Mortality in Pond-Cultured Shrimp *Penaeus monodon* in the Philippines Associated with *Vibrio harveyi* and White Spot Syndrome Virus

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ABSTRACT—Heavy mortalities were observed among pond-cultured *Penaeus monodon* in the provinces of Bohol, Misamis Occidental, Lanao del Norte and Zamboanga del Sur, Philippines. *Vibrio harveyi* was isolated purely from the hepatopancreas and lymphoid organs of affected shrimp and histopathological observations indicated a severe bacterial infection in the shrimp. Majority of the samples gave negative results in the one-step PCR for the detection of white spot syndrome virus (WSSV). However, nested PCR produced amplicons specific for WSSV DNA from most of the shrimp tested. These results suggest that shrimp were infected dually with *V. harveyi* and WSSV, but the major causative agent of the present mortalities was *V. harveyi*.

Key words: *Penaeus monodon*, *Vibrio harveyi*, WSSV, mixed infection, luminescent vibriosis, white spot syndrome

Luminescent vibriosis due to *Vibrio harveyi* has been reported to cause mass mortalities in *Penaeus monodon* reared in hatcheries and grow-out culture farms^{1,2}. Infected shrimps are usually found near the edge of ponds, weak and anorectic. During the night, heavily infected shrimps can be seen in the dark emitting a greenish light due to the massive number of luminescent vibrios inside the body.

White spot syndrome (WSS) is one of the most devastating viral diseases and causes serious epizootics and high mortality rate in cultured penaeid shrimp in Asia

and Americas. Although previous efforts³ reported the absence of White spot syndrome virus (WSSV) from cultured or wild *P. monodon* in the Philippines, the disease has recently been confirmed to be present in the country⁴.

During the first quarter of 1999, mortalities were observed in shrimp farms in various provinces in the Philippines such as Bohol in the Visayas, Misamis Occidental, Lanao del Norte and Zamboanga del Sur in Mindanao. In this paper, we report the dual infection with the luminescent *Vibrio* and WSSV in cultured *P. monodon* in the Philippines.

Materials and Methods

Sampling of diseased shrimp: During the first quarter of 1999, high mortality rate was experienced in one of the shrimp farms in Bohol. The post larvae were purchased from a private hatchery without screening of WSSV. The farm was practicing intensive culture system with a stocking density of 25 shrimp/m². Initial signs observed at 30 to 89 days of culture, depending on the pond, was a sudden drop in feed consumption. Shrimp were observed to stay near the edge of the ponds and some emitted greenish light during the night.

Twenty weak shrimp swimming near the edge of two infected ponds with an average body weight (ABW) of 0.4 g and 14 g were collected and immediately processed on site for bacterial isolation. Bacteria were isolated from the hepatopancreas and lymphoid organs while the gills and epidermis of the same shrimp sampled for bacterial analysis were fixed in 95% ethanol for DNA extraction.

In the 3 provinces of Mindanao, 7 affected farms were practicing extensive culture system with a stocking density of 3–5 shrimp/m². High mortality and slow growth rates were also observed in the affected farms. Three to 5 weak shrimp from each farm with ABW of 5 g to 10 g were collected and processed as above.

Bacteriological tests: Bacteria were isolated from weak shrimp by inoculating samples on Nutrient Agar (NA: BBL) supplemented with 1.5% NaCl and Thiosulphate Citrate Bile Salt Sucrose Agar (TCBS: BBL). After 18–24 h incubation at room temperature (28°C), pure colonies were selected, purified and maintained frozen at –80°C in Nutrient Broth (NB: BBL) supplemented with 1.5% NaCl and 10% glycerol. Morphological, physiological and biochemical characterization tests and identification were conducted on these isolates as before². The representative isolates were also subjected to slide agglutination test using rabbit antiserum raised against *V. harveyi* (PN-9801) isolated in our previous study².

Histopathological tests: Ten shrimp samples from

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each pond in a farm in Bohol and 5 from each farm in Mindanao were preserved in Davidson's fixative. Hepatopancreas was removed and processed routinely and stained with hematoxylin and eosin.

Detection of WSSV by PCR: DNA was extracted from the gills and epidermis taken from 30 shrimp samples (15 shrimp each from Bohol and Mindanao) following the method described by Nonaka *et al.* (1998)⁵ with slight modification. The tissues from 5 shrimp were pooled as 1 sample. The DNA samples were submitted to one-step and nested PCR tests using the WSSV-specific primer pairs designed by Kimura *et al.* (1996)⁶.

Results

Bacterial isolation and identification: Pure luminous bacterial colonies were isolated from the hepatopancreas and lymphoid organs of all the shrimps tested. The presumptive *Vibrio* count on TCBS was 10^5 cfu/hepatopancreas and both the total plate and luminous bacterial counts on NA exceeded 10^6 cfu/hepatopancreas. Ten representative luminous bacterial isolates from the hepatopancreas were examined for their morphological, physiological, and biochemical characteristics. Results of detailed characterization tests led to the identification of these isolates to *V. harveyi* as previously reported². The identity of representative isolates was also confirmed by slide agglutination test to be of the same serotype to *V. harveyi* isolated in our previous work².

Gross signs and histopathology: At the time of sampling, no macroscopic white spots were observed on the body of affected shrimp. Histopathological examination did not reveal signs of infection with WSSV, *Penaeus monodon*-type baculovirus (MBV) or Hepatopancreatic parvovirus (HPV) infections. However, the histological

Fig. 1. Histological section of hepatopancreas of infected shrimp from a farm in Bohol showing chronic inflammatory lesions in the form of melanized hemocytic nodules in the intertubular spaces. H & E stain, bar = 50 μ m.

Fig. 2. Histological section of hepatopancreas of infected shrimp from a farm in Mindanao showing chronic inflammatory lesions in the form of melanized hemocytic nodules. H & E stain, bar = 50 μ m.

sections of hepatopancreas showed clear evidence of inflammatory lesions in the form of hemocytic aggregation, nodule formation and melanization (Figs. 1 and 2).

One-step and nested PCR: Electrophoresis of the PCR products of first step amplification did not reveal the presence of WSSV DNA (982 bp product) in all the samples except 2 pooled samples of the gills and epidermis from Mindanao. However, in the nested PCR, clear amplification (570 bp product) was obtained in all the samples except 1 pooled epidermis sample from Bohol (Fig. 3).

Fig. 3. Agarose gel electrophoresis of PCR amplification products of WSSV. Lanes: (1) ϕ $\times$ 174-*Hae* III DNA marker; (2–3) positive controls; (4–6) pooled gill samples from Mindanao; (7–9) pooled epidermis samples from Mindanao; (10–12) pooled epidermis samples from Bohol; (13–15) pooled gill samples from Bohol; (16) negative control.

Discussion

This study confirmed the presence of dual *V. harveyi* and WSSV infection in affected shrimp. There are few reports that correlate bacterial infection with WSSV infection in penaeid shrimp. *Vibrio alginolyticus* was isolated from the hemolymph of moribund *P. monodon* during the outbreaks of WSSV infection in Taiwan⁷, and various *Vibrio* species were isolated from *P. monodon* infected

with WSSV in India⁸⁾. *Vibrio penaeicida* was isolated from PAV (=WSS) infected *P. japonicus* in Japan with varying incidences⁹⁾. There have been no detailed analysis on the role of the two pathogens, viral and bacterial, in those mortalities. However, bacteria were usually considered as secondary invaders⁸⁾.

In the present study, *V. harveyi* was the only bacterial species isolated with bacterial count exceeding 10⁶ cfu/hepatopancreas. This bacteriological result is further highlighted by the histopathological changes in hepatopancreas which appear as severe inflammatory lesions¹⁰⁾. Nested PCR produced amplicons specific for WSSV DNA from most of the shrimp tested. However, the majority of these samples gave negative results during the one-step PCR. These results suggest that the causative agent of the present mortalities was *V. harveyi*. However, as to whether WSSV infection has any effect on *V. harveyi* infection, as a predisposing factor, requires to be elucidated by carefully-planned infection experiment.

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