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Characterization of plasmids from multiple antibiotic resistant *Vibrios* isolated from molluscan and crustacean of Kerala

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Abstract

The present study was aimed to investigate the occurrence of multiple antibiotic resistance, the role of plasmids and their relationship with the multiple antibiotic resistance in 30 *Vibrios* isolated from selected seafoods of Kerala, India. The isolated *Vibrios* were screened for plasmid DNA and were tested for transformation and conjugation efficiencies. Antibiotic resistance studies revealed that the levels of Multiple Antibiotic Resistance of *Vibrio* strains to various antibiotics differed considerably and were found to be varied in the expression of their resistance pattern. All studied *Vibrio* strains were found to be resistant to antibiotics; amoxycillin, ampicillin and carbeniciliin. 87 % were resistant to rifampicin; 74% to cefuroxime; 67 to streptomycin; 53% to norfloxacin and ciprofloxacin and 47% to furazolidone and nalidixic acid. The nine strains isolated from crustaceans and from molluscans have been found to harbor 1-3 plasmids with size varies from 5.98 kb to 19.36 kb. The average transformation efficiency is about $5x10^{-8}$ and the conjugation efficiency is varied from $2.1x\ 10^{-3}$ to 10^{-9}. The study of antibiotic resistance pattern could be useful to test the extent of drug resistance in seafoods and help to devise a nationwide antibiotic policy.

Introduction

The members of the family *Vibrionaceae* are a significant component of the microflora includes more than 30 species, 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 (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). Marine *Vibrios* are of great interest in coastal and estuarine waters because of their high salt tolerance. 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).

Extensive use and misuse of antibiotics in medication, veterinary, agriculture and aquaculture have caused the wide spread increased nature of antibiotic resistant bacteria (Kummerer, 2004). However, according to Nogueira-Lima *et al*. (2006), evaluating the risks associated with the use of antibiotics in seafoods is difficult due to the lack of quantitative data from most countries involved in this activity. Over time *Vibrios* exposed to antibiotics inside or outside the shrimp farming environment can acquire antimicrobial resistance transferable by mobile genetic elements and horizontal gene transfer (Serrano, 2005). Thus, due to the presence of R-factors in the population, the resistance developed through gene regulation of plasmids and chromosomes may be transferred vertically (by heredity) or horizontally (Madigan *et al*., 2003). Plasmids have been found in heterotrophic bacteria (Lobava *et al*., 2002) and in *Vibrios* (Manjusha, 2011) and in most cases their involvement in resistance to many antibiotics

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has been proven (Toranzo *et al*., 1983). To our knowledge plasmid occurrence and their relationship with multiple antibiotic resistances, have not been reported from *Vibrio* strains isolated from seafoods of Kerala coastal waters.

In this background, the present study is designed to investigate the occurrence of multiple antibiotic resistance in *Vibrios* and to assess the presence of plasmids and their relationship in multiple antibiotic resistance of *Vibrio* strains isolated from certain molluscan and crustacean of Kerala coastal waters.

Materials and Methods

Sampling site

Molluscan (*Perna virdis* and Sepia) and Crustacean (Shrimp) samples were collected from coastal waters of Kerala were used as seafood samples for the study. (8°18'N 74°52E to 12°48'N 72°22'E).

Bacterial isolation and storage

Bacteria containing the tissue samples were serially diluted after homogenization and Thiosulfate Citrate Bile Sucrose Agar (Himedia Laboratories, Mumbai) was used for growing isolates of *Vibrios* by spread plate technique. Nutrient broth culture with 20% glycerol and 2% sodium chloride were prepared and stored at –800 C 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

Antibiotic resistance of bacteria were determined by the single disc diffusion method with the use of Mueller Hinton Agar , according to the Buer Kirby method (Arvanitodou *et al*., 1997). Bacteria were multiplied on agar slants (ZB) at 20^0C. 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 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. Disks containing the following antibacterial agents were plated on the plate and incubated over night: amoxycillin (Am, 10µg), ampicillin (A, 10µg), carbenicillin (Cb, 100µg), cefuroxime (Cu, 30µg), chloramphenicol (C-30µg), ciprofloxacin (Cf-5µg), chlortetracycline (Ct-30µg), cotrimaxazole (Co-25µg) doxycyclinehydrochloride (Do-30µg), furazolidone (Fr-50µg), gentamycin (G-10µg), meropenem (M-10µg), netilmicin (N-30µg), nalidixic acid (Na-30µg), norfloxacin (Nx-10µg), rifampicin (R-5µg), streptomycin (S-10µg), sulphafurazole (Sf-300µg), trimethoprim (Tr-5µg), tetracycline (T-30µg), neomycin (Ne-5µg) and amikacin (Ak-10µg). The results were interpreted based on the recommendations of National Committee for Clinical Laboratory Standards for antimicrobial susceptibility tests (Finegold *et al*., 1982). 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. In our study, *Vibrio* strains were considered as MAR strains, if they are showing resistance against more than three antibiotics (Eleonor, 2001).

Plasmid isolation

Plasmid extraction from bacterial strains was performed using mini prep alkali lysis method (Birnboim and Doly, 1979) with minor modifications. Luria- Bertani (HiMedia, India) broth supplemented with 2% NaCl was used for cultivation of all the strains. The extracted DNA was electrophoresed in a 0.7% agarose gel at 80 V for 1-3 hours.

Transformation

The extracted plasmids from the isolates were used for the 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. Transformation efficiency was calculated from the ratio of number of transformants to the number of competent cells used for transformation.

Conjugation

Conjugation was done with *Vibrio* containing the plasmid encoded resistance as the donor cells and the *E.coli* HB 101 strains as being the recipient (Liu *et al*., 1999). The recipient *E.coli* HB 101 has

a selectable streptomycin resistance marker. Donor and recipient cells were inoculated in LB broth and incubated overnight at 37^{0}C. After overnight incubation, donor and recipient cells were mixed in a 1: 3 proportion in a sterile bottle. The mixture was 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^{0}C for 48 h. After incubation, the filter paper containing bacteria were washed with normal saline. The conjugated bacterial suspensions were plated onto MacConkey agar containing ampicillin and streptomycin . The inoculated plates were incubated after 48 h at 37^{0}C. The exconjugants grown in the medium containing ampicillin and streptomycin were checked for their antibiogram pattern and the plasmid content. Conjugation efficiency was calculated from the ratio of the number of exconjugants to the number of donor cells used for conjugation.

Results

A total of thirty strains were segregated as *Vibrios* after morphological and biochemical identification of strains isolated from molluscans and crustaceans. Among these isolates fifteen *Vibrios* were isolated from mollusk: *V. parahaemolyticus*, *V. costicola*, *V. alginolyticus*, *V. mimicus* (2) , *V. proteolyticus* (2), *V. splendidus* (3), *V. marinus*, *V. nereis*, *V. orientalis*, *V. carchariae* and *V. mediterranei*; and fifteen from crustacean: *V. parahaemolyticus* (2), *V.hollisae*, *V. pelagius*, *V. carchariae*, *V. splendidus*, *V. cholera* (5), *V. cincinnatiensis*, *V. vulnificus* (2) and *V. costicola*.

Among these *Vibrio* isolates, 100% isolates showed the multiple antibiotic resistance (MAR) to at least one of the 22 tested antibiotics . In (Table 1 and 2) are presented the antibiotic profiles of *Vibrio* isolates. The bacteria were tested for susceptibility to 22 antimicrobials representing 15 antimicrobial drug classes. When the data were analyzed taking into account the source from which samples were obtained, 100% of the isolates from mollusc and from crustacean were found to be resistant to at least one antimicrobial agent .

The *Vibrio* isolates obtained from both crustacean and mollusc were found to be resistant to amoxycillin, ampicilln and carbenicillin. Resistance to nalidixic acid was detected in seven isolates from mollusk and in five isolates from crustacean. Most of the isolates were resistant to amoxycillin, ampicillin, carbenicillin and amikacin. 87% were resistant to rifampicin; 74% to cefuroxime; 67% to streptomycin; 53% to norfloxacin and ciprofloxacin and 47% to furazolidone and nalidixic acid. Most frequently expressed resistance phenotype in *Vibrio* isolates from mollusks and crustaceans were found to be amoxycillin, ampicilln, carbenicillin, amikacin and amoxycillin, ampicillin, carbenicillin, cefuroxime respectively. The results has been showed that over 50% *Vibrio* isolates were resistant to clinically used antibiotics such as amoxycillin, norfloxacin, rifampicin and ciprofloxacin. Some of the isolates from both samples (7/15 and 9/15) were exhibiting resistance to fourth generation antibiotics such as Chloramphenicol and Doxycycline hydrochloride from I s of serious concern.

Bacterial isolates from mollusc showed the highest frequency of resistance determinants >10

Table 1. Antibiotic resistance profile of *Vibrios* isolated from molluscans

Sl. no	Identification	Antibiotic Resistance Profiles	No of Rs
1	*V. costicola*	Ac, A, Cb, Cf, Nx, R, Tr	7
2	*V. alginolyticus*	Ac, A, Cb, Cu, R	5
3	*V. mimicus*	Ac, A, Cb, Cu, Cf, R	6
4	*V. mimicus*	Ac, A, Cb, S, R	5
5	*V. proteolyticus*	Ac, A, Cb, Cu, Fr, S	6
6	*V. splendidus*	Ac, A, Cb, Cu, Do, Fr, ,G, Na, Nx, R, S,	11
7	*V. marinus*	Ac, A, Ak, Cb, Cu, Cf, Do, Fr, R, T, Ne,	11
8	*V. nereis*	Ac, A, Ak, Cb, Cu, Do, Fr, G, M, Na, Nx, R, S, Cf, Sf, Tr, T,	17
9	*V. orientalis*	Ac, A, Ak, Cb, Cu, Do, G, M, Nt, R,, S, Sf	12
10	*V. carchariae*	Ac, A, Ak, Cb, C, Cu, Ct, Cf, Fr, G, M, Na, Nx, Nt, Ne, R, S, Sf, Tr,T	20
11	*V. splendidus*	Ac, A, Ak, Cb, Cf, Fr, G, M, Na, Nx, Nt, Ne, R, S, Sf, Tr	16
12	*V. splendidus*	Ac, A, Ak, Cb, Cu, Cf, S, Nt, Na, Nx, R, S, T,	13
13	*V. proteolyticus*	Ac, A, Ak, Cb, Cu, C, R, S, Na, Nx, Nt, R, S, T	14
14	*V. parahaemolyticus*	Ac, A, Ak, Cb, Cu, C, Cf, S,T, Tr	10
15	*V. mediterranei*	Ac ,A, Ak, Cb, Cu, Fr, M, Nt, Na, Nx, R, Sf,Tr, T	14

(No. of R= Number of antibiotics to which *Vibrio* isolates were resistant) (Ac-Amoxycillin, A-Ampicillin, Ak-Amikacin, Co-Cotrimaxazole, Cb-Carbenicillin,Cu-Cefuroxime, C-Cholramphenicol, 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 2. Antibiotic resistance profile of *Vibrios* isolated from crustaceans

Sl. no	Identification	Antibiotic Resistance Profile	No of R determinants
1	*V.hollisae*	Ac, A, Cb, Cu	4
2	*V. pelagius*	Ac, A, Cb, Cu, C, M, Nt, R, S	9
3	*V. carchariae*	Ac, A, Cb, Cu, C, M, Nt, R, S	9
4	*V. splendidus*	Ac, A, Cb, Cu, Cf, Ct, M, Na, Nt, R S,	11
5	*V. cholerae*	Ac, A, Ak, Cb, Cu, C, Cf, Do, Na, Nt, Nx, R, S, Tr	14
6	*V. cholerae*	Ac, A, Cb, Cu, M, Nt, R, T, Tr	9
7	*V. vulnificus*	Ac, A, Cb, Cu, C, Cf, Ct, Na	8
8	*V. cincinnatiensis*	Ac, A, Cb, Cu, C, Co, Cf, Ct, Na, R	10
9	*V. parahaemolyticus*	Ac, A, Cb, Cu, C, Cf, M, Na, Nt, R, S,T	12
10	*V. parahaemolyticus*	Ac, A, Cb, Na	4
11	*V .cholerae*	Ac, A, Cb	3
12	*V. parahaemolyticus*	Ac, A, Cb, Cu, C, R, S	7
13	*V. vulnificus*	Ac, A, Cb, Cu, C, Cf, Ct	7
14	*V. costicola*	Ac, A, Cb, Cu, Cf, M, Nt, R,S	9
15	*V. vulnificus*	Ac, A, Cb, Cu, R, C, M, S	8

(No. of R= Number of antibiotics to which *Vibrio* isolates were resistant)
Ac-Amoxycillin, A-Ampicillin, Ak-Amikacin, Co-Cotrimaxazole, Cb-Carbenicillin, Cu-Cefuroxime, C-Chloramphenicol, 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.

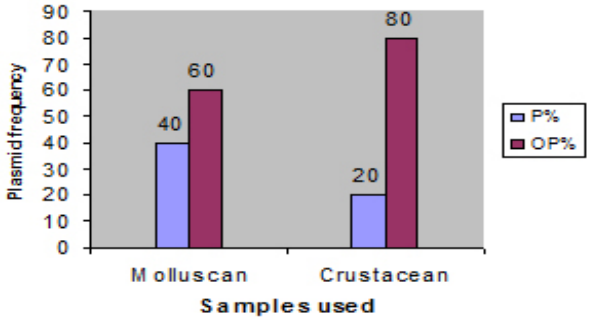

Figure 1. Percentage of plasmids in *Vibrios* isolated from seafoods samples

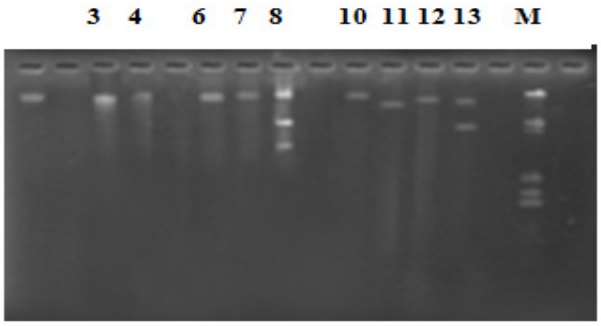

Figure 2. Plasmid profiling of MAR-*Vibrios* isolated from molluscan and crustacean

Plasmids isolated from different *Vibrio* species-Lanes 3, 4, 6, 7, 8, 10, 11, 12, 13 have pVMUS10, pVMUS15, pVMUS1, pVMUS7, pVSPF4, pVSY3, pVSY1, pVSY6, pVMUS 11 respectively and Lane M Marker

Resistance factors, are (10/15) followed by crustacean (4/15). The antibiotic resistance profiles for the *Vibrio* isolates were varying in different manner (Table 1 and 2). It was possible to verify that the antimicrobial resistance patterns of the *Vibrio* were not related to specific species. In general, evident differences in antimicrobial resistance patterns were observed among isolates from the seafood sample. The occurrence of simultaneous resistance to multiple antimicrobial drugs was observed in thirty *Vibrio* isolates. Commonly occurring resistance profiles identified among multiresistant isolates were. Ac+A+Cb; Ac+A+Cb+Ak; Ac+A+Cb+Ak,+Cu; Ac+A+Cb+Cu; Ac+A+Cb+Cu+C; Ac+A+Cb+Na .

The plasmid frequency is 40% in molluscans and 20% in crustaceans (Figure 1). Among the 15 MAR *Vibrios* strains isolated from molluscans, four contained single plasmid, while one *Vibrio orientalis* strain was with double plasmids. While three strains isolated from crustaceans harbored single plasmids and one strain *Vibrio* mediterranei from crustaceans have been found to harbor three plasmids in varying size of 5.98, 8.27 and 16.08 kb. The size of the extracted plasmids of *Vibrios* from both samples is found to be varied from 5.98 kb to 19.36 kb (Figure 2 and Table 3).

Table 3. Plasmid profiling of MAR-*Vibrios* isolated from mollusc and crustacean

Sl. No	Identity	Plasmid	Approximate plasmid size in kb	No.of plasmids
1	*V mimicus*	pVMUS10	14.93	1
2	*V. mediterranei*	pVMUS15	14.38	1
3	*V. alginolyticus*	pVMUS1	19.36	1
4	*V. costicola*	pVMUS7	16.69	1
5	*V. mediterranei*	pVSPF4	16.08,8.27,5.98	3
6	*V. costicola*	pVSY3	13.38	1
7	*V. carchariae*	pVSY1	15.36	1
8	*V. cincinnatiensis*	pVSY6	14.3	1
9	*V. orientalis*	pVMUS11	14.3,6.44	2

Changes in the antibiotic resistance patterns and the transformation efficiency of plasmids from multiple antibiotic resistant *Vibrio* isolates using *E. coli DH5* α are shown in Table 4. The average transformation efficiency was about 5 x 10^{-8}. (Table. 4) Both plasmids and the associated antimicrobial resistance were transformed into the recipient E. coli *DH5* α, which is sensitive to all the antibiotics tested. Subsequently, the plasmid associated antibiotic resistance pattern of the *Vibrio* strain was obtained from transformed *E. coli DH5* α strain. The resistance phenotype encoded in the plasmids could be transferred to *E. coli* transformant and as well as been expressed in the transformant. From the transformation studies of plasmids, it was evident that the plasmid encoded resistance markers are betalactamase (Ac, A and Cb), amikacin, cephalosporin, doxycycline, rifampicin, furzolidone ,trimothoprim and sulphamethoxazole.

Conjugation studies were also revealed that the plasmid encoded genes were transferable to a recipient *E.coli HB101*. The exconjugants were showing

Table 4. Transformation efficiency of *Vibrio* plasmids obtained from seafoods and the resistance pattern of *E. coli* DH5α transformants

Vibrio isolate	Plasmid name	R- pattern associated with donor *Vibrio* isolate	R- pattern of transformant *E.coli DH5* a	Plasmid encoded resistance	Transformation efficiency
V. mimicus	pVMUS 10	Ac, A, Cb, Cu, Cf, R	Ac, A, Cb, R (4)	Ac, A, Cb, R (4)	2.38 x 10^{-9}
V. orientalis	pVMUS 11	Ac, A, Ak, Cb, Cu, Do, G, M, Nt, R, S, Sf	Ac, A, Do ,G, M, R, S (6)	Ac, A, Do ,G, M, R, S (6)	5 x 10^{-9}
V. alginolyticus	pVMUS1	Ac, A, Cb, Cu, R	Ac, A,R (3)	Ac, A,R (3)	4.17 x 10^{-9}
V. mediterranei	pVSPF4	Ac ,A, Ak Cb,Cu, Fr M, Nt, Na, Nx, R, Sf, Tr, T	Ac, A, Ak, Cb, Cu, Fr, Tr, Sf (8)	Ac, A, Ak, Cb, Cu, Fr, Tr, Sf (8)	3.58x10^{-9}

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 5. Conjugation efficiency of *Vibrio* plasmids obtained from seafoods and the resistance pattern of *E.coli* HB 101 exconjugants

Vibrio isolate	Plasmid name	Plasmid encoded antibiotic resistance pattern (Donor)	R- resistance pattern of Exconjugant.	Conjugation efficiency
V. costicola	pVMUS 7	Ac, A, Cb, Cf, Nx, R, Tr	Ac, A, Cf, Cb, R,S	$2.18x10^{-9}$
V. alginolyticus	pVMUS 1	Ac, A, Cb, Cu, R	Ac, A, R ,S	$2.14x10^{-3}$
V. mimicus	pVMUS10	Ac, A, Cb, Cu, Cf, R	Ac, A, Cb, R,S	$2.07x10^{-6}$
V. marinus	pVMUS 15	Ac ,A, Ak Cb, Cu, Cf, Do, Fr ,R ,S,T, Ne	Ac, Ak, A, Cu, Cf, R, S	$2.1x10^{-3}$
V. orientalis	pVMUS 11	Ac, A, Ak , Cb, Cu, Do, G, M, Nt, R, S, Sf	Do,G,M,R,S, Sf, ,	$2.28 x10^{-3}$
V. mediterranei	pVSPF4	Ac ,A, Ak Cb, Cu, Fr M, Nt, Na, Nx, R, Sf, T, Tr	Ac, A, Ak, Cb, Cu, Fr,S, Sf ,Tr, T	$1.5 x10^{-6}$
V. pelagius	pVSY1	Ac, A, Cb, Cu, C, M, Nt, R, S	Ac, A, Cb, R,M,S	$11.66 x10^{-7}$
V. costicola	pVSY3	Ac, A, Cb, Cu, Cf, M, Nt, R, S	Ac, A, Cb,M,S	$5.83 x10^{-10}$
V.cincinnatiensis	pVSY6	Ac, A, Cb, Cu, C, Co, Cf, Ct, Na, R	Ac, Cb, Na, R,S	$6.83 x10^{-9}$

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-Nalidixicacid, Nt-Netilmycin , Nx-Norfloxacin, Ne-Neomycin, R-Rifampicin, S-Streptomycin, Sf-Sulfafurxazole, Tr-Trimethoprim, T-Tetracycline

the resistance pattern of plasmid. The conjugation efficiency varied from 2.1×10^{-3} to 10^{-9} (Table 5)

Discussion

Coastal environment, by its nature, presents a theatre of ecological diversity and evolutionary adaptation. *Vibrio* species occur widely in aquatic environments and are a part of normal flora of coastal seawater, estuarine and brackish waters. The abundance of *Vibrios* in crustacean and molluscans highlight the potential risk due to the increased seafood borne illness, and the presence of multiple antibiotic resistance (MAR) in these *Vibrios* would pose impediments in the treatment of these illnesses. High incidences of resistant bacteria in response to antibiotic usage have been reported in coastal maricultural areas (Herwig *et al*., 1997; Manjusha *et al*., 2005 and 2011). Large-scale marine aquaculture has been associated with environmental issues worldwide as a consequence of accelerated development and high stocking density. Chemicals and antibiotics are widely used to prevent or treat such infections. The increase in antibiotic resistance within clinical bacterial isolates is undermining the efforts of antibiotic therapy in the treatment of infectious diseases. At the same time, there has been a significant increase in the level of organic and inorganic pollutants, including antibiotic residues, entering the environment (Moura *et al*., 2010). Intensive use of antibiotics in clinical and agricultural settings has been suggested to promote an increase in antibiotic resistance bacterial populations (Aminov, 2009). In spite of the implications that this reservoir of resistance genes may spread to clinical pathogenic bacteria, the resistome has been relatively uncharacterized globally Notably, antibiotic resistance determinants found in potential pathogens comprised only a small portion of the total ARGs surveyed (Davies and Davies, 2010), which implies that the major reservoir for ARGs is in non-pathogenic environmental bacteria. This pool of ARGs was recently termed the environmental antibiotic resistome (Wright, 2007). A link between the environmental antibiotic resistome and the increasing antibiotic resistance problem in clinical pathogens seems plausible given the likely contact between clinical opportunistic pathogens, such as *Pseudomonas aeruginosa*, *Acinetobacter baumannii*, *Stenotrophomas maltophilia* and environmental microbes (Baquero *et al*., 2008; Martinez, 2009a). It is well established that ARB and ARGs existed prior to widespread antibiotic use (Hall and Barlow, 2004; Martinez, 2009a, Allen *et al*., 2010), however, the importance of the non-clinical environment in the increase of antibiotic resistance to clinical pathogens remains unclear (Martinez, 2009a; Davies and Davies, 2010). ARGs of clinical importance have been detected in various environmental non-pathogenic bacteria (Heuer *et al*., 2002; Riesenfeld *et al*., 2004) and from both soil and water ecosystems (Riesenfeld *et al*., 2004; Baquero *et al*., 2008). In several instances, the soil and water environments yielding significant populations of antibiotic resistant environmental isolates are from sites impacted by pollution with a variety of substances, including antibiotics, from human activities (Baquero *et al*., 2008).

The results of antibiotic resistance study indicates that majority of the *Vibrio* spp showed the antibiotic resistance to one or more antibiotics. Similar results were reported from our previous studies in *Vibrio* spp from clinical samples (Abraham et al., 1997) shrimp ponds (Eleonor and Leobert, 2001) water and shrimp tissue samples (Li *et al*., 1999). A very rapid adaptation of bacterial populations under different selective pressures is a commonly observed phenomenon.

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 (Radu *et al*., 1998; Lesmana *et al*., 2001), carbenicillin (27%) in penaeid shrimp

in Mexico (Radu *et al*., 1998; Roque *et al*., 2001), cefuroxime (66%), amikacin (55%), kanamycin (58%) and trimethoprim (76%) in *Sparus sarba* in China (Li *et al*., 1999). Interestingly, in our studies antibiotic resistance was also against chloramphenicol, tetracycline, chlortetracycline, nalidixic acid, gentamycin, sulphafurazole, trimethoprim that are commonly used in aquaculture farms through feeds during culture and hatchery production of seeds. The results of our studies are matching with similar other reports available on the resistances of chloramphenicol and tetracycline in *Sparus sarba* in China (Li *et al*., 1999). In our Kerala region most cases of multiple antimicrobial resistances among *Vibrio* spp. come from the mollusc (100%),with resistance to amoxycillin, ampicillin, and carbenicillin as the most frequent. Thus, in (2008) Costa *et al*. reported multiple antimicrobial resistances in 15.4% of their *Vibrio* isolates from pond water and shrimp farmed.

Plasmids are an important vehicle for carrying antibiotic resistance genes (Bennett *et al*., 2008) and the high organic load and large concentrations of diverse bacterial communities present in the environment presents a unique opportunity for the evolution and transfer of antibiotic resistance genes. 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. Antibiotic resistance plasmids can harbour genes that confer resistance to most if not all clinically significant antibiotic classes such as macrolides, tetracyclines, cephalosporins, fluoroquinolines, aminoglycosides and β-lactams (Bennett, 2008; Martinez, 2009a). The accumulation of different antibiotic resistance genes on plasmids may be enhanced in the environmental microbes (Bennett, 2008). 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. *Vibrio* spp 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 seafood samples to investigate the plasmid encoded antibiotic resistance profile , the extent of antibiotic resistance and distribution capability, which were revealed by assessing their transformation efficiency. Thus nine strains isolated from crustaceans and from molluscans have been found to harbor 1-3 plasmids with size varies from 5.98 kbs to 19.36 kbs. From the results of the plasmid extraction studies, among the MAR *Vibrio* bacteria strains isolated from seafoods have been shown the presence of plasmids in varying in number (1-3 plasmids per strain) and the size with molecular weights ranging from (5.9 to 19.36 kb). The strains isolated from molluscans are carrying four contained single plasmid, while other one was with multiple plasmids. Only three stains isolated from crustaceans harbored single plasmids and one with three plasmids. Similar results were reported reported by Li *et al*. (1999), Molina *et al*. (2002), Shafiani and Malik (2003) and Wang *et al*. (2006). This suggests that antibiotic resistance is encoded on a high molecular weight multiple plasmids, and can easily spreads in the community through food stuff generally consumed by the common man. Similar plasmid profiles in *Vibrio* spp were reported from earlier studies: *Vibrio* spp from cultured silver sea bream, *Sparus sarba* in China (Li *et al*., 1999), *V. ordalli* (Tiainen et al, 1995), V. vulnificus (Radu *et al*., 1998), *V. salmonicida* (Sorum *et al*., 1990) and most extensively in *V. anguillarum* (Pederson *et al*., 1999) and the plasmids were higher than those reported by Shafiani and Malik (2003) and Wang *et al*., 2006). However in the present study a large number of strains were devoid of plasmids but were resistant to all antibiotics on observations which indicates that resistance to these antibiotics is chromosomal. However the presence of plasmids in these isolates seemed to increase their antibiotic resistance (Ramesh *et al*., 2010). According to Qureshi and Qureshi (1992), adaptive responses of bacterial communities to several antibiotics observed in the present investigation may have possible implications for public health. Public health risk is further stressed by the occurrence of (70%) frequency of strains that are typically resistant to more than one antibiotic. Result obtained from this study indicates that antibiotics are a significant selection factor and probably play an important role in regulating the composition of bacterial communities in marine environments. Hence further studies on establishing the role of antibiotics and distribution of antibiotic resistance in seafoods are needed. However the presence of plasmids in these isolates seemed to increase their antibiotic resistance. In view of these studies, it is evident that the *Vibrio* strains isolated from seafoods were able to grow in presence of antibiotics. The property of antibiotic resistance in *Vibrios* may be an important in seafood industry polluted by antibiotics. This is the first report from Kerala where a comprehensive study on the plasmids present in *Vibrios* isolated from seafoods. Resistance to antibiotics is widespread in *Vibrios* and their relationship with transferable plasmids should be further studied.

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 *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). Most of the *Vibrio* isolates from the mollusk and crustcean were resistant to at least one of the tested antibiotics, and a significant percentage exhibited simultaneous resistance to multiple antibiotics, indicating a serious risk to public and animal health.

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 in that may harbor the antibiotic resistance genes in these isolates from seafoods 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). Kim *et al*. (2004) found genes of resistance to drugs of the tetracycline group to be ubiquitous in aquatic organisms and seawater, suggesting marine aquaculture environments may serve as a reservoir for such genes. The activated sludges and biofilms found in WWTPs are said to be rich in nutrients, have high load of organic and bacterial density, which is an ideal environ-ment for cell to cell contact and gene exchange (Dionisio *et al*., 2002; Zhang *et al*., 2009). The reservoir of antibiotic resistance mobile genetic elements (MGEs) in WWTPs include; conjugative transposable elements (transposons and insertion sequences) and integrative conjugative elements or integrons (Bennett *et al*., 2008; Allen *et al*., 2010). The combination of these elements with conjugative plasmids creates an environment where-by these plasmids can quickly acquire these MGEs via transposition or recombination and become mosaics of multiple resistance gene elements (Norman *et al*., 2009). Carattoli (2003) has reported this interaction as a factor for the rapid accumulation and spread of β-lactams resistance driven by related transmissible plasmids found in unrelated *Salmonella* strains.

R-plasmid-mediated resistance was also observed. The widespread resistance of *Vibrio* isolates to antibiotics such as oxytetracycline and ampicillinis mostly the result of careless use of drugs on shrimp farms. Further research will clarify how the presence of microorganisms carrying the drug resistance genes affects the incidence of infection in aquatic livestock and how it impacts human health and antimicrobial therapy. Surveillance of antimicrobial resistance and monitoring of drug use in aquaculture should been encouraged in order to improve the management of antibiotics to the benefit of public health and food safety associated with the activity. A correlation between environmental stress eg., pollution, resistance to antibiotics, pollutants and increased plasmid incidence in marine bacterial populations has been observed (Hada and Sizmore,1981; Baya,1986). The basis of antibiotic resistance development is due to mobile genetic elements such as plasmids and transposons. The selection of resistant mutant strains and the transfer of mobile genetic determinants like plasmids and transposons, promoted increased antibiotic resistance (Spengler *et al*., 2003).

The spread of antibiotic resistance among pathogenic bacteria thus posed a serious problem of therapeutic failure during the treatment of infectious diseases. The adaptation to antibiotics present in the aqueous environment is due to the acquisition and dissemination of simple antibiotic resistance genes by mobile genetic elements (Cruz and Davies, 2000). It is well known that plasmid is one of the most important mediators facilitating the vast spreading of antibiotic resistance among bacteria (Dale and Park, 2004).

The transfer of multiple resistances by plasmids is a major concern in aquatic bacterial chemotherapy. To face the challenge, much more research is needed regarding the incidence of multiresistant isolates and the use and effect of antibiotics in shrimp and humans (Manjusha *et al*., 2005). Research on antimicrobial resistance in *Vibrios* should be encouraged. Some species of the genus *Vibrio* are opportunistic pathogens. When infecting marine livestock they strongly impact productivity and pose a potential health risk to human consumers.

In summary, the prevalence of multiple drug resistant *Vibrio* spp. from seafoods 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. Over all results indicated that bacterial resistance in *Vibrio* strains from the seafoods is both plasmid mediated and chromosome mediated. Furthermore, *Vibrio* spp have the ability to transfer the plasmid-encoded resistance into other bacterial genera by means of transformation, conjugation. 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 seafoods in Kerala coastal waters. 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 antibiotics 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 seafood samples in Kerala suggested the potential threat to humans, and indigenous animals.

Therefore, the frequent assessment of bacterial resistance and their plasmid profiles in these coastal waters may give a better knowledge regarding the uncanny ability of the acquired drug resistance determinants in ubiquitous bacterial flora, *Vibrio* spp. Further detailed study on the antibiotic resistance profile and plasmid ecology of environmental isolates of *Vibrio* species from seafoods will be of special importance to understand the mechanism of genetic exchanges among Gram-negative bacteria in aquatic environments

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