Prevalence and distribution of Borrelia and Babesia species in ticks feeding on dogs in the U.K.

S. ABDULLAH 1, C. HELPS 2, S. TASKER 2, H. NEWBURY 3 and R. WALL 1

1Veterinary Parasitology and Ecology Group, School of Biological Sciences, University of Bristol, Bristol, U.K., 2Molecular Diagnostic Unit, Langford Vets and School of Veterinary Sciences, University of Bristol, Bristol, U.K. and 3MSD Animal Health, Milton Keynes, U.K.

Abstract. Ticks were collected during March–July 2015 from dogs by veterinarians throughout the U.K. and used to estimate current prevalences and distributions of pathogens. DNA was extracted from 4750 ticks and subjected to polymerase chain reaction and sequence analysis to identify Borrelia burgdorferi sensu lato (Spirochaetales: Spirochaetaceae) and Babesia (Piroplasmida: Babesiidae) species. Of 4737 ticks [predominantly Ixodes ricinus Linneaus (Ixodida: Ixodidae)], B. burgdorferi s.l. was detected in 94 (2.0%). Four Borrelia genospecies were identified: Borrelia garinii (41.5%); Borrelia afzelii (31.9%); Borrelia burgdorferi sensu stricto (25.5%), and Borrelia spielmanii (1.1%). One Rhipicephalus sanguineus Latreille (Ixodida: Ixodidae), collected from a dog with a history of travel outside the U.K., was positive for B. garinii. Seventy ticks (1.5%) were positive for Babesia spp. Of these, 84.3% were positive for Babesia venatorum, 10.0% for Babesia vulpes sp. nov., 2.9% for Babesia divergens/Babesia capreoli and 1.4% for Babesia microti. One isolate of Babesia canis was detected in a Dermacentor reticulatus (Ixodida: Ixodidae) tick collected from a dog that had recently travelled to France. Prevalences of B. burgdorferi s.l. and Babesia spp. did not differ significantly between different regions of the U.K. The results map the widespread distribution of B. burgdorferi s.l. and Babesia spp. in ticks in the U.K. and highlight the potential for the introduction and establishment of exotic ticks and tick-borne pathogens.

Key words. Babesia, Borrelia, Dermacentor, Ixodes, Rhipicephalus, disease, pathogen, vector.

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 distributions and prevalences of ticks and tick-borne pathogens are therefore of particular interest (Gray, 2008; Beugnet & Marié, 2009; Hansford et al., 2016a) and may be expedited by changes in climate, and increases in host populations and levels of animal movement (Hansford et al., 2016b). Two of the tick-borne pathogens of particular interest in this context in the U.K. 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 Ixodes hexagonus (Toutoungi & Gern, 1993). Nine pathogenic species of B. burgdorferi s.l. are described in Europe, including B. burgdorferi s.s., Borrelia garinii, Borrelia afzelii, Borrelia valaisiana, Borrelia lusitaniae, Borrelia spielmanii, Borrelia kurtzibachii, Borrelia bisetii and Borrelia bavariensis (Rauter & Hartung, 2005; Margos et al., 2010). Four
genospecies have been recently reported in Scotland, including \( B. \) \( afzelii \), \( B. \) \( garinii \), \( B. \) \( burgdorferi \) 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 and transovarial transmission appears to play only a minor role in the epidemiology of this pathogen (Nefedova et al., 2004). Lyme disease has serious consequences in humans. Reported human cases of Lyme disease increased 30-fold between 1999 and 2008 in Scotland (Health Protection Scotland, 2009). In dogs, a recent U.K. 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 et al., 2010) and therefore the prevalence of clinical Lyme disease in dogs represents a significant underestimation of the risk for 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 babesiosis. Four \( Babesia \) species are known to affect dogs; these include \( Babesia \) \( canis \), \( Babesia \) \( vogeli \), \( Babesia \) \( gibsoni \) and \( Babesia \) \( vulpes \) sp. nov. (the last of these was 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 manifestations range from 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 splenectomized or otherwise immunocompromised, and \( Babesia \) \( divergens \), 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 infected hosts, although the transovarial transmission of \( B. \) \( canis \) 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 that of its vector \( Dermacentor \) \( reticulatus \) (Földvári 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 U.K. for over 100 years (https://species.nbnatlas.org/species/NBN0SY0000039960) in relatively small, isolated populations. However, at least four established, predominantly coastal, populations have been recently confirmed (Jameson & Medlock, 2011). In the U.K., the number of cases of babesiosis in dogs imported from abroad has increased (Shaw et al., 2003). The first case of fatal babesiosis in a dog that had not left the U.K. was diagnosed in Kent and the causal agent was tentatively identified as \( B. \) \( vogeli \) (Holm et al., 2006). Subsequently, a cluster of cases of \( B. \) \( canis \) involving dogs in Essex with no history of foreign travel was reported (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 U.K. laboratories showed that 13 of 99 submissions in 2015 were positive for \( Babesia \) spp. and were considered to have come from dogs returning after travel outside the U.K. (Sánchez-Vizcaíno et al., 2016). In the first 3 months of 2016, 11 of 67 submissions were positive, indicating a sudden increase in cases and the geographical clustering of eight cases in Essex (Sánchez-Vizcaíno et al., 2016).

A novel zoonotic babesia, \( Babesia \) \( venatorum \) (Herwaldt et al., 2003), has been recorded in U.K. 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 U.K.; however, there have been reports of infestations in domestic properties in the U.K. of \( R. \) \( sanguineus \) thought to have been introduced by the import of dogs that were unprotected against ticks (Hansford et al., 2015a).

The evidence suggests that the distributions and prevalences of \( Borrelia \) spp. and \( Babesia \) spp. pathogens within the U.K. are currently highly labile and closer surveillance is therefore warranted. However, the relatively low prevalences and highly uneven geographical distributions 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 prevalences of \( Babesia \) spp. and \( Borrelia \) spp. in ticks collected from dogs presented to veterinary practices participating in a U.K.-wide national tick surveillance programme (Abdullah et al., 2016).

Materials and methods

Sample collection and DNA extraction

A national survey of ticks collected from dogs in the U.K. was undertaken in 2015, during which veterinary practices were asked to examine five dogs for ticks each week for 8 weeks following a previously described protocol (Abdullah et al., 2016). Ticks collected were submitted for identification and pathogen testing.

Each tick received by the investigators was given a unique identification number and stored at −20 °C pending analysis. Subsequently, ticks were identified to species, lifecycle 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 had not yet reached maximum expansion, and unfed ticks contained no blood. Each tick was cut longitudinally and transversely before DNA extraction. DNA extraction from ticks was performed using two commercially available extraction kits. Both extraction methods were compared using spectrophotometry (Nanodrop; Thermo Fisher Scientific, Inc., Waltham, MA, U.S.A.) and agarose gel electrophoresis and both were found to give a similar range of sample DNA concentrations. In the first 1600 tick samples, the DNeasy Blood & Tissue Kit (Qiagen GmbH, Hilden, Germany) was used. In the remaining samples, the high-throughput NucleoSpin® 96 Tissue Core Kit (Macherey-Nagel GmbH & Co. KG, Düren, Germany) was used. Both kits were used
following the respective manufacturer’s instructions. In assays involving unfed and partially fed ticks, whole ticks were used and the volume of reagent used for extraction followed the kit protocol. However, in assays involving fully fed ticks that contained large volumes of clotted blood, use of 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 and prevented the completion of extraction. To overcome this problem, only the anterior two-thirds of the fully engorged tick (containing the salivary glands) were used for extraction in an extraction protocol using 40 μL of Proteinase-K (instead of 30 μL) and 400 μL of tissue lysis buffer (instead of 240 μL) and in which samples were incubated at 56°C overnight. After overnight digestion, only half the lysate was transferred to spin columns (again, use of all the lysate created problems in the silicone columns). Two washes of wash buffer were applied to each column to clean them 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) quantitative polymerase chain reaction (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 set-up and assay inhibition. All ticks, except those that were 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′-AATAGGTTCAATAATAGCC TTAATAGC-3′) and BSLR (5′-CTAGTTTTGGCCATCTTCT TTGAAAA-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 Corp., Southampton, U.K.), 0.4 μL of 12.5 μM each of BSLF/BSLR primer mix and 2.6 μL water. Two microlitres 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 PS073; Eppendorf Ltd, Stevenage, U.K.). 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 ultraviolet 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 (NucleoSpin® 96 PCR Clean-up Core Kit; Macherey-Nagel GmbH & Co. KG) and sent for commercial DNA sequencing [Medical Research Council Protein Phosphorylation and Ubiquitylation Unit (MRC PPU), College of Life Sciences, University of Dundee, Dundee, U.K.] using Applied Biosystems Big Dye Version 3.1 chemistry on a model 3730 automated capillary DNA sequencer (Applied Biosystems, Inc., Foster City, CA, U.S.A.). Only forward sequencing was undertaken. Sequences were checked and edited, if necessary, using BioEdit Sequence Alignment Editor Version 7.2.5 (http://www.mbio.ncsu.edu/BioEdit/bioedit.html), and then compared with sequence data available in GenBank using BLASTn (http://www.ncbi.nlm.nih.gov/BLAST). Any sequences with homology of <97% 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 forward (5′-TTAAAGAAAAGGGACCTT AACCTG-3′), Babesia 1315 reverse (5′-CCGAAATACTACC GGATCAC-3′) and Babesia TaqMan probe (5′-FAM-CGATC GGTAGAGGCGAGCCG-GHG-3′) (Diagnostic Laboratories, Langford Vets, Bristol, U.K.). A primer/probe mix was made with 10 μm Babesia 944 forward, 10 μm Babesia 1315 reverse and 2.5 μm Babesia TaqMan probe. Positive (B. canis, 12,763 PCR product diluted at 10−11) 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 MgCl2 and 2 μL of water. 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 Technologies UK Ltd, Edinburgh, U.K.). 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-reactions (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-squared analysis (IBM spss Statistics for Windows Version 23.0; IBM Corp., Armonk, NY, U.S.A.) was used to compare Borrelia and Babesia regional prevalences using a system in which the U.K. was divided into eight geographic regions (Table 1). Distributions of tick samples and pathogens were mapped using qgis Version 2.8.1 (http://www.qgis.org/en/site/fusers/download.html) according to the dog owners’ postcodes.

Results

A total of 4750 ticks were analysed. Among these, 4737 were collected from dogs resident within the U.K. and 13 were collected from dogs that had been abroad. All but eight of the tick samples were adult females at various stages of engorgement, representing a wide geographic spread across the U.K. (Fig. 1). The numbers of each tick species collected from U.K.-resident dogs and included in the pathogen analysis were:
Table 1. Numbers of ticks analysed in the study, numbers positive for *Borrelia burgdorferi* s.l. and *Babesia* spp. and percentage prevalences with exact binomial 95% confidence intervals (CI) for different regions of the U.K.

| Region                | Total tick samples, n | Ticks positive for *B. burgdorferi* s.l., n | Prevalence of *B. burgdorferi* s.l., % | 95% CI  | Ticks positive for *Babesia* spp., n | Prevalence of *Babesia* spp., % | 95% CI  |
|-----------------------|-----------------------|---------------------------------------------|--------------------------------------|---------|-------------------------------------|---------------------------------|---------|
| 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: north        | 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%                                   | 0.000   | 0                                   | 0%                              | 0.000   |
| **Total**             | **4737**              | **94**                                      | **2.0%**                             | **0.004**| **70**                              | **1.5%**                         | **0.003**|

Fig. 1. Distribution of tick samples in the U.K. (each dot represents a sample location) submitted by veterinary practices and analysed for pathogens. [Colour figure can be viewed at wileyonlinelibrary.com].

4316 (91.1%) *I. ricinus*; 386 (8.1%) *I. hexagonus*; 23 (0.5%) *Ixodes canisuga*; nine (0.2%) *D. reticulatus*, and three (0.06%) *Haemaphysalis punctata* (Ixodida: Ixodidae). All of the eight nymphs were *I. ricinus*. The ticks on travelled dogs included one *D. reticulatus* and 12 *R. sanguineus*.

Fig. 2. Distribution of *Borrelia burgdorferi* s.l. species detected in ticks collected from dogs in the U.K. [Colour figure can be viewed at wileyonlinelibrary.com].

*Borrelia* distribution and prevalence

*Borrelia* ospA PCR and subsequent DNA sequencing showed that 94 of the 4737 tick samples from resident dogs (2.0%) contained *B. burgdorferi* s.l. DNA; these included 91 *I. ricinus* and three *I. hexagonus* samples. One *R. sanguineus* collected from a dog with a history of recent travel outside the U.K. was also found to be positive. Two of the 94 positive ticks were nymphs and the rest were adult females and included 72 partially fed, 16 unfed and three fully fed specimens. Prevalences of *B. burgdorferi* s.l. were 2.1% in *I. ricinus* and 0.8% in *I. hexagonus*. All other ticks tested were negative. *Borrelia* were found at sites throughout the U.K. (Fig. 2), broadly mirroring the distribution of tick samples submitted. Regional prevalences of *Borrelia* spp. ranged from 1.1% to 3.0% (Table 1) and showed no significant differences between
U.K. regions ($\chi^2 = 6.98$, d.f. = 7, $P = 0.43$). Sequence analysis of the 94 samples positive for \emph{B. burgdorferi s.l.} detected four genospecies, including 39 \emph{B. garinii} (41.5%), 30 \emph{B. afzelii} (31.9%), 24 \emph{B. burgdorferi s.s.} (25.5%) and one \emph{B. spielmanii} (1.1%). The one infected \emph{R. sanguineus} was found to be infected with \emph{B. garinii} (Table 2).

**Babesia distribution and prevalence**

The generic \emph{Babesia} spp. qPCR and subsequent DNA sequencing indicated that 70 of 4737 (1.5%) ticks collected from dogs contained \emph{Babesia} spp. DNA. All four tick species were found to be infected with \emph{Babesia} spp.; of the 70 positive samples, 62 (88.6%) were \emph{I. ricinus}, six (8.6%) were \emph{I. hexagonus}, one (1.4%) was \emph{I. canisuga} and one (1.4%) was \emph{D. reticulatus} found on a dog with a recent history of travel outside the U.K. No nymphs were positive for \emph{Babesia} spp.; all positive ticks were adult females and included 56 partially fed, three unfed and 11 fully fed specimens. \emph{Babesia} spp. were also widely distributed throughout the U.K., 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 did not differ significantly ($\chi^2 = 6.26$, d.f. = 7, $P = 0.51$). Of the 70 \emph{Babesia}-positive samples, 59 (84.3%) were positive for \emph{B. venatorum}, seven (10.0%) for \emph{B. vulpes} sp. nov., two (2.9%) for \emph{B. divergens/Babesia capreoli}, one (1.4%) for \emph{B. microti} and one (1.4%) for \emph{B. canis}. The \emph{B. canis} DNA was detected in an adult fully fed \emph{D. reticulatus} tick collected from a dog that had recently returned from France (Table 3). \emph{Borrelia} spp. and \emph{Babesia} spp. co-infections (\emph{B. garinii} and \emph{B. venatorum} in every case) were detected in three ticks, including two partially fed female \emph{I. ricinus} and one unfed female \emph{I. ricinus} (Table 3).

**Discussion**

In the present study, a large sample of ticks collected from dogs from all regions of the U.K. were tested and found to be infected at prevalences of 2.0% for \emph{B. burgdorferi s.l.} and 1.5%

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**Table 2.** Numbers and species of tick, lifecycle stage, \emph{Borrelia burgdorferi s.l.} species identified on partial \emph{ospA} gene sequencing and sequence identity with matching GenBank accession numbers for the analysed ticks.

| Ticks, $n$ | Tick species | Tick lifecycle stage | Borrelia species detected | Sequence homology, % | Accession no. |
|-----------|--------------|----------------------|---------------------------|----------------------|--------------|
| 2         | \emph{Ixodes ricinus} | Partially fed adult | \emph{B. afzelii} | 98% | AB253532 |
| 6         | \emph{Ixodes ricinus} | Partially fed adult | \emph{B. afzelii} | 97–99% | CP002950 |
| 2         | \emph{Ixodes ricinus} | Unfed adult | \emph{B. afzelii} | 97–98% | CP002950 |
| 1         | \emph{Ixodes ricinus} | Fed nymph | \emph{B. afzelii} | 99% | CP009059 |
| 2         | \emph{Ixodes hexagonus} | Partially fed adult | \emph{B. afzelii} | 98–99% | CP002950 |
| 1         | \emph{Ixodes ricinus} | Partially fed adult | \emph{B. afzelii} | 99% | CP009059 |
| 3         | \emph{Ixodes ricinus} | Partially fed adult | \emph{B. afzelii} | 100% | DQ007300 |
| 1         | \emph{Ixodes hexagonus} | Partially fed adult | \emph{B. afzelii} | 99% | DQ007302 |
| 10        | \emph{Ixodes ricinus} | Partially fed adult | \emph{B. afzelii} | 99–100% | DQ007303 |
| 1         | \emph{Ixodes ricinus} | Partially fed adult | \emph{B. burgdorferi s.s.} | 97–99% | CP009657 |
| 2         | \emph{Ixodes ricinus} | Unfed adult | \emph{B. burgdorferi s.s.} | 98–99% | CP009657 |
| 8         | \emph{Ixodes ricinus} | Unfed adult | \emph{B. burgdorferi s.s.} | 97–100% | DQ193525 |
| 5         | \emph{Ixodes ricinus} | Partially fed adult | \emph{B. burgdorferi s.s.} | 97–100% | DQ193525 |
| 1         | \emph{Ixodes ricinus} | Partially fed adult | \emph{B. burgdorferi s.s.} | 98% | JF262959 |
| 1         | \emph{Ixodes ricinus} | Partially fed adult | \emph{B. burgdorferi s.s.} | 98% | KC547473 |
| 2         | \emph{Ixodes ricinus} | Partially fed adult | \emph{B. burgdorferi s.s.} | 99% | X95361 |
| 4         | \emph{Ixodes ricinus} | Partially fed adult | \emph{B. garinii} | 98–99% | DQ135629 |
| 1         | \emph{Ixodes ricinus} | Partially fed adult | \emph{B. garinii} | 99% | JF331336 |
| 5         | \emph{Ixodes ricinus} | Partially fed adult | \emph{B. garinii} | 98–100% | JF331345 |
| 2         | \emph{Ixodes ricinus} | Fully fed adult | \emph{B. garinii} | 97–99% | JF331345 |
| 1         | \emph{Ixodes ricinus} | Unfed adult | \emph{B. garinii} | 97% | JF331345 |
| 1         | \emph{Ixodes ricinus} | Fed nymph | \emph{B. garinii} | 99% | JF331345 |
| 1         | \emph{Ixodes ricinus} | Unfed adult | \emph{B. garinii} | 99% | JF331346 |
| 2         | \emph{Ixodes ricinus} | Partially fed adult | \emph{B. garinii} | 99% | JF331361 |
| 3         | \emph{Ixodes ricinus} | Partially fed adult | \emph{B. garinii} | 98–99% | JF331369 |
| 2         | \emph{Ixodes ricinus} | Unfed adult | \emph{B. garinii} | 98–99% | JF331369 |
| 13        | \emph{Ixodes ricinus} | Partially fed adult | \emph{B. garinii} | 97–99% | JF331376 |
| 1         | \emph{Ixodes ricinus} | Fully fed adult | \emph{B. garinii} | 99% | JF331376 |
| 1         | \emph{Ixodes ricinus} | Partially fed adult | \emph{B. garinii} | 99% | KT619321 |
| 1         | \emph{Ixodes ricinus} | Partially fed adult | \emph{B. garinii} | 98% | X95354 |
| 1         | \emph{Ixodes ricinus} | Partially fed adult | \emph{B. garinii} | 98% | X95362 |
| 1         | \emph{Ixodes ricinus} | Partially fed adult | \emph{B. spielmanii} | 98% | CP001469 |
| 1         | \emph{Rhipicephalus sanguineus*} | Fully fed adult | \emph{B. garinii} | 98% | JF331361 |

*Tick found on a dog with recent travel history outside the U.K.*

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from various regions in England, most of which were collected from woodland, woodland edges or moorlands. Of a total of 954 ticks examined for B. burgdorferi s.l., 40 were found to be positive in PCR, giving a prevalence of 4.2%, but the authors were able to sequence and speciate only 24 samples, giving a prevalence of 2.5% (Hansford et al., 2015b). They also reported considerable variation (0–13.6%) in prevalence depending on the region of collection. Hansford et al. (2016c) sampled known hotspots for Lyme borreliosis in the U.K. and reported a prevalence of 18% in questing ticks (predominantly nymphs), but this was derived from very small sample sizes in many locations. A smaller-scale U.K. survey of ticks feeding on dogs, which used a sample methodology identical to that 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 U.K. ticks is considerably lower than prevalences reported from continental Europe, which range from 14% to 49% (Rauter & Hartung, 2005), although it may be higher in specific sites and habitats. The reasons for the generally lower prevalence in the U.K. are not known, but continued ongoing surveillance to monitor any future changes in the prevalence of this zoonotic spirochete would be prudent.

Borrelia burgdorferi s.l. comprises 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, 2011)]. Four different genospecies were detected in the current study, three of which were found to have fairly similar prevalences [B. garinii (41.5%), B. afzelii (31.9%) and B. burgdorferi s.s. (25.5%)], whereas only one case of B. spielmanii was detected. These relative prevalences are similar to those cited in previous reports (Rauter & Hartung, 2005; Estrada-Pena et al., 2011), in which meta-analysis found that B. afzelii and B. garinii were the most prevalent B. burgdorferi s.l. species in central Europe, followed by B. burgdorferi s.s. Different B. burgdorferi s.l. species are sustained by diverse transmission cycles involving different vertebrate host species but the same tick vectors (Margos et al., 2009). For example, B. garinii has been reported more commonly in birds, whereas B. afzelii circulates predominantly in rodent populations (Kurtenbach et al., 2002), which indicates that ticks feeding on dogs have fed previously on a variety of host species. The detection of B. spielmanii is the first record for the U.K. from an I. ricinus tick infesting a domestic dog that had not travelled recently. This Borrelia species is usually associated with rodents, especially dormice (Richter, 2006), and has been reported to cause erythema migrans and Lyme disease in humans (Maraspín et al., 2006). The detection of B. garinii in R. sanguineus in this study is of interest because the vector competency of this tick species for B. burgdorferi s.l. has not been confirmed, but tick samples collected in three sites in southern England and one in Wales were 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 4737 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 have not yet been identified and characterized (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, in which other endocellular symbionts would not be found. Thus, PCR and DNA sequence analyses of amplicons are necessary to accurately identify *Babesia* species and to avoid false positive results (Hildebrandt et al., 2013).

Sequence analysis of the 70 *Babesia*-positive amplicons found that 59 (84.3%) represented *B. venatorum*, seven (10%) represented *B. vulpes* sp. nov., two (2.9%) represented *B. divergens*/*B. capreoli* and one (1.4%) represented *B. microti*. The speciation of *Babesia* protozoa is complex and the pathogenicity of identified species is uncertain. Ten *Babesia* pathogens categorized as *B. vulpes* sp. nov. were detected and were also matched by BLAST on the National Center for Biotechnology Information (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) categorized 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. Differentiation between *B. divergens* and *B. capreoli* is made difficult by their morphological similarities and is further complicated by the high percentage of identity between their respective 18S rRNA gene sequences. *Babesia divergens* and *B. capreoli* have very few intra-specific differences in their 18S rRNA, which shows 99.83% identity, with differences only at positions 631, 633 and 1637 (Malandrin et al., 2010). The positions of primers and amplicon lengths used in this study did not allow the differentiation of these two species. Another similar pathogen is *Babesia odocoilei*. It infects white-tailed deer, elk and caribou in the U.S.A., 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

| Table 3. Numbers and species of tick, lifecycle stage, *Babesia* spp. identified on partial 18S rRNA gene sequencing and sequence identity with matching GenBank accession numbers for the analysed ticks. |
|---|---|---|---|---|---|
| **Ticks, n** | **Tick species** | **Tick stage** | **Babesia species detected** | **Sequence homology, %** | **Accession no.** |
| 45 | *Ixodes ricinus* | Partially fed adult | *B. venatorum* | 97–100% | KM289158 |
| 8 | *Ixodes ricinus* | Fully fed adult | *B. venatorum* | 98–100% | KM289158 |
| 3 | *Ixodes ricinus* | Unfed adult | *B. venatorum* | 99% | KM289158 |
| 1 | *Ixodes canisuga* | Partially fed adult | *B. venatorum* | 99% | KM289158 |
| 2 | *Ixodes hexagonus* | Partially fed adult | *B. venatorum* | 99% | KM289158 |
| 2 | *Ixodes ricinus* | Partially fed adult | *B. vulpes* sp. nov. | 98–99% | FJ1225390 |
| 1 | *Ixodes hexagonus* | Partially fed adult | *B. vulpes* sp. nov. | 99/99/99/99% | FJ1225390 |
| 1 | *Ixodes ricinus* | Partially fed adult | *B. divergens*/*B. capreoli* | 100% | FJ1225390 |
| 1 | *D. reticulatus* | Fully fed adult | *B. canis* | 99% | KT580785 |
| 2 | *Ixodes hexagonus* | Partially fed adult | *B. vulpes* sp. nov. | 98–99% | KT580785 |
| 1 | *Ixodes ricinus* | Partially fed adult | *B. vulpes* sp. nov. | 98% | KT580785 |
| 1 | *Ixodes ricinus* | Fully fed adult | *B. vulpes* sp. nov. | 100% | KM116004 |
| 1 | *Ixodes ricinus* | Partially fed | *B. divergens*/*B. capreoli* | 97% | KM116004 |
| 1 | *Ixodes ricinus* | Fully fed adult | *B. microti* | 99% | KM116004 |

*Two of these ticks had co-infection with *B. garinii*.

†One of these ticks had co-infection with *B. garinii*.© 2017 The Royal Entomological Society, *Medical and Veterinary Entomology*, 32, 14–22
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. Co-infection between Borrelia and Babesia has been reported previously (Krause et al., 1996; Jabłońska et al., 2016), but species combinations vary with geographical location (Swanson et al., 2006). Co-infections have been reported to produce more severe clinical symptoms and to 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 U.K. dogs (Swainsbury et al., 2016). In the present 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 U.K., 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 for the entry and establishment of this pathogen in the U.K. As pathogens were identified in fed ticks collected from dogs, it is possible that some were acquired with the current bloodmeal rather than being mature infections; this may have contributed to a slight overestimation of prevalence or of 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.

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