Isolation of *Vibrio vulnificus* Biotype I from Disease Outbreaks on Cultured Tiger Grouper *Epinephelus fuscoguttatus* Forsskal, 1775

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ABSTRACT—The first isolation of *Vibrio vulnificus* biotype I from disease outbreaks in cultured tiger grouper *Epinephelus fuscoguttatus* Forsskal, 1775 in southern Thailand has been described. Gross signs of diseased fish included dark coloration of body, anorexia, petechial hemorrhages in the skin of the tail and fins and ulceration of the skin. Hemorrhagic septicemia was observed in intestine, body cavity and spleen. The 205-bp amplified DNA fragment of the hemolysin gene (*vvhA*) was detected from all of bacterial isolates, indicating *V. vulnificus*. Bacteria showed characteristics of biotype 1, the human clinical isolate, as indicated by their motility, positive results for indole production, ornithine decarboxylation activity, acid production from D-mannitol and growth at 42°C. The human virulence potential of *V. vulnificus* isolates using three biomarkers, 16S ribosomal ribonucleic acid (rRNA), *vvhA* gene and virulence-correlated gene (*vcg*), showed that genotypes of *V. vulnificus* was the clinical-type with two profiles. The susceptibility of tiger grouper to *V. vulnificus* isolate was performed by intraperitoneal injection (i.p.) with 10⁶ CFU/mL bacterial suspensions. The experimentally injected tiger grouper had mortalities within 5 days post-injection and developed clinical signs similar to those found in disease outbreak. In a vaccination trial, the tiger grouper vaccinated with formalin inactivated whole-cell vaccine exhibited relative percent survival (RPS) of 68% following homologous isolate challenge. In conclusion, *V. vulnificus* biotype 1 strain that caused disease in tiger groupers is similar to *V. vulnificus* pathogenic to humans.

Key words: *Epinephelus fuscoguttatus*, tiger grouper, *Vibrio vulnificus*, biotype 1, biomarkers

Grouper aquaculture is growing rapidly in Thailand which is undertaken in floating net cage in the southern and eastern provinces. Several grouper species such as the tiger grouper *Epinephelus fuscoguttatus*, barramundi cod or humpback grouper *Cromileptes altivelis*, estuary grouper *E. coioides*, duskytail grouper *E. bleekeri*, giant grouper *E. lanceolatus* and the coral trout *Plectropomus leopardus* have been raised. Among them, tiger grouper is the most promising species as it has been successfully produced in the full-cycle culture with high growth rate in cages. Although one of the initial technical bottlenecks, larvae production, has been resolved, infectious diseases continue to challenge the successful culture of this species. In 2010, an outbreak of serious mortality occurred in cultured tiger grouper in Krabi and Phang-nga provinces, southern Thailand. Clinical signs of those affected ani-

mals showed their characteristics similar to vibrio infection.

Vibrios are Gram-negative, ubiquitous in marine and estuarine habitats, and comprise one of the major microbiota of these ecosystems. Many vibrios are serious pathogens for animal reared in aquaculture in which the disease outbreaks occur when fish are exposed to infectious agents in the presence of stress factors (Austin and Austin, 1999). It has been reported that *V. anguillarum*, *V. ordalii*, *V. salmonicida* and *V. vulnificus* biotype 2 are among the main bacterial pathogens in several marine fish species (Toranzo *et al.*, 2005). *V. anguillarum* is widely distributed causing a typical hemorrhagic septicemia in a wide variety of warm and cold water fish species of economic importance (Austin and Austin, 1999). Fish affected by this pathogenic bacterium show typical signs of a generalized septicemia with hemorrhages on the base of fins, exophthalmia and corneal opacity. Moribund fish are frequently anorexic with pale gills which reflect a severe anaemia. Oede-

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matous lesions, predominantly centered on the hypodermis, are often observed (Toranzo *et al.*, 2005). *V. vulnificus* produces diseases in human and aquatic animals and has been subdivided into 3 biotypes on the basis of differences in biochemical properties, such as indole production and mannitol fermentation, as well as serological and genetic traits, and host range (Tison *et al.*, 1982; Oliver, 1989; Amaro and Biosca, 1996; Dalsgaard *et al.*, 1996; Bisharat *et al.*, 1999). *V. vulnificus* biotype 1 and 3 are considered opportunistic human pathogens causing disease generally associated with handling or ingestion of raw shellfish, while biotype 2 is primarily eel pathogen and rarely affecting humans. (Amaro and Biosca, 1996; Biosca *et al.*, 1996; Fouz *et al.*, 2002; Fouz and Amaro, 2003; Jones and Oliver, 2009).

Recently, similar clinical signs were observed from moribund tiger grouper cultured in cages in Krabi province. This reoccurring of disease outbreak made serious losses to the farmers. In this present study, we investigate the recurrent disease outbreak on the tiger grouper. The clinical signs were described and the isolates were characterized phenotypically. The classification of strains, virulence and differentiation of their clinical and non-clinical (environmental) types were performed based on DNA polymorphisms at some loci. The findings of immunization trials was included in this study.

Materials and Methods

Sample fish

Moribund fish were collected from the cages in Krabi province from February to September in 2012 and subjected to laboratory at the Krabi Coastal Aquaculture Development Center for observation of clinical signs and bacterial isolation. Temperature and salinity during the period of disease outbreak were 28–30°C and 26–30 ppt, respectively.

Bacterial isolation and identification

Bacteria were isolated from external lesions, kidney and spleen of diseased tiger grouper using tryptic soy agar (TSA, Difco) supplemented with 1.5% NaCl and incubated for 24 h at 35°C. A colony from TSA was selected and streaked onto plates of thiosulphate citrate bile salt sucrose (TCBS) agar (Difco). The greenish colonies were selected and pure cultures were obtained. The bacterial isolates were then transferred to the Coastal Aquatic Animal Health Research Institute's laboratory for further investigation. They were characterized and identified using API 20E kit (BioMerieux) according to the manufacturer's instructions and the API profiles were compared with the API DataBase version 4.0. The inoculate TSA plates with the isolates were incubated at 42°C for 24 h. Motility was assayed on

Motility media (Sigma) supplemented with 1% NaCl.

DNA extraction

Genomic DNA extraction of the nine isolates (three strains from 2010, four strains from 2011 and two strains from 2012) was performed by boiling method (Park *et al.*, 2013) with minor modification. Briefly, bacterial cells were cultured on TSA plates containing 1.5% NaCl at 35°C for 24 h. One colony was suspended in 100 µL of sterile distilled water and boiled for 10 min at 100°C using heating block. After boiling, the suspension centrifuged for 5 min at 12,000 rpm, and the supernatant was collected and used as a template for PCR.

PCR assays

PCR assay was performed according to the method of Panicker *et al.* (2004) with slight modification. A 205-bp segment between nucleotides 786 and 990 of the open reading frame of *vvhA* (hemolysin A) gene was targeted for PCR amplification using oligonucleotide primers VVH785 (5'-TTCCAACCTTCAAACCGAAC-TATGAC-3') and VVH990 (5'-ATTCCAGT CGATGCGA-ATACGTTG-3') (Brasher *et al.*, 1998). PCR amplification was performed using GeneAmp® PCR System 9700 (Applied Biosystems). The initial denaturation was 96°C for 5 min, followed by 30 cycles of 94°C for 1 min, 57°C for 1 min and 72°C for 1 min. The final elongation step was programmed at 72°C for 7 min. The amplified DNA was analyzed in 1.8% agarose gel. *V. vulnificus* ATCC 27562 kindly provided by Dr. Pomwised Rattanarужи, Faculty of Science, Prince of Songkla University, was served as positive control and nuclease-free water replacing DNA was included as negative control.

Genotyping

V. vulnificus biotype 1 is subdivided into genotypes, clinical and environmental, based on DNA polymorphisms at some loci. In this study, we differentiate *V. vulnificus* virulent strains by using 16S ribosomal ribonucleic acid (rRNA), virulence-correlated gene (*vcg*) and cytotoxin/hemolysin gene (*vvhA*). The V1-V3 region of bacterial 16S rRNA gene was amplified from genomic DNA using PCR with a pair of eubacterial universal primers: 8UA (5'-AGAGTTTGTATCCTGGCTCAG-3') and 519B (5'-ATTACCGCSGCTGCTG-3') (Acinas *et al.*, 1999; Bertilsson *et al.*, 2002). PCR was performed as described by Nilson *et al.* (2003) and PCR products were sequenced in ABI Prism Genetic Analyzer (Applied Biosystems). Genotypes of each isolate were determined depending on sequence homology to *V. vulnificus* strain CMCP6 (GenBank accession number NR_074889.1) or *V. vulnificus* strain JY1305 (GenBank accession number PRJNA49015) using BLAST software (<http://www.ncbi.nlm.nih.gov/blast/>). A 277-bp segment of the *vcg* gene was targeted for PCR amplification.

cation as described by Rosche *et al.* (2005). Two primer sets, P1 (5'-AGCTGCCGATAGCGATCT-3') and P3 (5'-CGCTTAGGATGATCGGTG-3'), and P2 (5'-CTCAATTGACAATGATCT-3') and P3 were used for identification of the C-type and E-type isolates, respectively. Genotyping of *vvhA* was performed using PCR assay according to Senoh *et al.* (2005). An 813-bp segment of the *vvhA* gene was targeted for PCR amplification using two primer sets, *vvhA*-1F (5'-AGATTAAGTGTGTTGCACA AGCGGTG-3') and *vvhA*-1R (5'-ACCGAAAACAGCGCTGAAGGAAGAACGG-TA-3'), and *vvhA*-2F (5'-AAATTAAGT GCGTGCTACACACA-AGTGGTG-3') and *vvhA*-2R (5'-ACTGAGAAGAGT-GCTGAAGGG ATTACCGTA-3'). The *vvhA* gene amplified with *vvhA*-1F and *vvhA*-1R was designated as type 1 and *vvhA* amplified with *vvhA*-2F and *vvhA*-2R was designated as type 2 (Senoh *et al.*, 2005).

Pathogenicity

To demonstrate the pathogenicity, 20 healthy tiger grouper with average weight of 25 g were injected intraperitoneally (i.p.) with 10^6 CFU/mL of EP05-12 bacterial suspension. Sterile 1.5% NaCl was injected in the same manner to the control group. Mortalities were recorded daily for 10 days post injection. Bacteria were isolated from moribund fish and biochemically identified using API 20E kit as previously described to confirm the cause.

The lethal dose (LD_{50}) concentration of *V. vulnificus* strain EP05-12 was determined. The isolate was taken from -80°C , re-cultured on TSA for 24 h at 35°C . The single colony was transferred to tryptic soy broth (TSB, Difco) incubated for 18 h at 35°C . The optical density of bacterial suspension was determined to obtain the value of 0.1 at 640 nm which composed of 10^8 CFU/mL, then a ten-fold dilution was made to obtain the bacterial concentration of 10^7 , 10^6 , 10^5 , 10^4 , 10^3 , 10^2 CFU/mL. Tiger grouper were intraperitoneally injected

with different concentration of those bacteria 10 fish each. The mortality was recorded for 10 days. The LD_{50} were calculated according to the method of Reed and Muench (1938).

Vaccination and experimental challenge

The formalin inactivated whole-cells (EP05-12) vaccine was prepared and 0.1 mL of vaccine was injected i.p. to 100 healthy juvenile tiger grouper with approximate weight ranging from 25 to 30 g at the final concentration of 10^9 CFU/mL. Control group was injected in the same manner with 0.1 mL PBS per fish. For specific immune response, blood was collected from the caudal vasculature of five fish each from both vaccinated and control fish at 2, 7, 14, 21, 30, 45 and 60 days post vaccination. Serum was collected and tested for antibody production against whole cells of EP05-12 strain using a standard microagglutination procedure (Roberson, 1990). Challenge test was performed on 60 days post vaccination. Forty fish of each group (vaccinated and control) were challenged with EP05-12 and observed for 10 days. The level of protection was calculated according to Amend (1981).

$$\text{RPS} = (1 - \% \text{ mortality in vaccinated fish} / \% \text{ mortality in control fish}) \times 100\%$$

Results

Clinical signs

The diseased tiger grouper exhibited clinical signs typical of bacterial septicemia, which included external hemorrhaging at pectoral fin and tail (Fig. 1a), cutaneous lesions with hemorrhages on the skin (Fig. 1b). Some fish had exophthalmia and corneal opacity (Fig. 1a). Internally, hemorrhages in body cavity and spleen, pale kidney and bloody discharges in the abdominal cavity were observed. Similar clinical signs were also observed from diseased tiger grouper col-

Fig. 1. Vibriosis in tiger grouper *Epinephelus fuscoguttatus*: a) hemorrhagic pectoral fin and tail including exophthalmia and corneal opacity. b) hemorrhagic skin lesions on the dorsal part.

lected in 2010 and 2011.

Phenotypic characterization

Most of bacterial isolates from kidney and spleen of diseased tiger grouper grown on TSA supplemented with 1.5% NaCl exhibited round and opaque colonies with 2–3 mm in diameter. However, bacterial isolates from skin lesions were mixed culture but colonies similar to those found from kidney and spleen were abundant. The round and opaque colonies had greenish coloration on TCBS, suggesting *Vibrio* sp. According to the API database, this profile would correspond to *V. vulnificus* with a probability of 99.7%. Most of the strains were motile as performed on Motility agar and grew at 42°C. In API 20E, all strains were positive for indole production, ornithine decarboxylation and acid production from D-mannitol, suggesting the isolates belong to biotype 1.

PCR assay

The PCR assay resulted in the expected 205-bp

amplified DNA fragment from all bacterial isolates tested including the positive control, indicating *V. vulnificus* (Fig. 2). Thus the isolates have been confirmed.

Genotypic analysis of *V. vulnificus*

Table 1 summarizes the genetic profiles of all nine isolates. Genotypes of *V. vulnificus* were designated on the basis of 16S rRNA, *vcg* and *vvhA* types which can be divided into one group with two profiles. Based on 16S rRNA genetic analysis, genotypes of the isolates were determined depending on sequence homology to *V. vulnificus* strain JY1305 representative of type A (GenBank accession number PRJNA49015) or *V. vulnificus* strain CMCP6 representative of type B (GenBank accession number NR_074889.1). The results indicated that all of nine isolates belonged to type B, the clinical-type. Similarly, all isolates were classified as the clinical type (C-type) in term of *vcg* type. For *vvhA* type, eight isolates were designated as type 1, the clinical type, only EP05-10 isolate possessed type 2, the non-clinical (environmental) type.

Fig. 2. *Vibrio vulnificus*. The 205 bp of the bacteria isolated from diseased tiger groupers. Lanes: M, 1 kb molecular weight standard; N, negative control; P, positive control, A, EP01-10; B, EP05-10; C, EP-10-10; D, EP-04-11; E, EP-06-11; F, EP-07-11; G, EP-12-11; H, EP-03-12; I, EP-05-12.

Table 1. Distribution of genotypes of *V. vulnificus* according to the represented isolates from diseased tiger grouper in 2010–2012

Isolates	16S rRNA		<i>vvhA</i>		<i>vcg</i>	
	Type B	Type A	Type 1	Type 2	C-type	E-type
EP01-10	+	–	+	–	+	–
EP05-10	+	–	–	+	+	–
EP-10-10	+	–	+	–	+	–
EP-04-11	+	–	+	–	+	–
EP-06-11	+	–	+	–	+	–
EP-07-11	+	–	+	–	+	–
EP-12-11	+	–	+	–	+	–
EP-03-12	+	–	+	–	+	–
EP-05-12	+	–	+	–	+	–

Fig. 3. Survival rates of tiger grouper after challenged with *V. vulnificus*. The relative percent survival rate (RPS) was 68%. [RPS = (1 – %mortality in immunized fish/%mortality in control fish) × 100]

Fig. 4. Antibody titers of immunized tiger grouper injected with formalin-killed vaccine on day 2, 7, 14, 21, 30, 45 and 60.

Pathogenicity

The susceptibility of tiger grouper to *V. vulnificus* isolate was investigated by intraperitoneal injection (i.p.) with 10^6 CFU/mL of bacterial suspension. Experimental injected fish showed mortalities within 5 days post-injection and developed clinical signs of hemorrhagic septicemia with lesions on skin and internal organs similar to those found in disease outbreak. LD_{50} at 10 days of *V. vulnificus* strain EP05-12 was 3.32×10^6 CFU/mL or 3.32×10^5 CFU/fish.

Vaccination

Tiger grouper vaccinated with formalin inactivated whole-cell vaccine exhibited a specific humoral response with antibody titers ranging from 1:64 to 1:256. In a vaccination trial, the immunized fish exhibited relative percent survival (RPS) of 68% following homologous isolate challenge (Fig. 3). This formalin-killed vaccine induced protective immunity last for 2 months (Fig. 4).

Discussion

This report described recurrence of disease outbreaks of *V. vulnificus* on cultured tiger grouper in Krabi province, Thailand. The infected fish exhibited dark-colored fish, hemorrhage around the pectoral fins and swollen cutaneous lesions with petechial hemorrhages on the skin. They were exophthalmia and corneal opacity. Clinical signs and macroscopic findings on diseased tiger grouper were similar to vibrio hemorrhagic septicemia reported in several species of groupers (Tendencia and Lavilla-Pitogo, 2004). Similar symptoms, such as intestinal necrosis, anemia, ascetic fluid, petechial hemorrhages in the muscle wall, liquid in the air bladder, were also reported from other marine fish caused by pathogenic vibrios (Amaro and Biosca, 1996; Biosca *et al.*, 1996; Fouz *et al.*, 2002; Fouz and Amaro, 2003; Toranzo *et al.*, 2005; Jones and Oliver, 2009). All strains gave an identical API 20E profile correctly identified as *V. vulnificus* with a probability of 99.7%.

The API 20E system has been introduced and widely used for routine laboratory diagnostics and identification of fish bacterial pathogens however they still have some limitation to some species. Biosca *et al.* (1993) noticed some false negative reactions especially with citrate utilization when they tested *V. vulnificus* strains by the API 20E system. They also suggested including an oxidative/fermentative (O/F) test to discriminate between *Burkholderia cepacia* and *V. vulnificus*. Sanjuán *et al.* (2009) reported that only 60% of their studied strains were correctly identified as *V. vulnificus* strains using API 20E system and the remaining strains gave a mixed profile or were misidentified. Popovic *et al.* (2007) suggested that the API 20E can be used in identification and differentiation of fish bacterial pathogens in some species for presumptive test only. In this study, our isolates were confirmed using PCR assay. Result showed that the isolates yielded 205-bp amplification product of the open reading frame of *vvhA* gene in the PCR assay which was identical to *V. vulnificus*. The set of primers used proved to be highly species specific to *V. vulnificus* (Brasher *et al.*, 1998; Panicker *et al.*, 2004; Cañigral *et al.*, 2010). All of nine isolates showed characteristics of biotype 1 as indicated by their motility, positive results for indole production, ornithine decarboxylation activity, acid production from D-mannitol and growth at 42°C. Tison *et al.* (1982) differentiated biotype 1 from biotype 2 using phenotypic characters such as positive indole production, ornithine decarboxylation, acid production from mannitol and growth at 42°C. Amaro *et al.* (1992) summarized the main phenotypic characters distinguishing the two biotypes were ornithine decarboxylation, indole production and acid from mannitol. However, Dalsgaard *et al.*, (1999) described the biochemical characteristics of biotype 2 *V. vulnificus* isolates from diseased eel showing properties similar to previously described for eel-pathogenic strains of *V. vulnificus*, except positive indole production. Sanjuán *et al.* (2009) divided *V. vulnificus* strains into three biotypes according to their difference in biochemical and biological properties. Biotype 1 strain causes human disease, while biotype 2 strain causes disease called warm-water vibriosis in fish (mainly eels) and shrimp, and biotype 3 strain has been associated with handling tilapia. Our isolates belong to the biotype 1 and cause disease in fish.

Genotyping systems based on DNA polymorphisms at some loci have been used to determine the virulence potential between the clinical-type and non-clinical (environmental) type *V. vulnificus* isolates (Nilsson *et al.*, 2003). In this present study, we investigated *V. vulnificus* biotype 1 isolated from diseased tiger grouper using three biomarkers: the 16S rRNA, *vvhA* and *vcg*. All nine isolates were classified as the clinical-type for all genotypes with two different profiles, the 16S rRNA type B, *vvhA* type 1 and *vcg* type C and the 16S rRNA

type B, *vvhA* type 2 and *vcg* type C. Several reports revealed that most of *V. vulnificus* human isolates (biotype 1) should be *vcg* type C, *vvhA* type 1, and 16S rRNA type B and most environmental isolates should be *vcg* type E, *vvhA* type 2, and 16S rRNA type A (Nilsson *et al.*, 2003; Rosch *et al.*, 2005; Senoh *et al.*, 2005; Sanjuán *et al.*, 2009). Yokochi *et al.* (2013) classified *V. vulnificus* isolated from patients and environment into three profiles based on the 16S rRNA, *vvhA* and *vcg* type C. Among the combination profiles observed, the profile 1 (16S rRNA type B, *vvhA* type1 and *vcg* type C) and profile 4 (16S rRNA type B, *vvhA* type2 and *vcg* type C), showed the same profiles as our finding.

The pathogenic assays revealed that *V. vulnificus* biotype 1 was moderate virulent for tiger grouper with challenged, moribund fish showing the typical signs of a hemorrhagic septicemia. Tison *et al.* (1982) suggested that only strains of biotype 2 were eel pathogens and biotype 1 could only be isolated from the water or the surface of eels. Dalsgaard *et al.* (1999) reported that *V. vulnificus* isolated from diseased eels in Denmark were biotype 2 strains but having indole-positive. This phenotypic character was differed from the strains described by Tison *et al.* (1982) showing indole-negative. Mahmud *et al.* (2010) reported that *V. vulnificus* strains isolated from tilapia collected from market, netted fish and water samples in Bangladesh were biotype 1 of the clinical type. The immunized fish exhibited a specific humoral response with antibody titers ranging from 1:64 to 1:256, showing relative percent survival of 68% following homologous isolate challenge. This formalin-killed vaccine induces protective immunity last for 2 months. Similar finding was observed in the study of Collado *et al.* (2000) who demonstrated a protective immune response against *V. vulnificus* serovar E in vaccinated eels under laboratory conditions. They showed that the immunized fish exhibited a specific immune response with titers ranged from 1:4 to 1:8 for fish injected with whole-cell bacterin.

In this present study, we demonstrate the causative agent isolated from diseased tiger grouper belongs to *V. vulnificus* biotype 1 and genetically differed from strains commonly associated with fish. Genetic virulent profiles of the isolates show the same profiles as those found in *V. vulnificus* human biotype 1. We therefore conclude that *V. vulnificus* biotype 1 strain caused disease in tiger grouper is similar to *V. vulnificus* pathogenic to humans.

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