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Detection of a mollicute-like organism in kuruma shrimp, *Penaeus japonicus*

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A filamentous form of mollicute-like bacterium was detected during a routine health survey of wild adult kuruma shrimp *Penaeus japonicus*. The kuruma shrimp were native to Japan and were imported to Korea. The histology showed no pathological changes. Transmission electron microscopy revealed extensive infections of hepatopancreatic epithelial cells by a pleomorphic, filamentous intracellular bacterium. The filamentous bacterium was of about 60 nm in diameter and 300 nm to more than 1 μm in length. The morphology of bacteria were filamentous and branched with terminal blebs, or knobs, on the branches. They lacked the cell wall, and were bounded by the plasma membrane. They contained typical prokaryotic ribosomes and fibrillar DNA-like strands. No additional internal structure has been observed. They are considered to be mollicutes, based upon the morphological appearance and upon the cellular location.

Key words : *Penaeus japonicus*, Intracellular bacteria, Mollicute, Hepatopancreas, Transmission electron microscopy

While international trade of penaeid shrimp has contributed to development of penaeid shrimp culture, it has resulted in introduction of infectious diseases into new geographical regions by transfer of infected postlarvae or broodstock to outside the original locations(Lightner, 1983 ; Lightner, 1985 ; Colorni *et al.*, 1987). The infectious diseases generally occurred as epidemics and subsequently settled into endemics(Brock and Lightner, 1990 ; Lightner, 1992 ; Vega-Villasante and Puente, 1993). Therefore prevention of the spread of contagious diseases of imported penaeid shrimp is very important for aquaculture development.

Mollicutes are very small prokaryotic parasites totally devoid of cell wall, bounded only by a plasma membrane(Razin and Freundt, 1984). They have been found in several marine and freshwater organisms ; however, few of them have been isolated and cultivated(Harshbarger *et al.*, 1977 ; Zimmer and Woolacott, 1983 ; Boyle *et al.*,

1987 ; Hackett and Clark, 1989 ; Kirchhoff *et al.*, 1987). Recently, a filamentous mollicute-like bacterium was first described in penaeid shrimp *Penaeus vannamei* cultured in Texas(Krol *et al.*, 1991). Attempts to culture this intracellular organism have been unsuccessful(Frelier *et al.*, 1993), thus its pathogenesis in shrimp still remains uncertain.

To prevent the introduction and spread of exotic diseases in Korea, imported stocks of aquatic animals for culture purposes are being proved pathogen-free and monitored for signs of diseases by National Fisheries Research and Development Agency(NFRDA). During a routine health survey of kuruma shrimp *Penaeus japonicus* imported from Japan, a filamentous microorganism was detected.

Materials and Methods

1. Shrimp

Seventeen kuruma shrimp *Penaeus japonicus* were submitted to Pathology Laboratory of NFRDA as a part of a routine health survey required for an documentation of imported stocks for culture purposes. The sexually mature kuruma shrimp, about 20 cm in length, that were native to Japan, were collected by a net on the eastern coast of Japan, and were transported in sawdust by airplane directly to the laboratory of NFRDA.

2. Light and Electron microscopy

The shrimp were cut longitudinally. Then samples were fixed immediately in Davidson's fluid, dehydrated through on ascending ethanol series, cleared in xylene and embedded in paraffin. Histological preparations were sectioned at 4 μm, stained with Hematoxylin and Eosin and carefully checked for lesions and pathogens with a light microscope.

Before histological fixation, pieces of the hepatopancreas were collected from three shrimp, then fixed in 2.5% glutaraldehyde in sodium phosphate buffer(pH 7.4) for 2 h at 4℃. The tissues were rinced twice in the same buffer, postfixed in 1% phosphate-buffered osmium tetroxide for 2 h at room temperature, dehydrated through a graded ethanol series, and embedded in Epon resin. One μm sections for light microscopy were stained in 2.5% toluidine blue in 1% aqueous sodium borate solution. Thin sections were collected on copper grid and double stained with 4% uranyl acetate and lead citrate prior to examination with a JEOL 1200 EX-2 transmission electron microscope at 80 kV.

Results

There were no external or internal signs of disease among the *Penaeus japonicus* examined. Using light microscopy the tissues appeared to be normal and showed no pathological changes. But transmission electron microscopy(TEM) revealed a subclinical infection with a filamentous microorganism in the hepatopancreatic epithelial cells of the shrimp(Fig. 1−6).

Hepatopancreatic epithelial cells of three shrimp examined by TEM were infected by a filamentous microorganism. This intracellular microorganism was a highly pleomorphic, apparently obligate intracellular pathogen, that infected all types of hepatopancreatic epithelial cells(Fig. 1a). The structure of this organism was filamentous and branched. Depending on orientation of the microscope

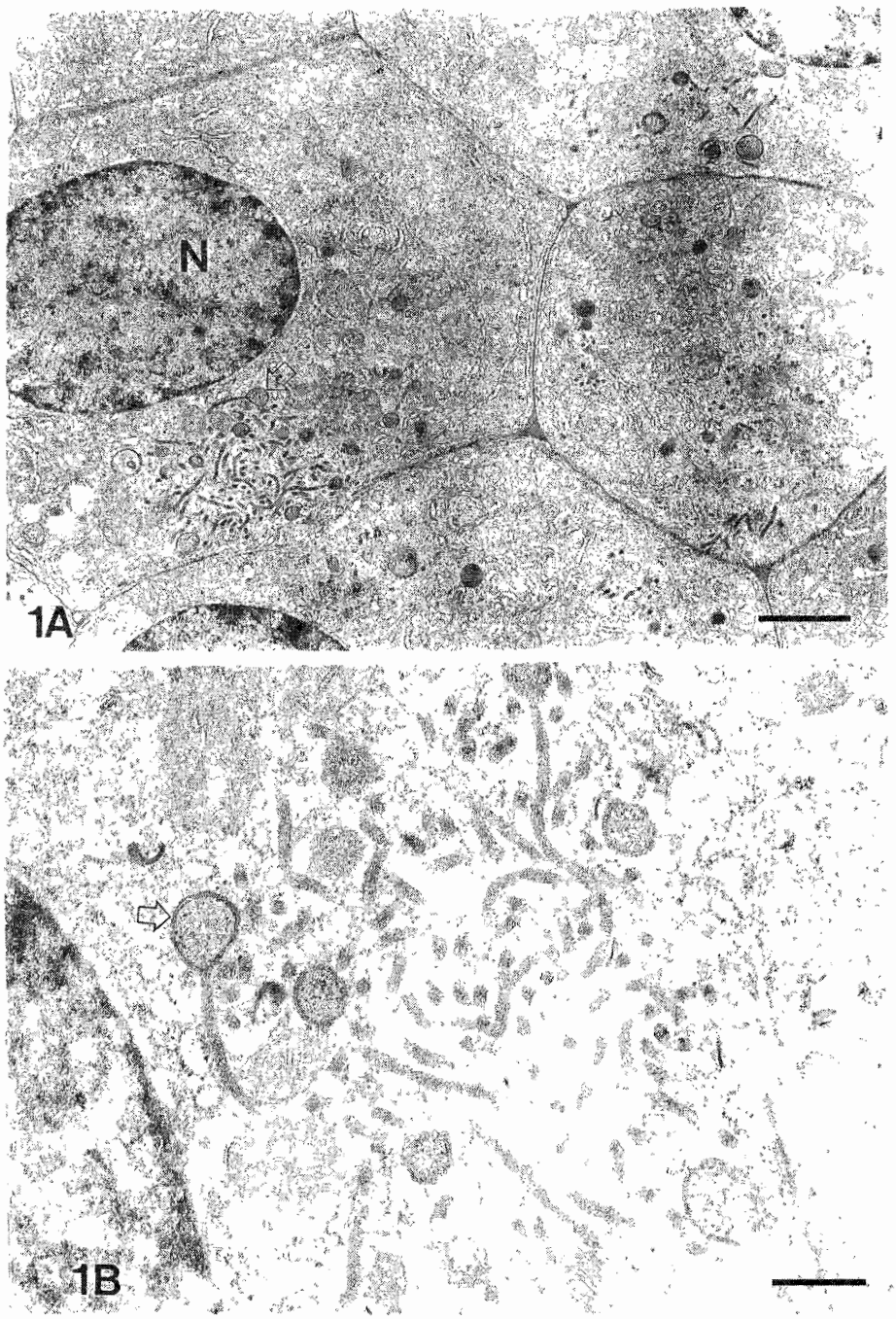

Fig. 1a. Transmission electron micrographs(TEM) of hepatopancreases of *Penaeus japonicus* infected with the filamentous mollicute-like organism(HMLO). Despite the infection, host cell nuclei(N) appear normal. Bar=2.0 μm. Fig. 1b. Higher magnification(×24,000) of Fig. 1a. HMLO has a round structure(arrows). Bar=0.5 μm.

section, filamentous forms of unique diameter with terminal blebs, or knobs, as well as small dense round form of 30 to 40 nm in diameter were respectively observed(Fig. 2). The filaments ranged from about 60 nm in width and from 300 nm to more than 1 μm in length. Another round structure was also observed, with a diameter of 200 to 300 nm(Fig. 1b). And it was possible to observe budding filaments from these round structure. Occasional forms were seen apparently containing spherical dilations(Fig. 2, 6).

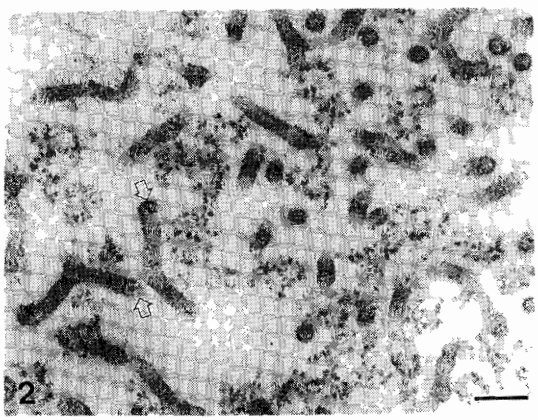

Fig. 2. TEM of branched form of HMLO with terminal knobs(arrows). Bar=0.2 μm.

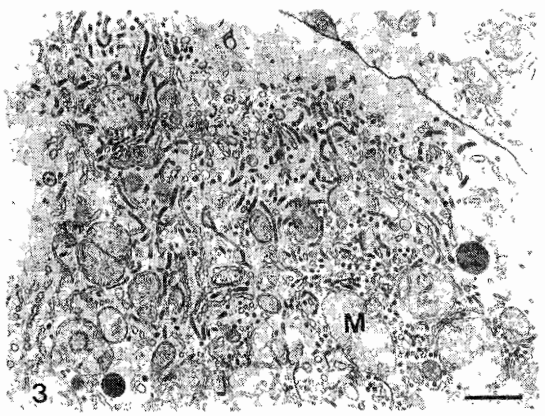

Fig. 3. TEM of HMLO-infected hepatopancreatocyte. Note the mitochondria(M). Bar=1 μm.

These microorganisms lacked a cell wall and were only bounded by a plasma membrane(Fig. 4). They contained typical prokaryotic ribosomes and fibrillar DNA-like strands(Fig. 5). No additional internal structure was observed. These prokaryotic organisms were found free in the cytoplasm of infected cells. There was no evidence of phagocytosis of these prokaryotic organisms within cells. When the cellular infection was heavy, the only organelles that appeared normally distributed in the cell cytoplasm were mitochondria and ribosomes(Fig. 3). Although different level of the bacterial infections in the shrimp, nearly all hepatopancreatic cells of the diseased shrimp were infected with bacteria. It was impossible to observe any host response on affected areas. No bacteria were observed free in hemolymph.

Fig. 4. TEM of HMLO in hepatopancreatocytic cytoplasm. Note the plasma membrane of HMLO (arrow). Bar=0.1 μm.

Fig. 5. TEM of HMLO in hepatopancreatocytic cytoplasm. Note HMLO contains typical prokaryotic ribosomes and fibrillar DNA-like strands(arrow). Bar=0.2 μm.

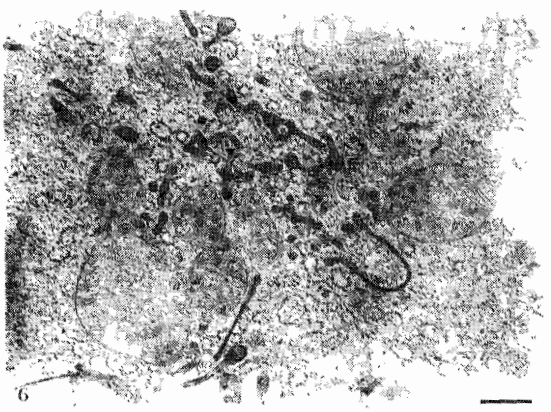

Fig. 6. TEM of HMLO in hepatopancreatocytic cytoplasm. Note HMLO contains spherical dilations (arrow). Bar=0.5 μm.

Discussion

The intracellular microorganism in hepatopancreatic cells of *Penaeus japonicus* lack a cell wall and are bounded only by the plasma membrane which suggest that these microorganisms are mollicutes(Razin and Freundt, 1984). All microorganisms occur free in the cytoplasm which distinguishes them from the small intracellular rickettsia-like bacteria of penaeid shrimp that typically occur in membrane-bound cytoplasmic inclusions in infected cells(Weiss and Moulder, 1984). The intracellular microorganism is a highly pleomorphic apparently obligate intracellular pathogen. The structure of intracellular microorganism is filamentous and branched. These facts are indicative of mollicutes. We named the organisms as hepatopancreatic mollicute-like organism(HMLO) as a means of distinguishing from other mollicutes until relationship is clarified.

A filamentous mollicute-like bacterium has been reported in penaeid shrimp *Penaeus vannamei* cultured in Texas(Krol *et al.*, 1991). When Krol *et al.*(1991) described the intracellular bacteria associated with necrotizing hepatopancreatitis(NHP) lesions in *P. vannamei*, they concluded that a filamentous mollicute-like bacteria, a helical form of a mollicute-like bacteria and a rod-shaped rickettsia-like bacterium were present in cytoplasm of heavily infected hepatopancreatic epithelial cells. But subsequent studies on NHP in penaeid shrimp by Frelier *et al.*(1992, 1993) and Lotz and Overstreet(1991) suggested that the filamentous mollicute-like bacterium reported by Krol *et al.*(1991) was in reality remnants of the host cell endoplasmic reticulum.

There are considerable similarities between HMLO and filamentous mollicute-like bacterium reported by Krol *et al.*(1991) : both bacteria were 60 nm in diameter ; both bacteria had terminal blebs, or knobs, on the branches ; both bacteria were associated with mitochondria and ribosomes in cytoplasm of infected cells. HMLO had the round structures about 200 to 300 nm in diameter which are uncommon in filamentous mollicute-like

bacterium (Krol *et al.*, 1991). With both infections, affected hepatopancreatic cells retained most of their normal architecture except for their organelles. But it is not sufficient that both bacteria in the different hosts are consider to be same bacteria only by their apparence and histology. Like Krol *et al.*(1991), we did not pay special attention to osmolarity of fixatives and buffers and both may drastically alter the actual size and shape the plastic organisms(Lemcke, 1972 ; Cole *et al.*, 1973 ; Razin and Freundt, 1984).

Mollicutes have no intracellular membrane or membrane-bound structure and thus it can be regarded as living membrane vesicles. Occasionally, HMLO have been found containing membrane-bound inclusions seemed to be internal vesicles. But these forms were shown to be involuted and they were thought that the plane of the section cut through the cell membrane twice.

In infected cell cytoplasm of *P. japonicus*, HMLO often associated with mitochondria. It explains why the mycoplasma have limited biosynthetic abilities, probably reflecting their small genome and parasitic mode of life(Razin and Freundt, 1984). Other studies reported that *Mycoplasma mycoides* and *Acholeplasma laidlawii* require a complex assortment of amino acids, nucleic acid precursors, lipids, vitamins and inorganic ions, and glucose as an energy source(Rodwell, 1979).

There was no mortality and little evidence of cell necrosis in affected areas despite the high infection rate of cells, therefore the virulence of HMLO remains uncertain. However, the discovery of mollicute-like organisms from the wild penaeid shrimp suggests a potential mechanism by which pathogenic mollicutes may be controlled and underscores continual need for health surveys prior to international transport of shrimps. Further studies will be needed to determine the identity of these bacteria and their pathogenesis in penaeid shrimp.

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보리새우(*Penaeus japonicus*)에 감염된 Mollicute-like Organism

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자연산 보리새우(*Penaeus japonicus*) 모하의 병리검사 결과 사상체의 mollicute와 유사한 미생물의 감염을 확인하였다. 검사에 사용된 보리새우 모하는 일본산이며, 한국으로 수입되었다. 일반 조직검사 결과 병리학적인 변화는 관찰되지 않았다. 투과전자현미경적 관찰 결과 간췌장 상피세포에서 세포내 다형태성 사상체 세균의 광범위한 감염을 발견하였다. 사상체 세균은 직경이 약 60 nm이며, 길이가 300 nm에서 1 μm 이상의 크기를 나타내었다. 이 세균의 구조는 사상체이며, 끝마디를 가진 가지로 분지 되는 특징을 보였다. 세포벽은 없으며, 원형질막으로만 둘러싸여 있었으며, 전형적인 원핵세포성 리보소옴과 선형 DNA와 유사한 가닥을 가지고 있었다. 그 외 다른 세포내 기관은 관찰되지 않았다. 이러한 형태적인 특징들과 세포내 감염부위로 볼 때 이 세균은 mollicutes로 판단된다.

Key words : *Penaeus japonicus*, Intracellular bacteria, Mollicute, Hepatopancreas, Transmission electron microscopy