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Original article

Diversity of piroplasms detected in blood-fed and questing ticks from several states in the United States

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ABSTRACT

Piroplasms in the genera *Babesia*, *Theileria*, and *Cytauxzoon* are tick-borne parasites that may be animal and human pathogens. Most piroplasms with known life cycles are transmitted by ixodid ticks; however, for many species, the vector is unknown. This study was conducted to determine the prevalence and diversity of piroplasms in ticks from several US states. Piroplasm-specific polymerase chain reaction (PCR) assays were used to test 1631 ticks from Georgia ($n=486$), Kentucky ($n=103$), Pennsylvania ($n=1$), Tennessee ($n=626$), and Texas ($n=414$). Ticks were either questing ($n=42$) or collected from animals ($n=627$) or humans ($n=962$). The 2 primary species tested were *Dermacentor variabilis* ($n=702$) and *Amblyomma americanum* ($n=743$), but *Amblyomma cajennense* ($n=99$), *Amblyomma maculatum* ($n=16$), *Ixodes scapularis* ($n=4$), *I. woodi* ($n=1$), and unidentified *Amblyomma* spp. nymphs ($n=64$) were also tested. A low prevalence of piroplasms was detected with 37 (2.3%), 35 (2.1%), and 9 (0.6%) ticks positive for *Theileria* spp., *Babesia* spp., or *Cytauxzoon felis*, respectively. Based on sequence analysis, at least 6 *Babesia* spp. were detected and 15 of the 35 (41%) *Babesia*-positive ticks were *A. americanum*, 19 (56%) were *D. variabilis*, and one (3%) was an *I. scapularis*. Nine *Babesia*-positive ticks were removed from humans from Kentucky ($n=1$), Georgia ($n=2$), Texas ($n=5$), and Pennsylvania ($n=1$). Three *Babesia*-positive ticks were questing *A. americanum* which represents the first report of *Babesia*-infected questing *Amblyomma* in the US. *Theileria* infections were only detected in *A. americanum*, and all sequences were similar to white-tailed deer associated *Theileria* spp. *C. felis* was only detected in *D. variabilis*. These data suggest that *A. americanum* may be a vector of *Babesia* spp., although experimental studies are needed to confirm vector competence. Finally, these data demonstrate a high diversity of piroplasms in both questing and partially fed ticks in the US; although, host-blood meals can be present in non-questing ticks.

Introduction

Piroplasms (Piroplasmida) are tick-transmitted Apicomplexan protozoa which include the 3 genera, *Babesia*, *Theileria*, and *Cytauxzoon*. While *Theileria* and *Cytauxzoon* spp. (Theileriidae) infect ungulates and felids, respectively, *Babesia* spp. (Babesiidae) infect a wide range of avian and mammalian hosts, including humans (Chae et al., 1999a,b; Nijhof et al., 2005; Criado et al., 2006; Hersh et al., 2012; Yabsley and Shock, 2013). Although some of these parasites can cause morbidity and mortality in animals and humans, the wildlife or domestic animal host and the presumed ixodid tick vector are unknown for many species of piroplasms (Kjemtrup et al., 2000b; Hersh et al., 2012).

In the United States, babesiosis became a reportable illness in 18 states in 2011 (Herwaldt et al., 2012). Although the majority of cases in the United States is due to *B. microti*, there are several emerging *Babesia* spp. in humans including *Babesia duncani*, *Babesia* sp. CA-type, *Babesia* sp. MO1, and an unclassified *Babesia* sp. (from Tennessee) (Herwaldt et al., 1996, 2003, 2004, 2011, 2012; Conrad et al.,

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2006; Gray et al., 2010; Fritzen et al., 2013). Additionally, babesiosis is a major disease of domestic dogs (Kjemtrup et al., 2000a), and at least 4 species have been reported from dogs in the United States including *Babesia gibsoni*, *Babesia canis*, *Babesia conradae*, and *Babesia* sp. Coco (Jefferies et al., 2007). *Cytauxzoon felis* is a significant pathogen of domestic cats in the southeastern and midwestern United States. Bobcats (*Lynx rufus*) are the primary wildlife reservoir for *C. felis*, and experimental studies have demonstrated that both *Amblyomma americanum* and *Dermacentor variabilis* are vectors (Blouin et al., 1987; Shock et al., 2011; Reichard et al., 2010). In addition, several genetic types of *Theileria* have been reported from wild and domestic ungulates and *A. americanum* in North America, but these parasites are rarely associated with clinical disease (Chae et al., 1999b; Stockham et al., 2000; Yabsley et al., 2005a,b; Reichard and Kocan, 2006; Scoles et al., 2011).

In the southeastern and midwestern United States, *D. variabilis* and *A. americanum* are vectors of numerous human and animal pathogens (Paddock and Yabsley, 2007; Goddard and Varela-Stokes, 2008; Stromdhal and Hickling, 2013). In recent years, the range of *A. americanum* has expanded, and it is reported as far north as Maine and as far west as Nebraska (Keirans and Lacombe, 1998; Diuk-Wasser et al., 2006; Bouzek et al., 2013). *Dermacentor variabilis* is common throughout the eastern United States and has a limited distribution in California and some parts of other western states (Merten and Durden, 2000).

To date, few large-scale studies of piroplasms in ticks have been conducted in the United States, although several studies have been conducted in European, Asian, and African countries (Inokuma et al., 2003; de la Fuente et al., 2004; Blaschitz et al., 2008; García-SanMartín et al., 2008; Bonnet et al., 2013; Ionita et al., 2013; Kim et al., 2013; Ros-García et al., 2013; Tian et al., 2013). The current study was conducted to better understand the prevalence and diversity of piroplasms in common United States tick species collected from animals, humans, or while questing. Studies of this type have been limited because several PCR protocols designed to amplify piroplasms also amplify tick DNA (Bondy et al., 2005; Yabsley et al., 2006), but other protocols may be more specific (Ionita et al., 2013; Kim et al., 2013). The primary aims for this study were to (i) determine the prevalence of piroplasms in ixodid ticks, (ii) characterize the *Babesia* spp. in ixodid ticks in the United States, and (iii) determine and compare the prevalence of *C. felis* in 2 known vectors (*A. americanum* and *D. variabilis*) in the study locations.

Materials and methods

A total of 1631 ixodid ticks from Georgia ($n = 486$), Kentucky ($n = 103$), Tennessee ($n = 626$), Texas ($n = 416$), and Pennsylvania ($n = 1$) was included in this study (Table 1). The 2 most common tick species were *D. variabilis* ($n = 702$) and *A. americanum* ($n = 743$), but fewer numbers of *Amblyomma maculatum* ($n = 16$), *Amblyomma cajennense* ($n = 89$), *Ixodes scapularis* ($n = 4$), *Ixodes woodi* ($n = 1$), and unidentified *Amblyomma* ($n = 64$) spp. nymphs were also tested. Ten randomly selected ticks from the original 74 *Amblyomma* spp. nymphs were all identified as *A. cajennense*. Although most of the ticks were collected from people ($n = 926$), 669 ticks in the study were questing ($n = 42$) or collected from domestic animals or wildlife ($n = 627$; Table 2).

DNA from the ixodid ticks included in this study was obtained from previous and ongoing studies on tick-borne pathogens. Ticks from Kentucky were collected from May to August of 2008 (Fritzen et al., 2011). Ticks from Tennessee were collected from April 2007 to September 2008 and in July and August of 2009 (Moncayo et al., 2010). Ticks from Georgia were collected from 2005 to 2006 (Elizabeth Gleim, unpublished). Ticks from Texas and Pennsylvania were collected from September 2008 until July 2012 (Phillip C. Williamson, unpublished). All DNA samples were maintained at −20 °C or −80 °C until testing.

Samples from Texas and Pennsylvania were screened with a PCR that amplified the entire internal transcribed spacer region (ITS-1) of *Cytauxzoon*, *Babesia*, and *Theileria* spp. using the following primers: ITS-15C (5′-CGATCGAGTGATCCGGTGAATTA-3′) and ITS-13B (5′-GCTGCGTCCTTCATCGTTGTG-3′) (Bostrom et al., 2008). The amplification reaction mix consisted of the following per 25 μl reaction volume: 11.75 μl molecular grade water, 2.5 μl 10× ThermoPol™ buffer containing $MgCl_2$ (New England BioLabs), 2 μl dNTP mix with 2.5 mM each dNTP, 0.5 μl each of 5 μM forward and reverse primers, 2.5 μl of 1 mg/ml BSA, 0.25 μl Taq DNA polymerase (New England BioLabs, 5 U/μl), and 5 μl of DNA extraction elute from each sample. A nested PCR reaction was then carried out using the following primers: ITS-15D (5′-AAGGAAGGAGAAGTCGTAACAAGG-3′) and ITS-13C (5′-TTGTGTGAGCCAAGACATCCA-3′). For the nested PCR reaction mix, 1 μl of amplified product was used as template and an additional 4 μl of molecular grade water was added to bring the total reaction volume up to 25 μl. The PCR cycling conditions for the initial and nested reactions were as follows: 94 °C for 1 min, followed by 35 cycles consisting of 94 °C for 30 s, 52 °C for 30 s, and 72 °C for 1 min, followed by a final extension step at 72 °C for 5 min.

Amplicons >200 bp from Pennsylvania and Texas were enzymatically treated to remove unincorporated dNTPs and primers by adding 4 μl of 1 U/μl ExoSAP-IT (Affymetrix, Santa Clara, CA). This was performed prior to using the amplification products as template for the cycle sequencing reactions. Products were incubated at 37 °C for 15 min followed by 80 °C for 15 min. Chain termination reactions were set up using BigDye® Terminator chemistry (Life Technologies, Carlsbad, CA), according to the following protocol per 15 μl reaction volume: 6 μl molecular grade water, 5 μl BetterBuffer BigDye® dilution buffer (Gel Company, San Francisco, CA), 1 μl BigDye® v.3.1 Ready Reaction solution, 1 μl 5 μM primer (forward or reverse), and 2 μl PCR product. Thermal cycling parameters for the reactions were as follows: 96 °C for 3 min, 25 cycles of 96 °C for 15 s, 50 °C for 10 s, and 60 °C for 4 min.

Following chain termination reactions, unincorporated primers were eliminated by spinning samples through Performa DTR Gel Filtration Columns (Edge Biosystems, Gathersburg, MD), according to the manufacturer's recommendations. Samples were then desiccated in a vacuum centrifuge at 45 °C for approximately 15–25 min until all liquid was removed. The pellets were resuspended in a 25-μl Hi-Di™ Formamide (Life Technologies, Carlsbad, CA) solution. Each sample was transferred to a 0.2-ml PCR tube and heat denatured at 95 °C for 3 min. This was followed by immediate cooling on an ice block for 3 additional minutes. The BigDye chain termination products were detected on a PRISM® 310 or 3130xl Genetic Analyzer (Applied Biosystems) during capillary electrophoresis.

For samples from Kentucky, Tennessee, and Georgia, the ITS-1 rRNA region was amplified using a nested ITS-1 PCR that detects all species of *Cytauxzoon*, *Babesia*, and *Theileria* (Bostrom et al., 2008) as previously described (Shock et al., 2011). All amplicons >200 bp from Kentucky, Tennessee, and Georgia were purified with a Qiagen gel extraction kit (Germantown, MD) and bi-directionally sequenced at the Georgia Genomics Facility (Athens, GA).

For samples that were determined to be positive for *Babesia* spp. by sequence analysis of the ITS-1 rRNA region, one or both of the following PCR protocols were conducted to obtain partial 18S rRNA gene sequences. A single PCR was used to amplify partial 18S rRNA gene region using primers Kim18SF (5′-GAAATTAGAGTGTTTC-3′) and KimRev2 (5′-ACCCTATTTAGCAGGTTAAG-3′). Briefly, 5 μl of the extraction elute from each sample was added to 20 μl of a master mix containing 11 μl of water, 2.5 μl of 25 μM $MgCl_2$, 5 μl of 5× GoTaq® Flexi Buffer, 0.25 μl of a mix containing 0.2 mM each dNTP (Promega), 0.5 μl of both forward and reverse

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