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ORIGINAL PAPER

Multiplex PCR system for identifying the carnivore origins of faeces for an epidemiological study on *Echinococcus multilocularis* in Hokkaido, Japan

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Abstract A multiplex PCR system was developed to identify the carnivore origins of faeces collected in Hokkaido, Japan, for epidemiological studies on *Echinococcus multilocularis*. Primers were designed against the D-loop region of mitochondrial DNA. Two separate primer mixtures (mix 1, specific forward primers to fox, raccoon dog and dog, and a universal reverse primer [prH]; and mix 2, specific forward primers to cat, raccoon and weasels and prH) were used so that the PCR products (160 bp, fox and cat; 240 bp, raccoon dog and raccoon; and 330 bp, dog and weasel) were distinguished by size. The multiplex PCR exhibited no cross-reactivity between carnivore species and did not amplify DNA from rodent prey. When 270 field-collected faeces were examined, 250 showed single PCR products belonging to specific target sizes, suggesting successful carnivore identification for 92.6% of samples. Taeniid eggs were detected in 11.1% of samples and coproantigen in 30.4%; whereas the prevalences of taeniid eggs and coproantigen were 12.9% and 34.0% in fox faeces, and 0% and 26.3% in cat faeces, respectively. These results suggest that the prevalence in different target animals can be evaluated individually and precisely using multiplex PCR system.

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Introduction

Field-collected faeces can provide valuable information about the animals in an area and it can be used for ecological studies in conservation biology and wildlife management (Foran et al. 1997; Kohn and Wayne 1997). Such information can also be used for epizootiological studies in which aetiologic agents or their derivatives are excreted in the faeces, enabling identification of infection from these samples (Fraser and Craig 1997; Morishima et al. 1999).

Echinococcus multilocularis is one of the most important zoonotic parasites and in humans it causes the lethal disease alveolar echinococcosis. The parasites are basically maintained in wildlife; foxes, other wild carnivores and occasionally domestic carnivores playing as the definitive host and voles playing as the intermediate host. The prevalence in foxes, which may be directly related to the risk of human infections, has been evaluated by the necropsy of captured or hunted animals (Eckert et al. 2001). In the last two decades, a new approach for evaluating infection has been developed using faeces collected in the field, in which parasite prevalence was estimated from the proportion of faeces containing parasite eggs or antigens (Nonaka et al. 1998; Raoul et al. 2001; Tsukada et al. 2000, 2002; Hegglin et al. 2003). This approach is far less disturbing to the local ecology than necropsy surveys, in which a certain proportion of animals have to be removed from the local ecosystem.

Even though studies with faeces represent an ecologically preferable approach, their reliability as an assessment of parasite prevalence remains controversial since faecal origin remains difficult to determine. For example, the criteria used for the identification of fox faeces include size, shape, colour and odour, as well as any traces such as tracks around the sample. Since none of these criteria are sufficient for unequivocal discrimination of fox faeces from that of other carnivore faeces, a certain level of bias always accompanied such survey results.

Recently, molecular techniques have been developed that enable faecal origin to be identified from faecal DNA, since faeces contain sloughed intestinal mucosal cells. Foran et al. (1997) and Paxinos et al. (1997) have developed a method to distinguish between various canid and felid species, including domestic dogs and cats, using restriction fragment length polymorphism of PCR products (PCR-RFLP). In general, PCR-RFLP requires relatively long PCR products for sequential digestion with restriction enzymes and thus, fresh faecal samples are preferred for this type of analysis, since the DNA must be in good condition (Foran et al. 1997). Long-range PCR tends to be unsuccessful for faeces collected in field, since these samples are rarely fresh and the DNA is often fragmented (Wasser et al. 1997; Frantzen et al. 1998; Murphy et al. 2000). Accordingly, for field studies on faecal samples of varied age, it is more appropriate to use target-animal-specific primers for PCR amplification of short products than to use PCR-RFLP. Moreover, this technique is also better suited to the examination of a large number of faecal samples.

Faeces contain a variety of components that are known to inhibit PCR and these can have a significant effect on the outcome of a PCR reaction (Monteiro et al. 1997). A number of techniques have been developed to improve extraction of faecal DNA and removal of PCR inhibitors. However, their efficiency varies depending upon the target animal and technique used (Huber et al. 2003; Palomares et al. 2002; Piggot and Taylor 2003; Pires and Fernandes 2003), and as yet there is no technique that is both applicable and reliable for all species.

In order to perform epizootiological studies of echinococcosis in Hokkaido, Japan, we selected a faecal DNA extraction method that results in minimal inclusion of PCR inhibitors. We then performed multiplex PCR system to identify the origins of carnivore faeces and used this technique for a field study.

Materials and methods

Faecal DNA samples

Faeces were collected from silver fox (*Vulpes vulpes fulvus*) at a fox fur farm (Kaji Mink Farm, Hokkaido, Japan) and from northern red fox (*V. v. schrencki*), raccoon dog (*Nyctereutes procyonoides*), raccoon (*Procyon lotor*), sable (*Martes zibellina*) and mink (*Mustela vison*) at Asahikawa Municipal Asahiyama Zoological Park and Wildlife Conservation Centre (Hokkaido, Japan). All faeces samples were stored individually in plastic bags at −20°C. In order to examine the efficiency of excluding PCR inhibitors in faeces, faecal DNA from silver foxes was extracted using the following four methods, QIAamp DNA Mini Kit (Qiagen; A-1, 2) and QIAamp DNA Stool Mini Kit (Qiagen; B-1, 2). Faecal DNA samples used in other experiments were extracted using method B-2.

Method A-1 was used for DNA extraction from whole faeces. Each faecal sample (200 mg) was placed in a microcentrifuge tube (2.0 mL) and mixed thoroughly in 1.6 mL SLP buffer (0.5 M Tris-HCl [pH 9.0], 0.05 M EDTA [pH 8.0] and 0.01 M NaCl), as described by Piggot and Taylor (2003). Following incubation at 70°C for 10 min, the mixture was centrifuged at 20,000×*g* for 1 min and then the supernatant (1.4 mL) was transferred to a fresh tube and recentrifuged at 20,000×*g* for 3 min. The supernatant (600 μL) was transferred to a new tube and then 15 mAU of Proteinase K (Qiagen) was added. We then added 600 μL AL buffer and incubated the mixture at 70°C for 10 min. The remaining extraction procedures were followed according to the manufacturer's instructions and DNA was extracted in 50 μL AE buffer.

Method A-2 was used for DNA extraction from a surface wash of faeces. About 1.5 cm of an intact faecal sample was placed in a plastic bag and frozen at −20°C. An appropriate amount of SLP buffer was added directly to the frozen samples so that approximately 1.4 mL of wash could be collected after removal of the faeces. Immediately after addition of the SLP buffer, the plastic bag was shaken vigorously 50 times and then the faecal sample was removed. The buffer in the plastic bag was transferred into a tube and incubated at 70°C for 10 min. The tube was centrifuged at 20,000×*g* for 3 min and then the supernatant (600 μL) was transferred to a new tube, to which 15 mAU of Proteinase K was added. The remaining procedures were performed as described for method A-1.

Method B-1 was used for DNA extraction from whole faeces. Each faecal sample (200 mg) was placed in a microcentrifuge tube (2.0 mL) and mixed thoroughly with 1.6 mL ASL buffer (provided in the QIAamp DNA Stool Mini Kit). The mixture was centrifuged at 20,000×*g* for 1 min and then 1.4 mL supernatant was transferred to a fresh tube (2.0 mL), to which an InhibitEX tablet was added. The mixture was mixed vigorously for 1 min and then incubated at room temperature for 1 min. The sample was centrifuged at 20,000×*g* for 3 min and 600 μL of the supernatant transferred to a fresh tube to which 15 mAU of Proteinase K was added. The remaining extraction procedures

were followed according to the manufacturer's instructions and DNA was extracted with 50 μL AE buffer.

Method B-2 was used for DNA extraction from a surface wash of faeces. The surface wash was performed as described in method A-2, except that ASL buffer was used instead of SLP buffer. The wash (1.4 mL) was then transferred into a fresh tube (2.0 mL), to which an InhibitEX tablet was added. The remaining procedures were performed as described for method B-1.

DNA concentrations were determined using a UV spectrophotometer (UV mini 1240, Shimadzu) with a DNA/protein software programme (Shimadzu).

Muscle, mucus and liver DNA samples

Muscle samples were collected from a fox and seven raccoon dogs captured during an effort to reduce agricultural loss due to foraging and trampling, from a marten (*Martes melampus*) and a grey-sided vole (*Clethrionomys rufocanus*) captured for this study, from a mouse and a rat purchased from a commercial breeder (SLC, Shizuoka), and from a cotton rat (*Sigmodon hispidus*) raised in our laboratory. All samples were stored at −20°C prior to DNA extraction using the QIAamp DNA Mini Kit Tissue Protocol. Mucus samples were obtained from a dog and a cat by swabbing the inside of the cheek with cotton-wool swabs. DNA was extracted using the QIAamp DNA Mini Kit Buccal Swab Spin Protocol. All animal experiments were conducted under the Guidelines for Animal Experiments of the Graduate School of Veterinary Medicine in Hokkaido University. In addition, DNA samples were extracted from the livers of various field rodents including *Clethrionomys rex*, *Clethrionomys rutilus*, *Apodemus argenteus*, *Apodemus speciosus* and *Apodemus peninsulae*, which were kindly provided by Dr. Hitoshi Suzuki, Graduate School of Environmental Science, Hokkaido University. DNA concentrations were determined as described above.

PCR for comparison of the efficiency of PCR inhibitor removal

In order to compare the efficiency of PCR inhibitor removal, we performed PCR amplifications of silver fox faecal DNA prepared by each of the four extraction methods. The primers prL (5′-CACCATTAGCACCCAAAGCT-3′) and prH (5′-CCTGAAGTAGGAACCAGATG-3′) were modified from primers L15997 and H16498, described by Gerloff et al. (1999), and designed to amplify part of the D-loop region present in all carnivores in this study. The reaction mixtures (20 μL) were prepared using a Taq PCR Core Kit (Qiagen) and comprised 2 μL template DNA, 0.8 μL of each primer (25 μM in Tris–EDTA [TE] buffer), 0.08 μL Taq polymerase (5 U/μL), 0.4 μL dNTP (10 mM), 2 μL 10× PCR buffer, 4 μL Q solution and 9.92 μL distilled water. Amplifications were performed in a thermal cycler (GeneAmp PCR system 9700, Applied Biosystems) using the following conditions: 94°C for 3 min, followed by 35 cycles of 94°C for 1 min, 56°C for 1 min and 72°C for 1 min, and a final incubation at 72°C for 5 min. The amplicons were examined by agarose gel electrophoresis.

If no amplification product was obtained, DNA extracted from fox muscle was added to the samples (1.2 ng of DNA in 2 μL of template DNA solution), using a concentration within the limits of detection by this method. In order to evaluate the effect of PCR inhibitors that may have been included in the faecal DNA solutions, the PCR reaction was then performed again using the mixed template. All amplifications included a positive control containing 1.2 ng fox muscle DNA as template, and a negative control containing no DNA.

DNA sequencing

Sequencing was performed on PCR products amplified from muscle samples of seven raccoon dogs using primers prL and prH. Amplicons were sequenced with a CEQ 8000 (Beckman Coulter) using Dye Terminator Cycle Sequencing with Quick Start Kit (Beckman Coulter).

Design of forward primers specific for target animals

Target animals included foxes, raccoon dogs, dogs, cats, raccoons and members of the weasel family (*M. melampus*, *M. zibellina*, *Mustela itatsi* and *M. vison*), which excrete faeces that are analogous to that of foxes. The respective DNA sequences for the mitochondrial D-loop region were obtained from GenBank (fox, AF09815; dog, AF008145; cat, U20753; raccoon, AF080182; *M. melampus*, AB152721; *M. zibellina*, AF336970; *M. itatsi*, AB052718; and *M. vison*, AB052720) and from this study (raccoon dog, AB292740). These sequences were aligned using Genetyx-Win ver. 4.0 (Software Development Co.), in order to design target-animal-specific forward primers that satisfied the following conditions: (1) target animals could be distinguished by the size of their amplification products; (2) there was a high level of variation between the sequences of different target animals; (3) primer lengths were between 20 and 30 bp; and (4) melting temperature (T_m) values were 56–58°C, temperatures equivalent to that of the reverse primer prH. The primers were modified further using more DNA sequences from target animals registered in GenBank, so that each primer sequence contained the lowest possible divergence for each target animal. In particular, absolute conservation of at least 3 bp, was maintained at the 3′ end of the primer (Table 1).

Table 1 Forward primers designed for target carnivores and diversity in the sequences within the same target carnivores

Carnivore	Forward primer	No. bp	T_m value	Size (bp) of products[a]	Max no. mismatches[b]	Conservation at 3′ end[c]
Fox	spFox (5′-GGAGCATATATGACTGCACG-3′)	20	56°C	165	4	8
Raccoon dog	spRdg (5′-GCAGGTACATATCCATGTATTGTC-3′)	24	56°C	232	0	24
Dog	spDog (5′-TTCCCTGACACCCCTACATTC-3′)	21	58°C	355	2	6
Cat	spCat (5′-CGATCTTCTATGGACCTCAACTAT-3′)	24	56°C	160	3	15
Raccoon	spRcn (5′-CCCCATATATAACCTTTAAACTACCC-3′)	26	57°C	245	1	16
Weasel	spWsl (5′-GACATTCTAACTAAACTATTCCCTGATTT-3′)	29	56°C	323-334	1	13

[a] Expected size of amplification products when PCR was performed with a corresponding forward primer and prH

[b] Maximum number of mismatched base pairs in sequence within target animals

[c] Number of consecutive base pairs that are conserved at the 3′ end of the sequence among the same target animals

Construction of multiplex PCR system for identifying carnivores

To use faecal DNA for the identification of target carnivores in Hokkaido, we designed a multiplex PCR system using two sets of primers (primer mix 1 contained spFox, spRdg, spDog and prH, and primer mix 2 contained spCat, spRcn, spWsl and prH). The concentration of each primer in each mix was 12.5 μM, in TE buffer. Reaction mixtures (20 μL) were prepared using a HotStarTaq Master Mix Kit (Qiagen) and comprised 2 μL template DNA, 1.6 μL primer mix, 10 μL PCR master mix, and 6.4 μL distilled water. Amplification was performed in a thermal cycler (GeneAmp PCR system 9700) using the following conditions: 95°C for 15 min, followed by five cycles of 94°C for 1 min, 56°C for 1 min and 72°C for 1 min, after which there were 30 cycles of 94°C for 1 min, 56°C for 30 s and 72°C for 1 min, and finally 72°C for 10 min. Amplification products were examined by agarose gel electrophoresis.

Detection of DNA in aged faeces by multiplex PCR

To evaluate the ability of the multiplex PCR to detect DNA in aged faeces, we performed multiplex PCR using DNA extracted from fox faeces that had been left to age for 1, 2, 4 and 8 weeks. The ageing process was performed on grass (natural conditions) during the summer (from June to August).

Field survey

Animal faeces with diameters <2.5 cm were collected on roads at Otaru City and Yoichi Town, Hokkaido, between May and November, 2004. In order to kill *E. multilocularis* eggs, the faeces samples were packed individually and stored at −80°C for at least 10 days. Samples were then stored at −20°C until use.

In order to determine the carnivore origins of each faeces sample, faecal DNA was extracted using method B-2, as described above, and then multiplex PCR system was performed. For some samples showing single-amplification products in the multiplex PCR system, PCR with primers PrL and PrH was further performed. For samples showing products with a size of a cat, since multiple products were obtained in PCR with PrL and PrH, PCR with spCat and PrH was performed. The products were sequenced with a 3130 Genetic Analyzer (Applied Biosystems) using BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems) and subjected to BLAST sequence similarity searching (National Centre for Biotechnology Information) in order to confirm the results of the multiplex PCR system.

Then, faeces were subjected to taeniid egg and coproantigen examinations, as reported previously (Morishima et al. 1999). Briefly, 0.5 g of faeces were weighed and put in plastic tubes. Then 1% formalin containing 0.3% Tween 20 were added to a total volume of 15 ml. The tubes were centrifuged at 1,000×*g* for 10 min. The resultant sediments were used for taeniid egg examination with centrifugal sucrose (specific gravity=1.27) flotation method, and the supernatants were used for coproantigen examination (sandwich enzyme-linked immunosorbent assay). Optical density (OD) values with more than the mean plus five standard deviations of negative controls (OD=0.289) were considered to be positive. The negative controls used were faecal samples from 605 companion dogs raised and kept only on the main island of Japan which is free from *Echinococcus* infection.

When taeniid eggs were detected, the eggs were isolated by a sieving/flotation technique (Mathis et al. 1996). DNA was then extracted from the eggs using a QIAamp DNA mini kit (Qiagen), followed by PCR amplification of the egg DNA using the primers EmSP1-A' (5′-GTCA TATTTGTTTAAGTATAAGTGG-3′) and EmSP1- B' (5′-CACTCTTATTTACACTAGAATTAAG-3′). These primers

were designed to amplify a partial fragment (243 bp) of the *E. multilocularis* cytochrome *c* oxidase subunit I (COI) gene. In a preliminary experiment, we found that the PCR-amplified DNA from five isolates of *E. multilocularis*, and it showed no cross-reactivity in in silico analysis with COI gene sequences from taeniid cestodes registered in GenBank, or in assays with DNA extracted from *Echinococcus granulosus* (G1 and G6), *Echinococcus vogeli*, *Taenia ovis*, *Taenia pisiformis*, *Taenia hydatigena*, *Taenia crassiceps* or *Taenia taeniaeformis*. The PCR amplification was performed in a GeneAmp PCR System 9700 using a HotStarTaq Master Mix Kit. The reaction mixture (20 μl) comprised the PCR mixture provided in the kit (10 μL), water (6.4 μL), each primer (0.8 μL; final concentration was 1 μM) and template DNA (2.0 μL). The PCR conditions were pre-incubation at 95°C for 15 min, followed by 40 cycles of 94°C for 60 s, 50°C for 90 s and 72°C for 60 s, and a final incubation at 72°C for 10 min. Amplicons were examined by agarose gel electrophoresis. A negative control containing no DNA was included in all tests.

For the samples positive for taeniid eggs, the same PCR reaction was also performed on the faecal DNA solutions described above.

Statistical analyses

Ninety-five percent confidence intervals (C.I.) of prevalences were calculated on the basis of binomial distributions using the software programme R (R Development Core Team, 2008).

Results

Comparison of four methods of faecal DNA extraction for excluding PCR inhibitors

Using primers prL and prH, PCR reactions were performed on faecal DNA extracted using the methods A-1, A-2, B-1 and B-2. These faeces samples were obtained from ten to 12 silver foxes. No amplification products were obtained from DNA extracted by method A-1. When 1.2 ng of fox muscle DNA was added to reactions containing DNA prepared by method A-1, only one sample showed an amplification product. In contrast, 11 of 12 samples showed amplification products when PCR was performed on DNA extracted by method A-2. DNA extracted by method B-1 showed an amplification product in five of ten samples and no product could be obtained in the five negative samples, even after the addition of 1.2 ng of fox muscle DNA. In contrast, all ten samples showed amplification products for DNA extracted by method B-2.

Variation of sequence in forward primer region within target animals

Variation of sequence in the forward primer region was evaluated using sequence data obtained from GenBank (37 foxes, one raccoon dog, 490 dogs, 27 cats, six raccoons, five *M. melampus*, eight *M. zibellina*, one *M. itatsi* and four *M. vison*) and from foxes in Hokkaido, which were registered in our recent study (25 foxes with accession numbers from AB292741–AB292765, including 17 haplotypes; Inoue et al. 2007; Table 1). Although some intra-species (intra-group for weasels) variations were observed within the sequences corresponding to primer regions, the mismatches were less than 4 bp. Sequences for the 3′ end of primers were well conserved within the corresponding target animals.

Multiplex PCR system on DNA from carnivores and their prey

Using primer mixes 1 and 2, we performed multiplex PCR amplifications on DNA extracted from target animals (Fig. 1). Single-amplification products were observed at the expected sizes using a combination of fox, raccoon dog or dog DNA with primer mix 1, and a combination of cat, raccoon or weasel DNA with primer mix 2. No cross-reactions were observed in other combinations. The same results were obtained regardless of the material (muscle, mucus or faeces) used for DNA extraction.

To evaluate detection sensitivity, we performed multiplex PCR on serial dilutions of DNA extracted from the muscles of a fox, raccoon dog and marten, as well as from the mucus of a dog and cat. The detection sensitivity of the multiplex PCR was 1–10 pg of DNA. Since carnivore faeces would likely contain the undigested debris of prey animals, we evaluated

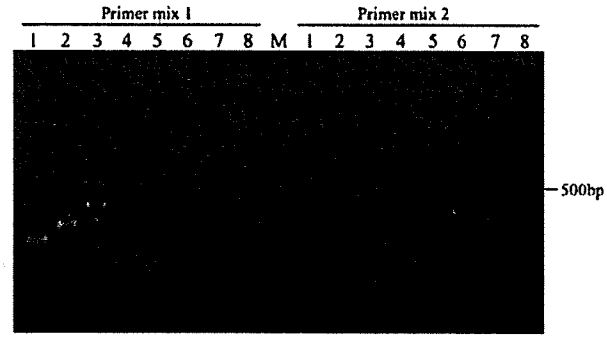

Fig. 1 Multiplex PCR amplification of the D-loop region of carnivore animals. Amplifications were performed with primer mix 1 (spFox, spRdg, spDog and prH) and primer mix 2 (spCat, spRcn, spWsl and prH). *Lane 1* fox (extracted from muscle), *2* raccoon dog (muscle), *3* dog (mucus), *4* cat (mucus), *5* raccoon (faeces), *6* mink (faeces), *7* sable (faeces) and *8* marten (muscle). *M* 100 bp DNA ladder

cross-reactivity of the multiplex PCR system to DNA from potential prey, including voles (*C. rufocanus*, *C. rex*, *C. rutilus*, *A. argenteus*, *A. speciosus* and *A. penninsulae*), mouse, rat, gerbil and cotton rat. Amplification was not observed with any of the rodent DNA.

Multiplex PCR on DNA extracted from aged samples

Multiplex PCR was performed with primer mix 1 on DNA extracted from fox faeces that had been left on grass under natural conditions for 8 weeks during the summer. During the experimental period, the minimum and maximum temperatures ranged between 9–24°C and 18–33°C, respectively. There were 15 days with >1 mm precipitation and the maximum precipitation was 26.5 mm.

Ten faeces samples were selected after each period of exposure (1, 2, 4 and 8 weeks). DNA was extracted from each sample and multiplex PCR performed. The amount of DNA obtained from faeces samples decreased gradually with ageing (starting at ca. 371.5 ng/sample and reaching ca. <65 ng/sample after 8 weeks of exposure). Nevertheless, amplification products were obtained from all samples including those collected after 8 weeks of exposure.

Field survey with multiplex PCR system

A total of 270 faeces samples were collected in the field. These samples were subjected to multiplex PCR system, in order to identify their carnivore origins. Single-amplification products of expected sizes were obtained from 250 samples (92.6%). The carnivore origins of these faeces samples are shown in Table 2. The origin of 20 samples could not be identified since four of these produced multiple PCR products and the remaining 16 did not result in amplification products. Five, three, four, two and four samples showing single products with sizes of fox, dog, cat, raccoon dog and weasel, respectively, were selected and the PCRs and sequencing were performed for subjecting to BLAST sequence similarity searching. All of samples showing single products with sizes of fox, dog, cat and raccoon dog showed 100% sequence identities with the respective species registered in GenBank. All of the samples showing single products with a size of weasel hit best to *M. melampus* with 97–98% sequence identities.

Results of parasite examinations are also shown in Table 2. In total, 30.4% (82/270, C.I., 24.9–36.2%) of faeces were positive for coproantigen. However, when individual species were considered, 34.0% (71/209, C.I., 27.6–40.8%) of fox faeces and 26.3% (5/19, C.I., 9.1–51.2%) of cat faeces were positive. Although six positives were found in samples in which the carnivore origin remained unknown, no positives were detected in any of the other animals identified by multiplex PCR system. In the taeniid egg examination, 11.1% (30/270, C.I., 7.6–15.5%) of all faeces tested were positive. However, 12.9% (27/209, C.I., 8.7–18.2%) of fox faeces contained eggs and three positives were found in samples in which the carnivore origin remained unknown. No positives were detected in any of the other animals identified by multiplex PCR system. Among the 30-taeniid egg-positive faeces, 27 were also positive for coproantigen (25 of 27 samples identified as being of fox origin).

In an *E. multilocularis*-specific PCR performed on egg and faecal DNA, all 27 faeces identified as being of fox origin produced PCR products from egg DNA, whereas only 22 generated products from faecal DNA (Table 3).

Discussion

Faeces are known to contain various PCR inhibitors that may not be completely excluded during DNA extraction and thus, these inhibitors can interfere with subsequent PCR reactions. Substances such as polysaccharides (Monteiro et al. 1997) and bile salts (Deuter et al. 1995) have been reported to act as potential PCR inhibitors. However, since faeces is supposed to contain rectum-derived cells on its surface, sampling methods such as scraping or swabbing of the faecal surface (Yamauchi et al. 2000; Davison et al. 2002; Pires and Fernandes 2003) or washing of faeces (Flagstad et al. 1999; Palomares et al. 2002; Piggot and Taylor 2003; Verma et al. 2003), have been proposed for collecting rectum-derived cells with the minimum inclusion of faecal materials. On the other hand, potato flour (Deuter et al. 1995) and magnetic beads (Flagstad et al. 1999) have

Table 2 Carnivore origins of field-collected faeces identified by multiplex PCR and results of coproantigen and taeniid egg examinations

	Origin							Total
	Fox	Cat	Weasel	Raccoon dog	Dog	Raccoon	Unidentified	
No. samples	209	19	16	3	3	0	20	270
No. positives in coproantigen examination	71	5	0	0	0	0	6	82
No. positives in taeniid egg examination	27	0	0	0	0	0	3	30

Table 3 Results of the *Echinococcus multilocularis*-specific PCR performed on egg and faecal DNA

Origin identified by multiplex PCR	Fox	Unidentified	Total
No. examined	27	3	30
No. positives in PCR on egg DNA	27	2	29
No. positives in PCR on faecal DNA	22	0	22

been reported to absorb inhibitory substances effectively. Guanidine thiocyanate has been also used as a deactivator of inhibitory substances because of its strong protein denaturation activity (Frantzen et al. 1998; Huber et al. 2003; Frantz et al. 2003). Commercial kits such as the QIAamp DNA Stool Mini Kit (Qiagen), use a combination of absorption and deactivation of these inhibitory substances.

In this study, we compared four methods for their ability to exclude PCR-inhibitory substances. No PCR products were generated from fox faecal DNA for which no attempts had been made to exclude inhibitory substances. Since products could not be obtained even after the addition of control fox DNA, it was suggested that PCR inhibiters played a role in the amplification failure. Although the QIAamp DNA Stool Mini Kit was somewhat effective at the exclusion of PCR-inhibitory substances, extraction of DNA from a surface wash of frozen faeces was more effective. However, surface wash alone was insufficient to completely eliminate inhibitory substances. Subsequently, we found that a combination of the QIAamp DNA Stool Mini Kit and surface washing of frozen faeces was the most effective method for excluding inhibitory substances.

For constructing the multiplex PCR system, we designed forward primers specific for the mitochondrial DNA of individual target animals. Faecal DNA obtained from field samples is likely to be fragmented and limited in abundance. Therefore, we chose to amplify mitochondrial DNA, since it represents a more robust target than nuclear DNA. In addition to being present in multiple copies per cell, mitochondrial DNA is circular, which renders it relatively resistant to DNase. Furthermore, PCR amplification of mitochondrial DNA is known to be more successful than that of nuclear DNA (Frantzen et al. 1998; Murphy et al. 2000, 2002) and amplification of a 150–400 bp product is more successful than that of a 700 bp product (Wasser et al. 1997; Murphy et al. 2000). Accordingly, primers were designed to amplify products of less than 400 bp.

Dalen et al. (2004) developed a multiplex PCR that can distinguish between the DNA of foxes, arctic foxes and wolverines. They performed the reactions using animal-specific forward primers designed against the mitochondrial D-loop region and the mammalian-common reverse primer (H16498) described by Gerloff et al. (1999). Following Dalen et al. (2004), we designed animal-specific forward primers against the mitochondrial D-loop region (Table 1). However, it was difficult to construct a single multiplex PCR that could distinguish six different animal groups simultaneously. Therefore, the forward primers were divided into two groups for multiplex PCR system with a modified mammalian-common reverse primer (prH). This method provided a similar sensitivity to all target animals, since the same region of DNA was being amplified. Moreover, PCR amplification with a mammalian-common D-loop primer set (prL and prH) makes it possible to determine whether a negative result is due to a sample being derived from a non-target animal (such as hares, deer or human) or due to a failure in DNA extraction or PCR inhibition. In fact, we found one multiplex PCR system-negative sample that did exhibit an amplification product using the prL and prH primers (data not shown).

Under natural conditions, faecal DNA may degrade gradually due to DNase activity or ultraviolet rays, and rectum-derived cells on the surface of faeces may be washed off by rain. DNA loss and degradation remain significant considerations for field studies dependent upon faecal DNA. Kovach et al. (2003) observed a significant reduction in successful PCR amplification of DNA from ageing faeces, and the degree of reduction varied among three species of lagomorphs. In contrast, Palomares et al. (2002) reported that ageing of faeces did not have a significant effect upon the successful PCR amplification of faecal DNA from Iberian lynx. In the present study, the total amount of DNA obtained from fox faeces was reduced by ageing under natural summer conditions for 8 weeks. However, this DNA was amplified successfully and the carnivore origins of 92.6% of faeces collected in the field were identified successfully.

Another consideration for field studies is potential cross-reactivity with the DNA from prey animals that may be present in carnivore faeces. Therefore, DNA was extracted from various rodents including field and experimental animals, and then examined with the multiplex PCR system. No cross-reactivity was detected with any rodent DNA. Relating to this consideration, Foran et al. (1997) reported that the digestive tract adequately degraded ingested animal matter and that cross-contamination did not occur in carnivore faecal DNA analysis.

In assessment of the prevalence of *E. multilocularis* in foxes using field-collected-faeces, coproantigen examination has been widely used (Raoul et al. 2001; Tsukada et al. 2002) because the method is considered as most appropriate for mass-screening purpose. The sensitivity and specificity of the coproantigen test used in this study had been evaluated using fox samples. The combined data from two studies comparing the test results of rectum faeces and necropsy results of wild foxes in Hokkaido (77 infected and 59 uninfected foxes) measured 92.2% in sensitivity and 96.6%

in specificity for the test (Morishima et al. 1999, Yimam et al. 2002). The test showed some cross-reactivity with patent *Taenia hydatigena* (Malgor et al. 1997) and *Taenia pisiformis* infections (unpublished data), however, these species are rare in Hokkaido. No cross-reaction was observed with *T. taeniaeformis* and *Taenia crassiceps* infections, which are relatively more prevalent in Hokkaido (Sakashita et al. 1995).

Multiplex PCR system provides a clear contribution to the assessment of field data. In the present survey, faeces with a diameter of less than 2.5 cm were collected and if size were used as the primary criterion for identification, the apparent prevalences of coproantigen and taeniid eggs in foxes were 30.4% and 11.1%, respectively. However, multiplex PCR system indicated that the actual prevalences were 34.0% and 12.9%, respectively. In addition, a rough evaluation of the prevalence of infection in other animals could be determined using multiplex PCR system data. Although other ambiguous criteria such as shape, colour and odour of faeces might be included for more reliable identification of faecal origin, the data from multiplex PCR system provides less equivocal identification and it also enables evaluation of specific carnivore prevalence and identification of their parasites. In addition, multiplex PCR system could be used to evaluate the reliability of previous ambiguous methods for estimating origins of faeces.

Of the 19 samples which origin was determined as cat by multiplex PCR system, five (26.3%) showed positive in coproantigen examination whereas taeniid eggs were not detected from any of those samples (Table 2). Since the chance of *E. multilocularis* to be mature and produce infective eggs in cats is considered to be low, egg examination has less value for assessment of the prevalence in cats. Considering that the necropsy survey of cats ($n=108$) conducted by the local government showed the prevalence of 5% in Hokkaido (data reported by the Hokkaido government), the present data may indicate the local high prevalence in cats. On the other hand, since the diagnostic reliability of the coproantigen examination has not been specifically determined for cat faeces, the present data may indicate the lower reliability of the method for cat faeces. Further studies are required for clarifying the significance of the observed result.

Faecal DNA did not provide a satisfactory means for evaluating the prevalence of *E. multilocularis*. According to PCR amplification of egg DNA, 27 faecal samples contained *E. multilocularis* eggs and these samples would be expected to contain debris from proglottids. However, only 22 samples gave positive PCR results from faecal DNA. In order to minimise the presence of PCR inhibitors, faecal DNA was extracted from a surface wash of frozen faeces and this procedure may have reduced the chances that *E. multilocularis* DNA would be co-extracted. Al-Sabi' et al. (2007) extracted DNA from whole faeces of foxes experimentally infected with *E. multilocularis*. They observed no PCR amplification in nine of 23 faeces samples containing parasite eggs in the low patent period and suggested that lack of amplification may have been due to PCR inhibitors present in the DNA extracts. Although PCR amplification of faecal DNA has the potential to detect infection more effectively, problems arising from the presence of PCR inhibitors remain to be overcome.

The multiplex PCR system developed will enable precise evaluation of current carnivore prevalence or changes in prevalence of target carnivores over time, thus contribute to epizootiological and control studies of *E. multilocularis*.

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NOTE *Parasitology*

A Vague Understanding of the Biology and Epidemiology of Echinococcosis by Dog Owners in Hokkaido, an Endemic Island for *Echinococcus multilocularis* in Japan

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ABSTRACT. A questionnaire survey was conducted by giving 14 statements about echinococcosis to 2,070 dog owners residing in Hokkaido in order to evaluate their understanding about the biology and epidemiology of *Echinococcus multilocularis*. Analysis of the answers revealed that dog owners understood the disease superficially, and there were several points of confusion in their understanding, especially regarding differences in the modes of transmission and disease development in dogs and humans. The results suggest the need for the proper education of dog owners to perform proper prophylactic measures against the disease.

KEY WORDS: canine, *Echinococcus*, epidemiology, parasitic zoonoses, zoonosis.

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Echinococcus multilocularis is distributed in the northern hemisphere, including Hokkaido, Japan. If humans accidentally ingest the parasite eggs and become infected, the parasite metacestodes develop in the liver and occasionally in other organs and cause a lethal disease, alveolar echinococcosis [10]. The parasite is primarily maintained in the sylvatic cycle, with foxes serving as definitive (final) hosts and voles serving as intermediate hosts. In endemic regions, dogs can also become a final host, serving as a potential infectious source to humans [1, 2, 4, 5, 7, 11]. Therefore, prophylaxis for dog infection is of high importance for risk management of the infection to humans, especially for dog owners [6].

In Hokkaido, the prevalence of infection in foxes has been approximately 40% during the last two decades [9]. By 2007, 531 human patients had been identified (data from the Hokkaido government). However, the routes of infection to humans have not been completely clarified [3, 10]. One possible route of infection that should be considered is via infected pet dogs. In our survey conducted from 1997 to 2007 to determine the prevalence of infection in pet dogs, 0.4% of the dogs examined (n=4,768) excreted taeniid eggs that were identified as *E. multilocularis* eggs by PCR examination of egg DNA [8]. To control echinococcosis, the Hokkaido government has been conducting surveys and countermeasures, including annual surveys on the prevalence of infection in foxes and other animals, development of diagnostic and therapeutic measures for human patients, and education of residents through schools and publications.

In this study, we conducted a simple questionnaire survey of dog owners who requested us to test their dogs for *E. multilocularis* infection in order to assess how precisely dog owners who have a potentially high risk of infection understand the biology and epidemiology of echinococcosis.

From 1997 to 2004, the questionnaire was conducted by giving 9 or 14 statements on the biology and epidemiology of echinococcosis to 2,070 dog owners residing in Hokkaido, who were asked to answer whether each statement is Right, Wrong, or Unknown (Table 1). More than 50% of the dog owners answered statements S1 to S8 correctly. Most of those statements contained descriptions of the basic biology and epidemiology of echinococcosis in Hokkaido, and the dog owners seemed to understand well the current situation of the disease in Hokkaido and the general mode of transmission. In contrast, less than 50% of the dog owners answered statements S9 to S14 correctly. In particular, less than 20% answered S13 and S14 correctly and more than 50% answered incorrectly.

Statements S4, S12, and S14 were related to the transmission of the parasite to dogs. Among the dog owners who answered S4 correctly, the percentages of owners that answered S12 correctly and incorrectly were 48.2% and 32.3%, respectively. For statement S14, the percentages were 24.2% and 60.4%, respectively. The results indicate that most dog owners understood that dogs get the infection by ingesting infected rodents; however, their understanding was vague, and many dog owners thought that dogs also get the infection from foxes, presumably by ingesting the parasite eggs excreted from foxes. One possible reason for this misunderstanding could be confusion regarding the two different modes of transmission to dogs and humans. In another words, many dog owners misunderstood an important characteristic of the parasite life cycle: transmission never directly occurs between two final hosts, such as fox and dog.

Regarding their understanding of the transmission to humans, 69.1% of the dog owners understood that humans

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Table 1. Questions about echinococcosis in Hokkaido given to dog owners and the percentage of correct answers

Statement No.	Statement	No. answered	Correct answer	Percentage correct answer	Percentage of incorrect answer
S1[a)]	Infected foxes inhabit Sapporo[b)].	448	Right	87.9	0.4
S2[a)]	The disease is now spread all over Hokkaido.	382	Right	87.2	3.9
S3	The parasite eggs in the feces of infected dogs can be an infectious source to humans.	2013	Right	86.4	3.4
S4	Dogs are infected by ingesting infected rodents.	2008	Right	80.1	10.3
S5[a)]	Rodents playing a role in the transmition of the disease are those found in houses.	445	Wrong	74.6	6.5
S6	Humans get the infection by ingesting the parasite eggs.	1998	Right	69.1	15.9
S7	Infected dogs have to be euthanized in Hokkaido.	1988	Wrong	60.9	3.3
S8[a)]	The parasite eggs can be killed by boiling them.	444	Right	52.7	15.5
S9	Infected dogs can be dewormed completely with an anthelmintic drug.	1985	Right	49.7	17.1
S10	The disease never transmits from human to human.	1995	Right	48.9	26.1
S11	Humans also get infection by ingesting infected pigs.	1986	Wrong	44.6	22.4
S12	Dogs are infected by physical contact with infected foxes.	1997	Wrong	44.5	33.0
S13	Infected dogs have parasitic legions in the liver.	1997	Wrong	19.7	50.5
S14[a)]	Dogs are infected by ingesting the parasite eggs excreted from foxes.	447	Wrong	19.0	62.4

a) Statements S1, S2, S5, S8, and S14 were given to only 451 owners.
b) The capital city of Hokkaido.

get the infection by ingesting the parasite eggs. However, this understanding was also vague, and 26.1% of the dog owners believed that the disease could be transmitted from human to human (see Table 1, S10). Moreover, 22.4% of the dog owners thought that the disease could be transmitted from infected pigs to humans (see Table 1, S11). In Hokkaido, approximately 2,000 infected pigs (prevalence: 0.2%) are detected annually during meat inspections (data from the Hokkaido Government). However, like humans, pigs get the infection by ingesting the parasite eggs and then develop lesions in the liver, thus serve as accidental intermediate hosts. Therefore, pigs never excrete the parasite eggs and transmission from pigs to humans never occurs. The surveyed dog owners misunderstood another important aspect of the parasite life cycle: transmission never directly occurs between intermediate hosts, as from human to human or from pig to human.

Further confusion was elucidated by the answers to statement S13; 50.5% of the dog owners thought that dogs develop parasitic legions in the liver, indicating that the dog owners thought that dogs develop the same legions as humans.

In conclusion, this study revealed that dog owners residing in Hokkaido, an endemic area of the disease, who have a risk of infection by their dogs superficially understood the biology and epidemiology of *E. multilocularis*. Their understanding about the difference in the mode of disease transmission to dogs and humans was not completely clear, leading them to misbelieve that dogs can get the infection in the same way as humans. This vague understanding was presumably due to the complicated nature of the parasite life cycle in that two different hosts play different biological roles, namely, final and intermediate hosts are required for completing the life cycle of the parasite. From the point of view on a risk management, the understanding on it is of primary importance for individual dog owner to perform effective prophylactic measures against parasite infection in his/her dogs. In order to do so, it is paramount that dog owners precisely understand the parasite life cycle and the events related to the risk of infection of dogs and humans. In this context, the role of veterinary practitioners is very important. We hope that the results of this simple questionnaire survey will be used by veterinary practitioners as a reference for explaining and enlightening dog owners of the disease process, then contribute to the performance of proper prophylactic measures against parasite infection by dog owners.

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Echinococcus multilocularis Infection in Pet Dogs in Japan

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Abstract

A survey of *Echinococcus multilocularis* infections in pet dogs in Japan from 1997 to 2007 was conducted by testing for coproantigen reactivity, fecal taeniid eggs, and egg DNA. In Hokkaido, the only island where *E. multilocularis* is endemic in Japan, 18 of 4768 dogs (0.4%) excreted taeniid eggs that were positive for *E. multilocularis* DNA by polymerase chain reaction (PCR). Most of the dogs testing positive for egg DNA were kept free-range, but three dogs had been kept inside their owners' houses. In addition, 15 dogs were suspected to be infected based on the results of a coproantigen test. One dog, which was transported from Hokkaido to Honshu, the main island of Japan, was excreting taeniid eggs that were positive for *E. multilocularis* DNA by PCR. These results suggest the importance of proper pet management in disease prevention, even for dogs kept indoors, and they point out a possible means by which the parasite may be introduced into non-endemic areas through transport of infected dogs.

Key Words: Diagnostics; Epidemiology; Parasitology; Zoonosis

Introduction

Echinococcus multilocularis is prevalent in Hokkaido, the northernmost island of Japan, with a prevalence in foxes of approximately 40% in the last two decades. By 2007, 531 human patients infected with *E. multilocularis* were reported in Hokkaido. The parasite is basically maintained in the sylvatic cycle, in which foxes are the definitive host and voles are the intermediate host. However, the habitat of foxes has been getting closer to or overlapping with that of humans, producing a potential risk of infection for humans and companion animals (Eckert et al. 2000, Tsukada et al. 2000, Romig 2002, Oku and Kamiya 2003).

In Central Europe, several studies have revealed that pet dogs were infected with *E. multilocularis* with a prevalence of 0.3%–7% in endemic regions (Deplazes et al. 1999, Gottstein et al. 2001). Deplazes et al. (2004) estimated that more than 10% of dogs would be infected at least once in their life, even in regions of low prevalence. In some endemic areas, such as Gansu and the Tibetan plateau in China and St. Lawrence Island in the United States of America, dogs play important roles both in the maintenance and in the transmission of echinococcosis to humans (Craig et al. 2000, Rausch and Fay 2002, Torgerson and Budke 2003, Budke et al. 2005a and b).

In Japan, despite the recent high prevalence of foxes in Hokkaido and an increasing awareness of the disease as a serious health risk, few data are available for evaluating the current infection rates in dogs in Japan. Infection with *E. multilocularis* in dogs was first recognized on Rebun Island, a small island in Hokkaido Prefecture, in 1954 (Yamashita 1997). In the 1960s, the parasites were also detected in dogs on the island of Hokkaido (Yorozuya et al. 1968). Since then, the local government has conducted necropsy surveys of dogs (including household pets and stray dogs) and has reported that adult *E. multilocularis* were detected in 99 (1.0%) of 9937 dogs necropsied during the period 1966–2006 (data reported by the Hokkaido government). However, most of these animals were examined before 1990 and therefore, the data do not reflect the recent prevalence of infection.

In the present study, to evaluate the current epidemiological status of canine echinococcosis in Japan, a survey of *Echinococcus* infection in pet dogs was conducted by examining coproantigen reactivity and the presence of taeniid eggs and egg DNA in fecal samples.

Materials and Methods

Fecal samples and questionnaire

We obtained dog fecal samples from local veterinarians, who collected them from August 1997 to August 2007. At the same time, dog owners completed a questionnaire to de-

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termine the possible source of transmission to the dogs. When positive or suspicious results were obtained in the primary examination, the veterinarians were asked to obtain further information from dog owners relating to the chance of transmission to the dogs, and to treat the dogs for worms by administering praziquantel and collect feces 1 day before and 1, 2, and 3 days after treatment. All feces were first incubated at 70°C for 12 hours to kill potentially viable *Echinococcus* eggs and were then immediately subjected to examination or stored at –40°C.

Taeniid egg and coproantigen tests

Taeniid egg and coproantigen tests were performed as described previously (Morishima et al. 1999). The coproantigen test detects a heat-resistant carbohydrate epitope so that the test can be performed with heat-sterilized samples (Kohno et al. 1995). Because dog feces for use as negative controls for the coproantigen test were not available during the initial phase of the survey, feces of 37 silver foxes kept in individual cages at a fox fur farm (Kaji mink, Fukagawa) were used as negative controls. Subsequently, the negative controls were feces from 605 pet dogs raised and kept only on the main island of Japan, which is free from *Echinococcus* infection. The prevalence of eggs of gastrointestinal parasites isolated from fecal samples of the negative controls was as follows: 24.3% *Toxocara canis* and 8.1% *Isospora* spp. in the silver foxes, and 1.7% *Toxocara canis*, 2.0% *Ancylostoma caninum*, and 4.8% *Trichuris vulpis* in the dogs. Two cutoff values were used to discriminate between negative and suspicious samples (mean [μ] + 3 SD of negative controls; OD = 0.137–0.219) and between suspicious and positive samples (μ + 5 SD; OD = 0.179–0.313).

Egg DNA test

When taeniid eggs were detected in the feces, they were isolated either by a sieving/flotation technique (Mathis et al. 1996) or collected manually from the cover glass used for taeniid egg examination in a stereomicroscope. Egg DNA was then extracted with a QIAamp DNA mini kit (Qiagen) according to the manufacturer's instructions, and the isolated DNA was amplified by polymerase chain reaction (PCR).

Each PCR was performed with *E. multilocularis*–specific primers for the U1 small nuclear RNA (U1 snRNA) (Yagi and Ohyama 1994), the mitochondrial 12S ribosomal RNA (12S rRNA) (Dinkel et al. 1998), or the mitochondrial cytochrome c oxidase subunit I (COI) gene. The primers used in the PCR for the COI gene were: EmSp1-A/B (5′-TCATATTTGTTTAAGTATAAGTGGT-3′) / (5′-TATTTACACTAGAATTAAGC-3′) or EmSP1-A′ /B′ (5′-GTCATATTTGTTTAAGTATAAGTGG-3′) / (5′-CACTCTTATTTACACTAGAATTAAG-3′), both of which were confirmed as *E. multilocularis*–specific because cross-reactivity was not detected *in silico* with COI sequences registered in GenBank or with DNA extracted from *E. granulosus* (G1 and G6), *E. vogeli*, *Taenia ovis*, *T. pisiformis*, *T. hydatigena*, *T. crassiceps*, *T. taeniaeformis*, other major parasites of dogs or host animals. Polymerase chain reactions with the EmSP1-A/B primers or with the EmSP1-A′/B′ primers were performed either in a GeneAmp® PCR System 9700 (Applied Biosystems) or in a MiniCycler (MJ Research) using a HotStarTaq Master Mix Kit (Qiagen). The reaction mixture (20 μL) was composed of the PCR Master Mix (10 μL), water (6.4 μL), 25 μM of each primer (0.8 μL), and template DNA (2.0 μL). The PCR conditions were preincubation at 95°C for 15 minutes, followed by 40 cycles of 94°C for 60 seconds, 43°C for 90 seconds when using EmSP1-A/B or 50°C for 90 seconds when using EmSP1-A′/B′ and 72°C for 60 seconds, followed by a final incubation at 72°C for 10 minutes. The amplicon (236 base pairs [bp] for EmSP1-A/B and 243 bp for EmSP1-A′/B′) was examined by agarose gel electrophoresis. A negative control without DNA was included in all tests.

The sequence of the amplified product of the COI gene was determined with a Beckman CEQ 8000 DNA analyzer and a GenomeLab DTCS Quick Start kit (Beckman Coulter). The sequences obtained were compared with the reference sequence registered in GenBank (accession number: AB018440) to confirm that the products obtained were derived from *E. multilocularis* DNA.

Results

Test results of dogs in Hokkaido

In all, 4768 dogs from Hokkaido were examined: The coproantigen test was not performed on 7 of the dogs (Table 1). Taeniid eggs were detected in 20 dogs, 18 of which tested positive in the coproantigen test; one tested suspicious, and the coproantigen test was not performed on one dog. An egg DNA test was performed on samples from the 18 dogs excreting taeniid eggs, and *E. multilocularis*–specific PCR products were obtained from all of the samples (Table 2). Direct sequencing was performed on the PCR products of the partial COI gene obtained from 9 dogs. The sequences obtained were identical to the *E. multilocularis* sequence registered in GenBank (AB018440). The numbers of taeniid eggs per gram of feces (EPG) of dogs testing positive for egg DNA were < 10 in two dogs, 10–99 in six dogs, 100–999 in three dogs,

TABLE 1. TEST RESULTS FOR TAENIID EGGS AND FOR *ECHINOCOCCUS MULTILOCULARIS* COPROANTIGEN IN DOGS IN JAPAN

	Taeniid egg test		*Coproantigen test*		*Taeniid egg & coproantigen tests*	
Region	*No. exam.*[a]	*No. pos.*[a]	*No. exam.*[a]	*No. pos.*[a]	*No. sus.*[a]	*No. both pos.*[a]
Hokkaido	4,768	20	4,761	41	31	18
Other[b]	348	3	348	3	1	3

[a]No. exam.: number of dogs examined; No. pos.: number of dogs testing positive; No. sus.: number of dogs testing suspicious; No. both pos.: number of dogs testing positive for both taeniid eggs and coproantigen.
[b]The islands of Honshu and Kyushu.

TABLE 2. RESULTS OF DNA TESTS PERFORMED ON TAENIID EGGS ISOLATED FROM DOG FECES

	Egg DNA test	
Region	*No. exam.*[a]	*No. pos.*[a]
Hokkaido	18	18
Other[b]	2[c]	1

[a]No. exam.: number of samples examined; No. pos.: number of samples testing positive.
[b]The islands of Honshu and Kyushu.
[c]One dog that tested negative for egg DNA was infected with *Taenia pisiformis*, which was confirmed by the morphology of excreted worms after arecoline treatment.

and > 1000 in seven dogs. Along with taeniid eggs, scoleces and segments of *E. multilocularis* were observed by local veterinarians in the feces of three dogs, and the excreted worms from one dog were morphologically identified as *E. multilocularis* by a veterinary parasitologist (Dr. Shin-ichiro Fukumoto in Rakuno Gakuen University, Ebetsu, Japan).

Results of the primary and follow-up examinations for the dogs testing positive are summarized in Table 3. All of the 18 dogs testing positive in the egg DNA test (groups I, II, and III) tested negative in the coproantigen and taeniid egg tests after deworming with praziquantel (5 mg/kg). One dog in group II did not test positive in the coproantigen test either in the primary examination or in the pre-deworming follow-up examination, although taeniid eggs were detected in both examinations. Caution must be taken in drawing conclusions from the dogs in group III, in which the animals primarily tested negative in the taeniid egg test. However, in the subsequent pre-deworming follow-up examination, taeniid eggs were detected in their feces, and they tested positive in the coproantigen test.

The infection status of dogs in groups IV and V was not clear. However, five of the dogs likely harbored active infections (Table 3). It is noteworthy that two dogs in groups IV and V, which were raised together, showed a possible history of re-infection. The dogs tested positive in the coproantigen test but negative in the taeniid egg test in the primary examination, and subsequently they were dewormed with praziquantel. Thereafter, they were examined periodically (every 3 months) to monitor for potential infection. One year later, one tested positive both in the coproantigen test and the taeniid egg test, and the other tested positive in the coproantigen test. Egg DNA tests were not conducted, but taeniid cestodes other than *E. multilocularis* are rarely found in Hokkaido. Thus the dogs were most likely re-infected with *E. multilocularis*.

Although the dogs in groups VI and VII tested positive or suspicious in the coproantigen test, these animals were considered to be uninfected, either because positive scores were not obtained in the pre-deworming follow-up examination (group VI) or because the test scores stayed positive even after deworming (group VII).

Test results of dogs in Honshu and Kyushu

In all, 348 dogs from the islands of Honshu (the main island of Japan) and Kyushu were examined, and 3 dogs tested positive both in the coproantigen and taeniid egg tests (Table 1). Egg DNA tests were conducted on samples from 2 of the 3 dogs, and one sample tested positive (Table 2). According to the questionnaire completed by the owner of the dog that tested positive, the dog was recently moved from Hokkaido. The dog that tested negative for egg DNA was infected with *Taenia pisiformis*, which was confirmed by the morphology of excreted worms after arecoline treatment. The third dog, for which no egg DNA test was conducted, was not examined further and, thus the infection status of that animal was not clear.

Other information for epidemiological consideration obtained from questionnaire

Data obtained from the responses in the dog owners' questionnaires indicated that there was no endemic focus in the geographical distribution of the 18 dogs testing positive for egg DNA in Hokkaido. Nine came from towns and villages ($n = 1325$) and nine came from cities ($n = 3290$) (153 dogs: unknown). The relative risk of infection in town and village dogs relative to city dogs was 2.3 (95% confidence interval: 0.99–6.24).

TABLE 3. RESULTS OF PRIMARY AND FOLLOW-UP EXAMINATIONS OF DOGS TESTING POSITIVE FOR *ECHINOCOCCUS MULTILOCULARIS* IN HOKKAIDO

		Primary examination		*Pre-deworming follow-up examination*		*Post-deworming follow-up examination*			
Group	*Number of dogs*	*Copro Ag*	*Taeniid egg*	*Copro Ag*	*Taeniid egg*	*Copro Ag*	*Taeniid egg*	*Egg DNA*	*Notes*
I	13	P/–	P	P/–	P/–	N	N	P	
II	1	S	P	N	P	N	N	P	
III	4	P/S	N	P	P	N	N	P	
IV	2	P	P	–	–	N	N	–	*1[a]
V	13	P	N	–	–	N/–	N/–	–	*2[a]
VI	9	P	N	S/N	N	S/N/–	N/–	–	
VII	2	P/S	N	P/N	N/–	P	N	–	

CoproAg: coproantigen test; Taeniid egg: taeniid egg test; Egg DNA: egg DNA test; P: positive; S, suspicious; N: negative; – not tested.
[a]Notes: *1: Positive reactions for both the taeniid egg and coproantigen tests are highly suggestive of *E. multilocularis* infection because taeniids cestodes other than *E. multilocularis* are rarely found in Hokkaido. *2: Two dogs in group V were raised with dogs in group I, and one dog in group V was raised with a dog in group IV.

Most of the 18 dogs were kept free-range, but 3 dogs were kept inside houses. Interestingly, the owners of 7 dogs had observed that their dogs showed some kind of interest in rodents (catching, eating, playing with a dead body, etc.). Two of the dogs kept indoors were set free during a walk, suggesting that they had a opportunity to become infected. The other dog had not been set free during a walk.

Discussion

Because 18 dogs were confirmed to be excreting *E. multilocularis* eggs by the egg DNA test and there were additional cases potentially infected with *E. multilocularis*, such as the dogs in groups IV and V (Table 3), the prevalence of *E. multilocularis* infection in the surveyed dogs in Hokkaido was at least 0.4 %, but may have been higher. Although the population surveyed was not representative of the entire population of pet dogs in Hokkaido, the survey revealed that pet dogs in Hokkaido were actually infected under a variety of exposure conditions.

Most of the dogs testing positve for egg DNA were kept free-range; thus the chance to catch and eat infected rodents was high. Some owners had, in fact, observed their dogs catching and eating rodents. In contrast, three of the dogs testing positive for egg DNA were kept inside of their owners' houses. Two were unleashed in large parks or woodlands during their daily walks, and thus they were assumed to have eaten an infected rodent then. The other dog was always kept on a leash during walks. The actual route of infection of this dog was not clear, but the owner indicated that he also owned a cat that frequently caught rodents and brought them back to the house. Thus the dog might have eaten an infected rodent provided by the cat. This potential route of infection is a new aspect to take into consideration in the prophylaxis of pet dogs.

The reason that the dog in group II in Table 3 tested negative for coproantigen in the pre-deworming follow-up examination is not totally clear. Any distinctive conditions of the fecal sample that may have affected the test result were not noted. The fecal sample from this dog carried 4 EPG at the primary examination and 2 EPG at the pre-deworming follow-up examination. Therefore, intensity of the parasite in the dog was assumed to be quite low. Because the minimum parasite burden required for the coproantigen test to detect infection is approximately 100 worms (Nonaka et al, 1996), this dog may have harbored too few worms to be detected by the coproantigen test.

The data in Table 3 concerning dogs in group III must be interpreted with caution. These dogs primarily tested negative for taeniid eggs, but taeniid eggs were detected in the subsequent pre-deworming follow-up examination. The dogs were probably in the pre-patent period when they were initially examined. In fact, some of the dog owners requested the test because they had observed their dogs eating rodents. In such cases, diagnoses should be made during the prepatent period, and accordingly, the coproantigen examination is of greater value for practical diagnosis.

It was assumed that the two dogs belonging to groups IV and V in Table 3 were re-infected one year after the deworming of the first suspected infection. This result suggests that an environment with high infection pressure for dogs exists in some areas of Hokkaido, and that regular deworming treatment of dogs may be required in such situations.

False positive reactions in the coproantigen test were observed in dogs belonging to groups VI and VII, and possibly those belonging to groups IV and V (Table 3). Accordingly, the rate of false positive reactions is 0.2%–0.5%. The sensitivity and specificity of the coproantigen test had previously been evaluated using fox samples. The combined data from two studies comparing the test results of rectum feces and necropsy results of wild foxes in Hokkaido (77 infected and 59 uninfected foxes) measured 92.2% in sensitivity and 96.6% in specificity for the test (Morishima et al. 1999, Yimam et al. 2002). Therefore, the specificity of the test for dogs may be higher than that for foxes. The test showed some cross-reactivity with patent *Taenia hydatigena* (Malgor et al. 1997) and *Taenia pisiformis* infections (unpublished data); however, these species are rare in Hokkaido. No cross-reaction was observed with *Taenia taeniaeformis* and *Taenia crassiceps* infections, which are relatively more prevalent in Hokkaido (Sakashita et al. 1995).

The results from this survey raised public awareness of canine infections, which in turn led to the modification of a Japanese law for infectious diseases in 2003, stipulating responsibilities of animal owners and veterinarians for the control and prevention of the zoonoses. Following this modification, a national reporting system for canine echinococcosis has been in force since October 2004. That system, along with the ongoing reporting system for human patients (in force since 1999), contributes to the determination of the actual risk of pet dogs in the transmission of echinococcosis to humans. In addition, this survey highlights the possibility that *E. multilocularis* may be introduced to the other islands of Japan by transport of infected dogs. Because a significant number of dogs are relocated from Hokkaido every year (Doi et al. 2003), counteractions (e.g., quarantine and deworming), as enforced in the United Kingdom and Norway, may be necessary for risk management in Japan.

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Disclosure Statement

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SHORT PAPER

Granulomatous Pericarditis Associated with Systemic Mucormycosis in a Finless Porpoise (*Neophocaena phocaenoides*)

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Summary

An adult male finless porpoise (*Neophocaena phocaenoides*) kept in an aquarium in Japan displayed loss of appetite and reduced body weight over several months. Necropsy examination revealed the presence of lesions in the pericardium, lung, and mediastinal and pancreatico-duodenal lymph nodes. Microscopically, these comprised regions of necrotizing granulomatous inflammation with multinucleated giant cells and surrounding fibrosis. Fungal hyphae were identified within macrophages and the extracellular tissue. Immunohistochemical labelling determined that these organisms were of the order Mucorales. A diagnosis of granulomatous pericarditis associated with systemic mucormycosis was made.

Keywords: finless porpoise; Mucorales; pericarditis; systemic mycosis

Mycotic pericarditis has been reported in humans and some animal species (Walsh and Bulkley, 1982; Jackman *et al.*, 1992; Faul *et al.*, 1999; Shubitz *et al.*, 2001; Tomita *et al.*, 2005), but to our knowledge there is only a single case report of the occurrence of this disease in marine animals. An Antarctic fur seal was reported with pericarditis associated with *Aspergillus fumigatus* infection by Thomas *et al.* (2001).

The natural source of mycotic organisms is soil, from which the aquatic environment may be contaminated. The organisms usually enter the body through the respiratory tract and can then rapidly disseminate to distant sites via the haematogenous route. Common sites of dissemination of fungi of the order Mucorales include lung, nasal tissue, eye and brain. The organisms are reported to have a tropism for vascular structures (Frater *et al.*, 2001). Systemic mucormycosis may originate from any of the primary sites of infection, particularly the lung, the nasal sinus or the alimentary tract (Thomas *et al.*, 2001).

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Mucorales, which are normally not pathogenic, are most likely to cause disease in individuals with immune suppression. Mucormycosis in marine animals has been attributed to a variety of species including *Apophysomyces elegans, Rhizomucor pusillus, Saksenaea vasiformis* (Thomas *et al.*, 2001), *Entomophthora coronata* (Sweeney *et al.*, 1976) and *Rhizopus* spp. (Wüschmann *et al.*, 1999). Here we describe a finless porpoise (*Neophocaena phocaenoides*) with granulomatous pericarditis associated with systemic mucormycosis.

An adult male captive finless porpoise (*N. phocaenoides*), kept in an aquarium for 410 days, displayed loss of appetite and reduction in body weight over several months. Serum biochemical examination revealed elevated level of γ-glutamyltranspeptidase (80 IU/l, reference range 30–50 IU/l), alanine aminotransferase (91 IU/l, reference range 28–60 IU/l), potassium (9.6 mmol/l, reference range 3.2–4.2 mmol/l) and total protein (117 g/l, reference range 60–78 g/l). Total white blood cell count was within normal limits (7.89×10^9/l, reference range $5.0–9.0 \times 10^9$/l). Despite treatment with liver supplements including

vitamin B and sulphur-containing amino acids, the animal died several months after the onset of the clinical signs and was sent to Tottori University for necropsy examination.

At necropsy, the animal was emaciated with marked reduction of blubber thickness. There were numerous white nodules, up to 2 cm in diameter, with a solid cut surface in the pericardial region, lung and mediastinal and pancreatico-duodenal lymph nodes. The pericardium was thickened and adherent to the epicardium. A solitary white mass, 22 × 12 × 13 cm in size, was noted between the pericardium and myocardium (Fig. 1). On sectioning, the mass was seen to contain thick green-white fluid.

Tissue samples were taken from the pericardial lesion in addition to the liver, spleen, kidney, heart, lung, stomach, small intestine, mediastinal and peritoneal lymph nodes and bladder. These were fixed in 10% neutral buffered formalin and embedded in paraffin wax. Sections prepared from these tissues (3 μm) were stained with haematoxylin and eosin (HE). Selected sections were stained with periodic acid-Schiff (PAS) and Grocott's methenamine silver (GMS). Immunohistochemistry (IHC) was performed on serial sections in order to further identify the fungal organism. The primary antibodies used were a mouse monoclonal antibody specific to fungi of the order Mucorales (Mab-WSSA-RA-1) (DAKO, Glostrup, Denmark) (Jensen *et al.*, 1996a) and a mouse monoclonal anti-*Aspergillus* spp. antibody (Mab-WF-AF-1) (DAKO) (Jensen *et al.*, 1996b). A commercially available streptavidin-biotin-alkaline phosphatase system was used for secondary detection of these antibodies (Histofine™ SAB-PO Kit; Nichirei, Tokyo, Japan) (Yokota *et al.*, 2004). As a negative control, phosphate buffered saline (PBS) was substituted for the primary antibodies and no reactivity was seen.

The lesions in the pericardium, lung and lymph nodes were characterized by necrotizing granulomatous inflammation with multinucleated giant cells. Fibrosis was prominent at the periphery of each nodular lesion. Hyphal elements were scattered randomly in the lesions and were present within macrophages as well as the extracellular tissue (Fig. 2). The hyphae were up to 40 μm in diameter, infrequently septate, thin-walled and displayed a variable pattern of branching (Fig. 3a, b).

On IHC, the intracellular cytosolic granular structures and cell walls of most of the hyphae were strongly and uniformly labelled by antibody specific for Mucorales (Fig. 3b). No labelling with *Aspergillus* spp. antibody was observed. Necrotizing vasculitis due to fungal invasion of the blood vessels was observed in the pericardial lesion.

Granulomatous lesions were most severe in the pericardium, but were also present in the lung, heart and multiple lymph nodes. It is possible that the pathogen initially entered the respiratory tract and extended directly to the pericardium or indirectly via the blood circulation. Mycotic infections often occur in hosts with immune suppression following chronic primary disease or stress (Sweeney *et al.*, 1976). In the present case there was no clear evidence of pre-existing immunosuppressive disease, but the stress associated with captivity may contribute to immune suppression in such animals.

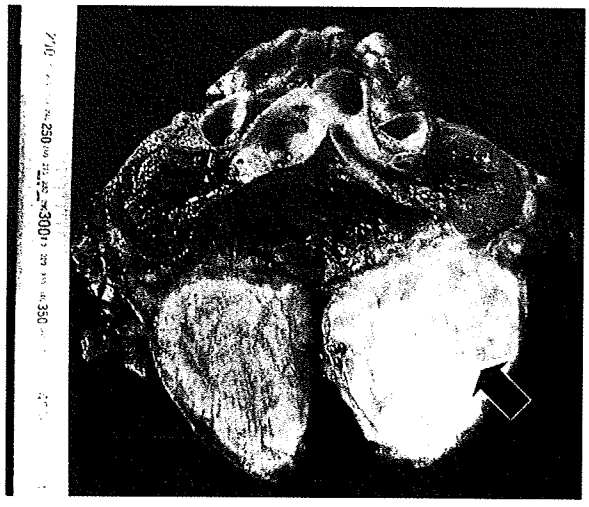

Fig. 1. Sectioned heart showing the presence of a large, solitary, cream-white mass associated with the pericardium of the left ventricular region (arrowed).

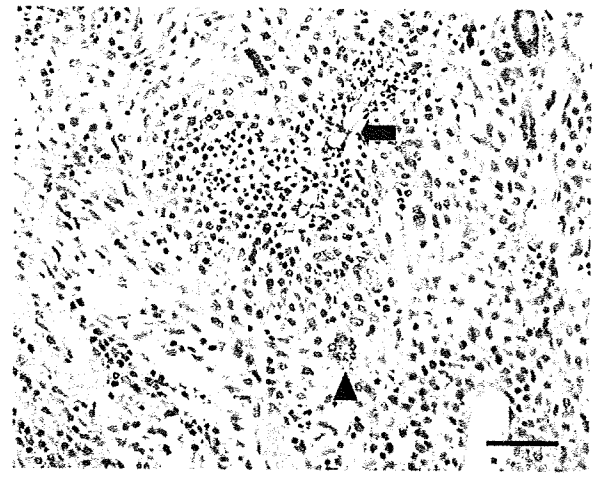

Fig. 2. Section from the pericardial lesion shown in Fig. 1 reveals the presence of granulomatous inflammation with multinucleated giant cells (arrowhead) and extracellular fungal hyphae (arrow). HE. Bar, 60 μm.

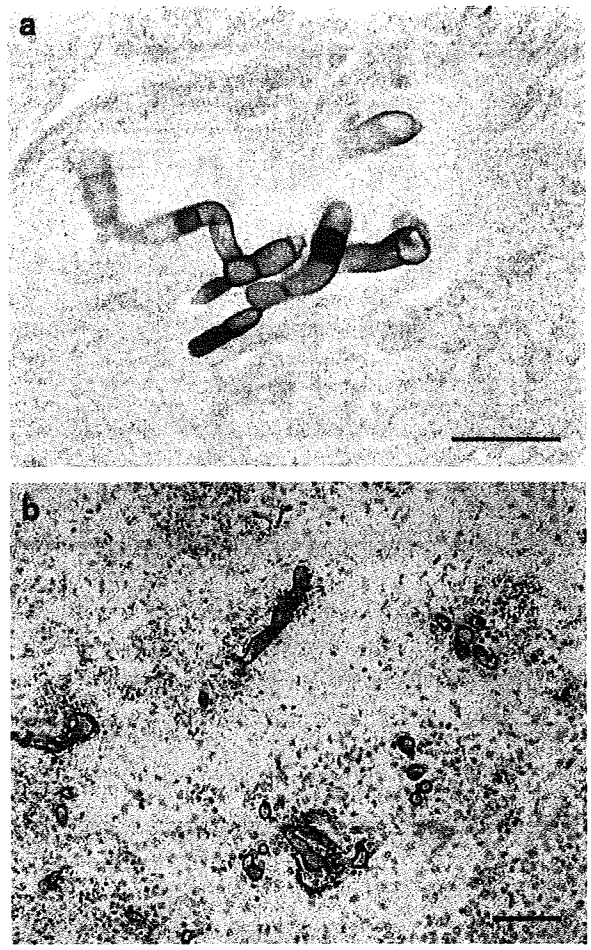

Fig. 3. (a) Detail of fungal hyphae within the pericardial lesion. Hyphae were up to 40 μm in diameter, infrequently septate, thin-walled and with a variable pattern of branching. GMS. Bar, 30 μm. (b) Labelling of the hyphae by antiserum specific for Mucorales. IHC. Bar, 60 μm.

The hyphae of Mucorales can often be distinguished from those of other invasive hyaline fungi by their greater width, obtuse branching pattern and sparse septation. Because of their thin hyphal walls, Mucorales hyphae are, however, susceptible to compression, resulting in twisting and folding with septa-like artefacts (Frater *et al.*, 2001). The rarity of septae in the organisms of the present case may relate to mechanical distortion, possibly induced by severe fibrosis within the lesion.

The primary antibody against Mucorales used in this study is known to be specific to members of this order (Jensen *et al.*, 1996a). The fungal hyphae diffusely observed within the granulomatous lesions of the present case varied in shape, were irregularly branched and showed strong labelling with antibody. These features, together with the observed tropism for vascular structures, allowed identification of the organism and classification of this as a case of disseminated mucormycosis.

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