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Molecular identification of selected tick-borne pathogens in wild deer and raccoon dogs from the Republic of Korea

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ABSTRACT

In this study, we investigated tick-borne pathogens in blood samples collected from 34 wild animals from six different regions in the Republic of Korea, including 18 Korean water deer (*Hydropotes inermis argyropus*), 15 Korean raccoon dogs (*Nyctereutes procyonoides koreensis*), and one roe deer (*Capreolus capreolus*). Polymerase chain reaction revealed *Anaplasma* spp. infection in 11 Korean water deer, one roe deer, and one Korean raccoon dog. *Theileria* spp. infection was detected in 14 Korean water deer and one roe deer. Co-infection with two pathogens (*Anaplasma* spp. and *Theileria* spp.) was identified in 10 Korean water deer and one roe deer. *Ehrlichia* and *Rickettsia* spp. infections were not detected in any of the animals. Genetic analysis showed that *Anaplasma* sp., *A. bovis*, *A. phagocytophilum*, *Theileria* sp., *T. cervi*, and *T. luwenshuni* were present in these animals. Our results showed that *T. luwenshuni* was the most prevalent species found in Korean water deer. Interestingly, our findings demonstrated that the Korean raccoon dog was a reservoir for anaplasmosis, indicating that transmission of *A. bovis* was not restricted to host species. The roe deer was found to be infected with a type F *T. cervi* strain. To the best of our knowledge, this study is the first to report *A. bovis* infection in Korean raccoon dogs, *T. cervi* in roe deer, and *T. luwenshuni* in Korean water deer. Our results indicated that wild animals represent possible reservoirs for these tick-borne pathogens, thus playing an important role in the transmission of tick-borne diseases (TBDs) in domestic animals, livestock, and humans. Furthermore, our findings highlight the risk associated with introducing new pathogens as well as the role of wild animals in the transmission and spread of these zoonotic TBD pathogens.

1. Introduction

Anaplasmosis and theileriosis are important tick-borne diseases (TBDs) that affect domestic and wild ruminants worldwide. The genus *Anaplasma* comprises six species that can be differentiated based on host cell tropism. The three species that infect red blood cells are known as erythrocytic *Anaplasma* species and include *Anaplasma marginale*, *A. centrale*, and *A. ovis* (Inokuma et al., 2001; Kocan et al., 2004; Renneker et al., 2013). The other three species, *A. phagocytophilum*, *A. bovis*, and *A. platys*, infect neutrophils, monocytes, and platelets, respectively (Dumler et al., 2001; García-Pérez et al., 2016; Ramos et al., 2014; Sreekumar et al., 1996). *A. phagocytophilum* and *A. bovis* infections have been reported in wild ruminants in the Republic of Korea (ROK) (Kang et al., 2011; Lee et al., 2009; Seong et al., 2015a, b); among these species, *A. phagocytophilum* is thought to possess the highest zoonotic potential.

Species of the genus *Theileria* are classified into two main groups depending on their cellular targets (lymphocytes or erythrocytes). *Theileria annulata* and *T. parva* are the causative agents of lymphoproliferative diseases, which are associated with high mortality and morbidity, and are commonly distributed in tropical regions (Tait and Hall, 1990; Gitau et al., 1999). *T. buffeli/orientalis/sergenti* causes benign theileriosis, and mild or asymptomatic diseases caused by this infectious agent have been reported in East Asia (Kang et al., 2012).

The *Theileria* parasite is transmitted by ixodid ticks and exhibits complex life cycles in both vertebrate and invertebrate hosts (Ica et al., 2007). In the ROK, *Theileria* sp. have been reported in several wild

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animal species, including Chinese water deer and Korean water deer (Han et al., 2009; Seong et al., 2015a), whereas infection by other *Theileria* species has not been observed in wild animals.

Wild animals serve as the primary reservoirs of TBDs. Moreover, these animals have begun to inhabit areas that are in close proximity to domestic animals and humans due to food scarcity and fear of predation; therefore, these animals play an important role in disease transmission from domestic animals to nearby wildlife populations (Daszak et al., 2000). To date, investigations on tick-borne pathogens in wild animals have been geared primarily toward evaluating their role in the epidemiology of livestock diseases. Thus, although TBDs can cause serious problems for domestic ruminants found in proximity to wild animals, only limited information is currently available regarding the prevalence of TBDs in wild animals in the ROK. Therefore, the goal of this study was to survey TBDs from wild animals in the ROK, investigate the epidemiology and diversity of these tick-borne pathogens, and evaluate the role of wild animals as zoonotic pathogen reservoirs.

2. Materials and methods

2.1. Ethical statement

All samples were collected with consent from provincial Wildlife Rescue and Conservation Centers for subjected animals. This study was approved by the Institutional Committee of Graduate Studies and Research at Chonbuk National University.

2.2. Sample collection

Blood samples were collected in 2015 from 34 animals found at six different Wildlife Rescue and Conservation Centers from Chungnam province, Chungbuk province, Jeonbuk province, Gyeongnam province, Gyeongbuk province, and Ulsan city in the ROK. These animals included 18 Korean water deer (*Hydropotes inermis argyropus*), 15 Korean raccoon dogs (*Nyctereutes procyonoides koreensis*), and one roe deer (*Capreolus capreolus*).

2.3. Polymerase chain reaction (PCR) for tick-borne pathogens

Genomic DNA was extracted from whole blood using a DNeasy Blood & Tissue Kit (Qiagen Inc., Valencia, CA, USA) according to the manufacturer's instructions. An AccuPower *Theileria* PCR Kit (Bioneer, Daejeon, Korea) was used to detect *Theileria* infection (forward primer [F], 5′-GTTATAAATCGCAAGGAAGTTTAAGGC-3′; reverse primer [R], 5′-GTGTACAAAGGGCAGGGACGTA-3′). This system amplifies a portion of the 18S ribosomal RNA (rRNA) gene with a predicted size of 239 bp under the following cycling conditions: 94 °C for 5 min, followed by 40 cycles of 94 °C for 20 s and 65 °C for 35 s, and a final extension at 72 °C for 5 min. For nested PCR amplification of *Theileria* spp., the V4 hypervariable region of the 18S rRNA gene was used (Chae et al., 1998; Zanet et al., 2014). An AccuPower Rickettsiales 3-Plex PCR Kit (Bioneer) was used to detect *Anaplasma* (F, 5′-TACCTCTGTGTTGTAGCTAACGC-3′; R, 5′-CTTGCGACATTGCAACCTATTGT-3′), *Ehrlichia* (F, 5′-CGGAATTCCTA GTGTAGAGG-3′; R, 5′-AGGAGGGATACGACCTTCAT-3′), and *Rickettsia* (F, 5′-TAGGGGATGATGGAATTCCTA-3′; R, 5′-CCCCCGTCA ATTCCTTTG AG-3′) infection. PCR was performed using specific primer sets targeting the 16S rRNA gene. The anticipated sizes of amplified PCR products for *Anaplasma*, *Ehrlichia*, and *Rickettsia* were 429, 340, and 252 bp, respectively, under the following cycling conditions: 95 °C for 15 min; followed by 40 cycles of 95 °C for 10 s, 58 °C for 30 s, and 72 °C for 30 s; and then a final extension at 72 °C for 5 min. The PCR products were separated by electrophoresis on 1.5% agarose gels and visualized after staining with ethidium bromide.

2.4. Nucleotide sequence and phylogenetic analysis

For further DNA sequence analysis, amplified 18S and 16S rRNA fragments were purified using a QIAquick PCR Purification Kit (Bioneer). After purification, the sizes of these target gene products were confirmed by 1.5% gel electrophoresis. The amplicons were then subjected to direct sequencing (Bioneer) using the same primers employed for each PCR. The obtained nucleotide sequences were analyzed using the Basic Local Alignment Search

Tool (BLAST) from the National Center for Biotechnology Information (NCBI) database to investigate the homologies of the *Theileria* and *Anaplasma* genes. The sequences were aligned using the ClustalX program. A phylogenetic tree was constructed based on nucleotide alignments using the neighbor-joining method (Saitou and Nei, 1987). Bootstrap analysis was conducted based on 1000 replicates using MEGA version 6 (Tamura et al., 2013). The representative sequences obtained in this study were registered to the GenBank database under accession numbers KU565343-KU565362.

3. Results

Wild animals from six different regions were tested for TBDs by PCR. As shown in Table 1, TBD-associated pathogens were identified in 17 blood samples from 34 animals belonging to three animal species (18 Korean water deer, 15 Korean raccoon dogs, and one roe deer). Tick-borne pathogens were detected primarily in Korean water deer (Table 1). Of the 17 infected animals, 15 were positive for *Theileria* spp. infection, and 13 were positive for *Anaplasma* spp. infection. *Ehrlichia* and *Rickettsia* spp. were not detected in any of the sampled animals. Interestingly, 11 of the 17 infected animals (10 Korean water deer and one roe-deer) were co-infected with two pathogens (*Anaplasma* spp. and *Theileria* spp.; Table 1). *Theileria* spp. were not detected in any of the 15 Korean raccoon dogs examined, and only one Korean raccoon dog was positive for infection with *Anaplasma* spp.

Based on 16S rRNA analysis, 13 animals were positive for *Anaplasma* spp. infection (Table 1). *Anaplasma* spp. infection was detected in Korean water deer, roe deer, and Korean raccoon dogs. Based on the sequencing of all 13 amplicons, 11 high quality sequences were obtained. These 11 sequences were classified into three groups based on phylogenetic analysis, including *Anaplasma* sp., *A. phagocytophilum*, and *A. bovis* (Fig. 1). *Anaplasma* sp. was detected in four Korean water deer and one roe deer. Interestingly, all *Anaplasma* sp. sequences isolated from Korean water deer were identical, even though they were isolated from different locations. In addition, the nucleotide sequence from the roe deer was 99.7% identical to that obtained from Korean water deer. Sequences from the *Anaplasma* sp. detected in the four Korean water deer (KU565351, KU565353, KU565354, and KU565355) and one roe deer (KU565352), clustered with similar species isolated from wild and domestic ungulates in the ROK, Japan, and China, but diverged from other *Anaplasma* species (Fig. 2). *A. phagocytophilum* infection was detected in five Korean water deer from three different locations (Gyeongnam, Gyeongbuk, and Chungnam provinces), and these sequences were 97.1–99.2% identical to each other. Based on phylogenetic analysis, the *A. phagocytophilum* isolates formed two clades, with two isolates (KU565359 and KU565360) diverging from the others (KU565358, KU565361, and KU565362; Fig. 2). The first two sequences (KU565359 and KU565360) differed genetically from *A. phagocytophilum* strains/isolates found in other countries, whereas KU565351 formed a clade with a Korean water deer isolate (KR045609) detected previously by our group (Fig. 1). The single Korean raccoon dog isolate was determined to be *A. bovis*. This isolate (KU565356) was closely related to *A. bovis* isolates from various hosts and geographic locations (Fig. 1). To the best of our knowledge, this is the first report of *Anaplasma* sp. infection in roe deer and *A. bovis* infection in Korean raccoon dog in the ROK.