Title:

Molecular detection of *Blastocystis* sp. subtype 14 in the Yezo sika deer (*Cervus nippon yesoensis*) in Hokkaido, Japan

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Abstract

This study describes the first report of *Blastocystis* sp. colonization in the sika deer (*Cervus nippon*) in Japan and in other animals in Hokkaido, Japan. *Blastocystis* sp. is one of the most widespread intestinal protist in a wide range of animals. *Blastocystis* sp. isolated from mammalian and avian species have been classified into 17 subtypes (STs). Some of the STs are zoonotic. The aim of this study was to evaluate *Blastocystis* sp. colonization in the Yezo sika deer (*Cervus nippon yesoensis*) in Hokkaido, Japan. The Yezo sika deer are currently overabundant and they are expanding their habitat to humans and livestock. A total of 132 deer fecal samples were subjected for molecular detection of *Blastocystis* sp.. Of these, 60 (45.5%) samples were positive using PCR, which targets the small subunit ribosomal RNA gene sequence. All *Blastocystis* sp. DNA sequences from the Yezo sika deer were genotyped into ST14, which were originally reported in cattle. These findings indicate that the current public health risks of *Blastocystis* sp. from the Yezo sika deer is low, although more detailed future analysis is required.

Keywords

Blastocystis sp., Yezo sika deer, subtype 14

40 *Blastocystis* sp. is an intestinal protist that can colonise a wide range of host
41 species from insects to mammals (Yoshikawa et al., 2016). Although asymptomatic
42 cases are common, it has been reported that *Blastocystis* colonization relates irritable
43 bowel syndrome (IBS) and symptoms such as diarrhea, constipation, abdominal pain
44 and flatulence in human patients (Dogruman-Al et al., 2009). *Blastocystis* sp. isolated
45 from mammalian and avian species have been classified into subtypes (STs) based on
46 the sequencing of the small subunit ribosomal RNA (SSU rDNA) gene (Alfellani et al.,
47 2013; Ramirez et al., 2016; Stensvold and Clark, 2016). At present, 17 known STs have
48 been reported. The host specificity of *Blastocystis* sp. seems to be low. ST1 to ST9 and
49 ST12 have been identified in humans (Cian et al., 2017; Ramirez et al., 2016; Rene et
50 al., 2009). Most of these STs in humans have also been detected in non-human hosts,
51 and *Blastocystis* sp. is considered as a zoonotic protist parasite (Stensvold and Clark,
52 2016).
53 The Yezo sika deer (*Cervus nippon yesoensis*) is the biggest wild ruminant
54 found on Hokkaido island, Japan. The population of the Yezo sika deer is overabundant
55 because the wolves, their natural predator, were endangered and there are no effective
56 population control measures put in place by the government. The current population of
57 the Yezo sika deer is estimated to be approximately > 650,000 in Hokkaido (Suzuki,

2019). This overabundant population can harm the ecosystem, and the Yezo sika deer is expanding its habitat into human populations. Furthermore, they continue to cause considerable damage, especially to livestock, crops, and forests (Takatsuki, 2009). These situations have resulted in inter-species infections by various kinds of pathogens from the Yezo sika deer to humans or livestock (Tei et al., 2003; Trimmel and Walzer, 2020). Therefore, it has become important to clarify the pathogen species carried by deer to prevent deer-borne infections in humans and livestock.

We aimed to determine whether the Yezo sika deer were colonised with *Blastocystis* sp., and which STs were dominant. To clarify these issues, we conducted a molecular epidemiological survey using individual traceable rectal fecal samples of the Yezo sika deer in the eastern part of Hokkaido.

From 2016–2017, 132 rectal fecal samples were collected from the Yezo sika deer hunted in the eastern part of Hokkaido, mainly in the Tokachi sub-prefecture (Table 1). The ELEZO Company hunters acted in accordance with the general hunting license. The hunters recorded the sex, age, date of the hunt, and location of the deer. Rectal fecal samples were collected within 2 h after hunt and stored at -30˚C until DNA extraction. DNA was extracted from approximately 0.2 g of a fecal sample using the

PureLink™ Microbiome DNA Purification Kit (Thermo Ficsher Scientific, Waltham, MA, USA). All DNA samples were adjusted to 5 ng/μl for the subsequent analysis.

Blastocystis-specific primers RD5 (ATCTGGTTGATCCTGCCAGT) and BhRDr (GAGCTTTTTAACTGCAACAACG) were used for parasite detection using Taq DNA polymerase (New England Biolabs, Ipswich, MA, USA) based on the study conducted by Scicluna *et al*. (Scicluna et al., 2006). PCR products were analyzed using agarose gel electrophoresis. In the PCR analysis, 60 of the 132 showed approximately 600-bp clear positive band representing the 18s small subunit ribosomal RNA of *Blastocystis*. All PCR positive products were subjected to sequencing and BLAST analyses using RD5 primer to confirm the real *Blastocystis* sp. positive. The positive ratio of the *Blastocystis* sp.was 45.5% (Table 1). There was no significant difference in positive rates when the areas were compared using the chi-square test.

Eight PCR positive samples were randomly chosen and subjected to sequencing analysis. *Blastocystis*-positive PCR products were purified using the QIAEX II Gel Extraction Kit (Qiagen, Hilden, Germany), and subjected to direct sequencing analysis using the RD5 and BhRDr primers and the BigDye™ Terminator v3.1 Cycle Sequencing Kit (Thermo Fisher Scientific). The deer *Blastocystis* sp. sequences obtained were compared using ClustalW. A phylogenetic tree was

93 constructed using the MEGA7 software (Pennsylvania State University, State College,
94 PA, USA). We performed the analysis using the neighbor-joining method and the
95 maximum composite likelihood substitution model. The Yezo sika deer *Blastocystis* sp.
96 sequence obtained was deposited in the GenBank database under accession number
97 MT373685. The eight sequences were identical in ClustalW comparison. In the
98 phylogenetic tree analysis, the *Blastocystis* sp. sequence derived from the Yezo sika
99 deer was clustered with the *Blastocystis* ST14 (Figure 1) (Fayer et al., 2012).
100 We demonstrated the presence of *Blastocystis* sp. in the Yezo sika deer using
101 molecular epidemiological survey. This is the first report describing *Blastocystis* sp.
102 colonization in Hokkaido and in the sika deer in Japan. The ST14 constituted majority
103 of the *Blastocystis* sp. from the Yezo sika deer. Masuda et al. reported that *Blastocystis*
104 ST14 was the major ST detected in cows of Kanagawa prefecture, Kanto district, Japan
105 (Masuda et al., 2018). Additionally, Masuda et al. reported that approximately half of
106 the *Blastocystis* sp.-positive cattle had breeding history in Hokkaido. This information
107 suggests that some of the cattle in Hokkaido are also colonised with *Blastocystis* ST14,
108 which was transferred to the Kanagawa prefecture via cattle transportation, although
109 there is no study describing *Blastocystis* sp. colonization in cattle in Hokkaido.
110 Therefore, *Blastocystis* sp. colonization in cattle in Hokkaido should be addressed in

111 future studies to clarify the ecology of *Blastocystis* sp. colonization in ruminants in
112 Hokkaido and in Japan as a whole.
113 *Blastocystis* ST14 was originally isolated from cattle (Fayer et al., 2012). There
114 is, however, no report describing the detection of ST14 in human patients to date. This
115 study was conducted in a limited period and was limited to the region of eastern
116 Hokkaido, mainly in the Tokachi sub-prefecture. To evaluate the comprehensive risk of
117 *Blastocystis* sp. colonization in animals, including humans, livestock, and wildlife in
118 Hokkaido, a continuous survey of *Blastocystis* sp. colonization in the Yezo sika deer
119 and other animals is required.
120 We did not isolate the *Blastocystis* parasite from the fecal samples using *in*
121 *vitro* culture in this study. We determined the Yezo sika deer *Blastocystis* STs using
122 direct sequencing analysis of the PCR products derived from the DNA samples
123 extracted from the feces of the deer. This molecular subtyping strategy is not suitable
124 for the detection of low population STs in a multiple-colonization case. The amplicon
125 derived from rare STs is masked by the sequences derived from the major STs.
126 Furthermore, the use of DNA from feces is not suitable for a more detailed molecular
127 characterization of the *Blastocystis* sp. because of the low ratio of *Blastocystis*-derived
128 DNA in the DNA samples. Therefore, in future studies, isolation and analysis of the

Blastocystis sp. parasite itself, obtained by *in vitro* culture although it is known that *in vitro* culture does not work well from ruminants, from the fecal samples should be employed to better understand the actual state of *Blastocystis* sp. colonization in the - Yezo sika deer.

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Figure caption

Figure 1. ST identification in the *Blastocystis* sp.-positive samples through sequencing and phylogenetic analysis.

The phylogenetic tree was constructed using the neighbor-joining method with the maximum composite likelihood model following *Blastocystis* 18S SSU rDNA sequence analysis. The percentage of replicate trees, in which the associated taxa clustered together in the bootstrap test (1000 replicates), has been shown next to the branches. The *Blastocystis* sp. sequence (MT373685) obtained from the Yezo sika deer in this study is shown in bold. Sequences of *Blastocystis* sp. isolated from the cattle in Kanagawa prefecture, Japan (Masuda et al., 2018) are indicated by •. *Eimeria tenella* has been used as an outgroup.