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Research Note

Detection of Lung Fluke Larvae in Serial Sections of the Freshwater Crabs, *Geothelphusa dehaani*, Experimentally Exposed to *Paragonimus miyazakii* Cercariae

JUNICHI GYOTEN

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Abstract

We developed a method of preparing serial sections of crustacean hosts containing *Paragonimus* larvae immediately after penetration (immature metacercariae). We studied 3 freshwater crabs, *Geothelphusa dehaani* exposed to *Paragonimus miyazakii* cercariae for 18 or 24 hrs. Sections prepared by this method gave satisfactory pictures of both host and parasite structures.

Key words: *Paragonimus*, Larva, Serial section, Crab

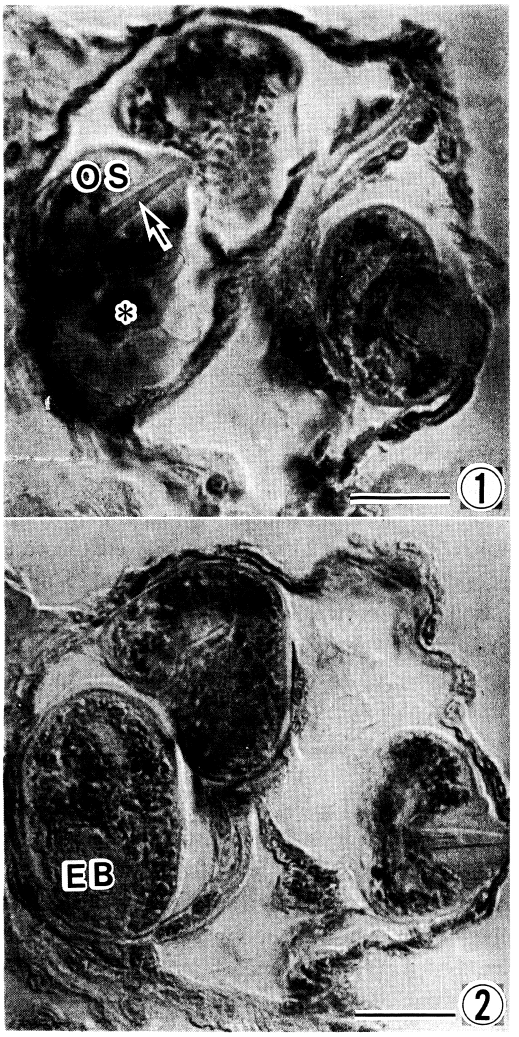
There are no commonly accepted views as to how *Paragonimus* cercariae enter crustacea. A few investigators studying hosts infected with cercariae, have attempted to determine the mode of cercarial penetration. Two prevailing theories, which assert percutaneous or peroral penetration, have been proposed (Yokogawa, 1953; Yoshida, 1961). However, neither of these theories have been proven, since the process of cercarial penetration into crustacean hosts has not been observed. This has mainly been due to the lack of adequate techniques for detecting larvae which have just penetrated the host. New techniques are required for further investigations into this issue. Therefore, the author devised a mean of preparing serial sections of crustacean hosts for detecting larvae, and the details are as follows.

P. miyazakii cercariae were collected from water containing many cercariae emerged from its snail hosts *Bythinella nipponica*, which were experimentally infected with *P. miyazakii* miracidia for over 80 days. The crab hosts, *G. dehaani*, were collected from a mountain stream at Komenono, Matsuyama City and were considered to be free from natural infection with *Paragonimus* spp., since none of 150 crabs previously collected were infected (Gyoten, 1986). Three of the crabs (2 females and 1 male, carapaces measuring 4.5–4.8×3.8–4.3 in mm) were exposed to 95, 100 and 103 cercariae respectively in dishes (60 mm in diameter) containing 10 ml of water. After 18 or 24 hrs, the crabs were placed in acetic alcohol (25 ml of acetic acid in 75 ml of absolute ethanol) for 3 days, to separate the chelipeds and walking legs from the body, then in 70% alcohol. The specimens were then decalcified and softened in 45% acetic acid for 5 hrs, and preserved in 10% formalin. The separated specimens were collected into 3 groups, the body parts, the left cheliped with 4 left walking legs and the right cheliped with 4 right walking legs. These were embedded in agar, then preserved in 10% formalin. Three days later, the groups were excised from the agar, dehydrated in graded alcohol, cleared in 2 changes of xylene, embedded in paraffin, and sectioned transversely at 10 μm thick. Sectioned specimens were stained with Delafield's hematoxylin, dehydrated in graded alcohol, cleared in xylene and mounted in Canada balsam. The unexposed controls were 2 crabs (1 female and 1 male, carapaces measuring 4.5–4.7×3.7–4.3 in mm) processed in the same manner.

A total of 1,353 serial sections of the 3 exposed crabs were examined microscopically for *Paragonimus* larval infection, and 74 fragments of *Paragonimus* larvae were found. There are no detectable

Department of Parasitology, Ehime University School of Medicine, Shigenobu-cho, Ehime Prefecture 791-02, Japan.
行天淳一（愛媛大学医学部寄生虫学教室）

fragments in sections of the 2 unexposed crabs. The fragments were easily distinguishable from the organs of the crabs by the presence of tegument and muscle cells stained light pink with Delafield's hematoxylin. Careful examination of the fragments revealed that some of them contained organs peculiar to *Paragonimus* larvae, such as a large stylet, a muscular oral sucker, a ventral sucker and a transparent excretory bladder (Figs. 1 and 2). These unique structures confirmed the larval origin of the fragments. In addition, as stylet sections were more prominent and unique than other larval organs and detectable in about two fragments per larva, they served as landmarks in several sections of crab organs. The 74 detected fragments were assembled into 18 total larval preparations, the number of which was corresponded to 6% of the exposed cercariae. This was larger than the recovery rate (3.4%) of *P. miyazakii* metacercariae in the cercarial infection experiment of Gyoten (1986). Three of the larval preparations were harbored in the pericardial cavity, 5 being in the liver, 7 in the body muscle and 3 in the leg muscle of the crabs, which were histologically distinguishable under the light microscope. One crab contained 2 larvae, whereas the other two contained 4 and 12.



Figs. 1 and 2. Sections of a crab, *Geothelphusa dehaani*, exposed to cercariae of the lung fluke *Paragonimus miyazakii* for 18 hrs. 1. Cross sections of the hepatic artery reveal *Paragonimus* larvae. The longitudinal section contained a stylet (arrow), oral (OS) and ventral sucker (*). 2. Serial sections of those shown in Figure 1. The longitudinal section contained a cell mass (EB) which is part of the I shaped excretory bladder. Scale bar = 25 μm.

Little is known about how *Paragonimus* cercariae enter crustacean hosts. Although there have been many key studies of crustacean hosts infected with cercariae (Ando, 1920; Yokogawa, 1953; Yoshida, 1961; Gyoten, 1986; Shibahara, 1991), even the portals of entry in the hosts have not been defined. Cercariae have remained undetectable while penetrating crustacean hosts, due to the lack of adequate methods of detection. To date, only a few microscopic studies on fresh crustacea have revealed larvae (Ando, 1920; Nakagawa, 1960; Stromberg, *et al.*, 1978; Gyoten, 1983). However, microscopic examination of fresh material cannot expose larvae located near the crustacean skeleton, which also cannot be studied under the light microscope, because it is opaque. To carefully examine entire crustaceans under the light microscope, serial sections of fixed crustacea should be prepared. To date, howevr, this issue has not been addressed. Thus, the author devised a means of preparing serial sections of crabs and applied it to crabs experimentally exposed to *P. miyazakii* cercariae. The procedure includes a series of immersions in acetic alcohol, 70% alcohol then 45% acetic acid, before fixation in 10% formalin for preparing serial sections. This process is part of Takao's method of preparing total specimens of flukes and tapeworms (Takao,

1983), which has derived from Snow's chromosome stain (Snow, 1963). However, these procedures facilitated the preparation of serial crab sections by decalcifying and softening the skeletons. The sections prepared using this method revealed satisfactory pictures of *Paragonimus* larvae such as cercariae, as well as the organs of the crab hosts. Thus, fragments of larvae and sections of organs were accurately identified and the habitats of the larvae after 18 to 24 hrs of exposure were as the pericardial cavity, liver, body and leg muscle of crabs. These habitats were the same as those where *P. miyazakii* mature metacercariae are generally harbored in (Hatsushika, 1967). Allowing for this, cercariae seem to migrate to definitive habitats within 18 hrs of exposure. Thus, when crabs exposed to cercariae are studied during this periods using the present method, the portals of entry and migration routes of cercariae should be definable. Consequently, this procedure will help studies on the relationships between *Paragonimus* larvae and their crustacean hosts.

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