PLANORBID SNAILS AS POTENTIAL MOLLUSCAN INTERMEDIATE HOST OF A HUMAN INTESTINAL FLUKE, *NEODIPLOSTOMUM SEOULENSIS* (TREMATODA: DIPLOSTOMATIDAE) IN KOREA

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Abstract. Three species of the pulmonate snails of the family Planorbidae have been reported from Korea; *Gyraulus convexiusculus*, *Hippeutis (Helicorbis) cantori* and *Segmentina (Polypylis) hemisphaerula*. Of these 3 species, only *H. cantori* is reported as the molluscan intermediate host of *Neodiplostomum seoulensis*, one of the important snail-borne human intestinal trematodes in Korea. However, *S. hemisphaerula* was also found to be an intermediate host for *N. seoulensis*. In field-collected snails, *H. cantori* and *S. hemisphaerula* were found shedding bifurcated cercariae of *N. seoulensis*, whereas no *G. convexiusculus* was found shedding cercariae. In experiments with laboratory-bred snails, only *S. hemisphaerula* was susceptible to miracidia of *N. seoulensis*. Tadpoles of *Rana nigromaculata* and *R. rugosa* were exposed to cercariae shed from field-collected and laboratory-bred *S. hemisphaerula*. All tadpoles of *R. nigromaculata* were found to be massively infected, but none of the tadpoles of *R. rugosa* were infected with larvae of *N. seoulensis*. Metacercariae from tadpoles of *R. nigromaculata* and the snake *Rhabdophis tigrinus tigrinus* were fed to rats, and eggs of *N. seoulensis* were detected in the rat feces one-week later. These rats were killed and adult *N. seoulensis* recovered from the small intestines. This is the first report of *S. hemisphaerula* as a molluscan intermediate host for *N. seoulensis* in Korea.

INTRODUCTION

A strigeid intestinal fluke, *Neodiplostomum seoulensis* (Seo, Rim et Lee, 1964) (Trematoda: Diplostomatidae) was first found in the small intestine of house rats, *Rattus norvegicus*, in Seoul, Korea and described as a new species by Seo *et al* (1964). The fluke was later found to be distributed nationwide in house rats (Seo *et al*, 1981) and human cases were reported (Seo *et al*, 1982; Hong *et al*, 1984; Hong *et al*, 1986).

The life cycle of *N. seoulensis* was studied in the laboratory and the field with special interest in larval development within the eggs and the intermediate hosts (Seo *et al*, 1988; Lee *et al*, 1986; Lee *et al*, 1986a, b). Summarizing the life cycle studies up to the present, *Hippeutis cantori* a Korean planorbid snail was listed as the first intermediate host (Seo *et al*, 1988) and Lee *et al* (1986a, b) reported that tadpoles of *Rana nigromaculata* play an important role as the second intermediate host. In addition to tadpoles, several species of Korean snakes, *Rhabdophis tigrinus tigrinus*, *Agkistrodon brevicaudus*, *Dinodon rufozonatum rufozonatum*, *Elaphe dione* and *E. rufodorsata*, were also listed as second intermediate or paratenic hosts (Hong *et al*, 1982; Cho *et al*, 1983; Seo and Hong, 1985). Chandler (1942) and Cook (1978) reported that the cercariae of *Fibricola* (syn. *Neodiplostomum*) *texensis* or *Fibricola cratera* shed from snails penetrate tadpoles, and become metacercariae (diplostomula). The cercariae have also been known not to invade adult frogs or snakes (Sudarikov, 1960). However, the metacercariae of *N. seoulensis* or other *Fibricola* spp were found in adult frogs or snakes (Hong *et al*, 1982; Sudarikov, 1960).

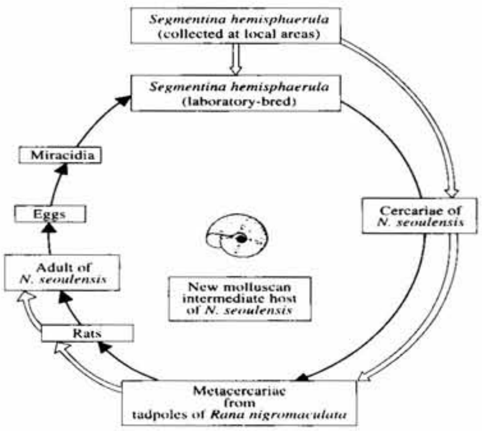
Three species of planorbid snails, *Gyraulus convexiusculus*, *Hippeutis cantori* and *Segmentina* *hemisphaerula* have been reported in Korea (Burch *et al*, 1987; Kwon, 1990; Chung *et al*, 1993; Chung *et al*, 1994; Chung *et al*, 1995), however the taxonomic status of Korean planorbids remains a problem. Although *H. cantori* is a known intermediate host it was uncertain until the present study whether the other two species of Planorbidae were susceptible to infection. Therefore this study was carried out to determine the susceptibility of all three species of Korean planorbid snails to infection with the miracidia of *N. seoulensis* and to establish the cycle of the parasite in the laboratory.

MATERIALS AND METHODS

The present life-cycle studies of *N. seoulensis*, using the *S. hemisphaerula* as a potential intermediate host are presented in Fig 1.

Study of first intermediate host

Collection of snails: The three species of Korean planorbid snails, *G. convexiusculus*, *H. cantori* and *S. hemisphaerula* were collected in the various localities of Korea. The localities, dates and habitats collected are listed in Table 1. The snails were identified using keys of Burch *et al* (1987).



(⇨) Cercarial cycle of *N. seoulensis* naturally shed from *Segmentina hemisphaerula*

(→) Cercarial cycle of *N. seoulensis* experimentally shed from laboratory-bred *Segmentina hemisphaerula.*

Fig 1 - Experimental procedures on the life cycle of *Neodiplostomum seoulensis.*

Table 1

Snail specimens of *Gyraulus convexiusculus, Hippeutis cantori* and *Segmentina hemisphaerula* collected from various localities in Korea.

Species	Localities collected	Date collected	Habitat	Catalog No.
Gyraulus	Kosam, Kyonggi-do	June 2, 1993	fish farm	IUMC 55
convexiusculus	Onyang-1, Chungnam	July 5, 1993	rice field	IUMC 50
	Onyang-2, Chungnam	July 5, 1993	pond	IUMC 56
Hippeutis	Kosam, Kyonggi-do	June 2, 1993	fish farm	IUMC 48
cantori	Onyang-1, Chungnam	July 5, 1993	rice field	IUMC 57
	Koksung, Jeonnam	Sept 1, 1993	ditch	IUMC 52
Segmentina	Yangsung, Kyonggi-do	June 2, 1993	pond	IUMC 49
hemisphaerula	Onyang-1, Chungnam	July 5, 1993	rice field	IUMC 58

* Snail specimens were recorded as Inha University Medical College (IUMC) voucher numbers.

Observation of cercariae naturally emerging from snails: The three species of planorbid snails collected from various local areas, were examined for the emergence of trematode cercariae. When the bifurcated cercariae were shed, 30 cercariae were used to infect tadpoles and recover metacercariae. Some of cercariae were also fixed in 10% formalin, stained with Semichon' s acetocarmine, and observed for the morphological features.

Susceptibility experiments with laboratory-bred snails: Snails free of trematode cercariae were reared in the laboratory. Each snail was exposed to five miracidia hatched from the eggs of *N. seoulensis*. The exposed snails were kept in an aquarium at 26ºC and fed with Tetra SML®, a tropical fish food. After the 5th day the snails were examined for release of cercariae under the fluorescent illumination (700 Lux) for two hours. The live cercariae were observed and some stained with Semichon' s acetocarmine for the morphological studies.

Study of second intermediate host

Tadpoles: The egg masses of the frogs, *R. nigromaculata* and *R. rugosa* were collected from a rural rice field and incubated in an aquarium at 18-20 ºC for one month to obtain tadpoles (Lee *et al*, 1986a). Month-old tadpoles of *R. nigromaculata* and *R. rugosa* were exposed to the *N. seoulensis* cercariae shed from *H. cantori* and *S. hemisphaerula*. No cercariae were shed from *G. convexiusculus* even after several exposures. All experimental tadpoles of *R. nigromaculata* became infected and two weeks after exposure were killed and examined for metacercariae. The entire body of the animal was mascerated with scissors, ground and then digested with artificial gastric juice (0.4% pepsin in 1% HCl solution) for one hour at 37ºC. The metacercariae were then isolated from the digested tissues. Some of the metacercariae collected were fixed in 10% formalin, stained with Semichon's acetocarmine for the morphological studies.

Snakes: In addition to the experimental tadpoles, six *Rhabdophis tigrinus tigrinus* were purchased from local collectors in Yonghyun-dong, Inchon in order to obtain metacercariae of *N. seoulensis*. After removing the snakes' head and stripping the skin, the stomach wall and the soft part of perigastric tissues were dissected (Hong *et al*, 1982). Soft tissue parts were digested with artificial gastric juice for one hour at 37ºC and the larvae collected. The metacercariae obtained from the snakes were given per os to rats to produce the adult worms for morphological comparisons with adult worms from rats given larvae from tadpoles. Eggs were also obtained from adults for miracidial experiments.

Study of infection of final host

Metacercarial infection of rats: A total of 200 metacercariae collected from tadpoles weekly from two weeks after the cercarial challenge and/or from naturally infected snakes were fed orally to each experimental laboratory rat (Sprague-Dowley strain, 120g body weight). Rat feces was examined daily for eggs of *N. seoulensis*. Immediately after finding eggs, the rats were sacrificed by spinal shock and dissected. The small intestines were removed and divided into duodenum, jejunum, ileum and the rest. The worms were collected under the dissecting microscope and their numbers determined. Worms were fixed with 10% formalin under cover slip pressure and stained with Semichon's acetocarmine. The stained worms were observed for species identification.

Embryonation of the eggs: Adult worms were recovered from the small intestine of rats, and eggs laid collected by shredding the worms. The eggs were rinsed 3 times with distilled water and transferred to the Petri dishes containing distilled water with a few drops of Fungizon® solution (Gibco Life Technologies Inc Grand Island, NY, USA). The Petri dishes were stored at 26ºC in a dark incubator with aeration. Some miracidia that hatched were stained with neutral red for observation of internal structures and for photography. Some hatched miracidia were used to challenge the three planorbid snail species targeted in this study.

RESULTS

Identification of snails

The three different Korean planorbid snails were mainly identified on the basis of external and internal morphological features (Chung *et al*, 1993). The spiral whorls roundly convexed above and below the periphery are shown in the snails of *G. convexiusculus;* but, the shells with sharply keeled periphery are those of *H. cantori*. The snails of *S. hemisphaerula* are characterized with internal lamellar barriers.

Cercarial infectivity of field-collected snails

Natural infections of *N. seoulensis* in the three species of planorbid snails collected from several local areas are shown in Table 2. Out of three species of planorbid snails, the natural emergence of *N. seoulensis* cercariae were observed in two species, *H. cantori* and *S. hemisphaerula*. *G. convexiusculus* were not infected with cercariae of *N. seoulensis*. Two snails out of 400 *H. cantori* (0.5%) collected from Kosam, Kyonggi-do were infected with the cercariae of *N. seoulensis*. However, no cercarial shedding was found in the other two populations of *H. cantori* collected from Onyang, Chungnam and Koksung, Jeonnam. Twelve snails out of 485 *S. hemisphaerula* (2.47%) collected from Yangsung, Kyonggi-do were naturally infected with the cercariae. However, the snails of *S. hemisphaerula* from Onyang, Chungnam were not infected with the *N. seoulensis* cercariae. The highest natural infection with *N. seoulensis* cercariae was observed in *S. hemisphaerula*.

Cercarial infection in tadpoles

Experimental infection of tadpoles was first done with the cercariae naturally shed from *S. hemisphaerula* and *H. cantori*. The cercariae attached to the skin of tadpoles by the suckers and penetrated into the junction of abdominal wall and tail of the tadpoles. The tail of the cercaria is discarded after penetration. All of the metacercariae of *N. seoulensis* were observed to be without a cyst wall throughout the period of observation. Numbers of metacercariae collected from tadpoles of *R. nigromaculata* are shown in Table 3. None of the tadpoles of *R. rugosa* harbored the metacercariae of *N. seoulensis*. On the 12th day after cercarial exposure, 336 immature metacercariae were collected from one tadpole of *R. nigromaculata*. These immature metacercariae were not used for the infection experiments to the laboratory rats. An average of 372 mature metacercariae were obtained from three infected tadpoles on the 14th day after cercarial exposure. However, the numbers of metacercariae on the 21st day after cercarial exposure averaged 341 from two tadpoles. The measurements of metacercariae obtained from tadpoles of *R. nigromaculata* are summarized in Table 8. The average size from 15 metacercariae was 309.4×211.6 μm. The metacercariae in the *R. nigromaculata* tadpoles infected with *N. seoulensis* cercariae from naturally infected *H. cantori* were also found at the 14th day after cercarial challenge (mean number: 320); however, the results obtained are not tabulated in this paper.

Metacercarial infection to the final host

A total of 200 metacercariae of *N. seoulensis* obtained on the 14th and 21st day after cercarial exposure to the tadpoles were fed orally to each experimental rat. All of the adult worms collected mainly from the upper part of the small intestines of the rats were identified as typical *N. seoulensis* (Seo *et al*, 1964; Seo, 1991). Almost all worms were located in both proximal and distal duodenum portions of small intestines of rats throughout the period of observation. Recovery rates of adult worms from the rats infected with the metacercariae of *N. seoulensis* are shown in Table 4. Sixteen (16) adult worms (8.0%) were collected from a rat infected with 14-day old metacercariae one week after metacercarial infection. The highest recovery rate (43.0%) was recorded in the rats infected with 14-day old metacercariae 3 weeks after metacercarial infection. An average recovery rate of 45.7% was recorded in the rats infected with 21-day old metacercariae. The highest recovery rate (49.0%) was found in the rats harboring 98 adult worms. The measurements of adult worms collected are summarized in Table 8.

Miracidial infection in laboratory-bred snails

Miracidia hatched from eggs of *N. seoulensis* were exposed to snails of three planorbid species suspected as the snail intermediate hosts in this study. On the fifth day after incubation of eggs, the miracidial eye spots appeared and short cilia were found on the surface of the miracidia. When the Petri dishes containing embryonated eggs were placed under the light sources on the ninth day, almost 20% of miracidia hatched out (Table 5).

Out of 35 five-week old snails of each species infected with miracidia, only one snail of *S. hemisphaerula* (2.9%) shed a large number of furcocercous cercariae (Table 6). The first emergence of 18 cercariae was detected on the 13th day after miracidial exposure. The daily and accumulative numbers of cercariae were determined and observed until 17 days after miracidial exposure. On the 17th day, 378 cercariae were observed and totally 1,060 cercariae were accumulated during the period of five-day observation (Table 7). Average measurements from 15 acetocarmine-stained cercariae from the laboratory-bred *S. hemisphaerula* are shown in (Table 8).

Table 2

Natural infectivity of *Neodiplostomum seoulensis* cercariae in the planorbid snails collected from local areas.

Species of snails	Local area collected	Date collected	No. of snails examined	No. of positive snails	Infection rate (%)
Gyraulus convexiusculus	Kosam, Kyoggi-do	June 2, 1993	100	0	0
Hippeutis cantori	Kosam, Kyonggi-do	June 2, 1993	400	2	0.5
	Onyang-1, Chungnam	July 5, 1993	8	0	0
	Koksung, Jeonnam	Sept 1, 1993	100	0	0
Segmentina hemisphaerula	Yangsung, Kyonggi-do	June 2, 1993	485	12	2.47
	Onyang-1, Chungnam	July 5, 1993	112	0	0

Table 3

Number of mature metacercariae in the tadpoles of *Rana nigromaculata* exposed to the *Neodiplostomum seoulensis* cercariae from *Segmentina hemisphaerula*[a].

Days after exposure	No. of tadpoles examined	Mean (Range) metacercariae
12	1	336 (-)[b]
14	3	372 (363-385)
21	2	341 (339-342)

[a] The tadpoles of *Rana rugosa* were not susceptible to the *N. seoulensis* cercariae shed from *S. hemisphaerula*.

[b] immature

Table 4

Recovery rates of adult worms from the rats infected with *Neodiplostomum seoulensis* metacercariae*.

Age of metacercariae in the tadpoles (day)	Infection period in the rats (week)	No. of worms recovered	Recovery rates (%)
14	1	16	8.0
	2	55	27.5
	3	86	43.0
	Mean	52	26.0
21	1	85	42.5
	2	98	49.0
	Mean	92	45.7

* 200 metacercariae were fed orally to each experimental rat.

Table 5

Developmental stages of the eggs of *Neodiplostomum seoulensis* in water.

Experimental condition : Aquaria with conditioned water at 26°C
Day 5 after incubation of eggs : Miracidia with eye spots
Day 9 after incubation of eggs : Hatching out of miracidia (20%)

Table 6

Susceptibility of laboratory-bred snails[a] of three planorbid snail species to 9-day old miracidia[b] of *Neodiplostomum seoulensis*.

Snail species	No. examined	No. shedding cercariae	Infectivity (%)
Gyraulus convexiusculus	35	0	0
Hippeutis cantori	35	0	0
Segmentina hemisphaerula	35	1	2.9

[a] Five-week old lab-bred snails were employed in this study.

[b] Five miracidia per each snail were infected to all the experimental planorbids.

Table 7

Daily and accumulative numbers of *Neodiplostomum seoulensis* cercariae shed from a snail of *Segmentina hemisphaerula**.

Days after miracidial exposure	No. of cercariae from a snail	Accumulative No. of cercariae from a snail
13	18	18
14	136	154
15	269	423
16	259	682
17	378	1,060

* 1) Cercarial shedding was observed under the stereomicroscope 2 hours after fluorescent illumination (700 Lux).
* 2) A cercaria-shedding snail died 23 days after miracidial exposure.

Table 8

Average measurements from 15 acetocarmine-stained cercariae, metacercariae and adult worms of *Neodiplostomum seoulensis*.

Item	Cercariae (μm)	Metacercariae (μm)	Adult worm (mm)
Head	101.4 x 43.1	-	-
Body	178.8	309.4 x 211.6	1.461 x 0.740
Tail	154.3	-	-
Oral sucker	-	39.6 x 36.1	0.068 x 0.069
Pharynx	-	28.0 x 16.2	-
Ventral sucker	-	31.4 x 35.8	0.070 x 0.079
Tribocytic organ	-	-	0.307 x 0.284
Anterior testis	-	-	0.217 x 0.616
Posterior testis	-	-	0.286 x 0.579
Ovary	-	-	0.115 x 0.262

DISCUSSION

Previous studies have shown that the house rat is the definitive host, the snail, *H. cantori*, the first intermediate host, tadpoles and adult frogs of *R. nigromaculata* the second intermediate hosts, and some species of snakes paratenic hosts of *N. seoulensis*. However, of the intermediate hosts, the taxonomy of the Korean freshwater planorbid snails is not well described. The taxonomic problems have been currently solved, in part, by the three different species in the planorbid group in Korea: *G. convexiusculus, H. cantori* and *S. hemisphaerula*, based on the cladistic analyses with the shell morphologies and internal, external anatomy data (Kim and Song, 1983; Kwon, 1990; Chung *et al*, 1993), on the mitochondrial DNA studies (Chung *et al*, 1994) and on the studies of allozyme variations among snail species (Chung *et al*, 1995).

The off-spring of three planorbid species reared in the laboratory were only used for the susceptibility experiments to guarantee the cercaria-free host snails. It required at least four to five weeks for the planorbid snails to grow to adults in the laboratory (Krull, 1931). Five-week old laboratory-bred snails were also employed in this study.

Until now *H. cantori* was the only known molluscan intermediate host of *N. seoulensis* (Seo *et al*, 1988) in Korea. However in this study it has been shown that *S. hemisphaerula* is the most susceptible molluscan intermediate host of *N. seoulensis* in Korea. It has been also confirmed in this study that the *G. convexiusculus* is not susceptible to infection with miracidia of the parasite. The *H. cantori* collected from the fields were shedding cercariae of *N. seoulensis* unlike *G. convexiusculus*, on the other hand laboratory-bred *H. cantori* were not susceptible to the miracidial infection.

Naturally infected metacercariae of *N. seoulensis* were found in the frogs of *R. nigromaculata*, while none of the *R. rugosa* harbored the metacercariae (Hong *et al*, 1982). The tadpoles of *R. nigromaculata* were also highly susceptible to the *N. seoulensis* cercariae in the laboratory experiments (Hong *et al*, 1985; Lee *et al*, 1986b). The tadpoles of both species were employed for the cercarial infection in this study. The tadpoles of *R. nigromaculata* were highly susceptible to the *N. seoulensis* cercariae (Hong *et al*, 1985); Lee *et al*, 1986b), while those of *R. rugosa* were not susceptible. Similar results were found in the adult frogs of *R. rugosa* (Hong *et al*, 1982). Further laboratory experiments on host-specificity of this newly found strigeid fluke are expected. The metacercarial larvae of *N. seoulensis* in the tadpoles became fully mature at least 14 days after cercarial infection in this study similar to results in former studies (Lee *et al*, 1986b).

It is considered that the snakes act as a paratenic host according to their food chain, rather than the second intermediate host of *N. seoulensis*. The metacercariae obtained from *Rhabdophis tigrinus tigrinus*, were also fed to the rats in this study to compare with the adult morphology of *N. seoulensis* developed from the metacercariae in the tadpoles of *R. nigromaculata.*

On the 7th day after metacercarial infection in rats, almost all worms contained eggs in the uteri, and the worms became adults in maximum size (Hong, 1982). The present results agreed with above findings. On the one hand, the main habitat of adult worms in the small intestine of the rats was the duodenum, just distal to the pylorus. It is also considered that the adults of *N. seoulensis* in the rats prefer to be in this habitat for rich nutrient sources from the duodenum as Smyth's assumption (Smyth, 1976).

The laboratory conditions of light and optimal temperature were thought to be essential for hatching out of miracidia from the eggs as well as for shedding of cercariae from the snail hosts. All the culture systems were kept at 26 ºC, and 700 Lux of fluorescent light illumination to study the embryonated eggs. The treatment of antifungal agents was a critical factor for killing fungi around the eggs in the culture systems.

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