

The first discovery of larval *Gnathostoma hispidum* (Nematoda: Gnathostomidae) from a snake host, *Agkistrodon brevicaudus*

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Abstract: The present study was performed to observe the infection status of several kind of animals with indigenous *Gnathostoma* in Korea, and morphological characteristics of gnathostome larvae detected from pit-viper, *Agkistrodon brevicaudus*, for the species identification. To know the existence of *Gnathostoma* in Korea, 3,450 loaches, 24 bullfrogs, several kinds of snakes, i.e., 55 *Elaphe rufodorsata*, 2 *Dinodon rufozonatum rufozonatum*, 62 *Rhabdophis tigrinus tigrinus* and 87 *Agkistrodon* spp., and 438 cats were examined. A total of 21 larval gnathostomes was detected from 12 pit-vipers, *A. brevicaudus*. They were 2.233×0.343 mm in average size and covered with about 210 transverse rows of minute cuticular spines. Their characteristic head bulbs were provided with 4 rows of hooklets of which average numbers in each row were 36.8, 39.0, 41.7 and 44.3, posteriorly. In the cross sections of midgut level, the intestinal wall consisted of a single layer of 19-25 elongate epithelial cells with a single nucleus. SEM observation of the larvae revealed unique features of head bulb, cuticular spines on transverse striations and a cervical papilla. On the basis of above morphological characteristics, they were identified as the advanced third-stage larvae of *Gnathostoma hispidum*. It was first confirmed that the pit-viper, *Agkistrodon brevicaudus* is the snake intermediate host of *G. hispidum*.

Key words: *Gnathostoma hispidum*, advanced third-stage larva, indigenous *Gnathostoma* larva, pit-viper, *Agkistrodon brevicaudus*, cross sectional morphology, SEM, tegumental ultrastructure

INTRODUCTION

The genus *Gnathostoma* is a clinically important helminth as a tissue inhabiting nematode. About 12 species, *G. spinigerum*, *G. hispidum*, *G. turgidum*, *G. doloresi*, *G. nipponicum*, *G. americanum*, *G. procyonis*, *G.*

miyazakii, *G. malaystae*, *G. vietnamicum*, *G. didelphis* and *G. brasiliense*, have so far been recognized as valid ones (Daengsvang, 1980; Miyazaki, 1991). However, as the causative species of human gnathostomiasis, only 4 ones, *G. spinigerum*, *G. hispidum*, *G. nipponicum* and *G. doloresi* have been reported. Especially, it has been recently known that a lot of Japanese people were infected with *G. hispidum* by eating raw flesh of loaches imported from China (Morita *et al.*, 1984; Taniguchi *et al.*, 1992).

In Korea, the larvae of *G. nipponicum* and *G.*

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hispidum were recently found from the loaches imported from China (Sohn *et al.*, 1993; Sohn and Lee, 1996). However, there have been no reports on the indigenous infection of larval gnathostomes except one by Kim (1973). Some workers surveyed cyclopoid copepods, tadpoles and loaches for the recovery of gnathostome larvae, however no worms were detected, and indigenous infection in human or definitive hosts have not been reported yet (Kim, 1973; Kim, 1983; Koga *et al.*, 1985).

Snakes have been reported as the intermediate host of some kinds of gnathostomes. However, in case of *G. hispidum*, no species of snakes have been verified as an intermediate host although frogs, loaches and crucian carps have been reported (Sohn, 1996). In this study, we found larval *G. hispidum* from the pit-viper, *Agkistrodon brevicaudus*, purchased from a local snake collector in Pusan, and their morphological characteristics were described. Discovery of *G. hispidum* larvae from the snake host is the first time so far as the world literatures are concerned.

MATERIALS AND METHODS

To know the existence of *Gnathostoma* in Korea, 3,450 loaches, *Misgurnus anguillicaudatus*, 24 bullfrogs, *Rana catesbeiana*, and several kinds of snake, *i.e.*, 55 *Elaphe rufodorsata*, 2 *Dinodon rufozonatum rufozonatum*, 62 *Rhabdophis tigrinus tigrinus* and 87 *Agkistrodon* spp., were examined from March 1994 to June 1997. They were all purchased from a wholesale house of freshwater fish and from a local snake collector in Pusan. All animals were transferred in our laboratory, and their muscles and viscera were isolated and artificially digested with pepsin-HCl solution in a 36°C incubator. Digested materials were washed with 0.85% saline, and examined under a stereomicroscope to collect gnathostome larvae. Additionally, 438 cats (stomach only) were examined from February 1995 to January 1996 to detect the adult *Gnathostoma*.

Among 21 larvae detected from the pit-viper, 12 ones were fixed with 10% neutral buffered formalin under a cover glass pressure, cleared

in alcohol-glycerin solution and mounted in glycerin-jelly. To observe the cross sectional morphology, four larvae were embedded in the liver tissue of mouse, fixed with 10% neutral buffered formalin and prepared for paraffin section. The samples were cut into cross sections of 5 μm in thickness at the various level of body and stained with hematoxylin and eosin. The remaining larvae were fixed with 2.5% glutaraldehyde, dehydrated in graded alcohol, dried in critical point dryer, and coated with gold. The specimens were observed under a DS-130C SEM (ISI Korea Co.) operated at 15 KV.

RESULTS

Results of the survey of indigenous gnathostome

No gnathostomes were detected from all kinds of animals examined except pit-vipers, *A. brevicaudus* (Table 1). Among 87 snakes of *Agkistrodon* spp. examined, 12 *A. brevicaudus* (13.8%) were infected with 1-3 larval gnathostomes. The infection status is shown in Table 2. Some worms were encysted with the fibrous membrane originated from host tissue (Fig. 1).

Description of the larval gnathostomes from pit-vipers

The whole body of larvae was covered with about 210 transverse rows of minute cuticular spines and 2.233 \times 0.343 mm in average size. A pair of lips protruded at the anterior end, and the muscular esophagus (about 0.765 mm long) and brownish intestine followed, and anus opened at the ventral side of posterior end. Two pairs of cervical sacs (about 0.339 mm long) were clearly observed in the region of esophagus (Figs. 2 & 3). The characteristic head-bulb (0.080 \times 0.192 mm in average size) was provided with four rows of hooklets. The average number of hooklets in the respective row was 36.8, 39.0, 41.7 and 44.3, posteriorly. Each hooklet had an irregular four-sided base (Figs. 4 & 5). The morphological features and measurements were compatible with those of *G. hispidum* reported by previous authors (Table 3).

Table 1. Results of a survey on the indigenous gnathostome in Korea

Host animal (Korean common name)	No. of animals examined	Time of examined	Results
<i>Misgurnus anguillicaudatus</i> (미꾸리)	3,450	Jun. and Jul. 1995	(—)
<i>Rana catesbeiana</i> (황소개구리)	24	Mar. and May 1994	(—)
<i>Elaphe rufodorsata</i> (무자치)	55	Mar. 1994 (35) ^{a)} Mar. 1995 (20)	(—)
<i>Dinodon rufozonatum</i> <i>rufozonatum</i> (능구렁이)	2	May 1994	(—)
<i>Rhabdophis tigrinus tigrinus</i> (유혈목이)	62	Mar. 1995 (30) Jun. 1996 (20) May 1997 (12)	(—)
<i>Agkistrodon</i> spp. ^{b)} (살모사)	87	May 1996 (31) Jun. 1996 (27) May 1997 (20) Jun. 1997 (9)	10 larvae (—) 8 larvae 3 larvae
<i>Felis domesticus</i> (고양이)	438	From Feb. 1995 to Jan. 1996	(—)

^{a)}No. of animals examined; ^{b)}All snakes examined were *A. brevicaudus* except 5 *A. saxatilis*.

Table 2. Recovery of larval gnathostomes from the Korean vipers

Time of examined	No. of snake examined	No. (%) of snake positive	No. of larvae detected		
			Muscle	Viscera	Total
May 1996	31	6 (19.4)	0	10	10 (1-3) ^{a)}
June 1996	27	0	0	0	0
May 1997	20	4 (20.0)	4	4	8 (1-3)
June 1997	9	2 (22.2)	1	2	3 (1-2)
Total	87	12 (13.8)	5	16	21 (1-3)

^{a)}range.

Cross sectional morphology of the larvae

The sections at the esophagus level showed a relatively thin body wall and a smaller diameter, and four characteristic sections of cervical sac (Fig. 6). The sections at the midgut level showed 12-16 muscle cells in each quadrant, a pair of large lateral cords and an intestine of which wall consisted of a single layer of 19-25 elongate epithelial cells. Most epithelial cells possessed a single nucleus

(Figs. 7 & 8).

SEM findings of the larvae

The whole body of larvae was covered with many transverse rows of cuticular spines except the head-bulb (Fig. 9). In the head-bulb, the mouth had a pair of lateral lips of equal size and of half moon shape. Each lip had a couple of labial papillae and a small amphid located between the two papillae. The hooklets on the head-bulb had single-pointed tips and curved posteriorly (Figs. 10, 11 & 12).

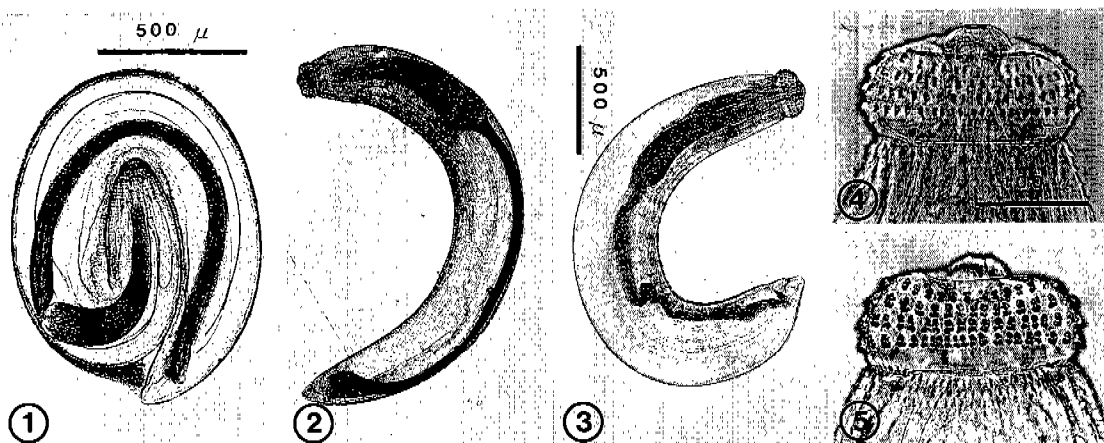
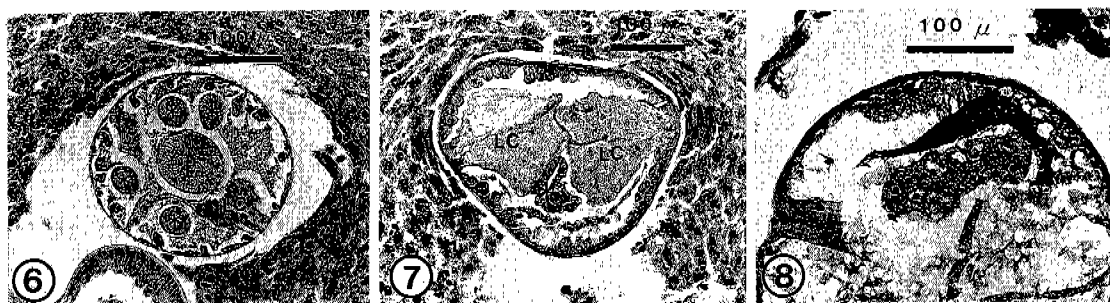


Fig. 1. An encysted larva of *Gnathostoma hispidum* from the muscle of a pit-viper, *Agkistrodon brevicaudus*. **Fig. 2.** The advanced third-stage larva (AdL₃) of *G. hispidum* recovered from the muscle of a snake. **Fig. 3.** The AdL₃ of *G. hispidum* recovered from the viscera of a snake. **Figs. 4 & 5.** Head-bulbs of the larvae bearing four transverse rows of hooklets.



Figs. 6-8. Cross sections of the AdL₃ of *G. hispidum* collected from the pit-viper. **Fig. 6.** Esophagus level showing the cervical sacs (*), esophagus (E) and lateral cord (LC). **Fig. 7.** Midgut level showing the intestine (*) and lateral cords (LC). **Fig. 8.** Magnification of midgut level with a section of intestine consisted of single layer of elongate epithelial cells. Most epithelial cells possessed a single large nucleus.

A dome-like cervical papilla was located between the 11th and 12th transverse striation. The cuticular spines on the transverse striations were sharp-pointed, larger and more densely distributed in the anterior area and gradually decreased in size and number posteriorly (Figs. 13, 14 & 15). Body surface in the adjacent area of anus was consisted of a highly wrinkled cuticle without spines (Fig. 16).

DISCUSSION

Human gnathostomiasis has been mainly occurred by *G. spinigerum*, and sometimes by

G. hispidum, *G. doloresi* and *G. nipponicum* (Morita *et al.*, 1984; Ando *et al.*, 1988; Nawa *et al.*, 1989). It has been known that the cutaneous gnathostomiasis consists of two clinical types of lesion. The one is the migratory intermittent edema-type eruption, the deeper form by *G. spinigerum*, and the other is the creeping type by the other three species of *Gnathostoma* (Miyazaki, 1960; Daengsvang, 1981). There has been a considerable increase of the creeping type eruption in Japan since 1980. It has been confirmed that a lot of Japanese patients were infected by ingesting raw loaches imported from China. All of the larval gnathostomes collected from

Table 3. Measurements^{a)} of the larval gnathostome from *Agkistrodon brevicaudus* and comparison with those of previous authors

Organs	Present study (1997) ^{b)}	Sohn and Lee (1996) ^{c)}	Takakura <i>et al.</i> (1985) ^{d)}
Body length	1.729-2.525 (2.233)	2.244-3.080 (2.660)	2.89 + 0.25
width	0.285-0.386 (0.343)	0.306-0.377 (0.346)	0.23 + 0.03
Esophagus	0.661-0.915 (0.765)	0.714-0.816 (0.755)	0.89 + 0.12
Cervical sac	0.285-0.407 (0.339)	0.306-0.459 (0.355)	—
Head-bulb			
length	0.072-0.102 (0.080)	0.087-0.122 (0.097)	0.069 + 0.008
width	0.158-0.225 (0.192)	0.184-0.204 (0.193)	0.155 + 0.012
No. of hooklets on head-bulb			
1st row	33-40 (36.8)	38-40 (39.0)	31-42 (38.5)
2nd row	35-42 (39.0)	40-44 (41.9)	34-44 (38.6)
3rd row	40-44 (41.7)	42-46 (43.9)	37-45 (41.6)
4th row	42-47 (44.3)	44-48 (45.6)	41-49 (44.3)
5th row	0-28 (4.3)	0-7 (1.5)	0-3 (0.3)

^{a)}Unit is mm (average); ^{b)}Ten larvae, ^{c)}10 and ^{d)}18 AdL₃ of *G. hispidum* were measured.

the imported Chinese loaches and the worms recovered from human cases with past history of eating raw Chinese loaches were identified as those of *G. hispidum* (Akahane *et al.*, 1982; Morita *et al.*, 1984).

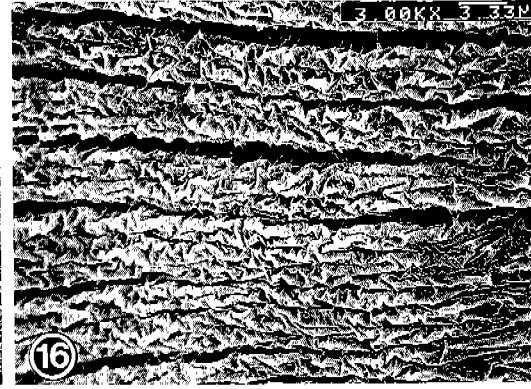
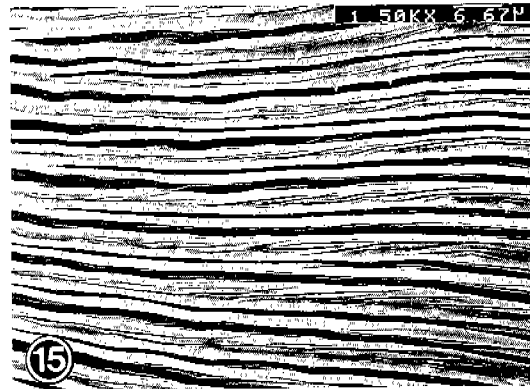
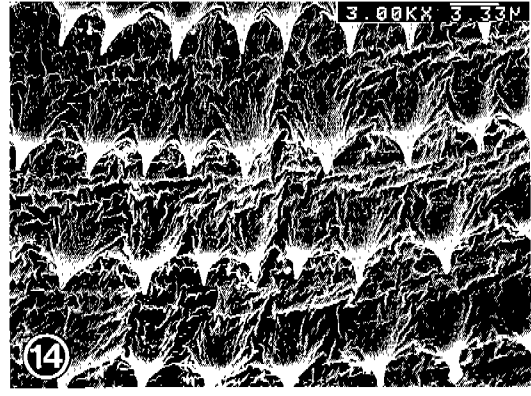
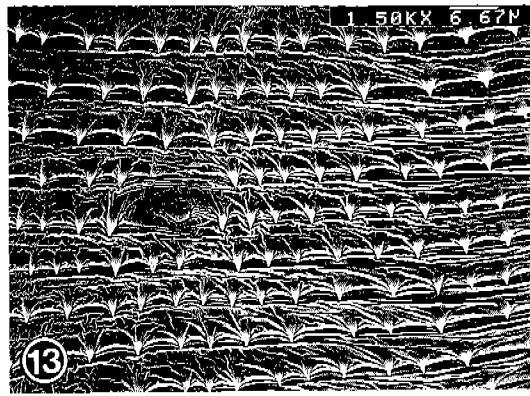
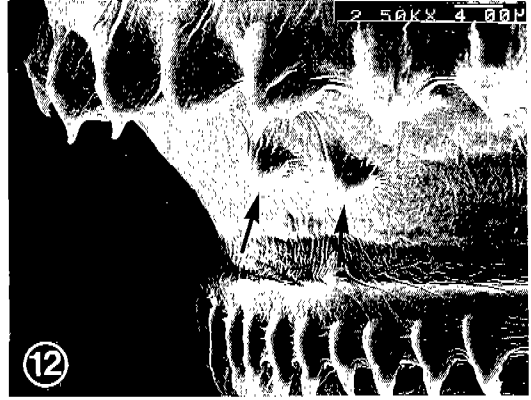
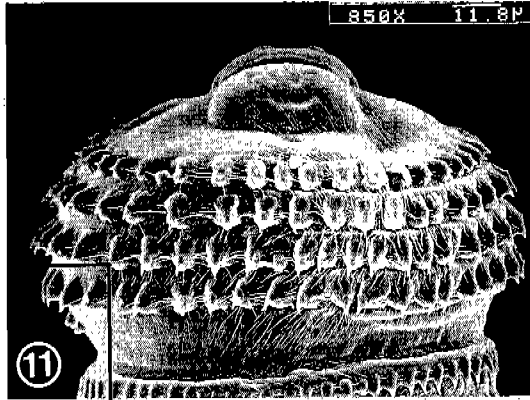
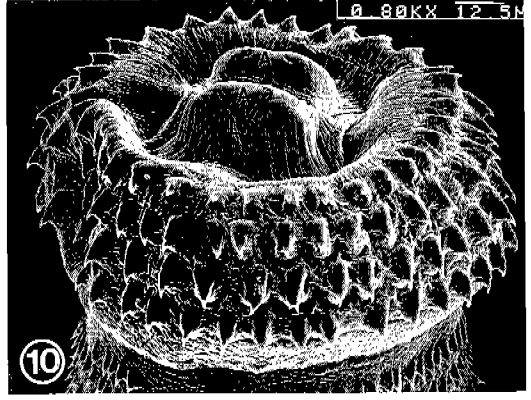
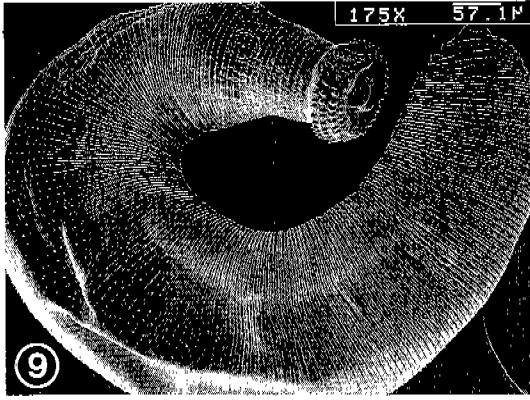
In Korea, gnathostome larvae were also found from imported Chinese loaches, and they were identified as the third-stage larvae of *G. nipponicum* and *G. hispidum* (Sohn *et al.*, 1993; Sohn and Lee, 1996). However, human cases have not been reported yet except the imported one by Lee *et al.* (1988). It's probably because Koreans do not eat the raw flesh of loaches unlike Japanese.

Several investigators have reported that larval gnathostomes parasitize in snakes. Chandler (1925) found the larvae of *G. spinigerum* in snakes, *Python reticularis*, *Naja bungarus* and *N. tripudianus* from India. Yamaguchi *et al.* (1956) found the larval *G. spinigerum* in *Natrix tigrina tigrina* and *Dinodon orientale* in Japan. Miyazaki and Ash (1959) found the larvae of *G. procyonis* in *Agkistrodon piscivorus* and *Natrix sipedon confluens* in USA. Miyazaki (1960) also found the larval *G. spinigerum* in the muscle of *Elaphe quadrivirgata* in Japan. In case of the larval *G. doloresi*, some Japanese workers discovered from the several species of snake, *Trimeresurus elegans*, *T. flavoviridis flavoviridis*, *T. okinavensis* and *Dinodon semicari-*

natus in Japan (Miyazaki and Kawashima, 1962; Tada *et al.*, 1969; Toshioka, 1970; Mako and Akahane, 1985). However, no species of snake have been reported yet as the intermediate host of *G. hispidum* so far as the literatures are concerned. Therefore, the present study first confirmed that the pit-viper, *A. brevicaudus*, is the snake intermediate host of *G. hispidum*.

Judging from the food habit of pit-vipers, infections with larval gnathostomes presumably occurred due to a secondary infection and they played a role as the transport or paratenic host. The most curious point is that what kind of animals were primarily infected with larval *G. hispidum* in Korea. Furthermore, it is uncertain whether the life cycle of this nematode is actively maintained in Korea or not, although it has been known that wild boars, the natural definitive host, inhabit in some place of deep mountains. Therefore, the natural life cycle of this nematode is the subject of a further study in Korea.

Miyazaki (1960) mentioned that the number and the shape of hooklets on the head-bulb are very useful for the species identification of the genus *Gnathostoma*. The hooklet features of the larval gnathostomes of this study were compatible with those of *G. hispidum* described by previous authors, however they were definitely different from those of other three



species distributed in the Far East (Table 5).

Studies for the species identification in cross sections of gnathostome larvae have been performed in Japan (Akahane *et al.*, 1986; Akahane and Mako, 1987; Ando *et al.*, 1991). Especially, it has been known that the cross sectional findings in the intestinal regions are obviously different among the four human infecting species of larval *Gnathostoma*, and the number of nuclei in an intestinal epithelial cell is the most important differential point. There are some useful differential findings listed in Table 6 for the species identification in the sectioned specimen of larval *Gnathostoma*. These findings were practically applied

in the biopsied specimen from human cases, and revealed the etiology of gnathostomiasis recently occurred in Japan (Ogata *et al.*, 1988; Taniguchi *et al.*, 1991 & 1992).

SEM findings in the present study revealed the shape and location of the labial papillae, amphids, cervical papillae, the shape and distribution of the hooklets on the head-bulb, and distribution of transverse striations and cuticular spines. These findings are not easily detectable under the light microscopy. Especially, the location of the cervical papillae is one of the important morphological features for identifying gnathostomes at the AdL₃ (Miyazaki and Ishii, 1952). In the present

Table 5. Comparison of the number of hooklets on head-bulbs in four species of larval *Gnathostoma* (AdL₃)

Species	1st row	2nd row	3rd row	4th row
<i>G. nipponicum</i> ^{a)}	29-36 (32)	30-37 (35)	31-41 (37)	—
<i>G. doloresi</i> ^{a)}	34-42 (38)	35-43 (40)	34-39 (36)	33-41 (37)
<i>G. spinigerum</i> ^{a)}	40-47 (43)	37-49 (45)	42-52 (47)	48-58 (52)
<i>G. hispidum</i> ^{b)}	32-38 (36)	37-41 (40)	39-44 (42)	42-48 (45)
Present study	35-39 (37)	38-41 (39)	39-44 (42)	42-45 (44)

^{a)}from Miyazaki (1952); ^{b)}from Koga *et al.* (1985).

Table 6. Morphological differences^{a)} in cross section at the midgut level in four species of larval *Gnathostoma* (AdL₃)

Species	No. of muscle cells in a quadrant	No. of intestinal cells	Morphology of intestinal cells	No. of nuclei in a cell
<i>G. nipponicum</i>	10-14	10-14	columnar	0-4 (one: 50%)
<i>G. doloresi</i>	11-15	18-28	spherical	0-3 (mainly 2)
<i>G. spinigerum</i>	10-15	21-29	columnar	0-7 (mainly 3-7)
<i>G. hispidum</i>	11-15	19-31	spherical	0-2 (mainly 1)
Present study	12-16	19-25	spherical	0-2 (mainly 1)

^{a)}Cited from Taniguchi *et al.* (1992) and slightly modified.

←

Figs. 9-16. Scanning electron microscopic (SEM) view of the advanced third-stage larvae of *Gnathostoma hispidum* from the pit-viper, *Agkistrodon brevicaudus*. **Fig. 9.** Whole body showing a head-bulb with hooklets, about 210 rows of transverse striations with cuticular spines and a cervical papilla (encircled). **Fig. 10.** Subfrontal view of the head bulb. An amphid (A) and two labial papillae (LP) are seen on each lip. **Fig. 11.** Four transverse rows of hooklets on the head-bulb. Each hooklet somewhat curved posteriorly. **Fig. 12.** Magnification of the boxed area in Fig. 11. Two hooklets are seen on the 5th row (arrows). **Fig. 13.** Body surface of the anterior part having cuticular spines on the transverse striations and a dome-like cervical papilla located between the 11th and 12th transverse striations. **Fig. 14.** Magnification of a part of Fig. 13. Body surface consists of a highly wrinkled cuticle and posteriorly curved spines. **Fig. 15.** Body surface of the middle part of which cuticular spines are more sparsely distributed on the transverse striations. **Fig. 16.** Adjacent area of the anus of which surface consists of a highly wrinkled cuticle without spines.

study, the papillae were located between the 11th and 12th transverse striations. This finding corresponds with that of Kondo *et al.* (1984). All findings obtained in SEM study should be helpful in a differential identification of larval gnathostomes.

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=초록=

한국산 살모사에서 최초로 발견한 돼지악구충의 제3기 유충

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국내 토착 악구충을 발견하기 위하여 1994년 3월부터 1997년 6월까지 미꾸리 3,450마리, 황소개구리 24마리, 무지개 55마리, 능구렁이 2마리, 유희목이 62마리, 살모사류 87마리 및 고양이 (stomach only) 438마리를 조사하였던 바, 12마리의 살모사에서 총 21마리의 악구충 유충이 검출되었다. 종 동정을 위하여 검출한 유충의 일부는 glycerin-jelly 봉입 표본 및 조직절편 횡단표본으로 제작하여 광학현미경으로 관찰하였고, 일부는 주사전자현미경으로 관찰하였다. 유충은 평균 2.233×0.343 mm 크기이었고, 작은 피극이 일정한 간격으로 배열되어 있는 약 210줄의 가로 주름을 가지고 있었다. 특징적인 head-bulb에는 소구 (hooklet)가 전방에서부터 평균 36.8개, 39.0개, 41.7개 및 44.3개씩 4줄 배열되어 있었다. 중장 부위의 횡단면에서는 19-25개의 상피세포로 이루어진 장벽이 관찰되었고, 대부분의 장상피세포는 1개씩의 큰 핵을 가지고 있었다. 주사전자현미경 관찰에서는 총체 전단의 입 부위, 특징적인 head-bulb, 가로주름 (transverse striation)과 피극 (cuticular spine), cervical papilla, 후단 부위 표피 등에서 특징적인 소견을 나타내었다. 이상의 형태학적 특징을 근거로 하여 한국산 살모사에서 검출한 악구충의 유충을 돼지 악구충 (*Gnathostoma hispidum*)의 advanced third-stage larva로 동정하였고, 이 연구를 통하여 살모사 (*Agkistrodon brevicaudus*)가 돼지악구충의 중간숙주임이 최초로 밝혀졌다.

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