Detection of *Babesia* DNA in blood and spleen samples from Eurasian badgers (*Meles meles*) in Scotland

PAUL M. BARTLEY*, CARI WILSON, ELISABETH A. INNES *and* FRANK KATZER

Moredun Research Institute, Pentlands Science Park, Bush Loan, Penicuik, EH26 0PZ, Scotland, UK

(*Received 19 December 2016; revised 15 March 2017; accepted 21 March 2017; first published online 8 May 2017*)

SUMMARY

Babesia are intraerythrocytic parasites of importance worldwide within the fields of human and veterinary medicine, as some *Babesia* sp., including *Babesia microti* are potentially zoonotic and can cause fatal disease in both humans and animals. The aims of this study were to use a nested PCR (amplifying the 18S rRNA gene) to determine the presence and species of *Babesia* parasite DNA found in blood ($n = 47$) and spleen ($n = 47$) samples collected from Eurasian badgers (*Meles meles*) in Scotland. The results showed 28/47 (59·6%) blood and 14/47 (29·8%) spleen samples tested positive for the presence of *Babesia* DNA. Initial sequence analysis of the *Babesia* DNA identified three distinct sequence types (submitted to GenBank KX528553, KX528554 and KX528555), which demonstrated ⩾99% identity to *Babesia* sp. parasites previously identified in badgers in Spain (KT223484 and KT223485). Phylogenetic analysis showed that the three isolates are closely related to *Babesia annae, B. microti* and other Piroplasmida species found in wildlife. Further sequence analysis of the samples demonstrated that the badgers were routinely infected with more than one parasite isolate and there was also evidence of genetic recombination between the *Babesia* parasite isolates (submitted to GenBank KY250472 – KY250477)

Key words: *Babesia*, European badger, *Meles meles*, blood, spleen, DNA, phylogenetics.

INTRODUCTION

Babesia are intraerythrocytic parasites of significant importance to both human and veterinary medicine worldwide. Ixodid ticks are considered to be the main vectors for the transmission of *Babesia* parasites (Homer *et al*. 2000). Both *Ixodes canisuga* and *I. hexagonus* have previously been commonly found on Eurasian (European) badgers (*Meles meles*) in Britain (Peirce and Neal, 1974), though recent data indicates that *Ixodes ricinus* is the most common ticks species in Britain (Jameson and Medlock, 2011). Pooled samples of *I. canisuga* ticks collected from badgers in Spain were found to be positive for *Theileria annae* DNA (also referred to as *B. annae*/*B. vulpes*), (Baneth *et al*. 2015; Millan *et al*. 2015) this is not however conclusive proof that the ticks were capable of transmitting the parasite or that they acquired the parasites from the badgers.

The Eurasian (European) badger is Britain's largest native (indigenous) carnivore species. Though badgers are not considered to be endangered in Britain, they are a protected species. Badger numbers fluctuate year on year and are adversely affected through illegal persecution (baiting and trapping) and habitat destruction (increasing urbanization). The last comprehensive survey of the Scottish badger population suggested that there are between 7300 and 11200 main badger setts in Scotland (Rainey *et al*. 2009). While in England and Wales there are reported to be 66 400–76 900 main badger setts (Judge *et al*. 2014). Combined, this is thought to equate to approximately 300 000 individual badgers in Britain (Young *et al*. 2015). Badgers feed predominantly on invertebrates (earthworms, slugs, snails, carabid beetles and insect larvae), but they will also consume cereal crops, occasionally hunt vertebrate prey (including small mammals, rabbits and birds) (Robertson *et al*. 2014) and scavenge carrion (Young *et al*. 2015).

There is currently little information available concerning the prevalence of haemoparasites in British wildlife. The only study of Piroplasmida in British badgers was conducted by Peirce and Neal in 1974, where samples were collected from Cornwall (South West England) and the paper described intraerythrocytic organisms resembling piroplasms, but was unable to clearly identify the infectious agent (Peirce and Neal, 1974). A study of British rodents (predated by badgers) by Healing (1981) demonstrated *Babesia* parasites in Giemsa-stained blood smears from wood mice (*Apodemus sylvaticus*), bank voles (*Clethrionomys glareolus*) and short tailed voles (*Microtus agrestis*), but again did not speciate the *Babesia* parasites found (Healing, 1981). More recent studies examining other British carnivores have included the identification of a *Hepatozoon* parasite closely related to *Hepatozoon canis*, in granulomatous lesions in the heart and skeletal muscles of Scottish Pine martens (*Martes martes*) (Simpson *et al*. 2005). While another study examining red foxes (*Vulpes vulpes*)

* Corresponding author: Moredun Research Institute, Pentlands Science Park, Bush Loan, Penicuik, EH26 0PZ, Scotland, UK. E-mail: Paul.bartley@moredun.ac.uk

doi:10.1017/S0031182017000476

Table 1. Primer names, specificity and sequences used for the detection of *Babesia* DNA in badger blood and spleen samples

Primer name	Specificity	Sequence (5′–3′)	Reference
BT1-F	Universal *Babesia* – *Theileria* – *Hepatozoon*	GGTTGATCCTGCCAGTAGT	(Criado-Fornelio *et al*. 2003)
BTH-1R	Universal *Babesia* – *Theileria* – *Hepatozoon*	TTGCGACCATACTCCCCCCA	
BTH-1F	Universal *Babesia* – *Theileria* – *Hepatozoon*	CCTGMGARACGGCTACCACATCT	
BT1-R	Universal *Babesia* – *Theileria* – *Hepatozoon*	GCCTGCTGCCTTCCTTA	
Bab-Mel-For	*Babesia*	ACATGCTCGAGACACATTAATT	This manuscript
Bab-Mel-Rev	*Babesia*	CTACTTCATTATTCCATGCTGT	
BT-Outer-R	*Babesia*	GGAAACCTTGTTACGACTTCTC	(Bartley *et al*. 2016)
BT-Inner-R	*Babesia*	TTCTCCTTCCTTTAAGTGATAAG	
600-F	*Babesia*	AGTTAAGAAGCTCGTAGTTG	
1200-F	*Babesia*	AGGATTGACAGATTGATAGC	
Type A – For	*Babesia*	GATTTGGCATCTTCTGGAT	This manuscript
Type A – Rev	*Babesia*	ACCCATCGGCCAAGGA	
Type B – For	*Babesia*	ATTGATTTGGCATCCTCTAATA	
Type B – Rev	*Babesia*	GATGCATACTGTAAGATTACCCAA	

from around Britain, found parasite DNA for *Babesia annae* in 46/316 (14·55%) of bloody lung exudate samples (Bartley *et al*. 2016). *Piroplasmida* DNA belonging to a *Babesia microti*-like organism (FJ225390) has been found in 20% ($n = 5$) of badgers from the Burgos Province of Spain, this parasite showed 97% identity to the published sequence of *B. annae* (AY150069) (Gimenez *et al*. 2009).

This current study aims to determine the prevalence and identify the species of *Babesia* parasite DNA present in blood and spleen samples collected from badgers in Scotland.

MATERIALS AND METHODS

Sample collection

Sixty-four badger carcasses were collected from around the Lothians and Borders regions of Scotland, following collisions with motor vehicles and stored at −20 °C (all carcasses were collected with the knowledge and permission of Scottish Natural Heritage) (Bartley *et al*. 2013; Burrells *et al*. 2013). Where possible, full necropsies were performed on the carcasses and individual tissue samples were collected using sterile scalpels and forceps. From 52 of the badger carcasses necropsied, samples of spleen ($n = 47$) and blood ($n = 47$) were collected. For 42 of the badgers both blood and spleen samples were available, while five animals had only spleen and a further five badgers provided only blood samples.

DNA extraction

The method used to extract DNA from samples was as previously described (Katzer *et al*. 2014). In brief, the Wizard® genomic DNA purification protocol (Promega, Madison WI, USA) was used, the method was modified to allow for the use of 0·4 g of starting material. The DNA samples were resuspended in a final volume of 200 μL DNase/RNase free water and stored at −20 °C prior to analysis. Extraction controls (water) were processed with every batch of spleen and blood samples. These not only acted as indicators of contamination, but were also used as additional negative controls for PCR.

PCR detection of Babesia DNA in blood and spleen samples from European (Eurasian) Badgers (M. meles)

A semi-nested PCR to detect the 18S rRNA gene was used to screen blood ($n = 29$) and spleen ($n = 28$) samples for the presence of piroplasmids (*Babesia* and *Theileria* and *Hepatozoon*) 18S rRNA DNA. The PCR used for the initial sample screening has been previously described (Bartley *et al*. 2016). In brief, a semi-nested PCR using universal *Babesia*/*Theileria* primers BT1-F and BTH-1R was used for the primary amplification and BT1-F and BT1-R (410 bp) for the second round amplification (Table 1) (Criado-Fornelio *et al*. 2003). Following sequence analysis of the initial screening results, *Babesia*-specific Primers (Bab-Mel-For and Bab-Mel-Rev) (601 bp) (Table 1) were designed (Primer3web v4·0·0) and used as second round primers to examine all blood ($n = 47$) and spleen ($n = 47$) samples.

The PCR reaction mixture has been previously described (Burrells *et al*. 2013). Briefly each reaction (20 μL) contained final concentrations of: Tris–HCl 45 mM, $(NH_4)_2SO_4$ 11 mM, $MgCl_2$ 4·5 mM, BSA 0·113 mg mL^{-1}, EDTA 4·4 μM and each of dATP, dCTP, dGTP and dTTP 1·0 mM, forward and reverse primers (Eurofins MWG Operon) 0·25 pm

https://doi.org/10.1017/S0031182017000476 Published online by Cambridge University Press

each and *Taq* polymerase 0·75 U (Bioline Ltd. London, UK) and 2 μL of each DNA sample. To increase the sensitivity of the assay each sample was analysed in quadruplicate.

Samples that gave positive results for all quadruplicates using the *Babesia*-specific (Bab-Mel) primers were also tested in another semi-nested PCR using the primers BT1-F and BT-Outer-R (Table 1) in the primary reaction and BT1-F and BT-Inner-R (Table 1) in the second round reaction, producing an 18S rRNA gene fragment of ~1·7 Kb. The reaction mixture and conditions for this PCR were as previously described (Bartley *et al*. 2016).

Following secondary amplification, 10 μL of each PCR product was analysed by agarose gel electrophoresis (2% agarose in 1× TAE buffer), stained with gel red (1:10 000) (Biotonium, Hayward, CA, USA) and visualised using UV light.

PCR clean up and sequencing of initial PCR amplicons

PCR amplicons were purified using the commercially available Wizard® SV Gel and PCR Clean-up System (Promega, Madison WI, USA), as per the manufacturer's instructions. The purified PCR products were eluted in a final volume of 30 μL DNase/RNase free water. The nucleic acid content of each sample was determined using spectralphotometry (Nanodrop ND 1000). For sequencing (Eurofins MWG Operon), 100 ng of each sample was sent with each primer (BT1-F, BTH-1F, BTH-1R and BT1-R), this created an overlapping forward and reverse consensus sequences for each sample.

Cloning of amplicons representing the 18S rRNA gene of Babesia in badger blood and spleen samples

Clones from PCR amplicons representing the *Babesia* 18S rRNA gene were generated using the BT1-F and BT-Inner-R primers as has been previously described (Bartley *et al*. 2016), with the following differences. White colonies were screened using the Bab-Mel-For and Bab-Mel-Rev PCR primers (Table 1), for the presence of the *Babesia* 18S rRNA gene insert. The purified plasmid DNA (Isolate II Plasmid Mini Kit, Bioline, London, UK) (100 ng per primer) was sent to be sequenced (Eurofins MWG Operon) using T7 and SP6 primers along with, 600-F, 1200-F, BTH-1F, BT1-R and BTH-1R (Table 1), this created an overlapping forward and reverse consensus sequences for each clone.

Comparison of Babesia DNA sequences found in badgers in Scotland

Primers were designed (Table 1) to span polymorphic regions within the *Babesia* 18S rRNA sequence, these primers allowed the detection and discrimination of *Babesia* parasite DNA type A (KX528553) and type B (KX528554) sequences found in this study. Spleen ($n = 47$) and blood ($n = 47$) samples were tested with combinations of types A and B-specific primers (type A forward – type B reverse or type B forward and type A reverse) to determine if genetic recombination occurred between the type A and B sequences. The PCR reaction mixture used for this analysis is identical to that described previously (Bartley *et al*. 2013). Optimal annealing conditions (59 °C) were determined using a gradient PCR, the general reaction conditions were as previously described (Bartley *et al*. 2016).

Sequencing and phylogenetic analysis

Consensus sequences were generated using SeqMan Pro$^{(TM)}$ (v 12·3·1) and compared against other published sequences using the **N**ational **C**entre for **B**iotechnology **I**nformation (NCBI) **B**asic **L**ocal **A**lignment **S**earch **T**ool (BLAST) tool, to produce a percentage identity. The **E**uropean **M**olecular **B**iology **L**aboratory – **E**uropean **B**ioinformatics **I**nstitute (EMBL-EBI) **Mu**ltiple **S**equence **C**omparison by **L**og- **E**xpectation (MUSCLE) software was used to create multiple sequence alignments of the results generated in this study against published sequences. Phylogenetic analysis was performed on a ~1·2 kb consensus using PhyML 3·0 software (ATCG, Phylogeny.fr). The Gblocks program was used for automatic sequence alignment curation, while PhyML was used for phylogenetic tree creation and the TreeDyn software was used to draw the phylogenetic tree (Anisimova and Gascuel, 2006; Dereeper *et al*. 2008; Guindon *et al*. 2010).

Statistical analysis

Proportion positive (prevalence), with confidence intervals (95% CI), was calculated for the presence of *Babesia* DNA in spleen and blood samples, as well as for the numbers of samples positive for type A or B *Babesia* sequences and the samples that were positive for both types A and B. All calculations were carried out using Minitab 15 (v15·1·0·0).

RESULTS

Detection of Babesia parasite DNA in blood and spleen samples from badgers in Scotland

The current study aimed to determine the prevalence and species of *Babesia* parasite DNA present in Scottish badgers. Spleen and blood samples were collected from badgers that had died as a result of collisions with motor vehicles. Due to the protected nature of badgers in the UK, this work was undertaken with the knowledge and permission of Scottish Natural Heritage, who oversee the welfare of the badger population in Scotland.

https://doi.org/10.1017/S0031182017000476 Published online by Cambridge University Press

During the initial screening of samples, forward and reverse consensus sequences were generated from 11 animals (six blood and five spleen) using the universal *Babesia* and *Theileria* PCR primers BT1-F and BTH-1R and BT1-F and BT1-R. These consensus sequences were analysed using NCBI BLAST. Five of these consensuses (881 bp) demonstrated 100% identity to KT223484 (badger type A isolate), a sequence from a *Babesia* sp. found in badgers from Spain, three samples demonstrated 100% identity to KT223485 (badger type B isolate), a second *Babesia* isolate also found in badgers from Spain. The remaining three samples demonstrated a third DNA sequence which had a high level of sequence identity to KT223485 (99·3% identity) and 95·6% identity to KT223484 but was clearly different from the other two DNA sequences identified in the Scottish badgers. No other Piroplasmida species were detected in the badgers.

Prevalence of Babesia DNA in spleen and blood samples from badgers

The PCR primers Bab Mel-F and Bab-Mel-R (Table 1) were designed to specifically amplify *Babesia* 18S DNA, which was identified during the initial sample screening using Pan-Piroplasm primers. The Bab Mel (F and-R) primers were used to determine the prevalence of *Babesia* parasite DNA in blood and spleen samples from badgers collected from across the Lothians and Borders regions of Scotland. The results are illustrated in Table 2, where it can be seen that 14/47 (29·8%) [95% confidence interval (CI) 16·7–42·9%] spleen and 28/47 (59·6%) (95% CI 45·6–73·6%) blood samples were positive for *Babesia* DNA using the Bab-Mel (F + R) primers (Table 1). With 9/42 (21·4%) (95% CI 9·0–33·8%) of the badgers having tested positive, where both spleen and blood samples were available for testing. A total of 33/47 (70·2%) (95% CI 57·1–83·3%) of the badgers tested positive for the presence of *Babesia* DNA in at least one sample.

The PCR amplicons of six samples (two blood and four spleen) were sequenced using the Bab Mel-(F + R) primers creating 530 bp overlapping forward and reverse consensuses. Two spleen samples demonstrated 100% identity to KT223484 [badger type A isolate (Spain)], while four samples (two blood and two spleen) demonstrated 100% identity to KT223485 [badger type B isolate (Spain)].

Comparisons of Babesia DNA sequences found in spleen and blood samples from badgers in Scotland

Table 2 illustrates the results from the PCR analysis using the type A and B primers (Table 1). Type A sequences were seen in 6/47 (12·8%) (95% CI 3·2–22·3) spleen and 18/47 (38·3%) (95% CI 24·4–52·2) blood samples. Type B sequences were seen

Table 2. Prevalence of *Babesia* sp. DNA in spleen and blood samples from Badgers in East/Central Scotland

Sample	N° Positive/N° Tested (% Prevalence) [95% confidence interval]	Both spleen and blood amples positive	Type A	Type B	Both types A and B	Total badgers positive for type A or B
Spleen	14/47 (29·8) [16·7–42·9%]	9/42 (21·4)[a] [9·0–33·8%]	6/47 (12·8) [3·2–22·3]	9/47 (19·1) [7·9–30·4]	5/47 (10·6) [1·8–19·4]	10/47 (21·3) 10·7–35·7
Blood	28/47 (59·6) [45·6–73·6%]		18/47 (38·3) [24·4–52·2]	22/47 (46·8) [32·5–61·1]	15/47 (31·9) [18·6–45·2]	25/47 (53·2) [38·1–67·9]

N° – Number.
[a] Calculation is based on the number of animals where both spleen and blood samples were collected at post-mortem examination and were available for testing ($n = 42$).

https://doi.org/10.1017/S0031182017000476 Published online by Cambridge University Press

in 9/47 (19·1%) (95% CI 7·9–30·4) spleen and 22/47 (46·8%) (95% CI 32·5–61·1) blood samples. Both type A and B sequences were found in 5/47 (10·6%) (95% CI 1·8–19·4) spleen and 15/47 (31·9%) (95% CI 18·6–45·2) blood samples. The overall results show that 10/47 and 25/47 individual badgers were positive for type A or B *Babesia* DNA in spleen and blood, respectively (see Table 2).

Since mixed genotypes were frequent the possibility of recombination within the same species was considered probable. To test this postulation all spleen ($n = 47$) and blood ($n = 47$) samples were tested using combinations of type A and B primers (type A forward – type B reverse or type B forward and type A reverse). Using these primer combinations PCR amplicons were seen for 4/47 spleen samples (8·5%) (95% CI 5·3–16·5) and 22/47 blood samples (46·8%) (95% CI 32·5–61·1). Six PCR amplicons from four animals were purified as described above and sent for sequencing (MWG Operon) and showed evidence of genetic recombination with type A and B sequences. A representation of the genetic recombinations seen in the six samples is illustrated in Table 3. As the type A and B primers were used for sequencing the purified PCR products, full-length consensus sequences were not always produced. Some observations in Table 3 were made based on a consensus (annotated by UPPER CASE letters), while when only a single strand was available these observation are annotated with lower case letters. The consensus sequences from all six PCR amplicons demonstrating genetic recombinations were submitted to Genbank, as *Babesia* sp. isolate Ba15(a) (KY250472), Ba15(b) (KY250473), Ba22(a) (KY250474), Ba22(b) (KY250475), Ba21 (KY250476) and Ba27 (KY250477).

Cloning and phylogenetic analysis of Babesia DNA found in blood and spleen samples

Amplicons from three samples produced using the BT1-F and BT-Inner-R primers were cloned, 12 *Babesia* 18S rRNA gene plasmid clones were created (four clones per sample), each was sequenced to create overlapping consensuses. The general consensus from each sample was submitted to GenBank, Badger type A UK1 (KX528553), Badger type B UK1 (KX528554) and Badger type B UK2 (KX528555) and used in the phylogenetic analysis.

The results from the phylogenetic analysis (Fig. 1) demonstrate that the three *Babesia* sequences identified during this study are clearly distinct from each other. The *Babesia* type A UK1 (KX528553) sequence grouped most closely with KT223484 (Badger type A, Spain) (98·8% identity over 1439 bp) and FJ225390 a piroplasm previously identified in badgers. The *Babesia* type B UK1 (KX528554) and *Babesia* type B UK2 (KX528555) sequences showed the greatest parsimony to each other (99·3% identity over 1557 bp) and KT223485

Table 3. Evidence of intragenic recombination events occurring between variant alleles of type A and B *Babesia* isolates

			SNP location/nucleotide										
Primers	Sample (Accession N°)	Consensus Length (bp)	585	586	604	650	1174	1231	1308	1313	1314	1316	1319	1320
–	Type A (KX528553)	1560	**T**	**A**	**C**	**C**	**C**	**G**	–	–	–	–	**G**	**G**
–	Type B (KX528554)	1560	**A**	**T**	**T**	**T**	**T**	**T**	**A**	**A**	**T**	**C**	**A**	**A**
FA – RB	Ba27 (KY250477)	742	**T**	**A**	**C**	**C**	**T**	**T**	**A**					
FA – RB	Ba21 (KY250476)	690	**t**	**a**	**t**	**T**	**T**	**T**	**a**					
FA – RB	Ba15(a) (KY250472)	718			**c**	**C**	**T**	**T**	**A**	**A**	**T**	**C**	**A**	**A**
FA – RB	Ba22(a) (KY250474)	707			C	**T**	**T**	**T**	**A**					
FB – RA	Ba22(b) (KY250475)	697			**t**	**T**	**C**	**G**	–	–	–	–	**g**	**g**
FB – RA	Ba15(b) (KY250473)	706	**a**	**t**	**T**	**T**	**C**	**G**	–	–	–	–	**g**	**g**

FA – RB*– type A forward – Type B reverse primers used to generate consensus.
FB – RA* – type B forward – Type A reverse primers used to generate consensus.
Upper case (A, C, G and T) sequence generated from forward and reverse consensus.
Lower case (a, c, g and t) sequence generated from a single direction only.
– =Indel.

https://doi.org/10.1017/S0031182017000476 Published online by Cambridge University Press

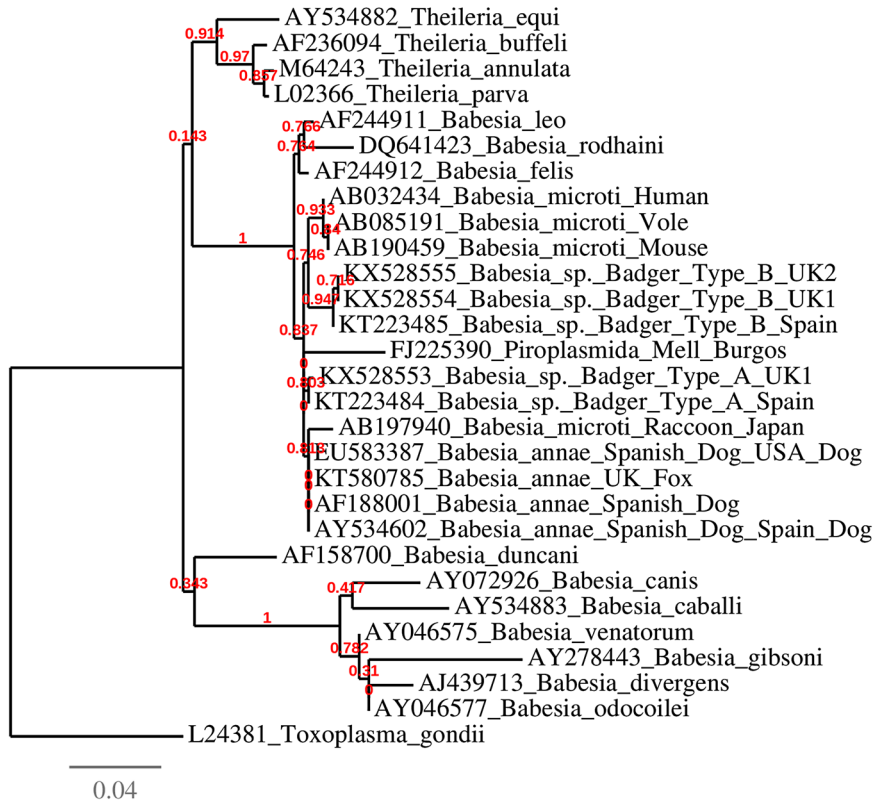

Fig. 1. Phylogenetic tree demonstrating differences in the 18S rRNA gene of *Piroplasmida* parasites. The results are based on the maximum-likelihood approximation of the standard likelihood ratio test score. The branch length is proportional to the number of base pair substitutions. Scale bar indicates the base pair substitution rate.

(Badger type B, Spain) than to the *Babesia* type A UK1 sequence (97·3 and 96·9% identity respectively over 1545 bp). All three UK *Babesia* sequences are situated in a clade with the *B. annae* isolates (KT580785 and AF188001) found in foxes and dogs, along with *Babesia*/*B. microti*-like parasites found in wildlife species including otters and raccoons as well as *B. felis* and *B. leo*.

DISCUSSION

This study has identified three distinct sequences of *Babesia* [Badger type A UK1 (KX528553), Badger type B UK1 (KX528554) and Badger type B UK2 (KX528555)] in the badger blood and spleen samples tested, however more types of *Babesia* may be present in the badger population as not all samples that tested positive for *Babesia* DNA could be assigned to either type A or B. This is the first molecular identification of *Babesia* DNA in blood and spleen samples from badgers in Britain. Previously, intraerythrocytic organisms resembling piroplasms have been observed in blood smears from badgers in Britain and Turkey (Peirce and Neal, 1974; Simsek *et al*. 2003) and parasite DNA has been isolated from badger blood samples in Spain (Gimenez *et al*. 2009). This current study is the first to report *Babesia* parasite DNA being isolated from the spleens of badgers. A recent study of spleen samples collected from red foxes (*V. vulpes*) in the state of Brandenburg, Germany demonstrated a high prevalence (47·5%) of *T. annae* (also referred to as *B. annae*) (Liesner *et al*. 2016). Our study demonstrates that sample selection for the detection of *Babesia* parasites is important. The use of spleen as well as blood increased the frequency of detection of the parasite, five animals would have appeared negative for the presence of *Babesia* DNA, had only blood samples been used. However, the collection of spleen samples is only possible during post-mortem studies. Ante-mortem studies of parasite prevalence would be reliant on blood samples, but would have the advantage of enabling researchers to examine the clinical condition of the animal and possibly collect any infesting ticks.

The results from this current study show a high prevalence 33/47 (70·2%) of *Babesia* sp. DNA in the badgers sampled from East/Central Scotland, although the overall sample size was low. The high prevalence demonstrated in this study would suggest that the infectious organisms have been present in the Scottish badger population for a considerable length of time, as the parasites appear to be widely dispersed across the entire region of East/Central Scotland. Previously reported incidence of piroplasm infections in badgers appeared to be rare, with isolated cases being reported in Britain

https://doi.org/10.1017/S0031182017000476 Published online by Cambridge University Press

(Peirce and Neal, 1974), Turkey (Simsek *et al*. 2003) and in Spain (Gimenez *et al*. 2009). However, all of these studies in badgers, including ours, have only examined small numbers of animals, so are unable to give a clear indication of the prevalence of *Babesia* infections in the resident badger populations within each country. The previous studies in Britain and Turkey also relied solely on direct microscopic examination of blood smears, with only the most recent study (Gimenez *et al*. 2009) providing a molecular identification of the infectious organism (Piroplasmida FJ225390). It is highly likely that the prevalence of *Babesia* infections being detected using PCR would be higher than that seen using blood smears due to the increased sensitivity and specificity of PCR analysis.

The three *Babesia* sequences identified in this study are closely related, but are distinct from one another and from the other published *Babesia* and Piroplasmida sequences found in other wildlife species. The greatest parsimony (fewest evolutionary changes) of the UK badger sequences KX528553 and KX528554 is to the *Babesia* sp. isolates found in Spanish badgers KT223484 and KT223485, respectively. The fact that parasite DNA with 98·75% identity is being isolated from the same host species in different countries would suggest that the sequences are highly conserved and host specific. The high level of host specificity is further supported by fact that the *B. annae* (KT580785) DNA found in British foxes (Bartley *et al*. 2016) was not found in the badgers, though both animal species will hunt similar prey species and were collected from similar habitats and locations across East Central Scotland. The results from this present study also demonstrate that more than one sequence can be found in an individual animal; with many of the animals examined in this study demonstrating the presence of multiple *Babesia* sequences. The presence of mixed infections with *Babesia* parasites are common and have been reported in *B. bovis* infections in cattle in Asia (Vietnam, Sri Lanka and Mongolia) (Liyanagunawardena *et al*. 2016).

Genetic recombination appears to be occurring between the parasites identified in this study. Combinations of the type A and B 18S rRNA gene sequences were identified in 22/47 animals and six of these were submitted to GenBank (KY250472 – KY250477). This would suggest that we have identified different strains of the same species of *Babesia* parasite and not separate parasite species. Genetic recombination is not uncommon amongst *Babesia* parasites and has been previously reported in the genes encoding merozoite surface antigens (MSAs) in isolates of *B. bovis* (Yokoyama *et al*. 2015).

The phylogenetic results from this present study (Fig. 1) indicate a close association between the *Babesia* isolates found in badgers and *B. annae* (KT580785) as well as a *B. microti* (AB197940) found in raccoon dogs and raccoons. This is in agreement with the observations made by Gimenez *et al*. (2009), who noted that the Piroplasmida (FJ225390) found in badgers in Spain was closely associated with *B. annae* and other *B. microti*-like organisms.

Further work is required to determine whether the *Babesia* isolates present in the badgers cause clinical disease, or whether they are of potential zoonotic importance. Further study is also required to determine the prevalence of *Babesia* infections in badgers throughout the rest of Scotland and across the entire badger population in the UK and the vectors (if any) that are transmitting the disease between hosts.

ACKNOWLEDGEMENT

The authors would like to thank Sarah Middleton for her contributions to this project.

FINANCIAL SUPPORT

The authors would like to thank Scottish Government's Rural and Environment Science and Analytical Services Division (RESAS) for supporting this work.

REFERENCES

Anisimova, M. and Gascuel, O. (2006). Approximate likelihood-ratio test for branches: a fast, accurate, and powerful alternative. *Systematic Biology* **55**, 539–552.

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

Bartley, P. M., Wright, S. E., Zimmer, I. A., Roy, S., Kitchener, A. C., Meredith, A., Innes, E. A. and Katzer, F. (2013). Detection of *Neospora caninum* in wild carnivorans in Great Britain. *Veterinary Parasitology* **192**, 279–283.

Bartley, P. M., Hamilton, C., Wilson, C., Innes, E. A. and Katzer, F. (2016). Detection of *Babesia annae* DNA in lung exudate samples from Red foxes (*Vulpes vulpes*) in Great Britain. *Parasites & Vectors* **9**, 84.

Burrells, A., Bartley, P. M., Zimmer, I. A., Roy, S., Kitchener, A. C., Meredith, A., Wright, S. E., Innes, E. A. and Katzer, F. (2013). Evidence of the three main clonal *Toxoplasma gondii* lineages from wild mammalian carnivores in the UK. *Parasitology* **140**, 1768–1776.

Criado-Fornelio, A., Martinez-Marcos, A., Buling-Sarana, A. and Barba-Carretero, J. C. (2003). Molecular studies on Babesia, Theileria and Hepatozoon in southern Europe – Part I. Epizootiological aspects. *Veterinary Parasitology* **113**, 189–201.

Dereeper, A., Guignon, V., Blanc, G., Audic, S., Buffet, S., Chevenet, F., Dufayard, J. F., Guindon, S., Lefort, V., Lescot, M., Claverie, J. M. and Gascuel, O. (2008). Phylogeny.fr: robust phylogenetic analysis for the non-specialist. *Nucleic Acids Research* **36**, W465–W469.

Gimenez, C., Casado, N., Criado-Fornelio, A., de Miguel, F. A. and Dominguez-Penafiel, G. (2009). A molecular survey of Piroplasmida and Hepatozoon isolated from domestic and wild animals in Burgos (northern Spain). *Veterinary Parasitology* **162**, 147–150.

Guindon, S., Dufayard, J. F., Lefort, V., Anisimova, M., Hordijk, W. and Gascuel, O. (2010). New algorithms and methods to estimate maximum-likelihood phylogenies: assessing the performance of PhyML 3·0. *Systematic Biology* **59**, 307–321.

Healing, T. D. (1981). Infections with blood parasites in the small British rodents *Apodemus sylvaticus, Clethrionomys glareolus* and *Microtus agrestis*. *Parasitology* **83**, 179–189.

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

Jameson, L. J. and Medlock, J. M. (2011). Tick surveillance in Great Britain. *Vector Borne and Zoonotic Diseases* **11**, 403–12.

Judge, J., Wilson, G. J., Macarthur, R., Delahay, R. J. and McDonald, R. A. (2014). Density and abundance of badger social

https://doi.org/10.1017/S0031182017000476 Published online by Cambridge University Press

groups in England and Wales in 2011–2013. *International Journal of Scientific Reports* **4**, 3809.

Katzer, F., Canton, G., Burrells, A., Palarea-Albaladejo, J., Horton, B., Bartley, P. M., Pang, Y., Chianini, F., Innes, E. A. and Benavides, J. (2014). Immunization of lambs with the S48 strain of *Toxoplasma gondii* reduces tissue cyst burden following oral challenge with a complete strain of the parasite. *Veterinary Parasitology* **205**, 46–56.

Liesner, J. M., Krucken, J., Schaper, R., Pachnicke, S., Kohn, B., Muller, E., Schulze, C. and von Samson-Himmelstjerna, G. (2016). Vector-borne pathogens in dogs and red foxes from the federal state of Brandenburg, Germany. *Veterinary Parasitology* **224**, 44–51.

Liyanagunawardena, N., Sivakumar, T., Kothalawala, H., Silva, S. S., Battsetseg, B., Lan, D. T., Inoue, N., Igarashi, I. and Yokoyama, N. (2016). Type-specific PCR assays for *Babesia bovis* msa-1 genotypes in Asia: revisiting the genetic diversity in Sri Lanka, Mongolia, and Vietnam. *Infection Genetics and Evolution* **37**, 64–69.

Millan, J., Proboste, T., Fernandez de Mera, I. G., Chirife, A. D., de la Fuente, J. and Altet, L. (2015). Molecular detection of vector-borne pathogens in wild and domestic carnivores and their ticks at the human-wildlife interface. *Ticks and Tick Borne Diseases* **7**, 284–290.

Peirce, M. A. and Neal, C. (1974). Piroplasmosis in British badgers (*Meles meles*). *Veterinary Record* **94**, 493–494.

Rainey, E., Butler, A., Bierman, S. and Roberts, A. M. I. (2009) Scottish Badger Distribution Survey 2006–2009: estimating the distribution and density of badger main setts in Scotland. Report prepared by Scottish Badgers and Biomathematics and Statistics Scotland.

Robertson, A., McDonald, R. A., Delahay, R. J., Kelly, S. D. and Bearhop, S. (2014). Individual foraging specialisation in a social mammal: the European badger (*Meles meles*). *Oecologia* **176**, 409–421.

Simpson, V. R., Panciera, R. J., Hargreaves, J., McGarry, J. W., Scholes, S. F., Bown, K. J. and Birtles, R. J. (2005). Myocarditis and myositis due to infection with Hepatozoon species in pine martens (*Martes martes*) in Scotland. *Veterinary Record* **156**, 442–446.

Simsek, S., Dumanli, N. and Koroglu, E. (2003). Piroplasmosis in a badger from Turkey. *Veterinary Record* **153**, 372.

Yokoyama, N., Sivakumar, T., Tuvshintulga, B., Hayashida, K., Igarashi, I., Inoue, N., Long, P. T. and Lan, D. T. (2015). Genetic variations in merozoite surface antigen genes of *Babesia bovis* detected in Vietnamese cattle and water buffaloes. *Infection Genetics and Evolution* **30**, 288–295.

Young, A., Marquez-Grant, N., Stillman, R., Smith, M. J. and Korstjens, A. H. (2015). An investigation of red fox (*Vulpes vulpes*) and Eurasian badger (*Meles meles*) scavenging, scattering, and removal of deer remains: forensic implications and applications. *Journal of Forensic Science* **60**(Suppl 1), S39–55.