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The value of external morphology in the identification of larval anisakid nematodes: a scanning electron microscope study

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Abstract. We studied larval nematodes of four genera of the Anisakidae using a scanning electron microscope (SEM). The anterior and posterior extremities and cuticular structures of the 3rd-stage larvae (L3) of *Anisakis* type I, *Pseudoterranova decipiens*, *Contracaecum* type B and *Hysterothylacium* were examined. The 4th-stage larvae (L4) of *Anisakis* type I, *P. decipiens*, recovered after infection into laboratory rats, and the L3 and L4 of *Anisakis* type I larvae from human were also examined in the same way. There were generic differences in the shape and size of the lip bulges, external papillary structures, the appearance of the boring tooth, the width and depth of the grooves and ridges of the cuticle and the caudal structures of the L3. In *Anisakis* type I and *P. decipiens* L3, changes were seen in the anterior extremity, cuticle and posterior extremity after molting to the L4. Similar changes can be expected in larvae infecting man. The L4 of *Anisakis* type I from rat and man were similar, while the L4 of *Anisakis* type I and *P. decipiens* showed differences. These ultrastructural differences might be of value in the identification of fragments recovered during endoscopy in man.

Introduction

The first report of anisakine larvae in human stools was by Hitchcock in 1950. Van Thiel et al. (1960) have published a detailed human case report in The Netherlands. Since then, several cases of human anisakiasis have been reported in Japan, The Netherlands, North America and Norway (Oshima 1972; Jackson 1975; Lichtenfels and Brancato 1976). Larvae of the genera *Anisakis*, *Pseudoterranova* (= *Phocanema*) and *Contracaecum* have been reported in man (Schaum and Müller 1967; Kates et al. 1973; Fujino

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et al. 1984) and can also infect experimental animal hosts (Shiraki 1969; Young and Lowe 1969; Gibson 1970).

Many studies have been done on the sectional and external morphology of these larvae with light microscope (LM) (Oshima 1972; Aihara 1973), but the few available transmission electron microscope (TEM) and SEM studies are on L3 and are incomplete (Valter 1978; Valter et al. 1982; Aji et al. 1982; Kliks 1983; Smith 1983; Fujino et al. 1984). To date very few studies on L4 are available (Fujino et al. 1984). As suggested by previous work (Myers 1975; Fredericksen and Specian 1981), further ultrastructural studies are warranted on the anterior and posterior extremities and cuticular morphology of the larvae to provide much needed information for their identification. Cuticular differences might help when only worm fragments are received for identification. SEM could be a reliable tool in identifying larvae recovered by endoscopy from patients and as an aid to diagnosis.

In our study we tried to elucidate the fine details of the L3 of *Anisakis simplex* (= *A.* type I) [*Anisakis* Dujardin, 1845 – type I according to Berland 1961], *Pseudoterranova decipiens* [*Pseudoterranova* Mozgovoi, 1950 – *P. decipiens* according to Gibson's 1983 classification], *Contracaecum* sp. (*C.* type B) [*Contracaecum* Railliet et Henry, 1912 – type B according to Koyama et al. 1969] and *Hysterothylacium* sp. (= *Thynnascaris* type III) [*Hysterothylacium* (= *Thynnascaris*) Ward and Magath, 1916 – closely resembles Cannon's type III, 1977] under LM and SEM. Special attention was paid to the external changes which occurred during the molt from L3 to L4 of *Anisakis* type I and *P. decipiens* in experimentally infected rats. These changes could also be expected in human infections. L3 and L4 of *Anisakis* type I recovered from humans were also studied. Although *Hysterothylacium* – a goeziine (= *Raphidascaridinae*) (Gibson 1983) is non-infective to man, we have included it in our study as it is found with *Anisakis* in fish and there are only a few ultrastructural studies (Soleim 1974; Valter 1978; Soleim and Berland 1981). An attempt was made to compare the anterior and posterior extremities and the cuticular structure of all specimens.

Materials and methods

Larval material. Anisakis type I, L3 were obtained from mackerel (*Scomber japonicus*) and *Hysterothylacium* L3 from squid (*Todarodes pacificus*), both marketed in Fukuoka, Japan. The Fisheries Experimental Station, Kushiro, Hokkaido, Japan, provided cod (*Gadus macrocephalus*) infected with *Contracaecum* type B L3 and Arabesque greenling (*Pleurogrammus azonus*) infected with *P. decipiens* L3.

The larvae were immediately collected from the digestive tract, abdominal cavity and viscera of the fish and squid. Thereafter, the abdominal muscles of the hosts were digested using a solution of 0.85% HCl and 1% pepsin at 37° C for 3–4 h and examined carefully for any remaining larvae. The larvae intended for animal infection were stored in Ringer's solution at 4° C and used within 2 h.

Specimens of *Anisakis* type I larvae, recovered from human patients and fixed in formalin, were received from the Ooiwa Gastroenterological Clinic in Fukuoka, Japan. All larvae were identified under LM by their morphological and morphometric characteristics prior to introduction into the rats.

Animal experiments. To obtain L4 of *Anisakis* type I, *P. decipiens* and *Contracaecum* type B, L3 were force fed to SD strain rats in groups. These rats were killed on days 1, 3, 5, 7 and 10, and a careful search for larvae was made in the digestive tract, abdominal cavity, viscera and muscles. *Hysterothylacium* L3 was not administered to rats as there were insufficient larvae.

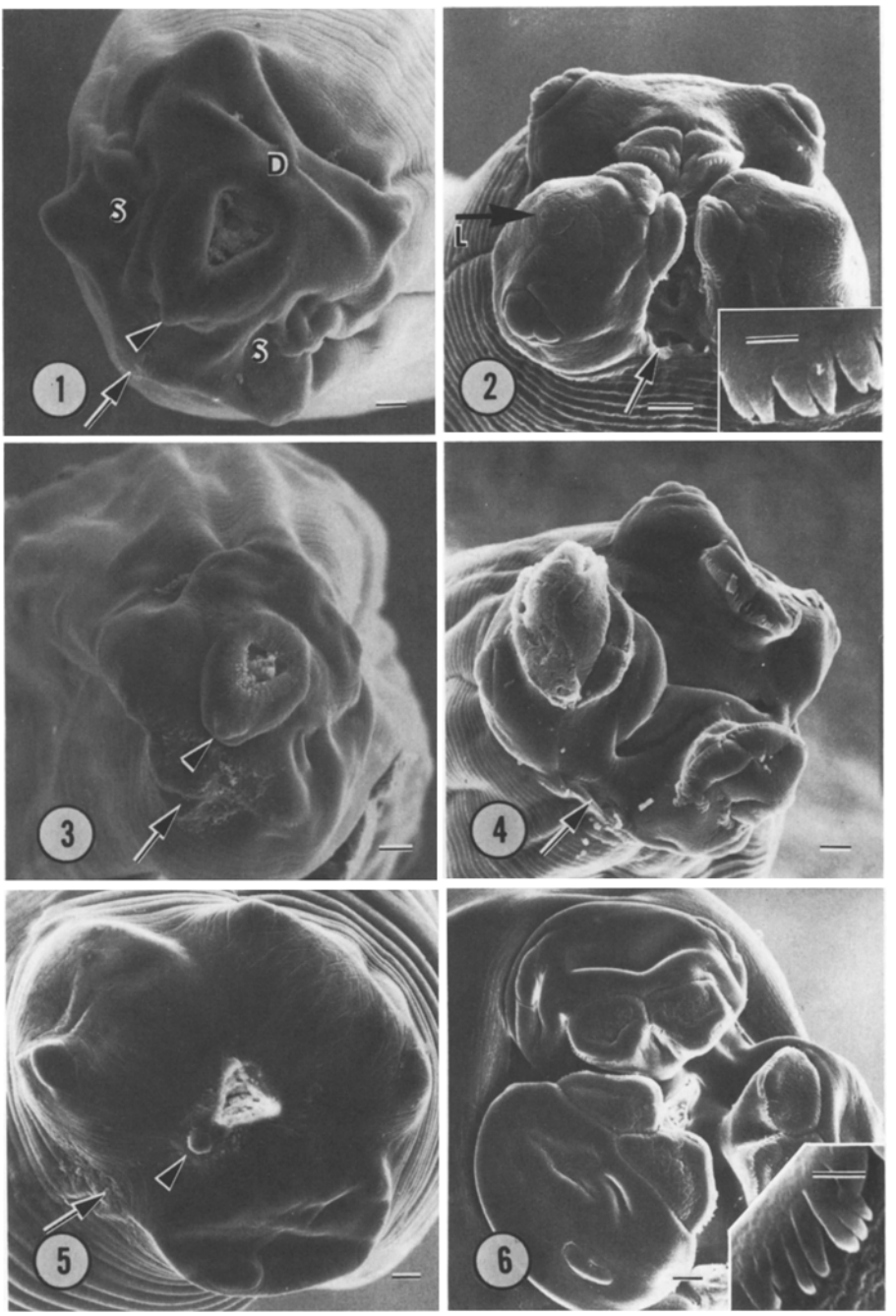
Preparation for SEM. Identification of molted and unmolted larvae was carried out under LM. The L3 and L4 thus obtained were divested of host tissue remnants by digesting them in a solution containing pancreatin and sodium bicarbonate at 37° C for 30 min. Thereafter, the specimens were rinsed thoroughly in Ringer's solution, followed by repeated changes in sodium cacodylate buffer. Half the specimens in each group were fixed in 10% formalin for 10 days or more, and the remainder was fixed in 2.5% glutaraldehyde in 0.1 M cacodylate buffer at pH 7.4 for 12 h. Both groups were then postfixed in 1% OsO_4 in the same buffer for 12 h, dehydrated in a series of graded ethanols, transferred to amyl acetate and dried in a Hitachi HCP-2 critical point dryer. The anterior and posterior extremities and mid cuticle of each specimen were mounted carefully on SEM stubs, coated with gold in a JEOL JFC-1100 sputter coater and observed under a JEOL JSM-U3 SEM at 15 kV.

Results

In *Anisakis* type I and *P. decipiens* molting to L4 was seen from the 3rd day onwards. *Anisakis* larvae penetrated the stomach and the intestinal wall but *Pseudoterranova* only penetrated as deep as the muscularis mucosa of the stomach. No larvae were recovered in the experiment in which *Contracaecum* larvae were fed to the rats.

External morphology

Anterior extremities of L3. The *Anisakis* L3 had three low lip bulges, one dorsal and two subventral, surrounding the triangular opening of the mouth (Fig. 1). Each subventral lip bulge contained one, the dorsal lip bulge contained two papilla-like structures, situated toward their bases. A prominent, ventral boring tooth was triangular in shape and directed outward. The excretory pore, positioned between the bases of the two subventral lip bulges, was a transverse slit. The anterior extremity of L3 recovered from humans (Fig. 3) was similar to that of the rat larvae. *P. decipiens* L3 was similar to that of *Anisakis* type I, except that the lip bulges were much more prominent and well demarcated (Fig. 5). The boring tooth was directed outward. The position and shape of the excretory pore was similar to that of *Anisakis* type I. The bases of the lip bulges exhibited structures resembling papillae, but higher magnification revealed them as swellings of the bases. The anterior extremity of the *Contracaecum* type B L3 had well-demarcated lip bulges: two subventral and one dorsal. The dorsal lip bulge appeared larger than the other two (Fig. 7). The four papillary structures were round elevations. In contrast to the previous two genera, the opening of the mouth cavity was slit-like and lay between the dorsal and the subventral lip bulges, extending right across the diameter. The ventrally located boring tooth projected inward; its shape differed from *Anisakis* and *Pseudoterranova.* The excretory pore was an oval opening positioned between the base of the



Figs. 1–6. Scanning electron micrographs. Dorsal lip bulge (*D*), subventral lip bulge (*S*), lateral papilla (*L*), boring tooth (*arrow head*), excretory pore (*arrow*). *Scale bar* = 10 μm

two subventral lip bulges. The anterior extremity of the *Hysterothylacium* L3 possessed three inconspicuous lip bulges bordering the triangular opening of the mouth. The shape of the ventrally projecting boring tooth was different from the other three genera (Fig. 8). There were no superficial structures which resembled papillae.

Anterior extremities of L4. The *Anisakis* L4 specimens from rats (Fig. 2) and humans (Fig. 4) showed three distinct lips. The dorsal lip was bigger than the two subventral ones, but each was bilobed with a median groove. The lips surrounded the triangular opening of the mouth cavity. The cephalic papillae were located toward the bases of the lips. They were very prominent and appeared to be double, with one half larger than the other. Some specimens had a lateral papilla on each subventral lip, seen as a small round elevated structure (Fig. 2). A single dentigerous ridge extended along the medial border of each bilobed lip and projected slightly toward the center. Each dentigerous ridge consisted of about 35 to 45 bifurcate and a few non-bifurcate denticles (Fig. 2). Denticles on the subventral lip were broad and short, and long and thin on the dorsal lip (Fig. 2, inset). The anterior extremities of the larvae from rats and humans did not show any clear structural differences. At the anterior extremity of *Pseudoterranova* L4 there were prominent lips, equal in size and shape. The bilobed medial region of the lips appeared much more prominent and comparatively larger than in the *Anisakis* type I. The dentigerous ridges surmounted the medial borders of the lips, exhibiting a well-defined "W" shape (Fig. 6). Each dentigerous ridge consisted of about 45 to 50 bifurcate and a few non-bifurcate denticles. The denticles on all three lips were similar, in contrast to *Anisakis* type I. They were also much longer and thinner than those of *Anisakis* (Fig. 6, inset). Four cephalic papillae were observed: two at the dorsal lip and one at the base of each subventral lip. The papillae were oval, flat and well demarcated by a shallow groove. A slight depression in the middle made them double structures.

Cuticular structures of L3. The *Anisakis* type I L3 had irregularly spaced, non-continuous, transverse, shallow grooves all over the body (Fig. 9). These

Fig. 1. *Anisakis* type I L3 anterior extremity

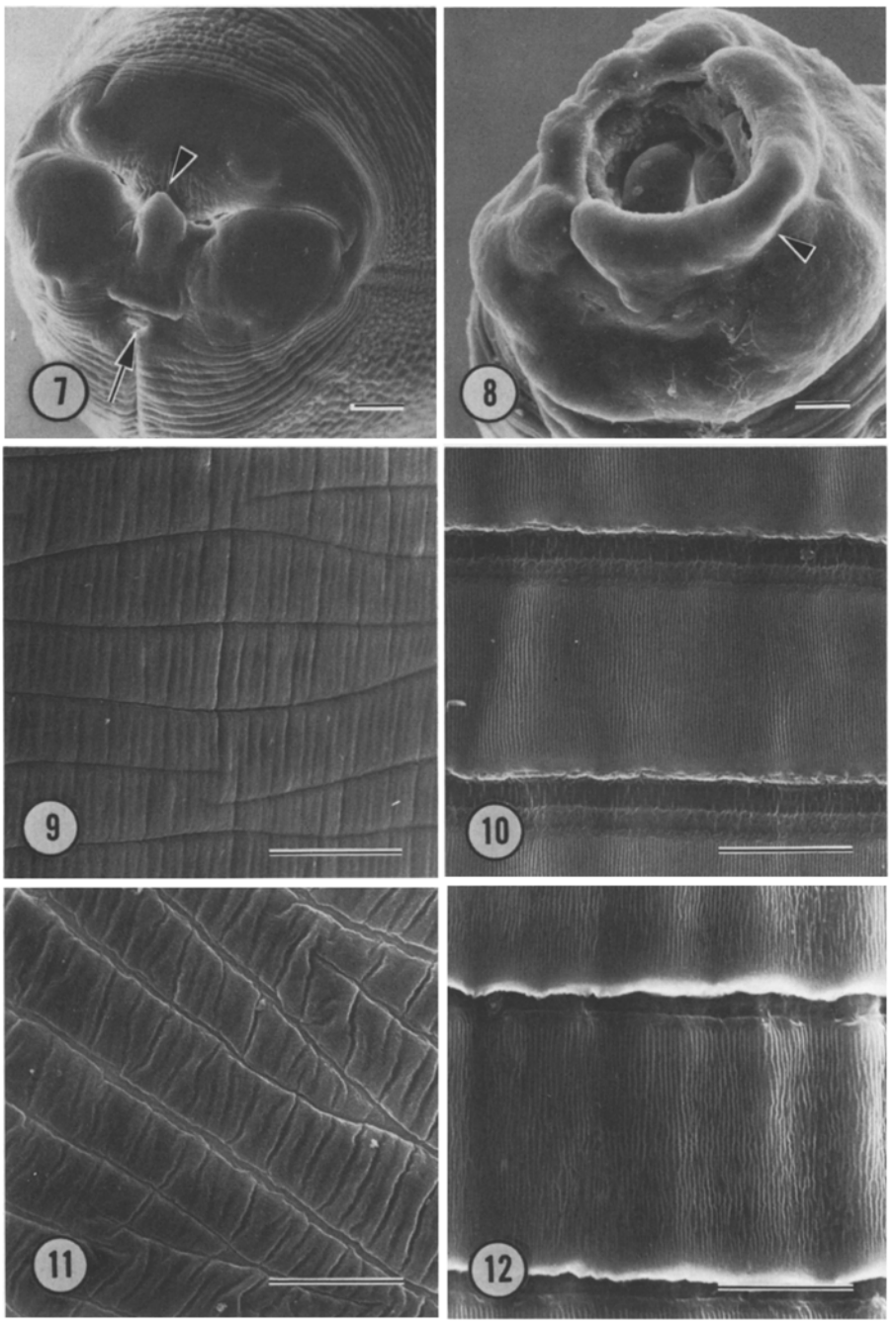
Fig. 2. *Anisakis* type I L4 anterior extremity recovered from rat. Double papillae are clearly seen. *Inset*: Enlarged view of the denticles of the dorsal lip. *Scale bar* = 2 μm

Fig. 3. *Anisakis* type I L3 anterior extremity recovered from human

Fig. 4. *Anisakis* type I L4 anterior extremity recovered from human

Fig. 5. *Pseudoterranova decipiens* L3 anterior extremity

Fig. 6. *Pseudoterranova decipiens* L4 anterior extremity. The "W" shaped dentigerous ridges are clearly seen. *Inset*: Enlarged view of the dorsal lip denticles. *Scale bar* = 2 μm



Figs. 7–12. Scanning electron micrographs. Boring tooth (*arrow head*), excretory pore (*arrow*). *Scale bar* = 10 μm

were close together toward the anterior and posterior ends. Between the transverse grooves were longitudinal, closely spaced, parallel striations caused by fine grooves and ridges. The L3 from humans (Fig. 11) also showed the same irregular cuticular pattern, but the transverse grooves were wider. A cuticular pattern similar to *Anisakis* was seen in *Pseudoterranova* L3 (Fig. 13), but with two types of transverse groove: one narrower and one broader, with a banded appearance. The *Contracaecum* L3 had somewhat regularly spaced, continuous, transverse grooves (Fig. 15). These were rather broad and had a double banded appearance. Parallel, irregularly spaced, longitudinal ridges were seen between them. The transverse grooves were closely arranged and well demarcated toward the anterior and posterior ends. *Hysterothylacium* L3 cuticle had very irregularly spaced, non-continuous, closely arranged transverse grooves (Fig. 16).

Cuticular structures of L4. The transverse grooves on the cuticle in the *Anisakis* type I L4 were wide, regularly spaced and continuous (Fig. 10). Higher magnification indicated that they were formed by two sub units: a shallow and a deep groove. The grooves were closely arranged toward the anterior and posterior ends. Longitudinal striations were regular, much more compact and finer than those in L3. The specimens from man (Fig. 12) showed similar features. *Pseudoterranova* L4 transverse grooves became broader, regularly spaced and continuous. The longitudinal striations were irregular compared to *Anisakis* type I, and appeared to be wrinkled at one end (Fig. 14).

Posterior extremities of L3. The majority of the *Anisakis* L3 possessed a straight, cone-shaped mucron at the posterior extremity and a few specimens had a partly curved, cone-shaped one (Fig. 17), but those of specimens from man were all straight and cone-shaped (Fig. 18). The round posterior extremity of the *Pseudoterranova* L3 bore a mucron, but in most of the specimens examined it was comparatively longer than in *Anisakis* (Fig. 21). The *Contracaecum* L3 posterior extremity tapered conically and lacked a mucron or a spine (Fig. 23). In some larvae it was found to be slightly curved. In *Hysterothylacium* L3 the body tapered gradually to end as a blunt, thin tail bearing a long spine (Fig. 24).

Fig. 7. *Contracaecum* type B L3 anterior extremity

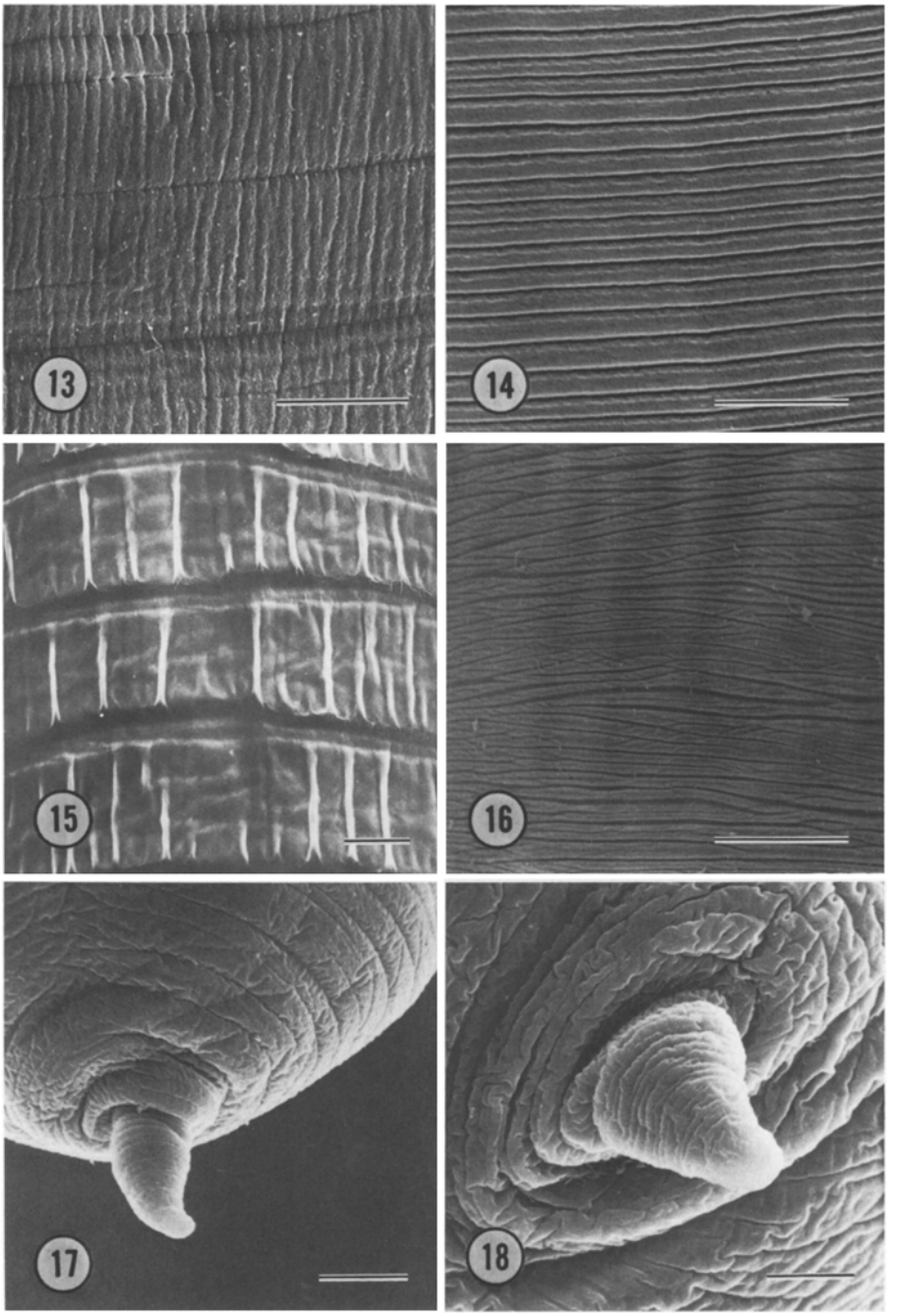
Fig. 8. *Hysterothylacium* L3 anterior extremity

Fig. 9. *Anisakis* type I L3 mid-cuticle. Note the irregular transverse grooves

Fig. 10. *Anisakis* type I L4 mid-cuticle, showing the regular transverse grooves

Fig. 11. *Anisakis* type I L3 mid-cuticle recovered from human

Fig. 12. *Anisakis* type I L4 mid-cuticle recovered from human



Figs. 13–18. Scanning electron micrographs. *Scale bar* = 10 μm

Posterior extremities of L4. Anisakis L4 from rats did not have a mucron but displayed one of two kinds of structure. Most specimens had a cone-shaped structure with a base wider than a mucron, studded with spherical elevations, giving it a rough appearance. The tip of the cone was depressed (Fig. 19). In a few specimens, there were three elevations in place of the mucron, the central one being slightly higher than the other two (Fig. 20). Most of the posterior extremities of the L4 had two phasmids situated symmetrically as round elevations surrounded by a distinct groove (Fig. 20, inset). Similar material from humans showed only an area studded with spherical elevations, although phasmids were observed in some. *Pseudoterranova* L4 had, instead of a mucron, a knob-like structure surrounded by a large number of well demarcated, spherical elevations (Fig. 22). The bilateral phasmids were flat, round, single structures (Fig. 22, inset).

The characteristics of these larvae are summarized in Table 1.

Discussion

To date the identification of larval anisakids has been based mainly on LM findings. But at times this method has been unsatisfactory in confirming identification and showing whether *Anisakis* and *Pseudoterranova* L3 have molted. Even the few available studies which use SEM and TEM have been on L3 and adults (Soleim 1974; Soleim and Berland 1981; Valter 1978; Valter et al. 1982; Carvajal et al. 1981; Fredericksen and Specian 1981; Smith 1983). There was no comprehensive ultrastructural study on the L4. Therefore in this study we infected laboratory rats with L3 from *Anisakis*, *Pseudoterranova* and *Contracaecum* and observed subsequent changes in the larvae. As in previous work (Young and Lowe 1969; Gibson 1970), maturation and molting of L3 *Anisakis* and *Pseudoterranova* to L4 occurred from the 3rd day after infection.

Anisakis larva penetrated the stomach and intestinal wall, but *Pseudoterranova* penetrated only up to the muscularis mucosa, becoming deeply attached ready to molt into L4. Previous experiments have shown that *Contracaecum* larval infections of animal hosts are difficult, and an attempt to infect human volunteers also failed (Kikuchi et al. 1969). So far only one case of human *Contracaecum* infection has been reported (Schaum and Müller 1967). We also failed to infect any rats with *Contracaecum*.

Fig. 13. *Pseudoterranova decipiens* L3 mid-cuticle. Note the two types of transverse grooves

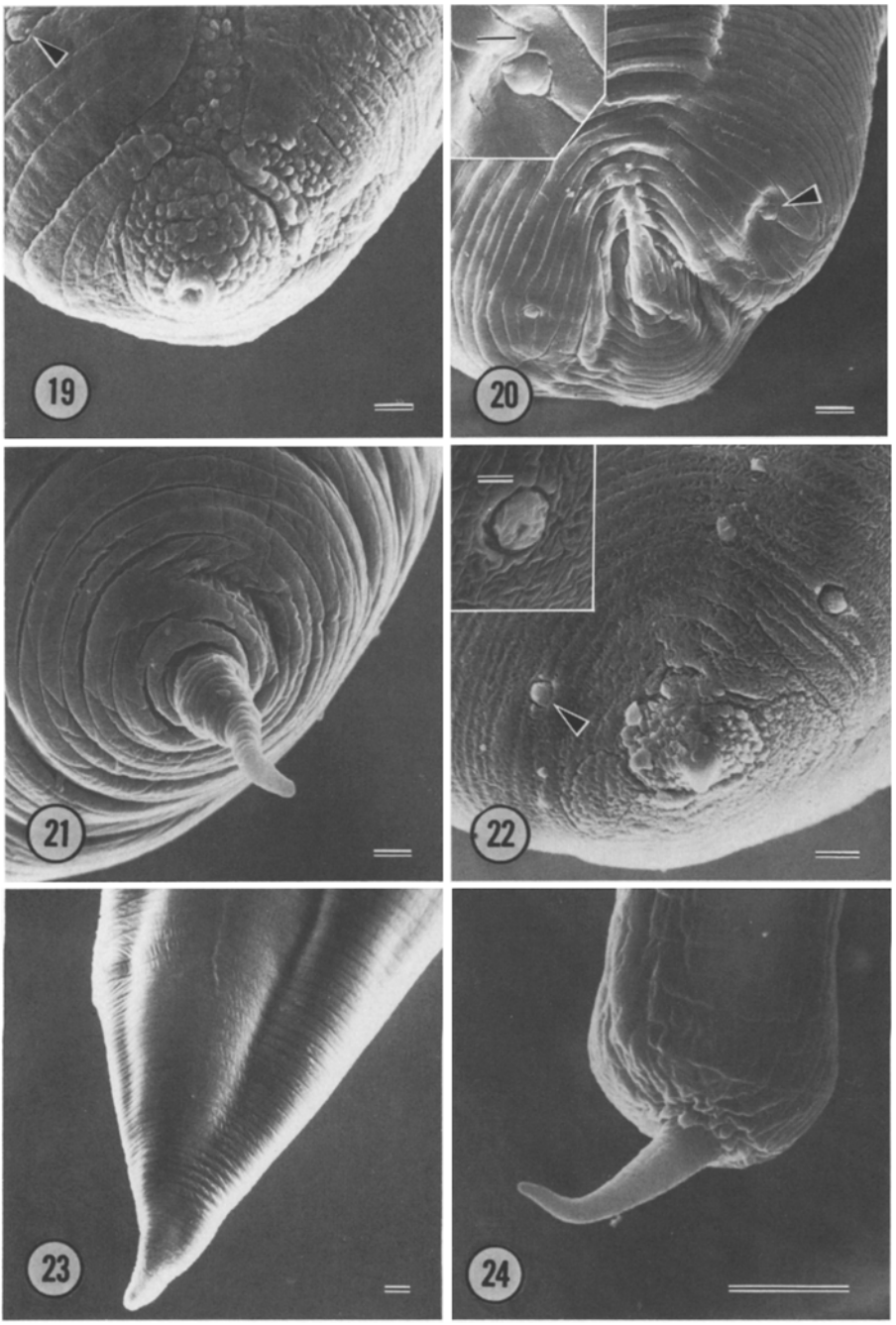
Fig. 14. *Pseudoterranova decipiens* L4 mid-cuticle

Fig. 15. *Contracaecum* type B L3 mid-cuticle

Fig. 16. *Hysterothylacium* L3 mid-cuticle, showing very irregular transverse grooves

Fig. 17. *Anisakis* type I L3 posterior extremity with curved mucron

Fig. 18. *Anisakis* type I L3 posterior extremity recovered from human



Figs. 19–24. Scanning electron micrographs. Phasmid (*arrow head*). *Scale bar* = 5 μm

Our findings, show structural differences among the L3 of the four genera, and also between the L4 of *Anisakis* type I and *P. decipiens*. The *Anisakis* L3 and L4 obtained from humans and rats had similar ultrastructural features.

Among the L3 of the four genera, the *Contracaecum* anterior extremity was distinctly different, bearing a well-differentiated cephalic structure and a slit-like transverse mouth opening. In contrast, the L3 of the other three genera possessed undifferentiated cephalic structures surmounting the triangular mouth opening. In addition, *Contracaecum* L3 had well-demarcated, round, elevated papillary structures whereas higher magnification did not reveal them in the other three genera. The boring tooth looked similar in *Anisakis* L3 and *Pseudoterranova* L3, but differed in shape in *Contracaecum* and *Hysterothylacium*. Marked differences in the cuticular pattern among the four genera were found in the present study. They are, however, open to question if one takes into account shrinkage due to fixation and dehydration.

Nevertheless, these findings may help diagnosis, especially when only fragments of worms are recovered from patients at endoscopy. Although previous work describe the cuticular pattern of these larvae under LM as having transverse and longitudinal striations, we have been able to show very clearly that the transverse striations are caused by grooves of various depths and widths and that the longitudinal striations are due to fine grooves and ridges. Valter et al. (1982) have said that the striations are caused by grooves, but the differential characteristics are not very clear. The posterior extremities of the L3 in these four genera differed with regard to the presence or absence of a mucron or a spine, the shape of the posterior end and its length.

The L4 of *Anisakis* and *Pseudoterranova* showed marked ultrastructural differences in the anterior extremity. Each had a typical lip shape and size including the medial bilobed portion, shape of the dentigerous ridges and shape, number and distribution of the denticles. Carvajal et al. (1981) in their study of the adults of *Anisakis* and *Phocanema* have also reported differences in size of the lips and the shape of the dentigerous ridges. The

Fig. 19. *Anisakis* type I L4 posterior extremity. A large number of spherical elevations are seen

Fig. 20. *Anisakis* type I L4 posterior extremity. The spherical elevations cannot be seen. *Inset*: Enlarged view of the phasmid. *Scale bar* = 2 μm

Fig. 21. *Pseudoterranova decipiens* L3 posterior extremity

Fig. 22. *Pseudoterranova decipiens* L4 posterior extremity. *Inset*: Enlarged view of the phasmid. *Scale bar* = 2 μm

Fig. 23. *Contracaecum* type B L3 posterior extremity

Fig. 24. *Hysterothylacium* L3 posterior extremity with long spine

Table 1. Summary of the differential characteristics among the anisakid larvae

Region	*Anisakis*		*Pseudoterranova*		*Contracaecum*	*Hysterothylacium*
	L3	L4	L3	L4	L3	L3
Anterior extremity						
Lip bulges (L3)/lips (L4)	Not prominent; $D^a > S^b$	Very prominent; D > S; medial lobes smaller	Slightly prominent; D > S	Very prominent; D = S; medial lobes larger	Prominent; D > S	Inconspicuous
Papillae	Elevated papilla like structures	Well demarcated; double; one larger	Elevated papilla like structures	Well demarcated; double	Well demarcated papillary structures	Not seen
Mouth cavity	Triangular	Triangular	Triangular	Triangular	Slit like	Triangular
Boring tooth	Triangular; directs outward	–	Similar to *Anisakis*	–	Triangular; broader base; directs inward	Curved prominence; directs outward
Dentigerous ridges	–	Projects in the center	–	"W" shape	–	–
Denticles	–	35–45 in number; shape different in D and S	–	45–50 in number; shape same in D and S	–	–
Cuticle						
Transverse grooves	Irregular; only one type	Regular; double banded	Irregular; two types	Regular	Somewhat regular; double banded	Very irregular
Longitudinal grooves and ridges	Irregular	Regular; clear pattern	Irregular	Irregular; one end wrinkled	Irregular	Not seen
Posterior extremity	Straight or curved mucron	Two types cone shaped; studded with spherical elevations/three elevations only	Straight; longer mucron than *Anisakis*	Knob like structure surrounded with spherical elevations	End conically	End as long thin spine

D^a = dorsal lip bulge/lip; S^b = subventral lip bulge/lip

cuticular transverse grooves were regularly spaced in both. The longitudinal striations were compact and clear in *Anisakis*, but no such clear pattern was observed in *Pseudoterranova* L4. Kliks (1983) has stated in a case report of *Phocanema decipiens* that the transverse striations (grooves) are irregularly spaced. The L4 posterior extremities of *Anisakis* and *Pseudoterranova* appeared to be similar except that the spherical elevations were well marked in *Pseudoterranova*. The peculiar appearance of the posterior extremities in about 10% of *Anisakis* specimens (Fig. 20) could have been due to sexual differences; further TEM and cross-sectional studies are needed for clarification. The majority of our specimens of L4 *Anisakis* and *Pseudoterranova* showed a pair of symmetrical phasmids near the posterior extremity.

Changes in internal structure when the L3 molts into the L4 have been reported by several authors using LM. Gibson (1970) and Fujino et al. (1984) have shown morphological changes in the internal structures such as the ventriculus and intestine and the development of the reproductive system in L4 *Anisakis* from rats and humans. As there are no reported LM histological and TEM studies on this aspect, further studies of L3 and L4 are being carried out in our laboratory.

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