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White Spot Syndrome Virus Disease of Shrimp and Diagnostic Methods

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Since 1993, the White Spot Syndrome Virus (WSSV) disease occurred in China among cultured shrimps resulting in mass mortality. Epizootiological surveys undertaken during the outbreak period of 1993-1994 indicated that all stages of *Penaeus chinensis*, *P. japonicus* and *P. monodon* were infected. Consequent to the transport of contaminated shrimp seedlings and seawater, the disease spread all over the farms of China. The disease was more rapidly transmitted at temperatures above 25℃. Challenge experiments showed the causative agent was highly virulent. White spots appeared on the carapace of both spontaneous and experimentally infected shrimps. Moribund shrimps contained turbid hemolymph, hypertrophied lymphoid organ and a necrotic mid-gut gland. Electron microscopy showed the presence of viral particles in the gills, stomach, lymphoid organ, and epidermal tissue of the infected shrimp. The virions were slightly ovoid with an envelope and averaged 350 × 150 nm; nucleocapsids measured 375 × 157 nm. With discontinuous sucrose gradient of 35, 50 and 60% (w/v), the virus was separated from hemolymph of the infected shrimp. The estimated molecular weight of genomic DNA was 237 Kb with *Eco*R I, 247 Kb with *Hin*d III and 241Kb with *Pst* I.

A total of 9 hybridoma colones secreting monoclonal antibodies (MAbs) were produced from mouse myeloma and spleen cells immunized with WSSV. The immunofluorescence assay of gill tissue showed that the MAbs reacted with diseased but not with healthy shrimp. The MAbs belonged to IgG1, IgG2b subclass and IgM class, all with kappa light Immuno-electron-microscopy with colloidal gold marker showed the presence of 5 MAbs epitopes on the envelope and one on the capsid of the virus. Baculoviral mid-gut gland necrosis showed the specificity of the MAbs produced.

For diagnosis 5 different methods were selected. Using Kimura primers for PCR, or MAbs for immunoblot, ELISA or FAT method, *in situ* hybridization was carried out to show the gene. All these methods detected WSSV in the organ samples of the diseased shrimp but not in healthy one.

Key words: White spot disease, Diagnostic methods

Introduction

Since 1978, shrimp culture industry has been rapidly developed in China. Over 200,000 mt (metric tonne) shrimp has been produced in 1992. During the years from 1978 to 1992, the shrimp production has been increased by 445 times. Most shrimp ponds are earthen with the depth of 1.5-2.0 m. Size of the ponds ranges from 2 to 10 ha, bur a few are larger than 20 ha. Majority of farms pump seawater from coastal areas, while others use natural tidal flow. Ponds are rarely equipped with aerators. The initial stocking density of juveniles is usually about 150,000/ha, and the average production about 1,500 kg/ha.

As the shrimp culture industry expanded, the incidence of disease also increased. To reduce the impact of the diseases, initial investigations were begun in 1979, but comprehensive studies on the shrimp diseases

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were not begun until 1982. These investigations focused on identifying the different diseases and their etiology, diagnosis, treatment, prevention and pathogenic ecology of mostly *Penaeus chinensis, P. penicillatus* and *P. merguiensis*. By 1980's the diseases were already more than 40 different kinds and were mostly caused by bacteria, fungi, white-black spot and *Paranophrys* in cultured shrimp (Meng, 1991). Although the negative impact was realized, the diseases were somewhat under control.

At the beginning of 1993, an unfortunate outbreak of the epizootic disease caused by virus spread to most shrimp farms. Mortality exceeding 80% occurred often within a week. This resulted in the lowest shrimp production since the industry was begun.

From other Asian countries also, reports on occurrence of the viral disease on cultured *Penaeus chinensis* (Zhan et al., 1995), *P. japonicus* (Inouye et al., 1994), *P. monodon* (Wongteerasupaya et al., 1995), and *P. pencillatus* (Chou et al., 1995) began to appear. For instance, the white spot disease caused by virus resulting in mass mortality of the cultured shrimp was reported in 1993 from Japan and Thailand (Inouye et al., 1994, Momoyama et al., 1994; Nakano et al., 1994; Takahashi et al., 1994; Wongteerasupaya et al., 1995). Recently there were also reports on the occurrence of disease from Indonesia and India (Durand et al., 1996). These were reported to be more serious, showing a wider host range and geographic distribution. Thus the shrimp culture industry in Asia began to face severe problem caused by the white spot viral disease.

Natural infection by the virus was observed in *P. chinensis, P. japonicus, P. monodon, P. indicus, P. merguiensis* and *P. penicillatus* (Momoyama et al., 1994; Takahashi et al., 1994; Cai et al., 1995; Chou et al., 1995; Wongteerasupaya et al., 1995; Zhan et al., 1995), however it was experimentally shown to be lethal in *P. vannamei, P. stylirostris* and *P. setiferus* (Durand et al., 1996). More recently its occurrence was observed in captured *Metapenaeus ensis* in Taiwan (Wang et al., 1997), cultured *M. ensis* in Japan (Momoyama et al., 1997) and in other crustaceans (Lo et al., 1996b). These reports have clearly shown that the host range of the virus was very much expanded.

To detect the viral disease, different methods such as monoclonal antibodies, polymerase chain reaction and gene probes were developed (Chang et al., 1996; Durand et al., 1996; Kimura et al., 1996; Lo et al., 1996a; Takahashi et al., 1996; Zhan et al., 1998b).

There were also many other reports describing the recent epidemic disease of shrimp (Inouye et al., 1994; Momoyama et al., 1994; Nakano et al., 1994; Takahashi et al., 1994; Chou et al., 1995; Wongteerasupaya et al., 1995; Zhan et al., 1995). The common symptoms recorded in more than 4 penaeid shrimp species included (i) lethargy, (ii) and reduction in food consumption and (iii) appearance of white spots on inner side surface of cuticle, especially the carapace (Takahashi et al., 1994; Chou et al., 1995; Zhan et al., 1995). Histologically, the characteristic symptom of the diseased shrimp showed nuclear hypertrophy due to the development and accumulation of intranuclear virions. In all the shrimps, the virus was shown to have the same morphology, i.e. non-occluded, slightly ovoid or rod-shaped, with an envelope. The target tissues were connective and epithelial tissues, particularly epithelial cells of the gills and stomach (Momoyama et al., 1994; Zhan et al., 1995). The virions were also observed in lymphoidal organ (Takahashi et al., 1994; Zhan et al., 1995). On the basis of tissues of the infected shrimp morphology of the virus and symptoms of the disease, the virus was called white spot baculovirus (WSBV) (Wang et al., 1995; Durand et al., 1996), *Penaeus chinensis* baculovirus (PCBV) (Zhan et al., 1995), systemic ectodermal and mesodermal baculovirus (SEMBV) (Wongteerasupaya et al., 1995), hypodermal and hematopoietic necrosis baculovirus (HHNBV) (Cai et al., 1995), rod-shaped nuclear virus of *Penaeus japonicus* (RV-PJ) (Inouye et al., 1994), penaeid rod-shaped DNA virus (PRDV) (Inouye et al., 1996), and white spot syndrome baculovirus (WSSV) (Durand et al., 1997). Now all these have been standardized

In our laboratory, we explored the epizootiology of WSSV disease that occurred in China, investigated for diagnosticetiology of the virus, and developed monoclonal antibodies. For diagnosis, different methods like

the immunoblot, FAT, ELISA, PCR, gene probes were applied to detect the virus.

Epizootiology

Epizootiological surveys: The epizootic white spot disease was first observed in August 1992 and its occurrence was also limited to a few cultured ponds of*P. japonicus* in Fujian Province (Yu, Kai-Kang, Ocean University, unpublished). The causative agent of the disease was unknown and shrimp loss was limited. In 1993, it spread to the coast of Shandong, Hebei and other Provinces, in which the shrimp was cultured. This transmission resulted from contaminated shrimp seedlings of *P. japonicus* transported from Fujian to other shrimp farms in different areas.

From May to August in 1993 and 1994, eight shrimp farms were investigated, one in Fujian, six in Shandong and one in Hebei. The disease began earlier in the south (Fujian in May) and later transmitted to the north (Hebei in August); hence, it was thought to be related to water temperature. Size of the diseased shrimp ranged from 6.0 to 9.0 cm in 1993. However, shrimps ranging from 2.6 to 3.6 cm in Farm No. 5 were also diseased in 1994. The cultured shrimps were *P. japonicus*, and *P. monodon* in 1993 and 1994 in farm No. 1, mainly *P. chinensis* and a few *P. japonicus* in farms No. 2~5, only *P. chinensis* in farms No. 6~8 in 1993. In 1994 in all farms only *P. chinensis* was cultured except in farm No. 1. Among 86 culture ponds examined during 1993, the disease occurred in 61 ponds (71% of the infected ponds). In 1994 disease occurred in 47 of 72 ponds (65% of the infected ponds). The mass mortality (within a week, more than 80%) occured most often in *P. chinensis*, which showed higher susceptibility to the disease than *P. japonicus* and *P. monodon*. The disease occurred first in the ponds of *P. japonicus* (contaminated postlarvae were introduced from Fujian, farms No. 1~5 in 1993). From these ponds it quickly spread to adjacent ponds, where *P. chinensis* was cultured (farm No. 2~5). This pattern was also indicated in farms No. 6 and 7. At farm No. 8, the disease did not occur until 1994 because it was an area with no other shrimp farms.

According to the surveys of 8 farms, the mortality remained high (often more than 50%) at temperatures above 25℃, but the disease seemed to have subsided (often less than 30%) below 20℃. A two-year investigation during the epidemic period indicated that the more water was exchanged (by draining away some pond water and then pumping water from canal into the pond) the more serious became the disease but when less or no water was exchanged, there was low mortality or no disease at all. Certainly, one of the spreading routes is through water (Zhan et al., 1998a).

Etiology

Gross symptom and light microscopy: The infected shrimps were lethargic and rejected feed. Moribund shrimps exhibited either abnormal red discolouration or colourelessness. Typically the external symptoms were the presence of white spots on the inner surface of the carapace. Light microscopic examination revealed that the spots were radials or ring-like appearance. The centers were thick and edges thin, and they can be dissolved in 5% acetate acid with the production of gas. The carapace can be peeled off easily. The moribund individuals displayed with turbid hemolymph, hypertrophied lymphoid organ and necrotic mid-gut gland. Histopathological examination showed the presence of hypertrophied nuclei especially in the tissues of gills, cuticular epidermis and stomach.

Virulence: When challenged with infection, the shrimps succumbed within 4 days after inoculation, but none of the controls suffered mortality. Infected shrimps showed white spots on inner surface of the carapace, like those of the natural infection, but they were smaller and fewer in number. Light microscopic observation showed the absence of bacteria in hemolymph.

Electron microscopy: In negatively stained preparation, intact enveloped virions appeared slightly ovoid, some with an appendage at one extremity and were about 375 × 157 nm. Unenveloped nucleocapsids measured, on an average, 395 × 83 nm; they exhibited a superficially segmented appearance. The segments, which were about 25 nm thick, were separated by

electron dense bands. Unenveloped nucleocapsids usually had 12~15 bands. The bands, which were about 2.3 nm, showed an angle of 90° to the long axis of the particle.

In the ultrathin sections of stomach, gills, epidermis tissue, lymphoid organ and others of the diseased *P. chinensis*, viral particles were present in hypertrophied nuclei. The slightly ovoid, mature virion, with an envelope measured about 350 × 150 nm and nucleocapsid averaged 325 × 120 nm in size. The envelope was about 6 nm in thickness; between the envelope and the nucleocapsids, there were electron lucent space about 7 nm. The capsid was about 5 nm thick around the core and less electron-dense than the core.

Virions were observed in different stages of development: some had envelopes with empty capsids, others had nucleocapsids but no envelope or two nucleocapsids within one envelope. Virus purification: The hemolymph of the infected shrimp was pooled and centrifuged 2000 g for 15 min at 4℃; the supernatant was laid onto top of discontinuous sucrose gradient of 35, 50, 60 (w/v) and ultracentrifuged at 80000 g (Hitachi 70 P~72, SRP28SA rotor) for 90 min at 4℃. The virus appeared between 50 and 60% of sucrose, and many virions were observed through electron microscope.

Nucleic acid analysis: The virus solution was diluted with TE buffer (10 mM Tris-HCl, 1 mM EDTA, pH 7.5) and incubated at 55℃ with proteinase K and SDS for 16 h. The DNA was extracted with phenol and chloroform, precipitated with ethanol, dissolved in distilled water, digested with restriction endonucleases (*Eco*R I, *Hind* III and *Pst* I) under suitable condition. The digested DNA was electrophoresed at 50 V for 6.5 h in 0.8% agarose gel with TBE buffer (0.09 M Tris, pH 8.5, 0.09M borate, 2.5 mM EDTA) and stained with ethidium bromide. The λDNA/*Hind* III was used as a marker The total MW of DNA estimated as 237 Kb with *Eco*R I, 247 Kb with *Hind* III and 241 Kb with *Pst* I.

Development of monoclonal antibodies

A total of 9 hybridoma clones secreting monoclonal antibodies (MAbs) were produced from mouse myeloma and spleen cells immunized with WSSV. An indirect immunofluorescence assay was standardized to evaluate the MAbs for their usefulness as rapid diagnostic tool for the identification of WSSV in cryosection and as tool for further study of the virus. Isotyping revealed that the MAbs were Ig G, and Ig M class, all with kappa light chains. Six MAbs were examined, using immuno-electron microscopy and colloidal gold as a marker, five MAbs were recognized as epitopes on the envelope of the virus and one MAb as an epitope on the capsid. The specificity of the MAbs produced was determined by the lack of reactivity to baculoviral mid-gut gland necrosis in the virus-infected tissues.

Diagnostic Methods

Immunoblot: The virus was prepared, purified and diluted at gradient of 100, and spotted on nitrocellulose membrane, as positive control. The gill and stomach tissues of naturally and experimentally infected shrimps were used as samples. The tissues were homogenized in 10 % (w/v) with PBS, and centrifuged at 2000 g for 15 min; the supernatants were diluted at gradient of 10 and then spotted on the same nitrocellulose membrane. The tissue of healthy shrimp, treated similarly as the diseased one, served as a negative control. The mixed culture fluids of 6 stains MAbs were used as primary antibody, and the secondary antibody was the goat anti-mouse Ig serum, conjugated to alkaline phosphatase; nitroblue tetrazolium (NBT) and 5-bromo-4-chloro-3-indolyphosphate p-toluidine salt (BCIP) served as substrate. The purification of the virus was determined until the dilution of 10^4, and samples from the infected shrimp at the dilution of 10^3. In samples from healthy shrimp, no colour appeared.

Fluorescent antibody technique: Cryosections were prepared from tissues of naturally or experimentally diseased shrimp, and from the tissues of healthy shrimp as control. Using FAT method, the specific fluorescence was observed in gills, stomach, heart, epidermis and lymphoid organ of the diseased shrimp

but not in the healthy shrimp.

Enzyme-linked immunosorbent assay: The samples were homogenized in PBS (10% w/v) and centrifuged as for the dot blot. Serially 5-fold diluted supernatant was added into microtiter wells and incubated at room temperature for 2 h or at 4℃ overnight. After washing 5 times with TBS-T, and blocking with the blocking agent, wells were filled with serially 2-fold diluted MAbs and incubated at 37℃ for 1 h. Following washing with TBS-T, sheep anti-mouse Ig serum, conjugated to peroxidase, was diluted 1 : 200 in TBS, added to each well and incubated at 37℃ for 1 h. After washing, wells were reacted with 0.1% of o-phenylenediamine dihydrochloride (OPD) in 0.1 M citrate buffer (pH 5.0) with 0.03% of H_2O_2 at room temperature for 10 min. Color development was stopped by adding 100 ㎕ of 3N H_2SO_4. The absorbance was measured at 492 nm using a microtiter plate reader (MPR-4A). With dilution of samples from the diseased shrimp, the virus positivity was determined at the dilution of 1250, but the control sample was virus negative. With dilution of antibodies (culture fluids of hybridomas), the virus was detected at dilution of 64 times.

***In situ* hybridization:** The nucleic acid was extracted from purified virus suspension, and digested with *Eco*R I. The digested DNA was electrophoresed in 0.8% agarose gel. The *Eco*R I DNA restriction fragments were directly ligated with DNA ligation kit (Takara Biomedicals, Japan), and transfomed to competent *Escherichia coli* (JM 109) cells. The cloned DNA fragments were labeled by incorporation of Dig-11-dUTP using Dig DNA labeling kit (Boehringer, Mannheim), according to the protocol suggested by the manufacturer. Diseased shrimp sections were used for *in situ* hybridization, following the procedure of Chang et al. (1996). Using the DNA probes, the virus positivity of the diseased shrimp tissues was examined. No reaction was found in the healthy shrimp tissue.

Polymerase chain reaction: A pair of primers of Kimura was used for PCR. The template DNA was extracted from spontaneously and experimentally diseased shrimp tissues. The amplification was performed with a GeneAmp PCR system 9700 (Perkins Elmer, USA), 30 ㎕ of PCR mixture consisted of 10 mM Tris-HCl, 50 mM KCl, 1.5 mM $MgCl_2$, 0.2 mM dNTP, 1.5 units of Taq DNA polymerase, 2 ㎛ primers and the template DNA. The PCR reaction was performed: First cycle of 93℃ for 3 min, 57℃ for 1.5 min, 72℃ for 5 min, and then 29 cycles of 93℃ for 1 min, 57℃ for 1.5 min, 72℃ for 1 min, plus a final 5 min extension at 72℃ after the 30 cycles. The PCR products were electrophoresed in 1.5% agarose gel and stained with ethidium bromide. Positive and negative controls were also carried out. DNA extracted from the tissues of diseased shrimp was utilized as template DNA for PCR assay. The amplification results demonstrated that the diseased shrimp was virus positive.

Discussion

Epizootiological surveys indicated that the WSSV disease is spread throughout shrimp farms in China. The disease was spread by the transportation of contaminated seedlings. The surveys also indicated that the virus spread rapidly by the sea water borne routes, when temperatures exceeded 25℃. This paper is concerned with horizontal transmission. The role of vertical transmission will be treated eslsewhere. Avoidance of the virus is the only available control measure, as there is no treatment for the disease. Clearly, a sensitive, accurate, and rapid diagnostic assay is urgently needed for the white spot disease and must be suitable for both in the laboratory and field.

We used 5 different methods to diagnose the WSSV disease. All of them are found effective. In practice, we propose that for different purposes, different methods should be used. Immunoblot is useful for detecting the virus; this is a simple method and can be carried out at the farm obtaining result within 4 h. It can detect relative amount of virus, if a control is available but can not detect target organ or cell. Like immunoblot, ELISA method can be used. Data from the ELISA are more accurate than immunoblot and can also be obtained by automatic microplate reader

instead of the unaided eye. But the ELISA can not detect the virus target. PCR method at nucleic acid level can be used for both quarantine and diagnosis at very low viral count, but can not be used for detecting virus target. FAT method is used for diagnosis and to detect virus target, for the reaction is *in situ*. Besides, this method avoids the influence of entoenzyme from shrimp, when relatively small sample is examined. Gene probe method can be used not only the same way, as FAT, but can also be used to detect the virus at the low count. This method is time-consuming but examines a relatively small sample.

Conclusions

Beginning 1993, the outbreak of epidemic disease of shrimp spread throughout the shrimp farms, as a result of transport of contaminated shrimp seedlings and sea water. Water temperatures above 25℃ accelerated the disease transmission. Causative agent of the disease is WSS virus; it is slightly ovoid with an envelope and averages 350 × 150 nm, and the nucleocapsid measures 325 × 120 nm in size. The targets of the virus are gills, stomach, lymphoid organ, epidermal tissue and others of the infected shrimp, the virus proliferates in nuclei. The nucleic acid of WSSV is about 240 Kb. The monoclonal antibodies produce anti-WSSV and have specificity; they belong to IgM, IgG1, IgG2b class and subclass and have the epitopes of five on envelope, one on capsid of virus. WSSV disease can be diagnosed by immnuoblot, FAT, ELISA using the developed MAbs, by *in situ* hybridization using the developed gene probes and by PCR using Kimura primers.

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