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Isolation, Identification and Determination of Antibiotic Susceptibility of *Vibrio parahaemolyticus* from Shrimp at Khulna Region of Bangladesh

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Abstract: To investigate the antibiotic susceptibility, *Vibrio parahaemolyticus* were isolated and identified from the sample of shrimps collected from different shrimp processing plants located at Rupsha, Khulna. Among the 27 samples, 10 strains were identified as *Vibrio parahaemolyticus*. Susceptibility of these *Vibrio parahaemolyticus* was assayed by disc diffusion method against 11 antibiotics. All these strains were highly sensitive to Tetracycline, Norfloxacin, Gentamicin, Riphampicin and Neomycin, but were resistant to Erythromycin, Penicillin, Ampicillin and Kanamycin. *Vibrio parahaemolyticus* showed both resistant and susceptible character against Cephalexin and Streptomycin. 7 strains were resistant and 3 were sensitive to Cephalexin on the other hand 8 were resistant and 2 were sensitive to Streptomycin, among the 10 *Vibrio parahaemolyticus* strains. The antibiotics named Tetracycline, Norfloxacin, Gentamicin, Riphampicin and Neomycin should be the choice to control diseases due to *Vibrio parahaemolyticus* as well as enteric bacteria.

Key words: Antibiotic, *Vibrio parahaemolyticus,* shrimp, therapy

INTRODUCTION

Human infection follows either direct contact with aquatic environment or indirectly via contaminated food and water. Food-borne *Vibrio* infections tend to occur more frequently in developed countries while transmission of *Vibrio* infections in developing countries is, by and large, water-borne. Further, the magnitude of food-borne infections in developing countries is still incompletely understood for the want of robust scientific information. Members of the genus *Vibrio* are autochthonous bacterial flora in the aquatic ecosystem and quite a few of them are associated with infections in humans and aquatic animals.

Among *V. cholerae*, the toxigenic serogroups O1 and O139 are the etiologic serogroups that cause cholera, which are responsible for epidemics and pandemics mainly due to poor water supply and personal hygiene. The other important and most common seafood-borne halophilic *Vibrio* is *Vibrio parahaemolyticus*. Since its discovery in 1953, many aspects on this pathogen were explored and established through extensive research (Fujino *et al*., 1953).

However, there is a burgeoning problem of food-borne illness throughout the globe. The relation between strains isolated from the environment and, those isolated from seafood and human clinical isolates are poorly understood. Studies conducted in many countries have shown that *Vibrio parahaemolyticus* is common flora in many coastal areas, fresh water bodies, plankton and in fishes.

A massive intensification occurred in shrimp aquaculture throughout the world, where stocking density and the rate of application of aqua-drugs and chemicals have been hugely accelerated. Use of antimicrobials in aquaculture essentially started with the reserch of Gutsell (2006), who recognized the

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potential usefulness of sulphanamides for combating furunculosis. Following this, chloramphenicol, oxytetracycline, kanamycin, nifurprazine, oxolinic acid, sodium nifurstyrenate, flumequin, ciprofloxacin and others were introduced (Austin and Austin, 2005). Indiscriminate use of chemicals and drugs often lead to problems like drug resistance, tissue residues, adverse effect on species biodiversity, etc. which ultimately affect the shrimp culture, human and environment. Several of these aspects have been well documented (Spanggaard *et al*., 1993; Herwig and Gray, 1997; Anderson and Levin, 1999; Tendencia and Pena, 2001).

Antimicrobial agents have been widely used in aquaculture worldwide to treat infections caused by a variety of bacterial pathogens of fish including *Aeromonas hydrophila*, *Aeromonas salmonicida*, *Edwardsiella tarda*, *Pasteurella piscicida*, *Vibrio anguillarum* and *Yersinia ruckeri*. This memorandum summarizes evidence that use of antimicrobial agents in aquaculture, as with other uses, selects for antimicrobial resistance in the exposed bacterial flora. Because antimicrobial agents use in aquaculture are administered by mixing them with feed which is dispersed in the water, use of antimicrobial agents in aquaculture directly doses the environment, which results in selective pressures in the exposed ecosystem. The emergence of antimicrobial resistance following use of antimicrobial agents in aquaculture has been identified in fish pathogens (World Health Organization, 1999; Midtvedt and Lingaas, 1992). For example, in several countries *Aeromonas salmonicida* is frequently resistant to multiple drugs including sulphonamides, tetracycline, amoxicillin, trimethoprim-sulfadimethoxine and quinolones (Barnes *et al*., 1994; Inglis *et al*., 1991), antimicrobial agents which are commonly used in aquaculture. The first isolation of *A. salmonicida* resistant to a specific antimicrobial agent has often been reported shortly after the introduction of the agent into aquaculture (Dalsgaard *et al*., 1994). Similar correlations between antimicrobial agents used in aquaculture and antimicrobial resistance are also reported among other fish pathogens (DeGrandis and Stevenson, 1985; Takashima *et al*., 1987).

Antimicrobial-resistant bacteria which result from use of antimicrobial agents in aquaculture can transfer these resistance determinants to other bacteria and many antibiotic resistance determinants in fish pathogens are frequently carried on transferable R plasmids (Watanabe *et al*., 1977; Aoki, 1997). Horizontal spread of plasmids from fish pathogens may therefore transfer resistance genes to other bacteria including those that are pathogenic to humans. Horizontal transfer of resistance genes on plasmids has been demonstrated between bacteria in the water of fish ponds and in marine sediments (Stewart and Sinigalliano, 2005). Plasmids carrying resistance determinants have also been transferred *in vitro* from fish pathogens to human pathogens including *Vibrio cholerae* (Hayashi *et al*., 1982) *Vibrio parahemolyticus* (Nakjima *et al*., 1983) and potential human pathogens including *Escherichia coli* (Sandaa *et al*., 1992; Son *et al*., 1997). Furthermore, plasmids carrying multiple antimicrobial-resistance determinants have been transferred in simulated natural microenvironments between bacterial pathogens of fish, humans and other animals, demonstrating that resistance determinants on plasmids can spread from fish pathogens to human pathogens (Kruse and Sorum, 1994). These studies indicate that dissemination of antimicrobial resistance determinants may be facilitated by the horizontal transfer of plasmids between related and diverse bacteria.

Bacteria on fish may also be transmitted to humans when the aqua cultured fish are eaten, or when other foods, which have been cross-contaminated by bacteria from fish, are eaten. For example, *Vibrio parahaemolyticus* is a common food borne disease in Japan where infections have been linked to the consumption of aquaculture fin fish (Ministry of Health and Welfare, 1999).

The progressive increase in antimicrobial resistance among enteric pathogens in developing countries is becoming a critical area of concern. The acute diarrheal diseases for which antimicrobial therapy is clearly effective include shigellosis, cholera and campylobacteriosis. However, for campylobacteriosis, the diagnosis is usually too late for antimicrobial therapy to be effective (World Health Organization, 1999; Midtvedt and Lingaas, 1992). Among the bacteria causing diarrheal

diseases, *vibrio parahaemolyticus* continue to be a major public health problem. So objectives of the present study are; (a) Isolation and identification of *Vibrio parahaemolyticus* from shrimp (b) Study of antibiotic susceptibility of *Vibrio parahaemolyticus* species.

MATERIALS AND METHODS

Isolation of *Vibro* Sp. From Shrimp

Shrimp samples were collected from different shrimp processing plant in Khulna district. For the collection of samples some clean, dry and sterile polythene bags were taken to the sampling site. Sufficient amount of fish was collected from each site; kept in the polythene bag and tagged. Measures were taken to avoid contamination as far as possible. Samples were processed immediately in the laboratory. Samples were kept refrigerated (4°C) until culture. Aseptically weigh a 25 g shrimp into a sterile stomacher bag. Cut large sample into smaller pieces before stomaching. After stomach, add additional Alkaline Peptone Water (APW) to bring the total amount added to 225 mL (10^{-1} dilution). Now APW was incubated for 24 h at 37°C.

Plating on Selective Agar Media

After incubation, a loop full of selective enrichment broth was transferred to the surface of the selective TCBS agar plate. The plates were then incubated at 37°C for 24 h and observed colonies of typical *vibrio parahaemolyticus*. After 24 h typical *vibrio* sp. showed as green colonies on TCBS agar. (Fig. 1A).

Maintenance and Preservation of the Selected Strains

Trypticase Soy Agar (TSA) slants were used for short-term preservation of selected strains. The strains were inoculated in TSA slant using a sterile loop and incubated at 37°C for 18-27 h. After growth, the slants were stored at freeze for short-term storage and for uses.

Identification of *Vibrio parahaemolyticus*

We have done several biochemical tests e.g., Arginine Glucose Slant (AGS), oxidase test, lysine decarboxylase test, arginine dihydrolase test, salt tolerance test, voges-proskauer test, carbohydrate

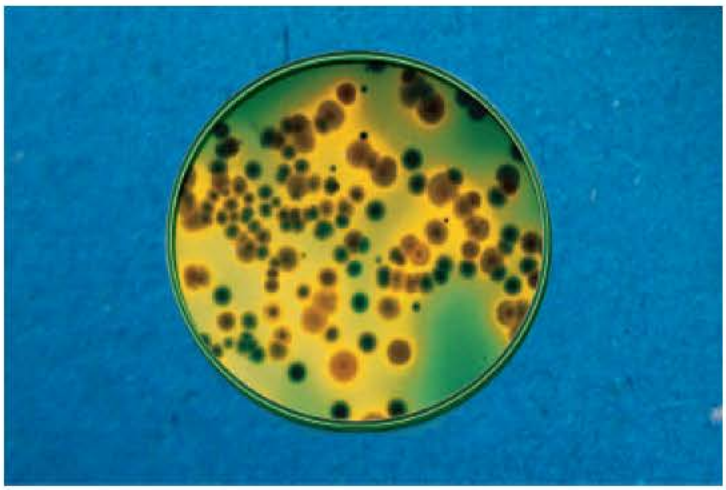

Fig. 1A: Colonies plated out from enrichment culture of shrimp TCBS agars and incubated at 37°C for 24 h, spread plating. Green colonies on TCBS agar were identified as *Vibrio parahaemolyticus*

fermentation test, gelatin hydrolysis test and urease test according to Berzes manual of microbiology for identification of *Vibrio parahaemolyticus* from *vibrio* sp.

Arginine Glucose Slant (AGS)

Each suspect T_1N_1 culture was inoculated to AGS by streaking the slant and stabbing the butt and Incubated AGS with loose cap overnight at 35±2°C. *V. parahaemolyticus* cultures showed an alkaline (purple) slant and an acid (yellow) butt, as arginine is not hydrolyzed. No gas or H_2S is produced.

Oxidase Test

The overnight T_1N_1 cultures were transferred to a filter paper that was saturated with oxidase reagent (1% N'-tetramethyl-p-phenylenediamine. 2 HCl) by using a platinum wire. A dark purple color developing within 10 sec indicates a positive test growth. *V. parahaemolyticus* shows oxidase positive.

Lysine Decarboxylase Test

A Lysine Iron Agar (LIA) slant was used instead of lysine broth to test for the production of lysine decarboxylase. Then the tubes were inoculated fresh cultures by stabbing the butt and streaking the slant. Organisms that produce lysine decaboxylase in LIA cause an alkaline reaction (purple color) throughout the medium. Organisms without these enzymes typically produce an acid butt (yellow). *Vibrio parahaemolyticus* gives a positive reaction in LIA.

Arginine Dihydrolase Test

An Arginine Glucose Slant (AGS) slant was used instead of arginine broth to test for the production of arginine dihydrolase. The tubes were inoculated fresh cultures by stabbing the butt and streaking the slant. Organisms that produce arginine dihydrolase in AGS cause an alkaline reaction (purple color) throughout the medium. Organisms without these enzymes typically produce an acid butt (yellow). *Vibrio parahaemolyticus* gives a negative reaction.

Salt Tolerance Test

The 0, 3, 6 and 8% salt broths were inoculated very lightly from fresh growth. T_1N_1 culture, lightly inoculate one tube each of T_1N_0; T_1N_3; T_1N_6 and T_1N_8 broths. The broths were incubated at 35 to 37°C and read at 18 to 24 h. In the absence of growth, they were incubated for up to 7 days.

Voges-proskauer Test

one milliter of a 48 h culture grown in phosphate medium was taken in a test tube. Then 10 drops of Barrits reagent A was added and culture was shaken. Immediately 10 drops of Barrits reagent B was added and the culture was reshaken every 3 to 4 times. The culture was observed after 15 min for the formation of pink complex color as an indicative of positive VP test. *Vibrio parahaemolyticus* gives a negative reaction.

Carbohydrate Fermentation Test

In the study, fermentation tests of sugars and sugar alcohol (lactose, arabinose and D-mannitol) were carried out. Tryptone broth was used as a basal medium for fermentation tests. 0.01% of phenol red was used as indicator. Fermentation tubes with 9 mL of basal medium provided with indicator were made. The medium was sterilized in autoclave at 121°C and 15 lb/inch ^{2} for 20 min. one milliter of filtered sterile 1% sugar was taken in each tube. One Durham tube was introduced in each of the fermentation tube before sterilization of the basal medium. The tubes were then inoculated in duplicate with fresh culture of the bacterial isolate and allowed to incubate at 37°C for 72 h. The change of color of the indicator to yellow indicated acid production and bubble in the Durham's tubes indicated gas

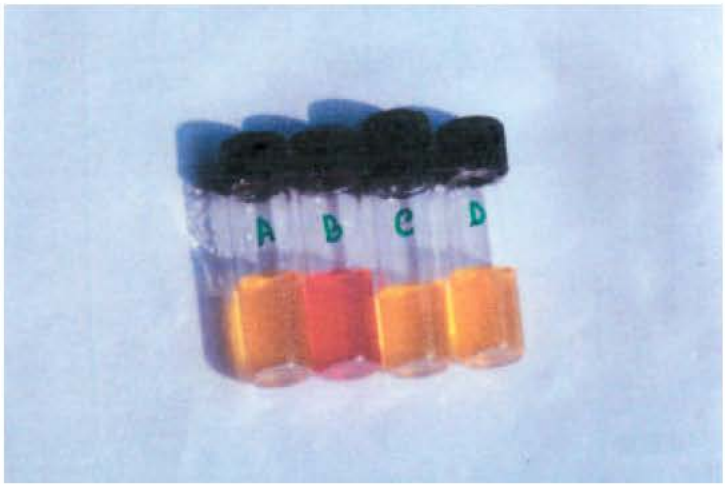

Fig. 1B: Urease test determine ammonia production A and C: Fresh medium color indicate negative result, B: Deep pink color indicates positive result, D: Negative control

production. No change of color indicated negative fermentation. *Vibrio parahaemolyticus* gives a positive reaction.

Gelatin Hydrolysis Test

Nutrient gelatin deep tubes were inoculated with the test organisms and kept at 37°C for 48 h. Following incubation the cultures were placed in a refrigerator at 4°C for 30 min. culture that remained liquid produced gelatinase and demonstrated gelatin hydrolysis. Culture that solidified on refrigeration lacked gelatinase and gave negative reaction. *Vibrio parahaemolyticus* gives a positive reaction.

Urease Test

Using sterile technique, each experimental isolate was inoculated into its approximately labeled urea agar tube by means of loop inoculation. Then the inoculated tubes were incubated at 37°C for 24 h. Deep pink color indicates splitting of urea resulting production of ammonia. Ammonia is alkaline and turns the indicator a bright pink color, which constitutes the positive result. (Fig. 1B).

Antibiotic Susceptibility of Isolated *Vibrio parahaemolyticus*

Antibiotic susceptibility testing was performed by the disk diffusion method using guidelines established by Kirby-Bauer and Stokes' recommended by the National Committee for Clinical Laboratory Standards (NCCLS) (National Committee for Clinical Laboratory Standards, 1997). 11 antibiotic discs with Erythromycin (15 μg) ; Penicillin (10 U) ; Streptomycin (10 μg) ; Tetracycline (30 μg) ; Norfloxacin (10 μg) ; Cephalexin (30 μg) ; Ampicillin (10 μg); Kanamycin(30 μg) Gentamicin (10 μg); Riphampicin (5 μg) ; Neomycin (30 μg) were used. We used commercially available antibiotics discs. For quality control, *E. coli* ATCC 25922 was tested under the same conditions and antimicrobial drugs.

Preparation of Müeller-Hinton Agar

Müeller-Hinton agar was prepared from a commercially available dehydrated base according to the manufacturer's instructions. Immediately after autoclaving, allowed it to cool in a 45 to 50°C water bath. The freshly prepared and cooled medium was poured into glass petri dishes on a level of

approximately 4 mm. A representative sample of each batch of plates was examined for sterility by incubating at 30 to 35°C for 24 h or longer.

Turbidity Standard for Inoculum Preparation

To standardize the inoculum density for susceptibility test, a $BaSO_4$ turbidity standard, equivalent to a 0.5 McFarland standard or its optical equivalent (e.g., latex particle suspension), was used. A $BaSO_4$ 0.5 M McFarland standards were prepared. A 0.5 mL aliquot of 0.048 mol L^{-1} $BaCl_2$ (1.175% w/v $BaCl_2$. $2H_2O$) was added to 99.5 mL of 0.18 mol L^{-1} H_2SO_4 (1% v/v) with constant stirring to maintain a suspension. The correct density of the turbidity standard was verified by using a spectrophotometer with a 1 cm light path and matched cuvette to determine the absorbance. The absorbance at 625 nm was 0.008 to 0.10 for the 0.5 McFarland standards. Then Barium Sulfate suspension was transferred in 4 to 6 mL aliquots into screw-cap tubes of the same size as those were used in growing or diluting the bacterial inoculums. These tubes were tightly sealed and stored in the dark at room temperature. Then the barium sulfate turbidity standard was vigorously agitated on a mechanical vortex mixer before each use and inspected for a uniformly turbid appearance.

Preparation of Inoculum for Performing the Disc Diffusion Test

Three to five well-isolated colonies of the same morphological type were selected from an agar plate culture. The top of each colony was touched with a loop and the growth was transferred into a tube containing 4 to 5 mL of tryptic soy broth. Then the broth culture was incubated at 35°C until it achieved or exceeded the turbidity of the 0.5 McFarland standards (usually 2 to 6 h). The turbidity of the actively growing broth culture was adjusted with sterile saline or broth to obtain turbidity optically comparable to that of the 0.5 McFarland standards under adequate light to visually compare the inoculum tube and the 0.5 McFarland standards against a card with a white background and contrasting black lines.

Inoculation of Test Plates

After adjusting the turbidity of the inoculum suspension, a sterile cotton swab was dipped into the adjusted suspension, within 15 min. The swab was rotated several times and pressed firmly on the inside wall of the tube above the fluid level to remove excess inoculum from the swab. The dried surface of a Müeller-Hinton agar plate was inoculated by streaking the swab over the entire sterile agar surface. The procedure was repeated by streaking two more times, rotating the plate approximately 60° each time to ensure an even distribution of inoculum. As a final step, the rim of the agar was swabbed. The lid was left ajar for 3 to 5 min, but no more than 15 min to allow for any excess surface moisture to be absorbed before applying the drug impregnated disks.

Application of Discs to Inoculated Agar Plates

The predetermined battery of antimicrobial discs was dispensed onto the surface of the inoculated agar plate. Each disc was pressed down to ensure complete contact with the agar surface. The discs were placed individually or with a dispensing apparatus, so that discs were distributed evenly and were no closer than 24 mm from center to center. 5 discs (4 antibiotics discs and one blank disc) were placed in each Petri dish. Then the plates were inverted and placed in an incubator set to 35°C within 15 min after the discs were applied.

Reading Plates and Interpreting Results

After 16 to 18 h of incubation, each plate was examined. The diameters of the zones of complete inhibition (as judged by the unaided eye) were measured, including the diameter of the disc. Zones were measured to the nearest whole millimeter, using a ruler, which was held on the back of the inverted

petri plate. The zone margin was taken as the area showing no obvious, visible growth that can be detected with the unaided eye. Faint growth of tiny colonies, which can be detected only with a magnifying lens at the edge of the zone of inhibited growth, was ignored. However, discrete colonies growing within a clear zone of inhibition were subcultured, re-identified and retested.

RESULTS AND DISCUSSION

In present study, total 27 samples were analyzed for isolation of *Vibrio parahaemolyticus* from locally available shrimp. After incubation of TCBS plates for 24 h at 37°C green colonies are screened out for biochemical identification. According to review of literature *Vibrio parahaemolytics* as well as *Vibrio mimicus, Vibrio vulnificus* and *Vibrio shegeloides* shows green colonies on TCBS after 24 h at 37°C. Among the samples only ten samples were positive for *Vibrio parahaemolyticus* suspected colony (Fig. 1A). To screen out *Vibrio parahaemolyticus*, several green colonies on TCBS agar was picked up and identified by a series of biochemical test.

To identify the *Vibrio parahaemolytics* isolates Arginine glucose slant, Oxidase, Lysine decarboxylase, Salt tolerance, Voges proskaur, fermentation (acid fermentation) test etc. were carried out. Typical *Vibrio parahaemolyticus* give positive Oxidase, Arginine glucose slant and Lysine decarbosylase test.

To differentiate the *Vibrio parahaemolyticus* from others, Salt tolerance and Acid from fermentation test was carried out. Typical *Vibrio parahaemolyticus* colonies do not grow in tryptone broth without any salt. But *Vibrio mimicus* and *Vibrio shigeloides* grew in tryptone broth without any salt. From Table 1 and 2, isolates only W_1P_2, W_2P_2, W_5P_2, W_8P_1, W_8P_2, $W_{14}P_1$, $W_{15}P_2$, $W_{18}P_1$, $W_{18}P_2$, $W_{23}P_1$ and $W_{23}P_2$ can grow in tryptone broth but cannot grow in 6% and 8% salt in tryptone broth. Except these isolates others cannot grow in tryptone broth without salt but can grow in 3, 6 and 8% salt tryptone broth. Typical *Vibrio parahaemolyticus* also cannot grow in tryptone broth without salt but can grow in 3, 6 and 8% salt tryptone broth.

Fermentation test for acid production is one of the most important tests to differentiate between *Vibrio parahaemolyticus* and *Vibrio vulnificus* species. Typical *Vibrio parahaemolyticus* has shown positive reaction and form acid from arabinose and D- Mannital fermentation and shown negative reaction in lactose fermentation.

Table 1: Biochemical identification of *Vibrio* sp. isolated from shrimp

												Growth in (W/V)				
							Acid from					NaCl				
Sample ID		AGS	1	2	3	4	Lactose	Arabinose	D- Mannitol	Gelatinase	Urease	0%	3%	6%	8%	Remarks
W_1	W_1P_1	KA	+	-	+	-	+	-	V	+	-	-	+	+	+	*V. vulnificus*
	W_1P_2	KA	+	-	+	-	-	-	+	+	-	+	+	-	-	*V. mimicus*
W_2	W_2P_1	KA	+	-	+	-	-	+	+	+	V	-	+	+	+	*V. parahaemolyticus*
	W_2P_2	KA	+	-	+	-	-	-	+	+	-	+	+	-	-	*V. mimicus*
W_5	W_5P_1	KA	+	-	+	-	+	-	V	+	-	-	+	+	+	*V. vulnificus*
	W_5P_2	KA	+	+	+	-	-	-	-	-	-	+	+	-	-	*V. shigeloides*
W_6	W_6P_1	KA	+	-	+	-	-	+	+	+	V	-	+	+	+	*V. parahaemolyticus*
	W_6P_2	KA	+	-	+	-	-	+	+	+	V	-	+	+	+	*V. parahaemolyticus*
W_8	W_8P_1	KA	+	-	+	-	-	-	+	+	-	+	+	-	+	*V. mimicus*
	W_8P_2	KA	+	+	+	-	-	-	-	-	-	+	+	-	-	*V. shigeloides*
W_{11}	$W_{11}P_1$	KA	+	-	+	-	-	+	+	+	V	-	+	+	+	*V. parahaemolyticus*
	$W_{11}P_2$	KA	+	-	+	-	+	-	V	+	-	-	+	+	+	*V. vulnificus*
W_{13}	$W_{13}P_1$	KA	+	-	+	-	+	-	V	+	-	-	+	+	+	*V. vulnificus*
	$W_{13}P_2$	KA	+	-	+	-	-	+	+	+	V	-	+	+	+	*V. parahaemolyticus*

+ = Positive result; - = Negative result; KA = Slant alkaline/ Butt acidic; V = Variable at different media; 1 = Oxidase; 2 = Argenine dihydrolase; 3 = Lycine decarboxylax and 4 = Vogas-Proskaur

Table 2: Biochemical identification of *Vibrio* sp. isolated from shrimp

Sample ID		AGS	1	2	3	4	Acid from: Lactose	Arabinose	D- Mannitol	Gelatinase	Urease	Growth in (W/V) NaCl: 0%	3%	6%	8%	Remarks
W_{14}	$W_{14}P_1$	KA	+	-	+	-	-	-	+	+	-	+	+	-	-	*V. mimicus*
	$W_{14}P_2$	KA	+	-	+	-	+	-	V	+	-	-	+	+	+	*V. vulnificus*
W_{15}	$W_{15}P_1$	KA	+	-	+	-	-	+	+	+	V	-	+	+	+	*V. parahaemolyticus*
	$W_{15}P_2$	KA	+	-	+	-	-	-	+	+	-	+	+	-	-	*V. mimicus*
W_{18}	$W_{18}P_1$	KA	+	-	+	-	-	-	+	+	-	+	+	-	-	*V. mimicus*
	$W_{18}P_2$	KA	+	+	+	-	-	-	-	-	-	-	+	-	-	*V. shigeloides*
W_{22}	$W_{22}P_1$	KA	+	-	+	-	-	+	+	+	V	-	+	+	+	*V. parahaemolyticus*
	$W_{22}P_2$	KA	+	-	+	-	-	+	+	+	V	+	+	+	+	*V. parahaemolyticus*
W_{23}	$W_{23}P_1$	KA	+	-	+	-	-	-	+	+	-	+	+	-	-	*V. mimicus*
	$W_{23}P_2$	KA	+	+	+	-	-	-	-	-	-	-	+	-	-	*V. shigeloides*
W_{26}	$W_{26}P_1$	KA	+	-	+	-	-	+	+	+	V	-	+	+	+	*V. parahaemolyticus*
	$W_{26}P_2$	KA	+	-	+	-	+	-	V	+	-	-	+	+	+	*V. vulnificus*
W_{27}	$W_{27}P_1$	KA	+	-	+	-	-	+	+	+	V	-	+	+	+	*V. parahaemolyticus*
	$W_{27}P_2$	KA	+	-	+	-	+	-	V	+	-	-	+	+	+	*V. vulnificus*

+ = positive result; - = negative result; KA = Slant alkaline/ Butt acidic; V = Variable at different media; 1 = Oxidase; 2 = Argenine dihydrolase; 3 = Lycine decarboxylax and 4 = Vogas-Proskaur

Table 3: Diameter of zone of inhibition (in mm) of identified *Vibrio parahaemolyticus* with antibiotic discs

Antibiotic discs	Mean diameter of zone of inhibition in mm: *E. coli* (ATCC 25922)	W_2P_1	W_6P_1	W_6P_2	$W_{11}P_1$	$W_{13}P_2$	$W_{15}P_1$	$W_{22}P_1$	$W_{22}P_2$	$W_{26}P_1$	$W_{27}P_1$
Blank disc	0	0	0	0.0	0	0	0	0.0	0.0	0	0
Streptomycin (10 µg)	18	0	0	0.0	0	0	17	16.0	0.0	0	0
Tetracycline (30 µg)	23	18	20	20.0	23	21	20	19.0	20.0	24	19
Norfloxacin (10 µg)	30	33	30	30.0	31	31	29	31.0	32.0	30	31
Erythromycin (15 µg)	0	0	0	0.0	0	0	0	0.0	0.0	0	0
Penicillin (10 U)	0	0	0	0.0	0	0	0	0.0	0.0	0	0
Cephalexin (30 µg)	17	18	17	0.0	0	0	19	0.0	0.0	0	0
Ampicillin (10 µg)	20	0	0	0.0	0	0	0	0.0	0.0	0	0
Kanamycin (30 µg)	22	0	0	0.0	0	0	0	0.0	0.0	0	0
Gentamicin (10 µg)	23	20	20	19.0	25	22	21	19.0	24.0	26	24
Riphampicin (5 µg)	10	12	9	12.0	14	14	12	9.0	13.0	11	12
Neomycin (30 µg)	22	22	20	21.5	23	21	20	22.5	21.5	23	21

Among these isolates, W_2P_1, W_6P_1, W_6P_2, $W_{11}P_1$, $W_{13}P_2$, $W_{15}P_1$, $W_{22}P_1$, $W_{22}P_2$, $W_{26}P_1$ and $W_{27}P_1$ showed positive reaction against Arabinose and D-Mannitol fermentation and also showed negative reaction in lactose fermentation. From the different biochemical test and according to Barge's manual of bacteriology 10 isolates were identified as *Vibrio parahaemolyticus*. All isolates identified as *Vibrio parahaemolyticus* were carried out to antibiotic susceptibility test.

Antibiotic susceptibility testing has two major functions in both human and veterinary medicine. Primarily, it can be used to guide the choice of antimicrobial treatment for the patient. Secondarily, it may be used at a surveillance tool to monitor antimicrobial resistance.

Ten selected isolates were carried out to antibiotic susceptibility test against preselected antibiotics. Antibiotics used in this experiment were: Erythromycin, Penicillin, Streptomycin, Tetracycline, Norfloxacin, Cephalexin, Ampicillin, Kanamycin, Gentamicin, Riphampicin and Neomycin. The results were prepared according to the zone of inhibition produced on plates. The results were shown in Table 3.

In present study, we observed significant differences between different isolates in diameters of zones of growth inhibition against Erythromycin, Penicillin, Streptomycin, Tetracycline, Norfloxacin, Cephalexin, Ampicillin, Kanamycin, Gentamicin, Riphampicin and Neomycin. In Table 4, we show

Table 4: Antibiotic susceptibility of identified *Vibrio parahaemolyticus* with antibiotic discs

	Interpretation of susceptibility									
Antibiotic discs	W_2P_1	W_6P_1	W_6P_2	$W_{11}P_1$	$W_{13}P_2$	$W_{15}P_1$	$W_{22}P_1$	$W_{22}P_2$	$W_{26}P_1$	$W_{27}P_1$
Blank disc	0	0	0	0	0	0	0	0	0	0
Streptomycin (10 μg)	R	R	R	R	R	S	S	R	R	R
Tetracycline (30 μg)	S	S	S	S	S	S	S	S	S	S
Norfloxacin (10 μg)	S	S	S	S	S	S	S	S	S	S
Erythromycin (15 μg)	R	R	R	R	R	R	R	R	R	R
Penicillin (10 U)	R	R	R	R	R	R	R	R	R	R
Cephalexin (30 μg)	S	S	R	R	R	S	R	R	R	R
Ampicillin (10 μg)	R	R	R	R	R	R	R	R	R	R
Kanamycin (30 μg)	R	R	R	R	R	R	R	R	R	R
Gentamicin (10 μg)	S	S	S	S	S	S	S	S	S	S
Riphampicin (5 μg)	S	S	S	S	S	S	S	S	S	S
Neomycin (30 μg)	S	S	S	S	S	S	S	S	S	S

R = Resistant; S = Susceptible

that all isolates were very sensitive to Tetracycline, Norfloxacin, Gentamicin, Riphampicin and Neomycin and all isolates were resistant to Erythromycin, Penicillin, Ampicillin and Kanamycin. Several isolates show intermediate antibiotic susceptibility pattern. Among the isolates, W_2P_1, W_6P_1, W_6P_2, $W_{11}P_1$, $W_{13}P_2$, $W_{22}P_2$, $W_{26}P_1$ and $W_{27}P_1$, show resistant to streptomycin and $W_{15}P_1$, $W_{22}P_1$ isolates were sensitive. Similarly, W_6P_2, $W_{11}P_1$, $W_{13}P_2$, $W_{22}P_1$, $W_{22}P_2$, $W_{26}P_1$ and $W_{27}P_1$, show resistant to Cephalexin and W_2P_1, W_6P_1, $W_{15}P_1$ isolates were sensitive.

The Kirby-Bauer agar disk diffusion places test results into the qualitative categories of sensitive, intermediate and resistant and may fail to detect subtle changes in antimicrobial resistance. As noted in the results from this study, most isolates tested, expressed antibiotic susceptibilities well within the 'sensitive' range.

If antibiotic use is the major selective pressure encouraging the development of antibiotic resistant, then reducing antibiotic use should result in decreased antibiotic resistance. A decrease in tetracycline resistance in *Salmonella* sp. isolated from man and pigs was observed after tetracycline was banned as a growth promoting in feed (Van Leeuwen *et al*., 1979). Similarly, when avoparcin was banned as a feed additive for poultry in Denmark in 1995, the prevalence of vancomycin resistant *enterococci* isolated from broilers has decreased from 80 to 5%, (Aarestrup *et al*., 1998). Enteropathogenic strains of *Vibrio parahaemolyticus* were isolated from shrimps, *Penaeus monodon* collected from the region of the Deltaic Sundarbans (West Bengal, India). About 63% of the isolated strains were resistant to ampicillin, cephalexin and kanamycin. However, all these strains were sensitive to nitrofurantoin, nalidixic acid, tetracycline and norfloxacin (Fig. 1C and D) Razvykh (1990). were studied sensitivity of 82 cultures of *Vibrio parahaemolyticus* to 8 antibiotics. It was shown that the majority of the strains were highly sensitive to levomycetin and gentamicin, sensitive to tetracycline, rifampicin, streptomycin, neomycin and kanamycin and resistant to ampicillin (Razvykh *et al*., 1990).

The disk diffusion method of determining antimicrobial sensitivity although widely used and economically attractive has its limitations. It is used mainly in a qualitative manner placing isolates in either a sensitive, intermediate, or resistant category. NCCLS, 1997 interpretative criteria for most antibiotics have been set using human pathogens and pharmokinetics. Only pirlimycin and penicillin-novobiocin have interpretive criteria that have been validated for bovine mastitis pathogens (Owens *et al*., 1997). Categorizing results for different *Vibrio parahaemolyticus* isolated from shrimp as simply sensitive, intermediate, or resistant may lead to erroneous conclusions (Fig. 1C and D). From Table 3, we chose to also compare results on the basis of zone diameter in millimeters for a more quantitative interpretation.

Fig. 1C: Results of the disk diffusion assay. This *Vibrio parahaemolyticus* isolate is sensitive to Gentamicin (GM), Riphampicin (RA), Neomycin (N) and Cephalexin (C)

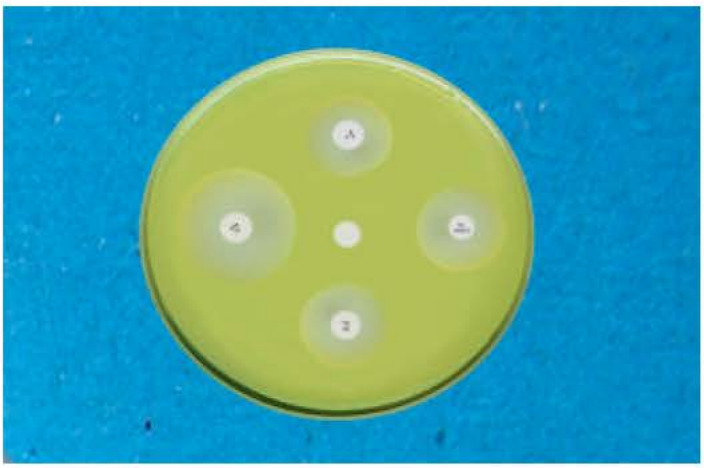

Fig. 1D: Results of the disk diffusion assay. This *Vibrio parahaemolyticus* isolate is sensitive to Tetracycline (ET), Norfloxacin (NOR) Gentamicin (GM) and Riphampicin (RA)

In essence, this study design resulted in a cross-sectional evaluation of antibiotic susceptibility patterns at one point in time. A longitudinal follow-up between different isolate would be of value to understand the dynamics of developing antibiotic resistance or the return of antibiotic sensitivity in endemic bacteria.

CONCLUSIONS

Vibrio parahaemolyticus isolated in this study show good susceptibility to most of the antimicrobial agents that are commercially available for the treatment of causing disease. From this study, there was a significantly larger number of resistant isolates for Erythromycin, Penicillin, Ampicillin and Kanamycin suggesting that antibiotic use in these shrimp's farms may be related to increased resistance.

To decrease antibiotic use and an increase in susceptibility; antibiotic use in our shrimp farm should be reevaluated. Guidelines for rational and limited antibiotic use should be set in place and communicated to both the veterinarian and shrimp producer so that development of antibiotic resistance can be prevented or reduced. Further studies are needed to examine the mechanisms of resistance.

REFERENCES

Aarestrup, F.M., F. Bager, M. Madsen, N.E. Jensen, A. Meyling and H.C. Egener, 1998. Surveillance of antimicrobial resistance in bacteria isolated from food animals to antimicrobial growth promoters and related therapeutic agents in Denmark. APMIS., 106: 606.

Anderson, D.I. and B.R. Levin, 1999. The biological cost of antibiotic resistance. Curr. Opin. Microbial., 2: 489-493.

Aoki, T., 1997. Resistance Plasmids and the Risk of Transfer. In: Bernoth Em, Ed. Furunculosis: Multidisciplinary Fish Disease Research. London, Academic Press, pp: 433-440.

Austin, B. and D.A. Austin, 2005. Bacterial Fish Pathogens: Disease if Farmed and Wild Fish. 6th Edn., Ellis Harwood, New York, pp: 384.

Barnes, A.C., T.S. Hasting and G.B. Amyes, 1994. Amoxicillin resistance in Scottish isolates of Aeromonas salmonicida. J. Fish Dis., 17: 357-363.

Dalsgaard, I., B. Nielsen and J.L. Larsen, 1994. Characterization of *Aeromonas salmonicida* sub sp. salmonicida: A comparative study of strains of different geographic origin. J. Applied Bacteriol., 77: 21-30.

DeGrandis, S.A. and M.W. Stevenson, 1985. Antimicrobial susceptibility patterns and R plasmid-mediated resistance of the fish pathogen *Yersinia ruckeri*. Antimicrobial. Agents and Chemother., 27: 938-942.

Fujino, T., Y. Okuno, D. Nakada, A. Aoyama, K. Fukai, T. Mukai and T. Uebo, 1953. On the bacteriological examination of Shirasu food poisoning. Med. J. Osaka. Univ., 4: 299-304.

Gutsell, J., 2006. Sulfa drugs and the treatment of furunculosis in trout. Science, 104: 85-86.

Hayashi, R., K. Harada, S. Mitsuhashi and M. Inoue, 1982. Conjugation of drug resistance plasmids from *Vibrio anguillarum* to *Vibrio parahemolyticus*. Microbiol. Immunol., 26: 479-485.

Herwig, R.P. and J.P. Gray, 1997. Microbial response to antibacterial treatment in marine microcosms. Aquaculture, 152: 139-154.

Inglis, V., G.N. Frerichs, S.D. Millar and R.H. Richards, 1991. Antibiotic resistance of Aeromonas salmonicida isolated from *Atlanta salmon, Salmo salar* L. in Scotland. J. Fish Dis., 14: 353-358.

Kruse, H. and H. Sorum, 1994. Transfer of multiple drug resistance plasmids between bacteria of diverse origins in natural microenvironments. Applied Environ. Microbiol., 60: 4015-4021.

Midtvedt, T. and E. Lingaas, 1992. Putative Public Health Risks of Antibiotic Resistance Development in Aquatic Bacteria. In: Chemotherapy in Aquaculture: From Theory to Reality. Michael, C. and D.J. Alderman (Eds.), Paris, Office International de Epizooties, pp: 302-314.

Ministry of Health and Welfare, 1999. Vibrio parahaemolyticus, Japan, 1996-1998. Infectious Agents Surveillance Report, 20: 159-160.

Nakjima, T., M. Suzuki, K. Harada, M. Inoue and S. Mitsuhashi, 1983. Transmission of R plasmids in *Vibrio anguillarum* to *Vibrio cholerae*. Microbiol. Immunol., 27: 195-198.

National Committee for Clinical Laboratory Standards, 1997. Performance Standards for Antimicrobial Disk Susceptibility Tests. Sixth edition. Wayne, PA: National Committee for Clinical Laboratory Standards. Approved Standard M2-A6., 17: 1.

Owens, W.E., C.H. Ray, J.L. Watts and R.J. Yancey, 1997. Comparison of success of antibiotic therapy during lactation and results of antibiotic susceptibility tests for bovine mastitis. J. Dairy Sci., 80: 313.

Razvykh, V.M., E.V. Friauf, N.I. Givental', L.F. Bogdanova and E.A. Ved'mina, 1990. Atibiotic sensitivity of *Vibrio Parahaemolyticus* isolated in the turkmene SSR. Antibiot. khimioter., 35: 23-4.

Sandaa, R.A., V.L. Torsvik and J.S. Grokoyr, 1992. Transferable drug resistance in bacteria from fish farm sediments. Can. J. Microbiol., 38: 1061-1065.

Son, R., G. Rusul, A.M. Sahilah, A. Zainuri, A.R. Raha and I. Salrnah, 1997. Antibiotic resistance and plasmid profile of Aeromonas hydrophilia isolates from cultured fish, Telapia (*Telapia mossambica*). Lett. Applied Microbiol., 24: 479-482.

Spanggaard, B., F. Jorgensen, L. Gram and H.H. Huss, 1993. Antibiotic resistance in bacteria from three freshwater fish farms and an unpolluted stream in Denmark. Aquaculture, 115: 195-207.

Stewart, G.J. and C.D. Sinigalliano, 2005. Detection of horizonal gene transfer by natural transformation in native and introduced species of bacteria in marine and synthetic sediments. Applied Environ. Microbiol., 56: 1818-1824.

Takashima, N., T. Aoki and T. Kitao, 1987. Epidemiological surveillance of drug-resistant *Pasteurella piscicida*. Fish Pathol., 20: 209-217.

Tendencia, E.A. and L.D. Pena, 2001. Antibiotic resistance of bacteria from shrimp ponds. Aquaculture, 195: 193-204.

Van Leeuwen, W.J., J. van Embden and P. Guinee, 1979. Decrease of drug resistance in Salmonella in the Netherlands. Antimicrob. Agents Chemother., 16: 237.

Watanabe, T.T., Y. Aoki, Y. Ogata and S. Egusa, 1977. R factors related to fish culturing. Annuals of the New York Academy of Sci., 182: 383-410.

World Health Organization, 1999. Joint FAO/NACA/WHO Study Group on food safety issues associated with products from aquaculture. WHO Technical Report Series. No. 883.