

Angiostrongylus cantonensis: Scanning Electron Microscopic Observations on The Cuticle of Moulting Larvae

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Abstract: *Angiostrongylus cantonensis* is a parasitic nematode that needs to develop in different hosts in different larval stages. Freshwater snails, such as *Pomacea canaliculata*, are the intermediate host, and rats are the definitive host. Periodic shedding of the cuticle (moulting) is an important biological process for the survival and development of the parasite in the intermediate and definitive hosts. However, there are few studies on the cuticle alterations between different stages of this parasite. In this study, we observed the ultrastructural appearance and changes of the cuticle of the 2nd/3rd stage larvae (L2/L3) and the 3rd/4th stage larvae (L3/L4) using a scanning electron microscope. We also first divided L2/L3 into late L2 and early L3. The late L2 lacked alae, but possessed a pull-chain-like fissure. Irregular alignment of spherical particles on the cuticle were noted compared to the L3. Alae appeared in the early L3. The old cuticle turned into a thin film-like structure which adhered to the new cuticle, and spherical particles were seen regularly arranged on the surface of this structure. Regular rectangular cavities were found on the surface of L3/L4. The caudal structure of L3/L4 was much larger than that of L3, but caudal inflation, such as seen in L4, was not observed. These results are the first to reveal the ultrastructural changes of the cuticle of *A. cantonensis* before and after moulting of L2/L3 and L3/L4.

Key words: *Angiostrongylus cantonensis*, moulting larva, scanning electron microscopy

INTRODUCTION

Angiostrongylus cantonensis was first found in the pulmonary artery of rats in China [1] and is the agent of human angiostrongyliasis [2]. The first stage larvae (L1) of this nematode can infect a snail intermediate host (*Pomacea canaliculata*) and develop to the third-stage larvae (L3) within about 3 weeks [3]. Although the rat is the permissive definitive host, humans can accidentally get infected by eating undercooked freshwater snails containing L3 larvae [4], which penetrate the host blood-brain barrier and move into the central nervous system [5]. In the permissive host, another 3 weeks or so is required for the L3s to develop into young adults and migrate to the pulmonary

artery, where they develop sexual maturity to lay eggs [6]. In the *A. cantonensis* life cycle, 2 moults are required for L1 to become infective stage L3 in the snail intermediate host, and another 3 moults are required to become adults in the final host [3].

Studies on a free-living nematode, *Caenorhabditis elegans*, revealed that the cuticle was synthesized during late embryogenesis, then shed and re-synthesized at each larval stage underneath the existing cuticle, which is subsequently shed by a moulting process [7]. Overlying the nematode cuticle is the lipid-rich epicuticle that is then covered by the glycoprotein-rich negatively-charged surface coat [8]. This labile accessory layer is associated with immune evasion in several parasitic nematodes [9].

Although *A. cantonensis* was first described in 1935, the biology of the worm is still not clearly understood. Since the cuticle plays such an important role in nematodes, we believe that changes associated with moulting of the cuticle is vital for the parasite. The purpose of this study is to observe the cuticular

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changes in *A. cantonensis* during the moulting from L2 to L3 (L2/L3), and from L3 to L4 (L3/L4) using scanning electron microscopy (SEM).

MATERIALS AND METHODS

Parasite preparation

The L2/L3 larvae of *A. cantonensis* were collected by a modified method of Parsons and Grieve [10]. The tissues of infected snails (*Pomacea canaliculata*) were minced and digested in a pepsin-hydrochloric acid solution at 37°C for 2 hr. L2/L3 Larvae were collected under a dissecting microscope.

Five BALB/c mice aged 6 weeks were supplied by the Center of Animal Experiments of Sun Yat-sen University, Guangzhou, China. The procedures involving animals were approved by the Animal Care and Use Committee of Sun Yat-sen University. Animals were raised in a room with air conditioning under a 12/12-hr light/dark cycle.

All the mice were infected with *A. cantonensis* L3 (20 per mouse) using a stomach tube fitted with 1 ml syringe. At day 5 post-infection, all the mice were sacrificed by cervical dislocation. The brains were removed from the cranial cavity and were teased into small pieces. L3/L4 were collected under a dissecting microscope.

SEM

All the harvested larvae were washed 3 times with 0.2 M PBS (pH 7.2) and fixed in 0.2 M PBS containing 2.5% glutaraldehyde at 4°C for 24 hr prior to electron microscopic observations. Specimens were washed 3 times in pH 7.2 PBS and 6 times in cold distilled water to remove glutaraldehyde and dehydrated through an ascending series of ethanol (50-100%). Then, ethanol was replaced with acetone and isoamyl acetate, and the worms were dried in a Hitachi HCP-2 critical-point drying machine using a transitional medium of liquid carbon dioxide. The specimens containing 2 L2/L3, 5 L3, 1 L3/L4, and 5 L4 were mounted on aluminum stubs and coated with platinum in an ion-sputtering apparatus (Hitachi E-102, Tokyo, Japan), and were examined and photographed in a scanning electron microscope (Hitachi S-2500, Tokyo, Japan).

RESULTS

Surface ultrastructure of L2/L3 from the snail host

L2/L3 larvae of *A. cantonensis* were divided into late L2 and

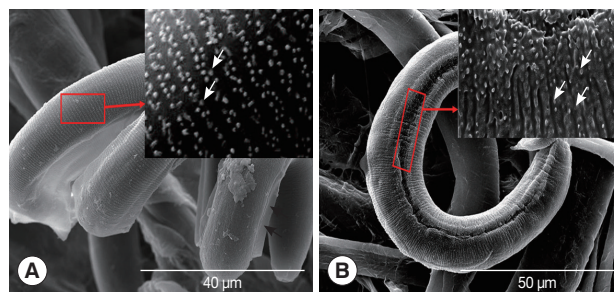


Fig. 1. SEM photographs of *Angiostrongylus cantonensis* early L3 and late L2. (A) Early L3 with alae (black arrows), a magnified view of the red frame area shows regularly arranged spherical particles (white arrows). (B) Picture of late L2 without alae, a magnified view of the red frame area shows irregularly arranged spherical particles (white arrows).

early L3 according to the time since recovery from the host snail. The SEM photograph of late L2 showed absence of alae, pull-chain-like fissure, and irregular alignment of the spherical particle structure on the cuticle of larvae comparing to L3 (Fig. 1A, B). The SEM photograph of early L3 exhibited an appearance of alae. The old cuticle turned into a thin film-like structure with regularly arranged spherical particles on it (Fig. 1A, B).

Surface ultrastructure of L3/L4 from the mouse host

L3/L4 larvae were recovered from the brains of mice 5 days after L3 infection and were first thought to be mutilated specimens under a light microscope. However, by SEM observation, regular rectangular cavities were found on the surface of worm, and the phasmod of the worm was different from either L3 or L4 stages (Fig. 2A, B). The caudal structure of the L3/L4 larvae was much larger than that of the L3 larvae, but caudal inflation was not observed (Fig. 2C, D).

DISCUSSION

Both L1 and L3 were extraordinarily active in PBS pH 7.2 at 26°C, and the surfaces of these 2 kinds of larvae were smooth. However, L2/L3 were extraordinarily inactive at the same condition, with their bodies forming a C-shape, and the surfaces of larvae were rough. After the 2 layers of cuticles were synthesized and before they were moulted together, we designated this stage as L2/L3 larvae. Ding et al. [11] reported SEM findings of various stages of this worm, and L2 was described to be difficult to distinguish the surface features whereas L3 and L4 were well described. However, the surface ultrastructure of moulting larvae was not reported.

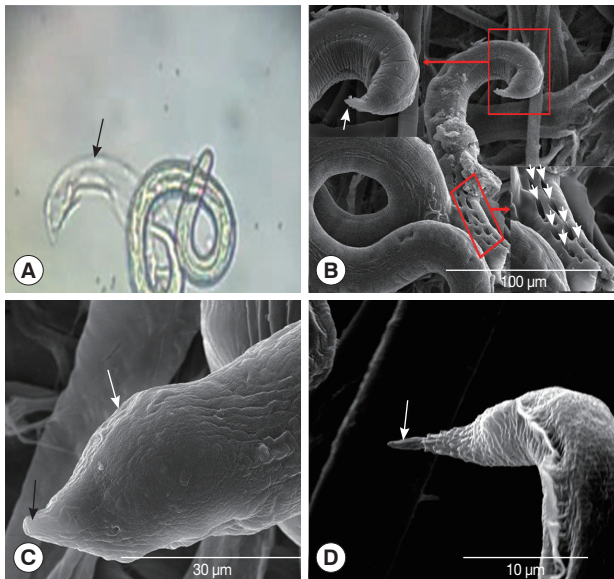


Fig. 2. SEM and light microscopic photographs of *Angiostrongylus cantonensis* L3/L4. (A) A light microscopic photo of L3/L4, the black arrow shows molted part of the worm. (B) A SEM photograph of L3/L4, regular rectangular cavities (RRC) and L3/L4 tail in red frames are shown by white arrows at a greater magnification. (C) L3/L4 tail at greater magnification. L4 tail with obtuse phasmid (black arrow) and caudal inflation (white arrow). (D) L3 tail with a sharp phasmid is shown by white arrow.

According to the SEM pictures, we could divide L2/L3 larvae into 2 types, the early L3 and the late L2. The surface of late L2 presented a pull-chain-like fissure, with an irregularly arranged spherical particles on the cuticle, and alae were absent. In contrast, the surface of early L3 larvae exhibited alae, and the old cuticle turned into a thin film-like structure with spherical particles arranged regularly on it. Japanese researchers had cultured L1 to L3 in vitro, and reported that L2/L3 under molting retained 2 cuticles formed from L1 and L2 [12].

Therefore, we propose a hypothesis about how L2/L3 moult their 2 layers of old cuticles. In late L2, the surface of the cuticle was deformed and turned into irregular spherical particles, and then the outside cuticle became a crevice-like structure. Gradually the larvae shed their outside cuticle like a jacket, thus the second layer of the cuticle inside became the outside cuticle. In early L3, the surface of the cuticle was deformed and turned into regular spherical particles which adhered to the new cuticle by a thin film-like structure. While the new cuticle was still growing, the old cuticle gradually degenerated around the helical worm body, like the pericarp shaved from an apple. The larvae recovered from the brain of mice 5 days after the infection were initially considered to be broken bodies of the

larvae under a light microscope. However, further observation by SEM showed regular cavities on the surface of the larvae, and the tail of the larva was like neither the tail of L3 described by Ho et al. [13] nor L4 described by Zeng et al. [14]. We inferred from this that regular cavities were results of moulting, and the larva was at an intermediate stage of L3 and L4.

A. cantonensis, unlike the free living nematode *C. elegans*, completes moulting processes in the host tissue, and this phenomenon makes its moulting quite difficult. In summary, our SEM observations on the moulting of *A. cantonensis* established a morphological foundation for further research in moulting mechanisms of *A. cantonensis*.

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