Baculovirus Infection of Penaeid Shrimp in Japan

Tokuo Sano
Laboratory of Aquatic Pathology, Department of Aquatic Biosciences
Tokyo University of Fisheries, 4-5-7 Konan, Minato Ku
Tokyo 108, Japan

and

Kazuo Momoyama
Yamaguchi Prefectural Naiikai Fisheries Experiment Station
Yamaguchi 754, Japan

Abstract

Baculoviral midgut gland necrosis (BMN) is a superacute viral disease, causing severe mortalities in kuruma shrimp larvae, _Penaeus japonicus_. With the goal of implementing realistic prophylactic measures, we developed diagnostic techniques for BMN, investigated the host range of BMNV, and tested the efficacy of a variety of chemical and physical agents as BMN virucides. The methods we developed can facilitate diagnosis in hatcheries. Washing fertilized eggs is the most effective prophylactic technique. In addition to _P. japonicus_, BMNV can infect _P. monodon_, _P. chinensis_ and _P. semisulcatus_ but not _Metapenaeus ensis_ or _Portunus trituberculatus_.

Introduction

The OIE (Office Internationale des Epizooties) has dealt with fish disease matters since 1960 through the Fish Diseases Commission (FDC), which produces an annual report on the main developments regarding current diseases, new pathogens, and diagnostic and control methods used worldwide. The FDC has created a separate section for fish diseases in the Animal Health Code. This is currently being revised and will contain eight pathogens (six viral agents and two bacterial agents); all will be placed on List B. List B contains 89 communicable animal diseases considered to be of socioeconomic and/or public health importance within host countries, and are significant in the international trade of animals and animal products. Recently, the FDC has expanded to encompass diseases of molluscs and crustacea (De Kinkeline et al., 1990). Consequently, eleven pathogens were placed on List B, including _Penaeus monodon_ baculovirus (MBV), _Baculovirus penaei_ (BP) and baculoviral midgut gland necrosis (BMN) virus.
This indicates the importance of baculoviruses to penaeid shrimp culture.

List B agents cause no serious socioeconomic problems for terrestrial homeotherms, nor are they a public health consequence. However, the baculovirus particles released from victims of BMN, both moribund animals and latently infected adults, undoubtedly constitute an "aquatic biohazard." "Aquatic biohazard" may be defined as a hazard for aquatic animals such as fish and shellfish caused by aquatic pathogens in List B (Sano, 1992) as opposed to the pathogens in List A that are dangerous for humans and/or terrestrial homeothermal animals.

Examples of aquatic biohazards include IPN (infectious pancreatic necrosis), VHS (viral hemorrhagic septicemia), and EHN (epizootic hematopoietic necrosis). Aquatic pathogens, in general, have the potential to spread widely and rapidly, and furthermore, to be transmitted intergenerically. The resulting diseases prevail as enzootic or panzootic and cause serious socioeconomic consequences. The goal of our studies on BMN is the extermination of this shrimp disease. Toward that aim, we have studied three aspects: diagnosis of BMN, the host range of BMNV, and countermeasures for BMN. This paper describes our results.

**Diagnostic Techniques for BMN**

BMN is a superacute communicable disease of kuruma shrimp in Japan (Sano et al., 1981). The most important factor allowing for the prompt implementation of prophylactic measures for BMN is rapid, simple and accurate diagnosis, applicable in situ on shrimp seed production farms, if possible. The following two diagnostic techniques we have developed meet these criteria.

**Fluorescent Antibody Diagnosis.** The principle of this technique depends on visualizing the specific fluorescence to the antigen of the baculovirus in the affected midgut gland smear or in the intestinal epithelium. The fluorescent antibody (FA) staining of the smear or the organ reveals that the number of juveniles showing ubiquitously visible fluorescence tends to increase with time after inoculation with the virus. Visible fluorescence in the nuclei of the epithelial cells was recognized at 18 h postinoculation (Sano et al., 1985).

**Dark Field Microscopic Diagnosis.** This diagnosis confirms nuclear hypertrophy of the midgut gland epithelial cells, which is pathognomonic for BMN virus infection, in fresh squash preparations under dark field illumination equipped with a wet-type condenser. Two to four days postinoculation at 25 - 30°C incubation temperature were considered to be satisfactory during the infectivity trial (Momoyama and Sano, 1988).

**Host Range of BMNV**

To determine the host range of BMNV, susceptibility trials were performed on
the larval stages of five species of crustaceans: giant tiger prawn (*Penaeus monodon*), fleshy prawn (*P. chinensis*), green tiger prawn (*P. semisulcatus*), greasy-back shrimp (*Metapenaeus ensis*), and blue crab (*Portunus trituberculatus*). BMNV was inoculated in accordance with the water-borne method (Momoyama and Sano, 1988). Giant tiger prawns developed severe infections (mortality), both the fleshy prawns and green tiger prawns had temporary infections (no mortality), and both the greasy-back shrimp and blue crabs were not infected with BMNV. Hence, the host range of BMNV includes *Penaeus monodon*, *P. chinensis* and *P. semisulcatus*.

**Countermeasures for BMN**

In order to establish realistic countermeasures for BMN, the virucidal effects of chemical and physical agents were studied. The results obtained are as follows.

**Virucidal Effects of Chemicals on BMNV.** BMNV is inactivated by exposure for 10 min at 25°C with the following chemicals and concentrations: 5-ppm active principle concentrations of chlorine; 25-ppm active principle concentration of iodine; 100-ppm benzalkonium chloride; 100-ppm benzethonium chloride; 0.5% Formalin; and 30% ethanol. Also, the virus is inactivated with the following treatments: ethyl ether for 18 h, at 4°C; 25% NaCl solution within 10 h and 12.5% NaCl solution within 24 h (Momoyama, 1989a; 1989c).

**Virucidal Effect of Physical Factors on BMNV.** BMNV is inactivated with ultraviolet irradiation of 4.1 x 10^5 μW x s/cm^2; summer sunlight exposure for 3 h at about 30°C; heating at 45°C for 120 min, at 50 and 55°C for 30 min, and at 60°C for 5 min; drying within 1.5 h at about 30°C (Momoyama, 1989b).

**Sterilizing Effects of Egg-washing on BMNV.** A prophylactic effect can be achieved by pouring enough sea water over the fertilized eggs of the shrimp or by soaking the eggs several times in an egg-washing tank. As a result of applying this measure in situ at a hatchery in Yamaguchi Prefecture, the incidence of BMNV has dwindled year by year since 1985 (Table 1). Figure 1 illustrates the collecting procedure for the fertilized eggs of shrimp.

**Discussion and Conclusions**

The two diagnostic methods we developed are simple, rapid and inexpensive, facilitating the diagnosis of BMN at hatcheries or in the field. Using these techniques, the virucidal effects of chemical and physical agents for BMNV were elucidated, and the host range of BMNV was determined. Furthermore, a preventive method for BMN, egg-washing, was developed and successfully prevented BMN in hatcheries where it has been implemented. As shown in Table 1, BMN has not occurred since 1987. Consequently, we determined that it was unnecessary to develop highly sensitive diagnostics such as a baculoviral DNA probe.
Figure 1. General procedure for collecting and washing shrimp eggs prior to incubation. (1) Broodstock spawn at night in spawning tank. The next day the spawner is removed, aeration is halted, and the eggs sink while the upper portion of sea water is drained. (2) The eggs are collected in a collection tank by means of a bottom drain pipe with gentle flowing sea water. (3) The eggs are collected with an outer 100 μm mesh net after they are first filtered through the inner 300 μm mesh net. (4) The eggs are washed with sterile sea water or by changing the water in the egg washing tank several times. The temperature of the rinsing water and the water in the egg washing tank should be the same as that in the spawning tank. (5) The eggs are incubated in a rearing tank with 40 - 50 cm-deep sea water that has been thermo-controlled and fertilized.
Baculoviral infection poses a danger to natural resources of penaeid shrimp. The most effective way to prevent the threat of BMNV is to exterminate the virus at the spot of a preliminary outbreak by virucidal measures. The recommended chemicals are chlorine and iodine, applied in the manner and doses indicated in this paper. Recently, Batts et al. (1991) reported that a 7.5-s exposure to a free iodine concentration of 0.14 mg/L inactivated 99.9% of infectious hematopoietic necrosis virus, suggesting that the water-borne route of virus transmission can be blocked by adding low iodine concentrations to the water supplies of hatcheries. However, whether these shorter exposure times and lower doses can also inactivate BMNV remains to be determined.

### Table 1. Incidence of BMN in a kuruma shrimp hatchery in Yamanaguchi Prefecture, 1982 to 1989.

<table>
<thead>
<tr>
<th>Year</th>
<th>No. of tanks examined</th>
<th>No. of BMN cases</th>
<th>BMN incidence (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1982</td>
<td>42</td>
<td>28</td>
<td>66.7</td>
</tr>
<tr>
<td>1983</td>
<td>39</td>
<td>13</td>
<td>33.3</td>
</tr>
<tr>
<td>1984</td>
<td>39</td>
<td>7</td>
<td>17.9</td>
</tr>
<tr>
<td>1985</td>
<td>48</td>
<td>3</td>
<td>6.3</td>
</tr>
<tr>
<td>1986</td>
<td>49</td>
<td>4</td>
<td>8.2</td>
</tr>
<tr>
<td>1987</td>
<td>38</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>1988</td>
<td>23</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>1989</td>
<td>24</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

### Literature Cited


Momoyama, K. 1989b. Inactivation of baculoviral mid-gut gland necrosis (BMN) virus by ultraviolet irradiation, sunlight exposure, heating and drying. Fish Pathol. 24: 115-118. (In Japanese with English abstract.)

Momoyama, K. 1989c. Tolerance of baculoviral mid-gut gland necrosis virus (BMNV) to ether, NaCl concentration and pH. Fish Pathol. 24: 175-177. (In Japanese with English abstract.)


Part II:

Contributed Papers -

Viral Diseases
Infection Route and Eradication of *Penaeus monodon* Baculovirus (MBV) in Larval Giant Tiger Prawns, *Penaeus monodon*

Chen, S.N., P.S. Chang and G.H. Kou
Department of Zoology, National Taiwan University
Taipei, Taiwan

Abstract

Our experiments showed that *Penaeus monodon* baculovirus (MBV) was transmitted orally. MBV may be eliminated from hatcheries by using MBV-negative broodstock or by washing nauplii or fertilized eggs several times in clean sea water, Formalin and iodophore.

**Introduction**

Of the six pathogenic viruses identified from penaeid shrimp, *Penaeus monodon* baculovirus (MBV) (Fig. 1), a nuclear polyhedrosis virus, is considered one of the most potentially serious pathogens to the larval stages of shrimp.

MBV has been found in shrimp from the Indo-Pacific and Pacific Coasts of Asia, Australia, Africa, and southern Europe and in North and South America (Anderson et al., 1987, Brock et al., 1983; Chen et al., 1989a; Lightner, 1983, 1988; Lightner et al., 1983, 1987, 1988). This virus infects a number of species of shrimp, including *P. vannamei*, *P. monodon*, *P. esculentus*, *P. semisulcatus*, *P. penicillatus*, *P. kerathurus*, *P. plebejus* and *Metapenaeus ensis* (Brock et al., 1983; Chen et al., 1989a; Lester et al., 1987; Lightner and Redman, 1981; Lightner, 1983, 1988; Lightner et al., 1983, 1985, 1987, 1988). The most serious infections were found in cultured giant tiger shrimp, *P. monodon* (Chen et al., 1989c; Lightner, 1988).

Epizootiological studies on MBV in *P. monodon* revealed an incidence rate higher than 50% in postlarvae, juveniles and broodstock obtained from Taiwan (Chen et al., 1989c) and Southeastern Asia (Chen et al., 1990). Results obtained from a pathogenicity study showed that environmental stressors may significantly increase mortality in MBV-infected larvae in hatcheries (Chen et al., 1989c). A hatchery experiment also showed that MBV may initiate mortality and growth retardation in MBV-positive postlarvae.
(Chen et al., 1989c; Chang and Chen, 1992). For this reason, MBV may result in variable larval production. To obtain a better quality of postlarval *P. monodon*, MBV should be eradicated from hatcheries.

The present paper attempts to describe the pathway of MBV infection. In addition, procedures for the eradication of MBV are also suggested.

**Materials and Methods**

To investigate the infection route of *Penaeus monodon*-type baculovirus (MBV) in *P. monodon*, nauplii and fertilized eggs derived from either MBV-positive or MBV-negative broodstock were used (Table 1). All experimental broodstock were collected from the open sea in southeastern Asia and imported into Taiwan.

For the broodstock, MBV infection was confirmed by the presence of occlusion bodies in shrimp feces. These were detected with the aid of 0.1% aqueous malachite green, 1% Gram’s or Giemsa’s staining solution and Olympus IM inverted and BH-2 light microscopes. Experimental larvae were maintained in hatchery ponds, with the exception of those used in Experiment 6. Each pond contained approximately 30 MT of 28- to 30-ppt sea water. Rearing temperatures ranged from 28 to 33°C.

Experiment 6 was conducted in plastic tanks. Each tank contained 5 - 10 MT of sea water and salinities and tempera-

![Figure 1a](image1a.png) **Figure 1a.** Section of hepatopancreas of *Penaeus monodon* infected by *Penaeus monodon* baculovirus (MBV). Note: Round occlusion bodies (arrows).

![Figure 1b](image1b.png) **Figure 1b.** MBV particles.
Table 1. The incidence of MBV in larval *P. monodon* cultured under conditions indicated (in hatchery or nursery)*

<table>
<thead>
<tr>
<th>Exp. Initial</th>
<th>Water</th>
<th>Sample Source and Treatment</th>
<th>MBV Infection Rate* (Positive/No. Examined)</th>
</tr>
</thead>
<tbody>
<tr>
<td>No. Stage</td>
<td>temp.</td>
<td></td>
<td>Z1</td>
</tr>
<tr>
<td></td>
<td>range (°C)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1a</td>
<td>N</td>
<td>28-30</td>
<td>MBV(-) broodstock</td>
</tr>
<tr>
<td>1b</td>
<td>N</td>
<td>28-33</td>
<td>MBV(+) broodstock</td>
</tr>
<tr>
<td>2a</td>
<td>N</td>
<td>28-30</td>
<td>MBV(-) broodstock</td>
</tr>
<tr>
<td>2b</td>
<td>N</td>
<td>28-33</td>
<td>MBV(-) broodstock</td>
</tr>
<tr>
<td>3a</td>
<td>N</td>
<td>28-30</td>
<td>MBV(+) broodstock</td>
</tr>
<tr>
<td>3b</td>
<td>N</td>
<td>28-30</td>
<td>MBV(+) broodstock</td>
</tr>
<tr>
<td>3c</td>
<td>N</td>
<td>28-30</td>
<td>MBV(+) broodstock</td>
</tr>
<tr>
<td>3d</td>
<td>N</td>
<td>28-33</td>
<td>MBV(+) broodstock</td>
</tr>
<tr>
<td>3e</td>
<td>N</td>
<td>28-33</td>
<td>MBV(+) broodstock</td>
</tr>
<tr>
<td>4a</td>
<td>N</td>
<td>28-30</td>
<td>MBV(+) broodstock</td>
</tr>
<tr>
<td>4b</td>
<td>N</td>
<td>28-30</td>
<td>MBV(+) broodstock</td>
</tr>
<tr>
<td>4c</td>
<td>N</td>
<td>28-30</td>
<td>MBV(+) broodstock</td>
</tr>
<tr>
<td>4d</td>
<td>N</td>
<td>28-30</td>
<td>MBV(+) broodstock</td>
</tr>
<tr>
<td>4e</td>
<td>N</td>
<td>28-33</td>
<td>MBV(+) broodstock</td>
</tr>
<tr>
<td>4f</td>
<td>N</td>
<td>28-33</td>
<td>MBV(+) broodstock</td>
</tr>
<tr>
<td>4g</td>
<td>N</td>
<td>28-33</td>
<td>MBV(+) broodstock</td>
</tr>
<tr>
<td>5a</td>
<td>N</td>
<td>28-30</td>
<td>MBV(+) broodstock</td>
</tr>
<tr>
<td>5b</td>
<td>N</td>
<td>28-33</td>
<td>MBV(+) broodstock</td>
</tr>
<tr>
<td>6a</td>
<td>E</td>
<td>28-30</td>
<td>MBV(+) broodstock</td>
</tr>
<tr>
<td>6b</td>
<td>E</td>
<td>28-33</td>
<td>MBV(+) broodstock</td>
</tr>
<tr>
<td>6c</td>
<td>E</td>
<td>28-33</td>
<td>MBV(+) broodstock</td>
</tr>
<tr>
<td>7</td>
<td>N</td>
<td>28-30</td>
<td>MBV(+) broodstock</td>
</tr>
</tbody>
</table>

* The experiments were performed in cement ponds in hatchery with approximately 30-40 MT of water, except those in plastic tanks.

* In every experiment, all the PL11 were moved to nursery ponds except those cultured in plastic tanks.

* The experiment was performed in a plastic tank with approximately 5-10 MT of water.

* The results were obtained from histopathological observations of hepatopancreas.

* The procedures for the treatment of nauplii and fertilized eggs are described in Table 3.

--- Not done

E: Fertilized egg stage  N: Nauplius stage  Z: Postzoeal stage  M: Mysis stage  P: Postlarval stage  MBV(-): Free of MBV  MBV(+): Positive for MBV
tures similar to those described above. Approximately 7,000 - 10,000 nauplii or fertilized eggs per ton were placed into each tank.

MBV infections in the hepatopancreata of developing stages of *P. monodon* larvae from zoea 1 to PL 40 were studied using tissue squashes and histopathological staining with 0.1% aqueous malachite green and hematoxylin and eosin (H&E), respectively, as described by Lightner (1983). For each stage, 5 - 32 randomly selected larvae were examined, and their MBV status was recorded.

To detect the mode of MBV transmission, nauplii derived from MBV-free broodstock were reared (in a similar tank) with the excrement from MBV-positive shrimp. The MBV status of these shrimp in the later stages was determined using the technique described above.

The eradication of MBV infection was achieved by the short-term washing of nauplii and fertilized eggs using filtered sea water with salinity of 28 - 30 ppt, 200 - 300 ppm Formalin and 20 - 30 ppm iodophore, as described in Figure 2.

During the course of larval rearing, aeration was continuous and the alga *Skeletonema costatum* was provided, as were artificial feeds (shrimp flake, plankton powder and micro-encapsulated feed, rotifers or brine shrimp larvae), to ensure a sufficient feed supply and complete growth of the experimental larvae. Feed debris and shrimp excrement were removed daily, and one-third of the sea water in each tank was exchanged at two-day intervals.

## Results and Discussion

### Pathway of MBV Infection

*Penaeus monodon* larvae produced from either MBV-positive or MBV-negative broodstock were examined histopathologically. The results in Table 1 and Figure 3 (Experiments 1 and 3) show that larvae produced from uninfected broodstock had no signs of infec-

(A) Nauplii

| Collect nauplii in plankton net | Running sea water 1 - 2 min | Formalin 300 ppm 30 s | Iodophore 50 ppm 30 s | Running sea water 1 - 2 min | Hatchery ponds |

(B) Fertilized Eggs

| Collect fertilized eggs | Running sea water 1 - 2 min | Formalin 200 ppm 30 s | Iodophore 20 ppm 30 s | Running sea water 1 - 2 min | Hatchery ponds |

*Figure 2. Procedures for the eradication of MBV in *P. monodon* hatcheries. Note: In a hatchery, nauplii are much easier to collect than fertilized eggs. Furthermore, fertilized eggs are much more sensitive to Formalin than nauplii are.*
Figure 3. Summary of the results obtained in Table 1. MBV status of larvae was determined by histopathological observation of hepatopancreases. For broodstock, MBV status was determined by examination of feces.
tion while reared in the hatchery at 28 - 33℃. However, when these larvae were moved to a nursery pond, MBV-positive individuals were found (Table 1 and Fig. 3, Experiments 1 and 3). When nauplii or fertilized eggs derived from MBV-infected broodstock were reared in the hatchery, MBV-positive shrimp were discovered at the mysis or postlarval (PL) stage, respectively (Table 1 and Fig. 3, Experiment 2).

MBV-infected PL2 were also found when nauplii produced from MBV-positive broodstock were reared in plastic tanks (Table 1, Experiment 6c). However, larvae produced from MBV-free broodstock reared in plastic tanks containing filtered water revealed no sign of infection up to the PL40 stage (Experiments 6a and b).

When nauplii produced from MBV-free broodstock were exposed to MBV-positive feces, MBV was detected at the mysis stage (Table 1 and Fig. 3, Experiment 5).

Although no one has found evidence of vertical transmission of MBV in shrimp larvae, the present study shows that MBV may be transmitted by oral ingestion of occluded or free MBV virions. Baculoviruses were also found to be transmitted orally in P. duorarum (Couch, 1974) and P. japonicus (Momoyama, 1981; Momoyama and Sano, 1989).

The above experiments also suggest that oral ingestion of feces from MBV-positive shrimp is the main source of MBV infection. If this is true, eradication of MBV at the hatchery level may be feasible; hence, experiments were conducted to determine the best means of eliminating MBV from P. monodon hatcheries.

Eradication of MBV Infection

To eradicate MBV infection in larval shrimp, various washing procedures were tested. When nauplii and fertilized eggs obtained from MBV-infected broodstock were treated as described in Figure 2, no MBV-positive larvae were found in the hatchery pond (Table 1 and Fig. 3, Experiment 4). One day after the larvae were moved from the hatchery to the nursery pond, however, MBV was diagnosed in some of the larvae. In contrast, MBV was not detected in the nauplii derived from MBV-free broodstock.

These results also support the hypothesis that MBV was transmitted by oral ingestion of MBV virions. Furthermore, they reveal that in a controlled environment, MBV can be eliminated by washing nauplii or fertilized eggs thoroughly with filtered sea water, 200 - 300 ppm Formalin and 20 - 50 ppm iodophore.

Our results also showed that washing with filtered sea water alone may only reduce the rate of MBV infection rate in larval shrimp (Experiment 7). Since it is easier to collect nauplii than fertilized eggs, and because the latter are much more sensitive to the chemicals employed, commercial hatcheries should treat nauplii instead of fertilized eggs.
In conclusion, there are two ways to eliminate MBV from hatcheries, thereby significantly improving the quality of *P. monodon* larvae:

- Use only MBV-free broodstock, and

- Wash fertilized eggs or nauplii with filtered sea water, Formalin and iodophore.

Studies on baculoviral midgut gland necrosis (BMN) virus in *P. japonicus* also showed that viral infection may be eradicated by eliminating broodstock excrement followed by washing fertilized eggs with clean sea water (Momoyama, 1991).

Pathogenic studies showed that in *P. monodon*, MBV infection may initiate damage or loss of hepatopancreatic tubule and midgut tissue leading to dysfunction of these organs or tissues (Chen et al., 1989b; Lightner et al., 1983). Consequently, molting was delayed so that the MBV-infected postlarvae were irregular in body size (Chang et al., 1992). It was also noted that larval shrimp infected with MBV contain fewer hepatopancreatocytes than juvenile or adult shrimp; destruction of these cells may cause a serious disease or mortality. In comparison with control shrimp, MBV-infected larvae showed relatively high mortality rates (Chang et al., 1992; Chen et al., 1989c).

Our recent study also confirmed that juvenile and adult *P. monodon* are more resistant to MBV than larval shrimp (Chang, 1992). These results, when considered in light of the high incidence rate of MBV among cultured shrimp in the world (Chen et al., 1989c, 1990; Lightner et al., 1985) suggest that the eradication of MBV in hatchery-reared larvae and the production of MBV-free larvae are important. We have also reached the conclusion that to produce shrimp larvae of superior quality, a broodstock quarantine system for MBV infection should be established, and the development of eradication measures for MBV should be emphasized.

**Acknowledgments**

This work was supported by a grant from the U.S. Department of Agriculture (Grant No. FG-TA-111) and the Council of Agriculture and the National Science Council (Grant No. NSC-81-0209-B002-05) of the Republic of China.

**Literature Cited**


Chang, P.S. and S.N. Chen. 1992. Effect of *Penaeus monodon*-type baculovirus (MBV) on
survival and growth of larval Penaeus monodon. Submitted for publication to Aquaculture.


Abstract

About 900 million juveniles of approximately 20 crustacean species are produced for sea farming and pond culture every year in Japan. *Penaeus japonicus*, the kuruma shrimp, is the most important species, comprising approximately 90% of all juveniles and nearly 100% of pond cultured crustaceans produced annually. Two viral diseases, baculoviral midgut gland necrosis (BMN) of *P. japonicus* and *Penaeus monodon* baculovirus (MBV) infection of *P. monodon*, the grass shrimp, have been recorded from these species in Japan. BMN is a severe infectious disease causing high mortalities in hatcheries. MBV, by contrast, has been detected only once in postlarvae that were produced for experimental purposes from spawners imported from Taiwan. The research conducted in Japan on BMN and MBV is reviewed in this paper.

Introduction

Since the adoption of the 200-mile economic zone system in many nations, sea farming is now strongly encouraged to increase production of coastal fisheries resources in Japan. Every year about 700 million juvenile crustaceans belonging to approximately 20 species are produced at public sea farming centers for stocking into coastal waters (Japan Sea Farming Association, 1990). The main species produced are *Penaeus japonicus*, the kuruma shrimp (84%), *Portunus trituberculatus*, the blue crab (7%), and *Metapenaeus ensis*, the greasy-back shrimp (7%) (Table 1). Three other penaeid shrimp, *Penaeus semisulcatus*, the green tiger shrimp, *P. chinensis*, the fleshy shrimp and *P. latisculatus*, the western king shrimp, are also produced for sea farming in certain regions.

Although juveniles of many crustacean species are produced for sea farming, *P. japonicus* is the only species reared to edible size in ponds in Japan. Since 1988, the number of seed *P. japonicus* imported from Taiwan for pond culture has increased substantially. Other penaeid shrimp, including non-native species, have never been cultured nor have they been imported, except for very rare experimental cases.

The annual production of pond-cultured *P. japonicus* from 1988 to 1990 was almost 3,000 MT, and about 200 million
Table 1. Crustacean species and number of juveniles produced for sea farming and pond culture in Japan in 1988.

<table>
<thead>
<tr>
<th>Crustacean species</th>
<th>Sea farming</th>
<th></th>
<th>Pond culture</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>No. (million)</td>
<td>Percent</td>
<td>No. (million)</td>
<td>Percent</td>
</tr>
<tr>
<td>Shrimp</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>Penaeus japonicus</em></td>
<td>557.1</td>
<td>83.8</td>
<td>194.2</td>
<td>99.9</td>
</tr>
<tr>
<td><em>Penaeus semisulcatus</em></td>
<td>9.4</td>
<td>1.4</td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>Penaeus chinensis</em></td>
<td>1.0</td>
<td>0.2</td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>Metapenaeus ensis</em></td>
<td>44.6</td>
<td>6.7</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Others (4 species)</td>
<td>0.3</td>
<td>0.0</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Crabs</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>Portunus trituberculatus</em></td>
<td>48.5</td>
<td>7.3</td>
<td>0.2</td>
<td>0.1</td>
</tr>
<tr>
<td><em>Portunus pelagicus</em></td>
<td>2.3</td>
<td>0.3</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Others (6 species)</td>
<td>1.7</td>
<td>0.3</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td>664.9</td>
<td>100</td>
<td>194.4</td>
<td>100</td>
</tr>
</tbody>
</table>

*Penaeus japonicus* juveniles are produced annually at public and private hatcheries for pond culture.

Because it is the major crustacean species used for sea farming and pond culture in Japan, research on crustacean diseases has focused on *P. japonicus*. To date, two viruses have been recorded in crustaceans in Japan. One is baculoviral midgut gland necrosis virus (BMNV) in *P. japonicus* (Sano et al., 1981); the other is *Penaeus monodon* baculovirus (MBV) in *P. monodon* (Fukuda et al., 1988). For many years, BMNV caused serious losses during the production of juvenile *P. japonicus*.

MBV, first detected in Japan, is thought to have originated in a foreign country. After the initial discovery, MBV was never found again in Japan, probably because it is not infectious to postlarval *P. japonicus*, and very few *P. monodon* are cultured in Japan. One reason only two penaeid shrimp viruses have been found in Japan may be that Japan rarely imports live shrimp from foreign nations. Another factor is the lack of comprehensive viral disease surveys. The current practice of importing large numbers of seed *P. japonicus* without screening for viruses, however, will allow foreign viruses to spread easily in Japan. IHHN virus is the most dangerous of the potential viral introductions and also the most likely to be imported. It is highly pathogenic to *P. japonicus* (Lightner, 1985) and it exists in Taiwan (Lightner et al., 1987), from which many seed *P. japonicus* are imported.

Research on penaeid shrimp viruses in Japan has dealt almost entirely with BMN (Momoyama, 1991) — only one paper was published on MBV (Fukuda et al., 1988). This paper summarizes the results of the studies on BMN of *P. japonicus* and MBV infection of *P. monodon* in Japan.
Baculoviral Midgut Gland Necrosis (BMN)

Outbreaks and Mortalities

BMN was first noticed at a private hatchery in Yamaguchi Prefecture in 1971. In 1972, the disease affected most hatcheries, causing drastic mortalities in the district. Since then, BMN has repeatedly caused 90% or higher mortalities during the mass production of P. japonicus seed in Japan (Momoyama, 1981).

Recently, the frequency of BMN outbreaks has decreased significantly, probably as a result of preventive measures now employed at hatcheries (Momoyama, 1991). BMN is no longer a serious problem in the mass production of P. japonicus juveniles, although a few outbreaks are still reported every year.

Histopathology

Diseased shrimp lose their appetite, grow slowly, swim weakly, and develop soft and white turbid midguts at the advanced stage of infection. Shrimp dying of BMNV infection are usually less than 9.0 mm in body length (Momoyama, 1981).

Histological examinations confirmed that the midgut and the intestine are the target organs. Disarrangement and exfoliation of epithelial cells are remarkable in the midguts of diseased shrimp. Nuclear hypertrophy and chromatolysis of infected epithelial cells are the most characteristic cytopathological changes of BMN (Momoyama, 1981; Sano et al., 1981). Occlusion bodies are not formed in the hypertrophied nuclei, differentiating BMNV from other penaeid shrimp baculoviruses (Couch, 1974; Lightner, 1985; Lester et al., 1987; Johnson and Lightner, 1988).

Electron micrographs of the hypertrophied nuclei and the midgut lumen reveal numerous rod-shaped particles having outer and inner envelopes; these are baculovirus virions. The average length and diameter of the virions is 310 nm and 72 nm, respectively (Sano et al., 1981; Sano et al., 1984).

Vibrios often invade and grow in the midgut lumen of moribund shrimp (Momoyama, 1981). They must play an important role in killing BMNV-infected shrimp, but numerous hatchery trials with antibiotics were unsuccessful.

Diagnosis

Three diagnostic techniques have been developed for BMN. Squash and stained preparation diagnosis is used to detect the homogeneous hypertrophied nuclei in squashed and stained preparations of the affected midgut. The Feulgen reaction makes the difference between normal nuclei (about 10 μm in diameter) and infected hypertrophied nuclei (about 20 to 30 μm in diameter) clearer (Momoyama, 1983).

Dark field microscopic diagnosis is used to detect infected hypertrophied nuclei
in fresh squash preparations using a dark field microscope with a wet-type condenser. Infected nuclei are clearly seen in white against the dark background due to the increased reflected and diffracted rays produced by the numerous virus particles in the nuclei (Momoyama, 1983). Because this method has the advantages of simplicity, rapidity, precision and low cost, it is the only diagnostic method used in shrimp hatcheries.

**Fluorescent antibody diagnosis** is used to detect BMN-specific virus antigen in smears or sectioned preparations of the midgut. Sano et al. (1985) demonstrated BMNV infection in postlarvae 18 h after inoculation by detecting fluorescence in the nuclei of the midgut epithelial cells. The method was also used to demonstrate the presence of BMNV in the midguts of spawners latently infected with the virus (Momoyama, 1988).

**Source of Infection**

Epizootiological investigations have indicated that spawners with latent BMNV infections may be the main source of infection in hatchery epizootics. Histological examinations revealed nuclear hypertrophy of the midgut epithelial cells in three out of 70 spawners examined. Fluorescent antibody techniques revealed the presence of BMN-specific virus antigen in the hypertrophied nuclei of the spawners (Momoyama, 1988).

Furthermore, histological examination of the midguts of young *P. japonicus* that had recovered from BMN and were then cultured at a farm showed a high rate (31.4%) of BMNV infection (Momoyama, 1988). Regular or frequent BMN outbreaks on farms where BMN survivors are cultured suggest that these shrimp are a source of infection to larvae.

**A Method of Experimental Infection**

Since there is no cell line available for penaeid shrimp virus culture, a reliable method to experimentally infect larval *P. japonicus* with BMNV has been developed to facilitate studies on BMN. Water-borne inoculation using the filtrate of diseased postlarvae stored at -80°C is one means of infection. The virulence of material frozen at -80°C persists at almost the same level for at least seven years (Momoyama, 1989a). Demonstration of infection in test animals is accomplished by dark field microscopy four days postinoculation (Table 2) (Momoyama and Sano, 1988).

**Susceptible Stages**

The relationship between age and susceptibility to BMNV was studied using the infection method described above.

Fertilized eggs and nauplii were refractory to the virus, showing no evidence of infection on the final day of the infection challenge. The zoea, mysis larvae, PL2 (two day-old postlarvae) and PL4 were “highly susceptible” to infection with BMNV, exhibiting higher mortality and lower growth rates compared to control shrimp. PL6 were classified as “susceptible;” they grew only
Table 2. A method for experimentally infecting larval *P. japonicus* with BMNV.

| Inoculum: 450 nm filtrate of BMNV-infected postlarvae (stored at -80°C) |
| Test shrimp: Mysis larvae (zoa to PL4 are also available) |
| Inoculation: Water-borne for two hours |
| Test period: Four days |
| Demonstration of infection: Dark field microscopic diagnosis |
| Rearing water temperature: 25 - 30°C |
| Food: Brine shrimp nauplii |

slightly slower than controls. PL8 and PL10, by contrast, were refractory, exhibiting no mortality and no loss of growth, although some animals developed slight infections (Sano et al., 1985; Momoyama and Sano, 1989).

The route of infection with baculoviruses in shrimp is considered to be by oral ingestion of virus-contaminated sediments or by cannibalism of diseased shrimp (Couch, 1978; Lightner et al., 1983). Overstreet et al. (1988) established experimental infections in larval and postlarval *P. vannamei*, the whiteleg shrimp, with *Baculovirus penaei* (BP) by feeding virus-laden rotifers or brine shrimp. In the infection trials using *P. japonicus* and BMNV, food was not added to the water thus, the animals could not feed during the inoculation period. However, peristaltic movements, which are frequently observed in the esophageal region of shrimp, suggest the intake of sea water containing BMNV particles through the mouth. This hypothesis is supported by the observation that azocarmine G accumulated in the stomach and intestinal lumen of shrimp placed in sea water containing this dye (Momoyama and Sano, 1989). If the concentration of the virus in the water is high enough, virus-laden food may not be necessary to establish baculoviral infections.

**Infection Cycle of BMNV**

Based on the results obtained in the epizootiological and water-borne susceptibility studies, the following infection cycle was proposed (Fig. 1) (Momoyama, 1991). Some survivors recover from BMNV disease and reach maturity without entirely eliminating the virus from the body (A). BMNV grows in the nuclei of the midgut epithelial cells of these broodstock (B). Then the virus particles are excreted into the environmental water with feces and collapsing cells after being released into the lumen of the midgut tubules from the necrotic epithelial cells (C). Shrimp older than nauplius become infected with the virus by orally ingesting it (D). If the shrimp are between the zoa and PL6 stages (E), even if a few shrimp live normally by defeating the virus attack (F), most shrimp become diseased (G) and some will die (H). If the shrimp are older than PL6 (I), most shrimp live normally with a slight infection (J). Some of the recovered shrimp (K) as well as the slightly infected shrimp (F,
J) grow up to be the source of the next infection (L), completing the cycle (M). Some instances of BMN outbreaks were caused by contamination from other rearing tanks in the hatchery (N).

**Inactivation and Survival of BMNV**

Inactivation of BMNV by chemical and physical factors, and survival time of the virus in sea water at different temperatures were examined by means of water-borne infectivity experiments. BMNV was inactivated by 10-min exposure at 25°C to any of the following disinfectants: 5-ppm chlorine; 25-ppm iodine; 100-ppm benzalkonium chloride and benzethonium chloride; 30% ethyl alcohol; and 0.5% formalin (Momoyama, 1989b). The virus was also inactivated with the following chemical treatments: ethyl ether for 18 h at 4°C; NaCl, 25% solution within 10 h and 12.5% within 24 h; pH 1.0 within 10 min, pH 1.5 and 2.0 within 30 min, pH 2.5 within 60 min, and pH 3.0 and 4.0 within 180 min (Momoyama, 1989c).

With regards to physical factors, BMNV was inactivated by: ultraviolet irradiation of $4.1 \times 10^5 \, \mu W \times s/cm^2$; summer sunlight exposure for 3 h; heating at 45°C within 120 min, 50 and 55°C within 30 min, and 60°C within 5 min (Momoyama, 1989d). In sea water, BMNV could not survive longer than 4 d at 30°C, 7 d at 25°C, 12 d at 20°C, and 20 d at 15°C (Momoyama, 1989a).

BMNV appears to be much more sensitive to chemicals and physical stresses than insect baculoviruses (Aruga, 1979),

![Figure 1. Infection cycle of BMNV.](image-url)
probably because BMNV lacks occlusion bodies. In addition, BMNV presumably evolved in the marine environment, which is more constant than conditions on land.

Prevention

Referring to the proposed infection cycle for BMN, two countermeasures are used against BMN epizootics in Japan. One prevents transmission via broodstock feces by rinsing the fertilized eggs with virus-free sea water and transferring them to a disinfected rearing tank. The other prevents infection from previous batches of shrimp by addition of 20-ppm chlorine to the rearing tank to disinfect the water, etc. and to kill infected populations of shrimp. Since 1985, egg rinsing has been conducted on an industrial scale, and BMN has never occurred in hatcheries where this precaution is taken (Momoyama, 1991).

MBV Infection of P. monodon

Detection of MBV In Japan

In 1983, a P. monodon seed production experiment was conducted at a private hatchery in Yamaguchi Prefecture using broodstock imported from Taiwan. The resulting postlarvae appeared healthy and exhibited no external clinical signs. However, light and electron microscopy revealed the presence of MBV in the midgut epithelial cells of the postlarvae. Because of the prevalence of MBV in Taiwan (Lightner et al., 1987) and the scarcity of P. monodon along the coast of Japan (Hayashi, 1981), Fukuda et al. (1988) concluded that the virus had been introduced from Taiwan with the spawners.

Pathogenicity of MBV to Postlarval P. japonicus

The pathogenicity of MBV to P. japonicus PL1 was examined by water-borne and oral inoculation infectivity trials. Since postlarvae did not show any evidence of infection such as 1) the formation of nuclear occlusion bodies in the midgut epithelial cells, 2) lack of growth, or 3) significant mortality by either inoculation method, P. japonicus was judged to be refractory to MBV (Fukuda et al., 1988).

Literature Cited