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Extraintestinal migration of *Pharyngostomum cordatum* metacercariae in experimental rodents

E.H. Shin, J.Y. Chai* and S.H. Lee

Department of Parasitology, Seoul National University College of Medicine, and Institute of Endemic Diseases, Seoul National University Medical Research Center, Seoul 110-799, Korea

Abstract

Extraintestinal migration patterns of *Pharyngostomum cordatum* (Digenea: Neodiplostomidae) were studied in experimental rodents such as mice, rats, and hamsters. When metacercariae isolated from grass snakes were infected orally to rodents, they penetrated the intestinal wall at days 2–3 post-infection (p.i.) and were discovered mainly in the diaphragm, intercostal muscles, and vital organ such as the lungs at days 7–28 p.i., without morphological changes. Interestingly, from several rodents which died suddenly at days 2–9 p.i., small to considerable numbers of metacercariae were found, not only in the lungs, but also in the heart and brain. Within the tissues, worms were freely motile until day 7 p.i., but later they were surrounded by host cells, and finally tissue cysts were formed. When metacercariae harvested from the snakes and intercostal muscles of rodents were infected orally to cats, they developed into adult flukes in the small intestine. The results show that *P. cordatum* undergoes considerable extraintestinal migration including the vital organs of its rodent hosts.

Introduction

Pharyngostomum cordatum (Neodiplostomidae), an intestinal trematode of wild cats, has been reported worldwide (Skrjabin, 1965). Tadpoles and adult tailless amphibians act as obligate intermediate hosts, and toads, snakes, tortoises, and shrews act as reservoir or paratenic hosts in nature (Kurimoto, 1976; Skrjabin, 1982). They grow into adults in the small intestine of cats, but not in the intestine of transport hosts such as mice, rats, hamsters, dogs and ducklings (Kajiyama & Nakamoto, 1982; Chai *et al.*, 1990). Instead, in rats, metacercariae of this fluke migrate into the diaphragm, intercostal muscles and thoracic cavity, and in chicks, ducklings, and owls they migrate into muscles, without further development (Wallace, 1939). A similar finding was reported from a raccoon dog (Ooi *et al.*, 1984), and even in cats, the definitive host, some worms do migrate into intercostal and other muscles (Wallace, 1939). However, invasion of metacercariae into the vital organs such as the lungs, heart, and brain in transport hosts has never been reported.

In our previous study with *P. cordatum* (Chai *et al.*, 1990), some experimentally infected animals, especially rodents, suddenly died for unknown reasons. This observation was not described in that paper (Chai *et al.*, 1990), but the death of the animals might have been related to extraintestinal migration of worms. The present study was therefore performed to observe in detail the extraintestinal migration patterns of *P. cordatum* metacercariae in rodent hosts and to verify their ability to invade vital organs such as the lungs, heart and brain.

Materials and methods

Animals

Seven-week-old male BALB/c mice and male Sprague-Dawley rats, raised under specific pathogen free (SPF) conditions, were purchased from the Korean Animal Center (Jinju, Korea). They were maintained at the animal facility equipped with SPF conditions in Seoul

*Author for correspondence
Fax: 82 2 765 6142
E-mail: cjy@plaza.snu.ac.kr

National University College of Medicine. Male golden hamsters (adults) and female cats (kittens) were purchased from a local market and brought to the laboratory. In order to control possible pre-existing helminth infections in hamsters and kittens, praziquantel (Shinpoong Pharm. Co., Seoul, Korea) at the dose of 10 mg kg^{-1} and albendazole (Shinpoong Pharm. Co., Seoul, Korea) at the dose of 6 mg kg^{-1} were given orally 2 weeks before experimental infection with *P. cordatum*.

Source and isolation of the metacercariae

The European grass snakes, *Rhabdophis tigrina*, naturally infected with metacercariae of *P. cordatum* (Chai *et al*., 1990), were purchased from a local snake collector in enzootic areas such as Inchon City, Kyonggi-do (Province), or Chunan City, Chungchongnam-do (Province), and kept hibernated at 4°C in a cold room until used.

Metacercariae were collected by a digestion technique using artificial gastric juice containing 0.5% pepsin (1:10,000) (Sigma Chemical Co., St Louis, Missouri, USA) and 0.8% HCl. The procedure was essentially the same as described for the isolation of *Neodiplostomum seoulense* metacercariae from snakes (Kook *et al*., 1998). Briefly, the snakes were killed and stripped of their skin. Then, the entire visceral mass and carcasses were removed and chopped before incubation in pepsin–HCl solution at 37°C for 90 min. The digested mixture was washed several times by filtration through coarse and fine stainless meshes (USA standard testing sieve; pore size 425 μm, 300 μm, 150 μm, and 106 μm). After the final filtration, metacercariae were transferred to a Petri dish and collected under a stereomicroscope. The whole procedure for the collection of metacercariae was done under cold conditions to maintain their viability.

Experimental infection of animals

To observe extraintestinal migration patterns of *P. cordatum*, mice, rats and hamsters, in batches of 15, were infected orally with 200 metacercariae in 0.2 ml in saline using a gavage needle. To obtain adult flukes, each of two kittens were inoculated orally with 200 metacercariae recovered from the snakes.

Separately from this, metacercariae were recovered from the intercostal muscles of infected rats, and used to infect two kittens to obtain adult flukes. Briefly, the intercostal muscles were removed and finely minced with a pair of scissors. Metacercariae were removed under a stereomicroscope, and batches of 200 were given orally to kittens.

Examination of body organs of rodents

On days 2, 7, 14 and 28 p.i., 3–5 mice, rats and hamsters were killed by cervical dislocation under ether anesthesia, and their body organs were removed and examined for migrating metacercariae. When animals died suddenly on unscheduled days, some of them were immediately necropsied. The organs examined were the intestine, diaphragm, intercostal muscles, lungs, heart, brain, liver, spleen and kidneys. Each organ was finely minced with a pair of scissors except for the intestine, which was longitudinally opened or cut into cross sections. Each piece of tissue was transferred onto a glass slide, and metacercariae surrounded by host cells, which were seen as yellowish-white spots of about 1 mm in diameter, were counted under a stereomicroscope. In order to observe morphological details of tissues containing worms, they were processed using a routine histological procedure. Briefly, a portion of the tissue surrounding the worm was fixed in 10% formalin, dehydrated in ethanol series, cleared in xylene, and embedded in paraffin. Sections of 5 μm thickness were made and stained with hematoxylin and eosin.

Examination of faeces of kittens

In order to monitor the presence as well as development of adult flukes in the intestine of kittens, faecal egg examinations were undertaken weekly from day 14 to day 35 p.i. After collection, faeces were transferred into a 50-ml plastic tube, and dissolved in 10 ml of tap water by vortexing. The suspension was smeared on a glass slide and eggs were identified under a microscope.

Statistical analysis

Student's t-test was performed to evaluate the significance of differences between groups, and the values of $P < 0.05$ were regarded as statistically significant.

Results

Rodent mortality

Considerable numbers of rodents infected with *P. cordatum* (4 of 15 mice, 2 of 15 rats, and 3 of 15 hamsters) died suddenly at early stages of infection, i.e. from day 2 to day 9 p.i. Among them, two mice (died at days 4 and 9 p.i.), one rat (died at day 3 p.i.), and one hamster (died at day 2 p.i.) were immediately necropsied to observe worm distribution in extraintestinal organs (table 1).

Recovery of P. cordatum *metacercariae from rodents*

Metacercariae (fig. 1) given orally were found to penetrate the intestinal wall (fig. 2) and peritoneal cavity (fig. 3) of the rodent hosts from as early as day 2 p.i. Subsequently they were found to have migrated into the diaphragm (figs 4 and 5) and intercostal muscles (figs 6 and 7), and even the vital organs such as the lungs (figs 8 and 9), heart and brain at days 2–9 p.i. (table 1). Worms did not show any visible changes in morphology. A small number of metacercariae remained in the intestinal lumen until day 3 p.i. (table 1).

However, when the organs of infected rodents were examined on day 14 p.i., metacercariae were found only in the diaphragm, intercostal muscles and lungs (table 2). The highest number was recovered from the intercostal muscles, followed by the lungs and diaphragm. Similar findings were observed on day 28 p.i. (data not shown). The percentage of worm recovery on day 14 p.i. was highest in mice, followed by rats, and hamsters (table 2),

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Table 1. Recovery of *Pharyngostomum cordatum* metacercariae from rodents which had died up to day 9 p.i.

Organs	No. of metacercariae recovered (%)*			
	Mice (n = 2)	Rat (n = 1)	Hamster (n = 1)	Total
Diaphragm	5	3	5	13 (1.6)
Intercostal muscles	55	21	5	81 (10.1)
Lungs	10	16	1	27 (3.4)
Heart	3	1	0	4 (0.5)
Brain	0	0	1	1 (0.1)
Intestine (lumen)	0	5	2	7 (0.9)
Total	73 (18.3)	46 (23.0)	14 (7.0)	133 (16.6)

* Per cent to the total number of metacercariae given. Each animal was infected with 200 metacercariae, and two mice suddenly died on days 4 and 9 p.i., respectively, the rat died on day 3 p.i., and the hamster died on day 2 p.i. No worms were recovered in the liver, spleen and kidneys.

but these differences were not statistically significant ($P < 0.05$).

Metacercariae, which migrated to extraintestinal organs of rodent hosts, were freely motile until day 7 p.i. After this time, the majority of metacercariae were surrounded by host inflammatory cells (figs 5 and 9), with worm cysts being formed from days 7 to 28 p.i. Cyst formation was most rapid and frequent in the diaphragm (figs 4 and 5) than in the intercostal muscles (figs 6 and 7) and lungs (figs 8 and 9). The larvae in the cysts remained intact without any noticeable morphological changes.

Recovery of P. cordatum *adults from kittens*

Metacercariae, isolated from snakes, developed into adult flukes of *P. cordatum* in the small intestine of kittens, showing eggs in the faeces after day 31 p.i. The average worm recovery (no. adult flukes recovered/no. metacercariae given) was 70.0% on day 35 p.i.

Metacercariae harvested from the intercostal muscles of rats also developed into *P. cordatum* adults (fig. 10) in the small intestine. The adult worm recovery on day 35 p.i. was, however, lower (average 43.0%) than that from kittens infected with metacercariae from the snake. Eggs were found in the faeces of these kittens after day 28 p.i. (fig. 11).

Discussion

The majority of *P. cordatum* metacercariae given orally to the three species of rodents migrated into the diaphragm and intercostal muscles, which is in agreement with the

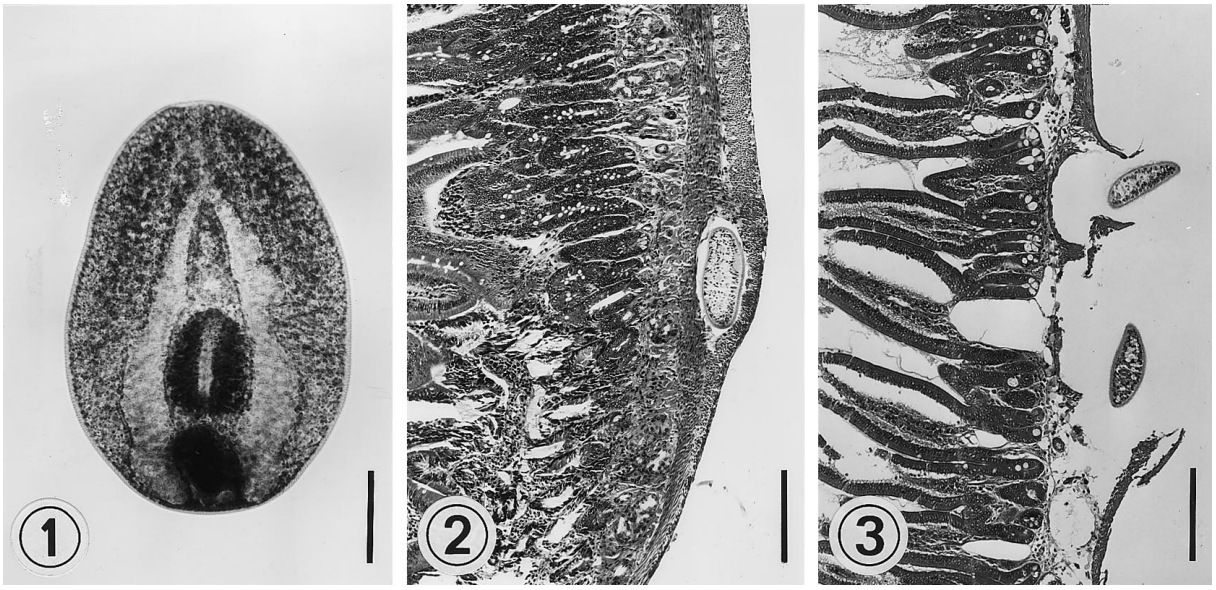

Fig. 1. Metacercaria of *Pharyngostomum cordatum* stained with acetocarmine recovered from a European grass snake *Rhabdophis tigrina*. The dark stained tribocytic organ (upper) and genital primordium (lower) are characteristically seen. Bar = 0.15 mm. Fig. 2. Intestinal wall section of a rat infected with *P. cordatum* (day 2 p.i.). A metacercaria is seen to have invaded into the mucosa, submucosa and muscle layer, and now near the serosa of the intestine. Haematoxylin and eosin stain. Bar = 0.5 mm. Fig. 3. Intestinal wall section of rat infected with *P. cordatum* (day 2 p.i.). Two metacercariae are seen entering the peritoneal cavity after tearing the intestinal serosa. Haematoxylin and eosin stain. Bar = 0.5 mm.

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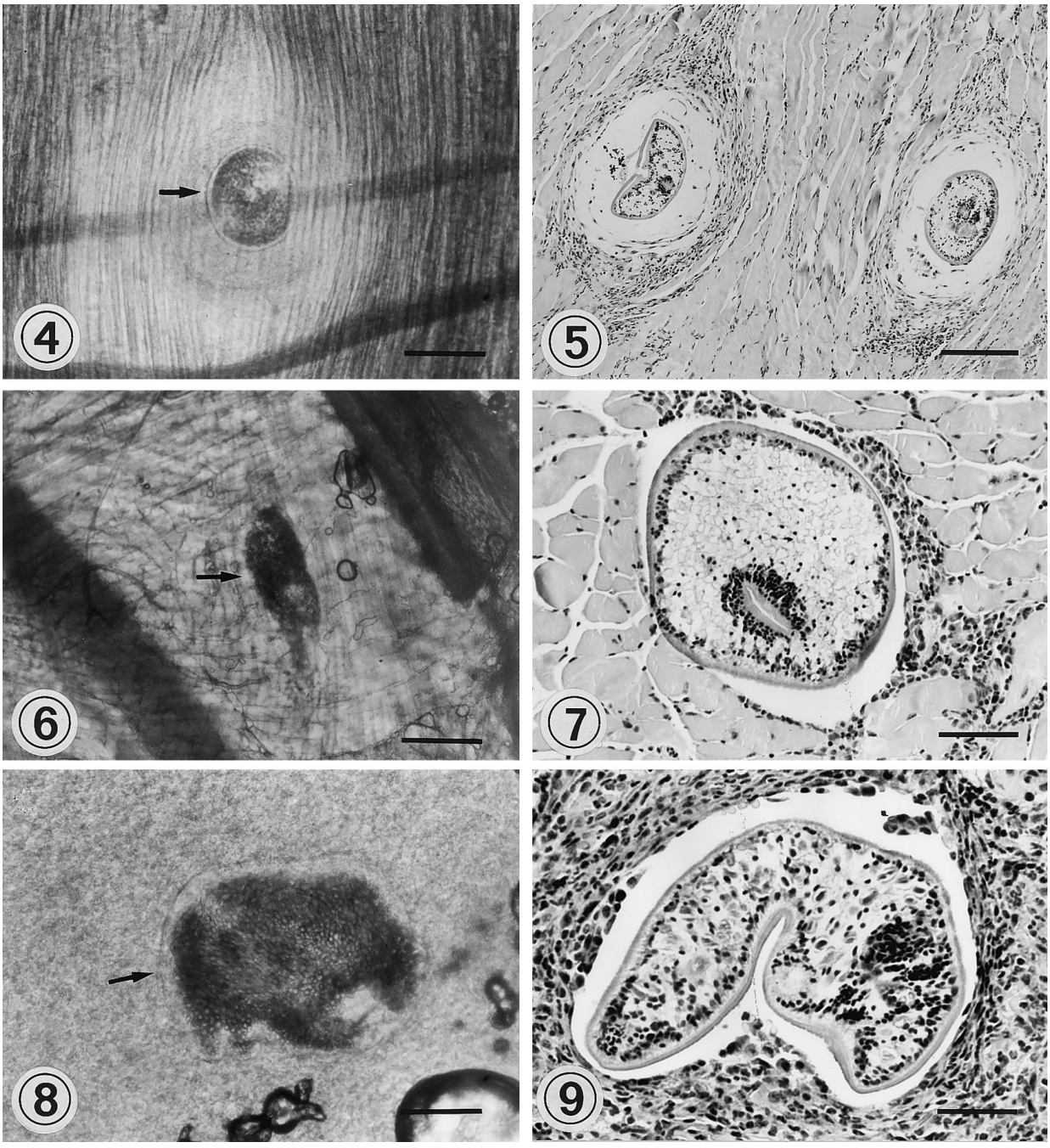

Figs 4–9. Unstained metacercariae of *Pharyngostomum cordatum* in extraintestinal organs of a BALB/c mouse on day 7 p.i. (figs 4, 6, 8) and those on day 14 p.i. stained with haematoxylin and eosin (figs 5, 7, 9). Fig. 4. Diaphragm containing a metacercaria (arrow) surrounded by a cyst of host origin. Bar = 0.4 mm. Fig. 5. Diaphragm with two metacercariae, both of which are surrounded by a thick layer of host cells. Bar = 0.5 mm. Fig. 6. Metacercaria (arrow) penetrating the intercostal muscle. Bar = 0.4 mm. Fig. 7. Section of an intercostal muscle containing a metacercaria. Bar = 0.2 mm. Fig. 8. Metacercaria (arrow) invading the lung parenchyma. Bar = 0.2 mm. Fig. 9. Lung invaded by a metacercaria with pronounced host inflammatory cell infiltration. Bar = 0.1 mm.

results of previous studies (Wallace, 1937, 1939; Ooi *et al.*, 1984). However, a small proportion was found to have invaded the vital organs such as the lungs, heart and brain, which appears to be a new finding. Identification of the metacercariae was based not only on their characteristic morphology with dense distribution of excretory granules, but also on the recovery of adult flukes at day 35 p.i. from kittens infected with those

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Table 2. Recovery of *Pharyngostomum cordatum* metacercariae from extraintestinal locations in rodents on day 14 p.i.

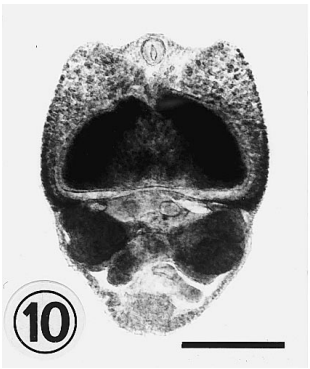
Organs	No. of metacercariae recovered from 5 animals* (%)†			
	Mice	Rats	Hamsters	Total
Diaphragm	13	0	4	17 (0.6)
Intercostal muscles	176	210	220	606 (20.2)
Lungs	80	48	0	128 (4.3)
Total	269 (26.9)‡	258 (25.8)‡	224 (22.4)‡	751 (25.0)

* Each animal was infected with 200 metacercariae.
† Per cent to the total number of metacercariae given.
‡ Differences between groups were not statistically significant ($P < 0.05$).
No worms were recovered in the heart, brain, liver, spleen, kidneys and intestine (lumen and wall).

recovered from the snake and from their extraintestinal locations within experimental rodents.

Another interesting finding was that the extraintestinal migration of *P. cordatum* metacercariae appeared to be harmful to the host, e.g. 20% of the animals infected with 200 metacercariae died suddenly on days 2–9 p.i. Systemic necropsy of two mice, one rat and one hamster revealed that the metacercariae had invaded not only the diaphragm and intercostal muscles but also the vital organs such as the lungs, heart, and brain. The sudden death of animals is likely to be related to worm invasion into vital organs.

Histological sections of the small intestine of rodents infected with metacercariae of *P. cordatum* clearly showed worms in the process of passing from the intestinal lumen through the intestinal wall to the peritoneal cavity. In this case, the migration of metacercariae resulted in a considerable degree of damage to the intestinal wall. On the other hand, many of the worms were trapped by a number of host cells at these extraintestinal locations, and were gradually surrounded by cysts of host origin. Cysts formed in the diaphragm, intercostal muscles and lungs are likely to cause difficulties in the respiratory capacity of the hosts. Cysts formed in the heart and brain resulted in a deterioration of the functions of each organ.



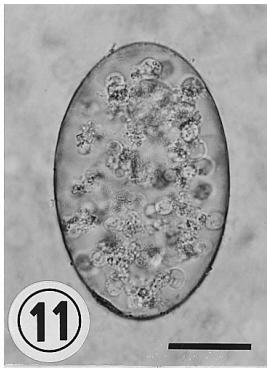


Fig. 10. Adult *Pharyngostomum cordatum* recovered on day 35 p.i. from the small intestine of a kitten infected with metacercariae harvested from intercostal muscles of a rat. Semichon's acetocarmine stain. Bar = 0.6 mm. Fig. 11. Egg of *P. cordatum* in the faeces of an experimentally infected kitten on day 31 p.i. Bar = 0.03 mm.

The exact cause of host mortality, however, remains to be clarified.

The results of the present study may also have clinical significances, since the possibility of human infections with *P. cordatum* cannot be easily excluded. The strigeoid flukes so far known to infect humans include several species belonging to three genera; *Alaria americana* (Fernandes *et al.*, 1976; Freeman *et al.*, 1976), *A. marcianae* (Beaver *et al.*, 1977), *Neodiplostomum seoulense* (Seo *et al.*, 1982; Hong *et al.*, 1984, 1986), and *Fibricola cratera* (Shoop, 1989). In the case of the latter two species, humans act as the definitive host, so that infections occur by adult flukes in the intestinal tract (Seo *et al.*, 1982; Shoop, 1989). However, in the case of *Alaria* spp., humans were a transport host and infection occurred at a number of extraintestinal locations. The clinical features associated with *Alaria* spp. infection include systemic and fatal diseases (Fernandes *et al.*, 1976; Freeman *et al.*, 1976), intradermal mass (Beaver *et al.*, 1977), ocular lesions (McDonald *et al.*, 1994), and respiratory symptoms and subcutaneous granuloma (Kramer *et al.*, 1996). The interesting case of systemic and fatal infection had numerous metacercariae of *A. americana* which invaded into the lungs and other organs (Fernandes *et al.*, 1976; Freeman *et al.*, 1976). This case is noteworthy because the pathological features were very similar to those observed from rodents infected with *P. cordatum* in the present study.

Human infections are likely to be caused by *P. cordatum* because firstly, *P. cordatum* has a close resemblance with *Alaria* spp. in its life cycle and the choice of hosts (Bosma, 1934) indicating a similarity in behavioural and immunological aspects of the host–parasite relationship. Secondly, humans are readily exposed to infective stages of *P. cordatum* by the consumption of improperly cooked meat of animals playing the role of intermediate, paratenic or transport hosts. In practice, some Korean people, who consume undercooked snakes such as *R. tigrina*, a paratenic host for both *N. seoulense* (Seo, 1990) and *P. cordatum* (Chai *et al.*, 1990), develop intestinal infections with *N. seoulense* (Seo *et al.*, 1982; Hong *et al.*, 1984, 1986). Even if infections with *P. cordatum* had occurred, the diagnosis might have been very difficult especially when worms had migrated into extraintestinal locations.

The present study has thus shown that *P. cordatum* is clearly capable of extraintestinal migration into the vital

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organs of rodent hosts, and might be related with the sudden death of some infected animals. The presence of human extraintestinal infection with *P. cordatum* is strongly implicated.

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