Ticks and Tick-borne Diseases 10 (2019) 101241

Contents lists available at ScienceDirect

Ticks and Tick-borne Diseases

journal homepage: www.elsevier.com/locate/ttbdis

Short communication

Co-infection of bacteria and protozoan parasites in *Ixodes ricinus* nymphs collected in the Alsace region, France

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ARTICLE INFO

Keywords:
Ixodes ricinus
Co-infection
Bacterial pathogens
Protozoan parasites
MALDI-TOF MS

ABSTRACT

Fifty nymphal *Ixodes ricinus* ticks collected in Alsace, France, identified by morphological criteria and using MALDI-TOF MS, were tested by PCR to detect tick-associated bacteria and protozoan parasites. Seventy percent (35/50) of ticks contained at least one microorganism; 26% (9/35) contained two or more species. Several human pathogens were identified including *Borrelia burgdorferi* s.s. (4%), *Borrelia afzelii* (2%), *Borrelia garinii* (2%), *Borrelia valaisiana* (4%), *Borrelia miyamotoi* (2%), *Rickettsia helvetica* (6%) and "*Babesia venatorum*" (2%). *Bartonella* spp. (10%) and a *Wolbachia* spp. (8%) were also detected. The most common co-infections involved *Anaplasmataceae* with *Borrelia* spp. (4%), *Anaplasmataceae* with *Bartonella* spp. (6%) and *Anaplasmataceae* with *Rickettsia* spp. (6%). Co-infection involving three different groups of bacteria was seen between bacteria of the family *Anaplasmataceae, Borrelia* spp. and *Bartonella* spp. (2%). Results highlight the panel of infectious agents carried by *Ixodes ricinus*. Co-infection suggests the possibility of transmission of more than one pathogen to human and animals during tick blood feeding.

1. Introduction

Ixodes ricinus, the European sheep tick, is widely found in temperate regions of the northern hemisphere. It has a high attraction to biting humans (Parola and Raoult, 2001) and well known in Europe as the vector of *Borrelia burgdorferi* sensu lato (s.l.), the etiologic agent of Lyme disease (Stanek et al., 2012), tick-borne encephalitis (Amicizia et al., 2013) and granulocytic anaplasmosis (Jin et al., 2012). The Alsace and Meuse areas, in north-eastern France, are the most endemic regions of the country with more than 100 cases per 100,000 inhabitants between 2009 and 2011 (HCSP, 2014). Moreover, *I. ricinus* ticks have been reported to harbour or transmit other emerging human pathogens such as *Rickettsia helvetica, Rickettsia monacensis* and *Babesia divergens* (Rizzoli et al., 2014). The emergence of tick-borne diseases in the past decade is worrying (Silaghi et al., 2016) and has underlined the need to strengthen epidemiological and vector surveys. A recent study conducted in the region of the French Ardennes on *I. ricinus* revealed high microorganism co-infection rates in adult ticks, raising questions about the possible co-transmission of these agents to humans or animals, and the potential consequences for human and animal health (Moutailler et al., 2016). The aim of the present work was to test nymphal *I. ricinus* stage infections by *Borrelia* spp. and other associated microorganisms circulating in an area of the Alsace region using molecular tools.

2. Materials and methods

Ticks were collected using the drag flag technique (Parola and Raoult, 2001) between May and June 2014 in the region of Murbach: N 47.918961 ; E7.210436 ; Altitude 520 m (Haut-Rhin, Alsace, France) in the frame of the study of Goldstein et al. 2018 (Goldstein et al., 2018). A convenient sample of 50 nymphs were selected for the current study, to

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https://doi.org/10.1016/j.ttbdis.2019.06.001
Received 29 November 2018; Received in revised form 8 May 2019; Accepted 1 June 2019

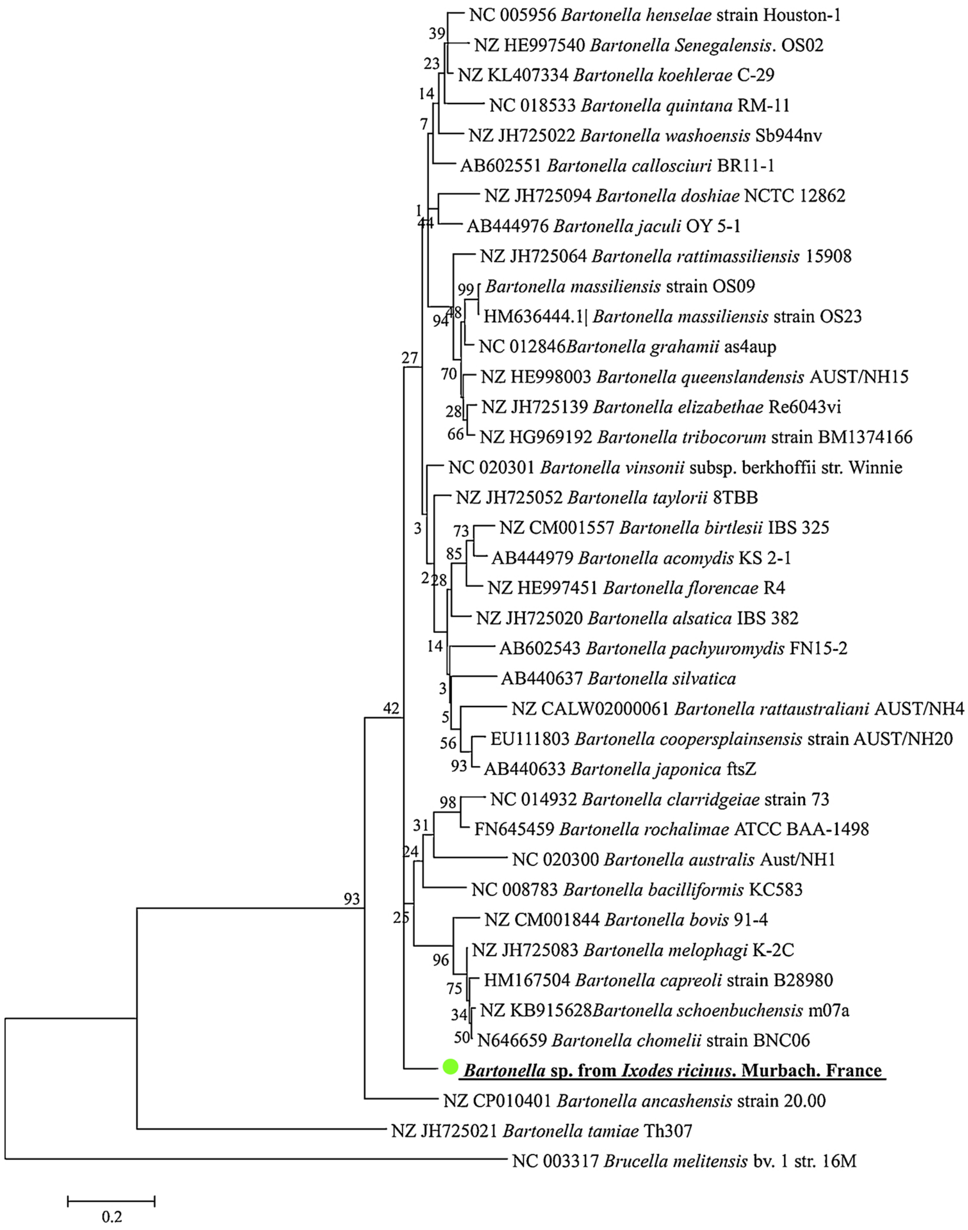

Fig. 1. Phylogenetic tree showing the position of *Bartonella* sp. amplified from *I. ricinus* in this study compared to other species. The sequences of the *Bartonella* sp. obtained in this study were aligned with *Bartonellaceae* family sequences available on GenBank using CLUSTALW implemented on BioEdit v3 (Hall, 1999). The sequence of *ftsZ* gene was first aligned, gaps and missing data were eliminated. The phylogenic tree was constructed using MEGA7 (Kumar et al., 2016). The evolutionary history was inferred by using the Maximum Likelihood method based on the Hasegawa-Kishino-Yano model. The percentage of trees in which the associated taxa clustered together is shown next to the branches. Initial tree(s) for the heuristic search were obtained automatically by applying Neighbor-Join and BioNJ algorithms to a matrix of pairwise distances estimated using the Maximum Composite Likelihood (MCL) approach and then selecting the topology with superior log likelihood value. A discrete Gamma distribution was used to model evolutionary rate differences among sites (4 categories (+*G*, parameter = 0.3117)). The tree is drawn to scale, with branch lengths measured in the number of substitutions per site. The analysis involved 39 nucleotide sequences. Codon positions included were 1st + 2nd + 3rd + Noncoding. All positions containing gaps and missing data were eliminated. There was a total of 335 positions in the final dataset.

launch a collaboration between two reference centers in France. Each specimen was morphologically identified using standard taxonomic keys (Hylliard, 1996). Proteomic identification using MALDI-TOF MS (Matrix-assisted laser desorption/ionization mass spectrometry) was conducted as previously described (Yssouf et al., 2013). DNA from these specimens was then extracted using the EZ1® Tissue Kit and BioRobot® EZ1®(QIAGEN, Hilden, Germany) according to the manufacturer's instructions. To assess the presence of the DNA of common microorganisms in ticks, all extracted DNA were screened by real-time polymerase chain reaction (qPCR), targeting specific genes of *Borrelia* spp. (*16S rRNA*), *Rickettsia* spp. (*gltA*), *R. helvetica* (*rfbE*), *Bartonella* spp. (*ITS*), *Bartonella henselae (Pap31),* Anaplasmataceae (*23S*), *Anaplasma phagocytophilum* (*polA*), *Coxiella burnetii* (*IS30A*) and *Babesia* spp. (*18SrDNA*), as previously described (Aubry et al., 2016). Negative controls (qPCR mix and DNA of uninfected *Rhipicephalus sanguineus* from laboratory colony) and positive controls (*Borrelia crocidurae, Bartonella elizabethae, R. monacensis, A. phagocytophilum*, *Babesia microti* and *C. burnetii* DNA originating from laboratory collection) were used in each respective qPCR. Ticks analyzed by qPCR were considered to be positive when the cycle threshold (Ct) value was less than or equal to 36, as used in previous studies (Aubry et al., 2016). All ticks which were identified as being positive by qPCR were confirmed by conventional PCR amplifications and sequencing. Sequences were analyzed as described previously (Aubry et al., 2016).

3. Results

All specimens were morphologically identified as the nymphal stage of *I. ricinus.* The protein spectra profiles from the MALDI-TOF MS analysis of tick legs were consistent and reproducible with high-intensity peaks in the range of 2–20 kDa (Additional file1). Of them, 34 tick specimens obtained log-score values (LSVs) higher than 1.8 when they were tested against the home-made reference MS spectra database. These LSVs (> 1.8) considered as correctly-identified specimens, corresponded to ticks from *I. ricinus*. For the remaining 16 ticks, the intensity and diversity of MS spectra were insufficient for relevant analysis.

Nearly 70% (35/50) of the *I. ricinus* collected were infected by at least one of the microorganisms tested by the qPCR (Additional file2). A total of 27/50 (54%) specimens harboured bacteria from the

Anaplasmataceae family. Positive samples were successfully amplified through the standard PCR using primers targeting 23S rRNA. However, after the sequencing, only four high-quality sequences were obtained. The sequences of the remaining 23 amplicons, were of poor quality. Blast analyses of the four high-quality sequences show 99.6% identity with "*Wolbachia* endosymbiont of *Drosophila incompta*" (GenBank accession no. CP011148). *Borrelia* spp. DNA was found in 20% (10/50) of the ticks. Sequencing analyses targeting the 16S rRNA gene long fragment led to the identification of seven sequences including two *B. burgdorferi* s.s. (GenBank accession no. CP019767 with 99.32 and 100% identity), two *B. valaisiana* (GenBank accession no. NR_036807, with 100% identity), one *B. afzelii* (GenBank accession no. KX688604, with 100% identity), one *B. garinii* (GenBank accession no. DQ147793, with 99.74% identity) and one *B. miyamotoi* (GenBank accession no. JF951382,100% identity). The three remaining sequences of *Borrelia* spp. were of poor quality and were removed from analyses. Three ticks were found to be positive for *Rickettsia* spp. *R. helvetica* was found in these three ticks using the *R. helvetica*-specific qPCR targeting the *rfbE* gene.

Bartonella spp. screening targeting the ITS gene found five positives. These five positive samples were only successfully amplified using primers targeting the long fragment of the *ftsZ* gene. None of the *rpoB, gltA* or ITS genes were amplified. The *ftsZ* sequences obtained from these five positive samples had less than 1% mismatches. Blast analyses showed 91.9% identity with uncharacterized *Bartonella* sp. strain B44608 (KY679153) and *B. vinsonii* subsp. *vinsonii* (AF467757) and 91.3% identity with *B. vinsonii* subsp. *berkhoffii* (KU292573). Phylogenetic analyses showed that this potentially new *Bartonella* sp. forms a separate clade between *Bartonella ancashensis* and the other *Bartonellaceae* species despite belonging to the latter (Fig. 1).

Lastly, from one tick the DNA of *Babesia* spp. was amplified using primers and probes targeting the 18S rDNA specific. A fragment of the *18S rRNA* gene was then amplified using conventional PCR primers. The resulting sequence was blasted and presented 100% identity with *Babesia* sp. "venatorum" (KU204792) and 98% with *B. odocoilei* (KC460321) detected in elk blood in Canada (Fig. 2). None of the tested ticks was positive for *A. phagocytophilum*, *B. henselae* or *C. burnetii*.

The overall prevalence of the pathogens found in this study was as follows: *B. burgdorferi* s.s. (4%), *B. afzelii* (2%), *B. garinii* (2%), *B. valaisiana* (4%), *B. miyamotoi* (2%), *R. helvetica* (6%),"*B. venatorum*" (2%), *Bartonella* spp. (10%) and *Wolbachia* spp. (8%).

Co-infection between two or three microorganisms was observed among the positive ticks. Indeed, co-infection by two microorganisms was found in 23% (8/35) of positive ticks. These include *Borrelia* spp. with *Anaplasmataceae* (2/35) and *Bartonella* spp. with *Anaplasmataceae* (3/35), followed by *Rickettsia* spp. with *Anaplasmataceae* (3/35). Co-infection with three microorganisms was found in 3% (1/35) of positive ticks. This multiple co-infection was found between *Borrelia* spp., *Bartonella* spp. and *Anaplasmataceae* (1/35)(Additional file2).

4. Discussion

Results reported in the present study indicate that *I. ricinus* ticks collected in Alsace, France were infected with multi-organism bacteria and protozoa (70%). However, the limited number of tested nymphs cannot reflect a complete epidemiological picture of all bacteria and protozoa circulating in or between *I. ricinus* ticks in this region. Thus, the full spectrum of bacteria infecting the collected *I. ricinus* has not been identified. That said, the results reported here present additional knowledge about *I. ricinus* associated microorganisms circulating in the north-eastern region of France.

The high rate of infection (70%) was previously reported in this same region (Ferquel et al., 2006) and in another neighbouring region (Moutailler et al., 2016). This study confirmed the presence of a broad spectrum of *Borrelia* species belonging to the Lyme disease group, namely: *B. burgdorferi* s.s., *B. afzelii*, *B. garinii* and *B. valaisiana,* already reported in this region (Ferquel et al., 2006; Moutailler et al., 2016). We also detected emerging pathogens such as *B. miyamotoi* and *R. helvetica* as well as a probable symbiotic bacteria belonging to the *Wolbachia* genus that has 99.6% identity with "*Wolbachia* endosymbiont of *Drosophila incompta"*. Protozoan parasites belonging to the *Babesia* genus were detected in 2% of ticks. However, phylogenetic analysis of the *18S rRNA* gene revealed that the *Babesia* detected here is part of a group of "*B. venatorum"* strains that form a separate clade. These strains have been detected in China (Jiang et al., 2015) and other European countries, such as Italy (Cassini et al., 2010), Slovenia (Duh et al., 2005), the Netherlands (Kik et al., 2011) and the Czech Republic (Venclikova et al., 2015), being isolated from *I. ricinus* ticks, roe deer or both. *Babesia "venatorum"*, previously called *Babesia* sp. EU1, is an emerging human and animal pathogen causing clinical features similar to those of *B. microti* in humans but its typical clinical course is unknown (Jiang et al., 2015). In France, it was isolated by culture from infected roe deer in the region of Deux Sèvres (Bonnet et al., 2007) and detected in *I. ricinus* ticks collected in the Île-de-France region (Paul et al., 2016) and in Britanny with a low prevalence of 0.65% and 0.35% in 2012 and 2013, respectively (Jouglin et al., 2017). A study conducted in a neighbouring region did not detect "*B. venatorum"* in *I. ricinus* ticks (Moutailler et al., 2016), suggesting a low prevalence or a recent introduction to the area.

Bartonella spp. was found in 10% of collected ticks. Only five positive samples in the qPCR were amplified using the primers targeting the *ftsZ* gene. The phylogenetic tree showed that this unique sequence of *Bartonella* sp. forms a separate clade between *B. ancashensis* and other *Bartonellaceae* species. Unfortunately, we did not successfully amplify this *Bartonella* sp. using other genes (La Scola et al., 2003). The lack of this additional data precludes the complete description of this potentially new *Bartonella* species. *Bartonella* associated with *I. ricinus* was previously reported (Chomel & Kasten 2010). DNA of *B. henselae*, the agent of cat scratch disease was detected in 17.6% of ticks in the French Ardennes (Moutailler et al., 2016) and in 9.8% of ticks collected in northern France (Halos et al., 2005). Interestingly, in France, *B. henselae*, *B. doshiae, B. tribocorum* and *B. schoenbuchensis* were isolated from blood samples of patients with poorly qualified syndromes after tick bites (Vayssier-Taussat et al., 2016).

Co-infections with two or three microorganisms were identified in 18% of *I. ricinus* nymphs (9/50), which accounted for 26% (9/35) of all infected nymphs. This co-infection could be the result of co-feeding or feeding on an infected host by several pathogens (Raileanu et al., 2017). *Anaplasmataceae* were involved in almost all combinations of co-infections. The association of three bacteria was seen between *Anaplasmataceae, Borrelia* spp. and *Bartonella* spp. (2%). In France, *B. burgdorferi* s.l. and *Bartonella* spp. have previously been simultaneously detected in 1.3% of nymphs collected in Lille (Halos et al., 2005). In other European countries, the prevalence of co-infection with different bacteria in *I. ricinus* nymphs has been reported. Indeed, the rate reported in Romania was 17.1% (Raileanu et al., 2017); in Italy, the prevalence was 5.71% (Otranto et al., 2014), whereas in western Switzerland 4.6% of the nymphal *I. ricinus* collected were found to be infected with several microorganisms (Lommano et al., 2012). In Spain, one nymph was co-infected with *B. valaisiana* and *Rickettsia* sp. (Palomar et al., 2012). It appears that differences in co-infection found in *I. ricinus* depend on the local epidemiology of infectious diseases and the availability of host reservoirs of those bacteria.

Co-infections by tick-borne pathogens in humans have been reported. These occurred from the bite of a single tick infected by several pathogens, or repetitive bites of several infected ticks (Diuk-Wasser et al., 2016; Krause, 2002). Co-infections in humans mostly involved Lyme borreliosis with either human anaplasmosis or babesiosis (Swanson et al., 2006). However, in Europe, co-infection with *Borrelia* spp., *A. phagocytophilum* and *Babesia* spp. was observed in patients with tick-borne encephalitis in Poland (Moniuszko et al., 2014). Co-infection with multiple pathogens may lead to an increase in the severity of the

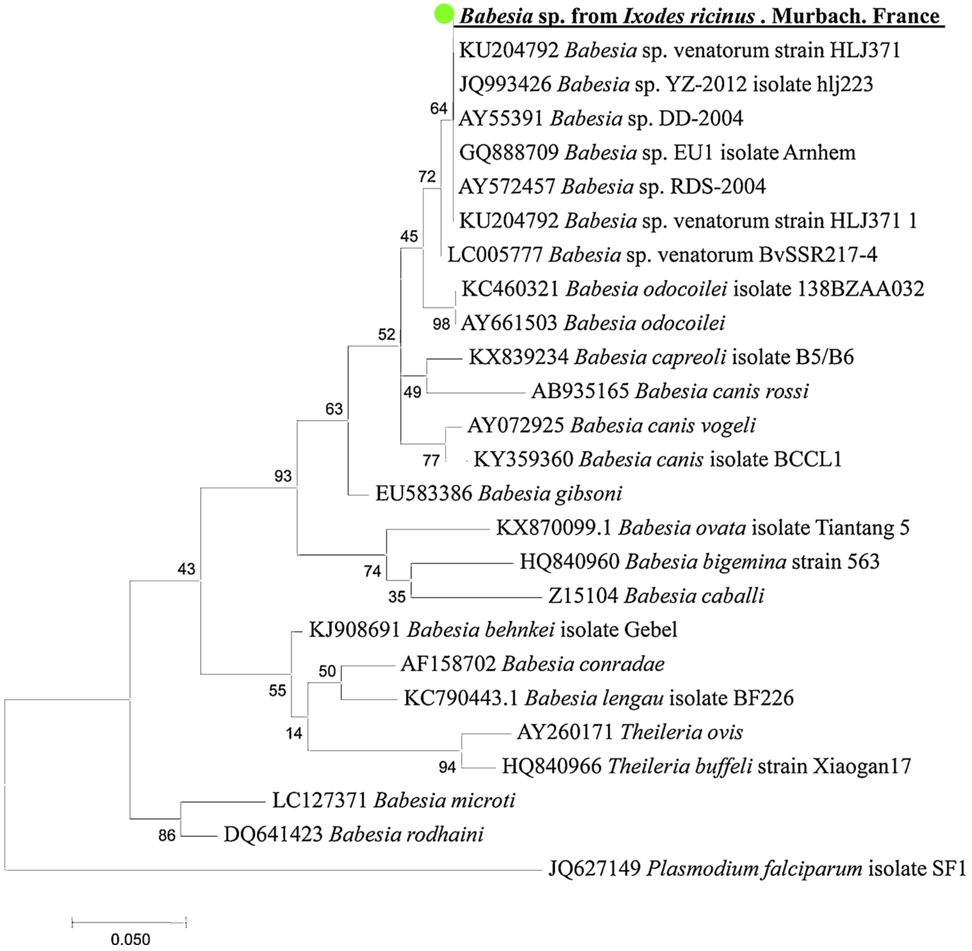

Fig. 2. Phylogenetic tree showing the position of *Babesia* sp. amplified from *I. ricinus* in this study compared to other species. The sequence of the *Babesia* sp. obtained in this study was aligned with *Babesiidae* family sequences available on GenBank using CLUSTALW implemented on BioEdit v3 (Hall, 1999). The sequence of *18S* gene was first aligned, gaps and missing data were eliminated. The phylogenic tree was constructed using MEGA7 (Kumar et al. 2016). The evolutionary history was inferred by using the Maximum Likelihood method based on the Hasegawa-Kishino-Yano model. The tree with the highest log likelihood (-1164.9283) is shown. The percentage of trees in which the associated taxa clustered together is shown next to the branches. Initial tree(s) for the heuristic search were obtained automatically by applying Neighbor-Join and BioNJ algorithms to a matrix of pairwise distances estimated using the Maximum Composite Likelihood (MCL) approach, and then selecting the topology with superior log likelihood value. The tree is drawn to scale, with branch lengths measured in the number of substitutions per site. The analysis involved 26 nucleotide sequences. All positions containing gaps and missing data were eliminated. There were a total of 192 positions in the final dataset.

disease and the development of an uncharacterized illness (Stanek et al., 2012). The risk of infections in people with a tick-borne pathogen other than Lyme borreliosis or tick-borne encephalitis after a bite from a co-infected tick is 2.5% (Hofhuis et al., 2017; Jahfari et al., 2016).

In the present study, we used MALDI-TOF MS to confirm the morphological identification of ticks we tested. Applying this technology, 68% (34/50) of ticks, for all of which a high-quality spectrum had been obtained, were successfully identified as *I. ricinus.* This innovative proteomic approach was successfully applied in identifying laboratory-reared ticks using only legs (Rothen et al., 2016; Yssouf et al., 2013), as well as ticks collected in the field (Kumsa et al., 2016). However, for some specimens, low-quality spectra were obtained. This may be due to several reasons including the storage method, homogenization method or the size of the nymph legs which are smaller than adult legs.

In conclusion, this study has led to the identification of several zoonotic pathogens including an uncharacterized *Bartonella* sp. carried by *I. ricinus* nymphs in the region of Alsace, northern France. A high level of co-infection was revealed in the ticks collected in this area, despite the small number of tested specimens. Infection or co-infection by multiple-organisms suggests the need to increase our vigilance to avoid tick bites, especially by nymphs which are more difficult to detect than adults.

5. Author contribution

Conceived and designed the experiments: NA, AL, PP. Performed the experiments: NA. Analyzed the data: NA, AL, PP. Contributed reagents/materials/analysis tools: NB, DR, HD, OM. Drafted the paper: NA. Critically reviewed the paper: PP, AL, NB, BJ.

Declaration of Competing Interest

The authors declare that they have no competing interests

Acknowledgements

"Ce travail a bénéficié d'une aide du gouvernement français au titre du Programme Investissements d'Avenir, Initiative d'Excellence d'Aix-Marseille Université - A*MIDEX"

"The project leading to this publication has received funding from Excellence Initiative of Aix-Marseille University - A*MIDEX, a French “Investissements d'Avenir” programme”.

We thank L. Zilliox and D. Napolitanoas well as the students working at the CNR Borrelia for technical assistance in tick collection.

Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.ttbdis.2019.06.001.

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