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Role of Hippoboscidae Flies as Potential Vectors of *Bartonella* spp. Infecting Wild and Domestic Ruminants

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The putative role of biting flies in *Bartonella* transmission among ruminants was investigated. Amplification of the *Bartonella* citrate synthase gene from 83 Hippoboscidae was detected in 94% of 48 adult *Lipoptena cervi* flies, 71% of 17 adult *Hippobosca equina* flies, 100% of 20 adult *Melophagus ovinus* flies, and 100% of 10 *M. ovinus* pupae. Our findings suggest that Hippoboscidae play a role in the transmission of *Bartonella* among ruminants. The vertical transmission of *Bartonella* in *M. ovinus* and the presence of *Bartonella* DNA in all samples suggest a symbiotic association between *Bartonella* and *M. ovinus*.

Bartonella spp. are intracellular small gram-negative bacteria transmitted by blood-sucking arthropods and considered to be emerging pathogens in humans and animals (1, 8, 9, 22). In recent years, these organisms have been identified in a wide range of wild and domestic mammals (4, 6, 7, 11), some of which have been associated with zoonoses. Recently, four new *Bartonella* species have been isolated from ruminants: *B. schoenbuchensis* and *B. capreoli* were recovered from wild roe deer (*Capreolus capreolus*) (3, 10), whereas *B. bovis* (3) and *B. chomelii* (20) were recovered from domestic cattle. There are no pathological outcomes associated with *Bartonella* infection in ruminants (5, 7).

Arthropod vectors involved in the transmission of *Bartonella* spp. among ruminants are still unknown. As blood-sucking ectoparasites of ruminants, flies of the family Hippoboscidae are good candidates for the transmission of *Bartonella*. Among Hippoboscidae, *Lipoptena*, *Hippobosca*, and *Melophagus* are the three main genera which parasitize mammals (14, 18). The deer ked (*Lipoptena cervi*), the predominant *Lipoptena* species in Europe, parasitizes cervids (15), whereas the louse fly (*Hippobosca equina*) parasitizes cows and horses and the sheep ked (*Melophagus ovinus*) is a permanent ectoparasite of sheep (*Ovis aries*) (18).

The aim of this study was to determine if Hippoboscidae could be putative vectors of *Bartonella* spp. in ruminants. We investigated whether *Bartonella* DNA could be detected in adult and pupal stages of Hippoboscidae collected from ruminants (domestic cattle and roe deer) known to be naturally infected with *Bartonella* spp. We extended the study to Hippoboscidae collected from sheep and horses for which no evidence of *Bartonella* infection had ever been demonstrated.

Collection and identification of Hippoboscidae. Eighty-three Hippoboscidae flies of different species, stages, and genders were collected and taxonomically identified under a binocular lens (18). Samples were obtained from the hosts or from our parasitology collection (Table 1). Each sample was stored in absolute ethanol.

DNA extraction and PCR. Each fly and pupa were washed three times in sterile water baths and once in a 70% ethanol bath and then dried. DNA was then extracted after the flies were crushed with a bead beater as previously described (16).

A 380-bp fragment of the citrate synthase (*gltA*) gene of the genus *Bartonella* was amplified in fly DNA extracts by PCR, using specific primers (BhCs781 and BhCs1137) as described previously (20, 26). All reactions were performed at least twice.

PCR-RFLP. PCR-restriction fragment length polymorphism (PCR-RFLP) was performed with 10 μl of the PCR product of the *gltA* gene obtained from 63 of 83 extracts of Hippoboscidae (45 *L. cervi*, 12 *H. equina*, and 6 *M. ovinus* extracts) by using restriction endonucleases TaqI, MseI, and DdeI (New England Biolabs, Beverly, Mass.) as previously described (17, 21). Patterns were compared to the PCR-RFLP profiles of six ruminant-related species or strains, i.e., *B. capreoli* (CIP 106691 and CCUG 43827) (3), *B. schoenbuchensis* strain R1 (DSM 13525 and NCTC 13165) (10), *B. bovis* strain R3, (CIP 106692 and CCUG 43828) (3), *B. chomelii* (A828^T and CIP 107869^T) (20), and A971, isolated from a Formosan Sika deer (*Cervus nippon taiouanus*) in a nature park in France (21).

Sequencing and sequence analysis. Ten amplified fragments (seven obtained from *L. cervi*, one obtained from *H. equina*, and two obtained from *M. ovinus*) were sequenced by GenomeExpress (Meylan, France). Another fragment, obtained from an *L. cervi* parasite for which a mixed profile was found by PCR-RFLP analysis, was first cloned in a pCR2-1 TOPO TA cloning vector according to the manufacturer's instructions (Invitrogen, Paisley, United Kingdom). Recombinant plasmid DNA was isolated from the selected white positive colonies with a Wizard *Plus* miniprep DNA purification system (Promega, Madison, Wis.).

Sequences were compared with known sequences listed in the GenBank nucleotide sequence databases by using the BLAST search option of the National Center for Biotechnology Information (NCBI) (http://www.ncbi.nlm.nih.gov).

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TABLE 1. Taxonomic identification, origin, and repartition of the 83 samples of *Hippoboscidae* tested, and results of specific PCR amplification of their *Bartonella* citrate synthase (*gltA*) genes

Species of Hippoboscidae	Evolutive stage	No. of flies	Host (no. of hosts)	Origin (yr of sampling)	No. of samples positive by PCR for *Bartonella gltA* gene/ total no. of samples (%)	
Lipoptena cervi	Adult	10	Wild roe deer (1)	Midi Pyrénéces (2000)	8/10	= 45/48 (94)
		14	Wild roe deer (unknown)	Unknown (1994)	13/14	
		24	Wild roe deer (1)	Bourgogne (2003)	24/14	
	Pupa	3	Wild roe deer (unknown)	Unknown (1994)	0/3	= 0/3 (0)
Hippobosca equina	Adult	2	Cow (1)	Ile de France (2001)	2/2	= 12/12 (100)
		10	Cow (3)	Rhone Alpes (2002)	10/10	
	Adult	5	Horse (1)	Centre (2003)	0/5	= 0/5 (0)
	Pupa	1	Horse (1)	Centre (2003)	0/1	= 0/1 (0)
Melophagus ovinus	Adult	6	Sheep (1)	Normandie (2003)	6/6	= 20/20 (100)
		4	Sheep (1)	Ile de France (2002)	1/1	
		2	Sheep (1)	Bourgogne (2003)	1/1	
		8	Sheep (2)	Roumanie (2004)	8/8	
	Pupa	3	Sheep (unknown)	Unknown	3/3	= 10/10 (100)
		2	Sheep (2)	Normandie (2003)	2/2	
		1	Sheep (1)	Bourgogne (2003)	1/1	
		4	Sheep (2)	Roumanie (2004)	4/4	

Detection of *Bartonella* DNA in flies of Hippoboscidae by specific PCR amplification of the citrate synthase gene (*gltA*). Among the 83 Hippoboscidae, 51 flies were identified as *L. cervi* (48 adults and three pupae), 18 were identified as *H. equina* (17 adults and one pupa), and 14 were identified as *M. ovinus* (eight adults and six pupae) (Table 1).

The presence of the specific amplified fragment was detected from 45 (94%) *L. cervi* adults, from 20 (100%) *M. ovinus* adults, and from 12 (71%) *H. equina* adults. All *H. equina* flies collected on cows ($n = 11$) were positive, whereas none of those collected on horses ($n = 6$) resulted in a PCR product DNA.

All 10 *M. ovinus* pupae resulted in a PCR product, whereas none of the 4 *L. cervi* and *H. equina* pupae displayed the expected amplified fragment.

Identification of *Bartonella* DNA present in flies of Hippoboscidae. PCR-RFLP analysis of *gltA* amplified from the 63 samples of flies of Hippoboscidae were performed and compared to the profiles of the ruminant-infecting *Bartonella* strains (Table 2). Using TaqI, MseI and DdeI, we identified four different profiles for known ruminant strains. Profile 1 was found in *B. schoenbuchensis* R1 and in 30 samples comprising 26 *L. cervi* and 4 *M. ovinus* samples. Four sequences obtained

TABLE 2. PCR-RFLP profiles, distribution, and sequence identification by partial sequence analysis of the *gltA* genes of ruminant-infecting *Bartonella*-reference strains obtained from different species of *Hippoboscidae*

PCR-RFLP profile type (associated strain[s])	Fragment size(s) (bp) after digestion with[a]:			Species of Hippoboscidae flies showing the profile type	No. of samples	No. of sequenced fragments	Closest *Bartonella* species (% DNA identity based on a fragment of the *gltA* gene)
	TaqI	DdeI	MseI				
1 (*B. schoenbuchensis* R1)	ND	300, U	180, U	*L. cervi*	26	4	*B. schoenbuchensis* R1 (100)
				M. ovinus			No sequence available
				Adult	1		
				Pupa	3		
2 (*B. chomelii*, *B. schoenbuchensis* R3, and A971)	ND	ND	180, U	*L. cervi*	4	1	*B. schoenbuchensis* R3 (100)
				H. equina	12	1	*B. chomelii* (100)
				M. ovinus			A971 cervid strain (99)
				Adult	1	1	
				Pupa	1	1	
3 (*B. bovis*)	150, 250	ND	150, 220	None			
4 (*B. capreoli*)	ND	100, 250	120, 180	None			
New (no strain associated)	ND	ND	200, 180	*L. cervi*	6	2	A971 cervid strain (95)
Mixed (profiles 1 and 2)	ND	ND, 300, U	180, U	*L. cervi*	7	1	
						Clone 1	*B. schoenbuchensis* R1 (100)
						Clone 2	*B. chomelii* (100)

[a] ND, sample was not digested; U, fragment was of undetectable size.

from *L. cervi* extracts showed 100% sequence similarity with the *gltA* gene of *B. schoenbuchensis* strain R1.

Profile 2 was found for *B. schoenbuchensis* R3 strain, *B. chomelii*, and strain A971, and profile 2 was also found in 16 samples (2 *L. cervi*, 2 *M. ovinus*, and 12 *H. equina* samples). Sequence analysis of the *gltA* gene fragment obtained from two *M. ovinus* samples presenting this PCR-RFLP profile showed 99% similarity with the A971 sequence, whereas the sequence obtained from *L. cervi* showed 100% similarity with the *B. schoenbuchensis* strain R3 sequence. The sequence obtained from one *H. equina* extract had 100% similarity with the *B. chomelii gltA* gene sequence.

Profiles 3 and 4 were found for *B. bovis* and *B. capreoli*, respectively, but for none of the fly samples.

Six *L. cervi* samples had a new RFLP profile different from those of the reference strains. Two fragments with this new RFLP profile showed 95% similarity with the A971 *gltA* sequence, 94% similarity with those of a *B. schoenbuchensis*-related strain (GenBank accession number AJ564635), and from 92 to 93% similarity to other ruminant-related *Bartonella* species (*B. capreoli* and *B. chomelii*).

A mixed profile between profiles 1 and 2 was obtained for seven samples (*L. cervi*). For this PCR-RFLP mixed profile, sequencing of cloned fragments allowed the distinction between two different sequences displaying 100% DNA similarity with *B. schoenbuchensis* and *B. chomelii*.

The present study is the first report of the presence of *Bartonella* in ruminant blood-sucking flies. Up to 84% of the flies of Hippoboscidae carried ruminant-related *Bartonella* DNA, which strongly suggests that Hippoboscidae might play a role in the transmission of *Bartonella* among ruminants.

PCR-RFLP profiles using the *gltA* gene were consistently similar to those of other ruminant-related *Bartonella* spp., as confirmed by sequence analysis. Among *L. cervi* samples, we observed different PCR-RFLP profiles. Most of the profiles and sequences obtained were related to *B. schoenbuchensis*. One sequence corresponding to an unrecognized PCR-RFLP profile shared less than 96% sequence similarity with those of the validated species or strains and therefore could not be identified by its *gltA* sequence (19). This finding was corroborated by the fact that undescribed *Bartonella* strains remain to be identified in ruminants (7, 21). *H. equina* flies presented *Bartonella* DNA related to the *B. chomelii* sequence (20). Flies of Hippoboscidae are common blood-sucking parasites of ruminants (18) and are already known for their vectorial role in the life cycles of pathogens such as *Trypanosoma melophagium* for *M. ovinus* and sheep (2, 23). Ruminants have the potential to become bacteremic with *Bartonella*, and we found that most of the flies feeding on ruminants were infected with *Bartonella* at a relatively high level (using quantitative PCR [data not shown]). Furthermore, a preliminary study conducted in our laboratory indicated that immunoglobulin G raised against ruminant-related *Bartonella* species could be detected in horses despite the fact that we did not culture *Bartonella* from horse blood. This fact suggests that *Bartonella* is transmitted from bovine to equine by a common vector and that Hippoboscidae are the most likely candidates. We propose that flies of the family Hippoboscidae may be vectors for *Bartonella*, as has been shown for horse-biting flies and in cases of equine infectious anemia (13) and for fleas transmitting *B. henselae* (12). Investigations to identify the localization of *Bartonella* within the flies are necessary to determine the exact role of Hippoboscidae in *Bartonella* transmission.

We extended the study to Hippoboscidae flies feeding on horses and sheep, which are animals for which no evidence of *Bartonella* infection has been reported. We cultured blood samples of more than 100 horses, 150 sheep, and 84 big horn sheep (*Ovis canadensis*), but we did not isolate *Bartonella* (reference 7 and our unpublished data). None of the *H. equina* organisms collected from horses presented *Bartonella* DNA, suggesting that the *Bartonella* DNA-positive louse flies likely acquired *Bartonella* after blood meals on infected cows.

Surprisingly, *Bartonella* DNA was present in all of the *M. ovinus* samples, even at the pupal stage. As *M. ovinus* is wingless, its transmission from one sheep to another occurs by contacts between ewes and lambs (18). The presence of *Bartonella* DNA in *M. ovinus*, along with the absence of *Bartonella* sp. isolation from sheep, might be explained by a symbiotic association between *Bartonella* and the ectoparasite without transmission to ruminants. The fact that 100% of the tested pupae were *Bartonella* DNA positive strengthens this hypothesis. Previous studies of tick endosymbionts showed that symbionts were closely related to bacterial pathogens transmitted by ticks and had lost the ability to infect vertebrates (24). Moreover, a 16S DNA sequence from *Wolbachia melophagi* (GenBank accession number X89110), which has been described as an endosymbiont of *M. ovinus* (25, 27), is closer to a *B. schoenbuchensis* 16S DNA (99% similarity) than to other *Wolbachia* 16S rDNA sequences (between 80 and 83.5% similarity with other insect endosymbiont *Wolbachia* species sequences), suggesting that this sequence is more closely related to *Bartonella* than to *Wolbachia*. As no *Bartonella* DNA was detected in *L. cervi* and *H. equina* pupae, the endosymbiontic nature of *Bartonella* is thus limited to *M. ovinus*.

The present study reports the first demonstration of the presence of *Bartonella* in ruminant blood-sucking flies and should lead to further investigations of *Bartonella* sp. transmission. Finally, the possible role of *Bartonella* sp. as a symbiont will change the way we consider *Bartonella* biology.

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REFERENCES

1. **Alexander, B.** 1995. A review of bartonellosis in Ecuador and Colombia. Am. J. Trop. Med. Hyg. **52:**354–359.
2. **Baker, J. R.** 1967. A review of the role played by the *Hippoboscidae* as vector of endoparasites. J. Parasitol. **53:**412–418.
3. **Bermond, D., H. J. Boulouis, R. Heller, G. Van Laere, H. Monteil, B. B. Chomel, A. Sander, C. Dehio, and Y. Piemont.** 2002. *Bartonella bovis* Bermond *et al.* sp. nov. and *Bartonella capreoli* sp. nov., isolated from European ruminants. Int. J. Syst. Evol. Microbiol. **52:**383–390.
4. **Birtles, R. J., T. G. Harrison, and D. H. Molyneux.** 1994. *Grahamella* in small woodland mammals in the U.K.: isolation, prevalence and host specificity. Ann. Trop. Med. Parasitol. **88:**317–327.
5. **Boulouis, H. J., and B. Chomel.** 1999. Actualités sur les bartonelloses. Bull. Acad. Nat. Med. **7:**1399–1412.
6. **Breitschwerdt, E. B., and D. L. Kordick** 2000. Bartonella infection in animals: carriership, reservoir potential, pathogenicity, and zoonotic potential for human infection. Clin. Microbiol. Rev. **13:**428–438.
7. **Chang, C. C., B. B. Chomel, R. W. Kasten, R. M. Heller, H. Ueno, K. Yamamoto, V. C. Bleich, B. M. Pierce, B. J. Gonzales, P. K. Swift, W. M. Boyce, S. S. Jang, H. J. Boulouis, and Y. Piemont.** 2000. *Bartonella* spp. isolated from wild and domestic ruminants in North America. Emerg. Infect. Dis. **6:**306–311.

8. **Chang, C. C., B. B. Chomel, R. W. Kasten, V. Romano, and N. Tietze.** 2001. Molecular evidence of *Bartonella* spp. in questing adult *Ixodes pacificus* ticks in California. J. Clin. Microbiol. **39:**1221–1226.
9. **Chomel, B. B.** 2000. Cat-scratch disease. Rev. Sci. Tech. Off. Int. Epizoot. **19:**136–150.
10. **Dehio, C., C. Lanz, R. Pohl, P. Behrens, D. Bermond, Y. Piemont, K. Pelz, and A. Sander.** 2001. *Bartonella schoenbuchii* sp. nov., isolated from the blood of wild roe deer. Int. J. Syst. Evol. Microbiol. **51:**1557–1565.
11. **Ellis, B. A., R. L. Regnery, L. Beati, F. Bacellar, M. Rood, G. G. Glass, E. Marston, T. G. Ksiazek, D. Jones, and J. E. Childs.** 1999. Rats of the genus *Rattus* are reservoir hosts for pathogenic *Bartonella.* J. Infect. Dis. **180:**220–224.
12. **Foil, L., E. Andress, R. L. Freeland, A. F. Roy, R. Rutledge, P. C. Triche, and K. L. O'Reilly.** 1998. Experimental infection of domestic cats with *Bartonella henselae* by inoculation of *Ctenocephalides felis* (Siphonaptera: *Pulicidae*) feces. J. Med. Entomol. **35:**625–628.
13. **Foil, L. D., C. L. Meek, W. V. Adams, and C. J. Issel.** 1983. Mechanical transmission of equine infectious anemia virus by deer flies (*Chrysops flavidus*) and stable flies (*Stomoxys calcitrans*). Am. J. Vet. Res. **44:**155–156.
14. **Hafez, M., and M. Hilali.** 1978. Biology of *Hippobosca longipennis* (Fabricius, 1805) in Egypt (Diptera: *Hippoboscidae*). Vet. Parasitol. **4:**275–288.
15. **Haigh, J. C., C. Mackintosh, and F. Griffin.** 2002. Viral, parasitic and prion diseases of farmed deer and bison. Rev. Sci. Tech. Off. Int. Epizoot. **21:**219–248.
16. **Halos, L., T. Jamal, L. Vial, R. Maillard, A. Suau, H. J. Boulouis, and M. Vayssier-Taussat.** 2004. Methods for hard and soft tick DNA extraction applicable to PCR detection. Vet. Res. **35:**1–18.
17. **Handley, S. A., and R. L. Regnery.** 2000. Differentiation of pathogenic *Bartonella* species by infrequent restriction site PCR. J. Clin. Microbiol. **38:**3010–3015.
18. **Kettle, D.S.** 1984. Medical and veterinary entomology, p. 285-292. Crook Helm, London, United Kingdom.
19. **La Scola, B., Z. Zeaiter, A. Khamis, and D. Raoult.** 2003. Gene-sequence-based criteria for species definition in bacteriology: the *Bartonella* paradigm. Trends Microbiol. **11:**318–321.
20. **Maillard, R., P. Riegel, F. Barrat, C. Bouillin, D. Thibault, C. Gandoin, L. Halos, C. Demanche, A. Alliot, J. Guillot, Y. Piémont, H. J. Boulouis, and M. Vayssier-Taussat.** 2004. *Bartonella chomelii* sp. nov., isolated from French domestic cattle (*Bos taurus*). Int. J. Syst. Evol. Microbiol. **54:**215–220.
21. **Maillard, R., M. Vayssier-Taussat, C. Bouillin, C. Gandoin, L. Halos, B. Chomel, Y. Piemont, and H. J. Boulouis,** 2004. Identification of. Bartonella strains isolated from wild and domestic ruminants by a single-step PCR analysis of the 16S-23S intergenic spacer region. Vet. Microbiol. **98:**63–69.
22. **Maurin, M., and D. Raoult.** 1996. *Bartonella* (*Rochalimaea*) *quintana* infections. Clin. Microbiol. Rev. **9:**273–292.
23. **Nelson, W. A.** 1981. *Melophagus ovinus* (Pupipara: *Hippoboscidae*): confirmation of the nonpathogenicity of *Trypanosoma melophagium* for sheep keds. J. Invertebr. Pathol. **37:**284–289.
24. **Noda, H., U. G. Munderloh, and T. J. Kurtti.** 1997. Endosymbionts of ticks and their relationship to *Wolbachia* spp. and tick-borne pathogens of humans and animals. Appl. Environ. Microbiol. **63:**3926–3932.
25. **Nöller, W.** 1917. Blut- und Insektenflagellaten Züchtung auf Platten. Arch. Schiffs Tropenhyg. **21:**53–94.
26. **Norman, A. F., R. Regnery, P. Jameson, C. Greene, and D. C. Krause.** 1995. Differentiation of *Bartonella*-like isolates at the species level by PCR-restriction fragment length polymorphism in the citrate synthase gene. J. Clin. Microbiol. **33:**1797–1803.
27. **Philip, C. B.** 1956. Comments on the classification of the order *Rickettsiales.* Can. J. Microbiol. **2:**261–270.