Anaplasma phagocytophilum DNA in So
Horses at Ciudad Juarez, Mexico

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Detection of *Theileria equi*, *Babesia caballi*, and *Anaplasma phagocytophilum* DNA in Soft Ticks and Horses at Ciudad Juarez, Mexico

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Abstract. Currently, ticks are second in transmission of pathological agents to humans, and in the veterinary field are ranked first. Thus, pathogens that might be in contact with human and animal populations, especially farm animals such as horses, *Equus caballus* (Linnaeus), should be identified. Two species of soft ticks in the Argasidae family, *Otobius megnini* (Duges) and *Ornithodoros turicata* (Duges), and one hard tick of the Ixodidae family, *Rhipicephalus sanguineus* (Latreille) were identified. DNA of pathogens *Theileria equi* (Laveran), *Babesia caballi* (Nuttall and Strickland), and *Anaplasma phagocytophilum* (Foggie) that have been reported in species of hard ticks but not soft ticks were identified. Overall, 144 blood samples from horses at Ciudad Juárez, Chihuahua, Mexico, were processed for DNA extraction, and analyzed by end-point or nested PCR to identify pathogens. The prevalence of *T. equi* was 6.9% (10/144) and 5.9% (3/51) in blood samples and soft tick samples, respectively; the prevalence of *B. caballi* was 2.8% (4/144) in blood samples and 5.9% (3/51) in soft ticks. There was one case of coinfection with both pathogens, and one blood sample tested positive for *A. phagocytophilum*, indicating a prevalence of 0.8% (1/124). The results suggested that soft ticks evaluated are potential vectors and might play a role in transmission of the pathogens.

Introduction

Vector-borne diseases have clinical and veterinary importance around the world. Currently, ticks are second in transmission of zoonotic agents to humans, and in the veterinary field are first (Shao et al. 2004, Gökdogan et al. 2016). Ticks are arthropods, obligate hematophagous ectoparasites that transmit infectious agents to animal and human populations (Benelli and Duggan 2018).

Ticks are divided into three families -- Argasidae (soft ticks), Ixodidae (hard ticks), and Nutalliellidae (Parola and Raoult 2001). There are more than 900 species of ticks in the world; 80% are hard ticks and 20% are soft ticks (Klompen and Oliver 1993, Gökdogan et al. 2016). The life cycle is divided into egg, larva, nymph, and

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adult stages, and each stage requires a host for survival (Kidd and Breitschwerdt 2003). Hard ticks and soft ticks have different feeding behavior. Hard ticks complete feeding in a few hours or days, while soft ticks complete feeding in minutes or hours (Boyle et al. 2014).

During feeding, ticks transmit pathogens by salivary secretions, feces, coxal fluids, or in a transovarian way to their offspring (Márquez-Jiménez et al. 2005). *Anaplasma phagocytophilum* (Foggie) is a zoonotic pathogen transmitted by a tick bite that infects neutrophils and is the etiological agent of granulocytic anaplamosis in horses, *Equus caballus* (Linnaeus), dogs, *Canis lupus familiaris* (Linnaeus), and humans (Silva et al. 2014). The pathogen can co-infect the host with other microorganisms such as *Babesia* spp. that is a hemoprotozoan parasite from the Apicomplexa family distributed throughout the world (Habibi et al. 2016).

Babesia caballi (Nuttall and Strickland) and *Theileria equi* (Laveran) cause equine piroplasmosis of equids such as horses, mules, *Equus asinus* x *caballus*, donkeys, *Equus asinus* (Linnaeus), and zebras, *Equus zebra* (Linnaeus), around the world (Xie et al. 2013). Piroplasmosis is characterized by symptoms such as fever, anemia, jaundice, depression, and anorexia (Battsetseg et al. 2001, Del Pino et al. 2016, Zhang et al. 2017). Piroplasmosis can economically affect the international trade of equids, because sick animals cannot travel or be marketed to countries called safety zones, where controls avoid parasitic infections (Farkas et al. 2013).

In Mexico, the Health Department reports vector-borne diseases of humans every year. From 2014 to the present, more than 1,000 cases of rickettsial diseases have been reported (Secretaria de Salud 2018). *T. equi, B. caballi,* and *A. phagocytophilum* commonly are detected by end-point and nested PCR (Massung et al. 1998, Battsetseg et al. 2001). In Mexico, some studies focused on identification of rickettsial pathogens in dogs (Rodriguez-Vivas et al. 2005, Zavala-Castro et al. 2006, Oliveira et al. 2010, Lira-Amaya et al. 2013, Almazan et al. 2016). Other studies focused on horses to evaluate the prevalence of *T. equi* and *B. caballi,* but only in hard ticks (Cantú-Martínez et al. 2012, Ayala-Valdovinos et al. 2017). The objective of this study was to identify morphologically and by using PCR the different species of ticks that naturally parasitize horses living in Cd. Juárez and the prevalence of tick-borne pathogens.

Materials and Methods

The study was done at the Babesia Unit of the National Center for Disciplinary Research in Veterinary Parasitology, Jiutepec, Morelos; the Molecular Biology and Clinical Veterinary Pathology Laboratory of the Institute of Biomedical Sciences at the Autonomous University of Ciudad Juárez; and the Clinical and Veterinary Parasitology Laboratory and Biotechnology IV Laboratories at the Faculty of Chemistry at the Autonomous University of Chihuahua.

Veterinarians of the Institute of Biomedical Sciences of the Autonomous University of Ciudad Juarez collected blood and tick samples. This work was reviewed and approved by the Ethical and Bioethical Committee of the Autonomous University of Juarez, Mexico, and done in compliance with Mexican and American guidelines for research on animals (Guide for the Care and Use of Laboratory Animals in National Resource Council 2011). Once samples were collected, the owners of the animals were informed, and written authorization was required to include their animals in the study. The sampling zone was Juarez in Chihuahua State, Northwest Mexico (31°43′59″ N; 106°28′59″ W; 1,120 m above sea level). For an animal to be

included in the study, the horse needed to have ticks on the day of sampling or according to previous reports (scars were identified from the bites produced by the animals), regardless of whether or not the horses showed clinical signs of disease. In total, 144 blood samples were collected from jugular puncture and stored at -20ºC. One hundred twenty-four horses lived permanently at Ciudad Juarez, and 20 had moved from Casas Grandes to Ciudad Juarez at least 3 months before the start of the study. The entire body of each horse was inspected for ticks, but they were found only in the ears. A total of 98 ticks was collected. Ticks were removed from horses by using entomological forceps or were captured in places where the horses lived. Only 51 ticks were analyzed for the study, and the remainder were identified only taxonomically. All ticks were preserved in 70% ethanol.

Ticks were analyzed with the aid of a stereoscope microscope (Zeigen, CDMX, Mexico) at the Parasitology Laboratory of the Chemistry College of the Autonomous University of Chihuahua. For reference, a manual of livestock ticks (Diamant and Strickland 1965) was used for animal disease and taxonomic identification.

Blood samples were preserved with EDTA in tubes at -20ºC until processed, and kept at 4ºC during extraction. In total, DNA from 144 blood samples was extracted using DNeasy Blood and Tissue kit (Qiagen, Valencia, CA) as specified by the manufacturer.

The ticks were homogenized by using a sterile tissue homogenizer (BioMasher II, Tokyo, Japan). DNA was extracted according to manufacturer instructions (Ultra Clean Blood Spin DNA Isolation Kit sample; MO Bio Laboratories, Inc., Carlsbad, CA). For every 50 mg of sample 1× lysis solution was used. If the purity of DNA at 260/280 was less than 1.8, extraction was repeated.

PCR for blood and tick samples was done in a C1000 Touch™ Thermal Cycler (Bio-Rad Laboratories, Hercules, CA). For end-point and nested PCR analysis for identification of pathogens, 12.5 µl of GoTaq® Green Master Mix (Promega Corporation, Madison, WI), 2 µl of a mixture of primer pairs at 10 µM concentration, and 10 µl of DNA per sample (7.89 ng average) from total horse blood or 5.5 µl of DNA from ticks (48.29 ng average) were used. Nuclease-free water was used to adjust the final volume to 25 µl. For nested PCR analysis, 12.5 µl GoTaq Master Mix, 2 µl of a mixture of primer pairs required for the nested PCR reaction at a concentration of 10 µM, and 1.5-2 µl of DNA product as template from the first PCR reaction were used. The final volume was adjusted with nuclease-free water to 25 µl. The sequences of the primer pairs are described in Table 1.

For *T. equi* and *B. caballi,* temperature-gradient analysis was used to identify the optimum working temperature, which was 63°C for annealing *T. equi* primers and 60.1°C for *B. caballi* primers. The Merozoite Antigen 1 (EMA-1) gene with 218 bp was evaluated for *T. equi*; for *B. caballi*, the gene targeted was a fragment from BC48, a Merozoite Rhoptry Protein with a final length of 430 bp (Battsetseg et al. 2001); and the 16S rRNA gene of 546 bp was targeted for identification of *A. phagocytophilum* (Massung et al. 1998). Electrophoretic analyses were done on 2% w/v agarose gels, at 95 volts for 60 minutes at room temperature. Once the analysis time had elapsed, to observe DNA bands, agarose gel was put on an ultraviolet transillumination unit.

Table 1. Primers and Protocols Used for Identification of Pathogens, Sequences of Primers, and Methodology for Identification of Pathogens by PCR and Nested PCR

Primer	Sequence	Fragment length (bp)	Organism	Protocol	Reference
BC48F1	ACG AAT TCC CAC AAC AGC CGT GTT	530	*Babesia caballi*	96ºC, 4 min, 94ºC, 1 min 56ºC, 2 min, 72ºC, 2 min 72ºC, 5 min, 40 cycles	Battsetseg et al. 2001
BC48R3	ACG ATT TCG TAA AGC GTG GCC ATG				
EMA5	TCG ACT TCC AGT TGG AGT CC	268	*Theileria equi*	95ºC, 10 min, 94ºC, 1 min 60ºC, 1 min, 72ºC, 1 min 72ºC, 5 min, 40 cycles	Battsetseg et al. 2001
EMA6	AGC TCGACC CAC TTA TCA C				
GE3F	CAC ATG CAA GTC GAA CGG ATT ATT C	932	*Anaplasma phagocytophilum*	95ºC, 2 min, 94ºC, 30 sec 55ºC, 30 sec, 72ºC, 1 min 72ºC, 1 min, 40 cycles	Massung et al. 1998
GE10R	TTC CGT TAA GAA GGA TCT AAT CTC C				
Nested primer					
BC48F11	GGG CGA CGT GAC TAA GAC ATG	430	*Babesia caballi*	96ºC, 4 min, 94ºC, 1 min 56ºC, 1 min, 72ºC, 1 min 72ºC, 5 min, 40 cycles	Battsetseg et al. 2001
BC48R31	GTT CTC AAT GTC AGT GAC ATC CGC				
EMA7	ATT GAC CAC GTC ACG ATG GA	218	*Theileria equi*	95ºC, 10 min, 94ºC, 1 min 63ºC, 1 min, 72ºC, 1 min 72ºC, 5 min, 40 cycles	Battsetseg et al. 2001
EMA 8	GTC CTT CTT GAG AAC GAG GT				
GE9F	AAC GGA TTA TTC TTT ATA GCT TGC T	546	*Anaplasma phagocytophilum*	95ºC, 2 min, 94ºC, 30 sec 55ºC, 30 sec, 72ºC, 1 min 72ºC, 1 min, 30 cycles	Massung et al. 1998
GE2R	CCA GCG TTT AGC AAG ATA AGA G				

Results

The overall prevalence of infestation by ticks was 36.1% from a total of 144 horses inspected (52/144). However, all horses in the study had scars from previous tick bites. All ticks were inside the ear canal. Another 46 ticks were collected from facilities in which the horses lived. The other 92 horses had visible marks produced by bites, but the ticks were not found during inspection. Only 88 ticks could be identified, because DNA had been extracted from the other 10. Species identified were 75% *Otobius megnini* (Duges) (66/88), 15.9% *Ornithodoros turicata* (Duges) (14/88), and 9.1% *Rhipicephalus sanguineus* (Latreille) (8/88). Images of analyzed specimens are in Figs. 1-3.

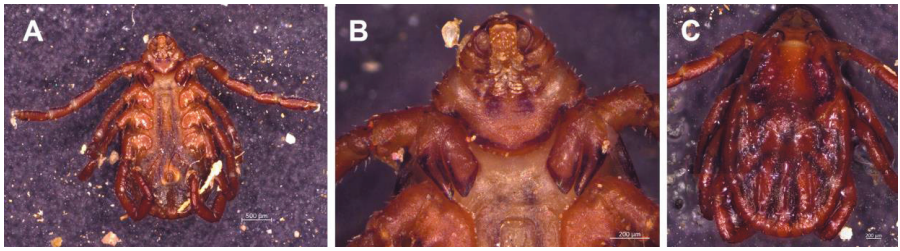

Fig. 1. Identification of *Rhipicephalus sanguineus*: ventral view (A), mouthparts enlarged (B), dorsal view (C).

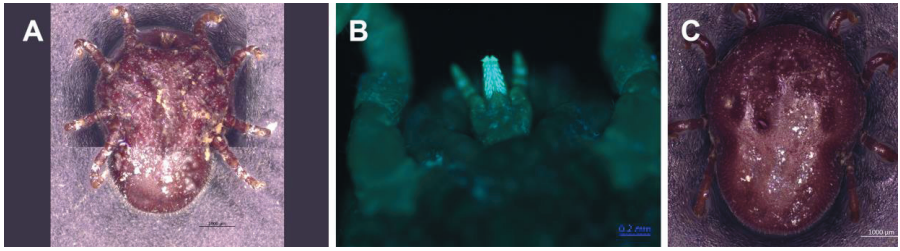

Fig. 2. Identification of *Otobius megnini*: ventral view (A), hypostome highlighted (B), dorsal view (C).

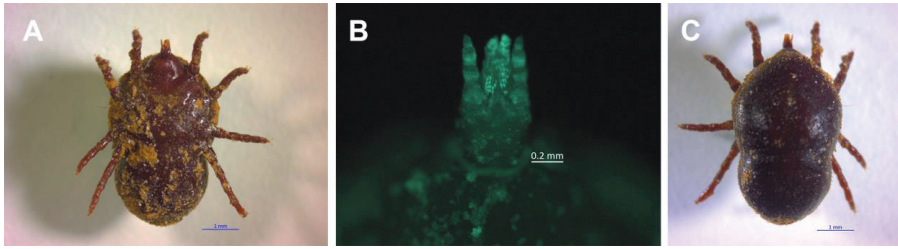

Fig. 3. Identification of *Ornithodoros turicata*: ventral view (A), mouthparts highlighted (B), dorsal view (C).

Average DNA concentration extracted from blood samples was 8.5 ng/µl with an average 260/280 purity of 1.92, ranging from 1.78-2.06. DNA was extracted only from 51 ticks (one tick was lost) captured on the host. The average DNA concentration of the extractions from ticks was 47.43 ng/µl, and these DNA samples also were analyzed by PCR end-point to identify pathogens.

Prevalence of *T. equi* in blood samples was 6.9% (10/144). The prevalence was 5.9% (3/51) in one *O. megnini,* one *O. turicata,* and one tick that could not be identified because of previous DNA extraction. Prevalence in blood samples was 2.8% (4/144) in *B. caballi* and 5.9% (3/51) in three *O. megnini* ticks. Only one case of coinfection with *T. equi* and *B. caballi* 0.7% (1/144) was found in blood samples. *A. phagocytophilum* was identified in samples of DNA extracted from equine blood. The prevalence was 0.8% (1/124), whereas no tick of those evaluated was positive.

Discussion

Fifty-one soft ticks from horses at Cd. Juárez, Chihuahua were analyzed. In Mexico, *Ornithodoros* spp. was found in southern states such as Chiapas, Coahuila bordering the State of Chihuahua to the east, Tabasco, Veracruz, and Yucatán (Sánchez-Montes et al. 2016, Guzmán-Cornejo et al. 2017). Ticks can parasitize various vertebrate Orders such as cattle, dogs, pigs, squirrels, turtles, and even snakes in North America (Dworkin et al. 2008, Barbour and Miller 2014, Kelly et al. 2014). Across the border with the United States, *O. turicata* was identified as early as the 19th Century at Guanajuato, Mexico (Donaldson et al. 2016). *Ornithodoros* spp. soft ticks transmit several types of pathogens, including *Borrelia* sp. (Christensen et al. 2017), *Babesia gibsoni* (Patton) in dogs (Battsetseg et al. 2007), and *Babesia vesperugini* (Dionisi), in common noctule bats, *Nyctalus noctula* (Schreber) (Liu et al. 2018), and could play a role in transmission of *Babesia* spp. to small terrestrial mammals from Brazil (Wolf et al. 2016). In addition, a key factor contributing to transmission of pathogens might be that soft ticks complete feeding during a period of minutes to hours, compared with hard ticks that feed more slowly for several hours to days (Krishnavajhala et al. 2017). *O. megnini* was also found in samples collected. The species previously was reported throughout the world. For example, in North America (Niebuhr et al. 2014), Europe (Lindström and Lindström 2017), and Asia (Kingston 1936), the species is associated with livestock and wild animals. Despite information from the 20th Century on the presence of *O. megnini* in southwestern California and Mexico (Jellison et al. 1948), there are no reports of pathogens at Chihuahua, nor of national distribution of parasites. Hence, the species needs further evaluation. Brown dog tick, *R. sanguineus*, also was found, which previously had been reported throughout Mexico, in the states of Yucatan (Pat-Nah et al. 2015), Morelos (Lira-Amaya et al. 2013, 2017), and Baja California, Coahuila, Durango, Sinaloa, and Sonora to the north (Tinoco-Gracia et al. 2009). Adaptation characteristics enable ticks to feed on hosts that do not belong in their natural trophic chain (Dantas-Torres 2010). *T. equi* has been reported in Mexico, in equines from the State of Jalisco (Ayala-Valdovinos et al. 2017), with prevalence of 19.7% in 1,000 samples of horse blood analyzed. In this work, hard ticks were the main species. Our results of *T. equi* in blood samples and in ticks are, to our knowledge, the first report in Chihuahua, and in soft ticks from equines. However, the positive tick results might be because the pathogen was in blood meal, rather than the tick being infected. Identification of the pathogen does not assume transmission to a host. The role of ticks in transmission of *T. equi* could be determined by using experimentally infected

ticks on healthy animals, as well as by evaluating salivary glands of vectors by using PCR. Molecular evaluation of blood (Posada-Guzmán et al. 2015, Manna et al. 2018) or ticks (Nader et al. 2018) showed the prevalence of *B. caballi* in Bulgaria, Costa Rica, and Italy. The first use of serological diagnostic methods for identification of *B. caballi* in horses from northern Mexico was done in the State of Nuevo León with seroprevalence of 27.4% (Cantú-Martínez et al. 2012). However, the results cannot be used for comparison because a different technique was used. In addition, hard ticks, not soft ticks, were studied. Positive samples of the rickettsial pathogen *A. phagocytophilum* were not detected in soft ticks, and only one blood sample tested positive for the pathogen. The pathogen infects various animal species such as dogs, equines, and humans (Hunyadi et al. 2017). Although prevalence in the present study was low, it matched previous reports of cosmopolitan distribution (Burgess et al. 2012, Slivinska et al. 2016, Saleem et al. 2018). In Mexico, seroprevalence of *Anaplasma* sp. was evaluated in companion animals such as dogs (Movilla et al. 2016), but not horses. Analysis at a national level determined the prevalence of rickettsial pathogens such as *A. phagocytophilum, Ehrlichia* sp., and *Rickettsia* sp. transmitted by hard ticks such as *Rhipicephalus, Dermacentor, Ixodes*, and *Amblyomma* (Sosa-Gutierrez et al. 2016), but have not been reported in ticks from the Argasidae family. In conclusion, results showed that horses from the northern part of Chihuahua might be at risk for contracting equine piroplasmosis from tick bites, and soft ticks that parasitize them could play a role in transmission of the pathogens evaluated.

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