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Plasmid associated antibiotic resistance in *Vibrios* isolated from coastal waters of Kerala

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Abstract: The present study was aimed to detect the plasmid profile, the role of plasmid associated multiple antibiotic resistance of *Vibrios* isolated from coastal waters of Kerala. The isolated plasmids from antibiotic resistant *Vibrios* were tested for the presence of integrons using polymerase chain reaction (PCR) to elucidate the presence of plasmid borne integron, a key element in horizontal gene transfer. 100 isolates of *Vibrios* from water samples of shrimp farms and coastal landing sites were tested for the antibiogram profile to 22 antibiotics and the presence of the plasmids. Antibiotic resistance studies revealed that 78% were expressing multiple antibiotic resistance (MAR), defined as the isolates having resistant to more than three resistance determinants. The levels of resistance exhibited by isolates to specific antibiotics vary between 94 % and 6%. In the plasmid profiling test, only 17 isolates (21%) harbored plasmid DNA which ranged in size from 1.4 to 25 kb, separating the isolates into various plasmid profiles. Interestingly it was observed from the plasmid profiling that 11 strains among them had a single plasmid, 4 strains were with two plasmids and one each of having three and four plasmids. The role of native plasmids in antibiotic resistance in *Vibrios* was confirmed by isolation and transformation of the plasmids in *E.coli* DH5α, followed by antibiotic resistance assay. Both the plasmids and the associated antibiotic resistance of selected isolates could be transferred to a recipient by transformation and conjugation. The study demonstrates the plasmid-mediated drug resistance as a contributor to the antibiotic resistance in *Vibrios* of the aquatic system and their ability to disseminate the resistance by means of transformation, conjugation and by horizontal gene transfer element like integrons. Antibiotic resistance marker and its location were confirmed from the resistant plasmids from *Vibrio* isolates by using curing protocols. As in many developing countries, antimicrobial resistance epidemiology is still in the infancy stage in the locality of the study. To our knowledge, there are no reports available on the plasmid mediated multiple antibiotic resistance in *Vibrio* isolates from coastal waters of Kerala and our study on plasmid profiling will provide a current baseline profile of plasmid mediated resistance of *Vibrios* from coastal waters in Kerala and thereby will provide a significant insight about Indian scenario.

Keywords: *Vibrios*, multiple antibiotic resistance, plasmid, int gene, PCR

Introduction

The members of the family *Vibrionaceae* are a significant component of the micro flora includes more than 64 species (Thompson *et al*., 2005) and many are pathogenic to humans and have been associated with food-borne diseases (Chakraborty *et al*., 1997). Among these species, *Vibrio cholerae* is not only the most feared but also the most extensively studied being associated with epidemic and pandemic diarrhoea outbreaks in many parts of the world (Kaper *et al*., 1995; Chakraborty *et al*., 1997). However, other species of *Vibrios* capable of causing disease in humans have received greater attention in the last decade, which include *Vibrio parahaemolyticus*, *Vibrio vulnificus*, *Vibrio alginolyticus*, *Vibrio damsela*, *Vibrio fluvialis*, *Vibrio furnissii*, *Vibrio hollisae*, *Vibrio metschnikovii* and *Vibrio mimicus* (Chakraborty *et al*., 1997). Several new *Vibrio* species, mainly in the phylogenetic neighborhood of *V. harveyi*, *V. halioticoli*, *V. splendidus*, *V. tubiashii*, and *V. fluvialis*, have been described in the last few years, with *V. neonatus*, *V. ezurae* (Saitou,1987), and *V. ponticus* (Macian *et al*., 2001) being the most recent ones. *V. harveyi*, *V. splendidus*, and *V. tubiashii* are frequently associated with disease in different species of fish and shellfish worldwide, while the *V. halioticoli* group comprises species that are potentially mutualist to abalones (Saitou, 1987). Some *Vibrio* strains are pathogenic and can cause Vibriosis, a serious infectious disease in both wild and cultured finfish and shellfish (Austin and Austin, 1993). In recent years, Vibriosis has become one of the most important bacterial diseases in maricultured organisms, affecting a large number of species of fish and shellfish (Woo and Kelly, 1995; Wu and Pan, 1997).

Antibiotics and other chemotherapeutic agents are commonly used in fish farms either as feed additives or immersion baths to achieve either prophylaxis or therapy. It was observed that individual and multiple antibiotic resistance were associated with antimicrobial use (McPhearson *et al*., 1991). Acquired antibiotic resistance in bacteria is generally mediated

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by extra chromosomal plasmids and is transmitted to next generation (vertical gene transfer) and also exchanged among different bacterial population (horizontal gene transfer). Extensive use of these antibiotics has resulted in an increase of drug-resistant bacteria as well as R-plasmids (Son *et al*., 1997).

Plasmid profiles determination is a useful and the earliest DNA-based method applied to epidemiological studies (Meyer, 1988). The profile identifications were used as serotype-specific reference patterns for detecting certain strain with possible variation in plasmid content which is very important in epidemiological study. Therefore, epidemiological surveillance of drug-resistant strains of *Vibrios* need to be undertaken to determine the origins and prevalence of multi drug resistance that is related or unrelated to the presence of R plasmids, and to find a way to prevent the spread of these drug-resistant strains in fish farms. In this background, the present study is designed to assess the presence of plasmids and their relationship with the antibiotic resistance in *Vibrio* strains isolated from seawater of different coastal sampling stations in Kerala, India.

Materials and Methods

Sampling site

Water samples were collected from brackish water shrimp farms and coastal sites of Kerala (8°18'N 74°52E to 12°48'N 72°22'E). Surface water samples were collected in sterile polythene bags and transported aseptically to the laboratory within 2-6 h.

Bacterial isolation and storage

The water samples were serially diluted and used for growing isolates of *Vibrios* by spread plate technique. Two media: Zobell's medium (Aaronson, 1970) and Thiosulfate Citrate Bile Sucrose Agar (TCBS) (Himedia Laboratories, Mumbai) were used for this purpose. The plates were incubated overnight at 37^0C. Single cell colonies from the plates were further sub cultured. Nutrient broth culture with 20% glycerol and 2% sodium chloride were prepared and stored at -80^0C as stock culture.

Identification of Vibrio

Isolated pure cultures of bacteria were grown on nutrient agar plates and used for identification using conventional biochemical tests (Mac Fadden 1976; West and Colwell, 1984). One-day-old cultures on nutrient agar were used as inocula. Gram stain reaction and cell morphology was observed as described earlier. The isolates were identified based on the standard scheme available for environmental *Vibrio* (Alsina and Blanch, 1994).

Antibiotic sensitivity test

Bacterial isolates were tested for anti-microbial sensitivity using the disc diffusion method (Bauer *et al*., 1966). The turbidity of the bacterial suspension was then compared with MacFarland's barium sulfate standard solution corresponding to 1.5 =10 cfu / ml. Any increase in turbidity is compared to the standard and were adjusted with normal saline. The standardized bacterial suspension was then swab inoculated on to Muller Hinton Agar. (Himedia laboratories, Mumbai) using sterile cotton swabs, which were then left to dry for 10 min before placing the antimicrobial sensitivity discs. Antibiotic impregnated discs 8-mm diameter was used for the test (Himedia laboratories, Mumbai). Disks containing the following antibacterial agents were placed on the plate and incubated over night: Amoxycillin (Am, 10 µg), Ampicillin (A, 10 µg), Carbenicillin (Cb, 100 µg), Cefuroxime (Cu, 30 µg), Chloramphenicol (C-30 µg), Ciprofloxacin (Cf-5 µg), Chlortetracycline (Ct-30 µg),Cotrimaxazole (Co-25 µg) Doxycyclinehydrochloride (Do-30 µg), Furazolidone (Fr-50 µg), Gentamycin (G- 10 µg), Meropenem (M- 10 µg), Netilmicin (N- 30 µg), Nalidixic acid (Na- 30 µg), Norfloxacin (Nx-10 µg), Rifampicin (R-5 µg) . Streptomycin (S- 10 µg), Sulphafurazole (Sf-300 µg), Trimethoprim (Tr-5 µg), Tetracycline (T-30 µg), Neomycin (Ne-5 µg), Amikacin (Ak-10 µg). After incubation, the diameter of the zone of inhibition was measured and compared with zone diameter interpretative chart to determine the sensitivity of the isolates to the antibiotics. The results were interpreted based on the recommendations of National Committee for Clinical Laboratory Standards for antimicrobial susceptibility tests (NCCLS, 2001). The procedure is intended for *in vitro* susceptibility testing of common rapidly growing and certain fastidious bacterial pathogens. *V. cholerae* and *E. coli* DH5alpha were used as positive and negative controls.

Plasmid isolation

Plasmid DNA was extracted from bacterial strains by using mini prep alkali lysis method (Birn Boim and Doly, 1979) with minor modifications. Briefly, it was included using twice the volumes of solutions II and III followed by a 15 min incubation on ice and number of phenol/chloroform/isoamyl alcohol (25:24:1) extractions. For plasmid extraction, bacteria were grown in Luria- Bertani (HiMedia, India) broth supplemented with 2% NaCl, with shaking. The strains were maintained as frozen stocks at –80°C

in marine broth (HiMedia, India) plus 20% (v/v) glycerol.

Plasmid Curing

Curing treatments were carried out using ethidium bromide (Molina-Aja *et al*., 2002). An overnight culture of plasmid contained resistant *Vibrio* strain (200 µl) was added into five different 5-ml cultures of LB broth containing 2% NaCl, previously adjusted to pH 7.5. Increasing concentrations of the curing agent were added to the five tubes cover the range from 50 to 500 µg/ml. The cultures was then incubated overnight at 37°C under constant agitation and observed for growth.

The cells from the culture tube that contains the highest concentration of curing agent permitting visible growth (usually in the range of 150-250 µg/ml) were serially diluted and plated on to Luria agar plates containing 2% NaCl and were grown up to single clones. These clones were tested for the antibiogram pattern, for the antibiotics to which they are originally resistant. Bacterial isolates, that showed change in the resistance pattern to the susceptible, were subjected for plasmid extraction.

Transformation

The isolated plasmids were used for transformation experiment using bacterial strain *E. coli* DH5α as recipient or host after making the cell competent with calcium chloride followed by the protocol mentioned in Sambrook *et al*. (1989), which helped the transformation of resistance plasmids from *Vibrios*. The bacterial strain *E. coli* DH5α was sensitive to all antibiotics studied and thereby after transformation plasmid encoded resistance was confirmed by checking the antibiogram profile of transformed *E. coli* DH5α strain. As an internal control, plasmid pUC18 was used as positive control for transformation studies. Transformation efficiency was calculated from the ratio of the number of transformants to the number of competent cells used for transformation.

Conjugation

Conjugations were done for all the *Vibrio* strains that contained the plasmid. Conjugation was done with *E. coli* HB 101 strains being the recipient and *Vibrio* containing the plasmid encoded resistance as the donor cells. The recipient *E. coli* HB 101 has a selectable streptomycin resistance marker (Liu *et al*., 1999). Donor and recipient cells were inoculated in LB broth and incubated overnight at 37^0C.Then the donor and recipient cells were mixed in a 1: 3 ratio in a sterile bottle. The mixture was then taken by a sterile 5 ml syringe and filtered through 0.2 µm filter paper. The filter paper containing the bacteria was then placed onto the Mac Conkey agar containing the antibiotics ampicillin and streptomycin at the rate of 50 µg/ml and 25 µg/ml respectively. The plates were incubated overnight at 37^0C for 48 h. After incubation, the filter paper containing bacteria were washed with normal saline. The conjugated bacterial suspensions were plated onto Mac Conkey agar containing ampicillin and streptomycin after serial dilution upto 10^{-8}. The inoculated plates were incubated after 48 h at 37^0C. Only the exconjugants containing both antibiotic resistance markers were grown in the medium containing ampicillin and streptomycin. The conjugated bacteria present in the plate containing both the antibiotics were checked for their antibiogram pattern and for their plasmid content. The recipient *E.coli* HB 101 cells were also plated after serial dilution onto Mac Conkey agar containing streptomycin and incubated at 24-48 h at 37^0C. Conjugation efficiency was calculated using the following formula; Conjugation efficiency = (No. of transconjugants on Mac Conkey with ampicillin and streptomycin)/(No. of recipient *E.coli* HB 101 cells on Mac Conkey with streptomycin) = X cfu/ml (Liu *et al*., 1999).

PCR amplification for integrons

PCR reaction was performed for detecting the presence of int genes of the integrons using the isolated plasmids as template to reveal the presence of horizontal gene transfer element in the plasmids. PCR reactions were performed in a total volume of 20 µl per tube, containing 2 µl plasmid DNA, 1.5 mM $MgCl_2$, 10 µl 1x Readymix *Taq* PCR (containing 1.5 U Taq DNA polymerase, 10 mM KCl, 0.001% gelatin, 0.2 mM dNTP), and 1 µl of following primers: 5'GGCATCCAAGCAGCAAG and reverse:5'AAGCAGACTTTGACCTGA (Stokes and Hall,1989). PCR amplifications were carried out in a ThermoCycler (Eppendorf PCR System) with the PCR program consisting of the initial denaturation at 94°C for 4 min followed by 34 cycles at 94^0C for 30 sec, at 62^0C for 90 sec and a final elongation at 72^0C for 10 mins. The PCR products were electrophoresised in 1% agarose gels and viewed under a gel documentation system (Amersham Pharmacea Biotech ,USA).

Gel electrophoresis

All plasmids and amplification products were combined with 4 µl of loading buffer (Bio-Rad) and 10 µl of these mixtures were applied to a horizontal agarose gel (Sigma Agarose, USA, 1% (w/v)) in

1× TAE Buffer (Bio-Rad) containing 0.5 µg/ml of ethidium bromide. Electrophoretic separation was at 100V for 40 min and a molecular weight marker (100 bp PCR ladder, Genei,Banglore) was included. The gels were visualized under UV transilluminator and recorded as jpeg file by using Gel Documentation System, (GelDoc2000, Bio-Rad). Image analysis was performed using Quantity One® software (Bio-Rad).

Results

A total of 350 bacterial isolates were examined after preliminary screening by Gram staining, cytochrome oxidase and oxidative fermentative tests and the100 isolates of *Vibrio* were selected and subjected to various preliminary morphological and biochemical identification and the biochemically identified *Vibrio* strains were used for the further study. Of the total 100 *Vibrio* isolates, 22% were susceptible to all antibiotics tested and 78% were showing multiple antibiotic resistance (MAR). The levels of resistance exhibited by the isolates to specific antibiotics are as follows: Highest incidence of antibiotic resistance was observed against Amoxycillin (94%), followed by Ampicillin and Carbenicillin (90%); Cefuroxime and Streptomycin (65%) followed by Neomycin and Amikacin (59.57%); Rifampicin (58.00%); Furazolidone (42%) and Meropenem (35%). The percentage of resistance was lower against Trimethoprim and Gentamycin (29%); Sulphafurazole (28%); Netilmycin and Norfloxacin (26%), Ciprofloxacin and Tetracycline (22%); Nalidixic acid 19%; Doxycycline hydrochloride (17%), Chloramphenicol (13%), Chlortetracycline, (9%) and Cotrimaxazole (6%) (Table 1).

Plasmid profiling revealed that out of 78 resistant strains analyzed for plasmid isolation, seventeen strains 21% harbored plasmids (1.4 to 25 kb size) and 79% of the *Vibrio* strains were without plasmids. Eleven strains were with a single plasmid, four strains were with two plasmids, and one strains each of having three and four plasmids. These plasmids are showing various plasmid profiles in their size and molecular weight and are presented in Table 2. Analysis of the relationship between the presence of plasmids and the expression of antibiotic resistance to various antibiotics exhibited by the *Vibrios* are summarized in (Table 1). The results were demonstrated that the *Vibrio* strains carrying plasmids in relation to the antibiotic resistance are as follows; amoxycillin (16 strains), ampicillin (16 strains), carbencillin (14 strains), cefuroxime and streptomycin (9strains), rifampicin (8 strains), amikacin (5 strains), neomycin (7 strains). meropenem (2 strains), nalidixic acid (2 strains), norfloxacin (1 strains), chloramphenicol (2 strain), ciprofloxacin (1 strain), co-trimaxazole (1 strain), doxycycline hydrochloride (3 strain), furazolidone (5 strains), gentamycin (2 strain), netilmycin(2 strains), chlortetracycline (1strain), furazolidone (1 strain), gentamycin (1 strain), netilmycin (1 strain), sulphafurazole (2 strain), trimethoprim (2 strain), and tetracycline (1 strain).

Based on the results obtained for antimicrobial results and plasmid profiles (Table 2), *Vibrio* plasmids were selected for transformation into *E. coli* DH5α. The results of transformation efficiency are shown in Table 3. Both plasmids and the associated antimicrobial resistance were transformed into the recipient *E. coli* DH5α, which was sensitive to all the antibiotics screened earlier. Subsequently, plasmid associated resistance pattern of the *Vibrio* strain was obtained from transformed *E. coli* DH5α strain with a range of transformation frequency of 10^{-5} to 10^{-8}. From our results of the studies of plasmids in *Vibrios* it was observed that the resistance markers in the plasmid encoded are betalactamase, amikacin, cephalosporin, Nalidixic acid and Rifampicin, which are transferred to *E.coli* as well. All the *Vibrio* strains lost the plasmids when treated with concentration of 300 µg/ml ethidium bromides. *Vibrio* strains were susceptible to antibiotics and plasmids were lost in all strains, after curing (Table 4).

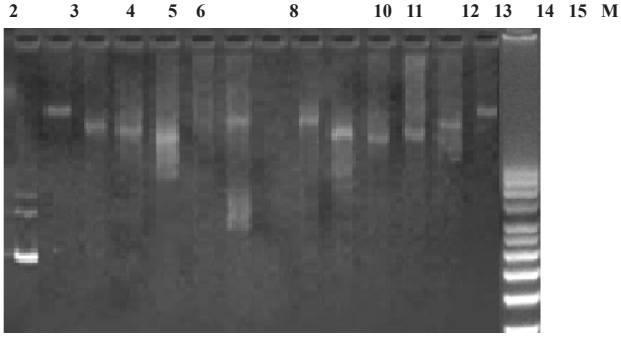

Figure 1. Gel image of plasmid profiles of the selected *Vibrio* isolates Plasmids isolated from different MAR *Vibrio* species- Lanes 2 has pUC 18 from *E.coli*; 3,4,5,6,8,10,11,12,13,14,15 of *V. mimicus* pVCL5; *V. damselae* pVCVA8; pVP5 *V .carchariae*; *V. metschnikovii* pVP17; *V .mediterranei* pVKG1; *V .mediterranei* pVO14; *V. vulnificus* pVMM1; *V. furnissii* pVMM2; *V. alginolyticus* pVMM3; *V. anguillarum* pVMM4; *V. vulnificus* pVMM5 respectively; M, supercoiled DNA ladder as marker

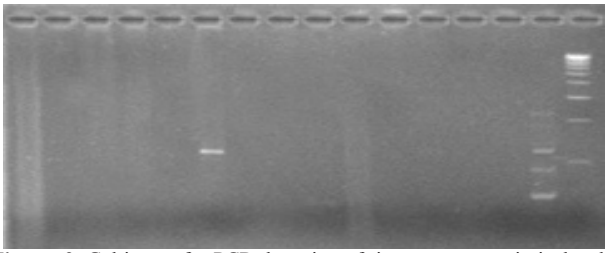

Figure 2. Gel image for PCR detection of integron genes in isolated R-plasmids of selected *Vibrios*
Lane M shows the molecular marker 100 bp ladder; Lane 6 is pVP17 (*V. metschnikovii*) positive for the integron *int* genes ;Lane P is the positive control of *V. cholerae* El Tor CO366 genomic DNA positive for integron

Table 1. Relationship between the presence of plasmids and expression of antibiotic resistance to various antibiotics of *Vibrio* isolates from water samples

Antibiotics	Number and % of strains exhibiting		Number of strains	
	Resistance	Susceptible	With Plasmids	Without plasmids
Amoxycillin	73 (94%)	5	16.00 (22%)	62.00 (78.00%)
Ampicillin	70 (90%)	8	16.00 (23%)	62.00 (77.00%)
Amikacin	46 (59.57%)	32	5.00 (11) %	73.00 (89.00%)
Carbenicillin	70(90%)	8	14.00 (20)%	64.00 (80.00%)
Cefuroxime	51(65 %)	27	9.00 (18) %	69.00 (82.00%)
Chloramphenicol	10 (13%)	68	2.00 (20) %	76.00 (80.00%)
Ciprofloxacin	17 (22%)	61	1.00 (6) %	77.00 (94.00%)
Chlortetracycline	7 (9%)	71	1.00 (14) %	77.00 (86.00%)
Co-Trimoxazole	5 (6%)	73	1.00 (20) %	77.00 (80.00%)
Doxycycline Hydrochloride	13 (17%)	65	3.00 (23) %	75.00 (77.00%)
Furazolidone	33 (42%)	45	5.00 (15) %	73.00 (85.00%)
Gentamycin	23(29%)	55	2.00 (9) %	76.00 (91.00%)
Meropenem	27 (35%)	51	2.00 (7) %	76.00(93.00%)
Netilmicin	20(26%)	58	2.00 (10) %	76.00 (90.00%)
Nalidixic Acid	15 (19%)	63	2.00 (13) %	76.00 (87.00%)
Norfloxacin	20 (26%)	58	1.00 (5) %	77.00 (95.00%)
Neomycin	46 (59.57%)	32	7.00 (15) %	71.00 (85.00%)
Rifampicin	45 (58%)	33	8.00 (18) %	70.00 (82.00%)
Streptomycin	51(65 %)	27	9.00 (18%)	69.00 (82.00%)
Sulphafurazole	22 (28%)	56	2.00(9.%)	76.00 (91.00 %)
Trimrthoprim	23 (29%)	55	2.00 (9 %)	76.00(91.00%)
Tetracycline	17 (22 %)	61	1.00 (6.00)	77.0 (89.00%)

Table 2. Plasmid profiling in *Vibrios* isolated from water samples

Sl.no	*Vibrio* sps	Plasmid	Approximate size	No. of plasmids
1	*V. anguillarum*	pVEK1	22.1, 6.2	2
2	*V. mediterranei*	pVN36	14.4, 6.6, 2.1, 1.4	4
3	*V. furnissii*	pVB9	27.7, 15.0, 6.7	3
4	*V. proteolyticus*	pVP10	25.1	1
5	*V. vulnificus*	pVMM1	12.3, 4.16	2
6	*V. costicola*	pVPD3	23	1
7	*V. mimicus*	pVCL5	18.31	1
8	*V. damselae*	pVCVA8	16.2	1
9	*V .carchariae*	pVP5	13.5	1
10	*V. metschnikovii*	pVP17	16.9	1
11	*V .mediterranei*	pVKG1	16.6, 2.9	2
12	*V .mediterranei*	pVO14	19.2	1
13	*V. vulnificus*	pVMM1	12.3, 4.16	2
14	*V. furnissii*	pVMM2	13.16	1
15	*V. alginolyticus*	pVMM3	13.58	1
16	*V. anguillarum*	pVMM4	16.11	1
17	*V. vulnificus*	pVMM5	25.4	1

Table 3. Transformation efficiency of *Vibrio* plasmids to *E. coli* DH5α and the resistance pattern of transformants

Donor *Vibrio*	Plasmid name	R-pattern associated with donor *Vibrio* isolate	R- pattern of transformant *E.coli* DH5α	R-pattern of plasmid	Transformation efficiency
V .carchariae	pVP5	Ac, A, Ak, Cb, Ne, S, Tr	Ac, A, Ak, Cb, S (5)	Ac, A, Ak,Cb,S (5)	3.13×10^{-8}
V. proteolyticus	pVP10	Ac, A, Ak, Cb, Cu, Ne, R, S	Ac,A,Cb,Cu,R, S (6)	Ac,A,Cb,Cu,,R,S (6)	7.15×10^{-7}
V. vulnificus	pVMM 1	Ac, A, Cb, Cu	Ac, A, Cu , Cb (4)	Ac, A, Cu, Cb (4)	5×10^{-8}
V. mediterranei	pVOMM14	Ac, A, Cb, C, Do, Sf	Ac, A, Cb, Sf (4)	Ac, A, Cb, Sf (4)	43.75×10^{-5}
V. mediterranei	pVN 36	Ac, A, Cb, S, R	Ac, A, Cb, R, S (5)	Ac, A, Cb, R, S (5)	5×10^{-7}
V. furnissii	pVB 9	Ac, A, Ak, Cb, Cu, Do, Fr, Na, Ne, R, S, T,	Ac, A ,Ak, Na, R (5)	Ac, A ,Ak, Na,R (5)	3.13×10^{-7}
V. mediterranei	pVKG 1	Ac, A, Ak, Cb, Cu, Fr, Nt, Ne, S	Ac, A, Cu ,Cb, (4)	Ac, A, Cu ,Cb, (4)	34.1×10^{-5}
V. anguillarum	pVEK1	Ac, A Cb,R	Ac, A Cb (3)	Ac, A, Cb (3)	5×10^{-5}

The numbers in parenthesis indicate the number of antibiotic resistance genes on the plasmid. Ac-Amoxycillin,A-Ampicillin,Ak-Amikacin,Co-Cotrimaxazole,Cb-Carbenicillin,Cu-Cefuroxime,C-Chlramphenicol, Cf-Ciprofloxacin, Ct-Chlortetracycline, Do-Doxy cyclinehydrochloride,Fr-Furazolidone,G-Gentamycin,M-Meropenem,Na-Nalidixic acid, Nt-Netilmycin , Nx-Norfloxacin, Ne-Neomycin, R-Rifampicin, S-Streptomycin, Sf-Sulfafurxazole, Tr-Trimethoprim, T-Tetracycline

Table 4. Results of the curing treatment of *Vibrio* strains isolated from water samples

Vibrio sps.	Plasmid	R Pattern before curing (Plasmid borne)	R Pattern after curing *(Chromosomal borne*)	Plasmid before curing	Plasmid after curing
V. mimicus	pVPCL5	Ac, A, Ak, Cb, Cu, Fr, Nt, Ne, S	Ac, Fr, Ne, Cu	18.31	lost
V. .damsela	pVCVA8	Ac, A, Ak, Cu, C, Co, Cf, Ct ,Do, Fr, G, M, Nt, Ne Na, Nx, R, S, Sf, Tr	Ac ,A, Ak, Cu, C, Ct ,Cf Co, Do ,G, Fr , M, Nt, Na, Ne, Nx	16.2	lost
V. carchariae	pVP5	Ac, A, Ak, Cb, Ne, S, Tr,	Ac, A, Tr, Ne	13.5	lost
V. metschnikovii	pVP17	Ac ,A, Cb, Cu, Ne, R,S,	Ac, A, Cb, Ne	9.9	lost
V. proteolyticus	pVP10	Ac, A, Ak, Cb, Cu, Ne, R,S,	Ac , A, Ak, Ne,	25.1	lost
V. anguillarum	pVEK1	Ac, A Cb, R	R	22.1, 6.2	lost
V. mediterranei	pVOMM 14	Ac, A, Cb, C, Do, Sf	Ac, A ,Cb. C, Do,	19.2	lost
V. vulnificus	pVOMM1	Ac, A, Cb, Cu	Ac	12.3,4.16	lost
V. furnissii	pVOMM2	Ac, A	Ac	13.16	lost
V. alginolyticus	pVOMM3	Ac, ,A, Cb, R	Ac, A	13.58	lost
V. anguillarum	pVOMM4	Ac, ,A	Ac ,A	12.11	lost
V. vulnificus	pVOMM5	Ac, A, Cb, S, M, Cu, Fr, T	Ac, A, Cb, S, Fr, T	22.7	lost
V. mediterranei	pVN36	Ac, A, Cb, R, S	Ac	14.4, 6.6, 2.1, 1.4	Lost
V. costicola	pVPD3	Ac, A, Cb, Cu, Fr, R, S, Ne	Ac, A, Cb, Cu,Ne	23	lost
V. furnissii	pVB9	Ac, A, Ak, Cb, Cu, Do, Fr, Na, Ne, R ,S, T,	Ac,A,Cb,Cu,Do, Fr ,Ne,S, T	27.7, 15.0, 6.7	lost
V. mediterranei	pVKG1	Ac, A, Ak, Cb, Cu, Fr, Nt, Ne, S	Ak, Fr ,Nt ,Ne, S	16.6,2.9	lost

Ac-Amoxycillin,A-Ampicillin,Ak-Amikacin,Co-Cotrimaxazole,Cb-Carbenicillin,Cu-Cefuroxime,C-Chlramphenicol, Cf-Ciprofloxacin, Ct-Chlortetracycline, Do-Doxycycline hydrochloride, Fr-Furazolidone, G-Gentamycin, M-Meropenem, Na-Nalidixic acid, Nt-Netilmycin , Nx-Norfloxacin, Ne-Neomycin, R-Rifampicin, S-Streptomycin, Sf-Sulfafurxazole, Tr-Trimethoprim, T-Tetracycline

Table 5. Conjugation efficiency and the resistance pattern of exconjugant (*E. coli* HB 101)

Vibrio Culture no.	Plasmid name	Antibiotic resistance pattern of Donor	R-resistance pattern of exconjugant *E.coli HB 101*	Conjugation efficiency
V. .damsela	pVCVA8	Ac, A, Ak, Cu, C, Co, Cf, Ct ,Do, Fr , G, M, Nt, Ne Na, Nx, R, S, Sf, Tr	Ac, A, R, S, Sf, Tr,	0.215×10^{-8}
V. carchariae	pVP5	Ac, A, Ak, Cb, Ne, S, Tr,	Ac, Ak, Cb ,S, Tr	0.444×10^{-4}
V. metschnikovii	pVP17	Ac ,A, Cb, Cu, Ne, R,S,	Ac, A,Cu, R,S	0.046×10^{-2}
V. proteolyticus	pVP10	Ac, A, Ak, Cb, Cu, Ne, R,S,	Ac, A, Cb ,Cu, R ,S	2.357×10^{-4}
V. anguillarum	pVEK 1	Ac, A Cb, R	Ac, A, Cb,S	4.25×10^{-3}
V. mediterranei	pVOMM14	Ac, A, Cb, C, Do, Sf	Ac ,A, Sf, S	15.333×10^{-5}
V. vulnificus	pVOMM1	Ac, A, Cb, Cu	Ac, A, Cb, S	0.222×10^{-4}
V. furnissii	pVOMM2	Ac, A	Ac, A,S	0.333×10^{-4}
V. alginolyticus	pVOMM3	Ac, ,A, Cb, R	Cb, R, S	6×10^{-5}
V. anguillarum	pVOMM4	Ac, ,A	Ac, S	10.42×10^{-5}
V. vulnificus	pVOMM5	Ac, A, Cb, S, M, Cu, Fr, T	M, Cu, S	5.5×10^{-4}
V. mediterranei	pVN36	Ac, A, Cb, R, S	Ac, A, Cb, S, R	1.071×10^{-6}
V. costicola	pVPD3	Ac, A, Cb, Cu, Fr, R, S, Ne	Ac, A, Fr, R, S	8×10^{-5}
V. furnissii	pVB 9	Ac, A, Ak, Cb, Cu, Do, Fr, Na, Ne, R ,S, T,	Ac, A, Ak ,Na, R,S , T	0.3×10^{-5}
V. mediterranei	pVKG 1	Ac, A, Ak, Cb, Cu, Fr, Nt, Ne, S	Ac ,A, Cb, Cu, S	1.78×10^{-5}

Ac-Amoxycillin,A-Ampicillin,Ak-Amikacin,Co-Cotrimaxazole,Cb-Carbenicillin,Cu-Cefuroxime,C-Chlramphenicol, Cf-Ciprofloxacin, Ct-Chlortetracycline, Do-Doxycycline hydrochloride, Fr-Furazolidone, G-Gentamycin, M-Meropenem, Na-Nalidixic acid, Nt-Netilmycin , Nx-Norfloxacin, Ne-Neomycin, R-Rifampicin, S-Streptomycin, Sf-Sulfafurxazole, Tr-Trimethoprim, T-Tetracycline

The conjugation efficiency, resistance pattern of the ex-conjugants and the plasmid extraction from the transconjugants were carried out in *E. coli* HB101. All the plasmids studied, except two, were found to be conjugative plasmids. After conjugation, the exconjugants possessing the characteristic resistance pattern and the ex-conjugants were recovered from MacConkey agar plates incorporated with Ampicillin and Streptomycin. Conjugation efficiency analysis showed that the *Vibrio* isolates from water sample conjugated with an efficiency varying from 10^{-2} to 10^{-8} (Table 5). The studies on the drug resistance patterns of the recovered transconjugants revealed that the resistance markers were transferred to the recipient strains of *E. coli* HB101. PCR based detection method was used for studying the presence

of integrons from the plasmids isolated from *Vibrio* isolates. It was observed that the plasmid pVP17 from strain *Vibrio metschnikovii* was positive for the presence of *int* genes of integrons, giving a PCR product of 800 bp size (Figure 2).

Discussion

An increase in the emergence of multi-drug resistant bacteria in recent years is worrying and that the presence of antibiotic resistance genes on bacterial plasmids has further helped in the transmission and spread of drug resistance among pathogenic bacteria. The growing problems with antimicrobial drug resistance are beginning to erode our antibiotic armamentarium to combat antibiotic resistance and thus limiting therapeutic options to present-day clinicians (Zulkifli *et al*., 2009). It has become increasingly apparent that a variety of important properties of microorganisms are plasmid mediated. The best-known example of the plasmid pool of bacteria is the plasmid mediated antibiotic resistance determinants, so called R-plasmids. The discovery of plasmid containing antibiotic resistant bacteria in polluted and relatively unpolluted areas prompted our research team to investigate the distributional limit of transferable resistance in the coastal waters. It has been long known that R factor plasmids are ubiquitous. *Vibrio s*pp. occur widely in aquatic environments and are a part of normal flora of coastal seawaters. Hence, we examined the presence of plasmids of *Vibrio* spp. collected from various coastal sampling sites and assessed the extent of antibiotic resistance and distribution capability, which were revealed by assessing their transformation efficiency.

Of the total 100 *Vibrio* isolates, 22% were susceptible to all antibiotics and 78% were resistant showing MAR. The results indicate that majority of the *Vibrio* spp. showed antibiotic resistance to one or more antibiotics. Similar results were reported from previous studies in *Vibrio* spp. from clinical samples (Abraham *et al*., 1997) shrimp ponds (Eleonor and Leobert, 2001) water and shrimp tissue samples (Liu *et al*., 1999).Highest incidence of antibiotic resistance was evident against Amoxycillin, Ampicillin, Carbenicillin, Cefuroxime, Streptomycin, Rifampicin, Furazolidine and Meropenem. These antibiotics are commonly used to prevent diseases in human beings. Therefore, terrestrial bacteria entering into seawater with antibiotic resistant plasmids might have contributed to the prevalence of the resistance in genes in the marine environment, which is concurrent with earlier reports (Chandrasekaran *et al*., 1998). However, there are few reports available on acquired antibiotic resistance against ampicillin (44%) in *Vibrios* from different sources (Son *et al*., 1998), Carbenicillin (27%) in penaeid shrimp in Mexico (Roque *et al*., 2001, Son *et al*., 1998), cefuroxime (66%), amikacin (55%), kanamycin (58%) and trimethoprim (76%) in *Sparus sarba* in China (Liu *et al*., 1999). It can be presumed that anthropogenic factors (hospital effluents) might have influenced in acquiring resistance in *Vibrio* spp. due to these antibiotics, as there are no reports available on the use of these drugs for aquaculture in India. However, more samples from terrestrial source need to be tested for antibiotic resistance and plasmid profile analysis to confirm our hypothesis. Interestingly, antibiotic resistance was also against Chloramphenicol, Tetracycline, Chlortetracycline, Nalidixicacid, Gentamycin, Sulphafurazole, Trimethoprim that are commonly used in aquaculture farms through feeds during culture and hatchery production of seeds. There similar reports available on the resistances of chloramphenicol and tetracycline in *Sparus sarba* in China (Liu *et al*., 1999). Hence, antibiotic resistant *Vibrios* could be a major threat to public health can be a significant reservoir of genes encoding antibiotic resistance determinants that can be transferred intra or interspecies.

It is well known that plasmid is one of the most important mediators facilitating the fast spreading of antibiotic resistance among bacteria (Dale and Park 2004). Since plasmids are easily transferable from bacterium to bacterium the environmental strains can undergo sudden changes in their plasmid carriage causing diversity in plasmid profile and the resulting antibiotic resistance pattern. Among *Vibrio* isolates, 21% were having plasmids of the sizes ranging from 1.4 to 25 kb. Eleven strains were with a single plasmid, four strains were with two plasmids, and one strain of each having three and four plasmids. However, plasmids of smaller molecular weight were also observed in some of the isolates. Similar plasmid profiles in *Vibrio* spp. were reported from earlier studies: *Vibrio* spp. from cultured silver sea bream, *Sparus sarba* in China (Liu *et al*., 1999), *V. ordalli* (Tiainen *et al*., 1995), *V. vulnificus* (Son *et al*., 1998), *V. salmonicida* (Sorum *et al*., 1990) and most extensively in *V. anguillarum* (Pederson *et al*., 1999). Hughes and Datta (1983) found that, although there was little antibiotic resistance among these strains, 24% contained plasmids, suggesting that, although plasmids are useful in spreading resistance, their presence does not necessarily mean an organism is resistant. However, over the year an increase in the use of antibiotics for the treatment of infectious diseases in fishes has resulted in gaining antibiotic resistance

and the expansion of R plasmids in commercial aquaculture (Aoki *et al*., 1977) owing to the selective pressure exercised by the chemotherapeutic agents when used over an extended period of time (Aoki *et al*., 1971;1981). It is reported that 34% of environmental *Vibrio*, *Aeromonas*, *E. coli*, and *Pseudomonas* isolates from Chesapeake Bay and Bangladesh were found to contain plasmids (McNicol *et al*., 1982). For *Vibrios* cases, the previous study showed that this bacteria species contained plasmid (Molina-Aja *et al.*, 2002). Sometimes there is a correlation between possessions of the plasmid with antibiotic resistance (Saunders, 1984; Son *et al*., 1998; Kagiko *et al*., 2001).

It was evident from the curing experiment that the loss of plasmids was observed in all of the *Vibrio* strains and demonstrated a change in their resistance pattern. In our studies plasmids has lost after curing because of treating with the ethidium bromide with shaking. This may be due to the fact that ethidium bromide reagent arrests further plasmid replication so that plasmid free segregants were formed and the subsequently formed vibrios were cured of their plasmids (Jeremy, 1998). The *Vibrio* strains that were cured of their plasmids were susceptible to these antibiotics. This results indicated that some of these resistance may be encoded on plasmids in some strains, while in some others they may chromosome mediated, as reported in earlier studies (Aoki *et al*., 1984) and a significant decrease in the minimum inhibitory concentration of the antibiotics in *Vibrio* isolates from cultured penaeid shrimp after curing (Molina Aja *et al.*, 2002). In our study, a large population of *Vibrio* stains (79%), was devoid of plasmids but showed an antibiotic resistance pattern, which indicated that in these bacteria, resistance might be mediated via chromosome. The studies of Son *et al*. 1998 also reported similar results in accordance with our results that there were plasmid less (53% of isolates), which showed the multiple antibiotics resistances pattern with high number of antibiotic which indicates that resistance to most of these antibiotics is of chromosomal origin or on mobile genetic elements that may help in the disseminations of the resistant genes to other bacteria of human clinical significance. Son *et al*. (1998) stated that generally epidemiologically unrelated isolates contains different plasmid profiles whereas related isolates could also display variation in plasmid profiles .

It was observed from the results of transformation experiment of *Vibrio* plasmids that the plasmid mediated bacterial resistance in *Vibrio* spp. is transferable to other bacterial genera (*E. coli*). Similar previous studies on transformation experiments were reported in plasmids of *Vibrio* isolates from *Sparus sarba* (Liu *et al*., 1999) and penaeid shrimp (Molina-Aja *et al*., 2002). Sizemore and Colwell (1977) found antibiotic resistant bacteria in most samples, including those collected 100 miles offshore and from depths of 8200 meters. Isolates considered autochthonous to the marine environment were examined for plasmids and used in mating experiments. Several of these were able to transfer plasmids to *E. coli* (Sizemore and Colwell, 1977), which is concurrent to our findings. Since these plasmids mobilize into *E. coli* DH5α suggest that the plasmids are of broad host range. Similar findings were reported in plasmids isolated from *Pseudomonas* spp. (Shahid, 2004).

Conjugation experiments were also showed that the resistance plasmids could be transferred from *E. coli* to *V. parahaemolyticus in vitro* (Guerry, 1975). The results of the conjugation using the *Vibrio* containing resistant plasmid as the donor and the *E. coli* HB 101 as the recipient, indicates that the majority of the plasmid associated resistant markers were transferred to the *E. coli* strain. Large sizes of plasmid were detected in almost plasmid positive isolates of *Vibrio* strains. Bacterial antibiotics resistance patterns sometimes associated with the presence of large plasmids and the ability of plasmids for conjugation process. Generally, plasmids which can be transconjugated usually possess a high molecular weight so the presence of plasmids that may harbor the antibiotic resistance genes in these isolates may increase their capacity to threaten human consumers since *Vibrio* strains carrying resistant genes qualified them as potential human pathogens (Zulkifli *et al*., 2009). Moreover, NCBI GenBank database, which currently lists some 1600 plasmid genomes (as of January 2009), shows that plasmids can be as small as 0.85 Kb. The smallest known conjugative plasmid currently is approximately 34 kb in size. Smaller plasmids, which do not possess conjugation machineries, often rely on mobilization or conduction (piggybacking on a transmissible plasmid by co-integration) for horizontal transfer (Anders *et al*., 2009).

Acquired antibiotic resistance in bacteria is generally mediated by extra chromosomal plasmids and is transmitted (vertical gene transfer) and also exchanged among different bacterial population (horizontal gene transfer). Plasmid borne integrons are a key player in being able to acquire, rearrange, and express genes conferring antibiotic resistance (Stokes and Hall, 1989). Irrespective of integrons, if located on a plasmid or chromosome, their structure and function are similar. Integrons and gene cassette arrays have been found in the chromosomes of

Vibrio, *Pseudomonas*, *Xanthomonas*, *Treponema*, *Geobacter*, *Dechloromonas*, *Methylobacillus*, and *Shewanella* species (Heidelberg, 2000; Holmes *et al*., 2003). In this study of PCR experiments for the detecting the presence of plasmid borne integrons, one of the plasmids isolate was positive for int gene, which is an indicative of the plasmid borne integrons, a key element in horizontal gene transfer. Their activity might have facilitated a community level response to intensive antibiotic use, which in turn helped in the emergence of integron-encoded, and multiple antibiotic resistances in disparate bacterial species. From the results, it is evident that there are integron mediated horizontal gene transfer may occur in rare cases, to augment the horizontal gene transfer responsible for antibiotic resistance from *Vibrio* spp to other genus.

In summary, the prevalence of multi-drug resistant *Vibrio* spp. is quite high in the locality of study and that the bacterial population is rather diverse based on the phenotypic and genotypic characterization of the isolates. Overall results indicated that *Vibrio* spp. present in aquatic system, acquire antibiotic resistance by means of plasmids and they are capable of transferring the resistance by means of transformation, conjugation and by other mobile elements like integrons. Furthermore, *Vibrio* spp. have the ability to transfer the plasmid-encoded resistance into other bacterial genera. The presence of plasmids in *Vibrios* may pose a potential health hazard, since plasmids from animals may be transferred to humans either directly or indirectly, if they are transferred to human pathogens; *Vibrio* spp. or *E. coli*. To our knowledge, there are no reports available on the plasmid mediated multiple antibacterial resistance in *Vibrio* isolates from coastal waters in India. Therefore, frequent assessment of bacterial resistance and their plasmid profiles in these coastal waters may give a better knowledge regarding the uncanny ability of acquired drug resistance determinants in ubiquitous bacterial flora, *Vibrio* spp. Non-pathogenic bacteria may also acquire resistance genes and serve as a continuing source of resistance for other bacteria, both in the environment, and in the human gut. As the effectiveness of antibiotics for medical applications decline, the indiscriminate use of in aquaculture and in humans can have disastrous conditions in future due to horizontal gene transfer and the spread of resistant organisms: Therefore, we must recognize and deal with the threat posed by overuse of antibiotics. The isolation of *Vibrio* species from coastal water samples in Kerala suggested the potential threat to humans, and indigenous animals. Further detailed study on the antibiotic resistance profile and plasmid ecology of environmental isolates of *Vibrio* species will be of special importance to understand the mechanism of genetic exchanges among Gram-negative bacteria in aquatic environment.

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References

Aaronson, S.1970. Experimental Microbial Ecology.2nd edn, New York: Academic Press 236 pp.

Abraham, T.J., Manley, R., Palaniappan, R. and Dhevendaran,K.,1997. Pathogenicity and antibiotic sensitivity of luminous *Vibrio harveyi* isolated from diseased penaeid shrimp. Journal of Aquaculture Tropics 12 (1): 1–8.

Anders, N., Lars,H., and Soren,J.S.,2009.Conjugative plasmids:vessels of the communal gene pool. Philosophical Transactions of The Royal Society Biological sciences 364:2275-2289.

Aoki, T., Egusa, S., Kimura, T. and Watanabe, T.1971. Detection of R factors in naturally occurring *Aeromonas salmonicida* strains. Applied Microbiology 22 (3): 716-717.

Aoki, T., Arai, T. and Egusa, S. 1977. Detection of R plasmids in naturally occurring fish-pathogenic bacteria, *Edwardsiella tarda*. Microbiology and Immunology 21 (2): 77-83.

Aoki, T., Kitao, T. and Kawano, K. 1981. Changes in drug resistance of *Vibrio anguillarum* in cultured ayu(*Plecoglossus altivelis*). Journal of Fish Diseases 4: 223-230.

Aoki, T., Kitao, T., Watanabe, S. and Takeshita, S. 1984 .Drug resistance and R plasmids in *Vibrio anguillarum* isolated in cultured ayu *(Plecoglossus altivelis)*. Microbiology and Immunology 28: (4) 1-9.

Aoki, T. 1992. Present and future problems concerning the development of resistance in aquaculture. In: Michel ,C.M.,Alderman,D.J.,(eds).Chemotherapy in aquaculture: from theory to reality p.254 –262. Paris: Office International des Epizootics.

Alsina, M. and Blanch, A. R.1994. A set of keys for biochemical identification of environmental *Vibrio* species. Journal of Applied Bacteriology 76: 79-85.

Austin, B., and Austin, D. A. 1993. Bacterial Fish Pathogens, 2nd edn.,Ellis Horwood, Chichester.

Bauer, A.W., Kirby, W.M.M., Sheris, J.C. and Turck, M.1966. Antibiotics susceptibility testing by standardized single disk method. American Journal of Clinical Pathology 45: 493-496.

BirnBoim and Doly. 1979. A rapid alkaline extraction procedure for recombinant plasmid DNA. Nucleic Acid Research 7:1513-1523.

Chandrasekaran, S., Venkatesh, B., and Lalithakumari, D.

1998. Transfer and expression of multiple antibiotic resistance plasmid in marine bacteria. Current opinion in Microbiology 37: 63–80.

Chakraborty, S., Nair, G.B., and Shinoda, S. 1997. Pathogenic *Vibrios* in the natural aquatic environment. Reviewof Environmental Health 12: 347-351.

Dale, J.W., and Park, S. 2004. Molecular genetics of bacteria. 4th ed. John Wiley & Sons Inc., Chichester, UK

Eleonor, A., and Leobert, D. 2001. Antibiotic resistance of bacteria from shrimp ponds .Aquaculture 195 : 193–204.

Finegold, S.M., and Martin, W.J. 1982.Bailey and Scott's Diagnostic Microbiology. 2nd ed. C.V. Mosby, Philadelphia.

Guerry, P.1975. The ecology of bacterial plasmids in Chesapeake Bay. University of Maryland, College Park, USA: University of Maryland, Ph.D thesis.

Heidelberg, J.F., Eisen, J.A., Nelson, W.C., Clayton, R.A., Gwinn, M.L., Dodson, R.J., Haft, D.H.,Hickey,E.K., Peterson, J.D., Umayam,L. A.,Gill, S.R., Nelson, K. E.,Read,T.D.,Tettelin,H.,Richardson, D., Ermolaeva, M.D.,Vamathevan, J. , Bass, S. , Qin, H., Dragoi,I., Sellers, P.,McDonald,L.,Utterback,T.,Fleishmann, R.D. , Nierman, W.C., White, O., Salzberg, S.L, Smith, H.O.,Colwell, R.R.,Mekalanos, J.J., Venter, J.C. and Fraser, C.M. 2000. DNA sequence of both chromosomes of the cholera pathogen *Vibrio cholerae*. Nature 406:477-483.

Holmes, A. J., Gillings, M.R., Nield, B.S., Mabbutt, B.C., Nevalainen, K.M.H., and Stokes, H.W.2003.The gene cassette metagenome is a basic resource for bacterial genome evolution. Environmental Microbiology 5: 383- 394.

Hughes, V. M., and N. Datta.1983. Conjugative plasmids in bacteria of the 'pre-antibiotic' era. Nature 302 :725-726.

Jeremy W Dale,1998.Molecular genetics of Bacteria, John Wiley and sons, Third avenue, New York, USA, 310pp.

Kagiko, M.M., Damiano, W.A. and Kayihura, M.M.2001. Characterization of *Vibrio parahaemolyticus* isolated from fish in Kenya. East African Medical Journal 78: 124-127.

Kaper, J. B., Morris, J. G. and Levine M. M. 1995.Cholera. Clinical Microbiology Reviews 8: 48-86.

Lesmana, M., Subekti, D., Simanjuntak, C.H., Tjaniadi, P., Campbell, J. R., Oyofo, B. A. 2001. *Vibrio parahaemolyticus* associated with cholera-like diarrhea among patients in North Jakarta, Indonesia. Diagonostic Microbiology and Infectious Diseases 39(3):71 -75.

Liu, J.Y., Rita W .T., Julia M. L., Ling, H. X., and Norman, W. Y. S., 1999. Antibiotic resistance and plasmid profiles of *Vibrio* isolates from cultured *Sparus sarba.* Marine Pollution Bulletin 39 :245 -249.

Mac Fadden, J. F. 1976. Biochemical Tests for the Identification of Medical Bacteria. WilliamsandWilkens, Baltimore, 310 pp.

Macian, M. C., Ludwig, W., Aznar, R., Grimont, P. D. A., Schleifer, K. H., Garay, E. and Pujalte, M.J. 2001. *Vibrio lentus* sp. nov., isolated from Mediterranean oysters. International Journal of Systematic and Evolutionary Microbiology 51:1449-1456.

McNicol, L. A., Barkay, T., Voll, M. J. and Colwell, R. R. 1982. Plasmid carriage in *Vibrionaceae* and other bacteria isolated from the aquatic environment. Journal of the Washington Academy of Sciences 72(7) : 60-66.

McPhearson, R. M., DePaola, A., Zywno, S. R., Motes, M. L. Jr., Guarino, A. M. 1991. Antibiotic resistance in Gram-negative bacteria from cultured catfish and aquaculture ponds.Aquaculture 99: 203-211.

Molina Aja, Alejandra, G., Alberto, A.G., Carmen, B., Ana Roque, Gomez-G. B. 2002. Plasmid profiling and antibiotic resistance of *Vibrio* strains isolated from cultured Penaeid shrimp. FEMS Microbiology Letters 213: 7-12.

National Committee for Clinical Laboratory Standards. 2001. Performance standards for antimicrobial susceptibilities testing—9th informational supplement. National Committee for Clinical Laboratory Standards, Wayne, Pa.

Pedersen, K.1999. The fish pathogen *Vibrio anguillarum*. Denmark:The Royal Veterinary and Agricultural University, Ph.D Thesis.

Roque,A., MolinaAja,A.,BolanMejia,C.,andGomez,G.B. 2001.Invitrosusceptibility to 15 antibiotics of *Vibrios* isolated from penaeid shrimps in Northwestern Mexico. International Journal of Antimicrobial agents 17: 383 -387.

Saitou, N.and Nei.M.1987.The neighbor-joining method: a new method for reconstructing phylogenetic trees. Molecular Biology and Evolution 4:406-425.

Saunders, J.R. 1984. Genetics and evolution of antibiotic resistance. British Medical Bulettin 40: 54-60.

Sambrook, J., Fritsch, E. F. and Maniatis, T. 1989. Molecular Cloning: A Laboratory Manual, 2nd Edition. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.

Shahid,M.A.2004.Plasmid mediated amikacin resistance in clinical isolates of *Pseudomona aeruginosa.* Indian Journal of Medical Microbiology 22: 182-184.

Sizemore, R. K. and Colwell, R. R. 1977. Plasmids carried by antibiotic resistant marine bacteria. Antimicrobial Agents Chemotherapy 12: 373-382.

Son, R., Rusul, G., Sahilah, A. M., Zainuri, A., Raha, A. R. and Salmah,I.1997. Antibiotic resistance and plasmid profiles of *Aeromonas hydrophila* isolates from cultured fish, Tilapia (*Tilapia mossambica*). Letters in Applied Microbiology 24: 479-482.

Son, R., Nasreldin, E.H., Zaiton, H., Samuel, L., Rusul, G. and Nimita, F. 1998. Characterisation of *Vibrio vulnificus* isolated from cockles (*Anadara granosa*):Antimicrobial resistance, plasmid profiles and random amplification of polymorphic DNA analysis. FEMS Microbiology Letters 165: 139-143

Sorum, H., Hvaal, A. B., Heum, M., Daae, F. L. and Wiik, R. 1990.Plasmid profiling of *Vibrio salmonicida* for epidemiological studies of cold-water Vibriosis

in Atlantic salmon (*Salmo salar*) and cod (*Gadus morhua*). Applied and Environmental Microbiology 56: 1033 -1037.

Stokes, H.W. and Hall, R. M.1989. A novel family of potentially mobile DNA elements encoding site specific gene integration functions: integrons. Molecular Microbiology 3:1669-1683.

Tiainen, T., Pedersen, K. and Larsen, J. L. 1995. Ribotyping and plasmid profiling of *Vibrio anguillarum* serovar O2 and *Vibrio ordalii*. Journal of Applied Bacteriology 79: 384 -392.

Thompson,F.L., Gevers, D.,Thompson,C. C., Dawyndt, P., Naser, S., Hoste. B., Munn, C. B. and Swings,J.2005. Phylogeny and Molecular Identification of *Vibrios* on the Basis of Multilocus Sequence Analysis. Applied and Environmental Microbiology 71(9): 5107-5115.

West,P.A.andColwell,R.R.1984. Identification of *Vibrionaceae*:an overview. In: Colwell,R.R.(Ed). *Vibrios* in the Environment,p.205–363.NewYork, USA: Wiley .

Woo, N. Y. S. and Kelly, S. P.1995. Effects of salinity and nutritional status on growth and metabolism of *Sparus sarba* in a closed seawater system. Aquaculture 135: 229-238.

Wu, H. B. and Pan, J. P. 1997. Studies on the pathogenic bacteria of the Vibriosis of *Seriola dumerili* in marine cage culture. Journal of Fisheries China 21: 171-174.

Zulkifli, Y., Alitheen, N.B., Raha, A.R., Yeap, S. K., Marlina, Son, R. and Nishibuchi, M. 2009. Antibiotic resistance and plasmid profiling of *Vibrio parahaemolyticus* isolated from cockles in Padang, Indonesia. International Food Research Journal 16: 53-58.