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Plasmid mediated antibiotic resistance in marine bacteria

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Abstract: *This research work was conducted in Uppanar estuary to ascertain the role of plasmids in the antibiotic resistance of bacteria. Water and sediment samples were collected for a period of three months. When tested against 20 antibiotics 22 MAR strains were isolated from the samples, which were found resistant to 5-13 antibiotics. They belong to 7 genera and 10 species. Gram-negative bacteria namely Neisseria mucosa, N. sicca, Branhamella catarrhalis, Klebsiella ozaenae, Citrobacter intermedius, Pseudomonas fluorescens and Enterobacter aerogenes were isolated. Gram-positive bacteria were of Bacillus subtilis, B. megaterium and Micrococcus luteus. When plasmid curing was done using acredine orange, the resistance against penicillin-G, ampicillin, tetracycline, amoxycillin, kanamycin, and chloramphenicol were totally lost in all strains, which confirmed the role of plasmid in these strains against antibiotics. Ten strains belong to different species were selected for the plasmid isolation and electrophoresis was done. Presence of plasmids in all strains was confirmed and the molecular weight was in the range of 2850 to 3170 bp. The study revealed that MAR strains are common in Uppanar estuary and they are plasmid mediated. This environment is seemed to be deteriorating at an alarming rate.*

Key words: *Antibiotics, Plasmid, Multiple antibiotic resistance*
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Introduction

Microbial resistance to antibiotics is a world-wide problem in human and veterinary medicine. It is generally accepted that the main risk factor for the increase in the antibiotic resistance is an extensive use of antibiotics (Lukasova and Sustackova, 2003; Mukherjee *et al.,* 2005). According to World Health Organization (WHO) the resistance to antibiotics is an ability of bacterial population to survive the effect of inhibitory concentration of antimicrobial agents (Paramasivam *et al*., 2007). Antimicrobial resistance in bacteria may emerge by several pathways. A bacterium of normally susceptible species might become resistant by mutation or acquisition of new genes. Some bacterial species are inherently resistant to certain antibiotics, where others are sensitive. Sensitivity has three requirements: a target for reaction, a mechanism for transport in to the cell before the antibiotic action takes place and absence of enzymes that could inactivate or modify the antibiotic. A change in any of these prerequisites could render an antibiotic-sensitive bacterium resistant to the drug (Levy, 1992). Studies on drug resistant bacteria has revealed that drug resistant bacteria do exist in the marine environment and that plasmid mediated drug-resistance does occur in marine bacteria (Colwell and Sizemore, 1974). The incidence of antibiotic resistance in pathogenic bacteria is rising (Ravikumar *et al.*, 2005). However there are only few works (Mukherjee *et al.,* 2005; Ravikumar *et al.,* 2005; Paramasivam *et al.,* 2007), available in Indian waters on genetic basis for the drug resistance. Hence the present study deals with the evaluation of multiple antibiotic resistant bacteria and the role of plasmid in antibiotic resistance.

Materials and Methods

Water and sediment samples were collected from Uppanar Estuary (Lat 11°43' N; Long 70°49' E). The average depth is about 3.5 m near the mouth and has gradually shallower (2.5 m) upstream. Water and sediment samples were collected for three months (October, November and December 2004) in sterile bottles and unused sterile plastic bags respectively. The total heterotrophic bacterial count (THB) was done using ZoBell Marine agar and multiple antibiotic resistant strains (MAR) were estimated using ZoBell Marine agar incorporated with four antibiotics namely ampicillin, chloramphenicol, kanamycin and penicillin, at a concentration of 10 μg/ml by adopting spread plate technique and plates were incubated for 24-48 hr at 28°C and results were expressed as CFU/ml/g (Dionisio *et al.,* 2002).

All the MAR strains were selected for their antibiotic resistance for 20 antibiotics namely amikacin (30 μg), amoxycillin (10 μg), ampicillin (10 μg), bacitracin (10 units), carbenicillin (100 μg), cephalothin (30 μg), cephotaxime (30 μg), chloramphenicol (30 μg), ciprofloxacin (5 μg), erythromycin (15 μg), gentamicin (10 μg), kanamycin (30 μg), nalidixic acid (30 μg), novobiocin (30 μg), norfloxacin (10 μg), oxytetracycline (30 μg), penicillin (10 μg), tetracycline (10 μg) and vancomycin (30 μg), which were supplied by Hi-Media, India Pvt., Ltd. Zone of inhibition was determined after 24 hr of incubation and multiple antibiotic resistant index (MAR index) was calculated for each species following the method of Kasper *et al.,* 1990. The MAR strains were identified up to their species level by following the scheme of Cappuccino and Sherman (2002) and confirmed with Bergey's manual of determinative bacteriology (Buchnan *et al.,* 1974).

All the MAR strains were screened for the incidence of plasmids responsible for the antibiotic resistance by adopting the method of Sambrook and Russel (2001). Molecular weight of the plasmids were determined by gel electrophoresis. The isolated

Table - 1: THB and multiple antibiotic resistant strains

Month	Sample	THB CFU/ml/g	MAR strains CFU/ml/g
October	Water	6.5×10^5	4.3×10^3
	Sediment	7.9×10^6	3.2×10^4
November	Water	8.3×10^5	5.6×10^4
	Sediment	8.8×10^6	6.3×10^5
December	Water	9.4×10^5	8.0×10^4
	Sediment	9.1×10^6	7.2×10^5

Table - 2: Colony morphology of the MAR strains

Name of the bacteria	Colony morphology
Bacillus megaterium	Round, glossy shade yellow colored colony with smooth and entire margin.
B. subtilis	Round cream colored smooth surfaced colony with entire margin.
Branhamella catarrhalis	Small round opaque white smooth surfaced colony with entire margin.
Citrobacter intermedius	Small round white smooth surfaced colony with entire margin.
Enterobacter aerogenes	Round translucent white smooth surfaced colony with entire margin.
Klebsiella ozaenae	Round white doom shaped glistering colony with stickiness.
Micrococcus luteus	Round yellowish green convex colony with granular surface.
Neisseria mucosa	Round white slimy surfed and become dry when exposed to prolonged incubation.
N. sicca	Round grayish slimy when exposed to prolonged incubation.
Pseudomonas fluorescens	Round green smooth surfaced colony with entire margin.

plasmids on the agarose gel was compared with standard molecular weight markers (Fig. 1). The marker used was EcoRI Hind III double digest and data were interpreted using Total Lab software (Nonlinear Dynamics, UK).

The role of plasmids in the antibiotic resistance was confirmed by curing the plasmid with acredine orange at a concentration of 500 µg/ml which was added to the nutrient broth and culture was incubated for 12 hr (Fugii *et al.,* 1997). Antibiotic resistance patterns of the strains before and after curing of plasmids was compared.

Results and Discussion

Total heterotrophic bacterial counts showed their maximum in the month of December in both water (9.4×10^5 CFU/ml/g) and sediment samples (9.1×10^6 CFU/ml/g). The MAR strains were also showed their maximum in December (8.0×10^4 CFU/ml /g and 7.2×10^5 CFU/ml/g) in water and sediment samples (Table 1).

A sum of 48 strains were isolated based on their colony morphology (Table 2). The biochemical and physiological characteristics of the MAR strains revealed that (Table 3) among

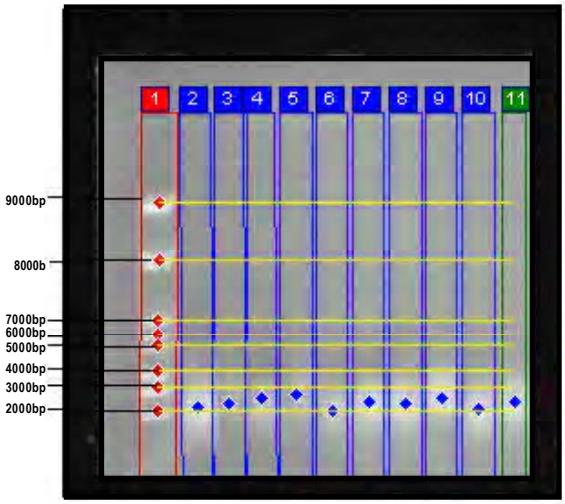

Fig. 1: Plasmids and their molecular weights

Lane 1. Molecular weight marker DNA/ECORI Hind III double digest. Lane 2. Plasmid of *Pseudomonas aeruginosa,* Lane 3. *Branhamella catarhalis,*Lane 4. *Neisseria sicca*, Laane 5. *Neisseria mucosa,* Lane 6. *Citrobacter intermedius,* Lane 7. *Bacillus subtilis*, Lane 8. *Micrococcus luteus,* Lane 9. *Klebsiella ozaenae,* Lane 10. *Enterobacter aerogenes,* and Lane 11. *Bacillus megaterium.*

them, when tested against 20 antibiotics, 22 were found to be MAR strains. MAR strains were belonging to 7 genera and 10 species. In these strains the gram negative strains dominated which included *Neisseria mucosa* (7), *N. sicca* (1), *Branhamella catarrhalis* (3), *Klebsiella ozaenae* (2), *Citrobacter intermedius* (1), *Pseudomonas fluorescens* (1) and *Enterobacter aerogenes* (2). Gram positive organisms were of *Bacillus subtilis* (3), *B. megaterium* (1) and *Micrococcus luteus* (1).

The calculated MAR index is given in Table 4. Among the 10 species tested for plasmid, *Nesseria mucosa* (Fig. 1) showed the plasmid with highest molecular weight (3170 bp) and lowest was that of *Citrobacter intermedius* (2850 bp).

$$\text{MAR index} = \frac{\text{Number of antibiotics to which the isolate was resistant}}{\text{Total number of antibiotics tested}}$$

In the present study total heterotrophic and multiple antibiotic resistant bacterial strains showed their maximum in December. Similar observations were made on the total heterotrophic bacteria in Cuddalore fishing harbour waters showed that increase in THB density during the monsoon period than the summer was reported by Thavasi and Jayalakshmi (2003). Untreated (or) insufficiently treated sewage along with faecal input through rain water run off might have contributed to the high level of the MAR bacteria during the study period. The prevailing low temperature in monsoon may be a reason behind it. The relation between R factors with thermosensitive replication (Smith, 1974) or themosensitive production of their transfer system (Rodriguze-Lemoine *et al.,* 1975) supported this view.

Table - 3: Biochemical characteristics of MAR strains

Characteristics	*Bacillus megaterium*	*B. subtilis*	*Branhamella catarrhalis*	*Citrobacter intermedius*	*Enterobacter aerogenes*	*Klebsiella ozaenae*	*Micrococcus luteus*	*Neisseria mucosa*	*N. sicca*	*Pseudomonas fluorescens*
Gram reaction	+	+	-	-	-	-	+	-	-	-
Shape of the cell	R*	R	C*	R	R	R	C	C	C	R
Spore formation	+	+								
Motility	+	+	-		+		-			+
Pigments							Y			G
Carbohydrate utilization										
Adonitol				-	+	-				
Arabinose	-			+	+	+				+
Cellobiose										
Esculin										
Fructose										
Galactose										
Glucose		+	-	+	+	-	-	+	+	-
Inositol				-	-	+				-
Lactose	-	+		+	+	+	-			
Mannitol				+	+	+				
Maltose										
Mannose										
Rhamnose										
Sorbitol				+	+	+				+
Sucrose				+	+					
Trehalose	+			+		+	-			
Xylose										
Starch	+	+								
Gelatin	+	+		-	+	-				
Fat	+	+	+							
Casein	+	+					+			
Catalase	-		+	+	+		+			
Cytochrome oxidase			+	-	-	-				
Nitrate reduction	-		+	+	+	+		+	-	+
Indole			-	+	-	-				
Methyl red				+	-	-				
Vogous Proskaur	-	+		-	+	-				
Citrate	+	+		+	+	-				
H_2S production			-	+	-	-				
Urease			-	+	-	-				
Litmus reaction										A
Decarboxylation										
Arginine					-		-			
Ornithine						-				
Lysine					+	-				

*R = rod, *C = cocci, - = negative, + = positive G = green, Y = yellow and A = Alkaline reaction

Comparatively more MAR strains were observed in sediment than water samples. Bottom sediments may act as long term reservoirs of drug resistant bacteria and drug residues (Koditschek *et al.,* 1974; Stewart and Koditschek, 1980; Timoney *et al.,* 1978). Moreover sediment is a favorable environment for microbes due to the prevailing stable environmental conditions and the accumulation of the nutrients.

Table - 4: Multiple antibiotic resistance index

Strain name	MAR index
Bacillus megaterium	0.3
B. subtilis	0.25
Branhamella catarrhalis	0.65
Citrobacter intermedius	0.3
Enterobacter aerogenes	0.25
Klebsilla ozaenae	0.35
Micrococcus luteus	0.25
Neisseria mucosa	0.45
N. sicca	0.5
Pseudomonas fluorescencea	0.5

The estuarine and coastal waters around Cuddalore area receive aquaculture effluents, which may contain drug residues in substantial amounts. In addition to that pharmaceutical (SPIC Pharmaceuticals and J.K. Pharmaceuticals) industries are also located in the SIPCOT area and the effluents are discharged in to the Uppanar estuary. Untreated sewage from Cuddalore town is also discharged directly in to the estuary. Thus this environment is receiving drug residues as well as microbial pathogens in multiple routes. The obtained results on antibiotic resistance also confirmed the availability of the drug residues (*i.e.*) all the strains were resistant to penicillin. The prime source Penicillin in this area is JK Pharmaceuticals and SPIC Pharmaceuticals where the production of Penicillin is going on.

Maximum antibiotic resistance was shown by *Nesseria mucosa* against 13 antibiotics followed by *Branhamella catarrhalis* and *Pseudomonas fluorescens*, Neisseria sicca against 12, 11 and 9 antibiotics respectively. The organisms were found to be most resistant to penicillin (100%) followed by amoxycillin and bacitracin (90.01%), while they were least resistant to cephataxime (22.7%) and nalidixic acid (22.73%). All strains were found to be sensitive to gentamycin, amikacin, norfloxacin and ciproflaxacin (100%). The varying antibiotic resistance might refer the usage pattern of these antibiotics, in and around this area. The 100% resistance against penicillin shown by all the isolates may due to their application since 1940 in the clinical field.

When acredine orange mediated plasmid curing was done, resistance against penicillin, ampicillin, tetracyclin, amoxycillin, kanamycin, chloramphenicol were lost in all strains, which confirmed the role of plasmids in antibiotic resistance. Plasmid replication is inhibited by various agents that can intercalate between the bases of DNA. Particularly acridines (acridine orange), without inhibiting the chromosomal DNA replication. Such inhibition can lead to loss of the plasmid (acridine curing) (Freifelder, 1987). Presence of plasmid was confirmed in all the ten species. The molecular weights of plasmids were in the range of 2850-3170 bp (Fig. 1). The molecular weight seemed to be strain specific rather than species specific. Similar results were obtained by Karbasized *et al.* (2003) in coliforms isolated from nosocomial infections in a hospital sample in Iran. In their study in several cases, different isolates showed similar plasmid profiles also. That may be due to the presence of transferable R plasmids among the bacterial population.

The molecular weight of the plasmids obtained in the present study seemed to be low compared to that of coliforms (≤56.4 kb) isolated from clinical samples (Karbasizaed *et al.,* 2003). No consistent relationship was found between MAR index and molecular weight of plamids. Likewise antibiotic resistant pattern and plasmid profile were also not coincided. This is not unexpected, since the same antimicrobial resistant pattern can be encoded by unrelated plasmids, transposons, phages and chromosomal genes (Dombrovskii, 1990). When plasmid was isolated in agarose gel electrophoresis and observed under UV transilluminator it was possible to get only feeble bands for the strains *Bacillus subtilis, Micrococcus luteus* and *Klebsiella ozaenae*. This may due to the presence of low copy number of the plasmids (Mesas *et al.,* 2004) in these strains.

Thus the plasmid mediated resistance in heterotrophic population suggested a free exchange (or) transfer of resistance between the sewage fed pathogens and the normal microflora in this ecosystem. Apart from this rout, in marine environment many antibiotic producing microorganisms are present. As a protection mechanism against these antibiotics, native bacteria may evolve in to resistant strains. Even antibiotic producing organism use similar antibiotic-resistance strategies, including alteration or replacement of target, inactivation for protection from their own antimicrobial compounds (Cundiffe, 1989). The results of the present study showed that, the plasmid mediated multiple antibiotic resistance is common in the normal microflora of this environment.

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