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Survey of the Sensitivity of *Vibrio* spp., Isolated from *Litopenaeus vannamei* to Different Antibiotics

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ABSTRACT

During reproduction and the culture period of white shrimp *Litopenaeus vannamei* (Boone, 1931), 453 cultured shrimp and 3756 samples from hatcheries of Iran were surveyed. All bacterial colony grown on the thiosulfate citrate bile salt sucrose agar (TCBS), are isolated and identified to the genus level with PCR and identified to the species level using a series of biochemical reactions. The *Vibrio* spp., isolated were survived for measure of sensitivity to seven broad-spectrum antibiotics. The *Vibrio* spp., were *V. alginolyticus*, *V. pharahemolyticus*, *V. fluvialis*, *V. splendidus*, *V. proteolyticus*, *V. anguillarum* II, *V. natriegenes*, *V. harveyi*, *V. nereis*, *V. gazogenes*, *V. pelagius* and *Vibrio* spp. The location of the research was Sistan and Baluchestan province of Iran at the shrimp culture site of Goatr. The results showed that the bacterial resistance to tetracycline, chloramphenicol, furazolidone, erythromycin, neomycin, ciprofloxacin and gentamicin were 20.69, 44.83, 58.62, 37.93, 45.45, 0 and 16.64% in hatcheries and 1.755, 0, 1.79, 58.93, 52.27, 0 and 27.90% in shrimp farms, respectively.

Key words: *Vibrio* sp., PCR, resistance, antibiotics, *Litopenaeus vannamei*

INTRODUCTION

Aquaculture is an important method to secure protein for humans. With the development of a global aquaculture industry, the rate of application of drugs and chemicals has been hugely accelerated for the purpose of outbreak control and disease prevention (Sasmal *et al*., 2004).

Antibacterial chemotherapy has been applied in aquaculture for over 50 years. Early attempts used sulphonamides in the treatment of furunculosis in trout and used tetracyclines against a range of Gram-negative pathogens (Austin and Austin, 1993).

After the discovery sulphonamides and antibiotics, optimists thought that it might be possible to eliminate many forms of infectious diseases in the world. However, the passing of time and irregular use of chemicals and drugs often lead to problems like drug resistance, tissue residue, adverse effects on species biodiversity, etc., that ultimately affect the cultured shrimp, humans and the environment. Several of these aspects have been well documented by Spanggaard *et al.* (1993), Herwig and Gray (1997), Andersson and Levin (1999) and Tendencia and de La Pena (2001). Vibrionaceae is a large and complex group of marine bacteria that can have a significant impact on the health of aquatic animals (Carson *et al.*, 2009). *Vibrio* spp., are Gram-negative, facultatively anaerobic, motile, curved, rod-shaped bacteria with a single polar flagellum. Vibriosis caused by members of genus *Vibrio* (Sung *et al.*, 1999) and the genus contains at least species pathogenic to humans, eight of which can cause or are associated with food-borne illness. The majority of food-borne illnesses are caused by *Vibrio cholerae*, *Vibrio parahaemolyticus* or *Vibrio vulnificus* (Oliver and Kaper, 1997). At least 2 million people get infected with bacteria that are resistant to antibiotics and antibiotic resistant infections lead to 23,000 deaths a year in the United States (CDC., 2013).

The present communication compares bacterial resistance to seven antibiotics among the bacterial flora of hatcheries and farms in Sistan and Baluchestan province in south-eastern of Iran, in 2010.

MATERIALS AND METHODS

Specimen collection: In this survey samples were taken from healthy and diseased *Litopenaeus vannamei* shrimp including: Broodstock, egg, nauplius, zoea, mysis and postlarve from hatcheries and cultured shrimp from farms located in Sistan and Baluchestan province (south-eastern of Iran, near the Iran-Pakistan border). Four hundred fifty-three cultured shrimp and 3756 samples from hatcheries were surveyed. The sampling was performed with a random cluster sampling method from May to Oct 2010. There were fifteen days between each sampling time. The live samples were transferred to the Offshore Fisheries Research Center (OFRC) lab in sterilised plastic dishes with 70% ethanol (C_2H_5OH) with portable aeration for analysis after less than 3 h (De Graindorge and Flegel, 1999). The temperature, salinity and pH of the water were recorded.

Physiological and biochemical characteristics: In the lab, samples from the full body of the larvae (20 larvae were pulled in one sample) and gill, hepatopancreas, hemolymph and muscle of cultured shrimp and broodstock were prepared. They were transferred in to Thiosulphate Citrate Bile Salt (TCBS) sucrose agar media culture plates and incubated at 27°C for 48 h (De Graindorge and Flegel, 1999; Dalsgaard *et al.*, 1995; Venkateswaran *et al.*, 1989). Colonies were grown up and transferred to plates of Tryptic Soy Agar (TSA, Merck) with 2.5% NaCl streaked for identification and TSA with 2.5% NaCl slant tubes for antibiogram tests and incubated for 48 h at 27°C (De Graindorge and Flegel, 1999). All the isolated bacteria were first identified to the genus level with PCR according to Tarr *et al.* (2007).

Through a series of biochemical reaction, the bacterial colonies were tested to identify bacterial taxonomic keys as proposed by De Graindorge and Flegel (1999), Bergey's Manual of Determinative Bacteriology (Holt *et al.*, 1994), Bergey's Manual of Systematic Bacteriology (Brenner *et al.*, 2008) and Baumann *et al.* (1973) in the veterinary office lab of Chabahar. The screening tests are shown in the Table 2.

DNA extraction and phylogenetic analysis of 16S rRNA: Genomic DNA was extracted following a modification of the method described by Lawson *et al.* (1998). DNA quality and concentration were determined by BioPhotometer measurements at 260 nm and the DNA density was determined to be 135.9 ng μL^{-1}. The extracted DNA was stored at -20°C until use.

The thermal cycling profile was as follows: A 3 min soak at 94°C followed by 30 cycles of 94°C for 45 sec, primer annealing at 57°C for 45 sec and extension at 72°C for 60 sec. The final cycle included an additional 5 min of extension time at 72°C.

Universal Polymerase Chain Reaction (PCR) primers F (5'-CGG TGA AAT GCG TAG AGA T-3') and R (5'-TTA CAT GCG ATT CCG AGT TC-3') were used for amplification of the 16S rRNA gene (Table 1).

Antimicrobial susceptibility testing: All isolated bacteria (from the Vibrionaceae phylum) were screened for their sensitivity to 7 broad-spectrum antibiotics using the agar disc diffusion method in accordance the National Committee of Clinical Laboratory Standards (NCCLS., 2002), using Muller-Hinton agar media with 2.5% NaCl (Phuong *et al.*, 2005). The antibiotic impregnated discs used in this study showed in the Fig. 1.

Information of the antibiotics which have been used in farms and hatcheries were collected by a questionnaire.

RESULTS

Environmental parameters: The water temperature, salinity and pH were 26-31°C, 41-56 ppt and 6.2-8.6 during the shrimp production period in 2010.

Table 1: Sequence of primers and product size used in the multiplex PCR

Target bacterium and gene	Primer sequence (5′-3′)	Product size (bp)	Reference
All *Vibrio* spp., 16S rRNA	F: CGGTGAAATGCGTAGAGAT	663	Tarr *et al.* (2007)
	R: TTACATGCGATTCCGAGTTC		

Table 2: Details of specific test performed on individual *Vibrio* colonies

Organism	Test																				Temperature	NaCl
serial No.	Gm	Ox	Cat	Mot	SW	ODC	LDC	ADH	Orn	Ind	Cit	Mr	VP	OF	Arab	Sor	Suc	Man	TCBS	VHA	(°C)	(%)
1	-	+	+	+	+	D	+	-	D	+	D	+	D	F	-	-	+	+	Y	-	15-42	1-10
2	-	+	+	+	+	+	+	-	+	D	D	+	-	F	D	-	-	+	G	-	20-40	3-8
3	-	+	+	+	-	-	-	+	-	D	+	-	+	F	+	-	+	+	Y	-	10-35	1-6
4	-	+	+	+	-	-	-	D	-	+	D	+	-	F	-	-	-	+	G	-	4-37	1-6
5	-	+	+	+	+	-	+	+	-	+	D	+	+	F	-	D	D	+	Y	-	20	1-10
6	-	+	+	+	-	-	-	+	-	+	+	-	+	F	+	-	+	+	Y	-	20-35	3-10
7	-	+	+	+	-	-	-	-	-	-	+	D	-	F	+	-	+	+	Y	-	4-37	3-6
8	-	+	+	+	-	+	+	-	+	+	+	+	-	F	-	-	+	+	Y	+	12-40	3-6
9	-	+	-	+	-	-	-	+	-	+	+	+	-	F	-	-	+	-	Y	-	20-35	3-6
10	-	+	+	+	-	-	-	-	-	-	+	D	-	F	+	+	+	+	+	-	20-42	3-6
11	-	+	+	+	-	-	-	-	-	+	+	D	-	F	-	-	+	+	+	-	20-35	3-8
12	-	+	+	+	D	D	D	D	D	D	D	D	D	F	D	D	D	D	Y/G	-	D	D

+: Positive, -: Negative, D: 26-75% positive, F: Fermentative, G: Green colony, Y: Yellow colony, /: or, -: Until (Holt *et al.*, 1994; Brenner *et al.*, 2008; De Graindorge and Flegel, 1999)

Phylogenetic analyses based on 16S rRNA: A total of 86 bacterial colonies were identified from *Vibrio* spp., with PCR and 32 bacterial colonies were not *Vibrio* genus.

Classification of the isolates: The results of the oxidase and catalase activity tests, growth in nutrient broth with 3 and 6% NaCl and motility tests were positive for every *Vibrio*. The isolated *Vibrio* spp., were based on biochemical characteristics into 12 groups (Table 2).

This study showed that isolated organisms were: (1) *V. alginolyticus*, (2) *V. pharahemolyticus* (3) *V. fluvialis*, (4) *V. splendidus*, (5) *V. proteolyticus*, (6) *V. anguillarum* II, (7) *V. natriegenes*, (8) *V. harveyi*, (9) *V. nereis*, (10) *V. gazogenes*, (11) *V. plagius* and (12) *Vibrio* spp., the bacterial colonies were identified from *Vibrio* spp., *V. alginolitycus* had most outbreaks (36.29%) and *V. anguillarum* II had least outbreaks (1.62%) in among bacterial samples. *V. cholerae* was not found.

Antimicrobial susceptibility: The results of sensitivity tests of the isolated *Vibrio* species from shrimp hatcheries and farms are presented in Fig. 1.

In total of the 29 *Vibrio* spp., (33.72%) surveyed in hatcheries, none were showed resistances to six or seven tested antibiotics.

The isolates *Vibrio* spp., from farms (n = 57) did not show resistance to all 7, 6, 5 and 4 antibiotics in one case. However, 10 cases showed resistance to three (14.54%), two (26.31%) and one (29.82%) antibiotics. The percentage of resistance to tetracycline, chloramphenicol, furazolidone, erythromycin, neomycin, ciprofloxacin and gentamicin antibiotics were 20.69, 44.83,

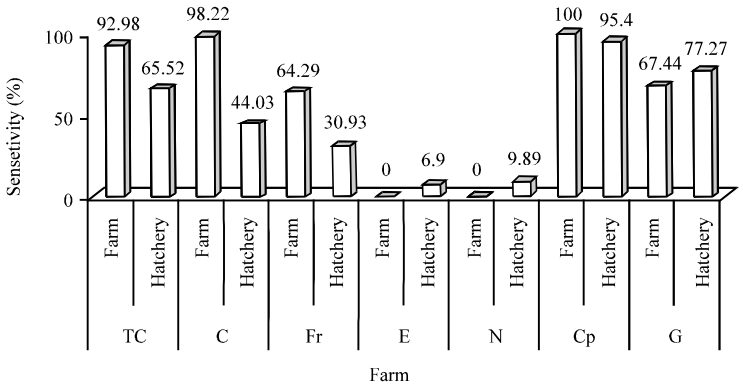

Fig. 1: Sensitivity of isolated *Vibrio* spp., from broodstocks, larvae and cultured shrimp of shrimp hatcheries and farms in Sistan and Baluchistan (2010). TC: Tetracycline (30 μg), C: Chloramphenicol (30 μg), Fr: Furazolidone (100 μg), E: Erythromycin (15 μg), N: Neomycin (30 μg), CP: Ciprofloxacin (5 μg) and G: Gentamicin (10 μg)

Table 3: Antibiotics which have been used in hatcheries and shrimp culture farms in Sistan and Baluchistan (2010)

Groups	Chemicals	Hatchery	Farm
Tetracyclines	Oxytetracycline	+	-
Amphenicols	Chloramphenicol	+	-
Nitrofuran	Furazolidone	+	-
Cephalosporins	Ciprofloxacin	+	-
Macrolides	Erythromycin	+	-
Aminoglycosides	Neomycin	+	-
	Gentamicin	+	-

+: Used, -: Unused

58.62, 37.93, 45.45, 0.00 and 16.64% respectively, in hatcheries and 1.75, 0.00, 1.79, 58.93, 0.00 and 27.90% respectively, in farms. It should be noted that the intermediate sensitivity to tetracycline, chloramphenicol, furazolidone, erythromycin, neomycin, ciprofloxacin and gentamicin antibiotics were 13.79, 10.34, 51.17, 45.45, 4.55 and 9.09%, respectively, in hatcheries and 5.26, 1.75, 1.79, 41.07, 47.73, 0.00 and 4.65%, respectively, in farms. There were variations in the degree of sensitivity of bacteria from hatcheries and farms to the 7 tested broad-spectrum antibiotics (Table 3).

DISCUSSION

The common biochemical commercial kits are unable to recognize *Vibrio* spp. and sometimes they are not able to distinguish between *Aeromonas* and *Vibrio* (Austin *et al*., 1997). Therefore, in this study, a PCR-based method was used (Tarr *et al*., 2007) for exact identification of the *Vibrio* isolates to the genus level. The *Vibrio* spp., most important in causing penaeid shrimp diseases are *V. parahaemolyticus*, *V. harveyi*, *V. vulnificus* and *V. alginolyticus* (De Graindorge and Flegel, 1999).

It is also assumed that the prevalence of *V. parahaemolyticus* and *V. alginolyticus* in the environment is correlated to parameters such as temperature and salinity. Higher densities of total and pathogenic *V. parahaemolyticus* and *V. alginolyticus* were observed with higher water temperatures (DePaola *et al*., 2000, 2003; Cheng *et al*., 2005), that could explain the seasonality of infections in shrimp which are more abundant in warmer months (Yeung and Boor, 2004). The risk of disease seems to increase with intensity of farming and thus the density of shrimp in the pond. Disease occurrence in shrimp ponds in Hainan, China was closely associated with excessive stocking and poor water quality (Spaargaren, 1998).

The water salinity (41-56 ppt) and pH (6.2-8.6) were higher than range of tolerance of *Litopenaeus vannamei* and the water temperature (26-31°C) were into normal range during the shrimp production period in this study. A reduction in immunity ability, together with decreases in phagocytic activity can be by stress of temperature, salinity and pH parameters in invertebrates (Bayne, 1990). Stress of temperature, salinity and pH water parameters could cause a reduction in immunity ability and the phagocytic activity decreases in invertebrates (Bayne, 1990).

It is also important to pay attention to transportation practices with regard to the increased prevalence of *Vibrio* spp. (density, salinity and temperature) (FAO and WHO., 2003; Sani *et al*., 2013).

The isolated *Vibrio* resistances to different antibiotics in hatcheries were more than farms. The abuse of antimicrobials, quantity and frequency, can result in the development of resistant strains of bacteria in hatchery system located in the province. The major problem is abuse of tetracycline as bacterial pathogens easily develop plasmid-mediated resistance to this drug (Tadjbakhsh, 1993), which can enhance the frequency of new bacteria resistant to tetracycline in the culture system (Hamilton-Miller, 1990). There are also reports of a significant correlation between Multiple Antibiotic Resistance (MAR) in bacteria that can transfer resistance to other bacteria (Kruse, 1994). This is an important point because tetracycline resistance has been evolving for a very longtime (millions of years) (Shotts *et al*., 1976). The hatchery owners should be warned about the rules of using this drug. Lavilla-Pitog *et al*. (2005) showed resistance of postlarvae infected with vibriosis to oxytetracycline and chloramphenicol (Lavilla-Pitog *et al*., 2005). There are also many reports from bacterial antibiotic resistance in farms and hatcheries shrimps all over the world (Jayaprakash *et al*., 2006; Carson *et al*., 2009; Molina-Aja *et al*., 2002; Rahawala *et al*., 2005;

Tendencia and De La Pena, 2001; Sasmal *et al*., 2004). This could be the result of absence of use or observation of the appropriate usage rules for these drugs in hatcheries of this zone. However the increasing sensitivity of isolated *Vibrio* spp., from cultured shrimp to tetracycline, chloramphenicol and furazolidone were due from the decrease or non-existence usage of these antibiotics in the farms. This is important that isolated bacteria from edible shrimps should not be resistance to furazolidone and chloramphenicol antibiotics because they should not use in aquaculture (Levy, 1989). Isolated *Vibrio* spp., resistance to erythromycin, neomycin and gentamicin in farms shrimps were more than in hatcheries shrimps. In regards to using the semi-intensive farming system in the studied zone and the fact that absent in this type farming system, some of the bacterial flora of the zone were resistance to above antibiotics (Abraham *et al*., 1997).

The increased resistance to neomycin in farm shrimps (52.27%) compared to hatcheries (37.93%) is an alert for producers and consumers of these shrimps.

Knowing the fact that neomycin is a drug with using prohibition in aquaculture (Levy, 1989), we suggest that there should be an investigation on neomycin residue in cultured shrimp tissues in the studied zone.

This study showed that *Vibrio* ssp., with MAR were present in the zone and it has reported less in the shrimp culture industry of Iran. This report is important, because antibiotic resistance transfer to other bacteria in the ecosystem (Tadjbakhsh, 1993). Also, there is a risk that a multi-drug resistance in non-cholera Vibrios transfer to *V. cholerae* O1 by persons working on shrimp industry, as happened in the 7th cholera pandemic in Ecuador in 1971 (Watanabe *et al*., 1971; Weber *et al*., 1994; Smith, 2007).

The results showed that ciprofloxacin (with sensitivity 100% in the isolated *vibrio* spp., farms and 95.45% in hatcheries) is the best drug against *Vibrio* spp., in the zone shrimp culture industry. Of course, irregular use and absence of the observation of usage rules for these drugs can cause decreased sensitivity to ciprofloxacin in the zone, as has happened in other zones around the world (Chopra, 1985; Courvalin, 1990).

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