① Immune protection elicited by irradiation attenuated cercariae

Non-specific immune response is usually elicited by the infection with normal cercaria of *S. mansoni* and the pathological damage caused by the schistosome eggs produced by adult worm. However, infection by radioactive ray irradiation attenuated cercaria was found to produce immune protection that is species specific and much work had been done in this field of research (Dean, 1983; Taylor and Bickle, 1986). Moreover, it was observed that different doses of irradiation of the vaccinating population of cercariae induced different degree of immune protection in the animal host. In mice and other experimental animals, the greatest host resistance to subsequent infection was induced by vaccination with cercariae that had been irradiated with high gamma ray irradiation dose (20-50 Krad). These irradiated cercariae were able to reach the lungs but their migration stop there, and thus the radiation dose was considered to be optimum for eliciting resistance to further infection (Mangold and Dean 1984; Bickle, 1984). Since it is generally difficult to analyze the degree of immune protection in immunized animals, the normal method is to compare the recovery rate of the worms obtained by portal perfusion of immunized animal group that had been subjected to challenge infection, with that of the untreated control animal group that were administered the same infecting dose as that of the challenge infection. However, by this method, it will not be possible to detect the immune protection that were induced when the worms were migrating from the skin to the lung, liver and the mensenteric vein. To overcome this problem, the site of infection at the skin as well as the lungs were resected and then macerated, followed by incubation in a culture medium for a certain period of time. The schistosome worms that were released into the culture medium were then enumerated for comparison among the various treated groups (Smithers and Gammage, 1980). Using this method, it became possible to track the migration of schistosomula of the challenge infection in the immunized mice. The site of attrition of schistosomula by the host immune response had been variously reported to be in the skin, the lung or in the other visceral organs after the worm had left the lung. Using both light and electrom microscopy to monitor the worm migration, there was a group of researchers that advocated the site of attrition to be the skin (Hsu *et al.*, 1983). Meanwhile, another group of workers argued that the site of attrition against the worm was either in the lung or other visceral organs that the worm migrated to after the lung (Crabtree and Wilson, 1986). Later, a method for studying the site of attrition of the worm was developed by transplanting the various developmental stages of the schistosome to suspected attrition site in the immunized host and examining the viability of the worm there. It was thus shown that in the immunized mice, the sites of attrition against the schistosome were in the skin or the lung. In rats, it was in the lung and in the guinea pig, it was in the lung and the liver. Thus, the site of attrition against the schistosome was found to differ among the animal species (McLaren *et al.*, 1985).

On the other hand, by radiolabeling the cercariae in the snail with $^{75}$Selenium, it became possible to track the migration of schistosome in its host using compressed organ autoradiography (Dobinson *et al.*, 1980). This method was used in the analysis of the immune protection of the host. In guinea pig immunized with 20 Krad irradiated attenuated cercariae, most of the schistosomula of the challenge infection reached the lung, indicating that almost none of the worms were killed in the skin. The number of worms was significantly reduced when they migrated from the lung to the liver in the immunized guinea pigs as compared to the number of worms recovered from the untreated control. At day 14-24 post-challenge infection, less worms were recovered

from the immunized animals (Kamiya, 1986). Among the immunized mice model, there had been an argument that the site of attrition of the worm in C578L/6 strain of mouse was in the lung or the post-lung destination of the migrating worm, and in CBA/Ca strain, it was in the skin. Using the compressed organ autoradiography method, the aforementioned site of attrition in the respective mouse strains were confirmed to be true (McLaren *et al.*, 1986; Kamiya *et al.*, 1987).

Next, the effect of radioactive ray irradiation on the schistosome was examined. The radiation dose used in the attenuation of schistosome cercariae was found to greatly influenced the maturity and the migration ability of the cercariae. When *S. mansoni* cercariae were irradiated with 2-3 Krad of X-ray, followed by infection into animal host, the schistosomula could migrate to the liver and survived there for about 6 weeks but most of them showed belated growth and were sexually sterile. Generally, the degree of immune protection elicited by the 2-5 Krad irradiated attenuated cercariae was very weak. The strongest degree of immune protection in the host was reportedly induced by immunization with 20 Krad or 50-60 Krad irradiated cercariae. Using the compressed organ autoradiography method as mentioned above, it was observed that the 20 Krad irradiated cercariae were able to migrate to the lung, whereas the 50 Krad irradiated cercariae remained only in the skin (Kamiya and McLaren, 1987). However, there was also a report that the 50 Krad irradiated cercariae were able to reach the lung while most of the 90 Krad irradiated worms remained in the skin (Mangold and Dean, 1984). This discrepancy among the reports might be due to the different strains of *S. mansoni* used and also the differences in the antigenicity of the worm used. Despite the difference in the observation, it has become clear that the attenuated cercariae must be able to reach the lung and stayed there for a certain period of time for the strongest degree of immune protection to be induced.

Moreover, the effect of irradiation on the expression of antigen by the schistosome had also been investigated. In mechanically transformed schistosomula, the higher the radiation dose that they had been exposed to, the less the number of surface antigens were expressed that could be detected by specific antibodies. This is probably due to the inhibition of protein synthesis and hindrance of the surface structure turnover as the result of irradiation. However, among the irradiated schistosome that managed to reach the lung, conspicuous expression of the 97KD candidate protective antigen (James and Sher, 1986) could be observed (Kamiya, 1986). Thus, it is possible that irradiation might have destroyed the ability of the schistosome to incorporate the host antigen onto itself. On the contrary, when there is a continuous release of the protective antigen in the lung for a certain period of time by the attenuated worm, then we can expect the induction of a strong degree of immune protection in the host.

Thus, the question arises as to what factor was responsible for the mechanism of the immune protection. It is interesting to note that in a pair of rats joined by parabiosis, if one of the rats were immunized with irradiation attenuated cercariae and expressed resistance to subsequent infection, the other non-immunized parabiotic rat will also express the same type of resistance as that of the immunized one. However, this immune protection was not expressed in B-cell deficient mice that had been treated with anti-$\mu$ chain antibodies to deplete the B cells. Thus, humoral immunity is thought to be involved in immune protection against *S. mansoni* infection (Suzuki and Damian, 1981; Damian *et al.*, 1981; Harn *et al.*, 1985; Bickle *et al.*, 1986).

Furthermore, in immune protection elicited by the irradiation attenuated cercariae, the lymphokine (cytokine) activated macrophage was found to serve as the effector cell in the killing of schistosomula (James *et al.*, 1984; McLaren and James, 1985; Kubelka *et al.*, 1986; James and Sher, 1990). However, the egg specific TH1 clone was

reported to play an important role in this macrophage-mediated killing mechanism (Kanazawa *et al.*, 1992). On the other hand, there was also a report on the existence of an immune evasion mechanism that the schistosome might manifest (Watanabe *et al.*, 1990), thus presenting the complexity of the problem.

② Immune protection elicited by ultraviolet ray attenuated cercariae

Using ultraviolet (UV) ray to attenuate the schistosome has not been a normal practice as compared to the use of radioactive ray irradiation. However, the UV light had been reportedly applied for attenuating the cercariae of *S. japonicum* and *S. mansoni* (Ruppel *et al.*, 1990; Kamiya *et al.*, 1993a; Kumagai *et al.*, 1992). Irradiation of *S. mansoni* cercariae with UVC (wavelength 254nm) at a dose of 15mJ/cm2 can prevent them from migrating from the host skin to the lung (Kumagai *et al.*, 1992). Cercariae that had been attenuated with the aforementioned UV dose, were found to elicit significant immune protection in mice and guinea pigs but not in Mongolian gerbils (Kamiya *et al.*, 1993b). Similar results for Mongolian gerbils were also seen when the animals were immunized with radioactive rays irradiated cercariae in which no immune protection was observed (Kamiya *et al.*, 1993a). Moreover, in the immunized guinea pig model, it was observed that the antigen presenting dendritic cells such as the epidermal Langerhans cell, played an important role in the induction of effective immune protection in the host (Sato and Kamiya, 1995).

**(4) Research on the diagnosis of schistosomiasis mansoni**

Many immunodiagnostic methods had been developed of schistosomiasis. During the seminar on "Application of modern technology for immunodiagnosis in schistosomiasis", held in Shanghai, China, in 1990, sponsored by WHO/TDR/ICGEB, there was a consensus that research on immunodiagnosis is an important area that needs to be vigorously pursued. From a historical perspective, comparatively much more research on immunodiagnosis of schistosomiasis japonica had been carried out than schistosomiasis mansoni in Japan.

Yoshimura (1968) and Aoki (1980) compared the protein profile and hemoglobinase of both *S. japonicum* and *S. mansoni*, and found that there are *S. mansoni* specific proteins. Since schistosomiasis is endemic mainly in developing countries, there arises a need to develop a simple and cheap diagnostic test that is robust and feasible for use under field condition. Thus, the modified circumoval precipitin test (COPT) became the answer for the aforementioned problem. Since it became clear that the schistosome egg glycoprotein was heat-resistant (Kamiya, 1981), it was possible to use formalin-fixed, alcohol-fixed, acetone-fixed and even naturally dried schistosome eggs as antigen in COPT (Kamiya, 1983, 1992; Kamiya *et al.*, 1991). Based on the reaction principle in COPT, a method to diagnose the presence of immune complex in the schistosome egg using histological section of formalin-fixed egg as antigen had been developed and became known as intraoval precipitin test (IOPT) (Kamiya, 1992). Moreover, for field application, skin test using *S. mansoni* VBS (veronal buffer saline) antigen or protease antigen (Senft Antigen) (Yokogawa *et al.*, 1984) and ELISA using schistosome egg antigen, had been employed with satisfactory results.

Presently, there are many factors that limit most of the immunodiagnostic tests for use in the field. For a feasible field test, the test must ideally includes the following conditions: simple and rapid, able to detect the target molecules in easily collected samples such as urine, use of test reagent that is stable and easily stored under tropical condition, a standardized procedure test that can clearly shows a positive or

a negative result, a test kit that is cheap and one that can evaluate the efficacy of therapy. To meet these field requirements, there is a need to use the recent modern technology to develop an ideal immunodiagnostic test. Foe example, to develop a test for detecting circulating antigen, there is a need to first produce an antigen such as a recombinant protein antigen that can elicit strong humoral response for the production of monoclonal antibody. It is hoped that the recent advances in biotechnology can contribute greatly towards the development of the test.

## (5) Conclusion

The above is an overview of the research on schistosomiasis mansoni and due to the great diversity of the research being carried out as well as the limited space allotted for this article, it has not been possible to clearly identify in detail the significance of each of the research results. Biological research on *S. mansoni*, especially on the interaction between the snail intermediate host and the schistosome, has been considered as very important from the epidemiological perspective of this disease. However, this topic was not touched upon in this article. With a phalanx of molecular biological tools at our disposal, it will be interesting to see which direction future research on schistosomiasis mansoni will be headed for.

### References


1) Abe, K., Kagei, N., Teramura, Y. and Ejima, H. (1993): Hepatocellular carcinoma associated with chronic *Schistosoma mansoni* infection in a chimpanzee. J. Med. Primatol., 22, 237-239.
2) Amano, T., Freeman, G. and Colly, D. G. (1990): Reduced reproductive efficacy in mice with schistosomiasis mansoni and in uninfected pregnant mice injected with antibodies against *Schistosoma mansoni* soluble egg antigens. Am. J. Trop. Med. Hyg., 43, 180-185.
3) Amano, T., Nakazawa, M., Oshima, T., Bosshardt, S. C. and Colly, D. G. (1996): Crossreactive idiotypes on rabbit anti-SEA antibodies stimulate anti-idiotype spleen and lymphnode cell responses of mice infected with *Schistosoma masnoni*. Parasite Immunol., 18, 21-28.
4) Takashi Aoki (1980): Antigenic property of hemoglobin-specific protease from *Schistosoma mansoni, S. japonicum* and *Fasciola* sp. from radioallegrosorbent test. Jpn. J. Parasitol., 29, 325-332. (in Japanese with English summary)
5) Bickle, Q. D. (1984): Studies on the relationship between the survival of *Schistosoma mansoni* larvae in mice and the degree of resistance produced. Parasitology, 84, 111-122.
6) Bickle, Q. D., Andrews, B. J. and Taylor, M. G. (1986): *Schistosoma mansoni*: Characterization of two protective monoclonal antibodies. Parasite Immunol., 8, 95-107.
7) Crabtree, J. and Wilson, R. A. (1986): The role of pulmonary cellular reactions in the resistance of vaccinated mice to *Schistosoma mansoni*. Parasite Immunol., 8, 265-285.
8) Damian, R. T., Greene, N. D., Suzyki, T. and Dean, D. (1981): Schistosomiasis mansoni in baboons. V. Antibodies and immediate hypersensitivity in multiply infected *Papio cynocephalus*. Am. J. Trop. Med. Hyg., 30, 836-843.
9) Damian, R. T. (1984): Immunity in schistosomiasis. A holistic view. In Marchalonis, J. J. (ed): Immunology of parasite and parasitic infections. Contemporary Topics in Immunology, 12, 359-420, Pleunum Press, New York.
10) Dean, D. A. (1983): A view. *Schistosoma* and related genera: Acquired resistance in mice. Exp. Parasitol., 55, 1-104.
11) Dobinson, A. R., James, R. R. and Christensen, N. O. (1980): Evaluation of L-$^{75}$Se-methionine as a radioisotopic marker for studying the migration of *Schistosoma mansoni* schistosomula in mice. Int. J. Nucl. Med. Biol., 7, 195-196.
12) Fujiwara, M., Makino, M. and Watanabe, H. (1988): *Schistosoma mansoni*: induction of severe glomerulonephritis in female BxSB mice following chronic infection. Exp. Parasitol., 65, 214-221.
13) Harn, D. A. (1984): *Schistosoma mansoni*: Parasite transfer of immunity by anti-egg monoclonal antibody. In August, J. T. (ed): Molecular Parasitology, 155-165, Academic Press, Orlando.
14) Hsu, S. Y. L., Hsu, H. F., Johnson, S. C., Xu, S. T. and Johnson, S. M. (1983): Histopathological

study of the attrition of challenge cercariae of *Schistosoma mansoni* in the skin of mice immunized by chronic infection and by use of highly irradiated cercariae. Z. Parasitenkd., 69, 627-642.
15) Iwamura, Y., Irie, Y. Kominami, R., Nara, T. and Yasuraoka, K. (1991): Existence of host-related DNA sequences in the schistosome genome. Parasitology, 102, 397-403.
16) James, S. L. and Sher, A. (1986): Prospects for a nonliving vaccine against schistosomiasis. Parasitol. Today, 2, 134-137.
17) James, S. L. and Sher, A. (1990): Cell-mediated immune response to schistosomiasis. Cur. Top. Microbiol. Immunol., 155, 21-25.
18) James, S. L., Natovitz, P. C., Farrar, W. L. and Leonard, E. J. (1984): Macrophages as effector cells of protective immunity in murine schistosomiasis: Macrophage activation in mice vaccinated with radiation-attenuated cercariae. Infect. Immun., 44, 569-575.
19) Kamiya, H. (1981): Immunodiagnosis of schistosomiasis by the application of whole parasites or parasite tissue sections. Akita Med. J., 7, 331-343. (in Japanese with English summary)
20) Kamiya, H. (1983): Circumoval precipitation (COP) test using air-dried eggs of *Schistosoma japonicum* and *S. mansoni*. Southeast Asian J. Trop. Med. Publ. Hlth., 14, 451-455.
21) Kamiya, H. (1986): Reinfection immunity in schistosomiasis, with special reference to immunity induced by radiation attenuated cercariae. Akita Med. H., 13, 481-500. (in Japanese with English summary)
22) Kamiya, H. (1992): Alternative preparation of *Schistosoma* egg antigens for circumoval and intraoval precipitin tests for use in endemic areas. In Immuno-diagnostic approaches in schistosomiasis, 71-81, Bergquist, N. R. (ed), John Wiley and Sons, New York.
23) Kamiya, H. and McLaren, D. J. (1987): *Schistosoma masnoni*: Migration potential of normal and radiation attenuated parasites in naive guinea pigs. Exp. Parasitol., 63, 98-107.
24) Kamiya, H., Smithers, S. R., and McLaren, D. J. (1987): Schistosoma mansoni: Autoradiographic tracking studies of isotopically-labelled challenge parasites in naive and vaccinated CBA/Ca mice. Parasite Immunol., 9, 515-529.
25) Kamiya, H., Liu, P. H., Ishigooka, S. and Blas, B. L. (1991): Application of alcohol or aceton-fixed *Schistosoma* eggs as an alternative to lyophilized eggs for circumoval precipitin test. Southeast Asian J. Trop. Med. Publ. Hlth., 22, 417-421.
26) Kamiya, H., Nakayama, H. and Yoshimura, K. (1993a): Defect of resistance in male gerbils, *Meriones unguiculatus*, vaccinated with gamma-irradiated cercariae of *Schistosoma mansoni* against a homologous challenge infection. J. Parasitol., 79, 616-618.
27) Kamiya, H., Ozaki, T., Nakayama, H. and Inaba, T. (1993b): Immunization potential of ultraviolet-attenuated cercariae of *Schistosoma mansoni* in rodent hosts. Parasitol Res., 79, 293-296.
28) Kamiya, H., Ozaki, T, Nakayama, H., Kumagai, M. and Inaba, T. (1994): Brain involvement in murine schistosomiasis mansoni. Jpn. J. Parasitol., 43, 294-300.
29) Kanazawa, T., Harris, T. S., Chikunguwo, S. M. and Stadecker, M. J. (1992): Larvicidal properties of macrophages induced by cloned murine schistosomal egg antigen-specific CD4 positive T-helper lymphocytes. Parasite Immunol., 14, 279-293.
30) Kubelka, C. F., Ruppel, A., Krammer, P. and Gemsa, D. (1986): Killing of schistosomula of *Schistosoma mansoni* by macrophages: induced by T-cell clone-derived by lymphokines and interferon-gamma. Parasitology, 92, 325-336.
31) Kumagai, M., Sato, H. and Kamiya, H. (1992): Migration kinetics of ultraviolet-attenuated *Schistosoma mansoni* in ICR mice. Jpn. J. Parasitol., 41, 300-305.
32) Mangold, B. L. and Dean, D. A. (1984): The migration and survival of gamma-irradiated *Schistosoma mansoni* larvae and duration of host-parasite contact in relation to the induction of resistance in mice. Parasitology, 88, 249-266.
33) McLaren, D. J. (1980): *Schistosoma mansoni*: The parasite surface in relation to host immunity, 1-229, John Wiley and Sons, Chichester, England.
34) McLaren, D. J. (1984): Disguise as an evasive stratagem of parasitic organisms. Parasitology, 88, 597-611.
35) McLaren, D. J. and James, S. L. (1985): Ultrastructural studies of the killing of schistosomula of *Schistosoma mansoni* by activated macrophages *in vitro*. Parasite Immunol., 7, 315-331.
36) McLaren, D. J., Kamiya, H. and Smithers, S. R. (1986): Where and how are schistosomes killed in the vaccinated mouse model? Parasitology-Quo Vadit? Program and Abstracts, Sixth International Congress of Parasitology, Howell, M. J. (ed.), Academy of Science, Canberra, 186.
37) McLaren, D. J., Pearce, E. J. and Smithers, S. R. (1985): Site potential for challenge attrition in

mice, rats and guinea pigs vaccinated with irradiated cercariae of *Schistosoma mansoni*. Parasite Immunol., 7, 29-44.
38) Nishimura, M., Asahi, M., Hayashi, M. and Takazono, I. (1985): Extracellular matrix in hepatic granulomas of mice infected with *Schistosoma mansoni*. Arch. Pathol. Lab. Med., 109, 813-818.
39) Nishimura, M., Hara, A., Nojima, H., Noda, S., Mashimo, M. and Horii, Y. (1991): Possible role of the hormonal form of vitamine D3 in the granuloma-associated angiotensin-coverting enzyme activity. Sarcoidosis, 8, 101-104.
40) Ruppel, A., Shi, Y. E. and Moloney, N. A. (1990): *Schistosoma mansoni* and *S. japonicum*: Comparison of levels of ultraviolet irradiation for vaccination of mice with cecariae. Parasitology, 101, 23-26.
41) Sakumoto, D., Yamaguti, N., Itano, K. and Inatomi, S. (1960): Studies on the structure of egg shell of the helminth with electron microscopy. Jpn. J. Parasitol., 9, 367. (in Japanese)
42) Sato, H. and Kamiya, H. (1995): Role of epidermal Langerhans' cells in the induction of protective immunity to *Schistosoma mansoni* in guinea-pigs. Immunology, 84, 233-240.
43) Smithers, S. R. and Doenhoff, M. J. (1982): Schistosomiasis. In Cohen, S. and K. S. Warren (eds.): Immunology of parasitic infections, 527-607, Blackwell Scientific Publications Ltd., London.
44) Smithers, S. R. and Gammage, K. (1980): Recovery of *Schistosoma mansoni* from the skin, lungs and hepatic portal system of naive mice and mice previously exposed to *S. mansoni*: Evidence for two phase of parasite attrition in immune mice. Parasitology, 80, 289-300.
45) Suzuki, T. and Damian, R. T. (1981): Schistosomiasis mansoni in baboons. IV. The development of antibodies to *Schistosoma mansoni* adult worm, egg and cercarial antigens during acute and chronic infections. Am. J. Trop. Med. Hyg., 30, 825-835.
46) Tanabe, M., Kaneko, N. and Takeuchi, T. (1989a): *Schistosoma masnoni*: Suppression of carbamoyl phosphate synthetase (ammonia) and ornithine carbamoyl transferase activities in the liver of infected mice. Exp. Parasitol., 68, 432-442.
47) Tanabe, M., Kaneko, N. and Takeuchi, T. (1991): *Schistosoma mansoni*: higher proline levels in the livers of infected mice. Exp. Parasitol., 72, 134-144.
48) Tanabe, M., Miura, S., Kobayashi, S. and Asami, K. (1983): Changes in activities of lysosomal enzymes in experimental schistosomiasis mansoni. Jpn. J. Parasitol., 32, 271-278.
49) Tanabe, M., Okazaki, M., Kobayashi, S., Kaneko, N., Sekiguchi, T., Tateno, S., Motta, S. R. and Takeuchi, T. (1990): Serological studies on schistosomiasis mansoni in the Northeast Brazil (I). Rev. Inst. Med. Trop. Sao Paulo, 32, 121-131.
50) Tanabe, M., Sekiguchi, T., Kaneko, N., Kobayashi, S. and Takeuchi, T. (1989b): Elevation of laminin and beta-subunit of prolyl 4 hydroxylase in the sera of human subjects with schistosomiasis mansni. Jpn. J. Exp. Med., 59, 109-119.
51) Tanabe, M., Iwamura, Y., Amanuma, H., Irie, Y., Watanabe, M., Watanabe, T., Uchiyama, Y. and Yasuraoka, K. (1989): Integration and expression of murine retrovirus-related sequences in schistosomes. Parasitology, 99, 31-39.
52) Taylor, M. G. and Bickle, Q. D. (1986): Irradiated schistosome vaccines. Parasitol. Today, 2, 132-134.
53) Watanabe, T., Yamashita, T. and Sendo, F. (1990): Suppression of macrophage-mediated antibody-dependent killing of Schistosoma mansoni larvae by concanavalin A stimulated spleen cell-derived factor(s). Parasite Immunol., 12, 309-319.
54) Yamashita, T. and Boros, D. L. (1990): Changing patterns of lymphocyte proliferation, IL-2 production and utilization, and IL-receptor expression in mice infected with *Schistosoma mansoni*. J. Immunol., 145, 724-731.
55) Yamashita, T. and Boros, D. L. (1992): IL-4 influences IL-2 production and granulomatous inflamation in murine schistosomiasis mansoni. J. Immunol., 149, 3659-3664.
56) Yokogawa, M., Kojima, S., Kobayashi, M., Oya, H., Aoki, T., Asami, K and Miura, S. (1984): Schistosomiasis manoni in Brazil: Intradermal tests with antigens of proteolytic enzyme and veronal buffered saline extract of *Schistosoma mansoni*. Jpn. J. Parasitol., 33, 489-494.
57) Yoshimura, K. (1980): How can helminths escape from host immune attack? Jpn. J. vet. Ass., 33, 489-494. (in Japanese)
58) Yoshimura, K. (1968): Disc electrophoretic comparison between *Schistosoma japonicum* and *S. mansoni* adult worms. Jpn J. Parasitol., 17, 382-394.

*Progress of Medical Parasitology in Japan*, Vol. 8, edited by Otsuru, M., Kamegai, S. and Hayashi, S., Chapter III. Meguro Parasitological Museum, Tokyo, 2003

# 5. Biology of *Echinococcus*

Yuzaburo Oku and Masao Kamiya

Laboratory of Parasitology, Graduate School of Veterinary Medicine, Hokkaido University, Kita 18 Nishi 9, Kita-ku, Sapporo, 060-0818 Japan

## Introduction

The genus *Echinococcus* comprises of 4 species but most of the research done in Japan was on *Echinococcus multilocularis*. Besides *E. multilocularis*, there were also many reports on *E. granulosus* by Japanese workers, but due to space constrain, this article will focus only on *E. multilocularis*.

Since the last publication of the book series, "Progress of Medical Parasitology in Japan" with a chapter on *Echinococcus*, much progress in the research on this subject had taken place. This includes the reports on human cases, epidemiology of the disease, immunodiagnosis and therapeutic research by researchers at Hokkaido University, Medical School and Asahikawa Medical College. Studies on immunology of the parasite using experimental animals, development of diagnostic methods for the definitive host, establishment of the alternative definitive host and mass treatment

at Hokkaido University, Faculty of Veterinary Medicine. Moreover, workers at the Hokkaido Institute of Public Health had also reported on the epizootiology of the parasite and diagnosis of the disease in humans. In addition, there were also several groups of investigators who had carried out various experiments and survey that were related to the parasite. Besides the universities and the institutes, the Hokkaido local government annually carried out a massive survey on the prevalence of echinococcosis in animals and also the mass screening for antibody against the parasite among the inhabitants. Since these surveys and screening in Hokkaido were conducted on a long-term basis, it was possible to monitor the dynamics of the infection on that island.

The Laboratory of Parasitology at the Hokkaido University Graduate School of Veterinary Medicine has also been designated by OIE (Office International des Epizooties) as an echinococcosis reference laboratory.

A review paper on echinococcois in Japan had been published by Ohbayashi (1975)

Kamiya (1997), Sakamoto (1997) and also by Hokkaido Institute of Public Health (1999).

### (1) Origin of *E. multilocularis* in Japan

The strain of *E. multilocularis* that is presently distributed in Hokkaido, Japan, was proposed to have originated from St. Lawrence Island, U.S.A., in the Bering straits, and had been introduced into Hokkaido or the surrounding islands by human activities (Yamashita, 1973). Okamoto *et al.* (1995) compared 391 base pairs of the mitochondria cytochrome C oxidase subunit I (CO I) gene of the Hokkaido isolates (that includes the parasite from 3 different host species captured at 5 different locations in

Hokkaido) and the St. Lawrence isolates of *E. multilocularis*, and found complete homology among all the Hokkaido isolates irrespective of the host animals or the location as well as with the St. Lawrence isolates. Their findings thus supported the hypothesis that the *E. multilocularis* in Hokkaido had its origin in St. Lawrence Island.

**(2) Prevalence of *E. multilocularis* in Hokkaido (Results of parasitological survey)**

① Spread of the endemic area

Hasegawa (1974) of Hokkaido Institute of Public Health proposed that the prevalence of *E. multilocularis* in Hokkaido could be reduced if one third of the red foxes inhabiting Hokkaido could be culled annually. Since 1972, this culling proposal was put into practice and *E. multilocularis* infection was apparently seen to have decreased. Thus, the culling of the foxes during that time was deemed justified but with the passage of time, the endemic area for echinococcosis had actually spread in Hokkaido as described below. It seems that the author did not take account for invasion of infected red foxes into the region.

Until about 2 decades ago, based on the results of the annual survey by the Hokkaido local government, the endemic area for echinococcosis in Hokkaido had been limited to only the eastern part of Hokkaido, particularly 10 cities and districts. However, after 1983, the endemic area had began to spread to other areas in Hokkaido (Kumagai, 1988). In 1983, of the 212 cities and districts in Hokkaido, 32 were designated as endemic area and in 1987, the endemic area had increased to 121 cities and districts. Today, the whole island of Hokkaido is considered as endemic area. This trend of increase in the endemic area of echinococcosis is also seen in Europe and America. Echinococcosis patients were no longer limited to those living in the rural area but had also included those living in the urban area. In 1997, a long-term resident of the city of Sapporo was diagnosed as being infected with multilocular (alveolar) echinococcosis (Hokkaido Commission for the Control of Echinococcosis). The spread of the endemic area was in Hokkaido confirmed not only by the finding of the infection in animals but also in human living in supposedly non-endemic area. However, due to the movement, especially long distances, by the inhabitants such as moving houses and taking long distance trips, the use of human patient as an indicator for determining the endemicity of echinococcosis in a certain area had its limitation. Thus, the result of necropsy of the indicator animals (red foxes and voles) collected at the various cities and districts in Hokkaido become a more reliable parameter for determining the endemicity of echinococcosis for that area. In 1983, Sakui and her colleagues detected *E. multilocularis* lesion in the liver of pigs at a slaughterhouse in north-eastern Hokkaido during routine meat inspection (Sakui *et al.*, 1984). From then on, detection of *E. multilocularis* lesion in pig had been used throughout the whole of Hokkaido to pinpoint the spread of the disease in the supposedly non-endemic area (Ohbayashi, 1986).

② Infection in humans

Until 1983, echinococcosis in Japan was thought to be distributed in only Rebun Island and Eastern Hokkaido. During those years, approximately about 5 new patients had been diagnosed annually by the Hokkaido Commission for the Control of Echinococcosis. However, after that year, there was a steady annual increase in the number of new patients, albeit some fluctuations (Fig. 1). Nevertheless, before 1984,

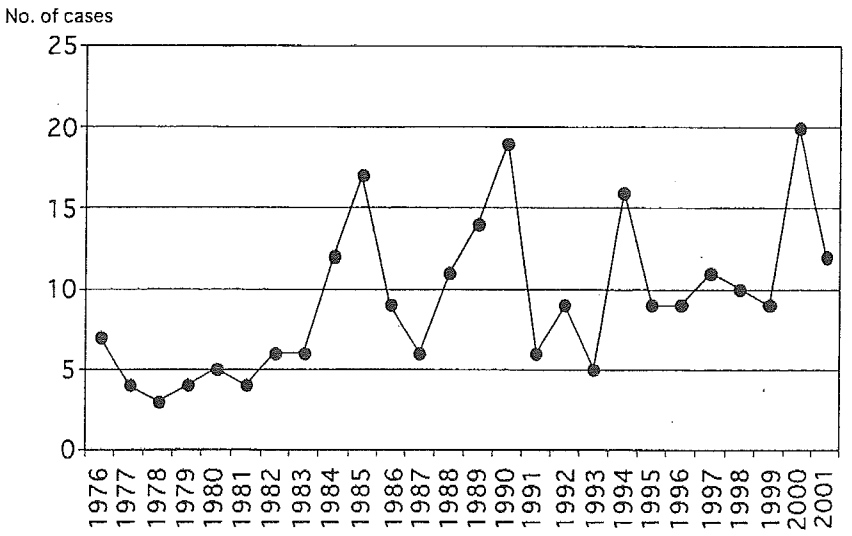


**Fig. 1.** Annual incidence of patients in Hokkaido (Data from Hokkaido local government)

diagnosis of echinococcosis in human was based on the result of serological tests (such as skin test, complement fixation test, indirect hemagglutination test and immunoelectrophoresis test), physical palpation of the abdomen (to check for hepatomegaly) and the plain X-ray image (to observed the calcified lesion) of the abdomen. After 1984, the ELISA test and ultrasound imaging were added as diagnostic tools for echinococcosis in Japan. This might be one of the factors leading to the increase in the number of newly designated echinococcosis patients in Hokkaido after that year (Uchino *et al.*, 1987). The residential area from where the patients came from were originally limited to eastern Hokkaido but in recent years, the ratio of patients who came from other areas in Hokkaido had been increasing (Fig. 2). To date, as of 2003, there had been a total of 424 persons registered as echinococcosis patients and in the 1990's about 120 of them were still living. The aforementioned patients were those who had undergone surgery and had their hydatid lesions removed follow by histological confirmation of the metacestode. However, according to the result of the mass screening of the inhabitants of Hokkaido by using ELISA for *E. multilocularis* antibody, the average seropositive rate was 0.30%. In 1995, of 83,863 persons tested, 169 (0.20%) were found to be positive for *E. multilocularis* antibody (Hokkaido Commission for the Control of Echinococcosis). Every year more than 50,000 persons in Hokkaido were serologically tested for *E. multilocularis* infection but the data on the seroprevalence of the inhabitants and the newly acquired infection were not made public.

Data on the prevalence of *E. multilocularis* in wildlife of Hokkaido is considered very important for predicting the number of potential patients in Hokkaido for the following several years to more than 10 years. The peresent Hokkaido local government have no control program against echinococcosis in wildlife. Doi (1995) calculated that within 15 to 20 years from now, there would be about 1000 echinococcosis patients in Hokkaido.

Besides Hokkaido, more than 64 cases of human echinococcosis had been reported in Honshu. Takahashi *et al.* (1986) of Hirosaki University reported 60 cases of echinococcosis in Honshu but none from Kyushu, Shikoku or Chugoku regions. After their report, a total of 22 cases of echinococcosis, including some new ones were also observed in Aomori (Yamaguchi *et al.*, 1988; Inaba *et al.*, 1992). Among the echinococ-

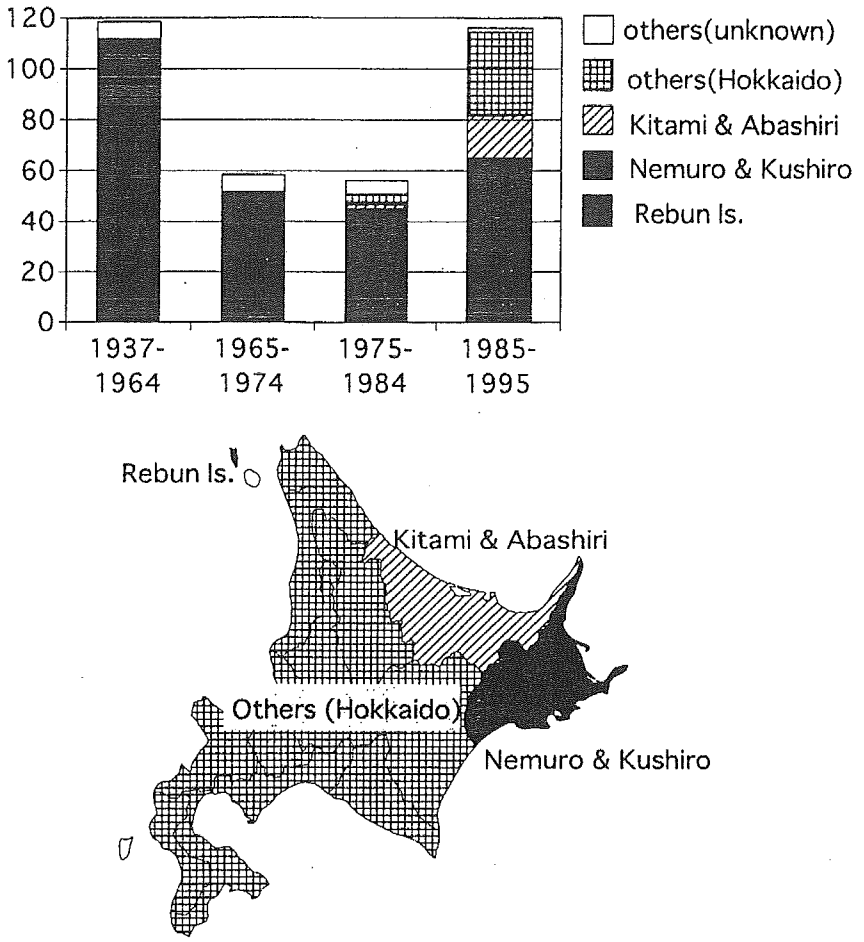


**Fig. 2.** Incidence of patients in various ares of Hokkaido (Data from Hokkaido local government)

cosis patients in Honshu, 16 (8 from Aomori, 2 each from Miyagi and Tokyo, and 1 each from Akita, Nagano, Fukui and Kyoto) had never visited Hokkaido and they were considered to have been infected locally. However, the actual route or source of transmission of echinococcosis for these patients remained unknown (Takahashi *et al.*, 1986).

③ Infection in definitive host

The definitive host of *E. multilocularis* in Hokkaido are known to be the red foxes, *Vulpes vulpes schrencki*, as well as the dogs and cats (Yamashita, 1973). Immature adult worm of *E. multilocularis* had also been collected from naturally infected raccoon dogs, *Nyctereutes procyonoides*, in Hokkaido (Yagi *et al.*, 1988a). Based on the high prevalence of *E. multilocularis* and their large number inhabiting Hokkaido, the red foxes are considered the major definitive host for the cestode in Hokkaido. Although the prevalence of *E. multilocularis* in cats and dogs in Hokkaido were very low, their role in the transmission of the parasite to humans cannot be overlooked due to their proximity and frequent contact with humans (Ohbayashi, 1986).

According to the results of the parasitological survey by necropsy of red foxes conducted by the Hokkaido local government from 1966 to 2001, a total of 23,117 foxes were examined and 4,284 were found to be positive for *E. multilocularis*, thus giving an average positive percentage of 18.5%. Although the anterior part of the small intestine of the foxes were examined from 1966 to 1983, main predilection site of *E.*

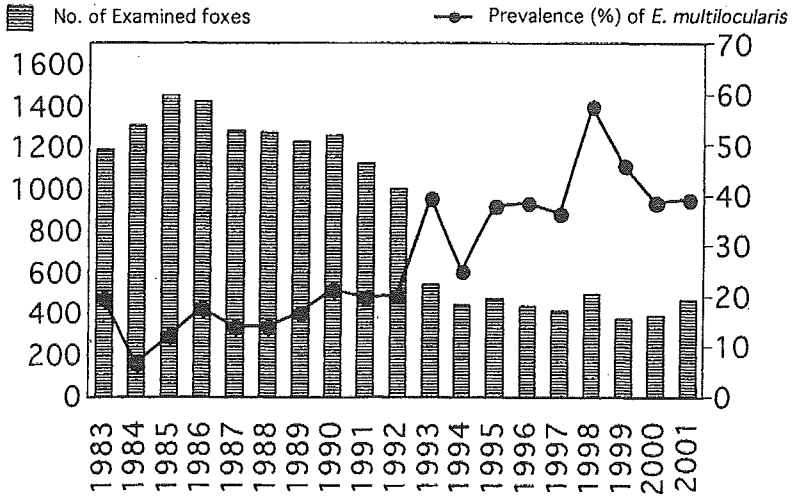


**Fig. 3.** Prevalence (%) of *E. multilocularis* in foxes in Hokkaido (Data from Hokkaido local government)

*multilocularis* is the central and poterior part of the small intestine. Therefore, the poterior part have been examined since 1984. After 1993, the prevalence of the cestode in the red foxes was nearly 40%, indicating that the disease had not only spread to new areas in Hokkaido but also increased in its prevalence among the foxes (Fig. 3).

The epidemiology of echinococcosis is not only influenced by the prevalence of the cestode in the red foxes but also by the number of red foxes present in a certain area. A scientifically sound survey of the dynamics of the fox population in a certain specific area has yet to be conducted. For example, the reported sighting of the red foxes in Sapporo city in 1994 was 56 cases, in 1995 it was 67 cases and in 1996, it was 95 cases. Thus, the increase in the number of red foxes scavenging in the urban area presents a new problem in the epidemiology of echinococcosis that needs to be dealt with in the future (Oku, 1997).

In order to assess the infection risk of alveolar echinococcosis among urban residents of Sapporo, Japan, a survey was conducted on the prevalence of *Echinococcus multilocularis* among urban foxes in the area (Tsukada *et al.*, 2000). The fox distribution, evaluated from footprints left on the snow in parks and woodlands and from the location of fox carcasses, was concentrated along the border of the urban area and in the southwestern part of the city, facing the mountain. Fox feces were collected around active fox dens and analysed by a coproantigen detection assay and fecal egg examination for *Echinococcus* infection. Tirty-three of 155 feces were coproantigen positive.

Since 1967 for the past 36 years, only 99 (1.0%) out of 9,874 dogs examined in Hokkaido were found to be infected with *E. multilocularis*. This prevalence was very much below that of the red foxes. A part of the dogs examined were stray dogs that had been caught and kept captive in the dog pound. Since these stray dogs had no master to feed them, there is a possibility that they might have been catching voles for food. Thus, there is a need to investigate the *E. multilocularis* infection in pet dogs (Oku, 1997). Takahashi *et al.* (1990) of the Hokkaido Institute of Public Health reported a case of *E. multilocularis* infection in a dairy farm dog and in 1997, *E. multilocularis* was detected in a pet dog reared in the suburb of Sapporo (Uchida *et al.*, 1997). The latter finding was also reported in the local newspaper. It is really a pity that in the past 10 years, the number of dogs examined by the Hokkaido local government had been small, that is, less than 10 per year, and thus the actual

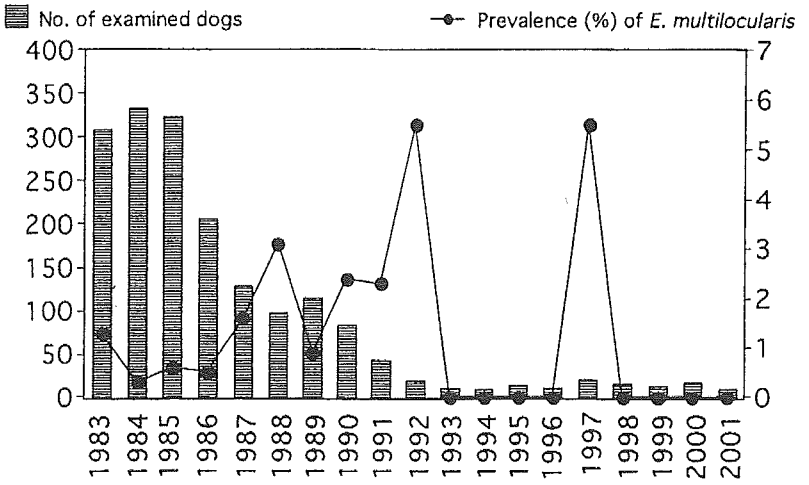


**Fig. 4.** Prevalence (%) of *E. multilocularis* in dogs (Data from Hokkaido local government)

prevalence of *E. multilocularis* in dogs in Hokkaido could not be determined (Fig. 4)*.

Of the 91 cats in Hokkaido that had been examined, 5 were infected with *E. multilocularis*. Despite the small number of cats examined, it indicated the possibility of a high prevalence of the cestode among the cats. However, all the *E. multilocularis* tapeworms collected from the cats were those of the immature form (Yagi *et al.*, 1984). Low susceptibility of cats had also been reported in other countries. But some patent infection of cats were found in Europe.

Of the 70 raccoon dogs that were examined by Hokkaido government, only 1 was found spontaneously infected with *E. multilocularis*. Only a few worms were recovered from the raccoon dog and all of them were immature worms. Thus, it was considered that although the raccoon dogs prey on the voles, they were not a very suitable definitive host for *E. multilocularis* (Yagi *et al.*, 1988a).

A survey was done to investigate the epidemiological status of *E. multilocularis* in red foxes and raccoon dogs in Otaru, Hokkaido, between June and September 1999 (Yiman *et al.*, 2002). Sixty-seven red foxes and 13 raccoon dogs were captured, and the 38 red foxes (56.7%) and 3 raccoon dogs (23.1%) were found to be infected. The total biomass of *E. multilocularis* in all infected red foxes and raccoon dogs were 2,817,000 and 1,515 worms, respectively. In one of the infected raccoon dogs, mature worms and eggs of *E. multilocularis* were found in the intestine and fecal sample, respectively.

*Echinococcus multilocularis* had not been detected in either the definitive or the intermediate animal hosts in Honshu until the finding of three cases of swine hepatic alveolar echinococcosis in Aomori (Kamiya and Kanazawa, 1999), and a dog that was brought from Hokkaido to Gunma prefecture in central Honshu (Kamiya, 2001). Although there had been a report of the finding of adult worm of *E. multilocularis* in a dog in Aomori, detailed examination of the worm showed that there was a misidentification of the worm (Takahashi *et al.*, 1986). Thus, the spread of the endemic area of the parasite to the Honshu in Japan and an increase in the number of human cases are speculated.

*In 2002 finally Hokkaido Government released the information on itemized rearing condition of 99 infected dogs in 2002. Of those 37 were own dogs.

④ Larval infection in the animal intermediate host

1) Infection in wild rodents

It has been long known that the most important intermediate host of *E. multilocularis* in Hokkaido is the Bedford vole, *Clethrionomys rufocanus bedfordiae* but the parasite had also been detected in *Clethrionomys rutilus mikado* (Yamashita, 1973). Spontaneous infection of *E. multilocularis* in its intermediate host in Hokkaido such as in *Clethrionomys rex*, *Apodemus argenteus*, *Mus musculus* and *Rattus norvegicus*, as well as in the insectivores, *Sorex unguiculatus* and *Sorex caetiens*, had been reported (Kamiya *et al.*, 1977; Inaoka *et al.*, 1984; Okamoto *et al.*, 1992; Iwaki *et al.*, 1993; Takahashi and Nakata 1995; Ohbayashi, 1996). Ohta (1984) published a book on the taxonomy and ecology of the wild rodents of Hokkaido. Generally, the Norway rat had been thought to be resistant to *E. multilocularis* infection. To confirm that the strain of *E. multilocularis* isolated from the spontaneously infected rat was of a special one, eggs of that strain were orally inoculated into laboratory rats. However, it was observed that that strain of *E. multilocularis* could not be successfully established in the inoculated laboratory rats (Iwaki *et al.*, 1995). As mentioned above, there was no difference in the DNA sequence of the COI gene of the strains of *E. multilocularis* that were isolated from the rat and from the Bedford vole (Okamoto *et al.*, 1995).

Although the population of *Apodemus speciosus* in Hokkaido is as high as the Bedford vole (Ohta, 1984), no spontaneous infection of *E. multilocularis* had ever been observed in that rodent (Ohbayashi, 1996).

Results of seasonal monitoring of the dynamics of *E. multilocularis* infection in the Bedford vole in Hokkaido showed that there was a high prevalence of *E. multilocularis* among the voles in spring and these voles were those that had survived through the previous winter (Takahashi and Uraguchi, 1996).

2) Larval infection in pigs and horses

Although *E. multilocularis* infection in pigs had previously been reported in Russia, this fact had been totally overlooked. Sakui *et al.* (1984) reported detecting histopathological lesions of *E. multilocularis* in 34 out of 58,567 pigs examined. Later, many more pigs were examined for *E. multilocularis* lesions during meat inspection, and after 1993, in conjunction with the increase of the *E. multilocularis* infection rate in the red foxes, there was also a rapid increase in the number of pigs found infected with the parasite (Fig. 5). Since approximately 1 million pigs were slaughtered in Hokkaido every year, the number of positive pigs probably depend on the contamination with eggs in the environment around pig farming, the management of the animals and the system for the parasite examination. In pigs, *E. multilocularis* was observed to grow poorly and no protoscoleces were observed in the alveolar hydatid cyst (Sakui *et al.*, 1984). Besides the evidence of the histopathological observation, *E. multilocularis* infection in pigs was further confirmed by transplanting the hydatid lesion from the pig into laboratory reared Mongolian gerbil and observing that the cyst grew to become viable and infective in the gerbil (Kamiya *et al.*, 1987). These reports supported the notion that *E. multilocularis* could infect livestock on a worldwide scale. Pigs slaughtered for their meat were considered ideal animals for monitoring the spread of the endemic area of echinococcosis in Hokkaido because of their short life span, easy identification of the farm that they were reared in and the animals were individually inspected during slaughter. In 1998, three cases of swine alveolar echinococcosis were detected from a farm in Honshu (Kamiya and Kanazawa, 1999).

*E. multilocularis* infection in a horse in Hokkaido was first reported by Miyauchi *et*

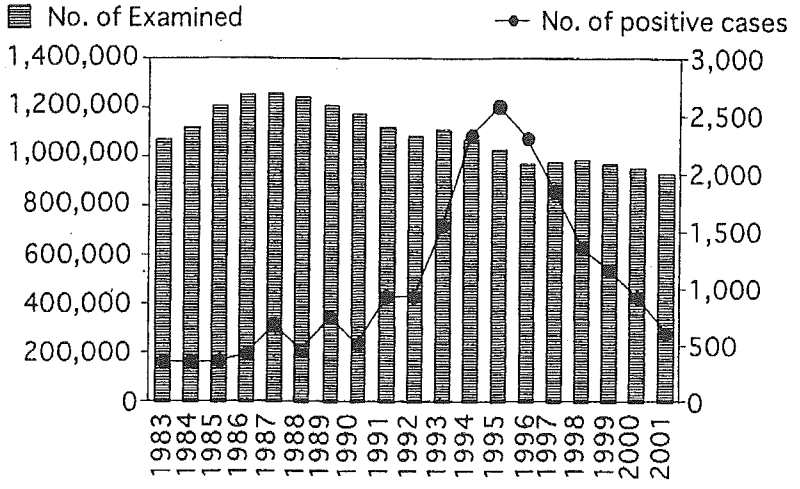


**Fig. 5.** Annual incidence of swine alveolar echinococcosis in Hokkaido (Data from Hokkaido local government)

*al.* (1984). Later, several similar cases in horses in Hokkaido were also described (Kaji *et al.*, 1993).

3) Larval infection in zoo animals

*Echinococcus multilocularis* infection in zoo animals had long been known in Europe. In the 1990's, an orangutan and a Japanese macaque in Kushiro zoo, as well as a gorilla and a ring-tailed lemur in Asahiyama zoo of Asahikawa city, were found to be infected with *E. multilocularis* (Kondo *et al.*, 1996). Furthermore, Japanese macaques in Obihiro zoo was found to die of multilocular echinococcosis (Oku *et al.*, 2001). All the aforementioned zoo animals were thought to have contracted the infection locally in Hokkaido. Among the various species of non-human primates, it was observed that there were differences in the growth of the alveolar hydatid cyst as well as the degree of lesion seen in the animal host.

### (3) Factors responsible for the transmission of *E. multilocularis*

① Transmission of *E. multilocularis* to the red foxes

It is well known that the red foxes eat a lot of Bedford voles and this has been implicated as the major source of transmission of *E. multilocularis* to the foxes (Abe, 1975). The culling and necropsy of the red foxes in Hokkaido was carried out only in winter and thus there is a limitation in the data on the seasonal variation of prevalence of *E. multilocularis* in the foxes. However, by determining the age of the foxes in relation to the infective rate, it was observed that there was a high rate of *E. multilocularis* infection among the foxes that were less than 1 year old (Takahashi *et al.*, 1987). Moreover, examination of the feces around the fox den showed the possibility that the young cubs were already infected with *E. multilocularis* shortly after their birth (Takahashi and Uraguchi, 1996).

② Viability of *E. multilocularis* eggs

The time length of the viability of *E. multilocularis* egg is known to be dependent upon the temperature in which the eggs were stored. Due to the biohazard nature of the eggs, only facilities with a high level of biosafety equipment were allowed to do experiments with the eggs. Thus, there had been no detailed description of the

viability time span of *E. multilocularis* egg with regard to its infectivity in the intermediate host. Ishige *et al.* (1993) of the Hokkaido Institute of Public Health, stored suspension of *E. multilocularis* eggs at temperatures ranging from 4℃ to 48℃ for various period of time (3hr to 256 days), followed by oral inoculation of the eggs into susceptible mice for bioassay of the eggs. They then counted the number of the early hydatid lesions in the inoculated mice. They observed that the *E. multilocularis* eggs lost their infectivity after storage at 20℃ for 32 days, but at 4℃, it took between 128 to 256 days for the eggs to become non-infective. This is the first report on the viability test of the *E. multilocularis* egg in relation to its infectivity.

③ Transmission of *E. multilocularis* to the voles

The prevalence of *E. multilocularis* in the voles is generally very low. The actual transmission route of the parasite to the vole is largely unknown. Takahashi *et al.* (1989) of the Hokkaido Institute of Public Health conducted an echinococcosis survey of voles inhabiting the areas around the fox dens. They found that there was a difference in the prevalence in voles caught around the fox dens that had cubs in it and those that did not. This showed that the areas around the fox dens that had the nursing cubs were contaminated with *E. multilocularis* eggs. This is attributed to the defecation by the cubs around the fox dens as stated above. The Bedford voles born in a certain year were usually infected with *E. multilocularis* during the summer to the autumn of that year. These infected voles usually overwintered until the following year and it was thought that they served as the source of *E. multilocularis* infection for the young cubs. Furthermore, to investigate the time length of the viability of the *E. multilocularis* eggs around the contaminated fox dens in nature, echinococcosis survey of the voles around the fox dens that had been abandoned by the fox was carried out.

The age of the voles caught were determined to look for any correlation between the infected voles and their age. It was found that the viability of *E. multilocularis* eggs around the fox dens did not extend for a long period of time. Kamiya (1986) also discussed the possibility of using competitive inhibition of parasitism among related taeniid species (cross-resistance among the parasite in the vole host) as a method to control the spread of echinococcosis.

④ Transmission of *E. multilocularis* to humans (analysis of risk factor)

To elucidate the route of transmission of *E. multilocularis* to human, it is important to first identify the high risk group people and then analyze the various factors contributing to the infection in that group of people. Researchers at Asahikawa Medical College had attempted such an analysis. In major dairy cattle and pig farming areas in Hokkaido, much farming-waste products and wet garbage were left unattended. Most of the farming garbage ended up as food for the foraging red foxes. Thus, people living in these areas where there are many foraging foxes roaming around were considered as the high-risk group (Doi *et al.*, 1987; Nakao *et al.*, 1988). In winter, when food source of wildlife is scarce, the red foxes in Hokkaido depended heavily on the farming waste products for food and this fact was revealed in an investigation on the food habit of the red foxes (Abe, 1975). However, Kondo *et al.* (1986) pointed out that the food eaten by the red foxes in Hokkaido in winter varied among the different areas depending on the availability of the food. The red foxes will eat whatever food it can easily get hold of in that area in winter.

Based on the frequency of contact with red foxes, the hunter, fur dealer and taxidermist were thought to be in the high-risk group of being infected with

multilocular echinococcosis. However, an ELISA test for *E. multilocularis* antibody in these professional group of people showed that there was no correlation between the prevalence of the disease and their profession (Inaoka *et al.*, 1987a; Doi *et al.*, 1987). Depending on the situation, high-risk group of people varied from one country to another.

To prevent the spread of *E. multilocularis* through the transmission of the parasite egg in drinking water, running water facilities were built throughout Hokkaido to provide the inhabitants with safe drinking water. However, to date, *E. multilocularis* eggs had not been detected in drinking water in Hokkaido. Nevertheless, there is a need to understand the defecation habit of the red foxes and the likely places where they tend to shed their feces. Uraguchi and Takahashi (1989) monitored the movement and activity of the red foxes using the spotlight census method. They observed that the red foxes frequently roam around the human residential area in Hokkaido.

### (4) Research on the development of *E. multilocularis*

A P3 level biosafety experimental facility was built at the Hokkaido Institute of Public Health in 1987 and this enabled experiments using *E. multilocularis* eggs to be carried out. Using that facility, research on *E. multilocularis* eggs as well as experimental infection with the parasite eggs in animal host had been reported (Yagi *et al.*, 1988b). On the other hand, researchers at Hokkaido University, Faculty of Veterinary Medicine, developed the alternative definitive host model for *E. multilocularis* using Mongolian gerbil and golden hamster. They thus demonstrated that by using these animal host model, biosafe experiments could be carried out even in a small laboratory (Kamiya and Sato, 1990a, b; Oku *et al.*, 2002).

① Development of *E. multilocularis* in the intermediate host

In Bedford voles orally inoculated with *E. multilocularis* eggs, protoscoleces formation was observed at 41 days postinfection, and at 142 days postinfection, 1g of the hydatid cyst was found to contain 146,000 protoscoleces. After that, the number of protoscoleces found per gram in the cyst plateau off and did not increase significantly. (Yagi *et al.*, 1989).

Although golden hamster had been previously reported to be resistant to echinococcosis, Kamiya (1974) demonstrated that the animal was to a certain degree susceptible to primary (infection by eggs) and secondary (infection by injection of metacestode tissue) echinococcosis. Inaoka *et al.* (1983) showed that the Chinese hamster was susceptible to secondary echinococcosis infection. No spontaneous *E. multilocularis* infection had been observed in *Apodemus speciosus* and Ooi *et al.* (1992) showed that infection in that rodent could neither be experimentally established. *E. multilocularis* had been found in naturally infected rat but that isolate of the parasite could not be shown to experimentally infect rat through its eggs (Iwaki *et al.*, 1995).

It is known that the red foxes feed on the dead voles in nature. Ohnishi *et al.* (1984) observed that the protoscoleces in the dead intermediate host were still viable after the carcass had been stored at 10℃ for 14 days.

Sakamoto and Gemmell (1978) observed various anomalies among the protoscoleces in hydatid cyst and suggested that formation of the protoscoleces became inhibited after long-term passage of secondary echinococcosis in Mongolian gerbil. They also proposed that the hydatid cyst became more virulent after long term laboratory passage due to the shorter survival period of the experimentally infected Mongolian gerbil (Sakamoto *et al.*, 1996).

Matsuhisa *et al.* (1996) inoculated protoscoleces, germinal cells or the small vesicles of the hydatid cyst directly into the portal vein of experimental animals and reported that those injected with protoscoleces did not form secondary hydatid cyst in the liver. This phenomenon was thought to be different from the predicted vesiculation of the protoscoleces when being cultured *in vitro* and they suggested that the protoscoleces were less towards that of the secondary echinococcosis.

② *In vitro* culture of the metacestode of *E. multilocularis*

Sakamoto *et al.* (1967) reportedly succeeded in culturing the germ cell layer of the hydatid cyst in a liquid culture medium, but after the third passage, proliferation of the cell began to decline and no cyst were formed. Although Furuya (1991) reported success in immortalizing the germinal layer cell of the *E. multilocularis* cyst, the cell growth became abnormal after injection into experimental animals and no cyst formation was observed. Although the success of culturing *E. multilocularis* cell *in vitro* might be due to the special characteristic of that parasite, research done along that line can be considered as innovative. Later, it was reported that hydatid cyst could be formed after the injection of cultured germinal layer cells into the experimental animals but the injected cells were cells of the primary cell culture and not those that had been immortalized (Yamashita *et al.*, 1997).

③ Growth of *E. multilocularis* to the adult tapeworm and the period of parasitism in the definitive host

Although both the dogs and red foxes are known to be the permissive definitive host of *E. multilocularis*, long-term observation of the cestode in these hosts were seldom carried out. This is due to the shedding of infective biohazardous *E. multilocularis* eggs in the feces of these animal hosts and that posed a great danger to researchers. Ito *et al.* (1990) conducted a long-term observation of *E. multilocularis* infection in its natural definitive host and found that the parasite eggs were shed in the host feces at day 26 postinfection for the host with the shortest prepapent period. From the dynamics of the eggs shed in the feces of the dogs and red foxes, they observed that the infection was persistent from 2-4 months and the possibility of a large amount of eggs being shed in the feces for a long period of time was very low. Moreover, they also observed a 7-13 days cycle for the increase and decrease in the number of eggs shed. Furthermore, in a collaborative research between Hokkaido Institute of Public Health and Hokkaido University, Faculty of Veterinary Medicine, a sandwich ELISA that detect and measure the amount of metabolic product (coproantigen) produced by the adult worm of *E. multilocularis* that were shed together with the feces, was developed. This test was also used to confirm that the adult worms of *E. multilocularis* did not parasitize for a long period of time and were expelled naturally after a short period of parasitism (Nonaka *et al.*, 1996). Using this fecal monitoring method, it became unnecessary to do a post-mortem examination of many definitive host animals for the diagnosis of echinococcosis. In addition, it also became possible to continue observing and monitoring the course of *E. multilocularis* infection in the same animal.

Gravid adult worms of *E. multilocularis* had been reportedly detected in cats in Europe and thus refuting the notion that eggs could not be formed in the worms parasitzing cats. However, Kamiya *et al.* (1985) observed that the cats were not a very suitable definitive host in their experimental infection of *E. multilocularis* in cats and suggested that it is quite rare for the worm to develop to gravid stage in cats in Hokkaido. Other carnivores that were found distributed in Hokkaido were the rac-

coon and mink. Experimental infection of *E. multilocularis* in the mink showed that the infection could not be established (Ooi *et al.*, 1992).

For the safe handling of the biohazard infective eggs of *E. multilocularis*, the worms were allowed to grow until the immature adult stage in the dog intestine and then removed for culture *in vitro* to obtain the eggs. Researchers in other countries had described such method of obtaining the parasite eggs. However, Kamiya and Ishigooka (1984) transplanted the immature adult worms from the dog intestine into the peritoneal cavity of Mongolian gerbil and let the worm grew until gravid in that host. Thus, they had developed a simpler method for obtaining the *E. multilocularis* eggs.

④ Development of the alternative definitive host model for *E. multilocularis*

Research on the growth of *E. multilocularis* related taeniid species had been previously carried out at Hokkaido University, Faculty of Veterinary Medicine. Thus, an attempt was made to develop an alternative definitive host for cestodes. It was observed that when cysticerci of *Taenia crassiceps* were orally inoculated into mice, some cysticerci developed in the direction of the adult tapeworm stage (Saitoh, 1987). This observation led to the development of the first alternative definitive host for *E. multilocularis*. Briefly, Kamiya and Sato (1990a, b) succeeded in growing the protoscoleces of *E. multilocularis* to gravid adult tapeworm in the intestine of prednisolone-treated Mongolian gerbil and golden hamster, and recovering the parasite eggs that were found to be viable and infective to the intermediate host. Later, this alternative definitive host model was improved by considering the use of the North American desert rodents and Chinese rodents (Ohtsubo, 1993; Oku *et al.*, 2002). It was observed that the worm recovery could be improved by using younger Mongolian gerbils and prednisolone should be administered to the host animals well before the initiation of oral inoculation with the protoscoleces. This animal model was used for the study of pathological changes during the parasite infection and the host immune protective response against reinfection (Inohara *et al.*, 1996a), as well as for the collection of the adult worm antigen and the modification of the coproantigen detection method (Nonaka et al., 1996). The development of this alternative definitive host for *E. multilocularis* was highly evaluated oversea as being a new experimental method.

In general, when *E. multilocularis* protoscoleces were injected into the peritoneal cavity of the intermediate host, they developed into the hydatid cyst. However, when Sato and Kamiya (1990) administered *E. multilocularis* protoscoleces into the pulmonary alveoli of hamster, they observed that the worm developed toward the adult tapeworm stage. Later, Inohara *et al.* (1996b) also observed the formation of proglottids and genital primordia in the worms after administering *E. multilocularis* protoscoleces to SCID (severe combined immuno deficiency) mice by various parenteral routes. Thus, although the worm that grew by this method did not develop to the gravid stage, this observation is unique because it demonstrated that the worm could develop in the direction of the adult worm despite being in a parenteral environment.

### (5) Immunology of *E. multilocularis* infection

Kamiya (1972) showed that the AKR mouse, which is deficient in the fifth component of the complement system, is a very susceptible animal for *E. multilocularis* infection but one that also demonstrate age resistance to the infection. Furthermore, it was shown that the susceptibility to *E. multilocularis* infection varied for different strains of mice, sex and age of the animals (Kamiya, 1973). Moreover, Kamiya and

Ohbayashi (1981) showed that protoscoleces formation were delayed in male mice that had been castrated. Kamiya *et al.* (1980b) suggested that complement lysis of the protoscoleces plays a very important role in determining the susceptibility of the host to *E. multilocularis* infection, especially among the different animal species as well as among the different strains of mice. They also demonstrated that the host complement lysis of the *E. multilocularis* protoscoleces occurred through the alternative pathway. Later, they observed the process of the lysis of protoscoleces by the complement through scanning electron microscopy (Kamiya *et al.*, 1982). Guinea pig is an animal that is very resistant to *E. multilocularis* infection and the serum complement of that animal reacted strongly in the lysis of the protoscoleces.

The importance of thymus or T cell in modulating the outcome of secondary echinococcosis was demonstrated by researchers at Hokkaido University, Faculty of Veterinary Medicine using nude and SCID mice. In the nude mice, *E. multilocularis* cyst developed in large spherical vesicular cyst which is not seen in normal mice (Kamiya *et al.*, 1980a). In the SCID mice, increase in larval mass and development of the larva of *E. multilocularis* were shown to be regulated by the T cell (Playford *et al.*, 1992, 1993). Furthermore, the inflammatory reaction surrounding the hydatid lesion such as the infiltration of lymphocytes, histocytes and eosinophils were also shown to be associated with T cells. To investigate the role of natural killer (NK) cell in the immune response to *E. multilocularis* infection, Oku *et al.* (1984) conducted an experimental infection of *E. multilocularis* in the NK cell deficient beige mice. However, they did not observed any difference in the growth of the hydatid cyst between beige and normal mice.

Ishige and Kizaki (1987) proposed that the susceptibility of the various strains of mice to *E. multilocularis* infection was dependent upon the H-2 gene and reported that mice with the k H-2 haplotype gene were resistant to the infection. However, Nakaya *et al.* (1997) reported that there was no evidence to suggest that resistance to *E. multilocularis* infection is related to the H-2 gene.

It had already been known that the mouse peritoneal macrophage has the ability to kill protoscoleces of *E. multilocularis*. Kanazawa *et al.* (1993) demonstrated that the protoscolicidal activity of the macrophage was mediated through nitric oxide (NO).

Kizaki *et al.* (1993) observed *in vitro* that the protoscoleces of *E. multilocularis* were able to induce immunosuppression through their action on $CD8^+$ cell.

As part of the collaborative research between Hokkaido University, Faculty of Veterinary Medicine and Hokkaido Institute of Public Health, kinetic of the antibody response in AKR mice was monitored after the mice were experimentally fed with *E. multilocularis* eggs (Matsumoto *et al.*, 1998). This experiment was significant because the route of infection used was the same as that in nature and until then, most of the similar research done were those that used secondary echinococcosis.

Sato and Furuya (1994) analyzed the antigen of *E. multilocularis* and found that the 30-35 kDa polysaccharide antigen represents the C antigen. Kohno *et al.* (1995) also produced several monoclonal antibodies against *E. multilocularis* and found that they recognized epitopes on different sites of the worm.

### (6) Immunodiagnosis of multilocular echinococcosis

*Echinococcus multilocularis* develops slowly in humans and symptoms were not observed until after the lesions had progressed to a very advanced stage. Therefore, it is very important to diagnose the disease at the initial stage when the lesion had not yet progressed to the advanced stage. The diagnosis of echinococcosis in humans is

dependent upon the immunodiagnostic method and the imaging diagnostic method. Researchers at the Hokkaido Institute of Public Health had been conducting research on the immunodiagnosis of *E. multilocularis* since the 1950's and had published many papers (Kumagai, 1988). The immunodiagnostic methods that had been carried in Hokkaido to diagnose multilocular echinococcosis in human include skin test, complement fixation test, indirect hemagglutination test (Kumagai *et al.*, 1973), immunoelectrophoresis test and RAST (Radio-allergo-sorbent test) (Kumagai *et al.*, 1976). However, at present the primary screening test used in Hokkaido to detect multilocular echinococcosis is ELISA (Sato *et al.*, 1983a, b). For the ELISA positive sample, the Western blotting method was carried out to confirm the positivity of the sample (Furuya and Kumagai, 1988). However, even the Western blotting method was not considered sensitive enough for the diagnosis because Sato *et al.* (1993) reported that among the 86 echinococcosis patients that they examined, 8% showed false negative. For the diagnosis of multilocular echinococcosis by ELISA, the Em2 antigen (a glycoprotein antigen) had been widely used throughout the world and also been commercialized in the form of a diagnostic kit. In Hokkaido, for the diagnosis of echinococcosis in humans, crude antigen is still used for the ELISA because sensitivity takes precedent over specificity (Sato *et al.*, 1983a). Furuya *et al.* (1990) analyzed many multilocular echinococcosis patient sera by Western blotting and observed the presence of many different bands. They classified patients whose sera showed the 30–35, 55 and 66 kDa bands or those showing the latter 2 bands as those of the complete form, while patients whose sera only showed the 30–35 kDa band as belonging to the incomplete form. Furthermore, they also examined the location of these antigens on the echinococcal tissue. Based on epidemiological data, they concluded that those patients who had long been infected with *E. multilocularis* tend to show the complete form of the Western blot sera profile, while those who had just been infected tend to show the incomplete form (Nagano *et al.*, 1995). Sato and Furuya (1994) reported that the 30–35 kDa antigen of *E. multilocularis* isolated by the Western blot method was both highly sensitive and specific.

On the other hand, Ito *et al.* (1993) suggested the use of specific *E. multilocularis* glycoprotein antigen, which they named Em16 and Em18. Together with the collaboration of oversea researchers, Ito *et al.* (1995) compared the reactivity of the sera of multilocular echinococcosis patients who showed progressive lesion (12 samples) and those with regressed lesion (3 samples) using the aforementioned antigens. They reported that by using Em18 in the Western blot test, they could differentiate the patients with progressive lesion from those with regressed lesion of echinococcosis, and thus claiming that their method is more effective than using Em2 in the ELISA test. Furthermore, Ito *et al.* (1997a) compared the specificity of their test against the sera of patients with unilocular and multilocular echinococcosis, and reported that the Em16 and Em18 were specific for only *E. multilocularis* (Ito *et al.*, 1997b). Moreover, by using partially purified Em16/Em18 to produce an Em16/Em18 ELISA, they showed that their test had a higher sensitivity and specificity as compared to the commercially available Em2 plus ELISA. Recently, they identify an antigenic relationship between Em18 and a 65-kDa immunodominant *E. multilocularis* surface protein previously identified as either EM10 or EmII/3 (Gottstein *et al.*, 1993; Sako *et al.*, 2002). Em18 was determined, revealing it to be a fragment of EM10. Em18 was found to be the product of degradation of EM10 by cysteine proteinase.