魚病研究 Fish Pathology, 30 (1), 39-45, 1995. 3

Dynamics of *Vibrio* sp. PJ in Organs of Orally Infected Kuruma Prawn, *Penaeus japonicus*

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(Received September 19, 1994)

Kuruma prawns (*Penaeus japonicus*) were experimentally infected with *Vibrio* sp. PJ by the oral administration. At 3, 6, 8, 10, 12, 24, 36, and 48 h post-inoculation, prawns were sampled to determine the cell number and distribution of the pathogen in various organs by viable cell count and the enzyme-labeled antibody technique (ELAT).

Vibrio sp. PJ was detected by culture method from the stomach and hemolymph at 3 h and from the hemolymph and almost all the organs sampled at 6 h post-inoculation. However, the pathogen started to disappear from all the organs from 8 h to 12 h. At 12 h, it reappeared in the hepatopancreas and lymphoid organs. Twenty-four hours after inoculation, the pathogen was detected from the hemolymph and all the organs except the stomach and gills, and at 36 h the pathogen was found distributed in all the organs. Principally the same distribution pattern of the pathogen was found by ELAT.

These results seem to indicate that the process in the pathogenesis of *Vibrio* sp. PJ infection in orally challenged kuruma prawns consist of five stages namely, establishment of the pathogen, distribution of the pathogen, clearance of the pathogen by host prawn, secondary multiplication of the pathogen, and systemic infection. The results also suggest that the pathogen multiplied in the stomach in the establishment stage and in the hepatopancreas and lymphoid organs in the secondary multiplication stage.

Key words: vibriosis, *Vibrio* sp. PJ, kuruma prawn, *Penaeus japonicus*, oral infection, ELAT

The increasing demand for kuruma prawn (*Penaeus japonicus*) and limited area suited for its farming led to the intensification of kuruma prawn culture in Japan. Though intensive culture could be efficient for productivity and economically advantageous, infectious diseases caused by viruses, bacteria and fungi are usually the limiting factors in the production (Momoyama, 1992).

Vibriosis caused by *Vibrio* sp. PJ is considered to be the most serious disease problem in the culture industry of kuruma prawn in Japan. There have been several studies conducted concerning vibriosis of kuruma prawn. Studies on the isolation of the pathogen and chemotherapy (Takahashi *et al.*, 1985 a, b), histopathology (Egusa *et al.*, 1988), and experimental vaccination (Itami *et al.*, 1989) were carried out. Recently, studies have been done on the ecology (de la Peña *et al.*, 1992), detailed characterization (de la Peña *et al.*, 1993), and classification (Ishimaru *et al.*, 1995) of *Vibrio* sp. PJ. However, pathogenesis of *Vibrio* sp. PJ infection in kuruma prawn has not yet been elucidated.

In this study, changes in viable cell number and distribution of *Vibrio* sp. PJ in organs of orally infected kuruma prawn were examined by using conventional culture method and the enzyme-labeled antibody technique (ELAT).

Materials and Methods

Bacterial strain and culture conditions

A strain KH-1 of *Vibrio* sp. PJ isolated from diseased kuruma prawn in 1989 was used in this study. Prior to the present study, the strain was passed 3 times through prawns to enhance the virulence. Stock cultures were maintained frozen at −80°C in ZoBell's 2216E broth supplemented with 10% glycerol (Cote, 1989), and were grown on ZoBell's 2216E agar at 25°C for 48 h prior to each experiment.

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Experimental animals

Before infection experiments, purchased prawns with an average weight of 17 g were acclimated to laboratory conditions in flow-through tanks supplied with aerated seawater of salinity of about 32 ppt. Prawns were fed a commercial diet and were kept at about 20°C for 2 weeks. Ten out of 100 prawns were randomly sampled for bacterial isolation from the lymphoid organs to ensure that the prawns were free from *Vibrio* sp. PJ.

Infection method

Oral intubation was used as the challenge method because this method was confirmed to be capable of killing prawns with a median lethal dose (LD_{50}) between 10^3 and 10^4 CFU/animal (unpublished data), which is only one order of magnitude higher than that of injection challenge (de la Peña *et al.*, 1993). In the present experiment, 10^4 CFU/animal of the pathogen was used as the challenge dose. Inoculated prawns were kept individually in 6 *l* rectangular plastic tanks supplied with aerated seawater. Eighty percent of the rearing water was changed daily and temperature was maintained at about 20°C. Prawns were not fed throughout the experiment. Samplings were done periodically (0, 3, 6, 8, 10, 12, 24, 36, and 48 h) to estimate the total bacterial number, *Vibrio* sp. PJ number and the distribution of the pathogen in the organs.

Viable cell count

After inoculation of the pathogen, 4 prawns were sacrificed in every sampling period. Each organ, i.e. hepatopancreas (=midgut gland), lymphoid organs, midgut, muscle, stomach, and gills, was weighed and homogenized with sterile 1/2 strength seawater. The homogenates were serially 10-fold diluted and spread onto ZoBell's agar. Hemolymph was also taken aseptically, then serially diluted and spread onto ZoBell's agar. After 2 days of incubation at 25°C, total bacterial and *Vibrio* sp. PJ colonies were counted, and CFU/g or m*l* of each organ was determined. *Vibrio* sp. PJ colonies were confirmed by slide agglutination with a rabbit antiserum prepared against formalin-killed *Vibrio* sp. PJ (KH-1).

Enzyme-labeled antibody technique (ELAT)

Two prawns were sacrificed for ELAT in every sampling time in parallel with the viable cell count. Prawn organs were fixed in Davidson's fixative following the method of Bell and Lightner (1988). The fixed organs were processed, embedded in paraffin wax and cut into 5 μm sections. These were stained by using an enzyme-labeled antibody technique following the methods of Watanabe (1981) and Muroga and De La Cruz (1987). The rabbit anti-KH-1 serum was used as the primary antibody. The second antibody labeled with peroxidase was purchased (peroxidase-conjugated swine Ig to rabbit Ig, DAKO, Denmark).

Results

Total bacterial and Vibrio sp. PJ count by bacterial isolation

Prawns were apparently healthy until 36 h post-inoculation (pi) with *Vibrio* sp. PJ. Lethargic or moribund prawns appeared at 48 h-pi and death was observed from 48 h- to 72 h-pi.

Fig. 1 shows the total bacterial count (average number of 4 prawns) from 6 different organs and hemolymph of kuruma prawns at each sampling time. Bacteria were isolated from all the samples examined throughout the sampling period. Gills exhibited the highest bacterial count (10^7–10^8 CFU/g) followed by stomach (10^6–10^7 CFU/g) and the numbers of total bacteria in these organs and hemolymph were almost constant throughout the experimental period. In many cases, the number of total bacteria in the hemolymph was the lowest (10^2–10^3 CFU/m*l*). The bacterial number increased in the latter half of the experiment in the midgut and lymphoid organs.

Fig. 2 shows the changes in viable cell number of *Vibrio* sp. PJ in different organs and hemolymph of kuruma prawn after oral inoculation. Although the ratio in colony number of *Vibrio* sp. PJ/total bacteria was quite low in some cases, especially in the stomach, a few colonies of *Vibrio* sp. PJ were detected from undiluted samples. The number of *Vibrio* sp. PJ varied significantly among 4 prawns examined at each sampling as shown in Fig. 2. Sampling at 3 h-pi revealed the presence of *Vibrio* sp. PJ in the stomach and hemolymph, and at 6 h in almost all the organs sampled. However, the pathogen started to disappear from all the organs from 8 h to 12 h. At 12 h, it reappeared in the hepatopancreas and lymphoid organs. Twenty-four hours after inoculation, the pathogen was detected from the hemolymph and all the organs except the stomach and gills, and at 36 h

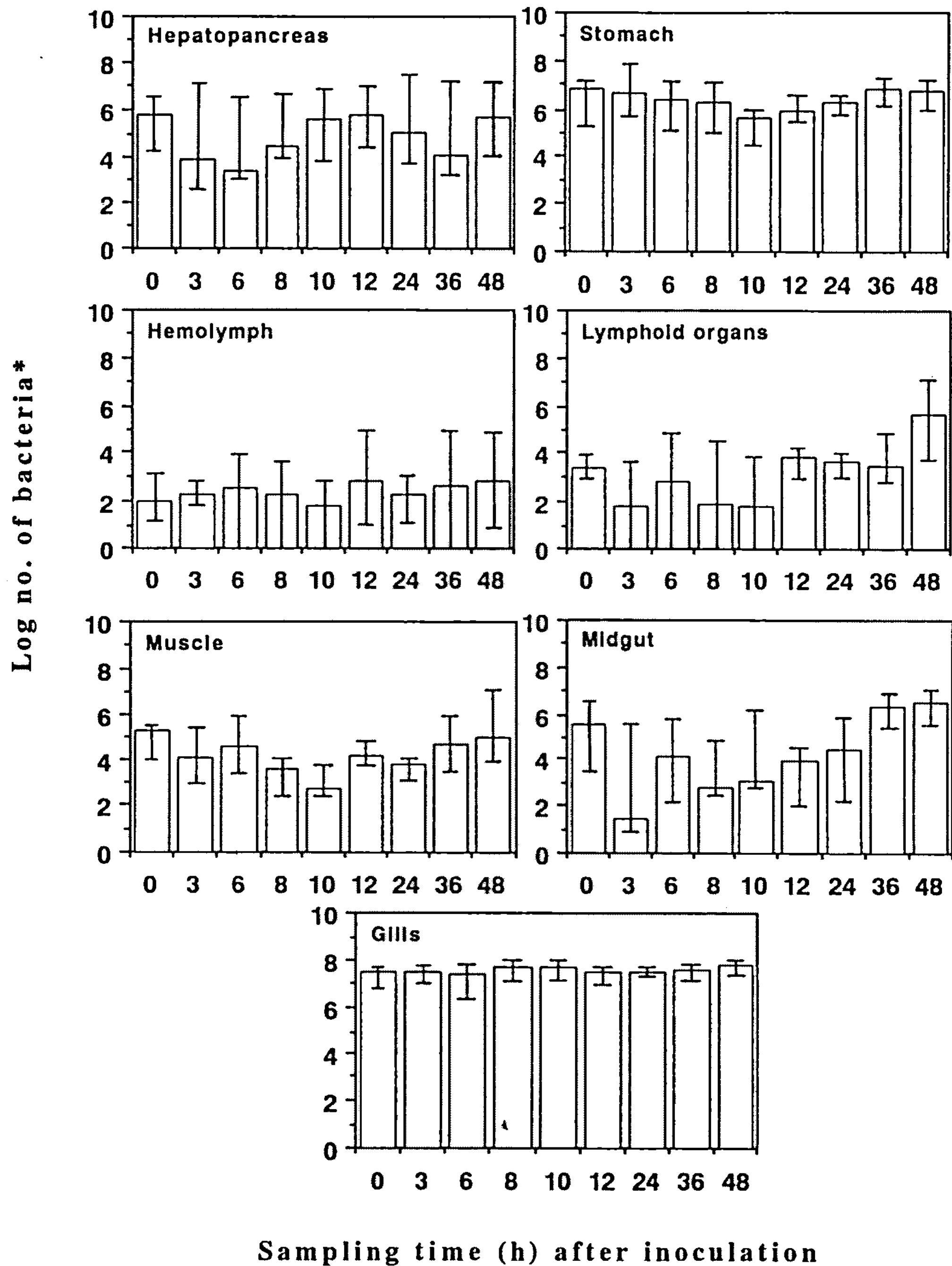

Fig. 1. Total bacterial count in six different organs and hemolymph of kuruma prawns infected orally with *Vibrio* sp. PJ.
*Average number of four prawns examined at each sampling time; vertical lines represent the range of 4 values.

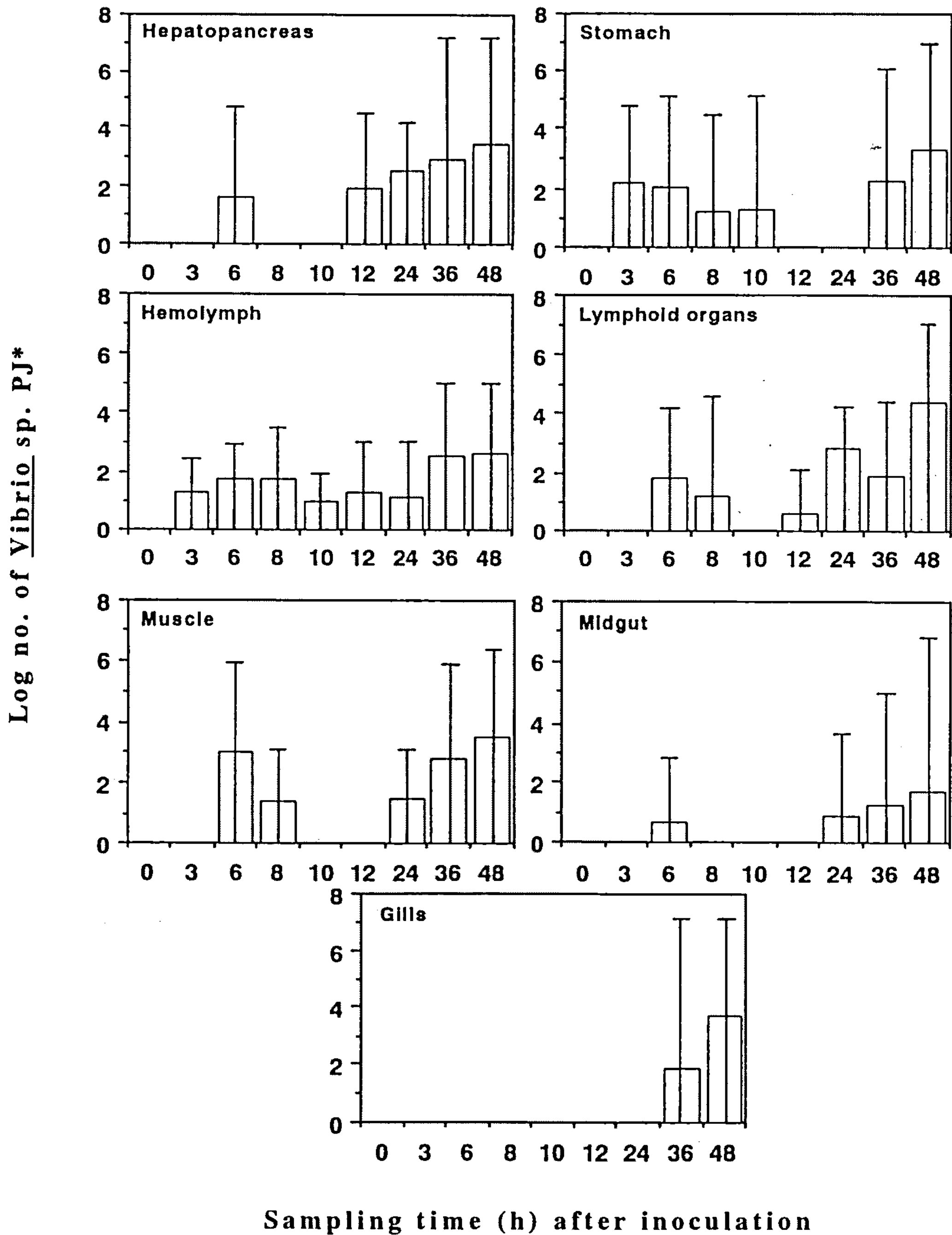

Fig. 2. *Vibrio* sp. PJ count in 6 different organs and hemolymph of orally infected kuruma prawns.
*Average number of four prawns examined at each sampling time; vertical lines represent the range of 4 values.

Table 1. Detection of *Vibrio* sp. PJ by ELAT in orally infected kuruma prawns

Organ	Presence of *Vibrio* sp. PJ								
	Sampling time (h) after inoculation								
	0	3	6	8	10	12	24	36	48
Stomach	−	+	+	+	+	−	+	+	+
Hepatopancreas	−	−	+	+	−	+	+	+	+
Lymphoid organs	−	−	−	−	−	+	+	+	+
Muscle	−	−	−	+	−	−	+	+	+
Gills	−	−	−	−	−	−	+	+	+
Midgut	−	−	−	−	−	−	+	+	+

Two prawns were used in each examination, and the result of the 2 samples completely coincided with each other.

the pathogen was found distributed in all the organs. The bacterial number increased significantly from 24 h to 48 h. The average number of *Vibrio* sp. PJ was 2.1×10^4 CFU/g (individual value: 0, 10^3, 10^7, 10^7) in the lymphoid organs at 48 h.

Distribution of Vibrio sp. PJ determined by ELAT

The results of ELAT of two prawns examined at each sampling time completely corresponded to each other and are summarized in Table 1. High correlation was observed in the detection of *Vibrio* sp. PJ from each organ between the results of culture method and ELAT, though some organs were found to be negative for the pathogen by the latter method. The pathogen was detected in the stomach at 3 h-pi, in the stomach and hepatopancreas at 6 h, in the muscle as well as stomach and hepatopancreas at 8 h. However, at 10 h the pathogen disappeared from all the organs except stomach. At 12 h, it reappeared in the hepatopancreas and lymphoid organs. From 24 h, the pathogen was detected from all the organ samples. Organ samples especially of the hepatopancreas at 36 h and 48 h revealed a prominent degeneration of the tubules and the pathogen was seen abundantly in the hemal sinuses. The pathogen could also be seen abundantly in the tubules of the lymphoid organs, in the lumen and columnar epithelium of the midgut, in the lumen of anterior and posterior chamber of the stomach, in the striations of the muscle, and in the secondary gill filaments. It can be noted that the pathogen was consistently detected from the stomach and hepatopancreas even before the systemic infection occurred.

Discussion

Tissue distribution of bacterial fish pathogens such as *Aeromonas hydrophila* (Hatai, 1972), *Pseudomonas anguilliseptica* (Nakai *et al.*, 1985) and *Vibrio anguillarum* (Muroga and De La Cruz, 1987) in each major host were studied. Similar studies were also conducted with crustacean pathogens in decapod crustaceans (Cornick and Stewart, 1968; McKay and Jenkins, 1970; White and Ratcliffe, 1982; Factor and Beekman, 1990). Martin *et al.* (1993) studied the bacterial clearance of penaeid shrimp (*Sicyonia ingentis*) with 4 strains of bacteria and concluded that shrimp rapidly removed injected bacteria from their hemolymph.

In the present study, it was demonstrated by culture method and ELAT that orally inoculated *Vibrio* sp. PJ, the pathogen of vibriosis in kuruma prawn, was present in the stomach and hemolymph at 3 h post-inoculation. The pathogen occurred in almost all the organs examined at 6 h and then disappeared from all the organs except hemolymph. It reappeared in the hepatopancreas and lymphoid organs at 12 h, and finally it was detected from all the organs and hemolymph at 36 h. From these results, the following 5 stages (establishment, distribution, clearance, secondary multiplication, and systemic infection) in the pathogenesis of *Vibrio* sp. PJ infection in orally challenged kuruma prawn were supposed.

The first stage is the establishment of the pathogen. The present result seems to indicate that the stomach is the most probable site for establishment, although the possibility that the bacteria propagated in the hemolymph can not be ruled out. In the second stage, the pathogen multiplying in the establishment site was immediately released into the hemolymph and distributed to other organs, i.e. hepatopancreas, lymphoid organs, midgut, and muscle. The hepatopancreas has direct access to the stomach chambers via lower cardiac grooves and primary hepatopancreatic duct (Bell and Lightner, 1988), and the midgut is just like an extension of the stomach. Thus, the pathogen could have invaded these two organs directly from the stomach, as already indicated by Chen *et al.* (1992) who studied the pathogenesis of *V. harveyi* infection in *P. monodon.*

In the third stage (8–12 h post-inoculation), the pathogen disappeared from all the organs (but not

from the hemolymph), suggesting that this stage can be called a clearance stage. The pathogen reappeared in the hepatopancreas and lymphoid organs at 12 h, in the midgut and muscle at 24 h, and in the stomach at 36 h. There was no increase in *Vibrio* sp. PJ number in the hemolymph from 10 to 24 h, therefore, during that period the same amount of bacteria released from the secondary multiplication site(s) into the hemolymph would be trapped and killed in a certain organ(s). The pathogen reappeared in the hepatopancreas and lymphoid organs just after the clearance stage, indicating that these two organs are most likely the secondary multiplication sites. It was suggested by Kondo *et al.* (1994)* that the clearance of bacteria is performed in the lymphoid organs in kuruma prawn, and multiple formation of melanized nodules encapsulating *Vibrio* sp. PJ was observed in the lymphoid organs (Egusa *et al.*, 1988). Thus, it can be assumed that the number of the pathogen in the lymphoid organs increased not only by multiplication but also by accumulation through trapping.

After the secondary multiplication stage, the pathogen was detected in all the organs with increased number indicating the occurrence of systemic infection which will lead to the death of the host.

As noted above, the stomach and the hepatopancreas/lymphoid organs were presumed to be the first and secondary multiplication sites, respectively, for *Vibrio* sp. PJ in orally infected kuruma prawns. However, in some prawns the pathogen was not detected or detected only in small numbers in these organs (Fig. 2). The detection of the pathogen would have been hampered by the large quantities of other bacteria in these organs (Fig. 1). Therefore, a more sensitive method for the detection of the pathogen should be applied to determine the exact sites for the multiplication of the pathogen in the prawns. In addition, it should be investigated whether the pathogen can grow in the hemolymph of the prawn or not.

Acknowledgements

The authors wish to thank Mr. T. Horie of Daiya-Yoshoku Co., Ltd. for providing prawns in this study and to the Ministry of Education, Science and Culture of Japan (Monbusho) for the support of this work in the form of scholarship to one of the authors (L. D. de la Peña), and a Grant-in-Aid for Scientific Research (06660238).

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