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ORIGINAL INVESTIGATION

Bartonella vinsonii subsp. *berkhoffii* and *Bartonella henselae* as potential causes of proliferative vascular diseases in animals

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Abstract *Bartonella* species are highly fastidious, vector borne, zoonotic bacteria that cause persistent intraerythrocytic bacteremia and endotheliotropic infection in reservoir and incidental hosts. Based upon prior in vitro research, three *Bartonella* sp., *B. bacilliformis*, *B. henselae,* and *B. quintana* can induce proliferation of endothelial cells, and each species has been associated with in vivo formation of vasoproliferative tumors in human patients. In this study, we report the molecular detection of *B. vinsonii* subsp. *berkhoffii*, *B. henselae*, *B. koehlerae,* or DNA of two of these *Bartonella* species simultaneously in vasoproliferative hemangiopericytomas from a dog, a horse, and a red wolf and in systemic reactive angioendotheliomatosis lesions from cats and a steer. In addition, we provide documentation that *B. vinsonii* subsp. *berkhoffii* infections induce activation of hypoxia inducible factor-1 and production of vascular endothelial growth factor, thereby providing mechanistic evidence as to how these bacteria could contribute to the development of vasoproliferative lesions. Based upon these results, we suggest that a fourth species, *B. vinsonii* subsp. *berkhoffii*, should be added to the list of bartonellae that can induce vasoproliferative lesions and that infection with one or more *Bartonella* sp. may contribute to the pathogenesis of systemic reactive angioendotheliomatosis and hemangiopericytomas in animals.

Keywords Infection · Pathology · Systemic angiomatosis · Hemangiopericytoma · Bacteria

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Introduction

Bartonella species are highly fastidious, vector borne, zoonotic bacteria that cause chronic intraerythrocytic bacteremia in reservoir and incidental hosts [1–5]. Bartonellae invade endothelial cells [6–9] and are considered unique in the bacterial kingdom in their ability to promote angiogenesis in an infected host. In human patients, specific vasoproliferative pathological lesions, including verruga peruana (*B. bacilliformis*), bacillary angiomatosis (*B. henselae* and *B. quintana*), and peliosis hepatis (*B. henselae*), have been associated with infection with specific *Bartonella* species [4, 10–12]. Bacillary angiomatosis (BA) and peliosis hepatis (PH) have been reported most often in immunocompromised HIV-infected patients, suggesting that suppression of immune regulatory mechanisms plays a role in the immunopathogenesis of *Bartonella*-induced vasoproliferation [12]. *B. henselae* appears to be the most frequent species infecting dogs [13], was the most frequently detected *Bartonella* species in the dogs with splenic hemangiosarcomas [14], and has been associated with PH in one dog [15].

Bartonella vinsonii subsp. *berkhoffii* was first isolated from a dog with endocarditis in 1993 [16]. Subsequently,

based upon distinct deletions and insertions within the 16S–23S intergenic (ITS) spacer region, three other genotypes were characterized in blood or tissue samples from foxes, coyotes, dogs and human patients [17–20]. On an evolutionary basis, *B. vinsonii* subsp. *berkhoffii* appears to have co-evolved with wild and potentially domestic canines [21], whereas opportunistic infection has been identified in cats, horses (Breitschwerdt, unpublished data), and human patients [22–25]. There is circumstantial evidence to support a role for *B. vinsonii* subsp. *berkhoffii* as a cause of vasoproliferative lesions in animals and perhaps in human patients. *B. vinsonii* subsp. *berkhoffii* genotype II was isolated from the blood of a dog with a hemangiopericytoma, and organism-specific bacterial DNA sequences were amplified and sequenced from blood and a tissue biopsy from a young man residing in the United States with hepatic epitheloid hemangioendothelioma (EHE) [26]. *B. vinsonii* subsp. *berkhoffii* genotype I DNA was also amplified and sequenced from blood and BA skin tissues from an iatrogenically immunocompromised dog [27]. Subsequently, *B. vinsonii* subsp. *berkhoffii* genotype I DNA was amplified from the blood of a woman from Australia with hepatic EHE, who was either sequentially or concurrently co-infected with *B. henselae* and *B. koehlerae* [28]. *Bartonella* spp. DNA, including *B. vinsonii* subsp. *berkhoffii*, has recently been amplified and sequenced from dogs with splenic hemangiosarcomas [14]. Collectively, these observations suggested that *B. vinsonii* subsp. *berkhoffii* might induce endothelial cell proliferations in dogs, as reported for three other *Bartonella* sp. in human patients. To date, a potential role for *B. koehlerae* in the etiopathogenesis of vasoproliferative lesions has not been investigated.

Bartonella-induced vasculoproliferations seem to depend on at least three overlapping mechanisms: direct mitogenic activation of endothelial cells [29], inhibition of endothelial cell apoptosis [30, 31] and angiogenic reprogramming of infected host cells leading to the activation of hypoxia inducible factor (HIF)-1, the key transcriptional regulator of angiogenesis [32, 33], and subsequent secretion of angiogenic cytokines such as vascular endothelial growth factor (VEGF) [34, 35]. Infected endothelial cells are obviously not a major source of VEGF production following *Bartonella* infection [34, 36]. *Bartonella* infection results in endothelial cell production of MCP-1, which is a potent chemoattractant for monocytes and macrophages [37]. In vitro infection of macrophage cell lines (THP-1) with *B. henselae* triggers the production of IL-1β and VEGF, and conditioned media derived from *B. henselae*-infected host cells (macrophages, epithelial cells) induced proliferation of endothelial cells in vitro [34, 35]. Collectively, these observations support a paracrine loop of angiogenesis induced by *Bartonella*, whereby infection results in a proinflammatory response, the recruitment of macrophages and monocytes to the site of infection, and the production of proangiogenic proteins that stimulate vascular proliferation [38]. In addition to the proangiogenic capabilities, *Bartonella* inhibits apoptosis of endothelial cells and monocytes [30, 31, 39]. Whereas the inhibition of endothelial cell apoptosis is clearly a Virb/D4-type IV secretion system–driven phenomenon mainly attributed to the secretion of *Bartonella* effector protein A (BepA) [31], inhibition of monocyte apoptosis by *B. henselae* is also associated with the activation of NF-κB which regulates the transcriptional activation of inhibitors of apoptosis proteins (IAPs)-1 and 2 [39].

In this study, we report the molecular detection of *B. vinsonii* subsp. *berkhoffii*, *B. henselae,* or DNA from both *Bartonella* species in naturally occurring hemangiopericytomas from a dog, a horse, and a red wolf (*Canis lupus rufus*) and *B. henselae*, *B. koehlerae* and/or *B. vinsonii* subsp. *berkhoffii* DNA in four cats and a steer with systemic reactive angioendotheliomatosis (SRA), a rare multi-systemic intravascular proliferative disorder [40, 41]. To date, hemangiopericytoma has been described in only one horse [42]; there are less than 15 published feline SRA cases, and only a single case report describing SRA in a steer [41]. In addition to these microbiological and pathological observations, we provide documentation that *B. vinsonii* subsp. *berkhoffii* can activate HIF-1 and induce production of VEGF in vitro, as has been demonstrated earlier for *B. quintana* and *B. henselae*. Thus mechanistically, *B. vinsonii* subsp. *berkhoffii* appears to be able to contribute to the development of vasoproliferative lesions.

Methods

Animal sample sources

Due to the infrequent nature of these vasoproliferative lesions in most animal species, paraffin-embedded tissues for PCR testing were requested from multiple institutional sources. The hemangiopericytoma paraffin tissue blocks from a previously described 13-year-old female-spayed Australian Heeler (designated dog 1) [26] and a more recently referred 13-year-old female English Shepherd (dog 2) were provided by Dr. Keith Linder, pathologist, North Carolina State University. The hemangiopericytoma paraffin tissue block from a previously described 14-year-old Arabian mare was provided by Dr. Kellye S. Joiner, pathologist at Auburn University [42]. The hemangiopericytoma paraffin tissue block from a 6-year-old female red wolf was provided by Ms. Sherry Samuels, Director, Museum of Life and Science in Durham, NC and Dr. Steve Rushton, pathologist at Rollins Diagnostic Laboratory,

Raleigh, NC. This cutaneous hemangiopericytoma was located on the sternum. Paraffin tissue blocks from the four cats (designated cats 1–4) with systemic angiomatosis were provided by Dr. Jim Cooley, pathologist, Mississippi State University, Starkville, MS, and Dr. Keith Linder, pathologist, North Carolina State University. The paraffin tissue blocks from a previously described steer with SRA were provided by Dr. Melanie A. Breshears, pathologist, Oklahoma State University, Stillwater, OK [41]. For SRA cases, lesional heart, pancreas, spleen, small intestine, brain and/or liver tissue were tested.

DNA extraction, PCR, and sequencing

DNA was extracted from the paraffin-embedded tissue blocks containing the respective tumors or SRA lesions. The 16S–23S intergenic transcribed spacer (ITS) region was amplified using *Bartonella* genus-specific primers as described previously [14, 17]. The tissue samples from each animal were processed at different time points in the laboratory, and extreme care was taken to prevent DNA carry over among tissues [43]. A blank reagent control (paraffin block without tissue) was included with each set of DNA extractions, and 0.001 pg/μL of *B. henselae* (Houston I strain) DNA was used as a positive control for all PCR reactions. DNA extracted from the blood of a healthy dog with no serological or BAPGM enrichment culture evidence of *Bartonella* infection was included as the negative control. To avoid DNA carryover in the laboratory, PCR sample preparation, DNA extraction, and PCR amplification and analysis were performed in three separate rooms with a unidirectional work flow. All PCRs were performed in the Intracellular Pathogens Research Laboratory, Raleigh, NC. DNA was not amplified in the negative control lane during the course of this study. PCR positive amplicons were sequenced (Eton Biosciences, Research Triangle Park, North Carolina) to establish the species, strain, or genotype. Sequences were compared with those available in GenBank using BLAST and aligned using the program VectorNTI AlignX (Invitrogen™, Life Technologies Corporation, USA) to determine the percentage similarity.

Bacterial isolates for cell culture experiments

Bartonella vinsonii subsp. *berkhoffii* genotypes I, II, and III [16, 20, 44] were used for the cell culture infection experiments. *B. vinsonii* subsp. *berkhoffii* genotype I (NCSU isolate 93-CO-1, ATCC #51672) was isolated from a dog with endocarditis [16], *B. vinsonii* subsp. *berkhoffii* genotype II from the blood of a coyote (DS-535-GG54) [44], and *B. vinsonii* subsp. *berkhoffii* genotype III (NCSU isolate 06-CO-1) from the blood of a military working dog diagnosed with endocarditis [20]. Bacteria were grown on Columbia Sheep Blood agar (Becton–Dickinson, Heidelberg, Germany) at 37 °C at a 5 % CO2 concentration. Bacterial colonies were harvested after 3 days of growth and washed extensively with PBS (Gibco, Karlsruhe, Germany). Bacterial numbers were determined by measuring the optical density at a wavelength of 600 nm (OD of 1.0 = $\sim 5 \times 10^8$ bacteria).

Cell culture and infection of HeLa 229 cells

For infection experiments, HeLa 229 cells (human cervical carcinoma cells) were grown in RPMI 1640 supplemented with 2 g/L NaHCO3 (Biochrom, Berlin, Germany), 10 % heat-inactivated fetal calf serum (FCS; Sigma Aldrich, Taufkirchen, Germany), 1 % L-glutamine (Gibco, Karlsruhe, Germany), and 10 mg/mL streptomycin and 100 U penicillin (Biochrom, Berlin, Germany) as described earlier [32, 33].

For the determination of HIF-1 activation, 5.0×10^5 cells were seeded in 6-well plates. For the determination of secreted cytokines, 1.0×10^5 cells were seeded in 24-well plates. *B. henselae* was used at a MOI of 250 or 500 for HIF-1 activation (6 h) and VEGF secretion (48 h). Bacteria were centrifuged onto cultured cells for 5 min at 300 g. Uninfected cells were used as negative controls, and desferrioxamine (DFO, 200 μM, Sigma) was used as positive control [45].

HIF-1α immunoblotting

For the detection of HIF-1 activation by immunoblotting, proteins from cell cultures were extracted as described [46], separated by 8 % SDS-PAGE, and blotted onto polyvinylidene difluoride (PVDF) membranes (Millipore, Schwalbach, Germany). Monoclonal mouse anti-HIF-1α antibodies (Becton–Dickinson, Heidelberg, Germany) were used as primary antibodies and horseradish peroxidase (HRP)-conjugated rabbit anti-mouse IgG antibodies (Dako, Hamburg, Germany) as secondary antibodies. Signals were visualized with the enhanced chemiluminescent (ECL)-reagent (PJK, Kleinbittersdorf, Germany). For loading control, monoclonal mouse actin-specific antibodies (Sigma Aldrich) were used as described [46].

Quantification of VEGF

VEGF concentrations were measured from cell culture supernatants by ELISA (R&D systems, Wiesbaden, Germany) according to the manufacturer's instructions [32].

Statistical analysis

All cell culture experiments were performed at least three times and revealed comparable results. Differences

between mean values of experimental and control groups were analyzed by Student's t test. A value of $p < 0.01$ was considered statistically significant.

Results

Bartonella PCR amplification and DNA sequencing

Hemangiopericytomas

Bartonella vinsonii subsp. *berkhoffii* genotype I, II, and III DNA sequences were amplified from hemangiopericytoma tissues obtained from dog 1, the mare, and the red wolf, respectively (Table 1). The mare was co-infected with *B. henselae*, whereas only *B. henselae* DNA was amplified from the hemangiopericytoma from dog 2. As the histopathology from the red wolf has not been previously published, the following description is provided: The dermis and subcutis contained an unencapsulated, poorly circumscribed, multi-nodular mass composed of densely packed neoplastic spindle cells, which form short interlacing bundles and whorls around capillary and arteriolar-type blood vessels in scant collagenous stroma (Fig. 1). The neoplastic cells have variably distinct cell borders and little eosinophilic fibrillar cytoplasm. Nuclei range from irregularly round to oval, contain finely stippled, marginated, or condensed chromatin and 1 to 2 variably distinct nucleoli. Five mitotic figures were observed in ten high magnification (400×) microscopic fields. Multifocal coagulative necrosis was present.

Systemic reactive angioenotheliomatosis

A representative cardiac lesion from a cat with systemic reactive angioendotheliomatosis is illustrated in Fig. 2. *B. vinsonii* subsp. *berkhoffii* DNA was amplified and sequenced from SRA lesions from two of the four cat (cats 1 & 2) tissues tested (Table 2). Cat 1 was co-infected with *B. henselae*, whereas cat 2 was co-infected with *B. koehlerae*. Only *B. henselae* DNA was amplified from SRA lesions from cats 3 and 4 or from the SRA lesions in the steer.

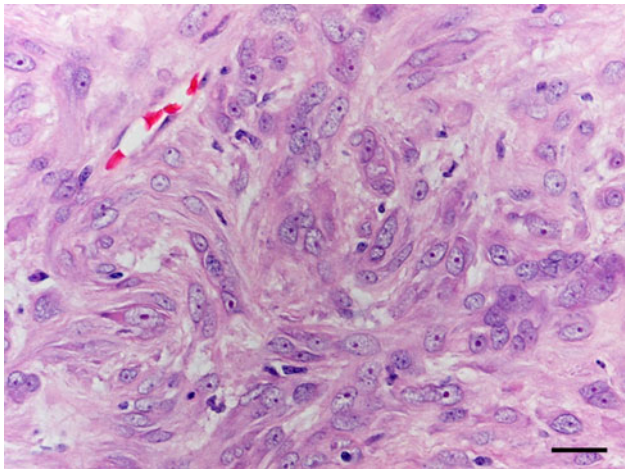

Fig. 1 Photomicrograph illustrates a subcutaneous hemangiopericytoma from a red wolf composed of neoplastic spindle cells that are moderately pleomorphic and are accompanied by numerous small blood vessels. Hematoxylin and Eosin. ×400 magnification. *Bar* = 20 μm

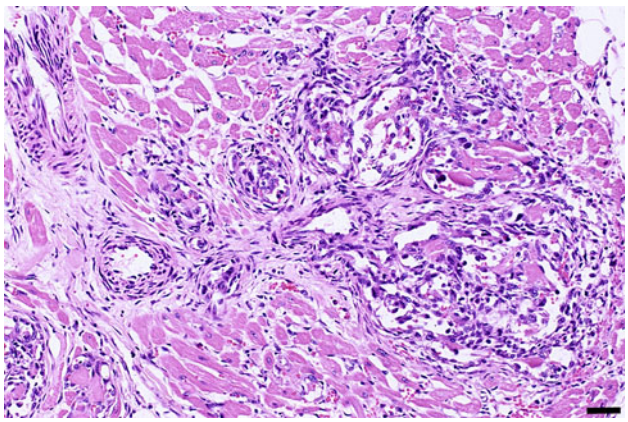

Fig. 2 Photomicrograph illustrates lesions of systemic reactive angioendotheliomatosis in the heart of a cat. Proliferative small vessels displace cardiac myocytes and contain small amounts of fibrin. Hematoxylin and Eosin. ×200 magnification. *Bar* = 100 μm

Table 1 *Bartonella* species DNA amplified and sequenced from blood or paraffin-embedded tissues from various animal species with hemangiopericytomas

Animal designation	Histopathological diagnosis	*Bartonella* spp.	Percentage similarity/GenBank accession number
Dog 1	Hemangiopericytoma	*B. vinsonii* subsp. *berkhoffii* genotype II	99.3 % similarity to DQ059763
Dog 2	Hemangiopericytoma	*B. henselae*	97.8 % similarity to AF369529
Horse	Hemangiopericytoma	*B. vinsonii* subsp. *berkhoffii* genotype I	99.3 % similarity to AF167988
		B. henselae	100 % similarity to AF369529
Red wolf	Hemangiopericytoma	*B. vinsonii* subsp. *berkhoffii* genotype III	97.6 % similarity to EU295657.1

Table 2 *Bartonella* species DNA amplified and sequenced from paraffin-embedded tissues from four cats and a steer with systemic reactive angioendotheliomatosis

Animal designation	Histopathological diagnosis	*Bartonella* sp.	Percent similarity/GenBank accession number
Cat 1	SRA	*B. vinsonii* subsp. *berkhoffii* genotype III	100 % similarity to EU295657.1
		B. henselae	99.8 % similarity to AF369529
Cat 2	SRA	*B. vinsonii* subsp. *berkhoffii* genotype III	99.6 % similarity to EU295657.1
		B. koehlerae	99.8 % similarity to AF312490.1
Cat 3	SRA	*B. henselae*	99.8 % similarity to AF369529
Cat 4	SRA	*B. henselae*	99.8 % similarity to AF369529
Steer	SRA	*B. henselae*	99.4 % similarity to AF369529

SRA systemic reactive angioendotheliomatosis

Activation of HIF-1 and induction of VEGF secretion by B. vinsonii subsp. berkhoffii

Activation of HIF-1 and subsequent secretion of VEGF are key events in the angiogenic cascade [47] and are also detectable in *B. henselae* infections [34, 39]. We analyzed whether infections with *B. vinsonii* subsp. *berkhoffii* also would result in such an angiogenic reprogramming of host cells. For this purpose, HeLa 229 cells were infected with *B. vinsonii* subsp. *berkhoffii* genotypes I, II, and III at MOI of 250 (data not shown) and 500 for 24 or 48 h. Infections with *B. vinsonii* subsp. *berkhoffii* genotypes I and II resulted in a strong activation of HIF-1 at 6 h post-inoculation of HeLa 299 cells (Fig. 3; as determined by HIF-1α immunoblotting). Similar results were obtained upon infection with *B. vinsonii* subsp. *berkhoffii* genotype III (data not shown). Accordingly, all three *B. vinsonii* subsp. *berkhoffii* genotypes were capable of inducing VEGF production from infected HeLa 229 cells (∼5.7-fold for *B. vinsonii* subsp. *berkhoffii* genotype I, ∼6.3-fold for *B. vinsonii* subsp. *berkhoffii* genotype II (Fig. 3b) when compared with the uninfected controls. Similar results were obtained for *B. vinsonii* subsp. *berkhoffii* genotype III (∼3.7-fold).

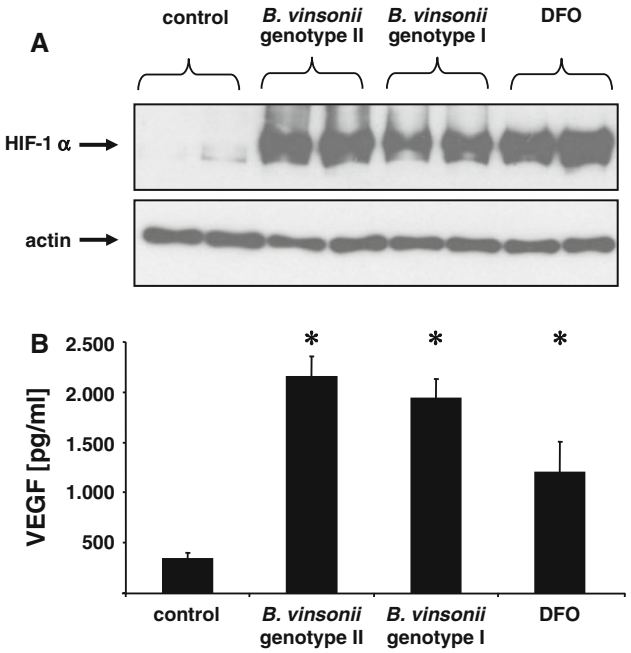

Fig. 3 Angiogenic host cell reprogramming by *B. vinsonii* subsp. *berkhoffii*. **a** HIF-1 activation by *B. vinsonii* subsp. *berkhoffii* genotype I and II. HIF-1α was detected 6 h upon infection by immunoblotting using anti-HIF-1α-Abs (loading control: actin). **b** Induction of VEGF secretion upon infection of HeLa 229 cells. Supernatants were taken 48 h after infection and analyzed by ELISA. *Significant difference between *B. vinsonii* subsp. *berkhoffii* infected cells ($p < 0.01$); negative control: uninfected cells; positive control: DFO (desferrioxamine; 200 μM)

Discussion

Based upon prior research, *B. bacilliformis*, *B. henselae,* and *B. quintana* can induce in vitro proliferation of endothelial cells and cause in vivo formation of vasoproliferative tumors in human patients [4, 7]. The findings in this study strengthen the hypothesis that *B. vinsonii* subsp. *berkhoffii* should be added to the list of *Bartonella* spp. that can contribute to the induction of vasoproliferative lesions in animals. Recent in vivo evidence supporting a role for *B. vinsonii* subsp. *berkhoffii* in vasoproliferative lesions has been generated by amplifying and sequencing the bacteria's DNA from a spectrum of vasoproliferative lesions in dogs, including hemangiopericytoma [26], bacillary angiomatosis [27], and hemangiosarcoma [14]; from cats with systemic reactive angioendotheliomatosis (this study); and from EHE human patients [26, 28]. PCR amplification or isolation of *B. vinsonii* subsp. *berkhoffii* from a dog, a horse, and a red wolf with hemangiopericytomas suggests that this bacterium may contribute to the development of this tumor across animal species. Also, amplifying DNA of three different *B. vinsonii* subsp.

berkhoffii genotypes (I, II, and III) in vasoproliferative lesions derived from multiple animal species would be an unexpected random event. In the context of feline infection, *B. vinsonii* subsp. *berkhoffii* genotype II bacteremia has been described in one cat with recurrent osteomyelitis [43], and infection with *B. vinsonii* subsp. *berkhoffii* genotype III is reported for the first time in two cats with SRA in this study. Since *B. vinsonii* subsp. *berkhoffii* has been associated with a wide spectrum of disease manifestations in dogs and human patients, including arthritis, endocarditis, osteomyelitis, and neurological manifestations [13, 18, 19, 21, 24, 43, 48], development of vasoproliferative lesions is most likely multi-factorial and, therefore, not solely related to infection with *B. vinsonii* subsp. *berkhoffii*. Based upon existing data, genotype IV has only been found in the north central United States and Canada; thus, a limited geographic distribution for this genotype may explain why it has not been isolated to date or found in animals with vasoproliferative lesions [18] (Breitschwerdt, unpublished data).

It is also of potential pathological relevance that DNA from more than one *Bartonella* species has been found in human patients with epithelioid hemangioendothelioma [28], in dogs with splenic hemangiosarcomas [14], and in the hemangiopericytoma from the Arabian mare and two of the four SRA cats in this study, suggesting that co-infection may represent an additive risk factor for the induction of a vasoproliferative lesion in vivo. As previously described, DNA extraction of two *Bartonella* sp. from a patient sample can result in selective amplification of only one species [22]; thus, co-infection may have been more frequent than was documented in this and previous PCR-based studies. Also, false-negative PCR results can be caused by prolonged periods of formalin fixation, which induces DNA cross-linking, and from administration of antibiotics prior to sample collection, which could reduce bacterial load in the tissue sample. As a limitation of this study, our laboratory previously reported *Bartonella* sp. DNA carryover during the collection and processing of paraffin-embedded animal tissue samples [23]; thus, DNA carryover during necropsy collection or processing of tissues for histopathology cannot be ruled out. However, infection with *Bartonella bovis* is prevalent in beef cattle in the United States, whereas amplification of *B. henselae* DNA from the steer in this study, although not unprecedented, occurs very infrequently and most likely represents an opportunistic infection in cattle [49]. Whether *Bartonella* spp. directly cause tumor angiogenesis or alternatively these bacteria are attracted to pre-existing sites of neoplastic inflammation [50], thereby explaining detection of *Bartonella* spp. DNA in these animals, is yet to be determined. In support of causation, antibiotic administration has resulted in resolution of bacillary angiomatosis in immunocompromised human patients [12] and BA lesions in a therapeutically immunocompromised dog [27].

In vitro evidence from this study indicates that all three *B. vinsonii* subsp. *berkhoffii* genotypes are capable of inducing the activation of HIF-1 from HeLa 229 cells subsequently leading to the production of VEGF, a potent endothelial mitogen [34]. Although studied in greater detail in recent years, the molecular mechanisms by which *Bartonella* spp. induce vasoproliferation in animals and human patients remain incompletely understood [4, 5]. Unfortunately, as an animal model of bacillary angiomatosis has not been developed to date, most current knowledge has been gathered from studies using cell culture systems [4]. Increased expression of VEGF is found in patients with verruga peruana, bacillary angiomatosis, and peliosis hepatis [34, 51] and has also been reported in association with vascular tumors including hemangiosarcoma, epithelioid hemangioendothelioma and hemangiopericytoma [52–54]. Future studies focussing on patients with vasoproliferative diseases should combine VEGF measurements with highly sensitive isolation and PCR amplification techniques for the detection of *Bartonella* sp. infection.

In conclusion, this study provides additional evidence that a fourth species, *B. vinsonii* subsp. *berkhoffii*, should be added to the list of bartonellae that can induce vasoproliferative lesions in animals. In addition, co-infection with more than one *Bartonella* sp. may induce additive risk. Potentially, a common mechanism involving triggering of angiogenic reprogramming of host cells by *Bartonella* spp. (*B. henselae*, *B. vinsonii* subsp. *berkhoffii,* and others) might underlie a spectrum of vasoproliferative lesions in animals and human patients.

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