Antimicrobial Resistance of Vibrio parahaemolyticus Isolated from White Shrimp and Water Samples in Shrimp Farms, Phang-Nga Province

การดื้อยาของเชื้อ Vibrio parahaemolyticus ที่แยกได้ในตัวอย่างกุ้งขาวและน้ำจากฟาร์มกุ้งจังหวัดพังงา

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ABSTRACT

The antimicrobial susceptibility study was performed on 104 *V.parahaemolyticus* strians from matched-pair of white shrimp (52 isolates) and water (52 isolates) from 15 shrimp ponds, Phang-nga province, and determined by disc diffusion method. All strians were resistant to 8 of 12 tested antimicrobial drugs in high percent, 87.5% (91/104), while 12.5% (13/104) were susceptibility. All 91 drug resistant strians were still susceptible to ofloxacin (OFX), ciprofloxacin (CIP), norfloxacin (NOR), and chloramphenicol (C). Moreover, the antimicrobial resistance rate of *V.parahaemolyticus* were similar in both white shrimp (86.5%, 45/52) and water (88.5%, 46/52). In this study, only 3 isolates were resistant to quinolone (Q) and fluoroquinolone (F) groups. These strian were detected in the same shrimp farm (BI3), but different time of collection. Nalidixic acid (NA)-oxolinic acid (OX) resistance pattern was detected in one of each shrimp and water sample with MICs for NA was 256 µg/ml, and CIP was 1 µg/ml and 4 µg/ml, respectively. Enrofloxacin (ENR)-NA-OX resistance pattern was detected in one water sample with MICs for NA and CIP were 256 µg/ml and 4 µg/ml, respectively. This finding also implies an urgent need for monitoring and controlling the re-emerging Q and F resistance in *V.parahaemolyticus* strians in shrimp farms.

บทคัดย่อ

การศึกษาความไวต่อยาต้านจุลชีพได้ดำเนินการทดสอบกับเชื้อ *V. parahaemolyticus* จำนวน 104 สายพันธุ์ (52 คู่) จากตัวอย่างกุ้งขาวและน้ำที่ได้จากการจับคู่ในบ่อเดียวกัน และดำเนินการตรวจสอบโดยวิธี disc diffusion พบว่า ทุกสายพันธุ์ดื้อต่อยาต้านจุลชีพ 8 จาก 12 ชนิดที่ศึกษา พบว่าส่วนใหญ่มีการดื้อยาสูงร้อยละ 87.5 (91/104) และไวต่อยาต้านจุลชีพร้อยละ 12.5 (13/104) โดยเชื้อดื้อยาทั้งหมด 91 สายพันธุ์พบว่ายังคงไวต่อยา ofloxacin (OFX), ciprofloxacin (CIP), norfloxacin (NOR), และ chloramphenicol (C) นอกจากนี้ยังพบว่าการดื้อยาของ *V. parahaemolyticus* ทั้งในกุ้ง (ร้อยละ 86.5, 45/52) และน้ำ (ร้อยละ 88.5, 46/52) มีอัตราใกล้เคียงกัน ในการศึกษาครั้งนี้พบเชื้อที่ดื้อต่อยาในกลุ่ม Quinolone (Q) และ Fluoroquinolone (F) เพียง 3 สายพันธุ์ จากฟาร์มเดียวกัน (BI3) ที่เก็บในเวลาต่างกัน รูปแบบการดื้อยา nalidixic acid (NA) -oxolinic acid (OX) พบในตัวอย่างกุ้งและน้ำอย่างละ 1 สายพันธุ์ โดยมีค่า MICs สำหรับ NA เท่ากับ 256 ไมโครกรัม/มิลลิลิตร และ ค่า MICs สำหรับ CIP เท่ากับ 1 ไมโครกรัม/มิลลิลิตร และ 4 ไมโครกรัม/มิลลิลิตร ของตัวอย่างกุ้งและน้ำตามลำดับ สำหรับรูปแบบการดื้อยา enrofloxacin (ENR)-NA-OA พบเพียง 1 สายพันธุ์จากตัวอย่างน้ำ ซึ่งมีค่า MICs ของ NA และ CIP เท่ากับ 256 ไมโครกรัม/มิลลิลิตร และ 4 ไมโครกรัม/มิลลิลิตร ตามลำดับ การศึกษานี้ชี้ให้เห็นถึงความจำเป็นเร่งด่วนในการเฝ้าระวังและการควบคุมการอุบัติซ้ำของเชื้อ *V.parahaemolyticus* ที่ดื้อต่อยาในกลุ่ม Q และ F ในฟาร์มกุ้ง

คำสำคัญ : *V. parahaemolyticus* การดื้อยา Quinolones และ Fluoroquinolones กุ้งขาว น้ำ ฟาร์มกุ้ง
Key Words : *V. parahaemolyticus*, Quinolone and Fluoroquinolone antibiotics resistance, white shrimp, water, shrimp farm

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Introduction

Vibrio parahaemolyticus is a halophilic member of the family *Vibrionaceae* that inhabits estuarine areas of temperate and tropical marine environments worldwide. Some strains of this organism cause enteric infections in humans, which are mainly associated with the consumption of raw shellfish and undercooked seafood. It is an important cause of food-borne illness in the United States and Asia including Thailand (Udomsantisuk *et al*, 1976). *V. parahaemolyticus* infections have increased globally in recent year (Chowdhury *et al*, 2000). This organism is the leading cause of seafood-associated bacterial gastroenteritis in the United States and Japan (Mead *et al*, 1999; Fujino *et al*, 1953), and causes approximately one half of the food-borne outbreaks that occur in some Asian countries (Joseph *et al*, 1982). An increasing number of cases of *V. parahaemolyticus* infections have been reported recently in Europe (Martinez-Urtaza *et al*, 2004). Environmental strains of *V. parahaemolyticus* are typically not human pathogens. However, these strains cause disease in shrimps, oysters, mussels and other marine invertebrates (Puente *et al*, 1992; Montilla *et al*, 1994). International trade of seafood can disseminate vibrios that are pathogenic for human or aquacultured animal (Berry *et al*, 1994; Shih *et al*, 1996). Shrimp farming is a sufficiently large and mature industry to have an effective range of antimicrobial agents for most bacterial diseases in shrimp culture. However, at present, there were exists great concern over the widespread use of antibiotics in aquaculture (Le *et al*, 2005). In Thailand, the most commonly used antimicrobial agents in shrimp farming were norfloxacin and enrofloxacin (Holmostrom *et al*, 2003). The drugs were used to prevent and treat *Vibrio* infections, which may result in residue of antimicrobial agents in water and mud, and subsequently, the development of antimicrobial agents resistance in bacteria in the environment (Le *et al*, 2005; Holmostrom *et al*, 2003; Graslund *et al*, 2003).

The incidence of resistance to antimicrobial in bacteria has increased at an alarming rate in recent years and is now a major global public health problem (Huda *et al*, 2003). Moreover, drug resistance in bacterial cells is currently a serious clinical problem. In particular, it is extremely difficult to treat patients infected with multi-drug-resistant bacteria (Chen *et al*, 2002). In 1992, all *V. parahaemolyticus* isolates from raw seafoods in Thailand were assessed for their susceptibility to 9 antimicrobial agents by disc diffusion method. The resistance of these isolates to ampicillin, colistin, tetracycline, cotrimoxazole, nitrofurantoin, nalidixic acid and chloramphenicol were 90.7%, 81.3%, 6.0%, 3.3%, 2.0%, 1.3% and 1.0%, respectively (Pumiprapat, 1992). Moreover, 63% *V. parahaemolyticus* isolates from shrimps, *Penaeus monodon* collected from the region of the Deltaic Sundarbans, India were resistant to ampicillin, cephalexin, and kanamycin. However, all these isolates were susceptibility to nitrofurantoin, nalidixic acid, tetracycline, and norfloxacin (Bhattacharya *et al*, 2000). Quinolone-resistant strains have rarely been found among strains of *V. parahaemolyticus* isolated from the environment and clinical sources. Fluoroquinolone is one of the current drugs of choice for treating patients infected by this organism, because it is considered that this marine bacterium is not exposed to quinolones in its natural habitat (Okuda *et al*, 1999).

In Thailand, a few studies indicated that the pattern of quinolones, fluoroquinolones and other antimicrobial groups used among the shrimp farms could cause the risk of the development of multi-drug resistant *V. parahaemolyticus*. Therefore, the present study was aimed at the analyse multi-drug resistance patterns *of V. parahaemolyticus* isolate of from white shrimp and water samples in shrimp farms in Phang-nga province by disc diffusion and agar dilution methods.

Materials and Methods

Sample collection

A total of 104 white shrimps (*Litopenaeus vannamei*) (52) and water (52) were obtained from 15 shrimp farms in Phang-nga province during February 2007 to February 2008, and studied for *V. parahaemolyticus* isolation. Approximately, 200 g of shrimp samples were collected in sterile plastic bags, and 1,000 ml surface water samples in sterile bottles of the same shrimp ponds. All collected samples were kept in an ice box and transferred to laboratory. The samples were processed the *Vibrio* spp. investigation within 24 h of collection.

Isolation and identification

V. parahaemolyticus was determined in all studied samples by standard method (Elliot *et al*, 1992). Briefly, the samples were cultured onto thiosulfate-citrate-bile-salt-sucrose (TCBS, Difco, USA) agar, with or without prior enrichment culture in alkaline peptone water containing 3% NaCl, then incubated at 37°C for 18-24 h. The suspected green colonies were further biochemically identified. The pair-matched *V. parahaemolyticus* strains isolated from the samples of shrimp and water obtained from the same ponds at the same time of collection were collected and stored onto semi-solid stock medium containing 1% NaCl (Difco, USA). The bacteria were cultured in Mueller Hinton Broth (MHB, Difco, USA) containing 1% NaCl at 37°C for 18-24 h for antimicrobial susceptibility testing.

Antimicrobial susceptibility test

All *V. parahaemolyticus* isolates from shrimps and water samples were tested for their susceptibility to 12 antimicrobial drugs: quinolones and fluoroquinolones i.e. nalidixic acid (NA, 30 µg), oxolinic acid (OA, 2 µg), enrofloxacin (ENR, 5 µg), ciprofloxacin (CIP, 5 µg), ofloxacin (OFX, 5 µg) and norfloxacin (NOR, 10 µg), ,and other antimicrobial drugs: ampicillin (AMP, 10 µg), chloramphenicol (C, 30 µg), trimethoprim/ sulphamethoxazole (SXT, 25 µg), streptomycin (S, 10 µg), tetracycline (TE, 30 µg), and kanamycin (K, 30 µg). using the disc diffusion method as described by CLSI (CLSI, 2007). The results were interpreted based on the recommendation of Clinical and Laboratory Standards Institute for Antibiotic Susceptibility Tests (CLSI, 2007). All antimicrobial discs were purchased from Oxoid (Basingstoke, United Kingdom).

Assessment of minimum inhibitory concentration (MIC) of quinolones and fluoroquinolones

The MICs of quinolones and fluoroquinolones resistance *V. parahaemolyticus* to the commonly used classification of quinolone antibiotics, first generation (nalidixic acid) and second generation (ciprofloxacin), was determined using the standard agar dilution method in Muller Hinton Agar (MHA; Difco, USA) containing with 1% NaCl following the method as described by CLSI (2007). The inoculum size of tested bacteria was approximately 10^8 cfu/ml. MIC is defined as the lowest drug concentration preventing visible bacterial growth of the inoculum after incubation at 37°C for 18-24 h.

Results and Discussion

V. parahaemolyticus occurs widely in aquatic environments including in shrimp farms where the presence of antimicrobial agents at low concentration through leaching or continous usage may lead to the development of drug-resistant strains and multiple antibiotic resistance, particularly using quinolones and fluoroquinolones groups (Holmstrom *et al*., 2003).

In the present study, a total of 104 *V. parahaemolyticus* strains were isolated from 52 pair – matched isolates from white shrimp and water samples in the same shrimp farms. All *V. parahaemolyticus* strains were then determined for their resistance to 12 antimicrobial drugs. A total of 104 *V. parahaemolyticus* strains were resistant to all tested antimicrobial drugs in high percent (87.5%, 91/104), while 12.5%(13/104) were susceptibility (Fig. 1). *V. parahaemolyticus* had high rates of resistance to ampicillin (98.9%, 90/91), [low streptomycin (6.6%, 6/91), tetracycline (4.4%, 4/91), trimethoprim/ sulphamethoxazole (3.3%, 3/91), nalidixic acid (3.3%, 3/91), oxolinic acid (3.3%, 3/91), kanamycin (1.1%, 1/91) and enrofloxacin (1.1%, 1/91)]. In this study, 91 drug resistant isolates were susceptibility to fluoroquinolone group; ofloxacin, ciprofloxacin, norfloxacin, and chloramphenicol.

According to the sources of *V. parahaemolyticus* strains, it was found that the antimicrobial resistance patterns of *V. parahaemolyticus* were similar in both groups (white shrimp and water) (Fig.1). For white shrimp samples, 13.5% (7/52) of *V. parahaemolyticus* were susceptibility to all 12 antimicrobial drugs, while 86.5% (45/52), were resistant to 7 antibiotics: ampicillin (97.8%, 44/45), streptomycin (13.0%, 6/45) and 2 isolates resistant to trimethoprim/sulphamethoxazole and tetracycline (4.4%, 2/45, each), and one isolate was resistant to kanamycin, nalidixic acid, and oxolinic acid (2.2%, each). *V. parahaemolyticus* were susceptibility to ofloxacin, ciprofloxacin, norfloxacin, enrofloxacin, and chloramphenicol. The similar reports were available on the susceptibility of chloramphenicol in China (Li *et al.*, 1999). Moreover, *Vibrio* isolates from coastal and brackish water areas in India showing the highest antibiotic resistance was evident against amoxicillin, ampicillin, carbencillin, cefuroxime, rifampincin and streptomycin (Manjusha *et al,* 2005). These antibiotics are frequently used against different terrestrial organisms including human beings. In addition, Vaseeharan *et al*. (2005) reported that 90 isolates 100% of *Vibrio* spp. were resistant to ampicillin, and more than 50% of the isolates were resistant to ceftriaxone, ciprofloxacin, furazolidone and kanamycin. It can be presumed that anthropogenic factors (hospital effluents) might have influenced in acquiring resistance in *Vibrio* spp. due to these antimricrobial agents.

For 46 *V. parahaemolyticus* strains isolated from water samples, all 100% were resistant to 6 antibiotics. The highest percent of drug resistance were ampicillin (100%, 46/46), followed by tetracycline, nalidixic acid (quinolone), and oxolinic acid (fluoroquinnolone) (4.3%, 2/46; each), and one isolate each resistant to trimethoprim/ sulphamethoxazole and enrofloxacin (fluoroquinnolone) (Fig.1).

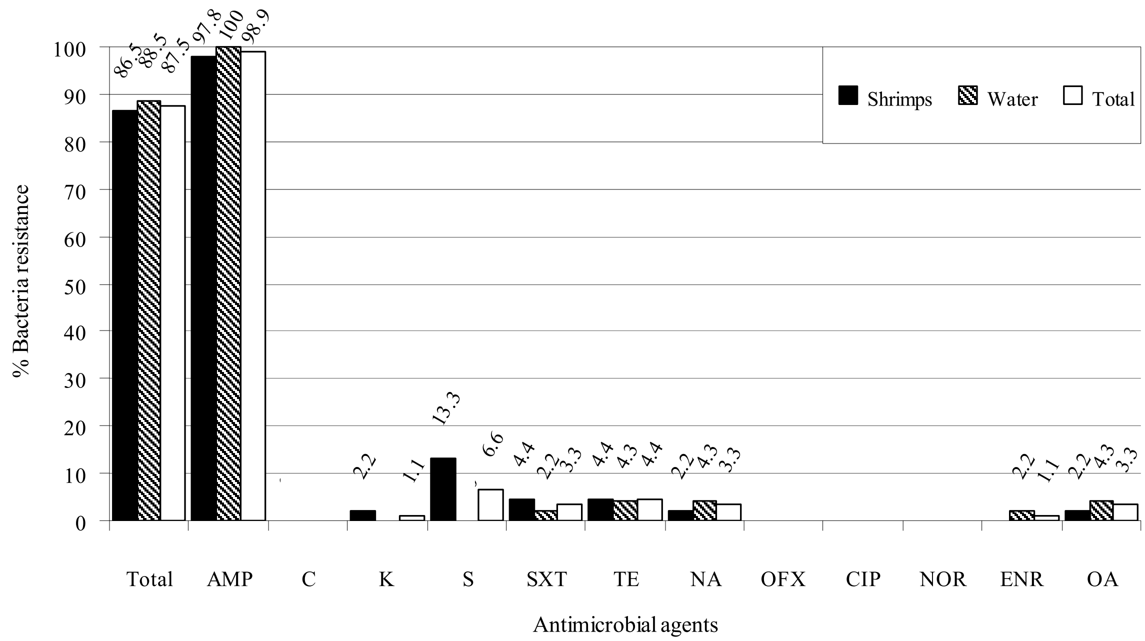

Fig. 1 Antimicrobial susceptibility of total *Vibrio parahaemolyticus* (N=104) isolates from white shrimp (52) and water (52) samples from shrimp farms by agar disc diffusion method (Total: all antimicrobial agents, AMP: ampicillin, K: kanamycin, TE: tetracycline, S: streptomycin, C: chloramphenicol, SXT: trimethoprim/sulphamethoxazole, ENR: enrofloxacin, CIP: ciprofloxacin, OFX: ofloxacin, NOR: norfloxacin, NA: nalidixic acid, and OA: oxolinic acid)

Table 1 shows the drug resistance of quinolones and fluoroquinolones groups found in *V. parahaemolyticus* strains compared to other antimicrobial drugs. A total of 91 drug resistance *V. parahaemolyticus* strains were resistant to other antibiotics in 96.7% (88/91) which was significantly higher than those to quinolones in 3.3% ($p<0.05$). Moreover, *V. parahaemolyticus* strains resistant to other antibiotics and quinolones in white shrimp and water samples were quite similar, and not significantly different ($p>0.05$). It was demonstrated that percent of *V. parahaemolyticus* isolated from water samples (4.3%, 2/46) resistant to quinolone and fluoroquinolone were not significantly higher than those from white shrimp samples (2.2%,1/45) ($p>0.05$).

The results of antimicrobial resistance patterns of 104 *V. parahaemolyticus* strains in shrimps and water samples are shown in Table 2. The prevalence of single drug resistant pattern (86.8%, 79/91) was significantly higher than those of multi-drug resistant patterns (13.2%, 12/91) ($p<0.05$). In this study, there were 4 antimicrobial resistant patterns; single resistance pattern, ampicillin was the highest percent in 86.8% (79/91), followed by double resistance pattern in 6.6% (6/91), triple resistance pattern in 4.4% (4/91) and quadruple resistance pattern in 2.2% (2/91). This result indicate that majority of *V. parahaemolyticus* in shrimp farms develop resistance against antibiotics until become multi-drug resistance strains.

Table 1 Antimicrobial susceptibility to quinolones and fluoroquinolones groups and other antibiotic groups of *Vibrio parahaemolyticus* strains from shrimp (N=45) and water (N=46) samples in shrimp farms

Antimicrobial group	No.(%) of resistant *Vibrio parahaemolyticus* strains found in		
	Shrimp	Water	Total
Quinolones and fluoroquinolones[a]	1 (2.2)	2 (4.3)	3 (3.3)
Other antibiotics[b]	44 (97.8)	44 (95.7)	88 (96.7)

[a] Enrofloxacin, Ciprofloxacin, Ofloxacin, Norfloxacin, Nalidixic acid, OA: oxolinic acid

[b]Ampicillin, Kanamycin, Tetracycline, Streptomycin, Chloramphenicol, Trimethoprim/sulphamethoxazole

According to the sample sources, antimicrobial resistance patterns of *V. parahaemolyticus* strains isolated from white shrimps samples were similar to those from water samples as shown in Table 2. Only single drug resistance pattern of *V. parahaemolyticus* strains from shrimp (82.2%, 37/45) was lower percent than those from water (91.3%, 42/46). The other remainding drug resistance patterns were quite the same percent, and only the combination of drug types were the same or different. For quinolones and fluoroquinolones resistance strains found in triple resistance pattern AMP- NA- OA and S- NA– OA, were isolated from shrimp and water samples, respectively. Quadruple resistant pattern; AMP- ENR- NA- OA was found only in a water sample (Table 2). For the similar antimicrobial resistant patterns of pair-matched samples between shrimp and water collected from the same shrimp farms were 67.3% (35/52) and showed quite high frequency (data not shown). The reasons could be postulated that acquired antibiotic resistance in vibrio is generally mediated by extra-chromosomal DNA or plasmids and is transmitted to next generation and also exchanged among different vibrio population between shrimp and water in the same shrimp ponds.

For quinolones and fluoroquinolones, MICs of the widely used nalidixic acid and ciprofloxacin for the resistant isolates of *V. parahaemolyticus* are shown in Table 3. Two isolates concurrently resisted to both nalidixic acid and oxolinic acid had the MIC for nalidixic acid 256 µg/ml each and ciprofloxacin (second generation) 1 µg/ml, and 4 µg/ml found in a shrimp and a water sample, respectively. The MICs for nalidixic acid 256 µg/ml, for ciprofloxacin 4 µg/ml were found in an isolate concurrently resisted to nalidixic acid, oxolinic acid, and enrofloxacin detected in a water sample. The quinolone and fluoroquinolones resistant isolates were detected in shrimp and water samples from the same shrimp farm (BI3) but different time of collection. *V. parahaemolticus* in this source may be developed the resistance by one of three mechanisms: alterations in the quinolone enzymatic targets (DNA gyrase), decreased outer membrane permeability or the development of efflux mechanisms (http://www.aafp.org/afp/20000501/2741.html).

Table 2 Antimicrobial resistance patterns of 104 *Vibrio parahaemolyticus* strains isolated from shrimp and water samples in shrimp farms

Antimicrobial resistance pattern	No. (%) of *V. parahaemolyticus* isolated from		
	Shrimps	**Water**	**Total**
Single	**37 (82.2)**	**42 (91.3)**	**79 (86.8)**
AMP	37 (100.0)	42 (100.0)	79 (100.0)
Double	**5 (11.1)**	**1 (2.2)**	**6 (6.6)**
AMP K	1 (20.0)	0 (0.0)	1 (16.7)
AMP TE	0 (0.0)	1 (100.0)	1 (16.7)
AMP S	4 (80.0)	0 (0.0)	4 (66.7)
Triple	**2 (4.4)**	**2 (4.4)**	**4 (4.4)**
AMP TE SXT	1 (50.0)	1 (50.0)	2 (50.0)
AMP NA OA	0 (0.0)	1 (50.0)	1 (25.0)
S NA OA	1 (50.0)	0 (0.0)	1 (25.0)
Quadruple	**1 (2.2)**	**1 (2.2)**	**2 (2.2)**
AMP TE S SXT	1 (100.0)	0 (0.0)	1 (50.0)
AMP ENR NA OA	0 (0.0)	1 (100.0)	1 (50.0)

AMP: ampicillin, K: kanamycin, TE: tetracycline, S: streptomycin, C: chloramphenicol, SXT: trimethoprim/ sulphamethoxazole, ENR: enrofloxacin, CIP: ciprofloxacin, OFX: ofloxacin, NOR: norfloxacin, NA: nalidixic acid, and OA: oxolinic acid

Vaseeharan *et al*. (2004) found a MIC range of ciprofloxacin at 0.32-0.43 mg/L to control effectively the *Vibrio* and *Aeromonas* species. Previous reports showed ciprofloxacin to be the most active drug of the quinolones applying to aquacultures. In addition, Zanetti *et al.* (2001) reported that the MIC of ciprofloxacin was 0.38 mg/L to control *Vibrio* spp. isolated from the environments, which was lower than that in our study. This high MICs recovery may reflect an adaptation of vibrios to resist to those drugs used for a long period during shrimp farming or those drugs used after treatment or contaminated in shrimp feeds have still persisted in the farm. Moreover, the widespread use of fluoroquinolones among the farms, e.g. norfloxacin and ciprofloxacin, is a particular cause for concern, considering their importance for treatment of a broad range of human pathogens (Holmstrom *et al.*2003, WHO, 1998).

Table 3 Characterization of quinolones and fluoroquinolones-resistant *Vibrio parahaemolyticus* isolates from shrimp farms

Code number	Resistance pattern	Source	Location	MIC (µg/ml)	
				NA	CIP
D-SH297/TCG1-2	NA OA	Shrimp	BI3	256	1
D-W608/TCG1	NA OA	Water	BI3	256	4
D-W707/TCG1-2	ENR NA OA	Water	BI3	256	4

CIP: ciprofloxacin, ENR: enrofloxacin, NA: nalidixic acid and OA: oxolinic acid

Conclusions

V. parahaemolyticus is an organism of concern in shrimp cultures where antimicrobial agents are used for the control of bacterial diseases in shrimp culture ponds. The results of this study showed that antibiotic resistance and multi-resistance occurred at high frequency among *V. parahaemolyticus* isolated from white shrimp (86.5%, 45/52) and water (88.5%, 46/52) of shrimp farms in Phang-Nga province, and the occurrence of antimicrobial resistance patterns of *V. parahaemolyticus* isolates from shrimp samples was similar to those from water samples indicating drug resistance genes may be transferred among vibrios in shrimp farms. In addition, the emergence of quinolone (nalidixic acid) and fluoroquinolone (ciprofloxacin) resistant *V. parahaemolyticus* was found in the same shrimp farm during investigation. The high MICs of both antimicrobial drugs are ominous and may be a prelude to other pathogenic *Vibrio* spp. acquiring resistance in shrimp farm, which will create major problems in food borne diseases outbreaks with these drug-resistant vibrios. In addition, this findings also implies an urgent need for a monitoring system of antimicrobial drugs management used in shrimp farming to improve the health of consumers and aquaculture industry problems.

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