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Short Communication

Molecular Detection of *Babesia divergens* from an Outbreak of Babesiosis in Holstein Cows, England

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Abstract

Bovine babesios is a sporadic disease within the United Kingdom causing mortality and morbidity within the national herd. Despite numerous reports in cattle, there have been no published reports of its molecular characterization or genetic confirmation of the *Babesia* species since its first description in England. This study describes the molecular detection and species identification of *Babesia divergens* in a case of babesiosis from an outbreak of the disease on a farm in northern England. Phylogenetic analysis demonstrated that *B. divergens* 18S RNA sequence in England is 100% identical to *B. divergens* in Ireland and France. The sequence derived is publically available and can be used to compare future cases of bovine babesiosis, especially if the pathogenesis of disease changes in response to the emergence of other *Babesia* species.

ABBREVIATIONS

B: *Babesia*; PCR: Polymerase Chain Reaction; UK: United Kingdom

INTRODUCTION

Bovine babesiosis is a tick-borne protozoan infection that causes morbidity and mortality in cattle. The disease is characterized by fever, listlessness, and dehydration resulting from Haemolytic anaemia caused by the destruction of erythrocytes. This is evident in the red coloration in urine, hence the common name for the disease, red water. The most common *Babesia* species causing disease in Western Europe is *Babesia divergens*. Infections occur sporadically throughout Europe and may extend as far south as North Africa [1]. Its distribution is associated with that of its tick vector, *Ixodesricinus*. However, additional species that can infect cattle include *B. major*, *B. bovis*, *B. bigemina*, *B. ovata*, *B. occultans* and *B.venatorum* (formly *babesia* sp.EU1) [2]. Co-infection with *B. divergens* and *Anaplasma phagocytophilum*, the causative agent of tick-borne fever also transmitted by *I. ricinus*, appear to be very common, therefore blood samples from affected animals should be screened for both pathogens. In addition to infection in cattle, *B. divergens* is zoonotic with a number of documented cases reported across Europe in splenectomized individuals [1].

B. divergens was first described in England by McFadyean and Stockman [3], who named it *Piroplasmida divergens*. This initial study established that this species was morphologically distinct from the *Babesia* first described by Victor Babes and a *babesia* species detected in cattle in Devon [4] and now believed to be *B. major* [5]. Since this time *B. divergens* has been diagnosed in the United Kingdom (UK) by examination of Giemsa stained smears from cattle blood and size of the erythrocytic form of the parasite. A positive result is based on the presence of the trophozoite or merozoite forms of the parasite in erythrocytes and the length of the merozoite form has been used to distinguish different *babesial* species. Surprisingly, there have been no published reports of molecular characterisation of *B. divergens* in cattle from England or publically available DNA sequence from English derived cases. The only report from the UK is a fatal case of babesiosis in a reindeer (*Rangifer tarandustarandus*) where a fragment of 18S

ribosomal RNA gene was used to confirm the infecting organism [6]. A recent report has also used molecular methods to detect *B. divergens* in I. ricinus removed from a domestic dog [7]. There are *B. divergens* sequences from bovine babesiosis cases in Ireland where the incidence of clinical babesiosis is monitored and have been shown to be in decline [8]. In order to address this we have used molecular methods that have been applied to the detection of *Babesias* and *Theilerias* in England [9,10,11] to confirm infection with *B. divergens* in a recently described case of bovine babesiosis from a farm in England [12].

MATERIALS AND METHODS

DNA was extracted from 100 µl of EDTA treated blood taken from the first cow using the DN easy Blood and Tissue Kit (QIAgen, Manchester, UK) following the manufacturer's instructions. *Babesia* parasites were detected using a pan-piroplasm PCR that amplifies a partial (423 base pair) fragment of the 18S ribosomal gene using primers PIRO-A (AATACCCAATCCTGACACAGGG) and PIRO-B (TTAAATACGAATGCCCCCAAC) [13]. Amplified products were sequenced using flanking primers and derived sequence edited in Laser gene version 12.1 (DNASTAR) and assigned to a particular species based on BLAST (NCBI) search, when agreement was ≥ 98 % to sequences of known *Babesia* species in GenBank. Once identification was achieved, Neighbour-joining analysis was conducted using I MEGAv.6 [14]. Bootstrap values were calculated to test the robustness of the cluster using 1000 pseudoreplicates. Values greater than 70% were considered significant.

RESULTS AND DISCUSSION

In September of 2016, a pregnant female Holstein cow collapsed. A subsequent veterinary inspection suggested milk fever (hypocalcaemia) with a temperature of 37.2°C and the cow was treated with calcium intravenously. It subsequently gave birth to a still-born calf and shortly afterwards began to spasm and exhibited behavior including leg paddling and eye rolling. The legs became hyper-extended and the cow died shortly afterwards. A blood sample was taken from the animal. The sample haemolysed within the tube and had a packed cell volume (PCV) of 4% (normal range for cattle 24-48%). The animal tested negative for *A. phagocytophilum*by polymerase chain reaction (PCR), and haematological examination of Giemsa-stained blood smear identified red blood cell inclusions as being consistent with *Babesia* infection. Based on this, a diagnosis of babesiosis was made and attributed to infection with *B. divergens*. During September ten cows from a total of 120 cattle on the property were affected. A second cow was also confirmed positive for babesiosis by blood smear. Of the affected cows three aborted despite preventative treatment with Imizol® following the manufacturer's recommendations, and two died, including the case above. According to the owner, the herd had been grazed on tick infested land and ticks were regularly reported on the cattle during milking. However, the most recent cases of red water fever were reported four years previously.

Pan-piroplasm PCR successfully amplified a fragment of the correct size from DNA extracted from a blood sample obtained from the cow (Figure 1). The amplicon was sequenced to generate 364 base pairs with 100% identity to *B. divergens* from isolates derived from a number of European countries. Phylogenetic comparison with a range of *B. divergens* sequences from Scotland, Ireland and France confirmed the species designation and demonstrated that it was distinct from other small *Babesia* species (Figure 2). The sequence has been submitted to GenBank with accession number KY296360.

Numerous tests have been developed for diagnostic as well as epidemiological investigation of babesiosis in livestock. In Great Britain, the main approach is to match clinical evidence with detection of the parasite in stained blood smears [15]. Alternatively, serological detection is used such as *Babesia*-specific immunofluorescence antibody tests (IFAT). *Babesia divergens* was first described by McFadyean and Stockman [3] based on the morphology of the merozoite form, with dimensions of 1.5 - 2.0 by 0.4µm, leading to its designation as a small *Babesia* species. This has meant that past detection of *babesia* have been based purely on morphometric methods leading to uncertainty over the precise species designation [16,17]. However, more than 100 species of *Babesia* have been described [18], some

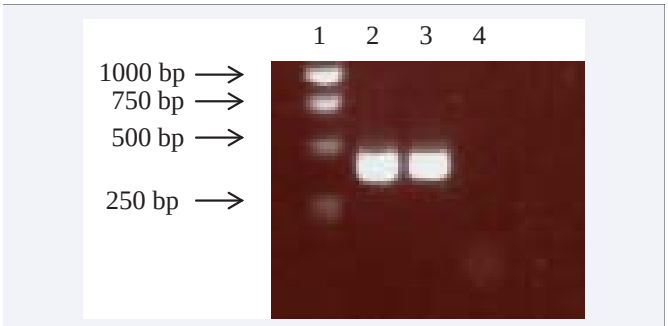

Figure 1 Gel image showing amplification of babesia genome using primers directed against the 18S ribosomal RNA gene. The gel shows 1) DNA markers (1 Kb marker, Promega, UK), 2) Babesia positive control, 3) DNA extracted from the blood of the Holstein cow, 4) No template control.

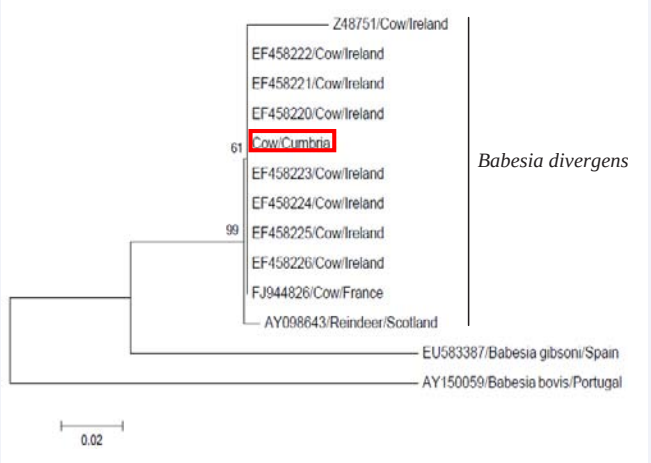

Figure 2 Phylogeny of *Babesia divergens* from the British Isles. The sequence from England is boxed in red. Neighbour-joining analysis based on 364 base pair partial sequence of the 18S ribosomal RNA gene. Bootstrap values are the result of 1000 replicates.

of which are present in the UK tick population [7] and have a similar morphology during the erythrocytic stage of infection in their primary mammalian host. Also large *Babesia* species have been detected in the UK such as *B. major* [19] and *B. motasi* [10] that can infect livestock. A further issue has been the dearth of research activity on UK-endemic piroplasms since the 1980s.

CONCLUSION

B. divergens has been detected in English livestock for over 100 years although this is the first time that the infecting species has been confirmed by molecular methods. The tick vector, *Ixodesricinus* is present across the country although disease is associated with areas of high tick abundance. There is evidence for endemic stability in livestock [20] and in the UK this is manifest as occasional outbreaks of bovine babesiosis when immunologically naïve adult cattle are moved onto fields where infected ticks are present. This is likely to be the case in the cattle herd reported in this study.

The application of piroplasm PCR-sequencing offers the opportunity to both detect and identify species of *Babesia* in cases of haemolytic anaemia in cattle and other livestock [21]. Further study is required to assess the sensitivity of this approach and its ability to detect both acute piroplasm infection and carrier status of British livestock.

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