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Theileria infection with severe anemia and unhealed fracture in a sika deer *Cervus nippon aplodontus* (Cervidae: Cetartiodactyla)

Rie Maruko [a,1], Toshihiro Tokiwa [b,*,1], Junji Nakai [c], Shin-ichi Nakamura [d]

[a] Nara Deer Preservation Foundation, 160-1 Kasuganocho, Nara City, Nara, Japan
[b] Laboratory of Veterinary Parasitology, Nippon Veterinary and Life Science University, 1-7-1 Kyonancho, Musashino City, Tokyo, Japan
[c] United Nara Ayameike Animal Hospital, 2-1-19-1 Hikidacho, Nara City, Nara, Japan
[d] Kyoto Institute of Nutrition & Pathology Inc., 7-2 Furuiketani, Tachikawa, Ujitawaracho, Tsuzuki County, Kyoto, Japan

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ABSTRACT

An adult female sika deer (*Cervus nippon aplodontus*) inhabiting Nara Park, Nara, Japan, had broken bone injuries from a car accident. During its treatment, we found that the sika deer had severe anemia and the fracture remained unhealed throughout. Peripheral blood smear revealed piroplasms in the erythrocytes, which were identified as merozoites of undescribed *Theileria* species, widely found in sika deer in Japan. This is the report of a clinical case of *Theileria* infection, accompanied by severe anemia in a sika deer.

Members of the genus *Theileria* Bettencourt et al., 1907 are apicomplexan parasites, of which domestic and free-ranging mammals are the intermediate hosts, and ticks are the final hosts. Some species, such as *T. cervi, T. capreoli, T. uilenbergi, T. luwenshuni, and T. tarandirangiferis* parasitize animals in the family Cervidae [1–3]. Although most of them are thought to be low pathogenicity lineages, *T. cervi* could cause disease in juvenile, immunosuppressed or malnourished deer [1,4]. In Japan, at least 3 different lineages have been recorded in sika deer (*Cervus nippon* ssp.); (1) undescribed species, including *Theileria* sp. sika1, and *Theileria* sp. Thrivae [1–3,5–10], (2) *Theileria capreoli* group including *T. capreoli*-like, *T.* sp. Sola, and *T.* sp. sika2 [6–8,11], and (3) *T. luwenshuni* group [6,12]. Here, we describe the course of *Theileria* infection in a free-living sika deer (*C. n. aplodontus*) from Nara Park, Japan, and record the pathological, morphological and genetic characteristics of the species infecting it.

A free-living, approximately 12 years old female sika deer from Nara Park, weighing 42 kg, suffered an open fracture of the right metacarpal bone in a traffic accident. The wild sika deer in Nara city is designated as a National Treasure in Japan, and protected by Law for the Protection of Cultural Properties. Therefore, this deer was admitted to the shelter at the Nara Deer Preservation Foundation, Nara, Japan on April 11, 2020 (day 0). The open wound at the site of fracture was small, and no other trauma was noticed. Consequently, the wound was dressed under anesthesia, with twice a week changes, and the fractured bone was set with an external splint. On the day 13, right hind limb lameness developed suddenly, and radiograph revealed an oblique fracture of the right femoral diaphysis. As for this fracture, we decided to wait for spontaneous healing through rest. On the day 23, a new radiograph showed the entirely unhealed fracture of the right metacarpal bone. However, we did not see any lesions nor other clinical signs except for lameness. On the day 58, peripheral blood analyses on a MEK-6458 cell counter (Nihon Kohden, Japan) corrected for sika deer, and automated clinical chemistry analyzer Dri-Chem 4000 V (Fuji Film, Japan), showed a significantly lower hematocrit (Ht) value of 0.128/L (12.8%), low hemoglobin and red blood cell count, and higher serum protein, creatinine and blood urea nitrogen (BUN) content (Supplemental Table S1). Initially, we suspected renal failure, but symptoms, like polydipsia and polyuria were absent. On the day 72, Ht value dropped to 0.070/L (7.0%), and the BUN and creatinine increased slightly. Giemsa stained peripheral blood film revealed erythrocyte aggregation, size inconsistency, basophilic spotted erythrocytes, and piroplasms of various shapes in 10.5% (21/200) of the erythrocytes. Rod-shaped piroplasms being 2–3 μm in length were predominant, round ones 1.5–2.0 μm wide spherical forms and multiplying forms were also observed (Fig. 1). On the day 86, the sika deer showed mild anorexia, reduced body weight (35.4 kg), and completely unhealed fracture. Although the general

* Corresponding author.
E-mail address: tokiwa@nvlu.ac.jp (T. Tokiwa).
[1] These authors contributed equally to this work.

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health was good, blood tests showed falling Ht value of 0.069/L (6.9%) and erythrocytes with persistent piroplasms. On August 11 (day 122), the sika deer died, and it weighed 30.5 kg, with the fractures still unhealed.

Necropsy showed that the sika deer was highly emaciated, with extremely depleted subcutaneous fat. There was clotting blood in the heart, but most of it was plasma component, with very little blood cell component. Pericardial fat showed serous atrophy. Spleen and lymph nodes were also atrophied, and the spleen, liver, and kidneys were severely discolored. In the liver, a few liver flukes (*Fasciola* sp.) were found. Histopathological examination showed very few erythrocytes in various organs, including the heart, spleen, lymph nodes, bone marrow, lung, liver, kidneys, and adrenal glands. Bone marrow had a large number of erythroid and myeloid precursors, and megakaryocytes. Moderate deposition of hemosiderin was visible in the spleen, and fewer lymphocytes in the spleen and lymph nodes. Kidneys had a mild interstitial fibrosis, urinary casts, and thickening of the basement membranes of glomeruli and Bowman's capsules. In the liver, there was severe fibrosis in the portal area, and liver flukes were found in the bile ducts. Lungs had nematode larvae in the alveolar walls, macrophage infiltration in the alveolar space, and pulmonary edema. *Sarcocystis* spp. were found in the myocardium. Because of the unhealed fracture, even with no infection at the site and a stable splint, we presumed that piroplasms induced the anemia, unhealed fracture, and the overall debility, ultimately leading to death due to respiratory failure caused by pulmonary edema.

To confirm the identification of piroplasm, a fragment of its 18S ribosomal RNA (18S) gene was sequenced. Genomic DNA was extracted from the deer blood on the day 72, using QIAmp DNA Blood Mini Kit (Qiagen, Germany). The 18S gene was amplified by PCR using the primer set BTH18S_1stF (5'-GTGAAACTGCGAATGGCTCATTAC-3′) and BTH18S_1stR (5'-AAGTGATAAGGTTCACAAAAGTTCCC-3′) [13]. Reactions were set up using TaKaRa Ex Taq polymerase (TaKaRa, Japan). The amplification program consisted of initial denaturation at 95 °C for 1 min, followed by 35 cycles at 95 °C for 45 s, 55 °C for 45 s, and 72 °C for 1 min, with a final extension step at 72 °C for 2 min. The products were electrophoresed on 2% agarose gel, purified with ExoSaP-IT (Applied Biosystems, USA), and sequenced on a 3730× DNA analyzer (Applied Biosystems), after labeling with BigDye terminator (Applied Biosystems), using PCR primers and sequence primer (InnerSeq: 5′-AGTCTGGTGCCAGCAGC -3′) [13]. The 1564 bp long sequence was deposited in the DNA Data Bank of Japan under accession no. LC623877. Comparison with the sequences available in the International Nucleotide Sequence Databases at the NCBI using BLAST program, showed that the present sequence was novel, and shared the highest similarity (99.87%) with that of an undescribed *Theileria* species, found in the sika deer from Iwate and Yamaguchi, Japan (accession nos. AB602882, AF529273, AF529272), and *T. cervi,* found in the sika deer from China (AF959223-5, HQ184411). The present sequence was aligned with the reference sequence from the public database using MAFFT version 7 with the Q-INS-I setting [14]. Sequences of *T.* sp. Sola (LC060448), and *T.* sp. Thrivae (AB981978, AB981977) were excluded from this analysis, as both the ends were obviously unmatched. Phylogenetic analyses using MEGA X software [15] allowed the construction of a phylogenetic tree using maximum likelihood method according to the Tamura-Nei model, with gamma distribution plus Invariant sites. The reliability of the internal branches was assessed through bootstrap analysis of 1000 replicates. The present sequence formed a monophyletic group with *Theileria* sp. of the sika deer from Yamaguchi, Iwate, and Hokkaido, of the *Haemaphysalis* ticks from Kagoshima, Japan, and *T. cervi* from China with high bootstrap value (95%) (Fig. 2). Phylogenetic trees showed polyphyly of *T. cervi*, consisting of lineages from the sika deer from China (accession nos. KT959223-5, HQ184411) and monophyletic group with those from the white-tailed deer (*Odocoileus virginianus*) and elk (*Cervus canadensis*) from the USA (accession nos. U97054, U97056, AF086804, AY735134, AY735117). As the white-tailed deer are the type host for *T. cervi,* the former lineage detected in China is probably a species misidentification. Based on the present results, the intraerythrocytic piroplasm seen here was identified as undescribed *Theileria* sp. recorded in Japan and China.

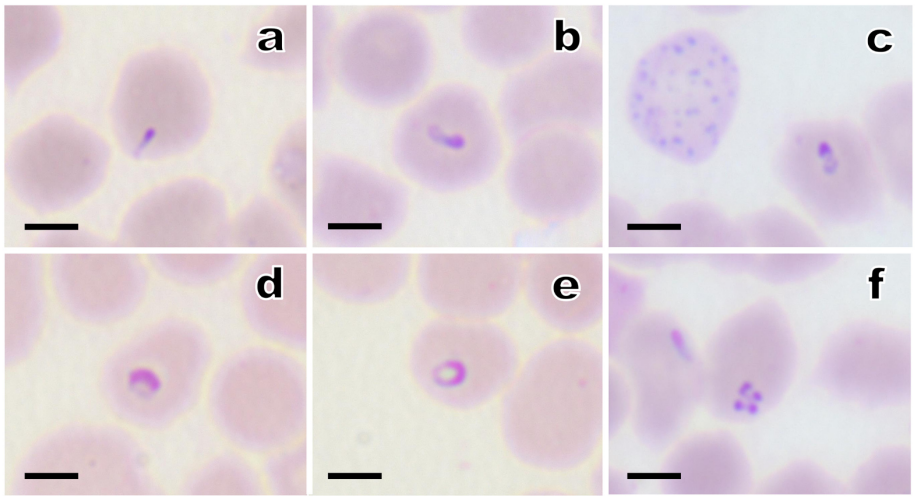

Fig. 1. Piroplasms of *Theileria* sp. in the erythrocyte of a sika deer. A & B. Rod-shaped merozoites. C. Infected erythrocyte and basophilic-stippling erythrocyte. D & E. Spherical piroplasms. F. Multiplying form. Giemsa stain. Bar = 5 μm.

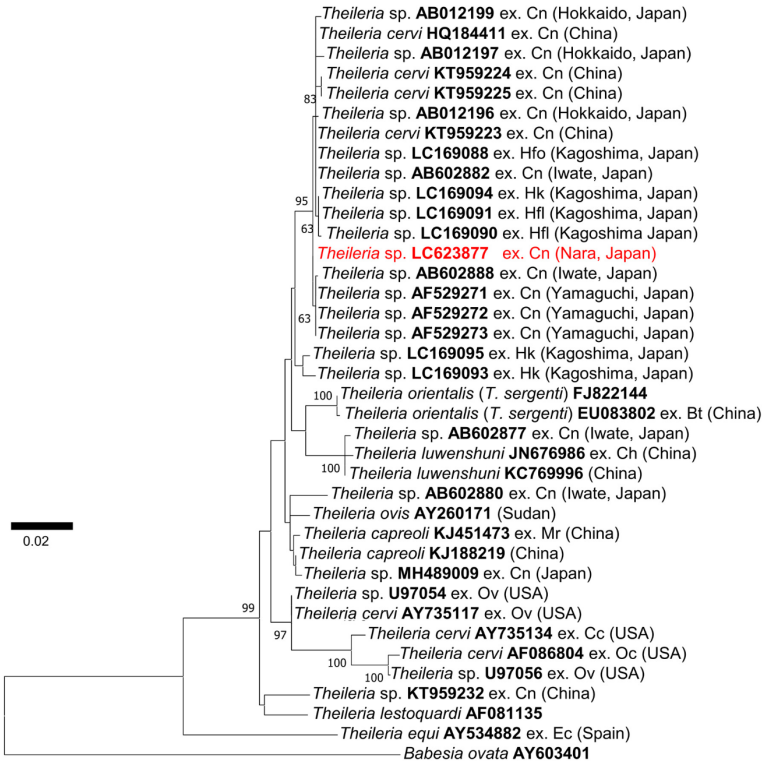

Fig. 2. Phylogenetic tree of cervine *Theileria* and closely related species using 18S gene sequence. *Babesia ovata* was used as an outgroup. Nodes are labelled with support from bootstrap values (60<). Scale bar represent substitution per site. Bt: *Bos taurus,* Cc: *Cervus canadensis,* Ch: *Capra hircus,* Cn: *Cervus nippon,* Ec: *Equus caballus,* Mr: *Muntiacus reevesi,* Ov: *Odocoileus virginianus,* Hfl: *Haemaphysalis flava,* Hfo: *Haemaphysalis formosensis,* Hk: *Haemaphysalis kitaokai.*

Bovine theileriosis caused by *T. parva* or *T. annulata* is characterized by large number of schizont-infected lymphocytes, resulting in leukosis-like disease, and a generalized hyperplasia of lymphoid tissues [1]. Other species can cause subclinical or mild infections, characterized by a rapid multiplication of intraerythrocytic piroplasms, leading to anemia and icterus [1]. Cervine *Theileria* species are essentially low-pathogenic, except in the animals immune-compromised by stress or disease [1,4,16,17], but Bessho & Nakamura (1987) reported clinical theileriosis in a sika deer captured in Kyoto prefecture [18]. White-tailed deer in the USA is also reported to show a large proportion of parasitized erythrocytes (about 70%), with extensive deposition of hemosiderin in the liver and spleen, indicating hemolytic anemia, due to *T. cervi* infection [19]. In the present case, although the lymph nodes were atrophied, and did not have schizonts, clinical findings were consistent with the cervine theileriosis.

Current treatment options for theileriosis are limited. For the domestic animals with severe anemia, symptomatic treatments, such as infusion, are considered, rather than administration of antiprotozoal drugs. Although a previous study reported a good response to 8-aminoquinoline preparation in the sika deer with *Theileria* infection [18], unfortunately, due to the discontinuation of domestic production and sales, this drug was not available. Diminazen aceturate, which is used for bovine theileriosis in Japan, may be one of the options for cervine theileriosis. However, we chose not to use this drug because no data is available on its use and adverse effect in the sika deer.

A closely related lineage of *Theileria* has been reported in *Haemaphysalis* ticks, *H. kitaokai, H. flava,* and *H. formosensis*, from other parts of Japan [13]. There are about 1300 free-living sika deer in Nara Park, and *Haemaphysalis* ticks are commonly seen on them [20], suggesting that *Theileria* infection is easily established in the area. Detailed study of the status of *Theileria* infection among the sika deer and the ticks, will help in devising strategies to control and treat the infection.

Declaration of Competing Interest

None.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.parint.2021.102349.

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