Multiple Drug Resistant Isolates of *Vibrio* Carrying the Transferable R Plasmid from Shrimp Farms in Thailand

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ABSTRACT—One thousand forty-nine bacterial isolates, which were collected from shrimp farms in Thailand, were resistant to antimicrobial agents: ampicillin (ABPC), chloramphenicol (CP), kanamycin (KM), oxolinic acid (OA), streptomycin (SM) and tetracycline (TC). One hundred forty-two isolates were identified as *Vibrio*. These isolates were conjugated with *Escherichia coli* for detection of transferable R plasmid. Six patterns of transferable R plasmids, which encoded resistance to the combinations of one to five drugs (ABPC, CP, SM, sulfamonomethoxine, TC and trimethoprim), were found from 16 drug-resistant isolates of *Vibrio*. This is the first report of transferable R plasmids from *Vibrio* spp. in shrimp farms in Thailand.

Key words: *Vibrio*, antimicrobial resistance, transferable R plasmid, shrimp

Black tiger shrimp *Penaeus monodon* and pacific white shrimp *Penaeus vannamei* are some of the most important culture species in Thailand, with combined total production of about 509,000 tons in 2008¹⁾. However, as in other culture species, these shrimps are impacted by outbreaks of infectious diseases, which had led to serious economic damage. Antibiotics and other chemotherapeutics are widely used in shrimp farms to treat bacterial diseases. While the extensive use of antimicrobial agents as feed additives for prophylactic and therapeutic purposes has contributed significantly to shrimp production, it is also thought to be responsible for the increase of drug resistant bacteria^{2–5)}. In fact, shrimp pathogenic bacteria were found to have resistant genes of antimicrobial agents^{6,7)}. One of the major mechanisms by which drug resistance in bacteria arise is conjugative, transferred resistance between bac-

teria by the resistant plasmid, R plasmid. The transfer of drug resistance by R plasmid represents a major threat to the treatment of infectious diseases in both humans and animals, including shrimp, makes treating bacterial infections with antimicrobial agents very difficult shrimp^{4,5,8,9)}. There have been some reports of drug resistance and plasmid profiling of *Vibrio* strains in shrimp like the resistance to carbenicillin of bacteria isolated from penaeid shrimp in Mexico¹⁰⁾, the multiple drug resistance plasmids from shrimp farms in India¹¹⁾ and the transferable plasmid that is resistant to chloramphenicol (CP) in *V. harveyi* from white shrimp¹²⁾. In the present study, we isolated drug resistant strains of *Vibrio* spp. in shrimp farms in Thailand and determined whether they carry transferable R plasmids.

Materials and Methods

Screening of drug resistant bacteria from shrimp farms

All bacterial isolates were collected in 2006–2007 from different regions in Thailand. The samples were obtained from hepatopancreas, intestine and muscle of shrimp and water in shrimp farms. Bacteria were grown on Tryptic Soy Agar (TSA) (Difco) with 2% sodium chloride (NaCl) and an antimicrobial agent (Table 1). All plates were incubated at 25°C for 24–48 h. In this experiment, we didn't attempt to isolate sulfamonomethoxine (SMMX)-resistant bacteria using TSA with 2% NaCl. Through antagonism between 4-aminobenzoic acid (PABA) and sulfa drugs in microbes, the antimicrobial activity of sulfa drugs is decreased in the general growth media containing PABA.

Identification of *Vibrio* spp.

All drug resistant strains were identified using four biochemical methods: Gram-staining, cytochrome-oxidase test, oxidation/fermentation of glucose (O/F) test and growth ability test on TSA media containing various concentrations of NaCl (0%, 1% and 3%). Only resistant strains of *Vibrio* spp. carrying one or more transferable R plasmids were identified to species using API 20E and 20NE test (bio-Mérieux).

Detection of transferable R plasmid

Each strain of drug resistant *Vibrio* spp. was initially cultured in 2% NaCl-Tryptic Soy Broth (TSB) (Difco), while *Escherichia coli* RC85 nalidixic acid (NA) resistant *nal* and *E. coli* HB101 streptomycin (SM) resistant *str*, used as recipients, were grown in 2 × Yeast Tryptone (2 × YT) broth.

The broth cultures of drug resistant *Vibrio* isolates and *E. coli* RC85 *nal* were mixed 1:1 and incubated overnight at 37°C and then plated on BTB-lactose-LB agar containing 0.0045% bromothymol blue (BTB), 1% lactose, 50 µg/mL NA and one of the drugs to which the *Vibrio* spp. was resistant. Under these conditions, only

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Table 1. Screening of drug resistant bacteria from shrimp farms using single antimicrobial agent

Location	No. of bacteria resistant to antimicrobial agents (No. of <i>Vibrio</i> resistant to antimicrobial agents)						
	ABPC*	CP	KM	OA	SM	TC	Total
Chachoengsao	28 (2)**	7 (1)	76 (1)	62 (1)	86 (4)	55 (1)	314 (10)
Chantaburi	75 (24)	0 (0)	64 (24)	55 (1)	64 (9)	53 (4)	311 (62)
Phatthalung	1 (0)	0 (0)	1 (1)	0 (0)	0 (0)	1 (0)	3 (1)
Samutsakhon	20 (0)	14 (0)	65 (4)	69 (0)	66 (3)	29 (3)	263 (10)
Satun	6 (4)	0 (0)	7 (2)	2 (0)	4 (2)	3 (0)	22 (8)
Songkhla	8 (8)	0 (0)	8 (3)	1 (0)	0 (0)	8 (3)	25 (14)
Trad	18 (4)	0 (0)	21 (13)	16 (0)	29 (18)	27 (2)	111 (37)
Total	156 (42)	21 (1)	242 (48)	205 (2)	249 (36)	176 (13)	1,049 (142)

* ABPC: ampicillin, CP: chloramphenicol, KM: kanamycin, OA: oxolinic acid, SM: streptomycin, TC: tetracycline.

** The number of *Vibrio* spp. is indicated in parenthesis.

These bacteria were isolated in Tryptic soy agar with 2% NaCl containing 10 µg/mL ABPC, 5 µg/mL CP, 10 µg/mL KM, 1 µg/mL OA, 10 µg/mL SM, or 5 µg/mL TC.

E. coli RC85 *nal* that acquired an R plasmid from *Vibrio* would grow. The transconjugants were purified three times on a similar medium to confirm the presence of a transferred R plasmid. The purified colonies were then tested for their resistance to other drugs by culturing them in the same media described above but containing a different drug to which the *Vibrio* spp. were resistant. The resulting transconjugant *E. coli* RC85 *nal* from *Vibrio* were retransferred to *E. coli* HB101 *str* by mixed (1:1) cultivation in 2 × YT broth at 37°C. The mixed cultures were plated on BTB-lactose-LB agar containing 1,000 µg/mL SM and another drug depending on their resistance markers. The transconjugants were purified three times on a similar media¹³⁾.

Determination of antimicrobial agent susceptibility in transferable R plasmid

The R plasmid-carrying *E. coli* RC85 *nal* were further cultivated in Müller-Hinton agar (MHA) (Difco) to determine the minimum inhibitory concentration (MIC) of six antimicrobial agents: ampicillin (ABPC), chloramphenicol (CP), SM, tetracycline (TC), sulfamonomethoxine (SMMX) and trimethoprim (TMP). It is reported that the R plasmids, which were detected from fish pathogenic bacteria, were encoded with SMMX and/or TMP¹⁴⁾. The detected R plasmids were tested to whether they encode these drugs or not.

The MIC was determined using the standard agar dilution method following Clinical and Laboratory Standards Institute guidelines¹⁵⁾. The drug resistance marker of R plasmid was determined by comparing *E. coli* RC85 *nal* carrying plasmid and carrying non-plasmid. We also tested the antibiotic susceptibility of *E. coli* HB101 *str* carrying a transferable R plasmid to four quinolones: oxolinic acid (OA), enrofloxacin (ERFX), norfloxacin (NFLX) and ofloxacin (OFLX) by the same method. The difference in MICs between *E. coli* HB101 *str* carrying R plasmid and no R plasmid was more evident when measured using a one-step dilution

method (e.g. 0.01, 0.02, 0.03, ... 0.05 µg/mL).

Results and Discussion

Assuming that bacteria that can grow in a medium containing an antimicrobial agent were resistant isolates, from a total of 1,049 isolates resistant to the antimicrobial agents, 142 were characterized to be *Vibrio* spp. (Table 1).

Sixteen of the 142 antimicrobial-agent-resistant *Vibrio* isolates were found to carry transferable R plasmids. All these *Vibrio* spp. from which R plasmids were detected were isolated from shrimp but not from the pondwater. MICs of *Vibrio* spp. carrying R plasmid against eight antimicrobial agents and the R plasmid transconjugants against six antimicrobial agents are shown in Table 2 and 3, respectively. The detected transferable R plasmid showed resistance to the combination of ABPC, CP, SM, SMMX, TC and/or TMP (Table 4). The resistance markers of transferable R plasmids from *Vibrio* are classified into six patterns from one to five drugs. The survival, propagation and transferring of R plasmids in the resistant *Vibrio* strains suggest that resistance to a particular antimicrobial agent can be transferred, and that the proliferation of *Vibrios* resistant to antimicrobial agents such as OTC, SA, and the combination of SA and TMP/OMP may have been brought about the indiscriminate use of these agents for treating bacterial infection in shrimp farms in Thailand. Although most of the R plasmids isolated in the present study encoded CP resistance (Table 3), only one strain isolated in Chachoengsao showed CP resistance when grown in the medium at a concentration of 5 µg/mL CP (Table 1). It is possible that MIC varies according to bacterial species (i.e. *Vibrio* and *E. coli*) even if the same R plasmid is maintained (Tables 2 and 3).

Recent reports have shown that the presence of R plasmid encoding resistance to quinolones have been found in human pathogens¹⁶⁾. *E. coli* HB101 *str* carry-

Table 2. The MIC value of *Vibrio* isolates carrying R plasmids

ID. of isolates	<i>Vibrio</i> identified	MIC (μ g/mL) of antimicrobial agents*							
		ABPC	CP	KM	OA	SMMX	SM	TC	TMP
1 SCW2JP-A**	<i>V. parahaemolyticus</i>	8	0.25	16	0.25	2	128	0.5	1
2 SCAa5.1	<i>V. vulnificus</i>	32	0.13	128	0.06	16	4	0.125	2
3 442OA	<i>V. alginolyticus</i>	0.25	0.25	8	4	128	16	0.5	1
4 464SM	<i>V. alginolyticus</i>	16	128	8	0.25	512	512	32	512
5 SCW2JP-S	<i>V. parahaemolyticus</i>	8	0.25	16	0.25	2	128	0.5	1
6 TVSOW72	<i>V. parahaemolyticus</i>	32	1	16	0.25	512	32	64	1
7 STW8,5-A	<i>V. parahaemolyticus</i>	16	16	8	0.5	512	128	0.5	512
8 STW14,6	<i>V. alginolyticus</i>	64	1	16	0.25	4	32	0.5	2
9 134K	<i>V. parahaemolyticus</i>	4	0.5	16	0.25	2	32	16	0.5
10 AVGW56	<i>V. parahaemolyticus</i>	32	16	8	0.25	512	256	0.5	2
11 STW8,2	<i>V. parahaemolyticus</i>	16	16	16	0.25	512	256	0.5	2
12 STW8,5-S	<i>V. parahaemolyticus</i>	16	16	8	0.5	512	128	0.5	512
13 STW9,3	<i>V. parahaemolyticus</i>	16	16	16	0.25	512	256	32	2
14 STW15,1	<i>V. parahaemolyticus</i>	4	8	8	0.25	512	128	16	1
15 TVGW56	<i>V. parahaemolyticus</i>	32	16	32	0.25	512	256	16	1
16 AST4	<i>V. parahaemolyticus</i>	8	8	16	0.25	128	256	8	256

* ABPC: ampicillin, CP: chloramphenicol, KM: kanamycin, OA: oxolinic acid, SMMX: sulfamonomethoxine, SM: streptomycin, TC: tetracycline, TMP: trimethoprim.

** All *Vibrio* spp., carrying transferable R plasmids, were isolated from cultured shrimp.

Table 3. The MIC value of *Escherichia coli* RC85 *nal* carrying R plasmids from *Vibrio* spp.

ID. of isolates	<i>Vibrio</i> identified	Location	MIC (μ g/mL) of antimicrobial agents*					
			ABPC	CP	SMMX	SM	TC	TMP
<i>E. coli</i> RC85 <i>nal</i>			4	4	4	4	4	0.25
1 SCW2JP-A**	<i>V. parahaemolyticus</i>	Chantaburi	16	8	4	4	2	0.5
2 SCAa5.1	<i>V. vulnificus</i>	Chantaburi	16	8	4	4	2	0.5
3 442OA	<i>V. alginolyticus</i>	Chachiengsao	4	4	4	512	2	0.5
4 464SM	<i>V. alginolyticus</i>	Chachiengsao	4	4	4	512	2	0.5
5 SCW2JP-S	<i>V. parahaemolyticus</i>	Chantaburi	4	4	4	512	2	0.5
6 TVSOW72	<i>V. parahaemolyticus</i>	Songkhla	8	4	4	512	2	0.5
7 STW8,5-A	<i>V. parahaemolyticus</i>	Trad	64	4	4	4	4	0.5
8 STW14,6	<i>V. alginolyticus</i>	Trad	32	4	4	4	4	0.25
9 134K	<i>V. parahaemolyticus</i>	Samutsakhon	4	8	4	16	256	0.5
10 AVGW56	<i>V. parahaemolyticus</i>	Chantaburi	4	128	512	512	4	0.25
11 STW8,2	<i>V. parahaemolyticus</i>	Trad	8	128	512	256	4	0.5
12 STW8,5-S	<i>V. parahaemolyticus</i>	Trad	8	128	512	256	4	0.5
13 STW9,3	<i>V. parahaemolyticus</i>	Trad	8	128	512	512	128	0.5
14 STW15,1	<i>V. parahaemolyticus</i>	Trad	8	128	512	512	64	0.25
15 TVGW56	<i>V. parahaemolyticus</i>	Chantaburi	4	128	512	256	128	0.5
16 AST4	<i>V. parahaemolyticus</i>	Satun	4	128	512	256	128	512

* ABPC: ampicillin, CP: chloramphenicol, SMMX: sulfamonomethoxine, SM: streptomycin, TC: tetracycline, TMP: trimethoprim.

** All *Vibrio* spp., carrying transferable R plasmids, were isolated from cultured shrimp.

ing R plasmid (STW8,5-S) had an MIC against quinolones (OA, ERFX, NFLX, OFLX) from 1.5 to 2.5 times that of a non R plasmid carrying strain at 0.2, 0.008, 0.021 and 0.021, respectively. However, another *E. coli* HB101 *str* containing an R plasmid transferred from *Vibrio* was found to have the same MIC values as a non-plasmid carrying strain. The present data is insufficient to show whether shrimp bacteria pos-

sesses R plasmid that are resistant to quinolones. Hence, we are presently conducting trials to answer this question.

The resistant isolates of *Vibrio* spp. carrying transferable R plasmids included twelve isolates of *V. parahaemolyticus*, three isolates of *V. alginolyticus* and one isolate of *V. vulnificus*. This is in agreement with the previous report that *V. parahaemolyticus* was the pre-

Table 4. Drug resistance markers of transferable R plasmid detected in *Vibrio* spp.

Drug resistance marker(s)*	No. of isolates
ABPC	4
SM	4
SM, TC	1
CP, SM, SMMX	3
CP, SM, SMMX, TC	3
CP, SM, SMMX, TC, TMP	1
Total	16

* ABPC: ampicillin, CP: chloramphenicol, SM: streptomycin, SMMX: sulfamonomethoxine, TC: tetracycline, TMP: trimethoprim.

dominant species in hepatopancreas and was the main species responsible for mass mortality in shrimp²⁾. In the present study, we identified the different *Vibrio* spp. through API test. However, there is a need to conduct more tests to determine whether these isolates, particularly the *V. parahaemolyticus* and *V. vulnificus* isolates, are the same biovars that cause diseases in humans.

To our knowledge this is the first report to demonstrate the presence of multiple transferable R plasmids in *Vibrio* spp. from shrimp farms. Resistance to antimicrobial agents is widespread in *Vibrio* spp. and their relationship with resistance genes should be further studied.

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