Presence and identity of Babesia microti in Ireland

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Short communication

Presence and identity of *Babesia microti* in Ireland

A. Zintl [*,a], A. McManus [b], M. Galan [c], M. Diquattro [d], L. Giuffredi [e], N. Charbonnel [c], J. Gray [f], C. Holland [g], P. Stuart [h]

[a] UCD Veterinary Sciences Centre, University College Dublin, Ireland
[b] MunsterTechnological University, Kerry Campus, Ireland
[c] CBGP, INRAE, CIRAD, Institut Agro, IRD, University of Montpellier, Montpellier, France
[d] Biological Engineering Department, University of Toulon, France
[e] VetAgro Sup, Lyon, France
[f] UCD School of Biology and Environmental Science, University College Dublin, Ireland
[g] School of Natural Sciences, Trinity College Dublin, Ireland
[h] Munster Technological University, Kerry Campus, Ireland

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ABSTRACT

Babesia microti is a tick-transmitted protozoan parasite of wildlife that can also cause serious disease in humans. It is now well established that *B. microti* represents an assemblage of different strains or species, only some of which are important zoonotic pathogens. Therefore, in order to assess the potential public health risk associated with *B. microti* in any given location, it is important to determine the strains that are present. This is the first study on the presence and identity of *B. microti* in Ireland. Overall, 314 wood mice (*Apodemus sylvaticus*), 243 bank voles (*Myodes glareolus*) and 634 questing *Ixodes ricinus* nymphs collected in various locations across Ireland were screened for the presence of *B. microti* by metabarcoding and nested PCR, respectively. Overall 8 rodent spleen samples (1.4%) were positive for *B. microti*, while all tick samples tested negative. Rodent isolates were identified as the 'Munich' strain which rarely causes human disease and is chiefly transmitted by the mouse tick, *Ixodes trianguliceps*. Together with reports from the UK these results suggest that *B. microti* does not represent a significant public health risk in Britain or Ireland.

1. Introduction

Babesia microti is an intraerythrocytic protozoan parasite of wild rodent, insectivore and carnivore hosts, transmitted by ixodid ticks. The main reason why the parasite has received considerable attention in recent decades is that it is also infectious to humans, causing fever, fatigue, headache, haemolytic anaemia, and, in severe cases, renal and multiorgan failure (Hildebrandt et al., 2021). Human babesiosis due to *B. microti* is most important in specific regions of the USA, where the number of babesiosis cases due to this pathogen averaged 1828 per year between 2011 and 2019 (Swanson et al., 2023). In contrast just eleven autochthonous *B. microti* cases have been reported in Europe to date (Hildebrandt et al., 2021). Similarly isolated cases of human *B. microti* infections have been identified in Asia, Australia and South America (Hussain et al., 2022; Kumar et al., 2021).

Close scrutiny of *B. microti* in recent years has revealed it to be an assemblage of (at least) five clades or strains (Goethert, 2021) with distinct biological and genetic characteristics. Just one of these strains, known as *B. microti* sensu stricto or US-type, is considered a major zoonotic pathogen. Its tick vector is *Ixodes scapularis*, and white-footed mice (*Peromyscus leucopus*) and other small wildlife species in the USA serve as reservoir hosts. Parasites that resemble this strain genetically have also been detected in a small number of human cases in Europe where *Ixodes ricinus* evidently serves as their main vector (Hildebrandt et al., 2021). Two other clades, one of which infects carnivores (e.g. foxes, skunk, otters and raccoons), the other small wildlife and felids in Japan and South Africa (Hobetsu/Otsu strain), have never been associated with human babesiosis (Goethert, 2021). For the two remaining clades, 'Kobe' and 'Munich', the zoonotic potential is uncertain. 'Kobe' was originally isolated from a Japanese blood-transfusion recipient and large Japanese field mice (*Apodemus speciosus*) captured near the residential area of a blood-transfusion donor (Nakajima et al., 2009). The 'Munich' strain has been identified in various rodent species (including *Apodemus flavicollis, Microtus agrestis, Microtus arvalis, Microtus*

* Corresponding author.
E-mail address: annetta.zintl@ucd.ie (A. Zintl).

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oeconomus, Myodes glareolus, Myodes rufocanus, Myodes rutilis and *Tamias sibiricus*) captured in Croatia (Beck et al., 2011), Finland (Kallio et al., 2014), France (Jouglin et al., 2017), Poland (Sinski et al., 2006; Tolkacz et al., 2017), Russia (Rar et al., 2016) and the USA (Goethert, 2021), often at high prevalence rates. In contrast, it has only been identified in seven autochthonous human babesiosis cases reported from Europe, including six patients from Poland (Moniuszko-Malinowska et al., 2016) and one from Spain (Arsuaga et al., 2016). The main tick vector of the 'Munich' strain is thought to be the nidicolous mouse tick, *Ixodes trianguliceps* (Goethert, 2021). In fact to our knowledge there are just two reports of 'Munich'-like isolates in questing *I. ricinus* ticks, both from northern Poland (Pieniazek et al., 2006; Welc-Falęciak et al., 2012). *B. microti* isolates described in the first of these studies differed from the 'Munich' strain by a single SNP in a 1,774bp fragment of the 18S rRNA gene (Pieniazek et al., 2006). In the second study just 437bp of the 18S rRNA gene were sequenced, but they showed 100% homology with the 'Munich' reference strain (AB071177) (Welc-Falęciak et al., 2012).

In the UK, only one (imported) human *B. microti* case has ever been

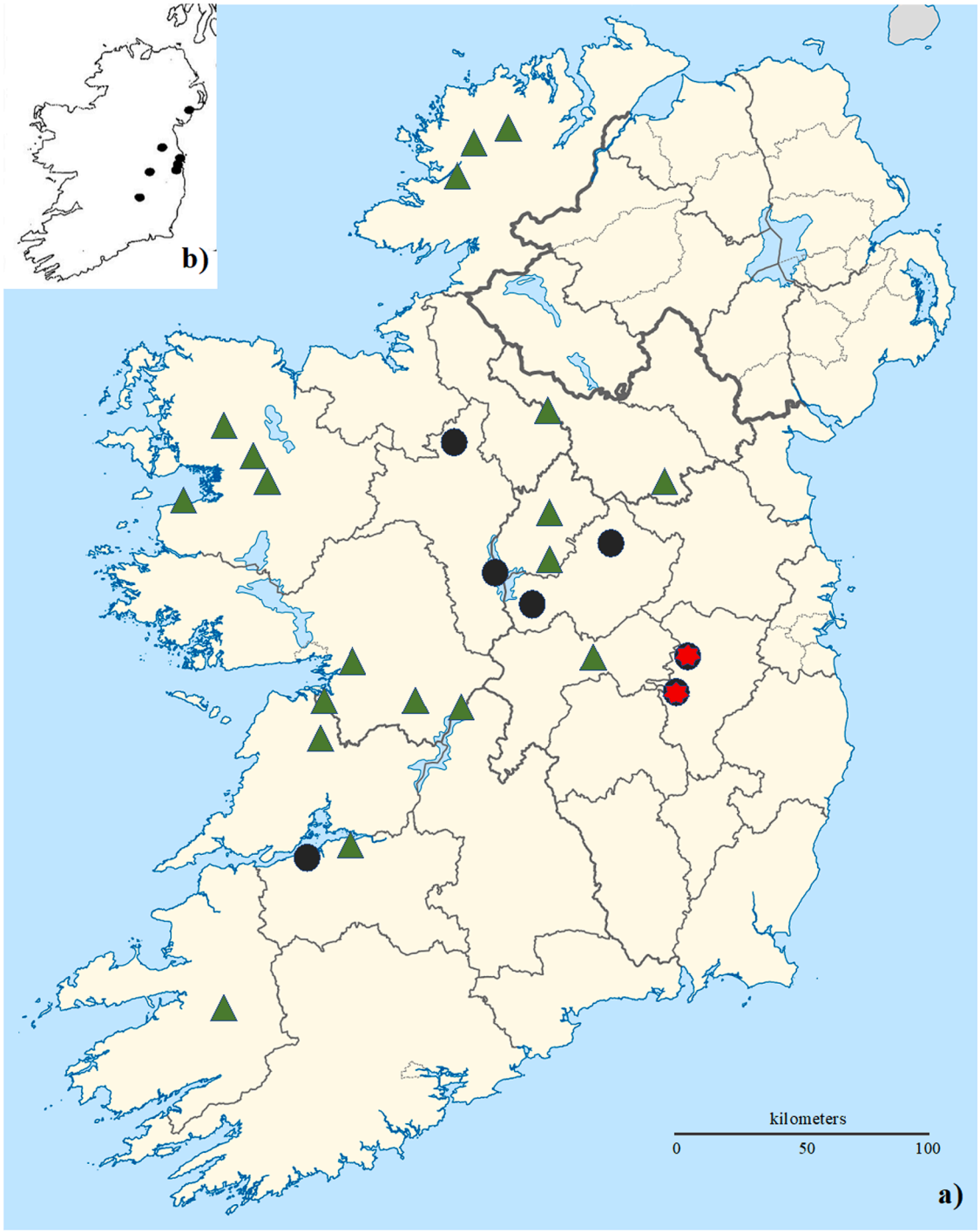

Fig. 1. (a) Map of Ireland showing the capture sites for rodents (black circles) and *I. ricinus* nymphs (green triangles) included in this study. Red stars indicate rodent capture sites with *B. microti*-positive ('Munich' strain) samples. The insert (1b) indicates the known distribution of *Ixodes trianguliceps* in Ireland according to Kelly et al (2001). Reproduced with the kind permission of the Irish Naturalists' Journal. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

reported (McGregor et al., 2019). What is more, *B. microti* has never been detected in questing *I. ricinus* ticks, although between the studies described by Bown et al. (2008), Harrison et al. (2011), Olsthoorn et al. (2021) and Sands et al. (2022), a total of 4,338 questing *I. ricinus* ticks (including 4,328 nymphs) have been screened. Yet, the fact that the parasite is readily detected in field voles (*M. agrestis*) and common shrews (*Sorex araneus*) (Bown et al., 2011), and in engorged ixodid ticks picked off cats (Davies et al., 2017), dogs (Abdullah et al., 2018), and wood mice (Olstoorn et al., 2021) would suggest that *B. microti* is common and widespread throughout the UK, indicating that the strains present in the UK may have limited infectivity for humans and/or *I. ricinus* ticks.

To date there are no data on the presence of *B. microti* in Ireland. In contrast to the UK, where there are 15 species of wild rodent species, Ireland has a depauperate mammalian fauna including just eight rodent species (Lysaght and Marnell, 2016). The present study aimed to investigate the presence of *B. microti* strains in the two dominant rodent woodland species, wood mice (*A. sylvaticus*) and bank voles (*M. glareolus*) and in questing *I. ricinus* nymphs collected in various locations throughout Ireland.

2. Materials and methods

2.1. Collection of rodents

Rodents analysed in this study, including 314 wood mice and 243 bank voles, were a subsample of rodents trapped in seven woodland sites throughout Ireland in 2016 (Stuart et al., 2020) and 2017 (Fig. 1a). They were trapped using single Longworth traps with hay bedding, baited with peanuts and set every 5–10 m on straight line transects and left overnight. Immediately following euthanasia of the animals with isoflurane (1000 mg/g, carried out under Irish Health Products Regulatory Authority licence), spleens were placed in RNAlater® storage solution (Sigma-Aldrich, Saint Louis, MO, USA) then stored at -20 °C until further analysis. Ethical approval for this work was obtained from Trinity College Dublin, Ireland, Animal Research and Ethics Committee.

2.2. Screening of rodents by metabarcoding

Genomic DNA was extracted from the spleen using the DNeasy® 96 Tissue Kit (Qiagen, Germany). Spleen DNA samples (n= 557) were screened for bacteria using universal primers (16S-V4F 5'-GTGCCAGCMGCCGCGGTAA-3' and 16S-V4R 5'-GGACTACHVGGGTWTCTAATCC-3') targeting the V4 hyper variable region of the 16S rRNA gene (251 bp) via Illumina MiSeq (Illumina) sequencing. These PCR primers have been shown to detect apicoplastic 16S rRNA in the eukaryotic phylum Apicomplexa (Abbate et al., 2020). PCR amplification, indexing, multiplexing, sequencing and de-multiplexing and finally taxonomic identification were carried out as described by Galan et al. (2016). For each sample two separate MiSeq sequencing analyses were performed resulting in two independent technical replicates. Raw Illumina sequencing data for the 16S rRNA gene has been made available on Zenodo (10.5281/zenodo.8028161) for the 8 rodent positive samples. The *Babesia* apicoplastic 16S sequence has been uploaded to GenBank under Accession number OR134899. Collection of rodent spleen samples and metabarcoding analysis was carried out as part of an EU-funded project entitled BioRodDis - Managing biodiversity in forests and urban green spaces: Dilution and amplification effects on rodent microbiomes and rodent-borne diseases.

2.3. Confirmation of B. microti-positive rodent isolates by conventional PCR

All rodent isolates that tested positive for *Babesia* sp. by metabarcoding were confirmed by nested PCR using the classical protocol developed by Persing et al. (1992) which targets a fragment of the 18S rRNA gene. The outer primers, Babl (5′-CTTAGTATAAGCTTTTATACAGC-3′) and Bab4 (5′-ATAGGTCAGAAACTTGAATGATACA-3′) amplify a 238bp fragment, the nested primers Bab2 (5′-GTTATAGTTTATTTGATGTTCGTTT-3′) and Bab3 (5′-AAGCCATGCGATTCGCTAAT-3′) a 154bp fragment of the 18S gene. The PCR reaction mix consisted of 1x GoTaq Flexi PCR buffer, 1.5mM $MgCl_2$, 0.2 mM of each deoxynucleotide triphosphate, 500 nM of the forward and reverse primers, 1.25 U Promega GoTaq Flexi DNA Polymerase (Promega, USA) and 5 µl DNA template (first PCR) or 2 µl primary PCR product (in the nested PCR) in a total reaction volume of 50 µl. The PCR conditions (94 °C for 5 min; followed by cycles of 1 min of denaturation at 94°C, 1 min of annealing at 55 °C, and 2 min of extension at 72 °C and a final elongation at 72C for 5 min) were identical in the two PCR assays except that the first assay was run for 35 cycles, the second for 40 cycles. The positive control consisted of a DNA construct of 208bp which contained all primer target sites but resulted in a nested amplicon of 114bp length (Eurofins Scientific). Positive and non-template controls with nuclease-free water instead of DNA template were included in all PCR assays.

For sequence analysis, PCR amplicons from the first PCR assay were purified using the QIAquick PCR purification kit (Qiagen, Germany) and sent for Sanger sequencing, in both directions using the external PCR primers, Bab1 and Bab4 (Eurofins Genomics, Germany). Positive isolates were further characterised by PCR amplification of a 1,300bp 18S rDNA fragment using primers PIRO-A (5'-AATACCCAATCCTGACACAGGG-3') and Primer B (5'-CCCGGGATCCAAGCTTGATCCTTCTGCAGGTTCACCTAC-3') (Goethert and Telford, 2003). The reaction mix and PCR conditions were the same as for the nested PCR. High quality sequence data were obtained by sequencing amplicons in both directions using sequencing primers 'For. 4' (5'-AAAGGAATTGACGGAAGGG-3') and 'Rev. 3' (5'-ACAGTTAAATACGGATGCCC-3') (Kjemtrup et al., 2000), in addition to PIRO-A and Primer B. Consensus DNA sequences derived by aligning forward and reverse sequences were compared against published sequences using BLAST and Clustal Omega. Sanger sequencing data obtained with primers Bab1 and Bab4 and PIRO-A and Primer B are available under Accession numbers LC770218 and LC770217 respectively.

2.4. Screening of questing I. ricinus nymphs for the presence of B. microti

Ixodes ricinus nymphs analysed in this study were a subsample of ticks collected by blanket dragging during surveys carried out between 2017 and 2019 as described by Zintl et al. (2020). They included 634 ticks collected from 11 (out of 26) counties of Ireland in a variety of habitats including farmland, woodland, bog and limestone pavement (Fig. 1a). DNA was extracted from individual nymphs using the QIAGEN QIAamp® DNA Mini Kit following homogenisation with stainless steel beads (Precellys® Montigny-le Bretonneux, France) and overnight incubation in proteinase K (provided with the kit) as described in Zintl et al. (2020). The presence of *B. microti* DNA was assessed using the nested PCR protocol described earlier.

2.5. Statistical analysis

The highest prevalence at which there was a 5% chance of finding no positives in a sample of a given size was calculated using the following formula: $p \leq 1\text{-}(0.05)^{1/n}$ where p= prevalence and n= sample size.

3. Results and discussion

3.1. Presence and identity of B. microti in rodent spleen samples

Bown et al. (2011) reported *B. microti* infection rates of over 30% in field voles (*M. agrestis*) and common shrews (*S. araneus*) trapped in Northern England, indicating that the protozoan parasite is common among certain small mammals in the UK. Neither of these two species

occur in Ireland. Instead this study focused on wood mice and bank voles as the most common rodent species in woodlands. Metabarcoding analysis of the rodent spleen samples resulted in 10,312,614 sequences of the 16S rRNA v4 region after data filtering, including 100,062 sequences from an Apicomplexa 16S rRNA MOTU (Molecular Operational Taxonomic Unit). These sequences matched three *B. microti* isolates in GenBank by 97% (Accession number CP113813, CP113819 and CP113825). Overall the MOTU was detected in both technical replicates of eight rodent samples (including three bank voles and five wood mice), with six to 93,333 sequences per individual. Nested PCR analysis aimed at the 18S rRNA gene resulted in well-defined products of the correct size, confirming the presence of *B. microti* in all eight samples. Moreover, sequence analysis of 192bp fragments amplified using primers Bab1 and Bab4 and 1,300bp fragments amplified using PIRO-A and Primer B revealed 100% homology with the 'Munich' strain of *B. microti* (AB071177). These results agree well with the fact that no human *B. microti* cases have ever been reported in Ireland, as the 'Munich' strain is apparently of limited zoonotic importance.

Our results indicate a much lower prevalence of *B. microti* in rodents in Ireland than in the UK (approx 1.2% in both bank voles and 1.6% in wood mice), however, these results have to be interpreted with caution as we used different screening methods. Interestingly, all positive rodent samples derived from two adjacent trapping sites in the east of the country (Fig. 1a). The fact that the recorded prevalence for wood mice was similar to that of bank voles is also noteworthy as the former are thought to only develop transient infections, while the latter support higher level and longer lasting infections and, as a result, usually have higher infection rates in the wild (Randolph, 1994; Bown et al., 2011; Harrison et al., 2011). Whether our results reflect a genuine idiosyncrasy in the epidemiology of *B. microti* in Ireland, potentially caused by the lack of variety in available host species remains to be investigated further.

3.2. Analysis of questing *I. ricinus* nymphs for the presence of *B. microti*

All 634 *I. ricinus* nymphs collected in 19 sites across Ireland tested negative for the presence of *B. microti*, increasing to a total of 4,962 the number of questing nymphs of this species to have been screened and found negative in Britain and Ireland (Bown et al., 2008, Harrison et al., 2011, Olsthoorn et al., 2021, Sands et al., 2022). According to our calculation, taken together, these results indicate that if *B. microti* is present in *I. ricinus* ticks in the British Isles, it infects 0.06% or less of this tick population (95% confidence). This compares to prevalence rates of between 0.33 and 11% reported in questing *I. ricinus* nymphal stages across Europe (Blanarova et al., 2016; Bonnet et al., 2014; Duh et al., 2001; Egyed et al., 2012; Foppa et al., 2002; Hildebrandt et al., 2010; Krawczyk et al., 2020; Sinski et al., 2006; Skotarczak et al., 2001; Welc-Falęciak et al,. 2012; Wojcik-Fatla et al., 2009) with a general trend of higher infection rates in Eastern Europe. It has been suggested that in Ireland, immature *I. ricinus* stages rely less on small mammal hosts than they do in continental Europe (Harrison et al., 2011) and this may result in a lower exposure rate to *B. microti*. However, considering that so far only the 'Munich' strain has been recorded here which is chiefly transmitted by *I. trianguliceps* (Bown et al., 2008) our results may also, yet again, confirm reduced infectivity of this strain for *I. ricinus*. Incidentally, in Ireland *I. trianguliceps* has chiefly been reported from the east of the country (Kelly et al., 2001, Figure 1b), its known distribution coinciding closely with the only two rodent capture sites where *B. microti*-positive rodent samples were detected.

4. Conclusions

This study provides the first record of *B. microti* in Ireland and demonstrates that *B. microti* can be maintained in woodland rodent communities composed of only *M. glareolus* and *A. sylvaticus*. Whether the limited availability of potential rodent host species or indeed, *I. trianguliceps* tick vectors, affects the epidemiology of *B. microti* in Ireland remains to be seen. To our knowledge this is also the first time *B. microti* isolates from Ireland or the UK have been identified to clade level. Together with published data from the UK, our results indicate that the 'Munich' strain may be the most common (only) strain present in the British Isles. To date this strain has only been detected in a very small number of human cases (Hildebrandt et al., 2021). Whether this is because most infections in humans are mild or asymptomatic or because its main vector, the nidicolous mouse tick, *I. trianguliceps* does not feed on humans is unclear. Either way, it is unlikely to represent a significant public health risk.

Author statement

The authors declare no conflict or bias.

CRediT authorship contribution statement

A. Zintl: Supervision, Conceptualization, Funding acquisition, Methodology, Data curation, Writing – original draft, Formal analysis. **A. McManus:** Methodology, Writing – review & editing. **M. Galan:** Methodology, Formal analysis, Data curation, Writing – review & editing. **M. Diquattro:** Methodology. **L. Giuffredi:** Methodology. **N. Charbonnel:** Supervision, Conceptualization, Funding acquisition, Writing – review & editing. **J. Gray:** Conceptualization, Data curation, Writing – review & editing. **C. Holland:** Supervision, Funding acquisition. **P. Stuart:** Supervision, Conceptualization, Funding acquisition, Writing – review & editing.

Data availability

Data will be made available on request.

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