Occurrence of a rickettsia-like prokaryote in the small abalone, *Haliotis diversicolor supertexta*, cultured in Taiwan

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Abstract

In this study, we describe for the first time the detection of a rickettsia-like prokaryote (RLP) from cultured small abalone, *Haliotis diversicolor supertexta*, in Taiwan. The description of the microorganism was performed by histopathology and transmissible electron microscopy from lesions in the intestine. Results revealed the size and shape of RLP were different from those of *Candidatus Xenohaliotis californiensis* and molecular analysis based on 16S rDNA of *Candidatus X. californiensis* was unable to amplify a significant DNA segment from samples, which suggests that the RLP of the small abalone differs from that of *C. X. californiensis* of the abalone, *H. cracherodii* Leach.

Introduction

Withering syndrome (WS) has been catastrophic to wild populations of black abalone, *Haliotis cracherodii*, and the lesions of moribund abalone are characterized by severe foot muscle atrophy eventually leading to death (Antonio et al., 2000). A rickettsia-like prokaryote, *Candidatus X. californiensis*, which was identified as the etiological agent, produces lesions in the mucosal epithelium of the gastrointestinal tract (Friedman et al., 2000, Moore et al., 2001). The RLP was observed within cytoplasmic vacuoles of abalone gastrointestinal epithelial cells and the bacterial inclusions were usually situated apical to the nucleus of the host cells. The bacterium was found in cells of the postoesophagus, transport ducts of the digestive gland and, less frequently, the intestine. The predominantly observed inclusion was spherical to oblong in shape and contained homogeneous and densely packed bacteria that stained deeply purple with haematoxylin and eosin. No morulae were observed (Friedman et al., 2000). This disease is also responsible for heavy losses of white abalone *H. sorensieni* and red abalone, *H. rufescens* Swainson (Haaker et al., 1995; Moore et al., 2000). Pathogenesis of WS is complex and differs among host species (Friedman et al., 2000). However, these bacteria were also found in WS-symptomatic and -asymptomatic abalone (Tinajero et al., 2002). Rickettsia like organisms have also been described in cultured *Haliotis discus* hannai on the northern China (Guo et al., 1999).

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The small abalone, *Haliotis diversicolor supertexta*, is commercially important in Taiwan as a primary cultured species. Abalone farms are located in northern, eastern, and southern coastal areas of the island. In recent years, the appearance of symptoms of WS characterized by a shrunken appearance of the foot muscle and retracted visceral tissues have been noted in the ponds, and mortality has reported in some farms (Liu et al., 2000). This paper describes a RLP by histopathology and transmission electron microscopy in tissues of abalone *H. diversicolor supertexta* showing mortality in an abalone farm located in southern Taiwan.

**Materials and methods**

**Sampling**

During a visit to an abalone farm in southern Taiwan in August 2004, 20 small abalone, 5-6 cm in shell length, were collected from the cages. This visit was part of an etiological study following mortality of spat abalone larvae in Taiwan. The foot muscle of all the abalone appeared shrunken, and visceral tissues were retracted. In the farm, abalone are cultured in land-based, flow-through tanks with forced air. Saltwater with a salinity of 33 ppt (parts per thousand) was pumped through pipes buried 1 m under the seabed, and the water temperature was approximately 24 °C. The abalone were fed with *Gracilaria* produced in ponds prior to introduction to the abalone farm.

A subsequent site visit was made to the grow-out farm with intertidal ponds in northern Taiwan, and 20 small abalone, 5-6 cm in shell length, were randomly collected from the ponds. All the abalone appeared normal without shrinkage of the foot muscle or retraction of the visceral tissues.

**Histopathology**

Abalone from both farms were dissected, and samples of visceral organs, muscle, and the mantle were fixed in 10% neutral formalin for 2 days, embedded in paraffin, sectioned at 5 μm, and stained with Mayer’s hematoxylin and eosin (H&E).

**Thin-section electron microscopy**

Samples of paraffin-embedded small intestines were retrieved from the paraffin block, deparaffinized, and reprocessed for electron microscopy. One milliliter of 2.5% glutaraldehyde (0.1 M cacodylate buffer, pH 7.2) was added to the samples, and fixed for 6 h, then rinsed and equilibrated 3 times with 0.1 M cacodylate buffer for 10 min each. One percent osmium tetroxide (0.1 M cacodylate buffer, pH 7.2) was carefully added to the samples and kept at room temperature for 2 h. Samples were rinsed and equilibrated 3 times with 0.1 M cacodylate buffer for 10 min each, then dehydrated using a gradient series of 35%, 50%, 70%, 85%, 90%, 95%, and 100% alcohol for 10 min each. Samples were immersed in a series of ethanol- Spurr’s resin mixtures for 2 h each. The ratio of ethanol to resin was changed from 3:1 to 1:1 to 1:3. Samples were embedded by 100% resin immersion. Then, the samples were polymerized for 24 h at 70°C. Semithin (1 mm) sections were stained with a 1% toluidine blue solution and observed under a light microscope to determine the cell morphology. Ultrathin sections (0.35 mm) were double-stained with 1% uranyl acetate and lead citrate and examined using transmission electron microscopy (JOEL JEM 1200 EX II) (Gardner et al., 1995).
DNA extraction and Polymerase chain reaction (PCR)

DNA extraction was performed using 30 mg of abalone intestine with a commercial kit (Viogene, Taiwan) following the manufacturer’s instructions. DNA was eluted with 100 µl of TE (Tris-EDTA) buffer and stored at -4 °C before use.

PCR amplification using three primer sets (RA5-1/RA3-6, RA5-6/RA3-8, and RA5-1/RA3-1) were performed as described by Andree et al. (2000). A 50 µl reaction mixture was prepared with 5 µl of 10x PCR buffer (10 mM Tris-HCl, 50 mM KCl, 1.5 mM MgCl₂, and 0.001% w/v gelatin), 400 µM dNTPs, 5µM tetramethylammonium chloride, 40 pmoles of each primer, approximately 60 ng of purified DNA, and 2 U of Taq polymerase, and was brought up to volume with sterile distilled water. PCR amplification was performed in a thermocycler (PTC-100, MJ Research, Inc., Watertown, MA) with initial denaturation of 94°C, 3 min, and then 40 cycles of 1 min of denaturation at 95°C, 30 s of annealing at 50°C, and a 30 s extension at 72°C, followed by incubation for 10 min at 72°C. Control reactions without temple were included.

PCR utilizing the universal primers (27f, 5' AGA GTT TGA TCM TGG CTC AG-3' and 1525r, 5'-AAG GAG GTG WTC CAR CC-3') designed for the amplification of most eubacterial 16S rRNA genes (Lane, 1991) were also performed with the DNA extract using the following conditions: initial denaturation of 94°C, 3 min, and then 35 cycles of 1 min of denaturation at 94°C, 1 min of annealing at 55°C, and 2 min of extension at 72°C, followed by an incubation for 10 min at 72°C. Positive control reactions include *Streptococcus agalactiae* and *Staphylococcus lugdunensis*, and negative control reactions without temple were included.

Using 6x loading buffer (2 µl), the PCR products (10 µl) were loaded horizontally onto a 1% agarose gel and subjected to electrophoresis for 40 min at 50 V in 0.5x TBE buffer. Gels were stained with ethidium bromide, and photographed under UV-light transillumination. A 100-bp DNA ladder was included as a molecular weight standard on each gel.
Results

Histopathology

The RLP appeared as intracytoplasmic basophilic inclusion vacuoles in the epithelial cells of the intestine (Figure 1), interstitium of the hypobranchial gland (Figure 2), and epithelia of the renal tubule of both healthy and atrophic abalone in both farms. They ranged from deeply stained small inclusions to large inclusions (approximately 160 μm in size) with precipitates. Variations in these precipitates may correspond to different developmental stages of RLP found in abalone, *Haliotis* spp. (Friedman et al., 2000). Tissue responses to RLP were rare.

Transmission electron microscopy

Ultrathin sections through the intestines revealed that prokaryotic cells were enclosed within membrane-bound vacuoles. These organisms were polymorphic, mostly rod-shaped, and 0.5 to 1.5 μm in size (Figure 3). The cell wall consisted of two membranes separated by an electron-lucent layer. The cytoplasm was electron-dense in the periphery with electron-lucent vacuoles in the centre. Binary fusion was noted in some organisms (Figure 4).

PCR

PCR analysis using RA5-1/RA3-6, RA5-6/RA3-8, and RA5-1/RA3-1 specific for *Candidatus X. californiensis*, did not amplify PCR products of the expected size. The eubacteria universal primers (27f/1525r) did not amplify a product from the abalone DNA template in the study but did amplify the expected products from the positive control organisms, *S. agalactiae* and *Staph. lugdunensis*.

Discussion

Recently, mass mortalities of abalone in grow-out farms have become a serious problem in Taiwan. A herpes-like virus has been demonstrated to be associated with high
mortality rates of cultured abalone in the northeast of the country (Chang et al., 2005). *Vibrio alginolyticus* and *V. parahaemolyticus* causing vibriosis can lead to the death of the small abalone (Lee et al., 2001), and *V. parahaemolyticus* was isolated from abalone with WS which experienced mass mortality in the high-water season (Liu et al., 2000).

In Taiwan, the oviferous adult female and male abalone collected from grow-out farms serve as breeders to fertilize the new generation during the spawning season. In an etiological study carried out after spat abalone mortality in hatcheries, grow-out farms were visited before the spawning season. The symptomatic appearance of WS was easily detected in cultured small abalone during the warmer-water season when the seawater temperature was higher than 30 °C. Abalone could recover from these symptoms when the seawater temperature decreased by a few degrees later on. Mortality of WS-symptomatic abalone was low or unapparent. The WS-symptomatic abalone were collected from a farm without mortality in the south, and, WS-asymptomatic abalone collected from another farm without mortality in the north area for comparison purposes. Histopathological examination revealed intracytoplasmic basophilic inclusion bodies in the epithelial cells of the intestine (Figure 1), and serous or mucous cells of the hypobranchial gland (Figure 2) were noted in both symptomatic and asymptomatic abalone. These lesions are similar to a rickettsial disease of abalone described in the U.S.A (Friedman et al., 2000). However, a survey carried out on yellow abalone, *H. corrugate*, and blue abalone, *H. fulgens*, revealed that the presence of RLP in epithelial cells of the digestive tract was not correlated with the external signs of WS (Tinajero et al., 2002).

Within the inclusion bodies, a prokaryotic organism was identified as a RLP using transmission electron microscopy. These organisms were pleomorphic, rod-shaped and were found within membrane-bound phagosomes of abalone gastrointestinal cells. Ultrathin sections through the intestines revealed that prokaryotic cells were enclosed within membrane-bound vacuoles. However, in the current study, the organisms were polymorphic with the majority rod-shaped (Figures 2 and 3). The size and shape differed from *Candidatus X. californiensis* (Friedman et al., 2000).

Furthermore, molecular analysis utilizing PCR based on the 16S rDNA of *Candidatus X. californiensis*, did not amplify PCR products of the expected size from the samples, suggesting that the RLP of the small abalone differs from that of the California abalone (Andree et al., 2000). *Candidatus X. californiensis* is the agent of WS in abalone, and the pathogenesis of WS is complex and differs depending on host species (Friedman et al., 2000). It is also probable that the susceptibility of different abalone species or individuals varies, or that an unknown condition or pathogen is also involved in WS development. Clinical studies have shown that water temperature may be involved in the development of RLP, further studies on this organism and associated lesions in small abalone during summer season are planned.
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References


Moore JD, Cherr GN & Friedman CS (2001). Detection of ‘Candidatus Xenohaliotis californiensis’ (Rickettsiales-like prokaryote) inclusions in tissue squashes of abalone (Haliotis spp.) gastrointestinal epithelium using a nucleic acid fluorochrome. Diseases of Aquatic Organisms 46, 147-152.
