(7) Hepatic multilocular echinococcosis

In most of the research carried out on *E. multilocularis* infection in the intermediate host, hydatid larval mass (suspension of minute vesicular cyst and protoscoleces) were injected intraperitoneally into the host to produce hydatid cyst in secondary echinococcosis. By this method of inducing the infection, the hydatid lesions were usually not limited to only locating in the liver as that seen in the human cases. Furthermore, to produce a hydatid lesion that is limited to only the liver in an experimental animal, one has to infect the animal with the eggs of the parasite. Since the parasite egg is biohazardous, this experiment could only be conducted in a special biosafety facility. A method to produce hydatid lesion that is found explicitly only in the liver was developed by injecting the homogenate of *E. multilocularis* larval tissue directly into the hepatic vein of either the cotton rats, rats or mice, or injecting the parasite tissue directly into the liver of the experimental animals (Ohnishi, 1984; Takagi *et al.*, 1987).

(8) Clinical signs and predilection site of *E. multilocularis* in humans

Since 1936, physicians at the Hokkaido University School of Medicine, First Department of Surgery had experienced many human cases of multilocular echinococcosis. Kasai *et al.* (1980) described their experience in the operation of 60 cases of echinococcosis patients from 1936 to 1978. They categorized the course of the disease into 3 stages; namely the incubation stage (6 cases), during which there were no clinical sign observed, the progressive stage (37 cases) and the terminal stage (17 cases). The following clinical signs that were observed in patients at the progressive stage and terminal stage of the disease were respectively, hepatomegaly, 89% and 94%, abdominal pain, 3% and 47%, jaundice, 19% and 70%, fever, 5% and 53%, ascites, 3% and 12%, and anemia, 5% and 29%. Thus, the frequency of the clinical signs was observed to be much higher for the terminal stage rather than the progressive stage patients. Moreover, as for the lesion in the liver, the small ones showed cyst completely filled up with stroma while the larger ones showed a necrotic core enclosed by a cyst-like structure. Liver function of the patient was not impaired until the terminal stage of the disease. Sato *et al.* (1996) made a statistical analysis of 156 cases of multilocular echinococcosis and found that the ratio of male to female patients was 85 : 67, with the male being more. The patients' age ranged from 7 to 72 years old, with an average of 45.2 ± 15.2 years old. Besides, the lesion found in the liver, metastasis to the lung (14 cases), brain (2 cases) and to the spleen (2 cases), were also seen. These metastases cases showed a positive correlation with the size of the lesion in the liver. Although it had been considered that humans were comparatively resistant to *E. multilocularis* infection with protoscoleces not being formed, Fujioka *et al.* (1993) reported finding protoscoleces in the histopathological sections of 8 out of 50 patients.

Takahashi *et al.* (1986) reported 60 cases of echinococcosis in Honshu island and found that the male patients were 2.5 times as many as the female patients. The age of the patients ranged from 21 to 76 years, with as average of 55.8 years. The site of predilection of the hydatid cyst in the aforementioned patients includes 55 patients with liver lesion, 14 patients with lung lesions, 6 with brain lesions and other organs affected includes the kidney, bone marrow, bone, mesentery, pleural septa and the spinal cord. Initial clinical diagnosis of 24 cases showed that the hepatic lesions were

misdiagnosed as hepatic tumor. Their major clinical complaints were abdominal pain and jaundice (13 cases), systemic malaise (12 cases), nausea and vomiting (9 cases), abdominal tumor (8 cases), hepatomegaly (7 cases) and others that includes the impairment of the organ function in which the parasite had metastase to. Calcification of the hydatid lesion in the patient was observed in 22 cases. In 12 cases, protoscoleces could be detected in the hydatid lesion.

Furuya *et al.* (1995a, b) discovered an *Encephalitozoon*-like protozoan in the hydatid lesion obtained from multilocular echinococcosis patients. They also found that of the 119 multilocular echinococcosis patients, 62 had antibody to that protozoan while among 159 healthy persons examined 8 were seropositive to that protozoan. Later, when the hydatid lesion were probe with a DNA primer in a PCR that was thought to be specific for *E. multilocularis*, the *Encephalitozoon*-like protozoan was also detected (Nagano *et al.*, 1996). This result showed that a taeniid tapeworm and an *Encephalitozoon*-like protozoan, both of which are taxonomically different, shared a homologous sequence of nucleic acid in their gene. Further elucidation of this still unknown protozoan awaits future investigation.

(9) Imaging diagnosis and biopsy for multilocular echinococcosis

Uchino *et al.* (1987) described their findings on the 99 human cases of multilocular echinococcosis that were treated at Hokkaido University School of Medicine, First Department of Surgery, as follows. By X-ray diagnosis of the abdomen, 30% of the patients were found to have calcified lesion of the hydatid cyst. The probability of detecting calcified lesion in the small hydatid cyst by this method was very low because the sensitivity level of this diagnostic method was low. By ultrasound imaging, a variety of lesions, such as calcified lesion, small vesicular cyst, necrotic lesion and liquefied lesion could be observed in the form of acoustic shadow coupled with strong echo in 97% of the echinococcosis patients. Since the ultrasound diagnostic apparatus were portable, this method of diagnosis has the highest applicability. By CT (computer tomography) scan, calcification of the lesion (high-density area) could be observed in all the patients. Moreover, non-uniform lesions such as those mixed with necrotic tissue and abscess could also be observed by this method. By angiography, it was observed that the lesion became devoid of blood vessels and the blood vessels surrounding the lesion became compressed and relocated.

Ogasawara *et al.* (1993) suggested MRI (magnetic resonance imaging) had been useful in the diagnosis and therapy of multilocular echinococcosis. However, they added that it was difficult to detect the calcified lesion sign even through the use of T1 and T2 weighted imaging. Thus, there is a need to reconsider and to modify the use of MRI in the diagnosis of multilocular echinococcosis.

Since it was thought that metastasis of the *E. multilocularis* lesion could occur during diagnostic biopsy, this procedure had been prohibited and made taboo. However, Namiki (1990) stated that in the case of hepatic lesion that is embedded deep within the tissue, intraperitoneal exploratory biopsy using the endoscope should not present any problem.

(10) Diagnosis of *E. multilocularis* in the definitive host

Yorozuya *et al.* (1968) examined the distribution of the *E. multilocularis* adult tapeworm along the small intestine in the definitive host and found that the most favorable site of predilection was the duodenum. Based on that report, only the

anterior portion of the small intestine of the foxes necropsied in Hokkaido was examined for *E. multilocularis* infection. However, after the report by Yagi *et al.* (1986) on the results of the survey and experimental infection of *E. multilocularis* in the red foxes, examination for the adult tapeworm of *E. multilocularis* in the foxes were changed to the posterior portion of the small intestine.

Diagnosis of tapeworm infection in the living definitive host is usually based on the identification of the parasite eggs that were being shed in the feces of the host. However, since the morphology of the eggs of taeniid species, including that of *Echinococcus* spp., were almost the same, it is very difficult to differentiate among them. Other method that had been used for the diagnosis of cestode infection in the definitive host is the detection of the excretory/secretory products by the adult tapeworm in the gut by using the immunological method. This type of coproantigen detection diagnostic method had been applied to other cestode in other countries. At the Hokkaido University, Faculty of Veterinary Medicine, monoclonal antibodies against adult worm of *E. multilocularis* were prepared for use in the coproantigen detection test to diagnose the infection in the definitive host. By using this test, which is based on the use of a sandwich ELISA, infection during the prepatent period could also be detected. Moreover, this test could also detect the coproantigen in the feces that had been treated with heat so as to render the parasite eggs non-infective. This is to reduce the biohazard for the laboratory worker doing the test. The coproantigen detection test basically used a monoclonal antibody (EmA9) specific for *E. multilocularis* adult worm (Kohno *et al.*, 1995) in a sandwich ELISA that detect the parasite coproantigen in the feces of experimentally infected definitive host as well as the alternative definitive host. This test could be used to diagnose the early phase of the infection when the parasite eggs were not yet produced. Moreover, the coproantigen detection test could also be used to diagnose the definitive host for echinococcosis when the parasite eggs shed had been reduced to so few that it had become very difficult to detect under the microscope (Kohno *et al.*, 1995; Sakashita *et al.*, 1995; Sakai *et al.*, 1996; Nonaka *et al.*, 1996). The stability of the coproantigen to chemical and physical treatment was evaluated and the test was found to be applicable in the field (Sakashita *et al.*, 1995). As stated above, this sandwich ELISA coproantigen detection method was also used to monitor the course of experimental infection of laboratory animal with *E. multilocularis* (Nonaka *et al.*, 1996). The validity of coproantigen ELISA using EmA9 for diagnosis of foxes was confirmed by comparing the results of autopsy, egg examination and coproantigen ELISA using rectal fecal samples (Morishima *et al.*, 1998; Yimam *et al.*, 2002).

Presently, this coproantigen detection test is being used to diagnose *E. multilocularis* infection in pet dogs and cats. It is also expected to be used as an indicator method for pinpointing the endemicity as well as the potential contamination by *E. multilocularis* eggs in a certain areas through the examination of the wild red foxes feces collected from that area. Since the antigen that wass recognized by the monoclonal antigen EmA9 is both stable and specific, it is considered as practical for actual use and had been highly evaluated in other countries. In addition, this coproantigen detection method had also been modified and found to be effective for the diagnosis of *E. granulosus* (Sakai *et al.*, 1995).

Furthermore, it was thought that identification of the *E. multilocularis* DNA in the feces of red foxes might be use as a diagnostic method. Since the eggs of taeniid species were apparently morphologically similar, it was found to be possible to identify *E. multilocularis* eggs using a specific primer to probe for the U1snRNA gene in a PCR to confirm the identity of the eggs (Yagi *et al.*, 1996).

(11) Therapy for multilocular echinococcosis (hydatidosis)

① Resection of the liver

Presently, the basic therapy for multilocular echinococcosis (hydatidosis) in human in Japan is still the resection of the infected liver. At Hokkaido University School of Medicine, First Department of Surgery, 156 patients been treated and many had undergone surgical operation to remove liver hydatid cysts since 1937 (Nakajima *et al.*, 1996). As described above, with the advent of early diagnosis of hydatidosis due to improvement of the diagnostic methods, the probability of detecting of small hydatid lesion became higher and thus the number of successful hepatic resection operation became higher. Since large quantity of the liver to be resected, the trend had been to resect a certain area or portion of the liver. Of the aforementioned 156 cases, 118 had undergone liver resection, with 71 of them having total resection of the lesion. However, in 5 cases after the liver resection operation, there was a recurrence of the liver hydatid cyst, including patients who had undergone an operation to remove a large chunk of liver tissue. Generally, during the progressive stage of the disease, it is very important to remove any hydatid lesion from the Glisson vessels of the hepatic hilus and the inferior vena cava. However, such total resection of the lesion is very difficult. In 47 cases, in which partial resection of the lesion was done, the prognosis was no different from those cases in which liver resection was deemed impossible due to the widespread hydatid lesion. Previously, in 24 cases in which it was thought that resection of the liver was not possible, bile duct drainage operation (hepatocholangiostomy) were performed. However, such operation was no longer conducted in recent years. In 11 cases, the hepatic resection surgery was terminated after the incision of the abdominal cavity. Ishizu *et al.* (1996) reported successful therapy in 11 out of 20 hydatidosis cases that were treated albendazole at 10 mg/kg/day at a regimen of 4 weeks medication followed 2 weeks intermission for 3 months to 3 years, in conjunction with surgical operation.

② Therapy other than surgery against hydatidosis

Sakamoto (1973) examined the efficacy of 37 different drugs against the protoscoleces of *E. multilocularis in vitro* based on the observation of the motility and morphological changes of the drug-treated protoscoleces. He found that halogenated salicylanide and bisphenol derivatives showed the strongest protoscolicidal effect. He also compared the efficacy of the drugs in mice experimentally infected with secondary echinococcosis (Sakamoto, 1979). Kanazawa *et al.* (1995) observed that the viability of the small vesicles of *E. multilocularis* cyst could be colorimetrically quantified using the MTT stain and proposed that this method could be used in the drug efficacy test.

Miyaji *et al.* (1992, 1993) measured the amount of the various polyamines in various taeniid species, including that of *E. multilocularis*. They reported that the proliferation of *E. multilocularis* cyst in experimentally infected mice could be suppressed after treatment of the infected mice with DFMO (difluoromethylornithine), an ornithine decarboxylase inhibitor.

It has been reported in American and European countries that concurrent use of surgical resection of lesion and chemotherapy with long term and high dose administration of albendazole and mebendazole, had been effective against hydatidosis in humans. This type of treatment had also been repeated in Japan. Inaoka *et al.* (1987b)

reported that intraperitoneal injection of albendazole into *E. multilocularis* infected Chinese hamster was the most efficacious when they compared the efficacy of albendazole and mebendazole. However, Kanazawa *et al.* (1994) observed that the efficacy of the drugs varied with the time at which the chemotherapy was initiated when they conducted the drug efficacy experiment using *E. multilocularis* infected Mongolian gerbils. Yazaki and Kohgo (1996) reported the suppression of the proliferation of hydatid lesion in 8 patients who had been treated with albendazole at low dose (400mg/day/twice) but for a long term (1 to 47 months).

The small vesicles of *E. multilocularis* were found to be comparatively resistant to X-ray treatment. The suppressive effect of X-ray on the proliferation of hydatid cysts could be observed only for those hydatid cysts that had been irradiated by more than 45,000 Rad and then infected into Chinese hamster to produce secondary echinococcosis. The complete suppressive effect of the X-ray treatment could been seen only at an irradiation dose of 55,000 Rad or above (Ohnishi, 1986).

Ohnishi and Kutsumi (1988) and Takahashi *et al.* (1993) tried heat-therapy using *in vito* test and infected jirds, respectively.

③ Anthelmintics against *E. multilocularis* adult worm

Sakamoto *et al.* (1971) reported that bithionol and bunamidine hydrochloride, at a dose of 200 and 40 mg/kg body weight, respectively, administered twice, were completely effective in deworming *E. multilocularis* tapeworm from the definitive host. Later, Sakamoto *et al.* (1979) examined the effective dose of praziquantel by administering once at a dose range of 0.1–10mg/kg body weight against *E. multilocularis* tapeworm and found that 100% efficacy could be achieved at a dose of 10 mg/kg. Bithionol, bunamidine hydrochloride and arecoline were found to be ineffective in killing the eggs of *E. multilocularis* (Sakamoto *et al.*, 1971).

(12) Control of *E. multilocularis* by baits distribution

The effect of bait-delivered anthelmintic to reduce the prevalence of *Echinococcus multilocularis* in wild red foxes was evaluated in Koshimizu, eastern part of Hokkaido, Japan (Tsukada *et al.*, 2002). Anthelmintic baits were distributed to each resident fox family. After one year of the anthelmintic bait distribution the prevalence of *E. multilocularis* in foxes evaluated either by the parasite egg examination or coproantigen ELISA decreased in the distributed section contrasting to that in the non-distributed section.

Conclusions

In recent years in Japan, parasitic diseases had been pushed into the spotlight again. In Hokkaido, the problem of multilocular echinococcosis had not yet been solved and had always been brought up now and again. This might be partially due to the neglect in controlling the definitive hosts that served as the source of infection for humans. On the contrary, most of the efforts in the control of multilocular echinococcosis in Hokkaido had been directed to the early diagnosis and early therapy of the echinococcosis patients. This was done without paying much attention to the control of the source of infection, as both the natural definitive and intermediate hosts of *E. multilocularis* in Hokkaido are the wildlife. It is very difficult to stop the spread of the parasite through human directed control measures. In fact, in recent years, the endemic area for this parasite had spread widely in Hokkaido and the situation had

changed from bad to worse. Moreover, there is also the danger that multilocular echinococcosis might spread to the main island of Japan, Honshu and turn it into an endemic area. Based on the results of the study on *E. multilocularis* infection in its definitive host conducted in Japan to date, it is possible to take concrete measures to prevent the spread of the source of infection of *E. multilocularis* to Honshu. The Emerging and other Communicable Diseases Surveillance and Control (EMC) division at WHO (World Health Organization) listed echinococcosis as one of the most important diseases in its 1996 to 2000 annual report.

In 1999, echinococcosis was classified as a disease in 4th category of the dangerous infectious diseases under the New Infectious Disease Prevention Law of Japan. Under that law, physicians are obliged to report any instances of human echinococcosis, but this does not apply to veterinarians who diagnosed echinococcosis in dogs and cats, despite finding the infective eggs from animals that are biohazardous. Therefore, it is necessary to quickly establish preventive measures after diagnosis of the pets.

Measures to control the spread of the source of infection of echinococcosis had already been taken in the American and European countries, but Japan is trailing behind is this aspect. Thus, it is proposed that a large scale survey and control measures for echinococcosis should be carried out with the support of the government in Japan.

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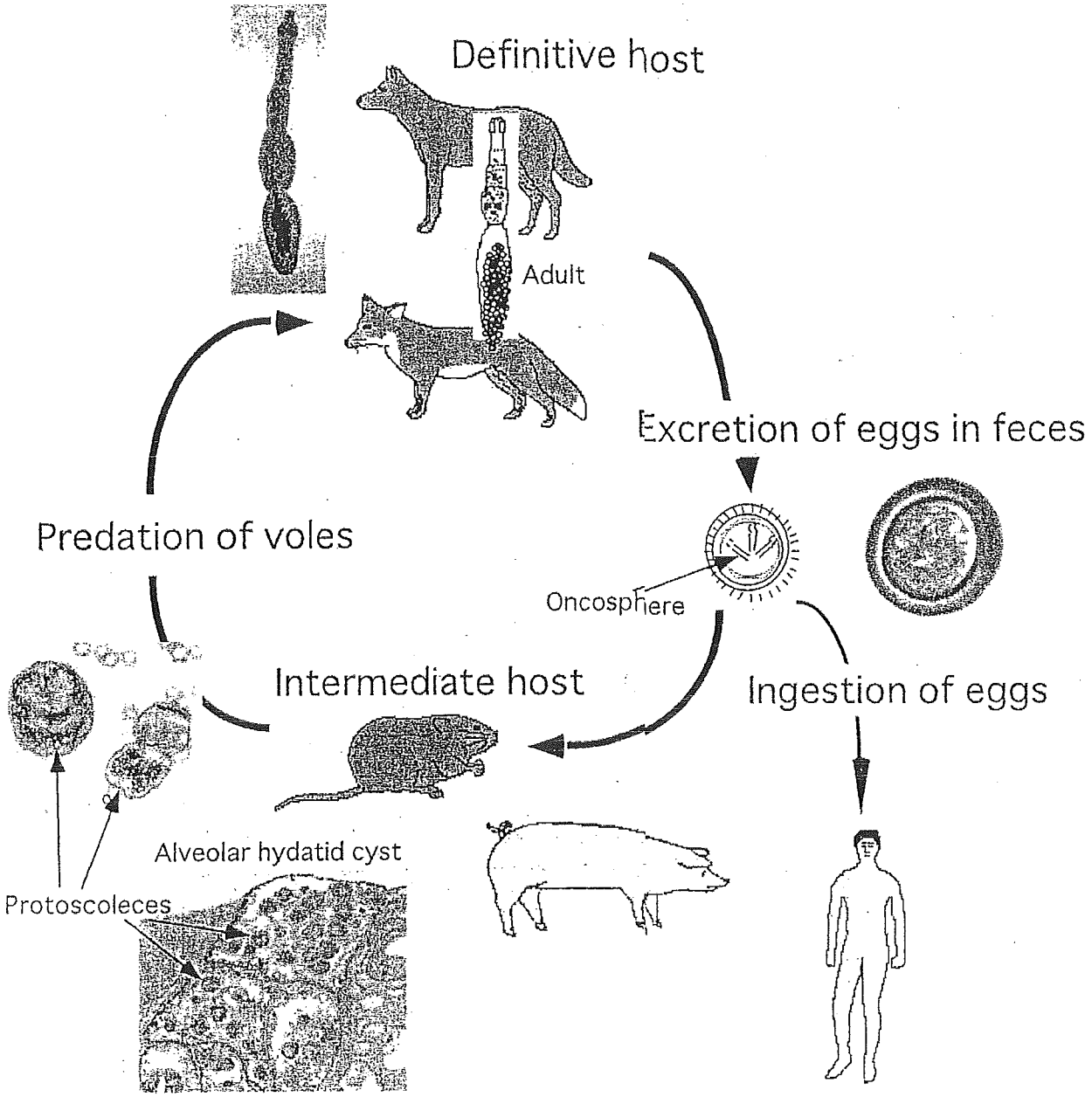

Fig. 1. Life cycle of *Echinococcus multilocularis*. The adults (3-6 mm), consist of a scolex, a neck and several proglottids, parasitize in the small intestine of the definitive hosts (carnivores such as foxes, dogs, wolves and cats) and produce eggs. Eggs are excreted with host feces and ingested by the intermediate hosts (voles). In the small intestine of the intermediate hosts, eggs hatch and oncospheres are released. The released oncospheres penetrate to the gut wall, reach to the liver via bloodstream, start a complexed asexual multiplication process and develop to alveolar hydatid cysts containing numerous protoscoleces. The definitive hosts are infected by ingesting the intermediate hosts and each protoscoleces develop into adult *E. multilocularis* in their small intestine. Humans, pigs and other animals are occasionally infected by ingesting the parasite eggs.

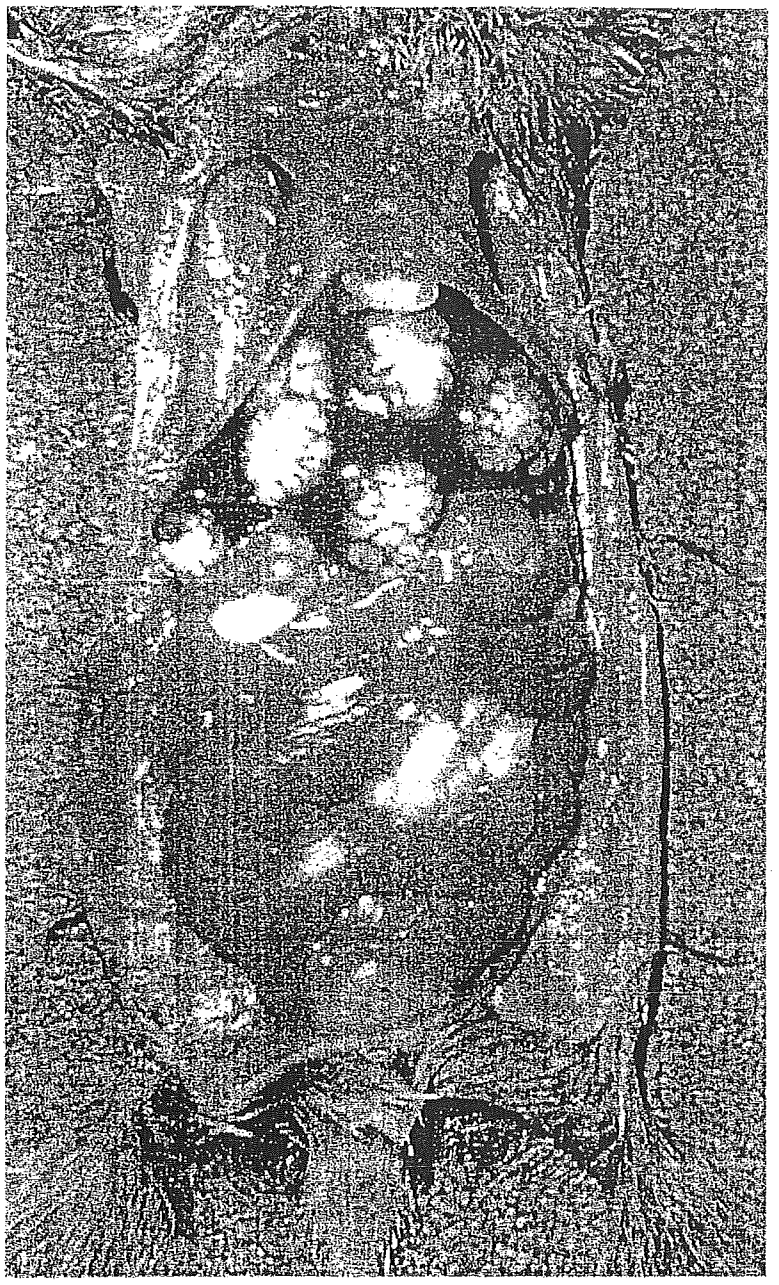

Fig. 2. Alveolar hydatid cyst found in a red backed vole (*Clethrionomys rufocanus bedfordiae*).

been expanding its distribution and at present, *E. multilocularis* is recognized all over Hokkaido island (Fig. 3). To monitor the change in prevalence, Hokkadio government has been performing necropsy surveys of foxes captured in winter at various sites of Hokkaido and showed that overall prevalence was 17.8% in 22,268 foxes surveyed during 1966-1999. However, in the last decade, the prevalence of the parasite in foxes has dramatically increased (58% in 1998) (Fig. 4). Our necropsy surveys conducted at the suburbs of Sapporo city showed similar high prevalences in foxes ; 54.9% in 1997-1998 [2] and 56.7% in 1999 [3]. Since the definitive hosts excrete the parasite eggs infective to humans, effective countermeasures against fox high prevalence are now emergent demand.

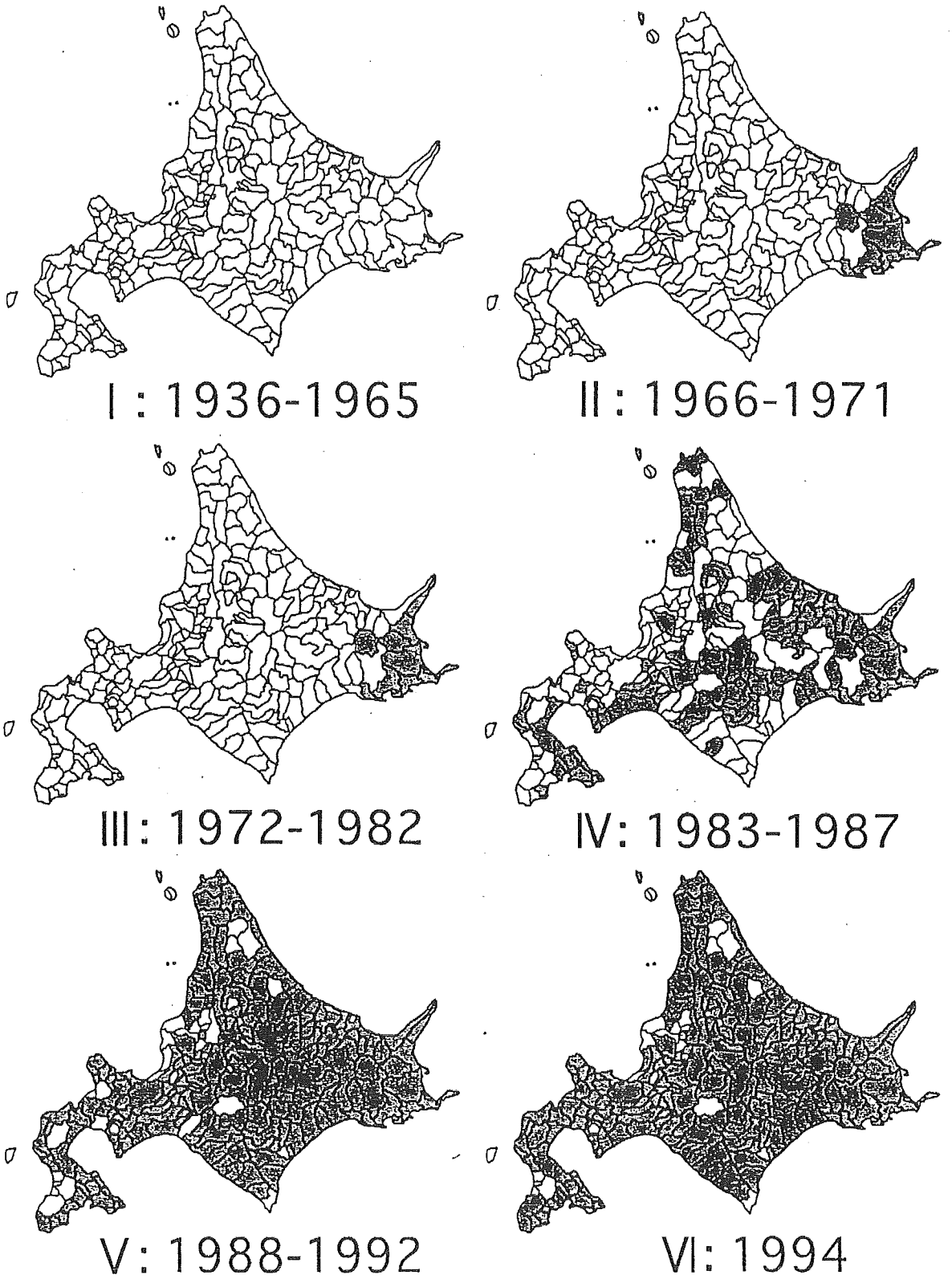

Fig. 3. Expansion of prevalent area of *Echinococcus multilocularis* in Hokkaido. I: 1936-1965 — The occurrence of the disease was restricted in the Rebun island; II: 1966-1971 — Patients and infected animals were found in the eastern part of Hokkaido; III: 1972-1982 — The prevalent area was considered restricted in the eastern part of Hokkaido; IV: 1983-1987 — Patients and infected animals were found in various regions; V: 1988-1992 — The prevalent area was further expanding and *E. multilocularis* was considered spread all the area of Hokkaido in 1992; VI: The prevalence of foxes showed an increase trend.

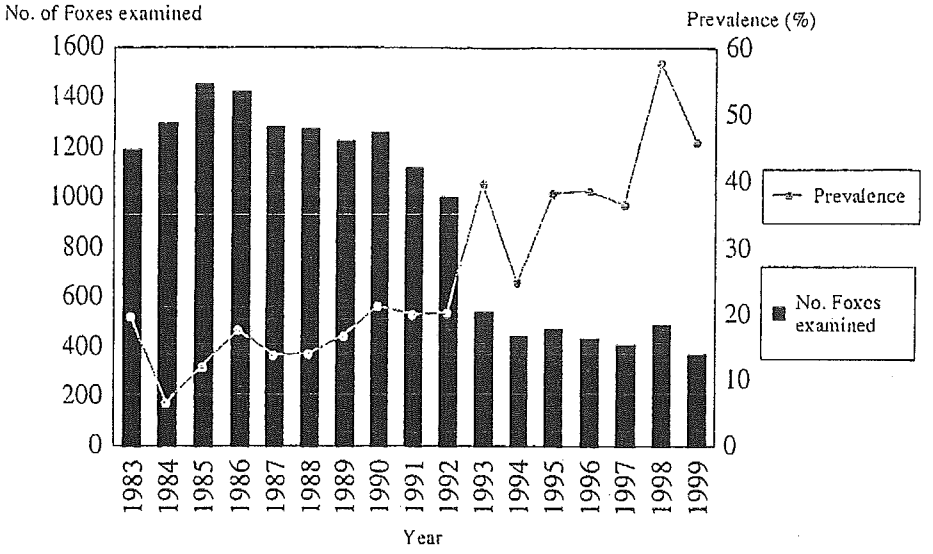

Fig. 4. Change in the prevalence in winter captured foxes in Hokkaido during 1983-1999 (data from Hokkaido Government).

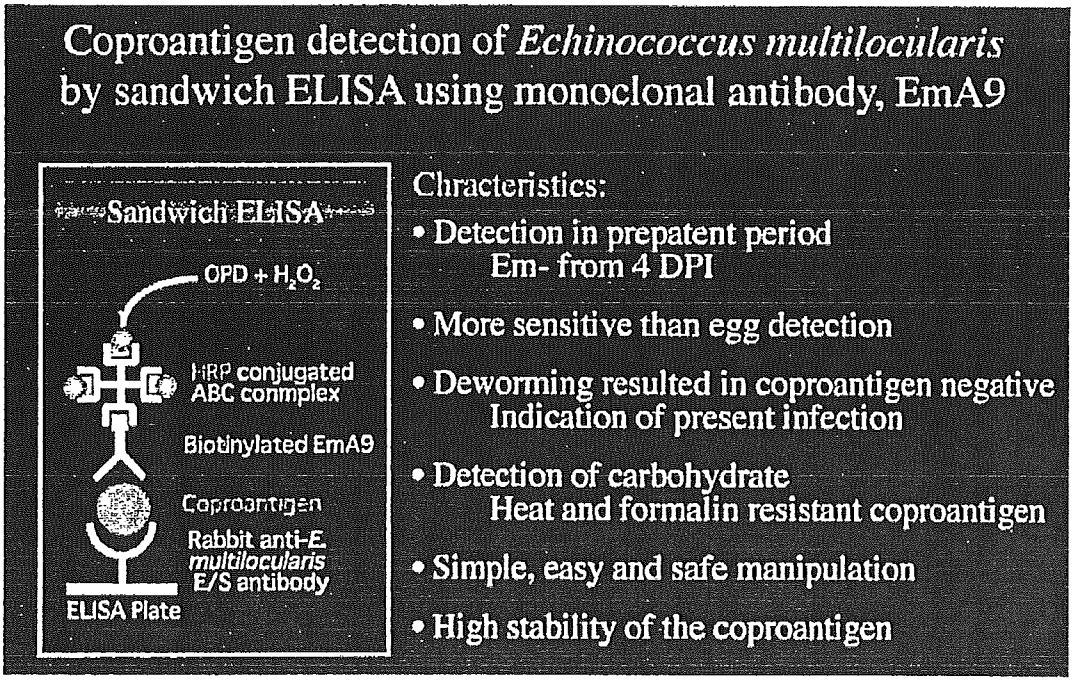

Fig. 5. Coproantigen detection of *Echinococcus multilocularis* by a sandwich ELISA using monoclonal antibody, EmA9, directed against *E. multilocularis* somatic antigen.

Our group at Hokkaido University developed a sandwich ELISA based diagnostic method for the definitive hosts of *Echinococcus* species [4,5]. The method detects the parasite excretory/secretory antigen in the fox (or dog) feces (coproantigen) (Fig. 5), whereby it is not necessary to autopsy the animal for diagnosis. Using this method, a survey was conducted in a pilot area (200 km^2) of Koshimizu facing the Sea of Okhotsk in Hokkaido during 1997 to 1998 [6]. Fox feces were collected around 36 fox breeding dens found at the beginning of

this survey and the seasonal variation of *E. multilocularis* prevalence in foxes was evaluated by the coproantigen detection method and fecal egg examination. This survey provided a baseline fox prevalence data for the next deworming trial (see below), showing that the prevalence of coproantigen positive feces was relatively high with no distinct seasonal fluctuations (51.6-66.7%). However, the prevalence of egg positive feces were varied, in which higher prevalence were found in summer and winter (31.1 and 38.7%) than spring and autumn (13.3 and 13.5%). The observed difference between coproantigen based and parasite egg based prevaleneces was estimated due to the difference of the sensitivities between the two methods and due to the difference of the seasonal intensities of the parasite in the fox population. Since higher intensities were found both in coproantigen (ELISA OD values) and egg detection (egg count) in juvenile than adult foxes, it was indicated that juveniles played a more important role in the environmental contamination with the parasite eggs. In Sapporo, another survey based on coproantigen detection was conducted on the foxes having their den sites in the parks or woodlands of urban area [7]. Infected foxes were found in Sapporo urban area and the suitable intermediate hosts, arvicolid rodents, were captured at the urban fringe although all the rodents captured were not infected with *E. multilocularis*. This survey suggested that the urban fringe offers a potential condition for the maintenance of *E. multilocularis* life-cycle. In France, the levels of endemicity in the different study sites were evaluated by the fox feces using coproantigen detection methods, concluding that coproantigen detection in field feces could serve for large-scale surveillance as an alternative to necropsy [8]. Those surveys suggested that the coproantigen detection in the field fox feces could provide a reliable information of diagnosis in the assessment of the change in the fox prevalence and in the assessment of the regional epidemicity of foxes.

The first deworming trial against *E. multilocularis* in wild foxes was conducted in Germany at the study area of 566 km^2 [9]. Batis containing 50 mg of the anthelmintic, praziquantel, were evenly and repeatedely distributed in the study area either by hand or aircraft. After 6 baiting campaings over a period of 14 months, the prevalence of *E. multilocularis* in foxes, initially 32%, had fallen to 4%. The effect was most pronounced in the central part of the baiting area, where no positive foxes were found in the last 2 months. However, in the marginal part of the baiting area, the reduction of the prevalence was moderate and the prevalence fluctuated between 5 to 20% after the baiting campaigns started. The observed difference in the baiting effects at central and marginal parts were attributed to the evaluation method of fox prevalence. In this campain, 2.2 foxes/km^2 were captured and necropsied to evaluate the change of fox prevalence. Because hunting pressure for foxes in the baiting area was high, making free space (niche) in the baiting area, foxes residing outside the baiting area tended to enter the baiting area. The observed higher prevalence in the marginal part was, therefore, due to the effect of those migrating foxes. This deworming trial showed that a bait distribution is effective for reducing the

fox prevalence, however, it also suggested that the scale of operation must be large enough for having the core area, in order to evaluate a true effect of bait distribution if the change of fox prevalence is evaluated by necropsy.

In 1998, our deworming program to manage echinococcosis in foxes was initiated at Koshimizu. This Koshimizu trial has several unique features. First, since feces were used for the evaluation of fox prevalence with coproantigen detection as an alternative to necropsy, a survey can be performed with a minimal ecological disturbance. Second, because of fox family based study, precise evaluation of bait consumption by foxes and even collection of feces in the study area can be achieved. The study area (200 km^2) was divided into two parts (Fig. 6), one (resided by 18 fox families) with bait distribution and the other (20 fox families) without bait distribution. Baits used in this study were fish sausage base : The manufactured fish sausages (90 g, 2 cm diameter × 12 cm long) were cut into 1.5 cm long and a half Droncit® tablet (50 mg praziquantel per tablet ; Bayer Co.) was embedded in each piece of fish sausage. To distribute baits, 5 bait holes (15 cm diameter × 30 cm depth) were made within 100 m apart from each fox breeding den in the bait-distributed area and the baits were put inside the hole (2 baits/hole). To check the bait uptake by foxes, a smooth slope with about 30 cm long and 20 cm wide was prepared in front of each bait hole. Consumption of baits by foxes was checked by fox footmarks left on the slope. Bait distribution was done every month in the first year and spring and autumn in the second year and only spring in the third year, with 4 consecutive days per month. To evaluate the change in the fox prevalence druing the campaign using the coproantigen detection and fecal egg examination methods [2], even number (3-5) of fox feces were collected every one or two

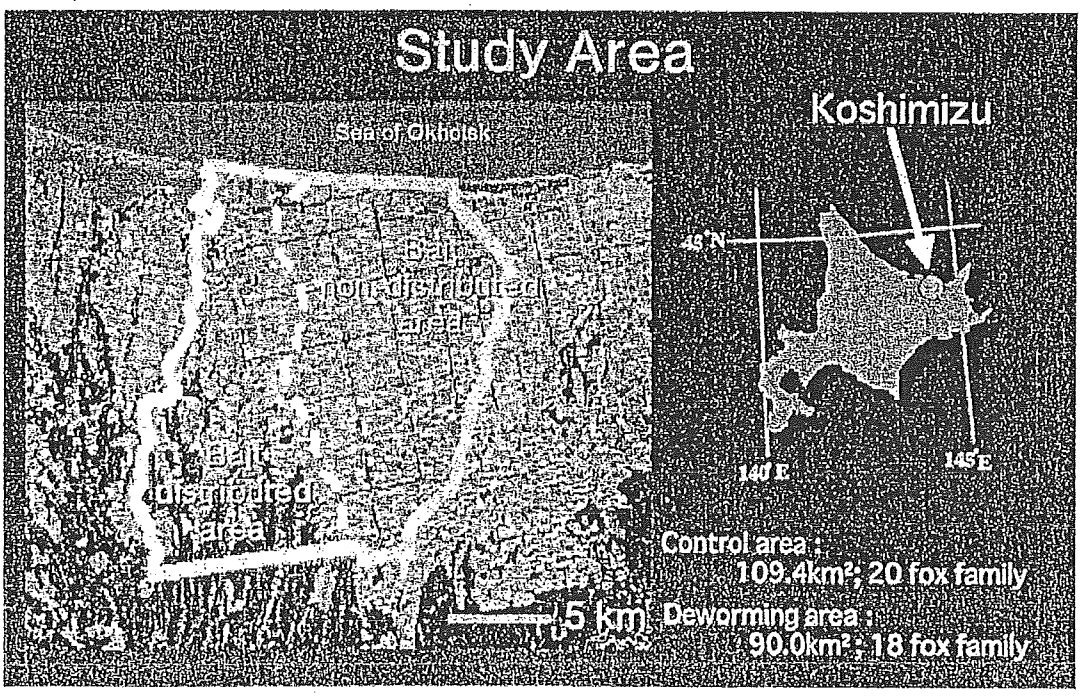

Fig. 6. Study area (Koshimizu, Hokkaido) of a deworming trial against foxes.