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ISOLATION OF *BARTONELLA* SPP. FROM EMBRYOS AND NEONATES OF NATURALLY INFECTED RODENTS

Michael Y. Kosoy,[1] Russell L. Regnery,[1] Olga I. Kosaya,[1] Dana C. Jones,[1] Eric L. Marston[1] and James E. Childs[1,2]

[1] Division of Viral and Rickettsial Diseases, National Center for Infectious Diseases, Centers for Disease Control and Prevention, Atlanta, Georgia, 30333, USA

[2] Corresponding author (e-mail: jfc5@cdc.gov)

ABSTRACT: Embryos and neonatal offspring of wild-captured cotton rats (*Sigmodon hispidus*) and white-footed mice (*Peromyscus leucopus*) were tested for the presence of *Bartonella* spp. Isolates of *Bartonella* spp. were obtained from 18 of 31 embryos and 7 of 19 neonates from bacteremic dams of the two species; no isolates were obtained from material from non-bacteremic dams. Sequence analysis demonstrated that the isolates from embryos and neonates matched the phylogenetic group of *Bartonella* spp. isolates obtained from the mother. No antibodies to homologous *Bartonella* spp. antigens were detected in maternal and neonatal blood or embryonic tissue. These findings suggest the possibility of vertical transmission of *Bartonella* spp. among natural rodent hosts.

Key words: *Bartonella* spp., embryos, immune tolerance, infection transmission, *Peromyscus leucopus*, rodents, *Sigmodon hispidus*, zoonotic disease.

INTRODUCTION

Several species of the genus *Bartonella* (formerly *Rochalimaea*), including *B. henselae*, *B. quintana*, and *B. elizabethae*, cause human disease in the United States. Human infection can lead to a wide spectrum of illnesses, ranging from cat-scratch disease to bacillary angiomatosis (Koehler, 1996). The number of species included in the genus *Bartonella* has increased over the last several years with the description and characterization of numerous *Bartonella* (formerly *Grahamella*) spp. isolated from wild-captured rodents from the United Kingdom and the United States (Birtles and Harrison, 1994; Kosoy et al., 1997).

Our understanding of the natural maintenance and transmission cycle of *Bartonella* spp. is still rudimentary. Arthropod vectors play a role in the transmission of *B. bacilliformis* (sandflies of the genus *Lutzomyia*), while *B. quintana* can be transmitted by the human body louse (*Pediculus humanis*; Byam and Lloyd, 1920). Cat fleas (*Ctenocephalides felis*) can be infected with *B. henselae* and may be important in the transmission cycle of *Bartonella* spp. among cats (Chomel et al., 1996) and possibly to humans (Zangwill et al., 1993).

The prevalence of bacteremia with *Bartonella* spp. within rodent communities is typically >50% (Birtles and Harrison, 1994; Kosoy et al., 1997). Bacteremic and non-bacteremic animals either lack, or have very low titers of, antibodies to these organisms (Kosoy et al., 1997). The mechanisms by which rodents acquire *Bartonella* spp. are unclear, although fleas have been suggested (Tyzzer, 1942).

MATERIALS AND METHODS

To assess the potential for vertical transmission, we attempted to isolate *Bartonella* spp. simultaneously from maternal, embryonal, and neonatal tissues of wild-caught rodents. Rodents were captured by using live traps (H. B. Sherman Traps, Tallahassee, Florida, USA) from Social Circle, Georgia, USA (33°66′N, 83°72′W), Amelia Island, Florida, USA (30°62′N, 81°46′W), and Morrow Mountain State Park, North Carolina, USA (35°36′N, 80°14′W), from May 1996 through May 1997.

Captured animals were anesthetized with methoxyflurane (Metofane, Pittman-Moore, Mundelein, Illinois, USA) and bled from the retroorbital plexus. Ten pregnant cotton rats (*Sigmodon hispidus*) and four pregnant white-footed mice (*Peromyscus leucopus*) were captured and deeply anesthetized, and 49 fetuses or uteri containing small embryos were removed and placed into sterile vials. In some instances only a single embryo was removed for testing, although the pregnant female may have

305

had additional embryos. In the laboratory, fetuses were separated from placental tissues within a biosafety hood, rinsed with sterile phosphate-buffered saline (pH 7.4), and removed from their amniotic sacs using aseptic technique. Estimated gestational age was obtained by measuring lengths of fetuses (Rugh, 1968). Four *S. hispidus* gave birth in the traps. These 19 newborn rodents were anesthetized, bled by cardiac puncture, and measured. Blood, placental and embryonic tissue samples were stored at −70 C until they were used.

Details of the procedures used to isolate *Bartonella* spp. have been published previously (Regnery et al., 1992a). Whole or diluted blood and 10% suspensions of embryonal tissue in brain heart infusion medium (Becton Dickinson, Cockeysville, Maryland, USA) were used for isolation. Aliquots of 0.1 ml of the blood or tissue suspensions were applied to heart infusion agar plates supplemented with 5% rabbit blood (Becton Dickinson). The plates were incubated at 32 C in an aerobic atmosphere of 5% CO_2 and held for 5 to 15 days. The cultures were examined daily for bacterial growth and colonies tentatively identified as *Bartonella* spp. were streaked onto a new agar plate. When visually uncontaminated *Bartonella*-like colony counts were >500 per plate, material was collected, placed in brain heart infusion medium, and stored at −70 C.

Isolated organisms tentatively identified as belonging to the genus *Bartonella* were prepared for polymerase chain reaction (PCR) by using QIAamp Tissue Kit (Qiagen, Inc., Chatsworth, California). Primers homologous to the citrate synthase (*glt*A) gene of *B. henselae* Houston I were used, as described previously (Norman et al., 1995), to amplify a 379-bp product. The PCR products were sequenced directly. Sequences were analyzed by STADEN (Staden, 1982) and Wisconsin Sequence Analysis Package 8.1 Unix (Genetic Computer Group, Inc., Madison, Wisconsin, USA) and aligned with *B. henselae*, *B. quintana*, *B. vinsonii*, and four newly described phylogenetic groups (A, B, C, and D) of the genus *Bartonella* (Kosoy et al., 1997). The *glt*A gene sequences have GenBank accession numbers of L38987, *B. henselae*; U28073, *B. quintana*; U28074, *B. vinsonii*; U84372, group A; U84375, group B; U84377, group C; and U84379, group D.

The indirect fluorescence antibody test was performed as previously described (Regnery et al., 1992b; Kosoy et al., 1997). Wells of 12-well spot slides were dotted with antigens of *B. henselae* (ATCC 49882), *B. quintana* (ATCC VR-358), *B. vinsonii* (ATCC VR-152), and four isolates representing phylogenetic groups (A, B, C and D) of *Bartonella* obtained from rodents.

Antigen wells were overlaid with serum, tissue suspensions, or mouse ascitic fluid produced in BALB/c mice with homologous reciprocal titers of ≥2,048. Mixtures of anti-mouse and anti-hamster conjugate (Kirkegaard & Perry Laboratories Inc., Gaithersburg, Maryland, USA) labeled with fluorescein isothiocyanate were used as appropriate. The same conjugates have given good results when used against a variety of sera from diverse rodent species, including *S. hispidus* and *P. leucopus*, in other studies (Kosoy et al., 1996). Sera and tissue suspension were screened at a dilution of 1:32.

RESULTS

Bartonella spp. were isolated from three of four pregnant white-footed mice and from six of 10 pregnant cotton rats. The colony counts of bacteria on agar after primary plating of maternal blood varied from 1,000 to 400,000 colony-forming units (CFU)/ml. Citrate synthase gene sequence analysis demonstrated that the three *Bartonella* isolates from pregnant *P. leucopus* belonged to phylogenetic group D, while the isolates from *S. hispidus* belonged to phylogenetic group A (five of six adult females and embryos) or C (one of five adults) (Table 1). *Bartonella* spp. were isolated from placental tissues obtained from eight of nine bacteremic dams, but not from any of the four nonbacteremic cotton rats (Table 1).

Bartonella spp. were isolated from embryos obtained from each of the bacteremic females of *P. leucopus* and from embryos recovered from four of the five bacteremic *S. hispidus* (Table 1). The colony counts of bacteria on agar after primary plating of the 10% embryonal suspensions varied from 4 to 200 CFU/ml. Sequence analysis demonstrated that the three *Bartonella* isolates from *P. leucopus* belonged to phylogenetic group D, while 15 isolates cultured from *S. hispidus* embryos belonged to phylogenetic group A (Table 1). No isolates were obtained from embryonic tissues and the placenta removed from the cotton rat which was bacteremic with *Bartonella* phylogenetic group C, or from 14 embryos removed from four nonbacteremic cotton rats.

TABLE 1.   *Bartonella* spp. in embryos of bacteremic white-footed mice (*P. leucopus*) and cotton rats (*S. hispidus*) obtained from Georgia and North Carolina, 1996–1997.

		Embryos			Phylogenetic group of *Bartonella*[a] (number of isolates sequenced) from:		
Species	Locality and date	Number in litter	Length (mm)	Positive/ tested	Maternal blood	Placental tissue	Embryonal tissue
P. leucopus	Morrow Mountain State Park, North Carolina, May 1996	3	12	1/1	D (1)	D (1)	D (1)
P. leucopus	Morrow Mountain State Park, North Carolina, May 1996	1	20	1/1	D (1)	D (1)	D (1)
P. leucopus	Morrow Mountain State Park, North Carolina, May 1996	2	17	1/1	D (1)	D (1)	D (1)
S. hispidus	Social Circle, Georgia, August 1996	4	11	4/4	A (1)	A (1)	A (4)
S. hispidus	Social Circle, Georgia, August 1996	6	40	0/6	A (1)	A (1)	none
S. hispidus	Social Circle, Georgia, March 1997	3	7	3/3	A (1)	A (3)	A (3)
S. hispidus	Social Circle, Georgia, April 1997	5	40	0/5	C (1)	none	none
S. hispidus	Social Circle, Georgia, May 1997	6	42	3/5	A (1)	A (1)	A (3)
S. hispidus	Social Circle, Georgia, May 1997	8	12	5/5	A (1)	A (1)	A (5)

[a] Phylogenetic group was determined by the similarity of the *gltA* gene to sequences obtained from rodent-borne *Bartonella* isolates of phylogenetic groups A, B, C, and D (Kosoy et al., 1997).

The gestation period in rodents of the genus *Peromyscus* averages 23 days (Layne, 1968), and the lengths of the infected embryos of *P. leucopus* (Table 1) corresponded to days 14 to 18 of gestational age. Gestation in cotton rats lasts approximately 27 days (Meyer and Meyer, 1944), and the lengths of the infected embryos of *S. hispidus* corresponded to days <14 to 25 of gestational age.

The four cotton rats which gave birth to neonates inside traps were bacteremic with 480 to 40,000 CFU/ml. Sequence analysis demonstrated that the *Bartonella* isolates from three cotton rats belonged to phylogenetic group A, while isolates obtained from one cotton rat were mixed and belonged to two phylogenetic groups, A and C (Table 2). Seven of 15 neonatal cotton rats from three of the four litters were bacteremic, while none of four were bacteremic from the fourth litter. *Bartonella* isolates belonging to phylogenetic group A were obtained from newborn rats from

TABLE 2.   *Bartonella* in neonatal cotton rats (*S. hispidus*) obtained from Social Circle, Georgia, June–July 1996.

Cotton rat, identification number	*Bartonella* in maternal blood, phylogenetic group[a]	Neonates in the litter		*Bartonella* in blood of neonates	
		Number	Weight (g)	Positive/ tested	Phylogenetic group[a] (number of isolates)
Gr161	A	5	5.5–6.0	2/5	A (5)
Gr172	A + C	4	6.0	4/4	A (3), C (1)
Gr101	A	6	6.0	1/6	A (1)
Gr87	A	4	4.5–5.0	0/4	none

[a] Phylogenetic group was determined by the similarity of *gltA* gene to sequences obtained from rodent-borne *Bartonella* isolates of phylogenetic groups A, B, C, and D (Kosoy et al., 1997).

three of the litters, and one isolate belonging to phylogenetic group C was obtained from a newborn rat from the litter of the female with the mixed infection (Table 2). In two of the three positive litters, isolates were obtained from only a fraction of the neonates tested, while Bartonella were isolated from all of the four neonates from the third litter (Table 2). The bacterial colony counts after primary plating of neonatal blood were similar to those of their respective dams, and varied from 400 to 40,000 CFU/ml among neonates with group A, and was 280 CFU/ml in the neonate with group C. There was no visible evidence of milk in the neonatal stomachs. No fleas were observed on rodents or in the plastic bags used for anesthetizing animals, however rodents were not brushed.

None of the sera from the 14 adult S. hispidus and P. leucopus, the 19 neonatal S. hispidus, or the tissue suspensions from 37 embryos of S. hispidus and P. leucopus had detectable antibodies to homologous Bartonella antigen at a 1:32 dilution.

DISCUSSION

This study provides the first report of the isolation of Bartonella spp. from embryonic and neonatal rodents. Isolation of the bacteria from fetuses of S. hispidus and P. leucopus suggests in utero transmission of Bartonella spp. may occur among natural hosts. Although fetuses were collected with precautions to avoid contact with maternal blood, the isolation of Bartonella spp. from the blood of neonatal cotton rats provides strong complementary evidence of intrauterine infection. The blood samples were collected from neonatal rats shortly after their birth (all <12 hr); some dams were delivering young when traps were opened. Transmission through other maternal excretions and secretions (e.g., saliva, milk, urine) could not have produced bacteremia within the brief interval from birth.

Isolation of bacteria from embryonal tissue suggests that Bartonella spp. can pass through the placental tissues. In some rodents, the placental tissue is reduced, and only the endothelial lining of the blood vessels separates the fetal blood from the surrounding maternal sinuses (Vaughan, 1978). This arrangement might facilitate transplacental passage of cells or organisms, especially during the second half of pregnancy. Both B. henselae and B. quintana readily infect human endothelial cells (Palmari et al., 1996), and rodent-associated Bartonella spp. have presumably similar cell tropism, providing them access to the embryonic circulation.

These data demonstrating bacteria in embryonic tissues and newborn rodents, along with the lack of antibody to homologous antigen in adult and neonatal blood, suggest a different parasite-host relationship between Bartonella spp. and rodents as compared with B. henselae in cats. A robust antibody response in infected cats leads to high antibody prevalence to B. henselae in cat populations (Childs et al., 1994). Bacteremic queens did not transmit B. henselae to their kittens in utero, but some kittens acquired maternal antibody (Abbott et al., 1997).

The ability to recognize "self"-molecules is thought to arise during the development of the fetal immune system by exposure of immature lymphocytes to self-components. Mice do not develop, or develop to only a limited degree, the ability to react immunologically against foreign tissue cells contacted in fetal life (Billingham et al., 1953). Active hematopoiesis with formation and development of cells of the immune system occurs in liver, spleen, and bone marrow beginning from day 9 and continuing until birth in the mouse fetus (Rugh, 1968). It is possible that antigens to Bartonella spp. introduced into rodents at an early embryonal stage could blunt the subsequent development of the humoral antibody response and account for the apparent lack of antibody to homologous antigen in bacteremic rodents. Vertical transmission could contribute to the very high prevalence of infection in rodent populations found during all seasons (Ko-

soy et al., 1997). Further laboratory experimentation will be required to resolve these questions.

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