worms (Zhang et al., 2001). Spleen cells of immunized mice produced an elevated level of IFN-γ in response to antigen stimulation. In our preliminary experiment in pigs, we also observed a significant anti-fecundity effect (Ohta et al., 2004).

In spite of the accumulating evidence for the efficacies of calpain as an anti-schistosome vaccine, it is still not clear how protective immunity is induced in calpain-immunized animals. To understand how anti-calpain immunity works, we examined the localization of calpain in different developmental stages of *S. japonicum*.

Schistosoma japonicum isolated in Yamanashi, Japan, was maintained by standard laboratory procedure in female BALB/c mice (SLC, Hamamatsu, Japan) and their snail hosts, *Oncomelania hupensis nosophora*. Worms were recovered by the perfusion method from the hepatic portal system of mice at 8 weeks after infection with 40 cercariae (Smithers and Terry, 1965). Antigens used in the present study were SWAP, SEA, and recombinant calpain molecules of *S. japonicum*. The methods for preparation of SWAP and SEA have been described previously (Rosane et al., 1996). Recombinant calpain was prepared as previously described (Zhang et al., 2001). This recombinant calpain is the region of 220–376 amino acid of the large subunit, and used for immunization and several assays. Furthermore, we prepared two fragments of recombinant calpain: (1) the region of 220–330 amino acid and (2) the region of 300–376 amino acid of the large subunit. These two fragments were used to determine a monoclonal antibody recognizing portion. In brief, all coding sequences were inserted to pGEX-2TK vector (Pharmacia, Uppsala, Sweden). This vector was then transformed into BL21 cells. The recombinant proteins were induced by addition of isopropanol β-D-thiogalactoside to a final concentration of 0.1 mM for 6 h. After induction, BL21 cells were collected and lysed with BugBuster reagent plus Benzonase (Novagen, Madison, USA). The insoluble fraction was then solubilized in sample buffer for SDS–PAGE to use in CBB staining and Western blotting.

Female BALB/c mice were intradermally immunized with recombinant calpain plus complete Freund's adjuvant for the first immunization, and Freund's incomplete adjuvant for subsequent immunizations. The calpain was administered at 2-week intervals and blood samples were collected before and after the immunizations. The presence of specific antibodies was detected via ELISA (see below). For the generation of monoclonal antibodies, spleen cells of immunized mice were fused with P3×63.Ag8.653 myeloma cells using polyethylene glycol 1500 (Boehringer–Mannheim, Germany). Hybridomas that secrete antibodies to calpain were selected and cloned by limiting dilution. Ascites was produced in BALB/c mice by injecting 5×10^6 hybridoma cells. Isotype was determined in ELISA using anti-isotype mAbs (Southern Biotechnology Associates, Birmingham, USA). For ELISA, 5 μg/ml of antigens was coated in microtiter plates (Nunc, Roskilde, Denmark). Wells were blocked with Tris-buffered saline-containing 1% casein, and incubated with antibodies. After washing, anti-mouse IgG (H + L) (KPL, Gaithersburg, USA) was added and incubated for 1 h. ABTS (2,2′-azino-di[3-ethyl-benzthiazoline sulfonate]) was used as substrate and optical densities were read in a Microplate Reader (Bio-Rad, Hercules, USA). Recombinant proteins and SWAP were fractionated on 10% SDS–polyacrylamide gels (TEFCO, Tokyo, Japan) and electrophoretically transferred to PVDF membrane (Bio-Rad). The membrane was blocked by 3% BSA in PBS, and then incubated with antibodies in Tris-buffered saline-containing 1% casein. After that, membranes were washed and incubated with peroxidase-conjugated anti-mouse IgG (H + L) (KPL). Detection of positive bands was done using Konica immuno-stain kit (Konica, Tokyo, Japan).

Adult worms of *S. japonicum* at 8 weeks after infection were prepared for use as frozen sections. Worms were incubated in RPMI 1640 (Sigma–Aldrich) supplemented with 10% FBS (Gibco-BRL, Grand Islands, USA) at 37 °C overnight to wash away the host components. After washing worms embedded in OCT compound (Sakura Finetechnical, Tokyo, Japan) were snap frozen, and were sliced with a cryostat (Lica, Nassloch, Germany) at a thickness of 6 μm. Sections

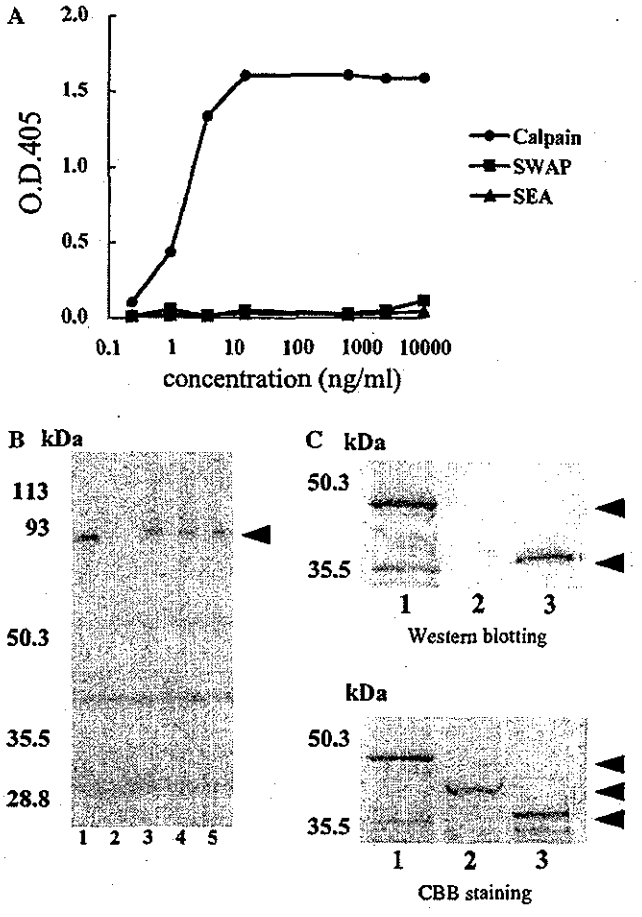

Fig. 1. Preparation and analysis of KG-2E11 by ELISA and Western blotting. (A) KG-2E11 generated from recombinant calpain-immunized BALB/c mice bound to calpain tightly, although SWAP was bound weakly and SEA was not by ELISA. (B) The soluble adult worm antigens (50 μg/well) were electrophoresed in SDS–PAGE. The bands on the gel were transferred to PVDF membrane and the membrane was treated with KG-2E11 (lane 1), normal mouse serum (lane 2) and several anti-calpain antisera, each of which was individual serum of a calpain-immunized mouse (lanes 3–5). All lanes except for lane 2 specifically recognized a molecule of 80 kDa, of which molecular mass was equivalent to a large subunit of *S. japonicum* calpain. (C) Determination of the epitope recognized with KG-2E11 was done by Western blotting analysis using three types of recombinant calpain. Recombinant calpain; the region of 220–376 amino acid (lane 1); the region of 220–330 amino acid (lane 2); and the region of 300–376 amino acid (lane 3) of calpain heavy chain were electrophoresed in SDS–PAGE. Positive bindings to KG-2E11 were observed in lane 1 and lane 3. We confirmed by CBB staining that each protein was transferred to PVDF membrane successfully.

were dried and preserved in acetone for 15 min at −20 °C. Immunostaining was done using a HistoScan kit (Biomeda, Foster City, USA). After incubation in blocking solution containing normal rat immunoglobulin (Sigma–Aldrich) for 30 min at room temperature, sections were incubated together with KG-2E11 mAb diluted 1:100 with blocking buffer for 3–9 h at 4 °C. An isotype control mAb (Dako, Glostrup, Denmark) was used as a negative control. After washing, each section was incubated with anti-mouse secondary antibodies (KPL) for 1 h at room temperature, and as a next step was treated with anti-goat polyclonal antibodies for 30 min (Biomeda). After treatment of antibodies, endogenous peroxidase was blocked by the use of 0.3%

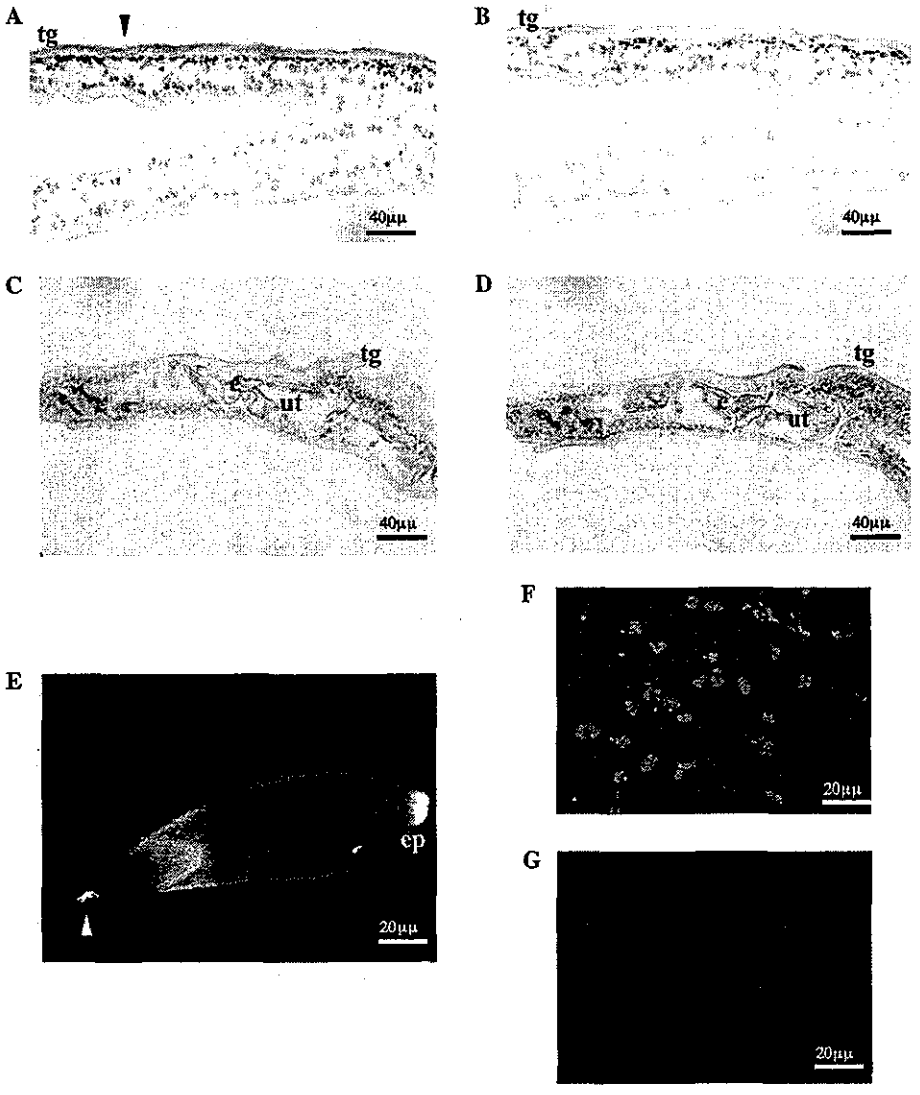

Fig. 2. Localization of calpain in *S. japonicum* with KG-2E11. KG-2E11 bound to the tegument (tg) of male worms (A), but did not bind to the tegument (tg) and the uterus (ut), including eggs (e), of female worms (C). Isotype control mAb was non-binding (B,D). Cercariae were stained with KG-2E11, and then the outlets of penetration glands (arrowhead) and the excretory pore (ep) were strongly positive (E). The secretions from cercariae were strongly stained by KG-2E11 (F), but they were non-binding by isotype control mAb (G).

H_2O_2 in methanol. Each section was then incubated with avidin–peroxidase for 15 min, stained with AEC staining solution for 10 min, and a counter staining with hematoxylin was done. For staining whole bodies, cercariae were fixed in acetone for 10 min at −20 °C. After washing with PBS, blocking was done by putting cercariae in 1% casein Tris buffer overnight at 4 °C. For staining "footprints" of *S. japonicum* cercariae, freshly released from crushed snails, were directly placed on silane-coated slide glasses for 1 h at room temperature. After these slide glasses were completely dried, and they were fixed in acetone for 10 min at −20 °C. KG-2E11 mAb was treated against both cercarial bodies and footprints for 3 h at room temperature. After incubation with biotin-conjugated antibodies (Vector laboratories, Burlingame, USA), both cercarial bodies and footprints were reacted with avidin-conjugated fluorescein (Vector laboratories) for 30 min at room temperature.

Mice were immunized with recombinant *S. japonicum* calpain to generate specific monoclonal antibodies. We obtained only one single stable clone, KG-2E11, which bound to recombinant calpain tightly (IgG2b, κ). In ELISA, KG-2E11 showed only weak binding to *S. japonicum* SWAP, and no detectable binding to SEA (Fig. 1A). Calpain, thus, seemed to be a minor component of whole adult worm proteins. However, it recognized an 80 kDa protein in SWAP, which corresponded to a large calpain subunit (Fig. 1B). KG-2E11 bound to the C-terminal portion of recombinant *S. japonicum* calpain in the tested two fragments of the heavy chain (Fig. 1C). This suggested that the KG-2E11 epitope is located in the region of 330–376 amino acid of calpain heavy chain.

Immunohistochemistry of adult worms with KG-2E11 showed that this mAb bound to the tegument of the male adult worms (Figs. 2A and B), whereas, it did not bind to the female tegument (Figs. 2C and D). Schistosomes had calpain molecules in the surface of the tegument in the present study as was reported previously (Siddiqui et al., 1993). On the other hand, KG-2E11 bound strongly to the outlets of cercarial penetration glands in *S. japonicum* cercariae (Fig. 2E). This suggested that calpain seemed to be secreted from cercariae. To confirm that calpain was secreted from the cercarial penetration glands, we incubated cercariae on slide glasses to let them secrete gland contents on the surface of the slide glasses. It has been reported that cercariae secrete mucoid substances and leave secretion spots ("footprints") on the bottom of the dishes (Linder, 1985). Many spots were observed

as footprints in the substances secreted from cercariae when we tested KG-2E11 binding (Fig. 2F). No positive binding was observed for isotype control mAb (Fig. 2G), indicating that the binding was not in a non-specific manner. Although, we still do not have solid evidence that the secreting substances contain native or partial fraction(s) of calpain, this could be a probable demonstration that cercariae are directly exposed to calpain-driven host immunity. Several researchers have reported that schistosome cercariae secrete substances containing proteases (Chavez-Olortegui et al., 1992; Fishelson et al., 1992; Landsperger et al., 1982; Stirewalt and Austin, 1973). Especially, the serin protease was localized in both pre- and post-acetabular glands of cercariae of *S. mansoni* (Fishelson et al., 1992; Marikovsky et al., 1990). Calpain is one of calcium-activated cystein proteases. At the time of penetration calpain may work as dermal alterations directly or indirectly dependent on calcium activation. It is important to investigate calpain secreted from cercaria to understand the mechanism of penetration. On the other hand, our observations that a large amount of calpain seems to be localized in the footprints strongly suggest important roles of calpain during survival and/or growth of the larval stage of schistosomes. Calpain release from newly transformed schistosomula of *S. mansoni* has been reported (Jankovic et al., 1996), and we have supported the expression of calpain in mechanical transformed schistosomula in *S. japonicum* (Ohta et al., 2004). Moreover, we found an elevated production of inducible nitric oxide synthase mRNA in the lungs of mice immunized with recombinant calpain (Zhang et al., 2001). This means that calpain from schistosomula moving on through the lung of host stimulates the effector cells of immunized-host. We suggest that the expression of calpain is found in all stages from cercaria, through the penetration, to adult worm. However, the functions of calpain in each stage are still unknown completely. The expression and secretion of calpain in cercariae is an important finding because cercaria and schistosomula seem to be targets for protective immunity characterized as a reduction of worm burden. It has been proposed that calpain-reactive Th1 cells recognize enzymes released from early migrating larvae (Jankovic et al., 1996), and that antigens derived from lung-stage schistosomulae stimulate secretion of IFN-γ (Mountford et al., 1995). The production of typel cytokines may cause a limited migration of schistosomulae (Wilson et al., 1986) and the killing of the larvae through the production of toxic nitrogen oxides (James et al., 1984; Oswald et al., 1994). To our knowledge, this is the first demonstration that calpain is localized in the penetration glands of cercariae, and is secreted from cercariae. The same situation might be supposed in schistosomula, although we still have no direct evidence. These findings could be clues for understanding the reasons why worm burden is reduced in mice immunized with calpain.

Acknowledgments

This study was supported in part by Grant-in-Aid for scientific research from the Ministry of Education, Culture, Sports, Science and Technology Japan (1539017), Grant for Emerging and Re-emerging Infectious Diseases, the Ministry of Health Welfare and Labour, Japan (H12-Shinko-18 and H15-Shinko-8), and a grant from the Japan–US Cooperative Medical Science Program (2002 and 2003).

References

Andresen, K., Tom, T.D., Strand, M., 1991. Characterization of cDNA clones encoding a novel calcium-activated neutral proteinase from *Schistosoma mansoni*. The Journal of Biological Chemistry 266, 15085–15090.

Chavez-Olortegui, C., Resende, M., Tavares, C.A., 1992. Purification and characterization of a 47 kDa protease from *Schistosoma mansoni* cercarial secretion. Parasitology 105, 211–218.

Fallon, P.G., Tao, L.-F., Ismail, M.M., Benett, J.L., 1996. Schistosome resistance to praziquantel: fact or artifact?. Parasitology Today 12, 316–320.

Fishelson, Z., Amiri, P., Friend, D.S., Marikovsky, M., Petitt, M., Newport, G., McKerrow, J.H., 1992. *Schistosoma mansoni*: cell-specific expression and secretion of a serine protease during development of cercariae. Experimental Parasitology 75, 87–98.

Hota-Mitchell, S., Clarke, M.W., Podesta, R.B., Dekaban, G.A., 1999. Recombinant vaccinia viruses and gene gun vectors expressing the large subunit of *Schistosoma mansoni* calpain used in a murine immunization-challenge model. Vaccine 17, 1338–1354.

Hota-Mitchell, S., Siddiqui, A.A., Dekaban, G.A., Smith, J., Tognon, C., Podesta, R.B., 1997. Protection against *Schistosoma mansoni* infection with a recombinant baculovirus-expressed subunit of calpain. Vaccine 15, 1631–1640.

James, S.L., Natovitz, P.C., Farrar, W.L., Leonard, E.J., 1984. Macrophages as effector cells of protective immunity in murine schistosomiasis: macrophage activation in mice vaccinated with radiation-attenuated cercariae. Infection and Immunity 44, 569–575.

Jankovic, D., Aslund, L., Oswald, I.P., Caspar, P., Champion, C., Pearce, E., Coligan, J.E., Strand, M., Sher, A., James, S.L., 1996. Calpain is the target antigen of a Th1 clone that transfers protective immunity against *Schistosoma mansoni*. Journal of Immunology 157, 806–814.

Landsperger, W.J., Stirewalt, M.A., Dresden, M.H., 1982. Purification and properties of a proteolytic enzyme from the cercariae of the human trematode parasite *Schistosoma mansoni*. The Biochemical Journal 201, 137–144.

Linder, E., 1985. *Schistosoma mansoni*: visualization with fluorescent lectins of secretions and surface carbohydrates of living cercariae. Experimental Parasitology 59, 307–312.

Marikovsky, M., Arnon, R., Fishelson, Z., 1990. *Schistosoma mansoni*: localization of the 28 kDa secreted protease in cercaria. Parasite Immunology 12, 389–401.

Mountford, A.P., Harrop, R., Wilson, R.A., 1995. Antigens derived from lung-stage larvae of *Schistosoma mansoni* are efficient stimulators of proliferation and gamma interferon secretion by lymphocytes from mice vaccinated with attenuated larvae. Infection and Immunity 63, 1980–1986.

Ohta, N., Kumagai, T., Maruyama, H., Yoshida, A., He, Y., Zhang, R., 2004. Research on calpain of *Schistosoma japonicum* as a vaccine candidate. Parasitology International 53, 175–181.

Oswald, I.P., Eltoum, I., Wynn, T.A., Schwartz, B., Caspar, P., Paulin, D., Sher, A., James, S.L., 1994. Endothelial cells are activated by cytokine treatment to kill an intravascular parasite, *Schistosoma mansoni*, through the production of nitric oxide. Proceedings of the National Academy of Sciences of the United States of America 91, 999–1003.

Redman, C.A., Robertson, A., Fallon, P.G., Modha, J., Kusel, J.R., Doenhoff, M.J., Martin, R.J., 1996. Praziquantel: an urgent and exciting challenge. Parasitology Today 12, 14–19.

Rosane, H.C.C., Padraic, G.F., Michael, J.D., 1996. Sm480: a high molecule weight *Schistosoma mansoni* antigen associated with protective immunity. Parasite Immunology 18, 149–157.

Scott, J.C., McManus, D.P., 2000. Characterisation and expression of a cDNA encoding the 80 kDa large subunit of *Schistosoma japonicum* calpain. Parasitology International 48, 205–214.

Siddiqui, A.A., Phillips, T., Charest, H., Podesta, R.B., Quinlin, M.L., Pinkston, J.R., Lloyd, J.D., Paz, M., Villalovos, R.M., Pompa, J., 2003a. Induction of protective immunity against *Schistosoma mansoni* via DNA priming and boosting with the large subunit of

calpain (Sm-p80): adjuvant effects of granulocyte–macrophage colony-stimulating factor and interleukin-4. Infection and Immunity 71, 3844–3851.

Siddiqui, A.A., Phillips, T., Charest, H., Podesta, R.B., Quinlin, M.L., Pinkston, J.R., Lloyd, J.D., Pompa, J., Villalovos, R.M., Paz, M., 2003b. Enhancement of Sm-p80 (large subunit of calpain) induced protective immunity against *Schistosoma mansoni* through co-delivery of interleukin-2 and interleukin-12 in a DNA vaccine formulation. Vaccine 21, 2882–2889.

Siddiqui, A.A., Zhou, Y., Podesta, R.B., Karcz, S.R., Tognon, C.E., Strejan, G.H., Dekaban, G.A., Clarke, M.W., 1993. Characterization of Ca^{2+}-dependent neutral protease (calpain) from human blood flukes, *Schistosoma mansoni*. Biochimica et Biophysica Acta 1181, 37–44.

Smithers, S.R., Terry, R.J., 1965. The infection of laboratory hosts with cercariae of *Schistosoma mansoni* and the recovery of the adult worms. Parasitology 55, 695–700.

Sorimachi, H., Ishiura, S., Suzuki, K., 1997. Structure and physiological function of calpains. The Biochemical Journal 328, 721–732.

Stirewalt, M.A., Austin, B.E., 1973. Collection of secreted protease from the preacetabular glands of cercariae of *Schistosoma mansoni*. The Journal of Parasitology 59, 741–743.

Wilson, R.A., Coulson, P.S., Dixon, B., 1986. Migration of the schistosomula of *Schistosoma mansoni* in mice vaccinated with radiation-attenuated cercariae, and normal mice: an attempt to identify the timing and site of parasite death. Parasitology 92, 101–116.

World Health Organization, 1998. The world health report 1997. World Health Organization, Geneva, Switzerland.

Zhang, R., Suzuki, T., Takahashi, S., Yoshida, A., Kawaguchi, H., Maruyama, H., Yabu, Y., Fu, J., Shirai, T., Ohta, N., 2000. Cloning and molecular characterization of calpain, a calcium-activated neutral proteinase, from different strains of *Schistosoma japonicum*. Parasitology International 48, 232–242.

Zhang, R., Yoshida, A., Kumagai, T., Kawaguchi, H., Maruyama, H., Suzuki, T., Itoh, M., El-Malky, M., Ohta, N., 2001. Vaccination with calpain induces a Th1-biased protective immune response against *Schistosoma japonicum*. Infection and Immunity 69, 386–391.

ELSEVIER

Journal of Hepatology 42 (2005) 47–53

Journal of Hepatology

www.elsevier.com/locate/jhep

Molecular evolutionary analyses implicate injection treatment for schistosomiasis in the initial hepatitis C epidemics in Japan

Yasuhito Tanaka[1], Kousuke Hanada[2], Etsuro Orito[3], Yoshihiro Akahane[4], Kazuaki Chayama[5], Hiroshi Yoshizawa[6], Michio Sata[7], Nobuo Ohta[8], Yuzo Miyakawa[9], Takashi Gojobori[2], Masashi Mizokami[1,*]

[1]*Department of Clinical Molecular Informative Medicine, Nagoya City University Graduate School of Medical Sciences, Kawasumi, Mizuho, Nagoya 467-8601, Japan*

[2]*Center for Information Biology, National Institutes of Genetics, Mishima, Japan*

[3]*Department of Internal Medicine and Molecular Science, Nagoya City University Graduate School of Medical Sciences, Kawasumi, Mizuho, Nagoya 467-8601, Japan*

[4]*First Department of Internal Medicine, Faculty of Medicine, University of Yamanashi, Yamanashi, Japan*

[5]*Department of Medicine and Molecular Science, Hiroshima University Graduate School of Biomedical Sciences, School of Medicine, Hiroshima, Japan*

[6]*Department of Infectious Disease and Control, Hiroshima University Graduate School of Biomedical Sciences, School of Medicine, Hiroshima, Japan*

[7]*Second Department of Internal Medicine, Kurume University School of Medicine, Fukuoka, Japan*

[8]*Department of Molecular Parasitology, Nagoya City University Graduate School of Medical Sciences, Kawasumi, Mizuho, Nagoya 467-8601, Japan*

[9]*Miyakawa Memorial Research Foundation, Tokyo, Japan*

***Background/Aims*: The mortality due to hepatocellular carcinoma (HCC) has ranged widely in various areas of Japan since 30 years ago and the incidence was particularly high in once *Schistosoma japonicum* (*Sj*)-endemic areas. Our aim was to estimate the spread time of hepatitis C virus (HCV) infection in the past with possible relevance to a higher incidence of HCC in once *Sj*-endemic than *Sj*-nonendemic areas.**

***Methods*: During 2001, 131 strains of HCV-1b were obtained from patients in three previously *Sj*-endemic areas, as well as *Sj*-nonendemic areas in Japan and a cross-sectional study was conducted on them with molecular evolutionary analyses.**

***Results*: A phylogenetic tree reconstructed on HCV-1b sequences in the NS5B region disclosed 2 independent clusters for *Sj*-positive and -negative groups with a high bootstrap value. The estimated effective number of HCV-infections indicated a transition from quiescence to rapid exponential growth in the 1920s among patients with schistosomiasis, which is 20 years earlier than that among patients without schistosomiasis.**

***Conclusions*: The estimated spread time in previously *Sj*-endemic areas in Japan coincides with injection treatment for *Sj* since 1921. A high incidence of HCC there would be attributed to a long duration of HCV infection since 1920s.**

***Keywords*: Hepatitis C virus; *Schistosoma japonicum*; Molecular evolutionary analysis; Hepatocellular carcinoma**

1. Introduction

Recently, the molecular clock has been successfully applied to long-term serial serum samples containing hepatitis C virus (HCV) from the US and Japan and estimated the spread time of HCV in the 1930s in Japan, which is 30 years earlier than that in the US in the 1960s [1]. Insofar as a long duration of HCV infection is the most important factor for the development of hepatocellular

Received 17 June 2004; received in revised form 1 September 2004; accepted 6 September 2004; available online 22 October 2004

* Corresponding author. Tel.: +81 52 853 8292; fax: +81 52 842 0021.
E-mail address: mizokami@med.nagoya-cu.ac.jp (M. Mizokami).

Abbreviations HCV, hepatitis C virus; Anti-HCV, antibody to HCV; HCC, hepatocellular carcinoma; *Sj*, *Schistosoma japonicum*.

0168-8278/$30.00
doi:10.1016/j.jhep.2004.09.023

carcinoma (HCC), it can be predicted that the incidence of HCC will increase in the US over the next 2–3 decades. Thus, a combination of classical epidemiological approaches and molecular evolutionary analyses would be particularly useful in the study of contagious diseases, typified by HCV infection.

The way how individuals contracted HCV infection has remained unclear in Japan. Recently, a Japanese report (Ministry of Health, Labour and Welfare: Distribution of age-adjusted mortality rate from liver cancer by prefecture between 1971 and 1975, Tokyo, 2001) indicated that the mortality due to HCC has already varied widely in various areas of Japan since 30 years ago; the incidence of HCC was much higher in Saga/Fukuoka, Hiroshima and Yamanashi Prefectures, which were once endemic for schistosomiasis japonica in the long past. Hence, a high incidence of HCC in the 1970s would be related to HCV transmitted by injection treatment for *Schistosoma japonicum* (*Sj*) conducted since 1921 in these areas. In fact, shared needles and syringes for intravenous injection treatment with antimonyl potassium tartrate or sodium antimony tartrate posed a significant risk for HCV transmission in endemic areas [2]. Indeed, the prevalence of antibody to HCV (anti-HCV) is high (36.5; 95% CI=28.1–44.9%) in patients with chronic schistosomiasis [2] and therefore, HCV infection is considered responsible for the development of HCC in patients with chronic schistosomiasis.

Since, once popular intravenous injection for schistosomiasis was a risk factor for HCV transmission, the spread time of HCV in the areas once endemic for *Sj* in Japan would deserve determination. In this study, molecular evolutionary analyses using principles of both population genetics and mathematical epidemiology [3] were applied to HCV-infected patients with and without a past history of chronic schistosomiasis in once *Sj*-endemic areas.

2. Meterials and methods

2.1. Sample collection

In Japan during 2001, 181 random serum samples positive for anti-HCV were obtained from patients with chronic liver disease in widely separated areas previously endemic for *Sj*, including Kofu in Yamanashi ($n=75$), Katayama in Hiroshima ($n=50$) and Chikugo in Saga/Fukuoka Prefectures ($n=56$). Schistosomiasis was diagnosed by ultrasonographic (US) and/or computer tomographic (CT) modalities or serological examinations [4]. Two kinds of serological tests, which can detect past history of schistosomiasis, were available in this study. In brief, IgG antibodies binding to two different *schistosome antigens, Sj* adult worm antigen and *Sj* egg antigen, were detected using an enzyme-linked immunosorbent assay (ELISA). As it is now accepted that ELISA titer of egg antigen-specific IgG is reliable for case-detection rather than IgG for adult worm antigen [4–6], the results based on the egg antigen-specific IgG were accepted in this study. Samples of more than 0.25 of optical density at 415 nm were determined to be positive, as previously confirmed [4–6]. The serum samples were tested for anti-HCV by Lumipulse II Ortho HCV (Ortho-Clinical Diagnostics K.K., Tokyo, Japan). As patients with *Sj* treatments were estimated to be old, relatively older patients were selected in the *Sj*-endemic areas to match age factor that might influence duration of HCV infection or HCC incidence. For a cross-sectional study, 30 serum samples were obtained from patients infected with HCV in Aichi Prefecture where *Sj* has not been endemic. The age- and sex- matced patients were also selected from the *Sj*-nonendemic areas excluding influece of these factors on HCC incidence. The study protocol conformed to the 1975 Declaration of Helsinki and was approved by Ethic Committees of institutions. Every patient gave a written informed consent to participate in the virological research of HCV. Information of injection treatment for *Sj* was obtained by means of self-administrated questionnaires or structured interviews. None had been treated with interferon therapy for HCV infection. HCC incidence was estimated by historical information from patients themselves and/or medical records during 2001. HCC was diagnosed by liver biopsy or combination of imaging modalities such as US, enhanced CT and angiography.

2.2. Genotyping and sequencing

Nucleic acids were extracted using a SepaGean RV-R Nucleic acid extracting kit (Sanko Junyaku Co., Ltd., Tokyo, Japan) in accordance with the manufacturer's protocol. They were reverse-transcribed to cDNA using SuperScript II Rnase H^- Reverse Transcriptase (Invirogen Corp., Carlsbad, California, USA) and random hexamer primer (Takara Shuzo Co. Ltd, Tokyo, Japan) by the method described previously [7].

A sequence spanning 339 nucleotides (nt) in the NS5B region was amplified by polymerase chain reaction (PCR) with primers described previously [1]. PCR products were directly sequenced with Prism Big Dye (Applied Biosystems, Foster City, California, USA) in an ABI 3100 DNA automated sequencer. To reduce the number of artificial substitutions arising in PCR, PLATINUM Pfx DNA Polymerase (Invirogen Corp.) with a very high fidelity was used. The sequences determined were utilized to confirm HCV genotypes and construct phylogenetic trees.

2.3. Test for clustering between Sj-positive and -negative groups

The phylogenetic tree was first constructed to examine the evolutionary history for *Sj*-positive and *Sj*-negative groups by the neighbor joining method [8]. Furthermore, to test whether either *Sj*-positive or *Sj*-negative group have evolved independently or not, we conducted an interior branch test for the neighbor-joining tree [9]. Thereafter, a *t*-test was conducted for the interior branch length and its standard error, which is computed using the bootstrap procedure.

2.4. Demographic model

A reconstructed tree was built on the NS5B sequence of 339 nt by a heuristic maximum-likelihood topology search with stepwise-addition and the nearest neighbor-interchange algorithms. Tree likelihood scores were calculated using HKY85 with the molecular clock enforced by PAUP version 4.0b8.

As estimates of the demographic history, a nonparametric function $N(t)$, known also as the skyline plot, was obtained by transforming coalescent intervals of an observed genealogy into a piecewise plot that represents an effective number of infections through time [3,10]. A parametric maximum-likelihood was estimated by several models with the computer software Genie v3.5 to build a statistical framework for inferring the demographic history of a population on phylogenies reconstructed on sampled DNA sequences [10]. This model assumes a continuous epidemic process in which the viral transmission parameters remain constant through time. Model fitting was evaluated by likelihood ratio tests of the parametric maximum-likelihood estimates [11,12].

2.5. Statistical method

Data for continuous variables were demonstrated as the mean ± standard deviation. The Fishers' exact test, Chi square test with Yates' correction and one-way ANOVA followed by the Scheffe's multiple comparison test were used to evaluate differences in the mean age, sex ratio

and incidence of HCC between groups, respectively. Differences with P values less than 0.05 were considered significant.

3. Results

Of 181 anti-HCV positive samples, 113 were classified into HCV genotype 1b (HCV-1b), which is predominant in Japan. Fifty-two of 181 samples (29%) were negative for HCV RNA or incomplete for sequencing and the remaining 16 samples (9%) of genotype 2a were excluded in this study due to a minor population. Of the HCV-1b strains, 47 were recovered from patients in Yamanashi, 31 in Hiroshima and 35 in Saga/Fukuoka Prefectures. Along with 18 HCV-1b strains in Aichi Prefecture serving as controls, a cross-sectional study was conducted on them with molecular evolutionary analyses. The patients in areas previously endemic for *Sj* revealed a significantly higher prevalence of chronic schistosomiasis [24/47 (51%) in Yamanashi (Kofu area), 21/31 (68%) in Hiroshima (Katayama area) and 19/35 (54%) in Saga/Fukuoka (Chikugo area)] than that in Aichi Prefecture (0/18 [0%], $P<0.0001$). There were no significant differences in the mean age or sex ratio among patients from these four areas (Fig. 1). Although the mean age of *Sj*-positive patients was just higher than that of *Sj*-negative patients in once *Sj*-endemic areas or matched-control patients in Aichi Prefecture, there were also no significant differences between these groups (Table 1).

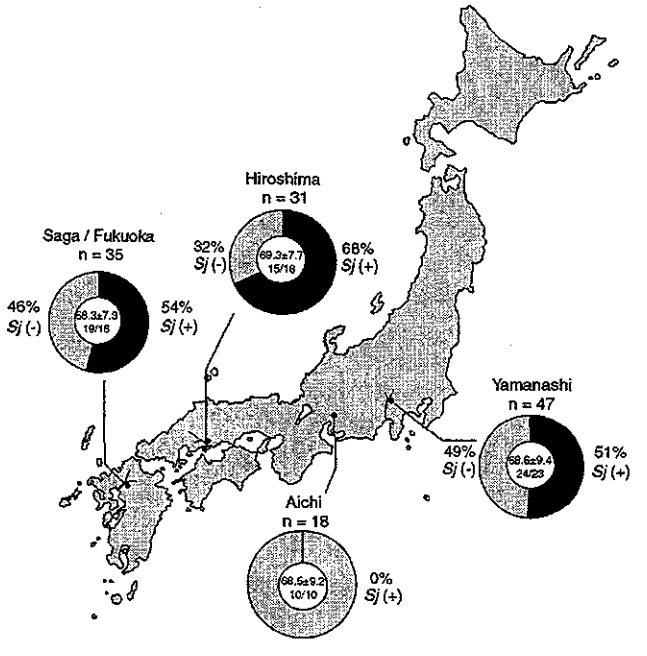

Fig. 1. Geographic distribution of *Schistosoma japonicum* (*Sj*) and characteristics of patients infected with HCV. *Sj* (+) and *Sj* (−) denote, respectively, presence and absence of infection with *Sj* diagnosed by ultrasonographic and/or computer tomographic methods or serological examinations. Pie graphs include the age (mean± standard deviation) and sex ratio (male/female).

Table 1
Characteristics of patients with and without schistosomiasis

	Schistosoma japonicum		Controls
	Positive ($n=64$)	Negative ($n=49$)	(Aichi) ($n=18$)
Mean age			
Total	69.9±7.7	67.4±8.7	66.5±9.2
Yamanashi	69.9±7.2	67.3±11.2	
Hiroshima	71.2±8.7	67.6±6.5	
Saga/Fukuoka	69.0±7.7	67.5±7.1	
Sex (male/female)			
Total	34/30	24/25	9/9
Yamanashi	13/11	11/12	
Hiroshima	10/11	5/5	
Saga/Fukuoka	11/8	8/8	
Incidence of HCC	25/55 (45%)	11/48 (23%)	3/18 (17%)

The incidence of HCC in *Sj*-positive patients was significantly higher than that in *Sj*-negative patients ($P=0.0226$) or controls ($P=0.0488$).
Abbreviations: HCC, hepatocellular carcinoma.

For cross-sectional study on the viral population size between HCV-infected patients with and without a past history of schistosomiasis, a phylogenetic tree for HCV-1b strains in the *Sj*-positive and -negative patients was constructed with use of the maximum-likelihood method enforced by the molecular clock as introduced in our previous report [1] and an independent study by Pybus et al. [3]; a substitution rate of 5.3×10^{-4} per site per year [1,3] was assumed for HCV. The phylogenetic tree disclosed 2 independent clusters for *Sj*-positive and -negative groups, with a high bootstrap value (81%) by the interior branch testing (Fig. 2), which is comparative with past epidemiological backgrounds in Japan. From distinct evolutionary histories in the two populations, the effective number of HCV-1b infections through time, $N(t)$, were assessed by the skyline plot. The parameters for several models in Genie v3.5 [3,10] were also examined. Time t was then transformed to year using the same rate, assuming the collecting time (year 2001) as the present. Fig. 3 shows the skyline plots and population growth for *Sj*-positive and -negative patients, according to a specific demographic model in Genie v3.5 with three parameters, piecewise expansion growth model, that was evaluated by the likelihood ratio testing [11,12]. Molecular evolutionary results thus obtained supported our previous study in which the divergence time of the most recent common ancestor of HCV-1b in each area in Japan was estimated before 1850 [1]. Our estimates of the effective number of HCV-infections showed a transition from constant size to rapid exponential growth in the 1920s among patients with chronic schistosomiasis in endemic areas, which is 20 years earlier than that among patients without schistosomiasis in the 1940s. Information on HCC was available for 121 of the 131 patients with HCV-1b. Although they were relatively small in number, the incidence of HCC was significantly higher in *Sj*-positive than -negative patients ($P=0.0226$) or controls ($P=0.0488$) (Table 1).

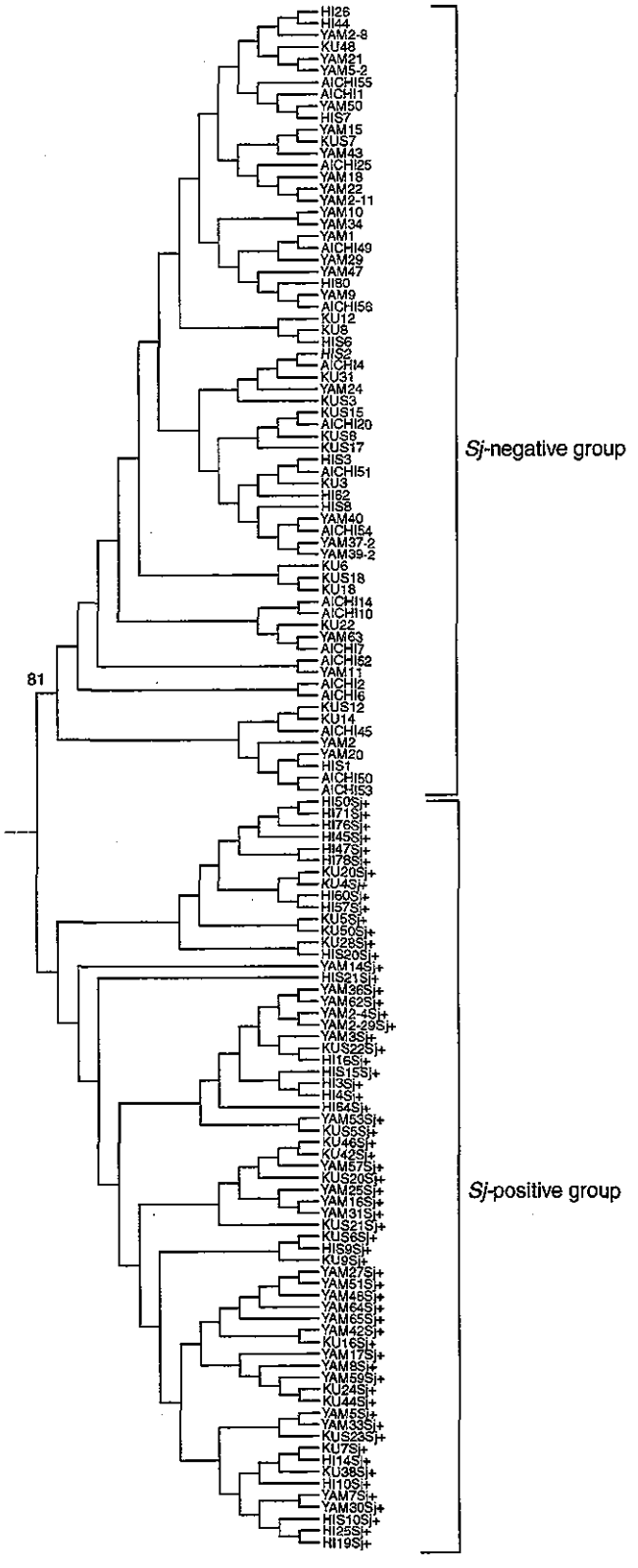

Fig. 2. A phylogenetic tree constructed on NS5B sequences of HCV-1b strains in *Schistosoma japonicum* (*Sj*)-positive ($n=64$) and -negative ($n=67$) groups. The numbers in the tree indicate bootstrap reliability by the interior branch test. *Sj*+ indicates *Sj*-positive strains. YAM; Yamanashi, HI/HIS; Hiroshima, KU/KUS; Saga/Fukuoka, Aichi; control strains.

4. Discussion

The specific demographic model based on the neutral theory [3,11,12], which has a constant size in the past and changes to exponential growth until the present, is applied to investigate the Japanese endemic of HCV. By means of the molecular evolutionary analyses, the spread time of HCV in *Sj*-positive patients was estimated 20 years earlier than that in *Sj*-negative patients from three areas in Japan where *Sj* was previously endemic (Yamanashi, Hiroshima, Saga/Fukuoka Prefectures). The spread time of HCV much earlier in *Sj*-positive than -negative patients indicates that the previous intravenous injection treatment with antimony compounds (antimonyl potassium tartarate or antimony sodium tartarate) on patients with schistosomiasis since 1921 [2] would have been a significant risk factor for HCV transmission in endemic areas through re-used needles and syringes. Indeed, it might be possible that HCV transmission from *Sj*-positive patients to *Sj*-negative patients occurs in the once *Sj*-endemic areas, but we could not find such strains in this study. One of the reasons is that residents in the village around the river, where schistosomiasis had been the most prevalent, might have been isolated from those in the other areas of the same Prefecture in the past due to the endemic disease 'schistosomiasis'. Interestingly, most Japanese strains from *Sj*-nonendemic areas in the database clustered with the *Sj*-negative group of the present study. Hence, factors other than the injection treatment for *Sj*, such as intravenous stimulants popular during and after World War II [13] and medical treatments including transfusion with blood units from paid donors in the past, would have imposed the risk for HCV transmission in most areas in Japan [14]. In addition, there would have been opportunities for HCV transmission through inadequately sterilized needles and syringes in general practices, which have contributed to a large reservoir of chronic HCV infection in Japan during the 1950s [13]. Such inadequately sterilized medical injections were still common in the less-developed world in the 20th century. WHO estimates that unsafe injections result in 2.3–4.7 million new HCV infections worldwide every year [15].

Although the spread time of HCV in *Sj*-positive group was earlier than that in *Sj*-negative group, there was no significant difference of mean age between the 2 groups. Two possibilities are considered. One is a sampling bias; as patients with *Sj* treatments were estimated to be old, relatively older patients were selected in the *Sj*-endemic areas to match age factor that might influence duration of HCV infection or HCC incidence. Second, the ages that patients had been infected with HCV were different between the 2 groups; the treatments for *Sj* in Japan were mainly conducted among relatively younger people including school children after screening of *Sj* [4,16,17], while the

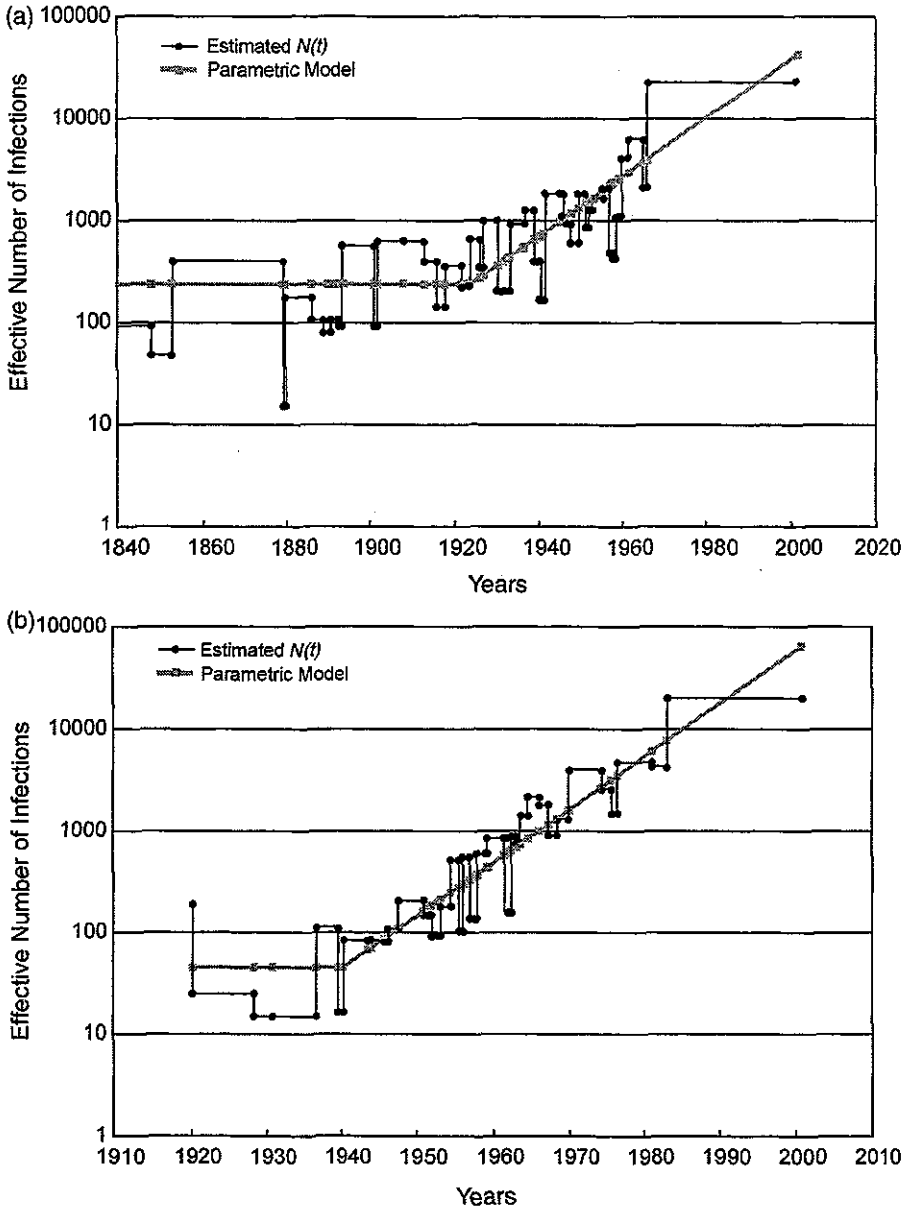

Fig. 3. The maximum-likelihood estimates of $N(t)$ on the effective number of infections with HCV genotype 1b in Japan for *Schistosoma japonicum* (*Sj*)-positive group (a) and *Sj*-negative group (b) separated in the phylogenetic tree (Fig. 2). The parametric model is indicated by the grey line and stepwise plots by the black line that represents corresponding nonparametric estimates of $N(t)$ (number as a function of time). Genetic distances are transformed into a time scale of year using estimates of the molecular clock in the NS5B region.

other risk factors such as blood transfusion were found in older people excluding at least children.

A disease possibly caused by schistosomal infection in Japan is documented in a book written some 300 years ago. In 1847, the clinical picture of this disease was precisely described by Yoshinao Fujii in the book 'Katayama-ki' that documented an endemic disease in Katayama area as Katayama's disease (equivalent to schistosomiasis). Water-borne epidemics of schistosomiasis prevailed in inhabitants around rivers (the tributaries of the Fuji river in Yamanashi, the Takaya river in Hiroshima and the Chikugo river in Saga/Fukuoka) in Japan, mediated by small shellfish (Miyairi-kai) serving as the natural host. More than 200,000 individuals were estimated to have been infected with *Sj* in Yamanashi Prefecture alone during 1965 through 1990 [16] and approximately 1,000,000 patients in the entire Japan since 1920s [17]. To cope with these endemics, more than 10 million intravenous injections with antimony compounds had been given in Japan since 1921 [17]. Thus, Japan would have started ahead of any other countries, in terms of HCV spread in association with schistosomiasis, wherein intravenous drugs were invented. Although acute schistosomal infection has disappeared in Japan since long ago, there are still elderly people with

chronic schistosomiasis in previously endemic areas, some of whom are developing HCC [2,14]. Substantial transmission among regions is supported by the lack of regional clustering of HCV sequences in this study.

A similar situation is reported in the Nile delta in Egypt where schistosomiasis once prevailed mediated by small shellfish [18] and the national campaigns for injection treatment with antimonyl potassium tartarate (tartar emetic) from the 1961 until 1986 are suspected to have given rise to the highest endemicity of HCV in the world ever, involving >20% of the national population there [19]. The prevalence of anti-HCV is extremely high (>70%) in patients with schistosomiasis there [18,20,21]. Highly prevalent HCV infection in the general Egyptian population accounts for most HCC cases in Egypt [22]. A question may arise whether schistosomiasis alone is responsible for the development of HCC. Patients co-infected with HCV and *Schistosoma mansoni* (*Sm*) may have a high incidence of viral persistence, accelerated fibrosis and development of HCC [23,24]. A recent population-based study between two large populations with district histories of *Sm* and hepatitis C infections, however, failed to indicate any interaction between *Sm* infection and the prevalence or severity of hepatitis C [25]. Moreover, no significant histological differences were found between anti-HCV-positive Egyptian patients with and without schistosoma [26]. Hence, the long duration of persistent HCV infection would be a more important factor for the development of HCC than the pathogeneticity of *Sm* itself.

Estimating the effective number of HCV infections has been very informative in looking back epidemic spreads of HCV infection in the Unites States [1] and Egypt [12,27]. In addition, it would also be useful in predicting the population size and extent of HCV infection. Studies to foresee future spreads of HCV would be required to cope with and prevent healthcare problems where de novo infections are increasing. The advantage of molecular evolutionary analyses, its ability to accurately estimate the dynamics of HCV based on a limited number of isolates in particular [3], will extend its application anywhere in the world where clinical sequelae of persistent HCV infection pose increasing burdens on the public health of nations.

In conclusion, the evolutionary analyses indicated that the estimated spread time in previously *Sj*-endemic areas in Japan coincides with injection treatment for *Sj* conducted since 1921. The high incidence of HCC in *Sj*-endemic areas is most likely attributed to long duration of HCV infection there transmitted through injection treatments.

Acknowledgements

We greatly appreciate Dr Oliver G. Pybus (Department of Zoology, University of Oxford, Oxford, UK) for his enlightening advice on molecular evolutionary analyses using Genie v3.5. We thank Ms Yoshiko Kobayashi (Nagoya City University Graduate School of Medical Sciences, Nagoya, Japan) for technical assistance and Dr Tatsuya Ide (Kurume University School of Medicine, Fukuoka, Japan) and Dr Hirotaka Kono (Hiroshima University Graduate School of Biomedical Sciences, School of Medicine, Hiroshima, Japan) for providing us with valuable materials. The work was supported by a grant-in-aid from the Ministry of Health, Labour and Welfare of Japan (H13-kanen-2), grants-in-aid for Young Scientists (A) from the Ministry of Education, Culture, Science and Sports of Japan (16689016), The Hori Information Science Promotion Foundation and Miyakawa Memorial Research Foundation.

References

[1] Tanaka Y, Hanada K, Mizokami M, Yeo AE, Shih JW, Gojobori T, et al. Inaugural article: a comparison of the molecular clock of hepatitis C virus in the United States and Japan predicts that hepatocellular carcinoma incidence in the United States will increase over the next two decades. Proc Natl Acad Sci USA 2002;99: 15584–15589.

[2] Iida F, Iida R, Kamijo H, Takaso K, Miyazaki Y, Funabashi W, et al. Chronic Japanese schistosomiasis and hepatocellular carcinoma: ten years of follow-up in yamanashi prefecture, Japan. Bull World Health Organ 1999;77:573–581.

[3] Pybus OG, Charleston MA, Gupta S, Rambaut A, Holmes EC, Harvey PH. The epidemic behavior of the hepatitis C virus. Science 2001;292:2323–2325.

[4] Minai M, Hosaka Y, Ohta N. Historical view of schistosomiasis japonica in Japan: implementation and evaluation of disease-control strategies in yamanashi prefecture. Parasitol Int 2003;52: 321–326.

[5] Matsuda H, Tanaka H, Blas BL, Nosenas JS, Tokawa T, Ohsawa S. Evaluation of ELISA with ABTS, 2-2′-azino-di-(3-ethylbenzthiazoline sulfonic acid), as the substrate of peroxidase and its application to the diagnosis of schistosomiasis. Jpn J Exp Med 1984;54:131–138.

[6] Matsumoto J, Kirinoki M, Kawai S, Chigusa Y, Ilagan EJ, Ducusin BE, et al. Prevalence of schistosomiasis japonica among schoolchildren and animal reservoirs in oriental mindoro, Philippines. Jpn J Trop Med Hyg 1999;27:175–180.

[7] Ohno T, Mizokami M, Wu RR, Saleh MG, Ohba K, Orito E, et al. New hepatitis C virus (HCV) genotyping system that allows for identification of HCV genotypes 1a, 1b, 2a, 2b, 3a, 3b, 4, 5a and 6a. J Clin Microbiol 1997;35:201–207.

[8] Saitou N, Nei M. The neighbor-joining method: a new method for reconstructing phylogenetic trees. Mol Biol Evol 1987;4:406–425.

[9] Dopazo J. Estimating errors and confidence intervals for branch lengths in phylogenetic trees by a bootstrap approach. J Mol Evol 1994;38:300–304.

[10] Pybus OG, Rambaut A. GENIE: estimating demographic history from molecular phylogenies. Bioinformatics 2002;18:1404–1405.

[11] Lemey P, Pybus OG, Wang B, Saksena NK, Salemi M, Vandamme AM. Tracing the origin and history of the HIV-2 epidemic. Proc Natl Acad Sci USA 2003;100:6588–6592.

[12] Pybus OG, Drummond AJ, Nakano T, Robertson BH, Rambaut A. The epidemiology and iatrogenic transmission of hepatitis C virus in egypt: a Bayesian coalescent approach. Mol Biol Evol 2003;20: 381–387.

[13] Fukui S, Wada K, Iyo M. History and current use of methamphetamine in Japan. Proceedings of Japan–US Scientific

Symposium '90 on Drug Dependence and Abuse; 7–8 September 1990. Tokyo, Japan: Cocaine and methamphetamine behavioral toxicology, clinical psychiatry and epidemiology; 1991 [p. 219–37].
[14] Yoshizawa H. Hepatocellular carcinoma associated with hepatitis C virus infection in Japan: projection to other countries in the foreseeable future. Oncology 2002;62:8–17.
[15] Drucker E, Alcabes PG, Marx PA. The injection century: massive unsterile injections and the emergence of human pathogens. Lancet 2001;358:1989–1992.
[16] Kitani K, Iuchi M. Schistosomiasis japonica: a vanishing endemic in Japan. J Gastroenterol Hepatol 1990;5:160–172.
[17] Okabe K. Epidemiology of schistosomiasis japonica. Kurume Med J 1975;38:11–55.
[18] Kamel MA, Ghaffar YA, Wasef MA, Wright M, Clark LC, Miller FD. High HCV prevalence in Egyptian blood donors. Lancet 1992;340:427.
[19] Cohen J. The scientific challenge of hepatitis C. Science 1999;285: 26–30.
[20] Hibbs RG, Corwin AL, Hassan NF, Kamel M, Darwish M, Edelman R, et al. The epidemiology of antibody to hepatitis C in Egypt. J Infect Dis 1993;168:789–790.
[21] Frank C, Mohamed MK, Strickland GT, Lavanchy D, Arthur RR, Magder LS, et al. The role of parenteral antischistosomal therapy in the spread of hepatitis C virus in Egypt. Lancet 2000;355:887–891.
[22] Hassan MM, Zaghloul AS, El-Serag HB, Soliman O, Patt YZ, Chappell CL, et al. The role of hepatitis C in hepatocellular carcinoma: a case control study among Egyptian patients. J Clin Gastroenterol 2001;33:123–126.
[23] Angelico M, Renganathan E, Gandin C, Fathy M, Profili MC, Refai W, et al. Chronic liver disease in the Alexandria governorate, Egypt: contribution of schistosomiasis and hepatitis virus infections. J Hepatol 1997;26:236–243.
[24] Kamal S, Madwar M, Bianchi L, Tawil AE, Fawzy R, Peters T, et al. Clinical, virological and histopathological features: long-term follow-up in patients with chronic hepatitis C co-infected with S. mansoni. Liver 2000;20:281–289.
[25] Blanton RE, Salam EA, Kariuki HC, Magak P, Silva LK, Muchiri EM, et al. Population-based differences in *Schistosoma mansoni*- and hepatitis C-induced disease. J Infect Dis 2002;185: 1644–1649.
[26] Helal T, Danial M, Ahmed H. The relationship between hepatitis C virus and schistosomiasis: histopathologic evaluation of liver biopsy specimens. Hum Pathol 1998;29:743–749.
[27] Tanaka Y, Agha S, Saudy N, Kurbanov F, Orito E, Kato T, et al. Exponential spread of hepatitis C virus genotype 4a in Egypt. J Mol Evol 2004;58:191–195.

Detection of *S. japonicum* a1 gene by polymerase chain reaction

By

Lu Shaohong[1,2], Shigeo Otsuki[1], Hajime Matsuda[3], Kenji Hirayama[4], He Yongkan[5] & Nobuo Ohta[1]

[1]Department of Molecular Parasitology, Nagoya City University Graduate School of Medical Sciences, Nagoya 467-8601, Japan

[2]Institute of Parasitic Diseases, Zhejiang Academy of Medical Sciences, Hangzhou 310013, China

[3]Department of Tropical Diseases and Parasitology, Dokkyo Medical College, Tochigi 321-0293, Japan

[4]Department of Molecular Immunogenetics, Institute of Tropical Medicine, Nagasaki University, Nagasaki 852-8523, Japan

[5]Hunan Institute of Parasitic Diseases, Yueyang, China

Correspondings:

Nobuo Ohta, Department of Molecular Parasitology, Nagoya City University Graduate School of Medical Sciences, Nagoya 467-8601, Japan

Key words: *Schistosoma japonicum*, PCR, *Sjα1*, Diagnosis

Abstract: We developed an effective PCR method to amplify *S. japonicum* a1 gene. Specific amplification of a 420 bp DNA fragment was achieved by using nest-PCR, the detection limit was 0.1fg when genomic DNA was used as template, no specific PCR product was obtained with DNA from *S. mansoni.* The detection of Sja1 from serum or blood samples of infected mice was successful, it is prospective to amplify *S. japonicum* DNA in human samples.

Introduction:

Schistosomiasis continues to be a serious problem of public health. Up today available diagnostic methods are far from ideal, stool examination by Kato-Katz lacks sensitivity especially in low prevalence, and the method also leads to frequently misdiagnosed cases in middle or high prevalence due to highly clustered distribution of *S. japonicum* eggs in stools. The antibody detection lacks specificity because of cross-react from patients harboring intestinal nematodes, the false positive reactions of antibody detection were observed about 15 to 21%. The detection of circulating antigens (CAg) might be useful for monitoring the disease, for it may reflect more reliable than antibody titers the viability and quantity of parasites in the host, but the sensitivities of the detection of CAg are just about 60%. The circumoval precipitine test (COPT) has remained only as a confirmatory diagnostic test in spite of its high sensitivity and specificity, since it is a very labor-intensive and expensive test.

In an attempt to surpass these diagnostic limitations polymerase chain reaction (PCR) may constitute a good tool for the diagnosis of the *Schistosoma* sp. infection. LA Pontes *et al* first reported using PCR on detection of a *S. mansoni* tandem repeat DNA sequence. Although the PCR demands a more sophisticated laboratory and more complex operational effort when compared with the Kato-Katz parasitological examination, it is 10 times more sensitive than the Kato-Katz examination when detection of *S. mansoni* and it is probably more sensitive than the circulating antigen detection. In the present study we have first evaluated the possible use of the PCR to detect Sja1 element, which is a short interspersed element (SINE)-like retroposon that

occurs in high copy number (10,000) interspersed throughout the *S. japonicum* genome. The PCR method developed in this study may constitute an alternative to the available diagnostic techniques for the detection of *S. j* infection.

Materials and Methods

Preparation of DNA: DNA was purified from 200 μl of serum or whole blood using QIAamp DNA purification kit according to the manufacturer's instructions.

Amplification by PCR: Primers were designed to amplify the Sja1*:* 5'-CATAGAAGCGATGTAGTC-3'and 5'-TGGACTGAAACTGGTCAG-3' for first PCR,5'-GAAATATAAAGGAACCGGTGG-3'and5'-ATCCTAAGCGAATTGCCTC G-3' for second PCR. 1 μl of the extracted material was used as initial template. The first PCR was carried out using an initial denaturation at 94℃for 2 min, followed by 40 cycles of 94°C for 30 sec, 49°C for 20 sec, and 74°C for 30 sec. The second PCR consisted of 30 cycles of 94°C for 30 sec, 53°C for 15 sec, and 74°C for 30 sec. After amplification, 10 μl of the products was eletrophoresed on an agarose gel, the amplification products had their identity confirmed by sequencing performed.

Results:

Sensitivity of PCR. To assess the sensitivity of the PCR, *S.japonicum* adult worm DNA was quantified and tested for amplification after serial dilutions. As shown in Figure 1, Specific amplification of a 420 bp DNA fragment was achieved by nest-PCR, the minimum detectable DNA quantity was 0.1 fg.

Specificity of PCR. The specificity of the PCR was evaluated by means of equal quantities of DNA from *S. m*, *Trypanosome evansi* and *Toxoplasma gondii* under the same reaction conditions. *S. j* DNA was used as positive control for the amplification reaction. As shown in Figure 2, no specific amplification was achieved for *S. m* genomic DNA and DNA from *S. m* infected mice. No DNA amplification for *Trypanosome evansi* and *Toxoplasma gondii* (data not shown).

Detection of mice serum different time after infection. *S. j* DNA from all serum

samples including 4, 5, 8 and 11 weeks after infection with 30 cercariae were detected success. No bands were visible with negative control serum.

Detection of mice whole blood samples 4weeks after infection with different numbers of cercaria. The whole blood samples were tested to evaluate the possibility of PCR without separation of serum. Blood were taken from mice 4 weeks after infection with 10, 30 and 60 cercaria respectively, As shown in Figure 4, PCR was able to detect the *S.j* DNA in whole blood samples 4 weeks after infection with 10 cercaria. No bands were seen with the negative control serums.

Discusssion:

PCR has been acclaimed as an outstanding sensitive and specific diagnostic tool for many diseases, but this technique has not been used for demonstrating the presence of *S. japonicum* DNA.

We tested the possibility of the use of PCR for the detection of *S. japonicum* DNA in serum or whole blood samples. The high sensitivity of the assay was demonstrated by its ability to achieve amplification with minimum 0.1 fg of DNA, the PCR product can be visible simply stained with ethidium bromide instead of silver staining. The high sensitivity of the assay is due to the high copy number of the target sequence throughout the genome. And the nest-PCR method increased sensitivity and specificity. The specificity of the test was demonstrated by the absence of specific amplification when *S. mansoni* DNA and two protozoan species were used as templates. The PCR can be performed with serum or whole blood samples, so there is no need to separate the serum if centrifugation equipment is unavailable in field sample collection.

Our results showed that the PCR method developed here is successful to detect *S. japonicum* DNA starting from 4 weeks after infection, developmental period of *S. joponicum* from ceraria to mature worm is about 4 weeks, it is necessary to evaluate the PCR value before 4 weeks at the time no egg produced.

To further study the specificity of the method, other helminthic parasites such as

Ascaris, Taenia and Trichiuris need to be tested. And a kinetic profile of the DNA after treatment in a mouse model and the efficiency of the detection in human samples by PCR remain to be evaluated. The DNA amplification assay may constitute an alternative to the available diagnostic techniques for the detection of *S. japonicum* infection in the future.

References

Chitsulo,L., Engels, D., Montresor, A., Savioli, L., 2000. The global status of schistosomiasis and its control. Acta Trop 77:41-45.

Zhou J, Tao KH, Li YX, Qian WH, Zhang JH, Wang Y, Zhang ZS. 2004Detection of Schistosomia japonicum 5D gene by polymerase chain reaction and genechip technique Zhonghua Liu Xing Bing Xue Za Zhi. 25(2):154-7

Caldeira RL, Jannotti-Passos LK, Lira PM, Carvalho OS 2004 Diagnostic of Biomphalaria snails and Schistosoma mansoni: DNA obtained from traces of shell organic materials. Mem Inst Oswaldo Cruz. 99(5):499-5 02.

Hertel J, Kedves K, Hassan AH, Haberl B, Haas W. 2004Detection of Schistosoma mansoni cercariae in plankton samples by PCR. Acta Trop. 91(1):43-6.

Hatz CF. 2001The use of ultrasound in schistosomiasis. Adv Parasitol48:225-84.

Hillyer GV, Ramzy RM, El Alamy MA, Cline BL. 1980 Immunodiagnosis of infection with Schistosoma haematobium and S. mansoni in man. Am J Trop Med Hyg. 29(6):1254-7.

Brindley PJ, Laha T, McManus DP, Loukas A. 2003 Mobile genetic elements colonizing the genomes of metazoan parasites. Trends Parasitol. 19(2):79-87.

LA Pontes, E Dias-Neto, and A Rabello 2002. Detection by polymerase chain reaction of Schistosoma mansoni DNA in human serum and feces. Am J Trop Med Hyg. 66(2) :157-162

Pontes LA, Oliveira MC, Katz N, Dias-Neto E, Rabello A. 2003Comparison of a polymerase chain reaction and the Kato-Katz technique for diagnosing infection with Schistosoma mansoni Am J Trop Med Hyg. 68(6):652-6

Laha T, McManus DP, Loukas A, Brindley PJ. 2000 Sjalpha elements, short interspersed element-like retroposons bearing a hammerhead ribozyme motif from the genome of the oriental blood fluke Schistosoma japonicum. Biochim Biophys Acta. 24;1492(2-3):477-82.

Figures:

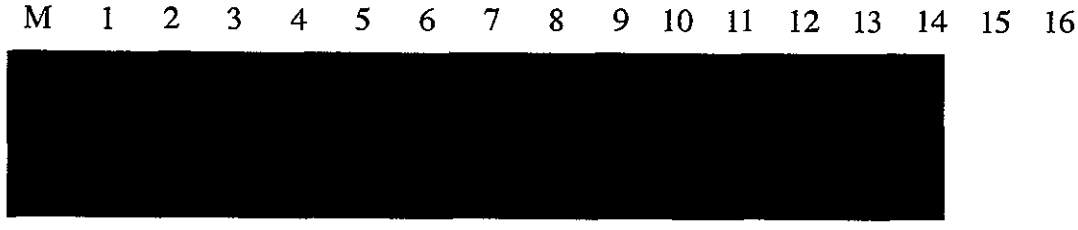

Fig. 1. Sensitivity of the PCR. 1~8: First PCR, 9~16: Second PCR. lanes 1,9: 100pg; 2,10: 10pg; 3,11: 1pg; 4,12: 100fg; 5,13: 10fg; 6,14: 1fg; 7,15: 0.1fg; 8,16: Negative

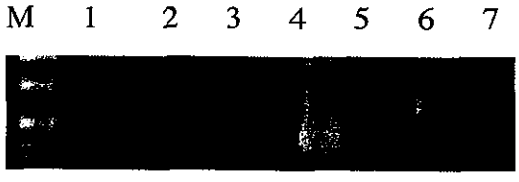

Fig. 2. Specificity of the PCR. 1~4: First PCR, 5~8: Second PCR, lanes 1,5: *S.j* genome Positive control; 2,6: negative control; 3,7: *S.m* genome: 4,8: *S.m* infected mice serum.

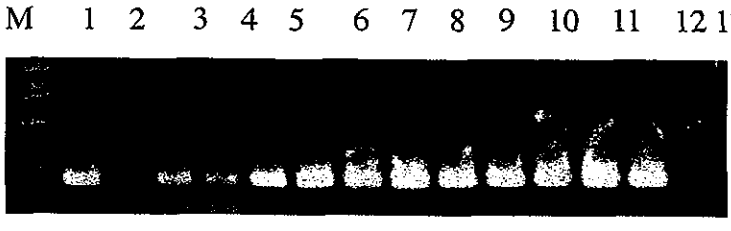

Fig. 3 Detection of mice serum different time after infection. Lanes 1, Positive control; 2: Negative control; lanes 3~5: 4 Wks after infection; lanes 6~8: 5 Wks after

infection; lanes 9~11: 8 Wks after infection; lanes 12~13: 11 Wks after infection.

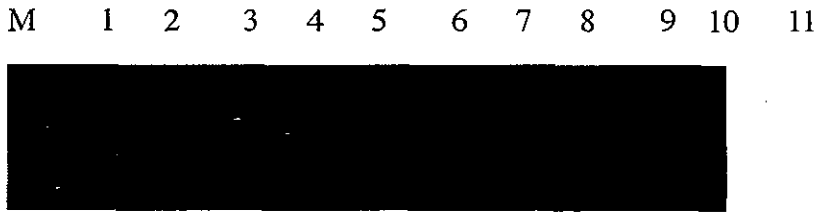

Fig. 4. Detection of mice whole blood samples 4wks after infection with different numbers of cercaria. lane1: Positive control; lane 2: Negative control; lanes 3~5: infected with 10 cercaria; lanes 6~8: infected with 30 cercaria; lanes 9~11: infected with 60 cercaria.

特集

北米・ヨーロッパでかかる寄生虫症

太田伸生　名古屋市立大学大学院医学研究科宿主・寄生体関係学分野　教授

SUMMARY

・北米・ヨーロッパでは先進国型寄生虫感染がみられる.
・最近の欧米の疾病構造の特徴から,寄生虫感染症の正確な実態把握ができていない.
・AIDSや高齢者などの日和見寄生虫感染症が多く見られる.
・発展途上国からの人間や物流など交流増加により熱帯寄生虫症も持ち込まれたり,輸血を通じた感染が起こっている.
・ミクロスポーラ症やサイクロスポーラ症などの新興寄生虫感染症のリスクは北米やヨーロッパへの渡航でも低くない.
・一部でマラリア流行の再興が起こってきた.

はじめに

日本人にとって，北米，ヨーロッパに渡航する際に寄生虫感染の可能性を考えることは少ないし，日常診療でそのような地域への渡航歴から寄生虫病を鑑別疾患に想起することは例外的な場合に限られるであろう．しかし，北米やヨーロッパでどのような寄生虫病が存在し，その感染の可能性はどの程度のものか，あるいは世界のヒトや物流の集散地として，持ち込まれた輸入病原体に邦人が感染する可能性はどうか，などの情報を把握しておくことは時に有用であろう．本稿では北米やヨーロッパに渡航する際に感染する可能性のある寄生虫病について概略を整理するとともに，最近の欧米の寄生虫感染の動向や特殊な感染経路などについて説明することを試みた．

I　北米の寄生虫症

北米とはメキシコ，アメリカ合衆国およびカナダを含むが，本稿では情報整備が進んでいるアメリカ合衆国の情況を中心に論じることにしたい．それだけでも亜熱帯から寒帯まで気候風土の異なる地域を包含しており，全体を一つに論じることは困難で，存在する寄生虫相も多様である．

ほかの先進諸国と同様に寄生虫感染症の実態は明らかでない．米国CDCではELISAによる寄生虫感染スクリーニングサービスを実施しているが，年間の依頼件数は5,000件以内で決して多くない．さらに確定診断の依頼は2,000件以下に留まっている．実際に寄生虫症が少ないのか，医師が寄生虫症を鑑別診断に考えないのかの判断はできないが，疫学調査結果によれば米国の寄生虫感染の実数が少ないとは考えられない．確実に把握されている寄生虫症を**表1**にまとめたが，注目されるものについて以下に説明を加えたい．

❶ クリプトスポリジウム症

本症は牛の腸管寄生原虫である*Cryptosporidium parvum*による代表的な水系感染症である．1993年にMilwaukee市全域で40万人以上の集団発生があり，重症下痢症による死亡者まで出た[1]．水道水からのクリプトスポリジウム検出率は17～55％に上っていたため，水道水の安全基準が見直された．しかし，**自然水の原虫汚染は依然として高く，自然公園や都市公園の噴水などから集団発生する事例が確認されている**[2]．

❷ バベシア症

マダニによって媒介されるバベシア症は，米国北東部を中心に*Babesia microti*による症例が年間数百例発生している．媒介マダニはライム病媒介種と同一であるため，米国東北部で発生したマダニ咬症では両方の検査が必要であろう．通常，重症化することはないが，免疫機能が低下した人，高齢者および脾臓摘出者では治療抵抗性となり死亡例もある．

❸ サイクロスポーラ症

新興寄生虫症であり，米国で汚染輸入フルーツ（ラズベリー）による下痢症の集団発生がみられた[3]．米国内の本原虫の分布，ヒト以外の保虫宿主の存在などよくわかっていない．

表1　欧米で感染の可能性のある寄生虫症

寄生虫症	北　米	ヨーロッパ
原虫		
マラリア	△（南部で定着？）	△（南部，ロシアで再興）
トリパノソーマ	＊（輸血でシャーガス病）	—
リーシュマニア	—	△（南西ヨーロッパ）
トキソプラズマ	◎	◎
クリプトスポリジウム	○（水道水，自然水）	○（水道水，自然水）
ランブル鞭毛虫	○（水道水，自然水）	○（水道水，自然水）
サイクロスポーラ	△（輸入食品）	？（情報なし）
ミクロスポーラ	△（日和見感染）	？（情報なし）
バベシア	△（東北部で*B. microti*）	？（*B. bovis*も？）
その他		○肉胞子虫（食肉）
蠕虫		
腸管寄生線虫	—	？（東欧で再興か）
イヌ/ネコ回虫	△（小児の感染）	？（情報なし）
旋毛虫	△（年間40例程度）	△（集団発生も）
肝蛭	—	—（畜産地域に）
広節裂頭条虫	△（カナダ，アラスカ）	△（北欧で）
エキノコックス	△（中西部以北で発生）	△（中～北部で）

◎：高頻度で見られるもの
○：感染の危険性が低くないと思われるもの
△：感染の可能性は高くないが確実に発生しているもの
—：存在しないか，ほとんど問題がないもの
＊：特殊なケースで問題となるもの