Detection of the Causative Bacterium of Vibriosis in Kuruma Prawn, *Penaeus japonicus*

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A one-year field survey on the presence of *Vibrio* sp. PJ, the causative agent of vibriosis in kuruma prawn (*Penaeus japonicus*), was carried out in prawn farms in Hiroshima and Yamaguchi Prefectures by a conventional bacterial culture method. The result revealed that the pathogen has already been widely distributed in the prawn culture environment. The population of the pathogen increased both in prawns and in their environment with a rise in water temperature.

Indirect fluorescent antibody technique (IFAT) was applied to smear specimens of the lymphoid organ of both experimentally and naturally infected kuruma prawns. This method was found effective as a rapid and reliable diagnostic method for the present disease, though the sensitivity was slightly lower than that of the culture method.

Vibriosis caused by a member of the genus *Vibrio* is considered to be the most important disease problem in the culture industry of kuruma prawn (*Penaeus japonicus*) in Japan because of considerable losses. Outbreaks of the disease in cultured kuruma prawn have been recorded since 1980, and studies on the characteristics of the causative bacterium (*Vibrio* sp.), histopathology, experimental treatment, and prophylactic vaccination have been done (Takahashi *et al.*, 1985a, b; Egusa *et al.*, 1988; Itami *et al.*, 1989). Recently, the pathogen (*Vibrio* sp. PJ) was found to be composed of antigenically homologous strains and widely distributed in prawn farms in Japan (de la Peña, Tamaki, Momoyama, Nakai and Muroga, unpublished data). The diseased prawns are usually treated with medicated (oxytetracycline and oxolinic acid) pellet-type feeds, however, the disease has not been completely controlled and is still the biggest problem for prawn farmers.

To establish control measures for the disease, either avoidance of the exposure to the pathogen, vaccination, medication, or environmental man-

ipulation for reducing disease triggering factors, ecological studies on the pathogen seem to be essential. In the present study, a one-year field survey was conducted to detect the pathogen from kuruma prawns and their environment by a conventional bacterial culture method.

Immunological procedures using antibodies labeled with fluorescent dyes or enzymes have been used extensively in the rapid diagnosis of infectious diseases and detection of many fish pathogens (Schill *et al.*, 1989). In this study, indirect fluorescent antibody technique (IFAT) was evaluated for the rapid diagnosis of the disease.

Materials and Methods

Field Survey by Culture Method

Sampling sites: Monthly samplings were done in 4 ponds of 3 kuruma prawn farms at Osaki Kamijima (Ponds A and B), Hiroshima Prefecture and Aio-cho (Pond C) and Ube City (Pond D), Yamaguchi Prefecture from May to December 1990 (except Pond C in May). In each pond, 4 corners and 1 center were covered

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for sampling of surface water and bottom mud and/or sand (hereafter simply mud).

Collection of samples: The water samples were collected in sterile flasks. Three 10-fold serial dilutions were made with sterile saline and 0.1 ml of each were plated onto ZoBell's 2216E agar and BTB-Teepole agar (Nissui) plates for bacterial examination. Mud samples were collected in individual sterile flasks using fine-mesh net. One gram of mud was homogenized in 10 ml sterile saline and three 10-fold serial dilutions were made. From each dilution, 0.1 ml were plated onto ZoBell's and BTB agar plates.

Five to ten apparently healthy prawns randomly caught from each pond were sacrificed at each sampling. Hemolymph, lymphoid organs, and gills were taken aseptically, then the former was streaked directly onto ZoBell's agar and the latter two were homogenized in sterile saline and plated onto the medium.

Microbiological examination: The plates inoculated with water/mud and prawn samples were incubated at 25°C for 48 h. *Vibrio* sp. PJ-like colonies, if any, were selected for pure culture based on their morphological appearance, and submitted to slide agglutination test using anti-*Vibrio* sp. PJ (KH-1: a strain from Kagoshima Pref.) rabbit serum. The agglutination-positive isolates were later confirmed to be *Vibrio* sp. PJ by biochemical characterization tests. Total viable cell count was also done on BTB and ZoBell's agar plates inoculated with water and mud samples obtained at Ponds A and B.

Indirect Fluorescent Antibody Technique (IFAT)

Bacteria: In addition to the strain KH-1, 8 strains of *Vibrio* sp. PJ (3 strains from Yamaguchi, 2 from Hiroshima, and each 1 from Oita, Ehime, and Kumamoto Prefs.) were used in this experiment. To test the cross reactivity with KH-1 strain, strains of different *Vibrio* species and other fish pathogenic bacteria were used. These include *Vibrio alginolyticus* (V-447), *V. campbelli* (ATCC 25920T), *V. parahaemolyticus* (HO-5), *V. harveyi* (ATCC 14129T), *V. anguillarum* (PT-87050), *V. ordalii* (V-105), *Aeromonas hydrophila* (ET-4), *A. salmonicida* (ATCC 14174), *Pseudomonas fluorescens* (03L), *Pasteurella piscicida* (SJ 9102), and *Edwardsiella tarda* (NUF 82).

IFAT: IFAT was done according to the method

of Chart *et al.* (1984) with some modifications. Duplicate smears of appropriate dilutions (ca. 10^7 CFU/ml) of each strain were made on non-fluorescent slides and introduced into IFAT. The smears were air-dried and fixed for 10 min in methanol, and again air-dried. They were overlaid with anti-KH-1 serum and incubated in a moist chamber at 37°C for 30 min, then washed 5 times with PBS (0.01 M phosphate, 0.15 M NaCl, 0.02% NaN₃, pH 7.2). Following air-drying, smears were overlaid with a commercially prepared fluorescein (FITC)-labeled goat anti-rabbit immunoglobulin (Kirkegaard and Perry Laboratories, Inc.) diluted to 1:100 then, incubated and washed as described above. Slides were air-dried and examined under a fluorescence microscope (Nikon EFD).

Preparation of experimentally infected prawns:

Nine healthy prawns (mean weight, 14 g) were intramuscularly injected with a 24 h culture of KH-1 at a dose of 1.9×10^8 CFU/animal. After 6, 24, and 36 h, each 3 prawns were sacrificed and two unchallenged prawns were also sampled (0 h). The lymphoid organs and muscle of both infected and control prawns were taken aseptically and weighed then, homogenized in 9 volumes of sterile saline. Duplicate smears of homogenates were made on non-fluorescent slides for IFAT. In the case of hemolymph, samples (30 μ l) were directly placed onto the slides. The intensity of resultant fluorescence was graded into 3 ranks (–, +, ++). Viable cell count of KH-1 strain in the tissues of the prawns was also made on ZoBell's agar.

Naturally infected prawns: Samplings were made in 1991 from August to October, when outbreaks of vibriosis were expected to occur. Thirty prawns were randomly collected from each of 2 private ponds (C and D: the same ponds as those surveyed in the previous year). Lymphoid organs were removed aseptically, homogenized in 0.5 ml of sterile saline, and smeared in duplicate on non-fluorescent slides. Presence of *Vibrio* sp. PJ in the homogenates was also determined by culture method on ZoBell's agar.

Results

Field Survey

During the field survey done from May to

Fig. 1. Detection of *Vibrio* sp. PJ by culture method from kuruma prawns in farms in Hiroshima (Ponds A and B) and Yamaguchi (C and D) Prefectures in 1990.

December of 1990, *Vibrio* sp. PJ was detected from healthy prawns of the four ponds in summer and autumn (July to December). Probably owing to repeated medications, significant mortalities due to vibriosis were encountered only in Pond D. The appearance of the pathogen in prawns coincided with the peak and/or the following declining period of water temperature (Fig. 1).

Fig. 2. Bacterial counts of pond water and mud in Ponds A and B on ZoBell's 2216E and BTB agar media.

The pathogen was also detected from rearing water from June to December except September and from mud in November, though only one out of five sites (4 corners and 1 center) was positive in each pond (Table 1). The pathogenicity of one isolate from water in June in Pond C was confirmed by injecting it into healthy prawns (LD_{50} : 1.7×10^1 CFU/g). In Fig. 2, the bacterial counts of pond water and mud from 2 ponds (A and B) on ZoBell's and BTB agar media are shown. Apparent seasonal changes in bacterial counts of water and mud

Table 1. Detection of *Vibrio* sp. PJ by culture method from pond water and mud in farms in Hiroshima and Yamaguchi Prefectures in 1990

Sample	Sampling date (Month)							
	May	Jun.	Jul.	Aug.	Sept.	Oct.	Nov.	Dec.
Rearing water								
Pond A	0 ^a	0	0	0	0	0	1	0
Pond B	0	0	0	1	0	1	0	1
Pond C	0	1	0	0	0	1	0	0
Pond D	0	0	1	0	0	1	1	1
Mud/Sand								
Pond A	0	0	0	0	0	0	0	0
Pond B	0	0	0	0	0	0	0	0
Pond C	—	0	0	0	0	0	0	0
Pond D	0	0	0	0	0	0	1	0

^a Number of sites positive for *Vibrio* sp. PJ among 5 sites examined (4 corners and 1 center).

were not observed during the research period from May to December.

IFAT

Nine selected strains of *Vibrio* sp. PJ including homologous KH-1 were all positive for IFAT

test with smear specimens using anti-KH-1 serum which was diluted 1: 1000. The sensitivity threshold of the assay was at about 3×10^4 CFU ($30 \mu\text{l}$ of 1×10^3 CFU/ml) of the homologous strain.

The specificity of IFAT using anti-KH-1 serum

Table 2. Specificity of indirect fluorescent antibody technique (IFAT) to some selected bacterial species at different dilutions of anti-*Vibrio* sp. PJ (KH-1) serum

Bacterium (strain)	Fluorescence at serum dilution of 1:				
	100	800	1000	1600	2000
<i>Vibrio</i> sp. PJ (KH-1)	+	+	+	+	—
<i>Aeromonas hydrophila</i> (ET-4)	—	—	—		
<i>Aeromonas salmonicida</i> (ATCC 14174)	—	—	—		
<i>Pseudomonas fluorescens</i> (03L)	—	—	—		
<i>Pasteurella piscicida</i> (SJ 9102)	—	—	—		
<i>Edwardsiella tarda</i> (NUF 82)	—	—	—		
<i>Vibrio alginolyticus</i> (V-447)	+	+	—		—
<i>Vibrio campbelli</i> (ATCC 25920T)	+	+	—	—	—
<i>Vibrio parahaemolyticus</i> (HO-5)	+	+	—	—	—
<i>Vibrio harveyi</i> (ATCC 14129T)	+	+	—	—	—
<i>Vibrio anguillarum</i> (PT-87050)	+	+	—	—	—
<i>Vibrio ordalii</i> (V-105)	+	+	—	—	—

* Weak fluorescence.

Table 3. Detection of *Vibrio* sp. PJ (KH-1) in experimentally infected kuruma prawns using culture method and IFAT

Hours after injection	Number of prawns	Number of cells isolated (CFU/ml or g)/result of IFAT		
		Hemolymph	Lymphoid organ	Muscle
0	2	0 /—	0 /—	0 /—
6	3	5.6×10^4 /+	9.7×10^3 /+	7.1×10^3 /+
24	3	1.1×10^5 /++	5.8×10^6 /++	4.5×10^4 /++
36	3	4.3×10^5 /++	6.6×10^6 /++	1.4×10^6 /++

Prawns (average weight, 14 g) were intramuscularly injected with KH-1 strain (1.9×10^6 CFU/animal) and reared at 21°C. Anti-KH-1 serum was used as first antibody at 1: 1000.

Table 4. Detection of *Vibrio* sp. PJ by culture method and IFAT from lymphoid organs of kuruma prawns in 2 ponds in Yamaguchi Prefecture

Sampling date (1991)	Pond and average weight of prawn (g)		Percentage of prawns positive for <i>Vibrio</i> sp. PJ	
			Culture method	IFAT
Aug. 7	C	4.3	6.7 (2/30)	3.3 (1/30)
	D	8.5	3.3 (1/30)	0 (0/30)
Sept. 9	C	8.2	0 (0/30)	0 (0/30)
	D	13.3	10.0 (3/30)	3.3 (1/30)
Oct. 10	C	15.5	6.7 (2/30)	6.7 (2/30)
	D	20.0	3.6 (1/28)	3.6 (1/28)

IFAT first antibody: anti-KH-1 serum, diluted 1: 1000.

was clearly demonstrated (Table 2). The *Vibrio* species tested gave a positive cross response at dilutions up to 1:800 but the specificity of the serum was revealed at 1:1000 dilution.

The results of the detection of *Vibrio* sp. PJ (KH-1) by IFAT and culture method in experimentally infected prawns are given in Table 3. There was a good correlation between the isolation of KH-1 strain and the result of IFAT test. In this *in vivo* experiment, the sensitivity limit of the assay was almost the same as in *in vitro* test; 2×10^1 CFU ($30 \mu\text{l}$ of 7.1×10^2 CFU/ml).

The result of the three consecutive monthly samplings using culture method and IFAT in the 2 private ponds to detect *Vibrio* sp. PJ from naturally infected prawns was given in Table 4. *Vibrio* sp. PJ was detected by culture method from every group of specimens except for pond C at the second sampling, though detection rate was always low. Five out of 9 samples positive for culture method were also positive for IFAT test.

Discussion

From the one-year field survey on the presence of *Vibrio* sp. PJ in the prawn farms in Hiroshima and Yamaguchi Prefectures, the pathogen proved to be distributed in apparently healthy kuruma prawns and their culture environment. Based on this result and long survivability of the pathogen in sea water (de la Peña *et al.*, unpublished data), it can be said that *Vibrio* sp. PJ has already been established in the prawn culture environment. The population of the pathogen increased both in prawns and their environment at the peak (Ponds A and B) or the following declining period (Ponds C and D) of water temperature. These periods coincide with the season when deterioration of environmental conditions advances and vibriosis prevails in prawn farms. The deterioration seems to start at the middle of the culture period (July or August) judging from observations of the accumulation of organic wastes and hydrogen sulfide at the pond bottom. The time differences in occurrence of *Vibrio* sp. PJ between the two prefectures can be attributed partly to the difference in the time table of stocking. Ponds A and B (Hiroshima) were stocked earlier than Ponds C and D (Yamaguchi) by about two

months thus, deterioration of environmental conditions in ponds in Yamaguchi Prefecture may have progressed later than in ponds in Hiroshima Prefecture. In mud, *Vibrio* sp. PJ was rarely detected probably because of the conditions unfavorable for the bacterium.

Some or most of the prawns positive for the detection of *Vibrio* sp. PJ (Fig. 1) were suspected to be at the initial stage of infection. In other words, the detection rate obtained here is thought to be lower than the true carrier rate, because we have often experienced that more than half of healthy appearing prawns purchased at these farms became ill with vibriosis during and after acclimation to laboratory conditions unless appropriate medications were done. This indicates that the carrier state of prawns will develop into overt infection through stress due to handling and adverse environmental conditions (Johnson, 1983). Detection rate of *Vibrio* sp. PJ from carrier prawns will be enhanced by introducing a special method like enrichment pre-culture. Another method includes bacterial isolation after giving stress as done for the detection of *Aeromonas salmonicida*-carriers in salmonids (Bullock and Stuckey, 1975).

Since *Vibrio* sp. PJ has been established in prawn farms, it seems quite difficult to eradicate the pathogen from culture environment and chemotherapy appears to be a stopgap remedy. Thus vaccination and removal of stress factors are possible ways for the control of the disease. In order to minimize the damage due to vibriosis by modifying culture techniques or manipulating environmental conditions, we must determine the exact stress factors which actually predispose prawns to the acquisition of infection or produce overt infection in carriers.

Disease should be precisely diagnosed in the early stage of infection or even in carrier stage because correct diagnosis is the most critical step in disease control program (Baticados, 1988). Fluorescent antibody technique (FAT) has been described for several fish pathogens such as *Edwardsiella tarda*, *Enterococcus seriolica* (*Streptococcus* sp.), *Nocardia kampachi*, *Pasteurella piscicida*, and *Vibrio anguillarum* (Schill *et al.*, 1989). In the present study, all the tested strains of *Vibrio* sp. PJ exhibited positive reaction even at 1:1000 dilution of anti-

KH-1 serum in indirect fluorescent antibody technique (IFAT). This finding confirmed the earlier result of our study that there is a major O-antigen common to all the *Vibriosp.* PJ strains tested (de la Peña *et al.*, unpublished data). Though the detection rate of the pathogen was slightly lower in IFAT than in culture method, IFAT was confirmed to be available for the diagnosis of this vibriosis because of its rapidity.

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