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HISTOPATHOLOGICAL EXAMINATION OF BIVALVE MUSSEL *HYRIOPSIS CUMINGII* LEA ARTIFICIALLY INFECTED BY VIRUS

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Abstract: The pathogen *Hyriopsis cumingii* Plague Virus isolated from the diseased *H. cumingii* Lea has been artificially transferred to the healthy *H. cumingii* Lea. The infected *H. cumingii* Lea has displayed some typical pathological changes. Observations by histopathology have showed that most of organs including the digestive gland, stomach and intestine are the primary targets of the plague virus. Lesions have been observed, accompanied with a number of empty areas, cell vacuolization and some cells falling-off. Arenavirus-like particles have been detected by using transmission electron microscopic (TEM) examination on investigated organs of the artificially infected bivalve mussel *Hyriopsis cumingii*. Virions are spherical with a size of 120 nm in diameter, and the outer layer of the intact arenavirus-like particles have small protuberance and moderate electron-dense nucleicapsid. Under electronic microscope, the most obvious cellular pathologic feature is the large vacuole areas of cytoplasm, and there is the reduction or devoid of organelles in the cytoplasm. Varying degrees of denaturalization and necrosis have been found in the intestinal mucosa epithelial cells. We have also observed that in cytopathic cells of digestive gland, the nuclei has been discovered to be amorphous and hypertrophied. There are denaturalization and necrosis in the epithelial cell of gill and the nucleus membrane of granulocytes have been dissolved.

Key words: *Hyriopsis cumingii* Lea; Arenavirus-like; Microstructure; Ultrastructure; Histopathology
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Bivalve mussel (*Hyriopsis cumingii* Lea), is a traditionally farmed bivalve mussel species in south China and plays an important role in margarita production[1]. Recently, more and more epizootic diseases have been found in farmed bivalve mussel species in China, partially because of the high density on farms and poor husbandry conditions[2]. The pathogens were thought to be virus, although some of them may have been parasites or bacteria [3—8].

The *Hyriopsis cumingii* Lea virus disease, which is often referred to as the *Hyriopsis cumingii* Lea plague disease (HCPD), was first reported in 1980s in China by agent of *Aeromonas punctata*[4], *Virus. fluzialis* IV and *H. halodurans*[9]. However, in our survey of HCPD, parasites are absent in smears and wet parts of 'plague diseased' bivalve mussel tissues, and after treatment by using antibiotics of penicillin and streptomycin, bacteria have not been consistently isolated from diseased bivalve mussel, which suggested that HCPD would be caused by viral agents. According to the former research, HCPD was caused by two kinds of viral agents: one is RNA virus, namely arenavirus which is discovered in the cytoplasm and the other is DNA virus, the herpes-like virus is mainly discovered in the nuclei[10]. Light and transmission electron microscopic (TEM) analysis of tissues from diseased bivalve mussels showed that the HCPD was associated with an arenavirus agent termed *Hyriopsis cumingii* Lea *plague Virus* (HcPV)[6,7]. The HcPV was later found to be spheral and spheral-like with different sizes[8]. All in all, we have found all these studies mainly focused on the agent caused HCPD, few histological, ultrastructural characteristics and few evidence on the plague disease of bivalve mussel *Hyriopsis cumingii* Lea infected by plague virus (HcPV) were reported.

In this report, we have presented the evidence of the existence of viral particles in HCPD-infected bivalve

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mussels. The plague virus assembly characterization, the relevant pathology, and ultra-structure changes have also been analyzed. These results can offer valuable reference to subsequent studies on the etiology, epidemiology and the diagnosis of HCPD.

1 Materials and methods

1.1 Materials

During the period of HCPD outbreak in Hunan province, China, in 2005, diseased mussel samples [(n= 20, Length of 17–18 cm, height of 16–17 cm) and control samples which were healthy (n = 30, length of 17–18 cm, height of 16–17 cm)] were collected from different bivalve mussel farms. The diseased samples had been stored in the 0.1 mol/L sterile phosphate buffer (50% glycerol, 0.1 mol/L $NaH_2PO_4{\cdot}2H_2O$ and 0.1 mol/L $Na_2HPO_4{\cdot}12H_2O$) at −80℃ for extracting virus, and the tissue samples of digestive gland, stomach, intestine, gills, mantle and foot muscle were collected from the mussels infected artificially by virus.

1.2 Preparation of viral fluid and recursive infection experiment

To isolate viral fluid, tissue samples (gill, intestinal, digestive gland, visceral mass, mantle and foot muscle) were firstly washed three times with saline, and homogenized in PBS (pH 7.4) on ice. After centrifugation, the supernatants were mixed with penicillin and streptomycin (2000–5000 U/mL). The mixtures were incubated for 16h at 4°C before bacteria check. After asepsis detection, the viral fluid was stored at −80°C for further recursive infection experiments. Twenty healthy control samples were injected with 0.2–0.3 mL viral fluid randomly and another ten negative control samples were injected with 0.2–0.3 mL sterile PBS. After recursive injection, the clinical symptoms of bivalve mussels were recorded. Some kinds of tissues from the artificially infected bivalve mussels were collected and produced the histological sections and ultrathin sections in a timely manner.

1.3 Histopathology and electron microscopy

For light microscopic histology and electron microscopic histology, small pieces (5–10 mm^3) of tissue samples from diseased *Hyriopsis cumingii* Lea were fixed in Davidson's AFA fixative (31% ethyl alcohol, 22% formalin and 11.5% glacial acetic acid) and processed for paraffin embedding. Sections were cut in 5 μm and stained with haematoxylin and eosin (H&E), followed by basic pathological observation under the light microscope (Olympus CX31-32C02 20800, Japan). For transmission electron microscopic preparation, sample pieces (1–2 mm^3) of digestive gland, stomach, intestine, gill, mantle and foot muscle were fixed in 3.0% glutaraldehyde in 0.1 mol/L cacodylate buffer (pH 7.4) for 24h at 4°C, followed by three rinses (10min each) with 0.1 mol/L PBS buffer. Specimens were post-fixed in 1% osmium tetroxide in 0.1 mol/L cacodylate buffer (pH 7.4) for 1.5h at 4°C, rinsed in the same buffer, and then dehydrated in graded ethyl alcohol and embedded in Epon 812 resin (Serva, NY, USA). Ultra-thin sections were cut by using a Reichert-Jung Ultra-cut microtome (Winterville, GA, United States) with a diamond knife, mounted on copper grids, stained with 2% uranyl acetate and lead citrate, then examined and photographed with a transmission electron microscope (JEM-1230EX JEOL, Tokyo, Japan) operating at 60 kV. Virus particles were measured using TEM.

2 Results

2.1 Histopathology

Histological examination of diseased *Hyriopsis cumingii* Lea has showed that the primary target of the plague virus are the digestive gland, stomach and intestine. Lesions, accompanied with numbers of empty areas, cell vacuolization and some cells falling-off, have been observed by histolysis in these tissues.

Observation under light microscope has showed that the main pathological changes are present in the digestive organs of the artificial infected mussels while absent in those of healthy mussels (Fig.1). In the artificial infected *Hyriopsis cumingii* Lea, gland tubule of digestive glands has become spongiosis, tubular cavity has appeared shrunken, nuclei have been crushed to the brim of cells with basophilic cytoplasm. The observation has also showed that there are many vacuoles in the digestive cell of gland tubule with some epithelia detachment, and the whole gland tubule has become erosion with cell vacuolated seriously (Fig. 1A and B). In the stomach, cilium and striated border have been discovered to be detachment from columnar cells with blurred structure, mucilage cells to be manifold and some epithelia to be detached from epithelial layer, and cells of mucous layer arranged irregularly and structure of submucosa to become sparse (Fig. 1C and D). In the intestines, connective tissue of submucosa has been found to be sparse cilium and striated border to be detached from epithelium of enteric cells, intestinal villi with blurred limits to appear shrunken, shortened and even disappeared. Both the number of epithelial cells and the thickness of the epithelial layer have been found to decrease, accompanied with nuclei arranged irregularly (Fig. 1E and F).

Besides digestive organs, we have also found that there are pathological changes in the mantles, foot muscles and gills. In control samples, connective tissue of mantles, foot muscle and gills are integrated, cells are arranged regularly with clear structure (Fig. 2 B, D, F). In the diseased *Hyriopsis cumingii* Lea, the muscular fibers of mantles are arranged irregularly with blurred structure. Connective tissue are fragmented and degenerated with

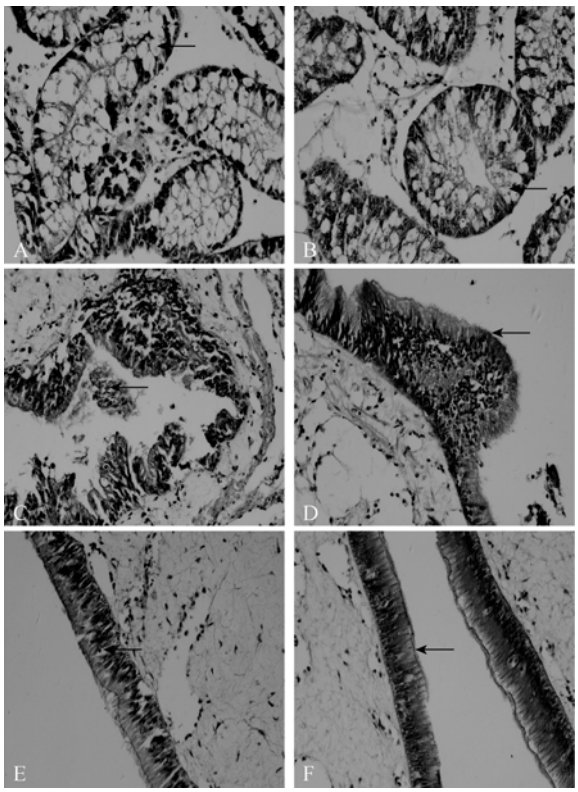

Fig. 1 Histological examination of the digestive organs from infected (A, C and E) and healthy bivalve mussel (B, D and F), *Hyriopsis cumingii* Lea. The histological sections (5–7 μm thick) were stained with haematoxylin and eosin (H&E), examined and photographed under a light microscopy (×400 magnification)

A. The digestive gland of diseased bivalve mussel. Gland tubule has become erosion with cell vacuolization (arrow); B. The digestive gland of a healthy bivalve mussel, showing normal histological structure; C. The stomach of diseased bivalve mussel. Cilia and microfloss have detached from columnar cells and mucilage cells have manifolded and some epithelia have detached from epithelial layer (arrow); D. The stomach of a healthy bivalve mussel, normal histological structure has been observed; E. The intestine of diseased bivalve mussel. Epithelium of diseased bivalve mussel. Cells have arranged irregularly and columnar cells have fragmented (arrow); F. The intestine of a healthy bivalve mussel, normal histological structure has been observed

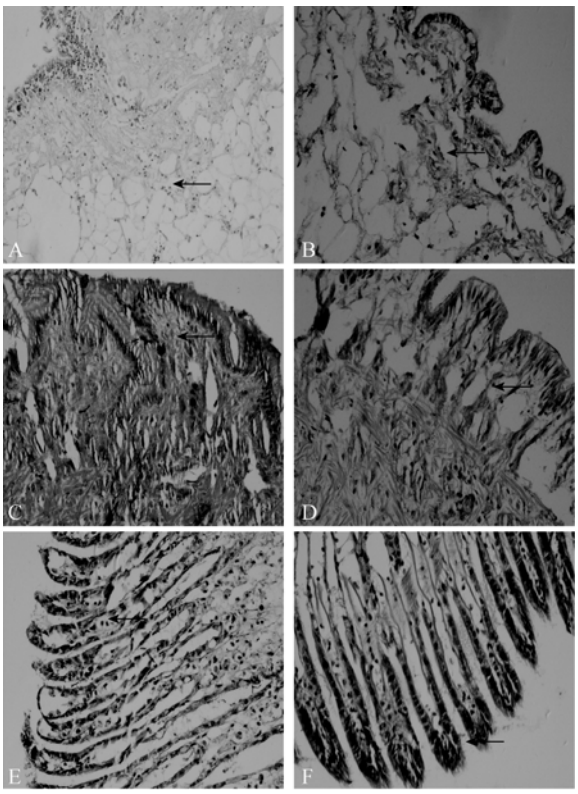

Fig. 2 Histological examination of the other organs from infected (A, C and E) and healthy bivalve mussel (B, D and F), *Hyriopsis cumingii* Lea. The histological sections (5–7 μm thick) were stained with haematoxylin and eosin (H&E), examined and photographed under a light microscopy (×400 magnification)

A. The mantle of diseased bivalve mussel. Muscle fiber arrange irregularly, connective tissue are fragmented and degenerated and numbers of vacuoles have been observed (arrow); B. The mantle of a healthy bivalve mussel (×400), showing normal histological structure; C. The muscle fiber of diseased foot muscle are fragmented with empty areas or vacuoles and the enlarged cells have become basophilic (arrow); D. The foot muscle of a healthy bivalve mussel, normal histological structure has been observed; E. The gills of diseased bivalve mussel, a number of enlarged cells have been observed in the epidermis and inter-lamellar epithelia of the filaments (arrow); F. The gills of a healthy bivalve mussel, normal histological structure has been observed

empty areas or vacuoles and basophilic particles. Some epithelia are detached from epithelial layer of mantle (Fig. 2A and B). Muscular fibers are fragmented with empty areas or vacuoles in the foot muscle, the epithelia of mucous layer are enlarged and the enlarged cells have become basophilic (Fig. 2C and D). In gills, enlarged cells have been found in the epidermis and inter-lamellar epithelia of the filaments which are arranged irregularly. Some epithelia are detached from filaments. Connective tissue are fragmented and degenerated with empty areas or vacuoles (Fig. 2E and F).

2.2 Ultrastructural Characteristics of virus and Ultra-pathology

No rickettsia, chlamydia, bacteria and other parasitic organisms have been found, observing in digestive gland, stomach, intestine, gill, mantle and foot muscle by TEM, whereas viral particles with different sizes have been found prevalently in epithelium cells of those tissues.

Typical spherical virus particles have been found in the digestive gland, stomach, midbowels, gills, mantle and foot muscle of the diseased bivalve mussels. Numerous virions aggregate around the cytoplasm of the infected cells and virus particles show a spherical aspect in the ultrathin sections. The outer layer of intact virus is small protuberance and moderate electron-dense nucleicapsid. Most of viruses are spheral, unenveloped, with diameters between 80–120 nm (Fig. 3).

The distribution of the virus in diseased bivalve mussel varies in the different tissues and cell types.

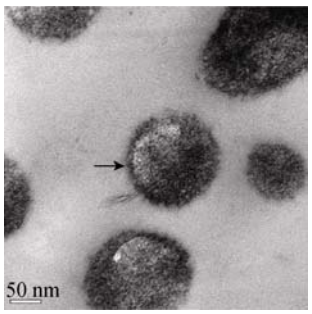

Fig. 3　Examined and photographed under a TEM (×300000, magnification), we have found that image of arenavirus–like virus in diseased visceral Mission *Hyriopsis cumingii* Lea
The outer layer of intact virus is small protuberance (arrow) and moderate electron-dense nucleicapsid. Most of virus were spheral, unenveloped, with diameters between 80– 120 nm. Scale bar=50 nm

Virions have been mostly observed in cells of digestive gland, gill, intestinal epithelium, connective tissue between muscular fibers from diseased bivalve mussel. The cytopathic cells of intestine epithelial cells are not arranged tightly, coursing large spaces between them, producing their deformity and dissolvement and resulting in an unintegral cell structure. (Fig. 4A). Particles from the visceral mission cytoplasm were different in electronic density and size (Fig. 4B). In gills, the endoplasmic reticulum are swollen and then attached to the karyotheca to form a separate cisternae between nucleus and the cytoplasm. The nuclear has been deformed with severe chromatin damage. Most of the endoplasmic reticulum have been observed to be swollen and fractured (Fig. 4C), ribosomal has been found to decrease in its number and scattered in the cytoplasm, rough endoplasmic reticulum and mitochondria have been found to be swollen, besides that, the outer membrane and cristae of the mitochondrion to be deformed and dissolved (Fig. 4D). In cytopathic cells of digestive gland, the nuclei are amorphous and hypertrophied (Fig. 4E). Virus assembly is probably processed in the cytoplasm and released into the cytoplasmic vesicle (Fig. 4F) due to the presence of numbers of virons (Fig. 4G). It has been clearly showed that the digestive gland is a major target organ of the virus, mainly showing vacuolization occurring in some cytopathic cells, where the serious dissolvement of their nuclei is also caused by the infected viruses, (Fig. 4H). In muscles, connective tissues between muscular fibers have become sparse with vacuoles, endoplasmic reticulum around the nucleus has ruptured, dissolved and scattered (Fig. 4I). Although severe cytopathologic changes have been observed, few virions have been found in the mantle and foot muscle of diseased bivalve mussel (Fig. 4J).

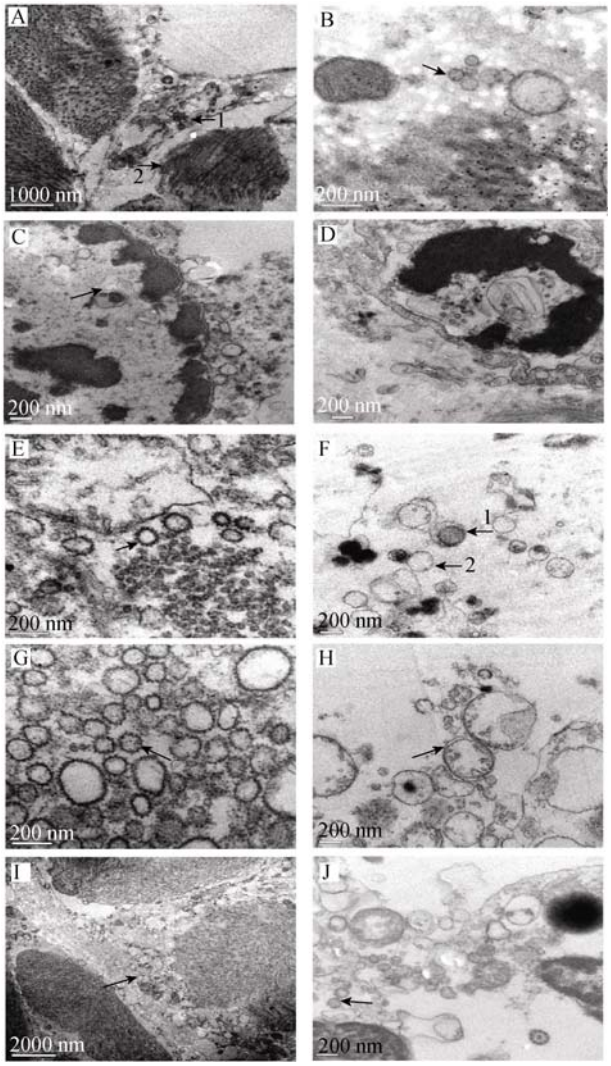

Fig. 4　Ultrastructural examination of the different tissues from infectd bivalve mussel, *Hyriopsis cumingii* Lea. The tissues sections (70–100 nm thick) were stained with uranyl acetate and lead citrate, examined and photographed under a electron microscope (×20000－×100000 magnification)
A. Cytopathic cells have been observed deformed and dissolved without intact cell structure. Connective tissue is loosen, cell dissolved (arrow 1); muscle fibers arranged neatly (arrow 2) (×20000, scale bar=1000 nm); B. There are many particles with different electronic density and sizes in visceral mission cytoplasm, and organelles are no integrity (arrow) (×100000, scale bar=200 nm); C. The nuclei are amorphous and hypertrophied, chromatin has been damaged serious, there are many vacuoles in the endoplasmic reticulum (arrow) (×50000, scale bar=200 nm); D. Endoplasmic reticulums are swolen, ribosomes have been found to decrease in its number and scattered in the cytoplasm (arrow) (×50000, Scale bar=200 nm); E. Nuclei are amorphous, hypertrophied, and some free virons have scattered in the cytoplasm (arrow) (×50000, Scale bar=200 nm); F. Virus particles have released from the cells (arrow 1), and cells highly vacuolize (arrow 2) (×50000, scale bar=200 nm); G. Numerous virons with different size have been seen in a digestive gland cell (arrow) (×100000, scale bar=200 nm); H. Cell pathological examination of the digestive gland from infected bivalve mussel, showing many vacuolization (arrow) and nuclei have dissolved, some virus particles have been released (×50000, scale bar=200 nm); I. The electron density of muscle fiber is deepen, and connective tissues between muscle fibers have become sparse with vacuoles (arrow) (×10000, scale bar=2000 nm); J. A small number of virus particles in the size of 80–100 nm in infected digestive gland cells (arrow) (×50000, scale bar=200 nm)

3　Discussion

Based on the results of microscope and TEM examination, we have found virions in HCPD-infected bi-

valve mussel organs/tissues which are spherical arenavirus -like virus with typical viral morphological characteristics. Many spherical viruses have been reported to be pathogens for mollusks [8,11—21]. These viruses belong to at least six families including iridoviridae [13], herpesviridae [17], poxviridae [19], retroviridae [18,20], reoviridae[11] and other viridae[12,21]. Lots of virions found in mollusk are spherical with[14,18,22] or without an envelope[23]. Herpesvirus replicates and assembles in the nucleus of infected cells[24]and Manila clam herpesvirus generally forms giant cellular structure of 106–126 nm in diameter in the cells[17]. The virus reported in this study assembles in the cytoplasm of infected cells and forms many vacuoles in the cells of infected mussel. We have found that the virus in the present study appear to be arenavirus-like viral particle, which is in disagreement with previous studies.

We have also discovered that organelles are enlarged or fragmented or dissolved in the digestive gland, gill, intestine, mantle and the foot muscle of the diseased mussel. The histopathological changes are similar to those in bay scallop *Argopecten irradians*, abalone *Haliotis diversicolor* Reeve, bivalve mussel *Hyriopsis cumingii* Lea, and Zhikong scallop *Chlamys farreri* infected by viruses[8,15,18,19,21,25,26]. Numerous intracytoplasmic vacuoles and the virus particles have been observed by us in the infected cells of the diseased mussel. Similar histopathological changes have been also observed in Zhikong scallop *Chlamys farreri* infected by a new spherical virus and in abalone *Haliotis diversicolor* Reeve infected by spherical virus[15,27,28]. These vacuoles were generally thought to be associated with biosynthesis of virus in the early infection phase[18]. Therefore, our finding have provided evidence that the *Hyriopsis cumingii* Lea plague is caused by a virus.

The shape, size and target tissue of HcPV reported in this study are similar to those spherical viruses detected in marine mollusk Zhikong scallop *Chlamys farreri* and abalone *Haliotis diversicolor* Reeve in culture[15,26]. However the diameter of virion (120 nm) is different from that of spherical viruses found in Zhikong scallop *Chlamys farreri* and abalone *Haliotis diversicolor* Reeve. TEM examination has showed that these viruses are composed of a nucleocapsid with small protuberance on the surface, which is similar to the virus found in abalone *Haliotis diversicolor supertexta* in culture[29]. As a primary functional unit, these small protuberances play a very important role in the viral attachment and invasion. Therefore we conclude that the virus discovered in this study is virulence because the virus can also cause the appearance of large vacuole areas of cytoplasm of infected cells and its devoid organelles.

In our experiment, the experimented samples which are the healthy mussels infected by viral fluid without other microbes or parasites, extracted from the diseased mussels, present the HCPD symptom, whereas the control samples which are injected with PBS present no symptom. So we think that the severe histopathological and cytopathological effects in various tissues/organs of infected bivalve mussels *Hyriopsis cumingii* Lea are obviously caused by the virus.

The isolated viral particles in this study will serve as essential material for further investigation of this virus such as the origin of the HCPD virus, its nucleotide characteristics, phylogenesis and route of transmission in the bibalve mussel and other hosts.

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人工感染三角帆蚌病毒性疾病的组织病理学观察

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摘要: 从自然发病的病蚌中提取出病毒, 经人工感染健康三角帆蚌后呈现出典型的病理变化。组织学观察显示消化腺、胃、肠、外套膜、斧足和鳃等均出现大量细胞空泡化、部分细胞肿大、上皮细胞排列不紧密, 结缔组织不完整, 部分细胞溶解甚至脱落等, 表明它们均为病毒损害的主要靶器官。透射电镜观察显示大量砂样病毒存在于患病三角帆蚌不同器官中。病毒粒子为直径约 120 nm 的圆球形, 表面具有明显的纤突, 无囊膜结构。被感染细胞呈现明显的病理变化, 包括细胞质大片空泡化, 细胞器相对减少或缺失, 细胞核电子密度加深, 肠黏膜上皮细胞不同程度的变性和坏死, 消化腺细胞核变形肿大, 鳃小片上皮细胞坏死脱落, 颗粒细胞的核膜溶解等。

关键词: 三角帆蚌; 砂样病毒; 显微结构; 超微结构; 组织病理学