EXPERIMENTAL INFECTION AND HORIZONTAL TRANSMISSION OF
Bartonella henselae IN DOMESTIC CATS

Marcelo de Souza ZANUTTO(1), Elza Masae MAMIZUKA(2), Roberto RAIZ-JÚNIOR(3), Thais Martins de LIMA(2), Constância Lima DIOGO(3), Thelma Suely OKAY(3) & Mitika Kuribayashi HAGIWARA(1)

SUMMARY

In order to study *B. henselae* transmission among cats, five young cats were kept in confinement for two years, one of them being inoculated by SC route with *B. henselae* (10^5 UFC). Only occasional contact among cats occurred but the presence of fleas was observed in all animals throughout the period. Blood culture for isolation of bacteria, PCR-HSP and FTSZ (gender specific), and BH-PCR (species-specific), as well as indirect immunofluorescence method for anti-*B. henselae* antibodies were performed to confirm the infection of the inoculated cat as well as the other naive cats. Considering the inoculated animal, *B. henselae* was first isolated by blood culture two months after inoculation, bacteremia last for four months, the specific antibodies being detected by IFI during the entire period. All contacting animals presented with bacteremia 6 months after experimental inoculation but IFI did not detect seroconversion in these animals. All the isolates from these cats were characterized as *Bartonella* (HSP and FTSZ-PCR), *henselae* (BH-PCR). However, DNA of *B. henselae* could not be amplified directly from peripheral blood by the PCR protocols used. Isolation of bacteria by blood culture was the most efficient method to diagnose infection compared to PCR or IFI. The role of fleas in the epidemiology of *B. henselae* infection in cats is discussed.

KEYWORDS: Cat; Felines; *Bartonella henselae; Bartonella quintana;* Hemoculture; Polymerase Chain Reaction; Indirect immunofluorescence.

INTRODUCTION

Cat-scratch disease (CSD) is transmitted to human beings by scratching, licking or bitting of cats harboring *B. henselae*. Also, fleas (*Ctenocephalides felis*) are thought to be involved in transmission. After infection of fleas, fecal elimination of viable bacteria occurs[11]. Bacteremia has already been reported in cats after intradermal inoculation of feces from contaminated fleas[8].

Classic CSD is characterized by regional lymphoadenopathy, which corresponds to the draining route of bacteria inoculation site. Atypical CSD may occur, mainly in immunodepressed patients, as bacillary angiomatosis and peliosis, endocarditis, recurrent fever and bacteremia with ocular, hepato-splenic, skeletal, cutaneous or pleuro-pulmonar symptoms[6].

The cat is the reservoir, and fleas are possibly the vectors of bacteria. The cat is the only animal undoubtedly involved in *Bartonella henselae* transmission[1,5]. Young animals less than one year old, are the animals presenting the highest transmission potential and are generally asymptomatic[1,20]. Occasional fever, lethargy, regional lymphoadenopathy,

edema and abscess formation at the site of inoculation[1,17,18,20], besides histological alterations characterized by lymphoplasmocytic infiltrate in liver, kidneys, lymphonodes and heart occur in chronically affected animals[13].

Infected felines bacteremia may last for a variable period of time, and can also be intermittent[1,5,8,13,14,17,18,20]. Isolation of *Bartonella* is done by blood culture even though bacteria growth in appropriate medium is fastidious. After natural or experimental infection, cats may develop humoral immunity with specific IgG antibodies production detected by enzyme-linked immunosorbent assays or IFI[1,5]. Serological tests specificity seems to be unsatisfactory due to cross reactions with *B. quintana* antigens (etiological agent of trench fever, bacillary angiomatosis and peliosis, transmitted to man by lice)[15,19]. Frequently, antibody anti-*B. henselae* and anti-*B. quintana*, can both be found in the same animal, pointing out the low specificity of IFI[16,20].

The identification of species and strains of *Bartonella* sp is based on molecular characterization of isolates or in infected clinical samples[2,5,8,9,12,13,14,17,18,20]. Therefore, PCR, a more sensitive method in comparison to hemoculture, might be used to detect *Bartonella* and type species[13].

(1) Departamento de Clínica Médica da Faculdade de Medicina Veterinária e Zootecnia da Universidade de São Paulo, São Paulo, SP, Brasil.
(2) Laboratório de Microbiologia do Departamento de Análises Clínicas da Faculdade de Ciências Farmacêuticas da Universidade de São Paulo, São Paulo, SP, Brasil.
(3) Laboratório de Investigação Médica (LIM36) da Faculdade de Medicina, da Universidade de São Paulo, São Paulo, SP, Brasil.
Correspondence to: Marcelo de Souza Zanutto, Rua Manoel Joaquim Pera 85, 05539-030 São Paulo, SP, Brasil. E-mail: mszanutto@hotmail.com or mzanutto@usp.br

Because of the unique role of cats in the epidemiology of CSD disease, the aim of this study was to analyze the susceptibility of young cats to *Bartonella henselae* infection, as well as its horizontal transmission, so that blood cultures were used to isolate *B. henselae*, three different PCR protocols were used to detect and characterize isolates, and IFI detected serum specific anti-*B. henselae* antibodies thus confirming seroconversion.

MATERIAL AND METHODS

Animals: Five healthy, naive cats were kept, from 2 months old until 2 years, in a confined environment, with sporadic contact among them. All animals were free of parasites, immunized against Panleukopenia, Herpesvirus, Calicivirus and Rabies. At the age of 6 months, one cat was challenged with 10^5CFU of viable *B. henselae* (Houston strain), by SC route and submitted to clinical evaluation every 2 – 3 days, for one year. Blood samples were collected assepticaly by jugular venipuncture for hemoculture (1.0 mL in Isolator pediatric tubes Oxoid®)[3], PCR (1.0 mL in EDTA tubes) and IFI (1.0 mL in gel separator tubes) on days 7, 14, 30, 54, 68, 90, 120, 150, 185, 210, 240 and 360 after inoculation. The remaining cats were submitted to physical examination and blood sampling at age 2, 4, 6, 12, 18, 20, 22 and 24 months.

Hemoculture: Blood (1.0 mL) was transferred from isolator pediatric tube (Oxoid®) into microtubes and centrifuged at 503 g for 3 minutes; 50 µL of sediment were placed on brain-heart infusion agar (Oxoid®) supplemented with 5% of defibrinated sheep blood[3]. Plates were kept at 35 °C in a 5% CO_2 atmosphere (CO_2 Incubator Sanyo® - model MCO-15 A). The presence of bacterial growth was assessed weekly during five weeks.

Polymerase Chain Reaction: DNA extraction was performed in blood samples according to the technique described by FERRIE *et al.*[7]. A scrapping of all colonies was used to extract DNA from the isolate. Briefly, 500 µL of a NaOH solution (50 mM) were added to the sample which was then incubated at 100 °C for 5 minutes; centrifuged at 15,300 g at 4 °C for 4 minutes; and 100 µL of TRIS-HCl (1M, pH 7.5) were added. The final concentration of DNA in the supernatant used was 1 µg/50 µL and 10 ng/50 µL for peripheral blood and isolates respectively.

HSP and FTSZ-PCR protocols, which amplify a segment of the gene that encodes a 60KDa heat shock protein (HSP), producing a 414bp segment[2], and a gene segment that encodes the cell division protein FtsZ, producing a 1,580 bp product, respectively[12], as well as, a pair of species-specific primers (BH-PCR) which amplifies a 354 bp fragment of *B. henselae* (also belongs to the FtsZ gene[12]), were used.

To a 50 µL of final volume, 200 µM dNTP, 0.4 µM of each primer, 2.5U of Taq DNA polymerase (Life Technologies®), 1X (200 mM Tris-HCl, 500 mM KCl, pH = 8.4) buffer, 1.5 mM of $MgCl_2$ (FTSZ and BH-PCR) and 2 mM (HSP-PCR) were added. Amplifications were performed in a PTC-150 Minicycler (MJ Research"): HSP-PCR began with a initial denaturation step (94 °C for 5 minutes), followed by 35 cycles of 94 °C for 1 minute, 55 °C for 2 minutes and 72 °C for 3 minutes, and a final extension at 72 °C for 10 minutes. FTSZ and BH amplifications were performed as follows: 30 cycles of 94 °C for 1 minute, 60 °C for 1 minute (FTSZ-PCR) and 55 °C for 1 minute (BH-PCR), and 72 °C for 3 minutes, followed by a final extension at 72 °C for 30 minutes.

Primers NS-31 and NS-41 that amplify a 652 bp fragment of a gene encoding a 18S ribosomal subunit of all eukaryotic organisms, were used to ensure the quality of extracted DNA[10].

As the positive control, DNA extracted from *B. henselae* Houston strain and *B. quintana* Oklahoma strain were used. Negative controls consisted of sterile distilled water instead of DNA.

HSP amplified products were submitted to a 2% agarose gel electrophoresis, and 1% agarose gels for FTSZ and BH (Life Technologies®), in a TAE X1 buffer, for 1 hour at 80V. Molecular weight standards of 100 bp and 1Kb were used (Life Technologies®). Gels were stained with ethidium bromide (0.5 µg/mL), visualized in a UV transiluminator (Hoefer - Amersham Pharmacia Biotech®), and photographed using a Polaroid Camera (Gel Cam Sigma®), T-667 films (Polaroid®).

Indirect Immunofluorescence Test (IFI): IFI was done according to CAMARGO[4]. *Bartonella henselae* Houston strain (co-cultivated in VERO cells, National Reference Center in Rickettsiosis, Marseille, France), was used as antigen, and sheep globulin anti-feline IgG (Sigma®), marked by fluoresceine isothiocyanate as the conjugate. Anti-*B. henselae* hyperimmune rabbit serum and the respective anti-rabbit IgG control (Sigma®) were used as positive controls. The antibody titer of a sample was considered to be the highest dilution in which fluorescence could be observed. Antibody titer ≥ 64 was adopted as the cut-off value for IFI reaction because sensitivity and specificity of the technique are adequate when titers are ≥ 64[19].

RESULTS

B. henselae inoculation by SC route in one of the cats (animal 1) resulted in bacteremia and the isolation of the agent by hemoculture two months (68 days) after inoculation. Morphology and staining characteristics of the colonies (Fig. 1), were suggestive of *Bartonella* sp, and identity of the isolate was confirmed by PCR, using gender specific primers (2 protocols) (Fig. 2) and species-specific primers (1
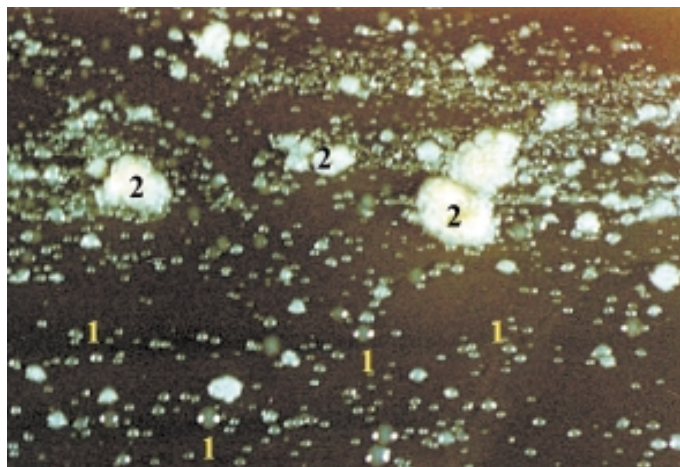

Fig. 1 - *B. henselae* colonies, after 14 days of culture. **1:** Circular brownish colonies,with a humid aspect, sticking to the agar; **2:** Whitish colonies of irregular, wrinkled and dry appearance.

protocol). Bacteremia lasted for 120 days, as seen in Table 1. Serum specific antibodies (IFI) were detected 30 days after inoculation, with IgG titers rising and falling in parallel with bacteremia (Table 1).

Bacteremia was observed in all cats that shared the same environment beginning four months after the inoculation of the first animal and present for a variable period of time (Table 2).
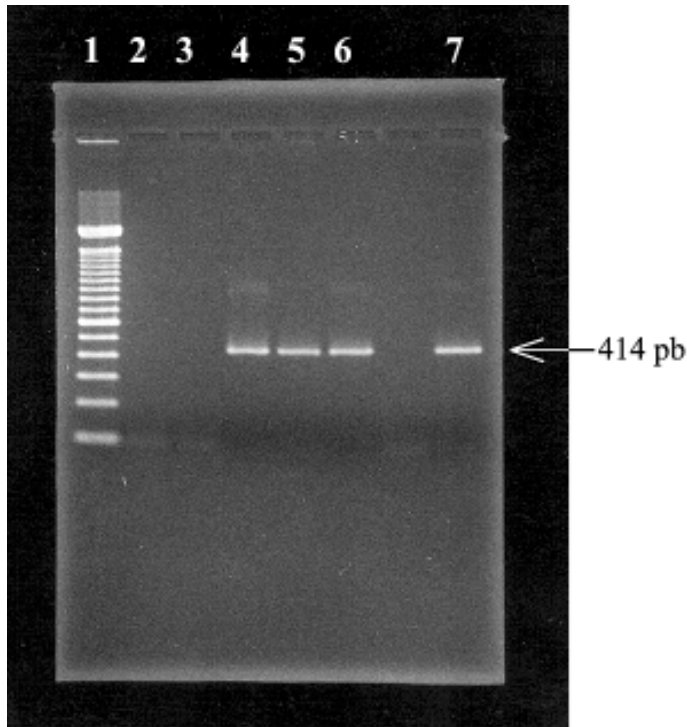

Fig. 2 - Electrophoresis in 2% ethidium bromide-stained agarose gel. HSP-PCR of bacterial culture DNA (414bp). Lane 1: 100bp ladder; Lane 2: negative control of reagents; Lane 3: negative control of extraction room; Lane 4: *B. henselae* Houston strain; Lane 5: *B. quintana*, Oklahoma strain; Lane 6: *B. henselae*, Marseille strain; Lane 7: positive control (*B. henselae*, Houston strain).

Although isolation of *Bartonella henselae* from peripheral blood of cats presenting with bacteremia was successful, PCR performed in the same samples did not yield any amplification.

Seroconversion occurred and IgG titers were determined by IFI only in the inoculated animal (Table 1). The other cats showed only weak reactions (titers < 64), and were considered non reagents.

DISCUSSION

Feline susceptibility to *B. henselae* infection was demonstrated by the isolation of the infectious agent from peripheral blood of the experimentally inoculated animal, and from other animals kept in contact, thus confirming the epidemiological role of cats[1,5,8,9,11,17,18].

Table 1
Hemoculture, PCR and IFI, before and after experimental SC inoculation of cat N° 1 with *B. henselae*, Houston strain (10^5 CFU), São Paulo, 1999

Daus after inoculation	Hemoculture	PCR in blood	IFI *B. henselae*
-120	(−)	(−)	32
-50	(−)	(−)	16
7	(−)	(−)	32
14	(−)	(−)	32
30	(−)	(−)	64
54	(−)	(−)	32
68	**(+)**	**(−)**	**256**
90	**C**	**(−)**	**256**
120	**(+)**	**(−)**	**64**
150	**(+)**	**(−)**	**256**
185	**(+)**	**(−)**	**256**
210	(−)	(−)	128
240	(−)	(−)	128
360	(−)	(−)	64

(+): *Bartonella henselae* Bacteremia; (−): Absence of bacteremia or absence of amplification of DNA of *B. henselae;* C: contaminated hemoculture

Table 2
Isolation of *Bartonella henselae* in experimentally inoculated cat and confined felines, PCR results in peripheral blood and IFI (from 2 to 24 months of age), São Paulo, 1998-2000

Age	2m			4m			6m			12m			18m			20m			22m			24m		
Cat n°	H	PCR	IFI	H	PCR	IFI	H	PCR	IFI	H	PCR	IFI	H	PCR	IFI	H	PCR	IFI	H	PCR	IFI	H	PCR	IFI
1	...			(−)	(−)	NR	(-)*	(−)	NR	(+)	(−)	256	(−)	(−)	64	...								
2	(−)	(−)	NR	(−)	(−)	NR	(−)	(−)	NR	C	(−)	NR	(+)	(−)	NR	(+)	(−)	NR	(−)	(−)	NR	(−)	(−)	NR
3	(−)	(−)	NR	(−)	(−)	NR	(−)	(−)	NR	(−)	(−)	NR	(+)	(−)	NR	(+)	(−)	NR	(−)	(−)	NR	(+)	(−)	NR
4	(−)	(−)	NR	(−)	(−)	NR	(−)	(−)	NR	(−)	(−)	NR	(+)	(−)	NR	(+)	(−)	NR	(+)	(−)	NR	(−)	(−)	NR
5	(−)	(−)	NR	(−)	(−)	NR	(−)	(−)	NR	(+)	(−)	NR	(−)	(−)	NR	†								

H: hemoculture; PCR: polymerase chain reaction; IFI: IgG anti-*B. henselae* Indirect immunofluorescence test; m: months; NR: non- reagent; (-)*: moment of the experimental inoculation; ...: not performed; †: death.

Bacteremia was observed two months after inoculation, more lately than reported by others[1,17,18,20]. The route of inoculation, type of bacterial strain, size and mode of inoculum preparation may have influenced the development of infection[1,13,14]. Apparently, infection occurs early and bacteremia lasts longer, when infected blood is inoculated[13].

All cats presenting with bacteremia were asymptomatic suggesting, as already reported by others[5,14,20], a low pathogenicity of *B. henselae* in cats. Seroconversion and bacteremia occurred simultaneously in the inoculated animal, but not in the others. In another study[6], titers < 100 had low positive predictive value for CSD diagnosis in man.

Although the rising and falling of antibody titers together with bacteremia suggests a relationship between bacteremia and production of antibodies, it does not mean that the host is protected against infection[5]. The highest antibody titers are found in cats with bacteremia, with predictive positive value for some[1,14], while others[5] consider that there is no relationship between threshold antibody titers and bacteremia. However, antibody-mediated immunity may be a way to control bacteremia, reducing its duration and the transmission of disease[14].

The absence of relationship between bacteremia and antibody titers may justify the transmission of infectious agent from the inoculated animal to the others as demonstrated by *B. henselae* isolation, although significant production of antibodies did not occur (titers < 64). These findings are in accordance with those of CHOMEL *et al.*[5] who found that some animals presenting with bacteremia did not present detectable IgG titers. Besides, non-reagent felines may be those that are partially tolerant to oral infection by *B. henselae*[8,11], what may have happened to the animals that were not inoculated which probably acquired the infection by ingestion of contaminated fleas or their feces.

Low IFI specificity to differentiate among *Bartonella* species[15], mainly with respect to *B. henselae* and *B. quintana*, is troublesome for interpretation of antibody titers in man. This fact underlines the need to characterize isolated species. Fortunately, felines infection by *B. quintana* has not been proved[20], and positive reactions, when *B. quintana* is used as the IFI antigen are interpreted as cross-reactions[16,19].

The three PCR protocols performed in the present study enabled characterization of the isolates as belonging to *Bartonella sp* (HSP and FTSZ protocols) and to the *henselae* species (BH-PCR protocol). However, the same technique applied to peripheral blood failed to produce positive amplifications in the same animals and samples in which bacteria were isolated by hemoculture. Low sensitivity of PCR has already been reported in the literature[15] and may be explained by an insignificant bacteria load, by heterogeneous distribution of bacteria in blood aliquots, and finally by a lack of sensitivity of the amplification protocols (primers, DNA extraction of whole blood instead of possibly only red cells, cycle programming – time and temperatures, rounds of amplification).

Our results corroborate those of other studies in that serological tests (IFI) have limited value in the assessment of bacteremia in animals. Besides, IFI is predictive of bacteremia only when titers are high, although an adequate negative predictive value is found when antibodies are absent. Moreover, we observed that PCR is not useful to detect *B. henselae* DNA in peripheral blood of felines and in the identification of eventual carriers of *B. henselae*, at least with the protocols used. Isolation of the agent, using hemoculture and characterization of the isolate by PCR, is the most efficient method to detect and type *Bartonella* species.

RESUMO

Infecção experimental e transmissão horizontal de *Bartonella henselae* em gatos domésticos

Procurou-se verificar a possibilidade de transmissão horizontal de *B. henselae* em 5 felinos confinados, dentre os quais apenas um foi inoculado experimentalmente por via subcutânea (SC) com 10^5 UFC. Todos os felinos apresentavam infestação por pulgas. Para a avaliação da infecção foram utilizados: isolamento bacteriano (hemocultura), detecção de DNA específico pela Reação em Cadeia da Polimerase (PCR), com os protocolos HSP, FTSZ e BH-PCR, e a pesquisa de anticorpos específicos por Imunofluorescência Indireta (IFI). Os protocolos da PCR foram também utilizados para a caracterização do isolado da hemocultura. A inoculação de *B. henselae* resultou na infecção assintomática do animal inoculado, comprovada através da soroconversão e de bacteremia de 4 meses de duração, com o isolamento da bactéria na hemocultura. Todos os animais contactantes apresentaram bacteremia 6 meses após a data de inoculação experimental. No entanto, não apresentaram reação de IFI positiva. Em nenhum momento foi possível detectar DNA de *B. henselae* no sangue circulante, com as PCR utilizadas. Não obstante, a PCR possibilitou a identificação da bactéria isolada como sendo do gênero *Bartonella* (HSP e FTSZ-PCR) e espécie *henselae* (BH-PCR). Conclui-se que o isolamento bacteriano por meio da hemocultura constitui-se no método mais eficiente para a identificação dos felinos infectados e bacterêmicos. Estes resultados também evidenciam a possibilidade do papel das pulgas na transmissão de *B. henselae* em gatos.

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