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Original Article

Antimicrobial resistance and *pirAB*-like profiles of *Vibrio parahaemolyticus* in Pacific white shrimp

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ABSTRACT

Antimicrobial susceptibility, plasmid-mediated quinolone resistance (PMQR) genes, the *pirAB*-like virulent gene and DNA gyrase A (*gyrA*) subtyping were studied to update the virulent profiles of *Vibrio parahaemolyticus* isolated from Pacific white shrimp (*Litopenaeus vannamei*) and pond water of shrimp farms. Sixty-six isolates were obtained from seven provinces in Thailand. Disc diffusion assay revealed the following antimicrobial resistant rates: ampicillin, 98.48%; doxycycline, 3.03%; oxytetracycline, 4.55%; erythromycin, 6.06%; florfenicol, 1.52%; and trimethoprim/sulfamethoxazole, 1.52%. The isolates were not resistant to quinolone agents (ciprofloxacin, enrofloxacin, norfloxacin, ofloxacin). Nine PMQR genes were screened in the isolates that presented quinolone intermediate susceptibility. Only the *qnrVC* quinolone-resistant gene was found in one multidrug-resistant (MDR) isolate that was collected from the pond water. Of all isolates, 39.39% carried the *pirAB*-like gene, which causes acute hepatopancreatic necrosis disease in shrimp. All *pirAB* positive isolates were collected from the shrimp samples but not from the water. Based on the *gyrA* sequence subtyping, the isolates were classified into five types. The isolates that carried the *pirAB*-like virulent gene were of three different types, whereas the MDR isolates were of the same type. There was no association between the *pirAB* and resistant profiles. Though *V. parahaemolyticus* in this study presented low antimicrobial resistance rates, the few isolates that presented MDR profiles and their capabilities to acquire a plasmid resistant gene indicate the risk of AMR dissemination in the environment.
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Introduction

The outbreak of acute hepatopancreatic necrosis disease (AHPND), originally known as early mortality syndrome (EMS), has caused serious global economic losses in the shrimp farming industry and has been reported in Thailand since late 2011 (Food and Agricultural Organization, 2013). *Vibrio parahaemolyticus* that carries *Photorhabdus* insect-related (Pir) toxins was proved to be the causative bacteria. These PirAB-like proteins are encoded by the 336-bp *pirA*-like (17198–17533) and 1317-bp *pirB*-like (17545–18862) that are located in the conjugative plasmid (accession number KM067908) (Han et al., 2015). The disease has been reported in Pacific white shrimp (*Litopenaeus vannamei*), Black tiger shrimp (*Penaeus monodon*) and Chinese white shrimp (*Penaeus chinensis*) (Tran et al., 2013).

Although *V. parahaemolyticus* is frequently presented in aquatic products, most strains of this species are nonpathogenic to humans. However antimicrobial-resistant (AMR) *V. parahaemolyticus* have been increasingly isolated from aquatic species. AMR bacteria have an impact not only because of the difficulty in animal treatment but also the serious concern for public health (Veldman et al., 2011). Antimicrobials that are used commonly for humans are the first priority to be investigated for AMR isolates contaminated in food products and environment.

Quinolone belongs to a group of antimicrobials that is widely used in humans, animals and aquatic species in Asia (Alday et al., 2012). Mechanisms of quinolone resistance include the alteration

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of gene coding for DNA gyrase sub-unit A (*gyrA*) and/or topoisomerase type IV sub-unit A (*parC*) at the quinolone-resistance determination region (QRDR) and the acquisition of plasmid-mediated quinolone resistance (PMQR) genes (Hooper, 1999; Ruiz et al., 2012). The PMQR genes have been found in different sizes of plasmids and in a variety of bacteria including Enterobacteriaceae and *Pseudomonas* spp. These indicate the simple transferability of resistance.

The objective of this study was to investigate the antimicrobial resistance profiles of *V. parahaemolyticus* in both *pirAB*-carried and non-*pirAB*-carried isolates from Pacific white shrimp and pond water from areas of acute hepatopancreatic necrosis disease (AHPND) outbreaks in Thailand. The resistant genes of PMQR and QRDR were examined. The subtyping of *gyrA* was applied for simple grouping of the isolates.

Materials and methods

Bacterial isolation

Between 2013 and 2015, 66 V. *parahaemolyticus* isolates from Pacific white shrimp ($n = 51$) and shrimp farm pond water ($n = 15$) were collected from seven provinces of Thailand comprising Chanthaburi ($n = 2$), Chon Buri ($n = 2$), Nakhon Pathom ($n = 18$), Phetchaburi ($n = 18$), Rayong ($n = 23$), Songkla ($n = 1$) and Suphan Buri ($n = 2$). The isolates were obtained from shrimp farms in AHPND outbreaks areas. The samples were submitted for bacterial identification at the Kamphaeng Saen Diagnostic Service, Faculty of Veterinary Medicine, Kasetsart University, Kamphaeng Saen Campus, Bangkok, Thailand and the Rayong Coastal Fisheries Research and Development Centre, Rayong, Thailand.

The isolates were cultured on chromogenic agar (CHROMagar Microbiology; Paris, France) and incubated at 30 °C for 24 h. Biochemical reaction tests were conducted to identify the species of *V. parahaemolyticus*, which comprised growth in NaCl (weight per volume (w/v) at 0%, 3%, 6%, 8% and 10%), API 20E diagnostic strips (bioMérieux; Durham, NC, USA) and a cytochrome oxidase test. The isolates were confirmed by amplifying the target transmembrane regulatory protein (*toxR*). The amplification conditions were 40 cycles consisting of denaturation at 94 °C for 1 min, annealing at 63 °C for 1 min and an extension at 72 °C for 1.5 min. The primer sequences were ToxR_forward 5′-GTC TTC TGA CGC AAT CGT TG-3′ and ToxR_reverse 5′-ATA CGA GTG GTT GCT GTC ATG-3′ (Kim et al., 1999).

Antimicrobial susceptibility test

The antimicrobial susceptibility of the isolates was tested using disc diffusion assay on Mueller-Hinton agar, supplemented with 1% NaCl, following the guidelines of the Clinical and Laboratory Standards Institute (2006a). Ten antimicrobial agents were assessed: ampicillin (10 μg), ciprofloxacin (5 μg), doxycycline (30 μg), enrofloxacin (5 μg), erythromycin (15 μg), florfenicol (30 μg), norfloxacin (10 μg), oxytetracycline (30 μg), ofloxacin (5 μg) and trimethoprim/sulfamethoxazole (1.25/23.75 μg) (Oxoid Ltd.; Basingstoke, UK). *Escherichia coli* ATCC 25922 was used as the control strain. The inhibition zones were evaluated according to the recommendations of Clinical and Laboratory Standards Institute (2006b).

Amplification of plasmid-mediated quinolone resistance genes

The extracted DNA was amplified to detect the presence of the nine PMQR genes described earlier: *qnrA*, *qnrB* and *qnrS* (Gay et al., 2006), *qnrC* and *qnrD* (Veldman et al., 2011), *oqxA* (Hansen et al., 2005), *qepA* (Richter et al., 2010), *acc(6')-Ib-cr* (Park et al., 2006) and *qnrVC* (Liu et al., 2013). The polymerase chain reaction (PCR) mixture was prepared to contain 10 × Tag buffer with $(NH_4)_2SO_4$ ($-MgCl_2$), 25 mM of $MgCl_2$, 10 mM of dNTPs, 5 U of Tag DNA Polymerase (Thermo Fisher Scientific Inc.; Waltham, MA, USA), 0.5 μM of forward and reverse primers and 1.5 μL of DNA template. The PCR cycle consisted of 30 s pre-denaturing at 95 °C, 30 s denaturing at 94 °C, 30 s of annealing at the different temperatures as required by each pair of primers, 30 s of extension at 72 °C and 5 min of final extension at 72 °C.

Amplification of pirAB-like genes

The *pirAB*-like gene was screened using PCR assay with an AP3 primer (Sirikharin et al., 2014). The 2.1 kb DNA fragments were sequenced between nucleotide residue positions 16766–18970 (accession number KM067908).

Quinolone-resistance determination region and DNA typing

Each QRDR was sequenced and analyzed for the nucleotides of *gyrA*, using the 485-bp primers set: GyrA_1W 5′-ACG CCC GTA AAC ATT GAR GAW GAG CT-3′ (forward) and GyrA_2W 5′-TGC GGC GGG ATG TTG GTT GCC ATA CC-3′ (reverse) that were designed for this study. Each of the PCR products was purified using a gel elution kit (GMbiolab Ltd.; Taichung, Taiwan) and sequenced using Sanger dideoxy sequencing (First BASE Laboratories Sdn Bhd; Selangor, Malaysia). The nucleotide sequences were aligned in BioEdit (Hall, 1999), translated to amino acid codons and point mutation detected by comparing with the sequence of *gyrA* reported in Okuda et al. (1999). Comparison of *gyrA* nucleotide sequences was performed for subtyping the bacteria. A phylogenetic tree was constructed using the neighbor-joining tree method with 500 bootstrap replicates to differentiate the *gyrA* type among the 66 isolates of *V. parahaemolyticus*.

Analytical methods

Primer design, nucleotide sequence alignment, amino acid translation and gene mapping were performed using the BioEdit sequence alignment editor, version 7.2.5 (Hall, 1999). A basic local alignment search tool (BLAST) was used to analyze all the nucleotide sequencing results (http://blast.ncbi.nlm.nih.gov). The molecular evolutionary genetic analysis (MEGA 6.0.6) software (Tamura et al., 2013) was used to construct the *gyrA* phylogenetic tree. The NCSS 2007 software (Hintze, 2007) was used for statistical analyses. Pearson's chi-square test and Fisher's exact test were used to analyze the correlation of subtype I and four other subtypes of *V. parahaemolyticus*.

Results

Among the 66 V. *parahaemolyticus* isolates from the shrimp and pond water samples, 26 (39.39%) carried *pirAB*-like genes that caused AHPND. All the positive *pirAB*-like isolates were from shrimp samples. The 2.1-kb nucleotide sequences of PCR products, which included the *pirAB*-like gene, of the 26 isolates were aligned in BioEdit and showed 100% identity to each other.

Antimicrobial susceptibility profiles of the isolates that were positive and negative for *pirAB*-like genes are presented in Table 1. The isolates conferred resistance to six antimicrobial agents (ampicillin, doxycycline, oxytetracycline, erythromycin, florfenicol, trimethoprim/sulfamethoxazole), with rates of 98.48%, 3.03%, 4.55%, 6.06%, 1.52% and 1.52%, respectively. Most of the isolates were resistant to ampicillin while low resistant rates to the other antimicrobials were recorded. Although the isolates did not show

Table 1
Correlation of gene encoded acute hepatopancreatic necrosis disease (*pirAB*)-like genes, isolate origins, antimicrobial resistance and quinolone intermediate susceptibility of *Vibrio parahaemolyticus* from shrimp and water samples.

pirAB[a] (n)	Origin (n)	Antimicrobial resistance isolates (n)						Quinolone intermediate isolates (n)			
		AMP	DOX	OXY	ERY	FFC	SXT	CIP	ENR	NOR	OFL
Positive (26)	Shrimp[b] (26)	26	0	0	0	0	0	4	0	4	4
Negative (40)	Shrimp (25)	24	0	0	2	0	0	2	0	1	1
	Water[c] (15)	15	2	3	2	1	1	1	1	0	0
TOTAL (66)		65	2	3	4	1	1	7	1	5	1
		98.5%	3.03%	4.55%	6.06%	1.52%	1.52%	10.61%	1.52%	7.58%	1.52%

Susceptibility breakpoints evaluated following Clinical and Laboratory Standards Institute (2006b).
AMP, ampicillin (10 μg); CIP, ciprofloxacin (5 μg); DOX, doxycycline (30 μg); ENR, enrofloxacin (5 μg); ERY, erythromycin (15 μg); FFC, florfenicol (30 μg); NOR, norfloxacin (10 μg); OXY, oxytetracycline (30 μg); OFL, ofloxacin (5 μg); SXT, trimetroprim/sulphamethoxazole (1.25/23.75 μg).
[a] PCR of the *pirAB*-like gene.
[b] Pacific white shrimp (*L. vannamei*) samples.
[c] Pond water of shrimp farms.

Table 2
Antimicrobial resistant profiles, gene encoded acute hepatopancreatic necrosis disease (*pirAB*), quinolone resistant gene (*gnrVC*) and DNA gyrase A (*gyrA*) subtypes of three multi-drug resistant *V. parahaemolyticus* isolates in water samples from Rayong province.

No	Source	Province	AMP	DOX	OXY	ERY	FFC	SXT	CIP	ENR	NOR	OFL	*pirAB*	*qnrVC*	*gyrA* type
1	Water	Rayong	R	R	R	I	S	I	I	I	S	I	N	P	IV
2	Water	Rayong	R	I	R	R	R	R	S	S	S	S	N	N	IV
3	Water	Rayong	R	R	R	R	S	I	S	S	S	S	N	N	IV

R = resistant; I = intermediate; S = sensitive; N = negative polymerase chain reaction; P = positive polymerase chain reaction.
Susceptibility breakpoints evaluated following Clinical and Laboratory Standards Institute (2006b).
AMP, ampicillin (10 μg); CIP, ciprofloxacin (5 μg); DOX, doxycycline (30 μg); ENR, enrofloxacin (5 μg); ERY, erythromycin (15 μg); FFC, florfenicol (30 μg); NOR, norfloxacin (10 μg); OXY, oxytetracycline (30 μg); OFL, ofloxacin (5 μg); SXT, trimetroprim/sulphamethoxazole (1.25/23.75 μg).

resistance to quinolone agents, they showed intermediate susceptibility profiles to ciprofloxacin (7, 10.61%), enrofloxacin (1, 1.52%), norfloxacin (5, 7.58%) and ofloxacin (1, 1.52%) as shown in Table 1.

To determine the molecular characteristics of quinolone resistance, the *gyrA* mutation and nine PMQR genes were investigated. The results revealed a *qnrVC* PMQR gene in one negative *pirAB*-like isolate that had intermediate susceptibility profiles to ciprofloxacin, enrofloxacin and ofloxacin (Table 2). The *qnrVC* PCR product was nucleotide sequenced and yielded a 492-bp fragment, which showed 100% identity to the gene that codes for a pentapeptide-repeat protein named QnrVC1 (Belotti et al., 2015). The nucleotide sequence of *qnrVC* was deposited in the GenBank (KU363013).

The nucleotide sequencing of *gyrA* and the amino acid translation of the isolates indicated that none of the amino acid changes in *gyrA* was associated with resistance to quinolones. The fragment DNA subtyping of the *gyrA* housekeeping gene classified the 66 isolates of *V. parahaemolyticus* into five subtypes: type I (48.48%), type II (3.03%), type III (13.64%), type IV (33.33%) and type V (1.52%). One-point or two-point mutations at locations of 237, 375, 450 or 456 of the *gryA* nucleotide sequence were found (Table 3). The *gyrA* subtypes and geographic regions in Thailand of the isolates are shown in Fig. 1. The positive *pirAB*-like isolates belonged to three types: type I (21 isolates), type II (2 isolates) and type IV (3 isolates) (Table 3). The proportion of *V. parahaemolyticus* type I compared to that of the other four subtypes revealed that the isolates carried a *pirAB*-like gene and were most likely to occur in type I ($\chi^2 = 17.90$, df = 1, $p < 0.0001$).

Discussion

In this study, *V. parahaemolyticus* isolated from shrimp in Thailand was relatively sensitive to antimicrobials. There was no significant ($p < 0.05$) difference in antimicrobial susceptibility profiles between *pirAB*- positive and negative isolates (Table 1). Nearly all isolates (65 of 66) were resistant to ampicillin. Three isolates that presented multidrug-resistant (MDR) patterns were collected from water samples in one province and were in *gyrA* subtype IV. Their R-Types were AMP- DOX-OXY-ERY-FFC-SXT, AMP-DOX-OXY-ERY, and AMP-DOX-OXY (Table 2). Possibly, the isolates might be from the same clone that developed resistance in the environment of that area.

Quinolone-resistant phenotypes and genotypes were the particular focus of this study. Fluoroquinolone is a drug choice threating *V. parahaemolyticus* infection in humans. Quinolone resistances had been increasingly found in *V. parahaemolyticus* (Elmahdi et al., 2016). Enrofloxacin is allowed to be used for therapeutics in aquatic species in Thailand but the extra-label quinolone use has been prohibited since 2016 (Government Gazette, 2018) In this study, the isolates were not resistant to ciprofloxacin, enrofloxacin, norfloxacin or ofloxacin. Therefore, the quinolone intermediate susceptibility isolates were investigated alternatively. The isolate that presented *qnrVC* had intermediate susceptibility to three of the quinolone agents tested and this isolate was an MDR strain (Table 2). The gene encoded Qnr protein belongs to the pentapeptide repeat family, which consists of uninterrupted pentapeptide repeats and plays a role in DNA gyrase protection (Richter et al., 2010) Based on the nucleotide BLAST results, the

Table 3
Point mutation of DNA gyrase A (*gyrA*) nucleotide five subtypes of 66 *Vibrio parahaemolyticus* isolates from shrimp and water and their gene encoded acute hepatopancreatic necrosis disease (*pirAB*)-like positive polymerase chain reaction.

Subtype	Nucleotide position of *gyrA*[a]				Number of isolates (*pirAB*-like positive)
	237	375	450	456	
I	A	A	C	A	32 (21)
II	A	C	T	A	2 (2)
III	G	A	T	A	9 (0)
IV	A	A	T	A	22 (3)
V	G	C	T	C	1 (0)
ATCC17802[b]	A	C	T	A	–

[a] Nucleotide position of *gyrA* start from ATG start codon.
[b] *V. parahaemolyticus* ATCC 17802 (CP014046) was a positive control strain.



Fig. 1. Locations of five subtypes (I, II, III, IV, and V) of *Vibrio parahaemolyticus* disseminated in seven provinces of Thailand. The isolates number are in parentheses (map sourced: ArcGIS Online https://www.arcgis.com/home/index.html).

492-bp fragment of *qnrVC* showed 100% identity to the sequences of *qnrVC1* in different bacteria, including those of the *V. cholerae* strain VC627 class I integron (EU436855), *Pseudomonas aeruginosa* strain Pa6 (KC000001) and Pa25 (JX861889) plasmids, *V. cholerae* non-O1/O139 strain DRV181 class 1 integron (HM015625), *Ralstonia pickettii* strain EXT149 (KJ547693) and uncultured bacterium plasmid pKAZ1 (KM506769). Furthermore, the fragment of *qnrVC* shared 97.56% identity to *qnrVC*6 reported in *V. parahaemolyticus* from China (accession number NG041633 and KC022804).

All *V. parahaemolyticus* samples that carried the *pirAB*-like gene were isolated from the shrimp samples. It was more likely to find *pirAB*-positive isolates from shrimp than from water because the bacteria prefer to colonize in the shrimp gastrointestinal tract where they can encode toxin that causes tissue destruction and dysfunction. The nucleotide sequences of the *pirAB*-like gene in the present study showed a 100% identity to those described in reports of other strains of AHPND-causing *V. parahaemolyticus*, including CV_CHN, 3HP, 5HP (Joshi et al., 2014), DE1_S1, DE2_S2, D06_S3 (Kongrueng et al., 2014), 13-028/A3 (Nunan et al., 2014) and M0605 (Tran et al., 2013). The present study confirmed that the profiles of the *pirAB*-like genes of different strains of *V. parahaemolyticus* are the same. To investigate more virulent genes, the human-virulent genes *tdh* and *trh* were amplified in this study. Fortunately, they were not found in any isolates (data not shown).

Aquaculture is another important route for dissemination of AMR into the environment. Awareness of AMR *V. parahaemolyticus* is not as well documented as other foodborne bacterial pathogens. Though *V. parahaemolyticus* strains that caused AHPND in shrimp do not infect humans, the increased antimicrobial use for treatment in infected shrimp farms might result in an increase in AMR bacteria. Even though *V. parahaemolyticus* in this study presented low antimicrobial resistant rates, the few isolates that presented MDR profiles and their capabilities to acquire plasmid resistant gene indicated the risk of AMR dissemination. Therefore, AMR surveillance in aquaculture should be performed closely to protect against the emergence and spread of resistant bacteria into the environment.

Conflict of interest

The authors declare that they have no conflict of interest.

Compliance with ethical standards

This article does not contain any studies with human participants or animals performed by any of the authors.

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