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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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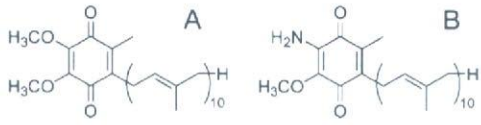

FIG. 1. Chemical structure of ubiquinone-10 (UQ_{10}) ($E_m' = +110$ mV) (A) and rhodoquinone-10 (RQ_{10}) ($E_m' = -63$ mV) (B).

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).

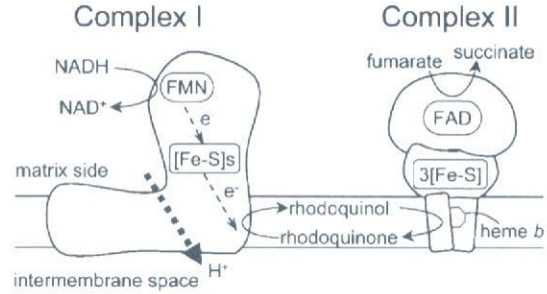

FIG. 2. Schematic representation of the NADH-fumarate reductase system in adult *A. suum*, which catalyzes the final step of the phosphoenolpyruvate carboxykinase-succinate pathway. In this system, the reducing equivalent of NADH is transferred to the low-potential RQ by the NADH-RQ reductase activity 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*-tetrazolyl 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.

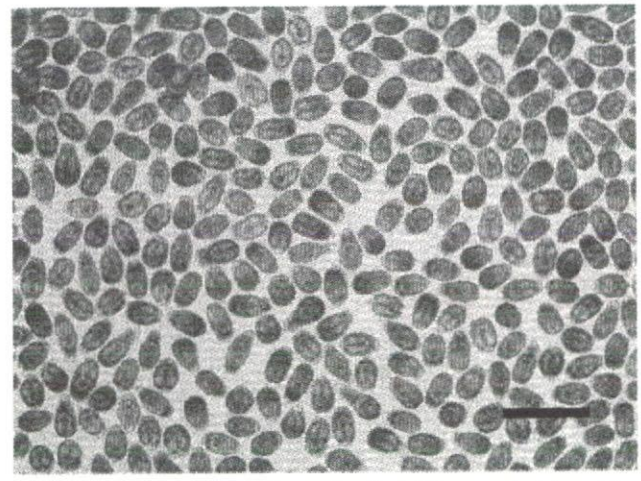

FIG. 3. Protoscoleces of *E. multilocularis* (Nemuro strain) used for the preparation of enriched mitochondrial fractions of the parasite and subsequent analyses. Bar, 500 μm.

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.

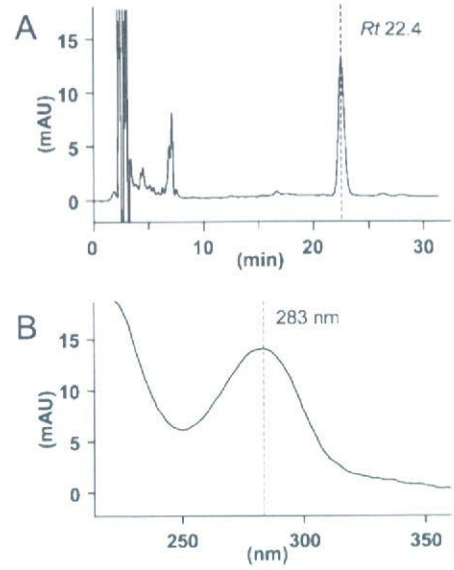

FIG. 4. (A) HPLC analysis of quinones extracted from the enriched mitochondrial fraction of *E. multilocularis* protoscoleces. Detailed experimental conditions are described in Materials and Methods. The highest peak had a retention time of 22.4 min (arrow). (B) Absorption of this peak was 283 nm, suggesting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

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.

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

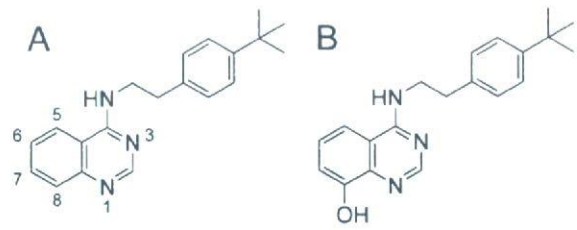

FIG. 5. Structures of quinazoline (A) and its 8-OH derivative (B) used for the enzyme inhibition assays and in vitro treatment of *E. multilocularis* protoscoleces.

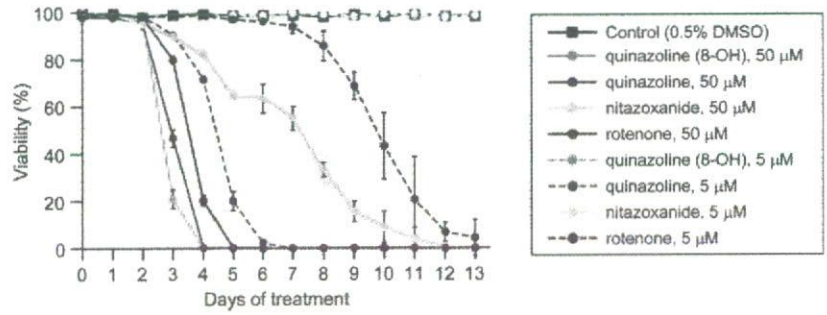

FIG. 6. Viability of *E. multilocularis* protoscoleces during in vitro treatment with quinazoline and its 8-OH derivatives, rotenone and nitazoxanide. Each compound was added to the culture medium at 5 or 50 μM. The results represent the means ± standard deviations of at least triplicate samples. DMSO, 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 metacestode 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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The use of tetracycline in anthelmintic baits to assess baiting rate and drug efficacy against *Echinococcus multilocularis* in foxes

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Abstract

Anthelmintic (praziquantel) baiting of wild red foxes against *Echinococcus multilocularis* infection was studied in a highly epizootic suburban area of Otaru, Hokkaido (the northern island of Japan) during the summer and autumn in the years 1999–2004. Acceptance of baits containing the biomarker tetracycline (TC) was evaluated. The prevalence of *E. multilocularis* infection in foxes before baiting (1999–2000) was 58% (88/153), whereas in the fourth year of bait distribution year (2004), it decreased to 11% (5/45). Analysis of TC marking in the teeth of foxes showed that 39% (77/195) of those captured after baiting were estimated to have consumed baits in the year of capture. Importantly, more juvenile (56%, 49/87) than adult foxes (26%, 28/108) were marked, indicating efficient baiting of juveniles, which tended to have a higher worm burden of *E. multilocularis*. Of 77 marked foxes, *E. multilocularis* and *Alaria alata* (monitored as the second indicator species of deworming) were not detected in 70 (90%) and 76 (99%) foxes, respectively. The results suggest effective deworming by bait consumption. However, it was also demonstrated that 9% of the marked foxes were infected or re-infected after bait consumption, suggesting high infection pressure and the importance of frequent baiting.

Keywords: *Echinococcus multilocularis*; Control method; Fox; Praziquantel; Tetracycline; Japan

1. Introduction

Alveolar echinococcosis (AE) is endemic in Hokkaido, the northern island (78,500 km^2; 5,660,000 inhabitants) of Japan, where the cycle of the cestode predominantly involves red foxes (*Vulpes vulpes*) and gray-sided voles (*Clethrionomys rufocanus*) (Oku and Kamiya, 2003). In Hokkaido, 500 cases of human echinococcosis have been diagnosed from 1937 to 2005 (according to the Hokkaido Government in 2005). During 1995–2005, 9–27 (mean 15.5) new human cases of AE were reported every year. The prevalence in foxes increased in the 1980s, and has been around 40% in the last decade (Kamiya et al., 2006). Considering this high endemicity, effective countermeasures against the source of infection are required.

Deworming trials against *E. multilocularis* infection in wild red foxes have been conducted by distributing baits containing praziquantel in Germany (Schelling et al., 1997; Tackmann et al., 2001), Switzerland (Hegglin et al., 2003), and Hokkaido (Tsukada et al.,

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2002; Takahashi et al., 2002), all of which showed successful reduction of infection prevalence in foxes. In these studies, changes in prevalence were evaluated in fox populations by necropsy or fecal survey (detection of coproantigen and eggs). However, the effect of baiting at the individual level has not yet been evaluated. Although bait acceptance by foxes has been monitored by bait disappearance and footmarks (Tsukada et al., 2002), or by camera traps (Hegglin et al., 2003, 2004), the relationship between bait consumption and parasite infection in each individual animal has not been investigated.

Tetracycline (TC) is widely used as a biomarker for monitoring bait consumption, especially in oral vaccination campaigns against rabies infection in wild carnivores in Europe and North America (Oleyar and McGinnes, 1974; Johnston et al., 1988; Brochier et al., 1991; Olson et al., 2000). Following consumption, TC is incorporated into calcific tissues of mammals, and its deposits are observed as a fluorescent line in the teeth and bone under ultraviolet light (Milch et al., 1957). Therefore, both bait consumption and parasite infection in individual foxes can be checked using baits containing TC, and subsequent necropsy.

In this field study, to evaluate the effect of anthelmintic baiting at the individual fox level against *E. multilocularis* infection, bait distribution using the biomarker TC was conducted at a highly epizootic area, Otaru, in Hokkaido.

2. Materials and methods

2.1. Study area

The study was conducted in the suburbs of Otaru City, located in the western part of Hokkaido, Japan (43°11′N, 140°59′E; Fig. 1). Otaru is a harbor city enclosed by the sea and forested hills, with a population of 140,000. The landscape of the study area (~110 km^2) is mainly forests and croplands but also includes houses, gardens, cemeteries, and public parks. A previous survey showed that 57% (38/67) of foxes were infected with *E. multilocularis* in this area from June to September in 1999 (Yimam et al., 2002).

2.2. Bait and biomarker

Bait matrix of ground fish flesh and fishmeal was mixed with a powder formulation of praziquantel (Hada Clean; Bayer Japan, Tokyo, Japan), and was formed into pellets. Each pellet weighed ~15 g and contained 50 mg praziquantel. As a biomarker, 100–160 mg tetracycline hydrochloride was incorporated into the bait pellets with paraffin mix (in 2001 and 2003), lard mix (in 2002), or gelatin capsular form (in 2004) to

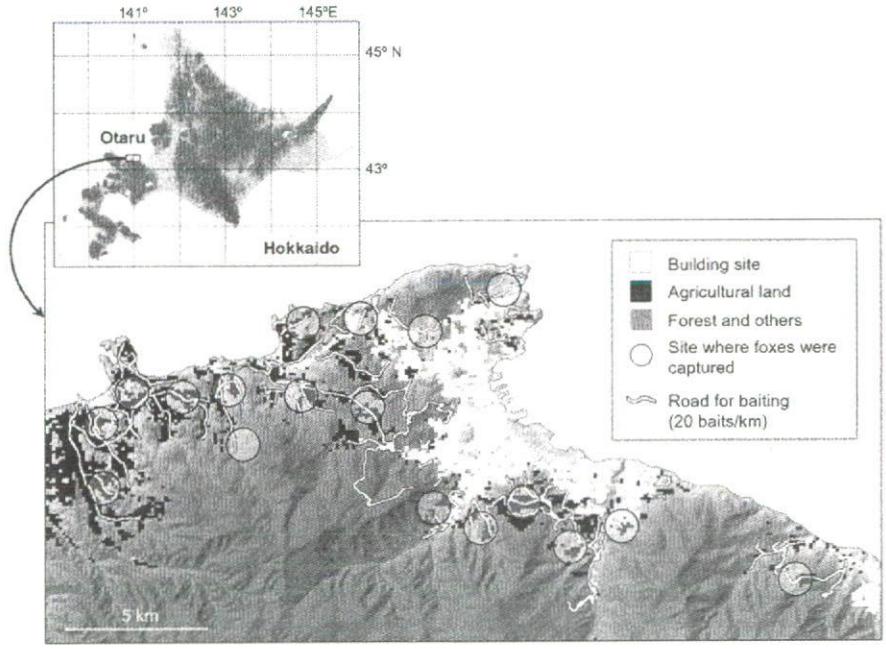

Fig. 1. Study area for an anthelmintic baiting trial in Otaru City, which is surrounded by forested hills and the sea. Baits were distributed along roads (20 baits/km) (shown by white lines). The site where red foxes were captured is indicated by an open circle.

Table 1
Area and frequency of anthelmintic bait distribution and use of biomarker tetracycline (TC)

Year	Area	Frequency	
		TC bait[a]	Non-TC bait[a]
2001	Western half	Twice (May and June)	Not distributed
2002	Whole	Twice (June and July)	Not distributed
2003	Whole	Once (June)	Six times (May, July to November)
2004	Whole	Four times (May to August)	Not distributed

[a] TC bait: bait containing TC, non-TC bait: bait not containing TC.

avoid decomposition of TC by water. Baits not containing TC were also used in 2003. Baits prepared were stored at −20 °C until use.

2.3. Bait distributions

Bait distributions were conducted two to seven times at intervals of ~1 month from May to November (2001–2004; Table 1). The baits were distributed by car along the roads at 20 baits/km (Fig. 1). In 2001, 2002, and 2004, baits containing TC (TC baits) were used in all distributions. In 2003, TC baits were used in only one (June) of seven distributions (May to November) to evaluate the bait consumption within one distribution.

2.4. Red foxes

In total, 440 fox carcasses provided by local hunters, which included 67 samples used by Yimam et al. (2002), were used for examinations. These foxes were trapped in the study area between June and September 1999–2004, as part of the local administration policy to mitigate crop damage. The numbers of fox carcasses in each year and its relationship to bait distribution are shown in Table 2. The age class (adult, ≥1 year old; juvenile, <1 year old) of each fox was determined by body size, tooth replacement (i.e., presence/absence of deciduous teeth or retromolar), and pulp cavity width in the canine teeth (i.e., the ratio of pulp cavity width to tooth width at gum line = >50%; Sasakawa et al., 1980). Age of the juveniles was considered to be 1–6 months old since fox puppies are born during March to April in Hokkaido. The carcasses were frozen at −80 °C for >2 weeks in order to inactive *E. multilocularis* eggs, and then at −20 °C until examination.

2.5. Detection of TC markings in teeth

Canine teeth were removed from 287 foxes captured during 2001–2004, when the bait distributions were conducted. TC markings were examined according to Johnston et al. (1987). Teeth were longitudinally sectioned to a thickness of 100–200 μm using a diamond blade cutter (Micro Cutter MC-201; Maruto, Tokyo, Japan). Upper canine teeth were used because of less curvature than lower canines, except when both upper

Table 2
Number of red fox carcasses and its relationship to anthelmintic bait distribution

Year	Number of fox carcasses (number of juvenile carcasses)			
	Non-baited area	Baited area		Total
		Before TC bait[a] distribution	After TC bait distribution	
1999	67 (31)	–	–	67 (31)
2000	86 (15)	–	–	86 (15)
2001	27 (11)	8 (1)	48 (18)	83 (30)
2002	–	35 (4)	35 (14)	70 (18)
2003	–	18 (2)	71 (41)	89 (43)
2004	–	4 (0)	41 (14)	45 (14)
Total	180 (57)	75 (7)	195 (87)	440 (151)

These foxes were captured during May to September in the years 1999–2004.
The details of bait distribution are shown in Table 1.
[a] Bait containing tetracycline (TC).

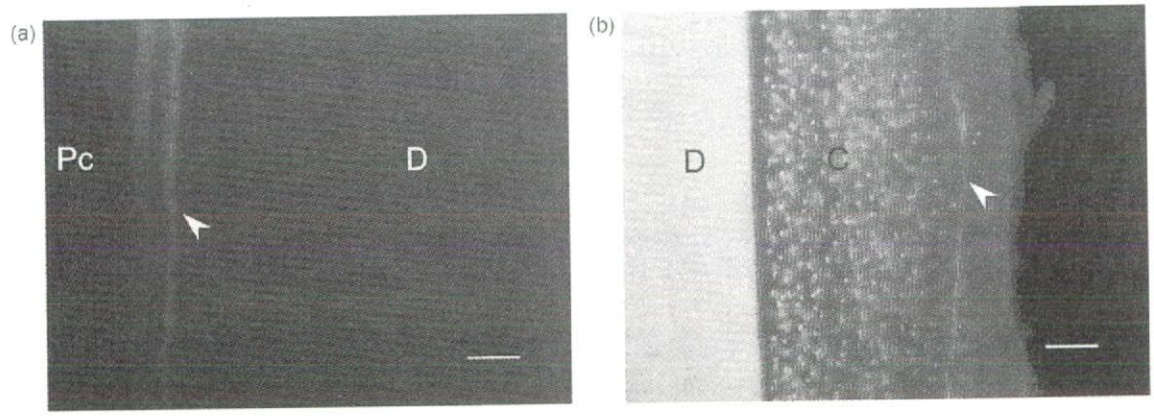

Fig. 2. Fluorescent yellow lines of tetracycline (TC) markings in canine teeth of foxes (arrowheads). (a) TC marking in the dentin (D) near the pulp cavity (Pc) of an adult fox, estimated to have been formed in the year when the fox was captured. (b) TC marking in the cementum (C) of an adult fox, estimated to have been formed in the year preceding capture. Scale bar = 100 μm. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

canines were lost or damaged. More than two sections were made of each tooth to observe the axis part of the tooth at as whole position as possible. Tooth sections were mounted on glass slides and examined under fluorescence microscopy for the presence of a golden yellow line of the TC biomarker, either in the dentin or cementum (Fig. 2).

Whether each line in adult foxes was formed in the year of capture or in the years preceding capture was estimated by the position of the line in the dentin and cementum. TC is deposited in growing teeth at the mineralization front. Therefore, a line in the dentin near the pulp cavity (i.e., the ratio of the distance between the line and the pulp cavity to the width of the dentin = <5%) or a line in the cementum near the outside edge of tooth (i.e., the ratio of the distance between the line and the edge to the width of the cementum = <5%) was estimated to have been formed in the year of capture (Fig. 2).

2.6. Detection of worms in the small intestine

The small intestine was removed from each fox and examined for the presence of adult worms of *E. multilocularis* and *Taenia* species. In addition, *Alaria alata* infection was tested for as the second indicator of deworming because the trematode is commonly found in foxes in Hokkaido (Kamiya and Obayashi, 1975) and can be dewormed with praziquantel. The small intestines were divided into three or six parts of equal length, and each part was cut longitudinally. The intestinal mucosa and contents were scraped and sedimented repeatedly with saline, using a 200-ml glass bottle, until the supernatant was clear. In 1999, whole sediments were examined under a stereoscopic microscope if <400 worms were present. If >400 worms were found, the worm burden was calculated from the count in one aliquot (Yimam et al., 2002). During 2000–2004, only a one-tenth volume of sediment (20/200 ml suspension) was examined under stereoscopic microscopy. The worm burden of *E. multilocularis* was calculated from the aliquot counts. The remainder was examined visually for *A. alata* and *Taenia* species.

2.7. Fecal egg examination

Feces were collected from the cecum or colon of 423 foxes. Then, 0.5 g of each sample was examined for the presence of taeniid eggs by the centrifugal floatation technique, using a sucrose solution with a specific gravity of 1.27 (Ito, 1980).

2.8. Statistical analysis

To determine percentages/prevalences, exact binomial 95% confidence intervals (CIs) were calculated using R version 2.4.1 (http://www.R-project.org). Differences in percentages and in worm burden were analyzed with Fisher's exact probability test and the Mann–Whitney *U*-test (Wilcoxon's rank sum test), respectively, using JMP version 5.1 (SAS Institute, Cary, NC, USA).

3. Results

3.1. TC marking

TC markings were observed in 104 (41%) of 252 foxes captured in the bait-distributed area after the first distribution of TC baits. No TC markings were detected

Table 3
Proportion of TC-marked foxes in relation to age class

Year	No. TC-marked/no. examined (% proportion)		
	Adult	Juvenile	All
2001[a]	2/30 (7)	7/18 (39)	9/48 (19)
2002	5/21 (24)	7/14 (50)	12/35 (34)
2003	11/30 (37)	22/41 (54)	33/71 (46)
2004[a]	10/27 (37)	13/14 (93)	23/41 (56)
Total[a]	28/108 (26)	49/87 (56)	77/195 (39)

The foxes were captured in the bait-distributed area after the first TC-bait distribution in each year.

[a] The rate of TC-marking was significantly different between adult and juvenile foxes with Fisher's exact probability test; $P < 0.01$.

in 27 foxes captured from non-bait-distributed area and 8 captured before TC bait distribution in 2001 (Table 2). Of 104 TC-marked foxes, 72 were estimated to be marked only in the year of capture; 27 were estimated to be marked only in pre-capture years; and 5 foxes were estimated to be marked both in the capture and pre-capture years. In 31 foxes estimated to be marked in pre-capture years, 30 were captured since the second year of bait distribution (2002), but 1 was captured in the first year (2001) of distribution. In the following analyses, only 77 foxes that were TC-marked in the year of capture were considered as TC-marked foxes. The percentage of marked foxes captured each year ranged from 19 to 56%, and overall was 39% (Table 3). The percentage of marked juvenile foxes was higher than that in adult foxes. The difference in the percentages was significant in 2001 and 2004 (Fisher's exact probability test).

3.2. Detection of parasite infections

In a total of 440 necropsied foxes, *E. multilocularis* was detected in 152 (34.5%), *A. alata* in 104 (23.6%), *Taenia taeniaeformis* in 2 (0.5%), and *Taenia crassiceps* in 1 (0.2%). Of 423 fecal samples collected from these foxes, taeniid eggs were detected in 73 (17.3%), of which 68 also contained *E. multilocularis* adults at necropsy. From the remaining five taeniid egg-positive foxes, *Taenia* species were not detected at necropsy. All of the *T. taeniaeformis* detected were immature, although mature *T. crassiceps* and mature worms of *E. multilocularis* were found in the same fox.

Of 77 TC-marked foxes, *E. multilocularis* and *A. alata* were not detected in 70 (91%) and 76 (99%) foxes, respectively. The foxes were captured 2–98 days (about half (40/77) were captured within 4 weeks) after the first TC bait distribution in each year. In seven TC-marked foxes harboring *E. multilocularis*, gravid segments of the worms were not observed. Feces were collected from 76 TC-marked foxes. As expected, no taeniid eggs were detected in these feces. In each year, the percentage of foxes harboring *E. multilocularis*, fecal taeniid eggs, or *A. alata* was lower in TC-marked than in unmarked foxes (Fig. 3).

In the pre-bait-distribution years 1999 and 2000, the overall percentage of foxes harboring *E. multilocularis* was 57% (95% CI, 44–69%) and 58% (95% CI, 47–69%), respectively. The percentages significantly decreased to 16% (95% CI, 9–25%) and 11% (95% CI, 4–24%) in 2003 and 2004, the third and fourth years of bait distribution, when the percentage of TC-marked foxes was 37 and 51%, respectively (Fig. 3). Similarly, the percentage of foxes positive for fecal taeniid eggs or *A. alata* decreased in the third and fourth year of baiting (Fig. 3).

No clear difference was observed in the worm burden of *E. multilocularis* in individual foxes before and after bait distribution, or between TC-marked and unmarked foxes. Overall worm burden in infected juvenile foxes (30–635500; mean, 117,987; median, 23,885) was larger than that in adult foxes (1–1165800; mean, 35,753; median, 1240) (Mann–Whitney *U*-test, $P < 0.01$).

4. Discussion

4.1. Evaluation of TC bait consumption by individual foxes

TC labeling of wild canids has been used in oral vaccination campaigns against rabies in which the percentage of animals that consumed bait in each population was estimated for its effect in controlling the disease (Selhorst et al., 2001). In the present analysis of TC-marking of teeth, 39% (77/195) of foxes were estimated to be marked in the year of capture and dewormed in the year of baiting. In previous studies on anthelmintic baiting of foxes, although bait disappearance was monitored visually (Schelling et al., 1997; Tsukada et al., 2002) and camera traps (Hegglin et al., 2003), the proportion of the local fox population that actually ingested the baits was not evaluated. Therefore, the present study, possibly for the first time, provides information about the actual number of foxes affected by anthelmintic baiting.

Nunan et al. (1994) found TC-like fluorescence in the teeth of 0.2% (5/3406) of red foxes collected prior to distribution of TC-containing rabies vaccine baits. Indeed, in the present study, one fox captured in the first year of baiting showed a fluorescent line that was probably formed in the previous year. Therefore, bias

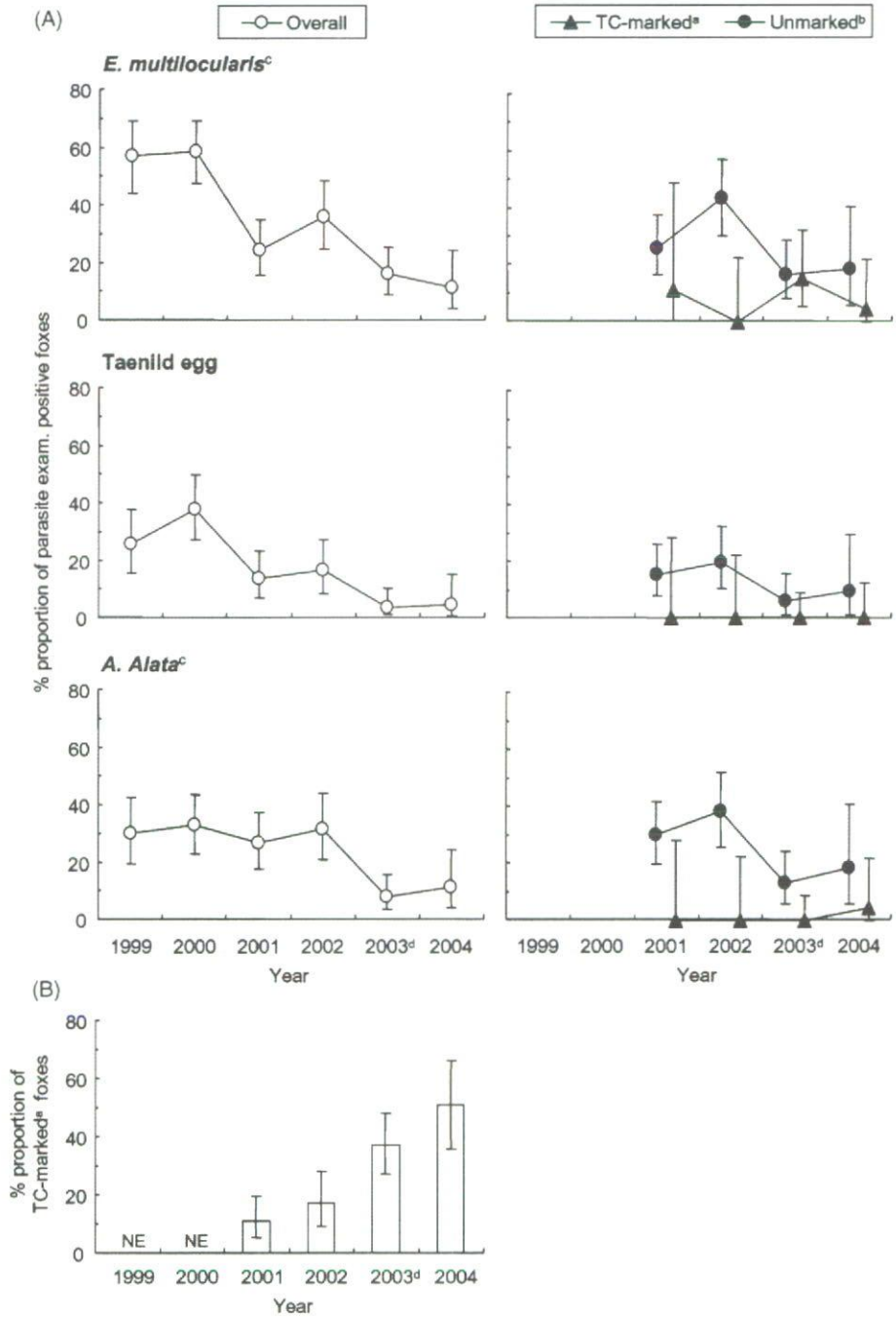

Fig. 3. (A) Proportion of foxes positive for *Echinococcus multilocularis* infection (upper), for fecal taeniid eggs (middle), and for *Alaria alata* infection (lower) in tetracycline (TC)-marked, unmarked, and all over foxes in each year. (B) Proportion of TC-marked foxes in each year. Error bars show the 95% CI. [a]TC-marked foxes were estimated to be marked in the year of capture. [b]Unmarked foxes include those captured in non-baited areas in 2001, and captured before annual TC bait distribution. The group also contains TC-marked foxes estimated to be marked solely in the pre-capture years. [c]Data from necropsy. [d]In 2003, non-TC-containing baits were also distributed (see Table 1). NE: not examined.

should be taken into account in the analysis of TC marking.

A higher marking rate was observed in juvenile than in adult foxes (Table 3), indicating effective baiting of juvenile foxes. This result agrees with the suggestion that distribution of baits during the summer will predominantly reach the young fox population because they are inclined to locate and consume baits more rapidly than adults (Vos, 2003). In the present study, the worm burden in juvenile foxes was higher than that in

adult foxes, both before and during bait distribution. Other studies in other parts of Hokkaido, Nemuro (Takahashi and Uraguchi, 1996), and Koshimizu (Morishima et al., 1999), have shown a higher prevalence and percentage of feces containing taeniid eggs in juvenile foxes. Similarly, a higher worm burden (Hofer et al., 2000) and prevalence (Tackmann et al., 1998; Losson et al., 2003) in juvenile foxes have been observed in endemic regions of Europe. Therefore, juvenile foxes are considered to play an important role in the transmission of *E. multilocularis*. Hence, the high percentage of marked foxes and effective deworming in juveniles observed in the present study would be quite effective in controlling the transmission cycle of *E. multilocularis*.

However, the lower marking rate in adult foxes implies that the baiting strategy was less attractive for them. The lower rate in adults can also be partially explained by less effective marking in adult foxes with the amount of TC used. According to Lawson et al. (1992), >75 mg TC is necessary to consistently mark adult foxes, whereas 50 mg marks almost all juvenile foxes. Although each bait contained 100–160 mg TC in the present study, TC was likely to be degraded by processing, preservation, and field exposure. Johnston et al. (2005) observed that the marking potential of TC in commercial rabies vaccine baits decreases to ~60% through the manufacturing and distribution chain. Therefore, the true percentage of adult foxes that consumed baits could be higher than that determined.

The percentage of foxes marked in the year of capture appeared to increase year by year. The percentage in 2003, when TC baits were distributed only once, was higher than that in 2001 and 2002, when TC bait distribution occurred twice annually. One possible explanation is the familiarization of foxes for uptake of baits. However, this could not be ascertained from our TC-marking data; no clear difference was observed when TC marking in the year of capture was compared between foxes marked and unmarked in pre-capture years.

4.2. Evaluation of deworming effect

E. multilocularis and *A. alata* infections were not detected in the majority of foxes marked in the year of capture, whereas the annual prevalence of these parasites in unmarked foxes was 18–43% and 18–38%, respectively (Fig. 3). These results indicate that the foxes consuming the baits were effectively dewormed. Among the marked foxes, which were captured 14–97 days after the first TC bait distribution in the year of capture, *E. multilocularis* was detected in seven animals. However, gravid worm segments were not found in any of these foxes. Since the body weight of these foxes ranged from 1.3 to 3.9 kg, and a dose of 5 mg/kg praziquantel shows 100% deworming for *E. multilocularis* (Rommel et al., 1976), a dose of 50 mg praziquantel was used for each bait, and was sufficient for complete deworming. Accordingly, these seven foxes were assumed to have been infected after consuming the baits. This suggests higher infection pressure for *E. multilocularis* in the period of this study, that is, during the summer and early autumn. In this season, fox families are territorially living around breeding den sites, and thus higher prevalence of *E. multilocularis* in voles (*C. rufocanus*) (Kamiya et al., 1977; Takahashi et al., 1989) and heavy contamination with *E. multilocularis* eggs (Morishima et al., 1999; Tsukada et al., 2002) are observed around fox dens. Therefore, this season is considered to be important for *E. multilocularis* transmission between foxes and voles in Hokkaido (Giraudoux et al., 2002). The high infection pressure observed in this study also supports this viewpoint. As a control measure, intensive (monthly) bait distribution during this period is recommended, at least for the initial phase of a baiting campaign.

A. alata was found in only one of the marked foxes. Foxes are infected with *Alaria* species by ingesting second intermediate (frogs) or paratenic hosts (e.g., snakes, and to a much lesser extent, voles), or transmammary (Shoop and Corkum, 1987). Although the main route of infection is unknown, food-habit analysis of foxes in Hokkaido has shown that the occurrence of reptiles and amphibians is much lower than that of rodents in the contents of feces and stomach (Tsukada and Nonaka, 1996; Tsukada, 1997). Therefore, we believe that the infection pressure for *A. alata* was lower than that for *E. multilocularis*.

The percentage of foxes positive for *E. multilocularis* infection and fecal taeniid eggs decreased from more than 50 and 25% in the pre-baiting years (1999 and 2000) to less than 20 and 5% in the third (2003) and fourth years (2004) of baiting, respectively (Fig. 3). In particular, even in unmarked foxes, the percentage in the fourth year of baiting was reduced to 14 and 7%, respectively. The reduction in the prevalence in unmarked foxes suggests a cumulative effect of bait distribution, which caused a decrease in the prevalence in intermediate hosts. From another point of view, annual variation in prevalence also has to be taken into account. It has been observed that the prevalence of *E. multilocularis* in local fox populations in Hokkaido have naturally changed, in relation to the

abundance of voles or snowfall in winter (Saitoh and Takahashi, 1998; Yokohata and Kamiya, 2004). In the present study, the prevalence in unmarked foxes in the first year of baiting (2001) was lower than that in pre-baiting years (1999 and 2000). However, the annual necropsy survey of foxes from 15 fixed area of Hokkaido in winter, which the Hokkaido Government has conducted, showed that the prevalence was not clearly changed during 1999–2004 (45.8% in 1999, 38.5% in 2000, 39.0% in 2001, 31.6% in 2002, 43.1% in 2003, and 46.0% in 2004; data from the Hokkaido Government in 2000–2005).

In this study, using TC biomarker, the acceptance of anthelmintic bait by individual foxes and subsequent deworming were confirmed. Furthermore, the relationship between bait consumption and parasite infection in each fox suggested re-infection cases after bait consumption and accumulative effect of controlling *E. multilocularis* infection by long-term baiting. The use of biomarkers in anthelmintic baiting campaign provides helpful information to plan an effective baiting program for controlling *E. multilocularis*.

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The vaccination potential of EMY162 antigen against *Echinococcus multilocularis* infection

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Abstract

Alveolar echinococcosis is caused by infection with the larval stage of *Echinococcus multilocularis*. We recently identified a cDNA clone, designated as *emy162*, that encodes a putative secreted protein. EMY162 shares structural features with the EM95 antigen, which is a host-protective antigen. The amino acid sequence of EMY162 shows 31.4% identity to EM95 whereas these antigens are distinguishable with respect to their predicted secondary structure and antigenicity on Western blot analysis. RT-PCR analysis revealed that the gene expression of *emy162* was significantly higher than that of *em95* at each life-cycle stage. Recombinant EMY162 antigen induced a significant level of host-protection (74.3%) in experimental infection with *E. multilocularis* eggs in mice. Notably, recombinant EMY162 antigen showed significant reactivity to the sera from alveolar echinococcosis patients. These results may help in the development of a practical vaccine to reduce the level of alveolar echinococcosis in humans.

Keywords: *Echinococcus multilocularis*; Alveolar echinococcosis; EMY162; Vaccine; EM95; Host protective antigen

Alveolar echinococcosis (AE) in humans is a zoonosis caused by infection with the metacestode stage of *Echinococcus multilocularis* [1]. The life cycle of the parasite is maintained between foxes, dogs or cats (definitive hosts), and various species of rodents (intermediate hosts). The adult worm lives in the small intestine of a definitive host and releases eggs in the host's feces. Human AE infection is caused by the accidental ingestion of eggs, which form large cysts, mainly in the liver and lungs. Although *E. multilocularis* infection levels in humans are generally low, untreated AE is a highly lethal disease due to the proliferation and metastasis of the parasite.

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 already been achieved in the genus *Taenia* that causes cysticercosis. Vaccination with either recombinant proteins or peptide epitopes of 45W, TO16, TO18, and TSA18 proteins resulted in a highly effective ptotection against *T. ovis* infection in sheep and *T. saginata* infection in cattle [2–5]. In addition, a recombinant vaccine has been developed to control cystic hydatid disease caused by *E. granulosus*. Lightowlers et al. [6] demonstrated that the recombinant EG95 vaccine is highly effective for the prevention of *E. granulosus* infection in sheep in Argentina, Australia and New Zealand. These experimental results indicate that the prevention of the disease by vaccination is possible, with a high degree of protective immunity against parasites.

We recently cloned a candidate gene with potential for use in the serological diagnosis of *E. multilocularis* infection in dogs from a cDNA library constructed from the mRNA of immature adult worms [7]. This gene, designated as

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emy162, encodes a 153-amino acid protein with a molecular mass of 17.0 kDa. The protein includes a signal peptide sequence, fibronectin type III motif, and a putative transmembrane domain, which are also found in the EM95 protein known to be a host-protective antigen against *E. multilocularis* infection [8]. EM95 antigen has been studied as the first candidate for vaccine development for intermediate hosts of *E. multilocularis*. In the present study, we focus on the fact that EMY162 has structural features in common with EM95.

In the present report, a vaccine trial was undertaken to determine whether EMY162 could induce host protective immunity against infection with *E. multilocularis*. In addition, we describe the molecular and genetic characterization of EMY162 and EM95 based on RT-PCR in several life-cycle stages and Western blot analysis with recombinant protein-specific antibodies and sera from AE patients.

Materials and methods

Cloning, expression, and purification of recombinant protein. DNA templates of *emy162* (Accession No. AB303298) were cloned from a cDNA library constructed from adult worm mRNA as described previously [7]. A cDNA clone encoding *em95* was kindly provided by Dr. M.W. Lightowlers (University of Melbourne, Victoria, Australia). For construction of ThioHis-EMY162, a DNA fragment of *emy162* encoding Val21 to Phe140 was amplified by PCR with the following primer sets: 5′-aga tct gta gac cca gag cta ata g-3′ with a BglII site and 5′-ctg cag gaa tcc gcc agc tct gtc a-3′ with a PstI site. For GST-EMY162, a DNA fragment was amplified with forward primer 5′-aga tct gta gac cca gag cta ata g-3′ and reverse primer 5′-ctc gag aat tag ggc gaa tcc gcc ag-3′. For the construction of ThioHis-EM95, the DNA fragment encoding Arg22 to Thr156 was amplified by the following primer sets: 5′-ggt acc aat tga gat aaa gac aac aga gag-3′ with a KpnI site and 5′-ctg cag cat gga agc ctg ttg cag aa-3′ with a PstI site. For GST-EM95, the forward primer was 5′-aga tct att gag ata aag aca aca gag-3′ with a BglII site, and the reverse primer was 5′-ctc gag cat gga agc ctg ttg cag aa-3′ with an XhoI site. The amplified fragments were treated with restriction endonucleases and cloned into pThioHis (Invitrogen) and pGEX-6P-3 (GE Bioscience) in frame, respectively. Cloning and expression of the recombinant DNAs were performed in *E. coli* strain Top10 (Invitrogen). The bacteria was cultured in Lennox broth at 37 °C, and protein expression was induced with 0.5 mM IPTG at 32 °C for 5 h. Recombinant antigen was purified with affinity chromatography and FPLC according to the method described previously [9].

RT-PCR analysis. Echinococcus multilocularis (Nemuro strain) was obtained from a dog–cotton rat life cycle maintained at the Hokkaido Institute of Public Health. Protoscoleces were collected from a cotton rat and washed with PBS. Some protoscoleces were treated with culture medium based on the method of Smyth and Davies [10] to obtain cultured metacestodes. 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 released from the intestinal contents by soaking in PBS to remove canine intestinal mucus, and then the worms were rinsed several times in PBS. After washing with PBS, all parasite materials were immediately soaked in RNAlater (Ambion, Inc.) and stored in liquid nitrogen. All experiments were performed in a specially designed safety facility (Biosafety level 3) at the Hokkaido Institute of Public Health.

Total RNA was isolated from each of the life-cycle stages of *E. multilocularis* by using an RNeasy Mini kit (Qiagen) according to the manufacturer's instructions. cDNA synthesis was performed by using an RT-PCR kit (Applied Biosystems). Reactions were performed in a total volume of 25 μl containing 250 ng of the total RNA samples. The RT mixtures were incubated at 48 °C for 30 min, and the reaction was terminated by heating at 95 °C for 5 min. Specific primer sets were designed from nucleotide sequences of *emy162* (Accession No. AB303298) and *em95* (Accession No. AY062921) as follows: 5′-gga aga tgg tac ttc gat tct gt-3′and 5′-cac gtg aat cca tcg gaa gt-3′ for *emy162* detection, and 5′-gtc tac aag aga caa act gc-3′ and 5′-tcc act agt cat tac ggt gc-3′ for *em95* detection. PCR was performed with 1 μl of cDNA in a total volume of 50 μl. The reaction was performed with a Gene Amp® PCR system 9700 (Applied Biosystems) under the following thermal cycling conditions: 5 min at 94 °C, followed by 40 repeats of 30 s at 93 °C, 30 s at 56 °C and 30 s at 70 °C, and finally 7 min at 70 °C.

Preparation of recombinant antigen-specific antibodies. Balb/c mice (7-weeks-old female) were immunized three times. Fifty micro grams each of recombinant antigens ThioHis-EMY162 and ThioHis-EM95 were administrated to mice with Freund's complete adjuvant. Thereafter, two boosters were given to the animals with incomplete adjuvant at 2-week intervals. Antibody production was assayed by immunoblot analysis.

Western blot analysis. Immunological cross-reactivity between EMY162 and EM95 recombinant antigens was analyzed by Western blot. Approximately 1 μg of GST-EMY162 and GST-EM95 were loaded onto SDS–PAGE. After SDS–PAGE, the separated protein bands were electroblotted onto a PVDF membrane and blocked by incubating in blocking buffer (PBS containing 10% skim milk and 0.1% Tween 20) for 1 h. The recombinant antigen bands were probed with anti-ThioHis EMY162 or anti-ThioHis EM95 antibody (1:1000 dilution in blocking buffer) by incubating the membrane for 1 h. After washing the membrane with 0.1% Tween 20 in PBS (PBST) three times, the membranes were incubated with anti-mouse IgG-alkaline phosphatase (AP) conjugate (1:5000 dilution in blocking buffer). The bound antibodies were detected with a BCIP/NBT immuno-detection kit (NEN Life Science).

Reactivity between recombinant antigen and sera from AE patients was performed by same method as described above except for the serum sample dilution. The recombinant protein was first probed with serum sample at a 1:400 dilution in blocking buffer and secondarily probed with AP-labeled goat anti-human IgG (1:2500 dilution in blocking buffer).

Vaccine trial of the recombinant EMY162 and EM95 antigens. Five female BALB/c mice (7-weeks-old) were immunized three times. Twenty micrograms of one of the recombinant antigens was administrated to mice with Freund's complete adjuvant 56 days prior to experimental infection. Boosters were given on days 21 and 42 with incomplete adjuvant. On day 56, which was 2 weeks after the final immunization, 200 parasite eggs prepared from the feces of an *E. multilocularis*-infected dog were administrated orally. All mice were sacrificed 4 weeks after infection, and necropsies were performed. The number of alveolar echinococcosis cysts in each mouse was counted.

Results and discussion

Sequence analysis of EMY162 and EM95

During the cDNA screening process in the development of immunodiagnostic antigen for the detection of *E. multilocularis* infection in dogs, which is routinely performed in our laboratory [7], we serendipitously found the novel cDNA clone (designated as *emy162*) via random cloning of the cDNA library constructed from the mRNA of immature adult *E. multilocularis*. The complete nucleotide sequence of *emy162* showed 52.8% identity with that of *em95*, but no gene with significant similarity was found in BLAST. As illustrated in Fig. 1A, it was predicted that these antigens shared common structural features that consisted of a signal peptide sequence at the N-terminal region (amino acids 1–17), one fibronectin motif in the central region (amino acids 35–115 for EMY162 and 30–113 for

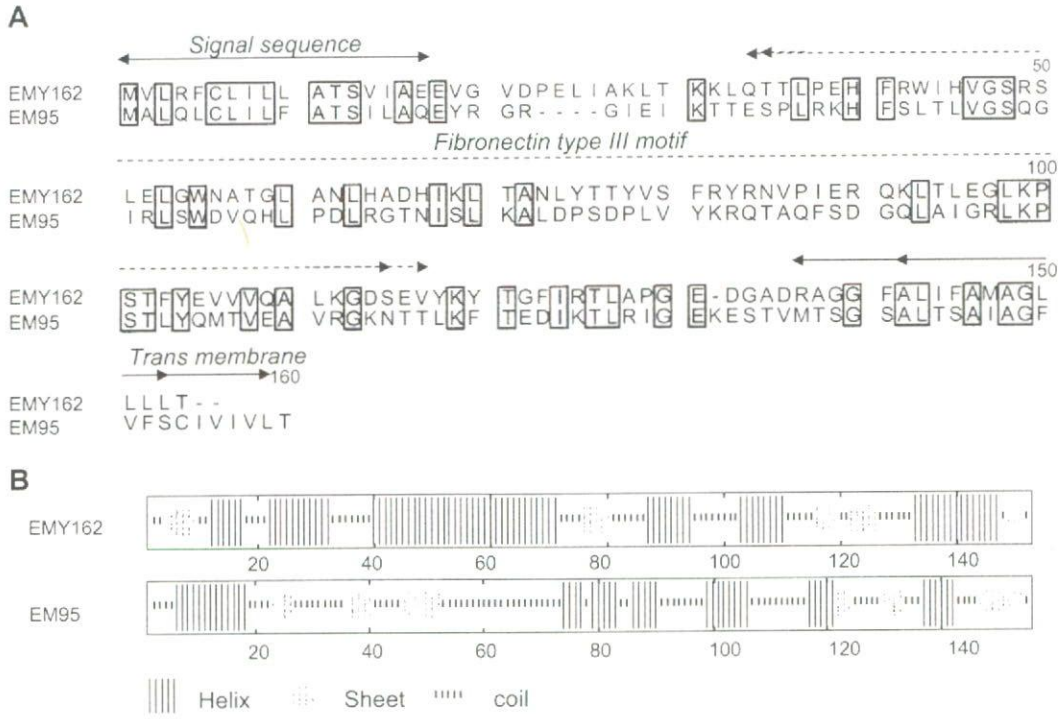

Fig. 1. Comparison of the amino acid sequences (A) and predicted secondary structures (B) of EMY162 and EM95. Alignment of the deduced amino acid sequences of EMY162 and EM95 was performed with DNASIS Pro (Ver. 2.0). Identical amino acid residues are in a shaded box. Common structural features are indicated by arrows. Secondary structure prediction was performed with on-line software based on the GOR IV method [13].

EM95), and a hydrophobic domain encoding a putative transmembrane domain in the C-terminal region (amino acids 136–153 for EMY162 and 138–155 for EM95). These results suggested that EMY162 belongs to the same family as EM95 and its related family proteins of *E. glanulosus*.

The deduced amino acid sequence of EMY162 was calculated to show 31.4% identity with EM95, and thus the sequences of the two antigens were considerably different. In all cases reported so far, the EM95 and EG95 family of proteins retain a high level of amino acid identity in their deduced amino acid sequences [11,12]. The EG95 family of *E. glanulosus* (EG95, EG95-1 to -6, EG95-QH-1 to -3, and EG95-XJ) shows 100–73% identity among their predicted amino acid sequences. EM95 shows 80–84% amino acid identity to the EG95-1 and EG95-6, respectively. The variant of EM95 [13] that was isolated from an *E. multilocularis* oncosphere-specific cDNA library shows 79% identity to EM95 at the amino acid level.

Application of the GOR IV on-line software [14] to the amino acid sequences of EMY162 and EM95 showed significant differences in the locations of α-helix, extended strand structures, and random coil structures. The secondary structure contents of EMY162 were predicted to be 52% α-helix, 15% extended strand, and 33% random coil, and those for EM95 were predicted to be 29% α-helix, 20% extended strand, and 51% random coil (Fig. 1B). These results suggest that there are significant differences in the molecular conformations of the EMY162 and EM95 antigens, although these molecules do share common structural features as described above.

RT-PCR analysis of emy162 and em95

To investigate the expression of *emy162* and *em95* in each life stage, we performed RT-PCR with total RNA extracted from metacestodes, immature adult worms and mature adult worms of *E. multilocularis*. We designed specific intron spanning primers for each gene. Genomic DNA was included in each assay to assess its contamination. As shown in Fig. 2, the RT-PCR product for *emy162* was detected at the predicted size (144 bp) in all cDNA samples prepared from each stage. The expression of *emy162* was similar to the previously reported expression of *eg95* [12]. In contrast, RT-PCR product for *em95* at the predicted size (183 bp) was detected only in the metacestode cDNA samples. No bands corresponding to the *em95* RT-PCR product were observed in cDNA samples from immature and mature adult worms. Thus, there appeared to be significant differences between the gene expression level of *emy162* and *em95* in the life-cycle stages. Although there is currently little information regarding the expression of the *em95* gene, Gauci et al. reported that *em95* was isolated as a cDNA clone from a cDNA library constructed from the mRNA of *E. multiloculars* protoscoleces [8]. Our result is consistent with this observation.

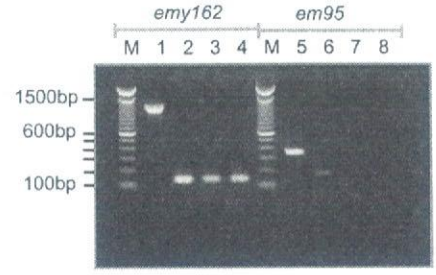

Fig. 2. RT-PCR analysis for expression of the *emy162* and *em95* genes in different life-cycle stages of *E. multilocularis*. Total RNAs from cultured metacestodes, immature adults, and mature adults were reverse transcribed into cDNA and applied to PCR. Molecular size markers are shown in lanes M. Lanes 1 and 5, genomic DNA for contamination control; lanes 2 and 6, metacestode; lanes 3 and 7, immature adult worm; lanes 4 and 8, mature adult worm.

Expression and purification of recombinant vaccine antigens

The EMY162 and EM95 antigens used for the vaccine trial were expressed in *E. coli* as soluble fusion proteins with a ThioHis tag. These recombinant antigens were extracted from bacterial cell lysates and purified with Ni-affinity resin and a Superdex 75 pg gel filtration column fitted on the FPLC. As illustrated in Fig. 3B, purified recombinant EMY162 and EM95 subjected to SDS–PAGE showed near single bands with a molecular mass of approximately 29 kDa. This estimated molecular size agrees with the sum of the ThioHis tag (15 kDa) and the predicted molecular sizes of EMY162 and EM95. A typical purification yielded 6.5 mg and 4.2 mg of pure ThioHis-EMY162 and ThioHis-EM95 from 1 L of culture medium, respectively.

Vaccine trial of recombinant EMY162 and EM95

Vaccine efficacy of EMY162 and EM95 was evaluated by the reduction of the number of *E. multilocularis* cysts in the livers and lungs of immunized mice. Five female BALB/c mice were immunized subcutaneously with the purified ThioHis fusion antigen plus adjuvant. After the final immunization, 200 parasite eggs were administrated orally to each group of mice. Fig. 3A shows the mean number of AE cysts in each group. The control group vaccinated with the ThioHis tag alone showed a 20% reduction in the mean number of AE cysts compared with that of the group immunized with adjuvant alone. The group of mice immunized with EMY162 developed significantly fewer cysts (74.2%) than the adjuvant control group

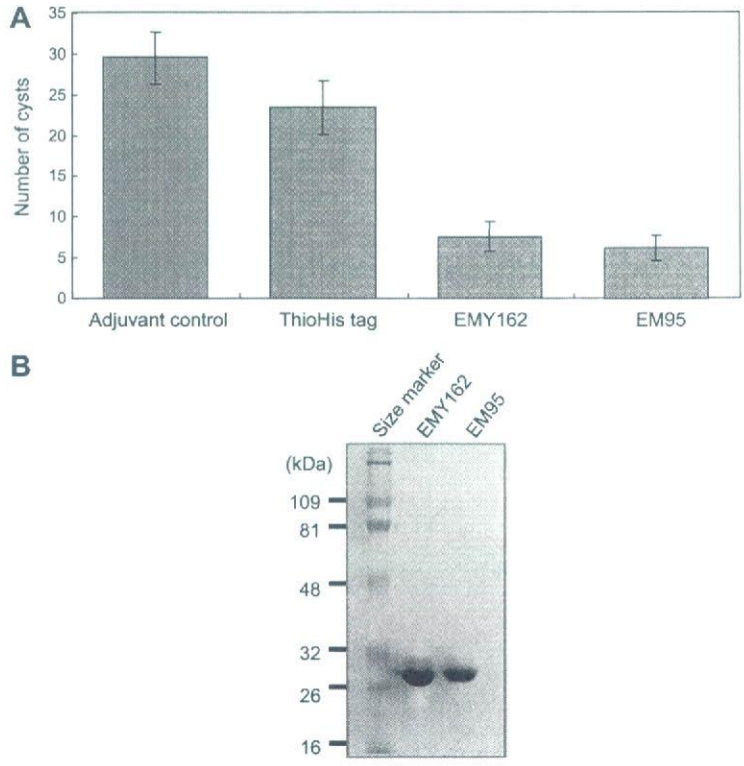

Fig. 3. Experimental infection of mice immunized with recombinant EMY162 and EM95 (A). SDS–PAGE analysis of the recombinant antigens is illustrated in the panel (B). Each mouse was immunized with purified recombinant EMY162 or EM95 with Freund's adjuvant three times. Two hundred parasite eggs were administrated orally after the final immunization. As controls, groups were immunized with adjuvant plus PBS or the ThioHis tag plus adjuvant. The values are the mean number of alveolar cysts ± SEM from five mice. $^{*}P < 0.05$ compared to the mean value of each group.