Abdullah, S., Helps, C., Tasker, S., Newbury, H., & Wall, R. (2018). Prevalence and distribution of *Borrelia* and *Babesia* species in ticks feeding on dogs in the U.K. *Medical and Veterinary Entomology*, *32*(1), 14-22. https://doi.org/10.1111/mve.12257

Prevalence and distribution of *Borrelia* and *Babesia* in ticks feeding on dogs in the UK

Abdullah[1*], S., Helps[2], C., Tasker[2], S., Newbury[3], H. & Wall[1], R.

[1]School of Biological Sciences, University of Bristol, Bristol, UK
[2]Molecular Diagnostic Unit, Langford Vets and School of Veterinary Sciences, University of Bristol, Bristol, UK
[3]MSD Animal Health, Walton Manor, Walton, Milton Keynes, UK

Running head: *Borrelia* and *Babesia* in ticks feeding on dogs

Abstract

Ticks were collected between March and July 2015 from dogs by veterinarians throughout the UK and used to estimate the current prevalence and distribution of pathogens. DNA was extracted from 4,750 ticks and subjected to PCR and sequence analysis to identify *Borrelia burgdorferi* sensu lato and *Babesia* species. From 4,737 ticks (predominantly *Ixodes ricinus* Linneaus), *B. burgdorferi* (s.l.) was detected in 94 (2.0%). Four *Borrelia* genospecies were identified: *Borrelia garinii* (41.5%), *Borrelia afzelli* (31.9%), *Borrelia burdorferi* sensu stricto (s.s.) (25.5%) and *Borrelia spielmanii* (1.1%). One *Rhipicephalus sanguineus* Latreille, collected from a dog with a travel history outside the UK, was positive for *B. garinii*. Seventy ticks (1.5%) were positive for *Babesia* spp.: 84.3% were *Babesia venatorum*, 10.0% were *Babesia vulpes* sp. nov., 2.9% were *Babesia divergens/capreoli* and 1.4% were *Babesia microti*. One isolate of *Babesia canis* was detected in a *D. reticulatus* tick collected from a dog that had recently travelled to France. The prevalence of *B. burgdorferi* (s.l.) and *Babesia* spp. did not differ significantly between different regions of the UK. The results map the widespread distribution of *B. burgdorferi* (s.l.) and *Babesia* spp. in ticks in the UK and highlight the potential for the introduction and establishment of exotic ticks and tick borne pathogens.

Keywords: *Ixodes*, *Dermacentor, Rhipicephalus, Borrelia, Babesia,* Vector, Pathogen, Disease

Correspondence: Swaid Abdullah, Veterinary Parasitology and Ecology Group, School of Biological Sciences, Life Sciences Building, University of Bristol, Bristol, BS8 1TQ, UK.
E-mail: swaid.abdullah@bristol.ac.uk

Introduction

Tick-borne disease has a major direct impact on animal health and welfare; in addition, companion animals, particularly dogs, can be considered as sentinels for the risk of human pathogen exposure (Lindenmayer *et al*., 1991; Smith *et al*., 2012). Changes in the distribution and prevalence of ticks and tick-borne pathogens are therefore of particular interest (Gray, 2008; Beugnet & Marie, 2009; Hansford *et al*., 2016a) and may be expedited by changes in climate, increases in host populations and increasing levels of animal movement (Hansford *et al*., 2016b). Two of the tick-borne pathogens of particular interest in this context in the UK are *Borrelia* and *Babesia*.

Lyme disease results from *Borrelia burgdorferi* (s.l.) species complex infection and is transmitted in Europe primarily by *Ixodes ricinus* (Ackermann *et al*., 1984), but can also be transmitted by *I. hexagonus* (Toutoungi & Gern, 1993). Nine pathogenic species of *B. burgdorferi* (s.l.) are described in Europe: *B. burgdorferi* sensu stricto (s.s.), *B. garinii*, *B. afzelii*, *B. valaisiana*, *B. lusitaniae*, *B. spielmanii*, *B. kurtenbachii*, *B. bissettii* and *B. bavariensis* (Rauter & Hartung, 2005; Margos *et al*., 2010). Four genospecies have been recently reported in Scotland: *B. afzelii*, *B. garinii*, *B. burgdorferi* (s.s.) and *B. valaisiana* (Millins et al., 2016). *Borrelia burgdorferi* (s.l.) infections circulate within reservoir populations of wild animals, particularly small mammals and ground nesting birds. They are transmitted trans-stadially within ticks, with trans-ovarian transmission appearing to play only a minor role in the epidemiology of this pathogen (Nefedova *et al*., 2004). Lyme disease is a serious problem for people and reported human cases of Lyme disease increased 30-fold between 1999 and 2008 in Scotland (Health Protection Scotland, 2009). In dogs, a recent UK study detected *B. burgdorferi* (s.l.) in 2.3% of ticks recovered (Smith *et al.*, 2012). Only 5-10% of dogs infected with *B. burgdorferi* (s.l.) develop clinical disease (Little, 2010), therefore, the prevalence of clinical Lyme disease in dogs significantly underestimates the risk of disease exposure.

Babesia spp. protozoans are found around the world and infect the blood cells of many animal species (Telford *et al*., 1993) causing the disease called babesiosis. Four *Babesia* species are known to affect dogs; *B. canis*, *B. vogeli*, *B. gibsoni* and *B. vulpes* sp. nov., (the latter previously described as *Babesia microti*-like) (Matijatko *et al*., 2012; Baneth *et al*., 2015). The clinical signs and severity of disease vary with different *Babesia* species infections as well as with the immune and health status of the animal, and range from a mild transient illness to acute disease associated with severe

haemolysis that rapidly results in death (Solano-Gallego & Baneth, 2011). Humans become susceptible to babesiosis only if splenectomised or otherwise immunocompromised, and *B. divergens*, a parasite of cattle, or *B. microti*, found in rodents, have been indicated as the most common causal agents (Gray *et al*., 2010). Ticks acquire *Babesia* spp. infections by feeding on an infected host and transovarial transmission of *Babesia canis* has been observed through up to five tick generations (Chauvin *et al*., 2009). For dogs, the most pathogenic and widespread of the species is *B. canis*, a large piroplasm endemic in most of continental Europe (Criado-Fornelio *et al*., 2000).

The distribution of *B. canis* is closely associated with its vector *Dermacentor reticulatus* (Foldvari *et al*., 2005) and therefore changes in the distribution of this tick are important. Historical records show that *D. reticulatus* has been found in the UK for over 100 years (http://data.nbn.org.uk) in relatively small, isolated populations. However, at least four established, predominantly coastal, populations have been recently confirmed (Jameson & Medlock, 2011). In the UK, there have been an increasing number of cases of babesiosis in dogs imported from abroad (Shaw *et al.*, 2003). The first case of fatal babesiosis in a dog that had not left the UK was diagnosed in Kent and the causal agent was tentatively identified as *B. vogeli* (Holm *et al*., 2006). Subsequently a cluster of cases of *B. canis* was reported involving dogs in Essex with no history of foreign travel (Hansford *et al*., 2016a). A later report of two additional cases suggests that *B. canis* is now endemic in this area. Retrospective *Babesia* test results from two UK laboratories showed that 13 of 99 submissions in 2015 were positive for *Babesia* spp., which were considered to be from dogs returning after travel outside the UK (Sanchez-vizcaino *et al*., 2016a,b). In just the first three months of 2016, 11 of 67 submissions were positive, indicating a sudden increase in cases with geographical clustering with eight cases originated from Essex.

A novel zoonotic babesia, *B. venatorum*, was identified (Herwaldt *et al*., 2003) and has been recorded in UK ticks (Smith *et al*., 2013). Another large piroplasm, *B. vogeli*, transmitted by *Rhipicephalus sanguineus*, is found in southern Europe around the Mediterranean and is an emerging pathogen in northern and eastern Europe (Irwin, 2009). *Rhipicephalus sanguineus* is not established in the UK; however, there have been reports of *R. sanguineus* infestations in domestic properties in the UK thought to have

been introduced by importing dogs that were unprotected against ticks (Hansford *et al.*, 2015).

The evidence suggests that the distribution and prevalence of *Borrelia* spp. and *Babesia* spp. pathogens within the UK are currently highly labile and closer surveillance is therefore warranted. However, the relatively low prevalence and the highly uneven geographical distribution of infections, mean that very large samples are required to ensure detection where they are present. The aim of this study, therefore, was to determine the prevalence of *Babesia* spp. and *Borrelia spp.* in ticks collected from dogs presented to veterinary practices participating in a UK-wide national tick surveillance programme (Abdullah *et al*., 2016).

Methods and Materials

Sample collection and DNA extraction

A national survey of ticks collected from dogs in the UK was undertaken in 2015, during which veterinary practices were asked to examine five dogs for ticks each week for eight weeks following a previously described protocol (Abdullah *et al*., 2016). Ticks collected were submitted for identification and then pathogen testing.

Each tick received by the investigators was given a unique identification number and stored at -20^{o}C pending analysis. Subsequently, ticks were identified to species, life-cycle stage and sex (Abdullah *et al*., 2016).

All ticks submitted over the first 13 weeks of the surveillance study described by Abdullah *et al*. (2016) were used in the present analysis. These were first classified by level of engorgement as: unfed, partially-fed, or fully-fed. Fully-fed ticks were those considered to have reached maximum engorgement in relation to scutal dimensions; partially-fed ticks were defined as those that contained some blood but which had not yet reached maximum expansion; unfed ticks contained no blood. Each tick was then cut longitudinally and transversely before DNA extraction. DNA extraction from ticks was performed using two commercially available extraction kits. Both the extraction methods were compared using spectrophotometry (Nanodrop) and agarose gel electrophoresis and both were found to give a similar range of sample DNA concentrations. DNA from first 1600 tick samples used QIAGEN, blood tissue kits (DNeasy Blood & Tissue Kit) and the remainder used the high throughput NucleoSpin® 96 Tissue Core Kit (Macherey-Nagel, Germany). Both the kits were used following the

manufacturer's instructions. For those that were unfed and partially-fed, whole ticks were used and the volume of reagent used for extraction followed the kit protocol. But for fully-fed ticks, which had large volumes of clotted blood, using the whole tick was not practical because even after overnight digestion in double the recommended volume of Proteinase-K and tissue lysis buffer, the digest clogged the silicone columns preventing the completion of extraction. To overcome this problem only the anterior two thirds of the fully engorged tick (containing salivary glands) was used for extraction and the extraction protocol used: 40 µl of Proteinase-K (instead of 30 µl) and 400 µl of tissue lysis buffer (instead of 240 µl) and the samples were incubated at 56 °C overnight. After overnight digestion, only half the lysate was transferred to spin columns (using full lysate again created problems in the silicone columns). Two washes of wash buffer were given to each column to clean them up properly before the ethanol wash and final elution. Finally, DNA was eluted in 100 µl of elution buffer and stored at -20 °C prior to further analysis. A canine glyceraldehyde-3-phosphate dehydrogenase (GAPDH) qPCR was multiplexed with the *Babesia* spp. qPCR, to detect canine DNA isolated from dog blood in each tick sample as a control for DNA extraction, qPCR setup and assay inhibition; all of the ticks, except the unfed, had amplifiable canine DNA demonstrating that the extraction and the PCRs were working appropriately.

Borrelia PCR and sequence analysis

Conventional PCR was used to detect *B. burgdorferi* (s.l.) in the DNA extract; primers BSLF (5′-AATAGGTCTAATAATAGCCTTAATAGC-3′) and BSLR (5'-CTAGTGTTTTGCCATCTTCTTTGAAAA-3') amplified a 250- 300 bp region of the *ospA* gene found in all *B. burgdorferi* (s.l.) (Smith *et al*., 2012). Master-mix was formulated as follows: 5 µl of 2x GoTaq Hot start mix (Promega, UK), 0.4 µl of 12.5 µM each BSLF/BSLR primer mix and 2.6 µl water. Two µl of extracted DNA were then added to 8 µL of master mix in 96 well PCR plates using a high throughput automated pipetting system (epMotion P5073, Eppendorf, UK). Water and *B. burgdorferi* (NG036 PCR product diluted at 10^{-10}) were used as negative and positive controls, respectively. Thermal cycling included an initial denaturation (95°C for 2 min), followed by 40 denaturation cycles (95 °C for 20 s), annealing (56 °C for 30 s) and extension (72 °C for 30 s). Amplified DNA was subjected to electrophoresis in a 1.5% agarose gel pre-stained with 0.05 µg/ml ethidium bromide and viewed under UV light. Positive samples were

identified as having a defined band of 250-300 bp on the gel and were later re-amplified in a 25 µl PCR for DNA sequencing.

Amplicons were prepared for DNA sequencing (Macherey-Nagel NucleoSpin® 96 PCR Clean-up Core Kit, Macherey-Nagel, Germany) and sent for commercial DNA sequencing (MRC I PPU, College of Life Sciences, University of Dundee, Scotland) using Applied Biosystems Big Dye Ver 3.1 chemistry on an Applied Biosystems model 3730 automated capillary DNA sequencer. Only forward sequencing was undertaken, the sequences were checked and edited, if necessary using BioEdit Sequence Alignment Editor Version 7.2.5, and then compared to available sequence data available (GenBank using BLASTn http:// www.ncbi.nlm.nih.gov/BLAST/). Any sequences with less than 97% homology were not considered.

Babesia PCR and sequence analysis

Babesia spp. were detected in DNA extracts using a probe based generic *Babesia* qPCR targeting the *18S rRNA* gene. The following primers were used for detection of *Babesia* spp.: Babesia 944 for (5'-TTAACGAACGAGACCTTAACCTG-3'), Babesia 1315 rev (5'-CCGAATAATTCACCGGATCAC-3') and Babesia TaqMan probe (5'-FAM-CGATCGGTAGGAGCGACGGGC-BHQ1-3') (Diagnostic Laboratories, Langford Vets, UK). A primer/probe mix was made as follows: 10 µM Babesia 944 for, 10 µM Babesia 1315 rev, 2.5 µM Babesia TaqMan probe. Positive (*B. canis*, 12763 PCR product diluted at 10^{-1}) and negative (water) controls were included in each 96 well PCR plate. The qPCR reaction was made with 2 µl of sample DNA and 8 µl of master mix, composed of 5 µl of 2x GoTaq Hot Start mix, 0.4 µl primer/probe mix, 0.6 µl 50 mM $MgCl_2$ and 2 µl H_2O. Thermal cycling conditions included an initial denaturation (95°C for 2 min; 45 cycles of 95 °C for 15 s, and 60 °C for 30 s) (Agilent MX3005P qPCR, Agilent, UK). Fluorescence data were collected at 520 nm at the end of each annealing/extension step. A cut off of 35 cycles was used to differentiate true *Babesia* spp. positives from possible cross-reaction (see discussion). Positive PCR samples were later re-amplified in a 25 µl PCR for DNA sequencing as described above for *Borrelia* spp.

Statistical analysis

Chi-square analysis (SPSS, version 2.3) was used to compare *Borrelia* and *Babesia* regional prevalences, with the UK divided into eight geographic divisions (Table

1). Distribution of tick samples and pathogen distributions were mapped using QGIS (version 2.8.1) using the owner's postcodes.

Results

A total of 4750 ticks were analysed. Among these, 4737 were from dogs resident within the UK and 13 were from dogs which had been abroad. All but 8 of the tick samples were adult females at various stages of engorgement, representing a wide geographic spread from across the UK (Fig 1). The number of each tick species collected from UK resident dogs and included in the pathogen analysis were: 4,316 (91.1%) *I. ricinus*, 386 (8.1%) *I. hexagonus*, 23 (0.5%) *I. canisuga*, 9 (0.2%) *D. reticulatus* and 3 (0.06%) *Haemaphysalis punctata*. All the 8 nymphs were *I. ricinus*. The ticks on travelled dogs included one *D. reticulatus* and 12 *R. sanguineus.*

Borrelia distribution and prevalence

Borrelia ospA PCR and subsequent DNA sequencing showed that 94 of the 4,737 tick samples from resident dogs (2.0%) contained *B. burgdorferi* (s.l.) DNA; these included 91 from *I. ricinus* and 3 from *I. hexagonus*. One *R. sanguineus* collected from a dog with recent travel history outside the UK was also found positive. Three of the 94 positive ticks were nymphs and the rest were adult females, including 72 partially-fed, 16 unfed and 3 fully-fed. *Borrelia burgdorferi* (s.l.) prevalence was 2.1% for *I. ricinus* and 0.8% for *I. hexagonus*, while all other tested ticks were negative. *Borrelia* were found at sites throughout the UK (Fig. 2), broadly mirroring the distribution of tick samples submitted. The regional prevalence of *Borrelia* spp. ranged from 1.1 to 3.0% (Table 1) with no significant difference between UK regions (χ^2= 6.98, d.f.=7, p=0.43). Sequence analysis of the 94 *B. burgdorferi* (s.l.) positive samples detected four genopecies, including 39 *B. garinii* (41.5%), 30 *B. afzelli* (31.9%), 24 *B. burdorferi* s.s. (25.5%) and 1 *B. spielmanii* (1.1%). The one infected *R. sanguineus* was found to be infected with *Borrelia garinii* (Table 2).

Babesia distribution and prevalence

The generic *Babesia* spp. qPCR and subsequent DNA sequencing indicated that 70 of 4,737 ticks collected from dogs contained *Babesia* spp. DNA, giving a prevalence of

1.5%. One of these was a *D. reticulatus*, found on a dog with a recent history of travel outside the UK was also found positive for *Babesia*. All four tick species were found to be infected with *Babesia* spp.; of the 70 positive samples, 62 (88.6%) were *I. ricinus*, 6 (8.6%) were *I. hexagonus,* 1 (1.4%) was *I. canisuga* and 1 (1.4%) was *D. reticulatus*. No nymphs were positive for *Babesia* spp.; all positive ticks were adult females including 56 partially fed, 3 unfed and 11 fully fed. *Babesia* spp. were also widely distributed throughout the UK, with a distribution broadly mirroring that of the tick samples submitted (Fig. 3). Regional prevalences varied from 0.5 to 2.4% (Table 1), and were not significantly different (χ^2= 6.26, d.f.=7, p=0.51). Of the 70 *Babesia* positive samples, 59 (84.3%) were *B. venatorum*, 7 (10.0%) were *B. vulpes* sp. nov., 2 (2.9%) were *B. divergens/capreoli*, 1 (1.4%) was *B. microti* and 1 (1.4%) was *B. canis.* The *B. canis* DNA was detected in an adult fully fed *D. reticulatus* tick collected from a dog that had recently returned from France (Table 3). *Borrelia* spp. and *Babesia* spp. coinfections (*B. garinii* and *B. venatorum* in every case*)* were detected in 3 ticks, including 2 partially-fed female *I. ricinus* and 1 unfed female *I. ricinus* (Table 3).

Discussion

In the present study, a large sample of ticks collected from dogs from all regions of the UK were tested and found to be infected at a prevalence of 2.0% for *B. burgdorferi* (s.l.) and 1.5% for *Babesia* spp. *Borrelia burgdorferi* (s.l.) were only detected in *I. ricinus* and *I. hexagonus*, whereas various *Babesia* spp. were detected in all four tick species analysed, highlighting the greater diversity of host-pathogen-vector relationships for *Babesia* spp. (Homer *et al*., 2000). *Borrelia spielmanii* was detected for the first time in a tick in the UK, which could possibly reflect its low prevalence. This study also highlights the continued potential for the introduction of ticks into the UK with travelled companion animals and the establishment of exotic pathogens, such as *B. canis*.

The prevalences of *B. burgdorferi* (s.l.) recorded here are broadly similar to other studies undertaken in the UK. For example, Bettridge (2013) showed that the prevalence of *B. burgdorferi* (s.l.) in *I. ricinus* is highly variable across various regions of the UK with prevalences that varied between 1 to 7.5% depending on the region and also the site from which the ticks were collected. Deciduous and mixed woodland had significantly higher prevalence than other habitats. The study indicated that the presence of high number of ticks was not necessarily related to high prevalence of the

pathogen. James *et al*. (2014) found that the prevalence of the *B. burgdorferi* sensu lato in Scotland varied between 0.8 to 13.9% in *I. ricinus* nymphs and again nymphs from mixed woodland were more likely to be infected than those collected from coniferous woodland. Hansford *et al.* (2014) also estimated the prevalence in questing ticks collected from various regions in England. Most of the ticks were collected either from woodland and woodland edges or moorlands. A total of 954 ticks were examined for *B. burgdorferi* (s. l.), out of which 40 were positive with PCR, giving a prevalence of 4.2%, but from these they were able to sequence and speciate only 24 samples giving a prevalence of 2.5%. They also reported considerable variation (between 0 to 13.6%) in prevalence depending on the region of collection. Hansford *et al*. (2016) sampled known hotspots for Lyme borreliosis in the UK and reported a prevalence of 18% in questing ticks (predominantly nymphs), but with very small samples sizes in many locations. A smaller-scale UK survey of ticks feeding on dogs, which used the same sample methodology as used here, reported a similar prevalence of 2.3% for *B. burgdorferi* (s.l.) (Smith *et al*., 2012; 2013). It is notable that in general the prevalence of *B. burgdorferi* (s.l.) in UK ticks is considerably lower than reported prevalences from continental Europe, which range from 14% up to 49% (Rauter & Hartung, 2005) although in specific sites and habitats it may be higher. The reasons for the generally lower prevalence in the UK are not known, but continued ongoing surveillance to monitor any future changes in prevalence of this zoonotic spirochete would be prudent.

Borrelia burgdorferi (s.l.) comprises of 19 species, five of which are reported to cause Lyme disease in humans: *B. afzelii*, *B. garinii*, *B. burgdorferi* s.s.*, B. bavariensis* and *B. spielmanii* (Stanek & Reiter). Four different genospecies were detected in the current study, three with relatively equal prevalence: *B. garinii* (41.5%), *B. afzelli* (31.9%) and *B. burdorferi* (s.s.) (25.5%), while only one case of *B*. *spielmanii* was detected. These relative prevalences are similar to previous reports (Rauter & Hartung, 2005; Estrada-Pena *et al*., 2011) where meta-analysis found that *B. afzelli* and *B. garinii* were the most prevalent *Borrelia burgdorferi* (s.l.) species in central Europe followed by *B. burdorferi* (s.s.). Different *Borrelia burgdorferi* (s.l.) species are sustained by diverse transmission cycles involving different vertebrate host species but the same tick vectors (Margos *et al*., 2009); *B. garinii* has been reported more commonly in birds whereas *B. afzelii* circulates predominantly in rodent populations (Kurtenbach *et al*., 2002), indicating ticks feeding on dogs have fed previously on a variety of host species. The detection of *B.*

spielmanii is the first record for the UK from an *I. ricinus* tick infesting a domestic dog that had not travelled recently. This *Borrelia* species is usually associated with rodents, especially dormice (Ritcher *et al*., 2006), and has been reported to cause *erythema migrans* and Lyme disease in humans (Maraspin *et al*., 2006). Detection of *B. garinii* in *R. sanguineus* in this study is of interest because vector competency of this tick species for *B. burgdorferi* (s.l.) has not been confirmed, but this tick collected in three sites in southern England and one in Wales has been found to carry *Borrelia* (Hubbard *et al*., 1998). Babesia detection in ticks using a highly sensitive probe-based qPCR (originally designed to detect *Babesia* spp. in dog blood) initially led to problems with cross-reactions with other tick-borne microorganisms. The qPCR identified 490 samples out of 4,737 DNA extracts that appeared to be 'positive' for *Babesia* spp., but after sequence analysis and BLAST, only 70 of these were confirmed as *Babesia* spp. The others were identified as a range of other organisms, mainly *Stenophora robusta*, uncultured eukaryote clone SGYH921 and some *Colpodellidae* spp.. Ticks carry a number of endocellular symbionts (Cheng, 1993) and several of these microorganisms are not yet identified and characterised (Raoult & Roux, 1997). This cross-reactivity reduces the accuracy of sensitive qPCR for pathogen detection in ticks. The same *Babesia* spp. qPCR does not give false positive cross-reactions when run on DNA extracted from dog blood where other endocellular symbionts would not be found. Thus, PCR and DNA sequence analyses of amplicons is necessary for exact *Babesia* species identification and for avoidance of false positive results (Hildebrandt *et al*., 2013).

Sequence analysis of the 70 *Babesia* positive amplicons found that 59 (84.3%) were *B. venatorum*, 7 (10 %) were *B. vulpes* sp. nov., 2 (2.9 %) were *B. divergens/B. capreoli* and one (1.4 %) was *B. microti*. The speciation of *Babesia* protozoa is complex and the pathogenicity of identified species is uncertain. Ten *Babesia* pathogens categorised as *B. vulpes* sp. nov. were detected, and these also matched by BLAST on the NCBI database with four different entries of *Babesia* piroplasms (*Piroplasmida* sp. mel1/Burgos/2007, *B. vulpes*, *Theileria annae* and *Babesia* cf. *microti*) with similar sequence identity scores. It was difficult to assign them specifically to any of these matches; recently Baneth *et al.* (2015) categorised these four *Babesia* piroplasms as a single species *B. vulpes* sp. nov, an approach also adopted in this study.

Two *Babesia* spp. amplicons were identified as *B. divergens* and *B. capreoli* with equal BLAST scores. The differentiation between *B. divergens* and *B. capreoli* is difficult

due to their morphological similarities and it is further complicated by the high percentage of identity between their respective 18S rRNA gene sequences. *Babesia divergens* and *B. capreoli* have very few intraspecific differences in their *18S rRNA* with 99.83% identity, with differences only at positions 631, 633 and 1637 (Malandrin *et al*., 2010). The position of primers and amplicon length used in this study did not allow these two species to be differentiated. Another similar pathogen is *Babesia odocoilei*. It infects white tailed Deer, elk and caribou in the United States, but is difficult to distinguish based on 18S rRNA gene sequences from *B divergens* and *B. capreoli*, and was not identified here (Holman *et al.,* 1994). *Babesia divergens* is a zoonotic pathogen with a wide host range, but has not so far been reported to cause disease in dogs; *B. capreoli* has been reported in wild cervids and its zoonotic potential is uncertain (Gray *et al*., 2010; Malandrin *et al*., 2010).

Three ticks were co-infected with *B. garinii* and *B. venatorum* and co-infection between *Borrelia* and *Babesia* has been reported previously (Krause *et al*., 1996; Jablonska *et al*., 2016) but the exact species combinations varies with the geographical location (Swanson *et al*., 2006). Co-infections have been reported to produce more severe clinical symptoms and introduce further complications in the diagnosis and treatment of disease (Krause *et al*., 1996).

A cluster of cases of *B. canis* infection with associated clinical signs of babesiosis has recently been reported in UK dogs (Swainsbury *et al*., 2016) and in this study *B. canis* was detected in one of the *D. reticulatus* ticks tested. In an earlier report (Abdullah *et al*., 2016), the dog from which this tick was obtained was not reported to have travelled outside the UK, but further investigation after finding this tick positive for *B. canis*, revealed that the dog had in fact recently returned from France. The detection underlines the ongoing risk of entry and establishment of this pathogen in the UK. Since pathogens were identified in fed ticks collected from dogs, it is possible that some were acquired with the current blood-meal rather than being mature infections; this may have contributed to a slight overestimation in prevalence or the presence of pathogens in unexpected vector species. Nevertheless, the data clearly suggest that dog owners need to be aware of the appropriate measures required to protect their dogs against tick infections at home and while travelling in other countries.

Acknowledgements

We are grateful to all the veterinary practices that submitted tick samples and to Andrew Bird, Nancy Matthews, Laura Harber, Issy Sykes, William Turner, George Van Horn, Roisin McDonough and Jay Towne for their assistance with DNA extraction and PCR analysis. We are grateful to Robert Armstrong for his many helpful editorial suggestions. SA was supported by a University of Bristol Zutshi-Smith PhD scholarship. This work was carried out with the approval of the University of Bristol ethics committee, UIN: UB/15/008

References

Abdullah, S., Helps, C., Tasker, S., Newbury, H. and Wall, R. (2016). Ticks infesting domestic dogs in the UK: a large-scale surveillance programme. Parasites & Vectors, **9**, 391.

Ackermann, R., Kabatzki, J., Boisten, H., Steere, A., Grodzicki, R., Hartung, S. and Runne, U. (1984). Spirochäten-Ätiologie der Erythema-chronicum-migrans-Krankheit. *DMW - Deutsche Medizinische Wochenschrift*, **109**, 92-97.

Baneth, G., Florin-Christensen, M., Cardoso, L. and Schnittger, L. (2015). Reclassification of Theileria annae as Babesia vulpes sp. nov. *Parasites & Vectors*, **8**, 207

Bettridge, J., Renard, M., Zhao, F., Bown, K.J., Birtles, R.J (2013). Distribution of *Borrelia burgdorferi* sensu lato in *Ixodes ricinus* populations across central Britain. *Vector Borne Zoonotic Diseases*, **13**, 139–146

Beugnet, F. and Marié, J. (2009). Emerging arthropod-borne diseases of companion animals in Europe. *Veterinary Parasitology*, **163**, 298-305.

Chauvin, A., Moreau, E., Bonnet, S., Plantard, O. and Malandrin, L. (2009). Babesia and its hosts: adaptation to long-lasting interactions as a way to achieve efficient transmission. *Veterinary Research*, **40**, 37.

Cheng, T.C. (1993). Is parasitism symbiosis? A definition of terms and the evolution of concepts. In: Toft, C.A., Aeschlimann A., Bolis L. (eds). *Parasite-host associations: coexistence or conflict.* New York: Oxford University Press. 15-36.

Criado-Fornelio, A., Gutierrez-Garcia, L., Rodriguez-Caabeiro, F., Reus-Garcia, E., Roldan-Soriano, M. and Diaz-Sanchez, M. (2000). A parasitological survey of wild red foxes (Vulpes vulpes) from the province of Guadalajara, Spain. *Veterinary Parasitology*, **92**, 245-251.

Estrada-Pena, A., Mangold, A.J., Nava, S., Venzal, J.M., Labruna, M.B., Guidon-Attali, C. (2011). A review of the systematics of the tick family Argasidae (Ixodida). *Acarologia*, **50**, 317–334

Földvári, G., Hell, É. and Farkas, R. (2005). Babesia canis canis in dogs from Hungary: detection by PCR and sequencing. *Veterinary Parasitology*, **127**, 221-226.

Gray, J. (2008). Ixodes ricinus seasonal activity: Implications of global warming indicated by revisiting tick and weather data. *International Journal of Medical Microbiology*, **298**, 19-24.

Gray, J., Zintl, A., Hildebrandt, A., Hunfeld, K. and Weiss, L. (2010). Zoonotic babesiosis: Overview of the disease and novel aspects of pathogen identity. *Ticks and Tick-borne Diseases*, **1**, 3-10.

Hansford, K.M., Fonville, M., Jahfari, S., Sprong, H., Medlock, J.M. (2014). *Borrelia miyamotoi* in host-seeking Ixodes ricinus ticks in England. *Epidemiology and Infection*, 1–9.

Hansford, K., Pietzsch, M., Cull, B., Medlock, J. and Wall, R. (2015). Overwintering of the brown dog tick in residential properties in England – raising awareness. *Veterinary Record*, **177**, 156-156.

Hansford, K.M., Fonville, M., Gillingham, E.L., Coipan, E.C., Pietzsch, M., et al. (2016). Ticks and *Borrelia* in urban and peri-urban green space habitats in a city in southern England. *Ticks and Tick-borne Diseases*, **8**, 353-361.

Hansford, K.M., Medlock, J.M., Swainsbury, C., Phipps, L.P., Maria Del Mar Fernandez De Marco, Hernández-Triana, L.M., Johnson, N., et al. (2016a). *Babesia canis* infection in ticks in Essex. *Veterinary Record*, **178**, 323-323

Hansford, K., Medlock, J., Atkinson, B. and Santos-Silva, M. (2016b). Importation of a Hyalomma lusitanicum tick into the UK on a dog. *Veterinary Record*, **179**, 415-415.

Health Protection Scotland. *Annual trends in notifiable diseases*, selected years from 1980–2009. http://www.hps.scot.nhs.uk/publichealthact/AnnualData2009.aspx.

Herwaldt, B., Cacciò, S., Gherlinzoni, F., Aspöck, H., Slemenda, S., Piccaluga, P., Martinelli, G., Edelhofer, R., Hollenstein, U., Poletti, G., Pampiglione, S., Löschenberger, K., Tura, S. and Pieniazek, N. (2003). Molecular Characterization of a Non– Babesia divergens Organism Causing Zoonotic Babesiosis in Europe. *Emerging Infectious Diseases*, **9**, 942-955.

Hildebrandt, A., Gray, J.S. & Hunfeld, K.-P. (2013). Human Babesiosis in Europe: what clinicians need to know. *Infection*, **41**, 1057–1072.

Holm, L.P., Kerr, M.G., Trees, A.J., McGarry, J.W., Munro, E.R. and Shaw, S.E. (2006). Fatal babesiosis in an untravelled British dog. *Veterinary Record*, **159**, 179–180.

Holman, P. J., T. M. Craig, D. L. Doan Crider, K. R. Petrini, J. Rhyan, and G. G. Wagner. 1994. Culture isolation and partial characterization of a Babesia sp. from a North American elk (*Cervus elaphus*). *Journal of Wildlife Diseases*, **30**, 460–465.

Homer, M.J., Aguilar-Delfin, I., Telford, S.R., Krause, P.J. and Persing, D.H. (2000). Babesiosis. *Clinical Microbiology Reviews*, **13**, 451–469.

Hubbard, M.J., Baker, A.S. and Cann, K.J. (1998). Distribution of Borrelia burgdorferi s.l. spirochaete DNA in British ticks (Argasidae and Ixodidae) since the 19th Century, assessed by PCR. *Medical and Veterinary Entomology*, **12**, 89–97.

Irwin, P.J. (2009). Canine babesiosis: from molecular taxonomy to control. *Parasites & Vectors*, **2** (Suppl 1).

Jabłońska, J. Żarnowska-Prymek, H., Stańczak, J., Kozłowska, J., Wiercińska-Drapało, A. (2016). Symptomatic co-infection with Babesia microti and Borrelia burgdorferi in patient after international exposure; a challenging case in Poland. *Annals of Agricultural and Environmental Medicine*, 23, 387–389.

James, M.C., Gilbert, L., Bowman, A.S., Forbes, K.J. (2014). The heterogeneity, distribution, and environmental associations of *Borrelia burgdorferi* sensu lato, the agent of Lyme borreliosis, in Scotland. *Front Public Health*, **2**, 129

Jameson, L.J. & Medlock, J.M. (2011). Tick Surveillance in Great Britain. *Vector-Borne and Zoonotic Diseases*, **11**, 403–412.

Krause, P.J., Telford III, S.R., Spielman, A., Sikand, V., Ryan, R., Christianson, D., Burke, G., Brassard, P., Pollack, R., Peck, J., Persing, D.H. (1996) Concurrent Lyme disease and babesiosis. Evidence for increased severity and duration of illness. *Journal of the American Medical Association*, **275**, 1657-1660.

Kurtenbach, K., De Michelis, S., Etti, S., Schafer, S.M., Sewell, H., Brade, V. & Kraiczy, P. (2002). Host association of *Borrelia burgdorferi* sensu lato – The key role of host complement. *Trends in microbiology*, **10,** 74–79

Lindenmayer, J. M., Marshall, D., & Onderdonk, A. B. (1991). Dogs as sentinels for Lyme disease in Massachusetts. *American Journal of Public Health*, **81**, 1448–1455.

Little SE, Heise SR, Blagburn BL, Callister SM, Mead PS. (2010). Lyme borreliosis in dogs and humans in the USA. *Trends in Parasitology*, **26**, 213–8

Malandrin, L., Jouglin, M., Sun, Y., Brisseau, N. and Chauvin, A. (2010). Redescription of Babesia capreoli (Enigk and Friedhoff, 1962) from roe deer (Capreolus capreolus): Isolation, cultivation, host specificity, molecular characterisation and differentiation from Babesia divergens. *International Journal for Parasitology*, **40**, 277-284.

Maraspin, V., Ruzic-Sabljic, E. and Strle, F. (2006). Lyme Borreliosis and Borrelia spielmanii. *Emerging Infectious Diseases*, **12**, 1177-1177.

Margos, G., Vollmer, S.A., Cornet, M., Garnier, M., Fingerle, V., Wilske, B., Bormane, A., Vitorino, L., Collares-Pereira, M., Drancourt, M., Kurtenbach, K. (2009). A new Borrelia species defined by multilocus sequence analysis of housekeeping genes. *Applied and environmental microbiology*, **75**, 5410-16.

Margos, G., Hojgaard, A., Lane, R., Cornet, M., Fingerle, V., Rudenko, N., Ogden, N., Aanensen, D., Fish, D. and Piesman, J. (2010). Multilocus sequence analysis of Borrelia bissettii strains from North America reveals a new Borrelia species, Borrelia kurtenbachii. *Ticks and Tick-borne Diseases*, **1**, 151-158.

Matijatko, V., Torti, M. and Schetters, T. (2012). Canine babesiosis in Europe: how many diseases? *Trends in Parasitology*, **28**, 99-105.

Medlock, J., Hansford, K., Bormane, A., Derdakova, M., Estrada-Peña, A., George, J., Golovljova, I., Jaenson, T., Jensen, J., Jensen, P., Kazimirova, M., Oteo, J., Papa, A., Pfister, K., Plantard, O., Randolph, S., Rizzoli, A., Santos-Silva, M., Sprong, H., Vial, L., Hendrickx, G., Zeller, H. and Van Bortel, W. (2013). Driving forces for changes in geographical distribution of Ixodes ricinus ticks in Europe. *Parasites & Vectors*, **6**, 1

Millins, C., Gilbert, L., Johnson, P., James, M., Kilbride, E., Birtles, R. and Biek, R. (2016). Heterogeneity in the abundance and distribution of Ixodes ricinus and Borrelia burgdorferi (sensu lato) in Scotland: implications for risk prediction. *Parasites & Vectors*, **9**, 595.

Raoult, D. and Roux, V. (1997). Rickettsioses as paradigms of new or emerging infectious diseases. *Clinical Microbiology Reviews*, **10**, 694-719.

Rauter, C. and Hartung, T. (2005). Prevalence of Borrelia burgdorferi Sensu Lato Genospecies in Ixodes ricinus Ticks in Europe: a Metaanalysis. *Applied and Environmental Microbiology*, **71**, 7203-7216.

Richter, D. (2006). Delineation of Borrelia burgdorferi sensu lato species by multilocus sequence analysis and confirmation of the delineation of Borrelia spielmanii sp. nov. *International Journal of Systematic and Evolutionary Microbiology*, **56**, 873-881.

Sánchez-Vizcaíno, F., Wardeh, M., Heayns, B., Singleton, D., Tulloch, J., McGinley, L., Newman, J., Noble, P., Day, M., Jones, P. and Radford, A. (2016). Canine babesiosis and tick activity monitored using companion animal electronic health records in the UK. *Veterinary Record*, **179**, 358-358.

Scharlemann, J.P.W., Johnson, P.J., Smith, A.A., Macdonald, D.W. and Randolph, S.E. (2008). Trends in ixodid tick abundance and distribution in Great Britain. *Medical and Veterinary Entomology,* **22**, 238–247.

Shaw, S. E., Lerga, A. I., Williams, S., Beugnet, F., Birtles, R. J., Day, M. J. & Kenny, M. J. (2003). Review of exotic infectious diseases in small animals entering the United Kingdom from abroad diagnosed by PCR. *Veterinary Record*, **152**, 176-177

Smith, F. and Wall, L. (2013). Prevalence of Babesia and Anaplasma in ticks infesting dogs in Great Britain. *Veterinary Parasitology*, **198**, 18-23.

Smith, F., Ballantyne, R., Morgan, E. and Wall, R. (2012). Estimating Lyme disease risk using pet dogs as sentinels. *Comparative Immunology, Microbiology and Infectious Diseases*, **35**, 163-167.

Solano-Gallego, L. and Baneth, G. (2011). Babesiosis in dogs and cats—Expanding parasitological and clinical spectra. *Veterinary Parasitology*, **181**, 48-60.

Stanek, G., Reiter, M. (2011). The expanding Lyme borrelia complex – clinical significance of genomic species? *Clinical Microbiology and Infection*, **17**, 487.

Swainsbury, C., Bengtson, G. and Hill, P. (2016). Babesiosis in dogs. *Veterinary Record*, **178**, 171-172.

Swanson, S., Neitzel, D., Reed, K. and Belongia, E. (2006). Coinfections Acquired from Ixodes Ticks. *Clinical Microbiology Reviews*, **19**, 708-727.

Telford, S.R., Gorenflot, A., Brasseur, P., Spielman, A. (1993) Babesial infections in humans and wildlife, p. 1–47. In J. P. Kreier (ed.), *Parasitic protozoa*. 2nd ed., vol. 5. Academic Press, Inc., New York, N.Y.

Tinoco-Gracia, L., Quiroz-Romero, H., Quintero-Martinez, M.T., Renteria-Evangelista, T.B., Barreras-Serrano, A., Hori-Oshima, S., Medina-Basulto, G., Vinasco, J., Moro, M.H. (2008) Prevalence and Risk Factors for Borrelia burgdorferi Infection in Mexicali, Baja California, a Mexico-US Border City. *The International Journal of Applied Research in Veterinary Medicine*, **6**, 161-165

Toutoungi, L.N. and Gern, L. (1993). Ability of transovarially and subsequent transstadially infected Ixodes hexagonus ticks to maintain and transmit Borrelia burgdorferi in the laboratory. *Experimental and Applied Acarology*, **17**, 581–586.

581 Table 1. The number of ticks analysed in the study, the number positive for *B. burgdorferi*
582 (s.l.) and *Babesia* spp. and percentage prevalences with exact binomial 95% confidence
583 intervals for different regions of the UK.
584
585

Region	Total number of tick samples	No. of ticks positive for *B. burgdorferi* (s.l.)	Prevalence (%)	95 % confidence interval	No. of ticks positive for *Babesia* spp.	Prevalence (%)	95 % confidence interval
Scotland – Highlands	266	8	3.0	0.021	5	1.9	0.016
Scotland – Lowlands	400	9	2.3	0.015	2	0.5	0.007
Wales	201	6	3.0	0.024	3	1.5	0.017
England – Southwest	1581	35	2.2	0.007	26	1.6	0.006
England – Southeast	942	13	1.3	0.007	11	1.2	0.007
England – Central	373	4	1.1	0.011	9	2.4	0.016
England – Northern	684	12	1.8	0.010	11	1.6	0.009
East Anglia	266	7	2.6	0.019	3	1.1	0.013
Unknown	24	0	0.0	0.000	0	0.0	0.000
Total	**4,737**	**94**	**2.0**	**0.004**	**70**	**1.5**	**0.003**

586

587

588 Table 2. The number and species of tick, life-cycle stage, *Borrelia burgdorferi* (s.l.) species
589 identified on partial *ospA* gene sequencing and sequence identity with matching GenBank
590 accession numbers for the analysed ticks.
591

Number of ticks	Tick species	Tick life-cycle stage	Species detected	Sequence identity (%)	Accession number
2	*I. ricinus*	Partially-fed adult	*B. afzelii*	98	AB253532
6	*I. ricinus*	Partially-fed adult	*B. afzelii*	97-99	CP002950
2	*I. ricinus*	Unfed adult	*B. afzelii*	97-98	CP002950
1	*I. ricinus*	Fed nymph	*B. afzelii*	98	CP002950
2	*I. hexagonus*	Partially-fed adult	*B. afzelii*	98-99	CP002950
1	*I. ricinus*	Partially-fed adult	*B. afzelii*	99	CP009059
1	*I. ricinus*	Partially-fed adult	*B. afzelii*	99	DQ007300
3	*I. ricinus*	Partially-fed adult	*B. afzelii*	100	DQ007302
1	*I. hexagonus*	Partially-fed adult	*B. afzelii*	99	DQ007302
10	*I. ricinus*	Partially-fed adult	*B. afzelii*	99-100	DQ007303
1	*I. ricinus*	Partially fed adult	*B. afzelii*	99	KT934527
5	*I. ricinus*	Partially-fed adult	*B. burgdorferi* s.s.	97-99	CP009657
2	*I. ricinus*	Unfed adult	*B. burgdorferi* s.s.	98-99	CP009657
8	*I. ricinus*	Unfed adult	*B. burgdorferi* s.s.	97-100	DQ193525
5	*I. ricinus*	Partially fed adult	*B. burgdorferi* s.s.	97-100	DQ193525
1	*I. ricinus*	Partially-fed adult	*B. burgdorferi* s.s.	98	JF262959
1	*I. ricinus*	Partially-fed adult	*B. burgdorferi* s.s.	98	KC954743
2	*I. ricinus*	Partially-fed adult	*B. burgdorferi* s.s.	99	X95361
4	*I. ricinus*	Partially-fed adult	*B. garinii*	98-99	DQ155629
1	*I. ricinus*	Partially-fed adult	*B. garinii*	99	JF331336
5	*I. ricinus*	Partially-fed adult	*B. garinii*	98-100	JF331345
2	*I. ricinus*	Fully-fed adult	*B. garinii*	97-99	JF331345
1	*I. ricinus*	Unfed adult	*B. garinii*	97	JF331345
1	*I. ricinus*	Fed nymph	*B. garinii*	99	JF331345
1	*I. ricinus*	Unfed adult	*B. garinii*	99	JF331346
2	*I. ricinus*	Partially-fed adult	*B. garinii*	99	JF331361
3	*I. ricinus*	Partially-fed adult	*B. garinii*	98-99	JF331369
2	*I. ricinus*	Unfed adult	*B. garinii*	98-99	JF331369
13	*I. ricinus*	Partially fed adult	*B. garinii*	97-99	JF331376
1	*I. ricinus*	Fully-fed adult	*B. garinii*	99	JF331376
1	*I. ricinus*	Partially-fed adult	*B. garinii*	99	KT963821
1	*I. ricinus*	Partially-fed adult	*B. garinii*	98	X95354
1	*I. ricinus*	Partially-fed adult	*B. garinii*	98	X95362
1	*I. ricinus*	Partially-fed adult	*B. spielmanii*	98	CP001469
1	*R. sanguineus**	Fully-fed adult	*B. garinii*	98	JF331361

592 *Tick found on a dog with recent travel history outside the UK
593

594

595 Table 3. The number and species of tick, life-cycle stage, *Babesia* spp. identified on partial 18S
596 rRNA gene sequencing and sequence identity with matching GenBank accession numbers for the
597 analysed ticks.
598

No. of Ticks	Tick Species	Tick Stage	Pathogen detected	Sequence homology (%)	Accession Number
45	*I. ricinus**	Partially-fed Adult	*B. venatorum*	97-100	KM289158
8	*I. ricinus*	Fully-fed Adult	*B. venatorum*	98-100	KM289158
3	*I. ricinus***	Unfed Adult	*B. venatorum*	99	KM289158
1	*I. canisuga*	Partially-fed Adult	*B. venatorum*	99	KM289158
2	*I. hexagonus*	Partially-fed Adult	*B. venatorum*	99	KM289158
2	*I. ricinus*	Partially-fed Adult	*B. vulpes* sp. nov.	98-99	FJ225390 KT223483 KT580785 KM116004
1	*I. hexagonus*	Partially-fed Adult	*B. vulpes* sp. nov.	99/99/99/99	FJ225390 KT223483 KT580785 KM116004
1	*I. ricinus*	Partially-fed Adult	*B. divergens/B. capreoli*	100	KM657258 KM657250
1	*D. reticulatus*	Fully-fed Adult	*B. canis*	99	KT008057 HQ662634 AY072926
2	*I. hexagonus*	Partially-fed Adult	*B. vulpes* sp. nov.	98-99	FJ225390 KT223483 KT580785 KM116004
1	*I. ricinus*	Partially-fed Adult	*B. vulpes* sp. nov.	98	FJ225390 KT223483 KT580785 KM116004
1	*I. ricinus*	Fully-fed Adult	*B. vulpes* sp. nov.	100	KT223483 LC127372
1	*I. ricinus*	Partially-fed	*B. divergens/B. capreoli*	97	KT279879 KM657258
1	*I. ricinus*	Fully-fed Adult	*B. microti*	99	LC127372

599 * Two of these ticks had coinfection with *B. garinii*
600 ** One of these ticks had coinfection with *B. garinii*
601
602
603

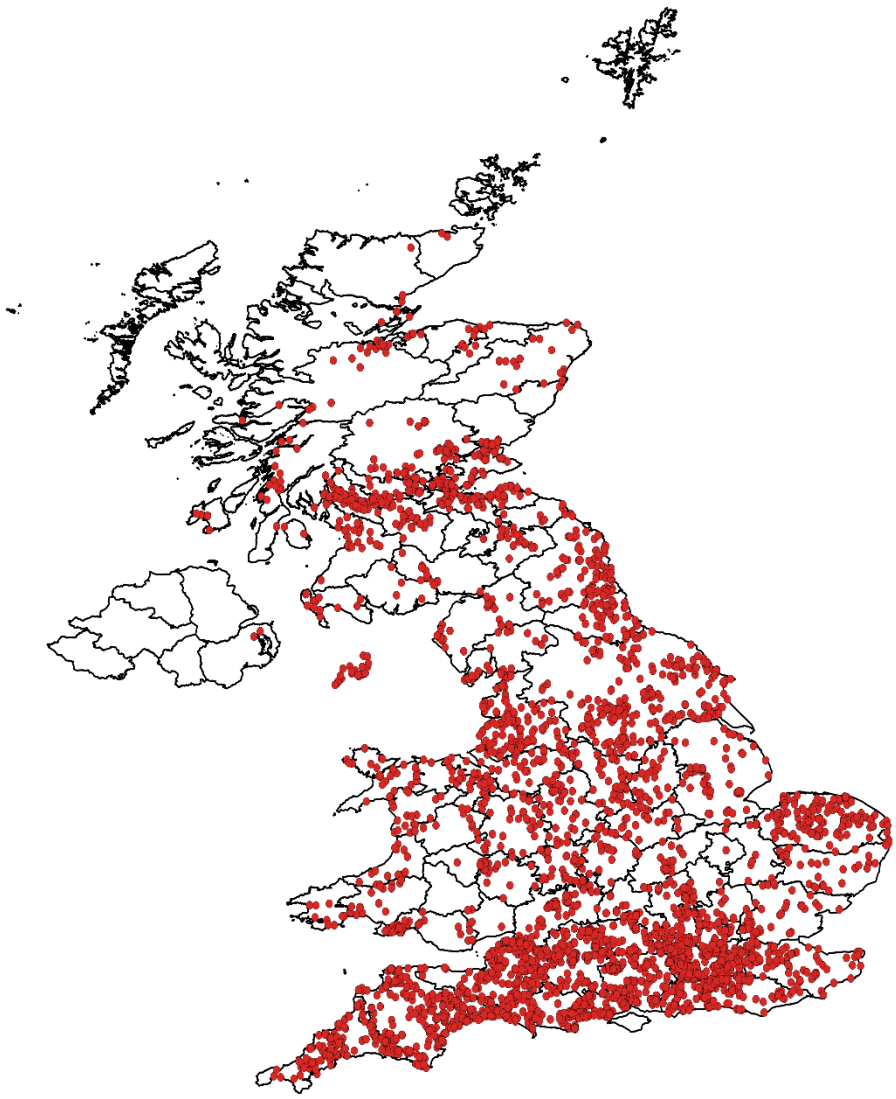
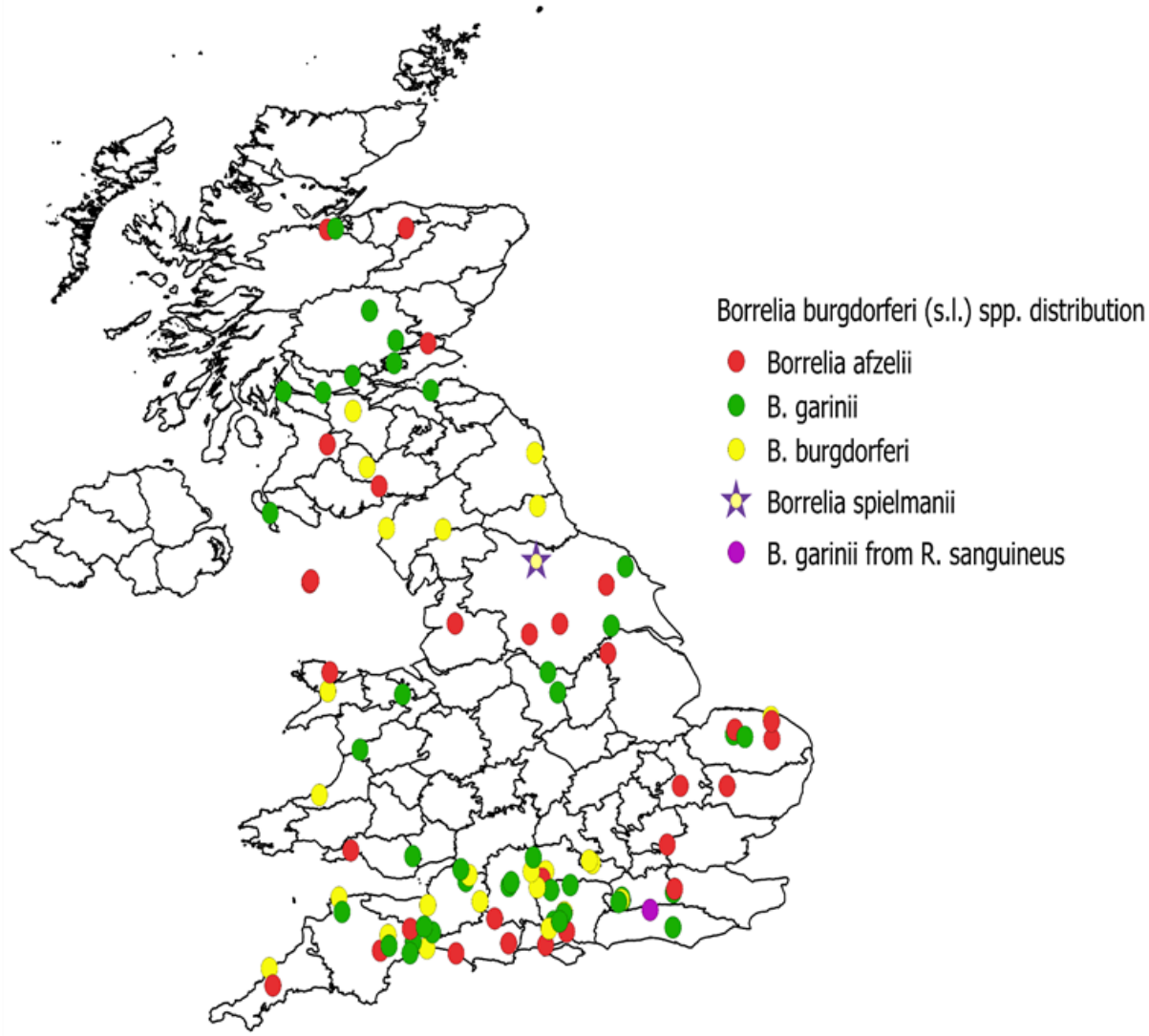


Fig 1. The distribution of tick samples (each dot represents a sample location) submitted by veterinary practices in the UK.



628

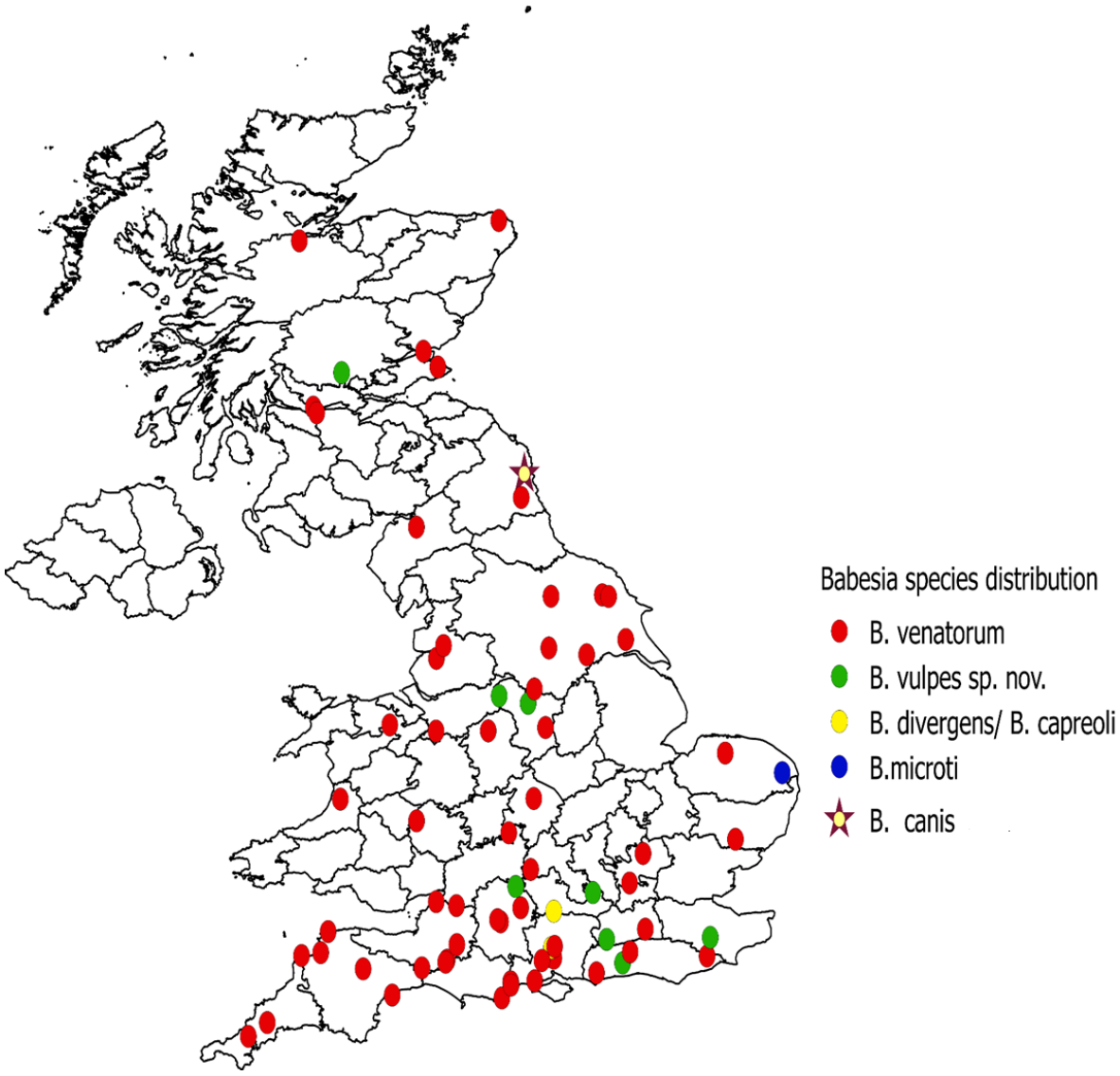
629

630 Fig 2. The distribution of *Borrelia burgdorferi* (s.l.) species detected in ticks collected from

631 dogs in the UK.

632

633



634

635

636 Fig. 3. The distribution of *Babesia* spp. detected in ticks collected from dogs in the UK.

637