

*Parasitology in Japan*

School-health-based parasite control initiatives: extending successful Japanese policies to Asia and Africa

Somei Kojima¹, Yoshiki Aoki², Nobuo Ohta³, Seiki Tateno⁴ and Tsutomu Takeuchi⁵

¹Center for Medical Science, International University of Health and Welfare, Ohtawara, Tochigi 324-8501, Japan

²Institute of Tropical Medicine, Nagasaki University, Nagasaki 852-8523, Japan

³Department of Parasitology, Tokyo Medical and Dental University, Tokyo 113-8519, Japan

⁴Bureau of International Cooperation, International Medical Center of Japan, Tokyo 162-8655, Japan

⁵Department of Tropical Medicine and Parasitology, Keio University School of Medicine, Tokyo 160-8582, Japan

Japan controlled its major parasitic diseases by the 1970s. Based on this experience, the Government of Japan proposed the Global Parasite Control Initiative in 1998 and established three research and training centres around the world. The Asian Centre of International Parasite Control (ACIPAC) is the first such centre, and completed five years of activities focused on school-health-based parasite control in the Greater Mekong Subregion in 2005. The lessons learned and experiences gained by ACIPAC should be applied to all health promotion programmes worldwide.

'Wormy world'

Malaria and other parasitic diseases still cause a huge amount of disease and disproportionately affect the poor: in particular, impoverished communities in low-income countries. Highly debilitating, rather than deadly, worm-induced diseases such as schistosomiasis and soil-transmitted helminthiasis (STH) remain a major health problem in tropical developing countries. Today, the picture is little better than when Stoll succinctly stated the situation in the title of his article 'This wormy world' in 1947 [1]. At the turn of the millennium, infectious diseases accounted for 32% of mortality and 41% of disease worldwide [2]. Today, ~200–450 million cases of malaria occur in the world annually, causing the death of 1–3 million people, predominantly African children [3]. More than 190 million people are estimated to be infected with schistosomiasis in 76 countries and territories. Although related mortality is lower than it was five decades ago [4], there are locations where 50–70% of the population is affected by geohelminths such as *Ascaris lumbricoides*, *Necator americanus*, *Ancylostoma duodenale* and *Trichuris trichiura*, as recently reported in the Great Mekong Subregion [5].

After the end of World War II, >70% of the Japanese population was infected with intestinal parasites, with conditions in many rural parts of the country resembling those in some developing countries today. However, in the

space of two to three decades, Japan eliminated most major parasitic diseases, including malaria, filariasis, schistosomiasis and STH. This achievement was facilitated by using a school-health-based approach to gain access to the community; this approach was implemented through triangular cooperation among government agencies, community-based non-governmental organizations (NGOs) and scientific experts. The enactment of the School Health Law (1958), which included mass examination and selective mass treatment targeted at schoolchildren, and the foundation in 1955 of a specialized non-profit organization, the Japan Association of Parasite Control (JAPC), greatly contributed to successful control measures [6]. The causative parasite of schistosomiasis japonica was discovered as a result of people's awareness of the disease and their request to the local government to clarify its aetiology [7]. Interventions to help control the disease included active case detection and mass chemotherapy, periodic distribution of molluscicides to kill the snail host, storage of night soil (which causes parasite egg degradation), environmental management such as cement lining of irrigation ditches, land reclamation and control of animal reservoirs (e.g. cows, stray dogs and wild rodents) [8–10]. The achievement in Japan shows that, to achieve the goal of parasite control, a comprehensive and coordinated programme of activities is required. The organization of voluntary associations in cooperation with national and local governments is essential to educate, motivate and engage communities in nationwide self-help efforts. The scientific community also has to be fully involved to ensure the production of and the best and most cost-effective use of diagnostics, therapeutics and preventive technologies and products. In addition, the private sector has an important role [11].

At the Group of Eight (G8) summits in Denver (USA; 1997) and Birmingham (UK; 1998), the late R. Hashimoto, Prime Minister of Japan at the time, emphasized the importance of parasitic-disease control as a means of improving public health, and stated the necessity for strengthening international cooperation towards global parasite control. Based on a report [12], the Government

Corresponding author: Kojima, S. (skojima@iuhw.ac.jp).
Available online 21 December 2006.

Box 1. Relevant websites

ACIPAC: <http://www.tmd.ac.jp/med/mzoo/acipac/index.html>
 European Commission: <http://www.europa.eu>
 Japanese Society of Parasitology: <http://jsp.tm.nagasaki-u.ac.jp/~parasite/>
 JICA: <http://www.jica.go.jp>
 Kenan Institute: <http://www.kiasia.org>
 Kenya Medical Research Institute: <http://www.kemri.org>
 Mahidol University: <http://www.mahidol.ac.th>
 Ministry of Foreign Affairs, Japan, Health and Development Initiative (2005): http://www.mofa.go.jp/policy/health_c/forum0506/hdi/pdf
 Noguchi Memorial Institute for Medical Research: <http://www.noguchimedres.org>
 Partnership for Child Development: <http://www.child-development.org>
 UNESCO: <http://www.unesco.org>
 UNICEF: <http://www.unicef.org>
 WHO: <http://www.who.int>

of Japan proposed to establish three centres for research and training, one in Asia and two in Africa. This was known as the Hashimoto Initiative (HI).

The Asian Centre of International Parasite Control (ACIPAC) (Box 1) was established in 2000 as a bilateral technical cooperation project in connection with the region-wide work of the Japan International Cooperation Agency (JICA) and in collaboration with Mahidol University and the Ministry of Public Health, Thailand. A further two centres were established in Africa, one in the Kenya Medical Research Institute, Kenya, and the second in the Noguchi Memorial Institute for Medical Research, University of Ghana, Ghana.

The ACIPAC approach

Mortality due to malaria is concentrated in sub-Saharan Africa, and the majority of deaths occur in children under five years of age. At the moment, treatment of children above this age is considered to be of secondary importance. However, in malaria-endemic areas, most children are infected with malaria parasites and, because they might not develop disease or because they exhibit only mild symptoms, continue to attend school; therefore, these children contribute to disease transmission. Thus, control measures aimed at school-age children should be effective at helping to prevent malaria transmission across the community.

In the south of Thailand, ~20–30% of schoolchildren are infected with STH (primarily ancylostomiasis) [13], whereas ~60% of schoolchildren in the mountainous region of the northern provinces are infected [14]. In neighbouring countries, morbidity is also high: for example, 70% for ascariasis and 86% for ancylostomiasis in Cambodia. A similar situation is observed worldwide. This demonstrates the impact of STH, not only on the health of schoolchildren but also on their education. In addition, these children might be a source of infection in the community. A cross-sectional study conducted in an area of southern Thailand revealed that schoolchildren with less knowledge of STH are likely to be infected more quickly and that boys, who dislike wearing shoes, have a higher intensity of hookworm infection than do girls [15].

Various health education programmes (including the prevention of malaria and other infectious diseases) that combine visible and easy-to-understand control measures are required as part of a successful strategy for parasite control. Although health education is not a tool with an immediate impact, it can have long-term benefits. Besides strengthening manpower in the health sector by mobilizing schoolteachers (e.g. to improve health education and to help administer anthelmintics), it is also useful to develop cooperative relationships between different ministries and sectors.

The ACIPAC mission and activities

ACIPAC started operations in 2000 with the overall goal of creating parasite control programmes, strengthened by human health resource development, in Southeast Asia. The outline of ACIPAC activities carried out during the past five years is summarized next.

The school-based approach advocated by ACIPAC is effective for parasite control in the region

ACIPAC advocated and promoted the school-based approach through international training courses, symposia and workshops. ACIPAC put an emphasis on the concept that schoolchildren should be considered as active health partners rather than simple recipients of health services (e.g. deworming, food and nutrient supply and health checks). Health messages conveyed through teachers would be relayed to the children and then to their siblings and friends. Schoolchildren would also make information and education communication (IEC) tools, with the idea that such hand-made tools would have a greater impact on parents than would those printed and distributed in large volumes by the authorities. To motivate the children to think and learn by themselves, health education *per se* must be changed from a top-down system.

Within the framework of the ACIPAC advocacy, the Office of Basic Education Commission (OBEC) of the Ministry of Education in Thailand developed model schools for malaria and STH prevention. OBEC, in collaboration with local teachers, also prepared user-friendly textbooks for children and manuals for teachers for the prevention and control of parasitic diseases. The English versions (Figure 1) were distributed to partner countries, international organizations and, upon request, NGOs.

In these model areas, schoolchildren developed IEC materials (e.g. posters and advocacy books) by themselves and brought them to the community for a demonstration. The children worked with teachers to identify mosquito breeding sites and to develop activities that have a positive impact on sanitation in communities. In addition, children were taught the proper use of bednets (to prevent malaria) [16], which was expected to lead to better care of siblings under five years of age who would otherwise be at greatest risk of death. The benefits of health education in the prevention of malaria has been shown in Thailand [17] and was achieved through behavioural changes in schoolchildren, using improved teacher training, interactive education and good teaching materials.

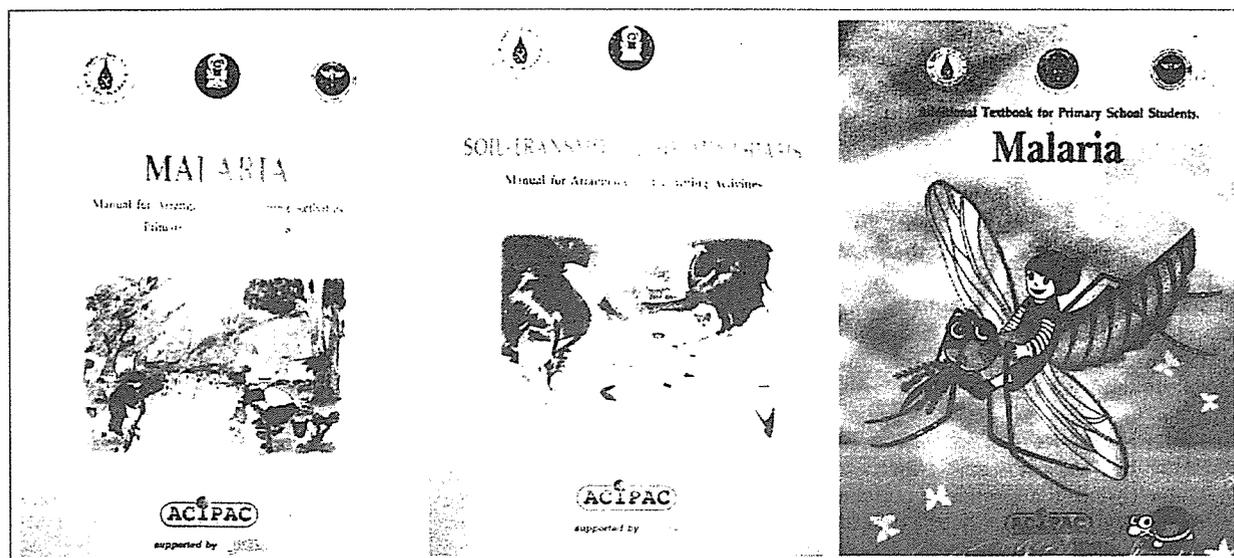


Figure 1. Teachers' manuals and a textbook for schoolchildren developed by ACIPAC in collaboration with OBEC, the Thai Ministry of Education, the Thai Department of Diseases Control, the Thai Ministry of Public Health, and Mahidol University, Thailand.

In response to the ACIPAC advocacy, the Ministry of Health in Laos stated its National Intestinal Helminth Prevention and Control Policies in 2003. In addition, ACIPAC ex-trainees started school-based control activities for malaria and dengue fever in Laos, thus showing the possibility of expanding the school-based approach to control other infectious diseases. The ex-trainees also increased coordination between the Ministries of Health and Education towards preparation of a national school health policy with support from the World Health Organization (WHO) and United Nations Educational, Scientific and Cultural Organization (UNESCO) [18]. In 2004, the Cambodian Government announced the establishment of the National Task Force for the Control of STH and Schistosomiasis, the Elimination Programme of Lymphatic Filariasis and a Helminthiasis Prevention and Control Policy.

These actions in partner countries indicate that the school-based approach advocated by ACIPAC has been accepted as an effective component of parasite control in the region.

Human resources for parasite control

ACIPAC has held international training courses for managers of school-based control programmes for malaria and STH four times during the past five years. In addition to trainees from Thailand and neighbouring countries, trainees from Kenya, Ghana and East Timor were enrolled. After finishing the course, trainees were requested to start small-scale pilot projects (SSPPs) in their respective countries, and the SSPPs were expected to be used for further development of human resources through in-country training for health personnel.

These training courses are considered unique in terms of the 'follow-up' of trainees because, by implementing SSPPs in each partner country, ACIPAC trainees also had important roles as trainers.

Small scale pilot projects

SSPPs were carried out in each of the partner countries. Other activities such as the provision of clean water and latrines were combined for some pilot schools, and teachers' manuals and comic books for schoolchildren were developed to facilitate health education.

Typical examples of activities performed were a 'model children' activity in Cambodia, cost sharing by the community for the construction of a water supply system in Laos and broadcasting (using loudspeakers) radio programmes about health education to the community in Vietnam. Model children were selected from upper forms (10–12 years old), and received two days of training on hygiene, the life cycles of the malaria parasite and of the soil-transmitted helminths and communication methods. These children then taught personal hygiene and prevention-of-infection methods to other children and kept detailed records of their activities. Children also developed IEC materials such as pictures and stories related to STH and malaria, which were used for delivering health messages to the community. In Laos, the communities of pilot project areas contributed 43.6% of the total budget for construction of water supply systems in schools. In these countries, KAP (knowledge, attitude and practice) surveys among schoolchildren showed changes in the children's behaviour after SSPP implementation compared with results in baseline surveys carried out before starting SSPPs.

Human and information networking

By implementing several activities and having meetings with those people enrolled in school health and parasite control, ACIPAC has made efforts to establish and strengthen the human and information network. For example, the homepage of the ACIPAC project was linked to the website of the Japanese Society of Parasitology. ACIPAC issued newsletters (Mekong Parasite News)

and printed magazines, which were distributed by related authorities in the partner countries.

Besides communication with both health and education sectors, one of the most important factors for the three centres established under the HI is the coordination of partnerships with other international agencies at the global and regional levels. ACIPAC also worked closely with other organizations and agencies such as the WHO Regional Offices (Western Pacific and Southeast Asia), the European Commission, United Nations Children's Fund (UNICEF), UNESCO, Southeast Asian Ministers of Education Organization (SEAMEO) and NGOs such as the Kenan Institute and the Partnership for Child Development to share experience of parasite control and school health activities.

Concluding remarks

ACIPAC has made an effort to establish the school-health-based approach to malaria and STH control mainly through human resource development, which can be applied to all health promotion programmes. Implementation of SSPPs resulted in the establishment of national policies on parasite control and/or school health in some partner countries, in addition to providing a good opportunity for the formulation of partnerships among health and education sectors and international partners. The lessons learned and experiences gained have helped shape the comprehensive approach encapsulated in the Japanese Health and Development Initiative, which, although global in scope, will focus strongly on Africa [19]. To achieve the Millennium Development Goals related to health issues, the Government of Japan has declared that Japan will provide assistance for education focusing on sanitation and prevention of infectious diseases such as HIV/AIDS and a range of parasitic diseases. This will be achieved by addressing local health issues at primary and secondary schools, at non-formal schools for out-of-school children, to those who have left school early and street children, and by providing literacy classes for adults. The two Africa-based centres will continue and will increase their efforts towards human resources development, orchestration of effective parasite control and improving living standards on the continent. Further details of the achievements by ACIPAC can be found in other publications [20–23]. Although the five-year achievements by ACIPAC were rather limited, the school-health-approach should be considered as an effective entry point to solve various issues related to providing comprehensive health care for children and their community.

Acknowledgements

On behalf of the Japanese section of ACIPAC, we express sincere thanks to our Thai colleagues, especially to Sornchai Looareesuwan, Jitra Waikagul, Pratap Singhasivanon, Praphasri Jongsuksuntigul and Pimpimon Thongthien for their outstanding contributions to the ACIPAC project, and to Pornchai Matangkasombut, President of Mahidol University and the Project Director of ACIPAC, and the authorities of the Governments of Thailand and Japan for their constant support. We also thank Andy Crump for critically reading the manuscript. We dedicate this article to the late Ryutaro Hashimoto with sincere appreciation.

References

- 1 Stoll, N.R. (1947) This wormy world. *J. Parasitol.* 33, 1–18
- 2 WHO (2000) *World Health Report: Health Systems – Improving Performance*. World Health Organization (<http://www.who.int/whr/> 2000)
- 3 Breman, J.G. *et al.* (2004) Conquering the intolerable burden of malaria: what's new, what's needed: a summary. *Am. J. Trop. Med. Hyg.* 71 (Suppl. 2), 1–15
- 4 Engels, D. *et al.* (2002) The global epidemiological situation of schistosomiasis and new approaches to control and research. *Acta Trop.* 82, 139–146
- 5 Brooker, S. *et al.* (2003) Mapping soil-transmitted helminthes in Southeast Asia and implications for parasite control. *Southeast Asian J. Trop. Med. Public Health* 34, 24–36
- 6 Kobayashi, A. *et al.* (2006) Historical aspects for the control of soil-transmitted helminthiasis. *Parasitol. Int.* 55, S289–S291
- 7 Minai, M. *et al.* (2003) Historical view of schistosomiasis japonica in Japan: implementation and evaluation of disease-control strategies in Yamanashi Prefecture. *Parasitol. Int.* 52, 321–326
- 8 Hunter, G.W., III and Yokogawa, M. (1984) Control of schistosomiasis japonica in Japan – a review 1950–1978. *Jpn. J. Parasitol.* 33, 341–351
- 9 Tanaka, H. and Tsuji, M. (1997) From discovery to eradication of schistosomiasis in Japan: 1847–1996. *Int. J. Parasitol.* 27, 1465–1480
- 10 Kojima, S. (2005) Schistosomes: Asian, In *Topley and Wilson's Microbiology and Microbial Infections: Parasitology* (10th edn) (Cox, F.E.G. *et al.*, eds), pp. 626–639, Hodder Arnold
- 11 Yokogawa, M. (1985) JOICFP's experience in the control of ascariasis within an integrated programme. In *Ascariasis and its Public Health Significance* (Crompton, D.W.T. *et al.*, eds), pp. 265–277, Taylor and Francis
- 12 Working Group on Global Parasite Control (1998) *The Global Parasite Control for the 21st Century. A Report on Global Parasite Control*. Government of Japan
- 13 Anantaphruti, M.T. *et al.* (2002) School-based helminthiasis control: I. A baseline study of soil-transmitted helminthiasis in Nakhon Si Thammarat Province, Thailand. *Southeast Asian J. Trop. Med. Public Health* 33 (Suppl. 3), 113–119
- 14 Waikagul, J. *et al.* (2002) A cross-sectional study of intestinal parasitic infections among schoolchildren in Nan Province, northern Thailand. *Southeast Asian J. Trop. Med. Public Health* 33, 218–223
- 15 Tomono, N. *et al.* (2003) Risk factors of helminthiasis among schoolchildren in southern Thailand. *Southeast Asian J. Trop. Med. Public Health* 34, 264–268
- 16 Kojima, S. and Takeuchi, T. (2006) Global parasite control initiative of Japan (Hashimoto Initiative). *Parasitol. Int.* 55, S293–S296
- 17 Okabayashi, H. *et al.* (2006) Keys to success for a school-based malaria control program in primary schools in Thailand. *Parasitol. Int.* 55, 121–126
- 18 Jimba, M. *et al.* (2005) Beyond deworming. *Lancet* 365, 751
- 19 Crump, A. and Yamamoto, T. Japan's Health and Development Initiative: a boon for Africa. *Science* (in press)
- 20 Kojima, S. *et al.* (2005) The Asian Center of International Parasite Control (ACIPAC): five years achievement. I. Introduction. *Southeast Asian J. Trop. Med. Public Health* 36 (Suppl. 3), 1–12
- 21 Waikagul, J. *et al.* (2005) The Asian Center of International Parasite Control (ACIPAC): five years achievement. II. ACIPAC human resources development. *Southeast Asian J. Trop. Med. Public Health* 36 (Suppl. 3), 13–16
- 22 Jongsuksuntigul, P. *et al.* (2005) The Asian Center of International Parasite Control (ACIPAC): five years achievement. III. School health for parasite control in Thailand: a review and current model activities. *Southeast Asian J. Trop. Med. Public Health* 36 (Suppl. 3), 17–27
- 23 Kobayashi, J. *et al.* (2005) The Asian Center of International Parasite Control (ACIPAC): five years achievement. IV. Activities in partner countries (Cambodia, Lao PDR, Myanmar and Vietnam): small scale pilot project (SSFP) and other impacts. *Southeast Asian J. Trop. Med. Public Health* 36 (Suppl. 3), 28–40

APPLICATION OF MULTIPLEX PCR FOR SPECIES DISCRIMINATION USING INDIVIDUAL METACERCARIAE OF *PARAGONIMUS* OCCURRING IN THAILAND

Hiromu Sugiyama¹, Yasuyuki Morishima¹, Achariya Rangsiruji², Suthewan Binchai², Punsin Ketudat² and Masanori Kawanaka¹

¹Department of Parasitology, National Institute of Infectious Diseases, Tokyo, Japan; ²Department of Biology, Faculty of Science, Srinakharinwirot University, Bangkok, Thailand

Abstract. A total of 6 lung fluke species have been documented in Thailand, of which *P. heterotremus* is the most important, since it affects humans. Although *P. westermani* is found as metacercariae in the same crab species as *P. heterotremus* in Thailand, human infections with *P. westermani* have not been confirmed. To accurately discriminate between the individual metacercariae of these two species, we established a multiplex PCR method. Using this method, two products each were amplified from the metacercarial DNA samples of *P. heterotremus* (ca. 310 and 520 bp) and *P. westermani* (ca. 140 and 520 bp). In contrast, 520-bp products alone were found to be generated from the DNA samples of *P. siamensis*, *P. bangkokensis* and *P. harinasutai*, 3 other species of lung flukes known to occur in Thailand. Digestion of these 520-bp products with the restriction enzyme *ScrFI* could unequivocally discriminate species by the number and size of the produced band(s): 3 bands (ca. 60, 210 and 250 bp) for *P. harinasutai*, 2 bands (ca. 250 and 270 bp) for *P. bangkokensis*, and an uncut band (520 bp) for *P. siamensis*. The established multiplex PCR used in combination with restriction enzyme digestion (PCR-RFLP with *ScrFI*) is effective for discriminating the 5 different species of lung flukes occurring in Thailand, even at the metacercarial stage.

INTRODUCTION

Six lung fluke species have been documented in Thailand (Srisont *et al.*, 1997; Blair *et al.*, 1999): *Paragonimus westermani*, *P. siamensis*, *P. heterotremus*, *P. bangkokensis*, *P. macrorchis* and *P. harinasutai*. Of them, *P. heterotremus* is the most important, since it affects humans. Although *P. westermani* occurs as metacercariae in the same crab species as *P. heterotremus* in Thailand, human infections with *P. westermani* have not been confirmed (Blair *et al.*, 1998). In order to accurately discriminate between individual metacercariae of these two species, we established a multiplex PCR method (Sugiyama *et al.*, 2005). In this study, we further evaluated the usefulness of the previously established multiplex PCR for species-level discrimination among *P. siamensis*, *P. bangkokensis* and *P. harinasutai*, 3 other species of lung flukes known to occur in Thailand.

MATERIALS AND METHODS

Parasite samples and DNA isolation

The metacercariae of *P. siamensis* (Fig 1) were

harvested from the freshwater crab, *Sayamia germaini*, captured in paddy fields in Prachin Buri Province, Thailand (Srisont *et al.*, 1997). The metacercariae of *P. bangkokensis* (Fig 2) were harvested from the freshwater crab, *Ranguna smalleyi*, captured in a mountain stream in Surat Thani Province, Thailand (Rangsiruji *et al.*, personal communication). The metacercariae of *P. heterotremus*, *P. westermani* (Thailand strain) and *P. harinasutai* (Fig 3) were harvested from the freshwater crab, *Larnaudia larnaudii*, captured in a mountain stream in Saraburi Province, Thailand (Kawashima *et al.*, 1989). DNA samples were prepared from the metacercariae as previously described (Sugiyama *et al.*, 2002).

DNA amplification and sequencing

For multiplex PCR amplification (Sugiyama *et al.*, 2005), the *P. heterotremus*-specific forward primer (PhTF1; 5'-TTCCCCAACGTGGCCTTGTGT-3', alignment positions 184 to 204 for the *P. heterotremus* second internal transcribed spacer (ITS2) region of the nuclear ribosomal DNA (rDNA)) and a newly designed *P. westermani*-specific forward primer (PwTF3; 5'-GTCTGCGTTCGATGCTGACCTACG-3', alignment positions 367 to 390 for the *P. westermani* ITS2 region) were used in combination with an interspecies-conserved primer pair, 3S (forward, 5'-G GTACCGGTGGATCACTCGGCTCGTG-3') and A28 (reverse, 5'-GGGATCCTGGTTAGTTTCTTTCTC CGC-3') (Bowles *et al.*, 1995). These primers were all

Correspondence: Hiromu Sugiyama, Department of Parasitology, National Institute of Infectious Diseases, Toyama 1-23-1, Shinjuku-ku, Tokyo 162-8640, Japan. Tel: +81-3-5285-1111; Fax: +81-3-5285-1173 E-mail: hsugi@nih.go.jp

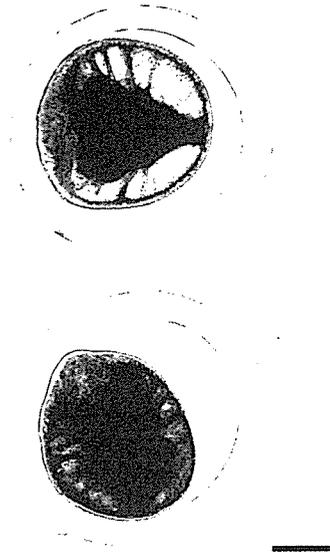


Fig 1- Photomicrograph of fresh *P. siamensis* metacercariae. The metacercariae were encysted by a thick wall and had a spherical shape. The wall thickness averaged 94 μm . The diameter of the cyst ranged from 668 to 736 μm with an average of 701 μm . Bar is 150 μm .

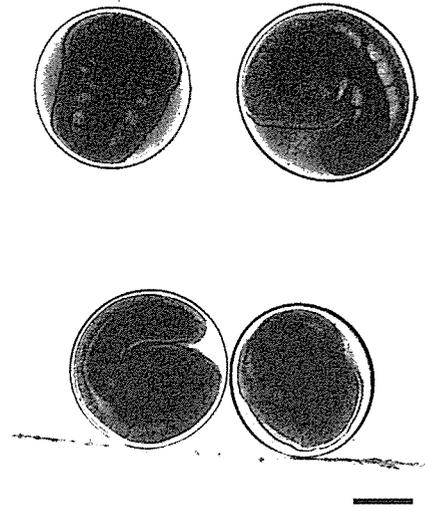


Fig 3- Photomicrograph of fresh *P. harinasutai* metacercariae. The metacercariae were encysted by a wall and exhibited a spherical-to-suboval shape. The wall thickness averaged 14 μm . The longitudinal and transverse diameters of the cyst ranged from 570 to 748 μm and 534 to 724 μm , respectively, with average dimensions of 655 x 634 μm . Bar is 150 μm .

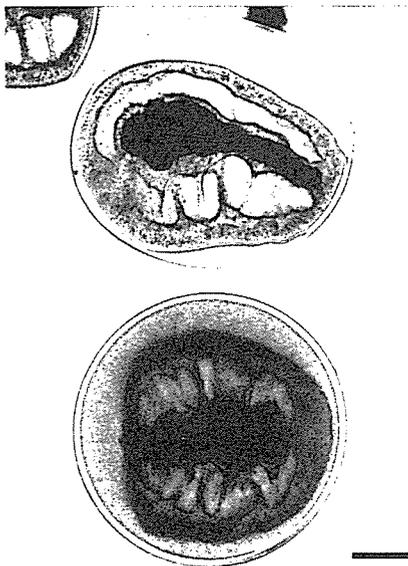


Fig 2- Photomicrograph of fresh *P. bangkokensis* metacercariae. The metacercariae were encysted by a wall and had a spherical-to-suboval shape. The wall thickness averaged 13 μm . The longitudinal and transverse diameters of the cyst ranged from 379 to 521 μm and 365 to 469 μm , respectively, with average dimensions of 437 x 422 μm . Bar is 150 μm .

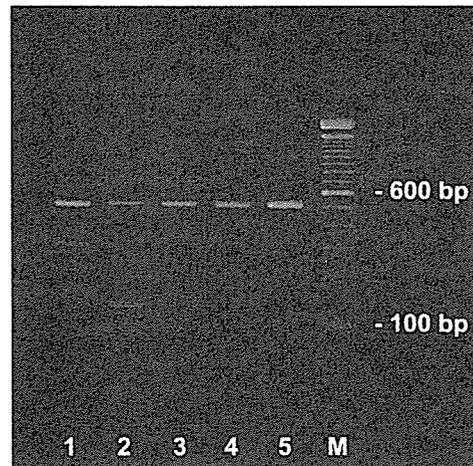


Fig 4- Results of multiplex PCR amplification of the metacercarial DNA samples from *P. heterotremus* (lane 1), *P. westermani* (lane 2), *P. siamensis* (lane 3), *P. bangkokensis* (lane 4) and *P. harinasutai* (lane 5). Two PCR fragments were amplified from the metacercarial DNA samples of *P. heterotremus* (ca. 310 and 520 bp) and *P. westermani* (ca. 140 and 520 bp). A single 520-bp fragment was produced for *P. siamensis*, *P. bangkokensis* and *P. harinasutai*. A 100-bp DNA ladder was used to estimate the sizes of the bands (lane M).

```

Ps 001: TGTCGATGAAGAGCGCAGCCAACTGTGTGAATTAATGCGAACTGCATACTGCTTTGAACA 060
Pb 001: .....T..... 060
Ph 001: .....T..... 060

Ps 061: TCGACATCTTGAACGCATATTGCGGCCACGGGTTAGCCTGTGGCCACGCCTGTCCGAGGG 120
Pb 061: ..... 120
Ph 061: ..... 120

Ps 121: TCGGCTTATAAACTATCGCGACGCCCAAAAAGTCGCGGCTTGGGTCTTGCCAGCTGGCGT 180
Pb 121: .....T..... 180
Ph 121: .....T..... 180

Ps 181: GATCTCCCCAATCAGGTCTCGTGCCTGTGGGGTGTGAGATCTATGGCGTTTCCCTAACAT 240
Pb 181: ...T...G...T.AC..T...T.G.....C.....C.....C..... 240
Ph 181: ...T...GG..T.AC..T...T.G.....C.....C.....C..... 240
                ScrFI

Ps 241: ACTCGGGCGCACCCACGTTGCGGCTGAAAGCCTTGACGGGGATGTGGCAACGGAATCGTG 300
Pb 241: GT[C....]..T....T...T.....[G....]..GT..... 300
Ph 241: GT[C....]..T....T...T.....[G....]..GT..... 300
                ScrFI                StuI

Ps 301: GCTCAGTAGATGAATTATGTGCGCGTTCGGTTGCTCCTGTCTTCATCTGTGGTTTATGTTG 360
Pb 301: .....GA..T.T.....C....A..A.....A.....G...C... 360
Ph 301: .....GA..T.T.....C....A..A.....A.....G...C... 360

                HincII
Ps 361: CGCGTGGTCTGTGTTTCGACGTTGACCTATCTATGTGCCATATGGTTCATTCTCCTGACCT 420
Pb 361: ..T....G..C..CT..T.C.....GAG.....G...C..... 420
Ph 361: ..T....G..C..CT..T.C.....GAG.....G...C..... 420

Ps 421: CCGATCAGACGTGAGTACCCGCTGAACTTAAGCATATCACTAA 463
Pb 421: ..... 463
Ph 421: ..... 463
    
```

Fig 5- Aligned sequences of the ITS2 region from *P. siamensis* (Ps), *P. bangkokensis* (Pb) and *P. harinasutai* (Ph) metacercariae. A dot in the *P. bangkokensis* and *P. harinasutai* sequences indicates identity with the *P. siamensis* sequence. The recognition sites of the *HincII* (GTT/GAC), *StuI* (AGG/CCT) and *ScrFI* (CC/CGG and CC/GGG) restriction enzymes are enclosed in boxes. The numbers refer to the lengths of the nucleotide sequences.

incorporated into a single-tube reaction. The multiplex PCR amplification was performed as previously described (Sugiyama *et al*, 2004) using 0.1 µM of PhTF1 and PwTF3 primers, 0.5 µM of 3S and A28 primers, 2.5 units of the Taq polymerase (Invitrogen, USA) and 10 ng of the DNA template. The resultant PCR products were separated by electrophoresis on 2% (w/v) agarose gels.

The amplified products were extracted from agarose gels and sequenced using the corresponding primers and the BigDye Terminator Cycle Sequencing Kit (Applied Biosystems, USA) on an automated sequencer (ABI310, Applied Biosystems). Sequence alignment and comparison were completed using the GENETYX-WIN (ver. 7.0, Software Development Co, Japan) program.

Restriction enzyme digestion of the multiplex PCR products [PCR-linked restriction fragment length polymorphism (PCR-RFLP)]

The amplified products (4 µl) were also treated

with five units of the restriction enzyme *HincII*, *StuI* or *ScrFI* (New England Biolabs, USA) at 37°C for 12 hours. The treated samples were then separated by electrophoresis on 3% (w/v) agarose gels.

RESULTS

Based on the established multiplex PCR method (Sugiyama *et al*, 2005), we confirmed that two products each were amplified from the metacercarial DNA samples of *P. heterotremus* (ca. 310 and 520 bp) and *P. westermanni* (ca. 140 and 520 bp) (Fig 4). On the other hand, 520-bp products alone were generated from the metacercarial DNA samples of *P. siamensis*, *P. bangkokensis* and *P. harinasutai* (Fig 4). Sequence analysis of the amplified products revealed that the aligned ITS2 region was 463 bp in length in each of the latter 3 species (Fig 5).

Similarity searches of the GenBank/EMBL/DBJ nucleotide databases revealed that the *P. siamensis*

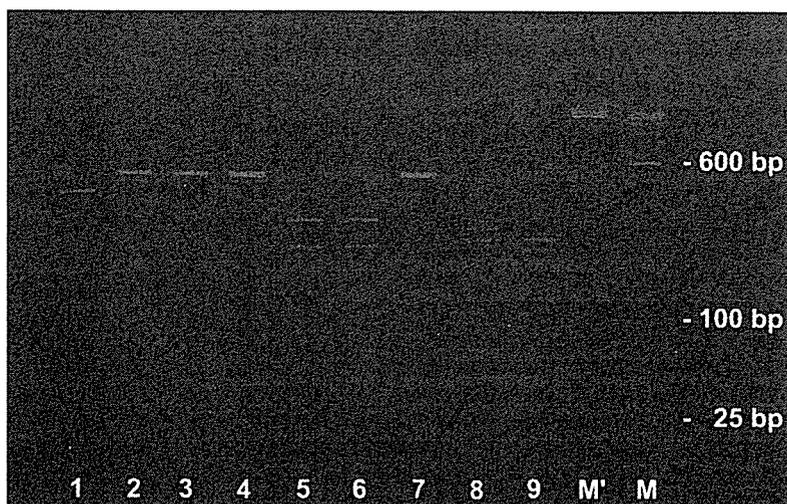


Fig 6- Results of RFLP analysis of the ITS2 products amplified from *P. siamensis* (lanes 1, 4 and 7), *P. bangkokensis* (lanes 2, 5, and 8) and *P. harinasutai* (lanes 3, 6, and 9) metacercarial DNA samples. The PCR product of *P. siamensis* (ca. 520 bp) was cleaved with *HincII*, producing two bands (ca. 110 and 410 bp, lane 1). The PCR products of the two other species remained uncut (lanes 2 and 3). In contrast, the PCR product of *P. siamensis* remained uncut with *StuI* (lane 4), while those of the two other species were cleaved to produce two bands (ca. 220 and 300 bp, lanes 5 and 6). Digestion with *ScrFI* discriminated *P. harinasutai* based on the restriction pattern of three distinctive bands (ca. 60, 210 and 250 bp, lane 9). The PCR products of *P. bangkokensis* were cleaved with *ScrFI* to produce two distinctive bands (ca. 250 and 270 bp, lane 8), while those of *P. siamensis* remained uncut (lane 7). Both 25-bp and 100-bp DNA ladders were used to estimate the sizes of the bands (lanes M' and M, respectively).

and *P. harinasutai* ITS2 sequences were identical to the sequences deposited under accession numbers AF159605 and AF159609, respectively. However, there was no sequence data in the GenBank/EMBL/DBJ for *P. bangkokensis*; therefore, we deposited the ITS2 region sequence under accession number AB248091.

Pairwise comparisons between *P. siamensis* and each of *P. bangkokensis* and *P. harinasutai* revealed 33 (7.1%) or 34 (7.3%) nucleotide differences, respectively. In contrast, only one (0.2%) nucleotide difference was found between *P. bangkokensis* and *P. harinasutai*.

For species discrimination by PCR-RFLP, the restriction enzymes, *HincII*, *StuI* and *ScrFI*, were selected on the basis of the theoretical restriction maps generated from the ITS2 sequences of *P. siamensis*, *P. bangkokensis* and *P. harinasutai* (Fig 5). Digestion with *HincII* discriminated *P. siamensis* by the restriction pattern of two distinctive bands of about 110 and 410 bp in size, while the 520-bp amplification products of the two other species remained uncut (Fig 6). In contrast, the PCR product of *P. siamensis* remained uncut by *StuI*, while those of the other two species were cleaved to produce two bands of about 220 and 300 bp.

Digestion with *ScrFI* could unequivocally discriminate *P. siamensis*, *P. bangkokensis* and *P. harinasutai* by the number and size of the produced band(s): 3 bands (ca. 60, 210 and 250 bp) for *P. harinasutai*, 2 bands (ca. 250 and 270 bp) for *P. bangkokensis*, and an uncut band (520 bp) for *P. siamensis* (Fig 6).

DISCUSSION

The phylogenetic relationships of the *Paragonimus* species occurring in Thailand have been demonstrated using genetic markers in the ITS2 region of the rDNA (Blair *et al*, 1998; Iwagami *et al*, 2000). In these studies, the ITS2 sequences were amplified by PCR with the primer pair 3S and A28, from DNA samples prepared from adult worms. Using this primer pair with two other species-specific primers in the previously established multiplex PCR method (Sugiyama *et al*, 2005), we demonstrated that 520-bp ITS2 sequences alone were generated from the individual metacercariae of *P. siamensis*, *P. bangkokensis* and *P. harinasutai*. Through pairwise comparisons of the sequences of the amplified products, these species were unequivocally discriminated from one another. We then utilized nucleotide differences to select the restriction enzymes *HincII*, *StuI* and *ScrFI* for PCR-RFLP analysis, which

allowed development of a more rapid and labor-saving discrimination method. Of the restriction enzymes examined, we confirmed that *ScrFI* allowed the most efficient discrimination among these species, based on the number and size of the produced band(s). It is noteworthy that analysis with this enzyme could unequivocally discriminate between *P. bangkokensis* and *P. harinasutai*, which have only a single base difference in the ITS2 region.

In this study, we demonstrated that the previously established multiplex PCR method (Sugiyama *et al.*, 2005), when used in combination with restriction enzyme digestion, is effective for discriminating the 5 different species of lung flukes occurring in Thailand, even at the metacercarial stage. This method may be applicable to *Paragonimus* occurring in other Asian countries, *eg.* China and India, where sets of *Paragonimus* species that have not yet been studied occur. Further collaborative studies, including evaluation of the usefulness of this method, are now in progress in these areas using locally obtained parasite samples.

ACKNOWLEDGEMENTS

We thank Miss Tapawittra Pongpawe for her technical assistance. This study was supported in part by a grant from the Nissan Science Foundation.

REFERENCES

- Blair D, Waikagul J, Honzako Y, Agatsuma T. Phylogenetic relationships among the Thai species of *Paragonimus* inferred from DNA sequences. In: Tada I, Kojima S, Tsuji M, eds. Proceedings of the Ninth International Congress of Parasitology. Bologna: Monduzzi Editore, 1998:643-7.
- Blair D, Xu Z-B, Agatsuma T. Paragonimiasis and the genus *Paragonimus*. *Adv Parasitol* 1999;42:114-72.
- Bowles J, Blair D, McManus DP. A molecular phylogeny of the human schistosomes. *Mol Phylog Evol* 1995;4:103-9.
- Iwagami M, Ho L-Y, Su K, *et al.* Molecular phylogeographic studies on *Paragonimus westermani* in Asia. *J Helminthol* 2000;74:315-22.
- Kawashima K, Sugiyama H, Ketudat P. *Paragonimus* infection in crabs in Thailand. In: Kawashima K, ed. *Paragonimus in Asia: biology, genetic variation and speciation (Paragonimus Research Report, Number 2)*. Fukuoka: Kyushu University School of Health Sciences, 1989:75-9.
- Rangsiruji A, Sugiyama H, Morishima Y, *et al.* A new record of *Paragonimus* other than *P. westermani* in southern Thailand. *Southeast Asian J Trop Med Public Health* 2006;37(suppl 3):64-8.
- Srisont D, Waikagul J, Yaemput S. *Paragonimus* in Thailand. In: Srisont D, Waikagul J, Yaemput S, eds. *Paragonimus*. Bangkok: Living Trans Media, 1997:65-91 (in Thai).
- Sugiyama H, Morishima Y, Kameoka Y, Kawanaka M. Polymerase chain reaction (PCR)-based molecular discrimination between *Paragonimus westermani* and *P. miyazakii* at the metacercarial stage. *Mol Cell Probes* 2002;16:231-6.
- Sugiyama H, Morishima Y, Kameoka Y, Kawanaka M. A multiplex PCR for discrimination between *Paragonimus westermani* and *P. miyazakii* at the metacercarial stage. *Southeast Asian J Trop Med Public Health* 2004;35(suppl 1):327-30.
- Sugiyama H, Morishima Y, Rangsiruji A, *et al.* Molecular discrimination between individual metacercariae of *Paragonimus heterotremus* and *P. westermani* occurring in Thailand. *Southeast Asian J Trop Med Public Health* 2005;36(suppl 3):87-91.

POSSIBLE DISCOVERY OF CHINESE LUNG FLUKE, *PARAGONIMUS SKRJABINI* IN MANIPUR, INDIA

Shantikumar T Singh¹, Deven L Singh² and Hiromu Sugiyama³

Departments of ¹Microbiology, ²Physiology, Sikkim Manipal Institute of Medical Sciences, Gangtok, India;

³Department of Parasitology, National Institute of Infectious Diseases, Tokyo, Japan

Abstract. To obtain more information about *Paragonimus* species prevalent in Manipur, India, hundreds of freshwater crabs, *Potamiscus manipurensis*, were captured from mountain streams in the Motbung Mountains in Senapati District, from December 1997 to January 1998. Crab extracts were prepared by digestion, differential filtration, and sedimentation. The filtered sediments were critically examined under a stereomicroscope. Isolated *Paragonimus* metacercariae were used for morphological study and animal experimentation. Forty-seven metacercariae were fed orally to a 3-month-old male puppy of local breed; at autopsy 155 days after inoculation, 12 adult worms were recovered; 2 were free in the thoracic cavity and 5 pairs were in lung cysts. Two adult worms were flattened and fixed in 70% ethanol and the remaining worms were put directly into 70% ethanol and preserved until July 2005. The former 2 worms were stained with borax carmine for morphological study at Sikkim Manipal Institute of Medical Sciences, Sikkim, India. Two adult worms in the latter group were sent to the National Institute of Infectious Diseases, Tokyo, Japan, for morphological identification and DNA sequencing. The morphological features of the borax carmine-stained worms were characteristic of *P. skrjabini*, although no genetic material for PCR amplification and sequencing could be extracted from the worm.

INTRODUCTION

Paragonimiasis, or lung fluke infection, caused by one of several *Paragonimus* species, primarily affecting the lungs but also other organs and tissues of the body, is one of the most important parasitic zoonoses. It is widely distributed in the world, and is endemic in Asia, Africa, and Latin America. The infection is acquired by ingestion of raw or improperly cooked freshwater crabs or crayfish containing encysted metacercariae. Rarely, it can be transmitted by eating undercooked meat of paratenic hosts, such as pigs and wild boar infected with larval worms. In India, human paragonimiasis was unknown until 1982, when the first human case was reported from Manipur (Singh *et al*, 1982). Subsequently, several cases of human paragonimiasis were described by Singh *et al* (1986), indicating that the disease was endemic in many parts of Manipur. Recently, an endemic focus of human paragonimiasis was detected in Arunachal Pradesh, another northeast state of India (Narain *et al*, 2003). Now, paragonimiasis is increasingly recognized as a public health problem, especially in the northeast region of India (Fig 1). Although there are many unsettled questions about the taxonomic identification, life cycle, and pathobiology of *Paragonimus* species

prevalent in India, *P. westermani* has been presumed to be the only species infecting animals and humans. The present study describes the discovery of a lung fluke species in India that was morphologically identified as *P. skrjabini*. This lung fluke species was reported to occur in China and has been regarded as an important species causing human infections (Chen, 1959; Chung and Tsao, 1962).

MATERIALS AND METHODS

Several hundred freshwater crabs, *Potamiscus manipurensis* (Fig 2) were collected from mountain streams in Motbung, Senapati District, Manipur, from December 1997 to January 1998 (Fig 1). After morphological examination, crab extracts were prepared by mincing, digestion with artificial gastric juice consisting of 1 g of pepsin (Sigma Aldrich) in 1 liter of 0.7% hydrochloric acid, and overnight incubation at 37°C. Filtered sediments were obtained by passing the digested extract through differential filters of wire sieves (mesh size 1 mm-700 µm) and rinsing with clean tap water 4-5 times. The final filtered sediments were prepared in physiological saline. The sediments were critically examined for *Paragonimus* metacercariae using a stereoscopic microscope. The metacercariae isolated from the sediments were transferred in glass vials containing physiological saline and stored at 4°C until further study.

A 3-month-old male puppy of local breed was experimentally infected orally with 47 metacercariae.

Correspondence: ST Singh, Department of Microbiology, Sikkim Manipal Institute of Medical Sciences, 5th Mile Tadong, Gangtok 737102, India.
Tel: 91-3592-270535; Fax: 91-3592-231496
E-mail: slg_sminms@sancharnet.in

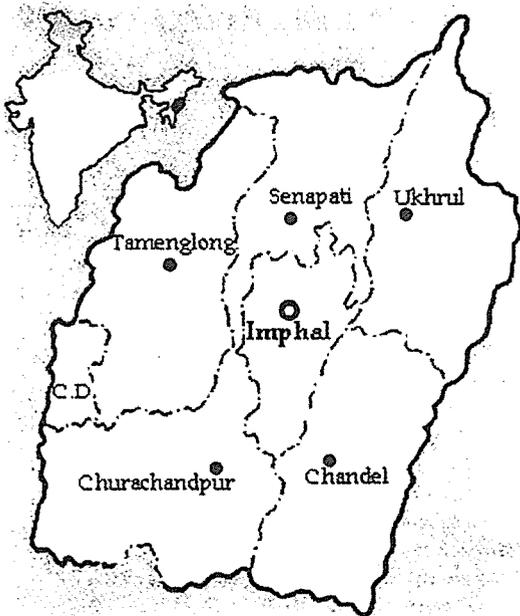


Fig 1- Map of India (upper left) indicating the location of Manipur State in the Northeast Region. Manipur State (center) comprises the capital city, Imphal, and 6 districts, including Senapati District, where *Paragonimus*-positive freshwater crabs were captured.

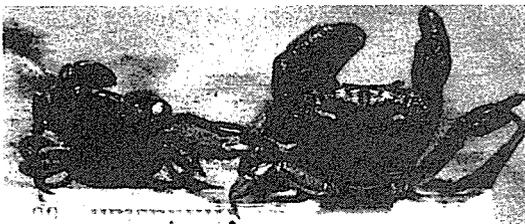


Fig 2- Freshwater crabs, *Potamiscus manipurensis*, playing a role as the second intermediate host of *P. skrjabini* in Manipur State, India.

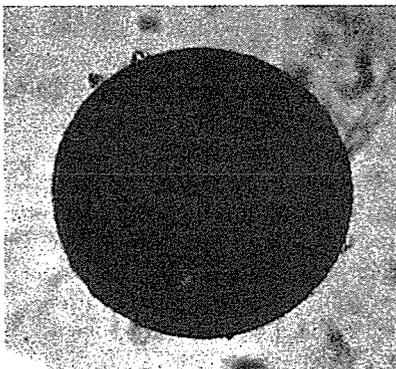


Fig 3- Microphotograph of a metacercaria.

Autopsy was performed on day 155 post-infection. The abdomen and thorax were cut open and examined thoroughly for pathological lesions and worms. The recovered worms and eggs were cleared in physiological saline and preserved in 70% ethanol for morphological identification and molecular characterization. Two worms were flattened between two glass slides, fixed in 70% ethanol, and the remaining worms were preserved in 70% ethanol until July 2005. The former 2 worms were stained with borax carmine and examined at Sikkim Manipal Institute of Medical Sciences, Gangtok, India; the 2 worms in the later group were sent the National Institute of Infectious Diseases, Tokyo, Japan, for parasitological confirmation and molecular characterization by PCR and DNA sequencing.

RESULTS

The metacercariae harvested from crabs were spherical in shape and possessed an 'I'-shaped excretory bladder (Fig 3). The average diameter of the inner cysts of the metacercariae measured 416 μ m. Some metacercariae were excysted during examination (Fig 4).

Autopsy on the test puppy showed hemorrhagic spots and brownish inflammatory patches on the surfaces of the lungs and pericardium. Two freely migrating worms were found in the thoracic cavity. In the lungs, 7 worm cysts were observed, of which 5 contained pairs of worms with reddish brown viscous fluid. Microscopy examination of the fluid, as well as brownish patches on the surface of the lungs, revealed numerous golden brown operculated eggs. The other 2 cysts, which localized deep in the lung parenchyma and consisted of thick fibrous walls, had no worm. Therefore, a total of 12 worms were recovered from the test puppy.

The unstained worms were spindle-shaped and measured 8-9 mm in length and 3-4 mm in width (Fig 5). The borax carmine-stained worms showed that the ovary ramified intricately like ginger stem, and the testes elongated like stumpy masses with fewer branches (Fig 6). The ovary and testes were almost the same in size. The ventral sucker, situated about the anterior third of the body, was slightly larger than the oral sucker. The vitellariae were thickly distributed from the lateral sides through the body to the midline. The uterus, faintly stained, was seen to contain eggs. The seminal vesicle and seminal receptacle were filled with spermatozoa. The cuticular spines were singly spaced and some showed splitting (Fig 7).

On the basis of the morphological characters of



Fig 4- Microphotograph of a larva excysted from a metacercarial cyst.

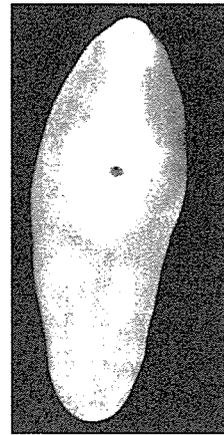


Fig 5- Photograph of an adult worm preserved in 70% ethanol.

the metacercariae and adult worms, the specimens were identified as *Paragonimus skrjabini*. No genetic material for PCR amplification and sequencing could, however, be extracted from an adult worm preserved in 70% ethanol.

DISCUSSION

In 1990, the first Indo-Japan joint research work on *Paragonimus* and paragonimiasis was launched in Manipur. The study aimed primarily to determine the crab host and *Paragonimus* species prevalent in various localities in Manipur. Freshwater crabs, *Potamiscus manipurensis*, collected at different localities, were found naturally infected with more than one type of *Paragonimus* metacercariae. All of the adult worms recovered from the experimentally infected animals were morphologically different from *P. westermani*. Unexpectedly the morphological features of the metacercariae and adult worms in the present study resembled those of *P. skrjabini*, which was first described from a viverrid, *Paguma larvata* (Smith) in Guangzhou City, Guangdong Province, China (Chen, 1959). Immature specimens have been reported from human cases of cutaneous paragonimiasis in the same province (Chung and Tsao, 1962), suggesting *P. skrjabini* is a zoonotic pathogen. Similar clinical forms have also been observed in Manipur.

The occurrence of another Chinese species *P. hueitu'ngensis* in Churachndpur District of Manipur was described by Singh (2002). Li and Chen (1992) proposed that *P. hueitu'ngensis* is synonymous with *P. skrjabini*, and one of the polymorphic forms of the *P. skrjabini* complex. The geographical distribution of the 2 species was thought restricted to China until our previous and present reports from Manipur, India.



Fig 6- Microphotograph of a borax carmine-stained adult worm showing the ovary and testes.



Fig 7- Microphotograph of the cuticular spines.

Considering the widespread distribution of the infected crab host and its popularity among crab-eaters in endemic areas, this species may be one of the important causes of paragonimiasis in animals and humans in Manipur. To confirm this speculation, more detailed life-cycle studies, morphological characterization, and molecular phylogenetics of this lung fluke is needed. The study will provide valuable epidemiological data on the distribution of the species, pathogenesis and phylogenetic relationships with species of the same genus occurring in Asian countries.

ACKNOWLEDGEMENTS

The authors thank the Director, Regional Institute of Medical Sciences, Imphal, Manipur and the Dean, Sikkim Manipal Institute of Medical Sciences, Tadong, Gangtok, India, for allowing us to conduct the study. We also thank Dr Achariya Rangsiruji, of Srinakharinwirot University, for her invaluable suggestions and review of the manuscript. The financial assistance provided to HS by the Tokyu Foundation for Better Environment is gratefully acknowledged.

REFERENCES

- Chen H-T. The occurrence of a new type of *Paragonimus* and some clinical problems related to lung flukes in China. Annual report 1958. Guangzhou: Chung Shan Medical College, 1959:192-3 (in Chinese).
- Chung H-L, Tsao W-C. *Paragonimus westermani* (Szechuan variety) and a new species of lung fluke: *Paragonimus szechuanensis*. Part II. Studies on the clinical aspects of *Paragonimus szechuanensis*: a new clinical entity. *Chin Med J* 1962;81:419-34.
- Li Y-S, Chen Y-Z. Discriminations on some species of *Paragonimus* from China. *Wuyi Sc J* 1992;9:269-75. (in Chinese, English abstract).
- Narain K, Devi KR, Mahanta J. *Paragonimus* and paragonimiasis - a new focus in Arunachal Pradesh, India. *Curr Sci* 2003;84:985-52.
- Singh I, Singh B Ng, Devi Subadani S, Singh Mohen Y, Razaque M. Pulmonary paragonimiasis. A case report. *Indian J Chest Dis Allied Sci* 1982;304-6.
- Singh TS, Mutum SS, Razaque MA. Pulmonary paragonimiasis: clinical features, diagnosis and treatment of 39 cases in Manipur. *Trans R Soc Trop Med Hyg* 1986;80:967-71.
- Singh TS. Occurrence of the lung fluke *Paragonimus hueitu'ngensis* in Manipur, India. *Chin Med J (Taipei)* 2002;65:426-9.
- Chen H-T. The occurrence of a new type of *Paragonimus*

原著論文

輸入キムチより検出された回虫様卵の分子同定

国立感染症研究所 寄生動物部

杉山 広・森嶋康之・川中正憲

東京医科歯科大学大学院 国際環境寄生虫学分野

太田伸生・赤尾信明

京都府立医科大学大学院 寄生病態学講座

有菌直樹・山田 稔・内川隆一・手越達也

名古屋市立大学大学院 医学研究科 宿主・寄生体関係学

吉田彩子

Key Words : キムチ, 回虫, 種同定, 食品媒介寄生虫, 輸入食品

緒言

厚生労働省の新興再興感染症事業「輸入蠕虫疾患の疫学および予防に関する研究」班（太田伸生班長）では、国内で流通・市販されている輸入キムチの寄生虫卵検査を実施し、29検体のうち11検体から何

らかの寄生虫卵を検出した¹⁾⁴⁾。検出した虫卵の中には回虫に極めて類似したものも見られたので、この虫卵についての種同定を目的に、塩基配列の解読・解析を試みた。

Molecular Identification of Eggs of Roundworm-like Parasite Found in Imported Kimchi

Hiromu Sugiyama* Yasuyuki Morishima* Masanori Kawanaka* Nobuo Ohta**
 Nobuaki Akao** Naoki Arizono*** Minoru Yamada*** Ryuichi Uchikawa***
 Tatsuya Tegoshi*** Ayako Yoshida*****

*Department of Parasitology, National Institute of Infectious Diseases

**Section of Environmental Parasitology, Tokyo Medical and Dental University

***Department of Medical Zoology, Kyoto Prefectural University of Medicine

****Department of Molecular Parasitology, Nagoya City University Graduate School of Medical Science

論文請求先：杉山 広 〒162-8640 新宿区戸山1-23-1 国立感染症研究所 寄生動物部

Clinical Parasitology Vol. 17 No. 1 2006

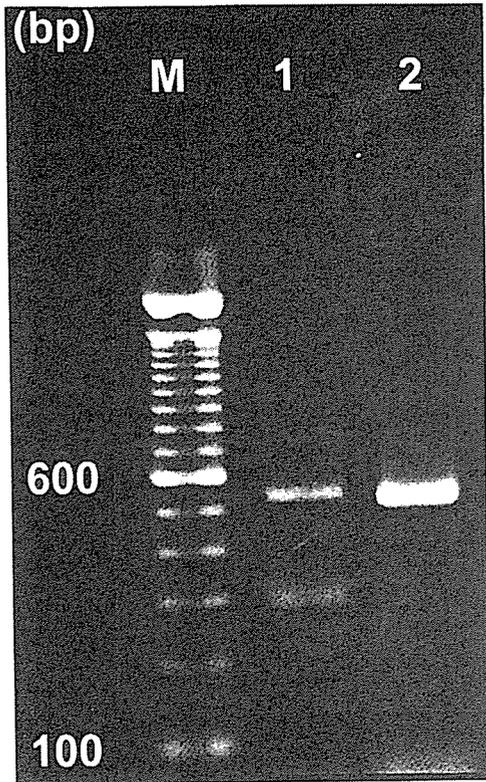


図1 キムチ由来虫卵の DNA (レーン 1), 人体症例由来の回虫卵 DNA (レーン 2) をテンプレートとする PCR 産物のアガロースゲル電気泳動所見。線虫類のリボソーム DNA・ITS-1 領域の増幅に汎用されている NC5 と NC13R とを用いて PCR 増幅させた。塩基配列の解読には、約 520bp のフラグメントをゲルから切り出し、これをテンプレートとした。フラグメントのサイズは DNA マーカー (レーン M, 100bp ラダー, Invitrogen) で推定した。

材料と方法

研究班で検出した虫卵 (2 個) から、まず DNA の抽出を試みた。方法は既報に準じた⁵⁾。すなわち、虫卵 (1 個ずつ) に SDS (最終濃度 0.5%) とプロテイナーゼ K (最終濃度 0.1mg/ml) を加えてタンパクを変性溶解し、エタノール沈殿により DNA を精製回収した。DNA 量を分光光度計で測定した後、リボソーム DNA の ITS-1 領域を標的として PCR 増幅した。プライマーには、線虫類からの PCR 増幅に汎用されている NC5 (フォワード, 26-mer) と NC13R (NC13 に相補的な配列のプライマー, リバース, 18-mer) とを用いた⁶⁾。得られた PCR 産物

```

SP 001:ATCGAGCAGAAAAA-CTCTCCGACGTGCACATAAGTACTATTTCGGGTATACG 059
AL 001:..... 059
As 001:.....A..... 060

SP 060:TGAGCCACATAGTAAATTGCACACAAATGTGGTATGTAATAGCAGTCGGCGTTTCITT 119
AL 060:..... 119
As 061:..... 120

SP 120:TTTTTT-GCCGGACAATTGCATGCGATTTCGTATGTGTTGAGGGAGAATAGGTGGCATGT 178
AL 120:..... 178
As 121:.....T...C..... 180

SP 179:TGGGCTTGTTAGAAAAGGCATGCCGTAGCGCTTATTTCCCGCTATTTCTGTAACAACGGT 238
AL 179:..... 238
As 180:..... 240

SP 239:GTCCATTTTGGCTCTACGCTTCACCGAGCTATCGCTGGACCGTCGGTAGCGATGAAAG 298
AL 239:..... 298
As 241:.....A..... 300

SP 299:GTGGAGAGAAAGCTCCTCCTTCGAGTCGAGTAGACTCAATGAGCCTCAGCTGGAGGCC 358
AL 299:..... 358
As 301:..... 360

SP 359:GCCAAAACCAAAAACACAATCACTTTTGAATAATCTATCTAATGAAAGATGCTAAATT 418
AL 359:..... 418
As 361:..... 420

SP 419:TTGTTAGTATCTCGAATTGTAAGATGAACAATACTTAGCGGGGATCACTCGGTTCCG 478
AL 419:.....N..... 477
As 421:.....T..... 480

SP 479:GG 480
AL 478:... 479
As 481:... 482
    
```

図2 キムチ由来虫卵 (および人体症例由来の回虫卵) のリボソーム DNA・ITS-1 領域の塩基配列 (SP) を示す。回虫 *Ascaris lumbricoides* (Al, GenBank Accession No. AB110019), 豚回虫 *Ascaris suum* (As, AB110022) と比較した。ドット (.) は回虫および豚回虫の登録配列がキムチ由来虫卵と同一であることを、ハイフン (-) はその部分の塩基が欠失していることを示す。

ル電気泳動し、さらにゲルから標的フラグメント (約 520bp) を回収した。これを PCR に使用した各プライマーでサイクルシーケンス用に蛍光標識し、塩基配列を解読した。配列の解析には、市販のプログラム (GENETIX-WIN) を活用した。人体症例由来の回虫卵 (含子虫卵に発育させた後に -80℃ で保存, 約 10,000 個) から同様に DNA を抽出し、PCR の後に塩基配列を解読して比較した。

結果

キムチ由来の回虫様卵 1 個から抽出される DNA 量を求めるため、抽出後の液量を 50 μL とし、波長 260nm で吸光度を測定した。その値は 0.0471 を示し、すべて核酸と仮定するならば、その量は約 118ng と算出された。この DNA と更にキムチ由来の他の回虫様卵 1 個から抽出した DNA を各々テンプレートとして PCR を行った。その結果、予想サ

イズと一致する約 520bp のフラグメントが増幅された (図 1)。これよりも小さいサイズのフラグメントも増幅されたので、約 520bp のフラグメントだけをゲルから切り出し、これを材料に塩基配列を解読した。その結果、プライマー領域を除いた PCR 産物のサイズは約 480bp となり、しかも両虫卵から得た配列は同一であることが分かった (図 2)。

人体症例由来の回虫卵の配列も約 480bp であった。キムチに由来する虫卵の配列と比較したところ、両者は完全に一致した。

この配列を用いて、ホモロジー検索を行った。その結果、回虫 *Ascaris lumbricoides* (GenBank Accession No. AB110019) の配列とは、一箇所を除いて完全に一致した (図 2, GenBank に登録されている回虫の配列に 1 塩基の欠失を認めた)。回虫に次いで相同性が高かったのは豚回虫 *A. suum* (AB110022) であった。しかしながら豚回虫とは 6 箇所の違いが認められた。すなわち、4 箇所塩基が相違し、2 箇所豚回虫に 1 塩基ずつの付加が見られた (図 2)。

以上の結果から、キムチに由来する回虫様の虫卵は、回虫 *A. lumbricoides* の虫卵であると判定した。

考 察

本研究で用いたキムチ由来の虫卵は、形態学的にも回虫卵と極めて良く類似することが示されていた⁴⁾が、虫種の確定には至っていなかった。そこでこの虫卵から DNA を抽出し、リボソーム DNA の ITS-1 領域を PCR 産物として増幅させ、塩基配列を解読して種の同定を試みた。その結果、虫卵はいずれも回虫と判定することができた。回虫と豚回虫との形態鑑別は難しく、加えて今回の検索材料はキムチからの検出虫卵で、鑑別は一層困難であった⁴⁾。このような加工食品由来の材料を対象として種同定・鑑別を行う場合には、本研究で示したよう

な遺伝子配列に基づく同定が極めて有用と考えられた。

線虫類の種の同定や鑑別には、ITS-2 領域の配列が指標として頻用されている³⁾。これに対して、回虫と豚回虫の鑑別には ITS-1 領域の配列が指標とされている²⁾⁶⁾。これは回虫と豚回虫では ITS-2 領域の配列が相互に一致するからで⁶⁾、両者の鑑別に際してはこの点に留意して標的領域を選択する必要がある。

謝辞：配列の解析に当たって助言を賜った慈恵会医科大学の石渡賢治先生に感謝いたします。

文 献

- 1) 平成 17 年 11 月 14 日付厚生労働省食品安全部監視安全課報道発表資料「韓国産および中国産のキムチに関する調査状況について」。(http://www.mhlw.go.jp/houdou/2005/11/h1114-1.html)
- 2) Ishiwata, K. *et al.* (2004) : Identification of tissue-embedded ascarid larvae by ribosomal DNA sequencing. *Parasitol Res*, 92, 50-52.
- 3) Jacobs, D. E. *et al.* (1997) : PCR-based methods for identification of potentially zoonotic ascaridoid parasites of the dog, fox and cat. *Acta Trop.*, 68, 191-200.
- 4) 太田伸生, 他 (2006) : 輸入キムチから検出された寄生虫卵. *Clin Parasitol*, 17, 67-69.
- 5) Sugiyama, H. *et al.* (2002) : Polymerase chain reaction (PCR)-based molecular discrimination between *Paragonimus westermani* and *P. miyazakii* at the metacercarial stage. *Mol Cell Probes*, 16, 231-236.
- 6) Zhu, X. *et al.* (1999) : Characterization of *Ascaris* from human and pig hosts by nuclear ribosomal DNA sequences. *Int J Parasitol*, 29, 469-478.

平成 16 年秋に集団発生した肺吸虫による食中毒事例 —原因の寄生虫学的精査—

国立感染症研究所 寄生動物部

杉山 広・森嶋康之・荒川京子・川中正憲

佐賀県衛生薬業センター

平野敬之・増本久人

佐賀県中部家畜保健衛生所

池添博士

Key Words : ウェステルマン肺吸虫, モクズガニ, メタセルカリア, 種同定, 集団感染

緒言

平成 16 年秋に佐賀県の某ホテル内にある中華料理店において、肺吸虫症の集団発生があった。原因食品はモクズガニの老酒漬(酔蟹)と判定され、ウェステルマン肺吸虫を原因とする食中毒として届出がなされた³⁾。このような事故の再発を予防するには、原因について寄生虫学的に精査し、事故発生の背景を明確にすることが重要と考えられる。そこで、原因食品となったモクズガニの捕獲場所を特定し、そ

こで捕獲されたモクズガニから肺吸虫メタセルカリアを検出し、この虫体について種の同定を試みた。その結果、本事例の原因を確定すると共に、再発予防に資する情報が得られたので報告する。

原因食品となったモクズガニの由来

本事例の原因食品はモクズガニと特定された³⁾ので、その由来を明らかにするために、関係者から聞き取りを行うと共に、仕入台帳や支払台帳(領収書)などについても提示を受けて検索した。その結

Outbreak of Paragonimiasis Caused by Ingestion of Drunken Crabs at a Hotel Restaurant in Saga Prefecture in 2004 : Species Identification of the Parasite Causing the Disease

Hiromu Sugiyama* Yasuyuki Morishima* Kyoko Arakawa* Masanori Kawanaka*
Takayuki Hirano** Hisato Masumoto** Hiroshi Ikezoe***

*Department of Parasitology, National Institute of Infectious Diseases

**Saga Prefectural Institute of Public Health and Pharmaceutical Research

***Saga Prefectural Chubu Livestock Hygiene Service Center

論文請求先: 杉山 広 〒162-8640 新宿区戸山 1-23-1 国立感染症研究所 寄生動物部

Clinical Parasitology Vol. 17 No. 1 2006



図1 モクズガニの鰓に寄生していた肺吸虫のメタセルカリア
いずれも球形を呈し、体肉内には赤色微細顆粒を多数認めた。Bar=200 μ m.

果、モクズガニはすべて一人の川漁師が、佐賀県北西部を流れる玉島川の五反田地区（河口から上流に3～4kmの範囲）で捕獲し、平成16年9月7日～10月28日にかけて、合計14回にわたり、当該ホテルに納入したことが明らかとなった。

モクズガニからの肺吸虫の検出

モクズガニを当該ホテルに納入した川漁師から、上述の地区で捕獲されたモクズガニ計69匹を、平成17年10月12日～11月16日にかけて、合計6回にわたり譲り受けた。このうち59匹については鰓だけを、また10匹については全身を調べ、肺吸虫メタセルカリアの検出に努めた。その結果、鰓を調べた群では11匹（寄生率19%）が、また全身を検索した群では2匹（寄生率20%）が、合計すると検査した69匹のうち13匹（寄生率19%）が、陽性であった。カニ1匹あたりのメタセルカリア数は、鰓を調べた群では1～12個、全身を検索した群では12個と167個であった。167個が検出されたカ

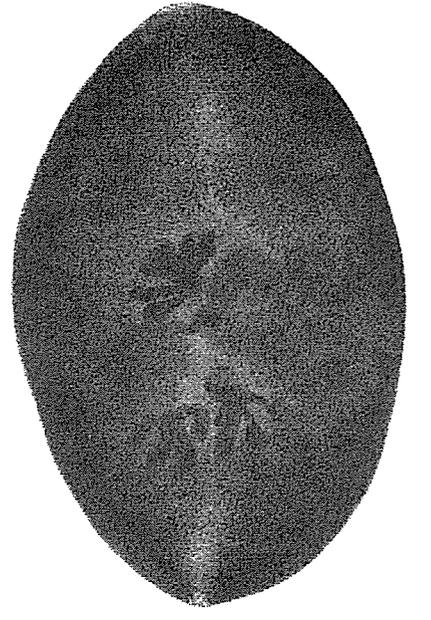


図2 試験感染ネコの肺から回収された成虫（染色封入標本）
卵巣は6本に簡単に分岐する。卵巣の基部に認めた受精嚢内には精子はなく、卵黄細胞を認めた。ウェステルマン肺吸虫（3倍体型）と同定された。Bar=2mm.

ニでのメタセルカリアの分布は、鰓39個、肝3個、生殖器1個、筋肉124個（胴体部63個、脚部61個）で、可食部である筋肉から最も多数のメタセルカリアが見出された。

肺吸虫の種同定

見出されたメタセルカリアを顕微鏡下に観察・計測した。メタセルカリアはいずれも球形を呈し、その直径は平均約378 μ m、体肉内には赤色の微細顆粒を多数認めた（図1）。

得られたメタセルカリア20個を試験ネコ（1頭）に投与し、217日後に剖検したところ、肺に形成された5個の虫嚢から各2隻ずつ、合計10隻の成虫が回収された。そのうち6隻をカルミン染色・封入標本として計測したところ、その大きさは体長が平均11.7mm、体幅は平均6.9mmであった。形態に基づく虫種の同定に重要な卵巣は、いずれも6本に簡

単に分岐していた。卵巢の基部には受精嚢を認めた
が、嚢内には精子が観察されず、卵黄細胞が認めら
れた(図2)。皮棘は単生であった。

以上のメタセルカリアおよび成虫の形態学的な特
徴から、今回検出した肺吸虫はいずれも3倍体型の
ウェステルマン肺吸虫と同定した。また、メタセル
カリアを出発材料にITS2領域(リボソーム)と16S
リボソームDNA領域(ミトコンドリア)の塩基配
列を解読し、形態に基づく結論を支持する結果を得
た(別途詳述の予定)。

考 察

本事例の原因食品であるモクズガニの由来を明ら
かにするために、食中毒事故の関係者へ聞き取り調
査等を行ったところ、カニは佐賀県北西部を流れる
玉島川で捕獲されたことが分かった³⁾。しかしなが
ら約45年前の調査では、玉島川のモクズガニは肺
吸虫陰性と報告されていた⁶⁾。そこで、玉島川で捕
獲されたモクズガニを改めて検査したところ、肺吸
虫の寄生率は約20%で、167個ものメタセルカリア
が寄生するカニも検出された。本調査の結果から、
今回の肺吸虫症事例の原因食品は、やはり玉島川の
モクズガニ(老酒漬)であることが確定となった。

原因となった肺吸虫の種名であるが、(シナ)モ
クズガニからは専らウェステルマン肺吸虫だけが
検出されており¹⁾、老酒漬(シナ)モクズガニによ
る既報の肺吸虫症例でも、原因はいずれもウェステ
ルマン肺吸虫とされている⁷⁾⁸⁾¹⁰⁾。本事例の原因も
ウェステルマン肺吸虫であると予想し、形態学的な
精査を加えた上で種を確定することにした。

わが国に分布するウェステルマン肺吸虫は、染色
体構成から2倍体型と3倍体型の2型に分類され
る⁴⁾。両型の主たる第2中間宿主は、2倍体型がサ
ワガニ、また3倍体型はモクズガニである⁴⁾。し
かしながら、サワガニから3倍体型を²⁾、またモク
ズガニから2倍体型⁹⁾を証明した報告もあるので、
宿主ガニの種類から型別を判定することはできな
い。両型の確実な鑑別には、メタセルカリアを終宿
主動物へ感染させて成虫を得、染色体標本で染色体
数や減数分裂像の有無を調べるか、染色封入標本と
して精子形成の有無を調べる必要がある⁴⁾。3倍体

型は2倍体型と生物学的性状が異なる。このため、
例えば人体例では一般的な少数感染の場合、肺での
虫嚢の形成と虫体の成熟、さらにそれに続く虫卵の
産生において、両型に差異が見られる⁵⁾。病態に直
結するこのような生物学的差異に注目すると、ウェ
ステルマン肺吸虫の型別判定は、虫種の鑑別と同様
に、大変重要な課題となる。

このような事由から本研究では、カニから検出し
た肺吸虫メタセルカリアを詳細に観察し、さらに肺
吸虫の好適終宿主であるネコに感染させて成虫を得
た。これを染色封入標本として観察したところ、虫
体はいずれもウェステルマン肺吸虫で間違いなく、
さらに精子形成を欠くので3倍体型と同定できた。
この結果、平成16年秋に集団発生した肺吸虫によ
る食中毒の原因は、届出のとおりウェステルマン肺
吸虫で³⁾、しかも3倍体型であることが確定された。

玉島川漁業協同組合の関係者に、地元住民の肺
吸虫に関する知識の有無を尋ねたところ、地元では
「モクズガニには肺ジストマ(肺吸虫)がいる」と認
識する者も多く、従って「モクズガニは必ず加熱調
理する」という回答を得た。しかしながら、原因施
設である料理店の調理担当者はこれを知らず、地元
産モクズガニは安全であると誤認して、今回の事故
を発生させた。このような形での肺吸虫症の再発を
予防するには、料飲店関係者に対して、生のモクズ
ガニ(あるいはサワガニ)は十分に加熱して提供す
るように、改めて徹底した啓発を行い、またこれを
継続する必要があると考えられた。

謝辞：情報の提供など、協力していただいた関係者各位
に感謝いたします。

文 献

- 1) Blair, D. *et al.* (1999) : Paragonimiasis and the Genus *Paragonimus*. *Adv Parasitol*, 42, 113-222.
- 2) 波部重久, 他 (1982) : 屋久島産のモクズガニおよびサワガニから得たウェステルマン肺吸虫のタイプ. *寄生虫誌*, 31, 27-32.
- 3) 平野敬之, 他 (2006) : 平成16年秋に集団発生した肺吸虫による食中毒事例について. *Clin Parasitol*, 17, 60-62.

- 4) 宮崎一郎 (1982) : アジアにおけるウエステルマンおよびベルツ肺吸虫の分布. 福大医紀, 9, 11-22.
- 5) 宮崎一郎 (1982) : 医学上重要なベルツ肺吸虫. 福大医紀, 9, 221-232.
- 6) 岡部浩洋, 他 (1961) : 佐賀県北部のウエステルマン肺吸虫. 久留米医誌, 24, 2342-2345.
- 7) 高橋正規 (1997) : 肺吸虫症による中毒患者の発生. 平成9年度厚生科学特別研究(新興・再興感染症研究事業)報告書「地衛研の連携による危機的健康被害の予知および対応システムに関する研究(研究代表者: 江部高廣)」, 110-112. (http://www1.iph.pref.osaka.jp/ophl2/upload/253/384kita9syu.html)
- 8) 時松一成, 他 (2001) : 感染経路が推測されたウエステルマン肺吸虫症の4例. 大分医会誌, 19, 34-38.
- 9) 横川宗雄, 他 (1986) : 沖縄県の肺吸虫について. 寄生虫誌, 35, 331-338.
- 10) 湯峰克也, 他 (2003) : 輸入上海ガニが原因と考えられるウエステルマン肺吸虫の集団感染例. 日呼吸会誌, 41, 186-190.