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Short communication

Plasmid mediated tetracycline resistance of *Vibrio parahaemolyticus* associated with acute hepatopancreatic necrosis disease (AHPND) in shrimps

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ABSTRACT

Antimicrobial resistance is one of the most important problems in public health, veterinary medicine and aquaculture. Importantly, plasmid mediated antibiotic resistance of pathogenic *Vibrio parahaemolyticus* from shrimp can potentially be transferred through transposition, conjugation and plasmid uptake to different bacterial species in aquaculture systems. In this study, we evaluated the antibiotic resistance pattern in *V. parahaemolyticus* strains associated with acute hepatopancreatic necrosis disease (AHPND) from penaeid shrimp and identified AHPND strains from Mexico showed a high level of resistance to tetracycline (≥5 μg/mL) and have the *tetB* gene coding tetracycline resistance. In particular, the *tetB* gene was carried in a single copy plasmid (named as pTetB-VA1) comprising 5162-bp with 40% G + C content from the strain (13-511/A1). The plasmid pTetB-VA1 consists of 9 ORFs encoding tetracycline resistant and repressor proteins, transcriptional regulatory proteins and transposases and showed a 99% sequence identity to other *tet* gene plasmids (pIS04_68 and pAQU2).

1. Introduction

Vibrio spp. are found in marine and estuarine environments throughout the world (Chakraborty et al., 1997). Also, in fish and aquatic crustaceans, this genus is present as a part of the normal intestinal microflora (Ruangpan and Kitao, 1991). Some *Vibrio* spp. such as *Vibrio anguilarum*, *Vibrio harveyi*, and *Vibrio parahaemolyticus* can cause bacterial diseases in shrimp (Lightner, 1993).

Recently, an emerging disease named acute hepatopancreatic necrosis disease (AHPND, also known as early mortality syndrome, EMS) has been causing significant losses in shrimp production in the Southeast Asian countries (Flegel, 2012; Leaño and Mohan, 2012; NACA-FAO, 2011; Lightner et al., 2012) and Mexico (Nunan et al., 2014). This disease is caused by *V. parahaemolyticus* and affects the hepatopancreas of infected shrimp (Tran et al., 2013). AHPND/EMS causes mortality, up to 100%, in *Litopenaeus vannamei* and *Penaus monodon* (Zorriehzahra and Banaederakhshan, 2015).

Tetracycline is one of the most commonly used antibiotics in aquaculture because of its efficiency and low cost (Neela et al., 2007). However, its extensive use has been associated with a rising level of resistance and this has resulted in the establishment of resistance determinants (Chopra and Roberts, 2001). Thus, the aim of this study is the identification of antibiotic resistant strains of *V. parahaemolyticus* associated with AHPND. We also investigated the genetic determinants (*tet* gene) of tetracycline resistance, which is located in the mobile element (5162-bp plasmid). Plasmid mediated antibiotic resistance is of great concern since these resistance determinants can potentially be disseminated among bacteria due to plasmid mobility.

2. Materials and methods

Seventy-eight (78) *Vibrio* strains were isolated from shrimp cultured in Vietnam, Mexico, India, USA, Philippines, Ecuador and Peru, and bacterial identifications were conducted at the University of Arizona Aquaculture Pathology Laboratory using 16S rRNA sequencing (Weisburg et al., 1991). The strains of *V. parahaemolyticus* were further confirmed by PCR targeting species specific genes (*tlh* and *toxR* genes) (Bej et al., 1999; Kim et al., 1999). Pathogenic strains, associated with AHPND/EMS, were selected by conventional PCR targeting *pir*A- and *pir*B-like genes (Han et al., 2015), and the pathogenicity were determined by laboratory infections through immersions or per os feeding, followed by histological examinations described by Tran et al. (2013). Tryptic Soy Broth with

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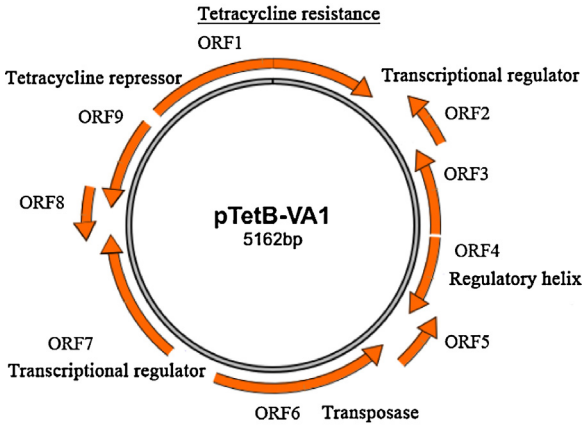

Fig. 1. A circular map of plasmid pTetB-VA1 (5162 bp) was constructed using Vector NTI program. Solid arrows show ORFs carried by the plasmid and the direction of the arrowheads represents the transcriptional orientation.

Table 1
Primers used for comparative qPCR and plasmid sequencing.

Name	Sequences	Size (bp)	
tetB-F	TTGCGGGAATTTGGCCTATCAATT	103	qPCR
tetB-R	GTTGAGACGCAATCGAATTCGGTAT		
toxR-F[a]	AATCCATGGATTCCACGCGTTATTT	103	
toxR-R[a]	CACCAATCTGACGGAACTGAGATTC		

Name	Sequences	Strand	Region (nt) in pTetB-VA1
*tetB*F	TCATTGCCGATACCACCTCAG	+	350–370
*tetB*F2	AAGTAGGGGTTGAGACGCAAT	+	575–595
*tetB*F3	GCTGACAATGGCGTTTACCT	+	1496–1515
*tetB*F4	GGTATTTTCAGCGGCTGTTC	+	2176–2195
*tetB*R4	GCGTAACAGATGCAGCCATA	–	3746–3765
*tetB*R3	GCTGTGGGGCATTTTTACTT	–	4657–4676
*tetB*R2	TAGCAACGCAGCGATAAAAA	–	482–501
*tetB*R	CCAACCATCATGCTATTCCATCC	–	718–740

[a] Primers were described by Han et al. (2015).

2.5% NaCl (TSB+) (Difco, Sparks, MD, USA) and Tryptic Soy Agar with 2.5% NaCl (TSA+) (Difco) were used for culturing at 28 °C.

The antibiotic susceptibility was determined by the disc diffusion method on Mueller-Hinton agar II (Difco) with 2.5% NaCl plates according to Clinical and Laboratory Standards Institute guidelines (CLSI, 2006). The antibiotic discs ampicillin (AMP, 10 μg), florfenicol (FF, 25 μg), oxytetracycline (OTC, 30 μg), tetracycline (TE, 30 μg) and nalidixic acid (NA, 30 μg) (BD BBL Sensi-Disk, BD Biosciences, Sparks, MD, USA) were used, and the results were recorded as resistant or susceptible by measuring the inhibition zone diameter according to the standard of CLSI (2006). For the tetracycline resistant strains, minimum inhibitory concentration (MIC) was determined according to the modified protocol of CLSI (2006), by a standard agar dilution method as follows: dilution of the tetracycline ranged from 1 to 100 μg/ml using a two-fold dilution series.

Bacterial DNA was extracted by boiling. Briefly, a single colony was resuspended in 100 μl of water, boiled for 10 min, pelleted, and the supernatant was collected. Screening of the tetracycline resistance genes (*tetA*, *tetB*, *tetC*, *tetD* and *tetE*) was conducted using a PCR-based technique according to the conditions described by Nawaz et al. (2006). PCRs were performed with PuReTaq Ready-To-Go PCR beads (GE Healthcare, Bio-Science Corp., Piscataway, NJ, USA).

One of the tetracycline resistant strains (13-511/A1), which contains the *tetB* gene, was selected for plasmid sequencing and analysis. The strain was grown overnight at 28 °C in TSB+ and used for plasmid preparation following the modified procedure of alkaline lysis (Kado and Liu, 1981). Bi-directional primer walking was

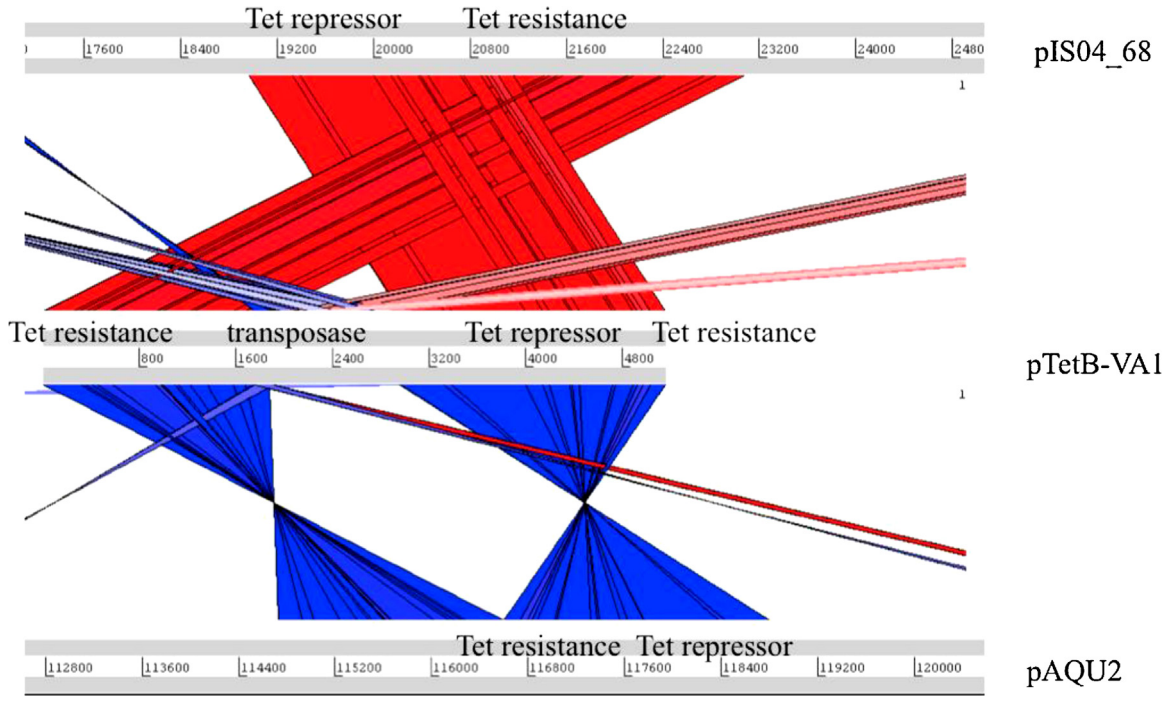

Fig. 2. Schematic representationof the comparative analysis of the sequence of the plasmid (pTetB-VA1) from *V. parahaemolyticus* (GenBank no. KM189195) with its related plasmids (a) pIS04-68 from *E. coli* (GenBank no. HG963476) and pAQU2 from *Vibrio* sp. (GenBank no. AB856327) using the Artemis Comparison Tool (ACT). Translated blast (TBLASTX; score cut-off, 40) was used to align translated genome sequences of plasmids. The blue and red lines represent the reverse and forward matches, respectively, and color intensity is proportional to the sequence homology. Nucleotide base-pairs were indicated between grey lines for each plasmid genomes. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

used according to the method described by Han et al. (2012). Its sequence was verified by DNA sequencing at University Arizona sequencing facility.

The eight primers used for primer walking are shown in Table 1. The full genome sequences of the plasmid from the strain (13-511/A1) were subsequently analyzed using the Vector NTI program (Invitrogen, Carlsbad, CA, USA) for the circular map and ORF prediction. Blast searches were conducted using the BLAST server of the National Center for Biotechnology Information website (http://www.ncbi.nlm.nih.gov), and the Artemis Comparison Tool (ACT) was used as a graphical pairwise DNA sequence comparison viewer (Carver et al., 2005).

This strain (13-511/A1) was further used for comparative qPCR analyses. All qPCR was performed with StepOnePlus real-time PCR system (Life Technologies, NY, USA) with a PerfeCta SYBR Green FastMix kit (Quanta Biosciences, Gaithersburg, MD, USA). Reactions comprised 1× SYBR Green qPCR mix, 300 nM primer pairs targeting the *tetB* gene (Table 1), and 1 μl of bacterial DNA. The following cycling conditions were employed: initial denaturation 95 °C for 3 min, followed by 40 cycles of 95 °C for 5 s, 60 °C for 30 s. Each sample was in duplicate and the *toxR* gene is used as an endogenous control (Table 1). The ΔCt values between the *tetB* gene and *toxR* gene were calculated by the StepOne vs.2.2.2 software using a comparative Ct method.

3. Results and discussion

Seventy-eight (78) *Vibrio* strains were collected in this study. Among these, 30 strains were identified as *V. parahaemolyticus* and 9 strains were further identified as AHPND pathogenic *V. parahaemolyticus* strains (Table 2).

AHPND/EMS is known for the speed with which it can cause high mortalities in shrimp farms (Zorriehzahra and Banaederakhshan, 2015). Several remedies to control this disease have been proposed, but a definitive solution is still unclear. Antibiotics are frequently used in aquaculture, as feed additives or by immersion bath to achieve prophylaxis or therapy (Devi et al., 2009). However, diagnoses are frequently presumptive, and treatments for causative pathogens are generally administered without confirmatory or susceptibility tests in many aquaculture systems (Hooper, 2001). The use of inappropriate antibiotics then leads, not only to therapeutic failures, but also to increasing prevalence of antibiotic resistant strains (Giraud et al., 2004).

The susceptibility of pathogenic *V. parahaemolyticus* strains to ampicillin, florfenicol, oxytetracycline, tetracycline and nalidixic acid was examined by the disc diffusion method (Table 3). Tetracyclines are the most commonly used antibiotics in clinical medicine, veterinary medicine and agriculture (Gilchrist et al., 2007; Neela et al., 2007). Of the pathogenic strains tested, the strains (13-511/A1 and 13-306D/4) from Mexico were resistant to oxytetracycline and tetracycline by disc diffusion method. Moreover, these strains (13-511/A1 and 13-306D/4) exhibited a high level of resistance to tetracycline (≥5 μg/mL) by a standard agar dilution method. Ampicillin resistance was observed in all tested strains, similar to what has been reported in other studies (Son et al., 1998; French et al., 1989).

One of the major mechanisms of tetracycline resistance in both gram-positive and gram-negative bacteria is that it is mediated by efflux systems that export the antibiotic out of the cell as fast as it enters (Chopra and Roberts, 2001). In order to find a relationship between the antibiotic resistance phenotypes and the presence of antibiotic resistance genes, we carried out PCR targeting tetracycline resistance genes (*tetA* to *tetE*), encoding for an efflux pump (Nawaz et al., 2006). The results revealed that both tetracycline resistant strains (13-511/A1 and 13-306D/4) contained the *tetB* gene (Table 3). In addition, the amplified PCR products were sequenced and aligned with the *tetB* gene sequences in GenBank and the amplified fragments showed (>99%) homology with the *tetB* gene of *Escherichia coli, Pasteurella multocida, Klebsiella ocitoca,* and *Salmonella enterica* (GenBank nos. KF362122.2, CP003328.1, CP003683.1, and JN983042.1). Other *tet* genes were not observed in the tested strains.

Table 2
Isolation of *Vibrio* spp. and collection of pathogenic strains associated with AHPND.[a]

Country of origin	No. of strains analyzed (n = 78)	16S rRNA sequencing	No. of *V. parahaemolyticus* strains[b]		No. of AHPND strains
			tlh	*toxR*	
Mexico	3	*V. parahaemolyticus* (2) *Vibrio* sp. (1)	2/2 (100%)	2/2 (100%)	2/2 (100%)
Vietnam	10	*V. parahaemolyticus* (9) *Vibrio* sp. (1)	9/9 (100%)	9/9 (100%)	7/9 (78%)
India	1	*V. parahaemolyticus* (1)	1/1 (100%)	1/1 (100%)	0/1 (0%)
USA	2	*V. parahaemolyticus* (2)	2/2 (100%)	2/2 (100%)	0/2 (0%)
Philippines	1	*V. harvei* (1)	0	0	0
Ecuador	28	*V. parahaemolyticus* (11) *V. harvei* (1) *V. alginolyticus* (1) *V. communis* (9) *V. owensii* (2) *V. hepatarium* (1) *V. shilonii* (2) *V. brasiliensis* (1)	11/11 (100%)	11/11 (100%)	0/11 (0%)
Peru	33	*V. parahaemolyticus* (5) *V. communis* (9) *V. vulnificus* (1) *V. alginolyticus* (1) *V. neptunius*/ *V. sinaloensis* (1)	5/5 (100%)	5/5 (100%)	0/5 (0%)

[a] Acute hepatopancreatic necrosis disease (AHPND).
[b] *Vibrio* (V.).

Table 3
Pathogenic *V. parahaemolyticus* isolates used in this study, antibiotic disc test and resistance genes screening.

Isolates	Species	Disease	Origin	Disc test					PCR
				Amp	FFC	OTC	TE	NA	*tetA* to *E*
13-511/A1[a]	*V. parahaemolyticus*	AHPND	Mexico	R	S	R	R	S	*tetB*
13-306D/4[a]	*V. parahaemolyticus*	AHPND	Mexico	R	S	R	R	S	*tetB*
12-194/g	*V. parahaemolyticus*	AHPND	Vietnam	R	S	S	S	S	–
13-028/A3[b]	*V. parahaemolyticus*	AHPND	Vietnam	R	S	S	S	S	–
14-188/1	*V. parahaemolyticus*	AHPND	Vietnam	R	S	S	S	S	–
14-188/2	*V. parahaemolyticus*	AHPND	Vietnam	R	S	S	S	S	–
14-188/3	*V. parahaemolyticus*	AHPND	Vietnam	R	S	S	S	S	–
14-188/4	*V. parahaemolyticus*	AHPND	Vietnam	R	S	S	S	S	–
14-188/5	*V. parahaemolyticus*	AHPND	Vietnam	R	S	S	S	S	–

[a] Nunan et al. (2014). Isolates collected from Mexico.
[b] Tran et al. (2013). Isolates collected from Vietnam.

Excessive use of antibiotics generates a strong selective pressure that has resulted in the transfer of resistance genes associated with plasmids or transposons among bacterial species (Kehrenberg et al., 2001). In gram-negative bacteria, plasmid mediated genes coding for tetracycline efflux proteins are widely distributed and these are normally associated with large and conjugative plasmids (Chopra and Roberts, 2001; Schmidt et al., 2001). In this study, the *tetB* gene coding for efflux pump systems was located in the plasmid, but in a small genome (5162-bp, with a G+C content of 40%) of the strain 13-511/A1. A circular map and predicted genes are shown in Fig. 1. The identified plasmid was designated as pTetB-VA1 and the complete nucleotide sequence of the plasmid has been deposited in the GenBank database (Genbank no. KM189195).

Artemis comparison tool (ACT) with the pTetB-VA1 and the most related plasmids (pIS04_68 and pAQU2) are shown in Fig. 2. Although pTetB-VA1 has a small genome, it shows a high sequence identity to these two plasmids, pIS04_68 (67,973-bp) from *E. coli* strain ISO4 (GenBank no. HG963476), and pAQU2 (160,406-bp) from *Vibrio* sp. 04Ya090 (GenBank no. AB856327). Additionally, the tetracycline resistance gene was found with the transposon (ORF5 and 6) in the plasmid. The ORF5 (*tnpR*) showed a 100% sequence identity with the corresponding genes of pIS04_68 and pAQU2, and the ORF 6 (*tnpA*) showed 90% homology to those of *V. campbellii* ATCC BAA-1116 chromosome I (GenBank no. CP006605.1), and *V. cholerae* plasmid pKA1 (GenBank no. AY858559.1).

We also performed qPCR to determine the copy number of the *tetB* gene compared to the *toxR* gene. Plasmids carrying the *tetB* gene have usually large genome (IncH type plasmid) and approximately 1–2 copy number of the *tetB* gene per chromosome (Taylor, 1989). Although the plasmid size is different, the copy number of the pTetB-VA1 (0.6-fold in relation to *toxR* gene) is similar to those of reported plasmids. The toxR protein is a transcriptional regulator for outer membrane proteins and has been used as *V. parahaemolyticus*-specific marker (Kim et al., 1999). There is only 1 copy of *toxR* gene found in chromosome I in the *V. parahaemolyticus* RIMD 2,210,633 and BB22OP.

In conclusion, we found 2 pathogenic tetracycline resistant strains associated with AHPND/EMS from shrimp. Identified tetracycline resistance was related with the *tetB* gene (plasmid mediated) among the tested strains. The *tetB* gene encoded plasmid (pTetB-VA1) from the strain (13-511/A1) is a single plasmid which showed high (99%) sequence similarities to reported plasmids. Also, it has tetracycline resistance gene with transposon. Although the results discussed in this study may not be directly related with the shrimp farming industry, identification of plasmid-mediated resistance in pathogenic AHPND strains may lead therapeutic failure in the aquaculture fields. In addition, these raise the possibility of an antibiotic resistance gene spreading among species in aquaculture systems, which underscores the importance of determining the antimicrobial susceptibilities of the microorganism before proceeding with a treatment.

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