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Title	Prevalence and intensity of <i>Echinococcus multilocularis</i> in red foxes (<i>Vulpes vulpes schrencki</i>) and raccoon dogs (<i>Nyctereutes procyonoides albus</i>) in Otaru city, Hokkaido, Japan
Author(s)	YIMAM, Alebel Ewunetu; NONAKA, Nariaki; OKU, Yuzaburo et al.
Citation	Japanese Journal of Veterinary Research, 49(4), 287-296
Issue Date	2002-02-28
DOI	https://doi.org/10.14943/jjvr.49.4.287
Doc URL	https://hdl.handle.net/2115/2926
Type	departmental bulletin paper
File Information	KJ00002400404.pdf



Prevalence and intensity of *Echinococcus multilocularis* in red foxes
(*Vulpes vulpes schrencki*) and raccoon dogs
(*Nyctereutes procyonoides albus*) in Otaru city, Hokkaido, Japan

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(Accepted for publication : January 9, 2002)

Abstract

A survey was done in an attempt to investigate the epidemiological status of *Echinococcus multilocularis* in red foxes and raccoon dogs in Otaru city from June to September 1999. Sixty-seven red foxes (*Vulpes vulpes schrencki*) and 13 raccoon dogs (*Nyctereutes procyonoides albus*) were captured, and postmortem examinations were conducted with them. Thirty-eight red foxes (56.7%) and 3 raccoon dogs (23.1%) were found to be infected with *E. multilocularis*. The total biomass of *E. multilocularis* in all infected red foxes and raccoon dogs were 2,817,000 and 1,515 worms, respectively. Nine of the infected red foxes harboring more than 100,000 worms accounted for 90.6% of the total biomass. No significant differences in the prevalence were observed between male and female, and juvenile and adult. However, the worm burden was higher in juvenile than in adult foxes. In one of the infected raccoon dogs, mature worms and eggs of *E. multilocularis* were found in the intestine and fecal sample, respectively. This result suggested that the raccoon dogs are probably playing a small role in the egg contamination of the environment. The validity of coproantigen ELISA for diagnosis of foxes was confirmed by comparing the results of autopsy, egg examination and coproantigen ELISA using rectal fecal samples.

Key words : *Echinococcus multilocularis*, ELISA, Japan, *Nyctereutes procyonoides albus*, raccoon dog, red fox, *Vulpes vulpes schrencki*

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Introduction

During the 90's, urban foxes had been reported in European countries^{1,2,13,20} and Japan¹⁸. In recent years, they are commonly seen in urban areas with their cubs, breeding in public parks and private gardens². Consequently, they probably contaminate the soil of gardens and sandpits in public parks with eggs of parasites, playing a role in the transmission of zoonotic parasitic diseases such as alveolar echinococcosis². In fact, the occurrence of *E. multilocularis* in urban foxes has been reported from European countries^{2,13} and Japan, including Sapporo^{7,18}. Red foxes (*Vulpes vulpes schrencki*) distribute throughout the island of Hokkaido, and their population is expected to increase^{4,11}. In recent years they have begun to inhabit the center of urban area¹⁸ and are frequently in contact with the human population¹⁰. Five to nineteen human cases of alveolar echinococcosis occurred each year in Hokkaido and a patient from the urban area was reported in Sapporo city¹⁸. However, the route of transmission to those patients has not yet been elucidated. It is thought that red foxes play a major role as the definitive host in Hokkaido¹⁴. Moreover, a survey of *E. multilocularis* in urban fox feces has revealed contamination with eggs in the central and peripheral areas of Sapporo city¹⁸. On the other hand, raccoon dogs (*Nyctereutes procyonoides albus*) have been reported as a carrier of *E. multilocularis* in Hokkaido²¹, however, their role in the transmission of the parasite has not been clarified.

At present conditions in which the wild carnivores are adapting to urban areas, it is an urgent requirement to determine the epidemiological status of *E. multilocularis* in wild carnivores, foxes and raccoon dogs. We carried out a survey of *E. multilocularis* in foxes and raccoon dogs in Otaru city which is

adjacent to Sapporo city.

In our laboratory, coproantigen ELISA has been developed for coprological diagnosis of *E. multilocularis* infection in foxes. This study was also conducted to confirm the validity of coproantigen ELISA for foxes by comparing the results of autopsy and fecal egg examination.

Materials and Methods

Host carcass processing and postmortem examination

Sixty-seven red foxes (35 females and 32 males ; 31 juveniles and 36 adults) and 13 raccoon dogs (6 females and 7 males ; 8 juveniles and 5 adults) were captured in different sites of Otaru city (Fig. 1) between June and September 1999. The animals were captured in order to mitigate agricultural loss as a result of their foraging and trampling. The carcasses were frozen at -80°C for more than 10 days and at -30°C for a month. Before postmortem examination, each carcass was allowed to thaw over night at room temperature. The small intestine was removed and divided into 6 parts of equal length (I-VI segments, proximal to distal part of the small intestine), and each part was then cut longitudinally and the intestinal mucosa was scraped. The scraped material from each segment was sedimented 3 - 4 times with saline (0.85%) for 15 min each time using a 200 ml-glass bottle. The whole sediment was examined for a segment with less than 100 worms/ 50 ml of the 200 ml sample. If a higher number of worms (>100 worms/ 50 ml) present, the worm burden was calculated from the count of one aliquot.

Fecal sample collection and preparation

Rectal fecal samples were collected from each animal and 0.5 g of each sample was separately placed in a 15 ml test tube, and heated at 70 °C overnight. Five ml of 1% for-

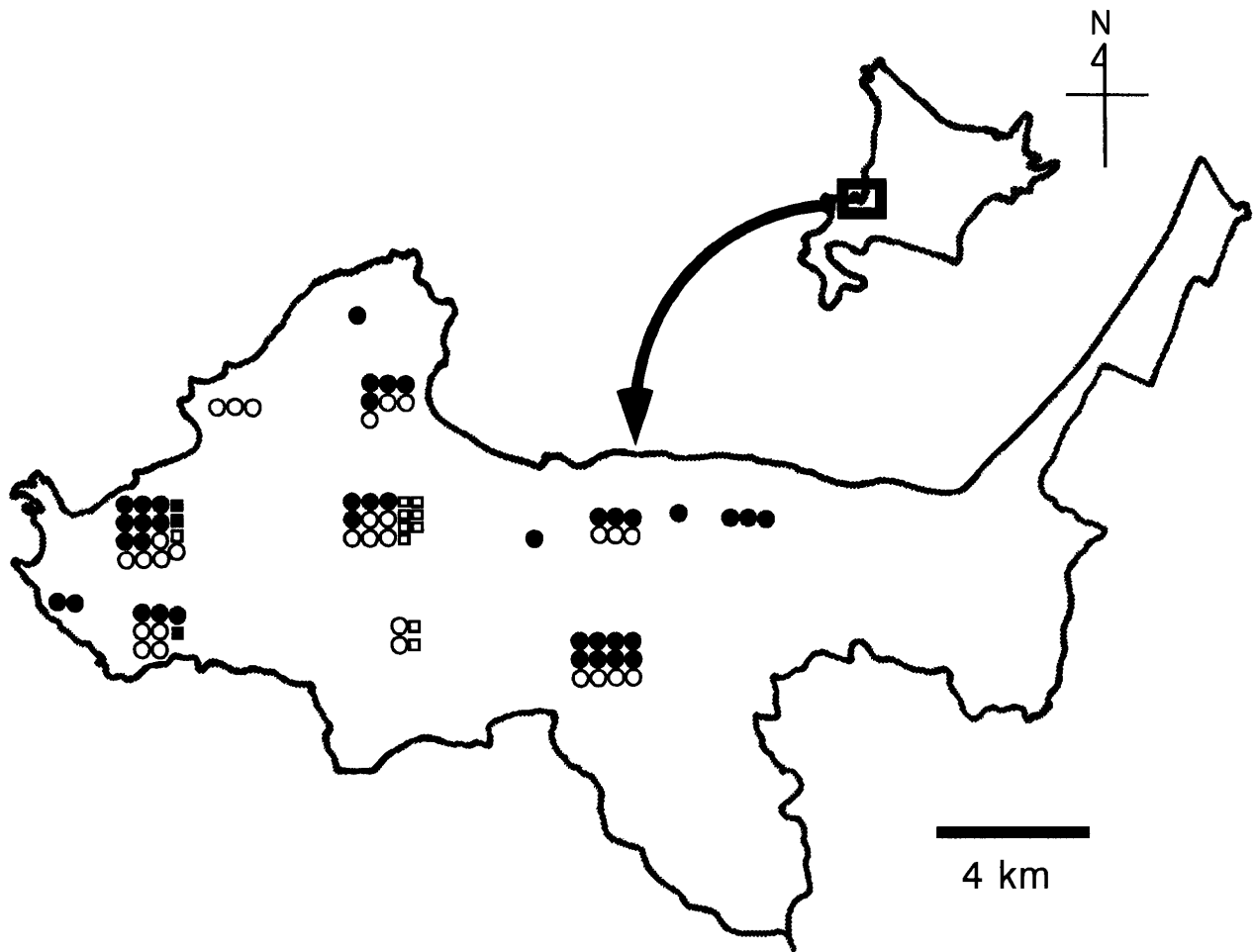


Fig. 1. Localities of *E. multilocularis*-positive and -negative red foxes and raccoon dogs in Otaru city. *E. multilocularis*-positive red foxes (●) ; *E. multilocularis*-negative red foxes (○), *E. multilocularis*-positive raccoon dogs (■) ; *E. multilocularis*-negative raccoon dogs (□)

malin solution containing 0.3% Tween - 20 (formalin-Tween) was added and shaken vigorously using a vortex shaker and then more formalin-Tween solution was added to the final volume of 15 ml. The samples were then centrifuged at 1,200 x g for 10 min. The supernatant was collected and stored at room temperature until use for coproantigen ELISA, while the sediment was used for parasite egg examination.

E. multilocularis coproantigen ELISA and cut-off value determination

Coproantigen detection and cut-off value determination was done following the proce-

dure described by Morishima (1999)⁸⁾. An aliquot (50 μ l /well) of anti-*E. multilocularis* excretory-secretory (ES) antigen rabbit IgG diluted with 0.05 M carbonate-bicarbonate buffer at a concentration of 1 μ g of protein/ml was added in U-type microtitre plates and incubated at 4°C for overnight. The plate was then washed 3 times with phosphate buffered saline (PBS, pH 7.4) containing 0.05% Tween - 20 (PBS-T) at room temperature and blocked with 100 μ l /well of 1 % bovine serum albumin (BSA) at room temperature for 1hr. After washing 3 times, 50 μ l of each fecal supernatant was added separately in each well and incubated at room temperature for 2 hrs.

After 4 times washing with PBS, biotinylated monoclonal antibody diluted with 0.5% BSA-casein in PBS-T solution was added and allowed to react for 1 hr at room temperature. The plate was washed 4 times and streptavidin, biotin and horseradish peroxidase (HRP) complex (Amersham) diluted with 0.5% BSA- 0.5% casein in PBS-T solution was added and reacted for 1 hr at room temperature. The plate was washed 5 times and an aliquot of 100 µl /well substrate solution (20 mg of o-phenylenediamine in 50 ml of 0.1 M citric phosphate buffer with 10 µl of H₂O₂) was added. The plate was incubated at 37°C for 30min under dark condition. The reaction was stopped with 50 µl of 4N H₂SO₄ and immediately the OD values at 490 nm were measured by the ELISA reader. All samples were tested in duplicate.

A cut-off value was calculated to be 0.106, which was the result of the mean OD-value plus 3 S.D., calculated from fecal samples of 28 *E. multilocularis*-free red foxes.

Fecal egg examination

Fecal egg examination was done with a sucrose solution of specific gravity 1.27 following the method described by Ito (1980)³.

Host age determination

The hosts were considered juveniles if they had deciduous teeth. In Hokkaido, the breeding season of red foxes is March and April. Animals examined were captured during June to September. Therefore, age of the juveniles captured were 2 to 6 months old.

Statistical analysis

The difference in prevalence was analyzed using Chi-square test (χ^2 - test). The worm burden and egg intensity (EPG: eggs per gram of feces) differences were analyzed using the Mann-Whitney U-test, while the correlation between worm burden and coproantigen ELISA OD values was analyzed using Pearson's correlation coefficient.

Results

Thirty-eight out of 67 (56.7%) red foxes and 3 out of 13 (23.1%) raccoon dogs were found to harbor *E. multilocularis* worms. The total biomass of *E. multilocularis* in all 38 infected red foxes was 2,817,000, while those of 3 raccoon dogs were 1,515 worms. The worm burden ranged from 1 to 550,000 worms with a median 6,400 in red foxes (Table 1), and from 288 to 910 worms with a median 317 in

Table 1. Prevalence and intensity of *Echinococcus multilocularis* in red foxes

Animals	Total examined	No. cases positive	Prevalence (%)	Intensity		
				Median	Mean	Range
Female	35	22	62.8	900	86,300	1 -372,000
Male	32	16	50.0	7,000	59,200	2 -550,000
Adult	36	18	50.0	3,500	53,300	1 -550,000
Juvenile	31	20	64.5	15,700	92,900	31 -372,000
All	67	38	56.7	6,400	74,200	1 -550,000

Statistical analysis :

A) Prevalence (Chi-square test)

Female vs Male : $\chi^2=0.322$; P=0.571

Adult vs Juvenile : $\chi^2=1.430$; P=0.232

B) Intensity (Mann-Whitney U-test)

Female vs Male : P=0.287

Adult vs Juvenile : P=0.021

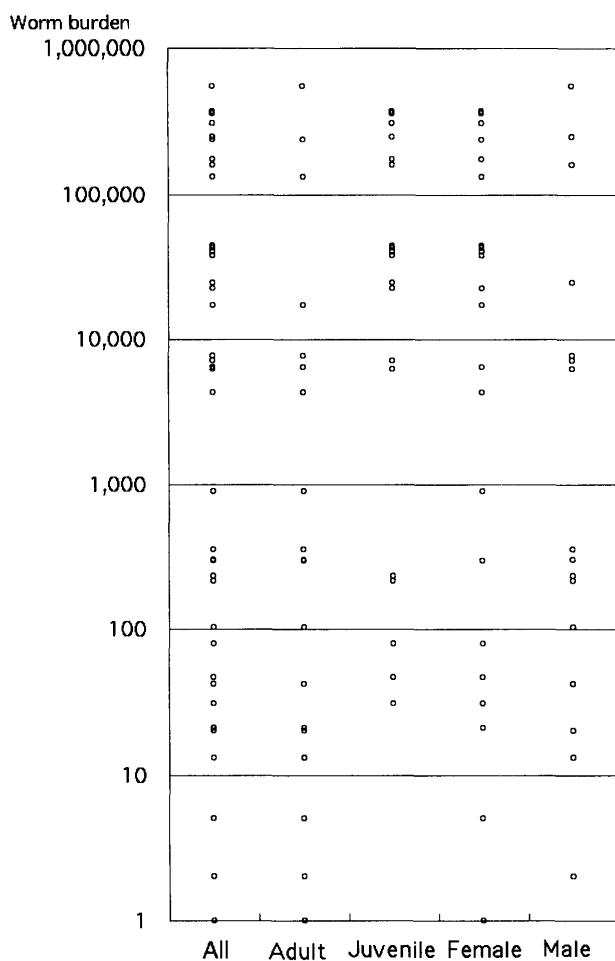


Fig. 2 . Worm burden of *E. multilocularis* in all, adult, juvenile, male and female red foxes (each point represents worm burden in an individual animal)

raccoon dogs. Of the total biomass of *E. multilocularis* in all 38 infected red foxes, 98.7% and 90.6% of the worms were harbored by 16 (42.1%) and 9(23.7%) foxes, respectively (Fig. 2).

Distribution of worms in the small intestine was not uniform. The highest occurrence in the small intestine was in segment IV (89.5%) followed by segment III (86.8%) and segment II (81.6%) in red foxes (Table 2). The highest worm burden and the highest median were in segment IV and segment II, respectively. A large number of detached proglottids were found in the segment VI of the small intestine and immature worms were collected from the large intestine of two foxes.

Of the 13 raccoon dogs examined, 2 juvenile and 1 adult harbored *E. multilocularis*. Gravid worms were found from segments I - III of the small intestine of a juvenile raccoon dog (worm burden : 810), while all of the worms found in 2 other raccoon dogs (worm burden : 288 and 317, respectively) were immature.

No difference in prevalence was found between male and female, and juvenile and adult foxes. A higher worm burden in juvenile

Table 2 . Distribution of *E. multilocularis* in the small and large intestine of infected red foxes (n=38)

Intestinal Segments	Worm burden(%)*	Median	Range	Occurrences**
Small intestine :				
I	452,000 (16.0)	78	0-227,000	76.3
II	355,000 (12.6)	730	0-99,000	81.6
III	545,000 (19.3)	420	0-310,000	86.8
IV	991,000 (35.2)	360	0-217,000	89.5
V	162,000 (5.7)	60	0-53,000	65.8
VI	313,000 (11.1)	0	0-240,000	44.7
Large intestine	1,000 (0.04)	0	0-800	5.3

* worm burden in each segments/total biomass

** Percentage of number. of positive foxes/total number. of infected hosts

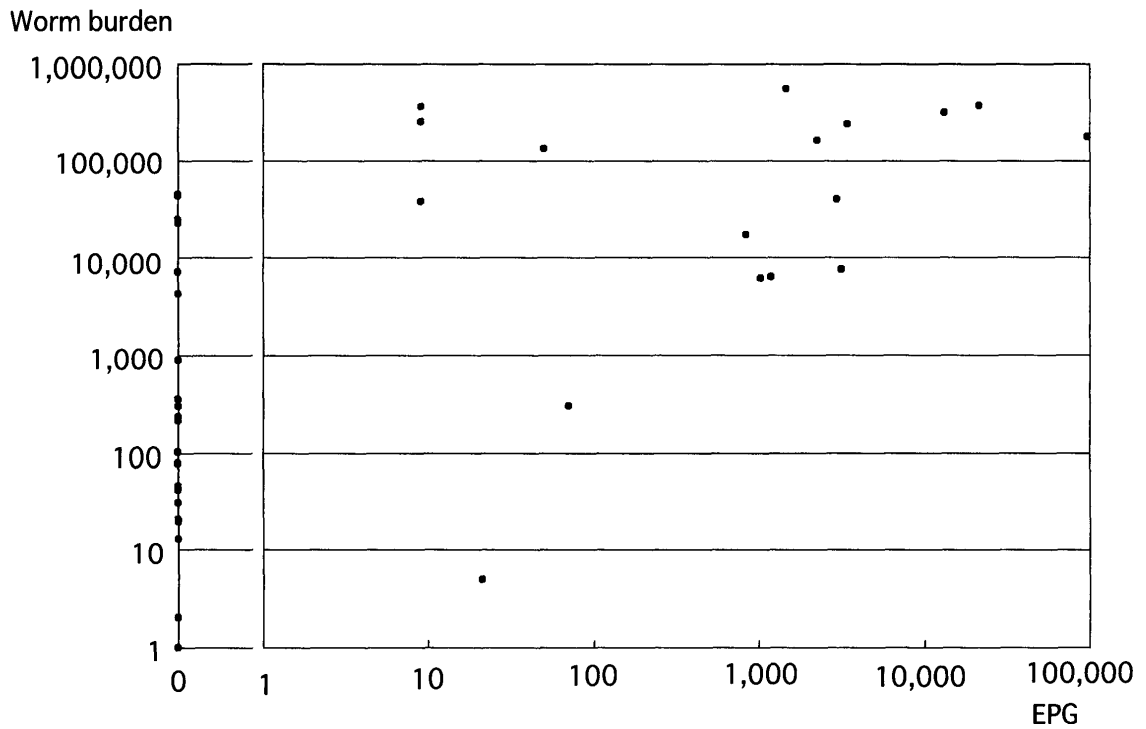


Fig. 3. Relationship between the worm burden of *E. multilocularis* and its egg count in red foxes (EPG: eggs per gram of rectal feces)

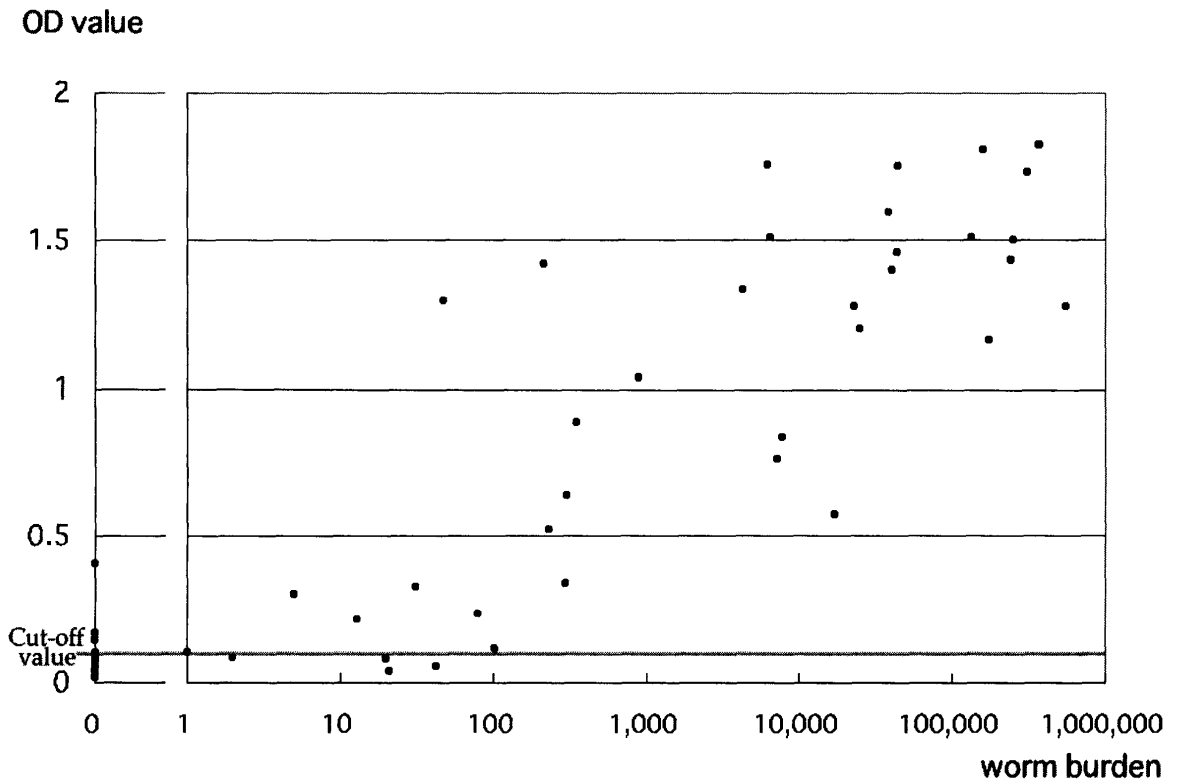


Fig. 4. Correlation between the worm burden and coproantigen ELISA OD values in red foxes infected with *E. multilocularis* Pearson's correlation coefficient, $r=0.521$
Cut off OD-value=0.106

Table 3. Comparison between coproantigen ELISA and necropsy examination

		Necropsy		
		+	-	Total
Coproantigen	+	34	2	36
-ELISA	-	4	25	29
Total		38	27	65
Sensitivity :		89.5% (34/38)		
Specificity :		92.6% (25/27)		
Positive predicative value :		94.4% (34/36)		
Negative predicative value :		86.2% (25/29)		

than adult foxes ($P=0.021$) was found, while no significant difference in worm burden ($p=0.202$) was found between male and female foxes (Fig. 2).

Examination of rectal fecal samples from 65 red foxes showed that 17 (26.2%) of the samples contained Taeniid eggs (Fig. 3). All of the egg-positive foxes were worm-positive in postmortem examination. The number of eggs (EPG) ranged from 0-97,000. In seven of the red foxes (3 juveniles and 4 adults), the EPG were between 1,000 and 10,000, while 3 of the juvenile foxes showed an EPG between 10,000 and 100,000. Six foxes harboring with 1,000 - 100,000 worms showed no eggs in their rectal feces (Fig. 3). Most of the worms from the 6 foxes were immature.

In one of 12 raccoon dog fecal samples analyzed, *E. multilocularis* eggs were found. The egg-positive raccoon dog harbored matured worms of *E. multilocularis* in the small intestine. The number of eggs (EPG) was 12.

Of 38 worm-positive red foxes (20 juvenile, 18 adults), thirty-four foxes were coproantigen-positive in ELISA (Fig. 4). All of the infected juveniles were coproantigen-positive and 4 of 18 adult foxes harboring *E. multilocularis* were found to be coproantigen ELISA-negative. The majority of foxes harbor-

ing immature worms showed comparatively lower OD-value. Two worm-negative foxes (2/27, 7.4%) were found to be coproantigen ELISA-positive (false positive), while four worm-positive foxes (4/38, 10.5%) were coproantigen ELISA-negative (false negative). The sensitivity and specificity of the assay was 89.5% and 92.6% (Table 3), respectively. Foxes harboring with less than 42 worms were coproantigen-negative. Analysis of the relationship between the worm burden and the coproantigen OD-value showed a mild correlation ($r=0.588$; $P<0.001$), while correlation between egg number and the coproantigen ELISA OD-values showed very low positive correlation ($r=0.140$).

The monthly change in the prevalence of *E. multilocularis* infection in red foxes did not significantly differ during the present survey; i.e., June (8/13), July (8/18), August (15/23) and September (7/13).

Discussion

Recent reports showed that the prevalence of *E. multilocularis* in the red foxes of Hokkaido has drastically increased to 57.4%¹². In the present study, a high prevalence in red foxes was confirmed in Otaru city.

The total biomass of *E. multilocularis* worms in 38 positive red foxes was 2,818,000 with the range of 1 - 550,000 (mean 74,200) worms. In a survey conducted in Sapporo and its environs (Ebetsu, Kitahiroshima and Noporo), a total biomass of 131,000 with a range of 1 - 34,000 (mean 3,300) worm was found in 39 positive red foxes⁷. Whereas in Zurich, Switzerland, a total biomass of 399,000 worms with the range of 1 - 57,000 (mean 3,000) worms was found in 133 positive red foxes². This highest worm burden of red foxes in Otaru suggested that Otaru was a high endemic area of *E. multilocularis*.

The distribution of worms in each seg-

ment of the small intestine was not uniform in red foxes and raccoon dogs. The worm burden and distribution in the intestine of the two host species differed fundamentally. In red foxes, the predilection sites of the worms were the second, third and fourth segments of the small intestine with particularly highest in the fourth segment, while in raccoon dogs the majority of worms of the total biomass were found concentrated in the first and second segments of the small intestine. Comparing the mean number of worm recovered between the two hosts indicates a large difference (74,000 and 505 worms in red foxes and raccoon dogs, respectively). The predilection site of worms for *E. multilocularis* in red foxes has been reported to be the distal part of the small intestine⁹. In the present study, segment IV of red foxes was observed to carry the highest worm burden and to be the highest occurrence site. A low number of immature worms were also found in the large intestine (unusual site of the parasite) in a few animals. This suggests that some immature worms were expelled from the hosts. Further study on the relationship of predilection sites and developmental stages is needed.

A higher worm burden in juvenile than adult red foxes suggests that the establishment and growth rate of the cestodes were affected by some immunological and physiological factors relating to the age of the host¹⁶. The higher worm burden in juvenile foxes is probably associated with a less developed immune response, and probably leads these foxes to play a major role in the dissemination of parasite eggs into the environment than adult foxes. However, the present results revealed no significant difference in EPG between adult and juvenile foxes. This is probably due to the low or absence of correlation between the worm burden and EPG in feces. The discrepancy between EPG and worm bur-

den may be derived from the period after infection and the cycles of destrobilation of the gravid segment. Some juveniles pass a short period after the uptake of intermediate hosts. Previous study on experimental infection of *E. multilocularis* in foxes showed that eggs appeared after 26 days post-infection¹². It has been indicated that heavily infected foxes play an epidemiologically significant role¹⁷. Those foxes might be spreader of the parasite, because they can excrete a large number of eggs into the environment.

Vos et al. (1994) reported a higher prevalence in juvenile foxes than adult foxes in Germany¹⁹, whereas Kritsky et al. (1978) noted no significant difference in the USA⁶. On the other hand, regional endemicity related with a high prevalence in juveniles was found under high-endemic conditions in Germany¹⁷. In the present study, which was conducted in summer captive red foxes, no differences in prevalence were found between juvenile and adult foxes.

It has been suggested that the detection limit of the coproantigen ELISA in the worm burden would be greater than 42 worms. As the worm burden increased, the coproantigen OD-value also increased. It indicated that the OD value was worm-burden dependent. Thus, the false negative of foxes harboring with comparatively low worm burden may associate with insufficient coproantigen in the fecal samples. Further study for promoting of the specificity and sensitivity of coproantigen ELISA assay is necessary. The validity of coproantigen ELISA for the diagnosis of foxes has been confirmed by comparing the results of autopsy, egg examination and the ELISA using rectal fecal samples.

Seasonal fluctuation in prevalence of *E. multilocularis* has been observed with highest prevalence in winter¹⁵. Higher prevalence is recorded in subadult male foxes collected

during winter in the urban area of Zurich²⁾. The prevalence of the present summer survey of red foxes was much higher than any data previously recorded in Otaru (0 - 40%). According to data reported by the Hokkaido government between 1984 and 1996, the emerging of *E. multilocularis*-positive red foxes in Otaru was found in 1991, and the prevalence has been gradually increasing among the red foxes. High prevalence of the parasite in red foxes might be indicating an increasing of infection risks to human.

One of the other important findings in the present study was the finding of the gravid worms of *E. multilocularis* from a raccoon dog in Hokkaido. Raccoon dogs were regarded as less susceptible to the parasite¹²⁾. The first available report on mature worms in raccoon dogs was published in Russia⁵⁾. In Hokkaido, however, *E. multilocularis* has also been reported in raccoon dogs but all the recovered worms were not completely mature²¹⁾.

Acknowledgement

The authors wish to acknowledge Mr. Takeshi Oode for providing the red foxes and raccoon dogs examined and Dr. Nobuhiro Nishi for storage of the samples. This study was supported by the Japanese Ministry of Health and Welfare for "The control of Emerging and Reemerging Disease in Japan", and by the Ministry of Education, Science, Sport and Culture, Japan.

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