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Investigation of *Theileria* sp. from Ticks and Roe Deer (*Capreolus pygargus*) in Jeju Island

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Abstract : Siberian Roe deer which inhabits Jeju Island is unique native species. Most of all the roe deer infect a lot of ticks, which can affect its population directly and can act as a vector to spread vector-borne diseases. The purposes of this study were to identify the ticks and detect the piroplamsosis on the roe deer in Jeju island. We collected ticks and blood samples in 23 roe deer rescued and treated at the Jeju Wildlife Rescue Center. As a result, we identified the one species of ticks, *Haemaphysalis longicornis* in roe deer and detected the closely related to *Theileria luwenshuni* in all blood samples (100%) and 8 pooled ticks (34.8%). These results indicate that there may be a high prevalence particularly of *T. luwenshuni* infection in Jeju wild roe deer and *H. longicornis* is a major vector of these diseases. It suggested that Jeju roe deer may act as reservoirs for these zoonotic pathogens.

Key words **:** Roe deer, *Haemaphysalis longicornis*, *Theileria luwenshuni*, Jeju.

Introduction

Roe deer have been classified to the smaller European roe deer (*Capreolus caprelous*) and larger Siberian roe deer (*Capreolus pygargus*). Siberian roe deer which inhabits Jeju island is unique native species, Jeju roe deer has significant differences in morphology compared with inland roe deer in Korea because of geographical isolation (15). Most of roe deer infected a lot of ticks, which can affect its population directly and act as a tick-borne diseases.

Theileriosis and babesiosis are tick-borne diseases reported in ruminants all around the world. *Theileria* spp. cause bovine theileriosis infecting lymphocytes as well as erythrocytes of the cattle and wild ruminants, and similar to *Babesia* undergo a complex developmental cycle in ticks. *Theileria* spp. have been reported in deer species such as reindeer, roe deer, red deer (11), white-tailed and sika deer (6,8,19), fallow deer (4) and elk (2,20). In wild ruminants, recently the sequence of the 18S rRNA gene of *T. capreoli* from the same deer species has been deposited in GenBank (AY726011) (1). New *Theileria* species were recently isolated by Garcia-Sanmartin *et al* (5) from wild ruminants in Northern Spain: *Theileria* sp. OT3 in red deer (*Cervus elaphus* L.), roe deer and chamois (*Rupicapra rupicapra* L.) and *Theileria* sp. 3185/02 in red deer and roe deer. The commonest theileriosis are caused by *T. annulata*, *T. sergenti/buffeli/orientalis* and *T. mutans*. Of these, *T. annulata* is the most virulent species, affecting large numbers of cattle. The benign *T. sergenti/buffeli/orientalis* is avirulent in cattle and clinical symptoms are rarely observed (16). The East Asia Theileiosis is most closely related to the *T. sergenti/buffeli/orientalis* group.

In Jeju, roe deer is a major wild ruminants distributed all over the area. There has been little information on the theileriosis in Jeju roe deer. In this study, to clarify the phylogenetic situation of *Theileria* spp., molecular analysis of *Theileria* spp. from Jeju roe deer were attempted by using molecular methods including polymerase chain reaction (PCR) and sequence analysis of the 18S rRNA gene.

Materials and Methods

Animals and samples

From January 2012 to April 2013, the roe deer (23 heads) rescued and treated at the Jeju Wildlife Rescue Center were used in this study. Blood was taken via jugular vein for Giemsa staining and DNA extraction. Ticks were collected from roe deer manually or by means of fine forceps.

Identification of Ticks

The ticks collected in same deer were stored in 4 ml cryovial containing 70% ethyl alcohol and identified under a dissecting microscope (Olympus CX31, Nikon Eclipse Ci) using standard keys and previous descriptions (21) at the Jeju

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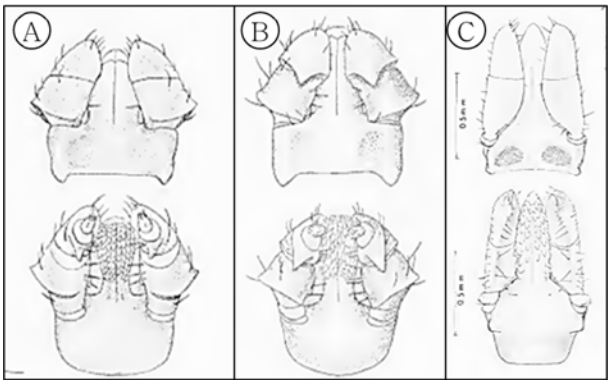

Fig 1. Apical part of female ticks (Ixodidae) previously collected roe deer in Korea (upper: dorsal part, lower: ventral part) (Yamaguti *et al*. 1971) (A) *H. flava*. Female. (B) *H. longicornis*. Female. (C) *I. nipponensis*. Female.

Wildlife Rescue Center's laboratory (Fig 1).

Blood film and Giemsa staining

Thin Blood smears from each blood sample were prepared and fixed with methanol, stained with Giemsa solution (stock solution : DW = 1 : 9) and then examined under a microscopy for the piroplasms in the erythrocytes.

DNA Extraction and 18S rRNA gene amplification

Total DNA was extracted from 300 µl of each EDTA blood sample using the G-Dex II TM blood DNA extraction Kit (Intron, Korea) and from fully engorged female ticks using the DNeasy Blood & Tissue Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. The DNA concentration was measured by spectrophotometer (NanoVue, GE Healthcare, USA) and adjusted around 100 ng/ul. PCR amplification was performed with the primer set cBabe-18sF: (5'-GTT GAT CCT GCC AGT AGT-3') and cBabe-18sR: (5'-AAC CTT GTT ACG ACT TCT C-3') that was specific to 18S rRNA gene of both *Babesia* and *Theileria* species. The PCR reaction consisted of 100 ng of genomic DNA, 10 µM of each primer, PCR buffer, 1.5 mM $MgCl_2$, 200 mM dNTPs and 1.5 U *Taq* polymerase (2X TOP simple TM DyeMIX aliquot-n*Taq* Kit, Enzynomics, Korea), made up to a final volume of 20 µl. The cycling conditions was 94°C for 5 min followed by 35 cycles at 94°C for 30 s, 59°C for 20 s and 72°C for 30 s with a final extension step of 72°C for 10 min. The PCR products were visualized using a 1.2% ethidium bromide-stained agarose gel. PCR products were purified using QIAquick PCR Purification Kit (Qiagen, Hilden, Germany), and purified PCR product was digested with 2 U of *Ava*II (Enzynomics, Korea) under the buffer solution at 37°C for 1 hour (PCR-RFLP). The digestion products were visualized in 1.8% ethidium bromide-stained agarose gel electrophoresis.

Sequencing and phylogenic analysis

The PCR products were purified using a commercial kit (MEGA-Bead gel extraction kit, Intron, Korea), and cloned into the T-vector TOPcloner (Enzynomics, Korea). Recombinant plasmid DNA was purified using Qiagen Plasmid mini kit (Qiagen, Hilden, Germany) and inserted PCR product was sequence analysis (Solgent, Korea). The sequences obtained (Partial 18S rRNA gene) were compared with the GenBank database by nucleotide sequence homology searches made at the network server of the National Center for Biotechnology Information (NCBI) using BLAST. Multiple sequence alignment were performed using the program AlignX (Vector NTI 8.0 suite, InforMax, USA) with an engine based on the Clustral W algorithm (17). Phylogenetic trees were constructed by neighbor-joining method with distance matrix calculation by Kimura's two parameters, operated by MEGA (Molecular Evolutionary Genetics Analysis) software version 5.1. Scale bar indicates the number of mutations per sequence position. The numbers at the nodes represent the percentage of 1,000 bootstrap resamplings.

Results

Ticks

The 141 engorged female ticks collected from deer were identified as *H. longicornis* (Fig 2). There were no other species of ticks in this study.

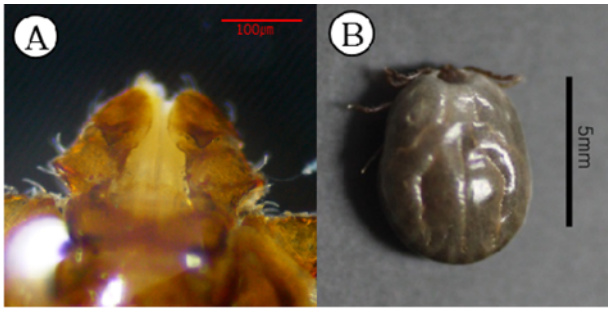

Fig 2. *H. longicornis* collected from roe deer (A) Apical part of female, (B) Engorged female.

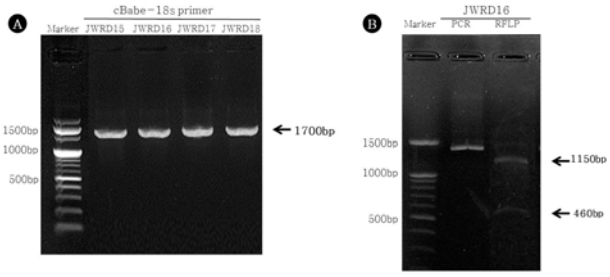

Fig 3. Electrophoresis analysis of DNAs amplified by PCR and PCR-RFLP targeted 18S rRNA of *Babesia/Theileria* spp. on 1.2% agarose gel (A) The sizes of amplified 18s rRNA product using cBabe-18s primer set are about 1700bp. (B) The PCR-RFLP fragment sizes of all PCR positive samples after digestion with *AvaII* are show one type; 80, 460, 1150 bp. The 80 bp is not seen because of small size. JWRD15,16,17,18; Jeju wildlife Rescued Deer sample No.

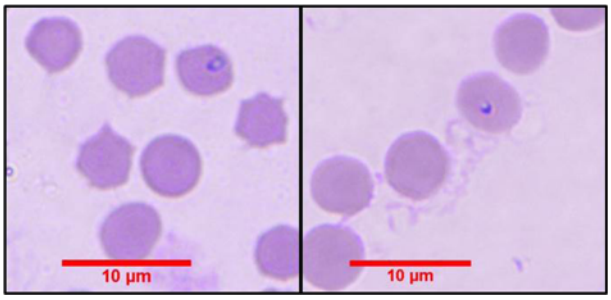

Fig 4. Giema-staining blood film showing morphological polymorphisms of *Theileria* sp. from PCR positive roe deer blood (size; 0.8~1.5 μm).

Theileria spp.

The primary PCR using cBabe-18S primer set amplified an ~1,700 bp band in case of *Theileria* species, while there were no bands amplified from negative controls (Fig 3A). All blood samples (100%) were detected about 1,700 bp band, while 8 out of the 23 pooled ticks were detected (34.8%). The PCR-RFLP fragment sizes of all PCR positive samples after digestion with *AvaII* are showed one type; 80, 460, 1150 bp. The 80 bp is not seen because of small size (Fig 3B).

Phylogentic analysis

The 18S rRNA gene sequences were edited and assembled to a final length of 1,700 bp. The gene sequences were submitted to GenBank under accession numbers: *T. cervi* (HQ-184411); *T. luwenshuni* (JX469518); *Theileria* sp. (FJ668376); *Theileria* sp. (FJ668373); *Theileria* sp. (FJ668374); *Theileria* sp. (AF036336); *Theileria* sp. China 1 (AY262119); *Theile-*

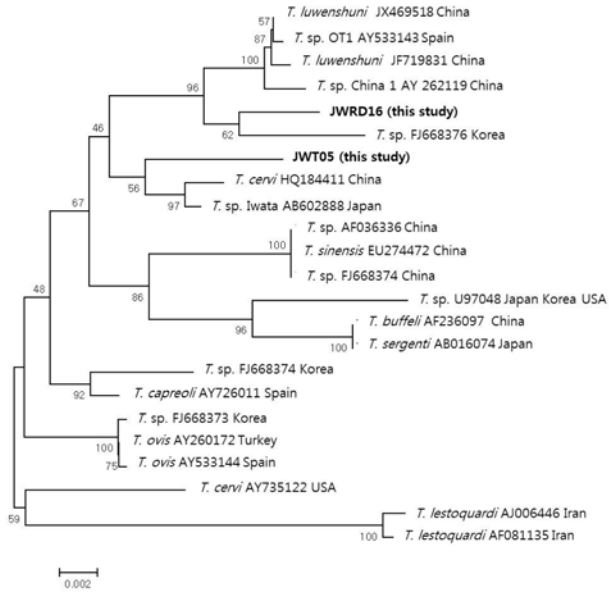

Fig 5. Phylogenetic tree of partial 18S rRNA gene sequences of various *Theileria* species and PCR-amplified 18S rRNA products from roe deer and ticks. Scale bar indicates the number of mutations per sequence position. The numbers at the nodes represent the percentage of 1,000 bootstrap resamplings. JWRD16; Jeju Wildlife Rescued Deer sample No, JWT05; Jeju Wildlife Tick sample No in this study.

Table 1. Similarity matrix between partial 18S rRNA gene sequences of various *Theileria* strains

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17
1	-	98.5	98.9	98.4	97.8	98.0	98.1	97.7	98.2	98.4	97.7	98.0	97.6	98.4	96.5	97.2	99.0
2		-	98.2	98.7	98.8	97.8	97.6	97.8	98.4	98.6	97.8	97.8	97.5	97.9	96.6	97.0	98.2
3			-	98.2	97.6	98.4	98.3	98.2	98.0	98.2	98.2	98.4	98.0	98.7	96.9	97.4	99.7
4				-	99.0	98.0	97.9	97.8	99.7	99.9	97.8	98.1	97.7	98.4	96.6	97.5	98.3
5					-	97.6	97.4	97.6	98.8	98.9	97.6	97.5	97.2	97.8	96.2	96.9	97.7
6						-	98.8	97.9	97.9	98.0	97.9	99.9	98.7	99.2	97.6	97.4	98.6
7							-	97.8	97.8	97.8	97.8	98.8	98.3	99.2	96.8	97.2	98.5
8								-	97.6	97.7	100.0	98.0	97.9	98.2	96.7	97.5	98.4
9									-	99.7	97.6	98.0	97.6	98.3	96.4	97.3	98.1
10										-	97.7	98.0	97.6	98.4	96.5	97.4	98.2
11											-	98.0	97.9	98.2	96.7	97.5	98.4
12												-	98.8	99.3	97.6	97.4	98.6
13													-	98.6	97.4	96.9	98.0
14														-	97.2	97.8	98.9
15															-	95.9	97.1
16																-	97.4
17																	-

1, *Theileria* spp. 18S rRNA of JWT05 (from ticks in this study); 2, JWRD16 (from roe deer in this study); 3, *T. cervi* (HQ184411); 4, *T. luwenshuni* (JX469518); 5. *Theileria* sp. (FJ668376); 6, *Theileria* sp. (FJ668373); 7, *Theileria* sp. (FJ668374); 8, *Theileria* sp. (AF036336); 9, *Theileria* sp. China 1 (AY262119); 10, *Theileria* sp. OT1 (AY533143); 11, *Theileria* sp. (AF036336); 12, *T. ovis* (AY260172); 13, *T. cervi* (AY735122); 14, *T. capreoli* (AY726011); 15, *T. lestoquardi* (AJ006446); 16, *Theileria* sp. (U97048); 17, *Theileria* sp. Iwata (AB602888)

ria sp. OT1 (AY533143); *Theileria* sp. (AF036336); *T. ovis* (AY260172); *T. cervi* (AY735122); *T. capreoli* (AY726011); *T. lestoquardi* (AJ006446); *Theileria* sp. (U97048); *Theileria* sp. Iwata (AB602888). The obtained sequences were compared with the 18S rRNA gene sequences of other *Theileria* spp. available in GenBank using the BLAST algorithm (Fig 5). The 18S rRNA gene sequence obtained from the 2 wild roe deer rescued at the Jeju Wildlife Rescue Center were identical to each other and had 98.4% sequence closely related to *T. luwenshuni* (JX469518) previously isolated in China (Table 1).

Giemsa staining

In thin blood smears, single small ring-like form was seen in 20 of 23 roe deer blood samples (87.0%). The size of a single parasite was 0.8~1.5 μm (Fig 4).

Discussion

Ten *ixodidae* species have been recognized on Jeju Island, Korea. *Haemaphysalis campanulata* Warburton, *H. phasiana, H. flava* (12), *Ixodes persulcatus*, *I. vespertilionis*, *I. pomeranzevi*, *I. turdus* and *I. nipponensis* (9) have scattered and accidental records. *Boophilus microplus* (10,21) and *H. longicornis* (9,12,13,21) have been regarded as common species, but Moon and Kim (13) suggested the decline of *Babesia microplus* on Jeju island possibly due to the tick controls and changed vegetation managements in ranches. Therefore, *H. longicornis* seems to be the most dominant and widely distributed species throughout Jeju island, most of ticks collected by flag dragging (12,14). In this study, we suggested that *H. longicornis* should be dominant tick (100%) on the wild roe deer and important vector as tick-borne protozoan diseases.

Theileria spp. are tick-borne protozoan diseases infecting various animals in the world. Recently, theileriosis has been prevalent in almost area of Korea, especially Jeju island is very serious. There are little documented *Theileria* spp. infections on the roe deer in Jeju. Piroplasm species differentiation is difficult using light microscopy. The small-subunit ribosomal RNA (rRNA) has proved to be an invaluable tool in the field of molecular phylogeny due to its ubiquity, size, generally slow rate of evolution and the most sequenced of all genes (2,18). In our study, we used the 18S rRNA gene specific primer set for amplification of *Theileria* spp. The PCR product were discriminated from *Theileria* spp. according to the 18S rRNA gene sequences and revealed *T. luwenshuni* (98.4%) in roe deer blood and *T. cervi* (98.9%) in tick samples. In PCR-RFLP, fragment sizes of all samples after digestion with *AvaII* are showed one type; 80, 460, 1150 bp. This pattern was closely related to *T. luwenshuni*. They are considered to be pathogenic parasites for small ruminants. In this study, the results confirmed that *T. luwenshuni* was detected and distributed in Jeju roe deer. Generally, *T. sergenti/bufleli/orientalis* was detected from cattle in inland and Jeju island (3). *T. ovis* and *T. capreoli* were also detected individually in wild Chinese water deer from inland (7), but *T. luwenshuni* from roe deer was only detected in Jeju. For the PCR positive samples, we confirmed the single small ring-like form within the erythrocyte (87.0%), but PCR method is an advanced technique that shows a higher sensitivity and specificity than the conventionally microscopic examination for detecting *Theileria* spp. from roe deer or ticks. There were no significant difference in blood and serum biochemical findings between *T. luwenshuni* infected and non-infected roe deer (data not shown).

Our results confirmed the quite high prevalence of infection of wild roe deer with *H. longicornis* as ectoparasite and *T. luwenshuni* as blood protozoa in Jeju island. Further studies regarding the other vectors, host-parasite relationships, clinical threatening and interaction between domestic and wild ruminants should be undertaken.

Conclusion

We collected ticks and blood samples from 23 roe deer rescued and treated at the Jeju Wildlife Rescue Center. We identified the one species of ticks, *H. longicornis* in roe deer and detected the closely related to *T. luwenshuni* in all blood samples (100%) and 8 pooled ticks (34.8%). These results indicate that there may be a high prevalence particularly of *T. luwenshuni* infection in Jeju wild roe deer and *H. longicornis* is a major vector of these diseases. It suggested that Jeju roe deer may act as reservoirs for these zoonotic pathogens.

Acknowledgments

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제주 노루와 진드기에서 타일레리아 감염 조사

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요 약: 제주도에 서식하는 노루는 육지부 다른 종으로 대부분이 진드기에 심하게 감염되어 있다. 제주노루가 진드기 매개질병에 감염 및 다른 야생동물이나 가축에게 질병을 전파할 수 있다. 이에 본 연구에서는 제주야생동물구조센에서 구조 및 치료되는 제주노루 23마리의 혈액과 진드기를 대상으로 진드기 종을 동정하고 주혈원충 감염을 확인하였다. 구조된 노루의 대부분은 심하게 진드기에 감염되었고 모두가 작은소참진드기(100%)였다. 감염노루 혈액과 진드기를 대상으로 주혈원충에 대한 PCR, RFLP 및 염기서열 분석한 결과 혈액시료 모두(100%)에서 *Theileria luwenshuni*로 확인되었으며, 진드기에서는 23개군중 8개군(34.8%)에서 확인되었다. 제주 노루 감염 진드기는 주로 작은소참진드기며, 이들이 *T. luwenshuni* 매개함을 확인하였다. 그러나 감염에 따른 혈액 및 혈청학적 검사에서 정상소견과 유의적 차이를 보이지 않았다. 앞으로 사람이나 가축으로 질병을 전파할 수 있는 진드기 매개질병 보균숙주로서 가능성이 있음으로 지속적인 조사 관찰이 필요하다.

주요어 : 노루, 작은소참진드기, *Thelieria luwenshuni*, 제주