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RESEARCH PAPER

Pathogen Isolation and Pathologic Observation on Explosive Epidemics of *Hyriopsis cumingii* Lea

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Abstract

To perform pathogenic and pathological investigations on explosive epidemics of *Hyriopsis cumingii* Lea in Hunan Province, China between 2005 and 2011. We isolated pathogenic bacteria SJ-2 from naturally infected *Hyriopsis cumingii* Lea, and the SJ-2 strain was artificial infected into the normal Hyriopsis cumingii Lea by axe foot injection method. The strain was identified according to conventional bacteriological isolation and identification and molecular biology methods, and pathological changes caused by the bacteria in diseased mussel tissues were observed. According to morphological and culture characteristics, physiological and biochemical tests and 16S rDNA molecular identification, SJ-2 strain shares 99% sequence identity with the type strain of *Aeromonas veronii (A. Veronii*). Results for antibiotic susceptibility tests showed that among 50 drugs, *A. Veronii* was highly sensitive to 15 drugs, moderately sensitive to 5 drugs, and showed drug resistance to left drugs. Macroscopical lesions, pathological changes and cytopathology were summarized. The pathogenic bacteria SJ-2 was isolated from *Hyriopsis cumingii* Lea affected by explosive epidemic disease, and was identified as *A. Veronii*. SJ-2 strain caused multi-organ lesions and function failure in corresponding organs in the *Hyriopsis cumingii* Leal, resulting in gradually decreased and disappeared normal physiological metabolism, and eventually death.

Keywords: Hyriopsis cumingii Lea, explosive epidemics, SJ-2 strain, aeromonas veronii, pathogenic bacteria, 16S rDNA, pathological changes.

Introduction

Hyriopsis cumingii Lea, commonly known as mussels and pearl mussels, is a kind of freshwater bivalve mollusks unique in China, and is widely cultured for commercial freshwater pearl production, playing a very important role in aquaculture business (B, 2015; Wang Y, 2009). There is large-scale cultivation of *Hyriopsis cumingii* Lea in Hunan, Hubei, Jiangxi, Anhui, Zhejiang, Shanghai and other provinces, particularly common in the Dongting Lake and some medium-sized lakes in Hunan Province (Pin-hong Y, 2005). With high-density, intensive development of China's freshwater pearl mussel aquaculture; currently, various diseases of pearl mussels occurred frequently and became one of the main obstacles affecting the development of pearl culture industry (QiaoLin L, 2009; ZHAO J, 2003). Since the 1980s, the explosive epidemic in *Hyriopsis cumingii* Lea occurred many times in the major production areas of freshwater pearls (Yaocai W H Z J L, 1991). Moreover, it was the most serious infectious mussel disease in the freshwater shellfish aquaculture and pearl culture in China with fast spreading speed and high mortality (Zhang G, 2005).

Bacteria, parasites and viruses have been regarded as the pathogens for the mussel diseases, many efforts aiming at preventing and controlling the explosive epidemic disease were performed on the pathogen level; and a previous study by Ni Dashu *et al*. suggested that *Aeromonas punctata subspnov hyriopsae* was one of the pathogens for the bacterial disease of *Hyriopsis cumingii* Lea (Ni D S, 1985). Qian Xuchu *et al*. isolated *Aeromonas hydrophila (A. hydrophila)* 83-2 and 83-8 strains from the digestive gland tissues of diseased mussels (Qian X C, 1984). By isolating the pathogen, Shen Zhirong concluded that "the mussel *Hyriopsis cumingii* plague" in agricultural production was a viral infectious disease, but without further research on the pathogenic viruses (Shen Z, 1986). Zhang Zhiguo *et al*. isolated the viral pathogen from diseased mussels from natural environment and the pathogen was preliminarily identified as a member of *Arenaviridae* according to the morphological studies (G, 1987). Histopathological on dynamic characteristics of

Hyriopsis cumingii plague has been evaluated by QiaoLin L et. al, including the pathological changes of mantle, gill and axe foot after artificial infection with the pathological tissue liquid (QiaoLin L, 2009). Lei Z and Baohong X *et al*. isolated the pathogen and performed preservation experiments and proposed that the pathogen belonged to *Arenaviridae*. In this study the pathogen of the explosive epidemic in *Hyriopsis cumingii* Lea in Hunan Province was isolated and identified, and a systematic pathological study on the naturally infected cases and artificial infected mussels was carried out to provide a theoretical basis for the further study of the disease.

Materials and Methods

Experimental Animals

Implanted mussels, aging two-year-old, were obtained from a commercial *Hyriopsis cumingii* Lea farm in Changde City, Hunan Province. There were 20 diseased mussels with obvious disease and dying status, and some healthy mussels with tightly closed shells, rapidly responding axe-shaped feet, strongly spraying water nozzle and stout and strong physique. The morphometric results of the mussels were as follows: the mean shell length was 75.0 ± 0.5 mm, shell height was 65.0 ± 0.3 mm and shell width was 15.0 ± 0.5 mm. Strains were isolated from diseased *Hyriopsis cumingii* Lea, and purified strains were frozen at -80℃in the Laboratory of Hydrobiology, Hunan Agricultural University. All animal experimental processes were performed in compliance with the regulations of the Animal Ethics Committee of College of Animal Science and Technology, Hunan Agricultural University.

External Symptoms and Anatomical Observation of Diseased Mussels

The external symptoms and mucus secretion of a diseased mussel were observed. The shells were opened with openers and scalpels and the pathological changes on the macroscopic level in the gill, mantle, axe-shaped feet, outer membrane of visceral sac, heart, kidney and adductor muscle of the diseased mussel were observed. The visceral sac was split, and the pathological changes in the digestive gland, gonad and gastrointestinal tract of the diseased mussel were observed. The body of additional diseased mussel was sterilized with alcohol swabs, the lesion was scraped with a sterile scalpel blade, and parts of gill filaments, mantles, axe-shaped feet, adductor muscle, heart, kidney, digestive gland, gonad and gastrointestinal tract were obtained for wet preparation and tissue section.

Isolation and Culture of Pathogenic Bacteria

The dying mussels with typical symptoms among the diseased *Hyriopsis cumingii* Lea were collected and tissues draw from the parts of gill filaments, mantles, axe-shaped feet, adductor muscles, hearts, kidneys, digestive glands, gonads and gastrointestinal tracts were put into centrifuge tubes under sterile condition. After being rinsed with sterile water, the tissues were cut into pieces aseptically, inoculated into tryptic soy broth (TSB) medium (BD Difco, Detroit, MI, USA) and incubated at 28 °C. Within 12-24 h, the color and characteristics of colonies were observed and individual dominant colonies were selected, purified and incubated. After twice purification, the colonies were incubated in tryptic Soy Agar (TSA) and thiosulfate Citrate Bile Salt Sucrose Agar (TCBS) medium (TIANGEN Co, Ltd, Beijing, China), respectively, to observe whether they were vibriones. The colonies were stored in a refrigerator at 4 °C for further tests. The bacteria were inoculated on tube slant and stored in a refrigerator at -80 °C with the addition of sterile glycerol (Qian W P *et al*, 2001).

Experimental Infection of SJ-2 Strains

Healthy mussels were cultured in aquariums for a week (50 per group). Each aquarium was equipped with a thermostat, a thermometer, an oxygenation machine. The criteria of healthy mussels were as follows: 1) tightly closed shells; 2) sprayed water powerfully; 3) white color in body; and 4) stout and strong physique. The experiment was carried out with the confirmed disease-free mussels. The isolated and purified strains were inoculated on a fresh agar medium individually. The lethal median dose (LD50) of *Hyriopsis cumingii* Lea was tested before the experimental infection of SJ-2 strains, and the concentration with 5×10^7 CFU/ml of SJ-2 strains was finally used in the experimental infection in healthy mussels. The axe-shaped feet of the healthy mussels were injected with 5×10^7 CFU/ml strains, with 0.5ml in each mussel. Healthy mussels injected with sterile saline (0.5ml in each mussel) were used as blank control group. After artificial infection, the mussels were continuously observed for several days with a water temperature maintained at 28 °C and continuous oxygen supply.

Histopathological and Morphological Observation

Paraffin-embedded tissue sections were used for histopathological observation. Tissues were fixed with Davidson's AFA (acetic acid, formaldehyde, alcohol) fixative, embedded in paraffin, sectioned and stained with hematoxylin and eosin (HE) according to a previous study (Baohong X, 2011). Microscopic observation and photograph were performed using an optical microscope (Olympus AX70, Japan). For the ultrastructural cytopathology and morphological observation of the bacteria, tissue samples for transmission electron microscope (TEM) were

prepared. Samples were fixed with glutaraldehyde and osmium tetroxide, and cut into ultrathin sections and doubly stained with lead acetate and lead citrate. The colonies were fixed with glutaraldehyde and negatively stained with phosphotungstic acid, and then observed and photographed under the TEM.

16S rDNA Gene Sequence Analysis of The Pathogenic Bacteria

Bacterial DNA was extracted with TIANGEN Bacterial genomic DNA extraction kit (TIANGEN Co, Ltd, Beijing, China) according to the instructions. Primers were synthesized by Shanghai Sangon Biotechnology Co. Ltd: forward primer, 27F: (5'-AGAGTTTGATCCTGGCTCAG-3') and reverse primer, 1492R: (5'-TACGGCTACCTTGTTACGACTT-3') (Mincer T J *et al*, 2002). Polymerase chain reaction (PCR) was performed by the reaction system with 25 μL (2×PCR Master Mix). The PCR products were separated with 0.8-1.0% agarose gel electrophoresis and were detected under the gel imaging system. The purification and extraction of the target gene were carried out according to the manuals of DNA extraction kit (TIANGEN Co, Ltd, Beijing, China). The selected clones were sequenced by Invitrogen (Shanghai, China) and the sequencing results were analyzed with DNAMAN software (Lynnon BioSoft, Vaudreuil, Canada). A phylogenetic tree containing the isolated strains was generated by the neighbor-joining method with MEGA 4.0 software (DNASTAR, USA).

Biochemical Measurement of The Pathogenic Bacteria

The physiological and biochemical indices of the purified stains were measured with API-20E identification system (BioMérieux, France), which was performed by the Laboratory Department of Xiangya No.2 Hospital, Central South University. The strain identification tube was used to further detect the identification characteristics of strains. The physiological and biochemical experiments of bacteria were conducted in accordance with the methods in *Bergey's Manual of Determinative Bacteriology* and the *Manual of Determinative Bacteriology*.

Susceptibility Tests of Pathogenic Bacteria

The isolated SJ-2 strains from diseased *Hyriopsis cumingii* Lea adjusted with sterile water into 0.5 McFarland standard bacterial suspensions ($1.0\sim1.5 \times 10^8$CFU/ml). 0.1ml bacterial suspensions was placed in the center of sterile plate and spread evenly with spreading rod. The antimicrobial susceptibility disks were well pasted into the plate of bacterial suspension. Each plate contains 5 to 6 disks with the interval no less than 24mm, and the interval between the paper center and the edge of plate was no less than 15mm. The plate with antimicrobial susceptibility disks were cultured in constant-temperature incubator at 28℃ for 24 hrs, and then measured the diameter of each inhibition zone. According to the size of the inhibition zone, susceptibility of bacteria was determined with the combination of the *Standard of antimicrobial range interpretation of susceptibility test (Kirby-Bauer Method).*

Results

Observations of External Symptoms and Pathological Anatomy

The mussels with the explosive epidemic disease showed acute onset, a shorter onset period, and frequent acute deaths. The artificially infected mussels showed symptoms within 48 h after being infected with the isolated strains, and presented with deaths within 72 h. The total mortality was 46-68% after continuous observation for 25 days. The symptoms of the artificially infected mussels were consistent with those of naturally infected ones. At the early stage of the disease, lots of mucus was excreted from the body of the diseased mussels. The spraying of water nozzle in the back shell of the diseased mussels was weak and diseased mussels showed decreased defecation. Slightly opened shells and exposed mantle edges were presented. With disease progress, the adductor muscles lost functions, the shells were no longer closed. Large amount of mucus was excreted and the mussel bodies were emaciated. The necropsy of the dying mussels showed that: the color of the gills turned from faint yellow to dark yellow, the outer edges of the gills became black and the epithelial tissues of the gills shed or even rotted. The edge membranes of the mantles showed dark brown and rotted. The axe-shaped feet were swelled with bubble-like protrusions and reddish, which couldn't retract from the outside of the shells or even rotted in severe cases. In the severe cases, the mussel bodies were emaciated, visceral sac sizes became smaller, and the adductor muscles became loose and failed to function. The visceral sac was dissected and we observed that there was no food in the gastrointestinal tracts, the color of the livers turned from dark green into gray and the color of gonads turned from faint yellow to milky white (Figure 1).

Morphological Characteristics of The Pathogenic Bacteria

The SJ-2 bacterial colonies on ordinary nutrient agar plates were circular, translucent milky white and slightly elevated, and had neat edges, moist and smooth surface. The bacterial colonies on TSA plates were circular, transparent milky white to greyish

white with diameters of 1.5-3.5 mm, and had smooth, moist surface and neat edges, which were larger than those on ordinary nutrient agar plates. The strains were Gram-negative on staining, shaped in short rod and single or paired in arrangement (Figure 2A). Under electron microscope, the cell size of bacteria was 0.5-0.6 μm×1.0-1.6 μm (Figure 2B). After flagella staining, a single polar flagellum could be observed under light microscope and transmission electron microscope. The smear examination and stab culture in semi-solid medium showed that the bacteria had motility ability (Figure 2C).

Physiological and Biochemical Characteristics of The Pathogenic Bacteria

The growth of the SJ-2 strains was observed in a salinity range of 0-9%, better growth in a salinity of 1-4%, and growth failure in a salinity of 10%. The optimum growth salinity for the strains was 3%. The pH value for strain growth is 5.0-9.0, and the optimum pH value is 7.0. The temperature-growth experiment illustrated that the strains could grow at 4-40 °C, the strain couldn't grow at the temperature below 4 °C or above 45 °C, and the optimum temperature is 28-30 °C. According to the results from the biochemical identification system and artificial biochemical test, the biochemical characteristics of the SJ-2 strains were shown in Table 1. The SJ-2 strain could form acid by the use of GLU, SUC, MAN, FRU and other saccharides except SOR, RAF, RHA, ARA, LAC, XYL, MAL, ADO and PEN. The decomposition tests of H_2S, LYS (lysine), ORN, MALO, ESC, SAL, INO, TAR, ACE, CET and DEC were negative and the decomposition tests of IND, ARG, VP, CIT, ONPG, OF/G, NIT, OXI, CAT, LEC, DNase, LIP, PRO and MR were positive. The strain could liquefy gelatin. The automatic determination results on the biochemical analyzer showed that the similarities between SJ-2 strain and type strain of the *Aeromonas veronii (A. Veronii)* were 99%. There was only one difference in the LYS decomposition test

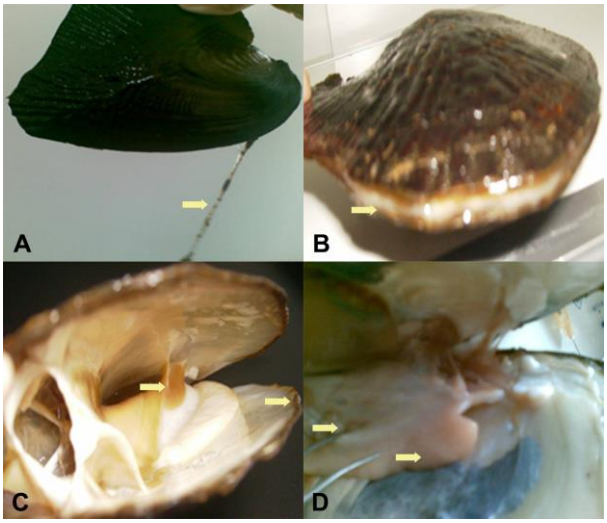

Figure 1. The main symptoms of diseased Hyriopsis cumingii Lea. A. Mucus outflowed; B. Shells opened slightly; C. The gills and the edges of mantles darken; D. The digestive glands turned yellow, axe-shaped feet swollen and redden.

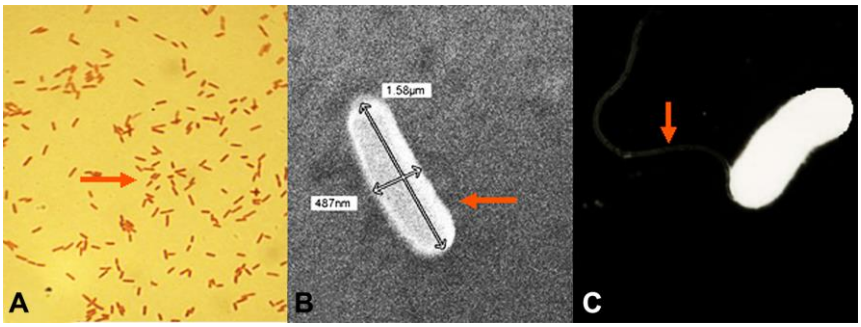

Figure 2. The morphological characteristics of SJ-2 strains and colonies. (A) Gram staining results observed under optical microscope (arrow indicates the bacterial cell, 1000 ×); (B) Observation under transmission electron microscopy (arrow indicates the the cell size, 18000 ×); (C) Observation under transmission electron microscopy (arrow indicates the flagellum, 18000 ×).

between THE SJ-2 strain and *A. Veronii*. So the identification of SJ-2 strain should be decided in combination with the determination results of 16S rDNA.

16S rDNA Gene Sequence Analysis and Phylogenetic Tree Construction of SJ-2 Strain

The DNA bands detected by agarose gel electrophoresis were clear and bright, indicating that the obtained DNA was of high quality. The target product of PCR amplification was about 1.5 kb, which was consistent with the molecular weight of the expected fragment amplified with the designed primers. The band of the recovery product in agarose gel electrophoresis was single and relatively bright (Figure 3A). The sequencing results showed that, without the primer binding region, the amplified 16S rDNA gene sequence from SJ-2 strain was 1453 bp (GenBank accession No.: GU294303). The homology of 16S rDNA sequences of SJ-2 strain and other registered strains in GenBank was aligned. A phylogenetic tree was constructed with a multiple sequence alignment (MSA) using ClustalW, and neighbor-joining by Bootstrap Test of Phylogeny using MEGA 4.0 (Figure 3B). The results showed that the SJ-2 strain was similar to genus *Aeromonas* in sequence clustering. In the alignment, SJ-2 strain was classified into the same cluster with *A. Veronii* (E65) and the phylogenetic relationship between them was the closest with a similarity of 99%.

Susceptibility Tests Result

As summarized in Table 2, among the selected drugs (n = 50), *A. Veronii* was highly sensitive to piperacillin, fortum, ceftriaxone, cefotaxime, ciprofloracin, lomefloxacin, levofloxacin, ofloxacin,

Table 1. Characteristics of biochemistry reaction of SJ-2 strains

Test item	SJ-2 stains	*A. veronii*
Glucose decomposition test (GLU)	+	+
Sucrose decomposition test (SUC)	+	+
Sorbitol decomposition test (SOR)	-	-
Raffinose decomposition test (RAF)	-	-
Amyl alcohol sugars rhamnose decomposition test (RHA)	-	-
Arabinose decomposition test (ARA)	-	-
Mannitol decomposition test (MAN)	+	+
Lactose decomposition test (LAC)	-	-
Xylose decomposition test (XYL)	-	-
Fructose decomposition test (FRU)	+	+
Maltose decomposition test (MAL)	+	+
Adonitol decomposition test (ADO)	-	-
Pentanediol decomposition test (PEN)	-	-
Urea/ urease decomposition test (URE)	-	-
Hydrogen sulfide test (H_2S)	-	-
Indole test (IND)	+	+
Lysine decomposition test (LYS)	-	+
Arginine decomposition test (ARG)	+	+
Ornithine decomposition test (ORN)	-	-
Malonic acid decomposition test (MALO)	-	-
Esculin hydrolysis test (ESC)	-	-
Salicin decomposition test (SAL)	-	-
VP glucose decomposition test (VP)	+	+
Citrate utilization test (CIT)	+	+
Inositol decomposition test (INO)	-	-
β-D-galactopyranoside (ONPG)	+	+
Tartrate utilization test (TAR)	-	-
Acetamide utilization test (ACE)	-	-
Bromide cetyl trimethyl amine test (CET)	-	-
Glucose oxidation-fermentation test (OF/G)	+	+
alkaline Decarboxylase test (DEC)	-	-
Nitrate reduction test (NIT)	+	+
Oxidase test (OXI)	+	+
Catalase test (CAT)	+	+
Lecithinase test (LEC)	+	+
DNase test (DNase)	+	+
Lipase test (LIP)	+	+
Protease test (PRO)	+	+
Gelatin liquefaction test (GEL)	+	+
Methyl red test (MR)	+	+

Notes: "+" positive; "-" negative

amikacin, norfloxacin, monobactams, ceftazidime/clavulanic acid, gatifloxacin, cefepime and tobramycin; moderately sensitive to streptomycin, polymyxin B, chloramphenicol, clarithromycin and azithromycin; and showed drug resistance to the left drugs.

HE Staining Results of Paraffin Tissue Sections

The *Hyriopsis cumingii* Lea was artificially infected with pathogenic *A. Veronii* and the pathological changes shared similar symptoms with naturally infected mussels mainly manifested in the digestive glands, gills, gonads (ovaries and testes), kidneys and intestines (Figure 4). Digestive gland cells infected with SJ-2 strains showed increased particles, swollen digestive tubules and smaller lumens; while digestive gland cells and capillary epithelial cells were separated, nuclei were dissolved, swollen, degenerated or even necrosed, more vacuoles appeared in cytoplasm and reticular cell membranes were observed (Figure 4A-B). The cells of the gill lamellae of the diseased mussels arranged loosely and the basal parts were dissolved, and multiple eosinophils overflowed in the middle of gill lamellae (Figure 4C-D). In diseased testes, spermatogenic acini were ruptured, there was no obvious boundary between them and the spermatogonia at all levels were distributed chaotically (Figure 4E-F). The ovarian follicular membranes were dissolved or disappeared, follicular cells were disintegrated and a large number of eosinophilic granules emerged. Oogonia disintegrated partially or even fell off and the cells had serious vacuolar degeneration (Figure 4G-H). In the diseased kidneys, the epithelial cells of urinary tubules were swollen, turned from cuboidal into columnar, and had slight vacuolar degeneration and cytoplasmic granular degeneration with unclear cell boundaries (Figure 4I-J). In the diseased intestinal tracts, irregularly arranged mucosal epithelial cells, larger cell gaps, condensed and deeply stained nuclei were observed, the cell membranae propria had edema calidum and the entire mucosal layer became thinner. Submucous connective tissues arranged loosely, in which basophils increased (Figure 4K-L).

Observation and Analysis of The Ultrastructures of Diseased Cells

Observation and comparison of ultrathin tissue sections of mantles, gills, kidneys, hearts and visceral sacs of healthy and diseased *Hyriopsis cumingii* Lea found that the manifestation in the infected tissues and organs were basically the same as those seen under the light microscope, especially, abundant in the digestive glands, gonads, gills and kidneys (Figure 5A-C). The diseased *Hyriopsis cumingii* Lea showed swollen cells, blurred or even ruptured cell membrane structure, and some cells aggregated to form syncytia structure with unclear boundaries. Cell nuclei were presented in irregular shape, and some nuclei significantly condensed to form meniscus-shaped or gradually dissolved with nuclear fragmentation. The nuclear membranes were incomplete. Most of the nucleoli were lost. Nuclear chromatin highly agglutinated and marginalized. The nuclear chromatin in the central part of the nuclei was extremely deficient, the electron density decreased, and many gaps are formed in the nuclei (Figure 5D-E). The electron density of cytoplasm decreased and cell organelles distributed sparsely. The outer membranes of mitochondria became swollen or dissolved and disappeared. In many cells, mitochondria also contained filamentous, irregular paracrystalline structures (Figure 5F-G). In the diseased cells, smooth endoplasmic reticula (SER) often were dilated and ruptured, while the rough endoplasmic reticula (RER) were expanded and presented with small tubuloalveolar proliferation. The ribosomes largely or entirely fell off and presented with dissolved state.

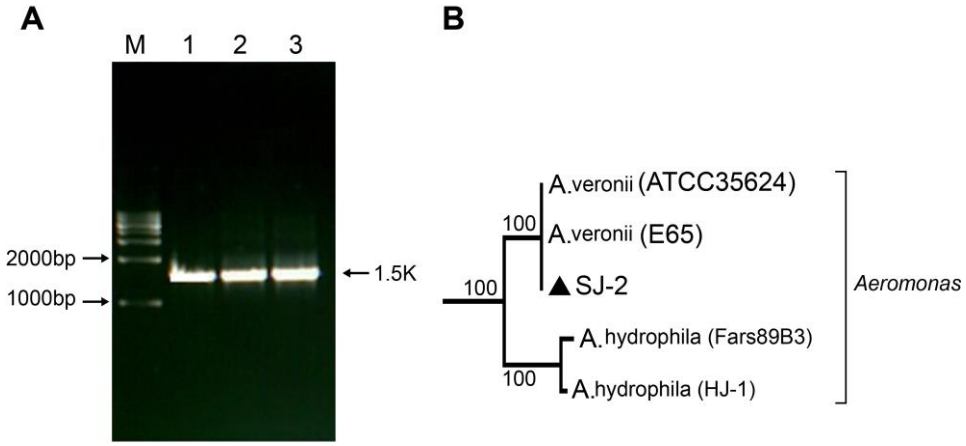

Figure 3. (A) Agarose gel electrophoresis of the recovery product: Lane M: 1 kb DNA ladder marker; Lane 1, 2, 3: the recovery products of amplified 16S rDNA of the SJ-2 strain; (B) Phylogenetic tree of SJ-2 strain based on 16S rDNA sequencing analysis: the figures over the branches indicated the boostrap values expressed as percentages of 100 replications.

Table 2. Susceptibility test results

		Aeromonas veronii	
Drugs	Pharmic content (ug/tablet)	Antibacterial ring diameter (mm)	Sensitivity
penicillin	6	0	R
oxacillin	1	0	R
ampicillin	10	0	R
V*cefazolin*	30	0	R
cefuroxime	30	0	R
ceftriaxone	30	0	R
gentamicin	10	12	R
kanamycin	30	0	R
tetracycline	30	10	R
erythromycin	15	0	R
jiemycin	2	0	R
vancomycin	30	0	R
1.25/23 *compound sulfamethoxazole*	75	0	R
amoxicillin/clavulanic acid	20/10	0	R
rifampicin	5	0	R
roxithromycin	15	0	R
trimethoprim	5	0	R
clindamycin	2	0	R
phosphonomycin	200	0	R
deoxytetracycline	30	10	R
cefotaxime/clavulanic acid	30/10	0	R
minocycline	30	0	R
cefoxitin	30	0	R
foroxone	300	0	R
furacilin	300	8	R
furadantin	300	0	R
ciprofloxacin	5	8	R
alcohol	75%	0	R
iodine	0.50%	8	R
carbolic acid	5%	0	R
streptomycin	10	11	I
polymyxin B	180	10	I
chloramphenicol	30	16	I
clarithromycin	15	16	I
azithromycin	15	16	I
piperacillin	100	32	S
fortum	30	24	S
ceftriaxone	75	28	S
cefotaxime	30	24	S
ciprofloracin	5	40	S
lomefloxacin	10	32	S
levofloxacin	5	26	S
ofloxacin	5	22	S
amikacin	30	18	S
norfloxacin	10	32	S
monobactams	30	24	S
ceftazidime/clavulanic acid	30/10	22	S
gatifloxacin	50	26	S
cefepime	30	22	S
tobramycin	10	18	S

Notes: "R" drug resistance; "I" moderately sensitive; "S" highly sensitive.

The whole structure of the endoplasmic reticula was blurred or even disintegrated (Figure 5H-I). Under electron microscope, the lysosomes at different levels and cellular debris in different digestion process could also be found (Figure 5J-K). The shape of diseased peroxisomes changed from round vesicle-like to irregular, the unit membranes were unclear and the electron density of the central core decreased significantly (Figure 5L-M). Golgi complexes expanded or even broke (Figure 5N-O). In the centrsome, the DIAD structure of centrioles became blurred (Figure 5P-Q). Myofibrilla lost their original fibrous structure, but was converged with a homogeneous flocculent distribution, the cross-band structure was blurred or disappeared and the nuclei in muscle cells were no longer close to the sarcolemmas

in distribution (Figure 5R-S). The microvilli on the free surface of normal epithelial cells were slender and tubular in shape, and compact and even in arrangement. When the epithelial cells were subjected to the infection, the microvilli showed significant pathological changes, including irregular expansion; fracture and dissolution, causing sharply decreased number of microvilli (Figure 5T-U).

Discussion

In Asia, disease caused by *A. hydrophila* is most widespread bacterial diseases in freshwater fish, and *A. hydrophila* infection may result in increased mortality in fishery products (Giri *et al.*, 2015; Ramesh *et al.*, 2015). In this study, a pathogenetic bacterial strain SJ-2 was isolated from the diseased mussel and the strain was basically consistent with the genus *Aeromonas* further confirmed by biological characteristics (Canals *et al.*, 2006; Hussain *et al.*, 2014). Specifically, we found that the morphological characteristics of the SJ-2 strain were gram-negative; physiological and biochemical tests revealed that the SJ-2 strain was 99% similar to *A. Veronii*; and only differences in LYS were found between SJ-2 strain and the type strain of *A. Veronii*. Based on 16S rRNA gene analysis, the 16S rDNA of SJ-2 strain was similar to *A. Veronii* with more than 100 sequences, and the similarities between them were 99%. Furthermore, the phylogenetic tree showed that SJ-2 strain was similar to *A. Veronii* in sequence clustering. The antibiotic susceptibility test suggested that among 50 antibiotics, *A. Veronii* was highly sensitive to piperacillin, fortum, etc.; moderately sensitive to streptomycin, polymyxin B, etc.; and showed drug resistance to the left drugs. Partially consistent with our findings, a pathogenetic bacterial strain X-1-06909 was isolated from naturally infected Siberian sturgeon in Beijing, and the isolated strain X-1-06909 was identified as *A. Veronii*, the strain were gram-negative and more sensitive to cefadroxil, neomycin etc. (Ma *et al.*, 2009). Zhang F *et al* isolated a dominant bacterial strain, 090212, from the diseased Miichthys miiuy, and the isolate 090212, Gram negative, was the pathogenic bacterium proved by artificial infection test, with the highest similarity to Vibrio harveyi (99% identity). The strain 090212 was sensitive to drugs such as Florfenicol and Tetracycline (Zhang *et al.*, 2010).

Aeromonas is a ubiquitous gram-negative bacterium that persists in the environment, and *A. Veronii* is the most common species distribution in *Aeromonas* isolates from diseased fish, healthy controls and water environment in China, but no significant difference exists in the isolates obtained from diseased fish and from healthy fish (Hu *et al.*, 2012; Rahman *et al.*, 2007). Pathogenic strains of *A. Veronii* resistant to multiple antibiotics were isolated from oscar Astronotus ocellatus showing signs of infectious abdominal dropsy (Sreedharan *et al.*, 2011). In this study SJ-2 strain was identified as *A. Veronii* according to the identification, phylogenetic analysis of 16S rDNA and the physiological, biochemical and morphological characteristics of SJ-2 strain. *A. Veronii* was not only a major pathogenic bacterium for human but also could cause diseases in a variety of aquatic animals such as freshwater fish, leech, Nile tilapia, Gilthead Sea Bream, bithyniidae mollusks, farmed rainbow trout, etc. (Abu-Elala *et al.*, 2015; Gashgari and Selim, 2015; Ku and Yu, 2015; Maltz *et al.*, 2015; Zhou *et al.*, 2013). Up to now, there was no report concerning *Hyriopsis cumingii* Lea were infected with *A. Veronii*. In this research, we isolated this bacterium from the bodies of *Hyriopsis cumingii* Lea affected with explosive epidemic. Experiments of artificial infection found that this bacterium was of high pathogenicity to healthy *Hyriopsis cumingii* Lea and that the symptoms and pathological

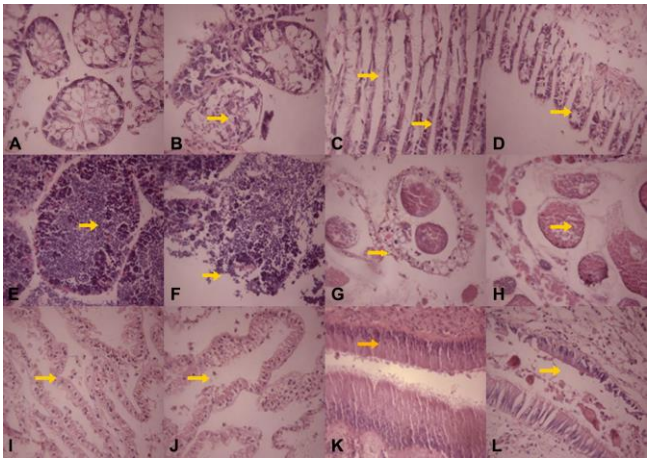

Figure 4. Paraffin tissue sections of dying Hyriopsis cumingii Lea by artificial infection (HE staining, 10 × 40 folds). (A) Digestive gland (healthy); (B) Digestive gland (SJ-2 infected); (C) Gill (healthy); (D) Gill (SJ-2 infected); (E) Testicle (healthy); (F) Testicle (SJ-2 infected); (G) Ovary (healthy); (H) Ovary (SJ-2 infected); (I) Kidney (healthy); (J) Kidney (SJ-2 infected); (K) Intestine (healthy); (L) Intestine (SJ-2 infected).

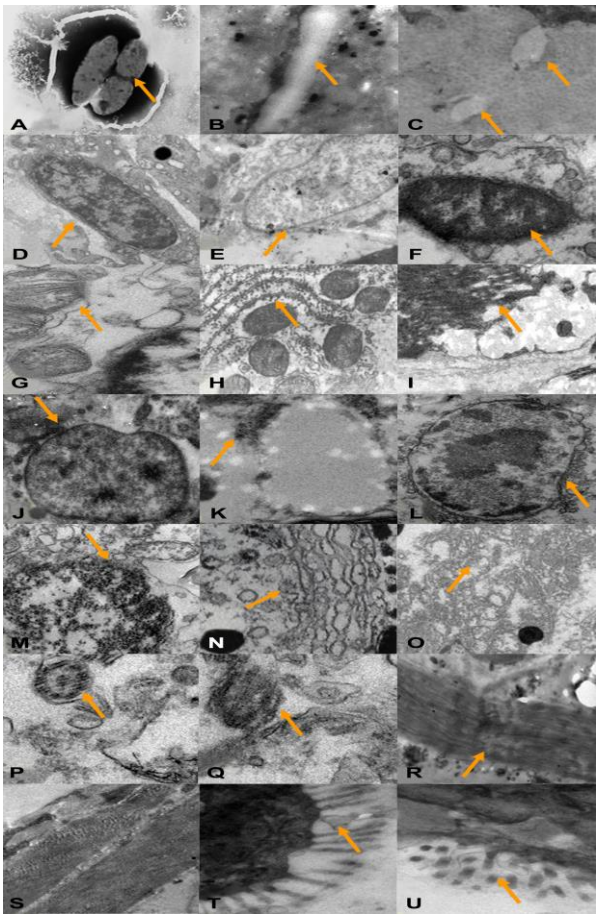

Figure 5. Ultrathin tissue sections of healthy and artificially infected *Hyriopsis cumingii* Lea.
(A) Pathogenic bacteria in the digestive glands of diseased *Hyriopsis cumingii* Lea (18,000 ×); (B) Pathogenic bacteria in the gonads of diseased *Hyriopsis cumingii* Lea (15,000 ×); (C) Pathogenic bacteria in the axe-shaped feet of diseased *Hyriopsis cumingii* (15,000×); (D) The nuclei of the intestinal epithelial cells of healthy *Hyriopsis cumingii* Lea (10,000 ×); (E) The nuclei of the intestinal epithelial cells of diseased *Hyriopsis cumingii* Lea (10,000 ×); (F) The mitochondria of healthy *Hyriopsis cumingii* Lea (50,000 ×); (G) The mitochondria of diseased *Hyriopsis cumingii* Lea (50,000 ×); (H) The endoplasmic reticula of healthy *Hyriopsis cumingii* Lea (50,000 ×); (I) The endoplasmic reticula of diseased *Hyriopsis cumingii* Lea (50,000 ×); (J) The lysosomes of healthy *Hyriopsis cumingii* Lea (60,000 ×); (K) The lysosomes of diseased *Hyriopsis cumingii* Lea (60,000 ×); (L) The peroxisomes of healthy *Hyriopsis cumingii* Lea (50,000 ×); (M) The peroxisomes of diseased *Hyriopsis cumingii* Lea (50,000 ×); (N) The Golgi complexes of healthy *Hyriopsis cumingii* Lea (50,000 ×); (O) The Golgi complexes of diseased *Hyriopsis cumingii* Lea (50,000 ×); (P) The myofibers of healthy *Hyriopsis cumingii* Lea (15,000 ×); (Q) The centrosomes of healthy *Hyriopsis cumingii* Lea (100,000 ×); (R) The centrosomes of diseased *Hyriopsis cumingii* Lea (100,000×); (S) The myofiber of diseased *Hyriopsis cumingii* Lea (15,000 ×); (T) The microvilli of healthy *Hyriopsis cumingii* Lea (50,000 ×); (U) The microvilli of diseased *Hyriopsis cumingii* Lea (50,000 ×); scale bar = 2000 nm.

characteristics of artificially infected mussels were similar to those of naturally infected ones. Thus, SJ-2 strain was suggested to be the main pathogenic bacterium for the explosive epidemic of *Hyriopsis cumingii* Lea.

Tissues of diseased mussels were observed under optical microscopes and electron microscopes, pathological changes in different degrees occurred in the organs, tissues and cells in the gills, kidneys, digestive glands, gonads and gastrointestinal tracts of the diseased mussels, among which gills, kidneys and digestive glands were affected most seriously. In line with our observations, it has been documented that the pathogens in shellfish were bio-accumulated in the gills and digestive glands, including stomach and digestive diverticula, which are good candidate sites for detection of pathogens (Wang and Shi, 2011). Gills were the respiratory organs of *Hyriopsis cumingii* Lea and gill lamellae were the basic structural and functional units of gills. The pathological changes of mussel gills would cause damages to the normal respiratory function, which would lead to respiratory failure of mussels (Gosling E, 2008). In this study, the gill lamellae of diseased mussels often became swollen or even fell off and the ultrastructure observation of the gill lamellae revealed that the respiratory epithelial cells ruptured and the organelles dissolved. Previous results highlighted the importance of the gills, not only as a point of entry of pathogens but also as a tissue capable of mounting an

immune response in rainbow trout Oncorhynchus mykiss after challenge with the Gram-positive pathogen Renibacterium salmoninarum (Campos-Perez *et al.*, 2000). However, significant pathological changes were not observed in the gill tissues regarding the pathological investigations on the tissues and organs of *Hyriopsis cumingii* Lea affected by the explosive epidemic caused by virus (Jianzhong S, 1993). A large number of pathogenic bacteria in the kidneys of the diseased mussels were observed by HE staining, the pathological changes of kidney tissue sections were mainly presented with the swelling of urinary tubule epithelial cells accompanied by a slight vacuolar degeneration and cytoplasmic granular degeneration. Proliferative kidney disease is a parasitic infection of salmonid fishes caused by Tetracapsuloides bryosalmonae, and the main target organ of the parasite in the fish is the kidney; an enhanced severity of renal pathology was also observed (Bettge *et al.*, 2009). The pathological changes of the urinary tubules caused disordered or destroyed osmoregulatory function of the diseased mussels, which could lead to dehydration of the mussels and excretory function of the body being affected. However, renal pathological changes were not reported in a related study (TiaoYi X, 2009). Lesions in a certain degree were found in the digestive glands of the diseased mussels, which manifested as particles in the cells of digestive glands increased and digestive tubules became swollen at early stage, while the cells of digestive glands and vascular epithelial cells separated and the nuclei dissolved at the late stage. Because the metabolism all required the regulation of digestive glands, disorder of digestive glands would affected the metabolism of organisms and detoxification function would decline or lose, which would result in that the inability for discharging toxic substances. In addition, the phagocytes in digestive glands couldn't clear up bacteria in time. Further, bacteria multiplied and the health of mussels was affected, and more researches on the digestive glands of diseased mussels should be performed.

In conclusion, the pathogenic bacteria SJ-2 was isolated from the digestive gland and the gastrointestinal tract of *Hyriopsis cumingii* Lea affected by the explosive epidemic disease in a commercial *Hyriopsis cumingii* Lea farm in Changde City, Hunan Province, and was identified as the *A. Veronii*. The SJ-2 strain caused multi-organ lesions and function failure in corresponding organs in the *Hyriopsis cumingii* Lea. As a result, the normal physiological metabolism gradually decreased and disappeared, which resulted in death.

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Competing Interests

We declare that we have no conflicts of interest.

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