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Title	Epizootiological survey of Trichinella spp. infection in carnivores, rodents and insectivores in Hokkaido, Japan
Author(s)	Kanai, Yuta; Inoue, Takashi; Mano, Tsutomu et al.
Citation	Japanese Journal of Veterinary Research, 54(4), 175-182
Issue Date	2007-02-28
DOI	https://doi.org/10.14943/jjvr.54.4.175
Doc URL	https://hdl.handle.net/2115/20016
Type	departmental bulletin paper
File Information	JJVR54-4-175.pdf

Hokkaido University Collection of Scholarly and Academic Papers : HUSCAP

Jpn. J. Vet. Res. 54(4) : 175-182, 2007

FULL PAPER

Epizootiological survey of *Trichinella* spp. infection in carnivores, rodents and insectivores in Hokkaido, Japan

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(Accepted for publication : January 4, 2007)

Abstract

In order to evaluate the present epidemiological situation of *Trichinella* infection in wild animals in Hokkaido, Japan, red foxes (*Vulpes vulpes*), raccoon dogs (*Nyctereutes procyonoides*), brown bears (*Ursus arctos*), martens (*Martes melampus*), rodents and insectivores captured in Hokkaido were examined for muscle larvae by the artificial digestion method from 2000 to 2006. Foxes (44/319, 13.8%), raccoon dogs (6/77, 7.8%) and brown bears (4/126, 3.2%) were found to be infected with *Trichinella* larvae and all other animal species evaluated were negative. Multiplex PCR and DNA sequencing revealed that larvae from a fox captured in Otofuke, in south-eastern Hokkaido, were *T. nativa*, and larvae from 27 animals including 21 foxes, 2 raccoon dogs and 4 brown bears captured in western Hokkaido were *Trichinella* T9.

Key Words : Epizootiology wild animals, Japan, *Trichinella nativa, Trichinella* T9, Zoonosis

Introduction

Trichinellosis is a zoonotic disease caused by nematodes of the genus *Trichinella*. Numerous mammals as well as birds and reptiles are known to harbor this parasite in their muscles. So far, the genus *Trichinella* is classified into eight species (*T. spiralis, T. nativa, T. britovi, T. pseudospiralis, T. murrelli, T. nelsoni, T. papuae* and *T. zimbabwensis*) and three genotypes (*Trichinella* T6, *Trichinella* T8 and *Trichinella* T9)[14)]. Since the discovery

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of *T. spiralis* in 1835, human trichinellosis had been considered to be associated with the consumption of pork[2]. However, the recent advances in the molecular techniques could reveal the presence of sylvatic sycles and sylvatic *Trichinella* containing *Trichinella* spp. other than *T. spiralis*. Today, it is known that *T. spiralis* is maintained mainly in domestic swine and the sylvatic *Trichinella* are maintained in the wild animals[17].

In Japan, the first case of trichinellosis was reported in a domestic dog in Hokkaido prefecture in 1957[11], thereafter, there have been three human outbreaks. The first occurred among local hunters in Aomori prefecture in 1974[22], caused by the consumption of black bear (*Ursus thibetanus*) meat, and the second and third outbreaks occurred at the restaurant in Hokkaido prefecture in 1979 and in Mie prefecture in 1981, respectively, caused by the consumption of brown bear (*U. arctos*) and black bear meat[22]. Since 1974, many wild animals have been examined to disclose the epizootiology of *Trichinella* infection in Japan ; however, by 1998, only two black bears[22], one red fox (*Vulpes vulpes*)[9] and one raccoon dog (*Nyctereutes procyonoides*)[20] in the northern part of mainland Japan, were found to harbor *Trichinella* muscle larvae.

In Hokkaido, the northern island of Japan, several kinds of mammals, including 198 foxes and 89 brown bears, were examined for *Trichinella* infection before 1999 but no animals were found to be infected[22,23]. In 1999, 5 of 43 (11.6%) red foxes examined were found to be infected with *Trichinella* larvae in Otaru, Hokkaido[24]. In addition, the presence of *T. nativa* and *Trichinella* T9 in Hokkaido was reported in 2006[6]. However, before the present study, only the six cases of *Trichinella* T9 from foxes in Otaru and Sapporo and one case of *T. nativa* from fox in Otofuke were reported in the limited area of Hokkaido. In this study, we investigated the prevalence of *Trichinella* infection in wild animals on a large scale and discussed the distributional pattern of *T. nativa* and *Trichinella* T9 in Hokkaido.

Materials and Methods

Animals and parasitological examination

From 2000-2006, 525 carnivores, including 319 red foxes (*V. vulpes*), 77 raccoon dogs (*N. procyonoides*), 126 brown bears (*U. arctos*) and 4 martens (*Martes melampus*) were shot or trapped by local hunters to prevent agricultural losses or for academic surveys in Hokkaido prefecture, Japan (Fig. 1). A total of 344 rodents and 27 insectivores were also trapped in this study (Table 1). Most of the foxes, rodents and insectivores and all of the raccoon dogs were captured in Otaru and Sapporo ; other animals were captured elsewhere in Hokkaido. Foxes, raccoon dogs and martens captured in Otaru were frozen at −80℃ for at least one week prior to muscle sampling in order to sterilize the eggs of *Echinococcus multilocularis* that are prevalent in Hokkaido. Some brown bears were frozen at around −30℃ for preservation prior to transportation to our laboratory. The other foxes and brown bears were delivered to the laboratory at low temperature but not frozen. Rodents and insectivores were examined as fresh samples.

Muscles were collected from the hind legs or tongue of all the animals, except for rodents and insectivores, from which the whole diaphragm, tongue and masseter were collected. At least 10 g of the muscles of carnivores or all of the collected muscles of rodents and insectivores (approximately 1-2g) were digested with artificial digestion fluid (200 ml of NaCl saline containing 1% pepsin and 1% HCl) at 37℃ for 2 hours according to a standard procedure[3]. Motile larvae detected were

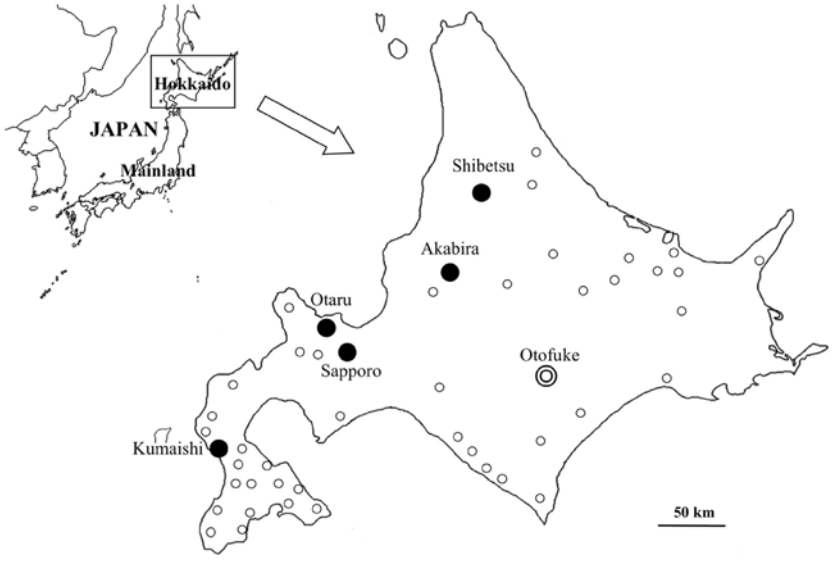

Fig. 1. Geographical distribution pattern of *Trichinella nativa* and *Trichinella* T9 in Hokkaido, Japan. The localities where foxes, raccoon dogs or brown bears were examined for *Trichinella* spp. are indicated by filled symbols (*Trichinella* T9 positive), double circle (*T. nativa positive*) or open circles (*Trichinella* not detected).

Table 1 Prevalences of *Trichinella* spp. infection in wild animals in Hokkaido, Japan from 2000-2006.

Host animals	Sampling places (no. positive / no. examined)			
	Otaru city	Sapporo city	Other areas	Total (%)
Carnivora				
Red fox (*Vulpes vulpes*)	41 / 254	1 / 39*	2 / 26*	44 / 319 (13.8)
Raccoon dog (*Nyctereutes procyonoides*)	6 / 77			6 / 77 (7.8)
Brown bear (*Ursus arctos*)		0 / 1	4 / 125	4 / 126 (3.2)
Japanese marten (*Martes melampus*)	0 / 4			0 / 4
Rodentia				
Gray red-backed vole (*Clethrionomys rufocanus*)	0 / 62	0 / 72		0 / 134
Northern red-backed vole (*Clethrionomys rutilus*)	0 / 3	0 / 4		0 / 7
Large japanese field mouse (*Apodemus speciosus*)	0 / 114	0 / 43		0 / 157
Small japanese field mouse (*Apodemus argenteus*)	0 / 28	0 / 13		0 / 41
Brown rat (*Rattus norvegicus*)		0 / 3		0 / 3
Black rat (*Rattus rattus*)		0 / 2		0 / 2
Insectivora				
Long-clawed shrew (*Sorex unguiculatus*)	0 / 12	0 / 14		0 / 26
Shrewmouse (*Sorex caecutiens*)	0 / 1			0 / 1

* One fox each captured in Sapporo and Otofuke[6] were included.

inoculated orally into gerbils for serial passage. Gerbils were kept in our laboratory under the Guidelines for Animal Experiments of the Graduate School of Veterinary Medicine in Hokkaido University.

Host age determination

The ages of foxes captured in Otaru were determined by counting the number of canine cementum annuli[10]. Extracted canines were cut into 3 mm pieces using a microcutter (Maruto, MC-201) and decalcified with Plank-Rychro solution (8.5% hydrochloric acid, 7% alumini chloridum and 5% formic acid in distilled water) for 48 hours. The canines were then deacidified with 5% sodium sulfate for 24 hours and washed in tap water for 24 hours. The decalcified canines were cut into 45 μm pieces using a freezing microtome. Sections were stained with Delafield's hematoxylin, mounted in Canada balsam and the number of annuli were counted under a microscope.

The raccoon dogs captured in Otaru and the foxes captured in Sapporo were divided into juveniles (<1 year) and adults (≥1 year) by the dental formula method as previously described[5,21].

Worm preparation and DNA extraction

Since our preliminary examination demonstrated that it was difficult to yield the PCR amplicon from DNA obtained from dead larvae after the artificial digestion of frozen muscles, worms were collected directly from muscles to avoid artificial digestion in this study. The presence of *Trichinella* spp. muscle cysts was confirmed by pressing *Trichinella*-infected muscles using Petri dishes. Muscle larvae were then collected with forceps and needles under a dissection microscope. Collected larvae were individually preserved in Tris-EDTA buffer at −30℃ until use. DNA was extracted from single larvae according to the previously described method[1]. Briefly, individual larvae were placed in a 200 μl tube containing 2 μl of 5mM Tris-HCl, overlaid by mineral oil and heated at 90℃ for 10 minutes. To the tube was added 1 μl of proteinase K (1 μg/μl, Takara) and 2 μl of water, followed by incubation at 37℃ for 2 hours. After incubation, the tube was heated at 90℃ for 10 minutes to inactivate the enzyme and preserved at −30℃ until use.

Trichinella larvae from 28 infected animals were subjected to molecular identification. Four individual larvae from each animal were analyzed separately by Multiplex PCR and DNA sequencing of the mitochondrial cytochrome oxydase subunit I (COI) gene as described previously[6,25].

Statistical analysis

Among the 254 foxes captured in Otaru in 2000, 2001 and 2004, 206 of predetermined sex and age were analyzed for the risk factors of *Trichinella* infection by logistic regression model. Sex and age of host and the year of capture were set as independent variables. Statistical analyses were performed using StatView® 5.0 (SAS Institute Inc.).

The prevalence of *Trichinella* larvae in adult foxes in Otaru and Sapporo was statistically analyzed by Fisher's exact test using the R software package version 2.0.1 (http : // www.r-project.org/). These two cities are located next to each other in Hokkaido. Foxes in Sapporo were captured on a plain where farms, houses and factories were scattered. Foxes in Otaru were mainly captured on cropland at the foot of wooded hills.

P-values ≤0.05 were considered statistically significant.

Results

A total of 44 foxes (infection rate = 13.8%), 6 raccoon dogs (7.8%) and 4 brown

bears (3.2%) were found to be infected with *Trichinella* spp. and all other animal species examined were negative (Table 1). Among the 54 infected animals, 47 were found in Otaru. Although no motile larvae were obtained from frozen samples, motile larvae were collected from non-frozen samples of three red foxes captured in Sapporo, Shibetsu and Otofuke, and two brown bears in Akabira. Gerbils inoculated with motile larvae were sacrificed a few months later and *Trichinella* larvae were collected from the muscle by artificial digestion.

Among the 254 samples of foxes captured in Otaru, 206 were of predetermined sex and age. Of these 206 foxes, none of the 60 juveniles were infected with *Trichinella* larvae, whereas 21.2% (31/146) of adult foxes were infected. The logistic regression model showed that age was the only significant variable associated with the prevalence and prevalence increased along with host age (odds ratio=2.006, 95% CI=1.501-2.681, $p<0.001$).

The prevalence of *Trichinella* infection in adult foxes in Otaru (20.9%, 31/148) was significantly higher than that in Sapporo (4.5%, 1/22) (Fisher's exact test, $p<0.05$).

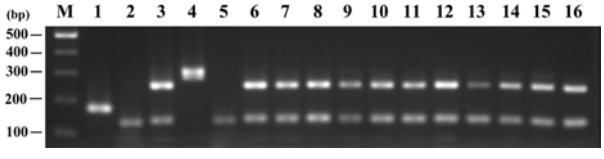

Fig. 2. Multiplex PCR products of *Trichinella* muscle larvae from various animals in Hokkaido, Japan. Lane 1: *T. spiralis* reference larva (ISS 413), lane 2: *T. nativa* reference larva (ISS 10), lane 3: *Trichinella* T9 reference larva (ISS 408), lane 4: *T. pseudospiralis* reference larva (ISS 13), lane 5: *T. nativa* from red fox (*), lane 6-12. *Trichinella* T9 from fox, lane 13-14: *Trichinella* T9 from raccoon dog, lane 15-16: *Trichinella* T9 from brown bear, M: 100bp DNA ladder.
*The result of *T. nativa* was reported previously[6].

On agarose gel electrophoresis of multiplex PCR amplicons, the muscle larvae detected from 21 foxes, 2 raccoon dogs and 4 brown bears showed two bands of 127 bp and 253 bp (Fig. 2, lanes 6-16), a specific pattern of the *T. britovi* complex (*T. britovi, Trichinella* T8 and *Trichinella* T9)[15]. The nucleotide sequence of part of the COI gene of larvae belonging to the *T. britovi* complex showed the highest similarity to *Trichinella* T9. As reported previously, the muscle larvae from a fox in eastern Hokkaido identified as *T. nativa*[6], but all of the present muscle larvae detected from 27 animals in western Hokkaido were identified as *Trichinella* T9 (Fig. 1). Among these 27 *Trichinella* T9 muscle larvae DNA sequences of 26 muscle larvae were identical while the other sample showed a single nucleotide difference. The former was completely identical to previously reported sequences of *Trichinella* T9 (DQ007898, AB091477) isolated in mainland Japan.

Discussion

The present study demonstrated that *Trichinella* infections were prevalent among foxes, raccoon dogs and bears in Hokkaido and *Trichinella* T9 distributed widely in the western part of Hokkaido.

Until 1999 when a relatively high prevalence of *Trichinella* infection among the fox population in Otaru city (11.6%, 5 out of 43 foxes) was reported[24], the prevalence of *Trichinella* infection in wild animals in Japan was considered low[22]. The present work demonstrated that the report in Otaru in 1999[24] was not a temporal phenomenon but a high prevalence was maintained in foxes and raccoon dogs in Hokkaido. In studies carried out about 20 years ago, *Trichinella* infections were not detected among 198 foxes and 88 brown bears examined in Hokkaido[22,23]. The previous researchers examined small portions

(approximately 5 g) of masseter muscles that were considered the common muscle site for *Trichinella* detection based on the study of pigs and rodents ; however, the site was later shown not to be a preferable site for *Trichinella* detection in carnivores[7]. Above mentioned defects of sampling in the previous studies might cause the underestimation of *Trichinella* infections in Hokkaido. The 198 foxes in the previous study were captured in areas different from in the present study, only the 3 foxes were captured in Otaru, in which most of the foxes were investigated in this study. The present result indicated that the prevalence differed in each sampling area (Table 1). Therefore, it is difficult to compare directly the present result with previous studies.

Statistical analysis by a logistic regression model showed that the prevalence of *Trichinella* infection in foxes increased with host age. Similar observations were reported in polar bears and lynxes and were assumed to be related with the increase of opportunities for acquiring *Trichinella* infection, and long survival of the larvae in the muscle[12, 18, 26].

The difference in the prevalence of *Trichinella* infection among foxes in Otaru and Sapporo is related to their food differences. The foxes captured in Sapporo were nesting in the anthropogenic structures, such as farms, barns or houses, and were considered to depend on more human products for their food compared with the foxes captured in Otaru, which were nesting near cropland[4]. In the Otaru area, there are more wild animals and animal cycles of *Trichinella*, such as fox-fox, -raccoon dog, -bear, or-vole transmission may maintain stably.

Besides foxes and raccoon dogs, four brown bears were infected with *Trichinella* T9. Before this study, only one case of *Trichinella* sp. infection in brown bear had been reported in Japan[22]. So far, bear meat has been the exclusive means of transmission for human trichinellosis in Japan (excluding suspected or imported cases). Recently, the cases of human trichinellosis associated with non-pork products increased in the United States and during 1997-2001, 51% of the cases were associated with bear meat[19]. Although the observed prevalence in brown bears was lower than that of foxes, bear meat seemed to be a more important source of human trichinellosis when considering Japanese dietary habits. In Japan, hunters and their relatives tend to eat bear meat[22, 23], but, as to the foxes, Japanese do not have a traditional culture for eating fox meat, although the human trichinellosis caused by consumption of fox meat was reported in Italy[16]. In addition, the large mass of brown bears may serve as a infectious source of infection for large numbers of humans even if the actual prevalence of the disease is low. Game meats, such as bears and deer which are possibly harboring the *Trichinella* larvae must be cooked well, since the freezing is not efficient to inactivate the *T. nativa* larvae, which was reported to survive at −18℃ for 4 years[8].

Trichinella nematodes from 23 red foxes, 2 raccoon dogs and 4 brown bears in 5 localities in western Hokkaido were identified as *Trichinella* T9, whereas the nematode from the south-eastern part was identified as *T. nativa* (Fig. 1). The distribution pattern of *Trichinella* spp. in the northern hemisphere is known to be separated according to climate zones[13]. Since the number of samples examined in this study was limited, it could not show whether the distribution of *T. nativa* and *Trichinella* T9 were separate or overlapped. Further survey, especially in eastern districts would elucidate the distribution pattern of *Trichinella* spp. in Hokkaido.

Acknowledgements

The authors would like thank local hunters and the late Mr. Takeshi Ohde for providing wildlife materials ; Dr. Masatsugu Suzuki (Hokkaido University) for his instruction of fox age determination ; Ms. Kana Yamamoto (Hokkaido Institute of Environmental Sciences) for her patience in preparing brown bear samples ; and Dr. Sumiya Ganzorig (Forum on Environment and Animals) for his valuable suggestions for the identification of the nematode. This research was supported in part by a grant for Research on Emerging and Re-emerging Infections Diseases, Ministry of Health, Labor and Welfare, Japan

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