ANTIBIOTIC RESISTANCE AND PLASMID PROFILES OF *VIBRIO* ISOLATES FROM MUTHUPETTAI MANGROVE ENVIRONMENT, SOUTHEAST COAST OF INDIA

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Abstract

A total of 209 pathogenic vibrios strains were isolated from Muthupettai mangrove environment, Southeast coast of India. All strains were identified to be 8 species (Vibrio cholera, Vibrio harveyi, Vibrio mimicus, Vibrio splendidus, Vibrio aestuarianus, Vibrio vulnificus, Vibrio parahaemolyticus and Vibrio metschnikovii) from all stations. The levels of resistance of bacteria to various antibiotics differed considerably. Among these 3 species (13 strains) multiple antibiotic resistance bacteria were identified from all isolates such as Vibrio cholerae (5 strains), Vibrio parahaemolyticus (5 strains) and Vibrio vulnificus (3 strains). The all strains were also able to resistance concentration of antibiotics up to 150µg/ml. The isolated strains were screened for plasmid DNA by agarose gel electrophoresis and tested for susceptibility to 10 antibiotics by the agar dilution method. 13 strains belonging to 3 species have been found to Muthupettai mangroves 1 – 3 plasmids, with sizes ranging from 11 – 112kb.

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Introduction

Mangrove wetlands along the coastal zone act as barrier against cyclones, protect coastal erosion and provide good nursery ground for number of commercially important aquatic organisms [1].

Bacteria of the genus *Vibrio* are commonly found in coastal, estuarine and mangroves waters. Some *Vibrio* strains are pathogenic and can cause vibriosis, a serious infectious disease in both wild and cultured finfish and shellfish[2]. In recent years, vibriosis has become one of the most important bacterial diseases in maricultured organisms, affecting a large number of species of finfish and shellfish[3, 4].

Antibiotics and other chemotherapeutic agents are commonly used in aquaculture farms either as feed additives or immersion baths to achieve either prophylaxis or therapy. However, extensive use of these drugs has resulted in an increase of drug-resistant bacteria as well as R-plasmids[5, 6]. Furthermore, many species of halophilic vibrios have become recognized as potential human pathogens causing serious gastroenteritis or severe wound infection upon exposure to contaminated seafood and/or seawater[7]. Elucidation of the antimicrobial susceptibilities of potential pathogenic vibrios will be important for prophylaxis and treatment of vibrio infections in human beings and in cultured marine organisms.

Diseases are, perhaps, the major cause of losses in the aquaculture industry, among

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them; vibriosis is one of the most frequent diseases affecting fishes, molluscs and crustaceans[8].

Plasmids have been found in vibrios[9,10], and in some cases, their involvement in resistance to many antibiotics has been proven[10,11].

To our knowledge, plasmid presence, profiling, or their relationship with antibiotic resistance, have not been reported from bacterial strains isolated from mangrove in the Muthupettai.

The aim of this study was to investigate the presence of plasmids and their relationship with antibiotic resistance in strains isolated from Muthupettai mangrove environment in Southeast coast of India.

Material and Methods

STUDY AREA: Muthupettai mangroves (Lat. 10° 25'N; Long. 79° 39'E) situated 400km south of Chennai lies along the south east coast of India. It has total area of 6800ha in which the water spread area covers approximately 2720 ha. It has two specialized habitats were noted viz. mangroves and lagoon (Figure 1).

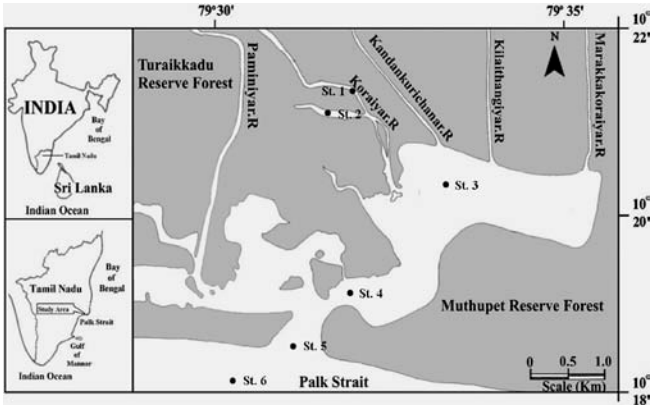

Figure 1. Map of Muthupettai mangrove environment showing different locations.

Many tributaries of the river Cauvery delta such as Paminiyar, Koraiyar, Kilaithangiyar, Kandankurichanar and Marakkakoraiyar flow through Muthupettai and nearby villages and form a lagoon before they reaches the sea, Bay of Bengal. *Avicennia marina* is the dominant mangrove species in Muthupettai and accounts for nearly 95% of the vegetative cover.

The sampling areas for present study viz.,1, Aquaculture discharge area; 2. Sethuguda; 3. Lagoon; 4. Sellimunai; 5. Sea mouth region and 6. Open sea.

A sediment samples at six stations were collected during April (2006) to March (2007) and transported on ice to the laboratory and processed within 18-24h.

Selective isolation and purification of *Vibrio* species

Sediment samples (1 g) were directly spread on Thiosulphate Citrate Bile Salts Sucrose (TCBS) agar plates (HiMedia Laboratories Pvt. Ltd, Mumbai) and incubated at 30 °C for about 12 h. Distinctive yellow and green colonies on agar plates were picked and streaked on new TCBS agar plates several times to purify bacterial isolates until pure cultures of isolates were obtained. A total of 209 bacterial isolates were randomly selected from Muthupettai mangroves. Only thirteen (MAR) strains were highly selective from each site for screening for the presence of plasmids [12].

Identification of *Vibrio* species

Morphological and biochemical properties of the bacteria were investigated according to Bergey's manual of determinative bacteriology [13].

Determination of antibiotic resistance

Antibiotic resistance of bacteria was determined by the single disc diffusion method with the use of Mueller-Hinton agar, according to the Kirby-Bauer method[14]. Bacteria were multiplied on agar slants (ZB) at 20°C. After 72h they were washed off the slants with 5 cm^3 of sterile buffered water and adjusted to a turbidity of 4 on the Mac Farland scale, which corresponds to 10^9 bacterial cells per 1 cm^3. Subsequently, 0.2 cm^3 of bacterial suspension prepared and introduced into steriled Mueller-Hinton medium cooled to 40°C. After mixing, the sample was poured onto Petri dishes and dried in a drier at 37°C for 1h. Paper discs impregnated with an antibiotic were than applied to the surface of the seeded medium. The blotting paper discs (13mm) were manufactured by HIMEDIA. The dishes were kept at 4 °C for 1h in order to allow antibiotic diffusion from the discs into the agar medium. The dishes were then incubated at 27 °C for 24h. Bacteria were classified as antibiotic resistant according to the manufacturer instructions. The following ten clinical antibiotics, with their concentrations given in parentheses were used in antibiograms: Ampicillin (AM, 10µg), Chloramphenicol (CP, 30µg), Gentamycin (GE, 10µg), Kanamycin (KM, 30µg), Nalidixic acid (NA, 30µg), Novobiocin (NB, 30µg), Penicillin (PL, 10µg), Rifampicin (RF, 10µg), Streptomycin (SM,

30µg) and Tetracycline (TE, 30µg). The results were used to calculate the antibiotic resistance index (ARI) for bacteria[15].

Time course for growth of the bacterial isolates

Exponentially grown cultures of the test organisms were inoculated into treated (30, 60, 90, 120 and 150 µg/ml of antibiotics) and untreated liquid culture medium and incubated at 28°C for different time intervals. A control was also run simultaneously. The growth was determined turbidometrically at different time intervals by measuring the optical density (OD) at 540 nm in a Spectronic-20 spectrophotometer[16].

Extraction of plasmid

Plasmid DNA of bacterial isolates was extracted using alkaline lysis method as described by Sambrook et al., (1989). QIAprep spin miniprep kit (Valencia, CA, USA) was also applied to confirm the plasmid extraction result by alkaline lysis method. The plasmid DNAs were loaded onto 0.7% horizontal agarose gels for separation and viewing. Gels were run at 5 Vper cm, strained in ethidium bromide, destained in water and photographed on a UV transilluminator [17].

Results

Vibrio spp. isolated from different stations of the Muthupettai mangroves environment were subjected to analysis for resistance to ten widely used antibiotics. Our observations were carried out in the month of April to March during the years 2006-2007.

Vibrio spp. isolated from the Muthupettai mangrove environment are characterized by large differences in the level of resistance to studied antibiotics (Figure 2).

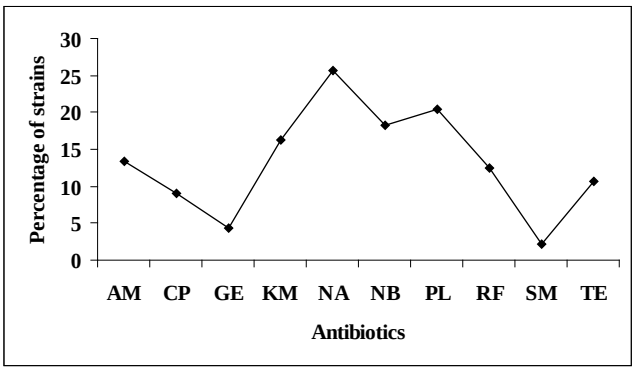

Figure 2. Resistance to different antibiotic among bacteria isolated from Muthupettai mangroves environment (percentages derived from the pooled date of all stations).

Over 15% of the bacterial microflora was resistant to clinically used antibiotics such as kanamycin, nalidixic acid, novobiocin and pencillin; less than 6% of the isolates were resistant to gentamycin and streptomycin.

Differences in the level of antibiotics resistance between the *Vibrio* spp. isolated from different station of the mangroves environment were determined (Table. 1).

	Stations					
Antibiotics	1	2	3	4	5	6
Ampicillin (AM)	16.53	14.57	13.40	12.83	11.80	10.60
Chloramphenicol (CP)	11.47	10.57	9.53	8.47	7.17	6.73
Gentamycin (GE)	7.47	5.83	4.17	3.57	4.90	5.63
Kanamycin (KM)	18.20	17.57	16.20	15.20	16.50	14.13
Nalidixicacid (NA)	29.20	28.37	24.80	26.77	24.20	21.17
Novobiocin (NB)	21.70	20.17	18.80	17.60	16.20	15.23
Penicillin (PL)	23.77	21.90	20.20	21.63	17.87	16.87
Rifampicin (RF)	14.83	13.63	13.17	11.27	11.87	10.07
Streptomycin (SM)	3.77	2.87	1.87	1.07	2.53	1.03
Tetracycline (TE)	13.17	11.90	10.20	9.50	10.13	8.87
ARI	0.16	0.15	0.13	0.13	0.12	0.11

Table 1. Resistance to the antibiotics of bacteria isolated from different stations (in %).

Most of the antibiotic resistant bacteria were found in the aquaculture pond discharge area of the mangrove environment (Station 1) (ARI 0.16) and the most sensitive bacteria were isolated from open sea (Station 6) (ARI 0.11).

At all studied station, most of the bacteria were resistant to kanamycin, nalidixic acid, novobiocin and pencillin, and most sensitive to gentamycin and streptomycin.

Figure 3 presents the results of the study of antibiotic resistance in yellow and green *Vibrio* spp. isolated from the Muthupettai mangroves environment.

Generally no differences between yellow and green bacteria were noted, more resistant kanamycin, nalidixic acid, novobiocin and pencillin and most sensitive to gentamycin and streptomycin.

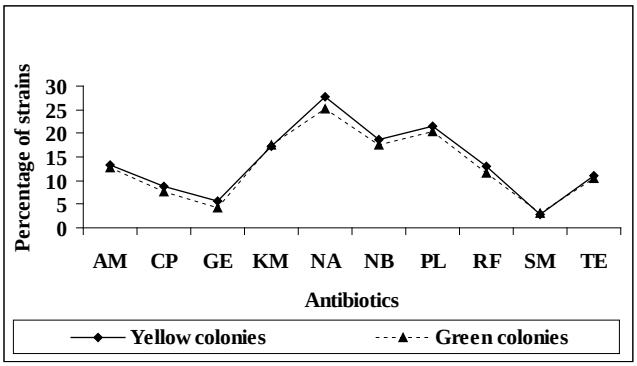

Figure 3. Differential resistance of yellow and green bacteria to studied antibiotics.

Chosen strains were analyzed for multiple antibiotic resistance (MAR) (Figure 4). About 11-15% of the studied bacteria were resistant to KM and NA. 3-10% of the studied bacteria showed an AM, CP, NB and PL MAR pattern (i.e. resistance to KM and NA of the 10 antibiotics tested). 0.5-2% of studied bacteria showed a SM and TE MAR.

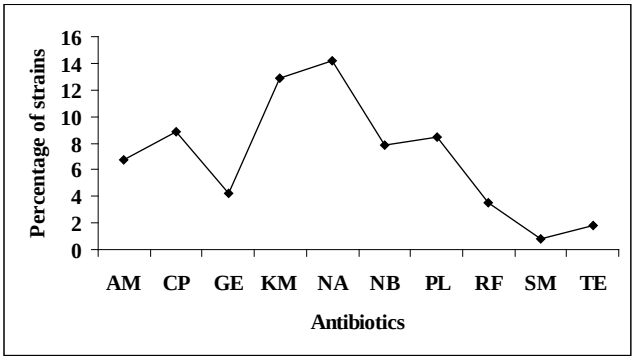

Figure 4. Multiple antibiotic resistance bacterial strains inhabiting Muthupettai mangrove environment.

A total of 209 strains were isolated based on colony morphology, gram-negative, motile by means of polar flagella, oxidase-positive, fermented glucose, yellow and green color from antibiotic resistance of TCBS selective agar plates for biochemical identification (Table 2). The majority of the isolated strains from station 1 were identified. The lowest number of isolates was identified from station 6. Highly resistance of the 13 (MAR) strains isolates were classified and identified as 3 species using MIC of resistance bacteria and plasmid profile.

All the thirteen multiple antibiotic resistance of bacterial strains (Table. 3) isolates of to *Vibrio cholerae* (5 strains), *Vibrio parahaemolyticus* (5 strains) and *Vibrio vulnificus* (3 strains) were tested for their resistance to the all antibiotics. The *Vibrio cholerae* strains resistance concentration up to 150µg/ml of 8 antibiotics, remaining antibiotics 120 µg/ml of SM and TE. The *Vibrio parahaemolyticus* strains resistance concentration up to 150µg/ml of 9 antibiotics, 120µg/ml of SM. The *Vibrio vulnificus* resistance concentration up to 150µg/ml of CP, KM, NA, NB and PL, 120 µg/ml AM, GE, RF, SM and TE.

	Stations						
Bacterial species	1	2	3	4	5	6	Total
Vibrio cholerae	13	11	15	12	11	13	75
Vibrio harveyi	10	8	4	3	6	3	34
Vibrio mimicus	5	3	7	-	1	-	16
Vibrio splendidus	5	-	-	-	-	-	5
Vibrio aestuarianus	3	-	3	-	-	1	7
Vibrio vulnificus	6	2	-	-	2	2	12
Vibrio parahaemolyticus	10	16	11	5	6	7	55
Vibrio metschnikovii	4	-	-	-	1	-	5
Total	56	40	40	20	27	26	209

Table 2. Antibiotic resistance of *Vibrio* species identified from mangrove environment.

Species	Strain no.	Number of plasmids	Plasmid size (kb)
Vibrio cholerae	7	1	42
	23	2	35, 59
	80	3	52, 91, 112
	153	1	76
	189	1	60
Vibrio parahaemolyticus	3	1	46
	57	1	46
	71	1	58
	187	2	39, 62
	203	1	44
Vibrio vulnificus	163	1	11
	180	1	11
	206	1	23

Table 4. Plasmid profiles of the 13 *Vibrio* species isolates.

Thirteen strains belonging to *Vibrio cholerae*, *Vibrio parahaemolyticus,* and *Vibrio vulnificus* have been found to Muthupettai mangroves 1 – 3 plasmids, with sizes ranging from 11 – 112kb (Table. 4). Ten strains were found to contain 1 plasmid, two strains contained 2 plasmids and 1 strain (strain no 80) contained 3 plasmids of different molecular weights. The antimicrobial resistant patterns of these strains which Muthupettai mangroves one or more plasmids were very similar, almost all of them were resistant to all antibiotics.

Discussion

Vibrio species occur widely in aquatic environments and are part of the normal flora of coastal seawater. They also exist as normal flora in fish and shellfish but also been recognized as opportunistic pathogens in many marine animals[2].

In the present study, cultivable antibiotic-resistant *Vibrio* species were widespread in the Muthupettai mangrove environment. Generally no differences between yellow and green bacteria were noted, most resistant to kanamycin, nalidixic acid, novobiocin and pencillin, and most sensitive to gentamycin and streptomycin. Similar results were obtained by [18] in southern Baltic Sea. This is not surprising since the intrinsic resistance of many marine bacteria to antibiotics is well documented[19- 21]. According to [22, 23], such a high level of antibiotic resistance in marine bacteria might result from terrestrial bacteria with antibiotic resistant plasmids entering the seawater; this fact may be responsible for the observed prevalence of resistance genes in the marine environment.

Bacteria occurring in many water basins show multiple antibiotic resistance (MAR), as has been reported by [18, 23-29]. Regarding MAR *Vibrio* species of Muthupettai mangroves, majority of the bacteria were resistant to KM and NA. That means that they are perfectly capable of detoxicating those antibacterial substances. The percentage of MAR was higher than those reported by [18, 19, 23, 27-31]. As pointed out by[32], differences in the percentage of bacteria resistant to various antibiotics may reflects the history of antibiotic application and hence serve as its indicator.

In the present work, all the 209 strains of *Vibrio* species featuring antibiotic resistance in the sample collected at different stations, which are affected by monsoon season heavy fresh water inflow, agricultural discharges, shrimp effluent pollution with indicators of sewage pollution and this result suggests that perhaps other anthropogenic sources of pollution are present and influencing the microbial communities at all sites. The majority of the isolated strains from station 1 were identified, which are affected by aquaculture pond discharge water in mangrove environment. Similar results were observed by[23]. The lowest number of isolates was identified at station 6. In the station, fresh water inflow, pollution sources and aquaculture effluent were low in marine environment. Highly resistance of the 13 (MAR) strains isolates were classified and identified as 3 species using MIC of resistance bacteria and plasmid profile.

The present study has shown that 3 *Vibrio* species isolates of to *Vibrio cholerae* (5 strains), *Vibrio parahaemolyticus* (5 strains) and *Vibrio vulnificus* (3 strains) were tested for their resistance to the all antibiotics. Growth pattern of 3 *Vibrio* species isolates in broth at different time intervals was studied. Growth of the isolates at the lowest concentrations (30µg/ml) was comparable to that of control. However, the growth declined at (120µg/ml) and dropped more sharply at 150µg/ml. *Vibrio parahaemolyticus* resistance of high concentration up to 150µg/ml of 9 antibiotics.

It is well known that plasmid is one of the most important mediators facilitating the fast spreading of antibiotic resistance among bacteria[33]. In order to examine if there is any plasmid involved in antibiotic resistance profile mentioned above, plasmid extraction with alkaline lysis and QIAgen miniprep kit were also applied in this study. From the results of the plasmid extraction experiment, bacteria gave large plasmids (1 - 3 plasmids per strain) with molecular weights ranging from 11 – 112kb. The similar results were reported by[12, 16, 17, 34, 35].

However, in the present study, a large number of strains were devoid of plasmids but were resistant to all antibiotics an observations which indicates that resistance to these antibiotics is chromosomal. However, the presence of plasmids in these isolates seemed to increase their antibiotic resistance.

According to[26], adaptive responses of bacterial communities to several antibiotics observed in the present investigation may have

possible implications for the public health. Public health risk is further stressed by the occurrence of a high frequency (77%) 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 mangroves environments. Hence, further studies on establishing the role of antibiotic substances in controlling mangroves sediment bacterial populations are needed.

Conclusions

In view of these studies, it is evident that the *Vibrio* strains isolated from Muthupettai mangroves sediment were able to grow in the presence of antibiotics. This property of antibiotic resistance in these bacteria may be important in the decontamination of mangrove sediment polluted by the antibiotics. This is the few report, where a comprehensive study on the plasmids present in *Vibrio* species isolated. Resistance to antibiotics is widespread in *Vibrio* species and their relationship with transferable plasmids should be further studied.

Declaration of Interest

The authors report no conflicts of interest. The authors alone are responsible for the content and writing of the article.

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Resistance of bacteria	MIC (µg/ml)	Antibiotics									
		AM	CP	GE	KM	NA	NB	PL	RF	SM	TE
Vibrio cholerae	30	-	-	-	-	-	-	-	-	2(5.0)	2(5.0)
	60	2(5.0)	1(2.5)	1(2.5)	1(2.5)	1(2.5)	2(5.0)	1(2.5)	1(2.5)	1(2.5)	1(2.5)
	90	1(2.5)	2(5.0)	2(5.0)	1(2.5)	1(2.5)	1(2.5)	2(5.0)	2(5.0)	1(2.5)	1(2.5)
	120	1(2.5)	1(2.5)	1(2.5)	1(2.5)	2(5.0)	1(2.5)	1(2.5)	1(2.5)	1(2.5)	1(2.5)
	150	1(2.5)	1(2.5)	1(2.5)	2(5.0)	1(2.5)	1(2.5)	1(2.5)	1(2.5)	-	-
Vibrio parahaemolyticus	30	-	-	-	-	-	-	-	-	1(2.5)	-
	60	1(2.5)	1(2.5)	2(5.0)	1(2.5)	1(2.5)	1(2.5)	1(2.5)	1(2.5)	1(2.5)	1(2.5)
	90	1(2.5)	2(5.0)	1(2.5)	1(2.5)	1(2.5)	2(5.0)	2(5.0)	1(2.5)	2(5.0)	1(2.5)
	120	1(2.5)	1(2.5)	1(2.5)	2(5.0)	1(2.5)	1(2.5)	1(2.5)	2(5.0)	1(2.5)	2(5.0)
	150	2(5.0)	1(2.5)	1(2.5)	1(2.5)	2(5.0)	1(2.5)	1(2.5)	1(2.5)	-	1(2.5)
Vibrio vulnificus	30	-	-	-	-	-	-	-	-	-	-
	60	-	-	-	-	-	-	-	-	-	-
	90	1(5.0)	-	2(10)	-	-	-	-	1(5.0)	2(10)	2(10)
	120	2(10)	2(10)	1(5.0)	1(5.0)	1(5.0)	2(10)	2(10)	2(10)	1(5.0)	1(5.0)
	150	-	1(5.0)	-	2(10)	2(10)	1(5.0)	1(5.0)	-	-	-

Table 3. Multiple antibiotic resistance of 3 *Vibrio* species isolates.

Values in parentheses indicates the percentage of the otal isolates

Total number of *Vibrio cholerae* isolates = 5

Total number of *Vibrio parahaemolyticus* isolates = 5

Total number of *Vibrio vulnificus* isolates = 3

Not detected = -