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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Characterization of *em*Y162 encoding an immunogenic protein cloned from an adult worm-specific cDNA library of *Echinococcus multilocularis*

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Abstract

A cDNA library based on mRNA from adult worms of *Echinococcus multilocularis* was constructed. One cDNA clone, *em*Y162, was isolated from this cDNA library. The putative protein from *em*Y162 cDNA consists of 153 amino acids and has a predicted molecular weight of 17.0 kDa. The amino acid sequences of EMY162 are predicted to be a hydrophobic N-terminus conserving a secretory signal, and a hydrophobic C-terminus encoding a transmembrane domain or glycosyl-phosphatylinositol membrane anchor, and to have single fibronectin type III-like domain. In addition, it was shown that the *emY162* gene (1738 bp) in the *E. multilocularis* genome DNA consists of three exons and two introns, and that *em*Y162 is expressed in all four stages (protoscoleces, cultured metacestodes, immature adult worms and mature adult worms). Moreover, immunity to recombinant EMY162, which comprises the fibronectin type III-like domain on the EMY162 protein, was examined. Immune responses to the recombinant EMY162 were studied by using serum from dogs infected with *E. multilocularis*. Strong IgG immune responses were detected in Western blots.

Keywords: *Echinococcus multilocularis*; Adult worm; cDNA library; *em*Y162 cDNA; Immunogenic protein; Recombinant EMY162 antigen

1. Introduction

Echinococcus multilocularis is a cestode parasite [1]. The larva, metacestode, develops in several mammalian intermediate host species, while the adult tapeworms develop mainly in dogs and foxes (definitive hosts). The life cycle of *E. multilocularis* generally occurs in foxes and rodents as intermediate hosts. Humans can be infected by accidental ingestion of the parasite eggs from an infected fox, or occasionally from infected dogs or cats. Invasion by *E. multilocularis* leads to destruction of the liver, and to damage of other organs via metastases [2]. Infection in humans causes alveolar hydatid disease [1]. Although human infection is uncommon in many countries, the disease has a high prevalence in the European latitudes north of the Alps [3] and south Gansu in China [4]. Similarly, the disease is endemic in the island of Hokkaido, Japan. In these areas the disease is a significant public health problem.

The basis of the strategy to reduce the risk of human infection is to break the cycle of transmission and avoid the production of infectious eggs. Protection against infection based on this strategy has been already achieved in the genus *Taenia*, which causes cysticercosis. Protective vaccination with either recombinant proteins or peptide epitopes of 45W, TO16, TO18 and TSA18 proteins had a high efficacy against *T. ovis* infection in sheep and *T. saginata* infection in cattle [5–8]. In addition, a recombinant vaccine has been developed for use in the control of cystic hydatid disease caused by *E. granulosus*. Lightowlers et al. [9] have demonstrated that the recombinant EG95 is a highly effective vaccine to prevent infection with *E. granulosus* in sheep in Argentina, Australia and New Zealand. These experimental results indicate that the prevention of the disease by vaccination, with a high degree of protective immunity against parasites, is possible. Vaccination of foxes and/or dogs (the definitive hosts) is postulated to be one of the most effective

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measures to control the spread of *E. multilocularis*. Gauci et al. [10] reported that EM95 recombinant protein induced significant levels of protection in mice (intermediate host) infected with *E. multilocularis* eggs. However, no information is available about the vaccination of dogs to prevent infection with *E. multilocularis* due to its biohazardous nature, and few studies have been conducted on immunity to *Echinococcus* infections in definitive hosts.

Meanwhile, many vaccine candidate proteins have been discovered that are secretory and anchored on the surface of parasites. They usually possess N-terminal hydrophobic signal peptides and C-terminal hydrophobic trans-membrane domains, and are involved in host–parasite interactions. These secreted and trans-membrane proteins participate in parasite functions including penetration and establishment in host tissues and modulation of host immune responses. These secretory proteins are candidates for development of an *E. multilocularis* vaccine, or for diagnostic use in canines. cDNAs encoding secreted and trans-membrane proteins from a parasite cDNA library have been isolated [11,12].

We attempted to clone cDNA of secretory proteins involved in immune defense systems in order to use them in the control of alveolar hydatid disease. One of the cDNA clone, *em*Y162, proved to be the first new secretory protein that acted as an antigen recognized by the canine immune system. In this paper, we report the analysis of *em*Y162 cloned from an adult worm-specific cDNA library, and the immune responses to recombinant EMY162 with the serum from *E. multilocularis*-infected dogs.

2. Materials and methods

2.1. Materials

E. multilocularis (Nemuro strain) was obtained from a dog–cotton rat life cycle maintained at the Hokkaido Institute of Public Health. Protoscoleces were taken from a cotton rat infected with the Nemuro strain and washed with PBS. Immature adult worms were collected on day 20 post-infection from a dog experimentally infected with *E. multilocularis* protoscoleces. Mature adult worms were also collected on day 60 post-infection. The worms were first released from the intestinal contents by soaking in PBS to remove canine intestinal mucus, then rinsed several times in PBS. The cultured metacestodes were obtained by an *in vitro* culture system basically following Hemphill and Gottstein [13] and Spiliotis et al. [14]. After washing with PBS, all parasite materials were immediately soaked in RNA Later (Ambion, Inc.) and stored in liquid nitrogen. In addition, 1×10^5 of *E. multilocularis* protoscoleces in 2 ml of PBS were used for oral infection. Five Beagle dogs (male, 16 months old) were bled at 40 days after infection, and sera were stored individually at −30 °C until examined for IgG response. The sera of 5 uninfected-Beagle dogs as the control were treated similarly. All experiments were performed in a specially designed safety facility, the Hokkaido Institute of Public Health (biosafety level 3).

2.2. cDNA library construction

Total RNAs from immature and mature adult worms were isolated using Isogen (Nippon Gene) according to the manufacturer's instructions. About 3 ml of parasite material was used for extracting total RNA. mRNAs in the total RNA were prepared using an Oligotex-dT30 (Super) mRNA Purification Kit (Takara Bio), and then reverse transcribed to cDNA using the SMART cDNA Library Construction Kit (BD Biosciences) for first-strand cDNA synthesis in a total reaction volume of 20 µl. The cDNA was inserted into the *Sfi*I site of the vector. The vector was used to prepare a cDNA library in bacteriophage λTriplEx2 (BD Biosciences) according to the manufacturer's recommendations. The library was screened by random cloning. Five hundred forty clones were identified from about 400,000 recombinant phages. The clone, designated *em*Y162 was excised from pTriplEx2 in host bacteria XL-Blue according to the manufacturer's recommendations (BD Biosciences).

2.3. Isolation of emY162 clone from cDNA library

The *em*Y162 clone was isolated as follows. Recombinant bacteriophages of the cDNA library were infected with XL1-Blue bacteria. After treatment for 15 min at 37 °C, the infected bacteria were spread on to a LB/$MgSO_4$ plate and then incubated at 37 °C overnight. Single plaques were transferred to SM solution and kept at 4 °C overnight. The SM solution containing the recombinant phage was treated with BM25.8 bacteria. After incubation for 1 h at 31 °C with shaking, the transformed bacteria were spread on a LB–carbenicillin (50 µg/ml) plate and incubated at 31 °C overnight. The cloned bacteria were grown in LB–carbenicillin (50µg/ml) medium at 31 °C overnight. The bacteria were harvested by centrifugation at 2600 rpm. Plasmid DNA was isolated by using QIAGEN Plasmid Tip 20 (Qiagen). The nucleotide sequences of the plasmid were analyzed on a Gene Analyzer, and the *em*Y162 clone was determined.

2.4. Amplification of emY162 gene in genome DNA

Genome DNA was extracted from protoscoleces of *E. multilocularis* using a DNeasy genomic Kit (Qiagen). One µl (1 ng/µl) of the solution was used as the template DNA for PCR. Primers were designed according to the DNA sequence of *em*Y162 compiled by Gene Works sequence analysis software: upstream primer: 5′-ggaagatggtacttcgattctgt-3′, downstream primer: 5′-tgagggcctgtaagttc-caact-3′. Two additional primers (5′-gagctaatagcaaagttg-3′ and 5′-cacgtgaatc-catcggaagt-3′) were also designed to sequence a fragment of *em*Y162 DNA. PCR amplification was carried out by a Gene Amp PCR System 9700 (Applied Biosystems) in 50 µl of reaction mixture with Taq DNA polymerase (Roche Diagnostics). PCR conditions were as follows: 94 °C for 2 min, then 30 cycles of 93 °C for 30 s, 55 °C for 30 s, and 68 °C for 4 min, and finally 68 °C for 7 min. The amplified DNA fragment was purified with TaKaRa Easy Trap v. 2 (Takara Bio) after separation by agarose gel electrophoresis. This was used as the template DNA for the sequencing reactions.

2.5. Detection of emY162 cDNA in four stages

The *em*Y162 cDNA was amplified by reverse transcriptase (RT)-PCR from each of the four stages (protoscoleces, cultured metasestodes, immature adult worms, and mature adult worms) of *E. multilocularis*. Total RNA from the four stages was isolated using an RNeasy Mini Prep Kit (Qiagen) according to the manufacturer's instructions. About 0.1 µg of total RNA was used for RT-PCR amplification of the *em*Y162 cDNA by the SMART cDNA Library Construction Kit (BD Biosciences). The same primers (5′-ggaagatggtacttcgattctgt-3′and 5′-cacgtgaatccatcggaagt-3′) used for amplification of the *emY162* gene in genome DNA were used for RT-PCR. The predicted size of the RT-PCR product was 144 bp. In addition, two primers (5′-gttgtgctatgtggcactcgact-3′ and 5′-caatccagacagagtatttgcgttc-3′) were also designed to amplify a fragment of β-actin cDNA of *E. multilocularis* to monitor the integrity of the RNA from each stage. PCR amplifications were carried out using Gene Amp PCR System 9700 in 50 µl of the reaction mixture with Taq polymerase (Roche Diagnostics). The conditions for PCR were as follows: denaturation at 94 °C for 2 min, then 35 cycles of 93 °C for 30 s, 55 °C for 30 s, and 68 °C for 30 s, and finally 68 °C for 7 min. Fragments were visualized by ethidium bromide staining after agarose gel electrophoresis.

2.6. Preparation of recombinant EMY162

The *em*Y162 DNA fragment was amplified from *em*Y162-pTriplEx2. An up-stream primer (5′-agatctgtagacccagagctaatag-3′) with a *Bgl*II site and a downstream primer (5′-ctgcaggaatccgccagctctgtca-3′) with a *Pst*I site were designed for amplification of a 360-bp fragment of *em*Y162 DNA. PCR amplification was carried out using the Gene Amp PCR System 9700 in 50 µl of reaction mixture with Taq DNA polymerase (TakaraBio). Amplified DNA was subcloned into a *Bgl*II/*Pst*I-digested ThioHis vector (Invitrogen) and then

transformed into *Escherichia coli* Top10 strain (Invitrogen). The bacterial culture was incubated overnight at 37 °C. After cultivation, recombinant EMY162 was induced with 0.5 mM isopropyl-D-thiogalacto-pyranoside for 4–5 h at 32 °C, and following centrifugation, suspended in B-PER plus a protease inhibitor. The recombinant EMY162 expressed as a fusion protein with ThioHis was treated with ProBond™Affinity Resin (Invitrogen). The fusion protein was eluted with 20 mM sodium phosphate buffer (pH 6.0) containing 500 mM imidazole and 500 mM sodium chloride, and purified by AKTA Explorer (Amersham Biosciences) with a Hiload Superdex 75 pg column equilibrated with 20 mM Tris–HCl buffer (pH 7.8) containing 500 mM sodium chloride. Purified protein was used in Western blotting.

2.7. Detection of IgG response by Western blotting

Western blotting was carried out as follows. Approximately 1 μg of recombinant EMY162 was loaded onto a 12% SDS-polyacrylamide gel. After electrophoresis, proteins were transferred to a PVDF membrane (Amersham Biosciences), and then treated with PBS containing 10% skim milk and 0.1% Tween 20 for 1 h at room temperature. The recombinant EMY162 protein was detected with diluted canine serum (1:400) at 40 days after infection with *E. multilocularis* and diluted AP-labeled rabbit anti-dog IgG (1:2500), using the BCIP/NBT Immuno-detection Kit (PerkinElmer) according to the manufacturer's instructions.

2.8. Analysis of DNA and protein sequences

Plasmid DNA containing the parasite cDNA was prepared using a DNA Purification Kit (Qiagen) and used as a template in DNA sequencing reactions. Sequencing was performed using the 3130*xl* Gene Analyzer with a Dye-terminator Cycle Sequencing Kit (Applied Biosystems). DNA sequencing of the *emY162* gene was also performed by the same means. DNA and protein sequences were compiled using Gene Works (v. 2.5.1, Teijin) sequence analysis software. The sequence was aligned to other gene sequences available in a Basic Local Alignment Search Tool (BLAST) search of GenBank databases.

3. Results and discussion

cDNA library was constructed from immature adult *E. multilocularis* worms. In the cloning experiment, 540 cDNA clones were isolated. Their nucleotide and predicted amino acid sequences were aligned to other sequences available in GenBank databases. The alignment hit one protein sequence relating to secretory proteins containing EG95 and EM95 proteins that act as antigen.

The *em*Y162 cDNA (GenBank accession number: AB303298) cloned from the immature adult worm cDNA library of *E. multilocularis* comprised 776 nucleotides with an open reading frame of 462 bases. Comparison of nucleotide alignments showing the presence of ATG at the 5′ end and TGA (stop codon) at the 3′ end indicated that this cDNA represents a complete copy of the mRNA of the *emY162* gene. This cDNA sequence does not appear to be a copy of any other gene sequence available in a BLAST search.

The deduced amino acid sequence of *em*Y162 cDNA showed similarity to the previously described antigenic secretory proteins EM95 and EG95-1 (30% and 36%, respectively) [10,15]. When a BLAST search was also conducted for homology to antigenic proteins of *T. ovis* and *T. saginata* parasites, the EMY162 protein showed less than 30% identity to all antigens [5,7,16]. In addition, the amino acid sequence of EMY162 showed some similarity to a part of tenascin, collagen-like protein, protein tyrosine phosphatase (receptor type), and fibronectin 1, according to a BLAST search of GenBank databases. Comparison with amino acid sequences of the cDNAs that

```
AAGATGGTAC TTCGATTCTG TCTTATTTTA CTGGCAACTT CAGTTATCGC TGAGGAAGTC GGGGTAGACC CAGGTAAGAT ACGTTTAAAA TTCAACTTGC   100
  M  V  L  R  F  C  L  I  L  L  A  T  S  V  I  A  E  E  V  G  V  D  P
ATCAGCATAC AATTTAAATT ATATAACAAG CTTGTATTCA TCTGCGTATG CATAGTATGT TGTGGTGGAT GCTTTTCAAC TCGTTAGTAG TTAACAGATG   200

ACCAGTTGGC TACTACCACT CATTTGATAA TATGTCACTG TCAAATCTAA AATGACGATT TTTAGCAGAA TCATTGACTC CATATTCCTA CTGCTGTCCT   300

CCTTCTGTAT CAGTTCGGGT AGATATTTGA GTCATCTGCG AAGTGTCATC CACGGGTAGG GAAATGCTTC AAAAATACAC CCTCACTTAG AAATGTAGTC   400

CCAAACACTG ATAGTAGACT GATTCACTGC ACCTCACGAC TACTTATTAT TGCACACAAT TTGTGTACCC TGCACTTGAT AGAACATTGC CTTGCTGATA   500

TTTCGCCTCA TTGTGTTCAC TCGCATAGTA CACTCCCACT TAAACAGCTC GTAAGGTAAA AGGCCTTCGG TATCTGGTCG CACCTACACA TCACCTCGTC   600

ACTGCGTGCA ACTTCAGCTG TAGCAAACCC ACCACTGACA CTATAACTCT CCGAAATCAG GATGTAGTGA CATCCATTTA CCTGCATGAT GGGTCCAGGA   700

TGACAGGAAG AAGTGCCTTG ACACACTTCA TATTGTTGGA ACACAATCCA CAAACTTAAC TTAAGCCTCA TGTTTAACCT TCTACCCAAG CATTCCGCTT   800

AATTTCGCTA AAAGCTTAGC CAAGAAGTGA CTGGAAAATT GGCTGAAATT GTTAATTTTG CAATGGGTTT GCTCCGGAAA AAAGGTTGCG CACGCGTGTG   900

TACACATGAC TGGCTATTGC GCATCAACGG ATGACACTCA GCAATTCTTA CCTCTGGTGG AACATACGGG TTAATTACTC CATCGTTTAG GGGAACACAG  1000

CAAAGTCCTG TTATCCACTG CCATCATTGT CATTATGTTT GGATAGAGGG TGCAACATTT CTGCCATTAA ATGGAATGGA ATCAACAAAA CATTATTGTT  1100

GAACAACGAA TTTTTCCGAT TCGTTTTTGC AGAGCTAATA GCAAAGTTGA CAAAGAAACT ACACAGACCA CTGCCAGAAC ACTTCCGATG GATTCACGTG  1200
                                E  L  I  A  K  L  T  K  K  L  H  R  P  L  P  E  H  F  R  W  I  H  V
GGTTCCCGCT CCCTTGAATT GGGTTGGAAT GCCACTGGTT TAGCCAATCT CCACGCGGAC CACATTAAAC TGACTGCAAA CCTTTATACA ACTTACGTTT  1300
G  S  R  S  L  E  L  G  W  N  A  T  G  L  A  N  L  H  A  D  H  I  K  L  T  A  N  L  Y  T  T  Y  V  S
CATTCAGGTA CAGAAATGTT CCTATCGAAC GTCAGAAACT CACTCTTGAG GGACTAAAGC CCAGTACATT CTACGAAGTG GTTGTGCAAG CACTCAAAGG  1400
  F  R  Y  R  N  V  P  I  E  R  Q  K  L  T  L  E  G  L  K  P  S  T  F  Y  E  V  V  V  Q  A  L  K  G
GGATTCCGAA GTTTATAAAT ACACTGGATT TATTAGAACA CTGGCTCCAG GTAAGCTAGC ATGCGATAGT AATGTGAACT TCACACCGAG GATGGATGCC  1500
 D  S  E  V  Y  K  Y  T  G  F  I  R  T  L  A  P
CACCAAACTT CACCTTTGGC GGCAGTTCCT GTGTTATTAC ACTAGACCAC AAGTTAGACG AACCACAAGG CGTCAGTAGC TAATGCTCAA ATTTTGCAAA  1600

GTTTGCCGTT GAGTGGCACT GGTGTGCTGC GTAACGACAC TGACAGAGTG TCATTTTCGC ATTGCAGGGG AAGATGGCGC TGACAGAGCT GGCGGATGCG  1700
                                                                G  E  D  G  A  D  R  A  G  G  C  A
CCCTAATTTT TGCAATGGCT GGGCTCCTAT TACTTACTTG AGCCTTCCCG TAAGCCAATG AAGGTGGTCA ACTGTTGCAT TAGTTGGAAC TTACAGGCCC  1800
  L  I  F  A  M  A  G  L  L  L  L  T  .
TCA                                                                                                          1803
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Fig. 1. Nucleotide sequences (1803 bp) of *emY162* gene in *Echinococcus multilocularis*. Predicted amino acid sequence of the encoded protein is shown below the DNA sequence.

produce the known secretory protein in parasites of the genus *Taenia* did not identify significant homology.

The EMY162 protein consists of 153 amino acids and has a predicted molecular weight of 17.0 kDa. This protein has a relatively high proportion of leucine residues (16%) and one putative N-linked glycosylation site at amino acid position 83. The predicted structure from the amino acid sequence of EMY162 consists of a hydrophobic N-terminus (amino acids 1 to 16) predicted to be a secretory signal, a hydrophobic C-terminus encoding a trans-membrane domain or glycosyl-phosphatylinositol membrane anchor (amino acids 130 to 153), and one fibronectin type III domain (amino acids 84 to 129) [17–19]. Bork and Doolittle [20], Bork et al. [21] and Campbell and Spitzfaden [22] have indicated that proteins with fibronectin type III domains include the immunoglobulin superfamily, cell adhesion, surface receptors and carbohydrate-binding proteins. Analysis of the predicted amino acid sequence has revealed the presence of a conserved motif. The motif defines a fibronectin type III domain, having 40% homology compared to fibronectin sequences available in a BLAST search of GenBank databases.

The *emY162* gene (GenBank accession number: AB303297) in genome DNA was amplified by PCR from the *E. multilocularis* genome DNA. A 1738-bp DNA fragment encodes amino acid sequences of the EMY162 protein. The *em*Y162 nucleotide sequences (1803 bp) are shown in Fig. 1. The *emY162* gene consists of three exons and two introns, with similarities in structure to secretory proteins such as the *em95* gene [10]. The intron splice sites of the *emY162* gene are conserved in comparison with the cDNA. Exons 1, 2 and 3 of the gene and cDNA are identical in the DNA sequence. The lengths of exons 1, 2 and 3 are 70, 318 and 74 bp, respectively. The intron length of the *emY162* gene does not appear to be as conserved as that of the *em95* gene [10]. The lengths of intron 1 and 2 are 1059 and 217 bp, respectively.

The length of exon 1 in the *emY162* gene is similar to that of the *em95* gene, while exons 2 and 3 differ in these genes [10]. The lengths of exon 2 and 3 of *em95* were 306 and 92 bp, respectively [10]. The exon lengths of the *emY162* gene do not appear to be as conserved. In addition, the length of intron 1 of *em*Y162 also differs from the length of intron 1 in *em*95. These analyses clearly indicate that the *em*Y162 isolated from the cDNA library based on mRNA from adult *E. multilocularis* tapeworms codes for a novel secreted transmembrane protein that is different from the gene family of secretory proteins such as em95.

Meanwhile, the same putative size (144 bp) was detected in all four stages examined. The RT-PCR products are shown in Fig. 2(A). The amplified sequence covers exon 1 and a part of the N-terminus in exon 2 of EMY162 protein that includes the

(A)

(B)

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P-emY162F1/Ra TGGAAGATGG TACTTCGATT CTGTCTTATT TTACTGGCAA CTTCAGTTAT  50
C-emY162F1/Ra TGGAAGATGG TACTTCGATT CTGTCTTATT TTACTGGCAA CTTCAGTTAT  50
I-emY162F1/Ra TGGAAGATGG TACTTCGATT CTGTCTTATT TTACTGGCAA CTTCAGTTAT  50
A-emY162F1/Ra TGGAAGATGG TACTTCGATT CTGTCTTATT TTACTGGCAA CTTCAGTTAT  50

P-emY162F1/Ra CGCTGAGGAA GTCGGGGTAG ACCCAGAGCT AATAGCAAAG TTGACAAAGA 100
C-emY162F1/Ra CGCTGAGGAA GTCGGGGTAG ACCCAGAGCT AATAGCAAAG TTGACAAAGA 100
I-emY162F1/Ra CGCTGAGGAA GTCGGGGTAG ACCCAGAGCT AATAGCAAAG TTGACAAAGA 100
A-emY162F1/Ra CGCTGAGGAA GTCGGGGTAG ACCCAGAGCT AATAGCAAAG TTGACAAAGA 100

P-emY162F1/Ra AACTACAGAC CACACTGCCA GAACACTTCC GATGGATTCA CGTG        144
C-emY162F1/Ra AACTACAGAC CACACTGCCA GAACACTTCC GATGGATTCA CGTG        144
I-emY162F1/Ra AACTACAGAC CACACTGCCA GAACACTTCC GATGGATTCA CGTG        144
A-emY162F1/Ra AACTACAGAC CACACTGCCA GAACACTTCC GATGGATTCA CGTG        144
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Fig. 2. Agarose gel electropherogram (A) and nucleotide sequences (B) of products amplified by reverse-transcriptase polymerase chain reaction (RT-PCR). (A) Total RNA from protoscoleces (P), cultured metacestodes (C), and immature (I) and mature adult (A) worms of *Echinococcus multilocularis* were used to amplify *em*Y162 cDNA with β-actin cDNA (379 bp) as the control. The position of the amplified *em*Y162 cDNA band of the expected size (144 bp) is indicated by an arrow. Molecular (M) size markers are shown in the left and right lanes. (B) Nucleotide sequences of 144-bp products of protoscoleces (P), cultured metacestodes (C), and immature (I) and mature adult worms (A) amplified by using RT-PCR with the primer pair of F1 and Ra are shown.

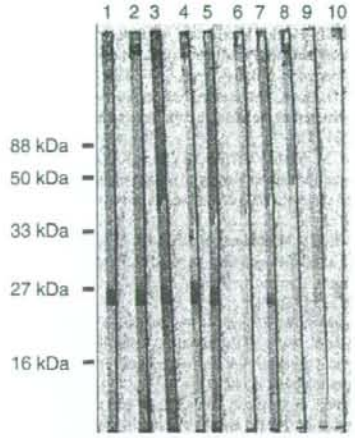

Fig. 3. Immunoblot analysis of recombinant EMY162 antigen using sera of dogs infected with *Echinococcus multilocularis*. Lanes 1–5: sera of dogs infected with the Nemuro strain (40 days after infection); lanes 6–10: sera of uninfected dogs.

peptide sequences (MVLRFCLILLATSVIAEEVGVDPE and LIAKLTKKLQTTLPEHF). The primers used in the RT-PCR analysis, according to the *em*Y162 mRNA sequence, span an intron of 1059 bp in the *emY162* gene. In a comparison of sequence alignment, PCR products from each of the four stages corresponded to positions 1 to 140 in *em*Y162 mRNA. The 144-bp RT-PCR products indicated that they contained cDNA sequences only, without introns. The results of RT-PCR indicated that *em*Y162 is expressed in all four stages. In addition, the nucleotide sequences of the 144-bp RT-PCR products are shown in Fig. 2(B). The sequences were all the same. No difference was detected in the peptide sequences of *em*Y162 mRNA expressed in all four stages.

As shown in Fig. 3, IgG responses to the recombinant EMY162 in dog serum at 40 days after infection were detected by using Western blotting. A blotting band was observed at a predicted molecular weight of 27 kDa, but it was not detected in the serum of the uninfected dogs used as the control. In this experiment, sera of five dogs that were infected with 1×10^5 *E. multilocularis* protoscoleces showed strong IgG response to recombinant EMY162. It is therefore possible that EMY162 could be used as a diagnostic antigen for serological evaluation of canine *E. multilocularis* infection.

Alignment of the amino acid sequences of *em*Y162, *em*95, *eg*95-5 and TSO45w-4B is shown in Fig. 4. The alignment data showed that amino acid differences are evident in the fibronectin type III-like domain and C-terminus, while these genes were 70% similar to amino acid sequences in the N-terminus. The significant differences are an insertion of four amino acids in the fibronectin type III-like domain and a deletion of seven amino acids in the C-terminus. In addition, the amino acid sequences of *em*Y162 were over 70% different in the linear immunogenic regions of *eg*95-5 and *em*95. The linear immunogenic regions of the EM95 and EM95 proteins include the peptide sequences TETPLRKHFNLTPV (peptide 6), SLKAVNPSDPLVYKRQTAKF (peptides 12/13), DIETPRAGKKESTVMTSGSA (peptides 21/22) and SALTSAIAGFVFSC (peptide 24) [23–25]. As shown Fig. 4, the amino acid sequences of the EM95 proteins are very similar to linear immunogenic regions of the EG95-5 protein. However, significant differences existed in the amino acid sequences of EMY162 protein as compared with two EM95 and EG95-5 proteins. In considering the use of EMY162 as a serodiagnostic

Fig. 4. Alignment of amino acid sequences of *em*Y162 and *em*95 from *Echinococcus multilocularis*, *eg*95-5 from *Echinococcus granulosus* and *TSO*45W-4B from *Taenia solium* (EM95, EG95-5 and TSO45W-4B sequences; GenBank accession numbers AJ420235, AF134378 and AF267119, respectively). The genes of each species have conserved amino acid sequences designated by gray and clear boxes.

tool, it will be essential to characterize host antibody responses, especially the kinetics of the specific antibody response after infection and subsequent chemical deworming, and class and subclass specificity against EMY162. In other parasitic infections in dogs, Deplazes et al. [26] and Nieto et al. [27] demonstrated that the analysis of IgG subsets in parasitized dogs provides evidence of a dichotomous response to infection: IgG2 is associated with asymptomatic protozoan infections and IgG1 is associated with helminth infections and diseases caused by protozoan infection.

Overall, *em*Y162 has features similar to those encoded by oncosphere antigens like *em*95, but does not share significant homology within its sequence. We demonstrated that EMY162 could target both mucosal and systemic immunity in dogs because it is predicted to be a protein with a fibronectin type III-like domain, while the serum of infected dogs showed strong IgG antibody responses to the recombinant EMY162. EMY162 could provide a potential route for the development of a practical vaccine to reduce the level of echinococcosis in canines. Future research will therefore focus on investigating the protective potential of the EMY162 protein or its peptide epitopes against infection with *E. multilocularis* eggs in intermediate and definitive hosts.

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Anaerobic NADH-Fumarate Reductase System Is Predominant in the Respiratory Chain of *Echinococcus multilocularis*, Providing a Novel Target for the Chemotherapy of Alveolar Echinococcosis▽

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Alveolar echinococcosis, which is due to the massive growth of larval *Echinococcus multilocularis*, is a life-threatening parasitic zoonosis distributed widely across the northern hemisphere. Commercially available chemotherapeutic compounds have parasitostatic but not parasitocidal effects. Parasitic organisms use various energy metabolic pathways that differ greatly from those of their hosts and therefore could be promising targets for chemotherapy. The aim of this study was to characterize the mitochondrial respiratory chain of *E. multilocularis*, with the eventual goal of developing novel antiechinococcal compounds. Enzymatic analyses using enriched mitochondrial fractions from *E. multilocularis* protoscoleces revealed that the mitochondria exhibited NADH-fumarate reductase activity as the predominant enzyme activity, suggesting that the mitochondrial respiratory system of the parasite is highly adapted to anaerobic environments. High-performance liquid chromatography–mass spectrometry revealed that the primary quinone of the parasite mitochondria was rhodoquinone-10, which is commonly used as an electron mediator in anaerobic respiration by the NADH-fumarate reductase system of other eukaryotes. This also suggests that the mitochondria of *E. multilocularis* protoscoleces possess an anaerobic respiratory chain in which complex II of the parasite functions as a rhodoquinol-fumarate reductase. Furthermore, in vitro treatment assays using respiratory chain inhibitors against the NADH-quinone reductase activity of mitochondrial complex I demonstrated that they had a potent ability to kill protoscoleces. These results suggest that the mitochondrial respiratory chain of the parasite is a promising target for chemotherapy of alveolar echinococcosis.

Echinococcosis is a near-cosmopolitan zoonosis caused by helminthic parasites belonging to the genus *Echinococcus* (family Taeniidae) (18). The life cycle of *Echinococcus* spp. includes an egg-producing adult stage in the definitive hosts and a larval stage in intermediate hosts including humans. The larval stage of the parasite produces a large number of infective protoscoleces that develop to adult worms after being ingested by the definitive host, or they produce a new parasite mass when liberated inside the intermediate host, causing metastases of the parasite lesions. The two major species of medical and public health importance are *Echinococcus granulosus* and *E. multilocularis*, which cause cystic echinococcosis and alveolar echinococcosis (AE), respectively.

Human AE is a life-threatening disease, and without careful clinical management, it has a high fatality rate and poor prognosis. Humans acquire AE infection by ingesting eggs from adult parasitic worms. Early diagnosis and treatment (mainly by radical surgery) of human AE are difficult because the disease progresses slowly and usually takes more than several years before clinical symptoms become apparent. An efficient chemotherapeutic compound is still not available. The first choice for the chemotherapy of AE is benzimidazole derivatives (18), but they are parasitostatic rather than parasitocidal against larval *E. multilocularis*. Therefore, the development of highly effective antiechinococcal drugs is urgently needed.

Biological systems for energy metabolism are essential for the survival, continued growth, and reproduction of all living organisms. "Typical" mitochondria are usually considered to be oxygen-consuming, ATP-producing organelles. In fact, typical mitochondria, such as those found in mammalian cells, require oxygen to function. They use pyruvate dehydrogenase for oxidative decarboxylation of pyruvate to acetyl coenzyme A, which is then completely oxidized to CO_2 through the Krebs cycle. Most of the energy is produced by oxidative phosphorylation: the electrons from NADH and succinate are transferred to oxygen by the proton-pumping electron transfer respiratory chain in which ubiquinone (UQ) (Fig. 1A) is commonly used as an electron mediator. The backflow of the protons results in ATP formation by the mitochondrial ATP synthase.

In parasitic organisms, on the other hand, the carbohydrate and energy metabolic pathways of adult parasitic helminths differ greatly from those of their vertebrate hosts. The most important factors in this respect are the nutrient and oxygen supply (reviewed in references 4, 12, and 13). Parasitic hel-

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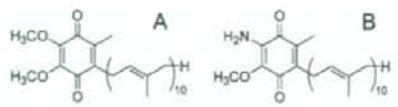

FIG. 1. Chemical structure of ubiquinone-10 (UQ_{10}) ($E_m' = +110$ mV) (A) and rhodoquinone-10 (RQ_{10}) ($E_m' = -63$ mV) (B).

minths have exploited a variety of energy-transducing systems during their adaptation to habitats in their hosts (7, 28). The parasitic nematode *Ascaris suum*, for example, resides in the host small intestine, where oxygen tensions are low, and exploits a unique anaerobic respiratory chain, called the NADH-fumarate reductase system, to adapt to its microaerobic habitat (Fig. 2) (2, 3, 14, 22; reviewed in reference 10). The NADH-fumarate reductase system is part of the unique respiratory system for parasitic helminthes and is the terminal step in the phosphoenolpyruvate carboxykinase-succinate pathway, which is found in many anaerobic organisms. Electrons from NADH are accepted by rhodoquinone (RQ) (Fig. 1B) via the NADH-RQ reductase activity of mitochondrial complex I and then transferred to fumarate through the rhodoquinol-fumarate reductase activity of mitochondrial complex II. The anaerobic electron transfer in complex I couples with proton transport across the mitochondrial inner membrane, providing ATP even in the absence of oxygen. This system, which does not normally function in mammalian mitochondria, is considered to be a good target for the development of novel anthelminthics (8, 9, 21). With regard to *Echinococcus* spp., the presence of both aerobic and anaerobic respiratory systems was previously suggested by a series of intensive studies (1, 16, 17), although the respiratory systems in this group of parasites are to be characterized in more detail.

In the present study, we prepared an enriched mitochondrial fraction from *E. multilocularis* protoscoleces and characterized the specific enzyme activities involved in mitochondrial energy metabolism as well as the quinone profile in the parasite's respiratory chain. Furthermore, based on findings reported previously by Yamashita et al. that quinazoline derivatives can inhibit the NADH-quinone reductase of mitochondria from *A. suum* (35), we tested several quinazoline-type compounds, with a view to developing novel antiechinococcal compounds.

MATERIALS AND METHODS

Isolation of *E. multilocularis* protoscoleces. We used the Nemuro strain of *E. multilocularis*, which is maintained at the Hokkaido Institute of Public Health (Sapporo, Japan). Mature larval parasites with protoscolex formation were obtained from cotton rats (*Sigmodon hispidus*) more than 4 months after oral infection with 50 parasite eggs. To isolate protoscoleces, the mature larval parasites were minced with scissors, pushed through a metal mesh, and washed repeatedly with physiological saline until host materials were thoroughly removed.

Preparation of enriched mitochondrial fractions. The enriched mitochondrial fractions of *E. multilocularis* protoscoleces were prepared essentially according to methods described previously for isolating adult *Ascaris* mitochondria (25, 26). Briefly, the isolated protoscolex sediment was suspended in 5 volumes of mitochondrial preparation buffer (210 mM mannitol, 10 mM sucrose, 1 mM disodium EDTA, and 50 mM Tris-HCl [pH 7.5]) supplemented with 10 mM sodium malonate. The parasite materials were homogenized with a motor-driven glass/glass homogenizer (six passes three to four times). The homogenate was diluted with the mitochondrial preparation buffer to 10 times the volume of the original protoscolex sediment and then centrifuged at 800 × *g* for 10 min to precipitate cell debris and nuclei. The supernatant was then centrifuged at 8,000 × *g* for 10 min to obtain the mitochondrial pellet. The pellet was resuspended in mitochondrial preparation buffer (without malonate) and centrifuged at 12,000 × *g* for 10 min. The resulting enriched mitochondrial fraction was suspended in mitochondrial preparation buffer (without malonate). The protein concentration was determined according to the method of Lowry et al. by using bovine serum albumin as a standard (15).

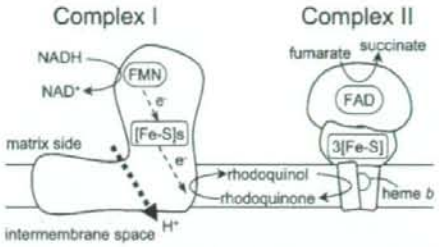

FIG. 2. Schematic representation of the NADH-fumarate reductase system in adult *A. suum*, which catalyzes the final step of the phosphoenolpyruvate carboxykinase-succinate pathway. In this system, the reducing equivalent of NADH is transferred to the low-potential RQ by the NADH-RQ reductase activity of mitochondrial complex I. This pathway ends with the production of succinate by the rhodoquinol-fumarate reductase activity of complex II. Electron transfer from NADH to fumarate is coupled to the site I phosphorylation of complex I via the generation of a proton-motive force. FMN, flavin mononucleotide; FAD, flavin adenine dinucleotide; [Fe-S]s and 3[Fe-S], iron-sulfur clusters.

Western blotting. An enriched mitochondrial fraction prepared from *E. multilocularis* protoscoleces and that from the liver of a cotton rat (used as the host animal for the parasite) were analyzed by Western blotting. Reactions were performed according to a method described previously by Towbin et al. (30). The proteins were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis on a 10% or 15% acrylamide gel and electrophoretically transferred onto a nitrocellulose membrane. The membrane was soaked in 1:5,000 anti-cytochrome *c* oxidase subunit IV antibody (component of the ApoAlert cell fractionation kit; Clontech Laboratories) in phosphate-buffered saline containing 0.05% (wt/vol) Tween 20 and 2% (wt/vol) skim milk. The membrane was incubated for 60 min at room temperature and then washed three times for 10 min with washing buffer, which consisted of 0.05% (wt/vol) Tween 20 in phosphate-buffered saline. Alkaline phosphatase-conjugated goat anti-mouse immunoglobulin G was then added as a secondary antibody, and the mixture was incubated for 30 min. After another wash with washing buffer, the membrane was soaked in reaction buffer (100 mM Tris-HCl [pH 9.5], 100 mM NaCl, 5 mM $MgCl_2$, 500 μg/ml of 4-nitroblue tetrazolium chloride, and 165 μg/ml of 5-bromo-4-chloro-3-indolylphosphate) to initiate the development of a colored product. Finally, the membrane was washed with distilled water to stop the reaction. For Western blotting, the amounts of parasite and cotton rat mitochondrial samples were normalized by the total protein amount or cytochrome *c* oxidase activity (see below).

Enzyme assays. All enzyme assays using the enriched mitochondrial fractions were performed in a 0.7- or 1-ml reaction mixture at 25°C. The reagents used in each assay were mixed with reaction buffer containing 30 mM potassium phosphate (pH 7.4) and 1 mM $MgCl_2$. The final mitochondrial protein concentration was 80 μg per ml of reaction mixture. For all reactions performed under anaerobic conditions, the reaction medium was supplemented with 100 μg/ml glucose oxidase, 2 μg/ml catalase, and 10 mM β-D-glucose and left for 3 min to achieve anaerobiosis. NADH oxidase activity in the isolated mitochondrial fraction was determined in the presence or absence of 2 mM KCN, 100 mM malonate, or both by measuring the absorbance of NADH at 340 nm ($\varepsilon = 6.2\ mM^{-1}\ cm^{-1}$). The reaction was initiated by the addition of 100 μM of NADH to the mixture. Succinate dehydrogenase (SDH) activity was determined by monitoring the absorbance change of 2-(4,5-dimethyl-2-thiazolyl)-3,5-diphenyl-2*H*-tetrazolium bromide (MTT; 60 μg/ml) at 570 nm in the presence of 120 μg/ml phenazine methosulfate and 2 mM KCN. The reaction was initiated by the addition 10 mM of succinate to the mixture. Succinate-quinone reductase activity was assayed under aerobic or anaerobic conditions in the presence of 0.1%(wt/vol) sucrose monolaurate by determining the amount of decyl UQ (dUQ) or decyl RQ (dRQ)

from the absorbance change at 278 nm (ε = 12.7 mM^{-1} cm^{-1}) or 287 nm (ε = 9.2 mM^{-1} cm^{-1}), respectively. Decyl rhodoquinol-fumarate reductase activity was measured under anaerobic conditions in a reaction mixture containing 0.1% (wt/vol) sucrose monolaurate. In this reaction, 60 μM dRQ was reduced to decyl rhodoquinol in the cuvette by adding 200 μM $NaBH_4$. The reaction was started by adding 5 mM fumarate to the mixture, and the oxidation of decyl rhodoquinol was monitored at 287 nm. NADH-fumarate reductase activity was determined by monitoring the oxidation of NADH (100 μM) at 340 nm under anaerobic conditions. The reaction was initiated by the addition of 5 mM fumarate as an electron acceptor. NADH-quinone reductase activity assays were carried out under anaerobic conditions using the same reaction mixture as that used for the NADH-fumarate reductase activity assay except that 60 μM dUQ or dRQ was used as an electron acceptor instead of fumarate. The enzyme activity was determined by monitoring the absorbance change of NADH at 340 nm. Ubiquinol oxidase activity was determined by monitoring the absorbance change of ubiquinol-1 (150 μM) at 278 nm (ε = 12.7 mM^{-1} cm^{-1}) in the presence or absence of 2 mM KCN. The activity of cytochrome *c* oxidase was determined as *N,N,N',N'*-tetramethyl-*p*-phenylenediamine dihydrochloride (TMPD) oxidase activity, which was measured by monitoring the absorbance change of TMPD (500 μM) at 610 nm (ε = 11.0 mM^{-1} cm^{-1}) in the presence or absence of 2 mM KCN.

Enzyme inhibition assays. Based on the findings of Yamashita et al. showing that that quinazoline-type compounds inhibit the NADH-quinone reductase activity of *A. suum* complex I (35), we determined 50% inhibitory concentration (IC_{50}) values of the quionazoline-type compounds against NADH-fumarate reductase activity of the parasite mitochondria and the NADH oxidase activity of bovine heart mitochondria (see "Enzyme assays"). The compounds used in the assays included quinazoline and its derivatives 6-NH_2, 6-NHCO($CH{=}CH_2$), 7-NH_2, 8-OH, 8-OCH_3, 8-OCH_2CH_3, and 8-$OCH(CH_3)_2$.

Analysis of the quinone profile of isolated mitochondria. Quinones were extracted from lyophilized mitochondria essentially according to a method described previously by Takada et al. (24). A lyophilized mitochondrial sample (2.9 mg protein) was crushed into powder before extraction, vortexed in 2:5 (vol/vol) ethanol/*n*-hexane for 10 min, and centrifuged at 20,000 × *g* for 5 min at room temperature. The supernatants were pooled, and the extraction of quinones was repeated twice. Pooled extracts were evaporated to dryness, dissolved in ethanol, and kept in the dark until high-performance liquid chromatography (HPLC) analysis. Quinones were applied to a reverse-phase HPLC column (Inertsil ODS-3 [5 μm and 4.6 by 250 mm]; GL Science) and eluted under isocratic conditions (1 ml/min) with 1:4 (vol/vol) diisopropyl ether-methanol at 25°C. The molecular species of the eluted quinones were identified by their retention times and by their spectral characteristics as measured with a UV-visible photodiode array (Shimadzu SPD-10-A). The concentration of quinones was determined spectrophotometrically. The major quinone detected was confirmed by mass spectrometry (MS) using an Applied Biosystems API-165 LC/MS system with electrospray ionization.

In vitro treatment of *E. multilocularis* protoscoleces. *E. multilocularis* protoscoleces were obtained as described above (see "Isolation of *E. multilocularis* protoscoleces"). The parasite materials were placed into culture medium suitable for the long-term maintenance of the protoscoleces in vitro (27). The parasite cultures were kept in a six-well plate at a density of approximately 500 protoscoleces per ml of culture medium, and half of the medium was replaced twice a week. This culture condition was also applied during in vitro treatment of the parasite. To examine the efficacy of chemical compounds against living *E. multilocularis* protoscoleces, the parasites were kept in the culture medium supplemented with 5 or 50 μM of each compound, including quinazoline and its 8-OH derivative, rotenone (a specific inhibitor of mitochondrial complex I) (19) and nitazoxanide (a compound with strong protoscolicidal action) (32). One control group was supplemented with 0.5% (vol/vol) dimethyl sulfoxide (vehicle) alone, and all conditions were assayed in triplicate. The viability of protoscoleces was determined by microscopic analysis of more than 170 protoscoleces per well for motile behavior and the ability to exclude trypan blue (32).

RESULTS

Preparation of enriched mitochondrial fractions. To characterize the mitochondrial respiratory chain of *E. multilocularis* protoscoleces, we prepared enriched mitochondrial fractions from the parasite. Approximately 80 g of larval *E. multilocularis* (containing approximately 10^5 protoscoleces per gram) was obtained from each cotton rat more than 4 months after oral infection with 50 parasite eggs. Approximately 20 g of the larval parasite was used per isolation of protoscoleces, yielding 2 ml of cleaned protoscolex sediment (Fig. 3). The enriched mitochondrial fractions were prepared from the protoscolex sediment as described in Materials and Methods. Each 1 ml of protoscolex sediment (containing 4.5 × 10^5 protoscoleces) yielded approximately 4 mg of mitochondria. Western blotting using an antibody to mammalian cytochrome *c* oxidase detected a specific band in the mitochondria from the liver of a cotton rat but not in mitochondria from *E. multilocularis* protoscoleces even when the amounts of both mitochondrial samples were normalized according to cytochrome *c* oxidase activity (data not shown). These results demonstrated that the enriched mitochondrial fractions from the parasite were sufficiently free of host components for use in enzyme assays and quinone analyses. In order to assess the quality of mitochondria, intactness was examined by the reactivity of NADH, which is a non-membrane-permeable substrate. NADH oxidase activity was not detected in the isotonic buffer, whereas it was fully activated in hypotonic buffer after a freeze-thaw treatment of the enriched mitochondrial fraction. Based on the results obtained, the method applied here for mitochondrial preparation seemed to be appropriate.

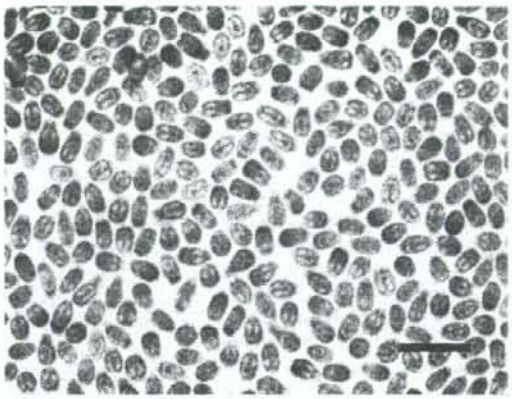

FIG. 3. Protoscoleces of *E. multilocularis* (Nemuro strain) used for the preparation of enriched mitochondrial fractions of the parasite and subsequent analyses. Bar, 500 μm.

Enzyme activities of *E. multilocularis* mitochondria. The specific enzyme activities involved in the mitochondrial respiratory chain of *E. multilocularis* protoscoleces are shown in Table 1. Parasite complex II exhibited an SDH activity of 103 nmol/min/mg. The specific activity of succinate-dUQ reductase was comparable to that of SDH activity (98.9 nmol/min/mg), whereas the succinate-dRQ reductase activity was lower (16.6 nmol/min/mg). The specific activity of decyl rhodoquinol-fumarate reductase, which is the reverse reaction of the succinate-RQ reductase activity of complex II, was determined to be 60.2 nmol/min/mg. The mitochondria of *E. multilocularis* protoscoleces exhibited NADH oxidase activity of 9.1 nmol/min/mg, which was almost eliminated by 2 mM KCN and 100 mM malonate. Ubiquinol-1 oxidase and TMPD oxidase activities were determined to be 4.4 nmol/min/mg and 12.6 nmol/

TABLE 1. Specific activities of mitochondrial respiratory enzymes in *E. multilocularis* protoscoleces

Assay	Sp act[a] (nmol/min/mg of protein) (mean ± SD)
SDH	103 ± 16
Succinate-quinone reductase	
dUQ (anaerobic)	98.9 ± 12
dRQ (anaerobic)	16.6 ± 3.5
Quinol-fumarate reductase (decyl rhodoquinol) (anaerobic)	60.2 ± 18
NADH oxidase	9.1 ± 2.1
NADH oxidase with:	
2 mM KCN	7.3 ± 1.5
100 mM malonate	4.4 ± 0.4
2 mM KCN and 100 mM malonate	1.7 ± 0.7
Ubiquinol-1 oxidase	4.4 ± 0.6
TMPD oxidase	12.6 ± 6.3
NADH-fumarate reductase (anaerobic)	45.0 ± 8.1
NADH-quinone reductase	
dUQ (anaerobic)	32.1 ± 2.7
dRQ (anaerobic)	61.3 ± 4.3

[a] Specific activities were obtained from at least three independently isolated mitochondria.

min/mg, respectively. These activities were completely inhibited by 2 mM KCN. Under anaerobic conditions, the specific activity of NADH-fumarate reductase was 45 nmol/min/mg, which was much higher than the NADH oxidase activity. The specific activity of NADH-dUQ reductase and NADH-dRQ reductase of complex I were determined to be 32.1 and 61.3 nmol/min/mg, respectively.

Quinone components in *E. multilocularis* mitochondria. To determine which quinones act as physiological electron mediators in the mitochondrial respiratory system of *E. multilocularis* protoscoleces, HPLC analyses were performed. As shown in Fig. 4A, the enriched mitochondrial fractions contained only one major quinone component at a retention time (*Rt*) of 22.4 min. The peak fraction exhibited a characteristic absorption maximum for RQs at 283 nm (Fig. 4B) (20). Subsequent MS analysis confirmed that the primary quinone of the parasite was RQ_{10} (electrospray ionization-MS *m/z* 848.8 $[M + H]^+$). The concentration of RQ_{10} was determined to be 0.73 nmol/mg of mitochondrial protein.

Effects of inhibitors on NADH-fumarate reductase in *E. multilocularis* mitochondria. To investigate the inhibitory effect of quinazoline (Fig. 5A) and its derivatives on the enzymatic activities in the anaerobic respiratory system of *E. multilocularis* mitochondria, we determined IC_{50} values against the NADH-fumarate reductase activity of the enriched mitochondrial fraction of the parasite. We found that all of the compounds inhibited the NADH-fumarate reductase activity of the parasite to some extent. Quinazoline and its derivatives including 6-NH_2, 6-NHCO($CH{=}CH_2$), 7-NH_2, 8-OH, 8-OCH_3, 8-OCH_2CH_3, and 8-$OCH(CH_3)_2$ exhibited IC_{50} values of 2.3, 2.1, 16, 62, 71, 48, 4,100, and 910 nM, respectively. Of the compounds tested, the 8-OH derivative (Fig. 5B) exhibited relatively selective inhibition against the NADH-fumarate reductase activity of *E. multilocularis* protoscoleces compared with the NADH oxidase activities of mammalian mitochondria: the IC_{50} values of quinazoline and its 8-OH derivative for the NADH oxidase activities of mammalian (bovine heart) mitochondria were 0.40 and 230 nM, respectively.

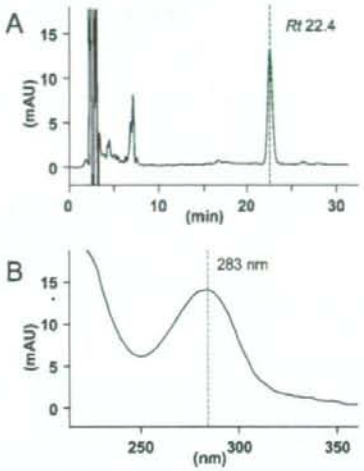

FIG. 4. (A) HPLC analysis of quinones extracted from the enriched mitochondrial fraction of *E. multilocularis* protoscoleces. Detailed experimental conditions are described in Materials and Methods. The highest peak had a retention time of 22.4 min (arrow). (B) Absorption of this peak was 283 nm, suggesting that it contained an RQ. mAU, milli-absorbance units.

Effects of inhibitors on living *E. multilocularis* protoscoleces. In order to examine the parasite-killing activities of the quinazoline-type compounds with different degrees of inhibitory effects against NADH-fumarate activities of *E. multilocularis* protoscoleces, we performed in vitro treatment of the parasite using quinazoline and its 8-OH derivative. The viability of the *E. multilocularis* protoscolex was progressively reduced during in vitro treatment of the parasites with 50 μM of the 8-OH derivative, and by day 5, all the parasites died (Fig. 6). The same compound did not have an obvious antiparasitic effect when used at a concentration of 5 μM. On the other hand, nonsubstituted quinazoline, which showed lower IC_{50} values with the enzymatic assay, eliminated the parasites on days 5 and 7 of in vitro treatment when used at 50 and 5 μM, respectively. Treatment with rotenone, a specific inhibitor of mitochondrial complex I (19), affected the viability of the parasite in a manner similar to that of the 8-OH derivative. The anti-echinococcal effect of nitazoxanide was relatively mild: even in

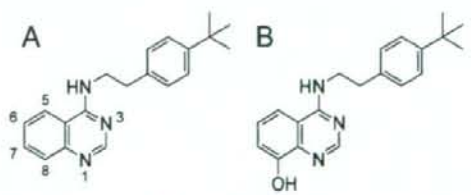

FIG. 5. Structures of quinazoline (A) and its 8-OH derivative (B) used for the enzyme inhibition assays and in vitro treatment of *E. multilocularis* protoscoleces.

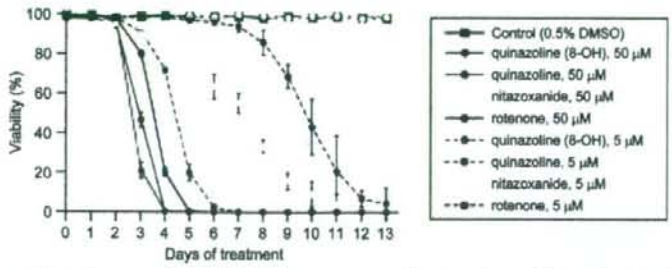

FIG. 6. Viability of *E. multilocularis* protoscoleces during in vitro treatment with quinazoline and its 8-OH derivatives, rotenone and nitazoxanide. Each compound was added to the culture medium at 5 or 50 μM. The results represent the means ± standard deviations of at least triplicate samples. DMSO, dimethyl sulfoxide.

the presence of 50 μM nitazoxanide, the viability decreased, but it did so only gradually, and it took 13 days before all the protoscoleces died. This compound did not affect parasite viability when used at 5 μM.

DISCUSSION

The most notable finding of the present study is that *E. multilocularis* protoscoleces possess a unique mitochondrial respiratory system that is highly adapted to anaerobic conditions. Specifically, the predominant enzymatic activity in the enriched mitochondrial fraction prepared from the parasite protoscoleces is the NADH-fumarate reductase system, which does not normally function in the aerobic respiratory chain of mammals. Thus, we infer that mitochondrial respiratory system of *E. multilocularis* would be a good target for the development of novel selective antiechinococcal compounds as demonstrated previously for other helminthic diseases (8, 21).

As early as 1957, Agosin found that *E. granulosus* protoscoleces have both aerobic and anaerobic respiratory systems and that glycolytic inhibitors are effective against both of them, indicating that they both depend on glycolysis (1). Subsequently, McManus and Smyth observed that protoscoleces cultured under anaerobic conditions produce more succinate than parasites kept under aerobic conditions, suggesting that the parasites survive under anaerobic conditions by utilizing the NADH-fumarate reductase system (16). Furthermore, McManus and Smyth reported that the specific activity of fumarate reductase in *Echinococcus* protoscoleces is lower than those of enzymes involved in the tricarboxylic acid cycle (17). These results, however, did not establish the importance of NADH-fumarate reductase activity in the mitochondrial respiratory system of the parasite because the other enzyme activities were not analyzed.

In the present study, we focused on the enzyme activities of the mitochondrial respiratory system of the parasite to determine whether the system is adapted to anaerobic conditions. Using the enriched mitochondrial fractions prepared from *E. multilocularis* protoscoleces, we showed that the activity of NADH-fumarate reductase in the respiratory system of the parasite is predominant compared with that of NADH oxidase, an enzyme involved in aerobic respiration in aerobic organisms such as mammals. Furthermore, direct measurements of complex II activities in both directions (i.e., succinate-RQ reductase and rhodoquinol-fumarate reductase activities) indicated that parasite complex II functions more favorably as a rhodoquinol-fumarate reductase in the presence of RQ/rhodoquinol. Thus, our results using isolated mitochondria of *E. multilocularis* protoscoleces coupled with assay systems for the determination of the parasite's enzyme activities revealed for the first time that the parasite mitochondria are highly adapted to anaerobic environments.

Analyses of the quinone components of *E. multilocularis* mitochondria revealed that RQ_{10} (Fig. 1B), whose redox potential is much more negative (E_m' [midpoint potential] = −63 mV) than that of UQ_{10} (E_m' = +110 mV) (Fig. 1A), was the primary quinone component of parasite mitochondria. In other parasitic helminths, like *A. suum* and *Hymenolepis diminuta*, RQ is an essential component of the NADH-fumarate reductase system (5, 11). In addition, van Hellemond et al. previously demonstrated that for all eukaryotes, the relative amount of RQ compared to the total amount of quinones correlates well with the importance of fumarate reduction in vivo (31). Similarly, during the development of the liver fluke *Fasciola hepatica*, there is a good correlation between the quinone composition and the importance of fumarate reduction in vivo (31). Therefore, RQ seems to be an essential component of fumarate reduction in eukaryotic respiration. Although menaquinone-related fumarate reduction in prokaryotes is well known (33, 34), there is no evidence that menaquinone serves this function in eukaryotes. In this study, enzyme assays demonstrated that the mitochondria from *E. multilocularis* possess NADH-fumarate activity as the predominant activity. In addition, the NADH-dRQ reductase activity was much higher than that of NADH-dUQ reductase, indicating that *E. multilocularis* complex I may interact preferentially with RQ rather than with UQ. Taken together, these results indicate that, as in other metazoan eukaryotes with anaerobic respiratory systems, *E. multilocularis* protoscoleces have a unique respiratory system that is highly adapted to anaerobic environments and in which RQ_{10} is used as the primary electron mediator.

Spiliotis et al. recently reported that the in vitro growth of larval *E. multilocularis* is more active under anaerobic than aerobic conditions (23). Thus, our findings for the respiratory system of *E. multilocularis* protoscoleces are consistent with the observations reported previously by Spiliotis et al. Larval *E. multilocularis* containing a large number of protoscoleces

lives in host tissues, mainly the liver, surrounded by thick connective tissues containing carbohydrate-rich laminated layers, which probably provide the parasite cells with an extremely-low-oxygen environment. Accordingly, it is not surprising that the parasite survives in the host by utilizing an anaerobic respiratory system.

Many anaerobic parasitic eukaryotes use the NADH-fumarate pathway, which is absent in mammals (2, 3, 10, 14, 22, 29). Therefore, this unique respiratory system is regarded as a promising chemotherapeutic target for the development of novel anthelminthics, as discussed in a recent review (9). In fact, Omura et al. previously found a natural compound, nafuredin, that is a potent inhibitor of the adult *A. suum* mitochondrial respiratory chain but much weaker against the mammalian mitochondrial respiratory chain (21). Yamashita et al. also found that quinazoline-type inhibitors were highly effective against adult *A. suum* complex I (35). Kinetic analyses using a series of quinazoline-type inhibitors revealed that *A. suum* complex I recognizes RQ_2 or UQ_2 in different ways, suggesting that mitochondrial complex I, which reacts preferably with RQs, could be a good target for chemotherapy. In the present study, we also tested several quinazoline-type compounds for their abilities to inhibit the anaerobic respiratory system of *E. multilocularis* protoscoleces. We found that all of the quinazoline-type compounds inhibited the NADH-fumarate reductase activity of *E. multilocularis* mitochondria to different extents. Furthermore, these compounds exhibited potent parasite-killing activities against *E. multilocularis* protoscoleces under in vitro culture conditions. Importantly, the nonsubstituted quinazoline, which has a higher inhibitory effect against NADH-fumarate oxidoreductase of the parasite mitochondria than the 8-OH derivative does, exhibited the parasite-killing activity even when used at 5 μM, whereas the 8-OH derivative did not do so at the same concentration. Such a correlation between the enzyme inhibition and the parasite-killing activities of these compounds suggests that the anaerobic NADH-fumarate reductase system of the parasite is a promising target for the development of antiechinococcal drugs.

Antiechinococcal drugs for chemotherapy of human AE should target not only protoscoleces but also the germinal layers of the *E. multilocularis* metacestode. The germinal layers in the larval parasite exhibit extremely unique characteristics. The parasite cells forming the germinal layers can differentiate into various tissues, including brood capsules and protoscoleces, and at the same time, they proliferate asexually as they remain in an undifferentiated state. This causes enlargement and, occasionally, metastasis of the lesions due to the formation of a large parasite mass. Therefore, for chemotherapy of AE, a complete cure cannot be achieved unless the germinal cells of the larval parasite are eliminated. Therefore, the mitochondrial respiratory system of germinal cells should be further characterized to aid in the development of a novel antiechinococcal compound(s) targeting the energy metabolism of larval *E. multilocularis*. However, it is presently quite difficult to obtain enough metacetode materials with homogeneous quality. Established methodologies for the in vitro cultivation of *E. multilocularis* metacestodes are now available (6, 23), and they will hopefully be applicable to large-scale preparations of metacestode materials in the near future.

During the life cycle of *E. multilocularis*, the parasite never undergoes active development and/or energy metabolism under aerobic conditions. The larval parasite lives mainly in the liver of intermediate host animals, whereas the adult worm dwells inside the small intestine of the final host, both of which are microaerobic conditions. Although the eggs of the parasite are exposed to air, they already contain a mature infective larva (oncosphere) waiting to be taken up by the next intermediate host. Therefore, the oncosphere does not develop or move under aerobic conditions. Taken together, these findings suggest that the respiratory system of *E. multilocularis* protoscoleces, as characterized in the present study, could represent the respiratory system used by the parasite throughout its developmental stages. Based on this speculation, the use of protoscolex materials in the first-step screening of candidate compounds by enzyme inhibition assays and subsequent in vitro parasite-killing assays appears to be reasonable, although it should be confirmed that the respiratory system of the *E. multilocularis* metacestode shares the same basic characteristics with that of the protoscolex stage of the parasite. We have already done preliminary experiments on the effects of the compounds used in this study, including the quinazoline derivative (8-OH), against in vitro-cultured metacestodes and found that the compounds exhibited high parasite-killing activities as evaluated by a modified MTT assay (data not shown). These results strongly suggest that our strategy is appropriate.

Highly effective chemotherapeutic compounds against human AE are not currently available despite the fact that the disease can be lethal unless the patient is appropriately treated during the early stage of the infection. Based on the findings presented here, it appears that the anaerobic respiratory system of *E. multilocularis*, which is distinct from that of host mammals, is a good target for the development of highly effective antiechinococcal drugs and, furthermore, that respiratory chain inhibitors (21, 35) are possible lead compounds for the development of antiechinococcal drugs.

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エキノコックス症

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1. はじめに

人獣共通感染症としてエキノコックス症を見た場合、「エキノコックス」という寄生虫の終宿主動物であるイヌと、「中間宿主」であるヒトとでは、感染した場合の病態が全く異なっていることを先ず注意することが必要である。エキノコックスの成虫が腸管内に寄生するだけのイヌにおいては見るべき症状を引き起すことはないが、エキノコックスの幼虫（包虫）が肝、肺、腎、脳などの臓器において発育するヒトでは、感染した場合、潜伏期を経て5～15年の後に極めて重篤な病気となる。ヒトには、成虫に感染しているキツネ、イヌなどの糞便内の虫卵が、偶発的に経口摂取されることで感染する。わが国のヒトのエキノコックス症には、その原因寄生虫種により単包性エキノコックス症（単包条虫）と多包性エキノコックス症（多包条虫）がある。前者に関しては、その生活環がわが国に土着している証拠がなく殆どが輸入症例と考えられているが、後者に関しては、1920年代以後北海道の一部に生活環の土着・伝播が認められてきた。しかるに近年、多包性エキノコックス症は北海道全域に広がり、北海道外への伝播域の拡大の兆しも現れて国民の健康に脅威を与える感染症となっている。そのために感染症法（1999年施行）では、エキノコックス症を全数把握疾患の四類感染症に指定し、医師に対して発生患者例の報告を義務付けた。そしてその後一部改正された感染症法（2004年10月）では、エキノコックスに感染したイヌについても獣医師による発生報告を義務付けた。著者らは2003年より現在に至るまで、厚生労働省科学研究費による研究班に所属して本症の調査と対策研究に従事してきた。このような立場から、以下にエキノコックス症に関する一般的な解説と、著者らが関わったエキノコックスに対する取り組みについて述べる。

2. エキノコックスとは

2-1. エキノコックス症の病因寄生虫

エキノコックス属を含むテニア科条虫は、「捕食者（終宿主）－被食者（中間宿主）」の間で伝播している。エキノコックス属条虫の終宿主はイヌ科動物であるが、主に家畜（羊、山羊、牛、豚、馬、ラクダなど）を中間宿主とする単包条虫（*Echinococcus granulosus*）と、野生動物（野ネズミ類）を中間宿主とする多包条虫（*E. multilocularis*）とが、公衆衛生上重要である。イヌ科動物の小腸に寄生する成虫は、単包条虫で2～7 mm、多包条虫で1.2～4.5 mmといずれも条虫としては非常に小さい。虫卵（30～35μm）は糞便とともに外界に排泄され、中間宿主に経口摂取されることによって、中間宿主の内臓で幼虫が発育する。中間宿主体内で幼虫は、包虫と呼ばれる嚢胞状の組織塊を形成して無性生殖により多数の原頭節を産生する。原頭節は終宿主動物の腸管内で成虫に発育する原基となるものである。単包条虫の幼虫（単包虫）の典型的なものは、単純な嚢胞で内部にほぼ透明な液を満たし嚢胞の内部に産生された原頭節が貯留する。多包条虫の幼虫は、単包条虫のそれとは異なり、直径1～5 mmの小さい包虫の集合体を形成することから「多包虫」と称されている。多包虫の割面はスポンジ状で、中に粘稠な液を含み全体として単包虫よりも硬い。多包条虫の好適な中間宿主であるエゾヤチ

ネズミの場合、感染後1～2ヶ月で数十万個以上もの原頭節が産生される。いずれの場合も終宿主・イヌ科動物への感染は、これらの幼虫を中間宿主の内臓とともに摂食することで起き、小腸で原頭節が成虫に発育・寄生して生活環が完結する。

2-2. エキノコックス症の伝播経路

エキノコックスのヒトへの感染は、虫卵を経口摂取することでのみ起きる。単包条虫は、主に「イヌー牧畜獣」という家畜の間で生活環が維持されている。牧羊犬などが、中間宿主動物の臓器を摂食することで幼虫を取り込み、単包条虫が腸管内で成熟・虫卵産生し、糞便と共に外界に排卵することによりヒトへ虫卵を伝播する。一方、多包条虫は、主として「キツネー野ネズミ」という野生動物の間で生活環が維持されている。しかし、飼いイヌも時には野ネズミを捕食し多包条虫を腸管内に寄生させるので、イヌとヒトとの密接な関係から、イヌはキツネよりも重要な病原体（虫卵）のヒトへの伝播者となる（図1に、多包条虫の生活環を示す）。

2-3. ヒトのエキノコックス症（単包虫症・多包虫症）の臨床症状

感染初期は、無症状で経過することが多い。単包虫症では、2/3のケースが肝に、1/5が肺に一次病巣を形成するといわれ孤立性の嚢胞が時間をかけて増大（1～30mm/年）することで諸症状を引き起こす。嚢胞がある程度増大したものでは、肝腫大や腹痛を認め周囲の諸臓器を圧迫し胆道閉塞や胆管炎を併発する。あるものは、破裂や崩壊によって消滅してしまうと考えられているが、破裂した場合は、嚢胞中にあった幼虫（原頭節）が他の臓器に転移して二次病巣を形成する。多くの場合、突然の嚢胞破裂によって症状が始めて現われる。

多包虫症では、殆ど全てのケースで肝に一次病巣を形成する。肝に生着した微小嚢胞が外生出芽によってサボテン状に連続した充実性腫瘤を形成し、進行すると肝腫大、腹痛、黄疸、肝機能障害などが現れる。さらに進行すると胆道、脈管などに浸潤し、閉塞性黄疸、病巣の中心壊死、病巣への細菌感染をきたして重篤となる。末期には腹水や下肢の浮腫が出現する。肝肺瘻をきたすと胆汁の喀出、咳嗽が認められ、脳転移をきたすと意識障害、けいれん発作などを呈する。外科的切除が根治的治療法であるが、何らの治療も行われなかった場合の死亡率は、70%とも言われ極めて高い。アラスカでの報告によれば、21人の感染者が発症診断後に生存した期間は平均して5.3年であり、全員が14年以内に死亡したとしている。

図1　エキノコックス症の感染経路

2-4. イヌのエキノコックス症（単包条虫症・多包条虫症）

既に述べたように、イヌへのエキノコックス成虫の感染は、エキノコックス幼虫（包虫）を保有する中間宿主の臓器を摂食することによって起きる。単包条虫の場合は羊、山羊、牛、豚、馬、ラクダなどで、多包条虫の場合は野ネズミ類である。感染した犬は、単包条虫で約2ヶ月、多包条虫で約1ヶ月後から虫卵を糞便中に排泄し始める。いずれに感染した場合でも、犬は稀に下痢を起す程度で、通常は症状を示さない。即ち、イヌでは本症の診断は臨床症状からは一般に不可能で、排泄された糞便を次の方法で検査することにより判明する。

1. 病原体（虫体の全部又はその一部（片節）の形態学的同定）
2. 虫卵など病原体の遺伝子検査（PCR法）
3. 病原体の抗原検出（ELISA法）

駆虫薬としては、エキノコックス成虫に対して極めて効果的なプラジカンテルがある。通常、1回の投与（5 mg/kg）で100%の駆虫効果がある。成虫の寄生持続期間は2～4ヶ月とされているが、幼虫摂取による再感染は容易に起こることが知られている。

2-5. エキノコックス症の地理的な分布

単包虫症に関しては、南アメリカ南部、地中海沿岸、旧ソ連の南部中央部、中央アジア、中国、オーストラリア、エチオピア及びタンザニアなどアフリカの一部が高度流行地として知られており、ヒツジが最も重要な中間宿主である。家畜の単包虫症に関しては、古くから撲滅対策が始められ成功を収めた国も少なくない。アイルランドでは19世紀中頃に住民の1/6が感染し、イヌの28%に単包条虫の寄生が見られたという。1890年に本症の撲滅法を制定し対策を始めた結果1950年代には寄生犬が認められなくなり、1960年代以降は新たな単包虫感染者は見られなくなった。ニュージーランドも、1960年代には世界で最も濃厚な汚染国として挙げられていたが、政府と民間をあげてその撲滅に取り組んだ結果、現在では人での単包虫症は殆どみられなくなった。

わが国では、1881年に熊本で日本最初のヒトの単包虫症が報告されて以来、現在までの症例総数は70数例に止まっている。患者の三分の一は国外での感染が示唆されているが、国内感染が疑われる患者の分布地域は主として、九州、四国、中国などの西日本であった。近年国内で見いだされる単包虫症例は、その殆どが有病地から来日した外国人症例である。

多包虫症の発生が多く報告されているのは、中央ヨーロッパ、ロシア全域、中央アジアの諸国、中国西部及びアラスカ西部である。独、仏、スイスなどでは、単包虫症よりも多包虫症が公衆衛生上のより大きな問題となっている。

わが国唯一の有病地である北海道には、もともとエキノコックス症は存在していなかった。20世紀になってからのヒトとモノの盛んな交流を背景として、多包条虫が北方諸島から侵入してきたものと考えられている。最初の流行は、毛皮の採取と野ねずみ駆除とを目的として移入したキツネに多包条虫感染個体がいたことから礼文島で発生した。1937年から1965年までの間に島民約8,200名のうち患者数114名を記録したが、1950年代以後の徹底した対策によりこの流行は終焉した。一方、1965年の患者発見から始まる根室・釧路を含む北海道東部地方での流行の発端は、北方諸島を中部千島まで人為的に移動させられたキツネが北海道に入り、その中に感染キツネが含まれていた事によるものと推定されている。この流行では1997年までに累計患者数146名を数え、現在でも毎年数名の新しい患者が見出されている。更に近年、北海道中央部・西部地方へ流行域が拡大しつつあり、1998年までに北海道で認定された患者数は累計で383名となり、以後、毎年5～20人の届出感染者がある。最近（1999年）青森県で、と畜検査されたブタの肝臓から多包虫病巣が発見された事から、本州への本種寄生虫の伝播が疑われるようになった。

3. エキノコックス症の北海道外への流行地域拡大を防止するために

北海道における多包条虫の流行に対しては、北海道エキノコックス症対策協議会による調査と対策が50年にも亘り継続的に行なわれてきた[1]。しかしながら、エキノコックス症（多包虫症）が、北海道外へと流行地を拡大する可能性が現実的なものとして考えられた感染症法（1999年）以後の段階においては、次に挙げる調査の実施が急務となった。

一つは、北海道に隣接する青森県においてエキノコックスの生活環が既に成立しているのかどうか、今ひとつは、北海道から道外各地へ移動するイヌの実態とそのエキノコックス感染状況の調査である。

3-1. 青森県におけるエキノコックス調査[2]

わが国における多包虫症の大部分は、多包条虫