Seropositivity to *Midichloria mitochondrii* (order Rickettsiales) as a marker to determine the exposure of humans to tick bite

Valentina Serra^a, Viktoria Krey^b, Christina Daschkin^{b,c}, Alessandra Cafiso^{id a}, Davide Sassera^d, Horst-Günter Maxeiner^e, Letizia Modeo^{id f}, Carsten Nicolaus^b, Claudio Bandi^{g,h} and Chiara Bazzocchi^{a,h,i}

^aDepartment of Veterinary Medicine, University of Milan, Milan, Italy; ^bBCA-research, BCA-clinic Betriebs GmbH & Co. KG, Augsburg, Germany; ^cCRELUX GmbH - a WuXi AppTec company, Martinsried, Germany; ^dDepartment of Biology and Biotechnology, University of Pavia, Pavia, Italy; ^eDepartment of Laboratory Medicine, St. Gertrauden Khs, Teaching Hospital of the Charite, Berlin, Germany; ^fDepartment of Biology, University of Pisa, Pisa, Italy; ^gDepartment of Biosciences, University of Milan, Milan, Italy; ^hPediatric Clinical Research Center “Romeo ed Enrica Invernizzi”, University of Milan, Milan, Italy; ⁱCoordinated Research Center “EpiSoMI”, University of Milan, Milan, Italy

ABSTRACT

Ixodes ricinus is the most common tick species parasitizing humans in Europe, and the main vector of *Borrelia burgdorferi* sensu lato, the causative agent of Lyme disease in the continent. This tick species also harbors the endosymbiont *Midichloria mitochondrii*, and there is strong evidence that this bacterium is inoculated into the vertebrate host during the blood meal. A high proportion of tick bites remains unnoticed due to rarity of immediate symptoms, implying the risk of occult tick-borne infections in turn a potential risk factor for the onset of chronic-degenerative diseases. Since suitable tools to determine the previous exposure to *I. ricinus* bites are needed, this work investigated whether seropositivity toward a protein of *M. mitochondrii* (rFlitD) could represent a marker for diagnosis of *I. ricinus* bite. We screened 274 sera collected from patients from several European countries, at different risk of tick bite, using an ELISA protocol. Our results show a clear trend indicating that positivity to rFlitD is higher where the tick bite can be regarded as certain/almost certain, and lower where there is an uncertainty on the bite, with the highest positivity in Lyme patients (47.30%) and the lowest (2.00%) in negative controls. According to the obtained results, *M. mitochondrii* can be regarded as a useful source of antigens, with the potential to be used to assess the exposure to ticks harboring this bacterium. In prospect, additional antigens from *M. mitochondrii* and tick salivary glands should be investigated and incorporated in a multi-antigen test for tick bite diagnosis.

KEYWORDS

Tick; serology; endosymbiont; Lyme disease

Introduction

Ticks are among the most important vectors of pathogens, causing human and animal diseases, in tropical, sub-tropical and temperate countries [1]. Approximately 10% of tick species are estimated to play a role in the transmission of a total of over 200 pathogens [2,3] including zoonotic bacteria (e.g. *Borrelia burgdorferi* sensu lato, *Rickettsia rickettsii*, *Coxiella burnetii*, *Ehrlichia canis*, *Francisella tularensis* [2]) and viruses (e.g. tick-borne encephalitis virus, Crimean-Congo hemorrhagic fever virus [2,4]).

In Europe, the most common tick-borne disease (TBD) is Lyme borreliosis (LB), caused by different genospecies of the spirochete *B. burgdorferi* s.l. [5]. These bacteria are transmitted by multiple tick species belonging to the *Ixodes* genus and infect several vertebrate species [6,7].

Ixodes ricinus, the main vector of LB in Europe [5], presents a wide geographical distribution throughout the European continent [8–10]. Moreover, this tick is responsible for the transmission of over 90% of the

TBDs occurring in this area [11], being also a competent vector of other pathogens, including spotted fever group *Rickettsia* species, *Borrelia miyamotoi*, *Anaplasma phagocytophilum*, *Babesia divergens* and *Babesia microti*. Human cases of parasitism by *I. ricinus* are rising, and many factors are involved in the current spread of this species [12,13] leading to a higher risk of tick bite exposure.

I. ricinus also hosts the alphaproteobacterium *Midichloria mitochondrii*, a symbiont belonging to the *Midichloriaceae* family (order *Rickettsiales*). The main route of transmission of this bacterium is maternal transmission to the progeny, although strong evidence supports the horizontal transmission as well [14–18]. This intracellular bacterium is present in all life stages of *I. ricinus*, being of notice that in the ovary and salivary glands of adult females the prevalence is of 100.00%, whereas in the adult males of the same species prevalence is around 44.00% [19–21]. There are indications that *M. mitochondrii* is transmitted to the vertebrate host during the blood meal, based on the seropositivity of both humans and animals exposed to *I. ricinus* bite

[16–18,22]. To reinforce these findings, *Midichloria* spp. DNA was detected by PCR in the blood of animals exposed to *I. ricinus* and other ticks [17,18,23], and there is indirect evidence for the replication of this endosymbiont once inoculated into the vertebrate host [22]. Regarding the tick bites performed by these arthropods to obtain a blood meal, they are characterized by the absence of pain and itch (which are blocked by anesthetic and anti-inflammatory components of the tick saliva), and rarity of immediate symptoms such as erythema migrans (EM). It is thus a general assumption that a high proportion of tick bites remains undetected (only 30.00% of patients with symptoms referable to tick-borne diseases report a history of tick bite [24] and just two-third of the cases enrolled with EM recall preceding tick bites [25]). On one hand, these features make it difficult to estimate the actual impact of these vectors, as undetected tick bites carry the risk of occult tick-borne infections (TBIs) in humans and animals, and their impact on public health is currently a matter of debate. On the other hand, it has been estimated that the exposure to tick bite only results in around 2.00% of the cases in EM and in approximately 0.20% of the cases in disseminated LB [26,27].

Several authors have hypothesized the possibility that occult TBIs are a risk factor for chronic-degenerative diseases with complex etiology (among which multiple sclerosis, rheumatological disorders, amyotrophic lateral sclerosis), with strong convincing evidence, to our knowledge, only for dilated cardiomyopathy [28–31].

Understanding whether tick bite represents a risk factor for chronic pathologies would require epidemiological studies on the association between tick exposure and a given pathology. There is thus the need for tools suitable to determine the previous exposure of a subject to tick bite. In addition, the complex diagnosis of most TBDs and in particular of Lyme disease (LD) could be improved by the acquisition of anamnestic information on previous tick exposure.

A tool capable of determining whether the patient has been parasitized could thus be incorporated into the diagnostic algorithms in situations of poor anamnestic information. The objective of this work was to evaluate whether seropositivity toward a protein of *M. mitochondrii* could represent a marker suitable to determine the exposure of humans to *I. ricinus* bite.

Material and methods

Sample collection

A total of 274 human sera previously tested with the *Borrelia* Blot (Anti-*Borrelia* Euroline RN AT IgG/IgM (Euroimmun, DN 2131–3201-G/N, Lübeck, Germany)) were retrospectively selected from the collection of a specialized tick-borne diseases laboratory (BCA-clinic of Augsburg; Germany). These samples (categorized as

Table 1. Number of sera samples analyzed for each country.

Country	No. of samples
Germany	114
France	33
Norway	28
Sweden	12
Netherlands	11
Denmark	10
Romania	9
Belgium	7
United Kingdom	7
Switzerland	6
Austria	5
Russia	5
Ukraine	5
Italy	4
Poland	3
Belarus	3
Finland	3
Spain	3
Czech Republic	2
Croatia	2
Ireland	1
Turkey	1

positive, borderline, or negative to IgG and IgM) were obtained from patients from several European countries (countries of origin and number of sera are detailed in Table 1).

Presence or absence of a report of tick bite was associated to the metadata referred to each serum sample. One hundred seventy-nine out of 274 patients reported memory of tick bite, while the remaining 95 patients did not declare a history of tick parasitism.

Anaplasma phagocytophilum, *Rickettsia* spp., and *Babesia* spp. antibodies were measured to complete the set of *Ixodes ricinus* associated pathogens in order to add some further support to determine a previous exposure to ticks, in addition to the memory of a tick bite and to referred symptoms. The kits used to determine the expanded serological status were: *A. phagocytophilum* IFA IgG/IgM (Focus Diagnostics, IF1450G/M, Cypress, USA/CA); *Rickettsia* IFA IgG/IgM (Focus Diagnostics, IF0100g/M); *Babesia* (*B. microti*) IFA Substrate Slide and *Babesia* IgG Detectable control (Focus Diagnostics, IF0904 and IF0910). The serological kits were used and interpreted according to the manufacturer's instructions.

Additionally, 50 sera were obtained from healthy donors that did not recall a tick bite and did not present any symptoms related to tick bite. These sera, collected from a blood service in Hamburg and previously tested negative for *Borrelia* s.l., *A. phagocytophilum*, *Rickettsia* spp., and *Babesia* spp. antibodies using the aforementioned kits, were used as negative controls. All sera samples were stored at -80°C until use. Written informed consent for the use of biological material was obtained from all patients prior to serum sampling.

ELISA assay

The *Midichloria mitochondrii* FliD protein recombinant form (rFliD [32]) was produced in *Escherichia coli* and used as substrate for ELISA assays.

ELISA flat-bottom 96-well plates were coated with 0.1 µg/well of rFlitD diluted in carbonate-bicarbonate buffer (Sodium carbonate anhydrous 15 mM and Sodium hydrogen carbonate 35 mM). After the incubation at 4°C over-night, the plate was washed three times for 5 min using 200 µl/well of washing buffer (PBS 1X and 0.10% Tween 20), and was incubated at 37°C for 1 hour with the coating buffer (PBS 1X and 3.00% Bovine serum Albumine). Sera were analyzed in duplicate at a dilution of 1:100, and the anti-human IgG HRP-conjugated antibody (Sigma-Aldrich)[®] was employed at 1:2000. After the preparation of the staining solution (citric acid 25 mM and Sodium phosphate dibasic 45 mM), the plate was incubated at dark with a solution (100 µl/well) consisting of 20 ml of staining solution plus 8 µl of hydrogen peroxide 30.00% and 1 o-Phenylenediamine dihydrochloride tablet 0.4 mg/ml (Sigma)[®] for 10 min. The optical density (O.D.) was measured at a wavelength of 450 nm using the 'Sunrise™ absorbance microplate reader' (Tecan)[®] and the data analysis software Magellan™ Standard (Tecan)[®].

The threshold of the reaction was established using sera from the 50 healthy blood donors and it was set as the mean optical density (O.D.) plus three times the standard deviation. Each sample was considered negative if its O.D. was less than the threshold value (0.32), and positive if its O.D. was higher or equal to the threshold.

To test whether proportion of seropositive samples was different between groups of patients, Chi-square test was performed considering a significant level at $P < 0.05$.

Results and discussion

A total of 274 sera from patients suffering from different symptoms potentially referable to tick-borne diseases (TBDs) were included in this study. Among these sera, the majority (179) had been collected from patients who remembered a tick bite, the remaining (95) from patients that did not recall a tick bite. Fifty sera were collected from healthy blood donors that resulted negative for serological tick-borne pathogens (TBPs) assays.

In order to identify which patients could have been parasitized by a tick, we analyzed all the 274 sera for positivity to TBPs, i.e. *Borrelia burgdorferi* s.l., *Anaplasma phagocytophilum*, *Rickettsia* spp., and *Babesia* spp. We emphasize that the kit used for *Rickettsia* spp. diagnosis is not specific for tick-borne rickettsiae, and could thus detect infections deriving from other vectors/sources. However, all the five subjects that tested positive to *Rickettsia* spp. reported a tick bite, which can be correlated with tick-borne rickettsia infection. We would thus suggest that the cross-reactivity with rickettsiae non-vectorized by ticks

had only minor impact on the obtained results, if any. The results of the serological analyses showed that 36.00% of sera were positive to *B. burgdorferi* s.l., 6.00% to *A. phagocytophilum*, 1.80% to *Rickettsia* spp. and 0.36% to *Babesia* spp. (see [Supplementary Table 1](#)). Based on the anamnestic information on the tick bite, on clinical diagnosis for Lyme disease (LD) and on the serological analyses results, we divided the patients into five groups: i) LD positive diagnosis (38 patients, 31 displaying the erythema migrans (EM) and Lyme positive serology; the remaining seven with positive serology and other clinical signs and symptoms); ii) seropositive to at least one TBP (81 patients, LD negative diagnosis); iii) reported tick bite (92 patients, seronegative to TBPs); iv) reported TBD-related symptoms (63 patients, TBDs negative diagnosis); v) negative controls (50 healthy donors). We emphasize that these five groups are to be intended as cohorts of subjects in which the probability of a bite by *Ixodes ricinus* is decreasing. In addition, it is also necessary to consider that subjects not reporting a tick bite could have been exposed to ticks and subjects whose serum was positive to TBPs could possibly be false-positive and/or may have been parasitized by a tick species other than *I. ricinus*. Even considering the above limitations, our results support the existence of these five different rankings in terms of the decreased risk of previous exposure to *I. ricinus*.

All sera were then analyzed for the presence of anti-*Midichloria mitochondrii* antibodies, using the anti-rFlitD ELISA assay. Rates of positivity for *M. mitochondrii* antigens in the five groups are shown in [Figure 1](#). Considering the results obtained from the five groups, there is a trend showing that the increase in seropositivity to *M. mitochondrii* is highly correlated with an increase in seropositivity and presence of clinical signs/symptoms for TBPs ($\chi^2 = 27.99$; $df = 4$; $p < 0.001$).

The highest prevalence (47.30%) was indeed found in LD patients in which tick bite can be considered certain/almost certain. The symbiont prevalence becomes lower as evidence of the bite becomes uncertain (memory of a bite, or just symptoms) and the lowest results were registered in the negative controls (2.00%). The positivity to rFlitD in subjects of the control group can be regarded as normal background cross reactivity or previous exposure to ticks or even to other possible *Midichloria* vectors. Seropositivity to *M. mitochondrii* in control subjects was also observed in a previous study conducted in Italy [16], although with a lower prevalence (1.20%). Although the observed difference is not significant, it is possible to speculate that the higher seroprevalence against rFlitD observed in control subjects in the current study could be expected in a country as Germany, where *I. ricinus* is more prevalent than Italy.

The possibility that seropositivity to *M. mitochondrii* could represent a serological marker for *I. ricinus* bite in

Figure 1. Detection of anti-*M. mitochondrii* antibodies in human patients suffering from different symptoms potentially referable to tick-borne diseases from different European countries. The patients are divided into five groups according to the anamnestic information of the tick bite, the clinical diagnosis for Lyme disease, and the results of serological analyses for tick-borne pathogens (TBP; i.e. *Borrelia* spp., *A. phagocytophilum*, *Rickettsia* spp., and *Babesia* spp.). Results are expressed as: M+ = sera positive to *M. mitochondrii*.

humans had already been suggested, based on a study limited to a total of 80 Italian patients with a diagnosis for LD [16]. Here we have extended this screening to a total of 274 subjects from several European countries, with different expected levels of *I. ricinus* exposure. In general, the tick species that most frequently parasitize companion animals and humans in Europe are *I. ricinus*, *Rhipicephalus sanguineus* sensu lato and *Dermacentor reticulatus* [33,34]. Thus, considering that *R. sanguineus* s.l. and *D. reticulatus* have never been shown to harbor bacteria of the *Midichloriaceae* family [35,36], positivity to rFlID is with a high chance an indicator of an *I. ricinus* bite.

Overall, our results support the potential utility of anti-rFlID seropositivity as a marker for *I. ricinus* bite in humans. Nevertheless, some additional studies still need to be performed to confirm our findings and to increase the methodology sensitivity. In fact, there is a non-complete overlap between the subjects at high, and reliable, previous exposure to ticks and positivity to *M. mitochondrii* (i.e. up to approx. 50.00% in LD patients). Several reasons can explain this observation: i) a tick species other than *I. ricinus* could have parasitized the patient (no ticks were recovered from the patients enrolled in this study); ii) only female ticks of *I. ricinus* are known to reach a 100% prevalence of the endosymbiont infection, as well as only adult females showed *M. mitochondrii* antigen and DNA in their salivary glands [16,37]; iii) the efficiency and mechanism of *M. mitochondrii* transmission to the host by the tick is still to be determined. Indeed, although the work conducted by Cafiso et al. [22] provides evidence for a positive antibody response against rFlID protein, even when a vertebrate host is parasitized by *I. ricinus* ticks

harboring reduced bacterial loads, evidence for sero-conversion is still lacking for immature stages. This consideration is noteworthy since in most cases humans are parasitized by *I. ricinus* larvae and/or nymphs [38]. In addition, we should also consider the possibilities that a portion of the subjects reporting a record for tick bite: i) had not been parasitized by a tick; ii) had been parasitized by a tick species other than *I. ricinus*; iii) the tick was not harboring *M. mitochondrii*; iv) the tick was harboring a *Midichloria* species that did not cross-react with anti-rFlID antibodies.

Additional techniques such as PCR have been used to demonstrate the circulation of *M. mitochondrii* DNA in several hosts parasitized by *I. ricinus* [17,18,22]. This approach could be a useful tool to investigate a recent tick bite when the host is intensively parasitized. On the other hand, if the bite of a single tick occurred weeks or months before the analysis, as in the case of the present work, the chance to detect the circulating bacterial DNA would be very low.

In summary, our study shows that *M. mitochondrii* can be regarded as a useful source of antigens for the development of a serological test for *I. ricinus* bite. We believe that this assay is a valuable contribution to future epidemiological studies, highly important to the acquisition of anamnestic information in patients.

In prospect, with the goal of increasing the sensitivity of a serological test for *I. ricinus* bite, additional antigens derived from *M. mitochondrii* and from the tick salivary glands could be pooled in a multi-antigen antibody detection assay, following, for instance, the method developed for the diagnosis of *Culicoides* spp. bite in horses [39].

Conclusions

This work provides further indirect evidence for the transmission of *Ixodes ricinus* symbiont *Midichloria mitochondrii* to humans during a tick blood meal in different European countries. The results of the current study, obtained for different European countries with an emphasis in German patients, are coherent with those of a preliminary study conducted on a smaller set of humans exposed to ticks in Italy [16]. In prospect, other antigens from *M. mitochondrii*, as well as from the tick itself, could be investigated and incorporated into a multi-antigen test for the diagnosis of the tick bite. The protein rFlid can be regarded as a good candidate to be included into such a type of multi-antigen test. Even though this study provides evidence for the presence of antibodies against the *I. ricinus* symbiont *M. mitochondrii* in human hosts, further studies are needed to determine the mechanism of transmission of these bacteria (or their antigens) from the tick to the human-host, as well as the period of their survival inside the host itself.

Acknowledgments

This work was partially financed by the Italian Ministry of Education, University and Research PRIN-2012 grant 2012A4F828 to Ch.B. and by Human Frontier Science Program grant RGY0075-2017 to D.S.

Disclosure statement

No potential conflict of interest was reported by the authors.

Funding

This work was supported by the Human Frontier Science Program [RGY0075-2017]; Italian Ministry of Education, University and Research PRIN-2012 [2012A4F828].

ORCID

Alessandra Cafiso <http://orcid.org/0000-0001-8739-3422>
Letizia Modeo <http://orcid.org/0000-0003-2291-7218>

References

- [1] Karaer Z, Guven E, Nalbantoglu S, et al. Ticks on humans in Ankara, Turkey. *Exp Appl Acarol*. 2011;54:85–91.
- [2] Jongejan F, Uilenberg G. The global importance of ticks. *Parasitol*. 2004;129(Suppl):S3–14.
- [3] Labuda M, Nuttall PA. Tick-borne viruses. *Parasitol*. 2004;129(Suppl):S221–S245.
- [4] Despommier DD, Gwadz RW, Hotez PJ, et al. *Parasitic diseases*. New York: Apple Trees Prod; 2000.
- [5] Mead PS. Epidemiology of Lyme disease. *Infect Dis Clin*. 2015;29:187–210.
- [6] Brisson D, Dykhuizen DE, Ostfeld RS. Conspicuous impacts of inconspicuous on the Lyme disease epidemic. *Proc Biol Sci*. 2008;275:227–235.

- [7] Tsao JI. Reviewing molecular adaptations of Lyme borreliosis spirochetes in the context of reproductive fitness in natural transmission cycles. *Vet Res*. 2009;40:36.
- [8] Bartosik K, Sitarz M, Szymanska J, et al. Tick bites on humans in the agricultural and recreational areas in south-eastern Poland. *Ann Agric Environ Med*. 2011;18:151–157.
- [9] Otranto D, Dantas-Torres F, Giannelli A, et al. Ticks infesting humans in Italy and associated pathogens. *Parasit Vectors*. 2014;7:328.
- [10] Petney TN, Pfäffle MP, Skuballa JD. An annotated checklist of the ticks (Acari: ixodida) of Germany. *Syst Appl Acarol*. 2012;17(2):115–170.
- [11] Süss J, Fingerle V, Hunfeld KP, et al. Durch Zecken übertragene humanpathogene und bisher als apathogen geltende Mikroorganismen in Europa – teil I Zecken und Viren. *Bundesgesundheitsbl-Gesundheitsforsch-Gesund-heitsschutz*. 2004;47:470–486.
- [12] Medlock JM, Hansford KM, Bormane A, et al. Driving forces for changes in geographical distribution of *Ixodes ricinus* ticks in Europe. *Parasit Vectors*. 2013;6:1.
- [13] Coipan EC, Jahfari S, Fonville M, et al. Spatiotemporal dynamics of emerging pathogens in questing *Ixodes ricinus*. *Front Cell Infect Microbiol*. 2013;3:36.
- [14] Sasser D, Beninati T, Bandi C, et al. “*Candidatus Midichloria mitochondrii*”, an endosymbiont of the tick *Ixodes ricinus* with a unique intramitochondrial lifestyle. *Int J Syst Evol Microbiol*. 2006;56:2535–2540.
- [15] Montagna M, Sasser D, Epis S, et al. *Candidatus Midichloriaceae* fam. nov. (Rickettsiales), an ecologically widespread clade of intracellular alphaproteobacteria. *J Appl Environ Microbiol*. 2013;79:3241–3248.
- [16] Mariconti M, Epis S, Gaibani P, et al. 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? *Pathog Glob Health*. 2012;106:391–396.
- [17] Bazzocchi C, Mariconti M, Sasser D, et al. Molecular and serological evidence for the circulation of the tick symbiont *Midichloria* (Rickettsiales: midichloriaceae) in different mammalian species. *Parasit Vectors*. 2013;6:350.
- [18] Serra V, Cafiso A, Formenti N, et al. Molecular and serological evidence of the presence of *Midichloria mitochondrii* in roe deer (*Capreolus capreolus*) in France. *J Wildl Dis*. 2018;54:597–600.
- [19] Lo N, Beninati T, Sasser D, et al. Widespread distribution and high prevalence of an alpha-proteobacterial symbiont in the tick *Ixodes ricinus*. *Environ Microbiol*. 2006;8:1280–1287.
- [20] Sasser D, Lo N, Bouman EA, et al. “*Candidatus Midichloria*” endosymbionts bloom after the blood meal of the host, the hard tick *Ixodes ricinus*. *J Appl Environ Microbiol*. 2008;74:6138–6140.
- [21] Epis S, Mandrioli M, Genchi M, et al. Localization of the bacterial symbiont *Candidatus Midichloria mitochondrii* within the hard tick *Ixodes ricinus* by whole-mount FISH staining. *Ticks Tick Borne Dis*. 2013;4:39–45.
- [22] Cafiso A, Sasser D, Romeo C, et al. *Midichloria mitochondrii*, endosymbiont of *Ixodes ricinus*: evidence for the transmission to the vertebrate host during the tick blood meal. *Ticks Tick Borne Dis*. 2019;10:5–12.
- [23] Di Lecce I, Bazzocchi C, Cecere JG, et al. Patterns of *Midichloria* infection in avian-borne African ticks and their trans-Saharan migratory hosts. *Parasit Vectors*. 2018;11:106.

- [24] Perronne C. Lyme and associated tick-borne diseases: global challenges in the context of a public health threat. *Front Cell Infect Microbiol.* [2014](#);4:74.
- [25] Mulder S, AJH VV, Bron WA, et al. High risk of tick bites in Dutch gardens. *Vector Borne Zoonotic Dis.* [2013](#);13:865–871.
- [26] Sprong H, van den Wijngaard KCC. Prevention of Lyme borreliosis after a tick bite. In: *Ecology and prevention of Lyme Borreliosis*. Wageningen Academic Publishers; [2016](#). p. 327–334.
- [27] Jahfari S, Hofhuis A, Fonville M, et al. Molecular detection of tick-borne pathogens in humans with tick bites and Erythema Migrans, in the Netherlands. *PLoS Negl Trop Dis.* [2016](#);10:1–15.
- [28] Hänsel Y, Ackerl M, Stanek G. ALS-like sequelae in chronic neuroborreliosis. *Wien Med Wochenschr.* [1995](#);145:186–188.
- [29] Halperin JJ, Kaplan GP, Brazinsky S, et al. Immunologic reactivity against *Borrelia burgdorferi* in patients with motor neuron disease. *Arch Neurol.* [1990](#);47:586–594.
- [30] Harvey WT, Martz D. Motor neuron disease recovery associated with IV ceftriaxone and anti-*Babesia* therapy. *Acta Neurol Scand.* [2007](#);115:129–131.
- [31] Lelovas P, Dontas I, Bassiakou E, et al. Cardiac implications of Lyme disease, diagnosis and therapeutic approach. *Int J Cardiol.* [2008](#);129:15–21.
- [32] Mariconti M, Epis S, Sacchi L, et al. A study on the presence of flagella in the order Rickettsiales: the case of '*Candidatus* Midichloria mitochondrii'. *Microbiol.* [2012](#);158:1677–1683.
- [33] Claerebout E, Losson B, Cochez C, et al. Ticks and associated pathogens collected from dogs and cats in Belgium. *Parasit Vectors.* [2013](#);6:183.
- [34] Geurden T, Becskei C, Six RH, et al. Detection of tick-borne pathogens in ticks from dogs and cats in different European countries. *Ticks Tick Borne Dis.* [2018](#);9:1431–1436.
- [35] Epis S, Sasser D, Beninati T, et al. *Midichloria mitochondrii* is widespread in hard ticks (Ixodidae) and resides in the mitochondria of phylogenetically diverse species. *Parasitology.* [2008](#);135:485–494.
- [36] Duron O, Binetruy F, Noël V, et al. Evolutionary changes in symbiont community structure in ticks. *Mol Ecol.* [2017](#);26:2905–2921.
- [37] Olivieri E, Epis S, Castelli M, et al. Tissue tropism and metabolic pathways of *Midichloria mitochondrii* suggest tissue-specific functions in the symbiosis with *Ixodes ricinus*. *Ticks Tick Borne Dis.* [2019](#);10:1070–1077. (Article in press).
- [38] Wilhelmsson P, Lindblom P, Fryland L, et al. *Ixodes ricinus* ticks removed from humans in Northern Europe: seasonal pattern of infestation, attachment sites and duration of feeding. *Parasit Vectors.* [2013](#);6:362.
- [39] van der Meide NMA, Savelkoul HFJ, Meulenbroeks C, et al. Evaluation of a diagnostic ELISA for insect bite hypersensitivity in horses using recombinant *Obsoletus* complex allergens. *Vet J.* [2014](#);200:31–37.