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

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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.

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, which could potentially be transmitted by the ticks, and uncoverts unexpected parasites in Western Europe.

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.
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), Hobbuhl (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 Malandrini, ORINIS, France; Emmanuel Cornillot, Montpellier University, France.

Sequence analysis and phylogenetic 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 [15,14]. 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* 18S rRNA 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.
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 suggest 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 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          | CTCATTGGTGAGCCGCGTA               | 83                 |
|                   |             | Bdi_R          | CTCTCCCAGATATGCTCTT               |                    |
|                   |             | Bdi_P          | AGAACCGAGGCCCCGTAACCCAGA          |                    |
| *Babesia sp.EU1*   | 18S rRNA    | BEU1_F         | GCGCGCTACACGTGATT                 | 91                 |
|                   |             | BEU1_R         | CAAAATACCCCCGTCAGG                |                    |
|                   |             | BEU1_P         | CATCGAGTTTAATCCTGTCCGCAAGG        |                    |
| *Babesia microti*  | CCTeta      | Bmi_F          | ACAATGGATTTTCACAGCAGAAA           | 145                |
|                   |             | Bmi_R          | CCCAGATTTCCGCAAATATATA            |                    |
| *Babesia major*    | CCTeta      | Bmaj_F         | CACTGTTGCGGCTGATCCAA              | 75                 |
|                   |             | Bmaj_R         | TCTCGAAGCCTCCACATGTT              |                    |
|                   |             | Bmaj_P         | AACACTGCTAACGCGAATGACCGAT         |                    |
| *Theileria parva*  | 18S rRNA    | Tpar_F         | GAGTATCAATGAGGGAAGAACG            | 173                |
|                   |             | Tpar_R         | CAGACAAAGGCAACTCGTC               |                    |
|                   |             | Tpar_P         | AAATAAGCCACATGACAGACCCGAA         |                    |
| *Theileria taurotragii* | 18S rRNA | Ttau_F         | GGTCTTGGACGTGACGTCGTT             | 80                 |
|                   |             | Ttau_R         | ACCGTCGTTGAGGGCAATC                 |                    |
|                   |             | Ttau_P         | TCGGAGCGGTTCCTGTGGATGT            |                    |

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 suggest 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         |
| *Babesia* major |                | 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* taurotragii | | 110157  | 97% (4e-87)         | 28S rRNA    | 197           | 1216        |
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 [23,33] 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

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**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.

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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. tauronigrus* (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. tauronigrus*, are detected in *I. ricinus*. *Rhizophus paludum* is the most common vector for *T. parva* and *T. tauronigrus*, but other *Rhizophus* species can also transmit these organisms, implying flexible vector specificity. Both species occur in Africa, where *T. parva* mainly infects cattle, whereas *T. tauronigrus* 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 parasite genome sequences in GenBank, but are not the most informative in phylogenetic analyses, our results are confirmed by other studies, in particular those demonstrating that the *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.

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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, Brasseau N, Hermouet A, Josquin M, Chauvin A (2009) Experimental in *vivo* transmission of *Babesia* sp. (EU1) by *Ixodes ricinus*. Vet Res 40: 21.
6. Bonnet S, Josquin 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, Le Rhun D, Lecuelle B, Levin ML, et al. (2011) Vector competence of the tick *Ixodes ricinus* for transmission of *Bartonella henselae*. PLoS Negl Trop Dis 5: e1186.
9. Guindon S, Gascuel O (2003) A simple, fast, and accurate algorithm to estimate large phylogenies by maximum likelihood. Syst Biol 52: 696–704.
10. L’Hostis M, Chauvin A (1999) *Babesia divergens* in France: descriptive and analytical epidemiology. Parasitologia 41 Suppl 1: 59–62.
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: e61499.
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 henselae*. 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. Feldstein J (1989) PHYLIP - Phylogeny Inference Package (Version 3.2). Cladistics 5: 164–166.
15. Feldstein J (1985) Confidence limits on phylogenies: an approach using the bootstrap. Evolution 39: 783–791.
16. Martinot M, Zadék MM, Hausmann Y, Gravely J, 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. Parasitologia 41 Suppl 1: 59–62.
18. Herwaldt BL, Capeci S, Gherlinzoni F, Aspock H, Slémenda SB, et al. (2003) Molecular characterization of a non-*Babesia divergens* organism causing zoonic babesiosis in Europe. Emerg Infect Dis 9: 942–946.
19. Haebelath K, Teuter AM, Brade V, Künger 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, Bidover 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* ticks 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. in *Ixodes ricinus* ticks in Belgium. Vector Borne Zoonotic Dis 11: 125–130.

29. Stagg DA, Young AS, Leitch BL, Grootenhuis JG, Dolan TT (1983) Infection of mammalian cells with *Theileria* species. Parasitology 86 (Pt 2): 243–254.

30. 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: 55–60.