Identification of parasitic communities within European ticks using next-generation sequencing

Sarah Bonnet, Lorraine Michelet, Sara Moutailler, Justine Cheval, Charles Hébert, Muriel Vayssier Taussat, Marc Eloit

▶ To cite this version:

Sarah Bonnet, Lorraine Michelet, Sara Moutailler, Justine Cheval, Charles Hébert, et al.. Identification of parasitic communities within European ticks using next-generation sequencing. PLoS Neglected Tropical Diseases, 2014, 8 (3), 10.1371/journal.pntd.0002753 . hal-02634857

HAL Id: hal-02634857
https://hal.inrae.fr/hal-02634857

Submitted on 27 May 2020

HAL is a multi-disciplinary open access archive for the deposit and dissemination of scientific research documents, whether they are published or not. The documents may come from teaching and research institutions in France or abroad, or from public or private research centers.

L'archive ouverte pluridisciplinaire **HAL**, est destinée au dépôt et à la diffusion de documents scientifiques de niveau recherche, publiés ou non, émanant des établissements d'enseignement et de recherche français ou étrangers, des laboratoires publics ou privés.

OPEN ACCESS Freely available online

Identification of Parasitic Communities within European Ticks Using Next-Generation Sequencing

Sarah Bonnet[1]*, Lorraine Michelet[1], Sara Moutailler[1], Justine Cheval[2], Charles Hébert[2], Muriel Vayssier-Taussat[1], Marc Eloit[2,3,4]

1 USC INRA Bartonella-tiques, UMR BIPAR ENVA-ANSES, Maisons-Alfort, France, **2** PathoQuest SAS, Paris, France, **3** Ecole Nationale Vétérinaire d'Alfort, UMR 1161 Virologie ENVA, INRA, ANSES, Maisons-Alfort, France, **4** Institut Pasteur, Laboratory of Pathogen Discovery, Paris, France

Abstract

Background: Risk assessment of tick-borne and zoonotic disease emergence necessitates sound knowledge of the particular microorganisms circulating within the communities of these major vectors. Assessment of pathogens carried by wild ticks must be performed without *a priori*, to allow for the detection of new or unexpected agents.

Methodology/Principal Findings: We evaluated the potential of Next-Generation Sequencing techniques (NGS) to produce an inventory of parasites carried by questing ticks. Sequences corresponding to parasites from two distinct genera were recovered in *Ixodes ricinus* ticks collected in Eastern France: *Babesia* spp. and *Theileria* spp. Four *Babesia* species were identified, three of which were zoonotic: *B. divergens*, *Babesia* sp. EU1 and *B. microti*; and one which infects cattle, *B. major*. This is the first time that these last two species have been identified in France. This approach also identified new sequences corresponding to as-yet unknown organisms similar to tropical *Theileria* species.

Conclusions/Significance: Our findings demonstrate the capability of NGS to produce an inventory of live tick-borne parasites, which could potentially be transmitted by the ticks, and uncovers unexpected parasites in Western Europe.

Citation: Bonnet S, Michelet L, Moutailler S, Cheval J, Hébert C, et al. (2014) Identification of Parasitic Communities within European Ticks Using Next-Generation Sequencing. PLoS Negl Trop Dis 8(3): e2753. doi:10.1371/journal.pntd.0002753

Editor: David H. Walker, University of Texas Medical Branch, United States of America

Received January 14, 2013; **Accepted** February 11, 2014; **Published** March 27, 2014

Copyright: © 2014 Bonnet et al. This is an open-access article distributed under the terms of the Creative Commons Attribution License, which permits unrestricted use, distribution, and reproduction in any medium, provided the original author and source are credited.

Funding: This study was partially funded by EU grant FP7-261504 EDENext and is catalogued by the EDENext Steering Committee as EDENext 097 (http://www.edenext.eu). The contents of this publication are the sole responsibility of the authors and don't necessarily reflect the views of the European Commission. Financial aid was also provided by the DIM Ile de France, the CoVetLab partner institutes, the ANSES, the Animal Health department of INRA and the INRA Metaprogram MEM and the PathoID project. The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Competing Interests: I have read the journal's policy and have the following conflicts: CH and JC are employees and ME is the chairman of PathoQuest, a spin-off of Institut Pasteur. This does not alter our adherence to all PLOS NTDs policies on sharing data and materials.

* E-mail: sbonnet@vet-alfort.fr

Introduction

Due to the combination of increased human and animal movement, socio-economic and environmental changes, as well as the complex interactions between reservoirs, pathogens, and human populations, more emerging diseases are being identified and the epidemiology of ancient diseases is changing, particularly that of vector-borne diseases [1]. After mosquitoes, ticks are the most common worldwide vector that can affect both humans and animals, and can transmit the highest variety of pathogens, including viruses, bacteria and parasites. Of these parasites, *Babesia* sp. or *Theileria* sp. are two well-known parasites responsible for several diseases that impact both human and animal health worldwide [2,3]. *Ixodes ricinus* is the most prevalent tick in Europe and the vector for several bacterial and viral pathogens [4], as well as three parasites: *B. divergens*, *B. microti* [4] and *Babesia* sp. EU1 [5,6]. To date, no other parasites have been reported to be transmitted by this tick species, even though these ticks feed on a very large spectrum of hosts potentially infected by several parasite species. However, the list of potential or known tick-borne pathogens is constantly evolving, and emergence or re-emergence of tick-borne diseases leads to the development of unknown health risks [4]. Therefore there is a real concern that tick-borne diseases will appear in areas previously free of such diseases, consequently new studies are required to catalog those parasitic communities hosted by, and potentially transmitted by ticks.

Traditionally, identification of microorganisms has relied on their cultivation in artificial environments, but it has become evident that ticks harbor a variety of microbes that may have obligate intracellular life histories and/or require highly specific medium for their cultivation, resulting in the impossibility of successfully culturing some microorganisms, especially parasites. Thus, the identification of tick-borne parasites increasingly relies on molecular detection approaches. Classically, pathogen detection in ticks is performed by PCR with specific primers. These are designed to amplify conserved microbial sequences in a predefined list of pathogens known to be transmitted by the specific collected tick species, in the specific geographical area of collection. However, this method is not at all suited to detect new or unexpected pathogens [7,8]. In addition, because of the relative paucity of available sequence data for tick-borne parasites, most of these techniques rely on the amplification of the 18S genes which are well conserved among parasites, implying an additional sequencing step in order to identify them at the species level.

Author Summary

Diseases transmitted by ticks have diverse etiology (viral, bacterial, parasitic) and are responsible for high morbidity and mortality rates around the world, both in humans and animals. The emergence or re-emergence of tick-borne diseases is increasingly becoming a problem as the geographical distribution of several tick species is expanding, as well as the numbers of potential or known tick-borne pathogens are constantly evolving. It is thus necessary to know which microorganisms circulate within communities of this major vector to ensure adequate epidemiological surveillance. In this study, we evaluated the potential of Next-Generation Sequencing techniques (NGS) to produce, without *a priori*, an inventory of both predicted and non-expected parasites carried by *Ixodes ricinus*, the most prevalent human biting tick in France. Our findings suggest that NGS strategies could be used to produce an inventory of live parasites residing in ticks from a selected area, thereby expanding our knowledge base of tick-associated parasites.

Finally, the amount of available DNA in a tick sample limits such detection to a limited number of PCR tests. Consequently, a detailed inventory of pathogenic agents carried by ticks must be carried out without *a priori*, necessitating novel approaches. Recently, the metagenomic profiles of the bacterial communities associated with the *Ixodes ricinus* tick have been assessed using Next Generation Sequencing (NGS) methods, which permits the characterization of the entire tick microbiome based on 16S rRNA sequencing [9,10]. However, such an approach does not allow identification of the bacteria at the species level, which is absolutely essential when distinguishing symbionts and commensals from the pathogenic bacteria carried by the ticks. To avoid this problem, we recently and successfully used a similar approach, but which sequenced the entire transcriptome of ticks, generating an in-depth picture of bacteria carried by *Ixodes ricinus* from Eastern France, and that led to the identification of both known and unexpected tick-borne bacteria [11]. In this study NGS with a similar protocol was used to produce an inventory of known and unexpected parasites carried by *I. ricinus* in the same area of Eastern France.

Materials and Methods

Study area and tick collection

A total of 1478 *I. ricinus* questing nymphs were collected by flagging in three forested areas of Eastern France (Alsace Department): Murbach (47°55′05″N, 7°8′46″E), Hohbuhl (48°27′33″N, 7°17′22″E) and Wasselonne (48°38′09″N, 7°21′45″E), a region with abundant ticks and a concomitant high risk of disease transmission. Ticks were pooled into groups of 15 individuals and crushed in 300 µl of Dulbecco's MEM (DMEM) medium supplemented with 10% fetal bovine serum. A pool of 15 *I. ricinus* nymphs from our pathogen-free colony was treated equivalently and used as a reference as previously described [11]. This control colony originated from female ticks collected in Murbach and was reared as previously described [12].

High throughput sequencing and data analysis

High throughput sequencing of tick pool samples was performed as previously described [11]. Briefly, total RNA, which indicates the occurrence of viable and replicating microorganisms, and total DNA, for specific real-time PCR, were separately extracted. Wild and pathogen-free RNA samples were sequenced to a depth of 100 million and 62 million for 101 bp paired-end reads respectively. As there is no publicly available *I. ricinus* reference genome, we removed those sequences corresponding to the ticks themselves, or to symbiotic or commensal bacteria naturally found in ticks, by subtracting sequences homologous to sequences from the pathogen-free reference sample using the SOAP2 aligner tool. Finally, 7 787 463 remaining reads out of 70 396 392 reads initially obtained from wild ticks, were used for *de novo* assembly, producing 174 841 contigs. Contigs were then assigned the closest known taxonomy according to their identity percentage (Blast search option of the National Center for Biotechnology Information, www.ncbi.nlm.nih.gov/BLAST), and distant alignments were not considered. Of the assigned reads, 6.65% of the cDNA derived sequences were of a parasitic origin, corresponding to 0.73% of the reads obtained from whole wild ticks. Among these sequences, contigs of significant interest were selected based on at least one of the following criteria 1) an identity percentage >95% with a particular parasite species, 2) known to be responsible for human or/and animal disease and 3) a high read number.

Confirmation of parasite targets with quantitative PCR

Real-time PCR was performed on DNA extracted from each pool of ticks to confirm taxonomic species assignment of NGS-derived contigs. Amplification was performed as previously described [11] and the primers newly designed for this study, based on the 18S rDNA, *hsp*70 and CCTeta sequences present in GenBank, are presented in Table 1. *Babesia* and *Theileria* DNA used for positive controls were kindly provided by Huseyin Bilgic, Faculty of Vet.Med, Turkey; Laurence Malandrin, ONIRIS, France; Emmanuel Cornillot, Montpellier University, France.

Sequence analysis and phylogenic tree analysis

For phylogenetic analysis, the 28S sequence data obtained via NGS (Table 2) were aligned and subsequently compared with parasitic species data from GenBank using the phyml v2.4.4 software [13,14]. Distance matrices were calculated using the General time reversible (GTR) model and bootstrap analysis was performed with 1000 replications [15]. *Plasmodium falciparum*, a close apicomplexa was used as an out-group.

Results and Discussion

To identify known, novel or unexpected parasites carried by ticks in France, *I. ricinus* were collected in Eastern France, a wooded region with high tick abundance. Using NGS techniques, 17 contigs were selected following the criteria previously described and are presented in Table 2. Parasites from two distinct genera were identified: *Babesia* spp. (13 sequences), and possibly *Theileria* spp. (4 sequences). Other eukaryotic sequences with significant identity to sequences present in the databank corresponded mainly to fungi (*Ascomycota*) and are not presented here.

Parasites from the *Babesia* genus

Three zoonotic *Babesia* species, *B. divergens*, *B. microti* and *Babesia* sp. EU1 were identified in *I. ricinus*, in addition to *B. major*, a parasite that only infects cattle. Transovarial transmission within ticks is characteristic of *Babesia* spp., implying that ticks constitute a real parasite reservoir in the field.

B. divergens. Following our criteria, three sequences related to *B. divergens* 18SrRNA were identified via NGS sequencing (Table 2), but no products were obtained after qPCR with specific primers aimed to amplify the *hsp*70 gene specific to this species.

Table 1. Primers and probes designed and used for the detection of *Babesia* species and *Theileria* species via quantitative PCR.

Pathogen	Gene target	Primer or probe	Sequence (5′→3′)	Amplicon size (bp)
Babesia divergens	hsp70	Bdi_F	CTCATTGGTGACGCCGCTA	83
		Bdi_R	CTCCTCCCGATAAGCCTCTT	
		Bdi_P	AGAACCAGGAGGCCCGTAACCCAGA	
Babesia sp.EU1	18S rRNA	BEU1_F	GCGCGCTACACTGATGCATT	91
		BEU1_R	CAAAAATCAATCCCCGTCACG	
		BEU1_P	CATCGAGTTTAATCCTGTCCCGAAAGG	
Babesia microti	CCTeta	Bmi_F	ACAATGGATTTTCCCCAGCAAAA	145
		Bmi_R	GCGACATTTCGGCAACTTATATA	
		Bmi_P	TACTCTGGTGCAATGAGCGTATGGGTA	
Babesia major	CCTeta	Bmaj_F	CACTGGTGCGCTGATCCAA	75
		Bmaj_R	TCCTCGAAGCATCCACATGTT	
		Bmaj_P	AACACTGTCAACGGCATAAGCACCGAT	
Theileria parva	18S rRNA	Tpar_F	GAGTATCAATTGGAGGGCAAG	173
		Tpar_R	CAGACAAAGCGAACTCCGTC	
		Tpar_P	AAATAAGCCACATGCAGAGACCCCGAA	
Theileria taurotragi	18S rRNA	Ttau_F	GGTCTTGGCACGTGGCTTTT	80
		Ttau_R	AGCCTGCTTTGAGCACTCTAA	
		Ttau_P	TTCGGACGGTTCGCTGTCTGGATGT	

doi:10.1371/journal.pntd.0002753.t001

This result suggests that the parasite exists in small numbers, which is under the PCR threshold of detection but detectable with NGS due to the high number of transcripts corresponding to the 18S rRNA gene. *B. divergens* is a bovine parasite transmitted by *I. ricinus*, and is thought to be responsible for most cases of human Babesiosis in Europe, and especially, but not exclusively, in splenectomized patients [2,16]. This parasite is the most widespread and pathogenic *Babesia* species infecting cattle in Northern temperate areas [17]. Traditionally, *B. divergens* has had a high serological prevalence in cattle from Western or Central France [17]. The discovery of this parasite in Eastern France may suggests that its geographical distribution is increasing, even within forested areas without cattle farms, which would require the existence of (an as-yet unidentified) reservoir hosts other than

Table 2. Illumina sequencing of parasitic sequences identified within *I. ricinus* ticks and sequence read number matched to reference genomes.

Suspected genus	Closest species	Contigs	% identity (e-value)	Target gene	Contig length	Read number
Babesia spp.	*Babesia divergens*	*131510*	100% (1e-55)	18S rRNA	181	254
		144996	96% (2e-110)	18S rRNA	318	3351
		154119	100% (2e-13)	18S rRNA	101	97
	Babesia microti	*107742*	100% (1e-31)	18S rRNA	137	271
		108532	99% (5e-70)	18S rRNA	222	1251
		120900	100% (6e-14)	18S rRNA	102	1896
		108977	97% (2e-100)	unknown	233	336
		153009	100% (1e-22)	unknown	130	852
	Babesia sp. EU1	*112965*	100% (3e-26)	18S rRNA	195	918
		150098	100% (2e-79)	18S rRNA	224	480
		172249	100% (4e-56)	18S rRNA	213	76
		114792	100% (3e-27)	18S rRNA	129	366
	Babesia major	*145999*	97% (3e-93)	28S rRNA	270	2351
Theileria spp.	*Theileria parva*	*127324*	94% (4e-34)	28S rRNA	163	237
		131568	96% (1e-39)	28S rRNA	188	149
		164638	97% (2e-29)	28S rRNA	139	149
	Theileria taurotragi	*110157*	97% (4e-87)	28S rRNA	197	1216

doi:10.1371/journal.pntd.0002753.t002

cattle. Further epidemiological studies are then now required in order to confirm that the parasite is now established in the studied area.

***Babesia* sp. EU1.** NGS analysis identified four contigs related to the *Babesia* sp. EU1 18S rRNA encoding gene (Table 2) and the DNA presence of this species was confirmed by qPCR. This species, implicated in human cases of Babesiosis in Europe [18,19], seems to phylogenetically lie in a sister group with *B. divergens* [18] in fact some serological cross-reactivity between *B. divergens* and *Babesia* sp. EU1 has been reported [20]. Roe deer were strongly suspected to be the wild reservoir of this parasite [6,21] and its transmission by *I. ricinus* was validated both *in vivo* [6,22] and *in vitro* [5]. In addition, *Babesia* sp. EU1 has been identified in *I. ricinus* in several European countries including Slovenia [23], Switzerland [24], the Netherlands [25], Poland [26], Italy [27], Belgium [28] and France [6,8], demonstrating a wide geographical spread across the continent. Increasing reports of *Babesia* sp. EU1 in ticks and wild ruminants makes this parasite an excellent candidate for the emergence of a new zoonotic tick-borne disease.

***B. microti*.** Five sequences related to *B. microti* 18S rRNA gene were identified following NGS analysis (Table 2) but were also not confirmed by qPCR aimed at amplifying the CCTeta gene. This result represents the first identification of this species in ticks from France. However, it is not surprising that this particular *Babesia* species was detected in wooded areas, as this rodent parasite is known to be transmitted by *I. ricinus*, and now seems to be widely established in Europe. Indeed, *B. microti* has been identified in *I. ricinus* in several European countries such as Switzerland [29], Poland [30], Slovenia [31], Germany [32], the Netherlands [25,33] and Belgium [28]. To date, only two cases of human Babesiosis caused by this parasite have been reported in Europe [34,35], but its zoonotic impact is well known in the United States [36]. Furthermore, autochthonous cases of *B. microti* infections have been diagnosed in Taiwan and Japan [37,38], emphasizing the increasingly greater world distribution of this parasite.

***B. major*.** NGS analysis revealed one contig with 97% similarity to the *B. major* 18S rRNA gene (Table 2). Despite a high number of reads obtained (2351), we also failed to confirm the presence of *B. major* DNA by qPCR for the CCTeta gene. *B. major* is a temperate-zone species able to infect cattle with lower pathogenicity than *B. divergens*, and has a far more limited geographical distribution which is linked to its tick vector, *Haemaphysalis punctata*. Whether finding RNA from this parasite in *I. ricinus* ticks is epidemiologically relevant, needs to be clarified with additional laboratory competency experiments. Indeed, even if no human cases have been reported for this parasite, its occurrence in *I. ricinus* ([39] and this study), a tick which frequently bites humans, as well as the fact that several *Babesia* species have

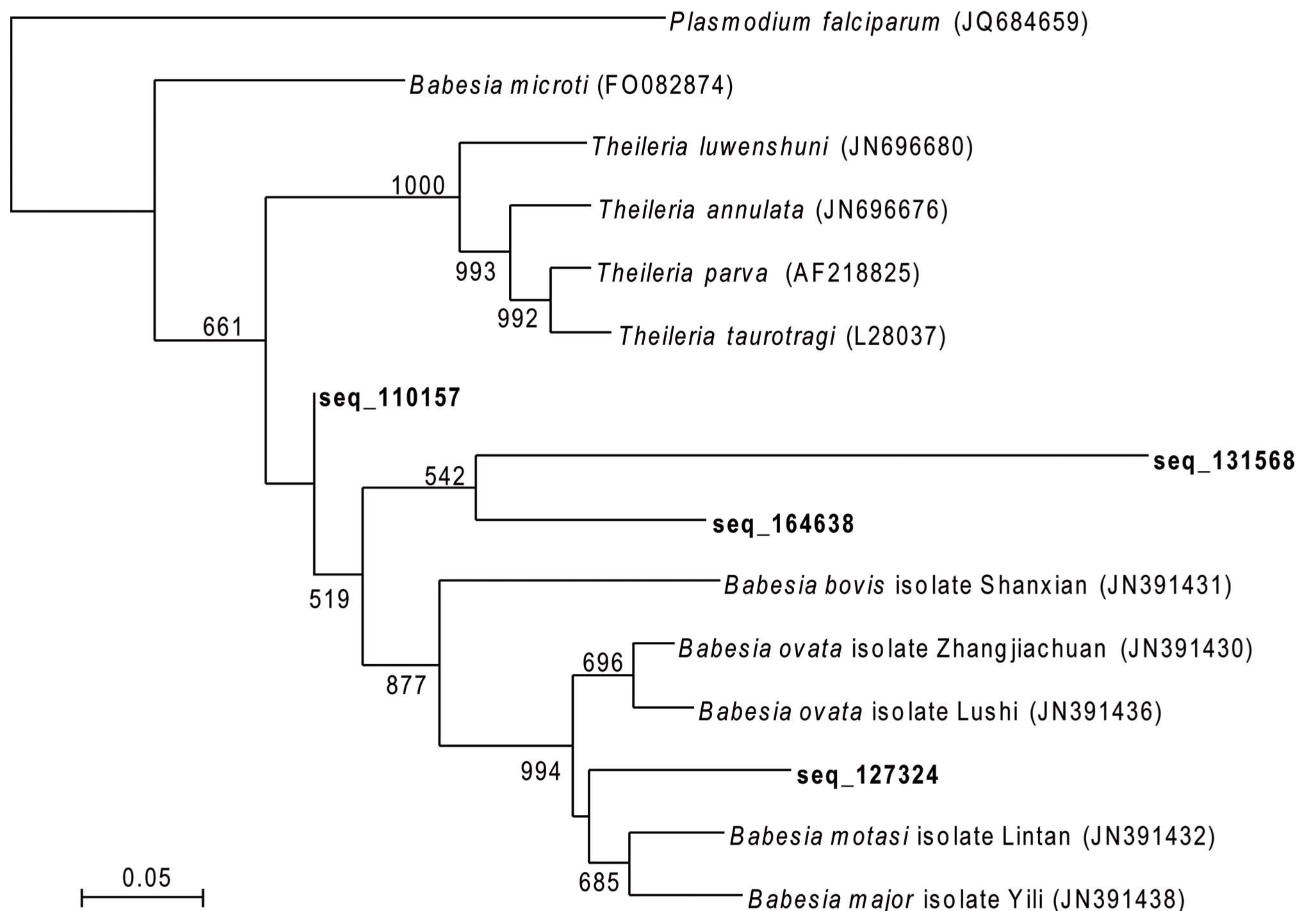

Figure 1. Maximum likelihood phylogenetic tree of partial 28S rRNA parasites sequences. GenBank accession numbers are given in parentheses. NGS Sequences are indicated in bold. Numbers represent bootstrap values (%) based on 1000 replications. Only bootstrap values higher than 500 are reported.
doi:10.1371/journal.pntd.0002753.g001

been shown to have wider vertebrate host ranges than previously thought [39,40], may justify surveillance of this parasite.

Parasites from the *Theileria* genus

Following our selection criteria, four sequences were identified as belonging to the *Theileria* genera (Table 2). Three were most closely related to *T. parva* with 94–97% 18S rRNA identity, but with relatively low e-values and numbers of associated reads (535 in total). The presence of *T. parva* DNA was however confirmed by qPCR also based on the 18S rRNA sequence. The fourth sequence appeared to be related to *T. taurotragi* (97% 18S rRNA identity) with higher e-values and read numbers (1216), but no amplification could be obtained after qPCR with specific primers for the 18S rRNA encoding gene. These results indicate that some related *Theileria* species, but different from *T. parva* or *T. taurotragi*, are detected in *I. ricinus*. *Rhipicephalus appendiculatus* is the most common vector for *T. parva* and *T. taurotragi*, but other *Rhipicephalus* species can also transmit these organisms, implying flexible vector specificity. Both species occur in Africa, where *T. parva* mainly infects cattle, whereas *T. taurotragi* was found to have a wider host range [41].

Phylogenetic analysis based on 28S NGS sequence data indicated that all four ambiguous sequences (127324, 131568, 164638 and 110157) seemed to belong to distinct and novel apicomplexa species (Figure 1). Only one sequence (110157), with the highest probability and read number, was confirmed to be related to a *Theileria* species. The other three seem to belong to *Babesia* species. However, considering that very few complete parasite genome sequences are available, parasite identification is mainly performed on the basis of 18S or 28S rDNA sequence analysis. These are the most highly represented parasitic sequences in GenBank, but are not the most informative in terms of species assignation. Moreover, this preliminary analysis was performed with short sequences (139–197 bp), which are not located at the same region within the 28S rDNA, therefore no definite species can be identified. Thus, further investigations are now required to clarify whether the identification of new *Theileria* or *Babesia* species in France, similar to tropical species, actually corresponds to an expanded geographical distribution of these species, and whether they have a potential pathogenic effect in mammals. Unfortunately, the absence of tick-borne parasite genome data causes difficulties in realizing such phylogenetic studies. However, in spite of the low level of robustness of theses phylogenetic analyses, our results are confirmed by other studies, in particular those demonstrating that the *B. microti* group is entirely divergent from either *Babesia* sensu stricto or *Theileria* species [42].

Conclusion

The inventory of parasitic RNA content in *I. ricinus* performed by NGS revealed the presence of expected viable parasites belonging to the *Babesia* genus, some of them being identified in France for the first time. However, the epidemiological relevance of these results must of course be interpreted with caution. Unfortunately, complete genomic data on tick-borne parasites is scarce, likely due to large genome complexity compared to the relatively small number of research teams in this field. In addition, their small genome size and the strong inter-species conservation of available sequences (essentially 18S rRNA), does not permit clear species identification. Moreover, unknown species with too distant alignment and the fewest database sequences could not be identified in this context. The increased number of sequences relative to tick-borne parasites in data banks should facilitate an increase in the power of NGS techniques to detect tick-borne parasites in the future. In addition, detecting pathogenic RNA within ticks does not imply that these pathogens are actually transmitted by this arthropod. Therefore competence and epidemiological studies are also required in order to verify whether *I. ricinus* is implicated in the transmission of those tick-borne diseases which are present or emerging in France. And finally, further studies are also required to confirm whether the unexpected *Theileria* species detected here is actually novel, and whether the detection of parasitic species similar to other tropical species in France, corresponds to increasing geographical species distribution.

Acknowledgments

We thank the *Tiques et Maladies à Tiques group (TMT)* of *Réseau Ecologie des Interactions Durables* for stimulating discussion and support.

Author Contributions

Conceived and designed the experiments: SB SM MVT ME. Performed the experiments: LM JC CH. Analyzed the data: SB LM. Contributed reagents/materials/analysis tools: SB LM SM JC CH ME. Wrote the paper: SB LM.

References

1. Colwell DD, Dantas-Torres F, Otranto D (2011) Vector-borne parasitic zoonoses: emerging scenarios and new perspectives. Vet Parasitol 182: 14–21.
2. Hunfeld KP, Hildebrandt A, Gray JS (2008) Babesiosis: recent insights into an ancient disease. Int J Parasitol 38: 1219–1237.
3. Morrison WI (2009) Progress towards understanding the immunobiology of *Theileria* parasites. Parasitology 136: 1415–1426.
4. Dantas-Torres F, Chomel BB, Otranto D (2012) Ticks and tick-borne diseases: a One Health perspective. Trends Parasitol 28: 437–446.
5. Bonnet S, Brisseau N, Hermouet A, Jouglin M, Chauvin A (2009) Experimental *in vitro* transmission of *Babesia* sp. (EU1) by *Ixodes ricinus*. Vet Res 40: 21.
6. Bonnet S, Jouglin M, L'Hostis M, Chauvin A (2007) *Babesia* sp. EU1 from roe deer and transmission within *Ixodes ricinus*. Emerg Infect Dis 13: 1208–1210.
7. Cotte V, Bonnet S, Cote M, Vayssier-Taussat M (2010) Prevalence of five pathogenic agents in questing *Ixodes ricinus* ticks from western France. Vector Borne Zoonotic Dis 10: 723–730.
8. Reis C, Cote M, Paul RE, Bonnet S (2011) Questing ticks in suburban forest are infected by at least six tick-borne pathogens. Vector Borne Zoonotic Dis 11: 907–916.
9. Carpi G, Cagnacci F, Wittekindt NE, Zhao F, Qi J, et al. (2011) Metagenomic profile of the bacterial communities associated with *Ixodes ricinus* ticks. PLoS One 6: e25604.
10. Nakao R, Abe T, Nijhof AM, Yamamoto S, Jongejan F, et al. (2013) A novel approach, based on BLSOMs (Batch Learning Self-Organizing Maps), to the microbiome analysis of ticks. ISME J 7: 1003–1015.
11. Vayssier-Taussat M, Moutailler S, Michelet L, Devillers E, Bonnet S, et al. (2013) Next generation sequencing uncovers unexpected bacterial pathogens in ticks in western Europe. PLoS One 8: e81439.
12. Reis C, Cote M, Le Rhun D, Lecuelle B, Levin ML, et al. (2011) Vector competence of the tick *Ixodes ricinus* for transmission of *Bartonella birtlesii*. PLoS Negl Trop Dis 5: e1186.
13. Guindon S, Gascuel O (2003) A simple, fast, and accurate algorithm to estimate large phylogenies by maximum likelihood. Syst Biol 52: 696–704.
14. Felsenstein J (1989) PHYLIP - Phylogeny Inference Package (Version 3.2). Cladistics 5: 164–166.
15. Felsenstein J (1985) Confidence limits on phylogenies: an approach using the boostrap. Evolution 39: 783–791.
16. Martinot M, Zadeh MM, Hansmann Y, Grawey I, Christmann D, et al. (2011) Babesiosis in immunocompetent patients, Europe. Emerg Infect Dis 17: 114–116.
17. L'Hostis M, Chauvin A (1999) *Babesia divergens* in France: descriptive and analytical epidemiology. Parassitologia 41 Suppl 1: 59–62.
18. Herwaldt BL, Caccio S, Gherlinzoni F, Aspock H, Slemenda SB, et al. (2003) Molecular characterization of a non-*Babesia divergens* organism causing zoonotic babesiosis in Europe. Emerg Infect Dis 9: 942–948.
19. Haselbarth K, Tenter AM, Brade V, Krieger G, Hunfeld KP (2007) First case of human babesiosis in Germany - Clinical presentation and molecular characterisation of the pathogen. Int J Med Microbiol 297: 197–204.

20. Duh D, Jelovsek M, Avsic-Zupanc T (2007) Evaluation of an indirect fluorescence immunoassay for the detection of serum antibodies against *Babesia divergens* in humans. Parasitology 134: 179–185.
21. Duh D, Petrovec M, Bidovec A, Avsic-Zupanc T (2005a) Cervids as Babesiae hosts, Slovenia. Emerg Infect Dis 11: 1121–1123.
22. Becker CA, Bouju-Albert A, Jouglin M, Chauvin A, Malandrin L (2009) Natural transmission of Zoonotic *Babesia* spp. by *Ixodes ricinus* ticks. Emerg Infect Dis 15: 320–322.
23. Duh D, Petrovec M, Avsic-Zupanc T (2005b) Molecular characterization of human pathogen *Babesia* EU1 in *Ixodes ricinus* ticks from Slovenia. J Parasitol 91: 463–465.
24. Casati S, Sager H, Gern L, Piffaretti JC (2006) Presence of potentially pathogenic *Babesia* sp. for human in *Ixodes ricinus* in Switzerland. Ann Agric Environ Med 13: 65–70.
25. Wielinga PR, Fonville M, Sprong H, Gaasenbeek C, Borgsteede F, et al. (2009) Persistent detection of *Babesia* EU1 and *Babesia microti* in *Ixodes ricinus* in the Netherlands during a 5-year surveillance: 2003–2007. Vector Borne Zoonotic Dis 9: 119–122.
26. Cieniuch S, Stanczak J, Ruczaj A (2009) The first detection of *Babesia* EU1 and *Babesia canis canis* in *Ixodes ricinus* ticks (Acari, Ixodidae) collected in urban and rural areas in northern Poland. Pol J Microbiol 58: 231–236.
27. Cassini R, Bonoli C, Montarsi F, Tessarin C, Marcer F, et al. (2010) Detection of *Babesia* EU1 in *Ixodes ricinus* ticks in northern Italy. Vet Parasitol 171: 151–154.
28. Lempereur L, De Cat A, Caron Y, Madder M, Claerebout E, et al. (2011) First molecular evidence of potentially zoonotic *Babesia microti* and *Babesia* sp. EU1 in *Ixodes ricinus* ticks in Belgium. Vector Borne Zoonotic Dis 11: 125–130.
29. Foppa IM, Krause PJ, Spielman A, Goethert H, Gern L, et al. (2002) Entomologic and serologic evidence of zoonotic transmission of *Babesia microti*, eastern Switzerland. Emerg Infect Dis 8: 722–726.
30. Skotarczak B, Cichocka A (2001) The occurrence DNA of *Babesia microti* in ticks *Ixodes ricinus* in the forest areas of Szczecin. Folia Biol (Krakow) 49: 247–250.
31. Duh D, Petrovec M, Avsic-Zupanc T (2001) Diversity of *Babesia* infecting European sheep ticks (*Ixodes ricinus*). J Clin Microbiol 39: 3395–3397.
32. Hartelt K, Oehme R, Frank H, Brockmann SO, Hassler D, et al. (2004) Pathogens and symbionts in ticks: prevalence of *Anaplasma phagocytophilum* (*Ehrlichia* sp.), *Wolbachia* sp., *Rickettsia* sp., and *Babesia* sp. in Southern Germany. Int J Med Microbiol 293 Suppl 37: 86–92.
33. Nijhof AM, Bodaan C, Postigo M, Nieuwenhuijs H, Opsteegh M, et al. (2007) Ticks and Associated Pathogens Collected from Domestic Animals in the Netherlands. Vector Borne Zoonotic Dis.
34. Meer-Scherrer L, Adelson M, Mordechai E, Lottaz B, Tilton R (2004) *Babesia microti* infection in Europe. Curr Microbiol 48: 435–437.
35. Hildebrandt A, Hunfeld KP, Baier M, Krumbholz A, Sachse S, et al. (2007) First confirmed autochthonous case of human *Babesia microti* infection in Europe. Eur J Clin Microbiol Infect Dis 26: 595–601.
36. Homer MJ, Aguilar-Delfin I, Telford SR, Krause PJ, Persing DH (2000) Babesiosis. Clin Microbiol Rev 13: 451–469.
37. Shih CM, Liu LP, Chung WC, Ong SJ, Wang CC (1997) Human babesiosis in Taiwan: asymptomatic infection with a *Babesia microti*-like organism in a Taiwanese woman. J Clin Microbiol 35: 450–454.
38. Saito-Ito A, Tsuji M, Wei Q, He S, Matsui T, et al. (2000) Transfusion-acquired, autochthonous human babesiosis in Japan: isolation of *Babesia microti*-like parasites with hu-RBC-SCID mice. J Clin Microbiol 38: 4511–4516.
39. Hilpertshauser H, Deplazes P, Schnyder M, Gern L, Mathis A (2006) *Babesia* spp. identified by PCR in ticks collected from domestic and wild ruminants in southern Switzerland. Appl Environ Microbiol 72: 6503–6507.
40. Chauvin A, Moreau E, Bonnet S, Plantard O, Malandrin L (2009) *Babesia* and its hosts: adaptation to long-lasting interactions as a way to achieve efficient transmission. Vet Res 40: 37.
41. Stagg DA, Young AS, Leitch BL, Grootenhuis JG, Dolan TT (1983) Infection of mammalian cells with *Theileria* species. Parasitology 86 (Pt 2): 243–254.
42. Nakajima R, Tsuji M, Oda K, Zamoto-Niikura A, Wei Q, et al. (2009) *Babesia microti*-group parasites compared phylogenetically by complete sequencing of the CCTeta gene in 36 isolates. J Vet Med Sci 71: 55–68.