induction was visualized with enhanced chemiluminescent (ECL+)-Reagent (PJK, Kleinbittersdorf, Germany) on X-Ray-Films (Sigma-Aldrich, München, Germany), which were developed in a developing machine (Curix 60, Agfa, Germany). The constitutively expressed Actin was used as a loading control. The protocol for the antibody-binding was identical to that of HIF-1 detection, with the exception of the use of 5% blocking buffer instead of 3% blocking-buffer, a higher dilution for the primary antibody and the use of chemiluminescent (ECL)-reagent (PJK). (Primary Antibody: mouse Anti-actin , Sigma-Aldrich, 1:10000 dilution; secondary Antibody: HRP- coupled rabbit anti-mouse IgG, Dako, 1:1000 dilution). Experiment was repeated twice.

Detection of VEGF in cell culture supernatants

Supernatants of infected cells were harvested 24 or 48 hours after infection and transferred to eppendorf tubes, centrifuged in a microcentrifuge at 14,000 rpm for 5 min at 4°C, transferred to fresh tubes and stored at –20°C until further use. VEGF concentration was measured using the human "Quantikine Immunoassay human VEGF ELISA-Kit" (BD), according to the manufacturer's instructions. Experiment was repeated twice.

Results

HeLa 229 cells were infected with *B. vinsonii* subsp. *berkhoffii* genotypes I, II and III at an MOI of 250 and 500 for 24 or 48 hrs. Supernatants were collected and the concentration of VEGF was determined by ELISA. *B. vinsonii* subsp. *berkhoffii* induced production of vascular endothelial growth factor from HeLa 229 cells in a dose dependent manner. All three genotypes tested showed increased VEGF production (Figure 1, Data not shown for *B. vinsonii* subsp. *berkhoffii* genotype III) when compared to the uninfected controls.

Hypoxia inducible factor-1α is a transcription factor, the activation of which leads to production of VEGF from cells. We tested the production of HIF-1α from the HeLa 229 cells

by *B. vinsonii* subsp. *berkhoffii* using Western blotting. Infection with all the three genotypes resulted in the production of HIF-1α from HeLa 229 cells (Figure 2, Data not shown).

Discussion

Bartonella sp. can induce proliferation of endothelial cells both *in vitro* and *in vivo* leading to the formation of vasoproliferative tumors (Dehio, 2005). *B. bacilliformis, B. henselae* and *B. quintana* are well recognized as the three species of *Bartonella* that have vasoproliferative properties. In this study we provide evidence that a fourth species, *B. vinsonii* subsp. *berkhoffii,* should be added to the list of vasoproliferative bartonellae. *B. vinsonii* subsp. *berkhoffii* was first isolated from a dog with endocarditis in 1993 (Kordick *et al*., 1996). Later 3 additional genotypes were isolated from coyotes and dogs (Maggi *et al*., 2006; Cockwill *et al*., 2007; Cadenas *et al*., 2008). *B. vinsonii* subsp. *berkhoffii* has been isolated/detected by PCR from humans, dogs, coyotes and cats (Maggi *et al*., 2006; Varanat *et al*., 2009), and the infection have been associated with a wide spectrum of diseases, including endocarditis, osteomyelitis, bacillary angiomatosis and neurological manifestations (Cockwill *et al*., 2007; Varanat *et al*., 2009; Breitschwerdt *et al*., 2010b; Yager *et al*., 2010).

The first indication that *B. vinsonii* subsp. *berkhoffii* can induce vasoproliferation was provided by the isolation/detection of this bacterium from vasoproliferative tumors like bacillary angiomatosis and epithelioid hemangioendothelioma (Breitschwerdt *et al*., 2009; Yager *et al*., 2010). Molecular mechanism of *Bartonella*-induced vasoproliferation remains unclear, and most of the knowledge gathered is from studies using cell culture models (Maeno *et al*., 1999; Kempf *et al*., 2001). *Bartonella* has direct mitogenic effect on the endothelial cell. This combined with the inhibitory effect on endothelial cell apoptosis, can lead to proliferation of endothelial cells (Kirby and Nekorchuk, 2002; Dehio, 2005).

VEGF is an important growth factor involved in *Bartonella*-induced angiogenesis (Kempf *et al*., 2001). Endothelial cells are poor source of VEGF (Maeno *et al*., 1999). *Bartonella* infection leads to the activation of NF-κB pathway leading to the recruitment of

inflammatory cells, including macrophages (Fuhrmann *et al*., 2001). *Bartonella* can colonize macrophages, leading to activation of HIF-1α (Kempf *et al*., 2005), which is a transcription factor for VEGF. The VEGF produced by macrophages act on the endothelial cells in a paracrine manner leading to endothelial cell proliferation (Resto-Ruiz *et al*., 2002). Increased expression of VEGF is found in verruga peruana, bacillary angiomatosis and peliosis hepatis (Kempf *et al*., 2001; Cerimele *et al*., 2003). In this study we provide evidence that *B. vinsonii* subsp. *berkhoffii*, activates HIF-1α and increase production of VEGF from the cells in vitro, in a similar manner to *B. henselae*. These findings further strengthen the argument that *B. vinsonii* subsp. *berkhoffii* should be added to the list of bartonellae, which are capable of inducing vasoproliferive tumors.

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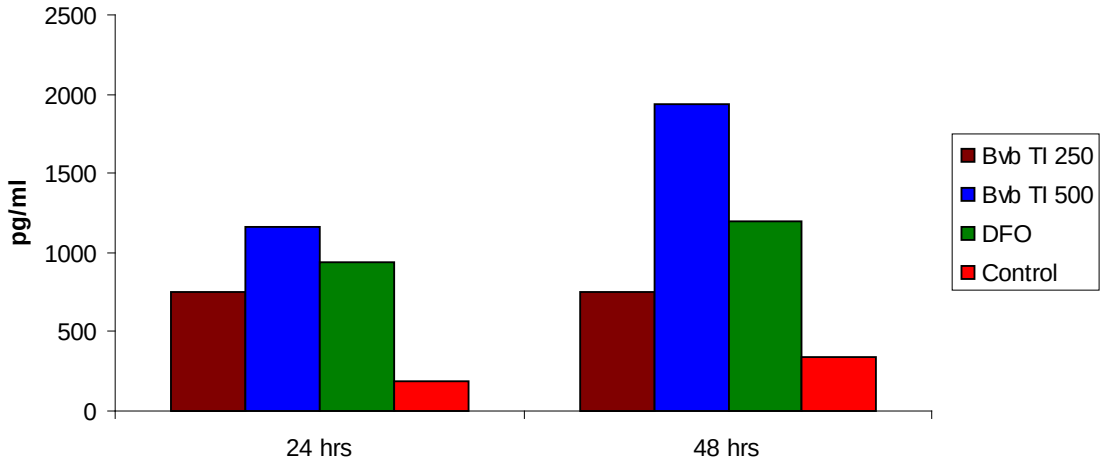
A. *B. vinsonii* subsp. *berkhoffii* genotype I



B. *B. vinsonii* subsp. *berkhoffii* genotype II

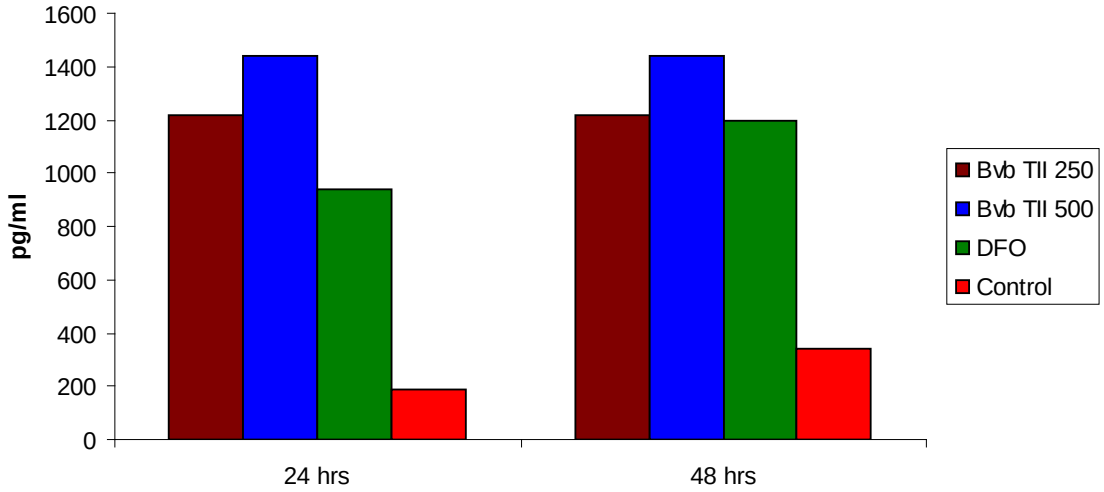


Figure 1. Induction of VEGF by *B. vinsonii* subsp. *berkhoffii*. HeLa 229 cells were infected with *B. vinsonii* subsp. *berkhoffii* for 24 or 48 hrs and concentration of VEGF was measured using ELISA. Bvb- *B. vinsonii* subsp. *berkhoffii*, DFO- disferrioxamine, control- uninfected cells

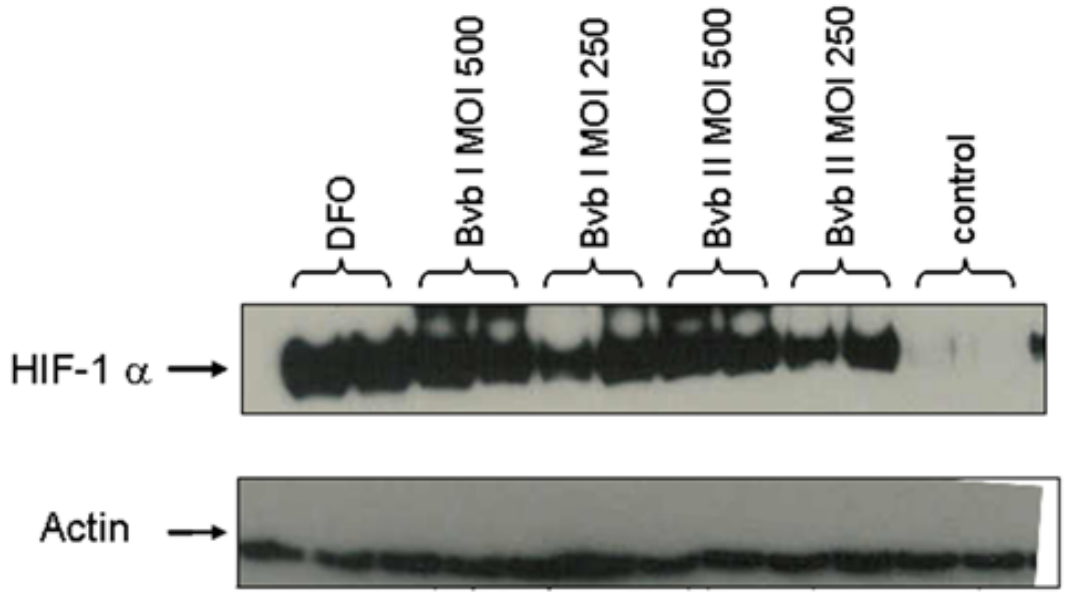


Figure 2. *B. vinsonii* subsp. *berkhoffii* activates hypoxia inducible factor-1 in HeLa 229 cells. Cells were infected with *B. vinsonii* subsp. *berkhoffii* for 6 hrs. Production of HIF-1 was determined by Western blotting. Bvb T I- *B. vinsonii* subsp. *berkhoffii* genotype I, Bvb T II- *B. vinsonii* subsp. *berkhoffii* genotype II, DFO- disferrioxamine, control- uninfected cells. Actin was used as a loading control.

Chapter 4.

Molecular prevalence of *Bartonella, Babesia* and hemotropic *Mycoplasma* sp. in dogs with splenic disease

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Molecular prevalence of *Bartonella*, *Babesia* and hemotropic *Mycoplasma* sp. in dogs with splenic disease

Molecular prevalence of *Bartonella, Babesia* and hemotropic *Mycoplasma* sp. in dogs with splenic disease

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Short title: Blood-borne pathogens in splenic diseases

Keywords: Bartonella, spleen, PCR

Abbreviations: lymphoid nodular hyperplasia (LNH), splenic hemangiosarcoma (HSA), fibrohistiocytic nodules (FHN), and polymerase chain reaction (PCR)

Abstract

Background: Among diseases that cause splenomegaly in dogs, lymphoid nodular hyperplasia (LNH), splenic hemangiosarcoma (HSA), and fibrohistiocytic nodules (FHN) are common diagnoses. The spleen plays an important role in the immunological control or elimination of vector-transmitted, blood borne pathogens, including *Bartonella, Babesia* and hemotropic *Mycoplasma* sp.

Objective: To compare the prevalence of *Bartonella, Babesia* and hemotropic *Mycoplasma* sp. DNA in spleens from dogs with LNH, HSA and FHN.

Materials and Methods: Paraffin embedded, surgically obtained biopsy tissues from LNH (N=50), HSA (N=50), FHN (N=37) were collected from the anatomic pathology archives. Spleens from specific pathogen free (SPF) dogs (N=8) were used as controls. *Bartonella*, *Babesia* and *Mycoplasma* sp. DNA was amplified using PCR, followed by DNA sequencing.

Results: *Bartonella* sp. DNA was more prevalent in FHN (29.7%) and HSA (26%) as compared to LNH (10%) (p=0.019, p=0.0373, respectively) or control spleens (0.0%). The prevalence of *Babesia* and hemotropic *Mycoplasma* DNA was significantly lower than *Bartonella* DNA in HSA (p=0.0005, p=0.006 respectively) and FHN (p=0.003 and p=0.0004 respectively). There was no statistically significant difference in DNA prevalence among the three genera in the LNH group.

Conclusions: The higher prevalence of *Bartonella* sp. in FHN and HSA warrants future investigations to determine if this bacterium plays a role in the development of these splenic diseases.

Introduction

The spleen, a highly vascular organ, plays an important role in the immunological control and elimination of blood borne pathogens.[31] Splenic macrophages phagocytose damaged and senescent erythrocytes, and facilitate elimination of extracellular and intracellular blood borne bacteria, protozoa and viruses through both innate and adaptive immune responses.[31] Splenic marginal zone macrophages express various receptors that

mediate antimicrobial immunity, including pattern recognition receptors (Toll-like receptors),[16] C-type lectin receptors (SIGNR1) and type-I scavenger receptors (MARCO).[28] SIGNR1 recognizes polysaccharide antigens,[22] whereas MARCO recognizes bacteria, including *Escherichia coli* and *Staphylococcus aureus*.[44]

Researchers have generated substantial evidence to support a role for infectious agents, including bacteria, viruses, Mycoplasma and protozoa as a cause or cofactor in the development of cancer in animals and human patients. Currently, the World Health Organization indicates that infectious organisms are responsible for nearly 20% of human cancers.[48] As a bacterial example, *Helicobacter pylori* causes MALT lymphoma, which is an antibiotic reversible "neoplastic" condition.[17,32]

Bartonella sp. establish persistent infection in erythrocytes, endothelial cells and professional macrophages, leading to NF-κB activation, promotion of a pro-inflammatory phenotype, and recruitment of inflammatory cells including neutrophils and macrophages.[10,14] Unique among bacteria, *Bartonella* can induce *in vitro* and *in vivo* proliferation of endothelial cells leading to vasoproliferative lesions in both immunocompromised human beings and dogs.[27,49] Splenic rupture and granulomatous splenitis have also been associated with *Bartonella* infection in people.[9,45]Although unproven, it is possible that splenic tissues provide a permissive environment for the growth and perpetuation of *Bartonella,* resulting in chronic, low-grade inflammation.

Babesia and hemotropic *Mycoplasma* sp. are vector-transmitted blood borne pathogens of dogs, for which the spleen influences disease pathogenesis.[15,23] Clinical presentations of canine babesiosis vary from subclinical to multi-organ failure and death.[20] The spleen is of central importance for the innate and adaptive immune response that controls *Babesia* sp. infection.[7] Infection with hemotropic *Mycoplasma* sp. has been reported in several mammalian species, including dogs and human patients.[15,41] The most commonly reported hemotropic *Mycoplasma* sp. in dogs includes *Mycoplasma hemocanis* and *Mycoplasma hematoparvum*, both of which have been associated with immune-mediated hemolytic anemia.[4] Splenectomy or other forms of immunosuppression also play an

important role in the infectious pathogenesis of hemotropic *Mycoplasma*.[15]

In this study we compared the prevalence of three vector-transmitted, intravascular, blood borne pathogens (*Bartonella* sp., *Babesia* sp. and hemotropic *Mycoplasma* sp.) in dogs with histologically confirmed splenic disease. We chose to study an angiogenic neoplastic condition (HSA), a non-angiogenic neoplastic condition (FHN), and a non-neoplastic splenic inflammatory condition (LNH). These lesions span a pathological spectrum involving endothelial proliferation, lymphoid and fibrohistiocytic proliferation and chronic lymphoid stimulation. Histologically normal splenic tissues from SPF dogs were used as tissue and laboratory processing controls.

Materials and Methods

Sample collection

Using the NCSU-CVM Pathology Data Base, paraffin embedded surgical biopsy samples from three groups of dogs were retrieved from pathology archive storage facilities. Group I included splenic FHN (n=37), group II HSA (n=50), and group III LNH (n=50). Group IV was consisted of histologically unremarkable spleens (n=8) from SPF dogs. For cases with more than one block available, the block containing the largest component of well-preserved mass (approximately 50% or more of the entire section) was chosen. Fresh tissues were prospectively collected from group IV SPF dogs at the time of euthanasia and processed into paraffin blocks during the course of this study. Although not processed at the same time, control samples were processed and paraffin embedded in the histology laboratory using the same equipment and techniques as the samples from the three study groups. All paraffin blocks were coded and processed for PCR in a blinded manner. Archival tissues used in this study were collected between 2004 and 2010. Splenic tissue samples were independently reviewed by a pathologist to confirm the histopathological diagnosis and the FHN grade. Following DNA extraction, all paraffin-embedded splenic tissues were tested by PCR for the presence of *Bartonella*, *Babesia* and hemotropic *Mycoplasma* sp. DNA.

DNA extraction

For each specimen, approximately 25 mg of tissue was manually excised from paraffin- embedded spleen, using a sterile disposable No: 10 scalpel blade. Tissues were processed in small batches and the work surface was thoroughly cleaned between each tissue block to avoid *Bartonella*, *Babesia* or hemotropic *Mycoplasma* sp. DNA carry over between the samples.[46] DNA was extracted using QIAamp DNA blood mini kit (Qiagen, Valencia, CA) following manufacturer's instructions. A blank paraffin block was used as a reagent control with each set of DNA extractions. DNA concentrations and purity were determined using a spectrophotometer (Nanodrop, Wilmington, DE). Extracted DNA was stored at -20^0C.

Polymerase chain reaction

Samples were tested for the presence of *Bartonella*, *Babesia* and hemotropic *Mycoplasma* sp. DNA,[5,21,30] utilizing the primers listed in Table 1. *Bartonella* genus primers targeted the 16S-23S rRNA intergenic transcribed spacer region (ITS 438s-1100as and ITS 325s-1100as) or pap31 (1s-688as) gene. *B. koehlerae* ITS species primers were also used (Bk1s-1125as). A 180bp region of the *Babesia* 18S rRNA gene was amplified using real-time PCR and primers *B.canis*f and Bcommonr. The *Babesia* species was determined using primers Bab455f and Bab772r followed by DNA sequencing. *Mycoplasma* sp. PCR was performed using the primers 322s and 938as targeting a 600bp region of the 16S rRNA gene. Reaction conditions used for each PCR are given in Table 2. Reaction mixtures contained 12.5 µl of the Takara premix Ex Taq (perfect real time) (Takara Bio USA Inc, Madison, WI), 7 µl of molecular grade water 0.25 µl each of forward and reverse primers (30 µM) and 5 µl of template DNA. Amplified products were analyzed on a 2% agarose gel stained with ethidium bromide. DNA extracted from the blood of a healthy dog was used as a negative control. Positive controls included 0.001 pg/µl (equivalent to 2.5 genome copies per µl) of *B. henselae* DNA and 0.01 pg/µl of *B. gibsoni* DNA. A positive control was not used for *Mycoplasma* PCR. All PCR positive amplicons were sequenced directly or by cloning in to

pGEM-T Easy vector (Promega, Madison, WI) to establish the species, strain or genotype. Three independent clones were sequenced from each PCR positive sample.

Statistical analysis

Statistical analysis was accomplished using SAS 9.2 software. The prevalence of *Bartonella* sp., *Babesia* sp. and *Mycoplasma* sp. DNA were compared between and among the study groups using chi square test of independence and the Fisher's exact test. The level of significance was set as $p< 0.05$.

Results

Study animals

Group I included FHN biopsy tissues from 22 male (60%) and 13 female (35%) dogs. Nineteen breeds were represented including Labrador retrievers (16%), Terriers (11%) and American cocker spaniels (8%). Ages of the animals in this group ranged from 6-15yrs with 35% of the animals being more than 12 years of age. Sex, breed and age were unknown for 2, 3, and 4 dogs, respectively.

Group II included HSA biopsy tissues from 32 male (64%) and 17 female (34%) dogs. Sex was unknown for one dog. Twenty one different breeds were represented, including Labrador retrievers (20%), Golden retrievers (18%) and Boxers (8%). Ages ranged from 5-15yrs with 42% of the dogs ranging between 9-12yrs of age at the time of diagnosis.

Group III included LNH biopsy tissues from 26 male (52%) and 20 female (40%) dogs. Nineteen breeds were represented in this group, including Labrador retrievers (24%), Terriers (12%) and poodles (8%). Ages ranged from 7-14yrs with 42% of the dogs ranging between 9-12 years of age. Sex, breed and age were unknown for 4, 4, and 5 dogs, respectively.

Group IV included histologically unremarkable splenic tissues from 5 male and 3 female SPF dogs that were euthanized at the conclusion of unrelated studies. Seven dogs were beagles, one was a blood hound and ages ranged from 2-7 years.

PCR analysis and sequencing

The prevalence of *Bartonella*, *Babesia* and hemotropic *Mycoplasma* sp. DNA in each study group, as determined by PCR and DNA sequencing, is summarized in Figure 1. All extraction, PCR negative controls, including the blank paraffin block reagent control, tested negative throughout this study.

Group 1, FHN:

The prevalence of *Bartonella*, *Babesia* and hemotropic *Mycoplasma* sp. DNA was 29.7%, 2.7% and 0.0%, respectively. Biopsies from FHN dogs were re-reviewed by a veterinary pathologist (KEL) and graded using previously published criteria.[40] There were 13 grade I, 14 grade II and 10 grade III fibrohistiocytic nodules. Prevalence of *Bartonella* sp. DNA was 23%, 42.9% and 20% in grades I, II and III FHN, respectively. Dogs were infected with *B. henselae* (SA2 strain) (n=4), *B. vinsonii* subsp. *berkhoffii* genotype I (n=2) and *B. koehlerae* (n=2). One dog was co-infected with *B. vinsonii* subsp. *berkhoffii* genotype III and *B. henselae* SA2 and another with *B. vinsonii* subsp. *berkhoffii* genotype III and *B. koehlerae*. For one amplicon the *Bartonella* sp. could not be established due to the lack of adequate clean sequence. One *B. henselae* SA2 ITS positive sample was also *B. henselae* SA2 positive based upon the Pap31 gene sequence. The *Bartonella* Pap31 gene was not amplified from any other FHN tissues. *Babesia gibsoni* was sequenced from the spleen of one dog, whereas no hemotropic *Mycoplasma* sp. DNA was amplified from group I splenic tissues.

Group II, HSA:

The prevalence of *Bartonella*, *Babesia* and hemotropic *Mycoplasma* sp. DNA in HSA dogs was 26%, 2% and 6%, respectively. Dogs were infected with *B. henselae* (SA2 strain) (n=7), *B. koehlerae* (n=1), and *B. vinsonii* subsp. *berkhoffii* genotype III (n=1). One dog was co-infected with two *B. henselae* ITS strains (SA2 and Houston I), a second with *B. henselae* SA2 and *B. koehlerae*, and a third with *B. henselae* SA2 and *B. vinsonii* subsp. *berkhoffii* genotype I. The DNA sequence from one amplicon had low similarity when compared to

available GenBank *Bartonella* sequences (closest similarity was 36% to *B. alsatica*). Two *B. henselae* ITS SA2 strains were PCR positive for *B. henselae* SA2 pap31 gene. By two independent DNA extractions, 1 HSA tissue was real time PCR positive for a *Babesia* sp., however, attempts to determine the species by amplifying a bigger fragment of the 18S rRNA gene was not successful. *Mycoplasma ovis* DNA was amplified and sequenced from three dogs.

Group III, LNH:

The prevalence of *Bartonella*, *Babesia* and hemotropic *Mycoplasma* sp. was 10%, 2% and 0%, respectively in splenic tissues from dogs with LNH. Dogs were infected with *B. koehlerae* (n=3) and *B. henselae* SA2 (n=2). One LNH tissue was real time PCR positive for *Babesia* sp., however, multiple attempts to determine the species by amplifying a bigger fragment of the 18S rRNA gene were not successful.

Group IV, Histologically unremarkable spleens:

All the splenic tissues from SPF dogs were PCR negative for *Bartonella*, *Babesia* and hemotropic *Mycoplasma* sp. Blood cultured from SPF dogs was using *Bartonella* alphaproteobacteria growth medium (BAPGM) followed by *Bartonella* sp. PCR was negative.[12] *Bartonella* antibodies were not detected by indirect fluorescent antibody testing.[12]

Statistical analysis

As SPF dogs were PCR negative for all three targeted genera, group IV was not included in the statistical analysis. When the prevalence of *Bartonella*, *Babesia* and hemotropic *Mycoplasma* sp. DNA was compared using a Chi square test of independence, there was a statistical difference among groups I, II and III. Subsequently, each group was compared with each of the other two remaining groups using a Fisher's exact test. The prevalence of *Bartonella* sp. DNA was higher in group I (FHN) compared to the group III (LNH)

(p=0.019) and higher in group II (HSA) as compared to the group III (LNH) (p=0.037), however, there was no difference in the prevalence of *Bartonella* sp. DNA between the FHN and the HSA groups (p=0.700). Prevalence of *Bartonella*, *Babesia* and hemotropic *Mycoplasma* sp. was also compared among the three groups. *Bartonella* infection was statistically higher than infection with *Babesia* and hemotropic *Mycoplasma* sp. in the FHN group (p=0.003 and p=0.0004, respectively), while there was no significant difference in *Babesia* and hemotropic *Mycoplasma* sp. (p=1) prevalence. *Bartonella* DNA was also statistically higher in the spleen of HSA dogs when compared to infection with *Babesia* and *Mycoplasma* sp. (p=0.0005 and p=0.006 respectively). There was no difference between the prevalence of *Babesia* sp. and hemotropic *Mycoplasma* sp. in the HSA group (p=0.31). There were no statistical differences in prevalence among *Bartonella, Babesia* and hemotropic *Mycoplasma* sp. in the LNH group (p>0.05). There were no significant differences in prevalence among each of the three FHN grades (p> 0.05).

Discussion

In this study, there was a higher prevalence of *Bartonella* sp. DNA in paraffin-embedded splenic biopsy tissues from dogs with HSA and FHN, as compared with splenic tissues from dogs with LNH. In the context of angiogenesis, three *Bartonella* sp. have been associated with vasoproliferative tumor-like lesions in human patients, including *B. bacilliformis* (verruga peruana), *B. quintana* (bacillary angiomatosis), *B. henselae* (bacillary angiomatosis and bacillary peliosis hepatis).[19,27] In dogs, *B. henselae* was amplified from peliosis hepatis and *B. vinsonii* subsp. *berkhoffii* was isolated from an immunosuppressed dog with bacillary angiomatosis.[26,49] Also, as *B. vinsonii* subsp. *berkhoffii* genotype II was isolated form a dog with a hemangiopericytoma and from a human patient with epithelioid hemangioendothelioma, viable intravascular bacteria have been documented in dogs and people with vasoproliferative tumors.[6] Based upon these preliminary facts, the detection of *Bartonella* sp. DNA in tissues from a subset of dogs with HSA was anticipated. Unexpectedly however, there was no difference in the prevalence of *Bartonella* sp. DNA

between HSA and FHN groups. Based upon *in vitro* studies using *B. henselae* as a prototypical representative of the genus, viable, intracellular infection has been documented in several macrophage-like cells, including monocytes, dendritic cells and microglial cells.[25,33,47] Therefore, based upon previous studies, *Bartonella* sp. may localize within splenic histiocytes and endothelial cells, but DNA could also be amplified from erythrocytes circulating through the spleen at the time of sample collection. Cellular localization of *Bartonella* sp. within the spleen is an important next step, however, efforts in our laboratory to localize the bacteria using Warthin-Starry silver staining, *B. henselae* immunohistochemistry and *in situ* hybridization on paraffin-embedded splenic tissues were only marginally successful (Data not shown).

The cause(s) of splenic FHN in dogs is unknown. Fibrohistiocytic cells are considered to be components of reticular meshwork in the splenic red pulp. These cells are intermixed with plasma cells, macrophages and lymphocytes.[40] Once erythrocytes are infected with a *Bartonella* sp., the bacteria remain within the infected cell for the entire life span of the cell.[36] Senile erythrocytes are removed from the blood stream by the filtering action of the reticular meshwork in the red pulp and are then phagocytized by the splenic macrophages.[31] Therefore *Bartonella* sp. DNA amplified from FHN, HSA and LNH groups could be related to phagocytized, dead bacteria in the splenic tissue. Alternatively, as *Bartonella* sp. can infect macrophages and prevent their apoptosis[25], it is plausible that chronic *Bartonella* infection of splenic histiocytes leads to their proliferation and formation of fibrohistiocytic nodules. Splenic nodules appear to form a continuum from lymphoid nodular hyperplasia to malignant splenic stromal neoplasms (malignant fibrous histiocytoma).[40] In the context of histiocytic infection, *Bartonella* sp. DNA was lower in Grades I (23%) and III (20%) FHN as compared to Grade II (42.9%), but these differences were not statistically significant. As the overall prevalence of *Bartonella* sp. DNA in the FHN group (29.7%) was slightly higher than HSA group (26%), prospective studies are required to determine if *Bartonella* sp. are a cause or cofactor in the progression of FHN in dogs.

Although the etiology and pathogenesis of canine HSA is incompletely understood and multifactorial, hypoxia, inflammation and uncontrolled angiogenesis are considered to be important pathogenic factors.[42]. Canine HSA can develop spontaneously in the absence of any known mutations, and is not a heritable condition, even though breed predilections have been reported.[39] Mutations of PTEN (phosphatase and tensin homolog) have been found in some canine HSAs[11], and alterations in the p16-cyclin D-Rb pathway have also been demonstrated.[51] In human beings, 25% of all adult cancers develop as a result of chronic inflammation with or without documentation of infection.[8] Canine HSA is characterized by the presence of inflammatory cells intermixed with the tumor cells. Macrophages and their cytokines promote tumor survival and facilitate metastasis.[8] *Bartonella* infection activates the NF-κB pathway leading to the recruitment of inflammatory cells including neutrophils, lymphocytes and macrophages to the site of infection.[14] Hypoxia activates hypoxia inducible factor Iα (HIF-Iα), which is a transcription factor for many downstream genes that promote angiogenesis, tumor growth and metastasis. *Bartonella* infection has been shown to activate the HIF-Iα resulting in increased expression of the growth factor VEGF.[24] Therefore *Bartonella* sp. increase VEGF concentrations in conjunction with a direct mitogenic effect on endothelial cells, while also inhibiting endothelial cell apoptosis and thereby potentially contributing to the development of vasoproliferative tumors.

Infection by bacteriophages is another unique feature of several *Bartonella* sp. Bacteriophages are found in *B. henselae*, *B. bacilliformis* and *B. vinsonii* subsp. *berkhoffii*.[2,3,29] Phage-associated chromosomal rearrangement has been postulated to play a crucial role in host specialization of *B. quintana*,[1] which appears to be a genomic derivative of the zoonotic pathogen *B. henselae* . In fact, the majority of genes found in *B. henselae* that are not found in *B. quintana* are located in four genetic clusters flanked by several phage integrases. This finding supports a potential role for bacteriophages as vehicles for *B. henselae* DNA rearrangements, which could prove important in promoting oncogenesis. However, based upon PCR amplification of the Pap31 bacteriophage associated gene, only 3 *B.henselae* SA2 strains had evidence supporting the presence of phages. Further studies are

required to investigate whether bacteriophages, inflammation, hypoxia or deregulated angiogenesis associated with chronic *Bartonella* infection contribute to HSA oncogenesis and metastasis.

We determined if *Babesia* and *Mycoplasma* sp.DNA, representing other intravascular, vector-transmitted erythrocyte-associated pathogens that might also sequester within splenic histoiocytes, were associated with FHN, HSA or LNH. The prevalence of *Babesia* and *Mycoplasma* sp. DNA in the FHN and HSA groups was significantly lower than the prevalence of *Bartonella* sp. DNA, whereas there was no difference between *Babesia* and *Mycoplasma* prevalence among FHN, HSA or LNH groups. In the context of vector borne intravascular pathogenic bacteria and protozoa, the spleen plays an important immunomodulatory role in controlling the infection, frequently referred to as infection immunity or premunition. Babesiosis occurs commonly in splenectomized individuals,[35] and in many cases, patients with occult babesiosis become symptomatic following splenectomy.[37] Splenic lesions are also a common finding in patients with systemic cat scratch disease caused by *B. henselae* infection. Splenic rupture was reported in a 65-year-old man due to *B. henselae*-induced splenitis[9] and persistent infection with *B. bacilliformis* was diagnosed in a chronically thrombocytopenic patient following splenectomy.[18] Using real time PCR targeting an 180bp segment of the 18S rRNA gene, amplification of *Babesia* sp. DNA was obtained from only one dog in each splenic pathology group. A 6 year-old Chihuahua with FHN was infected with *B. gibsoni*, whereas a 14 year-old Labrador retriever with LNH and a 13 year-old Golden retriever with HSA were both infected with a undetermined *Babesia* sp. Attempts to define the *Babesia* sp. by amplifying a larger gene segment were unsuccessful, potentially due to a low number of target organisms in splenic tissues sample or because of DNA degradation caused by formalin fixation.[13]

Mycoplasma ovis was amplified and sequenced from the spleen of three HSA dogs, whereas no *Mycoplasma* sp. DNA was amplified from the control, FHN or LNH groups. Recently, co-infection with *M. ovis* and *B. henselae* SA2 was reported in a veterinarian with a progressive neurological illness.[41] One of the three *M. ovis* infected dogs in this study was

also co-infected with a *B. henselae* SA2 strain. Both *B. henselae* and hemotropic *Mycoplasma* sp. are thought to be transmitted by the cat flea, *Ctenocephalides felis*.[38] In numerous animal species, *Mycoplasma* infections are typically characterized by a long period of latency. *In vitro,* mycoplasmas are able to transform normal cells to cancer cells.[43] *M. genetalium* and *M. hyorhinis* are capable of inducing malignant transformation of human epithelial cells and are thought to play a role in the development of various cancers including prostate cancer and gastric carcinoma.[34,50] We are not aware of a previous report *M. ovis* infection in dogs or whether *M. ovis* might cause oncogenic transformation of mammalian cells. It is possible that amplification of *M. ovis* DNA from these tissues represents DNA carryover during the tissue processing, however, this seems unlikely as sheep tissues are rarely processed in our histopathology laboratory and each of these tissues was processed at different time points in the histopathology laboratory. The mode of infection and significance of *M. ovis* DNA in dogs with splenic HSA is unknown, but warrants further investigation.

There are several limitations of this retrospective study. Obtaining optimal age and sex matched control tissues was not possible as splenectomy is performed only when there is overt evidence of splenic disease and splenic biopsies are infrequently obtained in the clinical setting. Presumably, the prevalence of *Bartonella, Babesia* and *Mycoplasma* DNA reported in this study represents an underestimate of the actual prevalence's of these intravascular infections due to the small quantity of tissue placed in paraffin blocks from massively enlarged spleens and the limited quantity of extracted DNA (host and pathogen) that can be incorporated into each PCR reaction. It is also possible that varying periods of formalin fixation would induce denaturation of DNA targets resulting in a false negative PCR result. Previously, our laboratory reported *Bartonella* sp. DNA carryover during the collection and processing of paraffin embedded tissues.[46] In that study, *Bartonella* sp. DNA was amplified from the necropsy room, tissue processor and from several microtomes. To minimize potential DNA carryover in this study, we only included surgical biopsy specimens. Individual sterile scalpel blades and a clean work area, not microtomes, were used to obtain tissue scrolls for DNA extraction. A blank paraffin block was used as a reagent control with

each set of DNA extractions. Also, at multiple time points using the same collection and extraction protocols used for splenic samples, we processed splenic tissues from a small number of SPF dogs with histologically unremarkable spleens. Neither *Bartonella, Babesia* nor hemotropic *Mycoplasma* DNA was amplified from negative control tissues, decreasing concern for laboratory-related DNA cross contamination.

In conclusion, we found a significantly higher prevalence of *Bartonella* sp. DNA in splenic tissues from dogs with FHN and HSA when compared to LNH. Also, dogs with FHN and HSA were more likely to have *Bartonella* sp. DNA in splenic tissues, as compared to *Babesia* or *Mycoplasma* sp. DNA. Prospective studies are needed to isolate viable *Bartonella* organisms by enrichment blood culture in dogs with FHN and HSA and to determine the cellular localization of the bacteria within splenic tissues. Also, performing *Bartonella* sp. PCR from FHN, and HSA tissues obtained from different geographical locations may further establish a temporal and spatial association between *Bartonella* infection and these splenic diseases in dogs.

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Table1. *Bartonella, Babesia* and hemotropic *Mycoplasma* sp. PCR primers used in this study.

Primer	DNA Sequence (5'-3')	Target Gene
Bspp 438s	GGTTTTCCGGTTTATCCCGGAGGGC	*Bartonella* sp. ITS
Bspp 325s	CTTCAGATGATGATCCCAAGCCTTCTGGCG	*Bartonella* sp. ITS
Bspp 1100as	GAACCGACGACCCCCTGCTTGCAAAGCA	*Bartonella* sp. ITS
Bspp 1000as	CTGAGCTACGGCCCCTAAATCAGG	*Bartonella* sp. ITS
Pap31 1 s	GAC TTC TGT TAT CGC TTT GAT TT	*Bartonella* sp. pap31
Pap31 688as	CACCACCAGCAAMATAAGGCAT	*Bartonella* sp. pap31
Bkoehl-1s	CTTCTAAAATATCGCTTCTAAAAATTGGCATGC	*B. koehlerae* ITS
Myco 322s	GCCCATATTCCTACGGGAAGCAGCAGT	Hemotropic *Mycoplasma* 16s
Myco938as	CTCCACCACTTGTTCAGGTCCCCGTC	Hemotropic *Mycoplasma* 16s
B.canis s	GCATTTAGCGATGGACCATTCAAG	*Babesia* sp. 18s
B.common as	CCTGTATTGTTATTTCTTGTCACTACCTC	*Babesia* sp. 18s
Bab455s	GTCTTGTAATTGGAATGATGGTGAC	*Babesia* sp. 18s
Bab772as	ATGCCCCCAACCGTTCCTATTA	*Babesia* sp. 18s

Table2. PCR conditions used in this study for the amplification of *Bartonella, Babesia* and *Mycoplasma* sp. target genes. All the reactions were performed using an Eppendorf Mastercycler epgradient (Eppendorf, Westbury, NY) , except for *Babesia* genus real time PCR which was performed in a Bio-Rad CFX96 real time system (Bio-Rad Laboratories, Hercules, CA).

Target gene	Reaction conditions	Number of cycles
Bartonella sp. ITS	94^0C X 30sec, 68^0C X 10sec, 72^0C X 15sec, 72^0C X 30sec	55
B. koehlerae ITS	94^0C X 15sec, 66^0C X 15sec, 72^0C X 18sec, 72^0C X 30sec	55
Bartonella sp. Pap 31	94^0C X 15sec, 62^0C X 15sec, 72^0C X 18sec, 72^0C X 1min	55
Hemotropic *Mycoplasma* 16S	94^0C X 15sec, 68^0C X 10sec, 72^0C X 15sec, 72^0C X 30sec	55
Babesia genus 18S	95^0C X 45sec, 58^0C X 45sec, 72^0C X 45sec, 72^0C X 5min	49
Babesia Sp. 445-772	95^0C X 45sec, 58^0C X 45sec, 72^0C X 45sec, 72^0C X 5min	50

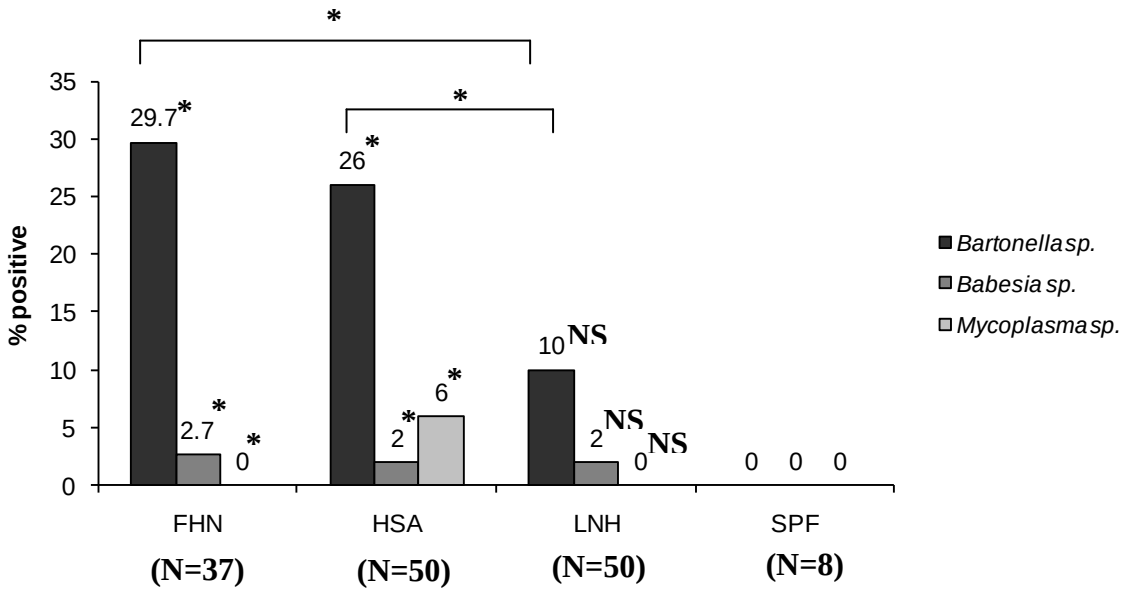

Figure 1. Molecular prevalence of *Bartonella*, *Babesia* and *Mycoplasma* sp. in splenic biopsies from dogs with fibrohistiocytic nodules (FHN), hemangiosarcoma (HSA), lymphoid nodular hyperplasia (LNH), and histologically unremarkable spleens from specific pathogen free dogs (SPF). Results are presented as percent positive. N= the number of dogs in each group. * $p < 0.05$. NS= no significant difference.

Chapter 5

Infection of Human Brain Vascular Pericytes (HBVPs) by *Bartonella henselae*

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M.Varanat, RG Maggi, KE Linder and EB Breitschwerdt. Infection of Human Brain Vascular Pericytes by *Bartonella henselae*.

Infection of Human Brain Vascular Pericytes (HBVPs) by *Bartonella henselae*

Introduction

Angiogenesis, the development of new blood vessels from existing vessels, is important physiologically (e.g. pregnancy, menstruation and wound healing) and pathologically (e.g. oncogenesis). Many infectious agents can induce angiogenesis in a host, including viruses, bacteria and fungi (Dehio, 2004; Ganem, 2010; Kontoyiannis *et al*., 2010). Infectious angiogenesis is well-studied in viral infections (e.g. Kaposi's sarcoma), whereas the mechanism of angiogenesis induced by *Bartonella*, the only genus of bacteria known to induce pathological angiogenesis, remains unclear. *Bartonella* sp. induce abnormal endothelial cell proliferation, leading to vasoproliferative disorders such as verruga peruana, bacillary angiomatosis and bacillary peliosis (Koehler and Tappero, 1993). It is known that *Bartonella* sp. can induce production of VEGF by host cells, including endothelial cells and macrophages, and that VEGF is expressed in the vascular tumor-like lesions induced by *Bartonella* sp. (Kempf *et al*, 2001; Leung *et al*, 1989).

There is increasing evidence of the role of infectious agents in tumorigenesis. The roles of *Helicobacter pylori* in inducing gastric lymphoma and gastric adenocarcinoma and the role of oncogenic viruses, such as Kaposi's sarcoma-associated herpes virus (KSHV) in humans, are well studied (Hansen *et al*., 2007; Moss and Malfertheiner, 2007). Recent research has provided evidence that members of the genus *Bartonella* may play a role in the pathogenesis of cancer (Lax and Thomas, 2002). Vasoproliferative tumors such as bacillary angiomatosis and verruga peruana induced by *Bartonella* sp. are benign and can be completely cured by antibiotic treatment (Rudikoff *et al*., 1989). Although a role in causation was not established, *B. vinsonii* subsp. *berkhoffii* was isolated by blood culture from a dog with hemangiopericytoma and a human with hepatic epithelioid hemangioendothelioma (Breitschwerdt *et al*., 2009). A recent study in our laboratory, we found a significantly higher

prevalence of *Bartonella* sp. DNA in splenic tissues of dogs with hemangiosarcoma when compared to dogs with non neoplastic lymphoid nodular hyperplasia of the spleen (Varanat *et al*., Unpublished data). These findings indicate that *Bartonella* sp. might play a role in the pathogenesis of vasoproliferative tumors in dogs and human patients.

Angiogenesis is an important step in tumor progression and metastasis. Along with endothelial cells, pericytes contribute to the formation of new vessels in angiogenesis. Pericytes are extensively-branched cells that wrap around endothelial cells on the abluminal side of capillaries (Diaz-Florez *et al*., 2009). Pericytes and endothelial cells share a basal lamina, and communicate with each other via gap junctions, tight junctions, soluble factors and surface adhesion molecules (Hirschi and D'Amore, 1996). Platelet-derived growth factor (PDGF), produced by the tumor cells and endothelial cells, induce the secretion of vascular endothelial growth factor (VEGF) from pericytes, which can protect endothelial cells from apoptosis (Reinmuth *et al*, 2001). Abnormal cell signaling between endothelial cells and pericytes contributes to tumor angiogenesis and metastasis. At the initiation of angiogenesis, the close association between endothelial cells and pericytes is disrupted, leading to the activation and detachment of pericytes thereby facilitating the migration of endothelial cells. Tumor hypoxia induces release of nitrous oxide and vascular endothelial growth factor, leading to vasodilation, increased vascular permeability and detachment of the pericytes from the endothelial cells (Raza *et al*., 2010).

In many respects, *Bartonella*-induced vasoproliferation resembles tumor angiogenesis, as VEGF plays a key role in both conditions. As the role of pericytes in *Bartonella*-induced vasoproliferation has not been reported, Human Brain Vascular Pericytes (HBVPs) were infected with *B. henselae* in this study to determine the effect of *B. henselae* infection on proliferation, apoptosis and angiogenic cytokine production.

Materials and Methods

Cell culture

Human brain vascular pericytes (ScienCell Research Laboratories, San Diego, CA) were grown in pericyte medium (ScienCell Research Laboratories, San Diego, CA) with 2% fetal bovine serum (FBS). Identity of the cells was confirmed by staining for α-smooth muscle actin (Diaz-Flores *et al*., 2009). An aliquot from each batch of the cells were tested for the presence of *Bartonella* sp. DNA by polymerase chain reaction as described elsewhere (Maggi *et al*., 2005).

Bacteria

B. henselae (intergenic spacer strain SA2) , isolated originally by blood culture from a female veterinarian with vasculitis, polyarthritis and neurological signs (Breitschwerdt *et al*., 2007), was grown in trypticase soy agar (TSA) with 10% sheep blood in a humidified incubator at 37^0C and 5% CO_2 for 7 days. Bacterial colonies were harvested and suspended in PBS. Bacteria numbers were determined by measuring optical density (OD) at 600 nm using a spectrophotometer. *E. coli* used in this study was kindly provided by Dr Paul Orndorff, NCSU. *E. coli* was grown on TSA agar with 10% sheep blood for 48 hrs.

Gentamicin protection assay

To demonstrate invasion of pericytes by *B. henselae*, a gentamicin protection assay was performed. Pericytes were cultured in poly-L-lysine-coated 6 well plates (BD Biosciences, San Jose, CA) for 48 hrs at a density of 5×10^4 cells per well. Pericytes were infected with $1.98\text{-}2.2 \times 10^8$ of agar-grown *B. henselae* and infected cells were incubated for 24, 48 and 72 hrs. At the end of each infection period, cells in 3 wells were treated with 100µg/ml of gentamicin sulfate (Sigma, St Louis, MO) for 3 hrs at 37^0C and 3 wells were left untreated. Cells were washed 3 times with DPBS (ScienCell Research Laboratories, San Diego, CA) to remove extracellular bacteria and residual antibiotic. Cells were harvested

from both gentamicin-treated and non-treated wells. Cells were lysed by adding 900 µl of sterile cell culture grade water (ScienCell Research Laboratories, San Diego, CA) followed by 100 µl of PBS. The number of viable bacteria was determined by plating serial dilutions onto TSA plates with 10% sheep blood agar. Plates were incubated at 37^0C with 5% CO_2 for 7 days, and the colonies were counted. The assay was done in triplicate and the experiment was repeated 3 times. One 6 well plate infection was carried out at 4^0C for 72 hrs and a gentamicin protection assay was carried out as described above, to determine if the cellular invasion is temperature dependent. One plate with uninfected cells was included in each experiment to confirm sterility.

Electron microscopy

HBVPs were grown in poly-L-lysine-coated $25cm^2$ tissue culture flasks and infected with 10^8 CFU of *B. henselae* for 48 hrs. Cells were harvested and centrifuged for 2 min at 500 rpm at room temperature. The supernatant was discarded, and the pellet was resuspended in McDowell and Trump's fixative (4% formaldehyde and 1% gluteraldehyde in phosphate buffer). After fixation for 1 hr at room temperature, the cells were pelleted by centrifuging at 500 rpm for 30 sec. The fixative was then removed by pipetting without disturbing the pellet. One ml of the 3% aqueous agar was added using a heated Pasteur pipette and quickly centrifuged at 500 rpm for 30 sec. After the agar had solidified, the end of the centrifuge tube was sliced off to separate the agar embedded sample. The sample was then cut into 1mm slices and stored in fixative at 4^0C until further processing. Samples were rinsed in 0.1 M phosphate buffer, treated with 1% osmium tetroxide, rinsed with distilled water, dehydrated in ascending grades of alcohol and embedded in Spurr resin. Semithin and ultrathin sections were cut using an ultramicrotome, post-stained by uranyl acetate and lead citrate, and examined using a Feico 208-S transmission electron microscope.

Proliferation assay

Cell proliferation was determined by measuring Bromodeoxyuridine (BrdU) incorporation with a BrdU cell proliferation chemiluminescent ELISA kit (Roche Applied Sciences, Mannheim, Germany) according to the manufacturer's instructions. Briefly, cells were seeded in a flat-bottomed 96 well micro titer plate (Sigma-Aldrich, St Louis, MO) at a density of 10,000 cells per well. Cell cultures were infected with *B. henselae* at a multiplicity of infection (MOI) of 100, 250 and 500 or heat-killed *B. henselae* or *E. coli* for 48 hrs. Cells in 3 wells each were treated with 10ng/µl of fibroblast growth factor (FGF) (BD Biosciences, San Jose, CA) or 20ng/µl of platelet derived growth factor (PDGF) (BD Biosciences, San Jose, CA). Cells in 3 wells were used as uninfected controls. BrdU (100µM) was added to the cell culture medium 24 hrs before the assay. After 24 hrs of BrdU incorporation, the media was removed and 200µl of the denaturing solution was added to each well and incubated for 30 min at room temperature. The supernatants were discarded, 100µl of anti-BrdU-POD antibody conjugate solution (1:100 dilution) was added to each well and incubated for 1.5 hrs at room temperature. Plates were washed 3 times with wash buffer. 100µl of the substrate solution was added to each well, and incubated at room temperature for 3 min on a shaker. Light emission was measured using a Perkin Elmer Victor3 luminometer (Perkin Elmer, Waltham, MA). The assay was done in triplicate wells, and the experiment repeated 3 times.

TUNEL assay for apoptosis

A TUNEL assay was performed using TiterTACS in situ kit (Trevigen, Gaithersburg, MD) according to manufacturer's instructions. Briefly, cells were grown in a flat-bottomed 96 well plate at a density of 10,000 cells per well. Cells were incubated overnight and infected with *B. henselae* at MOIs 100, 250 or 500 or with heat-killed *B. henselae* or *E. coli*. After infection, cultures were incubated for 48 hrs. Apoptosis was induced by the addition of 100nM of Actinomycin D for 12 hrs. For the TUNEL assay, the culture medium was

discarded and the cells were fixed with 3.7% buffered formaldehyde for 7 min at room temperature. After discarding the fixative, the cells were washed twice with PBS at room temperature and post-fixed in 100% methanol for 20 min at room temperature, followed by 2 washes with PBS. Cells were then treated with 50µl of proteinase K solution per well for 15 min at room temperature and washed with 200µl of distilled water per well, followed by PBS for 2 minutes. After blocking endogenous peroxidase by treating with 50µl of 3% hydrogen peroxide solution for 5 min at room temperature, plates were washed once with distilled water and incubated for 5 min with the labeling buffer. This buffer was discarded and 50µl of the labeling reaction mix was added per well and incubated at 37^0C for 1 hr. The reaction was stopped by the addition of 150µl of stop buffer. The cells were then washed twice with PBS and incubated for 10 min at room temperature with 50µl of Streptavidin-Horseradish Peroxidase (HRP) solution per well and washed 4 times in PBS with 0.1% Tween 20, followed by incubation with 100µl of TACS-Sapphire substrate for 30 min at room temperature in the dark. The reaction was stopped with 100µl of 5% phosphoric acid per well and the absorbance was measured at 450nm. The assay was performed in triplicate, and the experiment was repeated 3 times.

Measurement of VEGF and IL-8

20,000 HBVPs/well were grown in poly-L-lysine-coated 24 well plates. Cells were infected with *B. henselae* at an MOI of 250, 500 or 1000 or heat-killed *B. henselae* or *E. coli* or left uninfected. Cells were incubated at 37^0C and supernatant was collected at 12, 24, 48 and 72 hrs after infection and stored at -80^0C until use. VEGF and IL-8 concentrations in the cell culture supernatants were determined by enzyme linked immunosorbent assays using Human VEGF Duoset ELISA kit and human CXCL8/IL-8 Duoset ELISA kits (R&D systems, Minneapolis, MN), according to the manufacturer's instructions. The assay was performed in triplicate, and the experiment was repeated 3 times.

Statistical analysis

Statistical analysis was performed using SAS/STAT 9.2 for Windows (SAS Institute Inc., Cary, NC, 2008). Statistical significance was determined using Kruscal-Wallis test and Bonferroni post hoc test. Level of significance was set as a p value less than 0.05.

Results

Characterization of Human Brain Vascular Pericytes (HBVPs)

HBVPs were grown in pericyte medium. Cytospin preparations made from the cells were tested for the expression of smooth muscle actin using immunohistochemical staining. About 60-70% of the cells showed strong positive staining for smooth muscle actin (Data not shown). Cells were also tested for the presence of *Bartonella* sp. DNA by PCR prior to infection. Every batch of cells used in this study was negative for *Bartonella* sp. DNA.

***B. henselae* can invade HBV Pericytes.**

We set out to test whether *B. henselae* can infect and invade pericytes. We used a gentamicin protection assay and electron microscopy to demonstrate invasion of HBVPs by *B. henselae*. We found a progressive increase in the percentage of *B. henselae* invading HBVPs over a period of 72 hrs (Table 1). Maximum invasion was noted at 72 hrs and no invasion was noted 4^0C. Uninfected controls remained culture negative throughout the study. Invasion was also demonstrated using transmission electron microscopy (Figure 1). Extracellular bacteria were seen in close contact with the cell membrane (Figure 1A). Clumps of bacteria were seen being engulfed by invaginations of the cell membrane (Figure 1B). Clusters of pleomorphic rod-shaped bacteria were noted in the cytoplasm of the HBVPs as membrane bound inclusions (Figure 1C). Single bacteria were also seen free in the cytoplasm in some cells (Data not shown). Uninfected control cells did not contain any bacteria (Figure 1 D).

***B. henselae* suppresses the proliferation of HBVPs in a dose dependent manner.**

Next, we posed the question whether *Bartonella* can induce proliferation of the pericytes as previously demonstrated for endothelial cells (Maeno *et al.,* 1999). HBVPs were inoculated with *B. henselae* at MOIs of 100, 250 or 500 or with heat-killed *B. henselae* or *E. coli*. Cells were treated with FGF and PDGF as positive mitogenesis controls. Proliferation of HBVPs was measured using a BrdU incorporation assay. *B. henselae* did not have any stimulatory effect on the proliferation of pericytes; on the contrary, *B. henselae* inhibited proliferation of pericytes in a dose dependent manner when compared to uninfected cells ($p < 0.01$) (Figure 2). An inhibitory effect on proliferation was also shown by the heat-killed *B .henselae*, though there was no statistical significance when compared to the uninfected controls ($p=0.07$). *E. coli* did not have any effect on the proliferation of pericytes when compared to uninfected cells ($p=0.8$).

***B. henselae* does not affect on the apoptosis of HBVPs.**

B. henselae can inhibit apoptosis of endothelial cells (Dehio, 2005) which supports the development of vasoproliferative tumors. Our studies showed suppression of pericyte proliferation by *B. henselae*. We assessed whether decreased proliferation of HBVPs infected with *B. henselae* is due to induction of apoptosis. Actinomycin D was successful in inducing apoptosis in HBVPs (Figure 3). *B. henselae* infection did not have any significant effect on the apoptosis of HBVPs, while *E. coli* infection was found to have a cumulative effect on Actinomycin D-induced apoptosis in HBVPs ($p < 0.05$).

***B. henselae* induces production of VEGF by HBVPs.**

Angiogenic cytokines, such as VEGF and IL-8, play important roles in *Bartonella*-induced vasoproliferation (Kempf *et al*., 2001; McCord *et al*., 2006). *B. henselae* induced production of VEGF from HBVPs in a dose dependent manner with maximum VEGF production at 72 hrs post-infection (Figure 4A). At 12, 24 and 48 hrs post-infection, there

were only slight differences in the levels of VEGF induced by *B. henselae* when compared to uninfected controls. Heat-killed *B. henselae* also induced VEGF production from HBVPs, although at a lower level. *E. coli* did not induce significant production of VEGF by HBVPs at 72 hrs. There was slightly increased VEGF production by HBVPs infected with *E. coli* compared to uninfected cells at 12, 24 and 48 hrs when compared to uninfected controls. There was no significant induction of IL-8 by *B. henselae* (Figure 4 B), whereas *E. coli* induced IL-8 production from HBVPs.

Discussion

In this study, we found that *B. henselae* can infect human brain vascular pericytes (HBVPs) *in vitro*. *Bartonella* invades host cells by two mechanisms: endocytosis resulting in the formation of a *Bartonella*-containing vacuole (BCV) and invasome-mediated uptake resulting in the internalization of large clusters of bacteria (Dehio *et al*., 1997; Rhomberg *et al*., 2009). Our results demonstrate that endocytosis is the predominant mechanism by which *Bartonella* enter HBVPs. Small clusters of bacteria were found attached to, and becoming engulfed by invaginations of the cell membrane into the pericytes (Figure 1 A & B). We noted solitary and clusters of bacteria inside membrane-bound compartments within HBVPs after 48 hrs of infection. Whether *Bartonella* can multiply inside these membrane-bound compartments is not known (Dehio *et al*., 1997). We found a gradual increase in the number of intracellular bacteria over a period of 72 hrs using the gentamicin protection assay. This may be an indication of intracellular multiplication of *B. henselae*, but we cannot rule out bacterial multiplication outside the cell, leading to entry of new bacteria into cells.

Bartonella has direct mitogenic activity on endothelial cells (Maeno *et al*., 1999). We tested the effect of *B. henselae* infection on the proliferation of pericytes, and found that *B. henselae* had a suppressant effect. Reduced proliferation of pericytes was evidently not due to induction of apoptosis, since *Bartonella* infection did not have a significant effect on apoptosis of HBVPs when compared to uninfected controls. Cell death due to necrosis is also an unlikely mechanism, since the cells showed increased production of VEGF over a period

of 72 hrs post-infection. Angiogenesis begins with dissociation of pericytes from endothelial cells, facilitating migration and proliferation of endothelial cells, leading to the formation of new vessels (Diaz-flores *et al*., 2009). At this stage, the number of pericytes covering the parent vessels decreases (Diaz-Flores *et al*., 1992). Later, the newly-formed vessels are stabilized by the recruitment of pericytes (Benjamin *et al*., 1998). The mechanism by which *B. henselae* suppresses the proliferation of pericytes is not clear, but in the context of angiogenesis, the suppression of pericyte proliferation in the newly sprouting vessels may reduce pericyte coverage and enhance vessel proliferation.

VEGF and angiopoietin-2 (Ang-2) are important factors responsible for the endothelial cell-pericyte dissociation (Cao *et al*., 2007; Greenberg *et al*., 2008). In tumor angiogenesis, pericytes produce VEGF in response to hypoxia thereby facilitating dissociation of pericytes from endothelial cells and acting as an endothelial cell mitogen in a paracrine manner (Aiello *et al*., 1995). Under hypoxic conditions, VEGF can also act as a mitogen for the pericytes (Yamagishi *et al*., 1999). Infection of HBVPs with *B. henselae* resulted in a dose dependent increase in VEGF. The role of VEGF in the vasoproliferation induced by *Bartonella* is well-studied (Kempf *et al*., 2001). Endothelial cells themselves are poor producers of VEGF in response to *Bartonella* infection (Kempf *et al*., 2001). *Bartonella* infection activates the NF-κB pathway (Fuhrmann *et al*., 2001), leading to a proinflammatory phenotype which then results in the recruitment of inflammatory cells, including macrophages while the colonization of macrophages with *Bartonella* results in the activation of hypoxia inducible factor–I and secretion of VEGF, which then can act in a paracrine manner on endothelial cells, leading to their proliferation. VEGF expression is upregulated in bacillary angiomatosis, peliosis hepatis and verruga peruana lesions (Kempf *et al*., 2001; Cerimele *et al*., 2003). *B. bacilliformis* infection induces the production of Ang-2 from endothelial cells (Cerimele *et al*., 2003). Ang-2 competes with Ang-1 (which is responsible for establishing pericyte-endothelial cell interactions, leading to inhibition of endothelial proliferation and stabilization of vessels) for binding to Tie-2 receptors. The binding of Ang-2 to Tie-2 receptors reduces pericyte coverage, destabilizing the vessels and promoting

angiogenesis (Cao *et al*., 2000). *Bartonella* sp. can induce production of both VEGF and Ang-2 (Kempf *et al.*, 2001; Cerimele *et al.*, 2003), and the combined action of these two factors may lead to reduced pericyte coverage, promoting angiogenesis similar to hypoxia induced tumor angiogenesis.

IL-8 is also an angiogenic cytokine which is involved in *Bartonella*-induced vasoproliferation (McCord *et al*., 2006). IL-8 promotes angiogenesis by enhancing endothelial cell survival, proliferation and production of matrix metalloproteinases which helps in the initiation of angiogenesis by degradation of the basement membranes (Li *et al*., 2003). In our study, *Bartonella* infection had no effect on IL-8 secretion by pericytes, whereas *E. coli* induced significantly higher levels of IL-8 compared to the uninfected controls. Lipopolysaccharide (LPS) of Gram negative organisms is a known inducer of IL-8 (Johnson *et al*., 2010). *Bartonella* sp. can induce IL-8 secretion from human whole blood (Matera *et al*., 2003), monocytes, hepatocytes and endothelial cells (Mc Cord *et al*., 2006), and IL-8 is known to promote angiogenesis by inhibiting endothelial cell proliferation. The results from our study indicate that pericytes may not be a significant source of IL-8 in *Bartonella*-induced vasoproliferation.

In reservoir hosts *Bartonella* can cause long-lasting intraerythrocytic bacteremia (Schulein *et al*., 2001). In rats infected with *B. tribocorum*, the bacteria were cleared from the blood stream within a few hours. It is assumed that *Bartonella* colonize a 'primary niche' and multiply there before reappearing in the blood stream after approximately 5 days, and a periodic increase in the number of infected erythrocytes was noted at approximately 5 days interval. The identity of a 'primary niche' has not been proven experimentally, although endothelial cells and cells of the reticuloendothelial system are believed to be part of the primary niche (Dehio, 2005). The close proximity of pericytes to endothelial cells in conjunction with the macrophage-like properties of pericytes (Diaz-Flores *et al*., 2009) makes them a plausible contributor to the 'primary niche' for *Bartonella*.

Hemangiopericytomas are cancers thought to originate from pericytes. There is an ongoing controversy about the origin of these tumors among pathologists because they show

identical histomorphological characteristics to solitary fibrous tumors (Gengler and Guillou, 2006), and the new WHO classification includes these tumors in the category of solitary fibrous tumors (Fletcher, 2006). Yet some tumors are still diagnosed as hemangiopericytomas (Koch *et al*., 2008). *B. henselae* and/or *B. vinsonii* subsp. *berkhoffii* DNA has been detected in paraffin-embedded hemangiopericytoma biopsy tissues from dogs, a horse and a red wolf (Breitschwerdt *et al*., 2009; Varanat *et al*., unpublished data). Detection of the same infectious agent from the same pathological entity across three species indicates that this bacterium may act as a cause of, or co-factor in the development of this tumor. Based on these observations, one would expect that *B. henselae* would promote the proliferation of pericytes *in vitro*, but the results obtained in this study showed that *Bartonella* suppresses pericyte proliferation. Pericytes are a heterogenous population of cells with diverse characteristics and functions (Diaz-Flores *et al*., 2009) depending on the anatomical location. Pericytes in the brain may respond differently to *Bartonella* infection when compared to pericytes in the skin. Further studies are required to determine if *Bartonella* sp. can act as a co-factor in the development of these tumors.

Conclusions

Our study provides new insights in to the mechanism of *Bartonella*-induced vasoproliferation. For the first time, we showed that *B. henselae* can invade pericytes, leading to the suppression of pericyte proliferation and induction of VEGF production by pericytes. VEGF helps in the dissociation of pericytes from the endothelial cells and, combined with reduced pericyte coverage, can promote angiogenesis. This mechanism is identical to hypoxia-induced tumor angiogenesis. Future studies are required to determine if *Bartonella* sp. infection can enhance tumor angiogenesis, thereby promoting tumor progression and metastasis.

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Table 1. Invasion and intracellular replication of *B. henselae* in HBVPs based on Gentamicin protection assay. HBVPs were infected with *B. henselae* for 24,48 and 72 hrs. At the end of infection period cells were treated with gentamicin or left untreated. Assay was done in triplicate and experiment was repeated 3 times. Data from a representative experiment is shown. Data is presented as mean ± SD

Experiment	Length of incubation (Hrs)	Total bacteria recovered from the non-treated controls (log10 CFU/ml)	Intracellular bacteria from the gentamicin treated samples (log 10 CFU/ml)	% intracellular bacteria
37^0C	24	7.77 ± 0.08	5.69 ± 0.26	0.86 ± 0.30
	48	7.81± 0.07	6.32 ± 0.02	3.31 ± 0.70
	72	7.96 ± 0.05	6.91 ± 0.04	9 .0 ± 0.55
4^0C	72	6.69	0	0
Uninfected 37^0C	72	0	0	0

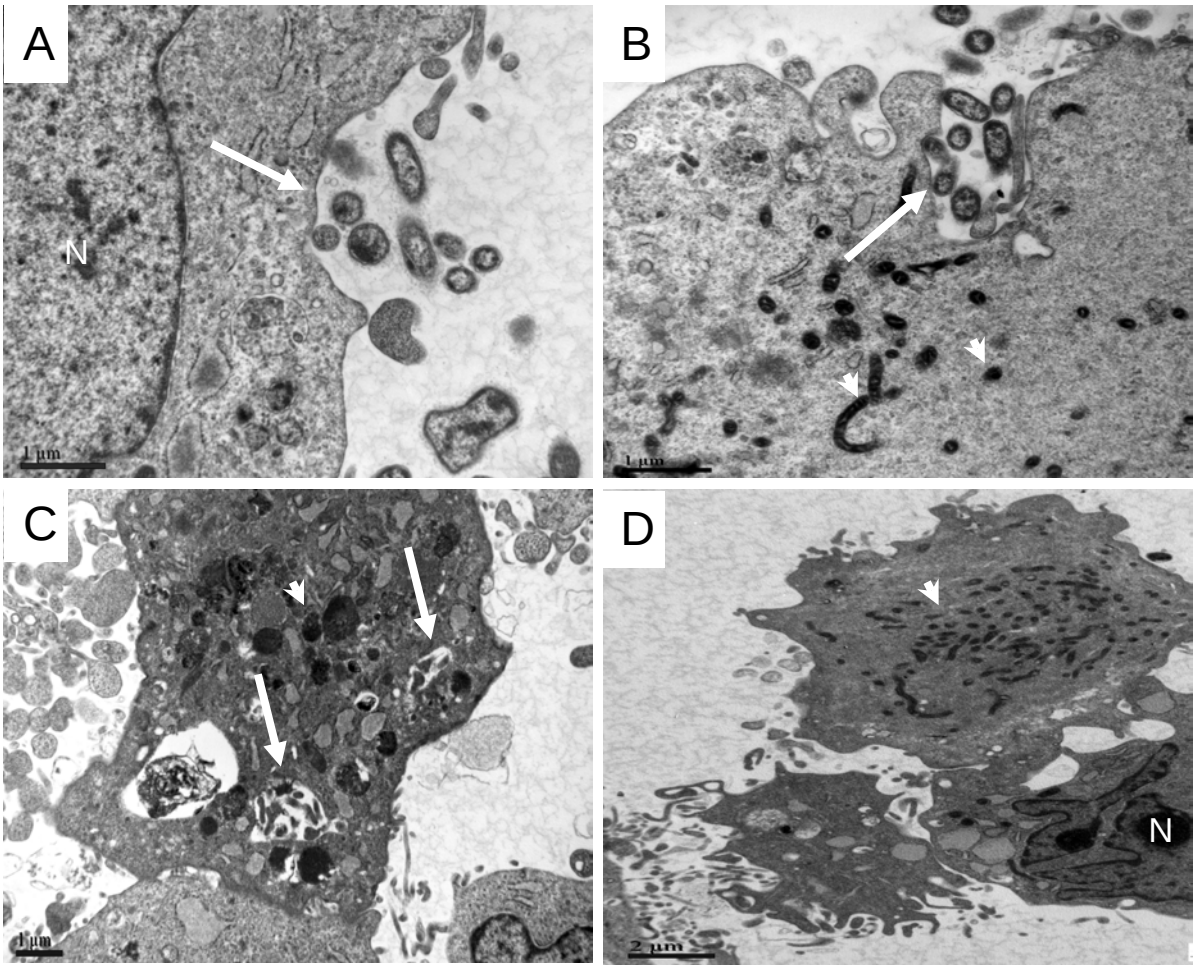

Figure 1. Electron micrographs of HBVPs infected with *B. henselae*. 48 hrs post infection A) Bacteria seen attached to the surface of the cells. B) Bacteria are engulfed by the invagination of the cell membrane. C) Aggregates of bacteria seen inside the cell in a membrane bound compartments D) Uninfected HBVP. Long white arrows indicate bacteria and short white arrows indicate mitochondria. N=Nucleus of the cell

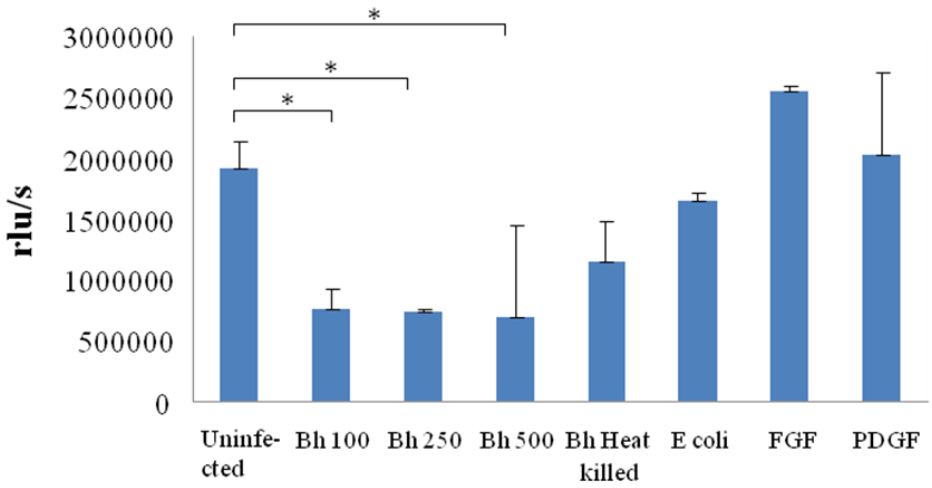

Figure 2. BrdU proliferation assay. HBVPs were infected with *B. henselae* at MOIs of 100, 250, 500 or heat killed *B. henselae* or *E. coli*. FGF and PDGF were used as positive controls for the proliferation. Cells were infected for 48 hrs and treated with BrdU for 24 hrs and BrdU incorporation was measured by chemiluminescent ELISA. Assay was done in triplicate and the experiment was repeated three times. Data from a representative experiment is shown. Data is represented as mean± SD. * $p< 0.05$

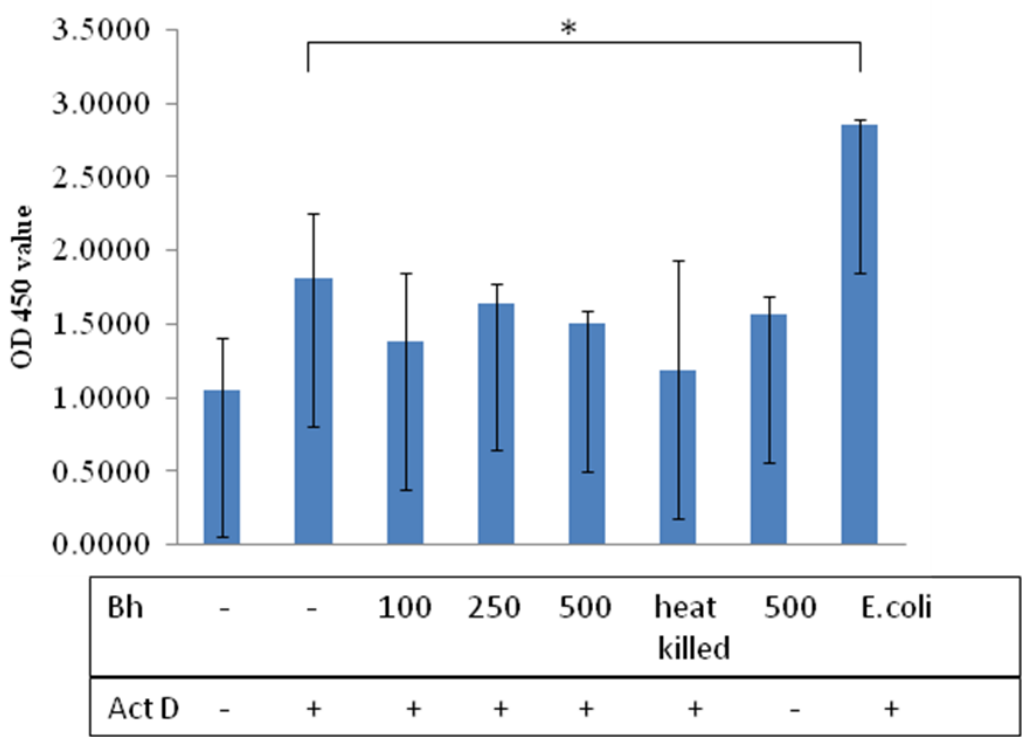

Figure 3. TUNEL assay for apoptosis. HBVPs were infected with *B. henselae* at MOIs of 100, 250, 500 or heat killed *B. henselae* or *E. coli*. Cells were infected for 48 hrs and apoptosis was induced by treating with actinomycin D for 12hrs and apoptosis was measured by colorimetric TUNEL ELISA. Assay was done in triplicate and the experiment was repeated three times. Data from a representative experiment is shown. Data is represented as mean± SD. * $p< 0.05$

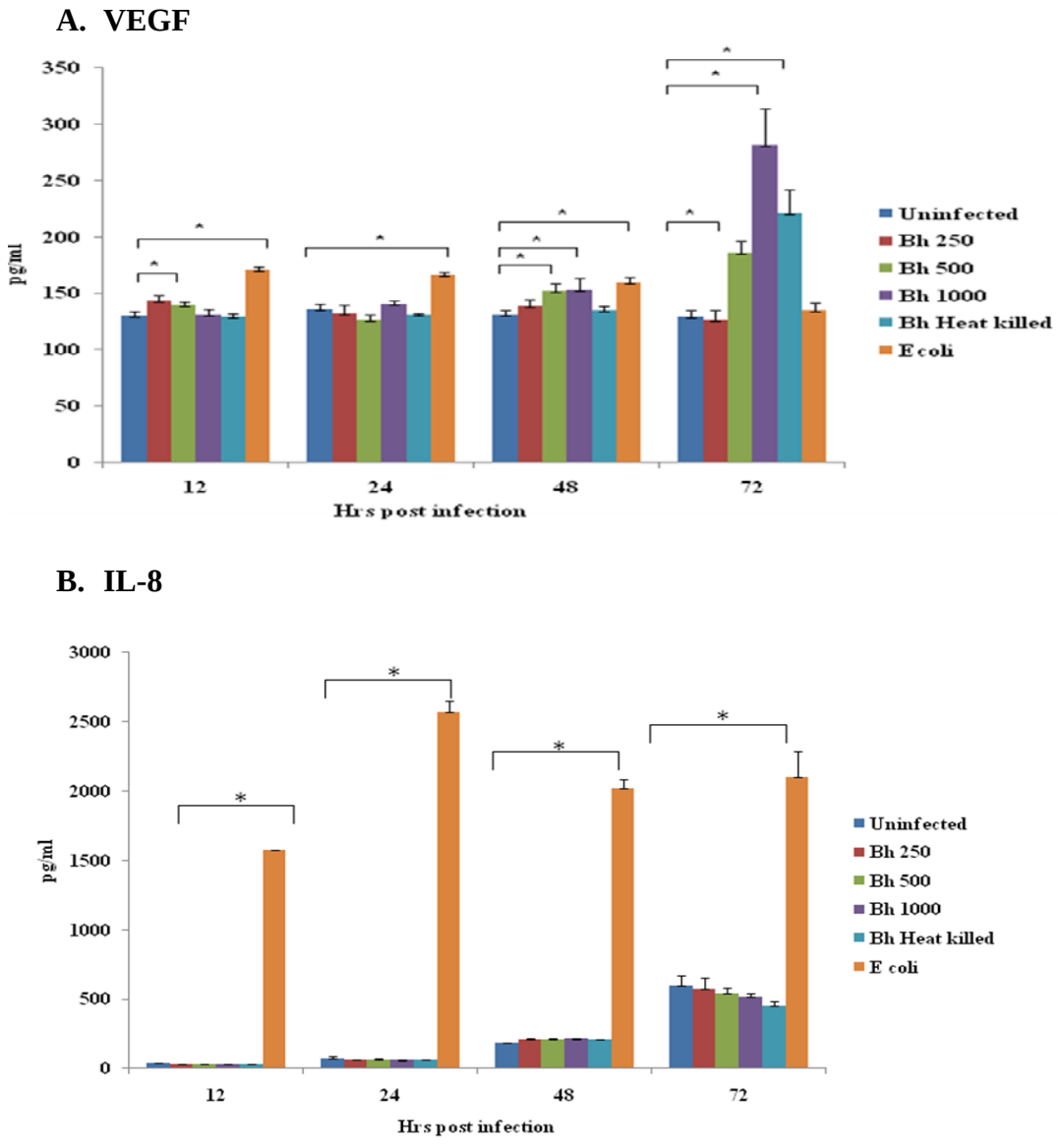

Figure 4. Cytokine production from HBVPs infected with *B. henselae.* HBVPs were infected with *B. henselae* at MOIs of 250, 500, 1000 or heat killed *B. henselae* or *E. coli*. Cells were infected for 72 hrs and supernatants were collected at time points of 12, 24, 48 and 72 hrs. Concentrations of VEGF and IL-8 concentrations were measured by chemiluminescent ELISA. Assay was done in triplicate and the experiment was repeated three times. Data from a representative experiment is shown. Data is represented as mean± SD. * $p< 0.05$

Chapter 6.

Conclusions and future directions

Conclusions and future directions

Vector-borne bacteria of the genus *Bartonella* remains understudied pathogens. *Bartonella* can induce vasoproliferative tumors such as bacillary angiomatosis, peliosis hepatis and verruga peruana, especially in immunocompromised patients. Tumors induced by *Bartonella* are benign and can be treated with antibiotics. The pathogenesis of *Bartonella*-induced vasoproliferation is not well understood. The studies presented in this thesis were aimed at improving our understanding of *Bartonella*-induced vasoproliferation. One of the reasons which slow down the efforts to dissect the molecular mechanisms involved in the *Bartonella*-induced vasoproliferation is the lack of a suitable animal model. Also *Bartonella* is highly fastidious and extremely difficult to culture in the laboratory, requiring special liquid enrichment media. As a result molecular methods like PCR have become very useful in the diagnosis of bartonellosis.

Archival paraffin-embedded tissues are readily available samples for diagnosis and retrospective research studies. We found potential DNA carryover, when paraffin-embedded tissues are used detection of *Bartonella* by PCR. *Bartonella* DNA was amplified from different parts of the microtome, necropsy room and from the tissue processor after routine cleanng. To minimize the chance of DNA carry over, biopsy specimens should be used whenever possible. Also avoid the use of microtome, by using a disposable scalpel blade to obtain samples. Including a blank paraffin block as negative control with each batch of DNA extractions is also important. Demonstration of *Bartonella* by silver staining, immunohistochemistry or fluorescent *in situ* hybridization (FISH) will help to rule out false positive PCR results. Silver staining is tedious and expensive to perform. Commercial antibodies are available only against *B. henselae,* making immunohistochemistry less valuable in case of other bartonellae. FISH utilizes, oligonucleiotides labeled with fluorescent dyes to localize specific nucleic acids. We developed probes to use for FISH on paraffin-embedded tissue. Eventhough the sensitivity of our probes were low, it may be useful for the detection of *Bartonella* in tissues when used in conjunction with other

diagnostic techniques like PCR. Future attempts should be directed towards improving the performance of the FISH probes and also to development of specific monoclonal antibodies against other *Bartonella* sp., which could be used for immunohistochemistry.

Three species of *Bartonella* (*B. bacilliformis*, *B. henselae* and *B. quintana*) are well known for their ability to induce vasoproliferative tumors. In this study we provide evidence that a fourth species, *B. vinsonii* subsp. *berkhoffii* can also induce vasoproliferation. *B. vinsonii* subsp. *berkhoffii* was detedted from blood and biopsy tissues from a dog with bacillary angiomatosis. *B. vinsonii* subsp. *berkhoffii* was also detected from the blood and tissues from a boy with epithelioid hemangioendothelioma and a dog with hemangiopericytoma. We also demonstrated that *B. vinsonii* subsp. *berkhoffii* can induce production of HIF-1 and VEGF, from HeLa 229 cells. This finding further support our hypothesis that *B. vinsonii* subsp. *berkhoffii* should be added to the list of bartonellae that can induce vasoproliferation. Further studies are warranted to determine if *B. vinsonii* subsp. *berkhoffii* have an inhibitory effect on the endothelial cell apoptosis, similar to *B. henselae* and *B. quintana.*

Spleen plays an important role in the control of blood-borne bacteria. We designed a case control study to determine the prevalence of *Bartonella*, *Babesia* and hemotropic *Mycoplasma* sp. in the splenic biopsy tissues of dogs with fibrohistiocytic nodules, hemangiosarcoma, lymphoid nodular hyperplasia and histologically normal spleens. We found a significantly higher prevalence of *Bartonella* sp. DNA in fibrohistiocytic nodules and hemangiosarcoma when compared to lymphoid nodular hyperplasia and histologically normal spleen. These findings indicate that *Bartonella* sp. may act as a cause or co-factor in the development of these conditions. Future studies are required to determine the role of *Batonella.*

In angiogenesis, along with endothelial cells pericytes also play a critical role. We infected Human Brain Vascular Pericytes with *B. henselae*. We found that *B. henselae* infection decrease proliferation of pericytes and also induce production of VEGF. Reduced pericyte coverge and increased VEGF production may help in the dissociation of pericytes

from the endothelial cells, promoting angiogenesis. These findings, along with the detection of *Bartonella* sp. from a variety of tumors, indicate that *Bartonella* may promote tumor angiogenesis, leading to tumor progression and metastasis. Future studies should be directed to determine, whether infection of tumor tissues with *Bartonella* could promote tumor angiogensis and metastasis.