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High *Trypanosoma cruzi* infection prevalence associated with minimal cardiac pathology among wild carnivores in central Texas

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ABSTRACT

Infection with the zoonotic vector-borne protozoal parasite *Trypanosoma cruzi* causes Chagas disease in humans and dogs throughout the Americas. Despite the recognized importance of various wildlife species for perpetuating *Trypanosoma cruzi* in nature, relatively little is known about the development of cardiac disease in infected wildlife. Using a cross-sectional study design, we collected cardiac tissue and blood from hunter-donated wildlife carcasses- including raccoon (*Procyon lotor*), coyote (*Canis latrans*), gray fox (*Urocyon cinereoargenteus*), and bobcat (*Lynx rufus*) – from central Texas, a region with established populations of infected triatomine vectors and increasing diagnoses of Chagas disease in domestic dogs. Based on PCR analysis, we found that 2 bobcats (14.3%), 12 coyotes (14.3%), 8 foxes (13.8%), and 49 raccoons (70.0%) were positive for *T. cruzi* in at least one sample (right ventricle, apex, and/or blood clot). Although a histologic survey of right ventricles showed that 21.1% of 19 PCR-positive hearts were characterized by mild lymphoplasmocytic infiltration, no other lesions and no amastigotes were observed in any histologic section. DNA sequencing of the TcSC5D gene revealed that raccoons were infected with *T. cruzi* strain TcIV, and a single racoon harbored a TcI/TcIV mixed infection. Relative to other wildlife species tested here, our data suggest that raccoons may be important reservoirs of TcIV in Texas and a source of infection for indigenous triatomine bugs. The overall high level of infection in this wildlife community likely reflects high levels of vector contact, including ingestion of bugs. Although the relationship between the sylvatic cycle of *T. cruzi* transmission and human disease risk in the United States has yet to be defined, our data suggest that hunters and wildlife professionals should take precautions to avoid direct contact with potentially infected wildlife tissues.

1. Introduction

The vector-borne protozoal parasite *Trypanosoma cruzi* is the etiologic agent of Chagas disease in humans and domestic canines. Vectors of *T. cruzi*, blood-feeding triatomine insects also called 'kissing bugs', are found throughout the Americas, including Texas (Kjos et al., 2009). Infection with parasites may occur after introduction of infected triatomine insect fecal material into a wound or mucous membrane, as well as orally (consumption of kissing bugs or their feces), congenitally, or through transfusion/transplantation (Bern et al., 2011).

Diverse wildlife species serve as reservoirs of *T. cruzi* across the parasite's range. Our understanding of the relative importance of wildlife reservoirs in the ecology and epidemiology of Chagas disease reflects vertebrate life history, especially as it relates to vector contact, and has been limited by the difficulty to collect large sample sizes of diverse wildlife taxa from across broad geographic areas. Although any mammalian species can potentially become infected with *T. cruzi* (Bern et al., 2011), the species that interact most frequently with kissing bugs have the opportunity to become infected or serve as the source of an infection. For example, in the southern United States, *Neotoma* spp. woodrats are well-recognized wild *T. cruzi* reservoirs, reflecting their association with nests commonly infested by triatomine nymphs and adults (Eads et al., 1963; Kjos et al., 2009). In South America, palm trees are an important ecological niche for contact between the *Rhodnius* genus of triatomines and opossums, which are a

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recognized reservoir of *T. cruzi* (Gaunt and Miles, 2000). With respect to the search effort, the ideal assessment of wildlife species' contributions to the enzootic transmission cycle would be to first have an unbiased assessment of triatomine feeding patterns, and then study those vertebrate species known to provide blood meals to the vector. A recent metaanalysis of triatomine feeding patterns suggests, however, that rather than innate preference for host species, host utilization by kissing bugs is dictated by the habitat they colonize (Rabinovich et al., 2011). Finally, with few exceptions (Barr et al., 1991a; Pietrzak and Pung, 1998; Roellig et al., 2009b), wildlife studies do not typically address how infection may relate to disease within infected individuals, likely because of sampling limitations.

The purpose of this study was to survey populations of raccoon (*Procyon lotor*), coyote (*Canis latrans*), gray fox (*Urocyon cinereoargenteus*), and bobcat (*Lynx rufus*) in central Texas to determine infection prevalence and circulating *T. cruzi* strain types. Further, we aimed to study the relationships among parasite infection in blood, infection in different areas of the heart, and the manifestation of cardiac pathology. Each of these species has previously been shown to be exposed to or infected with *T. cruzi* (Burkholder et al., 1980; Brown et al., 2010; Kribs-Zaleta, 2010; Rosypal et al., 2010; Charles et al., 2013). Central Texas is a region where at least four species of infected triatomine species occur (Kjos et al., 2009; Curtis-Robles et al., 2015) and where human and canine Chagas disease have been diagnosed (Texas Department of State Health Services, 2015a, 2015b; Tenney et al., 2014). These particular wildlife species were selected for study due to their high population densities across Texas and 'varmint' status among ranch owners, which results in efforts to reduce population size through recreational hunting that may pose a human health risk from hunter contact with infected wildlife tissues (Yeager, 1961; Pung et al., 1995).

2. Materials and methods

2.1. Sampling

We conducted a cross-sectional wildlife study in January 2014 at a hunting check station for a recreational nuisance predator hunt in central Texas. This event was organized by a group of private landowners as a predator calling competition of animals considered pests to Texas ranches. Animals legally harvested by teams of hunters over a 24-h period were brought to a central check station where teams were awarded for their harvest. Our team collected samples from animals for which harvest location information was available. Animals in our study were harvested from 25 counties in central Texas and included raccoons, coyotes, foxes, and bobcats. We performed a field necropsy, at which time we removed the heart from each animal within 24 h of death and stored at −20°C until further processing.

In the laboratory, hearts were examined grossly and dissected in order to obtain an approximate 1 cm^3 section of each of the apex and right ventricular free wall for molecular testing. In some cases, portions of the cardiac tissue had been destroyed during harvest, and so paired samples of right ventricle and apex were not always possible. When present, blood clots were collected from within the chambers of the heart during the dissection and frozen until further processing occurred. A section of right ventricular free wall was prepared in 10% formalin for histological examination. Additionally, any gross lesions were described and preserved as above for histological examination.

2.2. Molecular work

2.2.1. DNA extraction and T. cruzi detection

DNA was extracted from heart apex, right ventricle, and blood clot samples using the Omega E. Z.N.A. Tissue DNA kit (Omega Bio-Tek, Norcross, GA). No-template controls were included in each set of DNA extractions, and molecular grade water was included as negative controls in PCR reactions. In order to detect presence of *T. cruzi* DNA, a 166-bp segment of the *T. cruzi* 195-bp repetitive satellite DNA was amplified using a Taqman qPCR reaction with Cruzi 1, 2, and 3 primers and probe (Piron et al., 2007; Duffy et al., 2013). This approach has previously been shown as both sensitive and specific for *T. cruzi* (Schijman et al., 2011). Reactions consisted of 5 μL of template DNA, primers at a final concentration of 0.75 μM each, 0.25 μM of probe, and iTaq Universal Probes Supermix (Bio-Rad Laboratories, Hercules, CA), in a total volume of 20 μL in a Stratagene MxPro3000 instrument (Agilent Technologies, Santa Clara, CA), following previously described thermocycling parameters (Duffy et al., 2013), except with a reduced, 3-min, initial denaturation. The DNA extracted from *T. cruzi* strain Sylvio X10 (American Type Culture Collection, Manassas, VA), as well as DNA extracted from *T. cruzi*-positive kissing bugs collected in Texas (*Triatoma gerstaeckeri*, *Triatoma lecticularia*, and *Triatoma sanguisuga*), served as positive controls. After each reaction, the machine-calculated threshold was visually confirmed as reliable, and all reaction curves were visually checked for appropriate shape indicating successful amplification. Internal laboratory validation tests have defined cycle threshold (Ct) values indicative of positive (<31), negative (>33), and equivocal (between 31 and 33) status.

Samples classified as equivocal status after qPCR were subjected to confirmatory testing to determine sample status using the *T. cruzi* 121/122 primers to amplify a 330bp region of kinetoplast DNA (Wincker et al., 1994; Virreira et al., 2003). Reactions consisted of 1 μL template DNA, primers at a final concentration of 0.75 μM each, and FailSafe PCR Enzyme Mix with PreMix E (Epicentre, Madison, WI) in a final volume of 15 μL. PCR products were visualized on a 1.5% agarose gel stained with ethidium bromide. Any sample that yielded a band at the appropriate fragment size was interpreted as a positive sample, and those with no target fragments were considered negative in our calculations.

2.2.2. Strain-typing using TcSC5D PCR

All positive samples that yielded Ct values lower than approximately 25 on the qPCR were subjected to an additional PCR and subsequent DNA sequencing in order to determine the *T. cruzi* discrete typing unit (DTU). We performed a PCR to amplify the TcSC5D putative sterol oxidase gene (Cosentino and Agüero, 2012). Reactions consisted of 1 μL extracted DNA, 0.75 μM of each primer, and FailSafe PCR Enzyme Mix with PreMix E (Epicentre, Madison, WI) in a total volume of 15 μL. PCR products were visualized with gel electrophoresis as described above. Target amplicons were purified using ExoSAP-IT (Affymetrix, Santa Clara, CA) and bi-directionally sequenced on either an ABI Prism® 3130 Genetic Analyzer or ABI 3730 Genetic Analyzer (Applied Biosystems, Foster City, CA, USA) at the University of Florida DNA Sequencing Core Laboratory (Gainesville, FL). Resulting sequences and chromatographs were reviewed visually using Geneious version R7 (Kearse et al., 2012) and MEGA version 6 (Tamura et al., 2013) to confirm quality, align forward and reverse sequences, and examine the locations of key SNPs used to designate DTU (Cosentino and Agüero, 2012). Representative *T. cruzi* sequences were deposited to Genbank (Accession nos. KU705713-KU705715).