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Research paper

Detection of *Theileria orientalis* in mosquito blood meals in the United Kingdom

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ABSTRACT

Theileria spp. are tick-borne protozoan parasites that infect a wide range of wild and domestic animals. In this study, the utility of xenosurveillance of blood-fed specimens of *Culiseta annulata* for detecting the presence of piroplasms in livestock was investigated. Blood-fed mosquitoes were collected at Elmley National Nature Reserve, Kent, United Kingdom. All specimens were morphologically identified, and DNA barcoding was used to confirm the morphological identification. Both the vertebrate host species and Theileria genome was detected within the bloodmeal by real-time PCR. Sequencing was used to confirm the identity of all amplicons. In total, 105 blood-fed mosquitoes morphologically identified as *Cs. annulata* were collected. DNA barcoding revealed that 102 specimens were *Cs. annulata* (99%), while a single specimen was identified as *Anopheles messeae*. Two specimens could not be identified molecularly due to PCR amplification failure. Blood meal analysis revealed that *Cs. annulata* fed almost exclusively on cattle at the collection site (n = 100). The application of a pan-piroplasm PCR detected 16 positive samples (15.2%) and sequence analysis of the amplicons demonstrated that the piroplasms present in the blood meal belonged to the *Theileria orientalis* group. This study demonstrates how xenosurveillance can be applied to detecting pathogens in livestock and confirms the presence of *Theileria* species in livestock from the United Kingdom.

1. Introduction

The family Culicidae (Diptera) includes 112 genera and at least 3547 described species (Web Reference 1). In some species, the female requires a blood meal for egg maturation, and it is this requirement that makes members of this family important as biting pests and vectors of pathogens of humans and livestock (Becker et al., 2010). Mosquitoes transmit numerous protozoa, viruses and nematodes to humans, livestock, as well as both domestic and wild birds that are responsible for disease outbreaks all over the world (Forattini, 1998; Becker et al., 2010; Medlock et al., 2012; Schaffner et al., 2013). As a result of their medical, veterinary and environmental importance, mosquitoes are a target group in surveillance programmes for disease transmission and control across the globe.

The analysis of host selection and host preferences of mosquitoes and other haematophagous arthropods via the identification of their blood meals has become a focus of studies on the dynamics of vector-host-pathogen interactions (Kent, 2009; Kent and Norris, 2005). The understanding of these complex interactions could facilitate the implementation of control methods of vector-borne disease outbreaks (Alcaide et al., 2009; Chaves et al., 2010; Brugman et al., 2015). This non-invasive approach has recently been termed xenosurveillance (Grubaugh et al., 2015), and several studies have demonstrated that blood-fed mosquitoes could be used to detect influenza virus (Barbazan et al., 2008), Epstein-Barr virus and canine distemper virus (Ng et al., 2011). Current molecular techniques for blood meal identification and the increasing volume of open access databases for host species identification such as GenBank (Web Reference 2) and The Barcode of Life

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Databases (Web Reference 3) have facilitated this area of research (Ratnasingham and Hebert, 2007; Brugman et al., 2015).

In the United Kingdom (UK), molecular methods for host preference studies in mosquitoes have only been carried by Danabalan et al. (2014), and more recently by Brugman et al. (2015), targeting mosquito species inhabiting farmland and wetland areas. In addition, Brugman et al. (2015) detected myxoma virus in blood meals of two members of the *Anopheles maculipennis* complex (*Anopheles atroparvus* and *Anopheles messeae*) that had fed on the European rabbit (*Oryctolagus cuniculus*), and advocated the same xenosurveillance methodology for the targeted detection of pathogens in livestock and wildlife.

Piroplasms including *Babesia* and *Theileria* are tick-borne protozoan blood parasites that infect a wide range of both domestic and wild animals worldwide, including cattle and sheep (Mehlhorn and Schein, 1985). Piroplasms are transmitted by Ixodid ticks (Mehlhorn and Schein, 1985). However, there is some evidence for mechanical transmission by other biting arthropods including mosquitoes, tabanid flies, and lice (Turell and Knudson, 1987; Fujisaki et al., 1993; Hammer et al., 2015).

The samples analysed in this study were collected as part of a larger investigation of feeding preference of farm-associated mosquitoes. It was noted that *Culiseta (Cs.) annulata* fed predominantly on cattle suggesting a possible means of surveying livestock for blood-borne diseases using a non-invasive method. The aim of the present study was to test the utility of a targeted xenosurveillance approach for detecting piroplasms in blood-fed specimens of *Cs. annulata* collected within a livestock farm setting in the UK. The successful application of this targeted methodology could enhance the detection and surveillance of these parasites.

2. Methods

2.1. Collection of blood-fed mosquitoes

Blood-fed female mosquitoes were collected from Elmley National Nature Reserve, Isle of Sheppey (51.377445, 0.784068), Kent, UK, over 25 visits between June and October 2014. Elmley is a freshwater coastal marsh used to graze approximately 700 head of cattle (Fig. 1). Mosquitoes were primarily collected using a Prokopak aspirator (John W Hock, Gainsville, Florida, USA) from inside resting traps (plywood resting boxes $500 \times 500 \times 500$ cm, painted black on the outside and red on the inside) or from the inside of man-made structures at the site using an aspirator (Brugman et al., 2015).

Collected mosquitoes were placed into a cooler containing dry ice and transported to the laboratory. Blood-fed specimens were separated from non-blood-fed specimens on the same day as collection and stored at −20 °C until processed.

2.2. DNA extraction from mosquito abdomens

DNA extraction from engorged abdomens was performed following the protocols of Brugman et al. (2015). Briefly, abdomens of engorged mosquitoes were separated from the rest of the body on a chilled plate using forceps and placed into individual 1.5 ml Eppendorf tubes containing 200 μl phosphate buffered saline (PBS). The abdomens were pressed against the walls of the tube using the forceps to release the blood meal. The remaining head and thorax of each mosquito was kept and stored at −20 °C as voucher specimens. Each sample was incubated with 20 μl proteinase K and 200 μl buffer AL (QIAgen, Manchester, UK) for 30 min at 56 °C in a water bath. DNA extraction was carried out using the DNeasy Blood and Tissue Kit (QIAgen, Manchester, UK), following the manufacturer's instructions. All DNA extractions were stored at 4 °C until processing. Each DNA extract was used for three purposes as demonstrated previously (Brugman et al., 2015): Firstly, to confirm the species identity of collected mosquitoes, secondly, to identify the vertebrate source of the blood meal and, finally, for targeted pathogen detection, in this case of piroplasms.

2.3. Morphological and molecular identification of mosquitoes

Female mosquitoes were morphologically identified as *Cs. annulata* based on the key of Cranston et al. (1987). Due to the morphological similarities between several species of mosquitoes known to occur in the area, we tested the utility of DNA barcoding to support our morphological identification. We employed the *cytochrome c oxidase I* (*COI*) DNA barcoding approach (Hebert et al., 2003a,b) to confirm morphological identification, in accordance with the approach used in Brugman et al. (2015). PCR products amplified with primers LCO1490 and HCO2198 (Folmer et al., 1994) were visualized on a 1.5% agarose gel, and samples showing bands of the correct size of 658 base pairs (bp) were sequenced in both directions using the ABI PRISM® BigDye® Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, Life Technologies Ltd, Paisley, UK). The details of specimen records and sequence information (including trace files), are publically available on the Barcoding of Life Database (Web Reference 4) using the search term "DS-CSAN2014". All sequences have been submitted to GenBank (accession numbers: KU748414–KU748459, KU748461–KU748470, KU748472–KU748495, KU748497–KU748505, KU748507–KU748514).

Paired bi-directional sequence traces (sequenced with the LCO1490 and HCO2198 primers) for the *Cs. annulata* specimens were combined to produce a single consensus DNA barcode sequence. To achieve this, individual forward and reverse traces were oriented, edited, and aligned using the Sequencer (v.4.5; Genes Codes Corporation, Ann Harbour, MI), GenDoc (v. 2.6.02) and ClustalX sequence analysis programs. The full data set was analysed in MEGA v.6 (Tamura et al., 2013). The K2P distance metric was used to determine the intra- and inter-specific genetic distances, and a neighbour-joining (NJ) tree analysis was carried out to represent the specimen's clustering pattern. Bootstrap values were calculated to test the robustness of the cluster, which was obtained by conducting 1000 pseudoreplicates.

2.4. Identification of vertebrate host in the blood meal

Vertebrate host species in the blood meal were identified using a vertebrate specific, M13-tailed, triple-primer cocktail (VF1_t1 + VF1d_t1 + VFli_t1/VR1_tl + VR1d_t1 + VD1i_t1) targeting a 685 bp sequence of the *COI* gene (Ivanova et al., 2007; Brugman et al., 2015). Amplification products were sequenced in both directions using the ABI PRISM® BigDye® Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, Life Technologies Ltd, Paisley, UK). All sequences were edited using Lasergene version 12 (DNASTAR, Inc, Madison, Wisconsin, USA) and assigned to a particular vertebrate species when agreement was ≥98% to sequences of known species in GenBank (Martínez de la Puente et al., 2013; Brugman et al., 2015).

2.5. Piroplasm polymerase chain reaction

One hundred and five specimens were tested for the presence of piroplasms in their blood meal. Piroplasm genome was detected by quantitative real-time PCR. Samples were screened using primers PIRO-A and PIRO-B that amplified a 423 bp piroplasm sequence (Armstrong et al., 1998). The final PCR reaction mix (final volume 40 μl) consisted of: 13 μl H_2O, 20 μl SYBR®Green JumpStart™ Taq Ready Mix™ (Sigma-Aldrich, Dorset, UK), 1 μl of each primer (at