AN INVESTIGATION OF ENDEMIC AND EMERGING TICK-BORNE PROTOZOA AND RICKETTSIA IN SCOTTISH LIVESTOCK

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Author's Declaration

This thesis is entirely the product of my own efforts. The work on which it is based was my own, except where specifically stated in the text and in the acknowledgements section. It has not been previously submitted to any university for the award of a degree.

Alexander Gray

May 2017

Abstract

This project set out to determine the importance of tick-borne protozoan and bacterial pathogens in Scottish livestock. The study comprised several aspects including a national survey of large animal veterinary surgeons, an appraisal of cases of tick-borne disease observed by Scottish Disease Surveillance Centres, the development of a novel assay to detect piroplasms and a targeted cross-sectional study of livestock and deer in the north of Scotland.

A survey of the experiences of veterinary surgeons treating livestock in Scotland revealed cases of babesiosis (*Babesia divergens*) in cattle and tick-borne fever (*Anaplasma phagocytophilum*) in sheep. Examination of the records of the Scottish Agricultural College Consulting Disease Surveillance Centres for the years 2000 - 2013 revealed cases of babesiosis in cattle (n = 55) along with tick-borne fever and related disease in sheep (n = 116) and cattle (n = 6). Taking a combined passive and active surveillance approach, clinical material was obtained from a large number of healthy sheep, cattle and wild red deer (*Cervus elaphus*) together with a number of cattle and sheep suspected or confirmed as having a tick-borne disease. All samples were examined using a genus-specific nested PCR targeting the v4 region of the *Babesia*/*Theileria* 18S rRNA gene, which was developed and validated in the course of this study. This gene segment was confirmed as being capable of differentiating a diverse range of *Babesia* and *Theileria* spp. based on direct sequencing of PCR amplicons. A nested PCR assay targeting the 16S rRNA gene of *A. phagocytophilum* was also applied to each clinical sample and, if positive, the *msp4* locus was also amplified and sequenced.

Babesia ventatorum, a parasite typically associated with the roe deer (*Capreolus capreolus*) in Europe, was detected in 9 % of healthy sheep. Significantly, this is the first description of this parasite in sheep or in a vertebrate host in the United Kingdom. *Babesia divergens* was found in 11 % of wild red deer, confirming the presence of this parasite in this host species by molecular means for the first time in Scotland. Additionally a *Babesia odocoilei*-like parasite was found in 15 % of wild red deer, again for the first time in Scotland and only the second time in Europe. In cattle, *B. divergens* was confirmed as the cause of

three clinical cases of babesiosis and was also found in the blood of 6 % of healthy cattle in December.

Anaplasma phagocytophilum was found at a high prevalence in healthy sheep (73 %) and red deer (40 %) and at lower levels in healthy cattle (2.8 %). Comparison of *msp4* gene sequences confirmed identical or highly similar *msp4* genotypes in sheep and deer. Red deer were infected with larger numbers of *msp4* genotypes than sheep and infection with multiple genotypes increased over the course of a grazing season on tick-infested hill land. *Anaplasma phagocytophilum* is zoonotic and can also have negative welfare and economic impacts in both sheep and, to a lesser extent, cattle and so these findings are highly significant. An incidental finding was *Sarcocystis* sp. similar to *S. tenella* in 3 % of healthy sheep.

These results of this work clearly show what can be achieved by an active surveillance approach, using a 'catch all' molecular assay. In summary, this study discovered a novel, zoonotic pathogen in Scottish livestock and demonstrated that an endemic and arguably largely overlooked bacterium, *A. phagocytophilum*, is highly prevalent in the sheep population in tick-risk areas. Moreover, genotyping of this pathogen and *B. divergens* in both livestock and deer has provided new insights into potential reservoirs of infection for these organisms.

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Abbreviations and Symbols

16S/16S rRNA	16S (Svedberg units) ribosomal ribonucleic acid
18S/18S rRNA	18S (Svedberg units) ribosomal ribonucleic acid
A	adenine
ankA	*ankA* gene of *A. phagocytophilum*
APHA	Animal and Plant Health Agency
BLAST	Basic Local Alignment Search Tool
bp	base pairs
C	cytosine
dATP	deoxyadenosine triphosphate
dCTP	deoxycytidine triphosphate
dGTP	deoxyguanine triphosphate
DNA	deoxyribonucleic acid
DSC	disease surveillance centre
dTTP	deoxythymidine triphosphate
EDTA	ethylenediaminetetraacetic acid
ELISA	enzyme-linked immunosorbent assay
et al.	and others
FMD	foot and mouth disease
G	guanine
GARD	genetic algorithm for recombination detection
gltA	*gltA* (citrate synthase) gene of *A. phagocytophilum*
groESL	*groESL* (heatshock) gene of *A. phagocytophilum*
Hb	haemoglobin
HCT	haematocrit
HGE	human granulocytic ehrlichia/ ehrlichiosis
IFA/IFAT	indirect immunofluorescent antibody test
IgG	immunoglobulin G
IgM	immunoglobulin M
LAMP	loop-mediated isothermal amplification
MLST	multi-locus sequence typing
MOI	multiplicity of infection
msp2	major surface protein 2 (msp2/P44) of *A. phagocytophilum*
msp4	major surface protein 4 of *A. phagocytophilum*

n	number of samples
NCBI	National Center for Biotechnology Information
NSAIDs	non-steroidal anti-inflammatory drugs
OS	Old Sourhope strain of *A. phagocytophilum*
P	P value; statistical significance
p44	major surface protein 2 (msp2/P44) of *A. phagocytophilum*
PCR	polymerase chain reaction
PI	postinfection
PI-3	Parainfluenza-3 (virus)
PM	post mortem
qPCR	quantitative/ realtime polymerase chain reaction
R	Pearson correlation coefficient
RBC	red blood cells
RWF	red water fever
SAC	Scottish Agricultural College
sp.	species (singular)
spp.	species (plural)
SSU	small subunit
T	thymine
Taq	*Thermus aquaticus* polymerase
TBD	tick-borne disease
TBF	tick-borne fever
tris-HCL	tris(hydroxymethyl)aminomethane hydrochloride
v/V	hypervariable region
VIDA	veterinary investigation diagnosis analysis
VIO	veterinary investigation officer
VNTR	variable number tandem repeat

Acknowledgements

The work of this project would not have been possible without the assistance and encouragement of my supervisors Dr Willie Weir and Professor Brian Shiels (University of Glasgow) and Dr Frank Katzer (Moredun Research Institute). I am also grateful to my assessor Professor Jason Matthiopoulos for his advice and encouragement throughout, internal and external examiners Professor David Logue (University of Glasgow) and Professor Geoff Hide (University of Salford), respectively, for their constructive comments, and Dr Collette Britton for her seamless convening of the *viva voce* examination.

For their assistance in the gathering of information and sample material I extend my gratitude to the staff of the participating SAC Consulting Disease Surveillance Centres located across Scotland, the owners and staff of the estates where farm sampling took place, Mr Paul Morrison (Rogart Vets, Rogart), Professor Ruth Zadoks (University of Glasgow), Dr Mark Taggart and Dr Andrew French (University of the Highlands and Islands) and the veterinary surgeons who completed the veterinary survey. Dr Zerai Woldehiwet (University of Liverpool) and Dr Annetta Zintl (University College Dublin) kindly provided DNA from *Anaplasma phagocytophilum* and *Babesia divergens* respectively.

I also thank those who provided other advice and assistance including but not limited to Dr Pamela Johnston, Dr Francesco Marchesi, and Dr Hayley Haining (Veterinary Pathology, Public Health & Disease Investigation, University of Glasgow), Professor Maureen Bain, Dr Jane Kinnaird, and Mrs Margaret McFadyen (University of Glasgow), Dr Mara Rocchi (Moredun Research Institute), and Mr Phil Bosworth (Bredy Vets, Dorset).

I have been truly fortunate before and during the work of this project to have the continuing love and support of Andrea, our daughter Alice born in September 2015, and Madeleine. I thank my mother, also Alice, who died in November 2013, father Sandy, and brother Peter and family for their unflinching support throughout.

"There are some things which cannot be learned quickly, and time, which is all we have, must be paid heavily for their acquiring. They are the very simplest things and because it takes a man's life to know them the little new that each man gets from life is very costly and the only heritage he has to leave."

From *Death in the Afternoon* Ernest Hemingway 1932

CHAPTER ONE
Introduction

1.1 *Babesia* and *Theileria* spp.

1.1.1 Introduction

1.1.1.1 Taxonomy

Babesia spp. are tick-transmitted intraerythocytic apicomplexan parasites (Zintl *et al.*, 2003) which belong to the Order Piroplasmida along with the closely related genus *Theileria*. The Piroplasmida belong to the Class Aconoidasida within the Phylum Apicomplexa. This phylum includes many parasites of human and animals including *Toxoplasma gondii*, *Neospora canis*, *Sarcocystis*, *Cryptosporidium* and the malaria parasite *Plasmodium* (Figure 1-1).

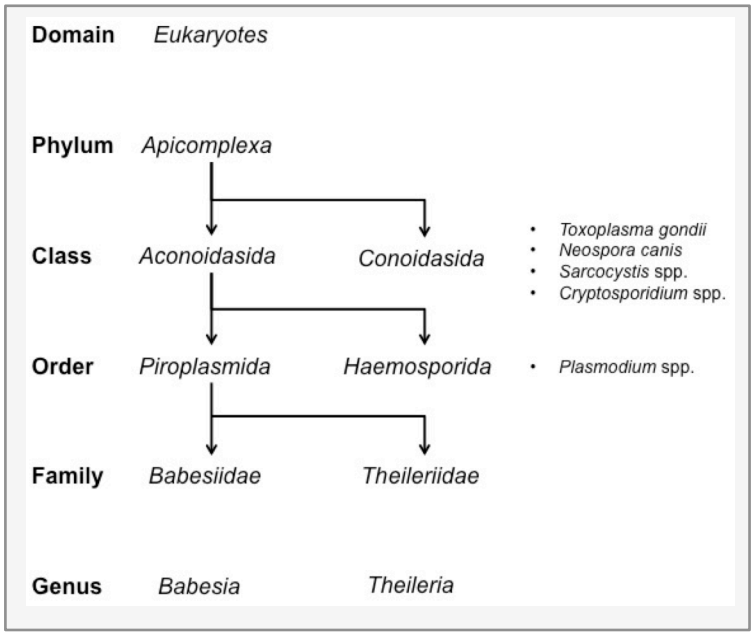


Figure 1-1 Taxonomic relationships of *Babesia* spp. to other apicomplexan parasites of veterinary and medical importance

The most economically important *Babesia* species worldwide are *Babesia bovis*, *Babesia bigemina* and *Babesia divergens* (Schnittger *et al.*, 2012). Both *B. bovis* and *B. bigemina* are restricted to subtropical and tropical regions due to the distribution of their tick vectors, while *B. divergens* is found in temperate zones. *Babesia divergens* is the only species of the genus currently recognised as a threat to UK livestock. It was first described in the United Kingdom over a century ago (M'Fadyean and Stockman, 1911) and subsequently its transmission by *Ixodes ricinus* was confirmed (Joyner *et al.*, 1963).

1.1.1.2 Life-cycle

The life-cycle of the parasite (Figure 1-2) can be divided into the stages occurring in bovine erythrocytes, characterised by asexual division, and those occurring in the tick vector, which includes a sexual cycle (Mackenstedt *et al.*, 1990; Mehlhorn and Schein, 1984).

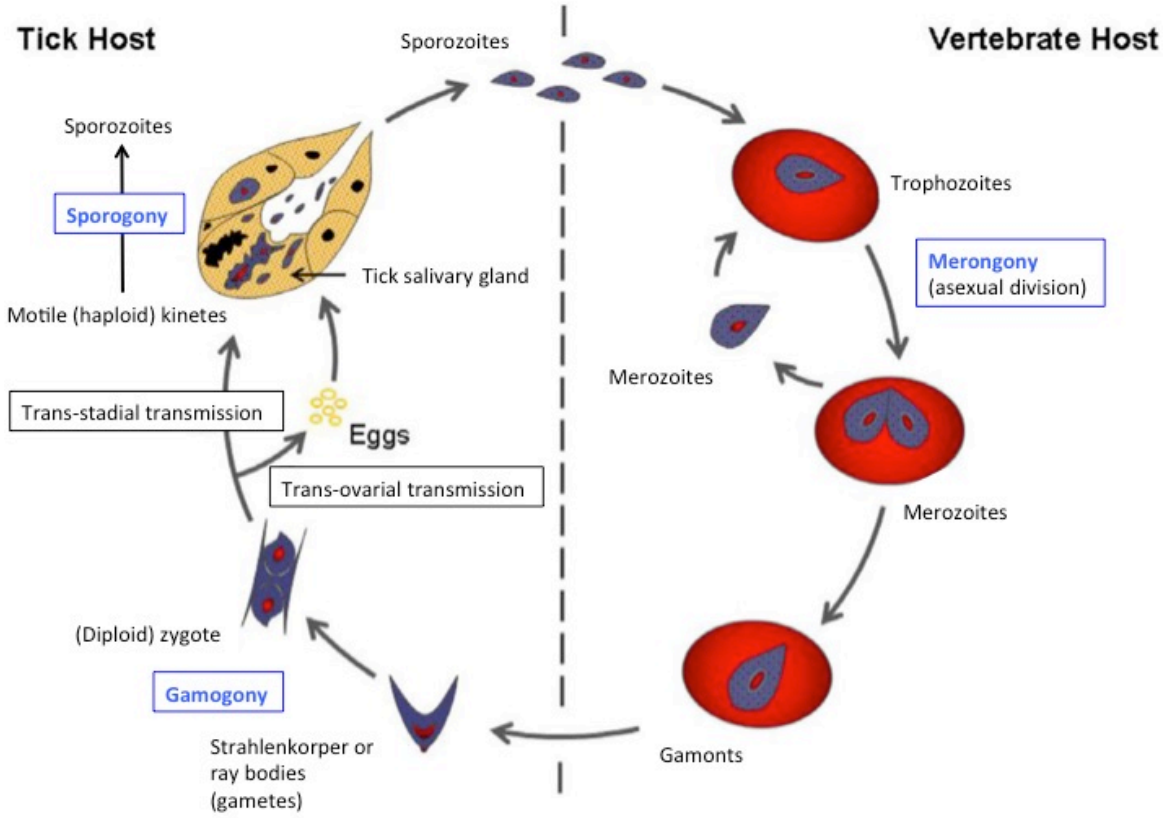


Figure 1-2 Typical life-cycle of the *Babesia* spp. parasite

Modified from (Schnittger *et al.*, 2012) and based on (Mehlhorn and Schein, 1984)

Infection can persist in the three-host hard tick vector, *Ixodes ricinus*, between life-cycle stages (trans-stadially) (Joyner *et al.*, 1963; Lewis and Young, 1980) and from the adult through the egg to the larvae (trans-ovarially) (Donnelly and Peirce, 1975).

1.1.2 *Babesia divergens* in cattle

1.1.2.1 Clinical signs

Red cell destruction by the parasite causes anaemia, jaundice and haemoglobinuria, the latter giving rise to the term 'red water' (Zintl *et al.*, 2003). Clinical signs range from mild sub-clinical infections to more severe or fatal presentations with an associated economic impact and welfare implications. A large-scale survey of Irish farmers and veterinary surgeons identified haemoglobinuria, dullness and tachycardia as common findings, with fever, constipation and tachypnea also reported (Zintl *et al.*, 2014).

1.1.2.2 Occurrence

Large-scale serological surveys have been undertaken in Northern Ireland (Taylor *et al.*, 1982) and Scotland (Adam and Blewett, 1978; Blewett and Adam, 1978b; Blewett and Adam, 1978a), which were based on the analysis of blood samples from the brucellosis eradication scheme operating at this time. In Scotland 11 % of the 22,044 cattle sera tested were positive for antibody and 27 % of herds were considered to be infected, with greater than 10 % of animals found to be seropositive (Adam and Blewett, 1978). A high proportion of the animals testing positive were of Irish origin and when these were removed from the analysis endemic foci were detected on the west coast, south-west coast, Perthshire and in the Nairn area in the north-east of Scotland. Interestingly, this distribution was similar to an earlier survey of clinical cases in Scotland (Foster, unpublished data, cited in Adam and Blewett (1978)). A smaller-scale serological study was carried out on the Island of Arran involving 244 cattle from eight beef and two dairy farms and a total of 28 % of the animals were found to be seropositive (Latif and Wells, 1973). The incidence of clinical disease has been estimated to vary from 0.07 % (Leech *et al.*, 1964) and 0.1 % (Leech *et al.*, 1960) in the United Kingdom to 0.26 % in Northern Ireland (Taylor, 1983) and 1.6% in Ireland (Gray and Harte, 1985). Local incidences can be higher, for example 0.66 % and

0.85 % in Devon and Cornwall respectively (Leech *et al.*, 1960) and 3-4 % in County Meath, Ireland (Gray *et al.*, 1983). Mortality rates of approximately 10 % are not uncommon (Gray and Harte, 1985). A spring peak in clinical disease followed by a second peak in autumn has long been recognised (Donnelly and Mackella, 1970; Gray *et al.*, 1983) although this is not observed universally (Collins *et al.*, 1970). Smaller herds are more likely to be affected (Gray and Harte, 1985; Taylor *et al.*, 1982) and beef cattle are more likely to be involved (Gray and Harte, 1985; Latif and Wells, 1973; Taylor *et al.*, 1982).

Interestingly, a recent large-scale survey of 721 Irish farms and 142 Irish veterinary surgeons revealed some changes in the occurrence of babesiosis in Ireland (Zintl *et al.*, 2014). For example, 91 cases of babesiosis were reported on 33 farms over twelve months, resulting in a calculated incidence of 0.06 %. Additionally, sales of imidocarb (Imazol GFK-Ireland), the licensed prophylactic and therapeutic agent for bovine babesiosis in both Ireland and the United Kingdom, reduced from 3,355 100 ml bottles in 2004 to 2,326 100 ml bottles in 2012, a reduction of 31 % (GFK-Ireland, personal communication, cited in Zintl *et al.*, 2014). The authors conclude that the reason for the reduction in incidence of bovine babesiosis is likely to be multifactorial. They suggest changing climate, reduction in suitable vector habitat due to pasture improvement and current trends in livestock management (for example reduced grazing, changes in frequency of transportation of cattle and reduced numbers of cattle maintained on marginal land prior to finishing ('store' cattle)) may be contributory. They also suggest that the widespread use of macrocyclic lactones directed towards other parasites may be unintentionally reducing tick numbers.

1.1.2.3 Diagnostic methods

Diagnosis of *B. divergens* infection is often based on case history and clinical signs (Collins *et al.*, 1970; Gray and Murphy, 1985) and can be confirmed by examining Giemsa-stained blood smears for the presence of the parasites within erythrocytes. Examination of blood from 157 cases in Ireland confirmed the presence of parasites in 135 of these (Sherlock *et al.*, 2000). This method is currently used in the Scottish Agricultural College (SAC) Disease Surveillance Centres (DSC) to confirm suspected infections before recording on the Veterinary Investigation Diagnosis Analysis (VIDA) database. Unfortunately, diagnosis based

on history, clinical signs and examination of blood smears has limitations. *Theileria mutans* was identified in cattle in south-east England on the basis of history, clinical signs and examination of blood smears (Barnett and Brocklesby, 1971; Brocklesby and Barnett, 1972; Hignett, 1953) but it is now clear that this was a member of the *Theileria buffeli/orientalis* complex. When describing *Babesia EU1/venatorum* for the first time in man, the authors suggested that without molecular investigation the cases caused by this species would have previously been incorrectly attributed to *B. divergens* (Herwaldt *et al.*, 2003).

The advent of molecular methods (and specifically a range of species and genus specific polymerase chain reaction (PCR) and quantitative/real time PCRs) (reviewed by Lempereur *et al.* (2017)) have, in addition to assisting the identification of species, also allowed detection of lower levels of parasitaemia than detectable by routine microscopy (Wang *et al.*, 2015). Both PCR and quantitative/real time PCR rely on the creation of numerous complementary copies of DNA by a heat resistant DNA polymerase in a reaction controlled by changes in temperature, and directed by oligonucleotide primers allowing a specific sequence to be targeted for amplification. If the target DNA is present, even at a low level, if can be amplified to detectable levels by repeating the cycle numerous times; subsequently repeating this using primers complementary to the amplicon already created (semi-nested or nested PCR) offers the opportunity to increase sensitivity further. Conventional and semi-nested/nested PCR differs from quantitative/real time PCR in the method used to detect the amplified DNA. Conventional PCR relies on visualisation of amplified DNA following separation based on the length of the amplified fragment by gel electrophoresis and staining using, for example, ethidium bromide. In contrast quantitative/real time PCR incorporates in the reaction mixture dyes that fluoresce, allowing reaction progress to me monitored and quantified in real time. This can be performed using a dye (for example SYBR^{R} green) that non-specifically integrates with double-stranded DNA or, alternatively, an oligonucleotide complementary to the amplified sequence bearing fluorescent and "quencher" molecules (TaqMan^{R} system). Separation of the oligonucleotide from the complementary *de novo* strand by the advancing DNA polymerase enzyme cleaves the smaller molecule, separating the fluorescent molecule from

the nearby quencher resulting in increasing fluorescence as amplification proceeds.

Molecular techniques are not without disadvantages. For example, because of the high level of sensitivity with PCR, careful attention must be paid to prevent cross-contamination. Moreover, the presence of parasite DNA should not be conflated with the presence of viable parasites (Wang *et al.*, 2015). Additionally, sequence-based species identification is wholly reliant on the correct annotation of sequences held in the database being used. For example, *B. divergens* was incorrectly identified in deer based on comparison with Genbank I8S rRNA sequences that were later shown to be incorrectly annotated (Slemenda et al., unpublished data, cited in Herwaldt *et al.* (2003); (Zintl *et al.*, 2011)).

1.1.2.4 Treatment and prevention

The drug imidocarb (Imazol, MSD Animal Health) is licensed for treatment in the United Kingdom and blood transfusion has been used successfully in clinical cases (Sherlock *et al.*, 2000; Zintl *et al.*, 2014). Prevention is based on tick control and chemoprophylactic use of imidocarb (Zintl *et al.*, 2003). Unusually, young cattle can be infected and develop immunity but not clinical signs while naïve adult animals can develop severe signs (Zintl *et al.*, 2005). This unusual phenomenon is termed 'inverse age resistance' and is a well-recognised feature of *B. divergens* infection, which leads to endemic stability within herds typified by infrequent clinical disease. However, if the epidemiological situation changes, for example by the movement of susceptible adult cattle into an endemically stable area, outbreaks of clinical disease may occur (Adam *et al.*, 1978; Latif and Wells, 1973).

1.1.3 *B. divergens* in red deer

In a study based on PCR amplification of two loci within the 18S rRNA gene of *Babesia* spp. in blood originating from 38 red deer culled in Ireland (Zintl *et al.*, 2011), six samples were found to be infected with a parasite with sequence indistinguishable from *B. divergens* U16370 at both loci examined (logged with Genbank as GU475472 and GU475473). Additionally, three animals were infected

with a *Babesia* sp. similar but not identical to *B. odocoilei* AY046577 (logged with Genbank as GU475474) (see 1.1.6.4 *Babesia odocoilei*-like parasites in red deer) and a further five were infected with a *Babesia* sp. with no matching sequence in the Genbank database (logged with Genbank as GU475475). Although *B. divergens* had been reported in red deer previously, this earlier identification was later found to be an error, due to presence of incorrectly annotated *B. divergens* 18S rRNA sequence in the Genbank database (Slemenda et al., unpublished data, cited in Herwaldt *et al.* (2003); (Zintl *et al.*, 2011)). In the absence of infection studies, complete identity with the reference *B. divergens* sequence U16370 is considered to be a prerequisite for identification of this species. However, since the accurate detection of *B. divergens* in Irish deer in 2011, it has also been found in red deer in Switzerland (Michel *et al.*, 2014) and Austria (Cezanne *et al.*, 2017) indicating that *B. divergens* is present in red deer in many locations in Europe.

1.1.4 *B. divergens* in humans

The first recorded human case attributed to *B. divergens* was described in 1957 (Skrabalo and Deanovic, 1957) and since then a number of species have been reported which cause disease in humans. In North America *Babesia microti* is the most common causative agent of human babesiosis, while in Europe *B. divergens* is less prevalent but may potentially result in more severe or fatal disease in splenectomised or immunocompromised individuals (Gray *et al.*, 2010). There is also evidence that immunocompetent humans can also be infected causing mild to moderate illness (Martinot *et al.*, 2011). In Scotland, a fatal case of human disease attributed to *B. divergens* occurred in 1978 (Entrican *et al.*, 1979b; Entrican *et al.*, 1979a).

1.1.5 Other *Babesia* and *Theileria* spp. identified in the UK

Significantly, in light of the results of this study (see 4.3 Results, discussed in 4.4.1 *Babesia venatorum* in Scottish sheep), a sheep was identified in Scotland that had a small *Babesia* sp. thought to be *Babesia capreoli* (Reid *et al.*, 1976; Purnell *et al.*, 1981). At the time, molecular methods for confirmation were not available, raising the possibility that a *Babesia* sp. not characterised by molecular means may still be present in present in Scottish sheep.

Additionally, the large *Babesia* sp. *Babesia motasi* has been identified and characterised in sheep in the United Kingdom (Lewis and Herbert, 1980; Lewis *et al.*, 1981; Alani and Herbert, 1988b; Alani and Herbert, 1988c), and both *Theileria ovis* (Lewis and Purnell, 1981) and *Theileria recondita* (Alani and Herbert, 1988a) have been identified based on morphological and transmission studies. To date, these three species have been identified only in Wales. They all share the tick vector *Haemaphysalis punctata* and are considered to be of low pathogenicity.Other tick-borne parasitic protozoa have been detected in the UK (but not Scotland), namely *Babesia major* (Brocklesby and Irvin, 1969; Brocklesby and Barnett, 1970; Barnett and Brocklesby, 1971) and a member of the *Theileria buffeli/orientalis* complex (Barnett and Brocklesby, 1971; Brocklesby and Barnett, 1972; Hignett, 1953). Both are considered to be of low pathogenicity and have the tick *Haemaphysalis punctata* as their vector.

1.1.6 Other *Babesia* and *Theileria* spp. identified in Europe

1.1.6.1 *Babesia venatorum*

Babesia venatorum, initially known as *Babesia* sp. EU1, is a zoonotic species first described in man (Herwaldt *et al.*, 2003). The main vertebrate host of *B. venatorum* in continental Europe is the roe deer (*Capreolus capreolus*). Infection was initially detected in Slovenia (Duh *et al.*, 2005b) then Italy (Tampieri *et al.*, 2008) and France (Bonnet *et al.*, 2007a; Bonnet *et al.*, 2009). This work indicated that *Ixodes ricinus* was the tick vector of *B. venatorum*. Most recently two larger-scale surveys of wild ruminants in Switzerland (Michel *et al.*, 2014) and Italy (Zanet *et al.*, 2014) have further confirmed the central role of the roe deer. *Babesia venatorum* has not been detected in red deer (Zintl *et al.*, 2011; Michel *et al.*, 2014) although an infected male *I. ricinus* was found on a red deer in Belgium (Lempereur *et al.*, 2012b). In this case it was suspected the infection was acquired from a previous host.

Infection with multiple *Babesia* species including *B. venatorum* has been demonstrated recently (Michel *et al.*, 2014) using a PCR (polymerase chain reaction) approach incorporating *B. venatorum*-specific primers (Hilpertshauser *et al.*, 2006). In addition to roe deer, other vertebrate hosts have also been identified for *B. venatorum* suggesting it is not limited to a single vertebrate

host. These other species have included alpine chamois (*Rupicapra rupicapra*) and alpine ibex (*Capra ibex*) (Michel *et al.*, 2014) and a forest reindeer (*Rangifer tarandus fennicus*) (Kik *et al.*, 2011).

In addition to the detection of *B. venatorum* in vertebrate hosts in Europe, it has also been detected extensively in its invertebrate tick vector. Infected ticks have been found infesting sheep in Switzerland (Hilpertshauser *et al.*, 2006), and cattle in Belgium (Lempereur *et al.*, 2012a) raising the possibility that cattle could be a reservoir host. To date, no record of detection in either cattle or sheep has been found. *Babesia venatorum* isolated from roe deer has been maintained in culture using sheep erythrocytes (Bonnet *et al.*, 2009), suggesting that the species had at least the potential to infect and survive in an ovine host.

Babesia venatorum has been identified in the United Kingdom in 4 of 742 ticks removed from companion animals (Smith *et al.*, 2013), while in Belgium it was first detected by examining ticks removed from companion animals (Lempereur *et al.*, 2011). In Norway, *B. venatorum* was found to predominate among ticks collected from the environment (Oines *et al.*, 2012) and was also detected in ticks removed from migratory birds (Hasle *et al.*, 2011), which would represent a realistic means of transferring infection across the North Sea to Scotland.

1.1.6.2 *Babesia major*

Babesia major has been found in cattle the United Kingdom (Barnett and Brocklesby, 1971; Brocklesby and Barnett, 1970; Brocklesby and Irvin, 1969) and has the tick *H. punctata* as its vector; the significance of highly similar sequences to those of *B. major* deposited in Genbank detected by molecular means in *Ixodes ricinus* collected from red deer (Hilpertshauser *et al.*, 2006) and the environment (Bonnet *et al.*, 2014) is unclear. Additionally, *B. major* has been reported in Europe (Uilenberg, 2006), specifically in France (Criado-Fornelio *et al.*, 2009; Bonnet *et al.*, 2014), Switzerland (Hilpertshauser *et al.*, 2006), and in Turkey (Altay *et al.*, 2008) and China (Liu *et al.*, 2008).

1.1.6.3 *Babesia capreoli*

Initially described as occurring in roe deer in Germany (Enigk and Friedhoff, 1962), *Babesia capreoli* was subsequently re-described and compared with the

closely related species *B. divergens* by Malandrin *et al.* (2010). They concluded that this was indeed a separate species that posed no threat to either livestock or humans and that it differed at three nucleotide positions of its 18S gene sequence from that of *B. divergens*.

1.1.6.4 *Babesia odocoilei*-like parasites in red deer

In addition to providing molecular confirmation of *B. divergens* in wild red deer, Zintl *et al.* (2011) also unexpectedly found a *Babesia* spp. sharing 98 % similarity in the amplified sequence (GenBank GU475474) with *Babesia odocoilei* (GenBank AY046577) in samples from three of 38 deer examined from Irish red deer. *Babesia odocoilei* is described as affecting white-tailed deer (*Odocoileus virginianus*) in the southern United States, initially by (Emerson and Wright, 1968; Emerson, 1970). The *B. odocoilei* reference sequence, GenBank AY046577, resulted from re-sequencing of the Engeling (Holman *et al.*, 1988) and Brushy Creek (Holman *et al.*, 2000) isolates of this parasite by Herwaldt *et al.* (2003).

1.2 Anaplasma phagocytophilum

1.2.1 Introduction

In the course of the experimental investigation of the transmission of "louping-ill" in sheep in the early 1930s, a tick-transmitted infective agent distinct from the louping-ill virus, capable of causing persistent infections typified by fever and low mortality ("tick-borne fever"), and suggested to facilitate clinically significant louping-ill virus infections, was demonstrated (Macleod, 1932; Gordon *et al.*, 1932a; Gordon *et al.*, 1932b). It was subsequently identified in granular leukocytes and monocytes of infected animals (Gordon *et al.*, 1940; Foggie, 1951). Since that time the bacteria has been known as *Rickettsia phagocytophila* (Foggie, 1951), *Cytoecetes phagocytophila* (Foggie, 1962) and *Ehrlichia phagocytophila* (Philip, 1974). Finally, following the recognition that it was the same organism as the human granulocytic ehrlichiosis (HGE) agent (Chen *et al.*, 1994) and *Ehrlichia equi* (Gribble, 1969), these were united as *Anaplasma phagocytophilum* (Dumler *et al.*, 2001) in the Order Rickettsiales and Family Anaplasmataceae (Figure 1-3).

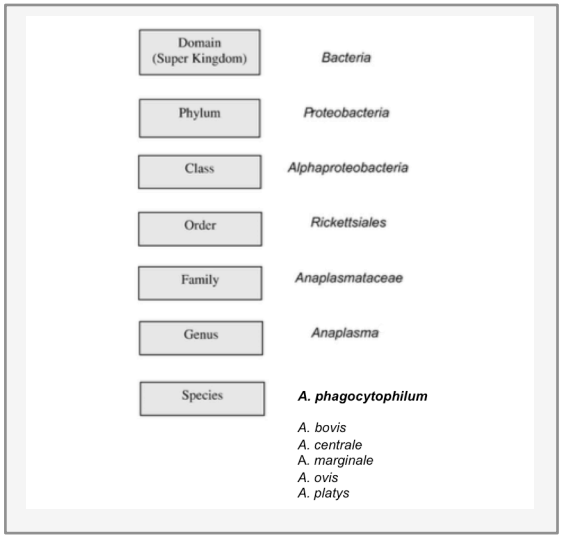


Figure 1-3 Position of *A. phagocytophilum* within Order Rickettsiales and Family Anaplasmataceae

Based on Dumler *et al.* (2001) and adapted from (Battilani *et al.*, 2017)

Other members of the genus *Anaplasma* affecting both domestic and wild ruminants include *Anaplasma bovis* (Donatien and Lestoquard, 1936), *Anaplasma centrale* (Theiler, 1911) and *Anaplasma marginale* (Theiler, 1910), affecting cattle and *Anaplasma ovis* (Bevan, 1912), affecting sheep. Additionally, *Anaplasma platys* (Harvey *et al.*, 1978) affects dogs. All species typically occur in tropical and subtropical regions, are transmitted by ticks of a variety of species and are obligate intracellular bacteria of blood cells. In contrast to *A. phagocytophilum* that infects granulocytes, *A. bovis* infects monocytes, *A. centrale*, *A. marginale* and *A. ovis* infect erythrocytes and *A. platys* platelets (Battilani *et al.*, 2017).

In the United Kingdom and Europe, *A. phagocytophilum* causes tick-borne fever in sheep and pasture fever in cattle. Both result in a similar clinical presentation which features high fever, recurrent bacteraemia, neutropaenia, lymphocytopaenia, thrombocytopaenia, and immunosuppression (Woldehiwet, 2006).

1.2.2 Vectors

In Europe the vector of *A. phagocytophilum* is the three-stage three-host hard tick *I. ricinus*. Early work established that the adults and nymphs of *I. ricinus* transmit the causal agent of tick-borne fever and that infection could not be transmitted from an adult female tick to its progeny but that it could be transmitted between stages of the development from larvae to nymph and on to adult (MacLeod and Gordon, 1933; MacLeod, 1936). As a result, although clearly the vector, *I. ricinus* is not considered as a reservoir host for *A. phagocytophilum* and maintenance of the bacteria therefore relies on persistence in the vertebrate host (Dugat *et al.*, 2015).

Under field conditions, rates of transmission of *A. phagocytophilum* from infected sheep to ticks, determined by PCR following moulting to the next tick instar, can be as high as 28 % for nymphs following moult from engorged larvae and 52 % for adult ticks following moult from engorged nymphs (Ogden *et al.*, 2003). Ogden *et al.* (2003) also found, as may have been expected, increasing host bacteraemia led to increased levels of transmission. An even greater positive effect was observed with increasing numbers of co-feeding adult female ticks. The authors suggested that sheep to tick transmission efficiency might be broadly increased by numbers of feeding ticks through modulation of both infection and numbers of both circulating and localised (cutaneous) neutrophils with a possible role for co-feeding transmission.

That sheep acquire resistance to *Ixodes ricinus* has been demonstrated experimentally (Abdulamir and Gray, 1987), however in field conditions the situation appears to be more complex, with alterations in host immune response at times of maximal adult feeding suggested to underlie apparently reduced resistance at these times (Ogden *et al.*, 2002b). As a result, a complex non-linear relationship is understood to exist between feeding tick numbers and onward transmission of *A. phagocytophilum*.

Molecular surveys in Scotland based on the PCR amplification of the 16S gene of *A. phagocytophilum* found prevalences of between 0.27 % and 2.0 % (Alberdi *et al.*, 1998) and 3.0 % (Walker *et al.*, 2001) in questing *I. ricinus*. These figures are comparable with those of the UK as a whole, where the range of infection

prevalence in questing *I. ricinus* was between 0.27 % (Alberdi *et al.*, 1998) and 9.0 % (Ogden *et al.*, 1998), based in both cases on PCR amplification of sections of the 16S rRNA gene.

1.2.3 Vertebrate hosts

In contrast to the other members of the genus, *A. phagocytophilum* is capable of causing disease, and has been detected by molecular means, in a relatively diverse range of vertebrate hosts and locations.

1.2.3.1 Sheep

Examination of sheep using molecular methods has revealed a wide range of prevalences of *A. phagocytophilum* infection. A longitudinal molecular survey of sheep in the UK (Ogden *et al.*, 2002a) found an infection rate of 38 % in sheep in North Wales. A comparable rate of infection (37.5 %) was found in Norway utilising nested PCR targeting the 16S and *msp4* genes (Stuen *et al.*, 2013b). Where molecular surveys have been carried out elsewhere in Europe, infection rates have been lower, for example 11.6 % in Denmark (Kiilerich *et al.*, 2009), 4 % in Germany (Scharf *et al.*, 2011) and between 3 % and 11.5 % in Italy (Torina *et al.*, 2008a; Torina *et al.*, 2008b; Torina *et al.*, 2010).

Early in the course of the investigation of tick-borne fever it was recognised that the blood of a sheep recovered from the initial febrile and clinically apparent phase of the infection remains infective to other sheep for protracted periods, for example between 35 and 70 days after recovery (MacLeod and Gordon, 1933). Additionally, it was found that ticks could transfer infection after feeding on a sheep five weeks following its recovery (MacLeod and Gordon, 1933). Blood from a naturally infected sheep was found to be capable of infecting susceptible sheep up to 25 months from the original field infection, confirming the presence of *A. phagocytophilum* for this period (Foggie, 1951). More recently *A. phagocytophilum* was detected by quantitative/real-time PCR (qPCR) (targeting the *p44* gene) in the blood of three previously infected sheep in excess of 300 days post-infection (Thomas *et al.*, 2012). Interestingly, this experiment found that following the initial clinically apparent phase of infection associated with a bacteraemia lasting approximately 15 days, 15 to 20 distinct

recurrent episodes of less severe and clinically inapparent bacteraemia followed lasting one to two days (61 % of recurrences) or three days or more (39 % of recurrences).

1.2.3.2 Red deer (*Cervus elaphus*)

In the United Kingdom, the blood of a small number of red deer was examined using quantitative/ real-time PCR (qPCR) targeting the *msp2* gene as part of an investigation of *A. phagocytophilum* in deer in the New Forest. Four of the five animals examined were positive by this method (Robinson *et al.*, 2009). Comparably high rates of infection of red deer have been found in Norway (87.5 %) (Stuen *et al.*, 2013b), the Czech Republic (86.0 %), Austria (66.7 %), Slovakia (53.1 %) and Poland (50.9 %).

Red deer have been experimentally infected with *A. phagocytophilum*. Following a moderate increase in temperature in the first 21 days of the infection no further clinical signs were observed, but the pathogen could still be detected in one animal 91 days post-infection (Stuen *et al.*, 2001). This suggests that, similar to sheep, persistent infection may occur in this species.

Importantly, early investigators were able to infect sheep with a strain originating in red deer from the Isle of Rhum, with similar results to those occurring with sheep-derived strains (Foggie, 1962) suggesting that there might be some commonality of the strains infecting both species. This was further investigated with similar results in Norway where susceptible lambs were infected by an *A. phagocytophilum* isolate originating from red deer and developed typical clinical signs of tick-borne fever (Stuen *et al.*, 2010).

1.2.3.3 Roe deer (*Capreolus capreolus*)

In the UK, including Scotland, roe deer are frequently infected by *A. phagocytophilum* (Bown *et al.*, 2009; Robinson *et al.*, 2009; Alberdi *et al.*, 2000).

Alberdi *et al.* (2000) examined blood and tissue from animals originating throughout United Kingdom. In this study IFAT serology was carried out on 102 roe deer blood samples, including 88 originating from Scotland, and 59 samples

(58 %) were found to be serologically positive for *A. phagocytophilum*, including 47 cases from Scotland (53 %). Additionally, conventional PCR targeting the *groE* gene of *A. phagocytophilum* identified 32/84 (38 %) positive blood samples and 24/82 (29 %) positive spleen samples. The situation for samples originating from Scotland was similar, where 26/70 (37%) of blood and 19/68 (28%) of splenic samples were found to be positive.

In a molecular survey of 279 roe deer originating from the Kielder forest in the North of England, utilising qPCR targeting the *msp2* gene, Bown *et al.* (2009) found 47.3 % of roe deer were infected by *A. phagocytophilum*. In a smaller survey, again utilising qPCR targeting the *msp2* gene, examining multiple deer species in the New Forest in southern England, one in five roe deer (20 %) was found to be infected by *A. phagocytophilum* (Robinson *et al.*, 2009).

In Europe, molecular surveys of roe deer have revealed variable but often high rates of infection in this species (reviewed by Stuen *et al.* (2013a)), including in excess of 90 % of animals tested in Germany (Scharf *et al.*, 2011; Overzier *et al.*, 2013).

Blood from both roe and fallow deer originating in the New Forrest has been used to infect susceptible sheep and cattle previously (McDiarmid, 1965), again suggesting some commonality between *A. phagocytophilum* strains infecting deer and domestic ruminants. Conflicting with this early evidence, more recent studies from Europe suggest that the strains found in roe deer are different from those affecting either humans, dogs, and horses, or domestic ruminant (Dugat *et al.*, 2015). This is based on comparison of PCR-amplified sequences of the *A. phagocytophilum groESL* operon (Rymaszewska, 2008), *ankA* gene (Scharf *et al.*, 2011), by multi-locus variable number tandem repeat (VNTR) analysis (Dugat *et al.*, 2014) and multi-locus sequence typing (MLST) (Chastagner *et al.*, 2014; Huhn *et al.*, 2014).

1.2.3.4 Other host species

No large-scale molecular survey of *A. phagocytophilum* in cattle has been carried out in the United Kingdom, however a number have been carried out in Europe with infection rates generally lower than those in sheep, red and roe

deer (reviewed by Stuen *et al.* (2013a)). This includes rates of 5.5 % in the Czech Republic (Hulinska *et al.*, 2004), 20.0 % in France (Laloy *et al.*, 2009), 4 % and 13 % in Switzerland (Hofmann-Lehmann *et al.*, 2004), 17 % in Italy (Torina *et al.*, 2008b) and 13 % and 19 % in Spain (de la Fuente *et al.*, 2005b; Naranjo *et al.*, 2006).

Early investigators succeeded in infecting goats with the agent of tick-borne fever and producing clinical signs typical of the disease (MacLeod and Gordon, 1933). *Anaplasma phagocytophilum* has been demonstrated in feral goats in southern Scotland by infection of susceptible sheep with blood from captured animals resulting in signs typical of tick-borne fever (Foster and Greig, 1969). Molecular surveys have revealed low rates of infection in goats in Switzerland (5.6 %) (Silaghi *et al.*, 2011) and Italy (up to 3.5 %) (Torina *et al.*, 2008a).

Additionally, *A. phagocytophilum* has been detected in the UK and Europe in a wide range of rodents, insectivorous mammals, birds and other domestic species such as dogs, cats and horses (reviewed by Stuen *et al.* (2013a)).

1.2.4 Zoonotic infection

Human infection by *A. phagocytophilum* was first described in six patients, two of whom died, in the eastern American states of Minnesota and Wisconsin (Chen *et al.*, 1994). In recognition of the presence of the infective agent in granulocytes (Figure 1-5) the disease was termed “human granulocytic ehrlichiosis” (HGE) and the infective agent “human granulocytic ehrlichia”. This terminology continued until it was recognised as being the same species as that causing equine ehrlichiosis in horses and tick-borne fever in ruminants and all were renamed *Anaplasma phagocytophilum* (Dumler *et al.*, 2001).

Clinical signs and laboratory findings in humans are non-specific and include fever and one or more of headache, myalgia, malaise, anaemia, leukopaenia, thrombocytopaenia or elevated hepatic transamidases (Chen *et al.*, 1994; Dahlgren *et al.*, 2015).

In the United States, where human anaplasmosis has been notifiable since 2000, in the years between 2008 and 2012 there were 8,896 human cases in 38 states,

with the highest reported number from the eastern states of Minnesota, Wisconsin and Rhode Island (Dahlgren *et al.*, 2015). Where reported, hospitalisation was required in 31 % of cases and the case fatality rate was measured to be 0.3 %.

Human anaplasmosis is less common in Europe having been reported mainly in Slovenia and Sweden (Strle, 2004) with fewer reports in other countries, such as France (Edouard *et al.*, 2012). In the United Kingdom antibodies to *A. phagocytophilum* have been detected in humans following tick bites, including one patient from the south-west of Scotland (Sumption *et al.*, 1995). Additionally, Hagedorn *et al.* (2014) describe a case of human anaplasmosis acquired in Scotland in an immunocompetent patient experiencing fever, malaise, myalgia and severe headache following tick bites. This case was diagnosed based on a four-fold or greater increase in Immunoglobulin M (IgM) and Immunoglobulin G (IgG) titres to *A. phagocytophilum*. *Anaplasma phagocytophilum* DNA could not be amplified from this patient's blood, but was amplified from the tick, which had been removed and retained by the patient. In this case a rapid response to doxycycline was observed. The latter case confirms that there are *A. phagocytophilum* variants present in Scotland capable of causing human anaplasmosis.

1.2.5 Clinical aspects of infection in sheep

Infection with *A. phagocytophilum* causes tick-borne fever in sheep, often occurring immediately after introduction to tick-infested pasture (Woldehiwet, 2006). This is typified by high fever with resultant dullness and reduced appetite together with recurrent bacteraemia, neutropaenia, lymphopaena (Figure 1-4), thrombocytopaenia and immunosuppression (Woldehiwet, 2006). Pathological changes are limited to splenomegaly (Gordon *et al.*, 1932b).

Although clinical signs associated with the pyrexia induced by tick-borne fever are relatively mild, for example dullness and reduced feeding, it has been recognised that in a hill environment this alone could have significant and potentially fatal consequences (Brodie *et al.*, 1986).

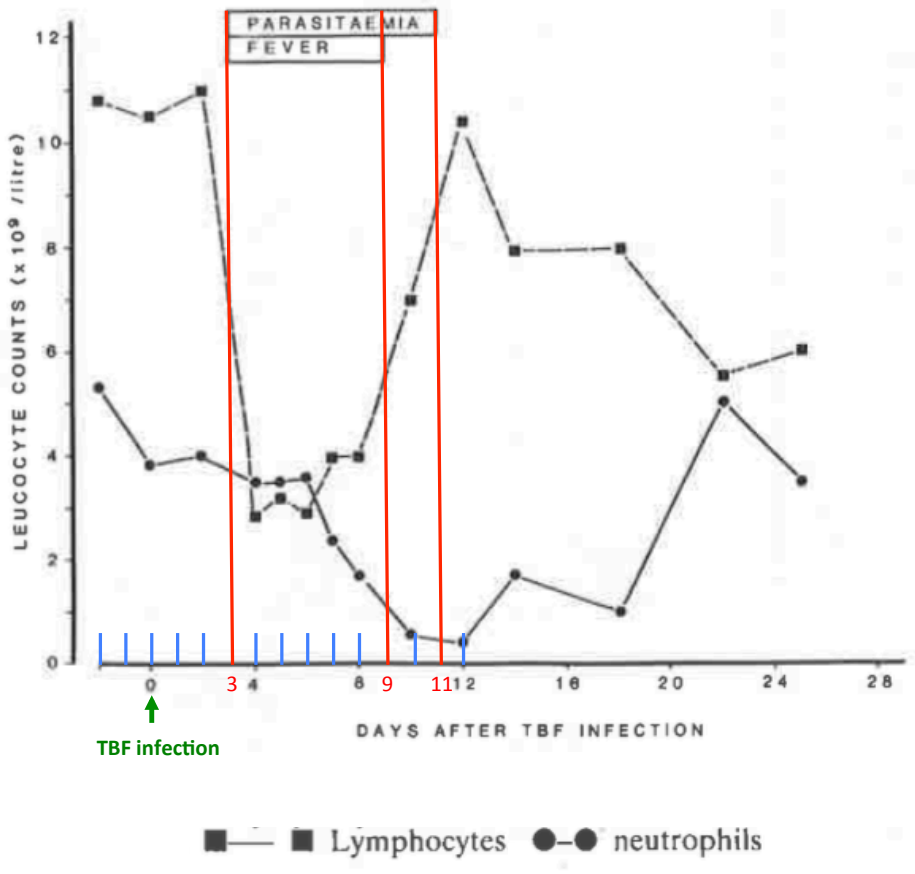


Figure 1-4 Typical response to *A. phagocytophilum* infection in sheep

(Modified from Brodie *et al.* (1986)). The onset of fever and bacteraemia (here referred to as parasitaemia, as was the convention at the time of publication) is associated with precipitous declines in neutrophil and lymphocyte counts.

As a result of the reduction in leukocyte and particularly neutrophil numbers and function, infection with *A. phagocytophilum* in domestic ruminants results in immunosuppression (Woldehiwet, 2006; Woldehiwet, 2010). This predisposes livestock to other bacterial and viral infections causing significant morbidity and mortality, again with associated welfare and economic costs. Bacterial infections include *Staphylococcus aureus* (causing tick pyaemia) and the Pasteurellaceae (specifically *Mannheimia haemolytica*) and viral infections louping ill virus, orf virus and parainfluenza-3 virus (PI-3).

1.2.5.1 Tick pyaemia

Tick pyaemia is the most common and most severe outcome following tick-borne fever (Woldehiwet, 2006). The association between tick infestation and the

development of multifocal abscessation is long standing. Whilst investigating louping ill in sheep in Northumberland, a disease characterised by the presence of abscesses in multiple organs in sheep infested by large numbers of ticks was described (M'Fadyean, 1894). More recently it has been recognised as frequently affecting lambs of between two and eight weeks of age (Foggie, 1962).

Tick pyaemia results from systemic dissemination of *Staphylococcus aureus* enabled by the immunosuppression resulting from tick-borne fever and leads to multifocal abscess formation. When joints or the vertebral column are affected, lameness and/or paralysis results (Foggie, 1962) while the presence of abscesses in other organs can result in thin, dull or poorly-thriving lambs (Brodie *et al.*, 1986).

In experimental infection, timing of inoculation with *Staphylococcus* spp. relative to infection with *A. phagocytophilum* appeared vital with regards to whether tick pyaemia resulted. Intravenous injection of *Staphylococcus* spp. either alone or simultaneously with *A. phagocytophilum* failed to produce tick pyaemia, while subsequent co-infection with *Staphylococcus* spp. at the onset of neutropaenia resulting from earlier *A. phagocytophilum* infection resulted in pyaemia (Foggie, 1956; Foggie, 1962; Foggie, 1957; Brodie *et al.*, 1986) suggesting the neutropaenia was prerequisite (Foggie, 1962).

In the search for the source of the Staphylococcal infection in field cases, it was found that *I. ricinus* was incapable of long-term internal carriage of *Staphylococcus* spp. following feeding on a septicaemic host (Foggie, 1947; Foggie, 1962) suggesting it did not act as a vector of this disease. Additional supportive evidence for this assertion was the difficulty encountered in culturing *Staphylococcus* spp. from engorged ticks feeding on animals suffering from tick pyaemia, and the absence of the bacteria from ticks collected on farms where tick pyaemia occurred (Foggie, 1947). *Staphylococcus* spp. could be retrieved from the mouth, nose, vagina or skin of the udder or inner thigh of ewes, or the skin of lambs with no difference in numbers carrying the bacteria between tick-free and infested farms (Foggie, 1947). Additionally, comparison of these isolates with *Staphylococcus* spp. from clinical cases of tick pyaemia revealed them to be identical in terms of pigment production, αß haemolysin production, coagulase production and lethality to mice (Foggie, 1947; Foggie, 1962)

suggesting the skin might be the source of the bacteria. Surprisingly, subcutaneous and intradermal injections of *Staphylococcus* spp. largely failed to produce multiple abscesses at distant sites consistent with tick pyaemia in either normal lambs or lambs rendered neutropaenic by *A. phagocytophilum* infection (Foggie, 1957; Foggie, 1962; Brodie *et al.*, 1986). Further attempts to replicate the multiple and disseminated abscessation found in tick-borne fever followed without success. Foggie (1959) performed an experiment where tick were allowed to attach and feed in areas of skin contaminated with both laboratory and field isolates of *Staphylococcus* spp. in both normal and neutropaenic lambs following *A. phagocytophilum* infection. He asserted that the *Staphylococcus* spp. found in tick-borne fever accessed the blood stream by means other than the tick attachment and feeding site (Foggie, 1959; Foggie, 1962). Most recently, Webster and Mitchell (1989) succeeded in experimentally reproducing tick pyaemia by infecting susceptible sheep with *A. phagocytophilum* followed by attachment of ticks previously contaminated with *S. aureus*.

Foggie (1962) estimated that on sheep farms in tick infested areas, the annual incidence was 5 % of the lamb crop with approximately 50 % of affected lambs dying and the remainder failing to fatten. On multiple farms in the south-west of Scotland and Arran, 24 lambs in a control group of 233 lambs either died or developed tick pyaemia (Brodie *et al.*, 1986). The authors of this study also estimated that 300,000 lambs of ewes extensively grazing hill land would develop tick pyaemia resulting in death or failure to produce any profit (Brodie *et al.*, 1986).

1.2.5.2 Louping ill virus

Early workers suspected that infection with the agent of tick-borne fever and louping ill simultaneously potentiated the effects of louping ill virus (Gordon *et al.*, 1932a). This was further investigated by Reid *et al.* (1986) who found that infection with louping-ill virus five days following infection with *A. phagocytophilum* resulted in the deaths of 17 of 18 animals in contrast to the relatively mild effects observed with infection by either agent in isolation. There was increased viraemia, more widely disseminated virus and a reduced humoral response in dually infected animals; additionally systemic fungal infection was found in seven of the 17 animals that died. The authors suggested that in areas

where both infections are endemic, concomitant infection with both agents in susceptible sheep would be unlikely. However, in susceptible sheep introduced to such an area, some of the losses attributed to louping ill virus might be explained by dual infection.

1.2.5.3 Respiratory disease

During early investigations it was observed that sheep occasionally died from pneumonic pasteurellosis following infection by *A. phagocytophilum* (Foggie, 1956). Further investigation confirmed that infection with *A. phagocytophilum* was association with more severe clinical signs and increased fatalities associated with the sheep respiratory pathogens PI-3 virus (Batungbacal and Scott, 1982b) and the bacterial pneumonia caused by *Pasteurella haemolytica* (now *Mannheimia haemolytica*) (Gilmour *et al.*, 1982). Interestingly, infection by *A. phagocytophilum* also appears to increase the risk of systemic pasteurellosis in sheep in Norway (caused by *Bibersteinia trehalosi*, previously *Pasteurella haemolytica* T and *Pasteurella trehalosi*) (Overas, 1983; Overas *et al.*, 1993).

Lambs experimentally infected with PI-3 virus show mild pyrexia and respiratory signs, however when *A. phagocytophilum* and PI-3 virus were given simultaneously, higher pyrexia and greater respiratory distress resulted (Batungbacal and Scott, 1982b). When administration of PI-3 virus was postponed until *A. phagocytophilum* bacteraemia had developed following experimental infection, respiratory signs were most severe and two of ten animals died. Further investigation of these animals revealed gross and microscopic changes suggestive of pneumonic pasteurellosis, and *Pasteurella haemolytica* (now *M. haemolytica* or *B. trehelosi*, not specified) was isolated.

1.2.5.4 Other infectious diseases

Cutaneous ecthyma (cutaneous pustular dermatitis or orf) is a zoonotic skin disease of sheep caused by the orf virus. Experimental infection with *A. phagocytophilum* followed by experimental infection with orf virus led to more severe disease than with the virus alone (Gokce and Woldehiwet, 1999).

1.2.5.5 Reproductive disease

Infection with tick-borne fever late in pregnancy is associated with abortion in susceptible adult sheep moved to tick infested ground (Brodie *et al.*, 1986; Jamieson, 1950; Jamieson, 1947; Harbour, 1945; Stamp *et al.*, 1950; Jones and Davies, 1995; Littlejohn, 1950) and can also lead to the death of the ewe. Additionally, the fertility of male sheep can be adversely affected by *A. phagocytophilum* (Watson, 1964).

1.2.5.6 Sub-clinical effects of infection in sheep

Both the primary and secondary consequences of infection by *A. phagocytophilum* outlined above have the capability to visibly affect both sheep welfare and productivity. Importantly however, it has been demonstrated that even where there are no clinical signs of disease, infection by *A. phagocytophilum* can lead to significant effects on productivity (Stuen *et al.*, 2002). Administration of long-acting oxytetracycline has been shown to lead to improved weight gain in lambs naturally exposed to *A. phagocytophilum* infection on tick-infested pasture suggesting that infection with *A. phagocytophilum* could be suppressing these gains (Brodie *et al.*, 1986).

1.2.5.7 Control in sheep

Methods of control focus on reducing tick infestation by use of acaricidal dips and topical treatments, and strategic use of long acting oxytetracycline (Brodie *et al.*, 1986; Woldehiwet, 2006), both of which are confirmed to reduced morbidity and mortality associated with *A. phagocytophilum* and associated secondary infections.

A commercially produced vaccine (Gavac^{TM}) has been available for some decades for vaccination of cattle against *Rhipicephalus* (*Boophilus*) *microplus*, utilising a recombinant tick mid-gut protein Bm86 (Canales *et al.*, 1997). More recently the focus for tick vaccine research has shifted to tick aquaporins, essential in water homeostasis especially during feeding, in *Rhipicephalus* (*Boophilus*) *microplus* (Guerrero *et al.*, 2014) and, especially relevant to the situation in the UK, *Ixodes ricinus* (Contreras and de la Fuente, 2017).

Breeding for reduced susceptibility to tick infestation has been highlighted as an important area for further research (Bioforsk - Norwegian Institute for Agricultural and Environmental Research, 2013).

Careful consideration of previous and potential exposure to ticks and the likelihood of exposure to ticks at the destination premises before the movement of pregnant sheep is advisable to avoid *A. phagocytophilum*-induced abortion storms and associated losses in pregnant sheep (Woldehiwet, 2006).

1.2.6 Clinical aspects of infection in cattle

Tick-borne fever was first identified in cattle in Somerset in 1948 (Hudson, 1950) where it was characterised as a transient and mild febrile disease associated with an abrupt reduction in milk yield and mild respiratory signs. Following confirmation by microscopy, transmission to susceptible animals followed. Pyrexia typically began on day six or seven post-infection (PI), lasted for three to four days and, in the majority of animals, recurred in subsequent episodes. Increased respiratory rate was associated with the pyrexic phase. These changes were consistent with later field reports of infection (Venn and Woodford, 1956; Tutt and Loving, 1955). Hudson (1950) found that animals became resistant to further challenge for a variable period but after six to twelve months the majority reacted to repeated challenge in an identical way to naïve animals. It was noted that cattle may experience disease in subsequent lactations following re-infection. Similar findings were recorded in Finland by (Tuomi, 1967a), with pyrexia beginning at a mean of 6.9 days PI and lasting for a mean of 3.5 days. A minority of animals experienced secondary episodes. Clinical signs, in addition to fever, were increased respiratory rate, coughing and reduced milk yield. Bacteraemia coincided with pyrexia, affecting neutrophils and eosinophils and occasionally monocytes near the end of this phase. The term “pasture fever” is used to describe this disease in cattle (Woldehiwet, 2006). In common with sheep, experimental and field infections with *A. phagocytophilum* carried out in Northern Ireland by Taylor and Kenny (1980) resulted in a reduced rate of growth in growing cattle. In cattle oxytetracycline was found to eliminate *A. phagocytophilum* following experimental infection (Tuomi, 1967b).

1.2.7 Diagnostic methods

1.2.7.1 Microscopy

During episodes of bacteraemia, *A. phagocytophilum* can be seen within leukocytes. Following staining of blood smears with Romanowsky stains (for example Giemsa, Wright or Leishman stains), organisms appear as one or more round to oval slate grey 1.5 – 6 µm diameter structures within neutrophils, eosinophils or rarely monocytes, consisting of macrocolonies of bacteria within intracytoplasmic vacuoles (termed "morulae") (Figure 1-5, Figure 1-6, and Figure 1-7).

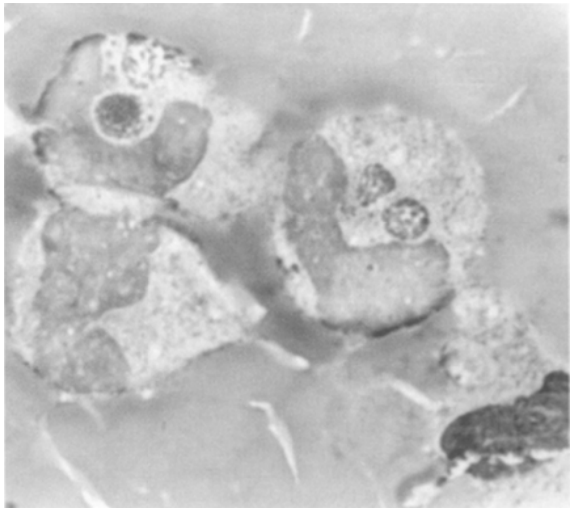


Figure 1-5 Microscopic appearance of *A. phagocytophilum* (Wright stain)

Image from (Chen *et al.*, 1994), illustrating *A. phagocytophilum* morulae in band neutrophils from the blood of a human patient.

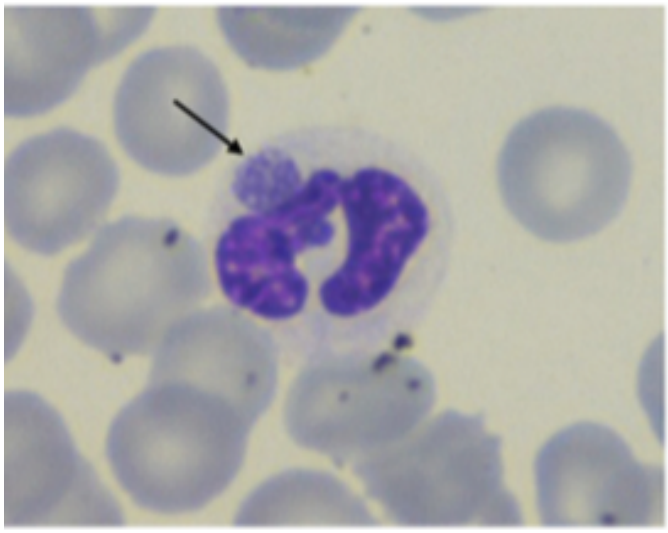


Figure 1-6 Microscopic appearance of *A. phagocytophilum* (May-Grunwald Giemsa stain)

Image from (Battilani *et al.*, 2017), illustrating an *A. phagocytophilum* morula in a neutrophil (black arrow).

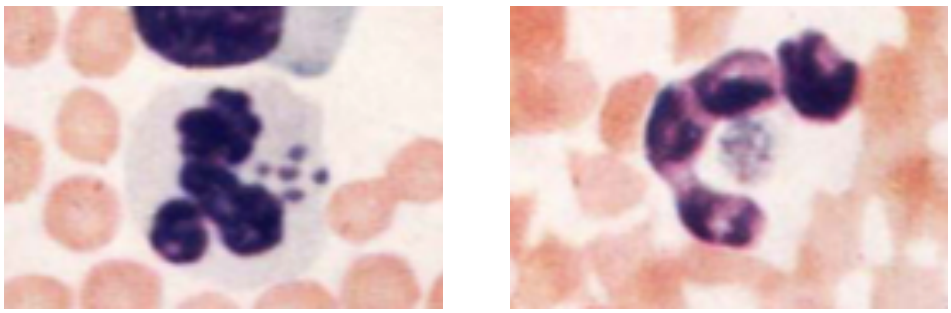


Figure 1-7 Microscopic appearance of *A. phagocytophilum* (Leishman stain)

Images from (Foggie, 1951), illustrating *A. phagocytophilum* in the cytoplasm of neutrophils.

1.2.7.2 Serology

Serological methods have been used in the detection antibody responses to *A. phagocytophilum*, however their use has been somewhat restricted due to the need for experimental infections of sheep or establishment of infection in tick cell lines to generate *A. phagocytophilum* antigen. Techniques developed include complement fixation (Woldehiwet and Scott, 1982b), countercurrent immunoelectrophoresis (Webster and Mitchell, 1988), indirect immunofluorescent antibody test (IFAT) (Paxton and Scott, 1989; Woldehiwet

and Horrocks, 2005) and enzyme-linked immunosorbent assay (ELISA) (Woldehiwet and Horrocks, 2005). With the exception of the ELISA developed by Woldehiwet and Horrocks (2005) that utilised *A. phagocytophilum* antigen originating from either an infected sheep or tick cell line, all ELISAs rely on antigen derived from the blood of infected sheep.

1.2.7.3 Polymerase chain reaction

Similar to the situation with *Babesia* and *Theileria* spp., detection of *A. phagocytophilum*, and investigation of its genetic diversity, relies heavily on molecular techniques including conventional, semi-nested and nested PCR, and quantitative/real-time PCR (see 1.1.2.3 Diagnostic methods). Target loci include the 16S rRNA locus, *groESL* operon, *ankA* gene and major surface protein-encoding genes *msp2* and *msp4* (Battilani *et al.*, 2017; Dugat *et al.*, 2015).

Nested PCR amplification of the 5' end of the *A. phagocytophilum* 16S gene using a nested PCR (primers ge3a and ge10r, and ge9f and ge2) has been described and experimentally demonstrated to be highly sensitive and specific for the detection of *A. phagocytophilum* in samples of human, animal and invertebrate vector origin (Massung *et al.*, 1998).

Recently amplification and sequencing of the *A. phagocytophilum msp4* locus, encoding part of the OMP1/MSP2/P44 outer or surface protein superfamily has been utilised to investigate variants of *A. phagocytophilum* circulating in Norwegian sheep and red deer (Ladbury *et al.*, 2008; Stuen *et al.*, 2013b).

Diagnostic testing for *A. phagocytophilum* in Scotland is currently carried out at the Moredun Research Institute Edinburgh and utilises a real-time PCR assay targeting a segment of the *msp2* gene (based on Courtney *et al.* (2004)).

Recently MLST has been described for *A. phagocytophilum* and utilised in the investigation of the pathogen in cattle, roe deer, horses and dogs (Chastagner *et al.*, 2014) and a range of other potential hosts (Huhn *et al.*, 2014). However, confounding factors such as a multiplicity of infection (MOI) and recombination have confounded efforts to apply MLST methodology (Huhn *et al.*, 2014).

1.2.7.4 Loop-mediated isothermal amplification

Most recently loop-mediated isothermal amplification (LAMP) (Notomi *et al.*, 2000) has been utilised for the detection of *A. phagocytophilum* in humans (Pan *et al.*, 2011), dogs (Lee *et al.*, 2012) and sheep and goats (Wang *et al.*, 2017). Targets for amplification have included the *msp2* gene (Pan *et al.*, 2011), the citrate synthase gene (*gltA*) (Lee *et al.*, 2012) and most recently the 16S gene (Wang *et al.*, 2017). In all cases LAMP was demonstrated to be highly specific and either as sensitive (Lee *et al.*, 2012), or more sensitive than conventional, nested or real-time PCR (Pan *et al.*, 2011; Wang *et al.*, 2017). The utility of the technique for detecting *A. phagocytophilum* in clinical samples was demonstrated by detecting the pathogen in 26 of 42 suspected or confirmed human cases by LAMP (Pan *et al.*, 2011); this compared to one by nested PCR and three by real-time PCR. It was also used to detect *A. phagocytophilum* in 53 of 94 sheep and goat blood samples (Wang *et al.*, 2017), compared to three by conventional and twelve by nested PCR.

CHAPTER TWO
National Survey of Tick-borne Disease

2.1 Introduction

In order to gain an insight into the impact of babesiosis, also know as red water fever (RWF), and tick-borne fever (TBF) and related diseases in Scottish livestock, a two-pronged approach was adopted. Firstly, a large-scale survey of veterinary surgeons working in large animal practice in Scotland was undertaken in order to gauge current opinion on the relative importance of the tick-borne diseases affecting the livestock they treat. Secondly, a cross-sectional study was undertaken of cases of TBD diagnosed by SAC Consulting/Scotland's Rural College (SAC/SRUC) Veterinary Investigation Officers (VIOs). SAC/SRUC VIOs work closely with large animal veterinary surgeons throughout Scotland assisting in the post-mortem and laboratory investigation of disease in livestock and generating passive surveillance data at a national level. Officers are based in eight Disease Surveillance Centres (DSCs) sited across the country (Figure 2-1). In addition to channeling diagnostic information back to large animal veterinary surgeons and onwards to livestock producers, diagnoses are recorded in the Veterinary Investigation Diagnosis Analysis (VIDA) database. These relationships are summarised in Figure 2-2.

Figure 2-1 Locations of SAC/SRUC Disease Surveillance Centres

This joint approach was intended to derive broad information about the present occurrence of TBD in Scotland while raising awareness of the project among large animal veterinary surgeons. It was anticipated that the first-hand knowledge of veterinary surgeons combined with DSC data would help identify 'hot spots' of TBD. It was important to identify such disease 'hot spots', as this would allow field-sampling sites to be identified for a targeted pathogen-discovery study. This was based on the assumption that, if conditions were

conducive to transmission of well-recognised TBD pathogens, previously unidentified pathogens may be found in the same area.

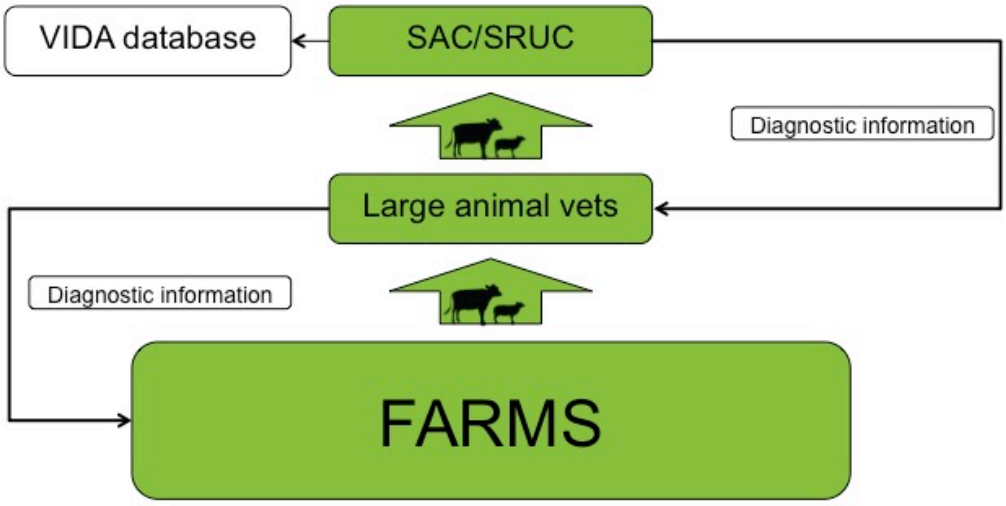


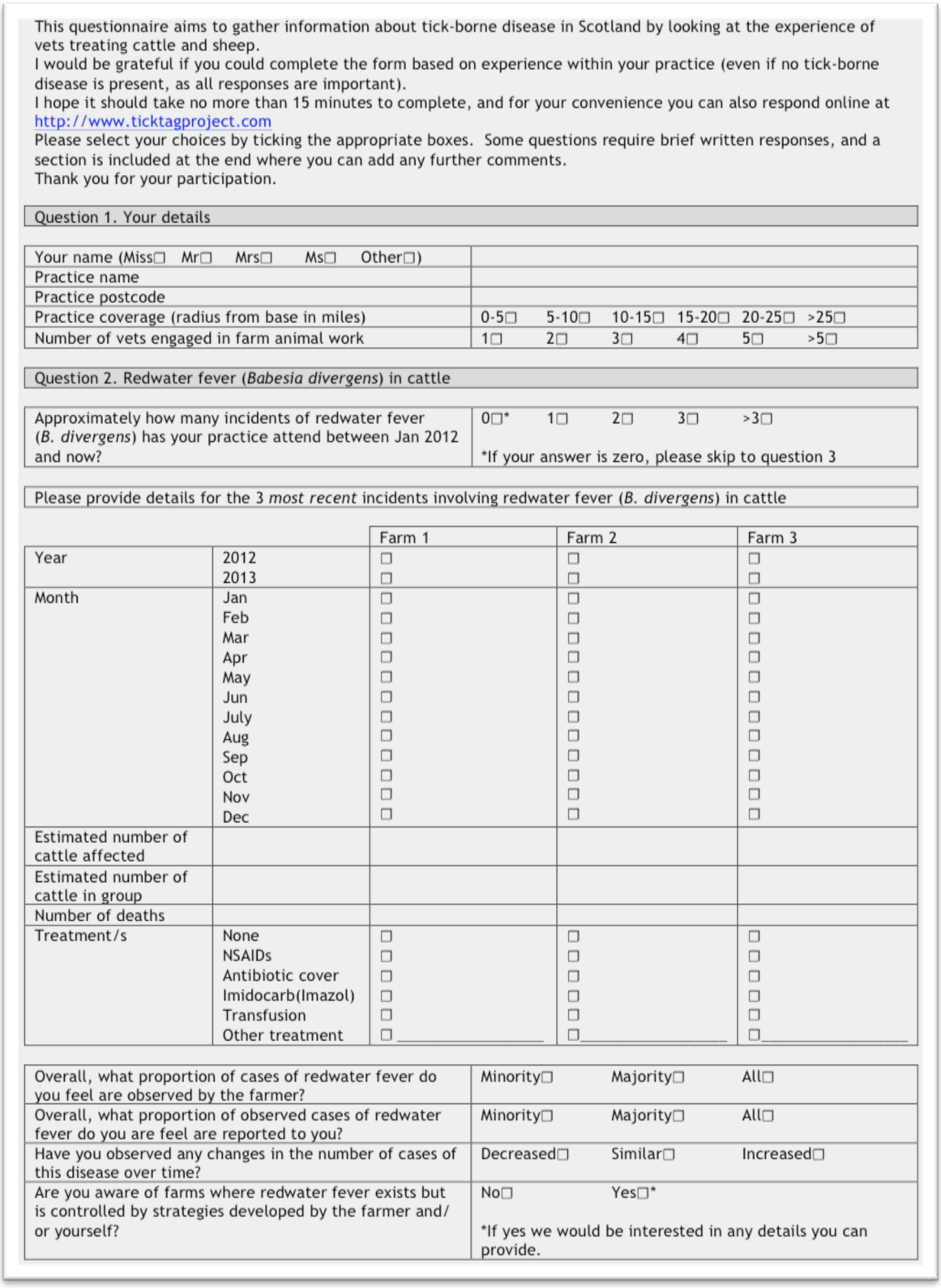
Figure 2-2 SAC/SRUC in relation to large animal veterinary surgeons and producers

Illustrating the large animal veterinary surgeons role in submission of animals to SAC/SRUC DSC post mortem rooms and flow of diagnostic information outwards to large animal vets and farms (VIDA Veterinary Investigation Diagnosis Analysis)

2.2 Materials and methods

2.2.1 National survey of large animal veterinary surgeons on tick-borne disease in Scottish livestock

Examination of practice records provided by the Royal College of Veterinary Surgeons allowed the identification of veterinary practices currently working with livestock. Each of the 100 practices identified was contacted by telephone to identify the member or members of staff primarily carrying out large animal work and the survey directed to these individuals. A letter and survey form (Figure 2-3) was sent to each practice together with information publicising a website which had been constructed to allow completion of the survey online (http://www.ticktagproject.com). If no response was received after a period of two months a reminder letter was sent.



This questionnaire aims to gather information about tick-borne disease in Scotland by looking at the experience of vets treating cattle and sheep.
I would be grateful if you could complete the form based on experience within your practice (even if no tick-borne disease is present, as all responses are important).
I hope it should take no more than 15 minutes to complete, and for your convenience you can also respond online at http://www.ticktagproject.com
Please select your choices by ticking the appropriate boxes. Some questions require brief written responses, and a section is included at the end where you can add any further comments.
Thank you for your participation.

Question 1. Your details

Your name (Miss☐ Mr☐ Mrs☐ Ms☐ Other☐)	
Practice name	
Practice postcode	
Practice coverage (radius from base in miles)	0-5☐ 5-10☐ 10-15☐ 15-20☐ 20-25☐ >25☐
Number of vets engaged in farm animal work	1☐ 2☐ 3☐ 4☐ 5☐ >5☐

Question 2. Redwater fever (*Babesia divergens*) in cattle

Approximately how many incidents of redwater fever (*B. divergens*) has your practice attend between Jan 2012 and now?	0☐* 1☐ 2☐ 3☐ >3☐ *If your answer is zero, please skip to question 3

Please provide details for the 3 *most recent* incidents involving redwater fever (*B. divergens*) in cattle

		Farm 1	Farm 2	Farm 3
Year	2012	☐	☐	☐
	2013	☐	☐	☐
Month	Jan	☐	☐	☐
	Feb	☐	☐	☐
	Mar	☐	☐	☐
	Apr	☐	☐	☐
	May	☐	☐	☐
	Jun	☐	☐	☐
	July	☐	☐	☐
	Aug	☐	☐	☐
	Sep	☐	☐	☐
	Oct	☐	☐	☐
	Nov	☐	☐	☐
	Dec	☐	☐	☐
Estimated number of cattle affected				
Estimated number of cattle in group				
Number of deaths				
Treatment/s	None	☐	☐	☐
	NSAIDs	☐	☐	☐
	Antibiotic cover	☐	☐	☐
	Imidocarb(Imazol)	☐	☐	☐
	Transfusion	☐	☐	☐
	Other treatment	☐ _____	☐ _____	☐ _____

Overall, what proportion of cases of redwater fever do you feel are observed by the farmer?	Minority☐	Majority☐	All☐
Overall, what proportion of observed cases of redwater fever do you are feel are reported to you?	Minority☐	Majority☐	All☐
Have you observed any changes in the number of cases of this disease over time?	Decreased☐	Similar☐	Increased☐
Are you aware of farms where redwater fever exists but is controlled by strategies developed by the farmer and/ or yourself?	No☐	Yes☐* *If yes we would be interested in any details you can provide.	

Figure 2-3 Survey sent to participating veterinary practices

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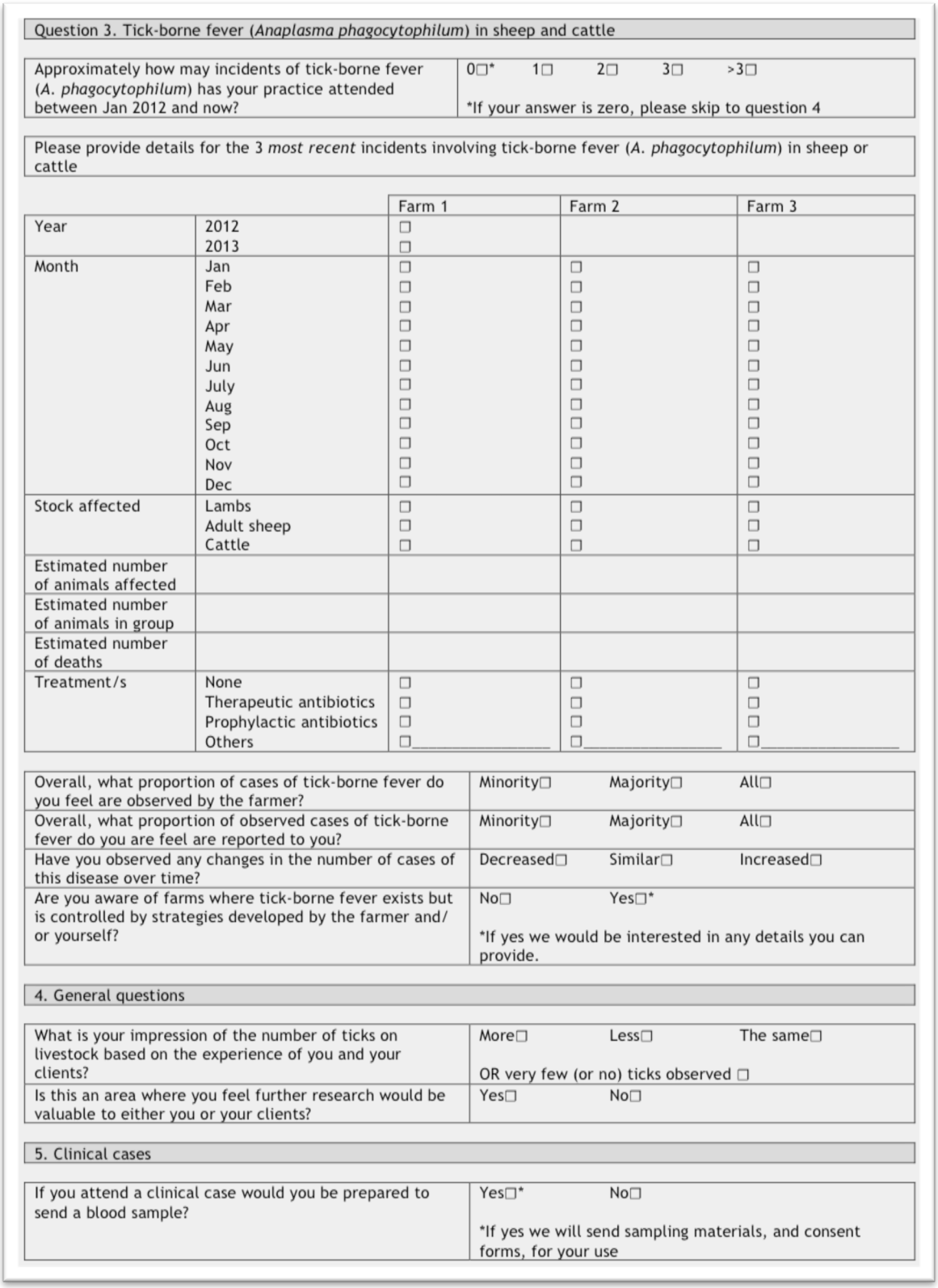

Question 3. Tick-borne fever (*Anaplasma phagocytophilum*) in sheep and cattle

Approximately how may incidents of tick-borne fever (*A. phagocytophilum*) has your practice attended between Jan 2012 and now?	0☐* 1☐ 2☐ 3☐ >3☐ *If your answer is zero, please skip to question 4

Please provide details for the 3 *most recent* incidents involving tick-borne fever (*A. phagocytophilum*) in sheep or cattle

		Farm 1	Farm 2	Farm 3
Year	2012	☐		
	2013	☐		
Month	Jan	☐	☐	☐
	Feb	☐	☐	☐
	Mar	☐	☐	☐
	Apr	☐	☐	☐
	May	☐	☐	☐
	Jun	☐	☐	☐
	July	☐	☐	☐
	Aug	☐	☐	☐
	Sep	☐	☐	☐
	Oct	☐	☐	☐
	Nov	☐	☐	☐
	Dec	☐	☐	☐
Stock affected	Lambs	☐	☐	☐
	Adult sheep	☐	☐	☐
	Cattle	☐	☐	☐
Estimated number of animals affected				
Estimated number of animals in group				
Estimated number of deaths				
Treatment/s	None	☐	☐	☐
	Therapeutic antibiotics	☐	☐	☐
	Prophylactic antibiotics	☐	☐	☐
	Others	☐_____	☐_____	☐_____

Overall, what proportion of cases of tick-borne fever do you feel are observed by the farmer?	Minority☐	Majority☐	All☐
Overall, what proportion of observed cases of tick-borne fever do you are feel are reported to you?	Minority☐	Majority☐	All☐
Have you observed any changes in the number of cases of this disease over time?	Decreased☐	Similar☐	Increased☐
Are you aware of farms where tick-borne fever exists but is controlled by strategies developed by the farmer and/ or yourself?	No☐	Yes☐* *If yes we would be interested in any details you can provide.	

4. General questions

What is your impression of the number of ticks on livestock based on the experience of you and your clients?	More☐	Less☐	The same☐ OR very few (or no) ticks observed ☐
Is this an area where you feel further research would be valuable to either you or your clients?	Yes☐	No☐	

5. Clinical cases

If you attend a clinical case would you be prepared to send a blood sample?	Yes☐*	No☐ *If yes we will send sampling materials, and consent forms, for your use

Figure 2-3 (continued) Survey sent to participating veterinary practices

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6. Further details and comments

7. Contact us

Please return the completed questionnaire to the following address:

We would also welcome any queries or comments, which can be forwarded to this address.

Thank you again for your participation.

Figure 2-3 (continued) Survey sent to participating veterinary practices

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As the responses arrived, the answers were recorded in a spreadsheet, with the following information for each:

1. Whether the practice had elected to participate based on the initial telephone contact, and if so whether it had returned the questionnaire.

2. Whether the practice had experienced a case of babesiosis or tick-borne fever during 2012 or 2013, and if so how many animals were involved and how they were treated. Comments were also examined for evidence that cases had been experienced historically but not during 2012 or 2013.

3. Whether the practice saw ticks on livestock, and if so whether numbers were perceived to have decreased, remained the same, or increased.

In addition to requesting clinical material, the letter asked veterinary surgeons to consider whether they were willing to identify individual farms where more detailed sampling could be undertaken.

2.2.2 SAC/SRUC tick-borne disease diagnoses 2000 - 2013

All diagnoses made by SAC/SRUC VIOs, including those relating to tick-borne disease, are compiled in SAC/SRUC VIDA database, with additional information about the animal and its location. Dr Franz Brulisauer, located at the Inverness DSC, kindly carried out interrogation of the VIDA database. Searches were carried out to identify cases submitted to the SAC/SRUC service where a diagnosis of RWF/babesiosis, TBF/tick-borne fever or the related disease tick pyaemia was recorded. Cases were also identified where a diagnosis of the viral infection louping ill was made. Case data including the date of the diagnosis, the DSC involved, the species of animal and the postcode of the premises of origin were collected.

Maps were generated to illustrate the spatial distribution of identified cases. This was done by creating .kml files at http://www.batchgeo.com that were uploaded to Ordnance Survey (GB) maps using the EDINA Digimap Ordnance Survey Service at http://digimap.edina.ac.uk. Graphs were also created to allow visualisation of the relationship between cases of RWF/babesiosis, TBF/tick-

borne fever and tick pyaemia and the Disease Surveillance Centre involved, the year of diagnosis and the month of diagnosis.

2.3 Results

2.3.1 National survey of large animal veterinary surgeons on tick-borne disease in Scottish livestock

Following examination of the list of practices recorded by the Royal College of Veterinary Surgeons, a total of 100 practices working with livestock in Scotland were identified. At the initial telephone contact three practices indicated they did not wish to participate. Of the remaining practices, 55 responded (43 in the first round, and a further 12 in the second round). The practices responding were widely distributed throughout the Scottish mainland and Islands (Figure 2-4).

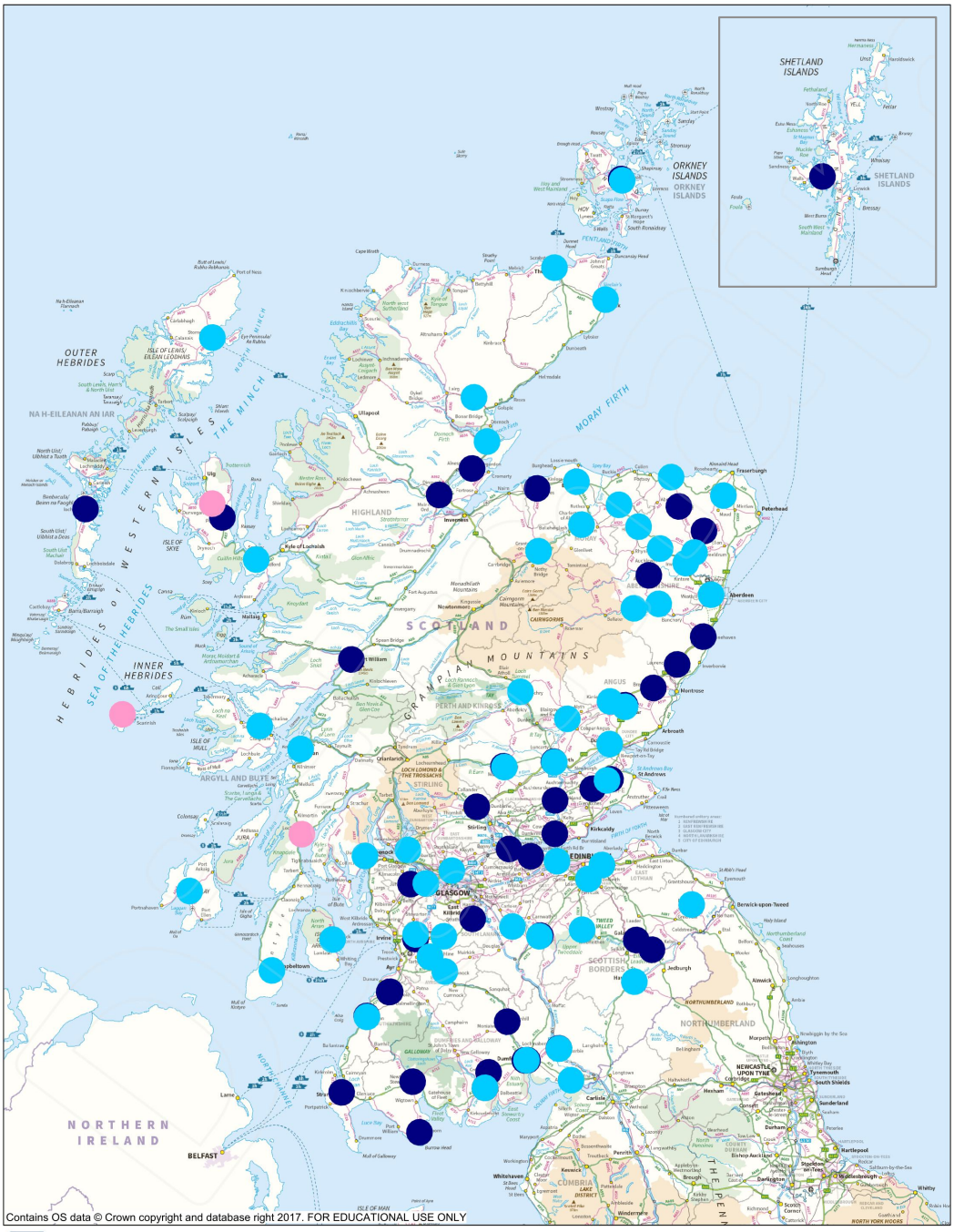


Figure 2-4 Veterinary practices participation and response to the survey

2.3.1.1 Red water fever

Region	Time period	Numbers affected / dead	Treatment	Propn. of cases observed	Propn. of cases reported to vet	Vet's impress. of case numbers	Control strategies employed by farmer	Vet's impress. of tick numbers
Aberdeen-shire	Apr 2012	2 of a group of 40 with 1 death	Imidocarb (Imizol, MSD Animal Health) Antibiotic cover Other (glucocorticoids)	Minority	Minority	Decreased	Tick control for cattle	More ticks observed
Argyll and Bute**	Jul 2012	1 of a group of 40 with no deaths	Imidocarb (Imizol, MSD Animal Health) Other (NSAID)	Majority	All	Not indicated	Spot on treatment for ticks and prophylactic use of Imizol on bought-in animals	More ticks observed
Perth and Kinross	Aug 2012	1 of a group of 20 with no deaths	Imidocarb (Imizol, MSD Animal Health) Antibiotic cover	All	All	Increased	Unaware of control strategies	More ticks observed
Highland*	Sep 2012	1 of a group of 10 with no deaths	Imidocarb (Imizol, MSD Animal Health) Antibiotic cover	Minority	All	Similar	Don't buy in stock from non tick endemic areas	More ticks observed
Highland*	Apr 2013	1 of a group of 30 with 1 death	Imidocarb (Imizol, MSD Animal Health) Antibiotic cover Other (NSAID)	Minority	All	Similar	Don't buy in stock from non tick endemic areas	More ticks observed
Argyll and Bute	May 2013	1 of a group of 10 with no deaths	Imidocarb (Imizol, MSD Animal Health) Antibiotic cover	Majority	Majority	Decreased	Avoiding cattle on affected areas and tick control were necessary	More ticks observed
West Dumbarton-shire	May 2013	2 of a group of 100 with 2 deaths	None	Minority	Minority	Similar	Aware of control strategies but not specified in response	More ticks observed
Argyll and Bute**	July 2013	1 of a group of 50 with 1 death	Imidocarb (Imizol, MSD Animal Health) Other (NSAID) Other (fluids, minerals)	Majority	All	Not indicated	Spot on treatment for ticks and prophylactic use of Imizol on bought-in animals	More ticks observed

Table 2-1 Responses from veterinary surgeons attending cases of red water fever

The same practice attending more than one case is indicated by * and **.

Six practices indicated that veterinary surgeons attended a single case or multiple cases of red water fever on eight occasions between April 2012 and July 2013. A summary of this information is presented in Table 2-1 and Figure 2-5. Four practices attended one case each (located in Aberdeenshire, Perth and Kinross, Argyle and Bute, and West Dumbartonshire) while two practices attended two cases each (located in Argyll and Bute and Highland). The cases occurred between April and September 2012 (n = 4) and April and July 2013 (n = 4). The disease was typically observed affecting a small number of animals

in a group, with deaths occurring on four of the eight occasions (a single animal on three occasions and two animals on a further single occasion). Treatment with Imidocarb (Imazol, MSD Animal Health) was undertaken on all occasions with the exception of one case where no treatment was used. Antibiotic cover was provided on five of the eight occasions and NSAIDs on three of the eight occasions. One animal received glucocorticoids and another supportive therapy with fluids and minerals. No animals were recorded as having received a blood transfusion. Of the six practices that had experienced cases, three felt the farmer observed only the minority of those affected, while two felt the majority of cases were observed and one that all were observed. Of the cases observed by the farmer, four practices felt either the majority (n = 1) or all (n = 3) affected animals came to their attention. The remaining two practices felt only the minority of cases observed by the farmer came to their attention. One practice did not indicate whether they felt the number of cases had changed, while of the remaining five practices, one felt the number of cases had increased, two felt the number was similar and two felt it had decreased. All practices that had experienced cases felt the number of ticks observed on livestock had increased overall. The practices where cases had occurred were able to provide some details about control strategies being used. These included treating cattle with products against tick infestation, avoiding grazing of cattle on affected areas and avoiding the purchase of susceptible stock.

2.3.1.2 Tick-borne fever

Region	Time period	Numbers affected / dead	Treatment	Propn. of cases observed	Propn. of cases reported to vet	Vet's impress. of case numbers	Control strategies employed by farmer	Vet's impress. of tick numbers
Highland	Apr 2012	12 lambs in group of 25 with 10 deaths	Antibiotics (therapeutic + prophylactic) Anti-tick treatment	Minority	Minority	Similar	Not aware of control strategies	More ticks observed
Na h-Eileanan an Iar**	Oct 2012	3 lambs in group of 70 with unknown number of deaths	Antibiotic (therapeutic)	Minority	Minority	Similar	Not aware of control strategies	More ticks observed
Highland*	May 2013	20 lambs in group of 300 with unknown number of deaths	Antibiotics (therapeutic + prophylactic) Anti-tick treatment	Minority	Minority	Similar	Yes but not specified	More ticks observed
Highland*	May 2013	20 lambs in group of 100 with unknown number of deaths	Antibiotics (therapeutic + prophylactic) Anti-tick treatment	Minority	Minority	Similar	Yes but not specified	More ticks observed
Highland*	May 2013	20 lambs in group of 100 with unknown number of deaths	Antibiotics (therapeutic + prophylactic) Anti-tick treatment	Minority	Minority	Similar	Yes but not specified	More ticks observed
Perth and Kinross	May 2012	10 lambs in group of 1500 with 5 deaths	Antibiotic (therapeutic) Anti-tick treatment	Minority	Minority	Similar	Anti-tick treatment at birth	More ticks observed
Na h-Eileanan an Iar**	Jul 2013	1 lamb in group of 50 with no deaths	Antibiotic (therapeutic)	Minority	Minority	Similar	Not aware of control strategies	More ticks observed
Na h-Eileanan an Iar**	Aug 2013	3 lambs in group of 93 with 1 death	Antibiotic (therapeutic)	Minority	Minority	Similar	Not aware of control strategies	More ticks observed

Table 2-2 Responses from veterinary surgeons attending cases of tick-borne fever or related disease

The same practice attending more than one case is indicated by * and **

Four practices indicated that veterinary surgeons attended a single case or multiple cases of tick-borne fever on eight occasions between April 2012 and July 2013. A summary of this information is presented in Table 2-2 and Figure 2-5. Two practices attended one case each (located in Highland, and Perth and Kinross) while two practices attended three cases each (located in Highland and Na h-Eileanan an Iar (Western Isles)). The cases occurred in April and October 2012 (n = 2) and May, July and August 2013 (n = 6). The number of affected animals varied between ten in a group of 1,500 to as many as twelve in a group

of 25. Deaths occurred on each occasion, with only one exception where a single animal in a group of 50 was treated successfully. On one occasion (mentioned above, where twelve lambs of a group of 50 were affected) the number of deaths was considerable, with ten of the twelve animals dying. In all responses, practices indicated that case numbers had remained stable recently. However, all responses suggested that only the minority of cases were observed and only a minority of these reported to the veterinary surgeon. This suggests that the true incidence of disease is much higher than that reported and this issue is likely to be reflected in the overall results of this survey. Antibiotics were administered therapeutically by all of the responding practices, with their use prophylactically mentioned in two of the responses. Topical anti-tick treatments were mentioned by three of the four practices whose responses indicated they had attended clinical cases. As with red water, all practices that had experience of clinical cases indicated tick numbers on cattle and sheep had increased. No practices reported having clinical cases of both red water and tick-borne fever during 2012/13.

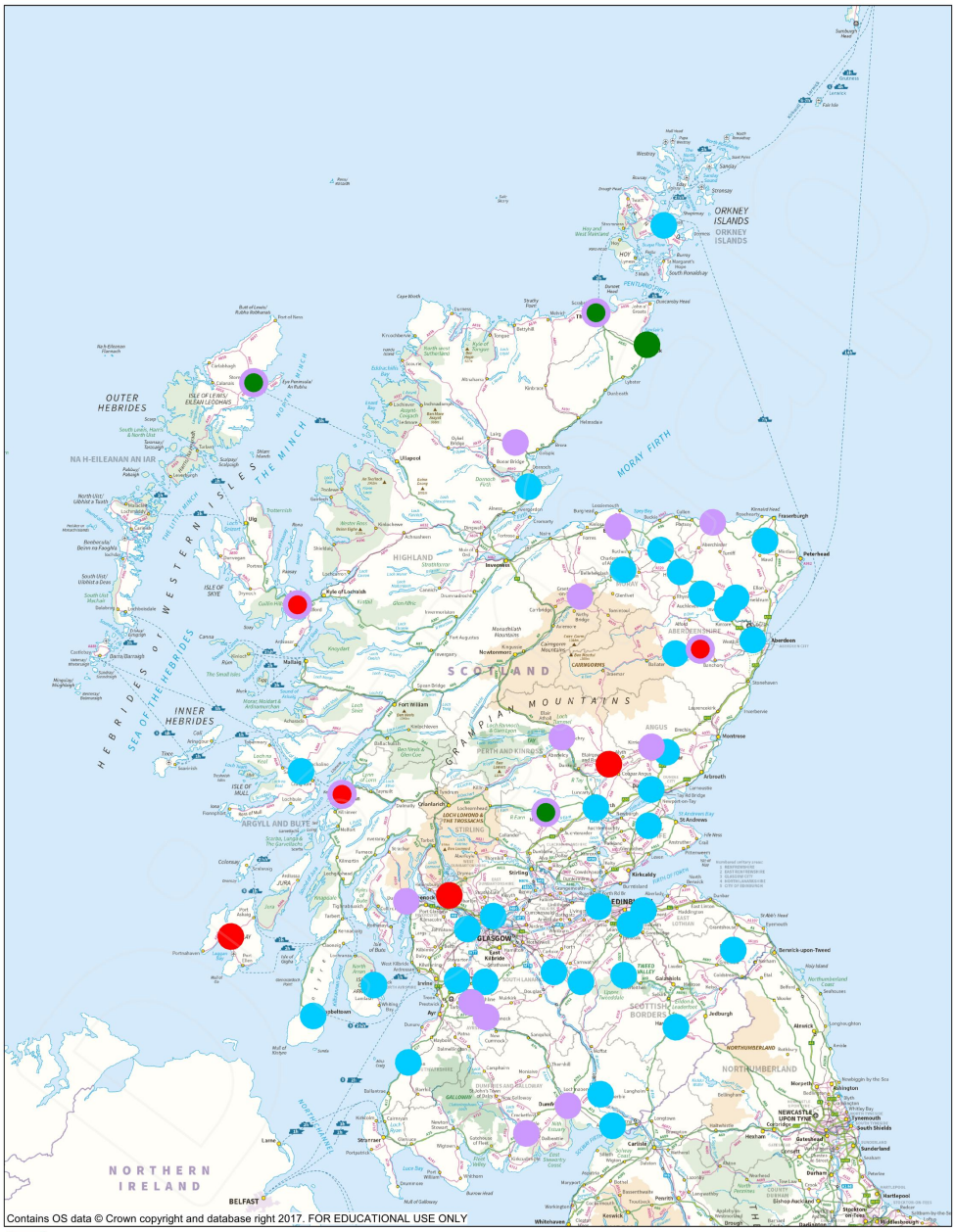


Figure 2-5 Veterinary practices experience of tick-borne disease

Some practices both volunteered a history of tick-borne disease (lilac dot) and attended a case of either red water fever or tick-borne fever (red dot or green dot respectively).

2.3.1.3 Additional points arising from the national survey of large animal veterinary surgeons

A number of themes emerged from examination of the veterinary surgeons' comments.

The survey also included a comments section where veterinary surgeons could record details about cases occurring before 2012 together with any other information they felt relevant. In this section, 17 practices voluntarily indicated they had a history of tick-borne disease prior to 2012. Figure 2-5 provides a summary of practices with no history of tick-borne disease, the 17 practices that had tick-borne disease before 2012/13 and the practices attending clinical cases of red water or tick-borne fever.

Increasing numbers of ticks affecting companion animals (dogs, cats, and horses) and humans were reported. Some areas had experienced ticks for the first time and this was associated with increasing deer numbers in two responses. Many farmers and veterinary surgeons were aware of holdings where clinical cases of red water had occurred and control strategies were enacted, for example not buying in stock, prophylactic use of imidocarb for introduced animals and the avoidance of affected pasture. One practice in Aberdeenshire reported a farm that deliberately exposed young stock to pasture where red water had occurred, thereby taking advantage of the reverse age immunity phenomenon. Tick-borne fever was widely acknowledged as endemic, but was considered to be greatly under-observed and under-reported. Tick control and the use of prophylactic antibiotics were reported as being used in control against TBF.

All responding practices were asked about their subjective impressions of tick numbers and the results are summarised in Figure 2-6. Responses were either 'no ticks observed' (in ten practices), 'less ticks' (in three practices), 'the same number of ticks' (in 18 practices), or 'more ticks' (in 24 practices). In the east of Scotland a mixture of all responses was obtained, suggesting changes in tick numbers possibly occurring in a localised fashion or reduced awareness of ticks and tick-borne diseases. Practices in the west were more likely to report increasing numbers of ticks.

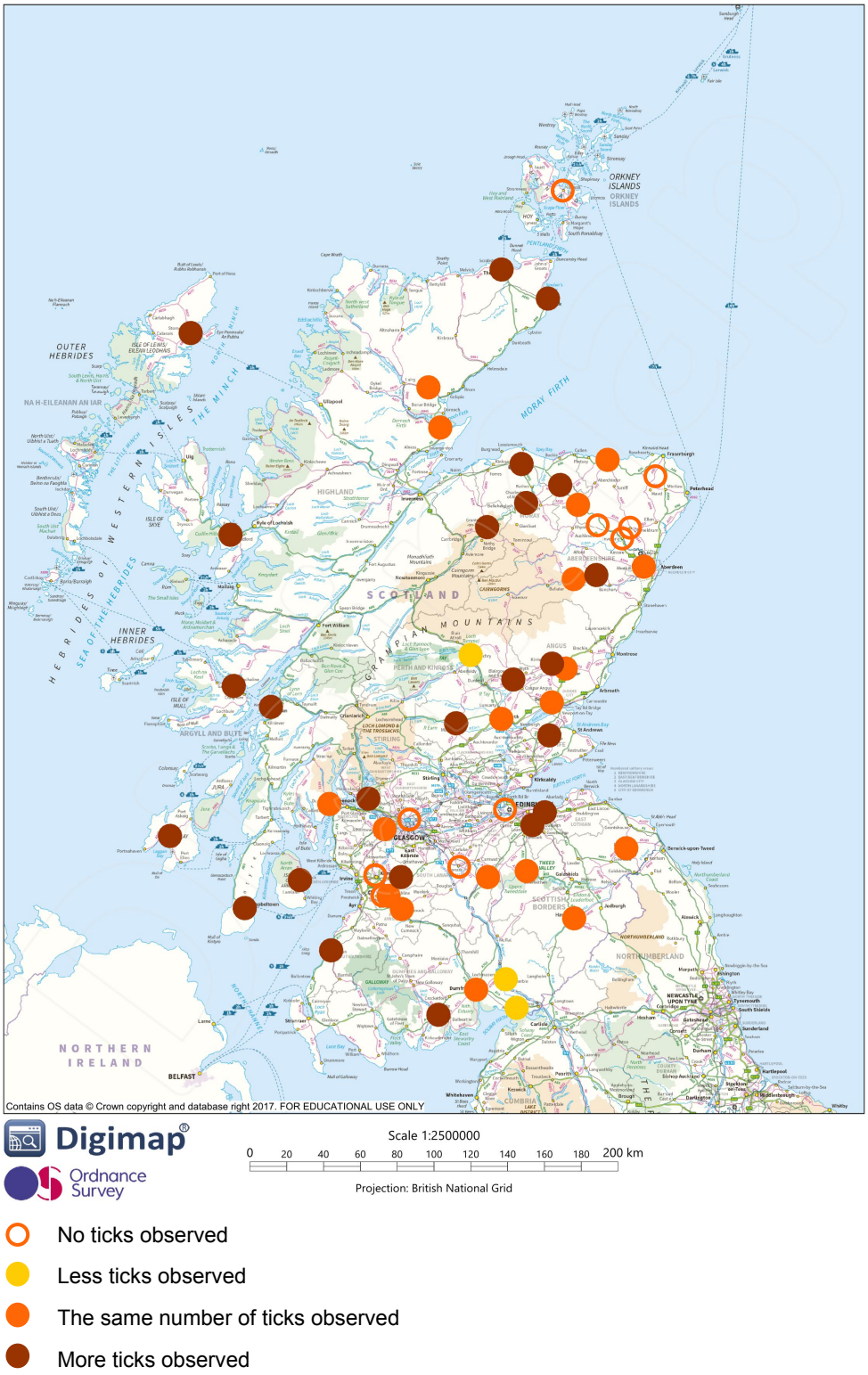


Figure 2-6 Numbers of ticks infesting cattle and sheep as perceived by veterinary surgeons

In addition to providing information about the occurrence of tick-borne disease in livestock at a national level, the survey also provided a useful means of identifying practices that were likely to contribute further to the study by identifying farms where tick-borne disease was prevalent (Chapters Four and Five).

2.3.2 SAC/SRUC tick-borne disease cases 2000 - 2013

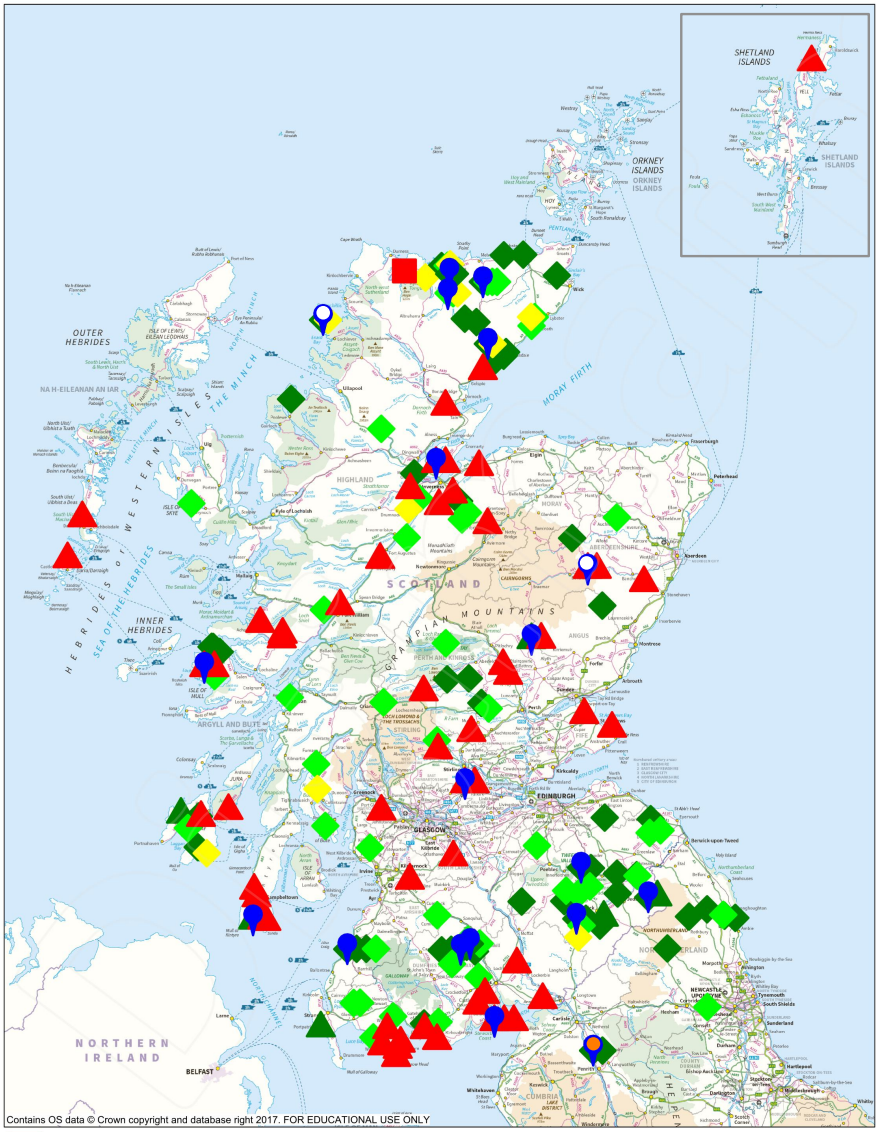
Interrogation of the SACSRUC VIDA database revealed 178 cases of tick-borne protozoan or bacterial disease between April 2000 and September 2013 investigated by SAC/SRUC Veterinary Investigation Officers. This constituted 152 premises and comprised 56 cases of babesiosis and 122 cases of tick-borne fever and related disease (Table 2-3). Both babesiosis and tick-borne fever were diagnosed based on supportive clinical signs and/or post-mortem results and/or by microscopic examination of stained blood smears. Additionally, a serological test for *A. phagocytophilum* was available during the period these diagnoses were reached which has subsequently been discontinued. As previously noted (1.2.7.3 Polymerase chain reaction), diagnostic testing for *A. phagocytophilum* in Scotland is currently carried out at the Moredun Research Institute, Edinburgh, and utilises a real-time PCR assay targeting a segment of the *msp2* gene (based on Courtney *et al.* (2004)), with a serological test in development.

	Cattle		Sheep		Deer		Total
Babesia	55	(98%)	0	(0%)	1	(2%)	56
All TBF related	6	(5%)	116	(95%)	0	(0%)	122
TBF only	6	(9%)	58	(91%)	0	(0%)	64
TBF/tick pyaemia	0	(0%)	48	(100%)	0	(0%)	48
TBF/louping-ill	0	(0%)	10	(100%)	0	(0%)	10
Total	61	(34%)	116	(65%)	1	(1%)	178

Table 2-3 SAC/SRUC babesiosis and tick-borne fever cases 2000 - 2013

The vast majority of cases of babesiosis were in cattle (55/56 (98 %)). No sheep were diagnosed with babesiosis and the remaining case (1/56 (2 %)) was in a red deer. Conversely, the majority of cases of tick-borne fever and related disease were in sheep (116/122 (95 %)). Of these sheep, similar numbers had tick-borne fever only (58/116 (50 %)) and tick-borne fever causing tick pyaemia (48/116 (41 %)), while a smaller number had concurrent louping-ill (10/116 (9 %)). There were no cases of tick-borne fever and related disease in red deer, and only a small number in cattle (6/122 (5 %)).

The distribution of all cases associated with tick-borne pathogens is illustrated in Figure 2-7. On the majority of premises (133/152 (87 %)) there was a single isolated case of babesiosis (n = 43) or tick-borne fever and related disease (n = 90).



	Cattle	Sheep	Deer
Babesiosis	▲		■
TBF	▲	◆	
TBF with tick pyaemia		◆	
TBF with louping-ill		◆	

Premises with multiple diagnoses

Premises with multiple diagnoses concurrently

Penrith APHA Veterinary Investigation Centre

Figure 2-7 All SAC/SRUC tick-borne disease cases 2000 - 2013

Premises where multiple cases of either babesiosis or tick-borne fever and related disease occurred are highlighted.

On a minority of premises (19 of 152 premises (13 %)) there were multiple cases of babesiosis and/or tick-borne fever and related disease. These occurred either in the same month (n = 2), suggesting a single outbreak, (Figure 2-7 and Figure 2-8, blue pins with white dots), or consecutively (n = 16) (Figure 2-7, blue pins and Figure 2-8). Additionally, one further premises with two consecutive cases of tick-borne fever or related disease was identified as Penrith APHA Veterinary Investigation Centre (Figure 2-7 and Figure 2-8, blue pin with orange dot) following submission of material to Scottish centres for diagnosis with the premises of origin not identified.

Four premises experienced multiple cases of babesiosis and ten multiple cases of tick-borne fever and related disease. At two premises there was a case of babesiosis and a case of tick-borne fever or related disease, both occurring in cattle.

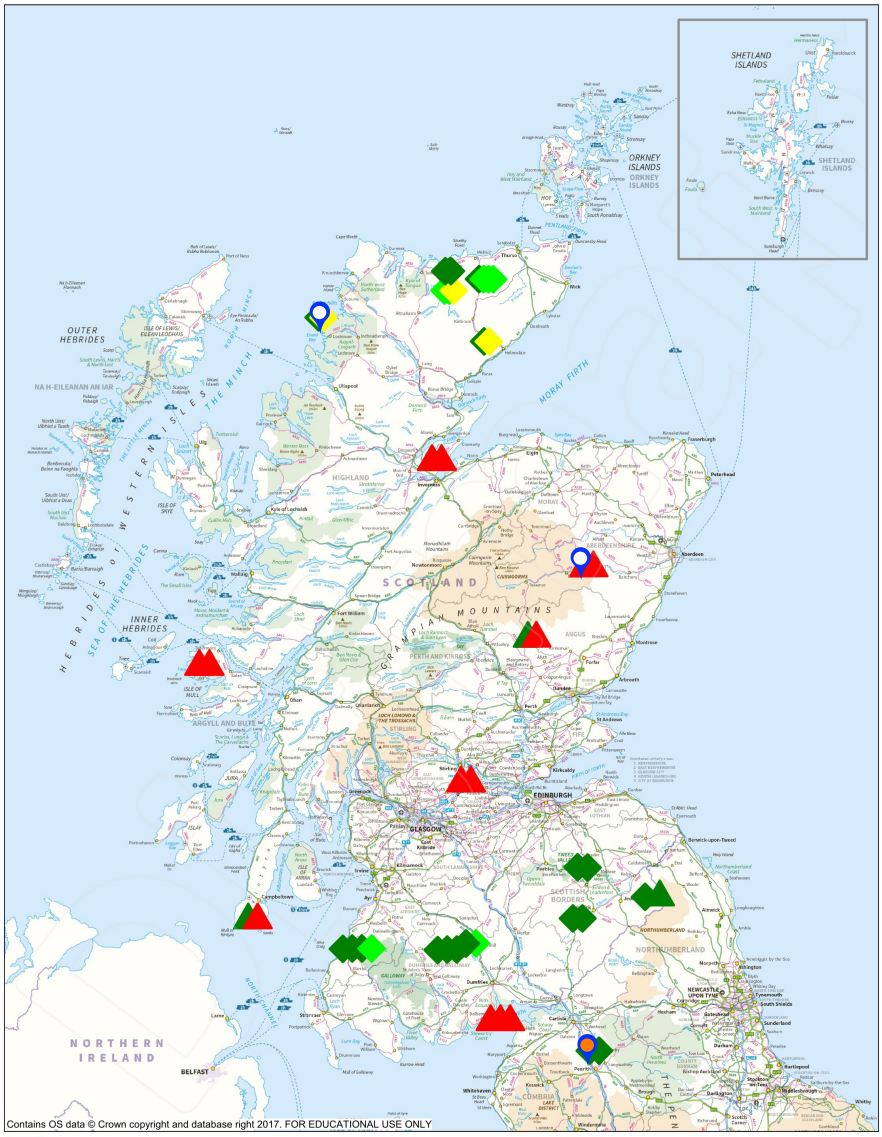


Figure 2-8 SAC/SRUC tick-borne disease cases 2000 - 2013, premises with multiple cases

Concurrent cases (blue pin with white dot) and consecutive cases of babesiosis and/or tick-borne fever and related disease; Penrith APHA Veterinary Investigation Office, blue pin with orange dot

Although cases occurred across Scotland, examination of the numbers of cases diagnosed by each Disease Surveillance Centre (Figure 2-1) reveals important differences (Figure 2-9). Babesiosis was most frequently diagnosed at Dumfries (14/56 = 25 % of cases of babesiosis), Inverness (25 % of cases of babesiosis) and Ayr (19.6 % of cases of babesiosis). Tick-borne fever and related disease was most frequently diagnosed at St Boswells (27/122 = 22.1 % of cases of TBF), Ayr (18 % of cases of TBF) and Thurso (18 % of cases of TBF).

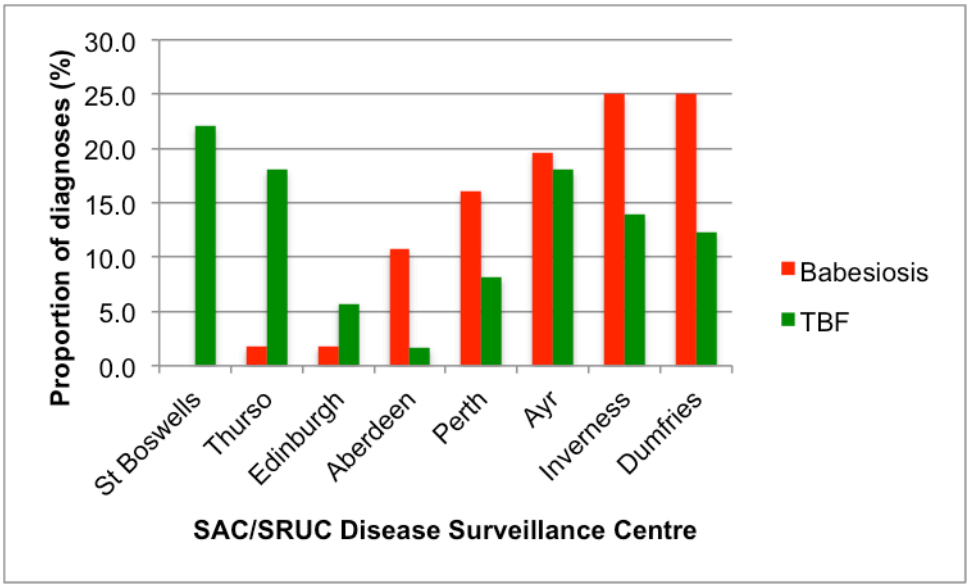


Figure 2-9 SAC/SRUC tick-borne disease cases 2000 - 2013 by DSC

Between the years 2000 and 2013 there was a mean of 12.7 cases of babesiosis and tick-borne fever and related disease per annum. Babesiosis was diagnosed less frequently (mean of four cases per annum) than tick-borne fever and related disease (mean of 8.7 cases per annum).

There were marked differences in the total number of cases of babesiosis in each year (Figure 2-10). Babesiosis was diagnosed most frequently in 2000 (n = 9), 2003 (n = 6), 2006 (n = 8) and 2010 (n = 7) with only one or two cases per annum in other years.

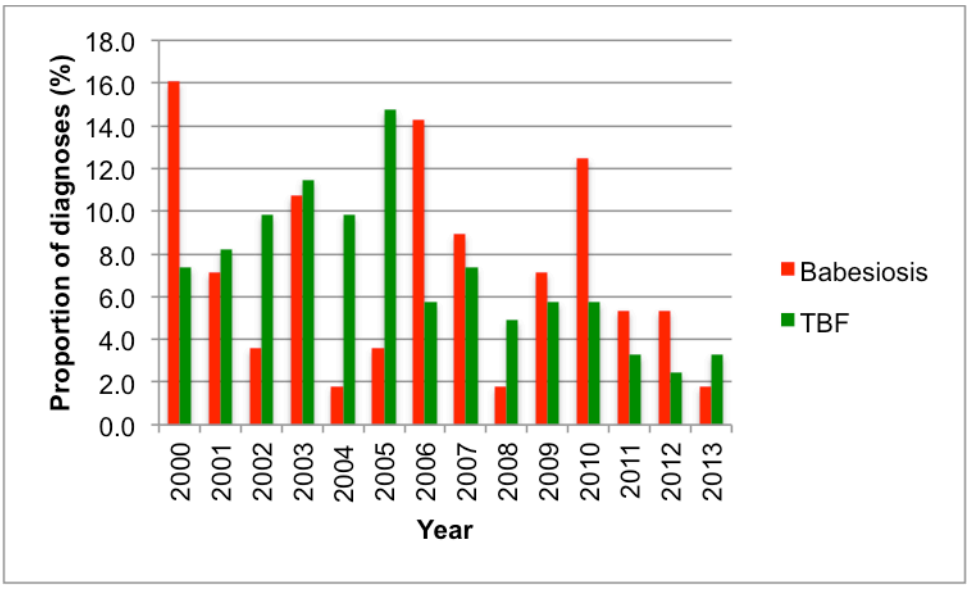


Figure 2-10 SAC/SRUC tick-borne disease cases 2000 - 2013 by year

Cases of tick-borne fever and related disease were most frequent in the five consecutive years 2001 (n = 10), 2002 (n = 12), 2003 (n = 14), 2004 (n = 12) and 2005 (n = 18) with the number of cases peaking in 2005. In total 66 cases of tick-borne fever and related disease occurred in these years (66/122 (54 %)). All DSCs reported cases of tick-borne fever and related diseases above the 2000 - 2013 mean for that DSC between 2001 and 2005 (in red, Table 2-4).

Site	2001	2002	2003	2004	2005	Mean (2000-2013)
Aberdeen	0	0	0	0	1	0.14
Ayr	3	5	1	3	5	1.57
Dumfries	1	0	1	2	3	1.07
Edinburgh	0	0	2	1	1	0.50
Inverness	2	1	3	1	3	1.21
Perth	1	2	0	0	2	0.71
St Boswell's	0	4	1	1	0	1.93
Thurso	3	0	6	4	3	1.57

Table 2-4 Tick-borne fever cases 2001 - 2005

Where the number of cases of tick-borne fever for the year exceeds the 2000 - 2013 mean for the DSC, the figure is highlighted in red.

Seasonal variation and peaks in the number of cases of both babesiosis and tick-borne fever and related diseases were observed between 2000 and 2013. This is illustrated in (Figure 2-11). Babesiosis occurred most frequently in August (18 % of cases), peaking in September (20 % of cases) and October (14% of cases). In the years between 2000 and 2013, babesiosis occurred in all months with the exception of January. Tick-borne fever and related disease occurred most frequently in April (17 % of cases), peaking in May (29 % of cases) and June (25 % of cases). In total 88 cases of tick-borne fever and related disease occurred in these months. Similar to babesiosis, in the years between 2000 and 2013 cases of tick-borne fever and related disease occurred in all months of the year with the exception of January and December.

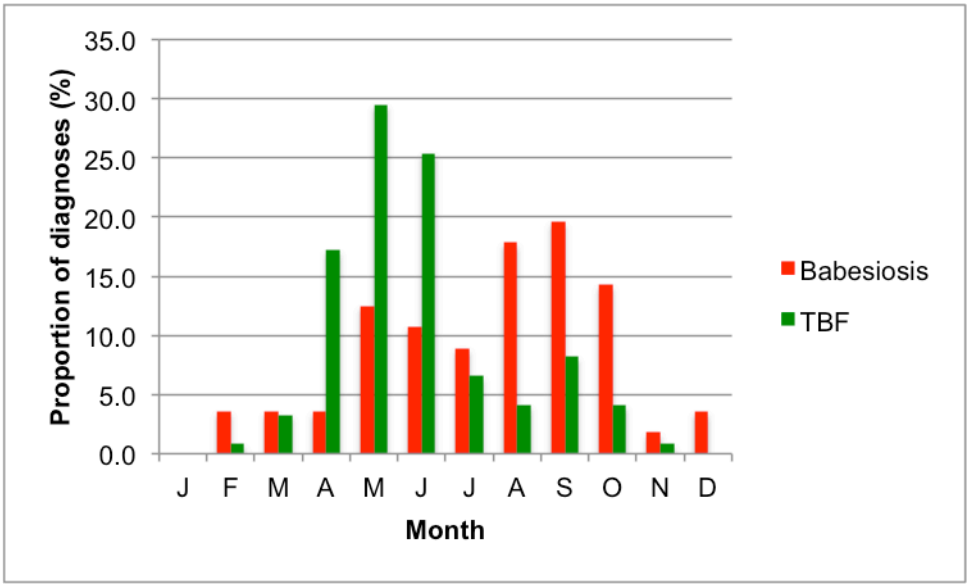


Figure 2-11 SAC/SRUC tick-borne disease cases 2000 - 2013 by month

2.4 Discussion

2.4.1 National survey of large animal veterinary surgeons on tick-borne disease in Scottish livestock

The survey was designed to query whether veterinary practices had encountered clinical disease in the past two grazing seasons and to investigate whether disease was historically recorded in each practice. It was clear from the comments that some practices that had not experienced clinical disease in the past two years but did have a previous history of cases. It was not clear if this

was due to the sporadic occurrence of disease or an actual reduction in numbers. Interestingly some responses suggested it was related to the increased use of topical anti-parasite treatments containing synthetic pyrethroids or macrocyclic lactones in recent years. There is evidence that macrocyclic lactone drugs, widely used for the prevention and treatment of a wide range of both endo- and ectoparasitic diseases, affect *Ixodes ricinus* (Gray *et al.*, 1996; Taylor and Kenny, 1990), and this is one of the possible explainations for reduced incidence of babesiosis in Ireland (Zintl *et al.*, 2014).

Environmental warming and increasing deer numbers have both been associated with increasing the numbers and broadening the range of *I. ricinus* (Dobson and Randolph, 2011; Gray *et al.*, 2009). This may explain the appearance of ticks in some previously unaffected areas reported in the survey, and even the appearance of ticks in association with deer, revealed in two responses. Deer can act as a reservoir for *B. divergens* infection (Zintl *et al.*, 2011) as well as a range of other *Babesia* species, including the zoonotic *B. venatorum*. In addition, once established in a tick population, *B. divergens* can remain present in the absence of susceptible hosts for up to four years. It achieves this by persistence between life-cycle stages and by transovarian transmission from the female tick's ovary through to the eggs and larvae of the next generation (Donnelly and Peirce, 1975; Joyner and Donnelly, 1979). The potential for infection of wildlife and persistence in the tick vector population means an environmental reservoir for this pathogen is likely to exist. Cattle protected from this reservoir by geographic location or use of drugs, that are therefore susceptible to infection, may suddenly become exposed by changing location or through cessation of drug usage. The loss to the farmer under such circumstances can be predicted to be significant through production losses, treatment costs, and livestock deaths.

The reports in the survey indicate occasions where veterinary surgeons have attended animals with severe clinical signs in the case of both red water fever and tick-borne fever. It is likely that red water and tick-borne fever occur as unobserved and/or unreported infections much more frequently, and the survey indicate this is believed to be especially true in the case of tick-borne fever. The economic impact of both diseases has not been assessed in Scotland although a very large number of animals are likely to be affected (for example in the case of tick pyaemia approximately 300,000 lambs per year across the country (Brodie

et al., 1986). The impact on animal welfare, especially due to tick-borne fever, is likely to be significant. The 18S PCR assay in development (described in Chapter Three) will help to identify which animals have *Babesia* spp. infections, which *Babesia* spp. are present in clinical cases, and which species may be present as a reservoir in wildlife. This may form the foundation for further work aimed at quantifying the economic impact and investigating the epidemiology of endemic TBD in Scottish livestock.

Treatments used for both red water fever and tick-borne fever were largely in line with those recommended (Radostits *et al.*, 2007a; Radostits *et al.*, 2007b). In comparison with a series of Irish cases (Sherlock *et al.*, 2000) and survey of Irish veterinary surgeons (Zintl *et al.*, 2014) where blood transfusion was used frequently as a treatment, this method was not used in the cases reported in the present survey. This may reflect differing approaches developed in the face of differing incidences of infection, infection pressure or economic climate and does not necessarily indicate a more lethal form of acute disease occurring in Irish cattle.

Imidocarb (Imazol, MSD Animal Health) was used in all cases with the exception of one where no treatment was used. This drug is widely acknowledged as an effective prophylactic treatment for introduced stock, and as a treatment for clinical disease. Unfortunately, it does have a significant drawback in its prolonged withdrawal period of 210 days for meat and milk, which can restrict its use especially in dairy animals (personal communication, Mr Phil Bosworth MRCVS, Bredy Vets, Dorset).

The results of the survey are a useful resource to gauge the perception of stakeholders towards tick-borne disease and to assess its current importance in terms of the UK livestock industry. Environmental changes coupled with trends towards reduced reliance on pharmaceuticals in agriculture make investigation of these diseases a priority issue for the maintenance of Scottish livestock production. For example, recent surveys in the USA highlight the potential for livestock to be under increased TBD threat due to changing environmental and epidemiological circumstances, despite extensive control strategies being in place. The possibility of novel or overlooked pathogens causing co-infections with recognised species may also be of importance. Specific areas that might be

useful to consider as novel control strategies include: effective vaccination, blocking transmission from specific hosts and the development of drugs with shorter withdrawal times and less residues. However, before significant investment in these areas is considered, it is important to accurately understand the range of TBD pathogens endemic in Scotland and the economic cost and epidemiology of the diseases they cause.

In the comments from veterinary surgeons it was noticed that some had observed that on certain holdings where clinical cases of red water used to be relatively frequent, episodes clinical disease are no longer experienced/reported/observed. This is of interest, as it seems to be opposed to the environmental changes favouring tick activity and the perception of increased tick numbers revealed by this survey. As explained above, one possible explanation is the widespread use of macrocyclic lactone-based oral drenches and topical treatments for a range of parasitic infections (not specifically ticks) on both sheep and cattle. On holdings with this history of decreased or absent clinical cases it would be interesting to see if tick-borne pathogens can still be detected in the livestock, although not causing clinical disease, or in the local tick population. A presence of pathogens in the ticks but not in the livestock might suggest a reservoir of infection is present, for example, in untreated livestock or more likely in the wild animal population. Further investigation would involve the identification of suitable locations with a high tick challenge but little reported TBD and cross-sectional sampling of the local livestock, tick and wild animal population.

2.4.2 SAC/SRUC tick-borne disease cases 2000 - 2013

To obtain additional information about the occurrence of tick-borne disease in Scottish livestock, data relating to 178 cases of babesiosis and tick-borne fever and related disease investigated and diagnosed by SAC/SRUC Veterinary Investigation Officers during the years 2000 - 2013 were examined.

2.4.2.1 Babesiosis

During the years 2000 to 2013, babesiosis was diagnosed on 56 occasions. Cattle were most frequently affected (55 cases (98 % of total)). Unexpectedly, a red

deer was also diagnosed with babesiosis on one occasion. Historical and clinical details were not available for this case leaving a number of important questions unanswered, for example was this a wild or farmed animal and what *Babesia* spp. was involved. Although diagnosis by microscopic examination of stained blood smears, as carried out by SAC/SRUC, has tremendous clinical utility it does not provide information about the *Babesia* spp. involved. To determine this a molecular approach is needed (Herwaldt *et al.*, 2003). The occurrence of this case, and the recent molecular confirmation of *B. divergens* in wild deer from Ireland, suggest investigation of *Babesia* spp. occurring in wild red deer is appropriate.

Babesiosis was diagnosed most frequently in 2000 (16 % of cases), 2003 (11 % of cases), 2006 (14 % of cases) and 2010 (12 % of cases) with only one or two cases per annum in other years. The survey of cases seen in practice covered the years 2012 and 2013, coinciding with years of low numbers of cases diagnosed nationally by SAC/SRUC staff. In spite of this, responses from large animal veterinary surgeons provided details about eight cases of babesiosis. Cases diagnosed by SAC/SRUC occurred most frequently in August, September and October, while the cases attended by large animal veterinary surgeons occurred in April (2/8 (25 %)), May (2/8 (25 %)), and July (2/8 (25 %)) and to a lesser extend August (1/8 (12.5 %)) and September (1/8 (12.5 %)).

2.4.2.2 Tick-borne fever

During the same period between 2000 and 2013, tick-borne fever was diagnosed on 122 occasions. In contrast to babesiosis affecting cattle almost exclusively tick-borne fever and related diseases were diagnosed largely in sheep (116 of a total of 122 cases (95 %)) and smaller numbers of cattle (6 if a total of 122 cases (5 %)) where it has been recognised previously. No cases of tick-borne fever were seen in red deer.

Cases of tick-borne fever and related disease diagnosed by SAC/SRUC VIOs occurred most frequently in April, May, and June and the majority of the eight cases attended by large animal veterinary surgeons (n = 5) occurred in these months. The other cases occurred in July (n = 1), August (n = 1), and October (n = 1).

Similar numbers of cases of tick-borne fever only (58/116 (50 %)) and tick-borne fever with tick pyaemia (48/116 (41 %)) were recorded. Tick-borne fever in isolation is a relatively mild disease clinically, although it can adversely affect extensively reared hill sheep (Brodie *et al.*, 1986), suggesting in cases where tick-borne fever was identified secondary infections were likely to be involved. These may include respiratory infections such as pneumonic pasteurellosis (Batungbacal and Scott, 1982b; Gilmour *et al.*, 1982), systemic pasteurellosis (Overas *et al.*, 1993; Overas, 1983)) or abortions may have occurred (Brodie *et al.*, 1986; Jamieson, 1950; Jamieson, 1947; Harbour, 1945; Stamp *et al.*, 1950; Jones and Davies, 1995; Littlejohn, 1950). Of these secondary infections, multifocal abscessation following systemic spread of *Staphylococcus aureus* (tick pyaemia) is reported to be the most common (Woldehiwet, 2006). Tick pyaemia commonly affects lambs between two and eight weeks of age (Foggie, 1962) explaining the increased numbers of cases in April, May, and June following lambing.

A lower number of cases were concurrently diagnosed with louping-ill (10/116 (9 %)). Experimental evidence suggests that the effects of louping ill infection in terms of the development of clinical signs and deaths are markedly worsened by co-infection with *A. phagocytophilum* (Reid *et al.*, 1986).

Cases of tick-borne fever and related disease were most frequent in the five consecutive years 2001 (n = 10), 2002 (n = 12), 2003 (n = 14), 2004 (n = 12) and 2005 (n = 18) with the number of cases peaking in 2005. This coincides with the 2001 foot and mouth disease epizootic, and the following years. This epizootic began with discovery of suspected infection in pigs in an Essex abattoir on 19/2/2001, confirmed on 20/2/2001, and subsequently traced to a farm feeding swill to pigs in Heddon on the Wall, Northumberland. A nationwide livestock movement ban was introduced on 23/2/2001 however, infection was already widely disseminated at this time, originating from Hexham and Longtown livestock markets. Dumfries and Galloway was especially severely affected following movements of livestock from Longtown market with 177 infected premises identified between the 1st of March 2001 and the 23rd of May 2001 (Thrusfield *et al.*, 2005). In response to the epizootic a widespread cull was initiated including livestock on infected premises, contiguous premises, and those within 3 km of infected premises in Dumfries and Galloway, resulting in

the slaughter of approximately 80,000 cattle, 564,000 sheep, 2600 pigs and 500 goats in this area. A period of restocking followed over the remainder of 2001 as animal movement restrictions were gradually relaxed, and in subsequent years. It is interesting to see that there was a peak in the number of cases of tick-borne fever and related disease in 2002 - 2005 (with 66 out of a total of 122 cases (54 %) during these four years). However, disease surveillance centres across Scotland participated in this increase in diagnoses, not only those adjacent to the restocking areas (Dumfries and St. Boswells to the east and Ayr to the north). It is therefore unclear whether this increase was due to alterations of livestock movements following the FMD epizootic or due to other unrelated factors, for example environmental conditions.

2.4.2.3 General considerations

Although there are seasonal peaks in the number of cases of both babesiosis and tick-borne fever, there is evidence that the tick vector *I. ricinus* is active in almost all months (with the exception of January). Zintl *et al.* (2014) found that previously accepted spring and autumn peaks in babesiosis cases (Donnelly and Mackella, 1970; Gray, 1980; Gray *et al.*, 1983), thought to be due to similar peaks in the activity of *I. ricinus* (Gray, 1980), had more recently been replaced with a near year round occurrence of the disease similar to that found in Scotland during this study.

There were 16 premises that experienced multiple consecutive cases of either babesiosis or tick-borne fever and related disease (or a combination of both) (Figure 2-7 and Figure 2-8) confirming that both diseases are capable of repeatedly afflicting the farms. The causes of these re-emerging clinical cases are unclear but the presence of a reservoir of infection in livestock, wildlife or ticks is suggested. Similarly it is unclear what management steps were taken to prevent disease and whether these were maintained to no avail before recurrence.

Samples collected by SAC/SRUC VIOs offer an opportunity to confirm that the *Babesia* spp. affecting Scottish cattle is *B. divergens*, currently diagnosed by the examination of stained blood smears. Although this confirms the diagnosis in a

clinical case, it does not discriminate between closely-related species (Herwaldt *et al.*, 2003).

Although *Babesia* and *Theileria* spp. of low pathogenicity have been described in the United Kingdom before this has always been in southern England or Wales in regions where the vector tick *Haemaphysalis punctata* is present. In sheep these include parasites identified without the advantage of current molecular methods as *B. motasi* (Lewis and Herbert, 1980; Lewis *et al.*, 1981; Alani and Herbert, 1988b; Alani and Herbert, 1988c), *T. ovis* (Lewis and Purnell, 1981) and *T. recondita* (Alani and Herbert, 1988a), and in cattle *B. major* (Brocklesby and Irvin, 1969; Brocklesby and Barnett, 1970; Barnett and Brocklesby, 1971) and a member of the *Theileria buffeli/orientalis* complex (Barnett and Brocklesby, 1971; Brocklesby and Barnett, 1972; Hignett, 1953). One exception in this group is a small *Babesia* sp. found in a sheep from Scotland and thought, at the time, to be *B. capreoli* (Reid *et al.*, 1976; Purnell *et al.*, 1981). Assuming that this parasite was transmitted by *I. ricinus*, as the workers that investigated it at the time did, this species may still be present in Scottish sheep suggesting that investigation by molecular means of *Babesia* spp. present in Scottish sheep would be appropriate.

Focusing on regions where endemic tick-borne disease is being diagnosed frequently (implying that the endemic tick *I. ricinus* is both present and transmitting disease to livestock) will maximise opportunities for the detection of hitherto undiscovered pathogens. Dumfries, Inverness and Ayr DSCs diagnosed the greatest number of cases of babesiosis (collectively 70 % of cases of this disease) and St Boswells, Ayr and Thurso the greatest number of cases of tick-borne fever and related disease (collectively 58 % of cases of these diseases). This suggests that material originating from these DSCs would provide a useful resource for identifying novel pathogens in Scottish livestock.

CHAPTER THREE
Babesia and *Theileria* spp. PCR Development and Initial PCR Deployment

3.1 Introduction

The cattle and sheep industries in Scotland have long been affected by tick-borne disease and these illnesses continue to be a cause of economic loss and to impact animal welfare. One of these is babesiosis or "red water fever" in cattle, caused by *Babesia divergens* (M'Fadyean and Stockman, 1911). In Scotland the widely distributed and most frequently encountered tick, *Ixodes ricinus*, transmits this pathogen (Joyner *et al.*, 1963). *Babesia divergens* is capable of infecting a number of other species including man (Gray *et al.*, 2010) and is known to have caused at least one human fatality in Scotland (Entrican *et al.*, 1979b; Entrican *et al.*, 1979a)

A clinical diagnosis of babesiosis is normally reached based on the previous occurrence of disease on a farm, the animal/s history and the clinical signs observed (Gray and Murphy, 1985; Zintl *et al.*, 2014). Clinical confirmation can be achieved by the examination of stained blood smears (classically with Giemsa but in a practice situation Diff-Quik is also suitable); this method is rapid and widely available. Parasite morphology can also be assessed by this method (recently reviewed by (Lempereur *et al.*, 2017)) but even with optimal preparation of the smears identification to species level is impossible.

Serological assays have been developed and utilised for the detection of antibodies to *B. divergens* and have been widely employed in epidemiological studies (Zintl *et al.*, 2003). Both indirect fluorescent antibody (IFA) and enzyme linked immunosorbent assay (ELISA) tests have been shown to have similar utility (Bidwell *et al.*, 1978) in the detection antibodies. They share the disadvantage of relying on the detection of antibodies that remain for variable periods following clearance the parasite (Zintl *et al.*, 2003) and require the generation of antigen (for example by infection of susceptible hosts) for use in the assays. Suitable susceptible hosts are splenectomised calves or the Mongolian gerbil (*Meriones unguiculatus*) (Gray and Kaye, 1991). Similar ethical and practical disadvantages affect xenodiagnoses, which entails the inoculation of a

susceptible animal, for example the Mongolian gerbil (Lewis and Williams, 1979) with blood from a suspected case to determine if infection results.

More recently conventional, nested, and qPCR techniques have been described for the detection of *Babesia* and *Theileria* spp. (recently reviewed by (Lempereur *et al.*, 2017)). The 18S rRNA gene of *Babesia* and *Theileria* spp. is a common target with both genus and species-specific primers described. This gene is an attractive target due to its high level of conservation within parasite species, well-defined phylogenetically informative variable regions that can be amplified by PCR using primers annealing to flanking conserved regions, and the large number of published sequences that assist in species identification by comparison. When genus-specific primers are utilised and the amplicon sequenced, unexpected species can be unmasked in addition to allowing subsequent phylogenetic comparison. However, a disadvantage of this approach is the masking of minority sequence in multi-species infections, which can be effectively demonstrated by the use of species-specific primers. These methods have the additional advantage of being generally more sensitive than microscopic examination of stained blood smears. Comparison of microscopic detection and detection by qPCR of *B. microti* from human patients has confirmed the greater sensitivity of this method (Wang *et al.*, 2015).

A reverse line blot hybridisation method, which has the capacity to simultaneously detect a larger number of species, has been described (Gubbels *et al.*, 1999). The utility of next generation sequencing (NGS) methodology in tick-borne pathogen discovery and cataloguing has recently been demonstrated by the detection of DNA in ticks collected in France from multiple zoonotic *Babesia* spp. (*B. divergens*, *Babesia* sp. EU1 and *B. microti*) and *B. major* along with sequences similar to tropical *Theileria* spp. Bonnet *et al.* (2014).

SAC/SRUC provide diagnostic services to Scottish producers through their veterinary surgeon via a network of eight DSCs (Figure 2-2). Examination of blood from cattle and other species, such as wild red deer (Table 2-3), to provide clinical confirmation of babesiosis is carried out by microscopic examination of stained blood smears. Based on the difficulty determining the species present this raises the question **could a species morphologically similar**

to but distinct from *B. divergens* could be causing clinical disease in Scottish livestock?

Although a number of unexpected *Babesia* and *Theileria* spp. have been found in the UK, these rely on *H. punctata* as their vector making their presence in Scotland less likely (see Chapter One). However a *Babesia* sp. was identified in a Scottish sheep (Reid *et al.*, 1976; Purnell *et al.*, 1981). This raises a second question: **could that or a similar parasite still be present but undetected in Scottish sheep (or cattle)?**

To address these questions, and the lack of a molecular survey of Scottish livestock, two steps were taken:

Firstly, a selection of previously published genus-specific (*Babesia* and *Theileria*) primers targeting the 18S gene were analysed to identify a primer pair which would amplify a phylogenetically informative region, capable of discriminating species at high resolution. To maximise the sensitivity of the assay, a nested PCR was decided upon and so a second set of primers bridging this amplicon was also selected. The novel combination of primers was optimised to ensure maximum sensitivity and the ability to amplify DNA from a diverse range of archived DNA samples.

Secondly, this nested 18S assay was applied to samples confirmed microscopically-positive for *Babesia* sp. or originating from sheep and cattle suspected to have a tick-borne disease (and therefore exposed to tick infestation). SAC/SRUC VIOs diagnose tick-borne disease frequently (Table 2-3 and Figure 2-7) and are well-placed to identify animals that have been exposed to ticks and/or are suspected on clinical grounds of having a tick-borne disease. VIOs at DSCs previously identified as experiencing a high proportion of cases of either babesiosis or tick-borne fever and related disease kindly provided such material to facilitate this approach (Figure 2-9).

An alternative source of samples from animals exposed to tick infestation was the livestock and wild ruminants on farms affected by tick-borne diseases as identified by veterinary surgeons responding to the National Survey of Large

Animal Veterinary Surgeons (Figure 2-4). Consequently, a second round of sample acquisition and processing is described in Chapter Four.

3.2 Materials and methods

3.2.1 *Babesia* and *Theileria* spp. PCR development

3.2.1.1 Sequence analysis

Published protozoan 18S SSU gene sequence data was downloaded from the NCBI database (https://www.ncbi.nlm.nih.gov). Sequences were aligned using CLC Genomics Workbench (Qiagen) and alignments trimmed such that all sequences were of a comparable length. Neighbour-joining trees were constructed and visualised using CLC Genomics Workbench.

3.2.1.2 Parasite material

In order to optimise the nested PCR protocol, DNA samples from a number of *B. divergens* strains was collected. This comprised four strains supplied by Dr Annetta Zintl (University College Dublin), namely BDIV1, BDIV2, BDIVT1 and BDIVT2. DNA was also extracted from the blood of a clinical case that had occurred on a farm in Dumfries and Galloway (BdivF) using a commercial kit (Wizard Genomic DNA Purification Kit, Promega) according to the manufacturer's instructions. To confirm that the nested PCR protocol could effectively amplify DNA from a range of piroplasm species, a panel of *Babesia* and *Theileria* DNA samples was assembled from the archive at University of Glasgow School of Veterinary Medicine. This included two strains of *Babesia bigemina*, two strains of *Babesia bovis*, *Theileria mutans*, *Theileria parva* and *Theileria annulata*. A bovine DNA (BL20 cell) extract (Promega Wizard extraction kit) and an *Ehrlichia ruminantium* DNA preparation were used as a negative controls.

The blood from the clinical case from Dumfries and Galloway (DNA extract BdivF) was submitted for haematological examination at the Veterinary Diagnostic Service (VDS) of the University of Glasgow School of Veterinary Medicine. As part of the assessment of the erythron, a blood smear was made and stained (May-Grunwald-Giemsa) and the cell monolayer examined

microscopically. Cell counting was assisted by using an eyepiece modified to delimit the size of the high power field to a small central region. The percentage of red cells containing parasites (parasitaemia) was calculated as the mean number of parasitised red cells per delimited field divided by the mean total number of red cells per delimited field x 100; the means were generated following counting cells in delimited central regions of seventy delimited high-power fields (x400 magnification).

3.2.1.3 PCR development and optimisation

Oligonucleotide primers were synthesised by a commercial service (Eurofins Genomics). PCR reactions were carried out in 96-well plates (AB1400, ThermoScientific) sealed with an adhesive film (AB0558, ThermoScientific) in a Techne TC512 PCR thermocycler. Typically, the four wells at each corner of the plate were not utilised in order to avoid evaporative loss of reaction mixture due to plate expansion and contraction during thermocycling. PCR reactions were carried out in a total volume of 20 µl. The components and their final concentrations are summarised in Figure 3-1.

2 µl	PCR buffer (GeneAmp 10X PCR Buffer (Life Technologies Applied Biosystems)) to give final concentrations of 10 mM Tris-HCl, 50 mM KCl, and 1.5 mM $MgCl_2$
0.4 µl	dNTP solution (Deoxynucleotide (dNTP) Solution Mix (New England Biolabs) or dNTP Mix (Promega) to give final concentrations of 0.2 mM dATP, 0.2 mM dCTP, 0.2 mM dGTP and 0.2 mM dTTP
2 µl	10 µM forward primer solution to give a final concn of 1 µM
2 µl	10 µM reverse primer solution to give a final concn of 1 µM
0.1 µl	*Taq* (AmpliTaq DNA Polymerase (Life Technologies Applied Biosystems)) to give a final concentration of 0.025 units/µl
2 µl	template DNA solution
11.5 µl	de-ionised water

Total reaction volume 20 µl

Figure 3-1 *Babesia* and *Theileria* spp. PCR reaction components and concentrations

5 µl of each PCR product was separated by electrophoresis on a 1 % agarose gel (UltraPureTM Agarose, Invitrogen) containing 1.5 µl ethidium bromide solution (10 mg/ml, Sigma) per 100 ml. A DNA ladder (either 100 bp DNA Ladder, Invitrogen (range: 100 to 1,500 bp; brighter bands: 600 bp and 2,072 bp) or 100 bp DNA Ladder, New England Biolabs (range: 100 to 1,500 bp; brighter bands: 500 bp and 1,000 bp)) was also loaded to allow determination of the approximate size of the amplicons. The DNA ladder and product bands were visualised under UV trans-illumination (Alpha Innotech FluoroChem 5500) and digital images acquired.

In order to optimise the annealing temperature for each reaction, gradient PCR was undertaken using the BdivF DNA extract as template; for outer and inner primer pairs twelve PCR reactions were carried out at incrementally increasing annealing temperatures (temperature range 15 °C centred at 62 °C) using the temperature gradient function of the thermocycler (Techne TC512). Thermocycler settings consisted of pre-heating the lid (105 °C) for four minutes

before an initial denaturation step at 94°C for five minutes. This was followed by 30 cycles of denaturation (94°C, 45 seconds), annealing (gradated temperature, 60 seconds) and extension (72°C, 60 seconds). A final extension step at 72°C for five minutes was also carried out. 5 µl of each PCR product were visualised as described above, and 1 µl the remaining product of the selected outer primer pair reaction was diluted 1:100 with water for use as the template for the inner reaction.

3.2.2 Field samples from SAC/SRUC Disease Surveillance Centres

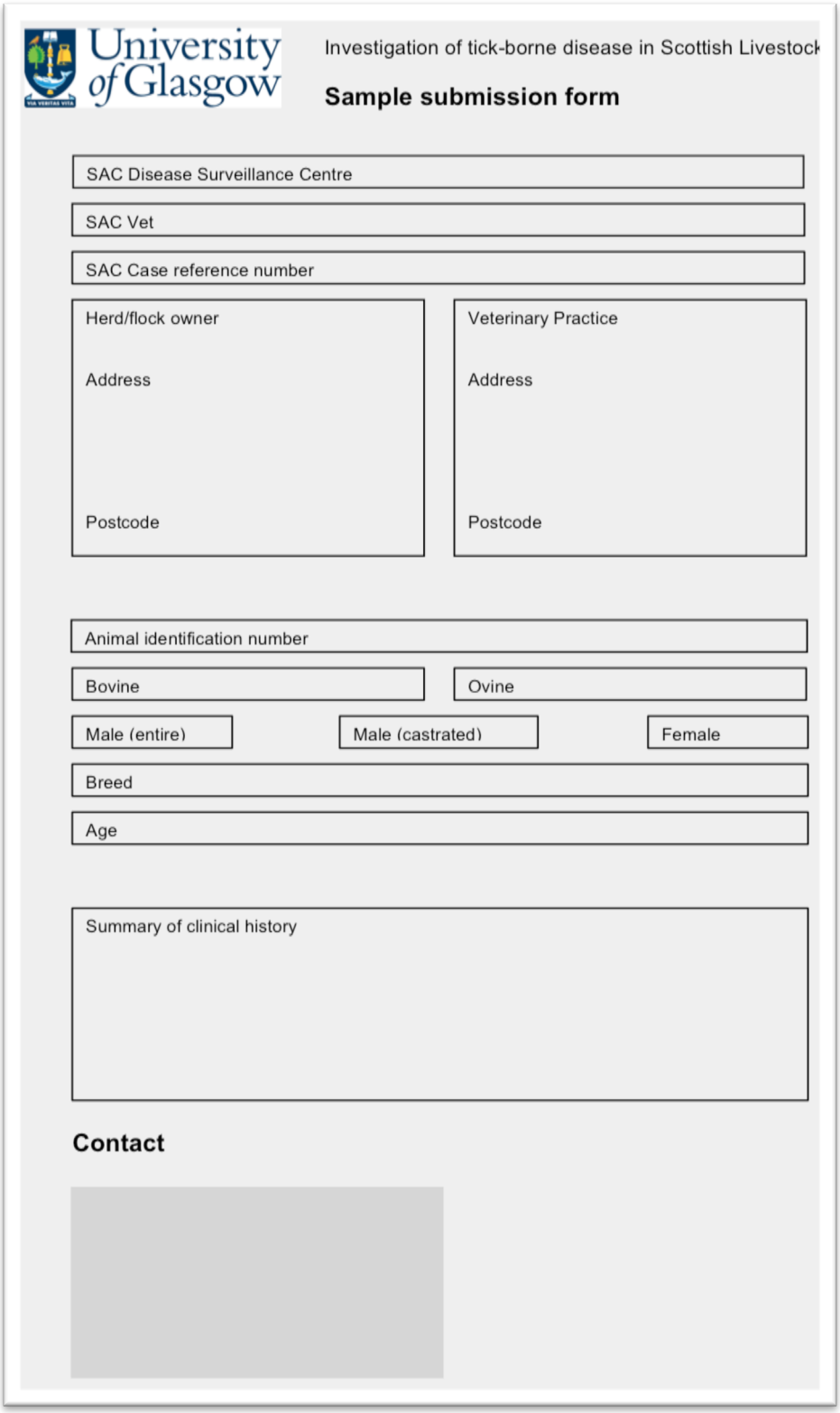
3.2.2.1 Field sample acquisition

During the 15 month period between September 2013 and December 2014, blood from animals diagnosed with babesiosis by microscopic examination of stained blood smears and blood and tissues from animals suspected of having a tick-borne disease were collected.

SAC/SRUC DSCs identified as frequently diagnosing the tick-borne diseases babesiosis and tick-borne fever and related disease (Figure 2-9) were contacted and visited; these DSCs were Ayr, Dumfries, Inverness and St. Boswells (Figure 3-2). Submission and PM room forms were available indicating which samples had been taken (Figure 3-3). Blood samples from field cases submitted by veterinary surgeons arrived in tubes containing anticoagulant (EDTA or Lithium heparin). In post-mortem cases, clotted blood in a plain sample tube and blocks of tissue of approximately 1 – 2 cm minimum dimension were taken. Samples were either posted or collected from the DSCs following storage at 4 °C or -20 °C.

Figure 3-2 Participating SAC/SRUC Disease Surveillance Centres

The four participating DSCs, Inverness, Ayr, St Boswells and Dumfries are shown in colour.



University of Glasgow

Investigation of tick-borne disease in Scottish Livestock

Sample submission form

SAC Disease Surveillance Centre

SAC Vet

SAC Case reference number

Herd/flock owner

Address

Postcode

Veterinary Practice

Address

Postcode

Animal identification number

Bovine

Ovine

Male (entire)

Male (castrated)

Female

Breed

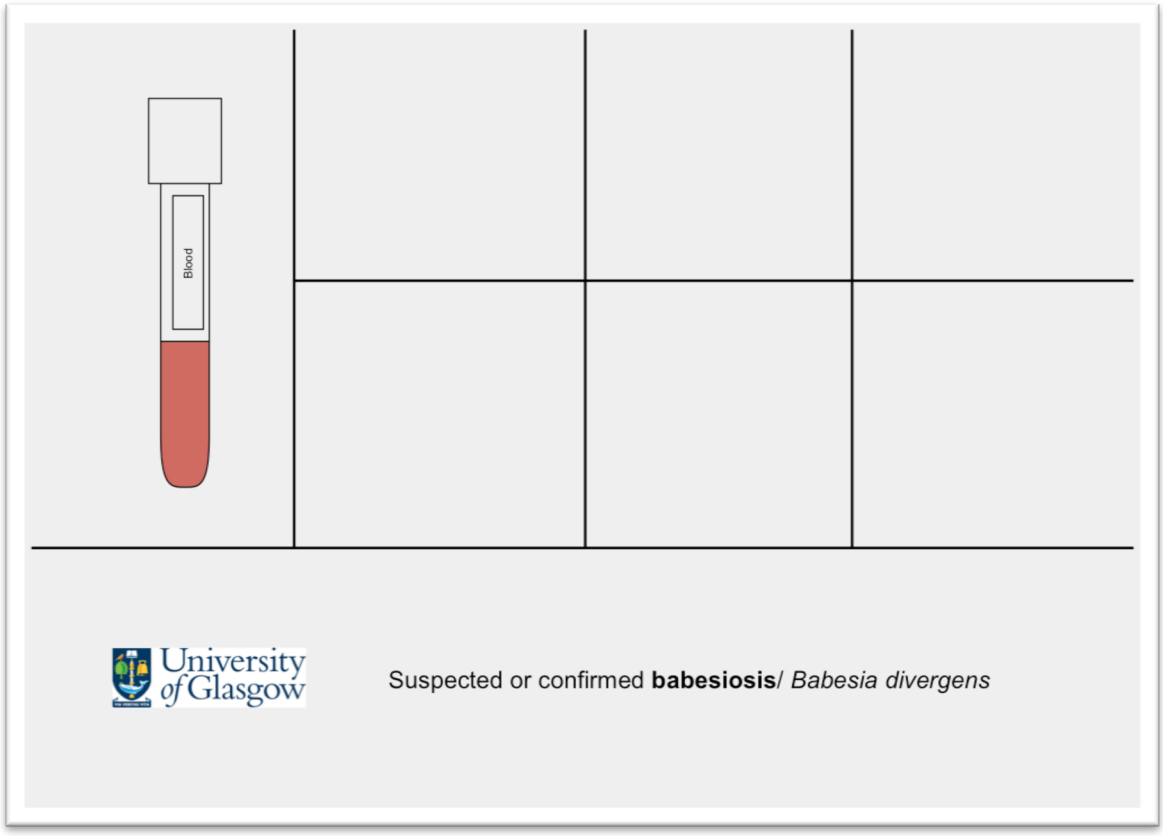
Age

Summary of clinical history

Contact

Figure 3-3 Sample submission form used by participating SAC/SRUC DSCs

(Continued overleaf)



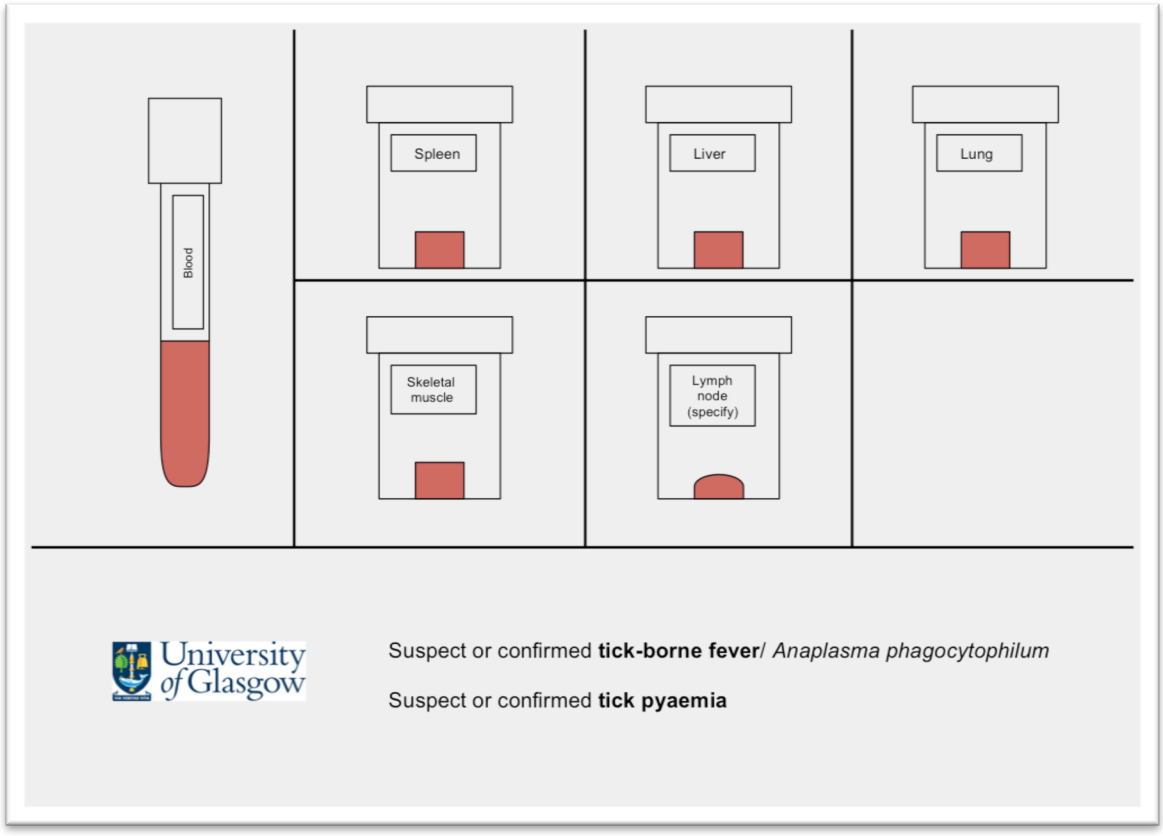


Figure 3-3 DSC sample submission form

(Continued)

3.2.2.2 DNA extraction

DNA was extracted from anti-coagulated livestock blood samples using a commercial kit (Wizard Genomic DNA Purification Kit, Promega) according to the manufacturer's instructions. DNA was extracted from solid tissues using the Promega protocol for DNA extraction from mouse tissues, which typically involves homogenisation of the tissue and proteinase K (Proteinase K (Fungal) 25530-015, Invitrogen) incubation/digestion. It is recognised that genomic (and presumably parasite) DNA extraction from clotted blood, using salt precipitation techniques (for example the Wizard Genomic DNA Purification Kit (Promega)), results in lower yields of DNA than from anti-coagulated samples (Clements *et al.*, 2008). Where only the sub-optimal alternative of clotted blood was available, for example when the sample originated from a dead animal at post-mortem examination, the protocol for solid tissues was followed.

Each DNA extract was used as template for the *Babesia* and *Theileria* spp. PCR assay, the development of which is described in this chapter.

3.2.2.3 Sequencing

Where SAC/SRUC field samples were found to be positive, suggesting the presence of a *Babesia* or *Theileria* spp., direct amplicon sequencing was undertaken. In brief, amplicons were purified from either gels (QIAquick Gel Extraction Kit, Qiagen) or PCR products (QIAquick PCR Purification Kit, Qiagen) and DNA concentration measured using a Qubit® 2.0 Fluorometer (Life Technologies) prior to dilution to 5 ng/µl. Sequencing was carried out in both forward and reverse directions using appropriate primers premixed with template DNA before submission to a commercial sequencing service (Eurofins Genomics). Forward and reverse sequences were aligned to generate a consensus sequence and trimmed to remove primer regions using CLC Genomics Workbench (Qiagen). All sequences generated were named according to the scheme illustrated in Figure 3-4.

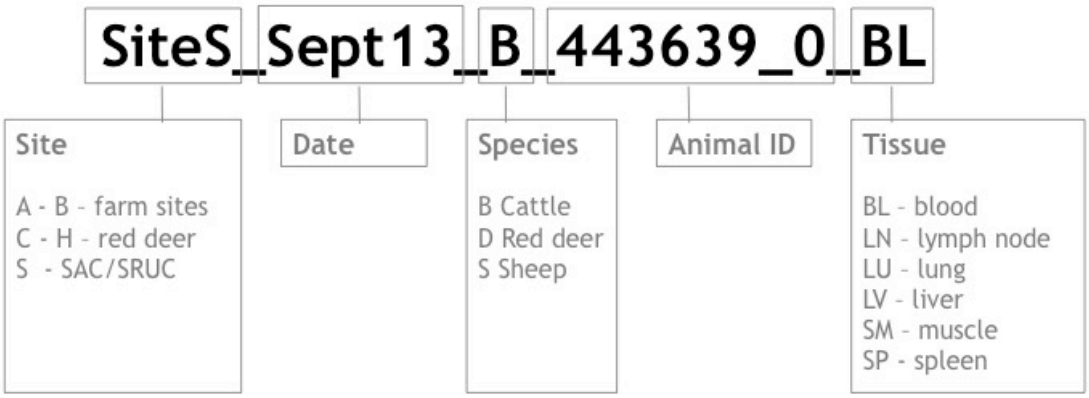


Figure 3-4 Convention used in naming sequences

In this example the sequence originated from the blood (BL) of a cow (B) identified by the number 443639_0 from SAC/SRUC (SiteS) obtained in September 2013.

Amplicon sequences were BLASTed against the non-redundant database at NCBI in order to identify the species present.

3.3 Results

3.3.1 *Babesia* and *Theileria* spp. PCR development

3.3.1.1 Primer selection

Fourteen *Babesia* spp. and six *Theileria* spp.18S SSU sequences were selected for multiple-alignment in order to facilitate the design of 'catch-all' PCR primers (Table 3-1). A target region for PCR amplification would be sought consisting of conserved primer sites, to ensure broad amplification, and a polymorphic central region, which could discriminate between different species.

Pathogen	Accession Number	Location	Species	Reference/s
***Babesia* spp.**				
Babesia bigemina	X59604	Mexico	Cow (*Bos taurus)*	Reddy *et al.* (1991)
Babesia bovis	L19077	South Africa	Vaccine strain	Allsopp *et al.* (1994)
Babesia capreoli	AY726009	France	Roe deer (*Capreolus capreolus)*	Malandrin *et al.* (2010)
Babesia conradae	AF158702	USA	Dog (*Canis lupus familiaris)*	(Kjemtrup *et al.*, 2000) (Kjemtrup *et al.*, 2006)
Babesia divergens	AY046576	Ireland	Cow	(Purnell *et al.*, 1976) (Herwaldt *et al.*, 2003)
Babesia sp. (EU1)	AY046575	Italy, Austria	Human (*Homo sapiens)*	(Herwaldt *et al.*, 2003)
Babesia gibsoni	AF175300	Japan	Dog	(Zahler *et al.*, 2000b) (Zahler *et al.*, 2000c)
Babesia gibsoni	AF175301	Sri Lanka, Malaysia	Dog	(Zahler *et al.*, 2000b) (Zahler *et al.*, 2000c)
Babesia major	EU622907	France	Cow	(Criado-Fornelio *et al.*, 2009)
Babesia major	GU194290	France	Cow	NA
Babesia microti	AF231349	Germany	Tick (*I. ricinus)*	(Zahler *et al.*, 2000a)
Babesia microti	AY789075	Poland	Tick (*I. ricinus)*	(Pieniazek *et al.*, 2006)
Babesia microti	U09833	Unknown	Mouse (*Mus musculus)*	NA
Babesia odocoilei	AY046577	USA	White-tailed deer (*Odocoileus virginianus)*	(Holman *et al.*, 2000) (Herwaldt *et al.*, 2003)
***Theileria* spp.**				
Theileria annulata	AY508470	Turkey	Cow	NA
Theileria annulata	M64243	India	Cow	(Gajadhar *et al.*, 1991)
Theileria buffeli	AB000272	Australia	Cow	(Chansiri *et al.*, 1999)
Theileria buffeli	Z15106	Kenya	Cow	(Young *et al.*, 1992) (Allsopp *et al.*, 1994)
Theileria mutans	AF078815	Kenya	Cow	(Chae *et al.*, 1999)
Theileria parva	L02366	Kenya	Cow	(Allsopp *et al.*, 1993)

Table 3-1 18S SSU rRNA genomic sequences selected for alignment

Following alignment and trimming to match the full 18S rRNA gene sequence of *Babesia divergens* AY046576, a dendrogram was constructed which, as suspected, confirmed that the entire 18S SSU rRNA gene could discriminate a diverse range of *Babesia* and *Theileria* spp. (Figure 3-5).

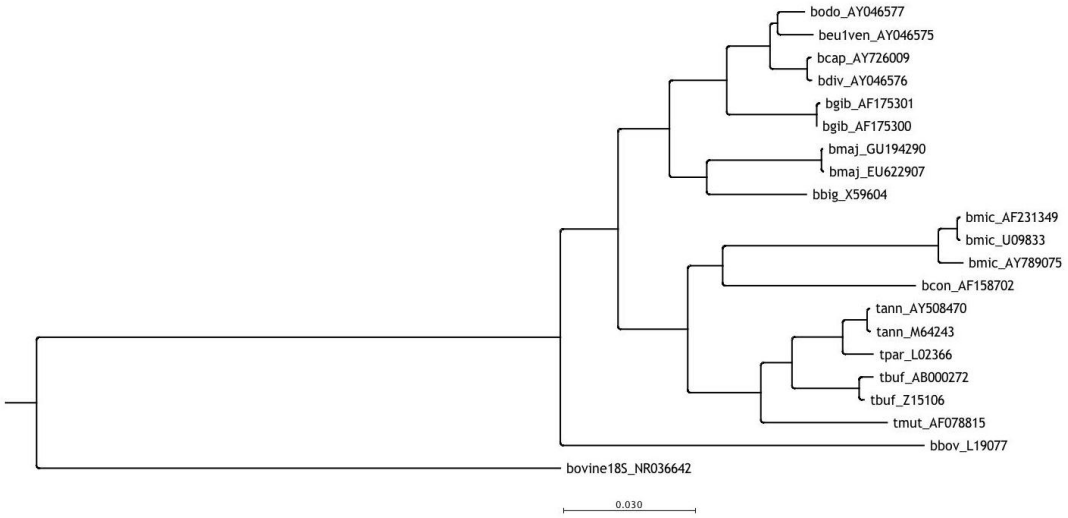


Figure 3-5 Neighbour-joining tree based on alignment of the entire 18S gene of selected *Babesia* and *Theileria* spp.

The bovine 18S gene sequence was used to root the tree.

The 1,800bp 18S rRNA gene sequence of the yeast *Saccharomyces cerevisiae* Z75578 was downloaded and annotated to highlight the variable regions (Hadziavdic *et al.*, 2014). This was trimmed to align to the 1,728bp sequence of *Babesia divergens* AY046576 (Herwaldt *et al.*, 2003) and the *S. cerevisiae* annotations were transferred to *B. divergens*. A diagram representing the *Babesia* and *Theileria* spp. 18S gene sequence alignment was created in the form of a heat-map illustrating the degree of conservation at each nucleotide position (Figure 3-6). Each nucleotide position in the alignment was coloured according to the degree of conservation of the majority nucleotide, with the exception of those where the position contained gaps in over 25 % of sequences (grey). The V4 region was identified as a potential target for amplification and sequencing, based on the likelihood of identifying conserved primer sites flanking a polymorphic central region.

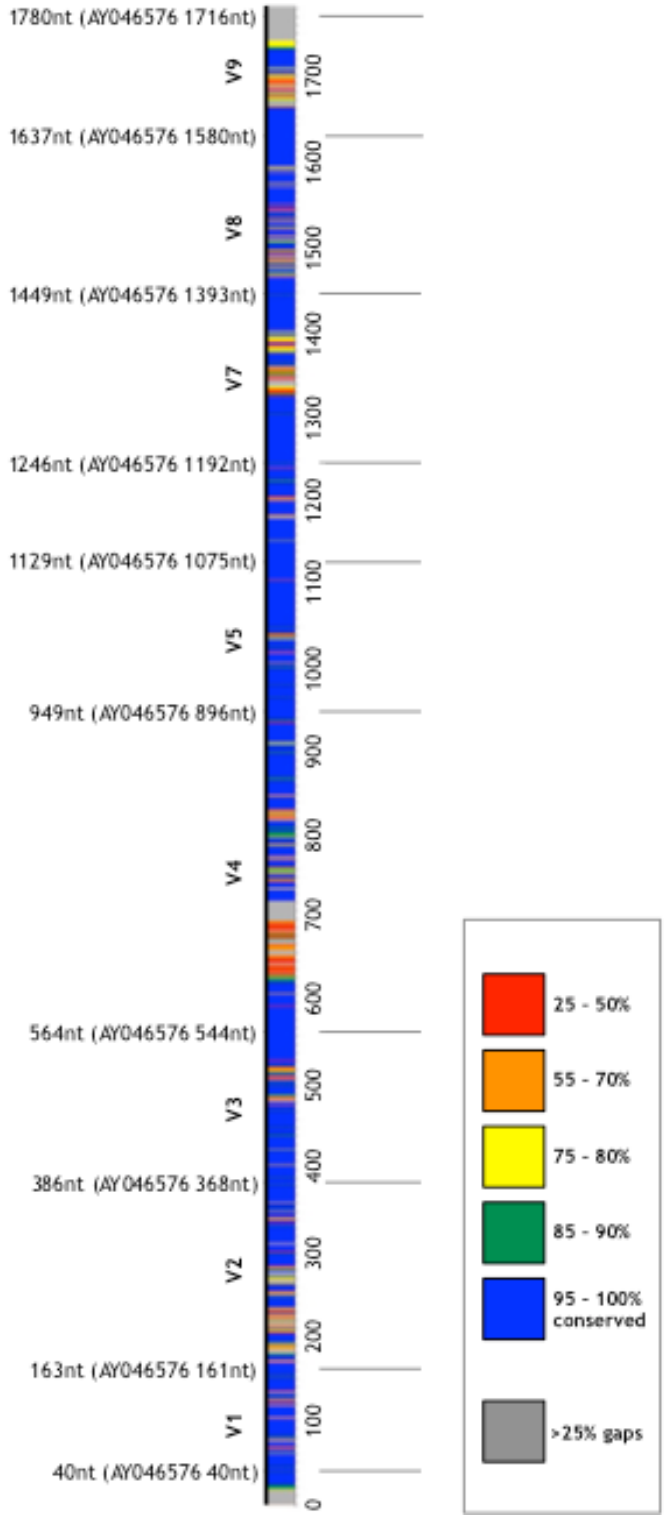


Figure 3-6 Conservation at nucleotide level amongst selected *Babesia* and *Theileria* spp. 18S gene sequences

Annotation of the variable regions V1-5 and V7-9 with co-ordinates shown relative to the *Babesia/Theileria* spp. alignment with the co-ordinates relative to the *B. divergens* AY046576 sequence in parenthesis.

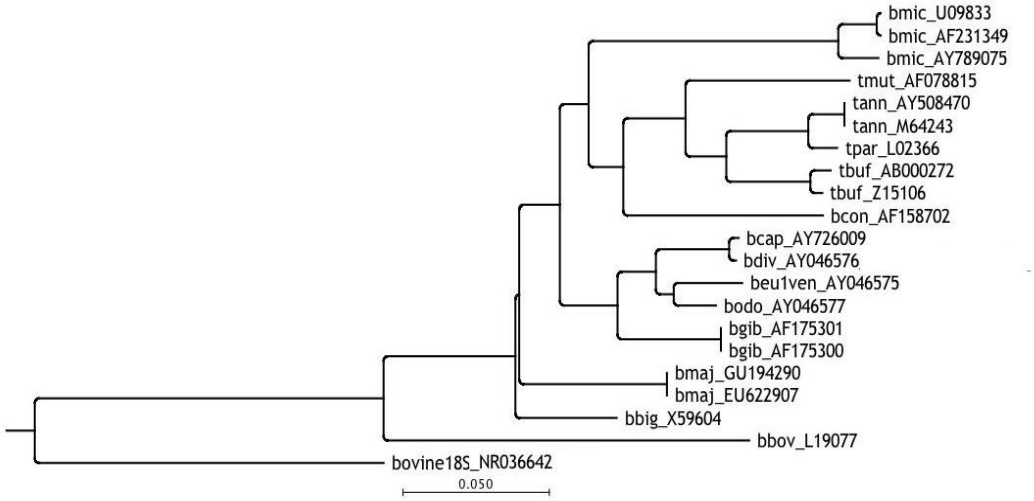


Figure 3-7 Neighbour-joining tree based on alignment of the V4 locus of the 18S SSU gene of selected *Babesia* and *Theileria* spp.

The bovine 18S gene sequence was used to root the tree.

The utility of the V4 region to differentiate *Babesia* and *Theileria* spp. was investigated by creating a second dendrogram based on this region alone (Figure 3-7). The dendrogram based on the V4 region was able to differentiate each species represented in the panel of sequences. As anticipated, it grouped all replicate sequences from the same species and the small number of *Theileria* sequences formed a monophyletic group.

The locations of previously published *Babesia* and *Theileria* spp. 18S primers are summarised in Table 3-2. These are illustrated relative to the *B. divergens* AY046576 sequence and the *Babesia* and *Theileria* spp. alignment. The majority were single primer pairs (eight primer pairs, two of which amplify the entire gene) along with two nested protocols, one semi-nested protocol and two protocols for sequencing either part or all of the gene.

	Original description	Primer name	Note	Primer sequence 5'-3'	*B. divergens*			Alignment	
					5'	3'	Size	5'	3'
1	Armstrong *et al.* (1998)	PIRO-A	F	AATACCCAATCCTGACACAGGG	411	432	22	430	451
		PIRO-B	R	TTAAATACGAATGCCCCCAAC	797	817	21	850	870
		Amplicon			411	817	407	430	870
2	Birkenheuer *et al.* (2003)	5-22F	NO F	GTTGATCCTGCCAGTAGT	7	24	18	7	24
		1661R	NO R	AACCTTGTTACGACTTCTC	1680	1698	19	1744	1762
		Amplicon			7	1698	1692	7	1762
		455-479F	NI F	GTCTTGTAATTGGAATGATGGTGAC	467	491	25	487	511
		793-772R	NI R	ATGCCCCCAACCGTTCCTATTA	786	807	22	839	860
		Amplicon			467	807	341	487	860
3	Bonnet *et al.* (2007b)	BAB GF2	F	GYYTTGTAATTGGAATGATGG	467	487	21	487	507
		BAB GR2	R	CCAAAGACTTTGATTTCTCTC	1006	1026	21	1060	1080
		Amplicon			467	1026	560	487	1080
4	Casati *et al.* (2006)	BJ1	F	GTCTTGTAATTGGAATGATGG	467	487	21	487	507
		BN2	R	TAGTTTATGGTTAGGACTACG	935	955	21	988	1008
		Amplicon			467	955	489	487	1008
5A	Criado-Fornelio *et al.* (2003)	BT1-F	SNO F	GGTTGATCCTGCCAGTAGT	6	24	19	6	24
		BTH-1R	SNO R	TTGCGACCATACTCCCCCCA	1031	1050	20	1085	1104
		Amplicon			6	1050	1045	6	1104
	Criado-Fornelio *et al.* (2003)	BT1-F	SNI F	GGTTGATCCTGCCAGTAGT	6	24	19	6	24
		BT1-R	SNI R	GCCTGCTGCCTTCCTTA	388	404	17	406	422
		Amplicon			6	404	399	6	422
5B	Criado-Fornelio *et al.* (2003)	BTH-1F	Seq F	CCTGMGARACGGCTACCACATCT	366	388	23	384	406
		BTH-1R	Seq R	TTGCGACCATACTCCCCCCA	1031	1050	20	1085	1104
		Amplicon			366	1050	685	384	1104
5C	Criado-Fornelio *et al.* (2003)	BT2-F	Seq F	GGAGTATGGTCGCAAGTCTG	1036	1055	20	1090	1109
		BT2-R	Seq R	CTTCTGCAGGTTCACCTACG	1701	1720	20	1765	1784
		Amplicon			1036	1720	685	1090	1784
5D	Criado-Fornelio *et al.* (2003)	BT1-F	18S F	GGTTGATCCTGCCAGTAGT	6	24	19	6	24
		BT2-R	18S R	CTTCTGCAGGTTCACCTACG	1701	1720	20	1765	1784
		Amplicon			6	1720	1715	6	1784
6	Georges *et al.* (2001)	RLB-F2	F	GACACAGGGAGGTAGTGACAAG	424	445	22	443	464
		RLB-R2	R	CTAAGAATTTCACCTCTGACAGT	817	839	23	870	892
		Amplicon			424	839	416	443	892
7	Gubbels *et al.* (1999)	RLB-F	F	GAGGTAGTGACAAGAAATAACAATA	432	456	25	451	475
		RLB-R	R	TCTTCGATCCCCTAACTTTC	901	920	20	954	973
		Amplicon			432	920	489	451	973
8	(Herwaldt *et al.*, 2003)	CRYPTOF	18S F	AACCTGGTTGATCCTGCCAGT	1	21	21	1	21
		CRYPTOR	18S R	GCTTGATCCTTCTGCAGGTTCACCTAC	1702	1728	27	1766	1792
		Amplicon			1	1728	1728	1	1792
9	Hilpertshauser *et al.* (2006)	Bab sppF	F	GTTTCTGMCCCATCAGCTTGAC	276	295	22	292	313
		Bab sppR	R	CAAGACAAAAGTCTGCTTGAAAC	692	714	23	743	766

		Amplicon			276	714	439	292	766
10A	Malandrin *et al.* (2010)	CRYPTOF	Seq 1 F	AACCTGGTTGATCCTGCCAGTAGTCAT	1	27	27	1	27
		CRYP-down	Seq 1 R	CTGCTGGCACCAGACTTGCC	521	540	20	541	560
		Amplicon			1	540	540	1	560
10B	Malandrin *et al.* (2010)	BABGF2	Seq 2 F	GYYTTGTAATTGGAATGATGG	467	487	21	487	507
		BABGR2	Seq 2 R	CCAAAGACTTTGATTTCTCTC	1006	1026	21	1060	1080
		Amplicon			467	1026	560	487	1080
10C	Malandrin *et al.* (2010)	CRYP-up2	Seq 3 F	GCCGCCTAGGGATTGGAGG	957	975	19	1010	1028
		CRYPTOR	Seq 3 R	GAATGATCCTTCCGCAGGTTCACCTAC	1702	1728	27	1766	1792
		Amplicon			957	1728	772	1010	1792
11	Yabsley *et al.* (2005)	5.1	NO F	CCTGGTTGATCCTGCCAGTAGT	3	24	22	3	24
		3.1	NO R	CTCCTTCCTTTAAGTGATAAG	1662	1682	21	1726	1746
		Amplicon			3	1682	1680	3	1746
	Gubbels *et al.* (1999)	RLB-F	NI F	GAGGTAGTGACAAGAAATAACAATA	432	456	25	451	475
		RLB-R	NI R	TCTTCGATCCCCTAACTTTC	901	920	20	954	973
		Amplicon			432	920	489	451	973

Table 3-2 Primers used to detect and sequence the *Babesia* and *Theileria* spp. 18S gene

F, forward; R, reverse; NO, nested outer; NI, nested inner; SNO, semi-nested outer; SNI, semi-nested inner; Seq, sequencing; 18S, amplification of the entire 18S gene. Primer sequences, size, and annealing positions are indicated relative to *B. divergens* AY046576 and the *Babesia* and *Theileria* spp. alignment. Expected amplicon sizes (including primers) for *B. divergens* AY046576 are highlighted in grey. The location of primers are represented on the alignment graphic (Figure 3-8), labeled 1-11.

Combining the visual representation of the *Babesia* and *Theileria* 18S gene alignment (Figure 3-6) and the primer annealing positions relative to the alignment (Table 3-2) allowed visualisation of primer annealing sites and amplified regions (Figure 3-8). Interestingly, excluding primer pairs amplifying the entire *Babesia* or *Theileria* spp. 18S gene and those intended for use in partial or complete sequencing of the gene, five of the six single primer pairs amplified the phylogenetically informative segment of V4 region. Additionally, both inner primer pairs of the nested protocols also amplified this region. Both observations are consistent with the potential of this region identified earlier and provide support for its targeting in the present study.

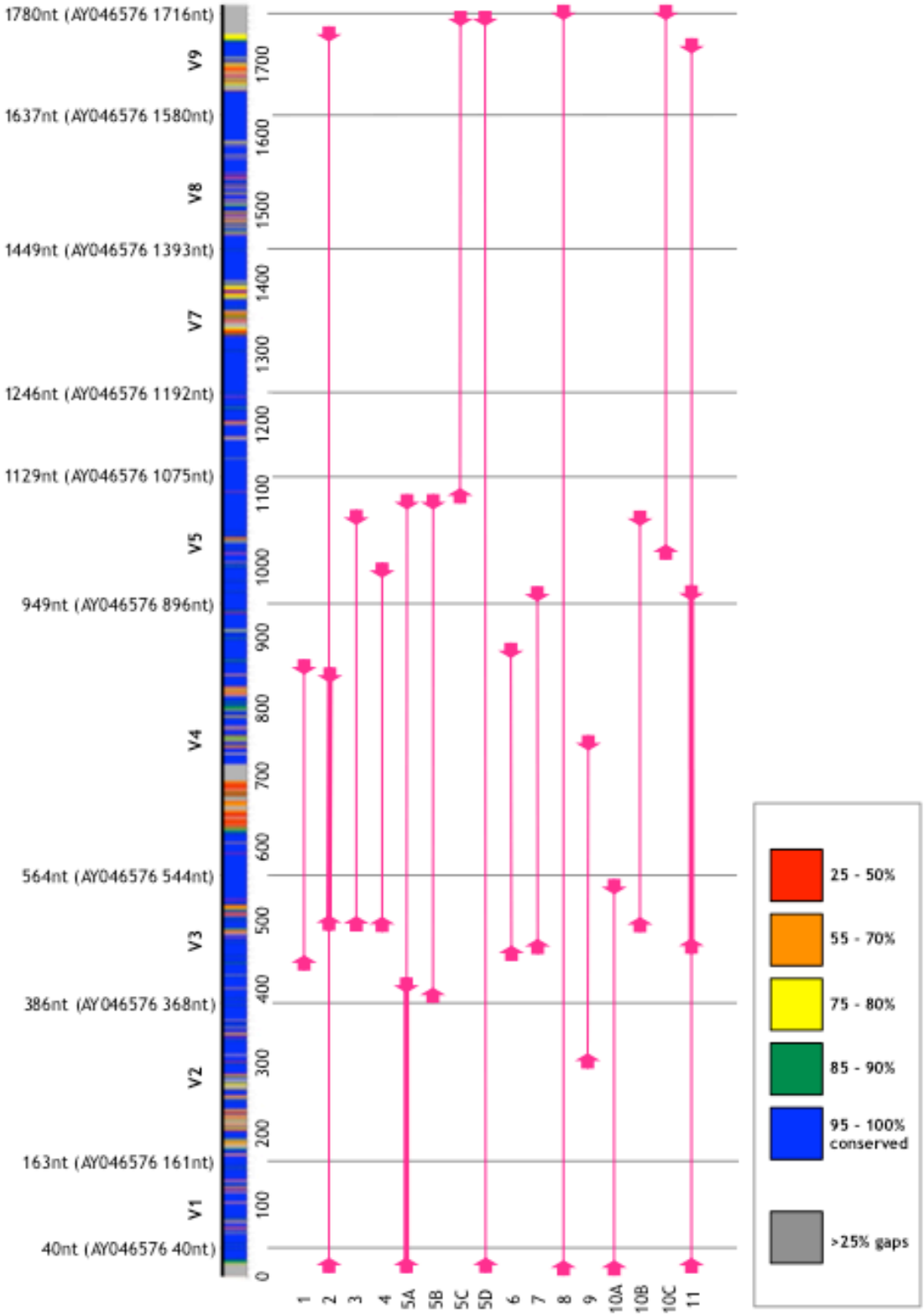
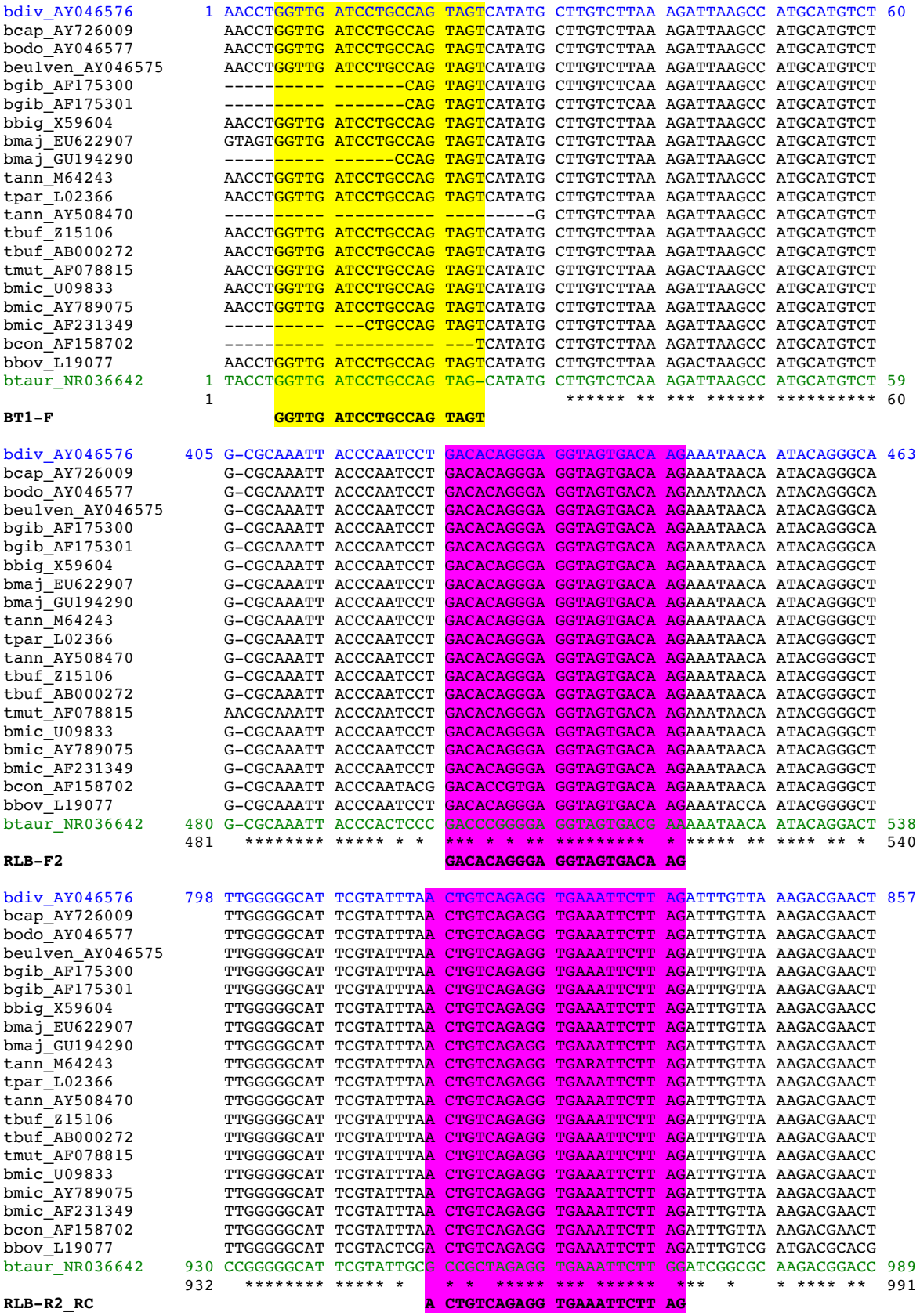


Figure 3-8 Linear diagram of the *Babesia* and *Theileria* spp. alignment with primers

Forward and reverse primers (numbered and described in detail in Table 3-2) are represented by short arrows with the intervening amplicon shown as a narrow (outer reaction) or thick line (inner reaction). Co-ordinates shown relative to the *Babesia/Theileria* spp. alignment with the co-ordinates relative to the *B. divergens* AY046576 sequence in parenthesis.

It was decided that a nested PCR protocol was required in order to maximise the potential sensitivity of the test. It was recognised that previously published outer and inner primer pairs could be incorporated into a novel assay if necessary. The choice of primers was based on manual examination of annealing sites against the multi-species *Babesia* and *Theileria* spp. 18S gene alignment, which also incorporated the bovine 18S sequence (Figure 3-9). The objective was to select existing primers with the potential to anneal as wide a range of *Babesia* and *Theileria* spp. as possible, but sufficiently different from the host sequence to maintain specificity. Primer annealing sites were manually examined for conservation across the alignment and excluded from consideration if they failed to broadly match all sequences. Additionally, shorter amplicons were favoured to reduce errors in amplification, minimise cycling times and to facilitate direct sequencing. A combination of primers was selected to fulfill these criteria and these form the basis for a novel nested protocol. The outer primer pair selected was BT1-F 5′-GGTTGATCCTGCCAGTAGT and BTH-1R 5′-TTGCGACCATACTCCCCCCA Criado-Fornelio *et al*. (2003) while the inner pair was RLB-F2 5′-GACACAGGGAGGTAGTGACAAG and RLB-R2 5′-CTAAGAATTTCACCTCTGACAGT Georges *et al*. (2001). Primer annealing sites are illustrated in Figure 3-9.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bcap_AY726009 AACCTGGTTG ATCCTGCCAG TAGTCATATG CTTGTCTTAA AGATTAAGCC ATGCATGTCT
bodo_AY046577 AACCTGGTTG ATCCTGCCAG TAGTCATATG CTTGTCTTAA AGATTAAGCC ATGCATGTCT
beu1ven_AY046575 AACCTGGTTG ATCCTGCCAG TAGTCATATG CTTGTCTTAA AGATTAAGCC ATGCATGTCT
bgib_AF175300 ---------- -------CAG TAGTCATATG CTTGTCTCAA AGATTAAGCC ATGCATGTCT
bgib_AF175301 ---------- -------CAG TAGTCATATG CTTGTCTCAA AGATTAAGCC ATGCATGTCT
bbig_X59604 AACCTGGTTG ATCCTGCCAG TAGTCATATG CTTGTCTTAA AGATTAAGCC ATGCATGTCT
bmaj_EU622907 GTAGTGGTTG ATCCTGCCAG TAGTCATATG CTTGTCTTAA AGATTAAGCC ATGCATGTCT
bmaj_GU194290 ---------- ------CCAG TAGTCATATG CTTGTCTTAA AGATTAAGCC ATGCATGTCT
tann_M64243 AACCTGGTTG ATCCTGCCAG TAGTCATATG CTTGTCTTAA AGATTAAGCC ATGCATGTCT
tpar_L02366 AACCTGGTTG ATCCTGCCAG TAGTCATATG CTTGTCTTAA AGATTAAGCC ATGCATGTCT
tann_AY508470 ---------- ---------- ----------G CTTGTCTTAA AGATTAAGCC ATGCATGTCT
tbuf_Z15106 AACCTGGTTG ATCCTGCCAG TAGTCATATG CTTGTCTTAA AGATTAAGCC ATGCATGTCT
tbuf_AB000272 AACCTGGTTG ATCCTGCCAG TAGTCATATG CTTGTCTTAA AGATTAAGCC ATGCATGTCT
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bmic_AF231349 ---------- ---CTGCCAG TAGTCATATG CTTGTCTTAA AGATTAAGCC ATGCATGTCT
bcon_AF158702 ---------- ---------- ---TCATATG CTTGTCTTAA AGATTAAGCC ATGCATGTCT
bbov_L19077 AACCTGGTTG ATCCTGCCAG TAGTCATATG CTTGTCTTAA AGACTAAGCC ATGCATGTCT
btaur_NR036642 1 TACCTGGTTG ATCCTGCCAG TAG-CATATG CTTGTCTCAA AGATTAAGCC ATGCATGTCT 59
1 ****** ** *** ****** ********** 60
BT1-F GGTTG ATCCTGCCAG TAGT
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bodo_AY046577 G-CGCAAATT ACCCAATCCT GACACAGGGA GGTAGTGACA AGAAATAACA ATACAGGGCA
beu1ven_AY046575 G-CGCAAATT ACCCAATCCT GACACAGGGA GGTAGTGACA AGAAATAACA ATACAGGGCA
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bmic_U09833 G-CGCAAATT ACCCAATCCT GACACAGGGA GGTAGTGACA AGAAATAACA ATACAGGGCT
bmic_AY789075 G-CGCAAATT ACCCAATCCT GACACAGGGA GGTAGTGACA AGAAATAACA ATACAGGGCT
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bcon_AF158702 G-CGCAAATT ACCCAATACG GACACCGTGA GGTAGTGACA AGAAATAACA ATACAGGGCT
bbov_L19077 G-CGCAAATT ACCCAATCCT GACACAGGGA GGTAGTGACA AGAAATACCA ATACGGGGCT
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481 ******** ***** * * *** * * ** ********* * ***** ** **** ** * 540
RLB-F2 GACACAGGGA GGTAGTGACA AG
bdiv_AY046576 798 TTGGGGGCAT TCGTATTTAA CTGTCAGAGG TGAAATTCTT AGATTTGTTA AAGACGAACT 857
bcap_AY726009 TTGGGGGCAT TCGTATTTAA CTGTCAGAGG TGAAATTCTT AGATTTGTTA AAGACGAACT
bodo_AY046577 TTGGGGGCAT TCGTATTTAA CTGTCAGAGG TGAAATTCTT AGATTTGTTA AAGACGAACT
beu1ven_AY046575 TTGGGGGCAT TCGTATTTAA CTGTCAGAGG TGAAATTCTT AGATTTGTTA AAGACGAACT
bgib_AF175300 TTGGGGGCAT TCGTATTTAA CTGTCAGAGG TGAAATTCTT AGATTTGTTA AAGACGAACT
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tbuf_Z15106 TTGGGGGCAT TCGTATTTAA CTGTCAGAGG TGAAATTCTT AGATTTGTTA AAGACGAACT
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tmut_AF078815 TTGGGGGCAT TCGTATTTAA CTGTCAGAGG TGAAATTCTT AGATTTGTTA AAGACGAACC
bmic_U09833 TTGGGGGCAT TCGTATTTAA CTGTCAGAGG TGAAATTCTT AGATTTGTTA AAGACGAACT
bmic_AY789075 TTGGGGGCAT TCGTATTTAA CTGTCAGAGG TGAAATTCTT AGATTTGTTA AAGACGAACT
bmic_AF231349 TTGGGGGCAT TCGTATTTAA CTGTCAGAGG TGAAATTCTT AGATTTGTTA AAGACGAACT
bcon_AF158702 TTGGGGGCAT TCGTATTTAA CTGTCAGAGG TGAAATTCTT AGATTTGTTA AAGACGAACT
bbov_L19077 TTGGGGGCAT TCGTACTCGA CTGTCAGAGG TGAAATTCTT AGATTTGTCG ATGACGCACG
btaur_NR036642 930 CCGGGGGCAT TCGTATTGCG CCGCTAGAGG TGAAATTCTT GGATCGGCGC AAGACGGACC 989
932 ******** ***** * * * ***** *** ****** *** * * **** ** 991
RLB-R2_RC A CTGTCAGAGG TGAAATTCTT AG

```
bdiv_AY046576    1006 GAGAGAAATC AAAGTCTTTG GGTTCTGGGG GGAGTATGGT CGCAAGGCTG AAACTTAAAG 1065
bcap_AY726009         GAGAGAAATC AAAGTCTTTG GGTTCTGGGG GGAGTATGGT CGCAAGGCTG AAACTTAAAG
bodo_AY046577         GAGAGAAATC AAAGTCTTTG GGTTCTGGGG GGAGTATGGT CGCAAGGCTG AAACTTAAAG
beu1ven_AY046575      GAGAGAAATC AAAGTCTTTG GGTTCTGGGG GGAGTATGGT CGCAAGGCTG AAACTTAAAG
bgib_AF175300         GAGAGAAATC AAAGTCTTTG GGTTCTGGGG GGAGTATGGT CGCAAGGCTG AAACTTAAAG
bgib_AF175301         GAGAGAAATC AAAGTCTTTG GGTTCTGGGG GGAGTATGGT CGCAAGGCTG AAACTTAAAG
bbig_X59604           GAGAGAAATC AAAGTCTTTG GGTTCTGGGG GGAGTATGGT CGCAAGTCTG AAACTTAAAG
bmaj_EU622907         GAGAGAAATC AAAGTCTTTG GGTTCTGGGG GGAGTATGGT CGCAAGTCTG AAACTTAAAG
bmaj_GU194290         GAGAGAAATC AAAGTCTTTG GGTTCTGGGG GGAGTATGGT CGCAAGTCTG AAACTTAAAG
tann_M64243           GAGAGAAATC AAAGTCTTTG GGTTCTGGGG GGAGTATGGT CGCAAGGCTG AAACTTAAAG
tpar_L02366           GAGAGAAATC AAAGTCTTTG GGTTCTGGGG GGAGTATGGT CGCAAGGCTG AAACTTAAAG
tann_AY508470         GAGAGAAATC AAAGTCTTTG GGTTCTGGGG GGAGTATGGT CGCAAGGCTG AAACTTAAAG
tbuf_Z15106           GAGAGAAATC AAAGTCTTTG GGTTCTGGGG GGAGTATGGT CGCAAGGCTG AAACTTAAAG
tbuf_AB000272         GAGAGAAATC AAAGTCTTTG GGTTCTGGGG GGAGTATGGT CGCAAGGCTG AAACTTAAAG
tmut_AF078815         GAGAGAAATC AAAGTCTTTG GGTTCTGGGG GGAGTATGGT CGCAAGGCTG AAACTTAAAG
bmic_U09833           GAGAGAAATC AAAGTCTTTG GGTTCTGGGG GGAGTATGGT CGCAAGTCTG AAACTTAAAG
bmic_AY789075         GAGAGAAATC AAAGTCTTTG GGTTCTGGGG GGAGTATGGT CGCAAGTCTG AAACTTAAAG
bmic_AF231349         GAGAGAAATC AAAGTCTTTG GGTTCTGGGG GGAGTATGGT CGCAAGTCTG AAACTTAAAG
bcon_AF158702         GAGAGAAATC AAAGTCTTTG GGTTCTGGGG GGAGTATGGT CGCAAGGCTG AAACTTAAAG
bbov_L19077           GAGAGAAATC AAAGTCTTTG GGTTCTGGGG GGAGTATGGT CGCAAGTCTG AAACTTAAAG
btaur_NR036642   1138 CCGGGAAACC AAAGTCTTTG GGTTCCGGGG GGAGTATGGT TGCAAAGCTG AAACTTAAAG 1197
                 1141   * **** * ********** ***** **** **********  ****  *** ********** 1200
BTH-1R_RC                                    TGGGG GGAGTATGGT CGCAA
```

Figure 3-9 *Babesia* and *Theileria* spp. 18S gene sequence alignment

Babesia and *Theileria* spp. 18S sequence alignment with *Babesia divergens* AY046576 highlighted in blue and host *Bos taurus* NR036642 18S gene sequence highlighted in green. Outer primer sites (BT1-F and BTH-1R) are highlighted in yellow, and nested primers sites (RLB-2F and RLB-2R) in pink.

Details of the two selected primers are summarised in Table 3-3 and illustrated graphically in Figure 3-3.

	Original description	Primer name	Note	Sequence 5'-3'	Bdiv AY046576 5'	Bdiv AY046576 3'	Size	Alignment 5'	Alignment 3'
5A	Criado-Fornelio *et al.* (2003)	BT1-F	SNO F	GGTTGATCCTGCCAGTAGT	6	24	19	6	24
		BTH-1R	SNO R	TTGCGACCATACTCCCCCCA	1031	1050	20	1085	1104
		Amplicon			6	1050	1045	6	1104
6	Georges *et al.* (2001)	RLB-F2	F	GACACAGGGAGGTAGTGACAAG	424	445	22	443	464
		RLB-R2	R	CTAAGAATTTCACCTCTGACAGT	817	839	23	870	892
		Amplicon			424	839	416	443	892

Table 3-3 Selected outer and inner primers

F, forward; R, reverse; SNO, semi-nested outer. Primer annealing positions are indicated relative to *Babesia divergens* AY046576 and the *Babesia* and *Theileria* spp. alignment. Expected amplicon sizes (including primers) for *Babesia divergens* AY046576 are highlighted in grey.

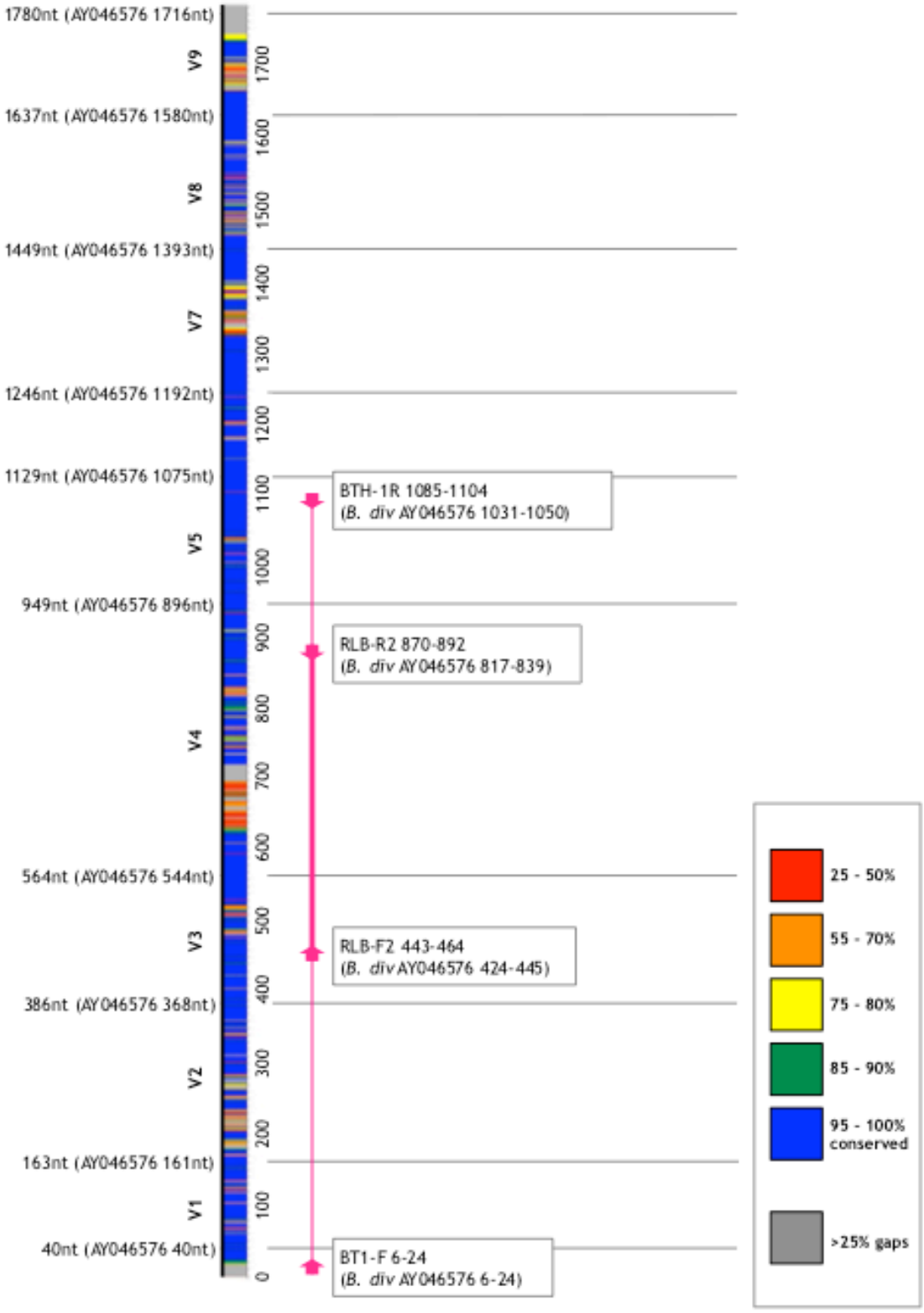

Figure 3-10 Graphical representation of the *Babesia* and *Theileria* spp. alignment with selected primers

Forward and reverse primers (numbered and described in detail in Table 3-3) are represented by short arrows with the intervening amplicon shown as a narrow (outer reaction) or thick line (inner reaction). Co-ordinates shown relative to the *Babesia/Theileria* spp. alignment with the co-ordinates relative to the *B. divergens* AY046576 sequence in parenthesis.

To confirm that the targeted region would provide sufficient sequence diversity to discriminate different *Theileria* and *Babesia* spp. on direct sequencing of the amplicon of the inner PCR reaction, a neighbour-joining tree was constructed (Figure 3-11) based on the predicted amplicon from a range of species. This confirmed the potential utility of this locus for the discovery and identification of piroplasm parasites.

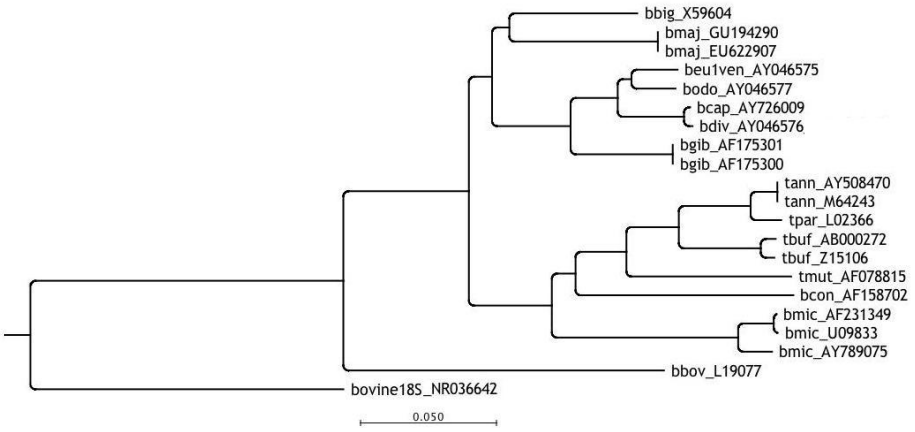

Figure 3-11 Neighbour-joining tree based on alignment of the RLB-F2/RLB-R2 amplified region of the 18S gene of selected *Babesia* and *Theileria* spp.

The bovine 18S gene sequence was used to root the tree.

3.3.1.2 PCR optimisation and testing

In order to determine the optimum annealing temperature for the selected primers, gradient PCR was performed (temperature range 15 °C centred at 62 °C) using the BdivF DNA extract as template. The results of this are shown in Figure 3-12 for the outer primer pair (BT1-F and BTH-1R) and in Figure 3-13 for the inner primer pair (RLB-F2 and RLB-R2). Based on the examination of these gels, an annealing temperature of 67 °C was selected the outer primer pair and 62 °C for the inner pair. The finalised *Babesia* and *Theileria* spp. 18S PCR protocol is summarised in Figure 3-14.

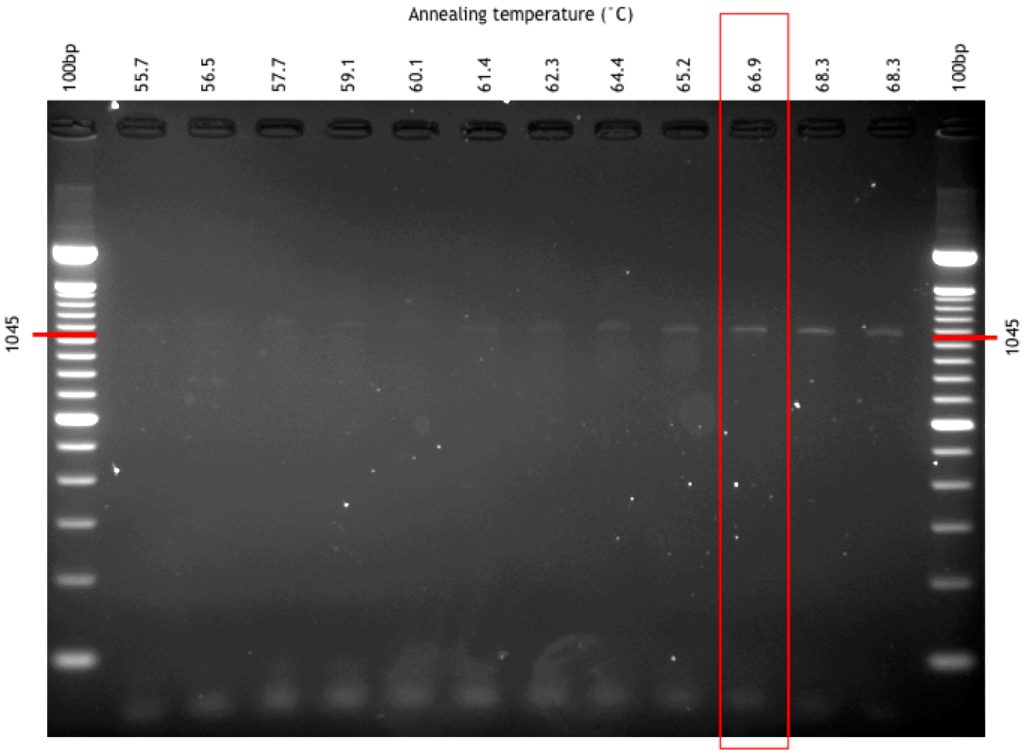

Figure 3-12 Gradient PCR of the outer primer pair (BT1-F/BTH-1R)

The selected lane and annealing temperature (67 °C) are highlighted by the red box and expected amplicon size (Table 3-2 and Table 3-3) indicated by red bars in the ladder.

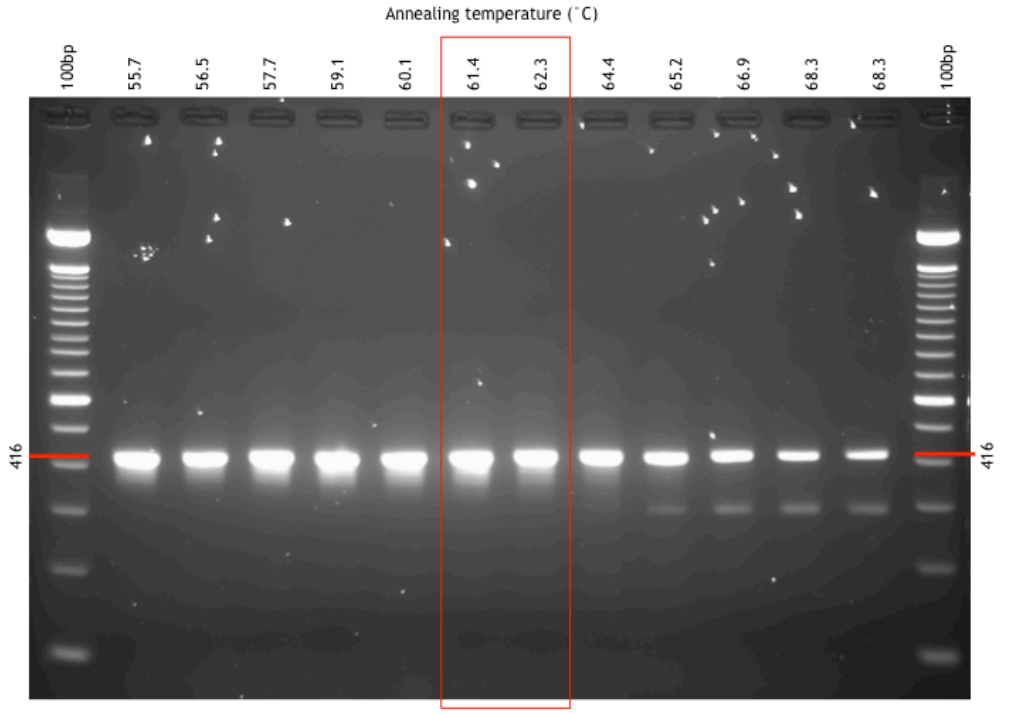

Figure 3-13 Gradient PCR of the inner primer pair (RLB-F2/RLB-R2)

The selected lane and annealing temperature (62 °C) are highlighted by the red box and expected amplicon size (Table 3-2 and Table 3-3) indicated by red bars in the ladder.

Outer reaction (primers BT1-F/ BTH-1R)

- Heated lid to 105°C 4 min before start of reaction
- Initial denaturation at 94°C for 5 min
- 30 cycles of:
    - denaturation at 94°C for 45s
    - annealing at 67°C for 60s
    - extension at 72°C for 60s
- Final extension at 72°C for 5 min

The primary reaction product is then diluted 1:100 for use as template in the nested reaction.

Nested reaction (primers RLB-F2/ RLB-R2)

- Heated lid to 105°C 4 min before start of reaction
- Initial denaturation at 94°C for 5 min
- 30 cycles of:
    - denaturation at 94°C for 45s
    - annealing at 62°C for 60s
    - extension at 72°C for 60s
- Final extension at 72°C for 5 min

Figure 3-14 Finalised *Babesia* and *Theileria* spp. 18S PCR protocol

In order to assess the sensitivity of the novel PCR protocol, a blood sample from a clinical case of babesiosis from Dumfries and Galloway caused by *B. divergens* (BdivF) was used to create dilution series. Assessment of the erythron of this clinical case resulted in the following parameters (with reference intervals shown):

- RBC 1.39 $x10^{12}$ cells/l (5.0 - 10.0)
- Hb 3.5 g/dl (8.0 - 15.0)
- HCT 6.5% (24% - 46%)

Examination of the stained red cell monolayer using an eyepiece modified to delimit a central area of each high power field (at x400 magnification) allowed counting of parasitised and total red cells in 70 fields. Using the mean for each figure, the level of parasitaemia was calculated:

- Mean total number of red cells per delimited field = 42.6
- Mean number of parasitised red cells per delimited field = 14.6
- Parasitaemia = 14.6 / 42.6 x 100 = 34.3 %

Using water as a diluent, a serial dilution of the BdivF DNA extract was created giving neat, 1:10, 1:100, 1:1,000, $1:10^4$, $1:10^5$, $1:10^6$ and $1:10^7$ dilutions. The products of the outer reactions using the BdivF DNA extract dilution series as a template is shown in Figure 3-15. The highest dilution at which a visible amplicon was produced was 1 in 1,000. The products of the nested reactions using the corresponding diluted primary reaction products as template are shown in Figure 3-16. The nested reaction resulted in a visible amplicon down to a dilution of 1 in 10^6 of the original BdivF DNA extract.

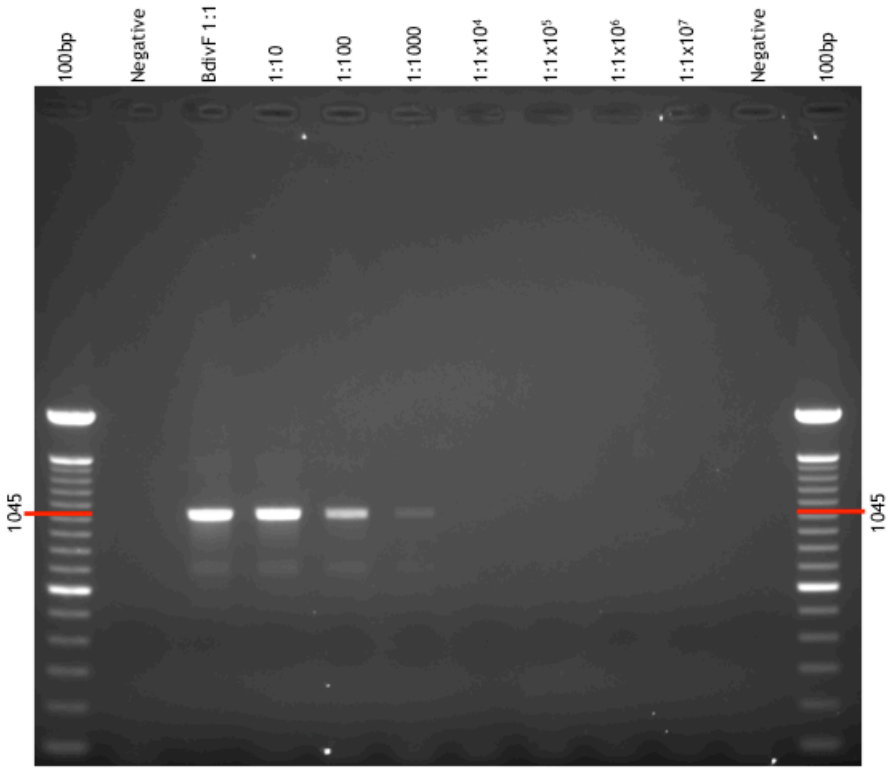

Figure 3-15 Dilution series for outer primer pair (BT1-F/BTH-1R)

Dilution series of BdivF DNA extract; red bars indicated expected amplicon size (1,045 bp, Table 3-3).

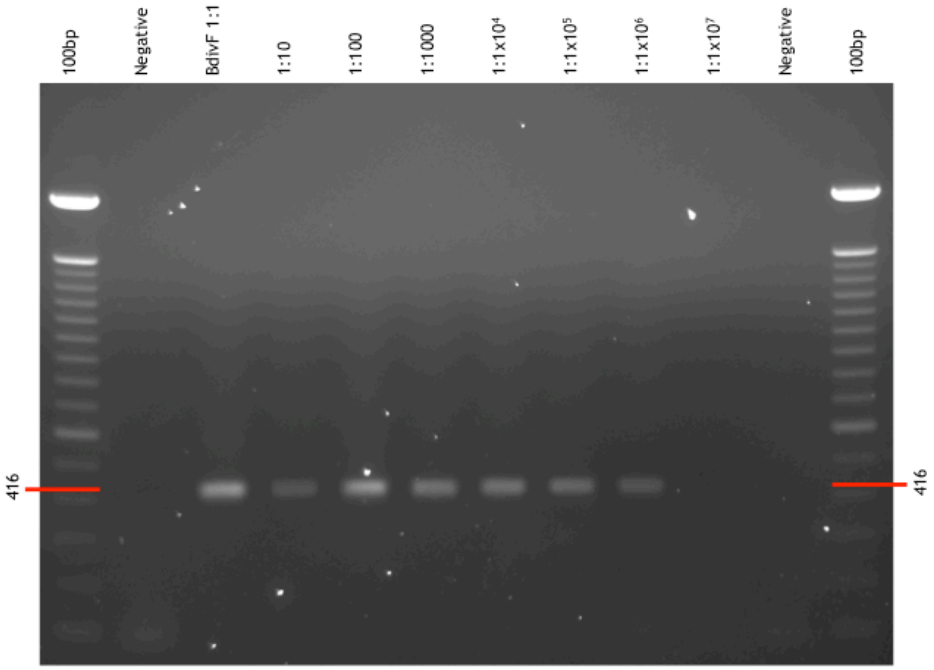

Figure 3-16 Dilution series for nested primer pair (RLB-2F/RLB-2R)

Dilution series of BdivF DNA extract; 1/100 dilution of primary product used as template. Red bars indicated expected amplicon size (416 bp, Table 3-3).

Because the number of red blood cells (1.39 x 10^{12} RBC/l (normal 5.0 - 10.0)), the level of parasite infection of these cells (34.3 %) and the maximum dilution of the extracted DNA solution at which the assay could amplify the target *B. divergens* sequence (1:1 x 10^6) were known for the BdivF blood sample, the theoretical lower limit of detection of the assay could be calculated:

*In the blood sample there were (1.39 x 10^{12} / 100) * 34.4 = 4.77 x 10^{11} infected cells/l. Therefore the assay could detect an equivalent of 4.77 x 10^{11} / 10^6 = 4.77 x 10^5 infected cells/l (or 477 infected cells/ml or 0.477 infected cells/µl). This animal was anaemic due to the infection. In a normal animal with 7.5 x 10^{12} RBC/l (middle of normal range) the calculated lower detection limit of 4.77 x 10^5 infected cells/ l would be equivalent to a parasitaemia of (4.77 x 10^5 / 7.5 x 10^{12}) * 100 = 6 x 10^{-6} % which equals 0.000006 %.*

Alignment of the selected primers with the bovine sequence suggested cross-amplification of bovine DNA was unlikely (Figure 3-9). This was confirmed by carrying out both outer and nested PCR reactions with DNA extracted from bovine BL20 cells in culture (Figure 3-17 and Figure 3-18). Similarly, no-template controls did not generate amplicons. Both outer and nested primers readily amplified the *B. divergens* DNA samples Bdiv1, Bdiv2, BdivT1 and BdivT2, in addition to BdivF, used in the optimisation process.

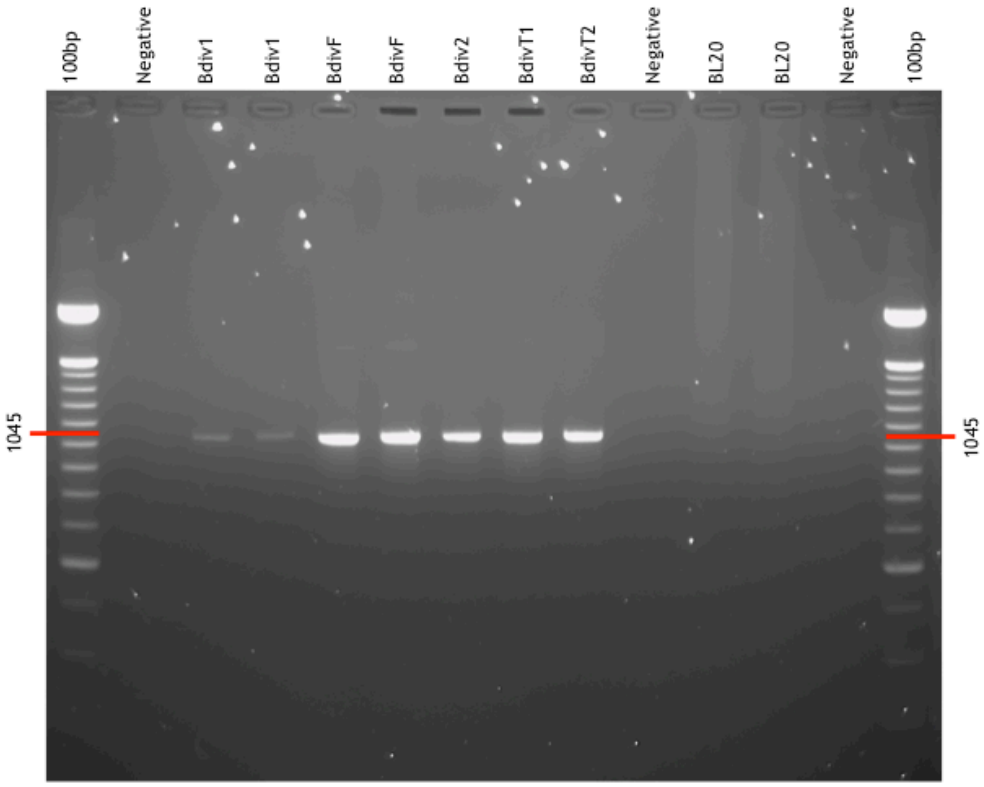

Figure 3-17 Control PCRs for outer primer pair (BT1-F/BTH-1R)

PCR templates were as follows: *B. divergens* (Bdiv1, Bdiv2, BdivT1, BdivT2 and BdivF); water (Negative); bovine (BL20). Red bars indicate expected amplicon size (1,045 bp) for *B. divergens* AY046576 (Table 3-3).

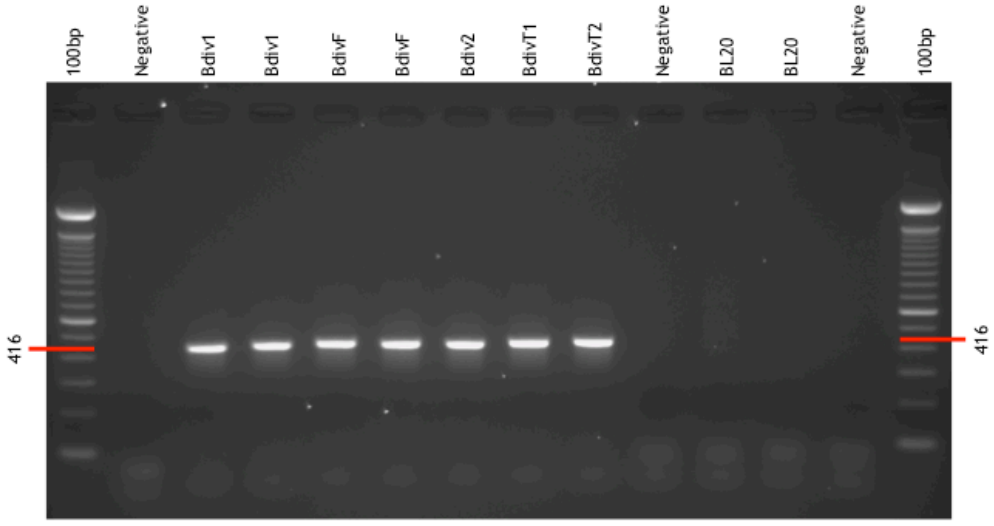

Figure 3-18 Control PCRs for inner primer pair (RLB-2F/RLB-2R)

PCR templates were as follows: *B. divergens* (Bdiv1, Bdiv2, BdivT1, BdivT2 and BdivF); water (Negative); bovine (BL20). Red bars indicate expected amplicon size (416 bp) for *B. divergens* AY046576 (Table 3-3).

Nested PCR using the selected primers and DNA extracted from a range of *Babesia* and *Theileria* spp. isolates resulted in amplification of all *Babesia* and *Theileria* spp. tested (Figure 3-19). One of the strains of *Babesia bovis* required dilution of the archived DNA extract template to generate an amplicon, which produced a less intense band than the others suggesting the presence of low levels of DNA and/or PCR inhibitory compounds. BdivF resulted in strong amplification as previously and both negative controls, *Ehrlichia ruminantium* and water, showed no amplification.

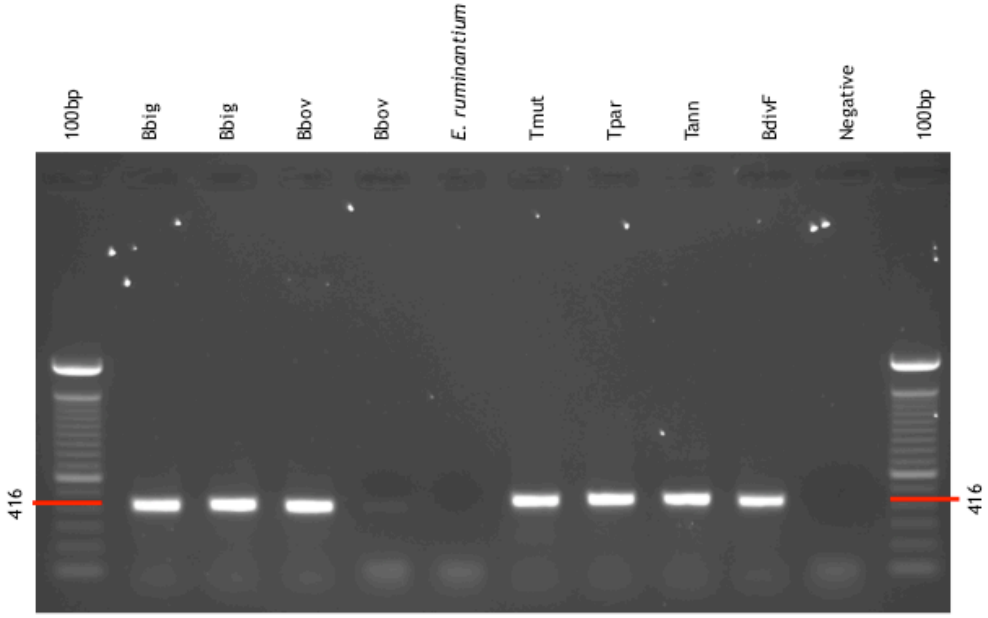

Figure 3-19 Amplification of template DNA from a range of Babesia and Theileria spp.

Babesia bigemina (Bbig), *Babesia bovis* (Bbov), *Theileria mutans (*Tmut), *Theileria parva* (Tpar), *Theileria annulata (*Tann), *Babesia divergens* (BdivF), *Ehrlichia ruminantium* and water (Negative). Red bars indicate expected amplicon size (416 bp) for *B. divergens* AY046576 (Table 3-3).

3.3.2 Analysis of field samples from SAC/SRUC Disease Surveillance Centres

Veterinary Investigation Officers at participating Disease Surveillance Centres identified 30 incidents (clinical events involving 1 or more cattle or sheep where tick-borne disease was confirmed by microscopic examination, suspected clinically, or suspected based on the presence of ticks) during the 15-month period between September 2013 and December 2014, from which they submitted blood and/or tissue samples (Table 3-4, and illustrated in Figure 3-20).

Submitted material	Samples submitted (Incidents (animals))	*Bab./The.* spp. PCR +ve (Incidents (animals))
Cattle (blood*)	3 (3)	3 (3)
Cattle (blood	9 (9)	0
Cattle (carcass)	1 (1)	0
All cattle	13 (13)	3 (3)
Sheep (blood)	2 (18)	0
Sheep (carcass)	14 (18)	0
Sheep (on-farm PM)	1 (2)	0
All sheep	17 (38)	0
Overall	30 (51)	3 (3)

Table 3-4 Material submitted from SAC/SRUC DSCs from incidents where a tick-borne disease was suspected, results of *Babesia* and *Theileria* spp. PCR

Submitted material by sample type and animal species showing *Babesia* and *Theileria* spp. PCR results. The number of incidents (clinical events where tick-borne disease was confirmed by microscopic examination (Cattle (blood*)), suspected clinically, or suspected based on the presence of ticks) is indicated together with the actual number of animals sampled in parenthesis.

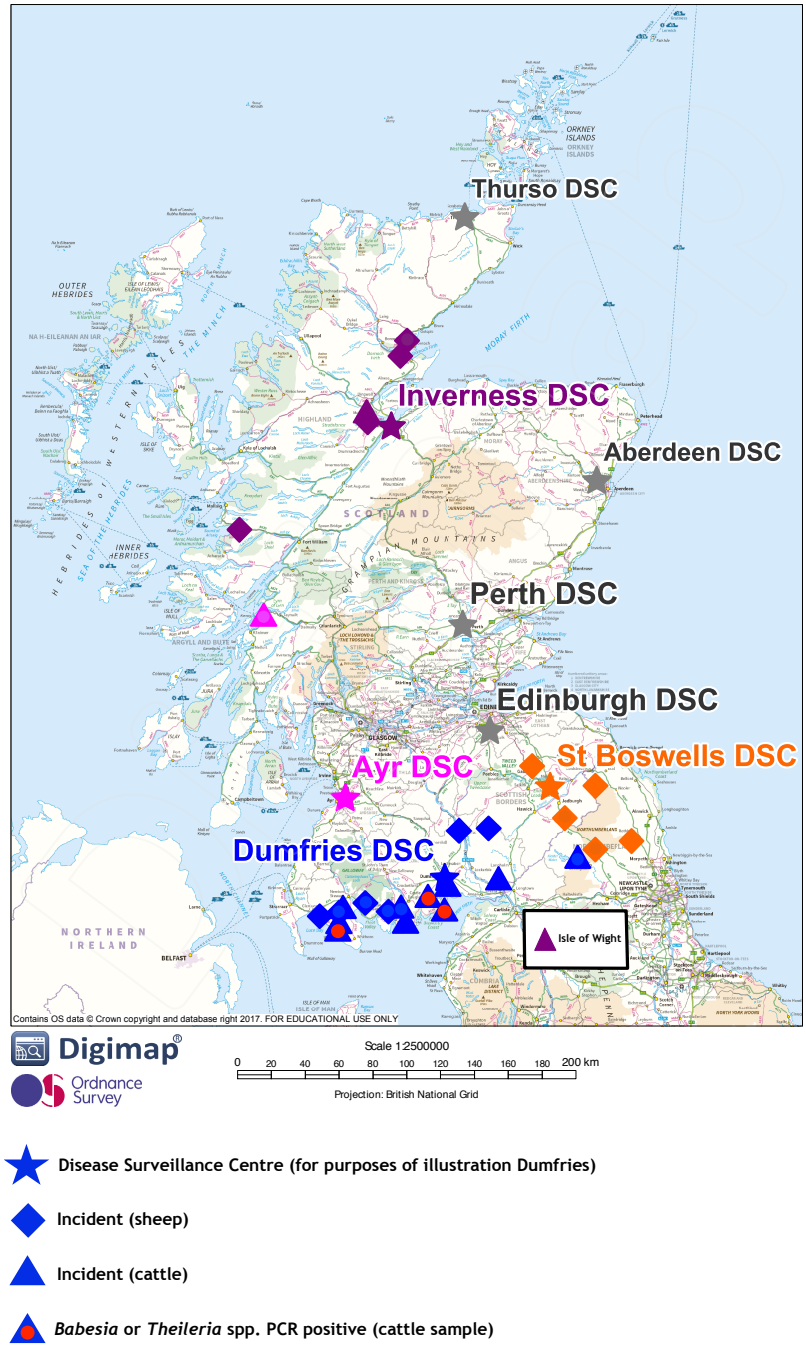

Figure 3-20 Spatial distribution of SAC/SRUC incidents of tick-borne disease from which samples were analysed, and where *Babesia*/ *Theileria* spp. PCR was positive

SAC/SRUC Disease Surveillance Centres (stars) labeled by colour, showing associated incidents involving sheep (diamonds) and cattle (triangles). A red dot within the symbol indicates that the incident resulted in a *Babesia*/*Theileria* spp. PCR positive result.

In total these 30 incidents involved 51 animals; 13 incidents involved cattle (13 animals) and 17 incidents involved sheep (38 animals). In 3 incidents the sample consisted of blood from cattle confirmed as having babesiosis following microscopic examination of stained blood smears; these samples were positive by *Babesia* and *Theileria* spp. PCR (Table 3-4, Cattle (blood*), and illustrated by red dots in Figure 3-20).

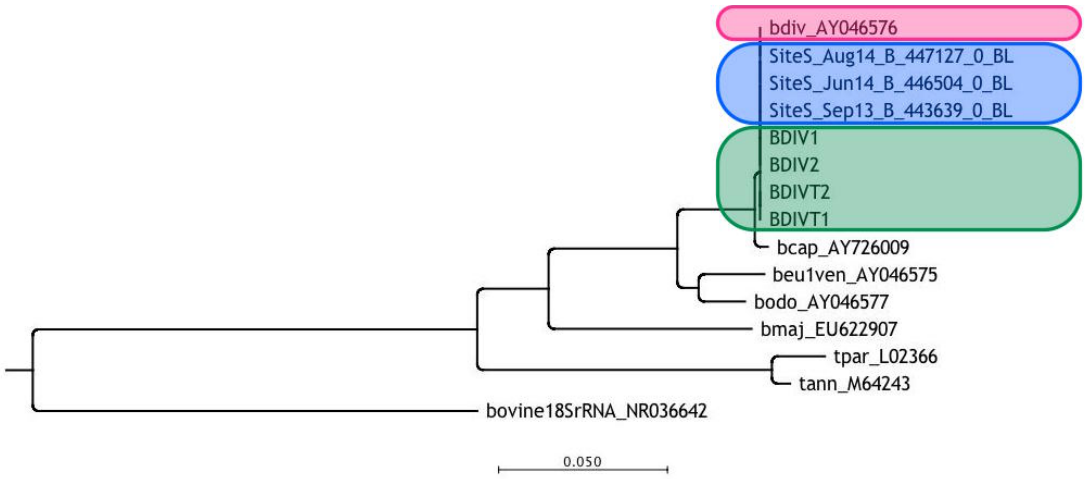

Figure 3-21 Alignment of amplicon sequences of *Babesia*/*Theileria* spp. PCR positive reactions from cattle confirmed as having babesiosis by microscopy

Neighbour-joining tree rooted with the bovine 18S gene sequence. Sequences from the present study are highlighted in blue, positive controls (BDIV1, BDIV2, BDIVT1, and BDIVT2) in green, and *B. divergens* AY046576 in pink. Other species include *B. venatorum* (AY046575), *B. capreoli* (AY726009), *B. odocoilei* (AY046577), *B. major* (EU622907), *T. parva* (L02366) and *T. annulata* (M64243).

The *Babesia*/*Theileria* spp. PCR amplicons were successfully sequenced and named according the convention previously described (Figure 3-4). The sequences were 371 nucleotides in length and were identical to each other, the *Babesia divergens* AY046576 reference sequence, and positive controls BDIV1, BDIV2, BDIVT1 and BDIVT2 (Figure 3-21).

Limited clinical details were available for these cases. For one of these cases occurring in June, only the animal's age and sex were provided (10 month old male). The other cases occurred in August (a 5 year old Holstein Friesian cow that was dull, pyrexic and had diarrhoea and haemoglobinuria) and September (a 17 month old Jersey cow recently moved to rough ground that was dull and had haemoglobinuria).

This sample set was also examined using the optimised 16S rRNA nested PCR described in Chapter 5 (results Table 5-2 (p. 131) and illustrated in Figure 5-7 (p. 132)). No co-infections were identified.

3.4 Discussion

3.4.1 Assay development

Although amplification of the entire 18S SSU gene and subsequent sequencing remains the gold standard for differentiating *Babesia* and *Theileria* spp. (Zintl *et al.*, 2011; Lempereur *et al.*, 2017) it has a number of disadvantages, for example increased difficulty of PCR of long sequences and the need for multiple sequencing primers and subsequent assembly. In order to circumvent these issues the first objective of this chapter was to determine if a shorter region of the gene could provide a similar level of resolution and if so, to select appropriate primers to construct a sensitive and specific nested PCR for application to sample material collected from the field. It was considered whether or not a short amplicon could be more appropriate for future investigations.

The 18S rRNA gene contains nine highly variable regions v1 to v9 (although the v6 region is relatively conserved in eukaryotes). Examination of an alignment of more than 50,000 eukaryote 18S rRNA gene sequences confirmed the v4 region as highly polymorphic and therefore phylogenetically informative (Hadziavdic *et al.*, 2014). In the course of this study the variable regions were defined relative to the 18S rRNA gene sequence of the yeast *Saccharomyces* cerevisiae (accession number Z75578). To examine the situation in *Babesia* and *Theileria* spp., and confirm the location and utility of the v4 region, parasite 18S rRNA gene sequences intentionally selected to represent a wide range of *Babesia* and *Theileria* spp. from diverse locations were aligned and compared. Consistent with the findings of Hadziavdic *et al.* (2014), the v4 region was capable of differentiating both distantly related and more closely related species, for example *B. divergens* and *B. capreoli* (Malandrin *et al.*, 2010). Primers were selected to anneal to conserved flanking regions but not to host species 18S rRNA sequence. This is recognised as a challenge (Qurollo *et al.*, 2017) due to the high proportion of sites conserved (a) between parasites and (b) between parasite and host. Fortunately inner and outer primers could be identified that fulfilled these requirements.

Experiments confirmed that a diverse range of *Babesia* and *Theileria* spp. were amplified and that host DNA was not. The lower limit of detection of the novel nested PCR combination was assessed as 4.77×10^5 infected cells/l (or 477 infected cells/ml or 0.477 infected cells/µl). However, this figure needs to be interpreted with caution as it was achieved by dilution of the extracted DNA, not the original blood sample with exogenous blood before DNA extraction. This may have resulted in a lower amount of PCR inhibitory substances than would result from DNA extraction from field-derived samples and therefore an artificially high level of theoretical sensitivity was achieved. Additionally, in the assessment of the original blood sample, only intracellular parasites were counted, meaning that the DNA from any extracellular piroplasms present would have increased the concentration of parasite DNA in the original DNA extract. Again, this could result in an artificially high estimate of sensitivity. A low number of extra-cellular parasites were noted on microscopy, which were assumed to be piroplasms released from infected red cells which haemolysed *in vitro*. With infected and non-infected cells both being liable to *in vitro* haemolysis, this would not impact upon the calculation of the percentage of parasitised red blood cells. However, the amount of parasite material in the sample may have been slightly underestimated, resulting in an unquantifiable but slight over-estimation of test sensitivity.

In summary, a novel nested combination of primers was selected based on amplification of the v4 region of the 18S rRNA gene that could clearly differentiate and identify *Babesia* and *Theileria* spp. following amplicon sequencing. Experimental work confirmed the assay's ability to amplify a wide range of species, its sensitivity and specificity. This novel nested combination can be used with confidence for the identification of recognised and novel *Babesia* and *Theileria* spp. from field samples.

3.4.2 Could a species morphologically similar to but distinct from *B. divergens* be causing clinical disease in Scottish livestock?

During the 15 month period between September 2013 and December 2014, blood from three cases of bovine babesiosis confirmed by microscopic examination of stained blood smears was examined. Each sample was found to contain

B. divergens. Amplicon sequencing confirmed the utility of this PCR assay to detect *B. divergens* in field-derived material, however no unexpected species were identified in these three samples.

The sequences were identical to GenBank AY046576 (Herwaldt *et al.*, 2003), suggested to be a prerequisite for molecular identification of *B. divergens* (Zintl *et al.*, 2011; Herwaldt *et al.*, 2003). It was found it be identical to a sequence from the Purnell isolate (Purnell *et al.*, 1976) originating in Ireland and another (GenBank Z48751) also originating in Northern Ireland.

Although the descriptions of the clinical signs were brief, and in one case limited to only the animal's age and sex, dullness and haemoglobinuria were common to the remaining two cases. This is consistent with the clinical signs reported most frequently by Irish farmers (Zintl *et al.*, 2014) (haemoglobinuria (32 %) and sluggishness (25%)) and veterinary surgeons (haemoglobinuria (37 %)). Other signs observed were fever, diarrhoea or in more advanced cases constipation and tachycardia. Interestingly this survey revealed that in Ireland microscopic examination of a stained blood smear was only carried out in 3 % of cases and submission of blood to a laboratory for examination in only 1 % of cases. If this is representative of the situation in Scotland then the numbers of cases diagnosed by SAC/SRUC VIOs is a significant underestimate of the actual number of cases, suggesting a large-scale survey of farmers would be useful to further investigate the current situation in Scotland. A veterinary surgeon attended 46 % of Irish cases of babesiosis, while in the survey of large animal veterinary surgeons in Scotland (2.3.1.1 Red water fever (p. 37)) veterinary surgeons attending a case of babesiosis felt that the majority or all cases of the disease came to their attention.

3.4.3 Could a *Babesia* or *Theileria* spp. be present but as yet undetected in Scottish sheep and cattle?

In spite of testing a moderate number of samples from cattle and sheep suspected of having tick-borne disease, no previously undetected species or sub-clinical infections with endemic *B. divergens* were found. Although *A. phagocytophilum* was identified in both sheep and cattle (discussed in Chapter Five), none of the three animals identified by microscopy and PCR as

being infected with *B. divergans* were co-infected with this bacterial pathogen. It was decided that in order to provide the best opportunity to discover currently overlooked pathogens in Scottish livestock, a major targeted sampling study was required. The next chapter details the planning and execution of such a study using intelligence garnered from the national survey of veterinary surgeons.

CHAPTER FOUR
Babesia and *Theileria* spp. PCR Deployment at Selected Scottish Sites

4.1 Introduction

Chapter Three explored the possibility that a previously undetected *Babesia* or *Theileria* spp. could be present in Scottish livestock. Blood from cattle diagnosed with babesiosis was investigated by molecular means and found to contain the endemic species *B. divergens*, however blood and tissue samples from animals exposed to tick infestation were not found to contain either *Babesia* or *Theileria* spp. With no evidence of a previously undetected *Babesia* or *Theileria* spp. was found during this initial work, and in light of the lack of a large-scale molecular survey of Scottish livestock, it was decided to expand the investigation to farms where tick infestation and tick-borne disease had historically been an issue. This was done with the assistance of a large animal veterinary surgeon responding to the National Survey of Large Animal Veterinary Surgeons (2.3.1 National survey of large animal veterinary surgeons on tick-borne disease in Scottish livestock (p. 35)) who was able to identify two potential farms where sampling could be undertaken, namely sites A and B. Both farm sites were in coastal locations to the north of Inverness and although in relatively close proximity to each other, separated by 15.5 miles (25 km), they were independent enterprises with no interchange of livestock between them.

At farm site A, a cross-sectional survey of clinically normal cattle and sheep sharing the same extensively grazed hill environment was undertaken. Blood samples were obtained from a large number of Luing cattle at this site following routine blood sampling for health scheme membership with the objective of determining whether the endemic parasite *B. divergens* or an unexpected parasite was present in these cattle. The description of a small *Babesia* sp. found in a Scottish sheep (Reid *et al.*, 1976; Purnell *et al.*, 1981) prompted the inclusion of Scottish Blackface sheep from farm site A in the survey. At the second farm, site B, samples were taken to allow a longitudinal study of clinically normal North Country Cheviot sheep before and after grazing similar extensively managed hill land to that at site A.

In addition to the lack of a molecular survey of Scottish livestock, a lack of information about *Babesia* spp. in Scottish red deer was also identified. This was highlighted by the recent molecular confirmation of *B. divergens* (Zintl *et al.*, 2011) and detection of a species similar to *B. odocoilei* (Emerson and Wright, 1968; Emerson, 1970) in wild red deer in Ireland (Zintl *et al.*, 2011). It was also clear from the diagnosis of a case of babesiosis in a red deer in Scotland by SAC/SRUC (2.3.2 SAC/SRUC tick-borne disease cases 2000 - 2013 (p. 44)); Table 2-3 and Figure 2-7) that a *Babesia* sp. capable of causing clinical disease in red deer is present in Scotland. Additionally, the potential of a *Babesia* sp. infecting deer, which may also cause illness in humans, is illustrated by the description of *Babesia* sp. EU1 (now *B. venatorum*) (Herwaldt *et al.*, 2003). However, the vertebrate host of this parasite appears to be the roe deer rather than red deer (Bonnet *et al.*, 2007a; Bonnet *et al.*, 2009; Michel *et al.*, 2014; Zanet *et al.*, 2014). For these reasons it was decided to obtain blood from clinically normal red deer culled at farm site A and an additional six sites (C-H) spanning the north-west highlands. For sites C to H, this was achieved with the assistance of Andrew French (University of Highlands and Islands). This chapter seeks to address two key questions:

1. ***Is a previously undetected Babesia sp. or spp. present in Scottish livestock?***

2. ***Which Babesia sp. or spp. are present in Scottish wild red deer?***

To answer these questions, blood samples were collected as described above and these were screened with the sensitive and specific nested 18S PCR assay developed in Chapter Three.

4.2 Materials and methods

The Ethics and Welfare Committee of the College of Medical, Veterinary and Life Sciences (University of Glasgow) approved blood sampling, consent and data management plans for this phase of the study (entitled “An Investigation Of Endemic And Emerging Tick-Borne Protozoa And Rickettsia In Scottish Livestock” Ref. 15a/13).

A collection of 318 blood samples were obtained from Scottish livestock and red deer; details of these samples are summarised in Table 4-1 with the locations of sampling sites illustrated in Figure 4-1. All the cattle and sheep were clinically well at the time of blood sampling. Similarly, the deer showed no evidence of disease and blood samples were obtained during post-mortem examination following culling. From site A, fresh anti-coagulated, fresh clotted and frozen anti-coagulated blood was obtained from sheep, cattle and culled wild red deer respectively. These animals co-existed on the same extensively managed moorland. At site B fresh anti-coagulated blood was obtained from 40 sheep in June and again in November of which 34 were sampled on both occasions. Frozen clotted blood from wild red deer culled in summer 2012 was obtained from sites C to H.

Site	Species sampled	Status	Number of animals sampled	Sampling period	Blood sample type
A	Sheep (SB)	Alive	47	Oct 2013	Fresh, EDTA
A	Cattle (L)	Alive	107	Dec 2013	Fresh, clotted
A	Red deer - female	PM	24	Oct 2013	Fresh, EDTA
B	Sheep (NCC)	Alive	40*	Jun 2014	Fresh, EDTA
B	Sheep (NCC)	Alive	40*	Nov 2014	Fresh, EDTA
C-H	Red deer - male	PM	60 (10 per site)	Summer 2012	Frozen, clotted

Table 4-1 Details of blood samples collected for this study

* includes 34 North Country Cheviot ewes sampled in both summer and autumn. The locations of sampling sites are illustrated in Figure 4-1. SB, Scottish Blackface; L, Luing; NCC, North Country Cheviot

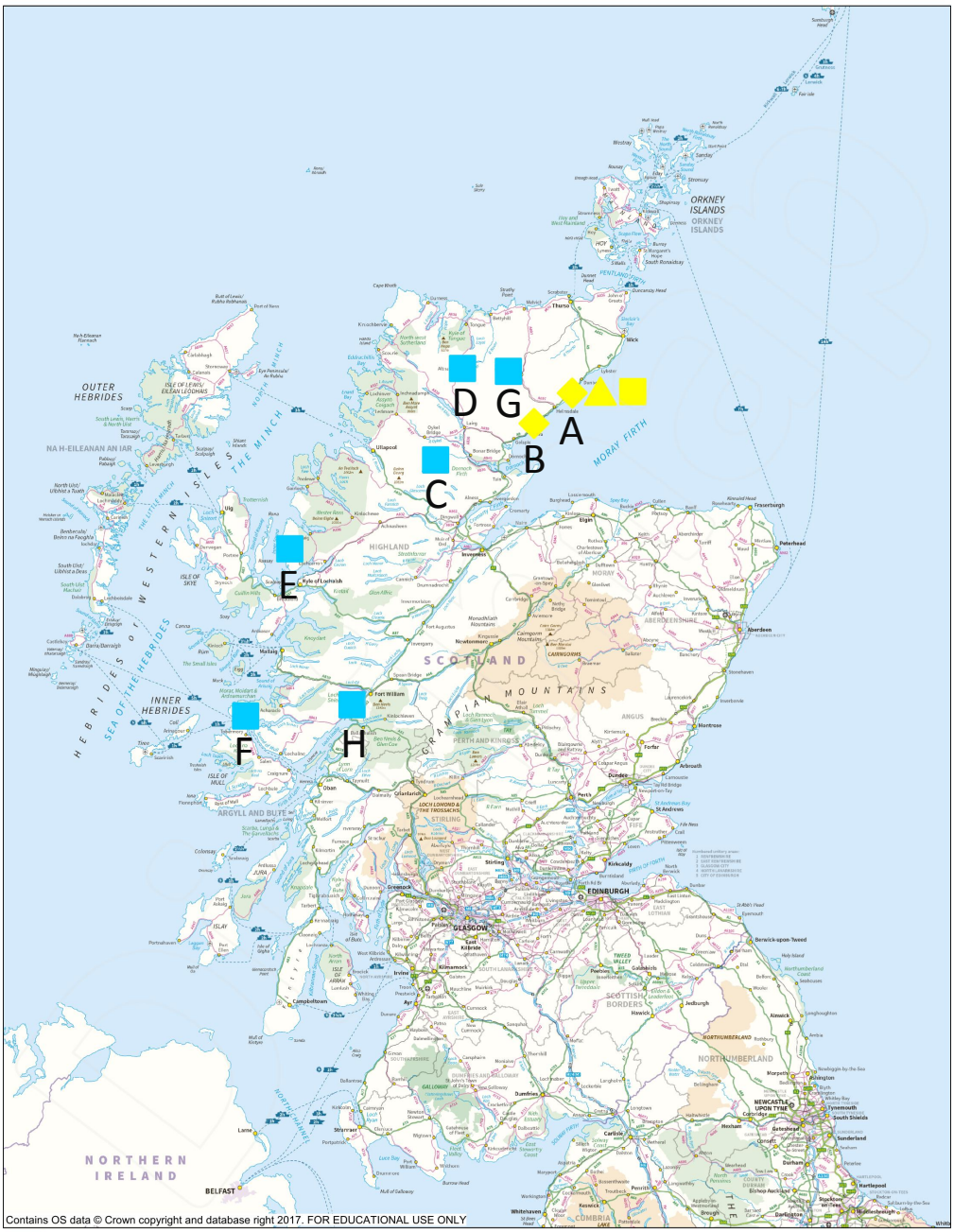

Figure 4-1 Location of sampling sites

Farm sites (A and B) are illustrated in yellow; red deer sampling sites (C - H) are illustrated in blue.

DNA was extracted from blood as previously described in 3.2.2.2 DNA extraction (p. 69). PCR amplification using *Theileria/Babesia* 'catch-all' primers was undertaken as detailed in Figure 3-14. Amplicons were separated using agarose gel electrophoresis and visualised as described in 3.2.1.3 PCR development and optimisation (p. 63). Amplicons from positive reactions were purified, sequenced and annotated using the methodology and nomenclature described in 3.2.2.3 Sequencing (p. 69) and Figure 3-4.

Amplicon sequences were identified by BLASTing against the NCBI non-redundant database (https://blast.ncbi.nlm.nih.gov). Sequences representing high-scoring BLAST 'hits' were downloaded from the NCBI database in FASTA format. Multiple sequence alignment was performed using CLC Genomics Workbench (Qiagen), which was also used to construct neighbour-joining trees. In all cases the bovine 18S SSU sequence was included and used to root the trees. Trees were visualised using CLC Genomics Workbench (Qiagen).

4.3 Results

4.3.1 Sheep at farm sites A and B

PCR analysis was undertaken on a collection of sheep blood samples from two farms located to the north of Inverness, termed Farm A and B. The results of this analysis are summarised in Table 4-2.

Sampling site	Site A	Site B	
Sampling period	**Oct 13**	**Jun 14**	**Nov 14**
Number of animals sampled	47	40	40
***Babesia/Theileria* spp. PCR positive**			
Overall	7 (15%)	2 (5%)	6 (15%)
Babesia venatorum	6 (13%)	1 (2.5%)	4 (10%)
Sarcocystis tenella	1 (2%)	1 (2.5%)	2 (5%)

Table 4-2 Sheep *Babesia* and *Theileria* spp. PCR results

At site A, the *Babesia/Theileria* spp. PCR was found to be positive for seven (15 %) of the 47 sheep sampled. At site B, where 40 sheep were sampled in June 2014 and in November 2014, the *Babesia/Theileria* spp. PCR was positive in two

sheep (5 %) at time of first sampling and six sheep (15 %) at the second sampling. All amplicons were successfully sequenced.

Following primer trimming, a 371-nucleotide alignment was generated from which a tree was constructed (Figure 4-2). Eleven of the sequences (six from site A and five from site B) were identical. BLAST analysis of this sequence identified 30 completely identical *Babesia* spp. 18S sequences in the NCBI Database (Table 4-3), which were annotated primarily as *Babesia* sp. EU1. This parasite was subsequently re-named *Babesia venatorum*.

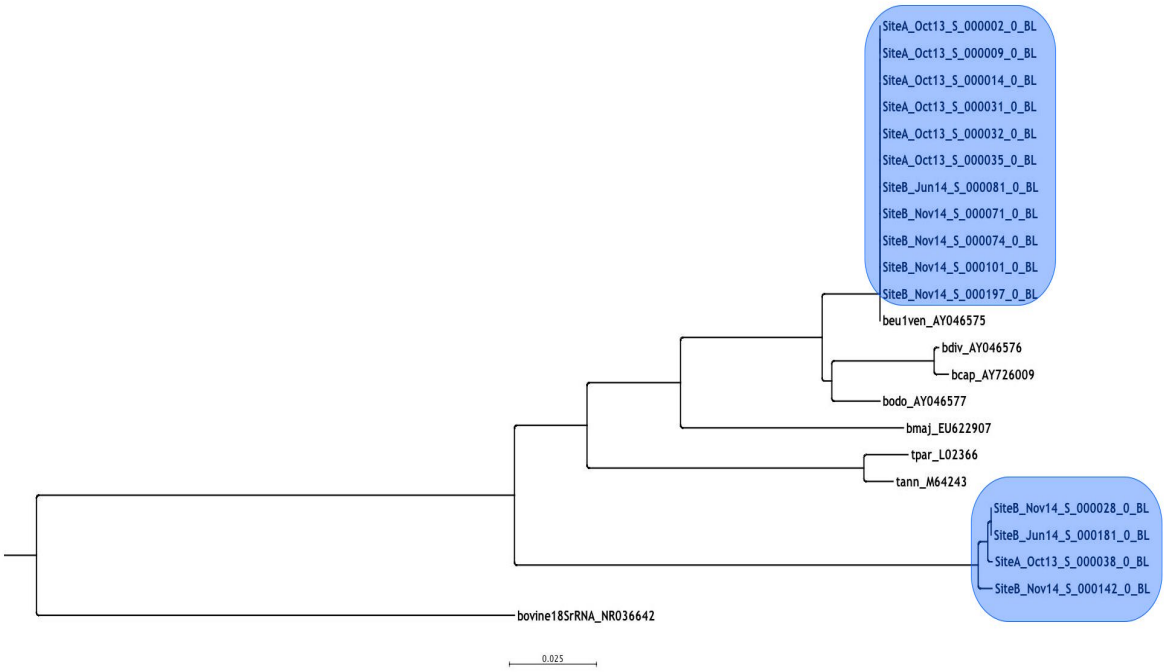

Figure 4-2 Neighbour-joining tree of 18S SSU rRNA amplicon sequences obtained from sheep at sites A and B

Sequences obtained in this study are highlighted in blue; published *Babesia* and *Theileria* sequences include *B. venatorum* (AY046575), *B. divergens* (AY046576), *B. capreoli* (AY726009), *B. odocoilei* (AY046577), *B. major* (EU622907), *Theileria parva* (L02366) and *Theileria annulata* (M64243). The bovine 18S sequence was used to root the tree.

Genbank Accession	Accession date	Parasite species	Host species	Host tissue	Location	Reference
AY046575	24/07/03	*Babesia* sp. EU1 clone BAB20	Human (*H. sapiens)*	Blood	Austria and Italy	(Herwaldt *et al.*, 2003)
AY553915	07/06/05	*Babesia* sp. DD-2004	Tick *(I. ricinus)*	NA	Slovenia	(Duh *et al.*, 2005a)
AY572457	09/09/04	*Babesia* sp. RDS-2004	Roe deer (*C. capreolus)*	Spleen	Slovenia	(Duh *et al.*, 2005b)
EF185818	30/07/07	*Babesia* sp. EU1 isolate rd 201	Roe deer (*C. capreolus)*	Blood	France	(Bonnet *et al.*, 2007a)
FJ215873	03/03/09	*Babesia* sp. EU1 isolate 7627	Tick *(I. ricinus)* (from cattle)	NA	France	(Becker *et al.*, 2009)
GQ888709	02/05/11	*Babesia* sp. EU1 isolate Arnhem	Forrest reindeer *(Rangifer tarandus)*	Blood	Netherlands	(Kik *et al.*, 2011)
GU647159	28/06/10	*Babesia* sp. EU1 strain 182	Tick *Ixodes ricinus*	NA	Italy	(Cassini *et al.*, 2010)
GU734773	05/01/11	*Babesia* sp. EU1 isolate Nov-Ip215	Tick (*I. persulcatus*)	NA	Russia	(Rar *et al.*, 2011)
HM113372	15/06/10	*Babesia* sp. EU1 isolate EG207	Tick *(I. ricinus)*	NA	Germany	Not published
HQ830266	18/01/11	*Babesia* sp. EU1 isolate rd2666	Roe deer (*C. capreolus)*	Blood	France	(Bastian *et al.*, 2012)
JQ929917	06/05/13	*Babesia* sp. EU1	Roe deer (*C. capreolus)*	Blood	Poland	(Welc-Faleciak *et al.*, 2013)
JQ993422	28/08/12	*Babesia* sp. EU1 isolate hlj53-1	Tick (*I. persulcatus*)	NA	China	Not published
JQ993425	28/08/12	*Babesia* sp. EU1 isolate hlj48	Tick (*I. persulcatus*)	NA	China	Not published
JQ993426	28/08/12	*Babesia* sp. YZ-2012 isolate hlj223	Tick (*I. persulcatus*)	NA	China	Not published
JQ993428	28/08/12	*Babesia* sp. EU1 isolate hlj231	Tick (*I. persulcatus*)	NA	China	Not published
JQ993430	28/08/12	*Babesia* sp. EU1 isolate hlj1002	Tick (*I. persulcatus*)	NA	China	Not published
JX287361	25/08/12	*Babesia* sp. '*venatorum*' strain xinjiang1	Human (*H. sapiens)*	Blood	China	(Sun *et al.*, 2014)
KC470050	21/01/13	*Babesia* sp. RWF-2013	Tick *(I. ricinus)*	NA	Poland	(Welc-Faleciak *et al.*, 2012)
KF773722	27/02/14	*Babesia* sp. '*venatorum*' strain 780_08	Roe deer (*C. capreolus)*	Spleen	Italy	(Zanet *et al.*, 2014)
KF773732	27/02/14	*Babesia* sp. '*venatorum*' strain 786_08	Roe deer (*C. capreolus)*	Spleen	Italy	(Zanet *et al.*, 2014)
KJ486557	19/06/15	*Babesia* sp. '*venatorum*' isolate Irk-Ip1440	Tick (*I. persulcatus*)	NA	Russia	(Rar *et al.*, 2014)
KJ486558	19/06/15	*Babesia* sp. '*venatorum*' isolate Kh-Ip210	Tick (*I. persulcatus*)	NA	Russia	(Rar *et al.*, 2014)
KM095110	06/10/14	*Babesia* sp. '*venatorum*' isolate 266G	Tick *(I. ricinus)*	NA	Czech Republic	Not published
KM095111	06/10/14	*Babesia* sp. '*venatorum*' isolate 283G	Tick *(I. ricinus)*	NA	Czech Republic	Not published
KM095112	06/10/14	*Babesia* sp. '*venatorum*' isolate 430G	Tick *(I. ricinus)*	NA	Czech Republic	Not published
KM095113	06/10/14	*Babesia* sp. '*venatorum*' isolate 449G	Tick *(I. ricinus)*	NA	Czech Republic	Not published
KM095114	06/10/14	*Babesia* sp. '*venatorum*' isolate 483G	Tick *(I. ricinus)*	NA	Czech Republic	Not published
KM244044	05/11/14	*Babesia* sp. '*venatorum*'	Human (*H. sapiens)*	Blood	China	(Jiang *et al.*, 2015)
LC005775	10/04/15	*Babesia* sp. '*venatorum*' isolate BvSSR174-6	Tick (*I. persulcatus*)	NA	Mongolia	Not published
LC005776	10/04/15	*Babesia* sp. '*venatorum*' isolate BvSSR217-3	Tick (*I. persulcatus*)	NA	Mongolia	Not published

Table 4-3 Identical sequences in the NCBI database to the *Babesia* 18S sequence obtained from sheep at sites A and B

Forty sheep were sampled in June and November at site B. Of these 34 were sampled at both time points. Temporal comparison of the presence of *Babesia venatorum* sequence in samples originating from these 34 animals from site B in June and November 2014 (Table 4-4) revealed the majority of ewes (85 %) were negative in both June and November 2014. No ewes were positive on both occasions. Four ewes (12 %) were negative in June and became positive in November 2014, while one ewe was positive on first sampling but negative on the second occasion.

Status in June 2014 / November 2014	Number of animals
Negative / remained negative	29 (85%)
Negative / became positive	4 (12%)
Positive / became negative	1 (3%)
Positive / remained positive	0 (0%)

Table 4-4 Comparison of *B. venatorum* PCR results in sheep at site B sampled in both June and November 2014

In addition the *B. venatorum* sequences, a separate group of four closely related sequences were identified in this study, comprising one from site A and three from site B (Table 4-2 and Figure 4-2). These were quite distinct from the *B. venatorum* sequences and were found to be noticeably longer at 450 nucleotides after primer trimming. BLAST analysis revealed high or complete identity to *Sarcocystis tenalla* and other closely related *Sarcocystis* spp. recently identified by molecular means in Europe (Table 4-5).

Study ID	Accession	Identity	Species	Host	Tissue	Location	Reference
SiteA_Oct13_S_000038_0_BL	KT873783	449/450	*Sarcocystis* sp. 8B-Fu795	Fox	SI	Germany	More *et al.* (2016)
	KP263755	449/450	*S. tenella* (Isolate 4)	Tatra Chamois	SM (LD)	Poland	Kolenda *et al.* (2015)
	KC209737	449/450	*S. tenella* (Isolate S9.1)	Sheep	SM	Norway	Gjerde (2013)
	KT873782	448/450	*Sarcocystis* sp. 8A-Fu795	Fox	SI	Germany	More *et al.* (2016)
	KT873748	448/450	*Sarcocystis* sp. Mh 12-23	Racoon Dog	SI	Germany	More *et al.* (2016)
SiteB_Jun14_S_000181_0_BL	KT873783	450/450	*Sarcocystis* sp. 8B-Fu795	Fox	SI	Germany	More *et al.* (2016)
SiteB_Nov14_S_000028_0_BL	KP263755	450/450	*S. tenella* (Isolate 4)	Tatra Chamois	SM (LD)	Poland	Kolenda *et al.* (2015)
	KC209737	450/450	*S. tenella* (Isolate S9.1)	Sheep	SM	Norway	Gjerde (2013)
	KT873782	449/450	*Sarcocystis* sp. 8A-Fu795	Fox	SI	Germany	More *et al.* (2016)
	KT873748	449/450	*Sarcocystis* sp. Mh 12-23	Racoon Dog	SI	Germany	More *et al.* (2016)
SiteB_Nov14_S_000142_0_BL	KT873739	450/450	*Sarcocystis* sp. Fu 12-732	Fox	SI	Germany	More *et al.* (2016)
	KP263758	450/450	*S. tenella* (Isolate 7)	Tatra Chamois	SM (D)	Poland	Kolenda *et al.* (2015)
	KP263754	450/450	*S. tenella* (Isolate 3)	Tatra Chamois	SM (LD)	Poland	Kolenda *et al.* (2015)
	KP263752	450/450	*S. tenella* (Isolate 1)	Tatra Chamois	SM (LD)	Poland	Kolenda *et al.* (2015)
	KT873782	448/450	*Sarcocystis* sp. 8A-Fu795	Fox	SI	Germany	More *et al.* (2016)

Table 4-5 Closely-related sequences in the NCBI database compared to the putative *Sarcocystis* sequence obtained from sheep at sites A and B

Host species include fox (*Vulpes vulpes*), Tatra Chamois (*Rupicapra rupicapra tatrica*), sheep (*Ovis aries*) and racoon dog (*Nyctereutes procyonoides*). Tissues are abbreviated as SI (small intestine), SM (skeletal muscle), LD (Latissimus dorsi) and D (Diaphragm).

Although this sequence was identified in sheep on site B in both June 2014 and November 2014, no single animal was found to contain this sequence at both time-points.

4.3.2 Cattle at farm site A

The *Babesia*/*Theileria* spp. PCR assay was applied to 107 cattle blood samples from site A and the results are shown in Table 4-6.

Number of cattle sampled	107
***Babesia/Theileria* spp. PCR positive**	7 (6%)
Babesia divergens	7 (6%)

Table 4-6 Cattle *Babesia/Theileria* spp. PCR results

The number of PCR positive results irrespective of species is provided in the *Babesia/Theileria* spp. PCR positive row. The subsequent row provides a breakdown of PCR positive result by the species identified.

A total of seven (6 %) of the 107 cattle sampled at site A were found to be PCR positive and amplicons were successfully sequenced in each case. All seven sequences were found to be identical with a length of 371 nucleotides after primer trimming. Following BLAST analysis, this sequence was found to completely identical to 26 *B. divergens* sequences in the NCBI database, including AY046576 (*B. divergens*), which has been suggested as a appropriate reference sequence for this species (Zintl *et al.*, 2011).

4.3.3 Red deer at sites A to H

The *Babesia/Theileria* spp. 'catch-all' PCR assay was applied to 24 female red deer from site A and 60 male red deer from sites C to H. The results are summarised in Table 4-7. No samples could be obtained from either roe deer or red deer present at Site B due to management practices at each site.

Sampling site	A	B	C	D	E	F	G	H	Overall
Number of animals sampled	24	-	10	10	10	10	10	10	84
***Babesia/Theileria* spp. PCR positive**	11 (46%)	-	3 (30%)	4 (40%)	0 (0%)	1 (10%)	2 (20%)	1 (10%)	22 (26%)
Babesia divergens	4 (17%)	-	3 (30%)	2 (20%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)	9 (11%)
***Babesia odocoilei*-like**	7 (29%)	-	0 (0%)	2 (20%)	0 (0%)	1 (10%)	2 (20%)	1 (10%)	13 (15%)

Table 4-7 Red deer *Babesia* and *Theileria* spp. PCR results

Sampling sites are illustrated in Figure 4-1. No deer samples were obtained from farm site B. Post-mortem blood samples were obtained from deer at sites C to H.

The *Babesia/Theileria* spp. PCR was positive for 22 of the 84 red deer (26 %) sampled, consisting of 11 of the 24 red deer (46 %) sampled at site A and 11 of the 60 red deer (18 %) sampled at sites C to H. All amplicons were successfully sequenced. No evidence of amplification of multiple sequences (that would suggest multiple infections) from the same template DNA was found.

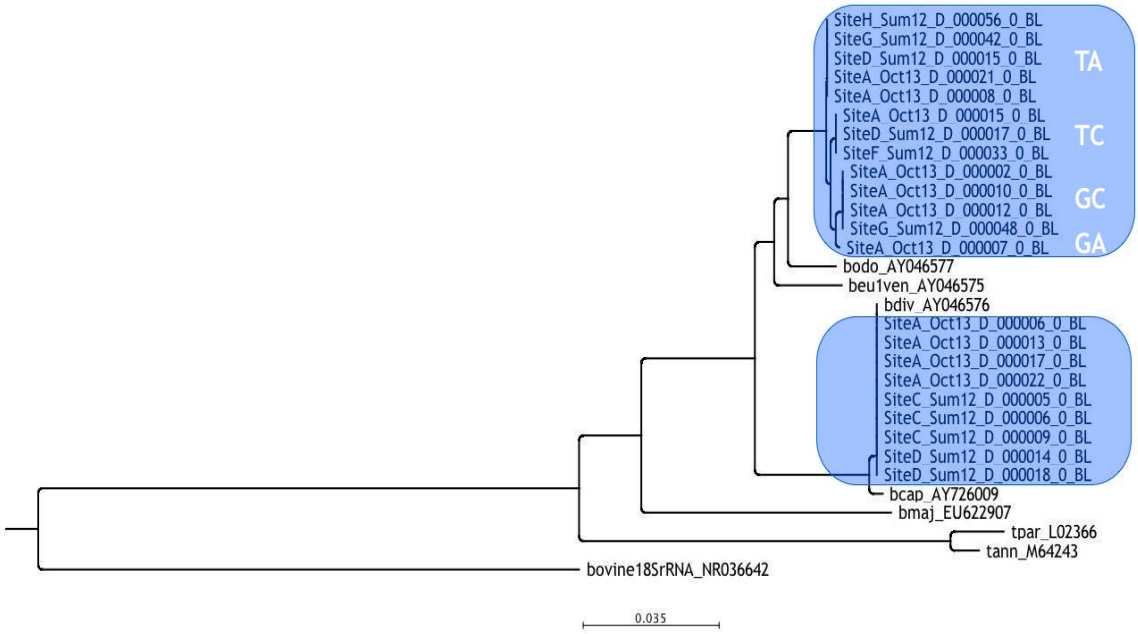

Figure 4-3 Neighbour-joining tree of amplicon sequences obtained from red deer

Sequences from this study are highlighted in blue and genotypes at positions 187 (T or G) and 217 (A or C) are highlighted in white within the *Babesia odocoilei*-like clade. Other sequences included are those originating from deer (*B. venatorum* (AY046575), *B. divergens* (AY046576), *B. capreoli* (AY726009), *B. odocoilei* (AY046577)), Europe (*B. major* (EU622907)) or are representatives of *Theileria* species (*T. parva* (L02366) and *T. annulata* (M64243)). The bovine 18S sequence was used to root the tree.

Nine of the sequences (representing 11 % of the deer sampled at sites A to H) were 371 nucleotides in length and were identical (Figure 4-3). BLAST analysis identified 26 completely identical *B. divergens* sequences in the NCBI Database, including *B. divergens* AY046576.

The remaining 13 sequences (representing 15 % of deer sampled at sites A to H) were 370 nucleotides in length and differed from each other at two nucleotide positions, namely 187 (T or G) and 217 (A or C) relative to the amplicon alignment. This resulted in four genotypes and this is illustrated in Figure 4-3.

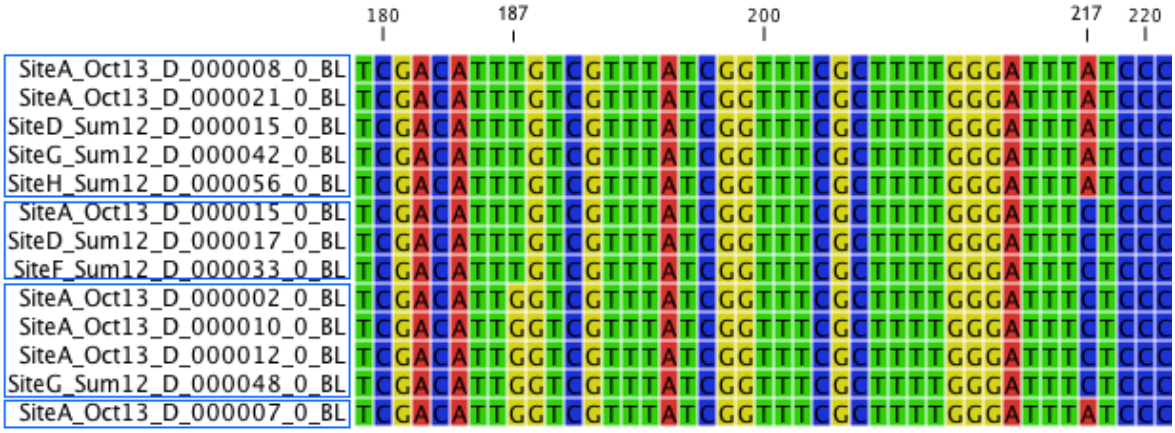

Figure 4-4 Alignment of *Babesia odocoilei*-like sequences identified in deer

This figure represents a section of the multiple sequence alignment between positions 179 and 221. The four sequence types, based on nucleotide positions 187 and 217, are highlighted in the blue boxes.

BLASTing the NCBI Database did not reveal any exact matches for any of the genotypes identified in this study, but demonstrated 97.3 - 98.1 % identity with *B. odocoilei* and other closely related *Babesia* spp. For this reason, the term *Babesia odocoilei*-like is used when referring to these sequences. The ten sequences with highest level of identity to each *B. odocoilei*-like sequence type found in this study are provided in Table 4-8. A dendrogram based on these sequences is presented in Figure 4-5.

Genotype	Accession	Identity	*Babesia* species	Host species	Location	Reference
TA	KU714605	364/370 (98.4%)	*Babesia* sp. MA-2016a isolate v5	Goat	Turkey	Not published
	AF158711	362/370 (97.8%)	*Babesia* sp. RD1	Reindeer	USA CA	Kjemtrup *et al.* (2000)
	AY046577	362/370 (97.8%)	*Babesia odocoilei* clone isolates E and B	White tailed deer	USA TX	Herwaldt *et al.* (2003)
	AY661502	362/370 (97.8%)	*Babesia odocoilei* isolate CA Bighorn sheep	Bighorn sheep	USA CA	Schoelkopf *et al.* (2005)
	AY661503	362/370 (97.8%)	*Babesia odocoilei* isolate NH Elk	Elk	USA NH	Schoelkopf *et al.* (2005)
	AY661507	362/370 (97.8%)	*Babesia odocoilei* isolate MN Musk Ox 1	Musk ox	USA MN	Schoelkopf *et al.* (2005)
	KC460321	362/370 (97.8%)	*Babesia odocoilei* isolate 138BZAA032	Elk	Canada	Pattullo *et al.* (2013)
	U16369	362/370 (97.8%)	*Babesia odocoilei* isolate Engeling	White tailed deer	USA TX	Holman *et al.* (2000)
	AF411337	361/370 (97.6%)	*Babesia* sp. isolate RD61	Reindeer	USA CA	Holman *et al.* (2002)
	KU714606	361/370 (97.6%)	*Babesia* sp. MA-2016a isolate v8	Goat	Turkey	Not published
TC	KU714605	363/370 (98.1%)	*Babesia* sp. MA-2016a isolate v5	Goat	Turkey	Not published
	AF158711	361/370 (97.6%)	*Babesia* sp. RD1	Reindeer	USA CA	Kjemtrup *et al.* (2000)
	AY046577	361/370 (97.6%)	*Babesia odocoilei* clone isolates E and B	White tailed deer	USA TX	Herwaldt *et al.* (2003)
	AY661502	361/370 (97.6%)	*Babesia odocoilei* isolate CA Bighorn sheep	Bighorn sheep	USA CA	Schoelkopf *et al.* (2005)
	AY661503	361/370 (97.6%)	*Babesia odocoilei* isolate NH Elk	Elk	USA NH	Schoelkopf *et al.* (2005)
	AY661507	361/370 (97.6%)	*Babesia odocoilei* isolate MN Musk Ox 1	Musk ox	USA MN	Schoelkopf *et al.* (2005)
	KC460321	361/370 (97.6%)	*Babesia odocoilei* isolate 138BZAA032	Elk	Canada	Pattullo *et al.* (2013)
	U16369	361/370 (97.6%)	*Babesia odocoilei* isolate Engeling	White tailed deer	USA TX	Holman *et al.* (2000)
	AF411337	360/370 (97.3%)	*Babesia* sp. isolate RD61	Reindeer	USA CA	Holman *et al.* (2002)
	KU714606	360/370 (97.3%)	*Babesia* sp. MA-2016a isolate v8	Goat	Turkey	Not published

Table 4-8 BLAST results for *B. odocoilei*-like sequences illustrating the top ten 'hits' in the NCBI database

(Table continued on next page)

Genotype	Accession	Similarity	Species	Host	Location	Reference
GC	KU714605	363/370 (98.1%)	*Babesia* sp. MA-2016a isolate v5	Goat	Turkey	Not published
	AF158711	360/370 (97.3%)	*Babesia* sp. RD1	Reindeer	USA CA	Kjemtrup *et al.* (2000)
	AF411337	360/370 (97.3%)	*Babesia* sp. isolate RD61	Reindeer	USA CA	Holman *et al.* (2002)
	AY046577	360/370 (97.3%)	*Babesia odocoilei* clone isolates E and B	White tailed deer	USA TX	Herwaldt *et al.* (2003)
	AY661502	360/370 (97.3%)	*Babesia odocoilei* isolate CA Bighorn sheep	Bighorn sheep	USA CA	Schoelkopf *et al.* (2005)
	AY661503	360/370 (97.3%)	*Babesia odocoilei* isolate NH Elk	Elk	USA NH	Schoelkopf *et al.* (2005)
	AY661507	360/370 (97.3%)	*Babesia odocoilei* isolate MN Musk Ox 1	Musk ox	USA MN	Schoelkopf *et al.* (2005)
	KC460321	360/370 (97.3%)	*Babesia odocoilei* isolate 138BZAA032	Elk	Canada	Pattullo *et al.* (2013)
	KU714606	360/370 (97.3%)	*Babesia* sp. MA-2016a isolate v8	Goat	Turkey	Not published
	U16369	360/370 (97.3%)	*Babesia odocoilei* isolate Engeling	White tailed deer	USA TX	Holman *et al.* (2000)
GA	KU714605	364/370 (98.4%)	*Babesia* sp. MA-2016a isolate v5	Goat	Turkey	Not published
	AF158711	361/370 (97.6%)	*Babesia* sp. RD1	Reindeer	USA CA	Kjemtrup *et al.* (2000)
	AF411337	361/370 (97.6%)	*Babesia* sp. isolate RD61	Reindeer	USA CA	Holman *et al.* (2002)
	AY046577	361/370 (97.6%)	*Babesia odocoilei* clone isolates E and B	White tailed deer	USA TX	Herwaldt *et al.* (2003)
	AY661502	361/370 (97.6%)	*Babesia odocoilei* isolate CA Bighorn sheep	Bighorn sheep	USA CA	Schoelkopf *et al.* (2005)
	AY661503	361/370 (97.6%)	*Babesia odocoilei* isolate NH Elk	Elk	USA NH	Schoelkopf *et al.* (2005)
	AY661507	361/370 (97.6%)	*Babesia odocoilei* isolate MN Musk Ox 1	Musk ox	USA MN	Schoelkopf *et al.* (2005)
	KC460321	361/370 (97.6%)	*Babesia odocoilei* isolate 138BZAA032	Elk	Canada	Pattullo *et al.* (2013)
	KU714606	361/370 (97.6%)	*Babesia* sp. MA-2016a isolate v8	Goat	Turkey	Not published
	U16369	361/370 (97.6%)	*Babesia odocoilei* isolate Engeling	White tailed deer	USA TX	Holman *et al.* (2000)

Table 4-8 (Continued) BLAST results for *B. odocoilei*-like sequences illustrating the top ten 'hits' in the NCBI database

The four sequence types identified in this study are denoted in terms of their genotype at position 187 and 217. For each of the four sequences identified in this study, the ten top-scoring BLAST 'hits' are detailed. These sequences were used to generate Figure 4-5. Host species include goat (*Capra aegagrus hircus*), reindeer (*Rangifer tarandus tarandus*), white tailed deer (*Odocoileus virginianus*), bighorn sheep (*Ovis canadensis nelsoni*), elk (*Cervus elaphus canadensis*) and musk ox (*Ovibos moschatus*).

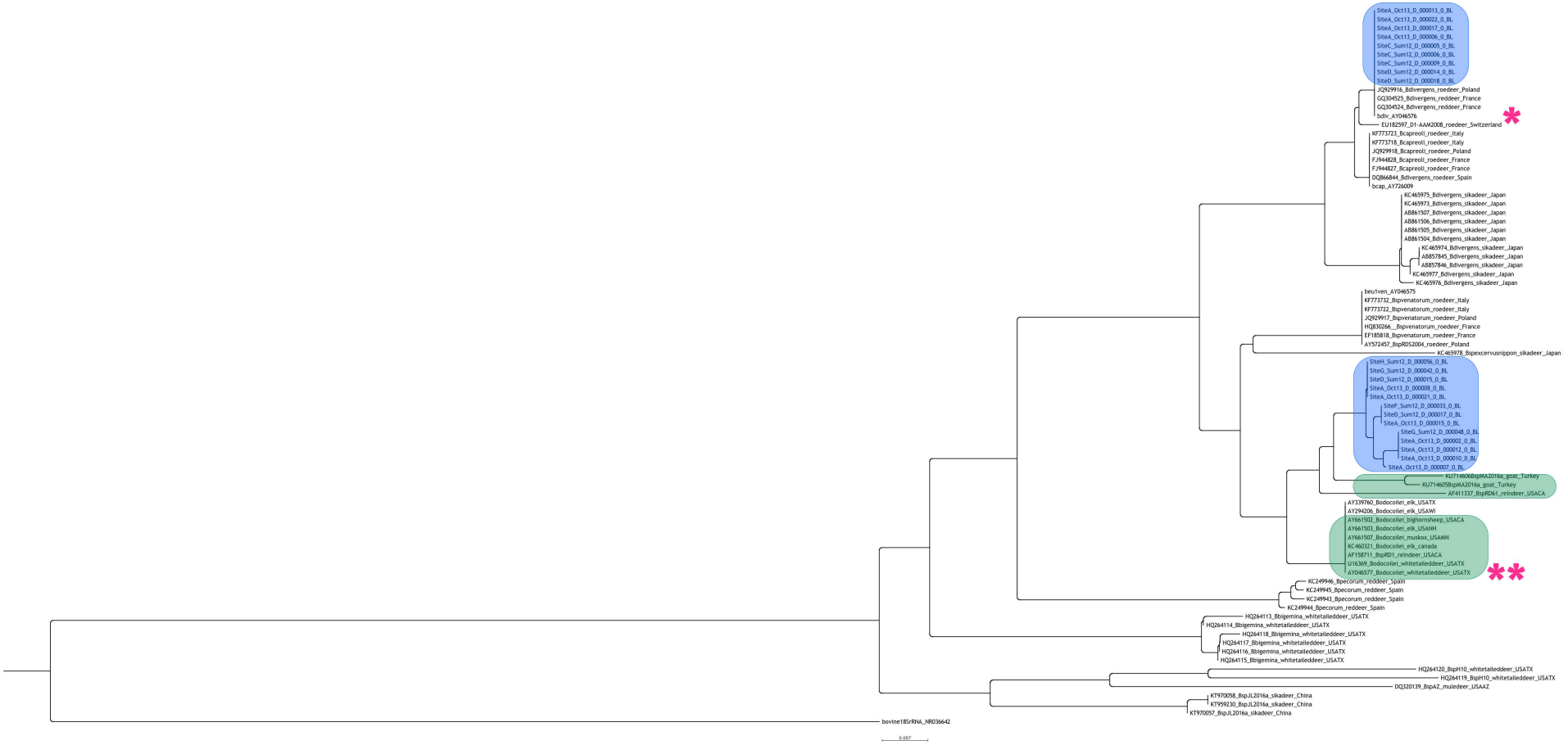

Figure 4-5 Neighbour-joining tree of amplicon sequences obtained from red deer

Sequences from the present study are highlighted in blue, BLAST results with greatest identity, and other *Babesia* spp. from deer. *Babesia divergens* AY046576 * and *Babesia odocoilei* AY046577 **. The bovine 18S sequence was used to root the tree. Blues box represent sequences from Scottish red deer in this study; green boxes represent sequences obtained from a range of hosts in the US.

A dendrogram (Figure 4-5) was constructed based on top BLAST 'hits' for the *B. odocoilei*-like sequences from the present study, other *Babesia* spp. identified in various deer species in the literature and the reference sequences *B. divergens* AY046576 and *B. odocoilei* AY046577. All sequences were trimmed to the *Babesia odocoilei*-like spp. from red deer from this study.

In this dendrogram the upper blue box represents *B. divergens* sequences from Scottish red deer, identical to a small number of other *B. divergens* sequences from deer, and *B. divergens* AY046576 (Herwaldt *et al.*, 2003). *Babesia venatorum* sequences originating from European roe deer cluster with *B. odocoilei*-like sequences from Scottish red deer (lower blue box) together with similar sequences originating from a range of hosts in the United States (green boxes).

To facilitate comparison with the results of a similar study carried out recently in Ireland (Zintl *et al.*, 2011), a multiple sequence alignment was performed consisting of GU475472 (identical to *B. divergens* AY046576, from 10/38 wild Irish red deer) and GU475474 (98 % identical to *Babesia odocoilei* AY046577, from 3/38 wild Irish red deer) with the sequences from red deer from this study, and a dendrogram generated (Figure 4-6). Zintl *et al.* (2011) utilised primers BAB GF2 and BAB GR2 (Bonnet *et al.* (2007a), amplifying nucleotides 467 to 1,026 of *B. divergens* AY046576 (Chapter Three, Table 3-2 and Figure 3-8). In contrast, the present study utilised RLB-F2 and RLB-R2 (Georges *et al.* (2001)), amplifying nucleotides 424 to 817 of *B. divergens* AY046576 (Chapter Three, Table 3-2 and Figure 3-8). The overlapping region forming the alignment corresponds to nucleotides 467 to 1,026 of *B. divergens* AY046576 and contains the nucleotide sites differentiating the various *B. odocoilei*-like sequences in the present study (Figure 4-4).

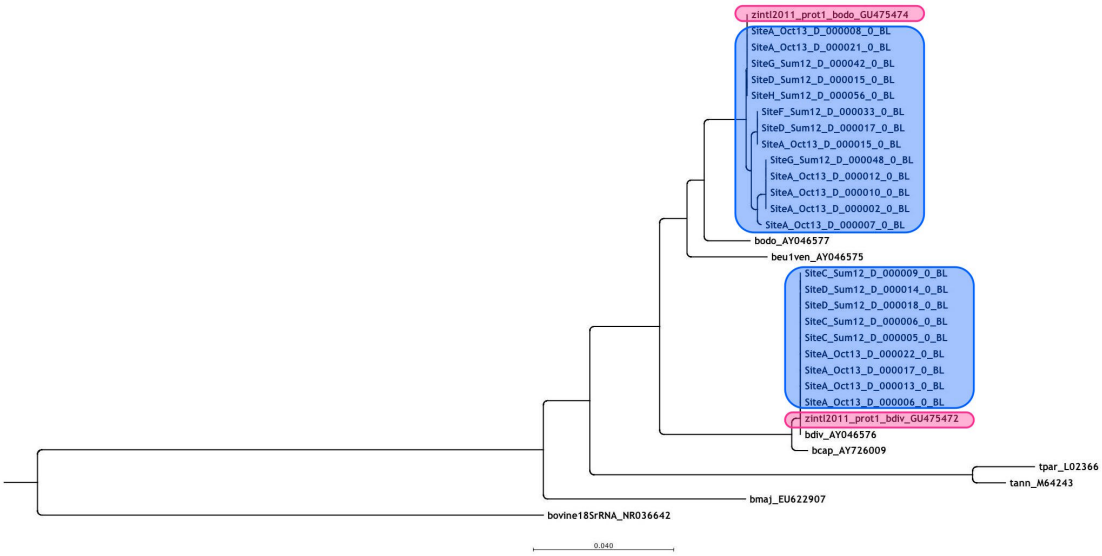

Figure 4-6 Neighbour-joining tree of *B. odocoilei*-like sequences incorporating sequences from a recent Irish study

Red deer-derived sequences generated in the present study are shown in blue. Sequences from a recent Irish study (Zintl *et al.*, 2011) are show in pink (GU475472 and GU475474).

Comparison of the overlapping portion of GenBank accession GU475472 (from 10/38 Irish red deer, identical to *B. divergens* AY046576) with sequences generated from Scottish red deer revealed nine Scottish red deer sequences to be identical to the Irish sequence (Figure 4-5).

Similarly, comparison of the overlapping portion of GenBank accession GU475474 (from 3/38 Irish red deer, similar to *B. odocoilei* AY046577) with sequences generated from Scottish red deer revealed five Scottish red deer sequences to be identical to the Irish sequence (Figure 4-6), and eight to differ only at the two previously identified polymorphic sites (Figure 4-3 and Figure 4-4) from the Irish sequence.

4.4 Discussion

4.4.1 Babesia venatorum in Scottish sheep

4.4.1.1 Discovery

A total of 127 blood samples from sheep at farm sites A and B were tested. Eleven of these contained an identical DNA sequence; BLASTing of this sequence revealed 30 identical matches in the NCBI database (Table 4-3). These included identical sequences from human patients in Austria and Italy identified as

Babesia sp. EU1 (subsequently *B. venatorum*) (Herwaldt *et al.*, 2003) and two additional sequences from humans (Jiang *et al.*, 2015; Sun *et al.*, 2014) from China. Other identical matches came from roe deer in France (Bonnet *et al.*, 2007a; Bastian *et al.*, 2012), Poland (Welc-Faleciak *et al.*, 2013), Italy (Zanet *et al.*, 2014) and Slovenia (Duh *et al.*, 2005b). One matching sequence originated in a forest reindeer (*Rangifer tarandus*) from the Netherlands (Kik *et al.*, 2011). The other identical sequences originated in the ticks *I. ricinus* (in Slovenia (Duh *et al.*, 2005a), France (Becker *et al.*, 2009), Italy (Cassini *et al.*, 2010), Poland (Welc-Faleciak *et al.*, 2012), the Czech Republic, and Germany) and *I. persulcatus* from Russia (Rar *et al.*, 2011; Rar *et al.*, 2014), China and Mongolia.

As suggested by the BLAST results above, the main vertebrate host of *B. venatorum* in continental Europe is the roe deer. Infection was initially detected in Slovenia (Duh *et al.*, 2005b), then Italy (Tampieri *et al.*, 2008) and France (Bonnet *et al.*, 2007a; Bonnet *et al.*, 2009) during the work that confirmed *I. ricinus* as the tick vector of *B. venatorum*. Most recently two larger-scale surveys of wild ruminants in Switzerland (Michel *et al.*, 2014) and Italy (Zanet *et al.*, 2014) have further confirmed the central role of the roe deer. *Babesia venatorum* has not been detected in red deer in this study and this corresponds with the work of others (Zintl *et al.*, 2011; Michel *et al.*, 2014) although an infected male *I. ricinus* was found on a red deer in Belgium (Lempereur *et al.*, 2012b). In this case it was suspected the infection was acquired from a previous host. Samples from roe deer have not been examined during this project and obtaining blood samples from roe deer culled at the sites where *B. venatorum* has been identified in sheep would provide further information about the role of this deer species as a host in the United Kingdom. Infection with multiple *Babesia* species including *B. venatorum* has been demonstrated recently (Michel *et al.*, 2014) using *species*-specific primers (Hilpertshauser *et al.*, 2006). Examination of the red deer samples from this study using *B. venatorum*-specific primers would be useful to confirm that the parasite is definitely not present but masked by the amplification of DNA from more prevalent species. In addition to roe deer, other vertebrate hosts have also been identified for *B. venatorum* suggesting it is not limited to a single vertebrate host. These other species have included alpine chamois (*Rupicapra*

rupicapra) and alpine ibex (*Capra ibex*) (Michel *et al.*, 2014) and a forest reindeer (*Rangifer tarandus fennicus*) (Kik *et al.*, 2011). The infection is also zoonotic having been described first in humans before being described in animals (Herwaldt *et al.*, 2003).

In addition to the detection of *B. venatorum* in vertebrate hosts in Europe, it has also been detected extensively in its invertebrate tick vector. Infected ticks have been found infesting sheep in Switzerland (Hilpertshauser *et al.*, 2006) and cattle in Belgium (Lempereur *et al.*, 2012a), raising the possibility that cattle could be a reservoir host, although to date no record of detection in either cattle or sheep has been found. *Babesia venatorum* isolated from roe deer has been maintained in culture using sheep erythrocytes (Bonnet *et al.*, 2009), suggesting that the species had at least the potential to infect and survive in an ovine host.

In Norway *B. venatorum* was found to predominate in environmental tick samples (Oines *et al.*, 2012) and was also detected in ticks removed from migratory birds (Hasle *et al.*, 2011) which would represent a realistic means of transferring infection across the North Sea.

4.4.1.2 Detection in sheep

Sheep have not previously been reported as being infected by *B. venatorum*. *Babesia* sp. infecting sheep in Europe are *B. motasi* and *B. ovis*, both of which tend to be identified in Southern Europe (reviewed by Lempereur *et al.*, 2017).

Historically, the large *Babesia* sp. *Babesia motasi* has been identified and characterised in sheep in the United Kingdom (Lewis and Herbert, 1980; Lewis *et al.*, 1981; Alani and Herbert, 1988b; Alani and Herbert, 1988c). In addition, *Theileria ovis* (Lewis and Purnell, 1981) and *Theileria recondita* (Alani and Herbert, 1988a) have been identified based on morphology and transmission studies. All have been identified in Wales only, share the tick vector *Haemaphysalis punctata* and were considered to be of low pathogenicity. However, in the 1970s a sheep was identified in Scotland that was infected with a small *Babesia* sp. thought at the time to be *Babesia capreoli* (Reid *et al.*, 1976; Purnell *et al.*, 1981). There is a possibility that this could have been

B. venatorum, as it is generally accepted that species are difficult to differentiate on morphological grounds alone (Herwaldt *et al.*, 2003).

4.4.1.3 Detection in the United Kingdom

In addition to this being the first description of *B. venatorum* in sheep, an extensive search of the literature also failed to identify any report of *B. venatorum* being identified in a vertebrate in the United Kingdom. However, *B. venatorum* was identified in the United Kingdom in 4 of 742 ticks removed from companion animals (Smith *et al.*, 2013), as it was in Belgium by the examination of ticks removed from companion animals (Lempereur *et al.*, 2011).

The clinical implications of the presence of *B. venatorum* in sheep in Scotland are currently unclear however this is a significant finding in terms of human health. The possibility that a splenectomised human patient from Drumnadrochit who developed fatal babesiosis (Entrican *et al.*, 1979b) could have been infected with *B. venatorum* was considered. However, xenodiagnosis was carried out in this case by inoculation of both Mongolian gerbils (*Meriones unguiculatus*) and splenectomised calves with the patients blood resulting in infections confirming this infection was caused by *B. divergens*.

4.4.1.4 Identification in the Scottish sheep population

Further investigation of the parasite identified in Scottish sheep as *B. venatorum* by amplicon sequencing and comparison with sequences derived from European ticks, animals and humans is warranted. Both Zintl *et al.* (2011) and Lempereur *et al.* (2017) suggested that molecular identification based on fragments of the 18S rRNA gene should be regarded with caution. However, the segment of the 18S gene used in the assay in the present study was carefully selected, and confirmed to amplify a highly phylogenetically informative region, and therefore these results can be taken with confidence. Careful morphological description and sequencing of the entire 18S rRNA gene of this parasite would both be appropriate next steps, although attempted establishment of *in vitro* cultures and ultimately experimental infection of susceptible animals would be the most comprehensive approach.

Roe deer were not included in this study. *Babesia venatorum* has frequently been identified in this species of deer in European studies, for example in Slovenia (Duh *et al.*, 2005b), Italy (Tampieri *et al.*, 2008; Zanet *et al.*, 2014), France (Bonnet *et al.*, 2007a; Bonnet *et al.*, 2009) and Switzerland (Michel *et al.*, 2014). This suggests that more strident efforts to obtain samples from this species either at farm sites A and B or more widely in Scotland are essential. Roe deer are increasingly cosmopolitan, living in close approximation humans who are susceptible to infection with this species of parasite.

4.4.1.5 Could *B. venatorum* be present elsewhere in Europe in sheep?

It is unclear why *B. venatorum* has not been detected in sheep previously. One possibility is that it is only present in a very localised area. Another is that many other molecular studies in both the UK and Europe have focused the search for *Babesia* and *Theileria* spp. in ticks. A practical advantage of this approach is that ticks are easily collected from the environment or from either companion animals or livestock. Ticks are often collected from areas where there is a perceived risk of the ticks biting humans rather than livestock and this may cause a sampling bias, lessening the chances of finding this pathogen. A further a disadvantage of this approach is that it does not directly address the question of the host. Because *Babesia* spp. are capable of surviving through generations of ticks, the presence of a pathogen in a tick attached to an animal does not imply that species is the host for that pathogen. This greatly reinforces the need for ongoing active surveillance of *Babesia* and *Theileria* spp. in livestock in the United Kingdom. Next generation sequencing could be employed in this role as it has been recently for the detection of pathogens in the tick vector (Bonnet *et al.*, 2014).

4.4.2 *B. divergens* in red deer and cattle

The endemic species *B. divergens* was detected in cattle and red deer sharing the same pasture at site A. The prevalence of *B. divergens* in cattle was low but the blood was obtained in December and, additionally, the samples were clotted/coagulated making DNA extraction more difficult. *Babesia divergens* has been confirmed by molecular means in red deer in Ireland (Zintl *et al.*, 2011) and European red deer (Duh *et al.*, 2005b; Michel *et al.*, 2014; Zintl *et al.*, 2011)

but had not been found previously in Scotland. The findings of the present study suggest that the parasite could retreat to a tick-deer cycle if excluded from the cattle population, for example by aggressive acaricidal therapy or depopulation, only to re-emerge at a later date causing clinical disease in exposed cattle. In addition to these implications for animal health, there are also implications for human health as this species is zoonotic (Entrican *et al.*, 1979b).

Interestingly, and in clear contrast to the confirmation by PCR of *B. divergens* infection diagnosed by microscopy in the three cattle with clinical signs of babesiosis (Chapter Three), the PCR positive animals were all clinically normal at the time of sampling/culling.

4.4.3 *B. odocoilei*-like parasite in red deer

Sequences similar to *B. odocoilei* AY046577 were amplified from the blood of 13 of the Scottish wild red deer sampled. Four genotypes were identified based on the nucleotides at positions 187 and 217; these were TA (n = 5), TC (n = 3), GC (n = 4) and GA (n =1). Overlapping parts of the TA genotype and GenBank GU475474 originating from Irish red deer (Zintl *et al.*, 2011) were identical. BLAST analysis revealed no identical sequences for any of the genotypes in the NCBI database, however the top ten BLAST hits for each of the four sequence types were essentially the same. These originated from parasites found in ruminants in the US and Canada, with the exception of two sequences that originated from parasites found in goats in Turkey.

The clinical significance of this *B. odocoilei*-like parasite in Scottish red deer is unclear, as is the importance of the four genotypes identified. One of the genotypes found in five deer was identical to that from three deer in Ireland and all shared a high level of similarity with sequences originating from ruminants in the US, Canada and Turkey.

4.4.4 *Sarcocystis* sp.

Closely related sequences were found in the blood of sheep from both farm sites A and B that were either identical or highly similar to *Sarcocystis tenella*. *Sarcocystis* spp. are Apicomplexan parasites closely related to *Toxoplasma gondii* and to a lesser extent *Babesia* and *Theileria* spp.

There are numerous *Sarcocystis* spp. that typically have sexual stages in the gastrointestinal tract of a carnivorous final host and encysted but infective stages in the tissues of a herbivorous intermediate host. In the case of *S. tenella*, these are dogs or foxes and sheep respectively. The parasite undergoes a small number of rounds of asexual division in the intermediate host's vascular endothelium and monocytes before encysting, and this may explain why it was found in the intermediate host's blood in this study. Although typically infection in both hosts is without clinical signs, in rare circumstances myeloencephalitis can occur in sheep (Caldow *et al.*, 2000).

Although this is the first molecular confirmation of this species in Scotland, amplification of DNA from this species represents a lower specificity of the PCR assay developed earlier (Chapter Three) than anticipated. This is explained by the close relationship between the targeted *Babesia* and *Theileria* spp. and *Sarcocystis* sp. In addition to its identity being confirmed by amplicon sequencing, the amplicon was larger than expected for *Babesia* spp., allowing it to be easily identified as a spurious result.

4.4.5 Summary

The work presented in this chapter is unique as it is the first large-scale molecular survey of *Babesia* and *Theileria* spp. parasites in livestock and wild red deer carried out in Scotland. DNA exactly matching that of a European species *B. venatorum* was amplified for the first time from the blood of sheep, and for the first time in a vertebrate host in Scotland. **This confirms that a previously undetected *Babesia* sp. is present in Scottish livestock** in addition to the endemic *B. divergens* also demonstrated in clinically normal cattle. **In wild Scottish red deer *B. divergens* was confirmed by molecular means for the first time in Scotland and the presence of a *B. odocoilei*-like parasite demonstrated in this species.** Unexpectedly, DNA of a *Sarcocystis* species very similar to *Sarcocystis tenella* was also amplified from sheep.

CHAPTER FIVE
Investigation of *Anaplasma phagocytophilum* in Scottish Livestock and Wild Red Deer

5.1 Introduction

In the United Kingdom, the association between tick infestation and susceptibility to infection in sheep is long standing (M'Fadyean, 1894). Over forty years after this early description, the bacterial agent underpinning this association was described by Gordon *et al.* (1940) and subsequently given the name *Rickettsia phagocytophila* (Foggie, 1951). Taxonomic re-organisation followed resulting in this changing to *Cytoecetes phagocytophila* (Foggie, 1962), *Ehrlichia phagocytophila* (Philip, 1974), and finally *Anaplasma phagocytophilum* (Dumler *et al.*, 2001).

Anaplasma phagocytophilum is transmitted transtadially by its tick vector *I. ricinus* (MacLeod and Gordon, 1933; MacLeod, 1936). Infection and disease, termed "tick-borne fever", often occurs following the movement of susceptible sheep or cattle (described initially in this species by Hudson (1950)) to tick infested pasture (Woldehiwet, 2006). *Anaplasma phagocytophilum* infects circulating neutrophils and eosinophils resulting in a well-characterised episode of bacteraemia, neutropaenia and immunosuppression (Woldehiwet, 2006). Associated with these changes is pyrexia that, in itself, can have an impact on extensively reared sheep (Brodie *et al.*, 1986). However, in sheep the resultant immunosuppression has the greatest impact by potentiating the effect of other pathogens, such as example louping ill virus (Reid *et al.*, 1986), parainfluenza-3 virus (Batungbacal and Scott, 1982b), orf virus (Gokce and Woldehiwet, 1999) and *Pasteurella* (Brodie *et al.*, 1986; Gilmour *et al.*, 1982; Overas, 1983; Overas *et al.*, 1993). The most common and severe of these is tick pyaemia; following systemic dissemination of *Staphylococcus aureus* multifocal abscessation leads to lameness, paralysis and failure to thrive in young lambs (Woldehiwet, 2006; Foggie, 1962). In Scotland, one study determined that up to 5 % of the total lamb crop was affected (Foggie, 1962) while another estimated this number as 300,000 lambs across the country (Brodie *et al.*, 1986). In Scotland staff at SAC/SRUC DSCs diagnosed 116 cases of tick-borne fever and related disease in the years between 2010 and 2013, and 48 (41 %) of these were tick pyaemia

(2.3.2 SAC/SRUC tick-borne disease cases 2000 - 2013 (p. 44)). However, this is likely to be a gross underestimate of the actual occurrence of disease as suggested by the survey of veterinary surgeons (2.3.1 National survey of large animal veterinary surgeons on tick-borne disease in Scottish livestock (p. 35)) where tick-borne fever was described as endemic, but greatly under-observed and under-reported. As a result *A. phagocytophilum* has a major impact on the welfare of Scottish livestock and the economic health of Scottish producers. Importantly, from the producers point of view there is also evidence that *A. phagocytophilum* can hinder the growth (productivity) of sheep (Stuen *et al.*, 2002) and cattle (Taylor and Kenny, 1980) without other clinical manifestations to draw attention to its presence. Additionally, there is evidence that variants exist in Scotland capable of infecting humans (Sumption *et al.*, 1995; Hagedorn *et al.*, 2014).

Despite its perceived importance, there is a paucity of information about rates of livestock infection with *A. phagocytophilum* in the United Kingdom, particularly using sensitive molecular diagnostic methods. One exception is a longitudinal study by Ogden *et al.* (2002a) that found a rate of infection in sheep of 38 % that is comparable with findings in Norway (Stuen *et al.*, 2013b). There is a similar lack of information about *A. phagocytophilum* infecting wild red deer (*Cervus elaphus*) in Scotland. This may be significant as, in addition to sharing extensive grazing with hill sheep, this species is suspected to be a reservoir host for the *A. phagocytophilum* found in domestic ruminants. *A. phagocytophilum* isolates originating in red deer have also been shown to be capable of infecting sheep (Foggie, 1962; Stuen *et al.*, 2010), and vice versa, with sheep isolates capable of establishing subclinical and persistent infections in red deer (Stuen *et al.*, 2001).

Despite the lack of current information about *A. phagocytophilum* in the field, there is a long history of experimental work on this pathogen in the UK. Much of this work have utilised a strain of *A. phagocytophilum* called “Old Sourhope” or OS (Batungbacal and Scott, 1982a; Batungbacal and Scott, 1982b; Batungbacal *et al.*, 1982; Woldehiwet and Scott, 1982a). This strain was initially isolated from sheep near Jedburgh in southern Scotland more than forty years ago (Foster and Cameron, 1970; Woldehiwet and Scott, 1982b)(Foster and Cameron 1970; Woldehiwet and Scott 1982). The advancement of molecular methods in the past

two decades means that tools are now available to detect *A. phagocytophilum* with a high level of sensitivity and to genotype strains at high resolution. Given the availability of these methods and the lack of recent information about the pathogen in field, it was decided to carry out a molecular survey of this evasive, immunosuppressive and potentially zoonotic bacterial species in Scottish sheep, cattle and red deer. This study was designed in order to answer the following four key questions:

1. What is the prevalence of *A. phagocytophilum* in an endemic focus?

2. Are *A. phagocytophilum* infections characterised by the presence of multiple genotypes of the pathogen?

3. Can *A. phagocytophilum* be detected in the wild red deer population and, if so, how do pathogen genotypes in deer relate to those found in livestock?

4. How do *A. phagocytophilum* genotypes in Scotland compare with those from other parts of the world?

5.2 Materials and methods

5.2.1 Field sampling

The large collection of clinical material examined for the presence of *Babesia* and *Theileria* spp. was also examined for the presence of *A. phagocytophilum* using a 16S-based PCR assay. This comprises SRUC/SAC diagnostic cases (Chapter Three) and blood collected during the targeted farm and red deer sampling study (Chapter Four), with a smaller number of additional samples obtained from red deer in the Western Highlands of Scotland which were not tested for the presence of *Babesia* or *Theileria* spp. DNA preparations made from samples that were positive on 16S PCR were then used as template for a second nested PCR targeting the *msp4* gene.

5.2.2 *Anaplasma phagocytophilum* PCR optimisation

5.2.2.1 PCR methodology and control DNA

PCR equipment and reagents, with the exception of primers, are essentially the same as those described in the section on *Babesia* and *Theileria* spp. PCR development and optimisation (3.2.1.3 PCR development and optimisation (p. 63)). Positive control DNA was extracted from the Old Sourhope (OS) strain of *A. phagocytophilum* (supplied by Dr Zerai Woldhiwet, University of Liverpool), henceforth referred to as *A. phagocytophilum* OS. Using this as a template and primers detailed in the following two sections, PCR amplification and amplicon sequencing generated an 16S sequence identical to Genbank accession numbers M73220 (Anderson *et al.*, 1991) and AY176587 (sequence type D) (Bown *et al.*, 2007) and an *msp4* sequence identical to EF442007 (sequence type iv) (Bown *et al.*, 2007). Water was used as a template negative control. Visualisation of PCR products was carried out by gel electrophoresis followed by UV illumination, again as described in 3.2.1.3 PCR development and optimisation (p. 63). Amplicons from positive reactions were purified, sequenced and annotated using the methodology and nomenclature described in 3.2.2.3 Sequencing (p. 69) and Figure 3-4.

5.2.2.2 PCR primers

A species-specific nested PCR protocol was selected to amplify a segment of the *A. phagocytophilum* 16S gene. The outer primer pair utilised were ge3a (5'-CACATGCAAGTCGAACGGATTATTC-3') and ge10r (5'-TTCCGTTAAGAAGGATCTAATCTCC-3') while the internal primer pair were ge9f (5'-AACGGATTATTCTTTATAGCTTGCT-3') and ge2 (5'-GGCAGTATTAAAAGCAGCTCCAGG-3') (Massung *et al.*, 1998). Based on the 16S nucleotide sequence of *A. phagocytophilum* OS (Genbank accession M73220 (Anderson *et al.*, 1991)), an outer amplicon of 931 nucleotides and a nested amplicon of 546 nucleotides is predicted, including the primer sequences.

A nested protocol was also used to amplify the *msp4* gene of *A. phagocytophilum*. The outer primer pair utilised were MSP4AP5 (5'-ATGAATTACAGAGAATTGCTTGTAGG-3') and MSP4AP3 (5'-TTAATTGAAAGCAAATCTTGCTCCTATG-3') (de la Fuente *et al.*, 2005a) while the

internal primer pair were msp4f (5'-CTATTGGYGGNGCYAGAGT-3') and msp4r (5'-GTTCATCGAAAATTCCGTGGTA-3') (Bown *et al.*, 2007). Based on the *msp4* sequence of *A. phagocytophilum* strain 'Norway variant2' (Genbank accession CP015376 (Al-Khedery and Barbet, 2014), an outer amplicon of 849 nucleotides and an inner amplicon of 381 nucleotides is predicted, including the primer sequences. The 'Norway variant2' strain originated from a sheep in Norway, so it was felt that it would be comparable with anticipated strains in Scottish sheep.

5.2.2.3 Statistical, genetic and phylogenetic analysis

Statistical analysis was primarily undertaken using Microsoft Excel, which was used to perform paired and unpaired T tests along with Pearson Correlations. Fisher's Exact Tests were performed using the online service, GraphPad (https://graphpad.com/quickcalcs/contingency1.cfm). Analysis of sequence polymorphism, including the calculation of segregating sites, nucleotide diversity and gene diversity was performed using DNASP (Rozas *et al.*, 2003). Sequence alignment was undertaken using ClustalX (Larkin *et al.*, 2007), which was also used to generate neighbor-joining trees. Maximum Likelihood trees were generated using RAxML (Stamatakis, 2014). Phylogenetic trees were visualised using Figtree (http://tree.bio.ed.ac.uk/software/figtree/). Phylogenetic networks were constructed using the Split Decomposition method of SplitsTree4 (Huson and Bryant, 2006). Recombination analysis was undertaken using GARD (Kosakovsky Pond *et al.*, 2006), the Genetic Algorithm for Recombination Detection, implemented by the online service Datamonkey (http://www.datamonkey.org). For comparative purposes, a series of published *msp4* nucleotide sequences was downloaded from GenBank generated from studies of (Ladbury *et al.*, 2008) (n = 24) and Stuen 2013 (Stuen *et al.*, 2013b) (n = 11).

5.3 Results

5.3.1 16S PCR optimisation

In order to determine the optimum annealing temperature for the outer (ge3a and ge10r) and inner primer pair (ge9f and ge2), gradient PCR was performed consisting of twelve reactions with annealing temperatures between 54.3 and 67.8 °C. Thermocycler settings consisted of pre-heating the lid (105 °C) for four

minutes before an initial denaturation step at 94 °C for five minutes. This was followed by 30 cycles of denaturation (94 °C, 30 seconds), annealing (gradated temperature, 30 seconds) and extension (72 °C, 60 seconds). A final extension step at 72 °C for five minutes was also carried out. The *A. phagocytophilum* OS DNA solution described above was used as template for the outer reaction, and the product of the selected outer reaction diluted 1:10 with water as template for the nested reaction. The reaction products were visualised as described in Section 3.2.1.3. The results of this are shown in Figure 5-1 for the outer pair and in Figure 5-2 for the inner pair. By choosing the highest annealing temperature producing a maximal amount of product an annealing temperature of 65 °C was selected for the outer reaction and 64 °C was selected for the inner reaction. The finalised *A. phagocytophilum* 16S PCR protocol is summarised in Figure 5-3).

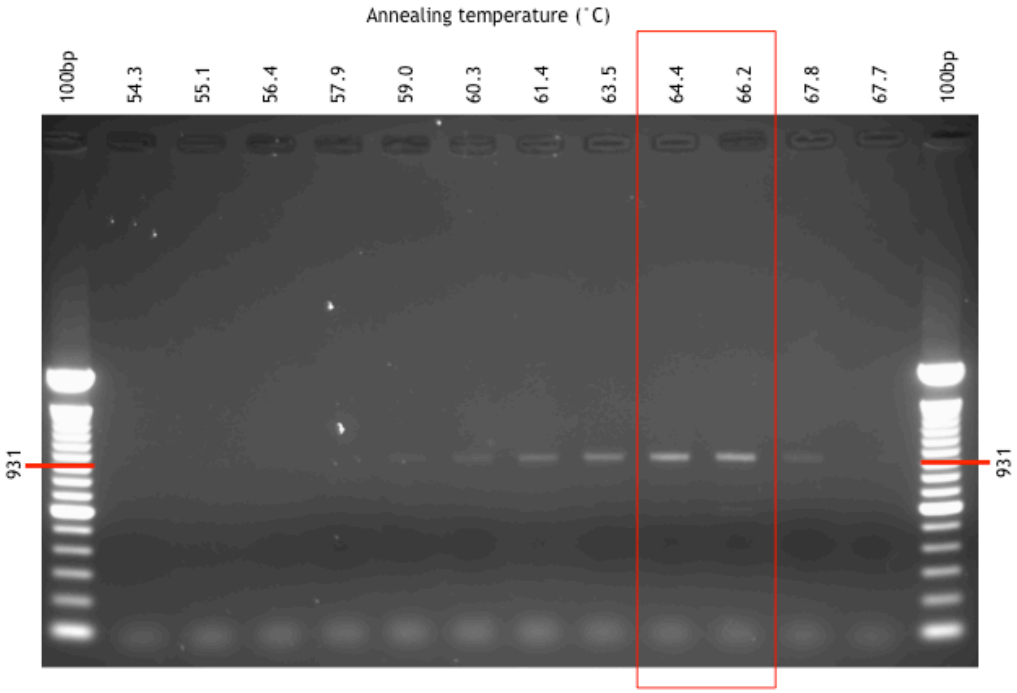

Figure 5-1 Gradient PCR of the outer primer pair (ge3a and ge10r)

The lanes most closely corresponding to the selected annealing temperature (65 °C) are highlighted by the red box and the expected amplicon size (931 nucleotides) is indicated by red bars in the ladder.

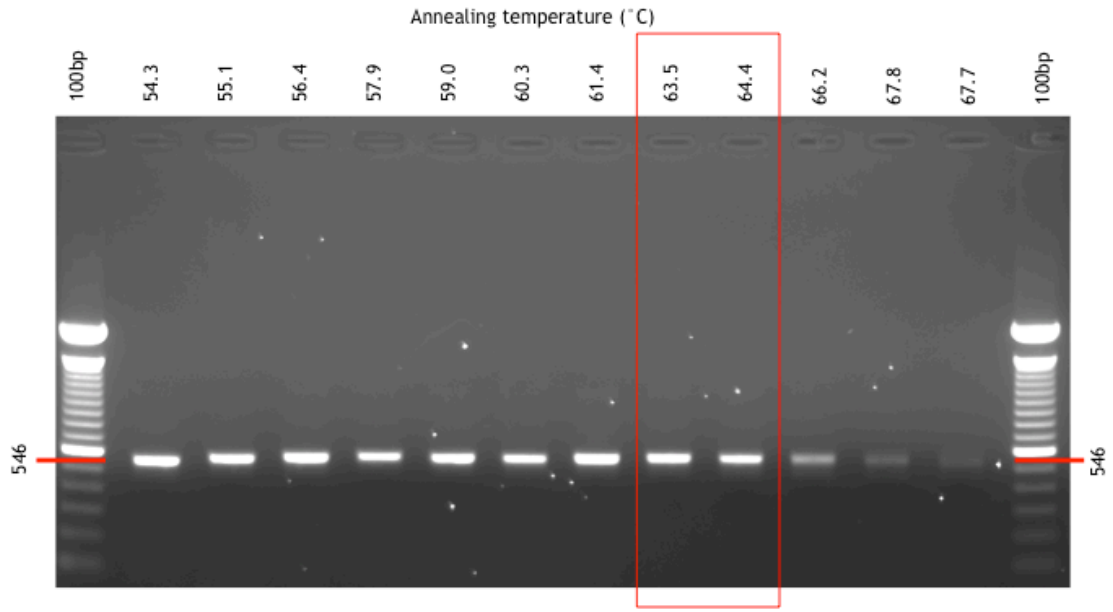

Figure 5-2 Gradient PCR of the nested primer pair (ge9f and ge2)

The lanes most closely corresponding to the selected annealing temperature (64 °C) are highlighted by the red box and the expected amplicon size (546 nucleotides) is indicated by red bars in the ladder.

Outer reaction (ge3a and ge10r)

- Heated lid to 105 °C 4 min before start of reaction
- Initial denaturation at 94 °C for 5 min
- 30 cycles of:
    - denaturation at 94 °C for 30s
    - annealing at 65 °C for 30s
    - extension at 72 °C for 60s
- Final extension at 72 °C for 5 min

The primary reaction product is then diluted 1:10 for use as template in the nested reaction.

Nested reaction (primers ge9f and ge2)

- Heated lid to 105 °C 4 min before start of reaction
- Initial denaturation at 94 °C for 5 min
- 30 cycles of:
    - denaturation at 94 °C for 30s
    - annealing at 64 °C for 30s
    - extension at 72 °C for 60s
- Final extension at 72 °C for 5 min

Figure 5-3 Finalised *A. phagocytophilum* 16S PCR protocol

5.3.2 *msp4* PCR optimisation

In order to determine the optimum annealing temperature for the outer primer pair (MSP4AP5 and MSP4AP3), gradient PCR was performed consisting of twelve reactions with annealing temperatures between 56.0 and 66.1°C. Similarly, to determine the optimum annealing temperature for the nested primer pair (msp4f and msp4r), gradient PCR was performed consisting of twelve reactions with annealing temperatures between 58.0 and 68.1°C. Other thermocycler settings were identical for optimisation of both of these primer pairs, and

consisted of pre-heating the lid (105 °C) for four minutes before an initial denaturation step at 94 °C for five minutes. This was followed by 40 cycles of denaturation (94 °C, 10 seconds), annealing (gradated temperature, ten seconds) and extension (72 °C, 50 seconds). A final extension step at 72 °C for five minutes was also carried out. The *A. phagocytophilum* OS DNA solution described above was used as template for the outer reaction, and the product of the outer reaction was diluted 1:1000 with water as template for the nested reaction. The reaction products were visualised as described in in 3.2.1.3 PCR development and optimisation (p. 63). The results of this are shown in Figure 5-4 for the outer primer pair and in Figure 5-5 for the nested primer pair. By choosing the highest annealing temperature producing a maximal amount of product an annealing temperature of 61°C was selected for the outer reaction and 60 °C was selected for the nested reaction. The finalised *A. phagocytophilum msp4* PCR protocol is summarised in Figure 5-6.

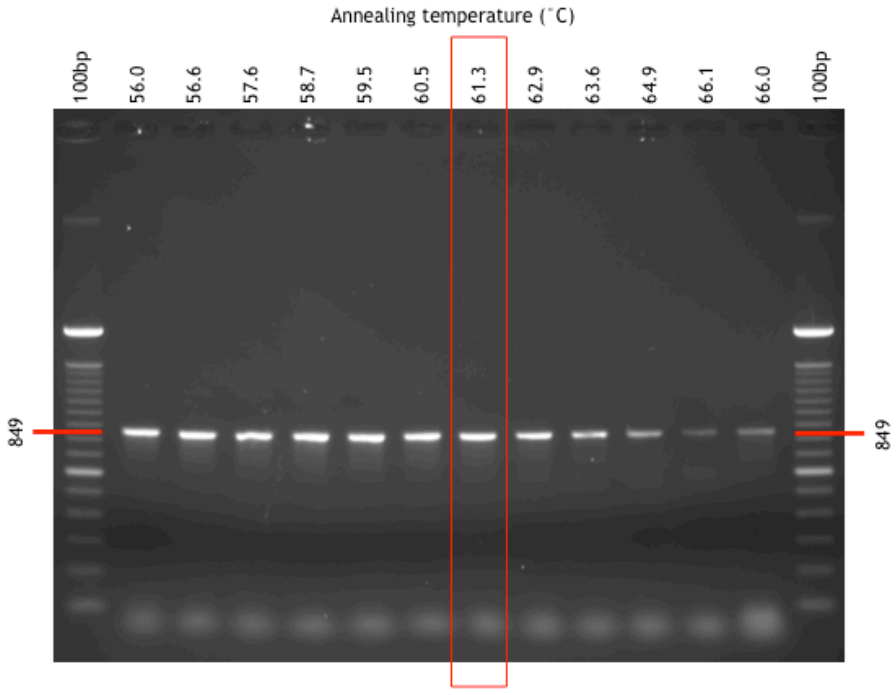

Figure 5-4 Gradient PCR of the outer primer pair (MSP4AP5 and MSP4AP3)

The lane corresponding to the selected annealing temperature (61 °C) is highlighted by the red box and the expected amplicon size (849 nucleotides) is indicated by red bars in the ladder.

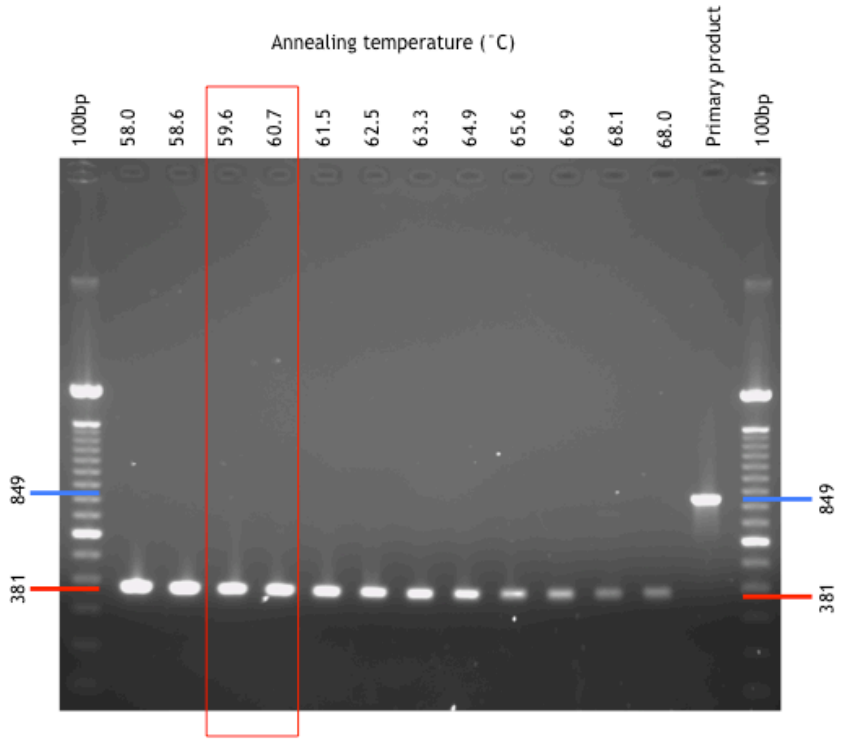

Figure 5-5 Gradient PCR of the outer primer pair (msp4f and msp4r)

The lanes most closely corresponding to the selected annealing temperature (60 °C) are highlighted by the red box and the expected amplicon size (381 nucleotides) is indicated by the red bars in the ladder. The expected amplicon size for the primary product (849 nucleotides) is indicated by blue bars in the ladder.

Outer reaction (MSP4AP5 and MSP4AP3)

- Heated lid to 105 °C 4 min before start of reaction
- Initial denaturation at 94 °C for 5 min
- 40 cycles of:
    - denaturation at 94 °C for 10s
    - annealing at 61°C for 10s
    - extension at 72 °C for 50s
- Final extension at 72 °C for 5 min

The primary reaction product is then diluted 1:1000 for use as template in the nested reaction.

Nested reaction (msp4f and msp4r)

- Heated lid to 105°C 4 min before start of reaction
- Initial denaturation at 94°C for 5 min
- 40 cycles of:
    - denaturation at 94°C for 10s
    - annealing at 60°C for 10s
    - extension at 72°C for 50s
- Final extension at 72°C for 5 min

Figure 5-6 Finalised *A. phagocytophilum msp4* PCR protocol

5.3.3 Summary of field samples examined

A summary of the 369 samples previously examined for *Babesia* and *Theileria* spp. in Chapters 3 (51 samples) and 4 (318 samples) is provided Table 5-1. This includes details of the specific sampling site, species of origin and health status of animals. A breakdown of the *Babesia* and *Sarcocystis* spp. already identified is also given, based on primary data presented in Table 3-4 and Table 4-1.

Additionally, a further set of frozen blood samples originating from male red deer culled during the summer of 2012 were included at this stage; these consisted of ten samples from each of three sites (designated sites I-K) in the West Highlands of Scotland (total n = 30), bringing the overall number of samples tested using the *A. phagocytophilum* 16S rRNA PCR to 399 (51 (Chapter 3), 318 (Chapter 4), and 30 (sites I-K, this Chapter)). These additional samples were not examined using the *Babesia* and *Theileria* spp. PCR developed and deployed earlier.

Site	Species	Status	No.	Sampling period	Sample type	Number *Babesia*/ *Theileria* positive
SAC/ SRUC	Cattle	Alive, TBD suspected (12) or PM, TBD suspected (1)	13	Sept 13 - Dec 14	Blood (12, Alive) or Tissue (1, Carcass)	3 (3 Bdiv)
SAC/ SRUC	Sheep	Alive, TBD suspected (18) or PM, TBD suspected (20)	38	Sept 13 - Dec 14	Blood (18, Alive) or Tissue (20, Carcass)	0
A	Sheep	Alive, healthy	47	Oct 2013	Blood, fresh, EDTA	7 (6 Bven, 1 Sarco)
A	Cattle	Alive, healthy	107	Dec 2013	Blood, frozen, clotted	7 (7 Bven)
A	Red deer (F)	PM, healthy	24	Oct 2013	Blood, frozen, EDTA	11 (4 Bdiv, 7 Bodo-lk)
B	Sheep	Alive, healthy	40	Jun 2014	Blood, fresh, EDTA	2 (1 Bven, 1 Sarco)
B	Sheep	Alive, healthy	40	Nov 2014	Blood, fresh, EDTA	6 (4 Bven, 2 Sarco)
C-H (10/site)	Red deer (M)	PM, healthy	60	Summer 2012	Blood, frozen, clotted	11 (5 Bdiv, 6 Bodo-lk)
I-K (10/site)	Red deer (M)	PM, healthy	30	Summer 2012	Blood, frozen, clotted	Not carried out

Table 5-1 Details of samples for testing with *A. phagocytophilum* 16S PCR

F, Female; M, male; Bven, *B. venatorum*; Sarco *Sarcocystis* spp.; Bdiv *B. divergens*; Bodo-lk *B. odocoilei*-like

DNA extraction had either already been undertaken as described in 3.2.2.2 DNA extraction (p. 69) or, for the additional 30 red deer samples (sites I-K), was carried out according to this methodology. The optimised *A. phagocytophilum* 16S PCR protocol was undertaken as described in Figure 5-3. A positive 16S PCR result confirmed the presence of *A. phagocytophilum* in the sample and for each positive sample, *msp4* nested PCR was also carried out as described in Figure 5-6. A subset of amplicons resulting from the initial 16S nested PCRs, and all *msp4* nested PCRs, were purified and direct sequencing carried out as described in 3.2.2.3 Sequencing (p. 69). Sequence naming was then carried out according to the scheme illustrated in Figure 3-4.

5.3.4 Prevalence of *Anaplasma phagocytophilum* in sheep and cattle confirmed or clinically suspected of having tick-borne disease by SAC/SRUC

Veterinary Investigation Officers at participating Disease Surveillance Centres identified 30 incidents (clinical events involving one or more cattle or sheep where tick-borne disease was confirmed by microscopic examination, suspected clinically, or suspected based on the presence of ticks) during the 15-month period between September 2013 and December 2014, from which they submitted blood and/or tissue samples (Table 5-2, and illustrated in Figure 5-7).

Submitted material	Samples submitted (Incidents (animals))	*A. phag.* PCR +ve (Incidents (animals))
Cattle (blood*)	3 (3)	0
Cattle (blood	9 (9)	3 (3)
Cattle (carcass)	1 (1)	0
All cattle	13 (13)	3 (3)
Sheep (blood)	2 (18)	1 (1)
Sheep (carcass)	14 (18)	6 (7)
Sheep (on-farm PM)	1 (2)	0
All sheep	17 (38)	7 (8)
Overall	30 (51)	10 (11)

Table 5-2 Material submitted from SAC/SRUC DSCs from incidents where a tick-borne disease was suspected, results of *A. phagocytophilum* PCR

Submitted material by sample type and animal species showing *A. phagocytophilum* PCR results. The number of incidents (clinical events where babesiosis was confirmed by microscopic examination (Cattle (blood*)), tick-borne disease was suspected clinically, or tick-borne disease was suspected based on the presence of ticks) is indicated together with the actual number of animals sampled in parenthesis.

Overall 30 incidents, involving 51 cattle or sheep, were investigated, resulting in 10 incidents where *A. phagocytophilum* 16S rRNA PCR was positive, involving 11 animals. Thirteen incidents affected cattle (involving a total of 13 animals), and 3 of these incidents (involving 3 animals) resulted in an *A. phagocytophilum* PCR positive result. A further 17 incidents affected sheep (involving a total of 38 animals), and 7 of these incidents (involving 8 animals) resulted in an *A. phagocytophilum* PCR positive result (Table 5-2, and illustrated in Figure 5-7).

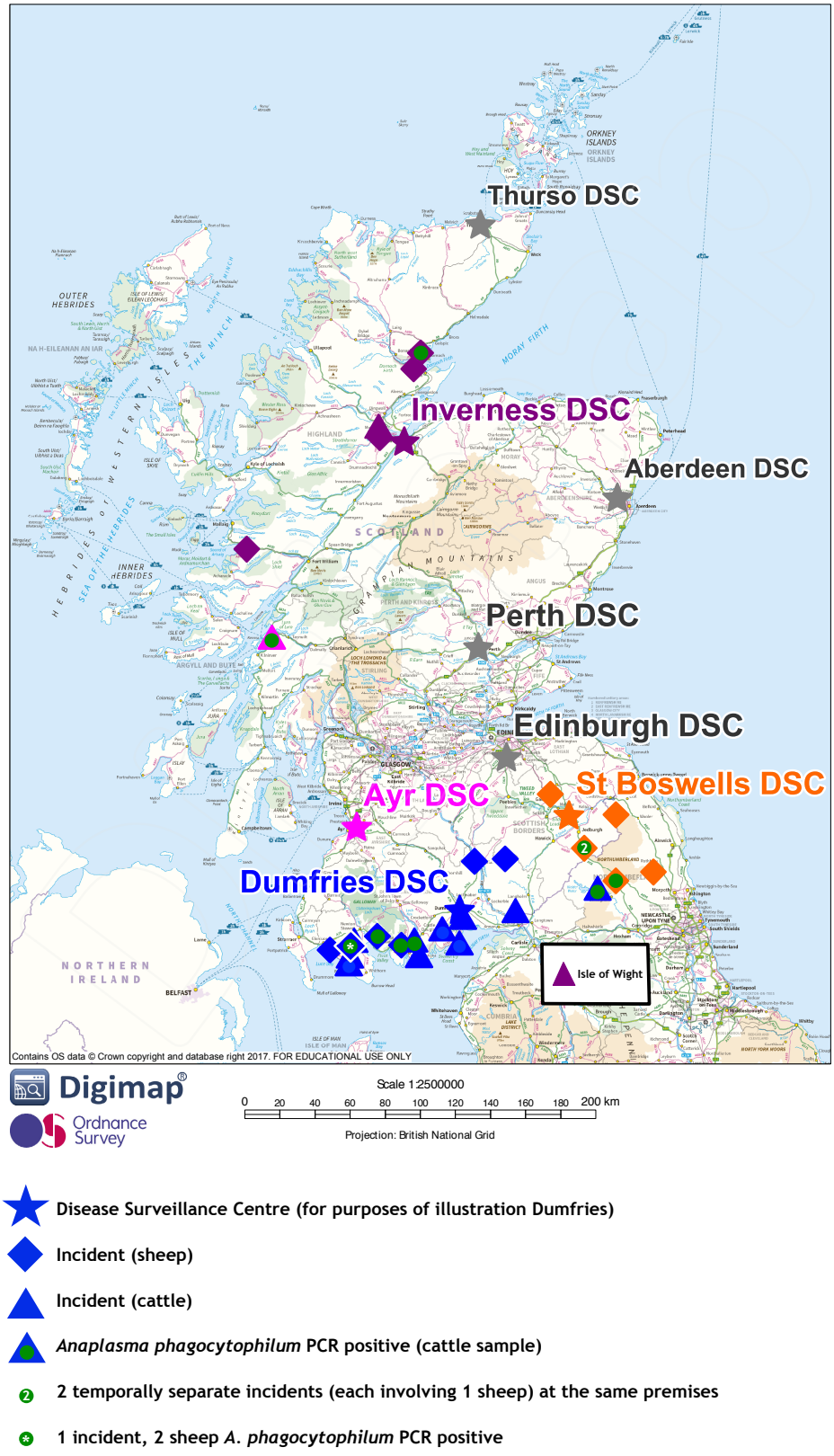

Figure 5-7 Spatial distribution of SAC/SRUC incidents of tick-borne disease from which samples were analysed, and where *A. phagocytophilum* PCR was positive

SAC/SRUC Disease Surveillance Centres (stars) labeled by colour, showing associated incidents involving sheep (diamonds) and cattle (triangles). A green dot within the symbol indicates that the incident resulted in an *A. phagocytophilum* PCR positive result. At one premises (superimposed "2") 2 incidents occurred at separate times, involving 1 sheep on each occasion, while at another (superimposed *) 1 incident resulted in *A. phagocytophilum* PCR positive results in 2 animals. All other incidents involved 1 animal.

5.3.5 Prevalence of *Anaplasma phagocytophilum* in healthy sheep, cattle and deer at sites C-H, and additional red deer sites I-K

Anaplasma phagocytophilum was detected in clinically healthy sheep, cattle and deer samples from all sampling sites (Table 5-3). In sheep the prevalence in autumn was high at site A and site B, at 81 % and 70 % respectively. There was little change in the prevalence of disease between the summer and autumn at site B. Very few cattle were found to harbour *A. phagocytophilum* with only 3 of 107 adult cattle sampled being PCR positive. A high proportion of the deer at site A were positive (67 %), which was considerably higher than prevalence in the deer sampled from various other locations in the West Highlands of Scotland (25%).

Origin	Species	Time of sampling	No. of animals sampled	No. of PCR positive samples	Prevalence
Site A	Sheep	Autumn 2013	47	38	80.9%
	Cattle	Autumn 2013	107	3	2.8%
	Deer	Autumn 2013	24	16	66.7%
Site B	Sheep	Summer 2014	40*	28	70.0%
	Sheep	Autumn 2014	40*	27	67.5%
West Highlands	Deer	Autumn 2012	90**	23	25.0%

Table 5-3 Molecular detection of *A. phagocytophilum*

* includes 34 North Country Cheviot ewes sampled in both summer and autumn, and ** 10 red deer samples from sites C-H (n=60) and 10 red deer samples from sites I-K (n=30)

The sheep sampled at site A consisted of 17 lambs and 30 ewes. Sixteen of the 17 lambs (94 %) were PCR positive for *A. phagocytophilum* while only 24 of the 30 ewes were positive (80 %). Although a higher proportion of lambs were found to be positive, this difference is not statistically significant (P = 0.3955, Fisher's Exact Test, one-tailed).

5.3.6 Analysis of field samples by *msp4* sequencing

5.3.6.1 "Simple" and "complex" sequences

Anaplasma phagocytophilum was detected in 135 animals in total (Table 5-3). This set of positive DNA samples was also used as a template for PCR

amplification of the *msp4* locus. An amplicon was successfully generated for each sample, which was then subjected to direct sequencing. Forward and reverse reads were generated and a total of 135 consensus sequences of *msp4* were generated. On examination of sequence traces, it was found that 104 of the sequences contained one or more site positions were there was evidence of more than one allele/nucleotide. Sequences were therefore classified as 'simple', where no ambiguities were present (n = 31) and 'complex' where one or more ambiguous positions were evident (n = 104). To investigate this issue, the distribution of ambiguous sites among sequences was investigated and this is presented in Figure 5-8, Figure 5-9, and Figure 5-10.

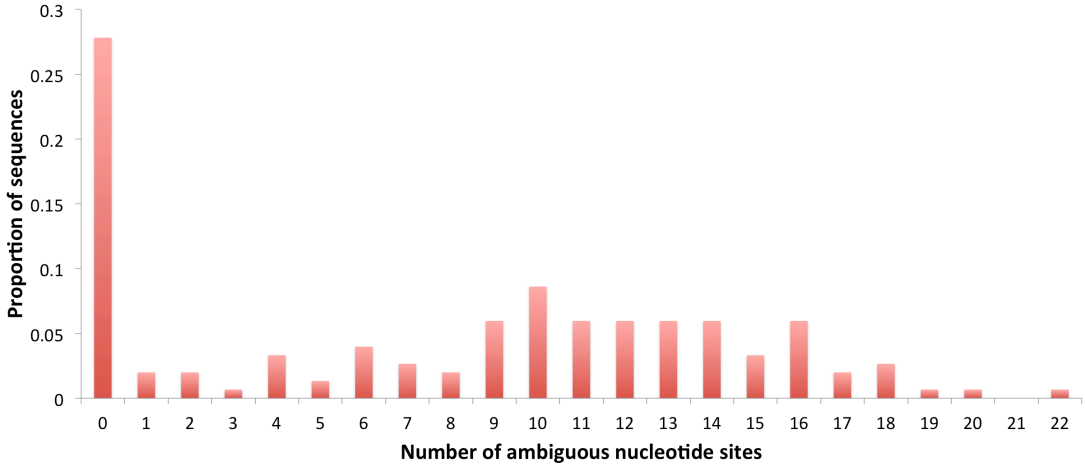

Figure 5-8 Distribution of ambiguous sites among sequences (n = 135)

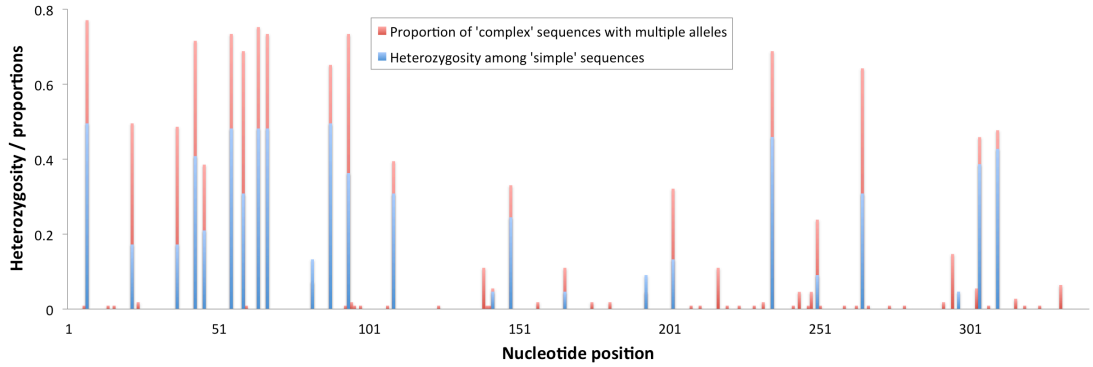

Figure 5-9 Heterozygosity in 'simple' sequences compared to the proportion of 'complex' sequences with ambiguities

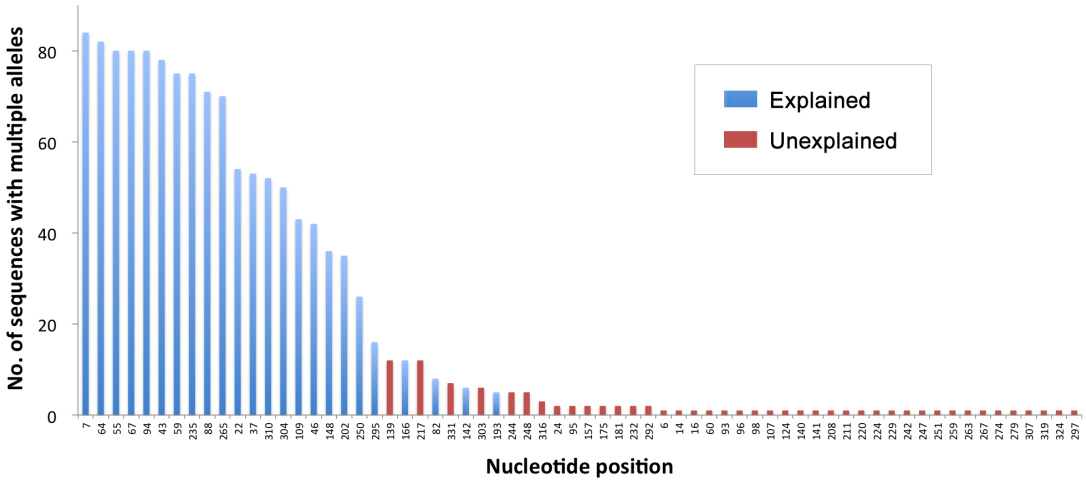

Figure 5-10 'Explanation' of ambiguous sites in 'complex' sequences on the basis of nucleotide polymorphisms among 'simple' sequences

From Figure 5-8 it can be seen that a considerable proportion of sequences (n = 31) have no ambiguities. The remainder have between 1 and 22 ambiguous positions, with a large number having between 9 and 16 ambiguous sites. When the distribution of ambiguous sites is analysed across the length of the gene (Figure 5-9) it is evident that the positions where a large number of complex sequences have ambiguities corresponds almost exactly to heterozygosity within the 'simple' sequence dataset. Thus, ambiguous sites are not randomly scattered throughout the gene, they are instead to be found almost exclusively in positions that are known to be polymorphic on the basis of the 'simple' sequences, which can be taken as a 'gold standard' for sequence fidelity. This overlap is highly statistically significant ($P < 0.0001$, Fisher's Exact Test, one-tailed). When the heterozygosity values of polymorphic sites were compared with the proportions of 'complex' sequences showing an ambiguity at the same site, a strong correlation was evident ($R = 0.9577$, Pearson Correlation). The nature of the polymorphisms in the 'simple' dataset (i.e. A/G or C/T etc.) was compared to the polymorphisms in the 'complex' dataset. For 24 sites, the allelic nature of the polymorphisms in the 'simple' and 'complex' datasets was identical (i.e. the same nucleotide alleles were present) and thus, the ambiguities in the 'complex' dataset could be explained. This is illustrated in Figure 5-10. It is clear that a large number of frequently encountered ambiguities can be explained. The unexplained ambiguities (red bars) are all at a relatively low frequency.

5.3.6.2 Multiplicity of infection

The presence of 'complex' traces can be explained by the co-occurrence of different *msp4* alleles, indicating co-infection of the host with multiple *A. phagocytophilum* genotypes. For this reason, the appearance of complex sequences was taken as evidence for multiplicity of infection (MOI). A summary of the results of the *msp4* sequences of field isolates is presented in Table 5-4.

Origin	Species	Time of sampling	No. of simple sequences	No. of complex sequences	Percentage of 16S PCR positive animals with MOI	Average no. of ambiguous nucleotides per sequence	Max no. of ambiguous nucleotides per sequence
Site A	Sheep	Autumn 2013	5	33	87%	8.61	18
	Cattle	Autumn 2013	1	2	67%	6.00	10
	Deer	Autumn 2013	1	15	94%	13.06	22
Site B	Sheep	Summer 2014	16	12	43%	3.61	13
	Sheep	Autumn 2014	6	21	78%	8.52	17
West Highlands*	Deer	Autumn 2012	2	21	91%	11.57	20
All sheep		Autumn	11	54	83 %	8.48 **	18
All deer		Autumn	3	36	92 %	12.18 **	22

Table 5-4 Summary of *msp4* sequencing results

* Includes a set of eight extra sequences sampled from three additional sites; ** Higher in deer than sheep (P = 0.0018, T test, two-tailed)

Deer samples showed the highest proportion of *A. phagocytophilum* samples with a MOI, 91 % of deer sampled from the West Highlands and 87 % at site A. Fewer *A. phagocytophilum* positive sheep had a MOI, 87 % at site A and 78 % at site B. Interestingly, when sampled earlier that year (in the summer) much fewer of the sheep showed a multiplicity of *A. phagocytophilum* infection (43 %). Two further metrics were calculated based on the presence of complex sequence: (a) the average number of ambiguous nucleotides per sequence and (b) the maximum number of ambiguous nucleotides per sequence. When analysed over each of the six groups studied, both of these metrics strongly correlate with the percentage of animals with a MOI (R = 0.939 and 0.800 respectively, Pearson Correlation). The groups of deer possess the highest number of ambiguous nucleotides per

sequence. Overall, comparing animals sampled in the autumn, a statistically higher number of ambiguous sites are found in deer compared to sheep (P = 0.0018, T test, two-tailed).

A similar prevalence of infection was found in sheep at site B in summer and autumn. To test whether the MOI varied between seasons, 26 sheep were identified that were found to be *A. phagocytophilum* positive on both occasions. Sheep were classed as having a single infection on the basis of a 'simple' *msp4* sequence and a multiple infection if they had a 'complex' *msp4* sequence. These results are presented in Table 5-5.

Summer status	Autumn status	No. of sheep
Single	Multiple	11
Multiple	Single	2
Multiple	Multiple	9
Single	Single	4

Table 5-5 Genotypic multiplicity of infection in 26 sampled sheep on two occasions at site B

Eleven of the 26 animals had evidence of a single genotype in summer and multiple genotypes in autumn, while the converse was true of only two animals. The hypothesis that, in general, individuals transitioned from harbouring single to multiple genotypes between summer and autumn was statistically supported (P = 0.0114, Fisher's Test, one-tailed). To further investigate this issue, when the number of ambiguous sites was compared in sequences from sheep in summer vs autumn, an average of 3.38 differences was found in summer, which rose to 8.46 in autumn, and this difference was also statistically significant (P = 0.0002, Paired T Test, two-tailed).

To determine whether there was a difference in the MOI in the lambs compared to the sheep at site A, the average number of ambiguous sites in sequences obtained from each was compared. Although the lambs had a higher value compared to sheep (9.56 vs 7.91) this was not statistically significant (P = 0.3312, T test, two-tailed).

5.3.6.3 Sequence diversity among *msp4* alleles

In order to analyse sequence diversity in the entire *msp4* dataset, the predominant allele/nucleotide at each position in each of the 104 complex sequences was determined. This allowed the most abundant allelic sequence to be approximated in each case. This, together with the set of 31 simple sequences, formed a dataset of 135 sequences, which were used to investigate *msp4* allelic diversity. The results of this analysis are presented in Table 5-6.

Origin	Species	Time of sampling	No. of sequences obtained	No. of alleles	Gene diversity	No. of segregating sites (S)	Nucleotide diversity (Pi)	Average number of nucleotide differences
Inverness North	Sheep	Autumn 2013	38	23	0.935	21	0.019	6.474
	Cattle	Autumn 2013	3	3	1.000	8	0.016	5.333
	Deer	Autumn 2013	16	15	0.992	20	0.020	6.883
Inverness South	Sheep	Summer 2014	28	18	0.958	24	0.023	7.870
	Sheep	Autumn 2014	27	24	0.989	23	0.021	7.236
West Highlands	Deer	Autumn 2012	23	21	0.992	24	0.019	6.372
All sheep		Autumn	65	42	0.960	24	0.020	6.762
All deer		Autumn	39	35	0.995	24	0.019	6.475
Overall		Summer / Autumn	135	86	0.979	29	0.020	6.996

Table 5-6 Summary of *msp4* allelic sequences

Among the 135 sequences, a total of 86 alleles was identified, with each sequence differing from each other sequence by around seven nucleotides on average. A total of 29 polymorphic sites were identified. This indicates that a large proportion of the low frequency ambiguous sites identified in the preceding section did not result in the calling of a novel and potentially spurious allele. Only a small proportion of variation was captured by the three sequences derived from cattle, which were distinct from one another over a total of eight polymorphic sites. In each group, the level of gene diversity was high and the overall gene diversity was calculated to be 0.979. This high level of diversity is consistent with the large number of distinct alleles sequenced. Diversity on a per-site basis (i.e. nucleotide diversity) was much lower and was around 0.020 for most of the groups.

5.3.6.4 Phylogenetic analysis

In order to investigate the phylogenetic relationship of the allelic sequences of *msp4* identified in this study, a maximum likelihood tree was constructed (Figure 5-11).

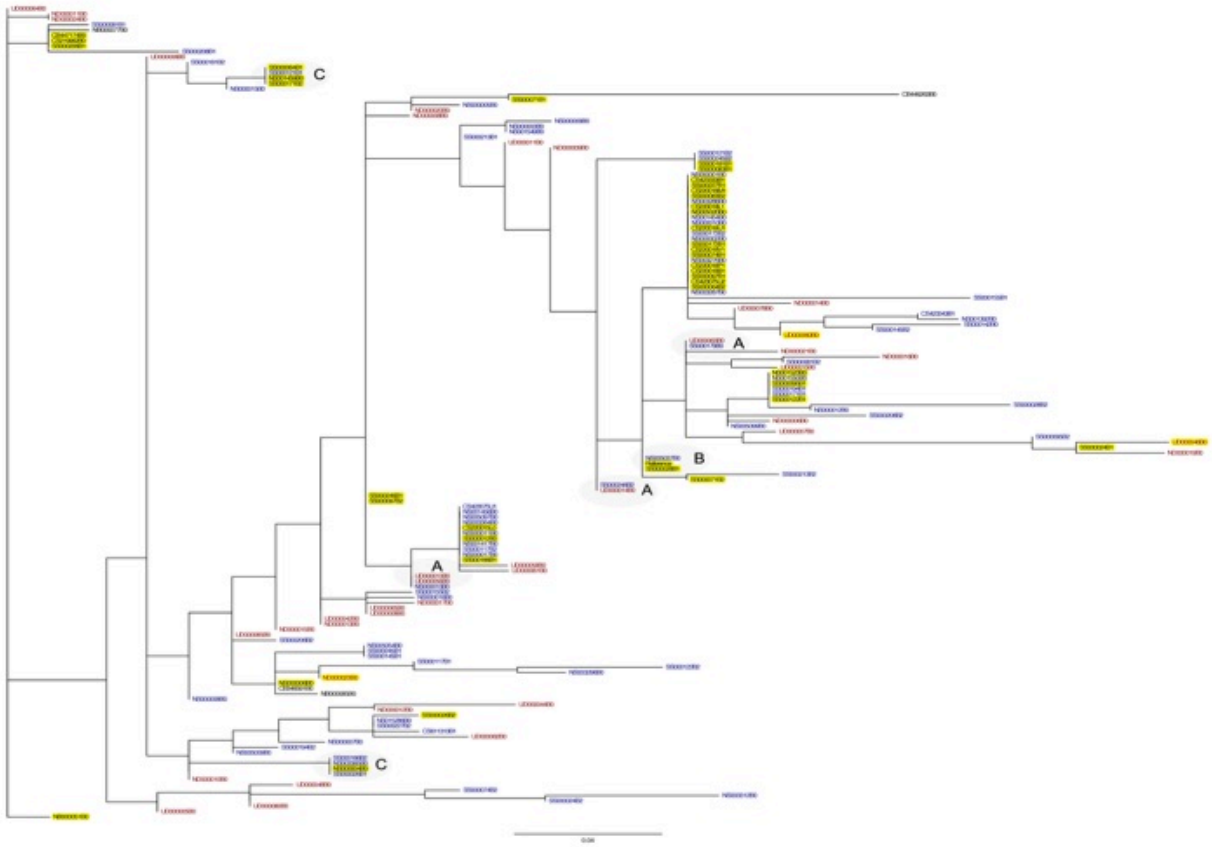

Figure 5-11 Maximum likelihood phylogenetic tree of *msp4*

Sheep derived sequences are shown in blue, deer sequences in red and 'simple' sequences (i.e. with no ambiguous sites) are show in yellow. For clarity, a number of sub-sets of identical alleles are markers which illustrate alleles which are (A) found in both sheep and deer; (B) identical to the reference sequence and (C) found in sheep at both site A and site B.

Examination of the tree reveals clearly that deer-derived and sheep-derived samples do not form separate clusters. Three identical alleles were found in the sheep and deer population (Figure 5-11, A). The *A. phagocytophilum* reference *msp4* sequence was found in sheep at both sites (Figure 5-11, B), which is one of a large number of alleles that was found at both sheep sampling sites (Figure 5-11, C). Clusters of 'simple' and 'complex' sequences were identified, supporting the concept that estimating the most abundant allele in mixtures will generate 'genuine' sequences. A large cluster of identical alleles highlights the

fact that some genotypes were commonly found in the dataset. Bootstrap analysis (not shown) indicates that the tree is generally unstable. This may be due, among other things, to recombination in the dataset and for this reason, phylogenetic network analysis was performed using all the *msp4* sequences generated in this study (Figure 5-12). The network exhibits considerable reticulation, consistent with presence of extensive recombination in the ancestry of the sequences analysed. To ensure this was not an artifact of approximating sequences with ambiguous nucleotides, a second phylogenetic network was constructed just using 'simple' sequences. This is illustrated in Figure 5-13, and again considerable reticulation is evident, strongly indicating the occurrence of recombination in the life history of this locus.

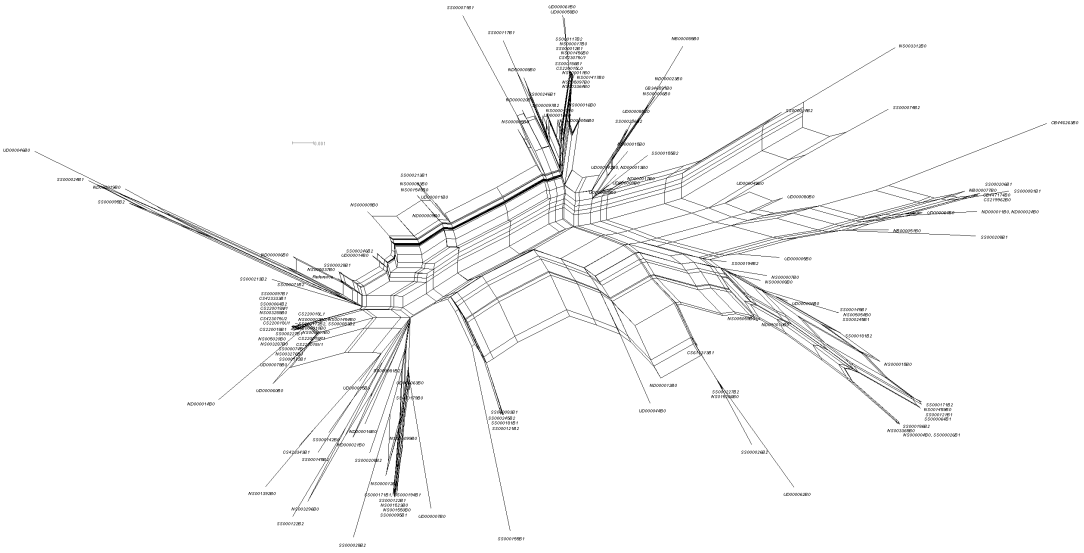

Figure 5-12 *msp4* Phylogenetic network representing all sequences identified

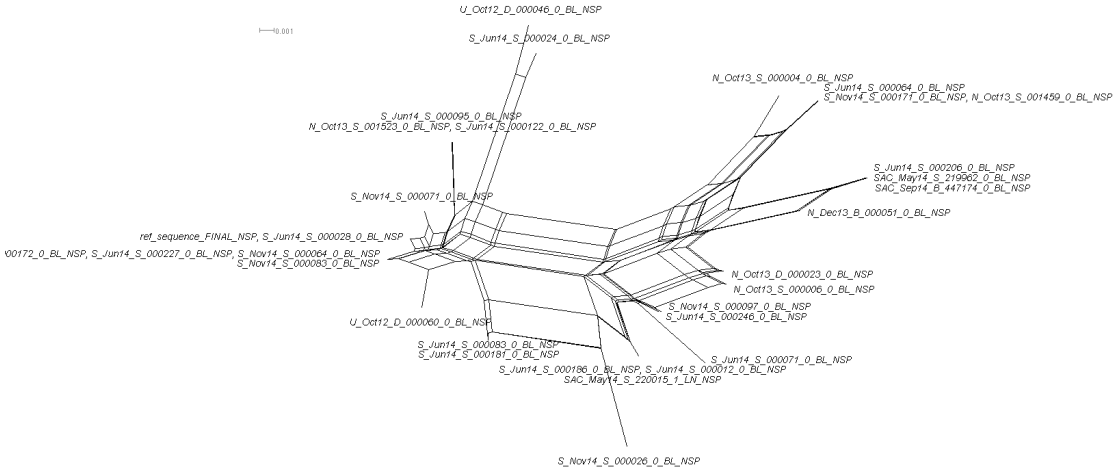

Figure 5-13 *msp4* Phylogenetic network representing all 'simple' sequences

A small number of sequences from SRUC/SAC cases are included.

To investigate whether evidence of specific recombination events could be detected to explain *msp4* sequence variation, analysis using the Genetic Algorithm for Recombination Detection (GARD) was performed. The results of this analysis are illustrated in Figure 5-14. Statistical support was obtained for the presence of four recombination breakpoints broadly in the central region of the *msp4* gene.

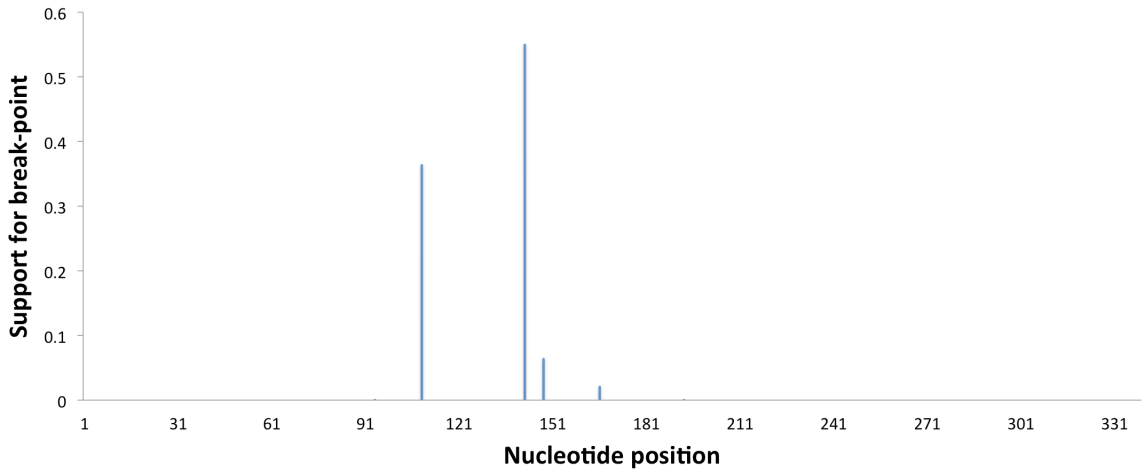

Figure 5-14 GARD analysis to detect recombination breakpoints

In order to appreciate the relationship between the *msp4* sequences identified in this study and those previously found in other parts of the world, a phylogenetic network was constructed based on the 31 'simple' *msp4* sequences together with published data from Ladbury *et al.* (2008) and (Stuen *et al.*, 2013b). This is illustrated in Figure 5-15, and an extensively reticulated network

is again apparent supporting the hypothesis that intra-genic recombination is a feature of *msp4*.

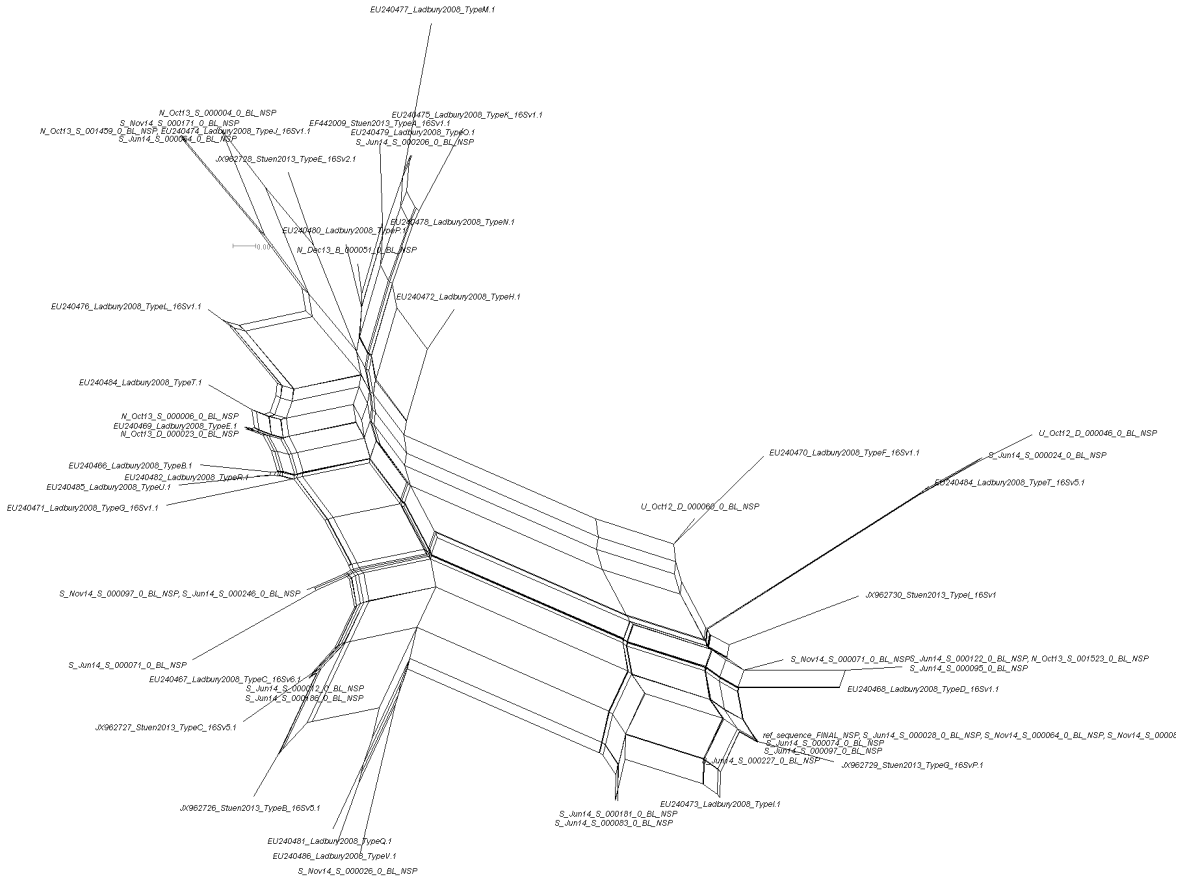

Figure 5-15 *msp4* phylogenetic network representing all 'simple' sequences and selected published foreign sequences

In order to better visualise the relationships between 'foreign' *msp4* sequences and the ones detected in this study, a neighbour-joining tree was generated (Figure 5-16).

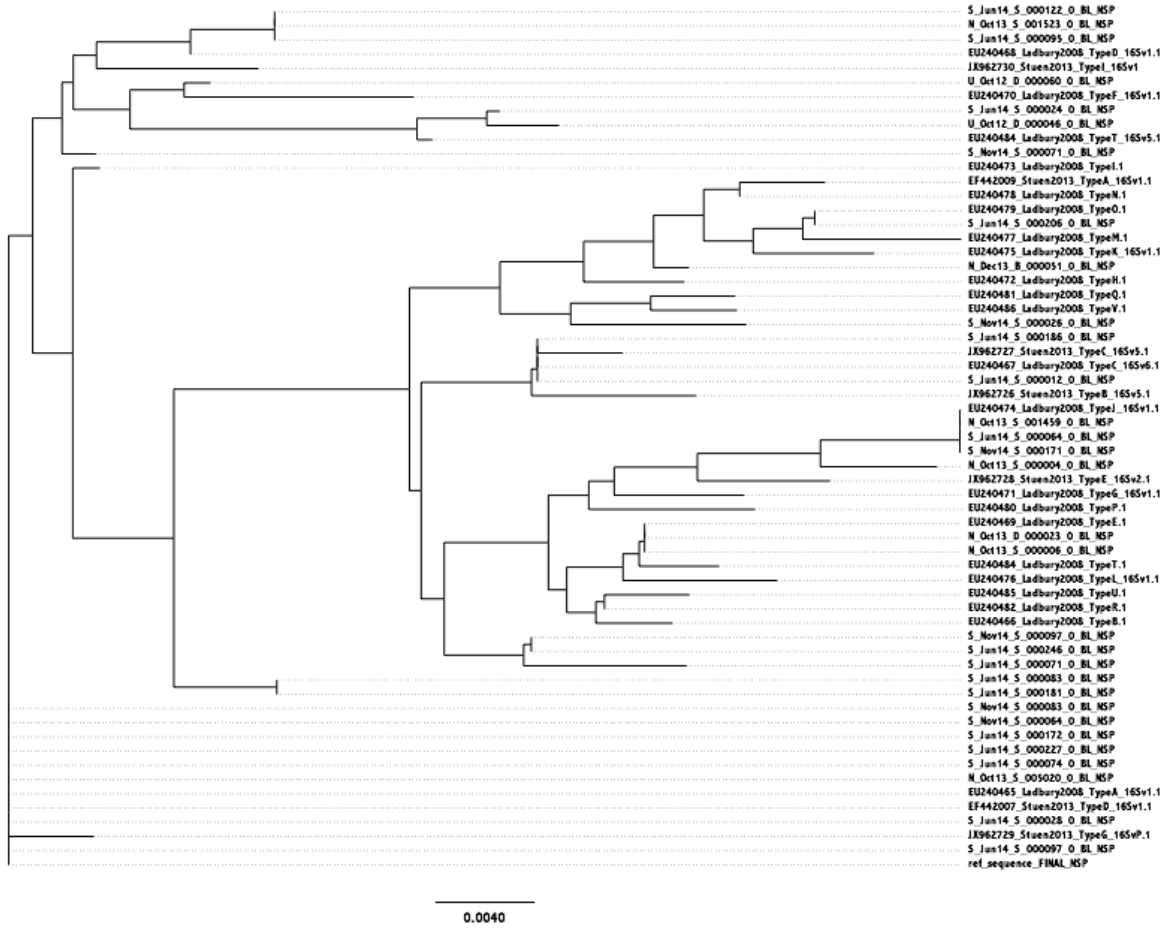

Figure 5-16 *msp4* neighbour-joining tree representing all 'simple' sequences and selected published foreign sequences

The sequences detected in Scottish livestock are scattered throughout the tree, interspersed with samples from Norway. There is no evidence of geographical sub-structuring among allelic sequences. A number of identical alleles can be identified towards the bottom of the tree, largely coming from the Inverness South site, but also including alleles from the two previously published studies.

5.4 Discussion

5.4.1 What is the prevalence of *A. phagocytophilum* in an endemic focus?

The prevalence of *A. phagocytophilum* was surprisingly high in sheep at farm sites A in autumn 2013 (80.9 %) and at farm site B in summer (70.0 %) and autumn 2014 (67.5 %). The only other molecular study of *A. phagocytophilum* infection prevalence in sheep in the UK was a longitudinal study carried out in North Wales that found an infection rate of 38 % (Ogden *et al.*, 2002a), approximately half of the prevalence rates uncovered in the present study in Scotland. In Europe, other studies have found prevalences of between 3 % and

37.5 % (Stuen *et al.*, 2013b; Kiilerich *et al.*, 2009; Scharf *et al.*, 2011; Torina *et al.*, 2008a; Torina *et al.*, 2008b; Torina *et al.*, 2010).

These infected animals represent a reservoir of infection for the upcoming lamb crop where clinically apparent disease can occur. This is typified by brief pyrexia and dullness which compromises a hill lambs ability to feed and maintain contact with its mother. Importantly, infection also results in marked immunosuppression making the lamb susceptible to other infectious diseases. The result is both increased mortality and, less obviously, decreased productivity, both of which are reduced by acaricidal and tactical antibiotic therapy (Brodie *et al.*, 1986).

Acute infection with *A. phagocytophilum* has been experimentally demonstrated to worsen clinical signs and increase mortality in animals co-infected with louping ill virus (Reid *et al.*, 1986) or *Mannheimia haemolytica* (Gilmour *et al.*, 1982; Brodie *et al.*, 1986). This also applies to *Staphylococcus aureus*, causing tick pyaemia, and other infectious disease. However, no information is currently available about whether sheep persistently infected with *A. phagocytophilum* also display modulated responses to these infections. That infection by *A. phagocytophilum* has negative effects on productivity in both cattle and sheep has been demonstrated (Stuen *et al.*, 2002; Taylor and Kenny, 1980). This is an interesting area for further investigation now that molecular techniques are readily available to detect infection with *A. phagocytophilum*.

The prevalence of *A. phagocytophilum* in cattle at site A was low (2.8 %), however the blood from these cattle was taken for routine health screening in December 2013 when ticks were not present due to severe weather, and the coagulated blood samples were also less suitable for DNA extraction. Additionally, in cattle *A. phagocytophilum* has been demonstrated to persist for short intervals. For example, the infection of susceptible animals by inoculation of blood from infected animals could only be achieved up to 14 days post-infection (Hudson, 1950) or during the clinical phase of infection (Tuomi, 1967a). This is in marked contrast to the situation in sheep. In this species persistence of infection has been demonstrated for up to 25 months (Foggie, 1951) or in excess of 300 days (Thomas *et al.*, 2012) in the absence of clinical

signs. This may be a factor in the much higher levels of prevalence in sheep found at these sites.

The survey of veterinary practices (Chapter Two) revealed that tick-borne fever came to the attention of veterinary surgeons infrequently despite the potential adverse outcomes of infection and surprisingly high levels of infection demonstrated in this study. High levels of undisclosed infection in livestock also have implications for human health, as *A. phagocytophilum* is recognised as an emerging zoonosis. Two cases have been reported in Scotland (Hagedorn *et al.*, 2014; Sumption *et al.*, 1995). Hagedorn *et al.* (2014) raise the worrying possibility that the infection is very likely to be under-diagnosed.

5.4.2 Are infections characterised by the presence of multiple genotypes?

Examination of the 135 *msp4* gene sequences resulting from this study reveals that 31 sequences contained no ambiguous nucleotide codes (i.e. 'simple' sequences), suggesting that a single *A. phagocytophilum msp4* genotype is present.

In the remaining 104 sequences there were varying numbers of ambiguous nucleotide codes (i.e. 'complex' sequences), interpreted as the presence of one or more *A. phagocytophilum msp4* genotypes in that sample, and hence the host. Thus, the majority of infections by *A. phagocytophilum* were characterised by the presence of multiple *msp4* genotypes co-infecting the host. While it is accepted that 'mis-calling' the major allele in complex sequences could result in artefactual sequence data, the vast majority of ambiguous sites in 'complex' sequences could be explained on the basis of nucleotide polymorphisms among 'simple' sequences, thereby supporting this approach. Fortunately, no indels were present in the *msp4* sequences examined and the alignment was completely in-frame. The presence of indels in the dataset would have resulted in frame-shifts in sequence traces from individual samples and would have made the interpretation of such traces virtually impossible. Alternative approaches to direct PCR sequencing should be considered in future studies. For example, the use of molecular cloning and sequencing would result in discrete alleles/amplicons being sequenced. While this was considered for the present

study, it was not deemed appropriate due to the time required and expense involved. A meta-genetic approach, involving high-throughput sequencing of amplicons could also be used to circumvent this issue.

Multiplicity of infection (MOI) within a cohort of animals could therefore be estimated as the proportion of individuals sampled yielding 'complex' sequences. At farm site B, where 40 North Country Cheviot ewes were examined for the presence of *A. phagocytophilum* in summer 2014 (before moving to extensively managed hill land) and again in autumn 2014 (after return from this area), prevalence remained similar (70 % and 67.5 % respectively). Of the 40 sheep sampled in summer and autumn 2014, 34 sheep were sampled on both occasions and 26 were infected with *A. phagocytophilum* at both time-points. It was found that the multiplicity of infection increased over this grazing period. This suggests that sheep are subjected to ongoing challenge and experience a build-up of *A. phagocytophilum* genotypes in the latter part of the year. However, as adult sheep were sampled on both occasions, the data also suggest that the extent of MOI wanes by the following summer. Whether this is due to strain-specific immunity or is related to competition among pathogen genotypes for host resources is unknown. However, in the absence of clinical signs, it is clear that these sheep remain functionally immune and further studies are warranted to investigate the nature of this non-sterile immunity in adult sheep.

5.4.3 How do genotypes in deer relate to those found in livestock?

In wild red deer at farm site A there was also a high prevalence of infection with *A. phagocytophilum* (66.7 %). Only one other molecular survey of red deer for infection with *A. phagocytophilum* could be found in the literature. This was carried out in the New Forest in southern England and found a prevalence of 80 %, although only five animals were examined. Other studies in Europe have uncovered prevalences in this species between 50.9 % and 87.5 % (reviewed by Stuen *et al.* (2013a)). It is unclear why the prevalence of infection by *A. phagocytophilum* was lower on the additional red deer sites C to H (25 %). However, this figure should be interpreted with caution as samples were not collected specifically for this study and so were taken in plain blood tubes, and

frozen and thawed on an unspecified number of occasions before DNA was extracted.

Although prevalence was slightly less in red deer at farm site A than sheep at either farm sites A or B, the multiplicity of infection found in deer, as indicated by several metrics, implies that an infected deer will contain a bigger 'swarm' of *A. phagocytophilum* genotypes than a sheep. This may be explained by a number of non-mutually exclusive hypotheses. For example, it may be simply due to their exposure to a greater number of genotypes, and this may be related to the areas they graze, assuming that their range is more extensive than or subtly different from local sheep. It is quite possible that deer, on average, may be bitten by more ticks than sheep, and a high tick burden was noted on a number of the deer which were culled and examined in the course of this study. In contrast to deer, sheep are present on hill land intermittently and also receive ectoparasiticide treatment, thus they may be subject to a lower tick challenge. An alternative hypothesis is that the immune response mounted by deer differs from that of sheep and that instead of MOI waning in the early part of the year, it may be maintained. Comparative studies are required to establish whether any of these factors underlie this difference between deer and sheep.

Examination of the maximum likelihood phylogenetic tree of *msp4* sequences (Figure 5-11) reveals three identical alleles in the sheep and deer population (Figure 5-11, A) and that deer-derived and sheep-derived samples do not form separate clusters. Thus, there is evidence to suggest that deer and sheep are exposed to a similar pathogen population. Stuen *et al.* (2013b) suggested that wild ruminants may not be infected with the same *msp4* genotypes as domestic livestock, although this was based on a smaller number of both sheep (n = 32) and red deer (n = 8) than in the current study. The results of the present study confirm that, in Scotland, a proportion of *A. phagocytophilum msp4* genotypes are shared between red deer and sheep. It has been suggested that both deer and sheep have persistent and high levels infection and therefore both may act as reservoir hosts (Dugat *et al.*, 2015). The high level of infection observed in this study in both species lends support to this concept.

Most recently, a multilocus sequence typing (MLST) approach has been published which might offer potential for analysis of a small number of selected samples

(Huhn *et al.*, 2014) alongside the 16S rRNA and *msp4* genes. However, a potential problem with either multilocus sequence or satellite-based techniques is the high proportion of animals displaying infection with multiple sequences. This problem could be partially overcome by adopting a meta-genomic strategy to resolve ambiguous sequence reads, however it would still be impossible to define multi-locus genotypes/haplotypes. Studies would therefore either have to focus on animals with a clonal infection or it would be necessary to culture and clone the pathogen directly. This would also allow a whole-genome approach to typing, which is becoming increasingly feasible for small, tractable pathogen genomes such as this.

5.4.4 How do genotypes in Scotland compare with those from other parts of the world?

Examination of the neighbour-joining tree representing all 'simple' sequences and selected published foreign sequences (Figure 5-16) reveals Norwegian and Scottish genotypes do not segregate according to location. It can be observed from the phylogenetic trees (Figure 5-11 and Figure 5-16) that some genotypes are more frequent than others and this may suggest the presence of a common genotype in the Scottish population. Interestingly, this genotype was also detected in the small number of *A. phagocytophilum* isolates from SRUC/SAC cases the south of Scotland. *msp4* is a clearly a highly diverse surface antigen and provides a high level of genotypic resolution. The extensive recombination in the ancestry of *msp4* (Figure 5-12, Figure 5-13 and Figure 5-14) suggests that much of the observed variation in this gene is ancient in origin. Therefore, with phylogenetic trees created on the basis of this gene being inherently unstable, genotyping systems based on this locus alone provide relatively little meaningful information about the molecular epidemiology of this pathogen on a broad level. It is likely, therefore, that multi-locus or whole-genome based approaches to characterising genetic diversity in this pathogen will be required before geographical and host sub-structuring can be fully quantified and firm epidemiological conclusions drawn.

CHAPTER SIX
General discussion

The tick-transmitted pathogens *Babesia divergens* (M'Fadyean and Stockman, 1911) and *Anaplasma phagocytophilum* (Gordon *et al.*, 1940) are endemic in Scottish livestock. Despite long being recognised as pathogens that can seriously impact animal welfare and cause significant economic loss, relatively little research into the situation in Scotland has been undertaken in the last two decades. During this period, methods for the sensitive and specific molecular detection of these organisms have been developed and applied in other countries around the world. The aim of this project was to further improve and apply this technology in order to characterise the tick-borne protozoa and bacteria in circulation in Scottish herds and flocks. In addition to examining samples for the presence of recognised species, a pathogen discovery approach was undertaken which was capable of identifying essentially any piroplasm protozoa present in livestock blood samples. However, in order to know how best to deploy this technology, an information-gathering approach was first undertaken.

The initial component of the project was a survey of veterinary surgeons treating cattle and sheep in Scotland, in order to gauge current perceptions of disease and to quantify the number of cases and the nature of clinical presentations (Chapter Two). This revealed that in Scotland, *B. divergens* primarily affects cattle, while *A. phagocytophilum* primarily affects sheep and to a lesser extent cattle. The widespread hard tick *Ixodes ricinus* transmits both *B. divergens* (Joyner *et al.*, 1963) and *A. phagocytophilum* (MacLeod and Gordon, 1933; MacLeod, 1936) in Scotland. Only *B. divergens* is transmitted trans-ovarially by *I. ricinus*, meaning this tick can act as a reservoir host for *B. divergens*. *Babesia divergens* causes babesiosis in susceptible adult cattle due to invasion and destruction of the host's red blood cells. Typical clinical signs are dullness and haemoglobinuria (Zintl *et al.*, 2014). Diagnosis is by assessment of history and clinical signs (Collins *et al.*, 1970; Gray and Murphy, 1985), and by examination of stained blood smears (Sherlock *et al.*, 2000; Zintl *et al.*, 2014).

Anaplasma phagocytophilum causes tick-borne fever in sheep and pasture fever in cattle (Hudson, 1950). Importantly, it causes immunosuppression resulting in

secondary bacterial and viral infections with the potential to cause reduced productivity, deaths and negatively affect the welfare of affected animals (Brodie *et al.*, 1986).

Diagnostic methods involving assessment of historical, clinical and postmortem findings, and assessment of stained blood smears are adequate for the routine diagnosis and initiation of treatment. However, in the case of babesiosis they provide no information about the *Babesia* spp. involved, meaning that potentially overlooked species could be present in Scottish livestock causing sub-clinical or clinical infection. To address this and the general paucity of information about the current prevalence of infection with *B. divergens*, other *Babesia* or *Theileria* spp. and *A. phagocytophilum*, nested PCRs based on the 18S SSU and 16S SSU rRNA loci respectively were optimised and applied to a wide range of blood and tissue derived from both healthy and clinically affected sheep, cattle and red deer.

Healthy sheep at two sites where tick-borne disease had been reported previously were found to be infected with *Babesia venatorum*, a parasite associated with the roe deer (*Capreolus capreolus*) in Europe, but first described in humans in Italy and Austria (Herwaldt *et al.*, 2003). *Babesia venatorum* has not been described in sheep previously either in Europe or elsewhere. Its presence in the UK was confirmed by its detection in ticks (Smith *et al.*, 2013), however this is the first time it has been detected in a vertebrate host in this country. In addition to the importance to the livestock industry in Scotland, this finding has implications in terms of public health. Tick-borne pathogens such as *Borrelia*, the cause of the Lyme disease, are well-recognised causes of disease in man and as such, governmental agencies, including Health Protection Scotland, perform the necessary risk assessments. The presence of a novel zoonotic pathogen in Scottish such as *B. venatorum* should similarly be formally addressed and a risk assessment undertaken in order to understand the threat to human health.

Arguably as significant as *Babesia* in terms of its impact on animal welfare and productivity is the very high prevalence of the endemic bacterial pathogen *A. phagocytophilum* detected in healthy sheep and red deer. This varied from 67.5 % to 80.9 % between groups of healthy sheep where blood samples were obtained at different sites. This level of prevalence is much higher than has been described in Europe, for example a maximum of 37.5 % in one survey in Norway (Stuen *et al.*, 2013b). This is mirrored by high levels of prevalence in red deer sharing one of the sites with a prevalence of 66.7 %. Examination of *msp4* gene sequences derived from the blood of both species suggested that there were genotypes that occurred in both hosts, unlike the situation in Norway where there was a more clear distinction between those present in sheep and those in red deer (Stuen *et al.*, 2013b). Reflecting similar recent molecular confirmation of *B. divergens* infecting wild red deer in Ireland (Zintl *et al.*, 2011) and elsewhere in Europe, *B. divergens* was found in healthy wild red deer culled in Scotland during this study. This finding suggests that transmission between this wild ruminant species and cattle is possible, although as suggested by Zintl *et al.* (2011) and Lempereur *et al.* (2017), sequencing of the entire 18S rRNA gene, morphological assessment and infectivity studies would be worthwhile to confirm this risk. It is clear that further work, including improvements in genotyping methodology, are required to properly investigate pathogens dynamics and to determine the likelihood of infection in one species being transmitted to the other via the tick vector. Recent advances in whole-genome sequencing make large-scale studies a realistic prospect in the near future.

In addition to *B. divergens*, wild red deer harboured a second *Babesia* sp., which was termed '*Babesia odocoilei*-like' in this study due to its close similarity to *Babesia odocoilei* (Emerson and Wright, 1968; Emerson, 1970). *Babesia odocoilei* infects white-tailed deer in the southern United States. Interestingly, given the molecular confirmation of *B. divergens* in red deer in Scotland mirroring the earlier molecular confirmation of *B. divergens* in Irish red deer (Zintl *et al.*, 2011), only one of four genotypes identified in Scotland was identical to one found by Zintl *et al.* (2011).

The discovery of a previously undetected zoonotic *Babesia* spp. in sheep, the molecular confirmation of *B. divergens* in red deer, the detection of a *B. odocoilei*-like parasite in red deer, and the revelation of a surprisingly high incidence of *A. phagocytophilum* infection in healthy sheep and red deer in Scotland all serve to underline the value of active surveillance. This not only applies to emerging disease but also to those assumed to be well-described in the UK, such as *Babesia* and *A. phagocytophilum*.

The work of this study and its findings outlined above suggest a number of avenues for future work. Following the identification of *B. venatorum* 18S rRNA sequence in sheep in Scotland complete sequencing of the 18S rRNA gene could be considered in the first instance, utilising one or more of the primer sets described in Chapter 3 (Table 3-2 and Figure 3-8). This would allow investigation of the similarity to other deposited *B. venatorum* sequences beyond that provided by the carefully selected locus amplified and sequenced in this study. In the field, investigation of larger numbers of sheep would provide a greater understanding of the spatial distribution of this parasite within Scottish sheep and specifically whether it is an issue restricted to the north east of Scotland, or is more widely distributed. The clinical significance of this infection in sheep is currently unclear and ultimately would require isolation of the parasite and subsequent experimental infection to elucidate its clinical significance. Roe deer play a central role as a vertebrate host for *B. venatorum* in continental Europe however the situation in Scotland is unclear, and was not investigated in the course of this study. Examination of blood and/or organs obtained from culled Scottish roe deer, using the *Babesia*/ *Theileria* spp. nested PCR primer set described in this study, could provide the initial step in addressing the question of whether roe deer play a similar central role in Scotland. Further investigation of the species of parasites in ticks, livestock, and deer may be facilitated by use of next generation sequencing platforms (as for example utilised by Bonnet *et al.* (2014)) and, especially in the relatively tractable bacterial genome of *A. phagocytophilum*, whole genome sequencing, which perhaps offer the most exciting opportunity for active surveillance of tick-borne pathogens.

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