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Collection methods of trematode eggs using experimental animal models☆

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ABSTRACT

Although observing the eggs of human parasitic helminth is essential for medical education in parasitology, opportunities for collection of the eggs are limited. Collection of the eggs using experimental animal models is needed for a sustainable supply. The metacercariae of three trematode species, *Paragonimus westermani*, *Clonorchis sinensis* and *Metagonimus yokogawai*, were collected from the second intermediate hosts: freshwater crabs and fishes, which were obtained using online shopping in Japan, and inoculated to experimental animal rat and dog. Consequently, eggs of the three trematode species were obtained abundantly from the feces of the animals. The eggs are being used for student training in several Japanese universities. In this article, we introduce the collection procedures for trematode eggs.

1. Background

Observing the eggs of human parasitic helminth is an important training item for education in parasitology in medical universities and colleges. Opportunities for collection of several kinds of helminth eggs are limited because the number of patients with these parasitic diseases has decreased. Furthermore, the eggs are frequently preserved in formalin and lost following each observation. New collection of helminth eggs is necessary to maintain constant supply to student training in parasitology.

2. Objective

The aim of this article was collection of many eggs of the three human parasitic trematode species, *Paragonimus westermani*, *Clonorchis sinensis* and *Metagonimus yokogawai*. We attempted collection of the metacercariae from second intermediate hosts obtained mainly using online shopping in Japan and inoculated them to experimental animals. The collection processes are presented in this article.

3. Methods

3.1. Collection of *P. westermani* eggs

The metacercariae were isolated from freshwater crabs, *Geothelphusa dehaani* from Chiba and Mie Prefectures in Japan. The procedure for isolation of metacercaria was as follows:

1. A suspension of finely minced crabs was successively passed through stainless-steel meshes of 1.0, 0.5 and 0.3 mm mesh size (Testing Sieve, Nonaka Rikaki, Tokyo, Japan) by washing with tap water.
2. The residues trapped by 0.3 mm mesh were recovered with tap water in 1 L conical-shaped glass.
3. Further tap water was added to the conical-shaped glass and it was kept standing for a while. The supernatant was discarded by decantation or aspiration. This step was repeated three times.
4. The precipitate was transferred to a glass dish and then the metacercariae (Fig. 1a) were collected using a capillary pipette under a dissection microscope.

The metacercariae were stored in 0.4% saline at 4 °C until use. The storage period was one to three months. Another second intermediate host, *Eriocheir japonicus*, from Yamaguchi, Oita or Kagoshima Prefecture could be purchased online but no metacercariae were detected.

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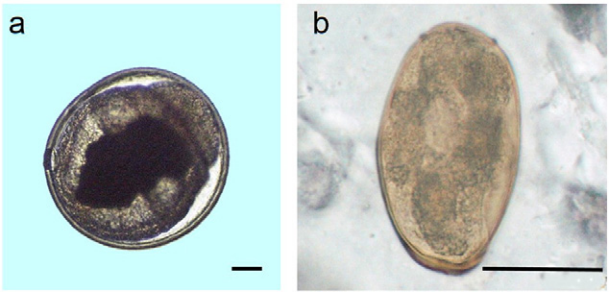

Fig. 1. *Paragonimus westermani* metacercaria and egg. The metacercariae were obtained from freshwater crabs, *Geothelphusa dehaani* (a). The eggs were detected in feces of an infected dog (b). Bars = 50 μm.

One hundred and five metacercariae were inoculated orally to a 24-week-old female beagle (Kitayama Labes, Yamaguchi, Japan). The fecal eggs (Fig. 1b) were detected at 70 days after the infection by AMS III method (a concentration method for the eggs) [1]. The karyotype was assumed to be diploid histologically [2]. The direct smear method could detect the eggs (1–2 eggs/field covered by a square cover slip [18 × 18 mm], 255 eggs per gram feces [epg]) on day 95. The number of detectable eggs increased on day 120 (5–10 eggs/18 × 18 mm, 1553 epg). Because the fecal egg output decreased on day 150 (Fig. 4a), fifty two metacercariae were further inoculated on day 155 after the primary inoculation. The eggs were detected abundantly (10–20 eggs/18 × 18 mm, 2201–3226 epg) by direct smear during 300–600 days after the primary inoculation (Fig. 4a). The eggs will be obtained from feces of this beagle in the future because the human infection period of *P. westermani* is about 10 years [2].

3.2. Collection of C. sinensis eggs

Freshwater fishes, *Pseudorasbora parva*, from Okayama Prefecture were purchased as a second intermediate host of *C. sinensis*. Some metacercariae were detected from the muscle compressed between 2 glass slides under an optical microscope. The number of metacercariae in *P. parva* in the winter was more than that in the summer. The procedure for artificial digestion to isolate metacercariae from fresh water fish was as follows:

1. The ground fishes with tap water were incubated in five volumes of artificial gastric juice containing 1% HCl and 0.3% pepsin (1:10,000 from porcine stomach mucosa, Wako Pure Chemical Industries, Osaka, Japan) at 37 °C for 2 h with vigorous shaking.
2. The digested materials were successively passed through a standard cotton gauze and stainless-steel meshes of 250 and 106 μm mesh size.
3. The residues trapped by 106 μm mesh were washed and recovered with physiological saline in a 300 mL conical-shaped glass.
4. Further physiological saline was added to the conical-shaped glass and it was kept standing for a while. The supernatant was discarded by decantation or aspiration. This step was repeated three times.
5. The precipitate was transferred to a glass dish and then the metacercariae were collected using a capillary pipette under a dissection microscope.

Some metacercariae (17 metacercariae per one *P. parva* on average) were considered morphologically to be *C. sinensis* [3]. To identify the metacercaria species, genomic DNA was extracted from individual metacercaria [4]. The ITS2 region of rDNA was amplified using primer pairs 3S with A28. The primer pairs can be applied to other trematode species [5,6]. Samples for DNA sequencing were prepared using an ABI PRISM BigDye Terminator Cycle Sequencing Ready Reaction kit (Applied Biosystems, USA) and sequencing was performed on 3100-Advant Genetic Analyzer (Applied Biosystems). As a result, the majority of metacercariae were identified as *Metronchis orientalis* [7] and other small population of metacercariae was identified as *C. sinensis*. The two metacercariae were morphologically similar except for the thickness of the cyst wall (Fig. 2a). The metacercariae were stored in phosphate- buffered saline at 4 °C until use. The storage period was approximately one month.

Ten metacercariae of *C. sinensis* were orally inoculated to a 8-week-old male rats (SLC, Shizuoka, Japan). The eggs of *C. sinensis* were detected at 25 days after the inoculation by the AMS III method (55 epg) (Fig. 2b). During days 36–190 after infection, the number of detectable eggs increased (139–1491 epg; Fig. 4b). Five adult flukes were recovered from the bile duct of this rat (Fig. 2c). The adult flukes were also identified as *C. sinensis* by sequencing of the ITS2 region and morphological appearance of the testes [8].

3.3. Collection of M. yokogawai eggs

The sweetfishes, *Plecoglossus altivelis*, as a second intermediate host of *M. yokogawai* were purchased from Wakayama, Kochi and Shimane Prefectures. Abundant metacercariae were detected from the muscles in the fish from Shimane Prefecture, but not in those from Kochi. Ground fishes with tap water were incubated in two volumes of artificial gastric juice containing 2% HCl and 0.3% pepsin at 37 °C for 2 h with vigorous shaking. The latter artificial digestion method was the same as for *C. sinensis*. The majority of metacercariae were assumed to be *M. yokogawai* from their morphological appearance (Fig. 3a). Furthermore, the sweetfish is a major second intermediate host of *M. yokogawai* [3]. Finally, approximately 20,000 metacercariae were obtained from 212 sweetfishes from Shimane Prefecture. The metacercariae were stored in phosphate-buffered saline at 4 °C until use. The storage period was approximately one month.

Ten thousand metacercariae were orally inoculated to a 12-week-old female beagle and fecal eggs (75 epg) were detected 9 days after

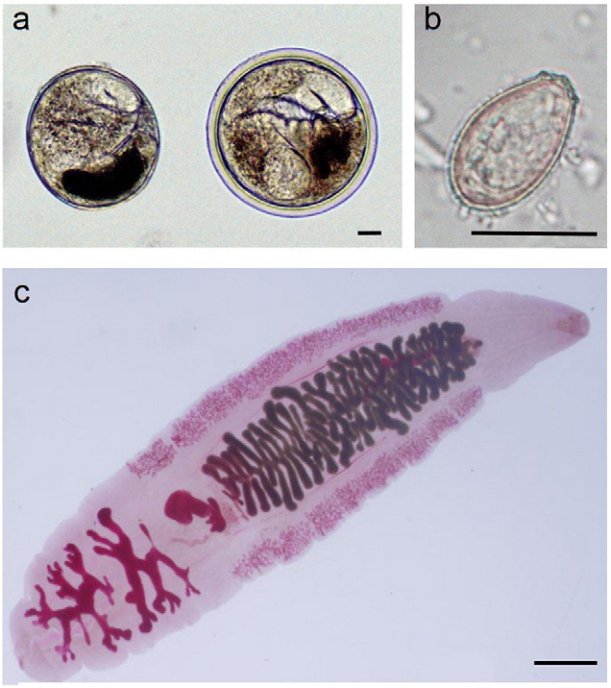

Fig. 2. *Clonorchis sinensis* metacercaria, egg and adult worm. The metacercariae were obtained from freshwater fishes *Pseudorasbora parva* (left, *C. sinensis*; right *Metronchis orientalis*) (a). The eggs were detected in feces of an infected rat (b). Bars = 20 μm. An adult fluke was recovered from bile duct of the infected rat. The fluke was stained with Carmine. Bar = 1 mm (c).