ISOLATION AND BIOLOGICAL CHARACTERISTICS OF *VIBRIO HARVEYI* AFFECTING HATCHERY-REARED *PENAEUS CHINENSIS* LARVAE*

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Abstract From April to May 1996, an epizootic occurred among *Penaeus chinensis* larvae, especially at the stage of zoeae, in some hatcheries located at Fengcheng, near Qingdao, Shandong Province. The mortalities were up to 80%. A total of 38 strains of bacteria, which were isolated or re-isolated from infected moribund larvae of *P. chinensis,* had morphological, physiological and biochemical characteristics of the genus *Vibrio*: Gram-negative, short rods and motile by means of a single polar flagellum. Growth on TCBS agar. Oxidase reaction, fermentation of glucose and sensitivity to the vibriostatic agent 0 / 129 were positive. The bacteria produced acid without gas from glucose. Among them, 26 strains seemingly belong to the same species, which were related to *Vibrio harveyi* on the basis of a comparison with the data of Bergey's Manual of Determinative Bacteriology (9th edition). The results have been proved by the identification results of Biolog system. A dominant *Vibrio* isolate was proved to be pathogenic to larvae of *P. chinensis* and was a causative agent for epizootic disease in larvae shrimp. Shrimp larvae challenged with 2.5×10^3—2.5×10^7 cfu / ml bacteria by immersion method showed significant mortalities in the nauplii, zoeae and $mysis_{1-2}$ larvae, but not in the postlarvae. At the zoeal stages, the isolate caused severe infection with up to 2.5×10^4 cfu / ml of bacteria in the rearing seawater, indicating that the causative agent was more virulent to zoeal larvae of *P. chinensis* than at other stages. This study is the first report in China on the pathogenicity of *V. harveyi* in the larvae of *P. chinensis.*

Key words *Penaeus chinensis* larvae Vibriosis *Vibrio* *Vibrio harveyi*

Subject classification number S945.1

Vibriosis caused by *Vibrio* spp. has become the economically most important disease in wild and cultured finfish and shellfish, affecting a large number of species. *Vibrio* infection has often been reported to occur in penaeid shrimp (Lightner, 1983, 1988; Takahashi *et al*, 1984, 1985; de la Peña *et al*, 1993; Mohney *et al*, 1994; Sahul Hameed, 1994). Several species of *Vibrio,* such as *V. alginolyticus, V. anguillarum* and *V. parahaemolyticus* (Lightner, 1983), *V. fisheri* and *V. fluvialis* (Sakata *et al*, 1987), *V. vulnificus* and *V. damsela* (Song *et al*, 1990, 1993) and *V. harveyi* (Karunasagar *et al*, 1994; Lavilla-Pitogo *et al*, 1990) have been described as pathogens for cultured penaeid shrimp. Vibriosis is responsible for heavy losses in production of penaeid shrimp in mariculture.

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Seed production has gained increasing importance for developing the shrimp culture industry worldwide. Recently, serious infections of *Vibrio* spp. at larvae and postlarvae stages of penaeid shrimp have been reported in some countries in the Indo-Pacific (Sae-oui *et al*, 1987; Tansutapanit *et al*, 1987; Lavilla-Pitogo *et al*, 1990; Baticados *et al*, 1991; Karunasagar *et al*, 1994; Sahul Hameed *et al*, 1996). Most of these *Vibrio* species are part of the autochthonous flora of marine waters, and therefore, the source of infection is suspected to be near-shore seawater (Lightner *et al*, 1992).

Outbreaks of vibriosis in cultured *Penaeus chinensis* caused by *V. cholerae* (non-ol) and *V. anguillarum* (Zheng, 1986a, 1986b, 1990), *V. alginolyticus, V. Parahaemolyticus* and *V. campbellii* (Xu *et al*, 1993) and *V. splendidus* (Chen *et al*, 1995) have been reported in China (Meng *et al*, 1982; Ye *et al*, 1986), but most of these studies focus on the grow-out penaeid shrimp, and few on the larval shrimp. Recently, serious diseases mostly caused by *Vibrio* spp. often occurred in most hatcheries in North China, this has become a severe barrier to prevent the development of the shrimp aquaculture. In order to reveal the reason of outbreak of vibriosis and develop successful control methods in shrimp hatcheries, the pathogenicity and characteristics of the causative agents were investigated, and the relevant factors associated with the health of hatchery-reared larval shrimp of *P. chinensis* were discussed in this study.

1 MATERIALS AND METHODS

1.1 Collection of samples

Samples of water and infected larvae of *Penaeus chinensis* were collected from various tanks of Fengcheng Hatchery from April to May 1996. The collected larvae were washed gently in sterile seawater for 3 times and transferred to a sterile screw-capped bottle containing sterile seawater. Water samples were collected using sterile bottles (250 ml). All samples were transferred to the laboratory and processed within 2 hours after collection.

1.2 Bacteriological analysis

About 50 infected larvae were randomly selected and placed in a sterile homogenizer with 1 ml of sterile 0.85% saline solution and homogenized. Then, 10-fold serial dilutions were made with 0.85% saline solution and 0.1ml of diluted samples were inoculated onto TCBS plates by the spread plate technique. To the water samples, 5μg / ml Tween-80 were added, the samples were shaken violently for 20 min, and then similarly diluted and inoculated onto 2216E plates.

After inoculation, the plates were incubated at 28℃ for 2—4 days. Bacterial colonies were carefully examined and counted. Morphologically similar and dominant bacterial colonies were selected and streaked onto 2216E plates for several times in order to obtain pure cultures. The purified cultures were maintained on 2216E agar slants for further study. On the other hand, the bacterial number in water samples were also investigated by acridine orange direct count (AODC) method described previously (Xu *et al*, 1993).

Bacterial isolates were identified using the Biolog System according to the mannual and characterized by various morphological, physiological and biochemical tests according to previously described methods (West *et al*, 1984).

1.3 Experimental pathogenicity

Experimental infection of shrimp larvae to confirm the pathogenicity of the isolates were performed in another hatchery located at Tushan Town, Laizhou City, where no diseases occurred dur-

ing the whole seed production season. Pathogenicity of one typical dominant isolate Z_3G_2 was studied for healthy nauplii(N), zoeae(Z), mysis(M) and postlarvae(PL) of *P. chinensis* by immersion method. All the experiments were carried out in white plastic containers (11-liter) with aeration, and all the temperature of containers were maintained constant by placing them in water baths. Around 500—1 000 larvae were reared in each container and fed a progression of *Nannochlorpsis* spp., Rotifers, Artemia larvae and egg yolk according to the different larval stages.

For the experimentally induced infection, the larvae of N, Z, M and PL were exposed to 2.5×10^6 cfu / ml bacterial cells for 24 h in duplicate. Meanwhile, another challenge test was done using the same isolate for Z_2 larvae with a different bacterial concentration (2.5×10^3, 2.5×10^4, 2.5×10^5, 2.5×10^6 and 2.5×10^7 cfu / ml). The larval survival rates were determined after different exposure time. The control consisted of larvae exposed to seawater only. In all the experiments, the larvae were examined carefully for diseases symptoms and the number of survivors in each container were counted to obtain the survival rate.

2 RESULTS

2.1 Bacterial counts in seawater and larvae samples

The total bacteria numbers in the tank water and in the body of different larval stages were counted by the methods of AODC and plate counts (Tab. 1). During the natural epizootics, the viable bacterial counts in the rearing tank water ranged from 7.0×10^3 to 3.45×10^5 cfu / ml on ZoBell's 2216 E agar, and from 6.94×10^6 to 6.865×10^7 cfu / ml counted by AODC. Correspondingly, the viable *Vibrio* sp. in larval body of different stages was from 6.0×10^3 to 4.7×10^6 cfu / larva on TCBS agar. The highest bacterial counts appeared at Z_2 stage in both the rearing tank water and larvae bodies. Moreover, light microscopy also revealed densely packed short rod bacteria in the smear of homogenized suspension of infected moribund zoeal larvae. Primary isolation revealed that up to 95% of the colonies were dark-green in color and uniform in morphology on TCBS agar; the isolates formed medium-sized (2—3 mm in diameter after 48 h at 28℃), low convex, smooth, entire, circular, cream-colored and semi-transparent colonies on ZoBell's 2216 E agar.

Tab.1 Bacterial counts in various samples (ND = no date)

Samples		Water		Larvae
Bacterial No.		2216E (cfu/ml)	AODC (cell/ml)	TCBS (cfu/larva)
Filtered water		<10	ND	ND
Stages	Egg	ND	6.94×10^6	ND
	N	7.0×10^3	1.36×10^7	6.0×10^3
	Z_1	1.51×10^5	4.225×10^7	3.4×10^5
	Z_2	3.45×10^5	6.865×10^7	4.7×10^6
	Z_3	1.07×10^5	6.12×10^7	2.1×10^6
	M_{2-3}	5.5×10^4	1.52×10^7	4.15×10^5
	PL_{5-6}	1.84×10^4	4.53×10^7	2.26×10^5

2.2 Characteristics and identification of isolates

In total, 36 representative bacterial isolates were obtained from the natural infected N, Z, M and PL and 2 from experimental infected larvae of *P. chinensis*. All were Gram-negative, short rods and motile by means of a single polar flagellum. Oxidase reaction, fermentation of glucose and sensitivity to the vibriostatic agent 0 / 129 were positive. The bacteria produced acid without gas from glucose. These selected features placed the organisms in the genus of *Vibrio* according to West *et al* (1984). Subsequently, characterization was concentrated on the 26 dominant isolates. All of the

Tab.2 Characteristics of the bacterial isolates from infected larvae of *P.chinensis* compared with reference strains

Characteristics		Isolates (n=24)	Re-isolates (n=2)	*V. harveyi*[1)]
Gram stain		−	−	−
bacterial shape		SR[2)]	SR	SR
colony color on TCBS		green	green	green/yellow
motility		+	+	+
swimming		−	−	−
flagella		SP[3)]	SP	SP
luminance		−	−	−
Growth Temperature (℃)	4	−	−	−
	10	d	d	ND
	37	+	+	+
	42	−	−	d
Growth in NaCl (%)	0	−	−	−
	6	+	+	+
	8	+	+	−
	10	−	−	−
0/129 sensitivity (μg/ml)	150	+	+	+
	10	d	d	+
catalase		+	+	+
oxidase		+	+	+
O/F test		F	F	F
citrate utilization		+	+	−
nitration reduction		+	+	+
V-P reaction		−	−	−
MR reaction		+	+	+
Indole production		+	+	+
H_2S production		−	−	−
Thornley's arginine dihydrolase		−	−	−
arginine decarboxylase		−	−	−
lysine decarboxylase		+	+	+
ornithine decarboxylase		+	+	+
phenylalaninase		+	+	d
TDA		−	−	−
gas from glucose		−	−	−
Acid source	arabinose	−	−	−
	inositol	−	−	−
	D-mannose	+	+	+
	D-raffinose	−	−	−
	rhamnose	−	−	−
	sucrose	−	−	d
	mannitol	+	+	+
	lactose	+	+	−
	saincin	+	+	+
	sobitol	−	−	−
	α-glucosamine	+	+	ND
	amygdalin	+	+	ND
	melibiose	−	−	−

26 isolates showed almost the same characteristics except for reaction in citrate and a few carbohydrates utilization tests, ONPG and sensitivity to 0 / 129 (10μg / ml) (Tab. 2). All the results revealed that the present isolates exhibited most relatedness to *V. harveyi* by a comparison with the data of Bergey's Manual of Determinative Bacteriology (9th ed.) (Tab. 2). The isolates were also identified as *V. harveyi* using the Biolog System.

2.3 Pathogenicity

The results of survival rates of N, Z, M and PL of *P. chinensis* after 24h exposure to 2.5×10^6 cfu / ml of Z_3G_2 were shown in Tab. 3. The bacterial isolate caused significant mortality in N, Z and early M larvae within 24h. The isolate caused 100% mortality in the Z_1 stage after 12 h exposure. In contrast, the isolate caused lower mortality in PL at the same bacterial concentration by immersion (Tab. 3).

As shown in Tab. 4, at a concentration of over 2.5×10^4 cfu / ml, the present isolate induced significant moralities (> 80%) after 24 h exposure by immersion. An increase in inoculation of the bacteria leads to more

rapid development of disease.

2.4 Confirmation of pathogenicity

The clinical signs observed in the experimentally infected larvae include poor swimming activity, anorexia and opaqueness of body, loss of setae and bending, twisting and settling to the bottom of the tank. These symptoms are the same as those observed in natural epizootics. The infected N and Z larvae settled to the bottom and moved only when disturbed. The infected M and PL always ascended spirally in a vertical direction to the surface and then sank to the bottom. The specific action of the representative bacterium of Z_3G_2 as a pathogen of *P. chinensis* larvae was confirmed by re-isolating the bacterium from moribund larvae and re-infecting the healthy larvae to satisfy Koch's postulates. Two re-isolate bacteria were identified according

续表2

Characteristics		Isolates (n=24)	Re-isolates (n=2)	*V. harveyi*[1)]
Single carbon source	propanol	−	−	d
	cellobiose	d	+	+
	α-citrulline	+	+	d
	ethanol	−	−	−
	D-gluconate	+	+	+
	L-arabinose	−	−	d
	L-glutamate	+	+	+
	L-leucine	−	−	−
	inositol	−	−	ND
	sucrose	−	−	d
	xylose	−	−	−
	α-ketoglutarate	d	+	+
Enzyme production	alginase	−	−	d
	amylase	+	+	+
	chitinase	+	+	+
	lipase	+	+	+
	gelatinase	+	+	+
	lecithnase	+	+	+
	caseinase	+	+	+
	urease	+	+	+
	ONPG	d	+	−

Note: 1) Data from Beryer's Manual of Determanative Bacteriology (9th ed.), 1994. 2) CR=curved rod. 3) SP=single polar. ND=no data; +=over 90% positive; −=over 90% negetive; d=11%—89% positive; F=fermentive

Tab.3 Survival rate of larvae and postlarvae *P.chinensis* after 24 h exposure to *V. harveyi*

Bacteria Dose(cfu/ml)	Survival rate (%)						
	N	Z_1	Z_2	Z_3	M_{1-2}	M_3	PL_{5-6}
Seawater (Control)	57.03	54.70	47.08	52.08	59.03	60.20	73.4
2.5×10^6	10.41	0	3.33	3.88	16.77	44.80	61.50

Tab.4 Survival rate of larvae (Z_2) *P. chinensis* at different times after exposure to different doses of *V. harveyi* (%)

Time (h)	Bacteria dose (cfu/ml)					
	Control	2.5×10^3	2.5×10^4	2.5×10^5	2.5×10^6	2.5×10^7
0	100.00	100.00	100.00	100.00	100.00	100.00
2	98.80	86.80	87.90	80.90	76.30	84.20
4	94.30	82.20	74.30	68.00	57.40	73.70
8	92.00	79.00	56.80	58.50	46.50	54.90
12	80.15	56.42	46.06	41.90	30.81	24.06
24	69.83	41.35	13.09	9.80	1.50	0

to the methods mentioned above (Tab. 2). These results indicate that the isolates are the causative agent of the epizootics.

3 DISCUSSION AND CONCLUSION

3.1 Classification and identification of isolates

Vibrios are the most common bacteria in coastal seawater, and some species have been reported as the pathogens of cultured *P. chinensis*. Mortality of Z and M of *P. chinensis* occurred regularly in the hatcheries in North China during the April and May in recent years and have caused serious losses (Meng *et al*, 1982; Meng, 1992). A total of 38 isolates were obtained from the infected larvae during the period of investigation. All the isolates examined were classified as being members of the genus of *Vibrio*. Twenty six of them seem to belong to the same of species, because the morphological, physiological and biochemical characteristics of these strains agreed well with each other except for growth at 10℃, sensitivity to vibriostatic agent 0 / 129 (10μg / ml), ONPG, citrate and a few carbohydrate utilizations. The present isolate was closely related to *V. harveyi* based on the morphological, physiological and biochemical characteristics examined in comparison with data described previously (Holt *et al*, 1994). However, it differed from *V. harveyi* by growth in 8% NaCl, acid from lactose and citrate utilization. Meanwhile, all isolates have been classified and identified using the Biolog System in Belgium, which results were closely coincides with this results. In view of all these results, the characteristics studied suggest the present isolate as a member of the genus *Vibrio*, with close resemblance to *V. harveyi*.

3.2 Pathogenicity

The bacteria initially isolated were confirmed to be the causative agents of epizootics in the hatchery-reared larvae of *P. chinensis* based on the results of pathogenicity experiments by immersion method. The present isolate administered via bacteria-bath at a dose of 10^6 cfu / ml was found sufficient to establish infection in N, Z and M_{1-2} larvae, but failed to induce infection in M_3 and PL. The mortality of Z_2 larvae exposed to different dosages (2.5×10^3—2.5×10^7 cfu / ml) of bacteria ranged from 100% to 40% within 24 h and the infection developed differently after different exposure time (2, 4, 8, 12 and 24 h). The mortality data show that the pathogenicity of the present isolate depends on the dosage, period of exposure and age of the shrimp, and that resistance to the present *Vibrio* isolate progressively increase as the larvae develop to the more advanced stages. Such variations in the level of mortality and susceptibility that correlate with age, dosage and time of exposure have been reported previously (Takahashi *et al*, 1984, 1985; Sahul Hameed, 1994; Sahul Hameed *et al*, 1996).

In general, most of the isolates of *Vibrio* sp. from diseased or seemingly diseased larvae shrimp may not always produce experimental infection, except when massive doses are inoculated (Lightner, 1988). Up to now, standard about the infected time and dosage for determining a pathogen is not available. On the other hand, because natural disease outbreaks always occur under different stress conditions, it is difficult to determine whether or not an isolate is pathogenic to penaeid shrimp. However, the present isolates have produced infection at lower dose (2.5×10^4 cfu / ml) by immersion. It is the first report of pathogenicity of *V. harveyi* to *P. chinensis* larvae in China.

3.3 Relationships between pathogenic *Vibrios* and stress factors to the disease outbreaks of larval shrimp

Most of the *Vibrio* spp. are opportunistic pathogens of penaeid shrimp, and vibriosis mostly

outbreaks under certain stress conditions. At the zoeal stages, an increase in bacterial count may be the result of infection, indicating the existence of stress factors to cause the onset of vibriosis in the hatchery-reared larvae of *P. chinensis*. In another disease-free hatchery in Laizhou, the bacterial counts were much lower and the different *Vibrio* sp. developed yellow colored colonies on TCBS agar, not the green ones observed in the infected hatchery (data not shown here). Stress which either reduces the resistance of the prawn or enhances the population and / or pathogenicity of the pathogen plays an important role in the disease process in prawns (de la Pefia *et al*, 1993). Minimizing stress may act as a prophylactic measure against the outbreak of vibriosis in penaeid shrimps hatcheries (Lightner, 1988).

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中国对虾幼体致病菌哈维氏弧菌的分离及其生物学特性研究*

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提要　于1996年4—5月，在青岛丰城地区一些对虾育苗场发生大规模爆发性传染病，主要感染中国对虾幼体，尤其是蚤状幼体。死亡率高达80%以上。从自然发病及人工感染患病濒死中国对虾幼体中分离出38株细菌，研究其形态特征，生理、生化特性及对中国对虾幼体的致病性。结果表明，38株分离物均为革兰氏阴性杆菌，菌体为杆状或短杆状，极生单鞭毛运动，不发光，氧化酶呈阳性，发酵葡萄糖产酸，TCBS平板上生长，菌落呈黄色或绿色，对弧菌抑制剂0/129(150g/ml)敏感，此均为弧菌属的典型特征，属于弧菌。其中26株细菌被归为同一类群，参照伯杰氏细菌学鉴定手册(1994年，第9版)鉴定表明，该菌同哈维氏弧菌(*Vibrio harveyi*)最为接近；另外，Biolog系统鉴定表明也为哈维氏弧菌，因此定名为哈维氏弧菌。利用浸泡感染法以2.5×10^{3}—2.5×10^{7} cfu/ml浓度的细菌感染不同发育时期(无节幼体期、蚤状期、糠虾期和仔虾期)的中国对虾幼体。结果表明，该病原菌主要感染中国对虾幼体的无节幼体晚期、蚤状期和糠虾早期并导致其大量死亡，而在仔虾期感染死亡率较低，并且2.5×10^{4} cfu/ml以上浓度的病原菌即可导致蚤状幼体严重感染死亡，由此可见，所分离的致病菌对中国对虾蚤状期幼体具有较强的致病力。人工感染试验结果表明，所分离的哈维氏弧菌为本次中国对虾幼体爆发性流行病的病原菌。

关键词　中国对虾幼体　致病菌　弧菌　哈维氏弧菌

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