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Molecular and Serological Evidence of the Presence of *Midichloria mitochondrii* in Roe Deer (*Capreolus capreolus*) in France

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ABSTRACT: *Midichloria mitochondrii* is a tick-borne intracellular bacterium of the order Rickettsiales, found with high prevalence in the sheep tick (*Ixodes ricinus*). *Midichloria mitochondrii* is capable of vertical transmission in the tick, but recently evidence of potential horizontal transmission to the tick hosts through the blood meal has been reported. We investigated the presence of the bacterium in the blood of roe deer (*Capreolus capreolus*) collected from an area known to be highly infested with *I. ricinus* ticks. We collected blood and sera samples for 3 yr in Gardouch (Haute Garonne, France) and subjected them to molecular screening through PCR and to serological investigation using enzyme-linked immunosorbent assay and western blot. Bacterial DNA was detected in the blood of four of seven animals, but only at one or two points in time, whereas all sera were positive for *M. mitochondrii* antigens at all times. Our results indicated that the presence of the bacterium in the blood is transient, but the antibody response appeared to be long-lasting, possibly due to constant exposure to tick bites, and thus to repeated injection of bacteria. The role of *M. mitochondrii* in the mammalian host, and its interaction with other tick-borne bacteria, remains unknown.

Key words: *Ixodes ricinus*, *Midichloria mitochondrii*, roe deer, tick-borne bacteria, vertebrate host.

European roe deer (*Capreolus capreolus*) are very common throughout Europe (Linnell and Zachos 2010). Based on their distribution and habits, roe deer offer a constant and reliable blood supply during the whole year for tick development and are thus regarded as a highly relevant host for ticks and as a reservoir for human tick-borne diseases (Vor et al. 2010; Vázquez et al. 2011). In addition, individual roe deer can transport ticks for long distances during dispersal (Debeffe et al. 2012), seasonal migrations (Cagnacci et al. 2011), and movements to search for mating partners (Richard et al. 2008). The most important tick associated with roe deer is *Ixodes ricinus*, a species responsible for the transmission of pathogenic bacteria, viruses, and protozoa to humans and animals. In addition to well-known pathogens, recent reports have shown the presence of several tick-associated bacteria in humans, dogs, sheep, sika deer (*Cervus nippon*), and goats (Parola et al. 2013), with roles that are still to be defined (Duron et al. 2017). Such a bacterium is *Midichloria mitochondrii* (Sassera et al. 2006), an intracellular bacterium of the recently described *Midichloriaceae* family of the order Rickettsiales (Montagna et al. 2013). These bacteria are located in the ovaries and salivary glands of *I. ricinus* (Mariconti et al. 2012; Epis et al. 2013). The latter localization suggests possible horizontal transmission as supported by the lack of cocladogenesis observed between *Midichloria* sp. bacteria and their tick hosts (Cafiso et al. 2016). Humans and dogs parasitized by *I. ricinus* are indeed seropositive to *M. mitochondrii* (Mariconti et al. 2012; Bazzocchi et al. 2013), and DNA of this bacterium was found in the blood of different mammalian species (Skarphédinsson et al. 2005; Bazzocchi et al. 2013). Our aim was to investigate the circulation of *M. mitochondrii* in roe deer, the host of choice for adult and nymphal stages of *I. ricinus*, and therefore considered an efficient animal sentinel for possible patho-

gens transmitted by this tick species (Wodecka et al. 2014).

Blood and sera samples were collected from seven roe deer in a 15 ha enclosure (9 ha woodlot and 6 ha of pasture) at the French National Institute for Agricultural Research experimental station at Gardouch in France (Haute Garonne). Each animal was sampled three times, in April of 2014, 2015, and 2016, with the exception of one deer that died before the third sampling. Ticks from each roe deer were collected, morphologically identified through standard dichotomous keys (Manilla 1998), and confirmed to be *I. ricinus*. Blood samples were withdrawn from the jugular vein; a portion was centrifuged for serum collection and another was used for DNA extraction. A qualitative PCR on the 12S ribosomal RNA gene of *C. capreolus* was carried out to check the quality of the DNA extraction (Fajardo et al. 2008). Detection of circulating *M. mitochondrii* DNA was performed using a nested PCR approach. External primers GYRB-RT-F1 (5′ AAGCTAAGAATTTGGCGTGATG 3′) and GYRBR6 (5′ GTTTTGGCTTCATTTG GATTTTC 3′) were designed on the *gyrB* gene sequence of *M. mitochondrii* and used to amplify a fragment of 776 base pairs (annealing temperature 57 C; final primers concentration 1 μM). The resulting products were subjected to a real time PCR using primers gyrBRT-f (5′ CTTGAGAGCAGAACCACCTA 3′) and gyrBRT-r (5′ CAAGCTCTGCCGAAA TATCTT 3′) described in Sassera et al. (2008) and amplifying a fragment of 146 base pairs internal to the previous amplified region. The second PCR was carried out using a real-time PCR approach to maximize the sensitivity and specificity of bacterial DNA detection. Blood samples were considered positive for the presence of *M. mitochondrii* DNA when a clear peak was present, the melting temperature of the reamplified samples corresponded to that of a positive control, and the resulting Sanger sequences showed 100% identity with a fragment of the published *gyrB* sequence of *M. mitochondrii* (accession no. AM159536.1; nucleotide position from 565 to 710). In parallel, the recombinant flagellar protein FliD (rFliD) of *M. mitochondrii* was used to determine anti-rFliD antibody levels in deer sera with an enzyme-linked immunosorbent assay (ELISA). The negative controls used to establish a cut-off value were the sera of 10 red deer from a virtually tick-free area (Merler et al. 1996) and the sera of two roe deer newly-born in an enclosure. The cut-off of the ELISA reaction was established at an optical density (OD) of 0.35 (mean OD on negative controls plus three times standard deviation) determined at a wavelength of 450 nm. All parasitized *C. capreolus* samples showed an average OD greater than 0.35, whereas the OD of all the samples obtained from tick-free animals were under the cut-off value. The ELISA results were confirmed by western blot (Mariconti et al. 2012).

Three deer were negative to *M. mitochondrii* DNA, two showed positivity at one of the three sampling times, and the remaining two were positive at two of three times (Table 1). These results confirmed that *M. mitochondrii* was released with the tick saliva during the blood meal. However, the negative results from the second sampling in some individuals that had been positive in the first one, and specifically the temporal pattern of roe deer 145 (positive–negative–positive), indicated that the presence of *M. mitochondrii* in roe deer blood was transient, and that this bacterium likely did not multiply heavily in the host blood. The two possible explanations for this finding are the presence of a transient infection or the movement of the bacteria to other body tissues. Interestingly, the pattern of immunization of the roe deer to *M. mitochondrii* was different compared to the results concerning the presence of the bacterium in blood samples. The ELISA and western blot serological assays showed seropositivity against *M. mitochondrii* in all samples from all 3 yr (Table 1). This result suggested that roe deer, when continuously parasitized by a large number of ticks, were continuously exposed to ongoing injection of *M. mitochondrii*. In this context, the presence of *M. mitochondrii* in tick saliva might play a role in the immune response and immunemodulation, possibly important for infection by tick-borne pathogens.

TABLE 1. Results of molecular (nested PCR for the amplification of a fragment of *gyrB* gene) and serological analyses (enzyme-linked immunosorbent assay and western blot) for the presence of *Midichloria mitochondrii* performed on roe deer (*Capreolus capreolus*) blood and sera collected in Spring 2014, 2015, and 2016 in France. Results are expressed as: + = positive sample; − = negative sample; NA = sample not available.

	Molecular analysis			Serological analysis		
Roe deer no.	2014	2015	2016	2014	2015	2016
144	+	−	−	+	+	+
145	+	−	+	+	+	+
146	−	−	−	+	+	+
147	−	+	+	+	+	+
148	+	−	NA	+	+	+
149	−	−	−	+	+	+
179	−	−	NA	+	+	NA

Because roe deer are the host of choice for nymphal and adult stages of *I. ricinus*, they are a good species for the study of some tick-borne pathogens, but not for all of them. Roe deer play an important role as reservoirs of *Anaplasma phagocytophilum*, two *Bartonella* species, *Babesia divergens*, and *Theileria* spp., but not for *Borrelia* spp. (Skotarczak et al. 2008; Chastagner et al. 2014). Deer are considered as dilution hosts for the agent of Lyme disease, because they maintain high tick intensities, which perpetuate tick populations, but they do not support transmission. The transient bloodstream presence of *M. mitochondrii* observed with our molecular analysis could be due to the function of roe deer as a dead-end transmission host, a situation similar to that of *Borrelia* spp. (Jaenson and Talleklint 1992; Nelson et al. 2000). Future studies could be focused on the role of roe deer in the circulation of *M. mitochondrii*, biological interactions of this bacterium with the host or with other bacteria transmitted by ticks (through the immune reaction of the host against bacteria), the possible replication of this bacterium inside the host, and the role of the bacterium in possible pathological alterations after its transmission.

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LITERATURE CITED

Bazzocchi C, Mariconti M, Sassera D, Rinaldi L, Martin E, Cringoli G, Urbanelli S, Genchi C, Bandi C, Epis S. 2013. Molecular and serological evidence for the circulation of the tick symbiont *Midichloria* (Rickettsiales: *Midichloriaceae*) in different mammalian species. *Parasit Vectors* 6:350.

Cafiso A, Bazzocchi C, De Marco L, Opara MN, Sassera D, Plantard O. 2016. Molecular screening for *Midichloria* in hard and soft ticks reveals variable prevalence levels and bacterial loads in different tick species. *Ticks Tick Borne Dis* 7:1186–1192.

Cagnacci F, Focardi S, Heurich M, Stache A, Hewison AJM, Morellet N, Kjellander P, Linnell JDC, Mysterud A, Neteler M, et al. 2011. Partial migration in roe deer: Migratory and resident tactics are end points of a behavioural gradient determined by ecological factors. *Oikos* 120:1790–1802.

Chastagner A, Dugat T, Vourc'h G, Verheyden H, Legrand L, Bachy V, Chabanne L, Joncour G, Maillard R, Boulouis HJ, et al. 2014. Multilocus sequence analysis of *Anaplasma phagocytophilum* reveals three distinct lineages with different host ranges in clinically ill French cattle. *Vet Res* 45:114.

Debeffe L, Morellet N, Cargnelutti B, Lourtet B, Bon R, Gaillard JM, Hewison AJM. 2012. Condition-dependent natal dispersal in a large herbivore: Heavier animals show a greater propensity to disperse and travel further. *J Anim Ecol* 81:1327–1337.

Duron O, Binetruy F, Noël V, Cremaschi J, McCoy KD, Arnathau C, Plantard O, Goolsby J, Pérez De León AA, Heylen DJA, et al. 2017. Evolutionary changes in

symbiont community structure in ticks. *Mol Ecol* 26: 2905–2921.

Epis S, Mandrioli M, Genchi M, Montagna M, Sacchi L, Pistone D, Sassera D. 2013. Localization of the bacterial symbiont *Candidatus* Midichloria mitochondrii within the hard tick *Ixodes ricinus* by whole-mount FISH staining. *Ticks Tick Borne Dis* 4:39–45.

Fajardo V, González I, Martín I, Rojas M, Hernández PE, García T, Martín R. 2008. Real-time PCR for detection and quantification of red deer (*Cervus elaphus*), fallow deer (*Dama dama*), and roe deer (*Capreolus capreolus*) in meat mixtures. *Meat Sci* 79: 289–298.

Jaenson TGT, Talleklint L. 1992. Incompetence of roe deer as reservoirs of the Lyme borreliosis spirochete. *J Med Entomol* 29:813–817.

Linnel JDC, Zachos FE. 2010. Status and distribution patterns of European ungulates: Genetics, population history and conservation. In: *Ungulate management in Europe: Problems and practices,* Putman R, Apollonio M, Anderson R, editors. Cambridge University Press, Cambridge, UK, pp. 12–53.

Manilla G. 1998. *Fauna d'Italia. Acari: Ixodida*, Vol. 36. Edizioni Calderini, Bologna, Italy, 280 pp.

Mariconti M, Epis S, Gaibani P, Dalla Valle C, Sassera D, Tomao P, Fabbi M, Castelli F, Marone P, Sambri V, et al. 2012. Humans parasitized by the hard tick *Ixodes ricinus* are seropositive to *Midichloria mitochondrii*: Is *Midichloria* a novel pathogen, or just a marker of tick bite? *Pathogens Glob Health* 106:391–396.

Merler S, Furlanello C, Chemini C, Nicolini G. 1996. Classification tree methods for analysis of mesoscale distribution of *Ixodes ricinus* (Acari: Ixodidae) in Trentino, Italian Alps. *J Med Entomol* 33:888–893.

Montagna M, Sassera D, Epis S, Bazzocchi C, Vannini C, Lo N, Sacchi L, Fukatsu T, Petroni G, Bandi C. 2013. '*Candidatus* Midichloriaceae' fam. nov. (Rickettsiales), an ecologically widespread clade of intracellular Alphaproteobacteria. *Appl Environ Microbiol* 79: 3241–3248.

Nelson DR, Rooney S, Miller NJ, Mather TN. 2000. Complement-mediated killing of *Borrelia burgdorferi* by nonimmune sera from sika deer. *J Parasitol* 86: 1232–1238.

Parola P, Paddock CD, Socolovschi C, Labruna MB, Mediannikov O, Kernif T, Abdad MY, Stenos J, Bitam I, Fournier PE, et al. 2013. Update on tick-borne rickettsioses around the world: A geographic approach. *Clin Microbiol Rev* 26:657–702.

Richard E, Morellet N, Cargnelutti B, Angibault JM, Vanpé C, Hewison AJM. 2008. Ranging behaviour and excursions of female roe deer during the rut. *Behav Process* 79:28–35.

Sassera D, Beninati T, Bandi C, Bouman EAP, Sacchi L, Fabbi M, Lo N. 2006. '*Candidatus* Midichloria mitochondrii', an endosymbiont of the tick *Ixodes ricinus* with a unique intramitochondrial lifestyle. *Int J Syst Evol Microbiol* 56:2535–2540.

Sassera D, Lo N, Bouman EAP, Epis S, Mortarino M, Bandi C. 2008. '*Candidatus* Midichloria' endosymbionts bloom after the blood meal of the host, the hard tick *Ixodes ricinus*. *Appl Environ Microbiol* 74: 6138–6140.

Skarphédinsson S, Jensen PM, Kristiansen K. 2005. Survey of tick-borne infections in Denmark. *Emerg Infect Dis* 11:1055–1061.

Skotarczak B, Adamska M, Sawczuk M, Maciejewska A, Wodecka B, Rymaszewska A. 2008. Coexistence of tick-borne pathogens in game animals and ticks in western Poland. *Vet Med* 53:668–675.

Vázquez LV, Panadero R, Dacal V, Pato FJ, López C, Díaz P, Arias MS, Fernández G, Díez-Baños P, Morrondo P. 2011. Tick infestation (Acari: Ixodidae) in roe deer (*Capreolus capreolus*) from northwestern Spain: Population dynamics and risk stratification. *Exp Appl Acarol* 53:399–409.

Vor T, Kiffner C, Hagedorn P, Niedrig M, Rühe F. 2010. Tick burden on European roe deer (*Capreolus capreolus*). *Exp Appl Acarol* 51:405–417.

Wodecka B, Rymaszewska A, Skotarczak B. 2014. Host and pathogen DNA identification in blood meals of nymphal *Ixodes ricinus* ticks from forest parks and rural forests of Poland. *Exp Appl Acarol* 62:543–555.

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