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Characterization of Plasmids from Multiple Antibiotic Resistant *Vibrio* sp. Isolated from Molluscs and Crustaceans

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This study investigated the role of plasmids and their relationship with the multiple antibiotic resistance of 30 *Vibrios* sp. isolated from molluscs and crustaceans sampled from the Kerala coastal waters of India. The biochemical identification and antibiotic resistance profiles were determined, followed by the plasmid profiles, conjugation and transformation efficiencies. The results showed a considerable difference in the level of bacterial resistance to various antibiotics; while all 30 strains were found to be MAR *Vibrios* sp. and their resistance patterns varied. All the strains were resistant to 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. In addition to their antibiotic resistance, the plasmid DNA of the MAR *Vibrios* strains isolated from the molluscs and crustaceans was also studied. Nine strains isolated from crustaceans and molluscs were found to harbor 1-3 plasmids with sizes varying from 5. 98 kb to 19. 36 kb. The average transformation efficiency was about 5×10^{-8} and the conjugation efficiency varied from 2.1×10^{-3} to 10^{-9}. A further study of antibiotic resistance patterns may be useful to test the extent of drug resistance in seafoods and help to devise a nationwide antibiotic policy.

Keywords: Multiple antibiotic resistance (MAR), plasmids, molluscs, crustaceans, *Vibrios*

Introduction

Members of the *Vibrionaceae* family represent a significant component of microflora including more than 30 species, and many are pathogenic to humans and have been associated with food-borne diseases [10]. Among these species, *Vibrio cholerae* is not only the most feared but also the most extensively studied, being associated with epidemics and pandemic diarrhoea outbreaks in many parts of the world [10]. However, several other species of *Vibrios* capable of causing disease in humans have also received attention over the last decade, including *Vibrio parahaemolyticus*, *Vibrio vulnificus*, *Vibrio alginolyticus*, *Vibrio damsela*, *Vibrio fluvialis*, *Vibrio furnissii*, *Vibrio hollisae*, *Vibrio metschnikovii* and *Vibrio mimicus* [10]. 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 [5]. In recent years, Vibriosis has become one of the most important bacterial diseases in maricultured organisms, affecting many species of fish and shellfish [44, 45].

The extensive use and misuse of antibiotics in medications, veterinary practice, agriculture and aquaculture, have caused antibiotic-resistant bacteria to become wide-spread [19]. However, according to Nogueira [28], evaluation of 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 [35]. Thus, due to the presence of R-factors in the population, the resistance developed through gene regulation of plasmids

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and chromosomes can be transferred vertically (by heredity) or horizontally [24]. Plasmids have been found in heterotrophic bacteria [10] and in *Vibrios* [26], and in most cases their involvement in resistance to many antibiotics has been proven [10]. To our knowledge there has been no previous report on the occurrence of plasmid occurrence and their relationship with the multiple antibiotic resistances of *Vibrio* strains isolated from seafoods sampled from Kerala coastal waters.

A study of antibiotic sensitivity was carried out, to detect the antibiotic resistance patterns of individual *Vibrio* strains when exposed to particular antibiotic concentrations. Antibiotics are frequently used against different terrestrial organisms, however, the presence of antimicrobial agents at low concentrations through leaching or continued usage may lead to the development of drug-resistant strains and multiple antibiotic resistance (MAR) in bacteria, which ultimately results in the transfer of resistance to pathogenic bacteria and reduced efficacy of antibiotic treatments for human and animal diseases. Thus, it was considered important to evaluate the possibility that aquatic environments may behave as a reservoir of antibiotic multi-resistant bacteria, increasing the risk of their transfer into shellfish, molluscs and then human pathogens. Furthermore, many species of halophilic *Vibrios* have already been recognized as potential human pathogens causing serious gastroenteritis or severe wound infections upon exposure to contaminated seafood and/or seawater. Therefore, the present study was undertaken to assess the multiple antibiotic resistance and characterization of resistant plasmids in *Vibrio* sp. from different aquatic samples.

With this background, the present study was designed to investigate the occurrence of multiple antibiotic resistance in *Vibrio* sp. and assess the presence of plasmids and their relationship to multiple antibiotic resistance of *Vibrio* strains isolated from certain molluscs and crustaceans sampled from the Kerala coastal waters.

Materials and Methods

Sampling site

Molluscan (*Perna virdis* & Sepia) and Crustacean (Shrimp) samples collected from the coastal waters of Kerala ($8^{o}18'$N $74^{o}52$E to $12^{o}48'$ N $72^{o}22'$E) were used as the seafood samples for the study. The seafood samples were collected in sterile polythene bags and transported aseptically to the laboratory within 2-6 h.

Bacterial isolation and storage

The bacteria contained in the tissue samples were serially diluted after homogenization and a Thiosulfate Citrate Bile Sucrose Agar (Himedia Laboratories, Mumbai) used to grow isolates of *Vibrios* with a spread plate technique. A nutrient broth culture with 20% glycerol and 2% sodium chloride was prepared and stored at -80^{o}C as the stock culture.

Identification of *Vibrio*

Isolated pure cultures of bacteria were grown on the nutrient agar plates and identified using identified conventional biochemical tests [23, 43]. The gram stain reaction and cell morphology were observed as described earlier. The isolates were identified based on the standard scheme available for environmental *Vibrios* [4].

Antibiotic sensitivity test

The antibiotic resistance of the bacteria was determined based on a disc diffusion using a Mueller Hinton Agar, according to the Buer Kirby method [3]. The bacteria were multiplied on agar slants (ZB) at 20^{o}C. 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 was compared with the standard and adjusted with normal saline. The standardized bacterial suspension was then swab inoculated on to the Muller Hinton Agar using sterile cotton swabs and left to dry for 10 min before adding the antimicrobial sensitivity discs. Antibiotic impregnated discs 8 mm in diameter were used for the test. Disks containing the following antibacterial agents were plated on the plate and incubated overnight: 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) and Amikacin (Ak, 10 μg). After incubation, the diameter of the inhibition zone was measured and compared with a zone diameter in-

terpretative chart to determine the sensitivity of the isolates to the antibiotics. The results were interpreted based on the recommendations of the National Committee for Clinical Laboratory Standards for antimicrobial susceptibility tests [14]. In this study, the *Vibrio* strains were considered as MAR strains, if they showed resistance against more than three antibiotics [14]. This procedure is intended for the *in vitro* susceptibility testing of common rapidly growing and certain fastidious bacterial pathogens. *V. cholerae* that has multiple-antibiotic-resistance and *E. coli* DH5 α that is susceptible to all the tested antibiotics were used as the positive and negative controls, respectively.

Plasmid isolation

The plasmid extraction from the bacterial strains was performed using the mini-prep alkali lysis method [8] with minor modifications. A Luria-Bertani (HiMedia, India) broth supplemented with 2% NaCl was used to cultivate all the strains. The extracted DNA was electrophoresed in a 0. 7% agarose gel at 80 V for 1-3 hours and photographed using a gel documentation system (Amersham Pharmacea).

Transformation

The plasmids extracted from the isolates were used for the transformation experiment using bacterial strain *E. coli* DH5α as the recipient [34]. The transformation efficiency was calculated from the ratio of the number of transformants to the number of competent cells used for transformation.

Conjugation

The conjugation was performed using *Vibrio* containing the plasmid encoded resistance as the donor cells and the *E. coli* HB 101 strain with a selectable streptomycin marker as the recipient [21]. Thus the purpose of the *E. coli* HB101 experiment was to investigate the conjugative ability of the plasmids carrying the resistance markers, since the HB 101 strain only included a selectable Streptomycin as its marker, and this strain has been specifically used in almost all previous conjugaton studies. This could also help to understand the inter-generic conjugation between plasmids of *Vibrio* strains and *E. coli.* Hence, after the conjugation, only those ex-conjugant strains incluing the plasmid resistance marker and the *E. coli* HB 101 with the streptomycin resistance could survive in a selection medium containing streptomycin (recipient character) and ampicillin. The donor and recipient cells were inoculated in a LB broth and incubated overnight at 37°C. Thereafter, the donor and recipient cells were mixed in a 1:3 proportion in a sterile bottle and the mixture was filtered through 0.2 μm filter paper. The filter paper containing the bacteria was then placed onto a MacConkey agar containing the antibiotics ampicillin and streptomycin at a rate of 50 μg/mL and 25 μg/mL, respectively and incubated overnight at 37°C for 48 h. Following the incubation, the filter papers containing the bacteria were washed with normal saline and the conjugated bacterial suspensions were plated onto MacConkey agar containing ampicillin and streptomycin. The inoculated plates were then incubated for 48 h at 37°C. The exconjugants that grew in the medium containing ampicillin and streptomycin were checked for their antibiogram pattern and plasmid content. The 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* as a result of morphological and biochemical identification of the strains isolated from the molluscs and crustaceans. Among these isolates fifteen *Vibrios* were isolated from the mollusks: *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 the crustaceans: *V. parahaemolyticus* (2), *V. hollisae, V. pelagius, V. carchariae, V. splendidus, V. cholera* (5)*, V. cincinnatiensis, V. vulnificus* (2), and *V. costicola.*

When thirty *Vibrio* isolates were tested for their susceptibility to antimicrobial agents, 100% of the isolates showed

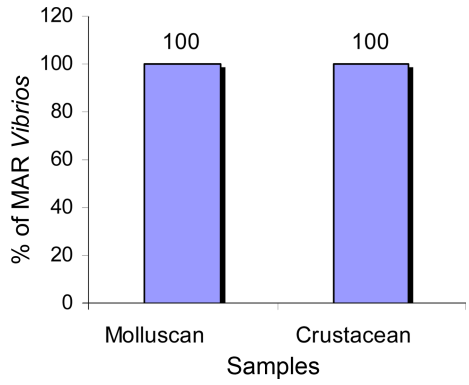

Fig. 1. Occurrence of antibiotic resistance in *Vibrios* isolated from molluscs and crustaceans (percentage).

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

Sl. No	Identification	Antibiotic Resistance Profiles	No of R Determinants
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)
(The abbreviations are as indicated by the manufacturer, and according to NCCLS, 2001)
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. cholera*	Ac, A, Ak, Cb, Cu, C, Cf, Do, Na, Nt, Nx, R, S, Tr	14
6	*V. cholera*	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)
(The abbreviations are as indicated by the manufacturer, and according to NCCLS, 2001)
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.

antibiotic resistance (MAR) to at least one of the 22 tested antibiotics (Fig. 1) Tables 1 & 2 present the antibiotic profiles of the *Vibrio* isolates. The bacteria were tested for susceptibility to 22 antimicrobials representing 15 antimicrobial drug classes, and when the data were analyzed taking into account the source from which the samples were obtained, 100% of the isolates from the molluscs and crustaceans were found to be resistant to at least one antimicrobial agent.

Resistance to amoxycillin, ampicilln and carbenicillin was found in *Vibrio* isolates from both the crustaceans and the molluscs while resistance to nalidixic acid was detected

in seven isolates from the molluscs and five isolates from the crustaceans. Most of the isolates were resistant to amoxycillin, ampicillin, carbenicillin and amikacin plus 87% were resistant to rifampicin, 74% to cefuroxime, 67% to streptomycin, 53% to norfloxacin and ciprofloxacin and 47% to furazolidone and nalidixic acid (Figs. 2 and 3).

The most frequently expressed resistance phenotypes in the *Vibrio* isolates from the molluscs and crustaceans were found to be amoxycillin, ampicilln, carbenicillin, amikacin and amoxycillin, ampicillin, carbenicillin, cefuroxime respectively. The results also showed that over 50% of the *Vibrio* isolates were resistant to clinically used antibiotics, amoxycillin, norfloxacin, rifampicin and ciprofloxacin. Some of the isolates (7/15 and 9/15) even exhibited resistance to fourth generation antibiotics such as chloramphenicol and doxycycline hydrochloride, which raises serious concern.

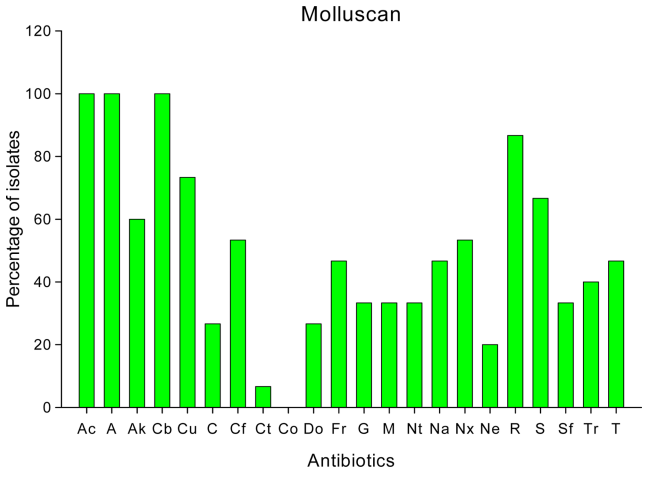

Fig. 2. Resistance to different antibiotics among *Vibrios* isolated from molluscans from Kerala Coastal waters (percentage)

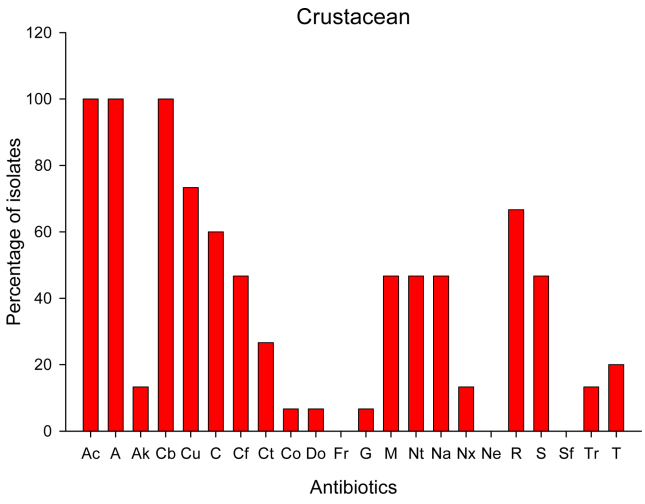

Fig. 3. Resistance to different antibiotics among *Vibrios* isolated from crustaceans from Kerala Coastal waters (percentage).

The bacterial isolates from the molluscs showed the highest frequency of resistance determinants >10 resistance factors, (10/15), followed by those form the crustaceans (4/15). The antibiotic resistance profiles for the *Vibrio* isolates were also varied (Tables 1 and 2), indicating that the antimicrobial resistance patterns were not related to specific species. In general, evident differences in the antimicrobial resistance patterns were observed among the isolates from the seafood samples. The occurrence of simultaneous resistance to multiple antimicrobial drugs was also observed in all thirty *Vibrio* isolates.

The commonly occurring resistance profiles identified among the multiresistant isolates were: Ac+A+Cb; Ac+A+Cb+Ak; Ac+A+Cb+Ak+Cu; Ac+A+Cb+Cu; Ac+A+Cb+Cu+C; and Ac+A+Cb+Na.

The plasmid frequency was 40% in the molluscs and 20% in the crustaceans (Fig. 4). Among the 15 multiple antibiotic resistant (MAR) *Vibrios* strains isolated from the molluscs, four contained single plasmids, while, the *Vibrio orientalis* strain contained two plasmids. Moreover among the *Vibrio* strains isolated from the crustaceans, three strains harbored single plasmids while the *Vibrio mediterranei* strain harbored three plasmids with sizes of 5.98, 8.27 and 16.08 kb respectively. The sizes of the plasmids extracted from the *Vibrios* strains isolated from both the molluscs and crustaceans varied from 5.98 kb to 19.36 kb (Fig. 5 and Table 3).

The changes in the antibiotic resistance patterns and transformation efficiency of the plasmids from multiple antibiotic resistant MAR *Vibrio* isolates when using *E. coli* DH5α are shown in Table 4. The average transformation efficiency was about 5×10^{-8} (Table 4). The plasmids and

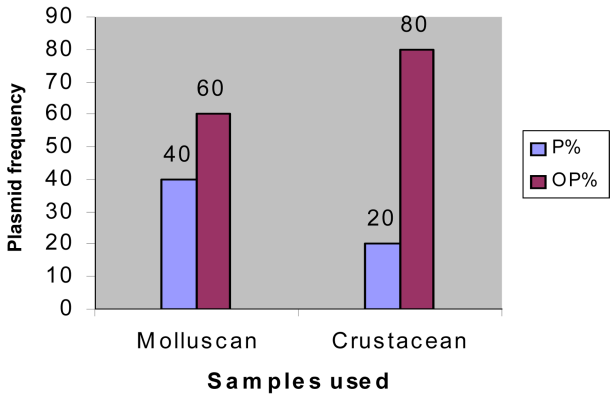

Fig. 4. Percentage of plasmids in *Vibrios* isolated from seafood samples.

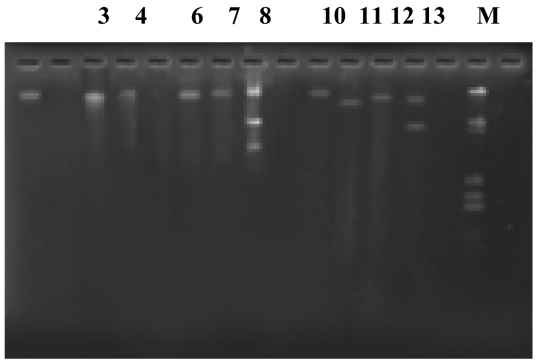

Fig. 5. Profile of single/double plasmids in MAR-*Vibrios* isolated from molluscs and crustaceans. Plasmids isolated from different Vibrio species: Lanes 3, 4, 6, 7, 8, 10, 11, 12 and 13 have pVMUS10, pVMUS15, pVMUS1, pVMUS7, pVSPF4, pVSY3, pVSY1, pVSY6 and pVMUS 11 respectively and Lane M Marker.

Table 3. Profile of single/ double plasmids in MAR-*Vibrios* isolated from molluscs and crustaceans.

Sl. No	*Vibrio*	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

associated antimicrobial resistances were all transformed into the recipient *E. coli* DH5α, which is sensitive to all the antibiotics tested. The plasmid associated antibiotic resistance pattern of the *Vibrio* strains was then obtained from the transformed *E. coli* DH5α strain. The resistance phenotypes encoded in the plasmids were transferred to the *E. coli* transformant as well as being expressed in the transformant. From the transformation studies of the plasmids, it became evident that the plasmid encoded resistance markers

Table 4. R-pattern of *Vibrio* plasmids and their transformation efficiency.

Vibrio isolate	Plasmid name	R- pattern associated with donor *Vibrio* isolate	R- pattern of transformant *E. coli* DH5 α	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)	2.38×10^{-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)	5×10^{-9}
V. alginolyticus	pVMUS1	Ac, A, Cb, Cu, R	Ac, A, R (3)	Ac, A, R (3)	4.17×10^{-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)	3.58×10^{-9}

The numbers in parenthesis indicate the number of antibiotic resistance genes in 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
(The abbreviations are as indicated by the manufacturer, and according to NCCLS, 2001)

Table 5. R pattern of *Vibrio* plasmids and conjugation efficiency.

Vibrio isolate	Plasmid name	Plasmid encoded antibiotic resistance pattern (Donor)	R- resistance pattern of exconjugant *E. coli* HB 101	Conjugation efficiency
V. costicola	pVMUS 7	Ac, A, Cb, Cf, Nx, R, Tr	Ac, A, Cf, Cb, R, S	2.18×10^{-9}
V. alginolyticus	pVMUS 1	Ac, A, Cb, Cu, R	Ac, A, R, S	2.14×10^{-3}
V. mimicus	pVMUS10	Ac, A, Cb, Cu, Cf, R	Ac, A, Cb, R, S	2.07×10^{-6}
V. marinus	pVMUS 15	Ac, A, Ak Cb, Cu, Cf, Do, Fr, R, S, T, Ne	Ac, Ak, A, Cu, Cf, R, S	2.1×10^{-3}
V. orientalis	pVMUS 11	Ac, A, Ak, Cb, Cu, Do, G, M, Nt, R, S, Sf	Do, G, M, R, S, Sf,	2.28×10^{-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	1.5×10^{-6}
V. pelagius	pVSY1	Ac, A, Cb, Cu, C, M, Nt, R, S	Ac, A, Cb, R, M, S	11.66×10^{-7}
V. costicola	pVSY3	Ac, A, Cb, Cu, Cf, M, Nt, R, S	Ac, A, Cb, M, S	5.83×10^{-10}
V. cincinnatiensis	pVSY6	Ac, A, Cb, Cu, C, Co, Cf, Ct, Na, R	Ac, Cb, Na, R, S	6.83×10^{-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 abbreviations are as indicated by the manufacturer, and according to NCCLS, 2001)

are betalactamase (Ac, A and Cb), amikacin, cephalosporin, doxycycline, rifampicin, furzolidone, trimothoprim and sulphamethoxazole.

The conjugation studies also revealed that the plasmid encoded genes were transferable to the recipient *E. coli* HB101, as the exconjugants showed the resistance patterns of the plasmids. The conjugation efficiency varied from 2 1×10^{-3} to 10^{-9} (Table 5).

Discussion

By their nature, coastal environments present a theatre of ecological diversity and evolutionary adaptation. *Vibrio* species occur widely in aquatic environments and are a normal part of the flora occurring in coastal seawaters, estuarine and brackish waters. However, the abundance of *Vibrios* in crustaceans and mollusks highlights a potential risk of increased seafood borne illness. Moreover, the presence of multiple antibiotic resistance (MAR) in these *Vibrios* would pose impediments in the treatment of such illnesses. High incidences of resistant bacteria in response to antibiotic usage have already been reported in coastal maricultural areas [17, 25, 26]. Large scale marine aquaculture has been associated with environmental issues worldwide as a consequence of accelerated development and high stocking density, where chemicals and antibiotics are widely used to prevent or treat such infections.

Among the 30 strains tested, all showed multiple antibiotic resistance. The results indicated that the majority of the *Vibrio* sp. showed antibiotic resistance to one or more antibiotics. Similar results have also been reported from our previous studies of *Vibrio* sp. from clinical samples [1], shrimp pond waters [14] and shrimp tissue samples [21]. As such the very rapid adaptation of bacterial populations would seem to be a commonly observed phenomenon.

The highest incidence of antibiotic resistance was evident against amoxycillin, ampicillin, carbenicillin, cefuroxime, streptomycin, rifampicin, furazolidine and meropenem. These antibiotics are all commonly used to prevent diseases in human beings. Therefore, terrestrial bacteria entering seawater with antibiotic resistant plasmids may have contributed to the prevalence of the gene resistances in the marine environment, which is concurrent with earlier reports [9]. However, few reports are available on the acquired antibiotic resistance to ampicillin (44%) in *Vibrios* from different sources, for e.g. [20, 32], carbenicillin resistance (27%) in penaeid shrimp in Mexico [32, 33], and cefuroxime (66%), amikacin (55%), kanamycin (58%) and trimethoprim (76%) resistance in *Sparus sarba* in China [21]. Interestingly, in our studies, the antibiotic resistance was also against chloramphenicol, tetracycline, chlortetracycline, nalidixicacid, gentamycin, sulphafurazole and trimethoprim, all of which are commonly used by aquaculture farms in feeds during the culture and hatchery production of seeds. The results of our studies are comparable to other similar reports available on the resistances to chloramphenicol and tetracycline in *Sparus sarba* in China [21]. In the Kerala region, most cases of *Vibrio* sp. with multiple antimicrobial resistance come from molluscs (100%), where resistance to amoxycillin, ampicillin, and carbenicillin is the most frequent. Thus, [11] the multiple antimicrobial resistances in 15.4% of their *Vibrio* isolates was reported to be found from pond water and farmed shrimp.

It has become increasingly apparent that a variety of important properties of microorganisms are plasmid-mediated. The best-known examples among the plasmid pool in bacteria are plasmid-mediated antibiotic resistance determinants, so called R-plasmids. The discovery of plasmid containing antibiotic resistant bacteria in polluted and relatively unpolluted areas prompted the present research team to investigate the distributional limit of transferable resistance in coastal waters. *Vibrio* sp. occur widely in aquatic environments and are a normal part of the flora in coastal seawaters. Hence, the presence of plasmids in *Vibrio* sp. collected from various seafood samples were examined to determine the plasmid encoded antibiotic resistance profile, the extent of antibiotic resistance and the distribution capability, as revealed by assessing the transformation efficiency. As a result, nine strains isolated from crustaceans and molluscs were found to harbor 1-3 plasmids with various sizes ranging from 5.98 kbs to 19.36 kbs. Among the strains isolated from the molluscs, four contained a single plasmid, while one contained multiple plasmids. Among the strains isolated from the crustaceans, three harbored a single plasmids while one harbored three plasmids. Similar results have also been reported in [21, 27, 36, 42]. This suggests that antibiotic resistance is encoded in multiple high molecular weight plasmids, and can easily spread through food generally consumed by human. Similar plasmid profiles in *Vibrio* sp. have been reported in earlier studies: *Vibrio* sp. from cultured silver sea bream, *Sparus sarba* in China [21], *V. ordalli* [40], *V. vulnificus* [32], *V. salmonicida*

[38] and most extensively in *V. anguillarum* [29] where the plasmids were higher than those reported in [36, 42]. However in the present study, while a large number of strains were devoid of plasmids, they exhibited resistance to all the antibiotics indicating that the resistance to these antibiotics is chromosomal. Notwithstanding the presence of plasmids in these isolates did seem to increase their antibiotic resistance [31]. According to Qureshi *et al*. [30], the adaptive responses of bacterial communities to several antibiotics observed in the present investigation may have possible implications for public health. This risk to public health is further stressed by the occurrence of (70%) frequency of strains that were typically resistant to more than one antibiotic. Therefore the present results indicates that antibiotics are a significant selection factor and likely play an important role in regulating the composition of bacterial communities in marine environments. Thus further studies on establishing the role of antibiotics and the 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 results, it is evident that the *Vibrio* strains isolated from seafoods were able to grow in the presence of antibiotics. As such this property of antibiotic resistance in *Vibrios* may be an important for seafood industry polluted by antibiotics. This is the first report from Kerala including 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.

From the results of the transformation experiment using the *Vibrio* plasmids, the plasmid mediated bacterial resistance in the *Vibrio* sp. was shown to be transferable to other bacterial genera (*E. coli*). Similar results from previous transformation experiments have been reported for plasmids in *Vibrio* sp. isolated from *Sparus sarba* [21] and penaeid shrimp [27, 37] found antibiotic resistant bacteria in most samples, including those collected 100 miles offshore and from depths of 8200 meters. When isolates considered autochthonous to the marine environment were examined for plasmids and used in mating experiments, several were able to transfer plasmids to *E. coli* [37], which is concurrent with the present findings. The mobilization of the resistance plasmids into *E. coli* DH5α suggests that these plasmids have a broad host range. Similar findings were also previously reported for plasmids isolated from *Pseudomonas* sp. [36]. In the present study most of the *Vibrio* isolates from the molluscs and crustaceans 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.

Previous conjugation experiments have shown that resistance plasmids can be transferred *in vitro* from *E. coli* to *V. parahaemolyticus* [15]. The results of the present conjugation using *Vibrio* sp. containing resistance plasmids as the donor and the *E. coli* HB101 as the recipient, indicated that the majority of the plasmid associated resistant markers were transferred to the *E. coli* strain. Large sized plasmids were detected in almost all the plasmid positive *Vibrio* isolates. Bacterial antibiotics resistance patterns are sometimes associated with the presence of large plasmids and the ability of plasmids to conjugate. Generally, plasmids that can be transconjugated have a high molecular weight. Thus the presence of plasmids harbouring antibiotic resistance genes in *Vibrio* isolates from seafoods may increase their capacity to threaten human consumers since *Vibrio* strains carrying resistant genes qualify them as potential human pathogens [46] Moreover, the 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 [2]. Kim *et al*. previously found the genes for resistance to drugs in the tetracycline group to be ubiquitous in aquatic organisms and seawater, suggesting that marine aquaculture environments may serve as a reservoir for such genes [18].

R-plasmid-mediated resistance has also been observed. The widespread resistance of *Vibrio* isolates to antibiotics such as oxytetracycline and ampicillin is mostly due to the careless use of drugs on shrimp farms. Yet further research is needed to clarify how the presence of microorganisms carrying drug resistance genes affects the incidence of infection in aquatic livestock and how it impacts human health and antimicrobial therapy. The surveillance of antimicrobial resistance and monitoring of drug use in aquaculture need to be encouraged to improve the management of antibiotics for the benefit of public health and food safety associated with the activity. A correlation between environmental stress, e.g. pollution, resistance to antibiotics and increased plasmid incidence in marine bacterial populations,

has already been observed [7, 16]. 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, promotes increased antibiotic resistance [39].

The spread of antibiotic resistance among pathogenic bacteria thus presents 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 [12]. It is already well known that plasmids are one of the most important mediators facilitating the vast spread of antibiotic resistance among bacteria [13]. Therefore, the transfer of multiple resistances by plasmids is a major concern in aquatic bacterial chemotherapy. To face this challenge, much more research is needed regarding the incidence of multiresistant isolates and the use and effect of antibiotics in shrimp and humans [25]. 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* sp. from seafoods was quite high in the locality of the present study and the bacterial population was quite diverse based on the phenotypic and genotypic characterization of the isolates.

The overall results indicated that bacterial resistance in the seafood isolated *Vibrio* strains was both plasmid mediated and chromosome mediated. Furthermore, the *Vibrio* sp. were found to have the ability to transfer their plasmid encoded resistance to other bacterial genera by means of transformation and conjugation. Thus, the presence of plasmids in *Vibrios* sp. may pose a potential health hazard, since plasmids from animals can be transferred to humans either directly or indirectly, if they are transferred to human pathogens *Vibrio* sp. or *E. coli*. To our knowledge, there have been no previous reports on the plasmid mediated multiple antibacterial resistance in *Vibrio* isolates from seafoods from Kerala coastal waters. Nonpathogenic bacteria can 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 declines, the indiscriminate use of antibiotics in aquaculture and in humans can create disastrous conditions in the future due to horizontal gene transfer and the spread of resistant organisms: Therefore, it is vital to recognize and deal with the threat posed by the overuse of antibiotics. As such the isolation of *Vibrio* species from seafood samples in Kerala suggests a potential threat to humans, and indigenous animals.

The frequent assessment of the bacterial resistance and plasmid profiles in certain coastal waters may provide better knowledge regarding the uncanny ability of the acquired drug resistance determinants in ubiquitous bacterial flora, *Vibrio* sp. Thus, further detailed studies 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. Notwithstanding, the results of the present study of plasmids isolated from seafoods, their transformation efficiency and the conjugation experiments can serve as baseline data for future research on the effect on the ecosystem and human antibiotic resistance. Therefore, the unscrupulous use of antibiotics against diseases should be avoided and restrictions on the use of antibiotics need to be implemented based on nationwide antibiotic policy for India.

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