Light and Transmission Electron Microscopic Studies of the Third Stage Larvae of *Anisakis Simplex*

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ABSTRACT

Objective: The purpose of this study was to demonstrate the structures of the third stage larva of *Anisakis simplex* in marine fish by using light microscope (LM) and transmission and transmission electron microscope (TEM).

Methods: L3 *Anisakis simplex* were processed for conventional light and electron microscopic studies.

Results: The body wall of the L3 *A. simplex* is composed of three layers, the cuticle, hypodermis and somatic muscular layer from the superficial to deep surface of the worm, respectively. The cuticle is thick, functions as a protective barrier and barring the antigenic molecules. Furthermore, TEM reveals that the cuticle is subdivided into cortical, middle and basal layers from outer to inner part of the cuticle. This layer is highly filamentous and the filaments arrange randomly in several directions. The hypodermis is a thin layer which functions as the cuticular productive layer. The lateral hypodermal cords are bilobed. The somatic muscular layer is composed of a single row of muscle cells, which lie along the long axis of the body. The gastrointestinal tract of the worm is a straight tube, lined with stratified epithelium and surrounded by the basal lamina. The intestinal epithelial cells contain various organelles, which its luminal surface presents numerous microvilli for absorption of nutrient molecules.

Conclusion: The cross section of LM and TEM can be used to distinguish the nematode species, as the LM reveals the lateral cords of L3 *A. simplex* are bilobed structures which are different from other species.

Keywords: L3 *Anisakis simplex*, LM, TEM

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INTRODUCTION

Anisakis simplex is a group of marine nematode which can be found in the body cavity, muscle, gastrointestinal tract and liver of marine fishes, birds and whales, dolphins,seals and porpoises (Muller R, 2002) The L3 larvae cause infection in human who consumes raw seafood such as sushi, sashimi, lomi-lomi, ceviche, and etc (Anderson RC, 2001). The patients can be treated only by using the endoscopy with biopsy forceps, for identify the worm morphology or detecting the patient immunological responses. The removal of the worm is essential to prevent the formation of eosinophilic granulomatous which is caused by allergic reaction. Eating habit is one of the diagnostic data from patient history, but the only correct way is to view the parasite morphology. The light microscopy is the easiest way and the transmission electron microscopy can be used to confirm the infection.

MATERIALS AND METHODS

Specimen collection

The third-stage larvae of *Anisakis simplex* were removed from peritoneal cavity and muscle of marine fish, washed several times in 0.85% NaCl and fixed in 10% formalin for light microscopic study and fixed in 2.5% glutaraldehyde in 0.1 M sodium cacodylate buffer for transmission electron microscopic study.

LM preparation

After formalin fixation, they were washed several times in distilled water and cut into small pieces. They were dehydrated in ethanol series from 70% to 100% for 10 minutes at each step and cleared twice in xylene for 10 minutes each at room temperature, infiltrated in

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the mixture of xylene and paraplast ratio 1:1 and pure paraplast, respectively. Then they were embedded in paraplast. The sections were cut at 6 µm thick in cross and longitudinal directions, mounted in permount, stained with routine hematoxylin and eosin, covered with cover slip and observed by light microscope.

TEM preparation

For the ultrastructural study of the L3 of *A. simplex* by transmission electron microscopy (TEM), the worms were fixed in 2.5% glutaraldehyde in 0.1 M sodium cacodylate buffer, pH 7.2 at 4°C. They were then washed three times with the same buffer, postfixed with 0.1% osmium tetroxide in 0.1 M sodium cacodylate buffer, pH 7.2 at 4°C for two hours, then washed three times with the same buffer. They were dehydrated in graded series of ethanol from 70% to 100% for 20 minutes at each step and infiltrated twice in propylene oxide for 40 minutes each. The solution was replaced twice with mixtures of propylene oxide and absolute alcohol ratio 1:2 overnight and 2:1 overday, respectively. They were embedded in Araldite. The ultrathin sections were cut at 50-70 nm that showing silver to gold interference, mounted sections on copper grids and stained with 5% uranyl acetate and lead citrate for 30 minutes each, respectively. Finally, the grids were observed under transmission electron microscope.

RESULTS

Light microscopic study of L3 *Anisakis simplex*

Cross section of L3 *Anisakis simplex* by light microscopic study

The cross section of the third stage larvae of *Anisakis simplex* (Fig 1A), reveals that the body wall is composed of three layers, the cuticle, hypodermis, and somatic muscular layers from outer to inner part of the worm, respectively (Fig 1B). The cuticle is the outermost layer, about 4-6 µm thick. It is composed of homogenous, and modulate acidophilic and covers the whole body wall. Furthermore, the outer margin of thickened cuticle forms numerous small spines project outward (Fig 1B). The function of the cuticle is to bare with the high hydrostatic pressure exerted by the fluid in the pseudocoelom. It also provides mechanical protection and resists digestion by the host.

The hypodermis lined underneath the cuticle (Fig 1B). The great function of this layer is to secrete substances forming the cuticle. This layer is about 2-3 µm thick, more basophilic than the cuticle, and has some parts projected into the body cavity, the hypodermal cords. There are one dorsal cord at mid dorsal region (Fig 1C), one ventral cord at mid ventral region (Fig 1D) and two lateral hypodermal cords at both midlateral regions (Fig 1E, 1F). The dorsal and ventral cords are narrow pedunculated in shape, while two lateral cords are larger, pedunculated and have a bilobate part protruding into the larval body cavity. Several large nuclei are observed in these cords. The dorsal and ventral cords are much more weakly developed and do not protrude beyond the muscular layer of the body wall.

The innermost layer is the somatic muscular layer (Fig 1B) that functions for movement purpose. It lies beneath the hypodermis and is composed of a single row of muscle cells. The muscle cells are elongate, tapering shaped and lying along the long axis of the body. This layer is separated into four quadrants by the hypodermal cords, which are projected in the pseudocoelom. The muscle cell comprises contractile and non-contractile portions (Fig 1G);

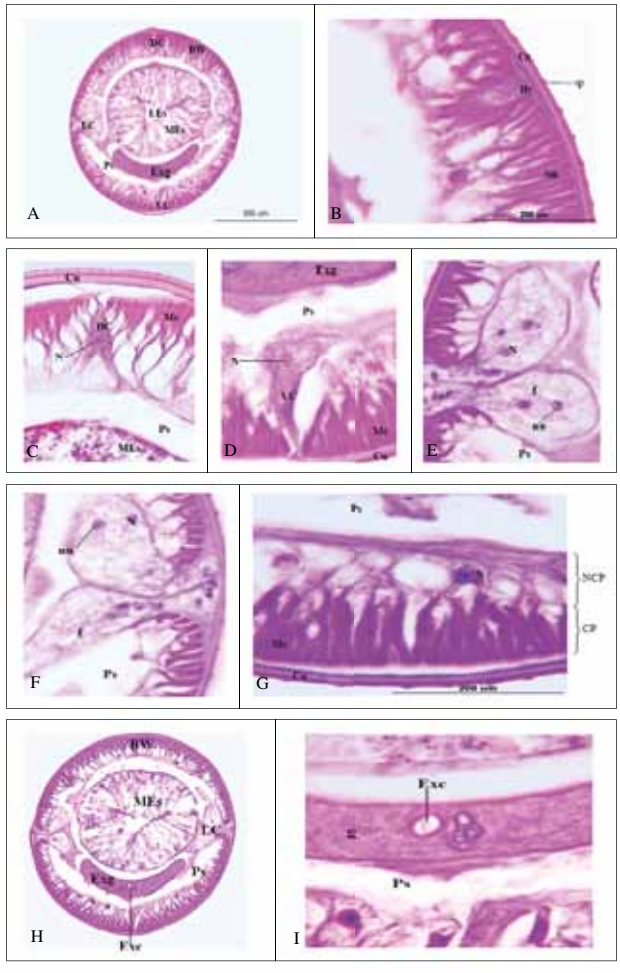

Fig 1A. Light micrograph shows cross section of L3 *Anisakis simplex*, Dorsal cord (DC), Ventral cord (VC), Lateral cord (LC), Pseudocoelom (Ps), Body wall (BW), Lumen of esophagus (LEs), Muscular esophagus (MEs), Excretory gland (Exg).

Fig 1B. Light micrograph shows the higher magnification of body wall which consists of 3 layers; Cuticle (Cu), Hypodermis (Hy) and Somatic muscular layer (Sm). The outer rim of the cuticle forms numerous small spines project outward (sp).

Fig 1C. Light micrograph shows the dorsal cord (DC) at the mid-dorsal position; Cuticle (Cu), nucleus (N), muscle cells (Mc), Pseudocoelom (Ps), Muscular esophagus (MEs).

Fig 1D. Light micrograph shows the ventral cord (VC) at the mid-ventral position; excretory gland (Exg), pseudocoelom (Ps), muscle cells (Mc), cuticle (Cu), and nucleus (N).

Fig 1E-F. Light micrograph shows both sides of the lateral cords. The lateral cords have a bilobate part protruding into the pseudocoelom (Ps), numerous large nuclei (N) with one dense nucleolus (nu), fine fibril (f).

Fig 1G. Light micrograph shows the somatic muscular layer. The muscle cells in somatic muscular layer involve contractile (CP) and non-contractile portion (NCP); pseudocoelom (Ps), nucleus (N), muscle cells (Mc), Cuticle (Cu).

Fig 1H. Light micrograph shows cross section of the excretory gland (Exg) locates in the ventral aspect of pseudocoelom (Ps). The rough basophilic granules (g) distributed the whole excretory gland. The excretory canal (Exc) is a duct that runs longitudinally along the entire length, muscular esophagus (MEs), Lateral cords (LC), Body wall (BW).

Fig 1I. Higher magnification of the excretory gland.

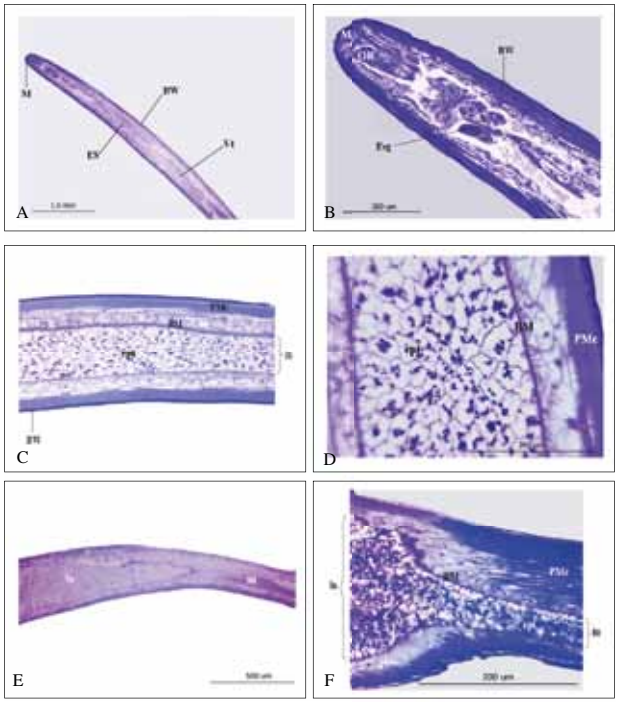

Fig 2A. Light micrograph shows longitudinal section of anterior part of L3 *A. simplex*; the mouth (M) at anterior end, the esophagus (Es), body wall (BW), ventriculus (Vt).

Fig 2B. Light micrograph shows longitudinal section of head of L3 *A. simplex*: Esophageal gland (Esg), body wall (BW), oral cavity (OR).

Fig 2C. Light micrograph shows longitudinal section of the body; the intestine (In), epithelial cells (epi), basement membrane (BM), body wall (BW), parallel fiber of muscle cell (PMc).

Fig 2D. Light micrograph shows the higher magnification of intestine. The epithelial cells (epi) of alimentary tract are stratified type with dot-like dense granules (g).

Fig 2E. Light micrograph shows longitudinal section of junction of intestine (In) and rectum (Rt).

Fig 2F. Light micrograph shows the higher magnification of longitudinal section of junction of intestine (In) and rectum (Rt). The rectum is a short canal and joins between the intestine and the anus. The epithelial cells of intestine are larger and denser than the rectum. The parallel fibers of the muscle cells (PMc) in contractile portion of somatic muscular layer are well developed in the tail of the worm.

the contractile portion lies close to the hypodermis and contains myofilaments, while non-contractile portion contains large concentric nucleus with prominence nucleolus and various organelles.

The body wall surrounds the body cavity, the pseudocoelom. The pseudocoelom separates the muscular layer from the internal organs such as gut and reproductive organs. The pseudocoelom of nematodes differs from the true coelomic cavity of other animal because the cavity lies between the cells of mesodermal origin such as muscle cells on the outer side and the cells of endodermal origin such as intestinal cells on the inner side. The reproductive organs have not yet developed in the L3 stage.

The gastrointestinal tract is a straight tube, lined with stratified type of epithelium and surrounded by basement membrane, appears round in cross section (Fig 1A).

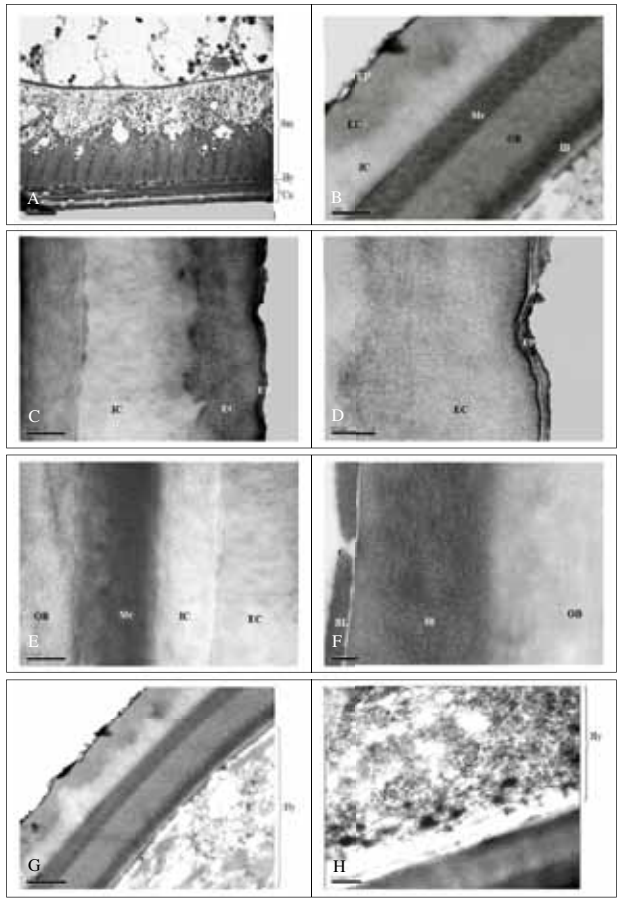

Fig 3A. Transmission electron micrograph shows the body wall of L3 *A. simplex*. The body wall comprise of three layers, the cuticle (Cu), hypodermis (Hy), and somatic muscular layers (Sm) from superficial to deep part of the worm, respectively.

Fig 3B. Transmission electron micrograph shows the cuticle and its sublayers; the external cortical (EC), internal cortical (IC), median (Me), outer basal (OB) and inner basal layers (IB), respectively. This is covered by epicuticle (Ep).

Fig 3C. Transmission electron micrograph shows the higher magnification of the subcortical layer of cuticle; Epicuticle (EP), external cortical (EC), internal cortical layer (IC).

Fig 3D. Transmission electron micrograph shows the higher magnification of the epicuticle (EP) appears as trilaminate covering the surface of external cortical layer (EC).

Fig 3E. Transmission electron micrograph shows the higher magnification of the external cortical (EC), internal cortical (IC), median (Me) and outer basal layer (OB).

Fig 3F. Transmission electron micrograph shows the higher magnification of the outer basal layer (OB) which is low electron dense than the inner basal layer (IB). The basal lamina (BL) separates the cuticle from the hypodermis and numerous canals (c) for exchange various materials.

Fig 3G. Transmission electron micrograph shows the hypodermis (Hy).

Fig 3H. Transmission electron micrograph shows the hypodermis (Hy) which is a thin strip of the cytoplasmic syncytium about 2-4 µm, lies between the basal lamina of the cuticle and the somatic musculature. The function of this layer is synthesis and maintenance of the cuticle.

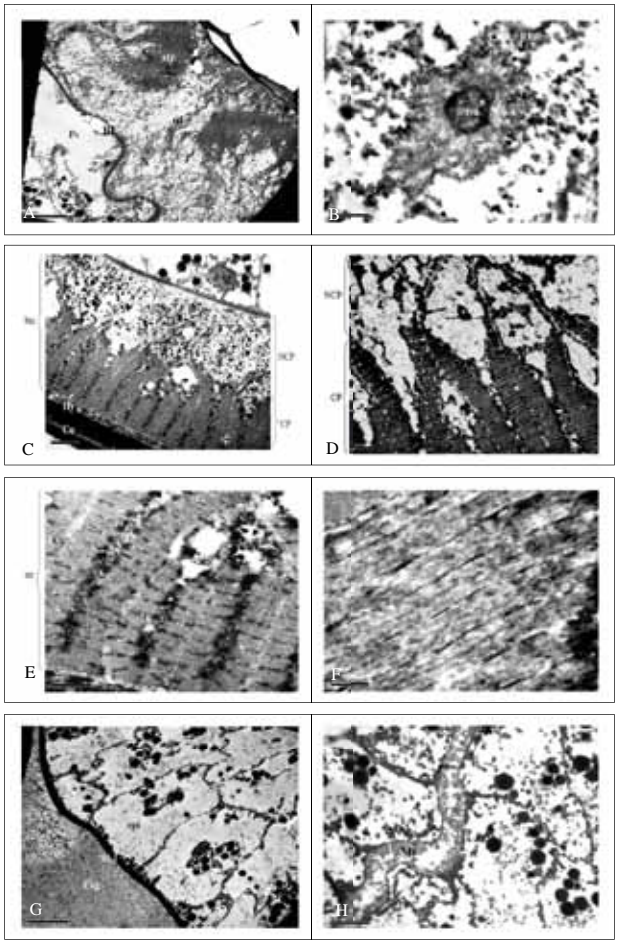

Fig 4A. Transmission electron micrograph shows various organelles in the lateral cord; nucleus (N), nerve fiber (nf), basal lamina (BL), striated muscles (str), ribosome (r) and pseudocoelom (Ps).
Fig 4B. Transmission electron micrograph shows the higher magnification of a large euchromatic nucleus (N) with a distinct nucleolus (nu) in the lateral cord; rough endoplasmic reticulum (RER).
Fig 4C. Transmission electron micrograph shows somatic muscular layer (Sm); the sarcoplasm of each muscle cell is divided into contractile portion (CP) and non-contractile portion (NCP); hypodermis (Hy), cuticle (Cu).
Fig 4D. Transmission electron micrograph shows the higher magnification of contractile portion (CP), non-contractile portion (NCP) and various organelles in somatic muscular layer, sarcoplasm (S).
Fig 4E. Transmission electron micrograph shows the cross section of the contractile portion; the striated muscles (str) are composed of actin and myosin that run longitudinal to the long axis of the body.
Fig 4F. Transmission electron micrograph shows the longitudinal section of the contractile portion. A thick filament is surrounded by numerous thin filaments.
Fig 4G. Transmission electron micrograph shows cross section of the intestine; the thick basal lamina of intestine (BLI), epithelial cells of intestine (epi), and excretory gland (Exg).
Fig 4H. Transmission electron micrograph shows branch of lumen of the intestine, lined with numerous microvilli (Mv) for absorb the nutrient molecules.

The lumen of esophagus is triradiated (Fig 1A), while the intestine and rectum appear irregular. The thickness of esophagus is less than intestine. These guts are lined with networks of columnar intestinal epithelial cells. In the intestinal cells, the nuclei are concentric and appear as dot-like dense basophilic granules within these networks. The excretory gland is elongate and crescent shaped occupied almost the ventral aspect of the pseudocoelom (Fig 1H, 1L). There are many basophilic granules distributed the whole gland. The excretory canal runs longitudinally along the entire length with numerous branches.

Longitudinal section of L3 *Anisakis simplex* by light microscopic study

In longitudinal section, the cuticle and hyperdermis are blended homogenously and cannot be distinguished (Fig 2A, 2B, 2C, 2D). The somatic muscular layer shows numerous parallel muscle fibers (Fig 2A-2F). The contractile portion are well developed in the tail end (Fig 2F). The gland cells near the oral cavity and esophagus function as enzyme secreting cells (Fig 2B). Digestive tract consists of oral cavity, esophagus, intestine, rectum and anus. The esophagus is a short tube with the dilated distal part called the ventriculus (Fig 2A). The intestine constitutes most of the length of the alimentary tract. (Fig 2C). The rectum is a short canal and joins between the intestine and the anus (Fig 2E, 2F). The lumen is surrounded by stratified epithelium type that found dot-like dense basophilic granules in each epithelial cell, size of epithelial cells in the rectum are smaller than in the intestine, and these epitheliums are lined with basement membrane (Fig 2D).

Transmission electron microscopic study (TEM) of L3 *A. simplex*

Cuticle

The thin sections of L3 *A. simplex* were studied by TEM. The body wall comprises of three layers, they are the cuticle, hypodermis, and somatic muscular layers from superficial to deep part of the worm, respectively (Fig 3A). The cuticle is approximately 5-6 µm thick but varies slightly among different regions and levels of the worm body. The thickest part is covering the lateral cord. The cuticle comprises 3 layers; they are the cortical, median and basal layers from outer to inner surface of the cuticle, respectively (Fig 3B). The fibrils arrange randomly with no clear demarcation between each layer, this feature is similar to many of the other nematodes.

The cortical layer is 2.5 µm and subdivided into the external cortical and internal cortical layers (Fig 3C). The external cortical layer is covered by a thin electron-dense osmiophilic layer about 0.05-0.08 µm thick and is termed the "epicuticle". At higher magnification, it appears as a trilaminate membrane which composed of two dark bands and a central light one (Fig 3C, 3D). However, the characteristic trilaminate membrane of this epicuticle can be observed only in some preparations. The external cortical layer lies immediately underneath the epicuticle, about 1-1.5 µm and is terminated at the base of each groove between the annulation. It is little filamentous in structure, the filaments arrange randomly in several directions. The internal cortical layer lies at the base of the annulations, about 0.5-1 µm thick and appears as a dense layer of fine parallel filamentous structures, not all of which are clearly discernible. Most filaments run parallel and oblique to the long axis of the body.

The median layer is 0.6-0.8 µm thick, comprises of fine dense filamentous structures arrange in parallel (Fig 3E). In a longitudinal section, the filaments appear as fine granules, but in a cross-section they appear as closely packed parallel bundles, which indicate that these filaments in the middle layer encircle the worm's body. The fibrils arrangement are clear demarcation between the internal cortical and outer basal layer.

The basal layer is about 2-2.3 µm thick and subdivided into two sublayers, they are the outer basal and inner basal layers with filamentous components of both sublayers arrange in opposite directions (Fig 3F). Filaments of the outer basal layer appear dense circular, while those of the inner basal layer are longitudinal. The filaments of both basal sublayers are more delicate, shorter and smaller than those locate in the more superficial layers. The thicknesses of outer and inner basal layers are 1.3-1.6 and 0.5-0.7 µm respectively. The basal lamina separates the cuticle from the underlying hypodermis. The basal lamina is a thin strip of about 0.15-0.25 µm, some regions there are pores of different sizes (Fig 3F).

Hypodermis and cords

The hypodermis is a thin strip of the cytoplasmic syncytium about 2-4 µm, lies between the basal lamina of the cuticle and the somatic musculature (Fig 3G, 3H). It is a particularly important and metabolically active part of the nematode's body walls, being responsible for synthesis and maintenance of the cuticle. It is characteristically thickened in four regions around the cross-section of the body, forming longitudinal column that extends through the length of the body, they are a dorsal, a ventral and two lateral hypodermal cords. These cords protrude into the pseudocelomic cavity between the somatic muscle cells and divide the latter into four quadrants.

The lateral cords, which commonly run the whole length of the body (Fig 3I). The lateral cord are the largest pedunculated and bilobed. The cytoplasm is highly complex and is roughly divisible into three zones. The narrow subcuticular region containing arrays of infoldings, in which are invaginations of the apical plasma membrane. The broader mid-region contains a nucleus, nerve fibers, cell organelles and abundant glycogen. The innermost region contains a network of infolded basal plasma membrane. The dorsal and ventral cords are pedunculated and smaller than the lateral cords; contain small vesicles, nucleus, ribosome, mitochondria

The hypodermis covering the somatic muscle cells is only a thin sheet of fused cytoplasmic processes of hypodermal cells. It is about 0.5 µm thick, and separated from the muscle by the basal lamina of variable thickness. This part of the hypodermal tissue also shows infoldings of the apical plasma membrane. (Fig 3G, 3H).

Somatic musculature layer

The somatic musculature layer of L3 *A. simplex* is well developed throughout the body (Fig 3K). The four hypodermal cords separate the somatic musculature layer into four quadrants. A thin basal lamina of low electron density separates between the hypodermis and the muscle cells, the intercellular matrix fill up the space between muscle cells. The sarcoplasm of each muscle cell is divided into contractile portion and non-contractile portion (Fig 3K, 3L).

The contractile portion is the outer zone, lies close to the hypodermis, contains myofibrillar elements, and rich in actin, myosin and glycogen particles The striated muscles run longitudinal to the long axis of the body. The mitochondria are fusiform in shape and may provide the energy for contraction. Each thick filament appears as a long thick rod surrounded by numerous thin filaments. This portion is divided in to bundles of myofilaments by irregularly-spaced invagination of plasma membrane.

The non-contractile portion is inner zone, contains various organelles such as the nucleus with one dense nucleolus, Golgi bodies, ribosome, rough endoplasmic reticulum, lipid and glycogen (Fig 3M, 3L).

The non-contractile portion is connected to the continuous layer of a thin basal lamina which separates the body wall from the pseudocoelom. The pseudocoelom is filled with fluid, the hemolymph which baths all the internal organs, and it contains scattered electron-dense precipitates that may represent the nutrient materials or other compounds of excretory and secretory significances. Alimentary tract

The alimentary tract of the worm is surrounded by the basal lamina (Fig 3O). At higher magnification, the basal lamina of alimentary tract is highly thin filamentous structure. The luminal surface has numerous fingers like microvilli (Fig 3P). In some locations, the cells bear only short and moderate number of microvilli. The epithelial cells of alimentary tract are stratified type (Fig 3O). The cytoplasm comprises of ribosome, short rough endoplasmic reticulum, and electron dense particle.

DISCUSSION

The ultrastructural studies of the infective third-stage larvae (L3) of Anisakis simplex by light and transmission electron micrographs showed a feature similar to many other nematodes that can be discussedfollowed.

The body wall observed by light micrographs is similar to other round worms. For example; it is the same as Ascaris lumbricoides (Marquardt WC et al, 2005) and Pseudoterranova decipipiens (Chai JY et al, 1995) by having three layers, the cuticle, hypodermis and somatic muscular layers. The cuticle is thick because it excerts several functions such as a protective barrier, resisting digestion by the host, protection against dehydration and antigenic source (William C et al, 2000). In the transmission electron micrographs; the cuticle comprises three main layers, they are the cortical, median and basal layers from outer to inner surface of the cuticle, respectively. It is highly filamentous in structure and the filaments arrange randomly in several directions, that corresponds to the studies of other nematodes by Mernmaung K et al. in Gnathustoma spinrgerum, Roongruangchai J et al. in Brugia pahangi and Vincent P et al. in Brugia malayi. The cortical layer is subdivided into the external cortical and internal cortical layers. This layer is covered by the electron-dense trilaminate membrane is called epicuticle, that exposed to the host immune system.

The hypodermis lies between the basal lamina of cuticle and somatic muscular layer. The basal lamina is discontinuous strip, numerous canals for transport materials into and out between the hypodermis to cuticular surface. The hypodermis is syncytial appearance, a partly important and metabolically active part of the nematode body wall, being responsible for secreting and maintaining of the cuticle. Hypodermal cells synthesize and secrete cuticular protein which is a part of the antigens. Furthermore, the hypodermal sheet is probably responsible for the exchange of materials. The hypodermis of Anisakid forms dorsal, ventral and lateral cords that protruded into the pseudocoe-

lom. The lateral cords are large bilobe, which are specific characteristic of *Anisakis simplex*.

The somatic musculature layer is composed of a single row of muscle cells which is separated into four quadrants by hypodermal cords. The muscle cells are elongate, tapering ending in shape and lying along the long axis of the body. This characteristic is similar to the other nematodes as Ascaris lumbricoides, Ancylostoma duodenale (Marquardt WC et al, 2005). The sarcoplasm bulges into the pseudocoel and the fibers extend up its sides. The sarcoplasm of each muscle cell is divided into contractile portion and non-contractile portion. The contractile portion contains, thick and thin myofilaments. The mitochondria may provide energy for movement of muscle fibers. The non-contractile portion contains large concentric nucleus with prominence nucleolus, the cytoplasm full of glycogen, ribosome, and rough endoplasmic reticulum.

The pseudocoelom or body cavity of nematode differs from the true coelomic cavity of other animals in that it is not completely lined with tissue of mesodermal origin. It is lined externally by a single layer of somatic muscle cells which are mesodermal origin, and lined internally by the cells of the alimentary tract and the reproductive organ, which are endodermal in origin. The pseudocoelom is filled with fluid or hemolymph which bathes all the internal organs and functions as part of the turgor pressure system exerting force continuously on the body wall. The internal pressure of nematodes is much higher than those found in the majority of invertebrates. It appears that this high pressure system is to be largely responsible for the uniform cylindrical structure of the nematode.

CONCLUSION

The detail structures of *Anisakis simplex* was demonstrated by light and transmission electron microscopies. The light microscopy reveals that the body wall comprises of three layers, the cuticle, hypodermis and somatic muscular layers. The transmission electron microscopy reveals more sublayers as the filamentous structures arranged in different direction. The outermost layer is the epicuticle, barring the antigenic molecules. The hypodermis is a thin layer except at the dorsal, ventral and both lateral hypodermal cords which protrude into the pseudocoelom and divide the somatic muscle into four quadrants. The lateral cords are bilobed structure and different from much of other nematodes, so can be used as species distinguished structure. The other structure is the somatic muscle cell of *Anisakis simplex* appears tapering end while in *A. lumbricoides* is bluntly ending. In TEM reveals that the cuticle separates from the hypodermis by the basal lamina which acquires pore or canal like fenestrated type of capillary while other species have no basal lamina.

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