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NOTE

Vibrio alginolyticus infection in the white shrimp *Litopenaeus vannamei* confirmed by polymerase chain reaction and 16S rDNA sequencing

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ABSTRACT: A Gram-negative, rod-shaped bacterium identified as *Vibrio alginolyticus* was isolated from diseased *Litopenaeus vannamei* (also called *Penaeus vannamei*) in Taiwanese culture ponds. The diseased shrimp displayed poor growth, anorexia, inactivity, reddish pleural borders of antennae, uropods and telson, opaque and whitish musculature, and mortality. In histological preparations, melanized hemocytic granulomas were observed in the connective tissue around hemal sinuses together with hemocytic aggregation in necrotic musculature. Six isolates of *Vibrio* were collected from diseased shrimp at 3 farms, and these were evaluated for characteristics including morphology, physiology, biochemistry and sensitivity to antibiotics. The results indicated that the isolates belonged to a single species that grew in 1 to 8% NaCl, at 10 to 40°C and on TCBS (thiosulfate-citrate-bile sucrose) agar, and that gave positive catalase, O/F (Oxidation/Fermentation), lysine decarboxylase, gelatinase and cytochrome-oxidase tests. Identification of CH003 (1 of 6 isolates) was confirmed by PCR assay for *V. alginolyticus* (expected amplicon 1486 bp). The 16S rDNA sequence (GenBank accession number AY373027) gave 99.9% sequence identity to *V. alginolyticus* (GenBank accession number X74690). The calculated 96 h LD_{50} dose of the isolated strain was 3.0×10^5 colony forming units (CFU) shrimp^{-1} (6.6×10^4 CFU g^{-1}).

KEY WORDS: *Litopenaeus vannamei* · Bacterial disease · *Vibrio alginolyticus* · PCR · 16S rDNA sequencing

INTRODUCTION

Over the past 15 yr, shrimp culture in Taiwan has been mainly based on indigenous species such as black tiger shrimp *Penaeus monodon*, kuruma shrimp *Maruspenaeus japonicus* (also called *Penaeus japonicus*) and red-tail shrimp *Fenneropenaeus penicillatus* (also called *Penaeus penicillatus*). The industry has suffered serious economic losses due to infectious diseases due to bacteria such as *Vibrio harveyi* (Liu et al. 1996a,b), *Photobacterium damsela* (Song et al. 1993) and *Vibrio alginolyticus* (Lee et al. 1996a,b), and viruses such as monodon baculovirus (MBV) (Lightner et al. 1987, Chen et al. 1989), white spot syndrome virus (WSSV) (Lo & Kou 1998) and yellow head virus (YHV) (Wang & Chang 2000). Disease outbreaks are associated with increases in the proportion of potentially pathogenic species in the *Vibrio* population of culture pond water (Lavilla-Pitogo et al. 1998, Sung et al. 2001). As a result, exotic white shrimp *Litopenaeus vannamei* (also called *Penaeus vannamei*) were introduced in 1985 (Lin et al. 1990) and have been widely cultivated since. Production reached 11 012 t in 2003 or about 5 times the combined production from indige-

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nous penaeid shrimp (2205 t). It was also higher than the production of farmed freshwater prawns *Macrobarchium rosenbergii* (10 045 t).

Traditionally, identification of a bacterial pathogen is dependent on its growth on selective media and characterization by morphological and biochemical tests (Baumann & Schubert 1984, Diggles et al. 2000). Recently, taxonomic studies have been developed based on 16S rRNA targeted polymerase chain reactions (PCR) (Kim & Jeong 2001), 16S rDNA genotyping using polymerase chain reaction/restriction fragment length polymorphisms (PCR/RFLP) (Urakawa et al. 1997), analysis of 16S–23S rDNA intergenic spacers (Kong et al. 1999), random amplified polymorphic (RAPD) DNA-PCR analysis (Aznar et al. 1993, Sudheesh et al. 2002) and DNA–DNA hybridization assays (Costa et al. 1998).

Since 2001, Taiwanese shrimp farmers have experienced disease problems linked to production declines in farmed *Litopenaeus vannamei* and associated with signs of disease different to those of Taura syndrome virus (TSV) (Yu & Song 2000). The disease signs include poor growth, anorexia, inactivity, whitish musculature and death. This paper reports on the isolation and characterization of *Vibrio alginolyticus* from the muscle of such diseased shrimp and its comparison to the reference strain *V. alginolyticus* ATCC17749. Experimental infections were also carried out with 1 isolate (CH003) to test pathogenicity.

MATERIALS AND METHODS

Isolation of bacteria. Six diseased shrimp showing whitish musculature and inactivity were collected from each of 5 farms in Pingtung, Taiwan, from May to September 2002. Bacteria were isolated from the muscle on tryptic soy agar (TSA supplemented with 2.5% NaCl, Difco) (TNA) plates incubated at 28°C for 18 h. For subsequent tests, they were incubated on either TNA or thiosulfate-citrate-bile sucrose (TCBS, Difco) supplemented with 2.5% NaCl.

To isolate bacteria, the shrimp cuticle was surface sterilized using 70% (v/v) alcohol and then cut with a sterile scalpel to expose the underlying discolored musculature. A sterile loop was touched to the discolored tissue and then streaked onto TNA, that was then incubated at 28°C for 24 h until visible bacterial colonies developed. One of the dominant colonies was then selected and re-streaked onto TNA to obtain pure cultures for identification tests.

Bacterial morphology and growth. Gram-stained smears from pure cultures of bacterial isolates were examined by light microscopy to determine cell size and morphology. After passage of test strains in tryptic soy broth (TSB, Difco) supplemented with 2.5% NaCl (TNB) and selection on TCBS agar supplemented with 2.5% NaCl for 24 h at 28°C, motility was tested by the hanging-drop method. Both colony color and growth in TCBS agar were recorded. The ability of the strains to grow in TNA at different temperatures was tested over a period of 7 d. Tolerance to NaCl was determined by the addition of different concentrations of NaCl (1 to 12%) to TNA cultures and examination for growth after 1 wk (Cheng & Chen 1998).

Biochemical characteristics. Test cultures were grown on TNA for 24 h at 28°C and then inoculated into test media for biochemical tests (Facklam & Carey 1985). Parallel tests were carried out with commercial API 20E Kits (ATB System, bioMerieux) for bacteriological identification. The reactions were compared with the reference strain *Vibrio alginolyticus* ATCC (American Type Culture Collection) 17749 (Sakazaki 1968).

For antibiotic sensitivity tests, isolates were spread on TNA plates and exposed to antibiotic discs containing 2,4-diamino-6,7-diisopropyl pteridine phosphate (O/129, 150 µg, Creative Microbiologicals), novobiocin (30 µg, BBL), neomycin (30 µg, BBL), nitrofurantoin (300 µg, BBL), sulfisoxazole (250 µg, BBL), chloramphenicol (30 µg, BBL), tetracycline (30 µg, BBL) and kanamycin (30 µg, BBL), respectively.

DNA isolation. Bacteria were grown at 28°C in TNB broth for 24 h and harvested by centrifugation at 7000 × *g* for 20 min at 4°C. The nucleic acid of pelleted bacteria was extracted using a Genomic DNA purification Kit (No. A1120, Promega) and stored at –20°C, until used for polymerase chain reaction (PCR) tests.

Polymerase chain reaction. The specific PCR primers for identification of *Vibrio alginolyticus* 16S rDNA were designed based on the method described by Ruimy et al. (1994) in the GenBank database (Benson et al. 1994) using the CLUSTAL program (Higgins & Sharp 1988). They were VA16F1 (5′-ATT GAA GAG TTT GAT CAT GGC TCA GA-3′) and VA16R1 (5′-CAG CTA TTA ACT ACA CTA-3′), and VA16F2 (5′-CCT TCG GGT TGT AAA GCA CT-3′) and VA16R2 (5′-TCC TCC CGT AGT TGA AAC TAC CTA CT-3′), respectively. The PCR reaction buffer contained 50 mM Tris-HCl buffer (pH 9), containing 50 mM KCl, 1% Triton X-100 (Boehringer Mannheim), 2.5 mM $MgCl_2$, 5 U Taq polymerase, 0.25 mM dNTPs, and 1µM of each primer. PCR reactions were performed as follows: 30 cycles of denaturation at 94°C for 1 min, annealing at 50°C for 1 min and elongation at 72°C for 2 min, followed by a 10 min extension at 72°C and cooling to 4°C. The PCR fragments were subjected to electrophoresis on a 1.5% agarose gel for length differences, and amplified DNA fragments were cloned into the pGEM-T Easy vector following the instructions provided (Promega). Recombinant bacteria were iden-

tified by blue/white screening and confirmed by PCR. Plasmids containing the insert were purified (Promega minipreps) and used as a template for DNA sequencing.

Sequencing of 16S rDNA. Nucleotide sequence analysis was performed using the dideoxynucleotide chain termination method (Sanger et al. 1977) on a DNA sequencer (Model 373A, Applied Biosystems). Plasmid DNA at 1 µg was used for sequencing with a Dye Terminator Cycle Sequencing Kit (Applied Biosystems) and subjected to electrophoresis on a 6% denaturing gel. Clones were sequenced with the M13 forward and reverse primers.

Infectivity trials. Strain CH003 was grown on TNA for 24 h at 28°C. Colonies were scraped off the plates and suspended in 0.85% NaCl, at a concentration of 2×10^8 CFU (colony-forming units) ml^{-1}. Bacterial concentration was calculated based on a standard curve created from a series of known concentrations of bacterial solution and absorbance. A series of 10-fold dilutions of this bacterial suspension was made with 0.85% NaCl solution, and 20 µl of each dilution was injected into the ventral sinus of the cephalothorax. Shrimp (4 to 5 g in the intermolt stage) were obtained from a commercial farm and acclimated in the laboratory for 1 wk prior to experimentation. The dose of bacteria per shrimp ranged from 4×10^4 to 4×10^6 CFU (see Table 3). A challenge test was conducted in triplicate with test and control groups comprising 10 shrimp for each replicate. Shrimp injected with equal volumes of sterile saline solution served as the control group. After injection, each group was held in a separate 60 l glass aquarium (10 shrimp in each replicate) containing 40 l seawater (20‰) at 28°C with aeration. Shrimp were fed with formulated shrimp feed (Shinta Feed Company) twice a day for up to 6 d. The numbers of moribund and dead shrimp were recorded daily and bacteria were isolated from the tissues of dead shrimp and characterized as described below. The LD_{50} was calculated based on the program of Trevors & Lusty (1985).

Histological observations. Muscle tissues of moribund shrimp were fixed in Davidson's solution (Bell & Lightner 1988). The fixed specimens were embedded in paraffin and cut into 5 µm sections, stained with Gram stain and with modified Mayer's hematoxylin and phloxine eosin stain before viewing under the light microscope (Sheehan & Hrachak 1980).

Table 1. Morphological and physiological characteristics of the isolated strains (n = 6) from diseased *Litopenaeus vannamei* in comparison to the reference strain *Vibrio alginolyticus*. ATCC17749. +: positive; –: negative; TSA: tryptic soy agar; TCBS: thiosulfate-citrate-bile sucrose agar; Y: yellow colonies

Character	Isolated strain	ATCC17749
Grain staining reaction	–	–
Cell morphology	Rod	Rod
Motility	+	+
Swarming on TSA (2.0% NaCl)	+	+
Growth on TCBS agar	Y	Y
Growth on MacConkey agar	+	+
NaCl tolerance		
TSA 1%	+	+
TSA 8%	+	+
TSA 10%	–	+
TSA 12%	–	–
Temperature tolerance		
4°C	–	–
10°C	+	+
40°C	+	+
50°C	–	–

RESULTS

Gross signs and histopathology of diseased shrimp

Shrimp mortality in ponds affected by disease outbreaks was up to 50% over 6 d. Typical signs of disease included anorexia, poor growth, inactivity, reddish pleural borders of antennae, uropods and telson, and opaque and whitish musculature. Upon histological examination, large numbers of bacteria were present in the musculature. Necrotic musculature was infiltrated with aggregating hemocytes.

Bacterial isolation and characterization

Six bacterial isolates were obtained from the diseased shrimp from 3 farms and identified as *Vibrio*. All were Gram-negative, short, motile rods that swarmed on TSA plates. They produced yellow colonies on TCBS agar and grew on MacConkey agar. All strains grew in media containing 1 to 8% NaCl and grew over a wide temperature range (from 10 to 40°C). Comparisons with reference strain *Vibrio alginolyticus* ATCC17749 are shown in Tables 1 & 2. All 6 strains were sensitive to 0/129, novobiocin, neomycin, nitrofurantoin, chloramphenicol, tetracycline and kanamycin. However, they were slightly resistant to sulfisoxazole.

16S rDNA sequence

The sequence of the 1486 bp 16 S rDNA PCR amplicon from Isolate CH003 was determined and deposited at GenBank under accession number AY373027. This sequence showed 99.9% identity with the sequence of the reference strain *Vibrio alginolyticus* ATCC17749

Table 2. Biochemical characteristics of isolated strains (n = 6) from diseased *Litopenaeus vannamei* in comparison to the reference strain *Vibrio alginolyticus* ATCC17749. +: positive; –: negative; OPNG: o-nitrophenyl-β-D-galactopyranoside

Test	Isolated strain	ATCC17749
Catalase	+	+
Fermentative	+/+	+/+
API 20E system		
Presence of:		
β-galactosidase (OPNG test)	–	–
Arginine dihydrolase	–	–
Lysine decarboxylase	+	+
Ornithine decarboxylase	–	–
Urease	–	–
Tryptophane deaminase	–	–
Gelatinase	+	+
Cytochrome-oxidase	+	+
Production of:		
H_2S	–	–
Indole	+	+
Acetoin	–	–
NO_2	+	+
Utilization of:		
Citrate	–	–
Glucose	+	+
Mannitol	+	+
Inositol	–	–
Sorbitol	+	+
Rhamnose	–	–
Sucrose	+	+
Melibiose	–	–
Amygdalin	–	–
Arabinose	–	–

(GenBank accession number X74690) and *V. alginolyticus* 16S rDNA (GenBank accession number X74691) (Ruimy et al. 1994). Results from morphology, biochemical tests and 16S rDNA indicated that the isolates were *V. alginolyticus*.

Experimental infections

Shrimp injected with isolate CH003 developed opaque and whitish musculature, and reddish pleural borders of antennae, uropods and telsons, and eventually died. A bacterial dose of 4×10^6 CFU induced 80% mortality in 72 h at 28°C. A dose of 4×10^4 CFU caused 23% mortality, with similar gross signs and histopathology in moribund shrimp over 48 to 144 h (Table 3). The calculated 96 h LD_{50} was 3.0×10^5 CFU shrimp^{-1}, which was equivalent to 6.6×10^4 CFU g^{-1} body weight. The infected shrimp displayed necrotic musculature infiltrated by hemocytes. Survival of control shrimp was 100% and muscle tissue was normal. Re-isolation of the injected bacteria from the moribund shrimp confirmed Koch's postulates.

DISCUSSION

In decapod crustaceans, several environmental factors, such as salinity, temperature and low dissolved oxygen, can cause opaque and whitish musculature. This opaque and whitish musculature commences at the tail and progresses to the head, which then turns to red (Lakshmi et al. 1978, Lightner 1983). These changes are reversible during the initial stages of development, if the causative stressor is eliminated. Body opaqueness has been observed in *Vibrio campebelli*-infected larvae of *Penaeus monodon* and *Fenneropenaeus indicus* (Hameed 1995). Body opaqueness, necrosis and lethargy have been observed in *Litopenaeus vannamei* larvae and postlarvae infected by *Vibrio harveyi*, *V. parahaemolyticus* and *V. penaeicida* (Aguirre-Guzmán et al. 2001). Similar gross signs and histopathology were seen in our field specimens and laboratory-challenged specimens.

Of the *Vibrio* species described in Bergey's Manual of Systematic Bacteriology, *V. alginolyticus*, *V. anguillarum*, *V. parahaemolyticus* and *V. harveyi* have been described as pathogenic to penaeid shrimp (Lightner 1983, Takahashi et al. 1985). *V. alginolyticus* isolated from moribund *Penaeus monodon* was reported to kill *P. monodon* and *Maruspenaeus japonicus* with an LD_{50} of 1.13×10^5 and 2.46×10^5 CFU g^{-1} body weight, respectively (Lee et al. 1996a,b). Our LD_{50} for *V. alginolyticus* in *Litopenaeus vannamei* was similar (3.0×10^5 CFU shrimp^{-1} or 6.6×10^4 CFU g^{-1} body weight).

Table 3. Experimental infection of *Litopenaeus vannamei* injected with isolated strain *Vibrio alginolyticus* (CH003). Cumulative mortality (%) data in the same column having different superscripted letters are significantly different ($p < 0.05$)

Bacterial dose (CFU shrimp^{-1})	No. shrimp	Time after challenge (h) 6	12	24	48	72	144
Saline	30	0	0	0	0	0	0
4×10^4	30	0	0^a	16.7 ± 3.3^b	23.3 ± 3.3^c	23.3 ± 3.3^c	23.3 ± 3.3^c
4×10^5	30	0	10.0 ± 5.8^a	46.7 ± 6.7^a	56.7 ± 3.3^b	56.7 ± 3.3^b	56.7 ± 3.3^b
4×10^6	30	0	3.3 ± 3.3^a	53.3 ± 3.3^a	76.7 ± 3.3^a	80.0 ± 5.8^a	80.0 ± 5.8^a

Vibriosis in penaeid shrimp is generally recognized as a secondary infection influenced by factors such as stress, environmental failures and high numbers of potentially pathogenic bacteria in the enviromment (Mohney et al. 1994, Lavilla-Pitogo et al. 1998). Changes in environmental factors such as salinity, pH and ammonia are capable of altering the virulence of *Vibrio harveyi* and *V. alginolyticus* (Prayitno et al. 1995, Liu & Chen 2004). For example, we previously showed that immune capacity and resistance to *V. alginolyticus* decreased in *Litopenaeus vannamei* exposed to ammonia (Liu & Chen 2004). Further work is needed on the effect of deteriorated pond water environments on shrimp immune capability and disease outbreaks. A topic of particular interest would be the effect of low salinity cultivation, since it is now commonly practiced with *L. vannamei* in Taiwan.

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