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MORPHOLOGICAL OBSERVATIONS OF *ECHINOCHASMUS JAPONICUS* CERCARIAE AND THE IN VITRO MAINTENANCE OF ITS LIFE CYCLE FROM CERCARIAE TO ADULTS

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ABSTRACT: The cercaria morphology of *Echinochasmus japonicus* was investigated using light and scanning electron microscopy. Cercariae, liberated from naturally infected snails (*Parafossarulus manchouricus*), had ovoid bodies and diminutive tails. The cercaria tegument was covered with minute spines. Four type II sensory papillae were observed on the dorsal side of the oral sucker, and type I papillae were distributed on the dorsal tegument surfaces. When cercariae were kept in the same bath as the freshwater fish, *Pseudorasbora parva,* which were free from trematode infections, parasites encysted only in the gills of fishes at day 4 postinfection (PI). The outermost metacercaria wall was fully formed in host tissues at day 7 PI. Adult worms were recovered from the intestines of rats, chicks, and ducks 28 days after experimental exposure to metacercariae. The head crown of the adult was armed with 24 collar spines, which were interrupted dorsal to the oral sucker, and the species was identified as *E. japonicus*.

Echinochasmus japonicus is a trematode of birds and mammals, and belongs to the Echinostomatidae. It was first described in experimental animals, i.e., dogs, cats, rats, mice, and birds (Tanabe, 1926); natural human infections have been reported in China and Korea (Chai and Lee, 2002). Its first intermediate hosts include the freshwater snail *Parafossarulus manchouricus* in Korea (Rhee et al., 1983), and *Parafossarulus striatulus* in China (Chen et al., 1990); freshwater fishes, i.e., *Pseudorasbora parva*, *Abbottina springeri*, and *Gnathopogon coreanus*, have been identified as second intermediate hosts in Korea (Rhee et al., 1984).

Echinochasmus japonicus is morphologically similar to *Echinochasmus liliputanus*, recently identified as a human parasite in China; however, it is characterized by a body size smaller than *E. liliputanus*, usually less than 1 mm (Seo et al., 1985; Xiao et al., 1992). The teguments of *E. japonicus* adults are covered with spade-shaped spines on the ventrolateral surface, extending to the subterminal region (Lee et al., 1987), whereas *E. liliputanus* has minute spines, arranged to the level of the posterior testis (Xiao et al., 1992). Random amplified polymorphic DNA analysis had shown that *E. japonicus* is different from *Echinochasmus fujianensis*, which has been proven to be the same species as *E. liliputanus* (Cheng et al., 1999).

Humans are infected by eating raw freshwater fishes harboring metacercariae (Seo et al., 1985). However, *E. liliputanus* has been reported to be transmitted to humans mainly by drinking unboiled water with cercariae in Anhui Province, China (Xiao et al., 1995). It is not clear whether humans can be infected directly by swallowing cercariae of *E. japonicus* in water. To evaluate the possibility of waterborne infection by *E. japonicus* in humans, studies on cercariae of *E. japonicus* are necessary. The present investigation describes the morphological characteristics of *E. japonicus* cercariae collected from the freshwater snail, *P. manchouricus*. Species identification was confirmed by examining adult worms from experimental animals.

MATERIALS AND METHODS

Cercariae release from snails

Snails, *P. manchouricus*, were collected from a reservoir near Jinju (35°03′N, 127°53′E), Gyeongsangnam-do, Korea. Ten snails were transferred individually to 50-ml conical tubes containing pond water, and kept at room temperature for 8 hr to allow cercariae release, which was observed using stereomicroscopy. Snails liberating cercariae were transferred individually to 15-ml conical tubes, and kept at room temperature for 8 hr. Cercariae were collected for further investigation.

Experimental infection of cercariae and metacercariae

Freshwater fishes, *P. parva*, were caught in an area that was nonendemic for echinochasmiasis in Korea. Randomly sampled fishes were examined using stereomicroscopy to confirm that the fish were free of trematode infections after artificial digestion of 10% of collected fishes in pepsin-HCl solution at 37 C for 3 hr. Fishes confirmed to be free of trematodes were then exposed to 8-hr-old cercariae of *E. japonicus* by keeping them both in the same water bath. The presence of metacercariae was investigated on days 1, 2, 4, 7, 14, and 28 postinfection (PI) in the fish heads, eyes, gills, scales, muscles, intestines, and fins.

To recover adult worms, 10 rats (6-wk-old, Sprague-Dawley), 5 chicks (2-wk-old, domestic), and 5 ducks (2-wk-old, domestic), were infected with metacercariae isolated from the fishes that had been experimentally infected with cercariae. Ninety, 50, and 50 metacercariae were orally intubated to each rat, chick, and duck, respectively, using a gavage needle. Animals were killed using ethyl ether at day 28 PI, and adult flukes were recovered from the intestines.

Morphological observations

The morphology of *E. japonicus* was examined by light microscopy at different developmental stages under cover-glass pressure, after fixing specimens in 10% formalin. Measurements of total body size, oral and ventral suckers, pharynx, ovary, anterior and posterior testes, and eggs were obtained.

The tegumental ultrastructure of *E. japonicus* cercariae was observed by scanning electron microscopy (SEM) (Sohn et al., 2002). Briefly, cercariae were washed several times with 0.1 M phosphate buffer (pH 7.4) and 3 times with 0.1 M cacodylate buffer (pH 7.2). They were then fixed in 2.5% glutaraldehyde solution at 4 C, washed in 0.1 M cacodylate buffer, dehydrated in a graded alcohol series (50%, 70%, 80%, 90%, 95%, and absolute), dried in a critical-point dryer, and mounted on stubs. After coating with gold in an ion coater (Eiko IB-3), they were examined using a SEM (ISI DS-130C, Tokyo, Japan) at an accelerating voltage of 10 kV.

RESULTS

Infection status of snails with trematode cercariae

Three types of cercariae were collected from the snails, *P. manchouricus*,: the cercariae of *E. japonicus* and those of 2

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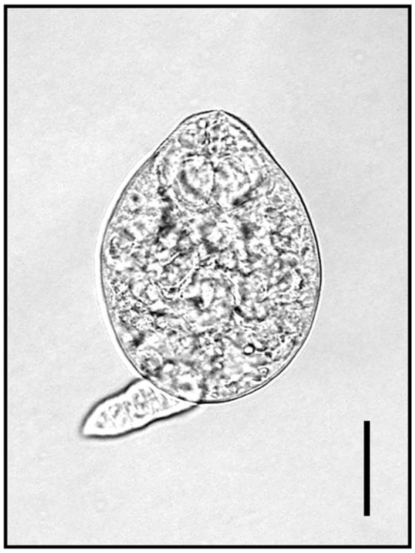

FIGURE 1. A cercaria of *Echinochasmus japonicus* liberated from the snail, *Parafossarulus manchouricus*. Bar = 30 μm.

unidentified species. The prevalence of snails shedding *E. japonicus* cercariae was 0.8%, and those of unidentified furcocercariae and lophocercous cercariae, were 1.4% and 0.2%, respectively. The 2 unknown species did not successfully develop into metacercariae in freshwater fishes exposed to cercariae.

Morphology of *E. japonicus* cercariae

Typically, the bodies of *E. japonicus* cercariae were oval, and 92.5 ± 2.0 by 71.3 ± 5.2 μm in size. The tail was shorter than its body at 45.8 ± 5.8 by 17.5 ± 1.5 μm (Fig. 1). Oral and ventral suckers were observed on its ventral surface. However, the head crown and collar spines, characteristic of adult worms, were not recognizable around the oral sucker.

The tegument was wrinkled transversely, and minute spines (smaller than 1 μm) were distributed over the whole body surface, except for the ventromedian area between the oral and ventral suckers (Figs. 2A–F). The oral sucker, when contracted, had a slitlike opening, where 4 nonciliated round swollen sensory papillae (type II) were located (Fig. 2B). Sensory papillae with cilia (Type I) were observed on the ventral surface just posterior to the oral sucker (Fig. 2C); the ventral sucker was muscular without tegumental spines or sensory papillae (Figs. 2E–F). The dorsal surfaces of cercaria were covered with minute tegumental spines, among which type I papillae with cilia longer than 5 μm were scattered (Figs. 3A–D). The cercaria tail was composed of wrinkled cytoplasmic processes, and adopted various shapes because of its high motility during the fixation process (Figs. 2G, 3E).

Encystment of *E. japonicus* cercariae in freshwater fishes

To identify the species of cercariae obtained from *P. manchouricus*, freshwater fishes free of trematode infections were exposed to cercariae. Metacercaria encystation was then monitored from day 1 to day 28 after exposure. Metacercariae were not detected until day 3 PI and, thereafter, they were found only in the gills. They were elliptical in shape and 77.7 ± 1.7 by 61.3 ± 1.7 μm at day 4 PI (Fig. 4A). However, cyst walls were fragile, and the metacercariae were easily liberated from cysts even by slight needle pressure after isolation. At day 7 PI, they were fully encysted; the outermost cyst wall was host-derived (Fig. 4B). Collar spines were observed around the oral suckers of metacercariae at day 14 PI.

Development of *E. japonicus* metacercariae into adults in experimental animals

Adult worms were recovered from rats (31.3%), chicks (26.0%), and ducks (18.0%) at 4 wk PI and were similar in size (average 327.3 by 216.8 μm). The measurements of adults isolated from experimentally infected rats are summarized in Table I. Adult worms were plump and pear-shaped, with an attenuated anterior and a rounded posterior (Fig. 4C). The anterior region was characterized by a head crown, where 24 collar spines were arranged in a row around the oral sucker; they were interrupted dorsal to the oral sucker (Fig. 4D). The ventral sucker was well developed and twice as large as the oral sucker. The cirrus sac was observed anterior to the ventral sucker. The uterus was short and located between the cirrus sac and ovary, which typically contained 0, 1, or 2 eggs. The eggs were immature, oval, 92.5 by 60.0 μm, and had a small operculum and a prominent knob at the abopercular end (Fig. 4E).

DISCUSSION

The cercariae of *E. japonicus* had a ovoid bodies and short tails, but no head crown or collar spines were recognizable, which is similar to morphological characters of *E. liliputanus* cercariae (Xiao et al., 2005). However, the distribution of sensory papillae around the oral sucker is different in the 2 species; the oral region of *E. japonicus* is covered with nonciliated and uniciliate papillae, whereas that of *E. liliputanus* is equipped with uniciliate and multiciliate papillae (Xiao et al. 2005). The SEM finding differentiates species of *Echinochasmus* from closely related species of *Echinostoma*, which has a well-developed head crown and collar spines, and a tail with several fin-folds, which is usually longer than its body (Fried and Fujino, 1987; Krejci and Fried, 1994). Tail fin-folds have been used to differentiate echinostome cercariae by some investigators, for example, the cercaria of *Echinostoma caproni* has 1 ventral and 2 dorsal tail fin-folds, whereas that of *Echinostoma trivolvis* has 2 ventral and 2 dorsal fin-folds (Krejci and Fried, 1994). However, the tails of *E. japonicus* cercariae had no fin-folds.

Two types of sensory papillae, i.e., ciliated (type I) and nonciliated round swellings (type II), were observed on the tegument of *E. japonicus* cercariae. Type I papillae were distributed on the ventral and dorsal surfaces of the tegument, whereas type II papillae were located only at the oral sucker. The distribution and type of sensory papillae resembled those of an adult *E. japonicus* (Lee et al., 1987), except for the mixed distribution of type I and II papillae on the oral sucker of adult *E. japonicus*. Type I papillae have been suggested to be tango-, rheo-, mechano-, or chemoreceptive, and type II papillae to be tango- and mechanoreceptive (Hong et al., 2004). The inhibition of in vitro encystment of *E. liliputanus* cercariae by treatment with silver nitrate suggested that the ciliated papillae may be involved in the cercariae encystment (Xiao et al., 2005).

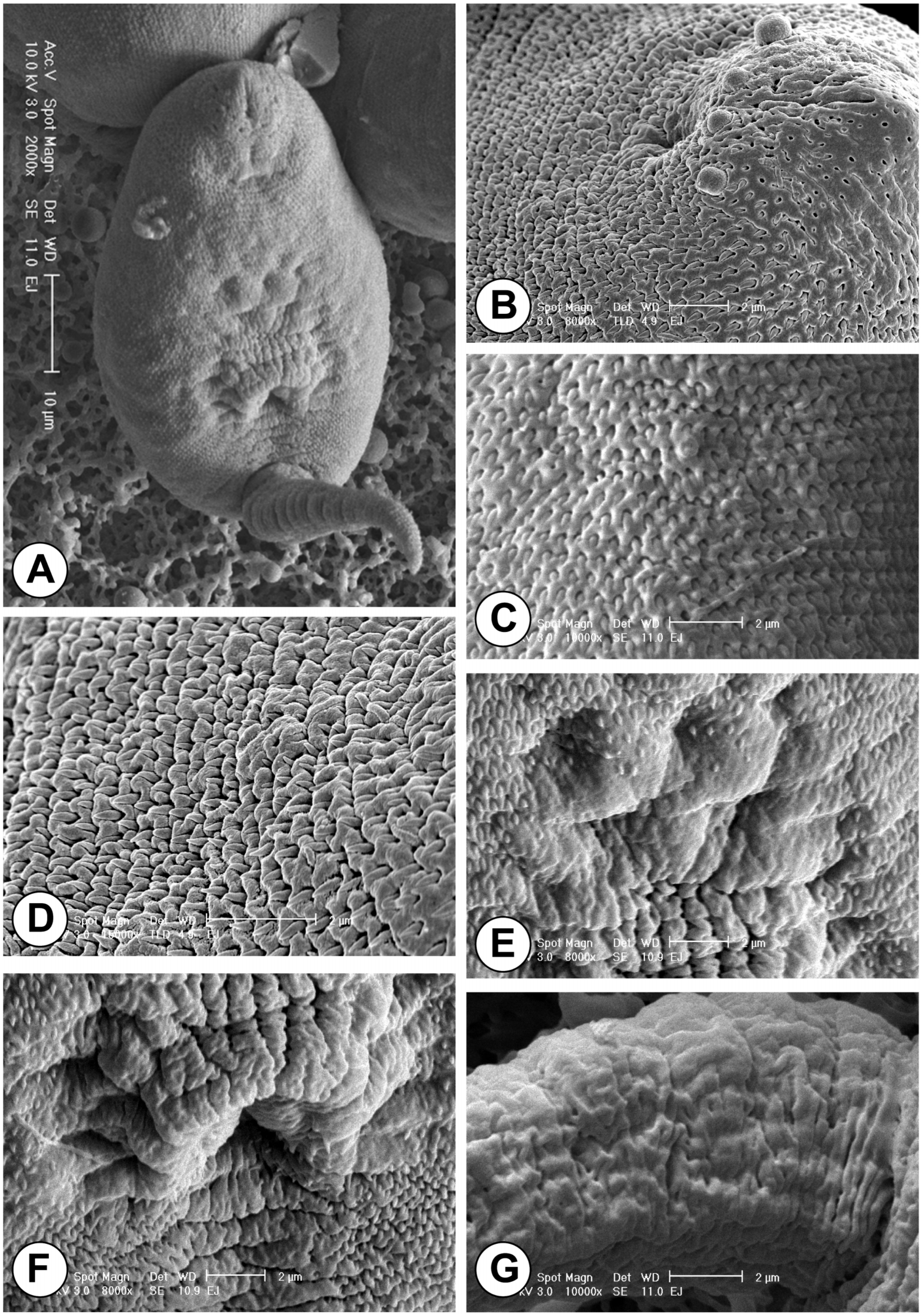

FIGURE 2. SEM observations of the ventral surface of *Echinochasmus japonicus* cercariae. (**A**) Whole ventral view. (**B**) Oral sucker. (**C**) Tegument on the ventroanterior surface. (**D**) Tegument on the ventroanterior surface between the oral and ventral suckers. (**E**) Tegument on the ventromedian surface above the ventral sucker. (**F**) Ventral sucker. (**G**) Ventral view of a tail.

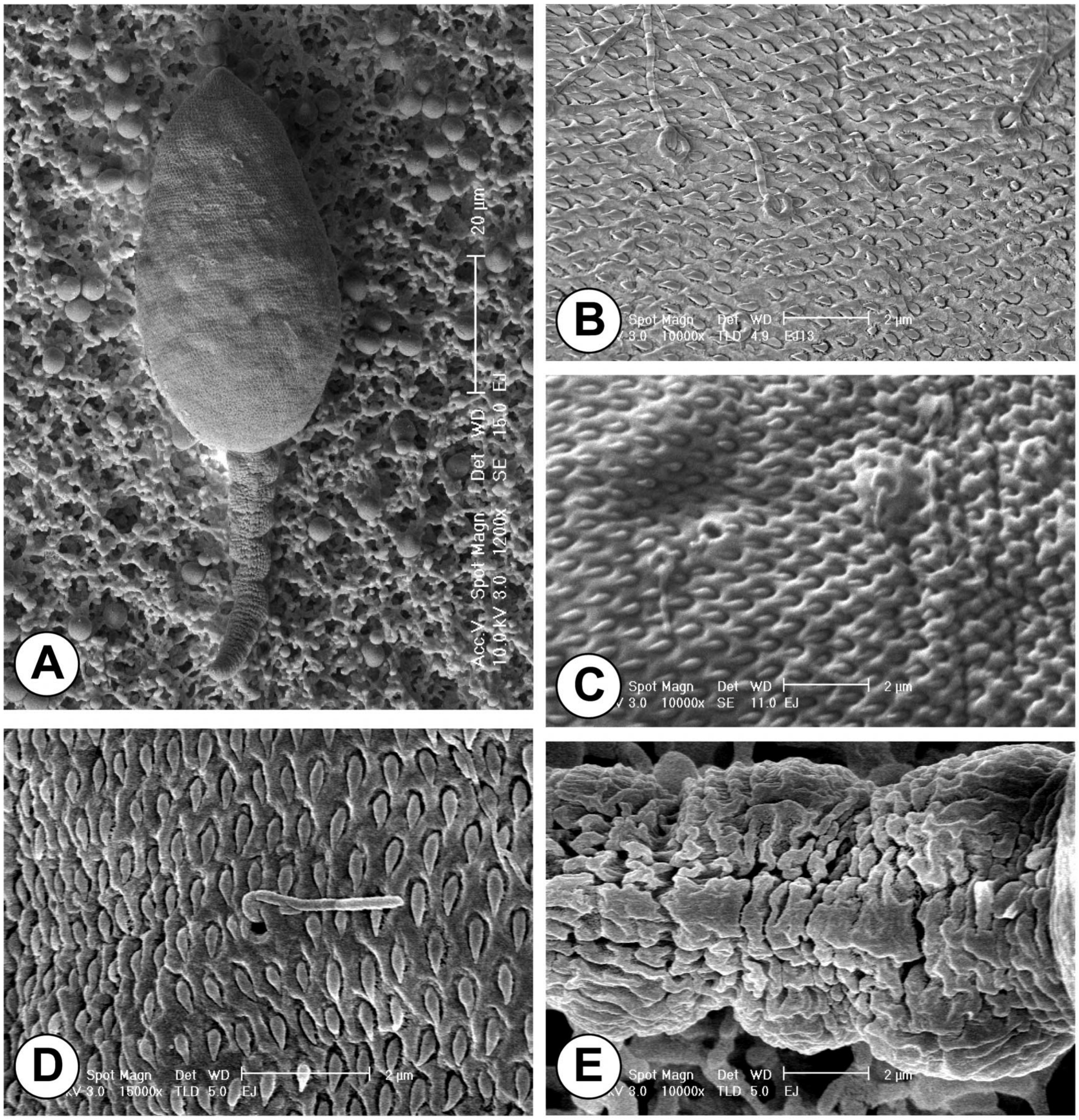

FIGURE 3. SEM observations of the dorsal surface of *Echinochasmus japonicus* cercariae. (**A**) Whole dorsal view. (**B**) Tegument on the dorsoanterior 1/3 surface. (**C**) Tegument on the dorsomedian surface. (**D**) Tegument on the dorsoposterior surface. (**E**) Dorsal view of a tail.

In the case of *Echinostoma* spp., sensory papillae other than type I or II have also been described in the cercaria stage. *Echinostoma trivolvis* cercariae are equipped with 2 kinds of ciliated papillae (Fried and Fujino, 1987), i.e., uniciliate papillae of variable length and multiciliate papillae containing up to 18 cilia per papilla. The latter have been suggested to be equivalent to the large nonciliated papillae observed in several species of echinostome cercariae by light microscopy (Fried and Fujino, 1987). Spherical bodies (or knobs) were reported to be abundant in the middorsal tegument of *E. trivolvis* cercariae (Krejci and Fried, 1994); however, their structural character and function remain to be evaluated.

Temperature is known to be a factor for survival and longevity of echinostome cercariae (Chen et al., 1990; Huffman and Fried, 1990; Pechenik and Fried, 1995). Cercariae shedding of *E. japonicus* from snails depends on water temperature (Chen et al., 1990); cercariae were found to emerge vigorously from infected snails at 27–32 C, but obviously decreased as the temperature fell, and completely stopped at 14 C. In contrast, the cercariae of *Echinostoma* sp. rarely survive beyond 2 days at ambient temperature; their survival is shorter at higher temperatures, e.g., 8 hr at 40 C (vs.) 75 hr at 10 C (Evans, 1985). The life-span and infective period of *E. trivolvis* cercariae are shorter at higher temperature by remarkably similar degrees, supporting the hypothesis that both survival and infectivity of cercariae are limited by the rate of energy expenditure (Pechenik and Fried, 1995). Similarly, the survival and infectivity of *E. caproni* cercariae were at a maximum at 12 C in artificial spring water (Fried and Ponder, 2003) and, when glucose was added to the water, the survival times of *Echinostoma* sp. cercariae were extended; however, their abilities to infect snails or to move in a linear direction was reduced (Ponder and Fried, 2004).

The cercariae of *E. japonicus* have been suggested to move

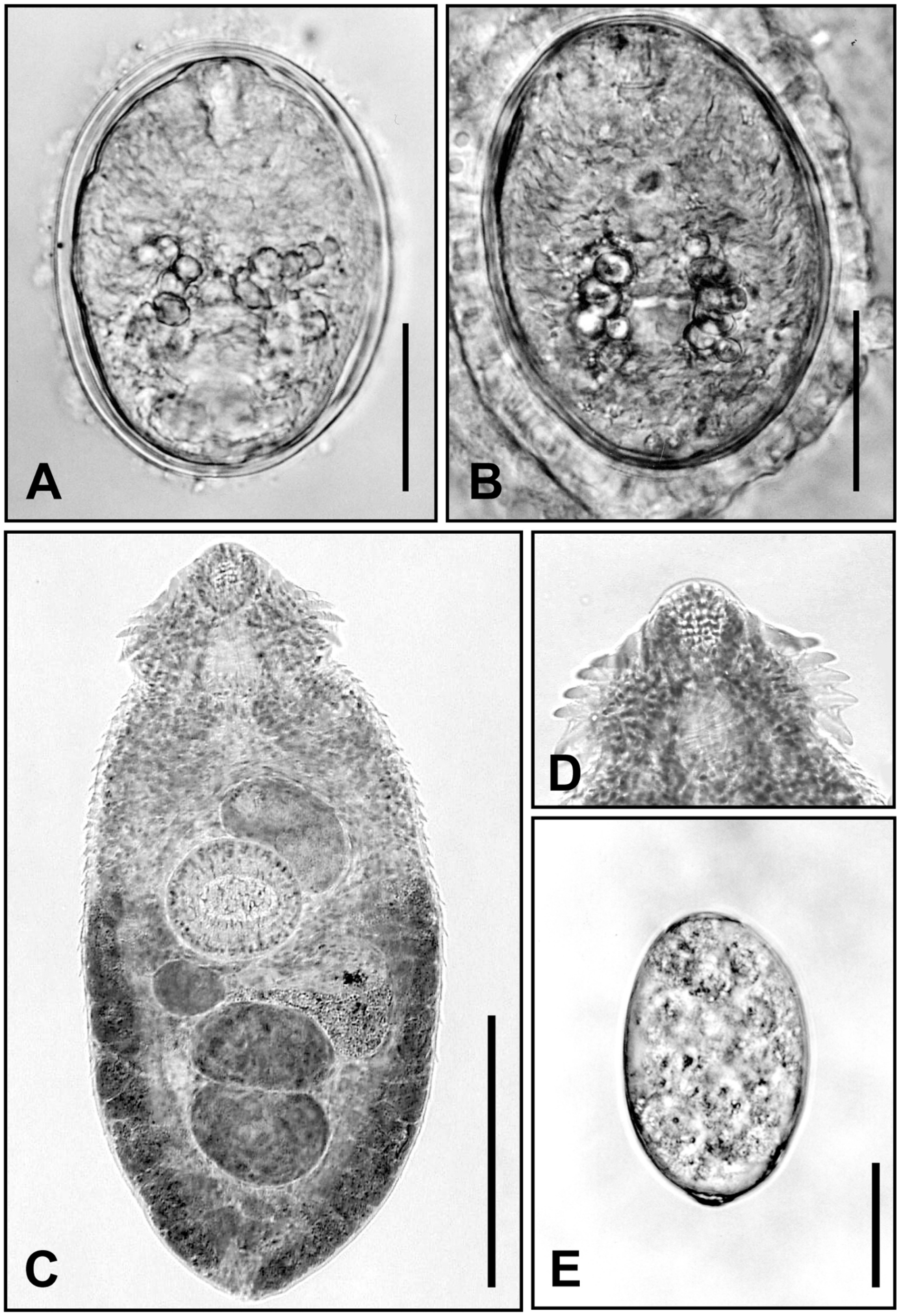

FIGURE 4. Metacercariae and an adult recovered from experimentally infected fishes and rats, respectively. (**A–B**) Metacercariae recovered from freshwater fish gills, *Pseudorasbora parva* experimentally infected with *Echinochasmus japonicus* cercariae at 4 and 7 days after infection. Bar = 30 μm. (**C**) An adult recovered from rats experimentally infected with *E. japonicus* metacercariae. Bar = 100 μm. (**D**) Magnification of the head crown and collar spines around the oral sucker. (**E**) An egg liberated from an adult worm. Bar = 30 μm.

toward light; therefore, they are more active in shallow water, where they contact smaller fishes more frequently (Chen et al., 1990). In the present study, the metacercariae of *E. japonicus* were found only in the gills of *P. parva*, which concurs with the finding of an earlier report (Chai et al., 1985). With tails that are shorter than their bodies, *E. japonicus* cercariae may not swim far after they are liberated from snails. Thus, they may not invade fish muscle actively, and instead enter fish passively, perhaps via water intake through gill baskets where they are entrapped and encyst.

Cercariae of *E. liliputanus* encysted only in the gills of goldfish, and encystment also occurred in vitro, especially in human gastric juice (Xiao et al., 2005). This finding suggests the possibility of infection of definitive hosts, including humans, by

Table I. Measurement of *Echinochasmus japonicus* adults recovered from experimentally infected rats 4 wk after infection.*

Organs		Average (μm)	Range (μm)
Body	length	327.3	282.5–402.5
	width	216.8	182.5–327.5
Oral sucker		37.0	35.0–42.5
Pharynx	length	35.3	32.5–37.5
	width	25.5	25.0–27.5
Ventral sucker		71.5	57.5–80.0
Ovary		45.3	27.5–50.0
Anterior testis	length	29.0	22.5–37.5
	width	107.5	90.0–122.5
Posterior testis	length	39.5	25.0–50.0
	width	97.5	77.5–112.5
Eggs†	length	92.5	92.5
	width	60.0	60.0

* Sprague-Dawley rats, 6 wk old, were experimentally infected with 90 metacercariae each. A total of 282 adults were recovered from the intestines, among which 10 adult worms were measured.

† A total of 20 eggs were measured, and they were all identical in size.

the cercariae, and may explain the major mode of human infections in China. People living in an area with poor water hygiene may be at a risk of infection with *E. japonicus*, even though they do not eat raw freshwater fishes. However, the possibility of human infection by *E. japonicus* cercariae needs further study.

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