Acute and Late *Bartonella henselae* Murine Model Infection

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Abstract

Bartonella spp. are fastidious gram-negative neglected bacilli with worldwide distribution. They are able to cause intraerythrocytic and potentially fatal infection. Cats and dogs are reservoirs of some species of these agents. Blood-sucking arthropods are potential vectors. Our aim was to evaluate the blood, skin, liver, and spleen in BALB/c mice by using molecular tests and confocal microscopy to demonstrate *Bartonella henselae* infection in the bloodstream and organs after 4 and 21 days of intraperitoneally injected bacterial suspension. We demonstrate that the occurrence of infection in organs precedes the detectable infection in blood. Therefore, late manifestation in blood may be another challenge in early detection and diagnosis of *B. henselae* infection.

Keywords: Bartonella, diagnosis, vector-borne, zoonotic

BARTONELLA SPP. ARE neglected reemerging pathogens that have been associated with a wide range of human diseases. The most known manifestation is the cat scratch disease (David et al. 2014). Furthermore, *Bartonella* spp. can cause chronic or even fatal infection. Cyclic bacteremia is a hallmark in cats and even in human beings as we observed in trench fever (Siciliano et al. 2015). Diagnosis is still a major problem in *Bartonella* sp. infection (Chomel et al. 2009a) and *Bartonella henselae* can lead to a range of manifestations, including bacteremia and endocarditis (Harms and Dehio 2012).

Harms and Dehio (2012) mention in their review that “upon inoculation the bartonellas are not capable of directly colonizing erythrocytes.” A primary niche seems to be necessary for a second step, the blood infection. In another study, acute BALB/c mouse infection was observed in liver, spleen, and kidney 7 days after inoculation with *Bartonella birtlesii* (Deng et al. 2012).

Therefore, our aim was to investigate the presence of *B. henselae* in blood, skin, liver, and spleen at acute and late infection in BALB/c mice.

We performed the experiment using 16 isogenic, female, 12-week-old BALB/c mice (20–22 g of weight). The mice

were randomly divided in two groups ($n=8$), the infected and the control groups. Four mice from each group were euthanized after 4 and 21 days postinoculation. Institutional Animal Care and Use Committee of the University of Campinas approved all experimental protocols (2847-1) before conducting the study.

A bacterial suspension was prepared using *B. henselae* (Houston 1, ATCC 49882T; American Type Culture Collection, Rockville, MD) with 10^6 Colony Forming Units (CFU)/mL in saline solution.

We infected eight mice by intraperitoneal injection of 30 μ L of the bacterial suspension on day zero of the experiment. The inoculum was about 10^4 CFU of the bacterium. We inoculated the same volume of sterile saline solution in the other eight mice (control group). After 4 and 21 days postinoculation, we euthanized and collected blood samples by intracardiac puncture and also collected skin, spleen, and liver samples from the infected and the control mice.

We extracted DNA from these samples using RTP Bacteria DNA Mini Kit (Strattec Molecular), as recommended by the manufacturer, and performed *Bartonella* genus-specific conventional PCR targeting 16S-23S ribosomal RNA intergenic spacer (Diniz et al. 2007). We also performed *B. henselae*

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species-specific nested PCR targeting *FtsZ* region using primers previously described by Kawasato et al. (2013). To prevent contamination, we followed the technique described by Pitassi et al. (2015). The average values of concentration and 260/280 ratio of DNA were adequate. The DNA samples, which were negative for *B. henselae*, were submitted to a conventional PCR for a constitutive gene expression, and the chosen region was a fragment glyceraldehyde-3-phosphate dehydrogenase (GAPDH). All of *B. henselae* negative samples were positive in the GAPDH PCR.

For confocal microscopy, skin, liver, and spleen samples were fixed in 4% phosphate-buffered saline (PBS) formalin for 24 h immediately after euthanasia. Fixed samples were dehydrated in ethanol, cleared in xylene, and embedded in paraffin blocks. Ten consecutive series of microtome sections, 6- μ m thick, were mounted on poly-L-lysine-coated slides (Sigma, St Louis, MO). After deparaffinization and hydration in an alcohol to water gradient, the slides were subjected to antigen retrieval by immersion in citrate buffer (pH 6.0) and heated in the microwave at maximum power for 1 min, at average power for 9 min, and washed in PBS (pH 7.2). After a blocking stage, 5% goat and 5% donkey sera, the samples were incubated with a mouse monoclonal antibody (H2A10) against *B. henselae* at a 1:100 dilution (ab 704-250; Abcam) and Goat Anti-Type IV Collagen 1:50 (134001; Southern Biotech) overnight followed by incubation of the secondary antibodies, Alexa Fluor[®] 555 (ab 150118; Abcam) Goat anti-Mouse (1:1000) and Donkey anti-Goat DyLight[®] 650 (ab 96934; IgG H&L) (1:100). The slides were mounted with VECTASHIELD (Vector Laboratories) and observed using Leica TCS SP5 II confocal microscope.

Our sensitivity in conventional PCR was 50 genome equivalent (GE) per tube, and in nested PCR it was 10 GE. Molecular tests from spleen, liver, and skin were positive 4 days after experimental infection in some samples (Table 1). Blood samples from all mice were PCR positive after 21 days post-*B. henselae* inoculation. A skin sample from one out of four infected animals was also confocal microscopy positive after 21 days.

Karem et al. (1999) used a BALB/c *B. henselae*-infection model. They followed the animals for 7 days with PCR and could not detect bloodstream infection. Liver, spleen, and kidney samples were positive from 72 h up to 7 days after experimental infection. In a previous study, we found similar results, after *B. henselae*-infected blood transfusion, the bloodstream infection was not detected with 4 days (Silva et al. 2016). In this current study, our aim was to evaluate BALB/c mice on 4 and 21 days after *B. henselae* intraperitoneal inoculation, and our findings confirm that the detection of *B. henselae* in blood only happened on day 21 after inoculation when the liver and spleen were already negative. *Bartonella* spp. are able to define a new niche after blood-step infection probably related to their genome repertoire and host characteristics (Chomel et al. 2009b, Harms and Dehio 2012). *Bartonella* spp. can infect heart valves (as in the endocarditis caused by *B. henselae* and *Bartonella quintana*), liver, spleen (bacillary angiomatosis and/or peliosis, caused by *B. henselae*), and skin (as in the Peruvian wart caused by *Bartonella bacilliformis* and bacillary angiomatosis caused by *B. henselae* and *B. quintana*) (Harms and Dehio 2012). It is likely that the microenvironment in the kidney, spleen, and liver provides a niche for homing of bacteria, where they grow. Subsequently, they are dispersed in the blood stream in a relatively larger amount, thus becoming feasible to detect. In conclusion, our data demonstrate that it was feasible to detect *B. henselae* in the bloodstream of infected subjects, but this occurred 3 weeks following the intraperitoneal inoculation. However, on day 4 postinfection, the inability to detect the bacteria in the bloodstream clearly demonstrates that it grows slowly initially in the tissues and is transmitted via circulation after a significant incubation period *in vivo*.

B. henselae bloodstream infection happened later in this mouse model. The number of DNA copies in the blood was probably low with 21 days of experimental infection because it was positive just with nested PCR. Further investigations, using real-time PCR, could quantify the bacteremia. The results of this translational study reinforce that the diagnosis

TABLE 1. DIAGNOSIS RESULTS OF THE MOLECULAR TECHNIQUES AND CONFOCAL MICROSCOPY USED TO DETECT *BARTONELLA HENSELAE* INFECTION

	Conventional ITS ^a PCR				Nested FtsZ ^b PCR				Confocal microscopy			
	Blood	Spleen	Liver	Skin	Blood	Spleen	Liver	Skin	Blood	Spleen	Liver	Skin
4 days												
C ₁₋₄	—	—	—	—	—	—	—	—	—	—	—	—
I ₁	—	—	—	—	—	—	—	—	—	—	—	—
I ₂	—	—	—	—	—	+	+	+	—	—	—	—
I ₃	—	—	—	—	—	+	—	—	—	—	—	—
I ₄	—	—	—	—	—	—	—	—	—	—	—	—
21 days												
C ₅₋₈	—	—	—	—	—	—	—	—	—	—	—	—
I ₅	—	—	—	—	+	—	—	—	—	—	—	—
I ₆	—	—	—	—	+	—	—	—	—	—	—	—
I ₇	—	—	—	—	+	—	—	—	—	—	—	+
I ₈	—	—	—	—	+	—	—	—	—	—	—	—

^a16S-23S rRNA gene intergenic transcribed spacer.

^bCell division protein.

C, control mice; I, infected mice.

of *Bartonella* sp. infection remains a challenge and that even molecular methods do not have enough sensitivity to detect low copy numbers of the bacteria. Time-dependent multistep platform must be used to minimize the impact of these limitations. Our study reinforces that liver and spleen may be a first step in *Bartonella* sp. infection and that bloodstream infection seems to be a second step in this infection.

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Author Disclosure Statement

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