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HEAVY METAL AND ANTIMICROBIAL RESISTANT BACTERIA ISOLATED FROM KARACHI COASTAL AREA AS AN INDICATOR OF POLLUTION

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ABSTRACT

In the present study 70 marine bacterial isolates were studied in order to speculate about pollution along the Karachi coastal area, through the isolation of heavy metal and antimicrobial resistant bacteria. The study revealed resistance characters in 62% gram positive and 38.3% gram negative bacterial strains. Heavy metal resistance was found in 30% of the total isolates which was mostly against zinc, nickel and chromium. Most of the marine bacterial isolates were found resistant to β-lactam (ampicillin) and aminoglycoside (kanamycin and streptomycin). Isolation and identification of these isolates has indicated occurrence of high level of pollution on the Karachi coast. Besides initial characterization through microbiological and molecular techniques, some of the selected strains were also identified through 16S rRNA gene sequencing. The nutshell of the study, analyzing the microbial flora verifies the hypothesis that states high rank pollution along the Karachi Coastline.

INTRODUCTION

Karachi is the largest metropolitan city and industrial hub of Pakistan. The city is located in the south of the country, along the coastline meeting the Arabian Sea. It is spread over 3,527 km^2 (1,362 sq mi) in area, almost four times bigger than Hong Kong. The Karachi coastline, which stretches over 135km, is facing severe pollution due to a combination of industrial, port, municipal and transportation activities in the area. The coastline is being overwhelmed with water-borne pollution being discharged in the shipping process into the marine environment (Beg, 1995) (Fig. 1).

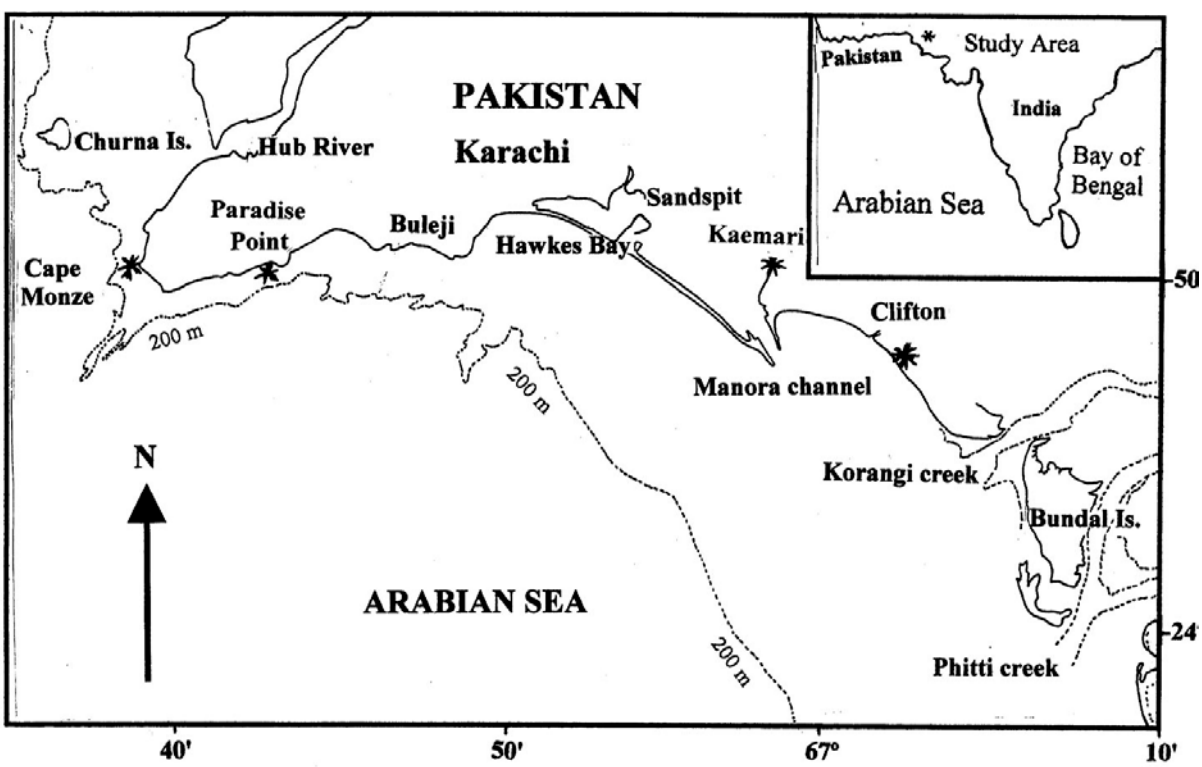

Fig. 1. Map showing Karachi coastline.

The use of microbiological properties as an indicator of pollution has long been identified as first step towards identification of potentially contaminated sites (Brookes, 1995). Coastal waters have shown the high quantity of total dissolved solid 2000ppm, low dissolved oxygen, petrochemicals, organic chemicals, aromatic and aliphatic solvents, plastic intermediates and its by-products, phenols, amines, heavy metals like lead, mercury, chromium, zinc, copper etc., (Wright *et al.*, 2006). These and many other chemicals make a complex cocktail of pollutants exerts both acute and chronic impacts directly or indirectly on human health,

marine biodiversity and fish eating birds. A study found that some of the marine life was contaminated with lead, which if consumed by humans through seafood, has been linked to anemia, kidney failure and brain damage (Pearce, 2007).

Coastal waters and sea near Karachi have not been regularly monitored on scientific basis for pollution load and changes in marine life. Initially Beg *et al*., (1995) reported the type of pollutants in Karachi harbor, and lately Saleem and Kazi (1998) reported contamination and distribution of heavy metals in the coastal and offshore sediments, but no report was available describing resistance level against toxic heavy metals and antibiotics of indigenous microbial flora inhabited in this region. Our study has unfolded and identified the bacterial species resistant to heavy metals and antibiotics consequential to the biological hazards. Besides that we have also attempted to get initial studies on molecular characterization of these isolates which include 16s rRNA gene analysis and plasmid isolation (Sandaa and Enger, 1994; Sobecky *et al*., 1997).

MATERIALS AND METHODS

Sampling and isolation: Surface sea water samples were collected manually from Cape Monze, Paradise point, Clifton beach, Kaemari and from offshore Open Sea. Two samples of sea weeds were also collected manually. For isolation of bacterial strains samples were enriched using conventional microbiological techniques in nutrient broth modified with filtered, glass distilled sea water for media preparation. Sea weeds were cut to small pieces for inoculation in 100ml modified nutrient broth (MNB). 48 hr enrichment was followed by spreading on MN-agar plates incubated at 30°C. Isolated colonies were purified.

Identification: The bacterial isolates were initially identified on the basis of colonial and cellular morphology then by API biochemical tests system (API 50CHB and API 20E bioMeriux, Farance).

Maximum tolerable concentration (MTC) of antimicrobials: MTCs of antimicrobials, amino glycoside (Streptomycin, Kanamycin), β-lactam (Ampicillin), Tetracycline, Chloramphenicol and Rifampicin was examined for 25, 50, 75, 100, 125, and 150 µg. ml^{-1} in MN-agar plates. Working solutions were prepared according to Miller and Poindexter (1994).

MTC of heavy metal salts: MTCs of aqueous heavy metal salts ($CuSO_4$, $NiCl_2$, K_2CrO_4, $ZnCl_2$, $CoCl_2$, and $PbCl_2$) was investigated on agar solidified artificial sea water (ASW-pH 8) plates containing variable concentrations (0.5, 1, 1.5 and 2mM) of heavy metals salts. Glucose/gluconate was supplied as carbohydrate source at the rate 2% w/v. The inoculated plates were incubated at 37° C for 48hr.

16s rRNA gene sequencing: Bacterial strains harboring resistance characters of interest were selected for identification on the basis of 16S rRNA gene sequence homology. The amplified product of 16S rRNA gene of CMG556 was sequenced using 16S- 3' primer with the ABI PRISM 377 Automated DNA Sequencer. Sequence data obtained was BLAST at http:/www.ncbi. nlm. nih.gov/blast/blast.cgi). CMG505, 527, 541and 571 was identified from Macrogen USA, The DNA sequence data obtained was BLAST for sequence homology http:/www.ncbi.nlm.nih.gov/blast/blast.cgi.

Plasmid DNA isolation: Isolated bacteria were investigated for presence of Plasmid DNA (Baya *et al*., 19986; Belliveau *et al*., 1991). Fresh LB (Luria Bertani Medium) grown cultures of Gram negative isolates (BirnBoim and Dolly, 1979) and Gram positive isolates were processed (Kado and Liu, 1981) for plasmid DNA extraction.

RESULTS

Sampling and isolation: A total of 70 marine bacteria were harvested from Sea surface water and two seaweeds (Valoniopsis pachynema and Cladophora uncinella) samples (Table 1). Gram staining revealed 62% bacterial strains as Gram-positive and 38% isolates as Gram negative. Some of the Gram positive isolates identified as endospore forming *Bacillus subtilis, B. polymexa, B. pumilus*, and *B. thuringiensis*. Gram negative isolates distinguished on the basis of biochemical identification test into various species belonging to family Pseudomonadaceae, Enterobactereaceae, Moraxellaceae and Xanthomonadaceae on the basis of API-biochemical tests and cellular morphology. Most of the Gram positive isolates exhibited characteristic periodic architecture of marine bacteria while other isolates showed variable opaque and translucent colony morphotype.

MTCs of antimicrobials: MTCs of six antimicrobials were found among 88.57% of the isolates. Results for Clifton II samples shown in Fig. 2, for Open Sea in Fig. 3, for Paradise Point in Fig. 4 and red sea weed in Fig. 5, whereas Kaemari samples results are shown in Fig. 6. Almost all isolates were found susceptible to chloramphenicol, tetracycline and rifampicin. CMG556 (*Pseudomonas* sp.) and CMG567 (*Proteus valgaris*) showed resistance to the six tested antimicrobials. However isolates of the Clifton beach samples ll showed more resistance towards antibiotics than the isolates from open sea water samples. Multiple antimicrobial resistance was more common in Gram negative than Gram positive isolates.

Table 1. Resistance characters of bacterial strains isolated from Karachi coastal sea water.

Sampling area	Strain code	API-identification	Resistance studied against	
			Antimicrobials ($\mu g.ml^{-1}$)	Heavy metals (mM)
Clifton beach sample I	CMG 501	*Pseudomonas cepacia*	Ap, Km	Cr
	CMG502	*N.I*	Ap, Km	-
	CMG503	*Pseudomonas cepacia*	Ap, Km, Sm	Cr
	CMG 504	*Aotinobacillus lignieresii*	Ap, Km, Sm	Cr
	CMG 505	*Arthrobacter* sp. *****	-	Cd, Ni,Co
	CMG 506	*P. stutzeri*	Ap, Km, Sm	Cu, Ni
	CMG 507	*Bacillus pumilus*	Ap, Sm	-
	CMG 508	*Bacillus polymyxa*	Ap, Sm	-
	CMG 509	*Bacillus polymyxa*	Ap, Sm	Cr
	CMG 510	*Bacillus polymyxa*	-	-
	CMG 511	*Bacillus subtilis*	Ap, Sm	-
	CMG 512	*Bacillus subtilis*	Ap, Sm	-
	CMG 513	*Bacillus subtilis*	Ap, Sm	-
	CMG 514	*Bacillus subtilis*	Sm	-
	CMG 515	*Bacillus subtilis*	-	-
Cape monze	CMG 516	*Pseudomonas cepacia*	-	-
	CMG 517	*Serratia marcescens*	Km	-
	CMG 518	*Enterobacter sakazakii*	Sm	-
	CMG 519	*Bacillus polymyxa*	Cm	-
	CMG 520	*Bacillus polymyxa*	Sm	-
	CMG 521	*Xanthomonas maltophilia*	Km	-
	CMG 522	*Pseudomonas mallei*	Km, Rif	Cu
	CMG 523	*Xanthomonas maltophilia*	Km	-
	CMG 524	*P. aeruginosa*	Km	-
	CMG 525	*P. aeruginosa*	Km	-
	CMG 526	*P. sp*	Km	-
Green sea weed	CMG 527	*Bacillus licheniformis******	Rif, sm	Co
	CMG 528	*N.I*	Sm	-
	CMG 529	*Bacillus polymyxa*	Ap, Sm	-
	CMG 530	*Bacillus subtilis*	Ap, Sm	-
	CMG 531	*Bacillus subtilis*	Ap, Sm	-
Kaemari	CMG 532	*Bacillus subtilis*	Ap	-
	CMG 533	*Bacillus subtilis*	Ap, Sm	-
	CMG 534	*N.I*	Ap	-

Table 1. (Cont'd.).

Open sea	CMG 535	*Morexella* sp.	Km, Sm	Cr
	CMG 536	*Bacillus subtilis*	Ap, Sm	-
	CMG 537	*Enterobacter cloacae*	Ap, Rif, Cm	Ni, Cd, Zn, Cr, Cu
	CMG 538	*P. pseudomonellei*	-	Zn, Ni, Co
	CMG 539	*P.* sp.	Km	-
	CMG 540	*P. aeruginosa*	Sm	-
	CMG 541	*Bacillus cereus******	-	-
	CMG 542	*N.I*	Ap	Zn, Ni, Cd, Co
	CMG 543	*Bacillus pumilus*	Ap, Km, Sm	-
	CMG 544	*Bacillus pumilus*	Ap, Km, Sm	-
	CMG 545	*E. coli*	Ap, Km, Sm	-
	CMG 546	*Bacillus polymyxa*	Ap, Km, Sm	-
	CMG 547	*N.I*	Km, Sm	-
	CMG 548	*N.I*	Km	-
	CMG 549	*Bacillus polymyxa*	-	-
	CMG 550	*Bacillus polymyxa*	Ap, Km	-
	CMG 551	*Bacillus pumilus*	Ap, Km, Sm	-
	CMG 552	*Bacillus subtilis*	Ap, Km	Co
	CMG 553	*B. polymyxa*	Km	-
	CMG 554	*Bacillus subtilis*	Ap, Km	-
	CMG 555	*Bacillus subtilis*	-	-
	CMG556	*Pseudomonas* sp.*	Ap,Cm,Km, Rif,Sm, Tc	Cd, Zn, Co, Ni
Paradise point	CMG 557	*Bacillus thuringiensis*	-	-
	CMG 558	*Bacillus thuringiensis*	Ap, Km	-
	CMG 559	*Bacillus polymyxa*	Ap, sm, Tc	-
	CMG 560	*Bacillus polymyxa*	Sm	-
	CMG 561	*Bacillus polymyxa*	Ap, Km, Sm, Tc	-
Red sea weed	CMG 562	*Pseudomonas* sp.	Ap,Cm,Km,Sm	Cu, Zn
	CMG 563	*Stomatococcus mucilaginosa*	Ap,Km,Sm	-
	CMG 564	*E. coli*	Ap,Km,Sm, Tc	-
	CMG 507	*Proteus mirabilis*	Ap, Sm	Ni
	CMG 508	*Proteus valgaris*	Ap,Cm,Km,Rif,Sm,Tc	Zn, Ni, Cd, Co
	CMG 59	*Bacillus subtilis*	Ap, Rif	Zn
	CMG 510	*P. fluorescence*	Ap,Cm, Rif	Zn
	CMG 511	*P. aeruginosa*	Ap,Cm,Km,Rif, Tc	Cd, Zn
	CMG 571	*Bacillus pumilus**	Ap,Cm,Km, Rif, Sm	Zn

Key: H.M heavy metals , * identified on the basis of 16S rRNA the accession nos. are; EU622829, EU622832, DQ410039, EU 622833, EU622830 respectively, Ap (Ampicillin); Sm (Streptomycin),Km (Kanamycin), Cm (Chloramphenicol), Rif (Rifampicin), Tc (Tetracycline), Co (Cobalt), Cr (Chromium), Cu (Copper), Cd (Cadmium), Ni (Nickel), Zn (Zinc), +ve positive Gram reaction, -ve negative Gram reaction, Phl phenol, Tol toluene, xyl xylene, N.I not identified, Approximate coordinates of sampling sites located from online *Google* Earth (2007): Clifton 24 ^{o} 45 °N, 67° 22 °E, Kaemari: 24° 01°N, 66° 32°E, Paradise point: 24° 45°N, 66° 43°E, Cape monze: 24° 15°N, 66° 2°E.

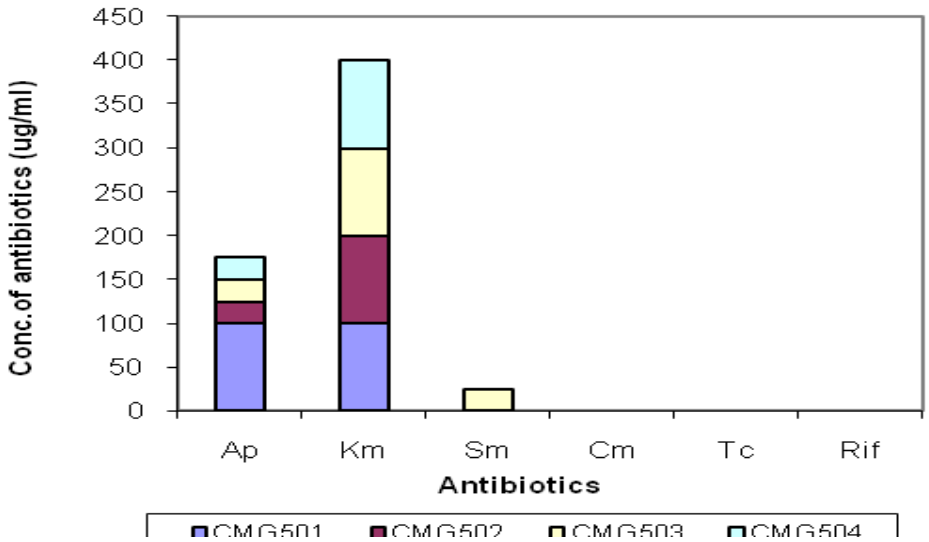

Fig. 2. MTC of antibiotics in marine isolates of Clifton beach water.

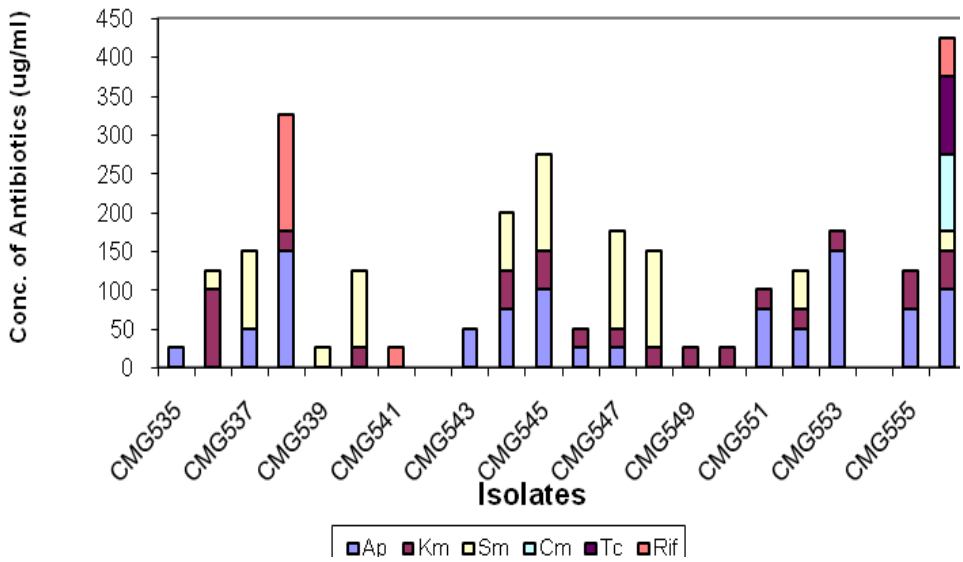

Fig. 3. MTC of antibiotics in marine isolates from open sea.

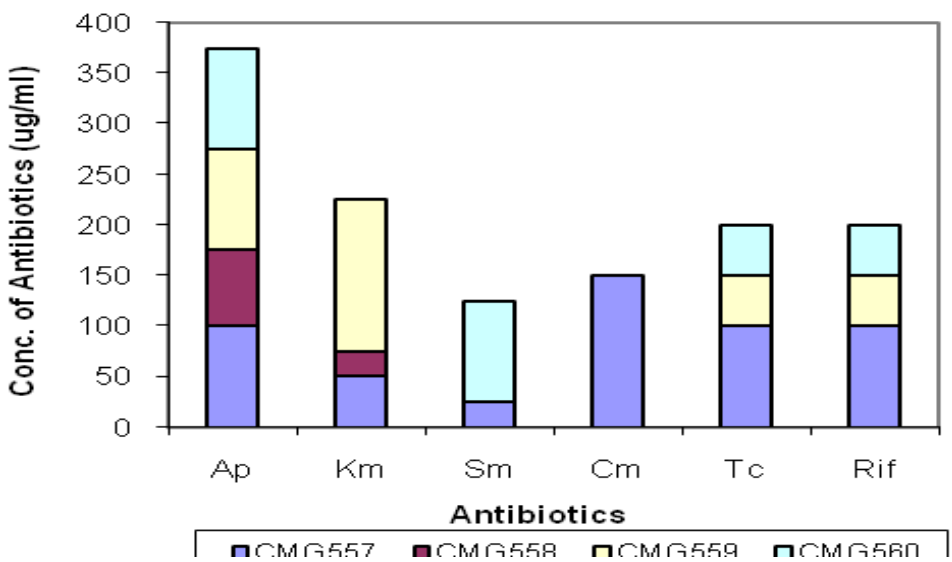

Fig. 4. MTC of antibiotics in marine isolates from Paradise point.

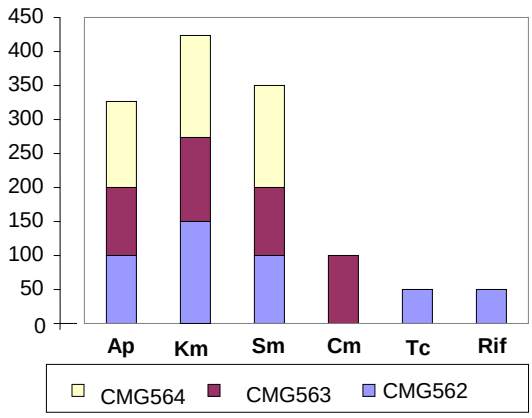

Fig. 5. MTC of antibiotics from red sea weeds of Paradise point.

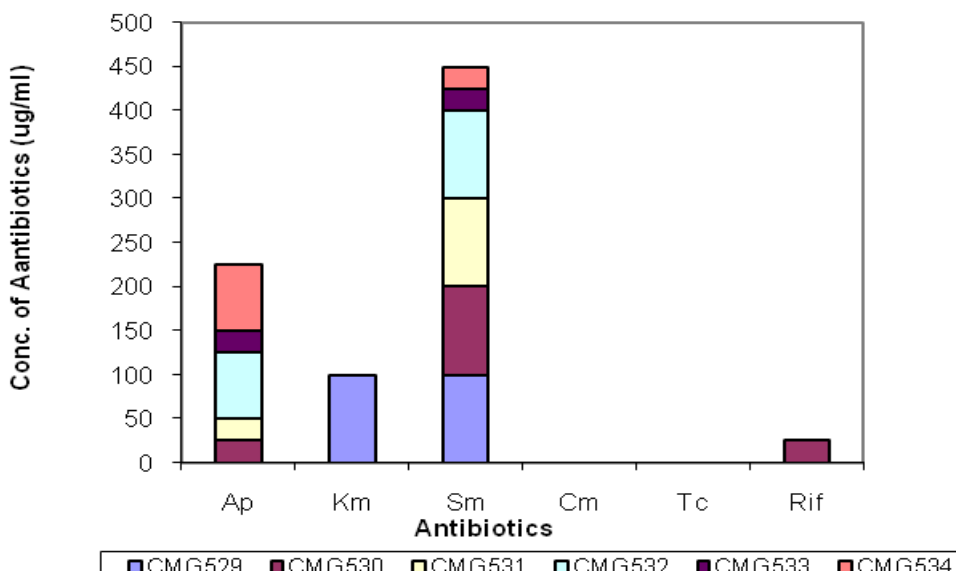

Fig. 6. MTC of antibiotics in marine isolates from Kaemari.

MTCs of heavy metals salts: Investigation of MTCs of heavy metals salts in marine isolates revealed 30% isolates resistant to very low concentrations of heavy metal salts which mainly belonged to Gram negative isolates. 12.76% showed resistance to two and rarely three heavy metals (HM) up to 2mM of aqueous metal salt. Resistance to Zn, Ni and chromium was frequent among the HM resistant isolates. Most of the HM resistant isolates belonged to Clifton beach samples II (Fig. 7) and Open Sea (Fig. 8). Isolates of Kaemari and Paradise point samples found susceptible to the tested HM. CMG571 (*Enterobacter cloacae*) and CMG556 from Open sea water sample showed resistance to multiple heavy metals (Cu, Cd, Ni, Zn).

16S rRNA gene sequencing: The percentage homology and Genbank accession numbers of the selected isolates is listed in Table 2.

Plasmid DNA: Five plasmid DNA molecules of variable sizes (Fig. 9) were successfully isolated in CMG537 (*Enterobacter cloacae*).

DISCUSSION

Metal pollutants pose a severe threat to ecological system due to their negative impact on most life forms. At high concentrations all heavy metals are toxic to all forms of life, including microbes (De Vicente *et al*., 1990). Since heavy metals are increasingly found in microbial habitats due to natural and environmental processes, microbes are known to possess a wide array of genetic composition that allows them to circumvent metallic stress (Adarsh *et al*., 2007; Appanna *et al*., 1996; Valdman *et al*., 2001). The increasing heavy metal

tolerance has another implication in the environment as it may contribute to the maintenance of antimicrobial resistance genes by increasing the selective pressure of the environment (Cooksey, 1993). The occurrence of multiple metal and antimicrobial resistance property in the microbial community poses a potential threat towards human and environmental health (D'Hondt *et al*., 2004; Miyake *et al*., 2003). There is concern that metal contamination functions as a selective agent in the proliferation of antimicrobial resistance (Austin *et al*., 2006).

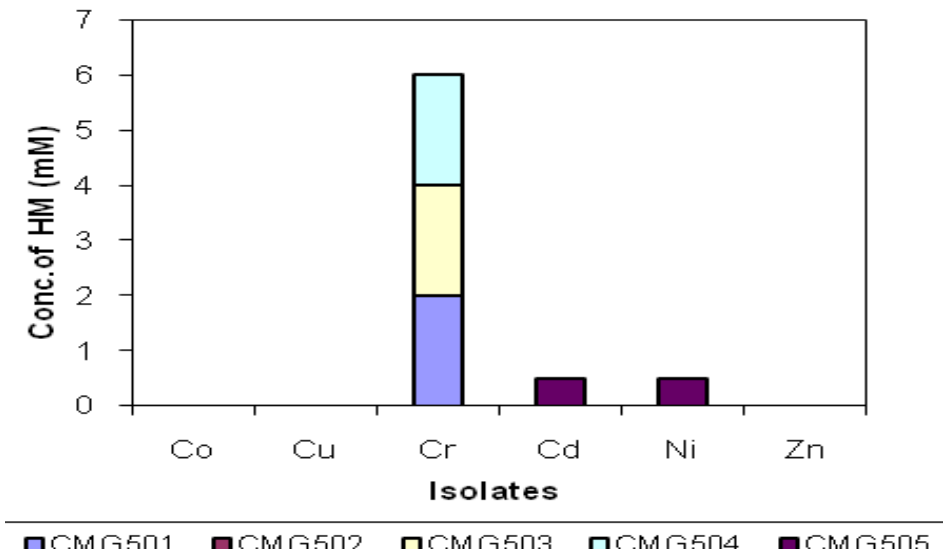

Fig. 7. MTC of heavy metals in marine isolates from Clifton beach water.

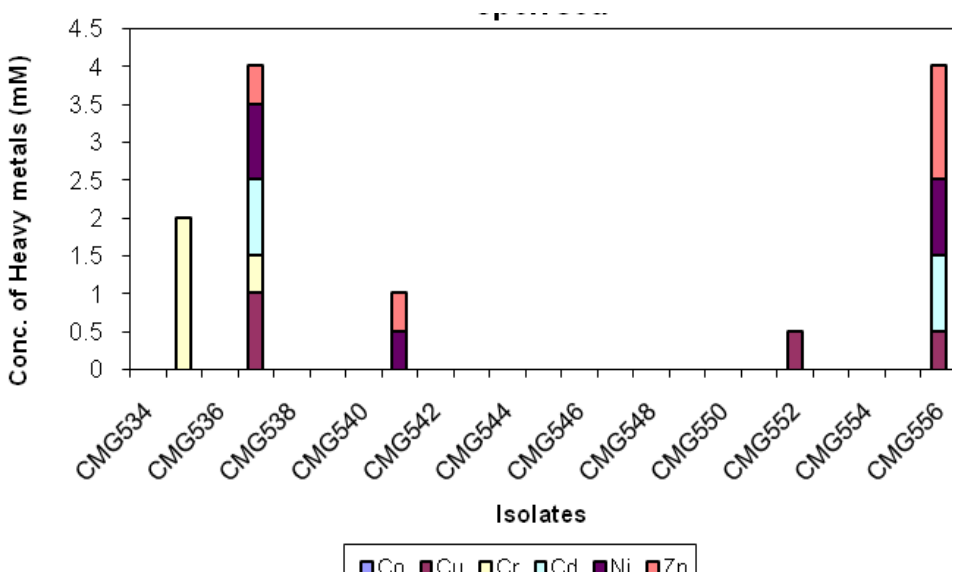

Fig. 8. MTC of heavy metals in marine isolates from open sea.

Table 2. List of isolates identified on the basis of 16S rRNA gene sequencing.

Isolation code	CMG code	Gram staining	Accession number	% Homology
G1a	CMG527	G+ve rods	EU 622833	99% *Bacillus licheniformis*
K6 (O2l)	CMG534	G+ve rods	EU622832	99% with *Bacillus cereus*
JR (O2S*n*)	CMG571	G+ve rods	EU622830	97% with *Bacillus polymyxa*
R2y	CMG505	G+ve cocci	EU622829	98% with *Arthrobacter* sp. FB-24
B1	CMG556	G-ve rods	DQ410039	98% with *Pseudomonas.* sp .and *P. stutzeri*

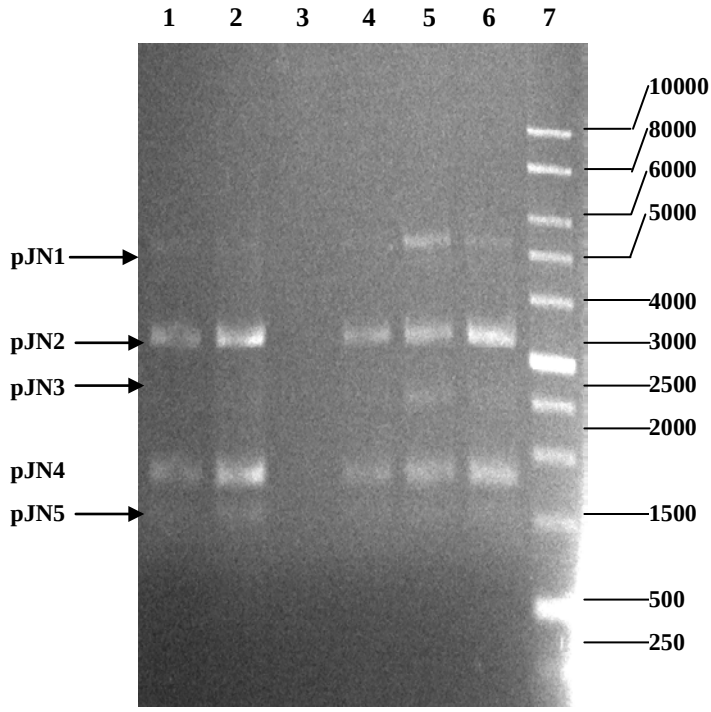

Fig. 9. Lane 1, 2, 3, 4, 5, 6 show wild type CMG535; Lane 7 shows 1Kb ladder (Gene Craft).

Coastal waters and sediments of Karachi have been found polluted with continuous input of organic and inorganic material from industrial, sewage and shipping activities since long. 70 marine bacteria were harvested by microbiological methods from surface sea water of popular recreation beaches, Karachi harbor Kaemari, Open Sea and sea weeds abundant along the Karachi coastline. The isolated bacteria were identified and characterized to reveal their resistance potential against antimicrobials and heavy metals.

Existence of high number of bacteria resistant mainly to β-lactam and amino glycoside antimicrobials indicate that the wide spread use of these antimicrobials in the environment has lead to evolve resistance in the microbial communities (Herwig *et al*., 1997; Mudry, 2002). This Abundance of multiple antibiotic resistant isolates in Clifton beach samples could be attributed to untreated release of domestic and city sewage into the beach water through a sewerage outlet has played an important role in the emergence of resistance system in the microbial communities (Cooksey, 1993).

Zobel the pioneer of marine microbiology says, most marine bacteria are Gram negative and motile Zobel (1946) however, in this study a major proportion of the isolates (62%) were found to be spore forming Gram positive, *Bacillus* species. Until recently few *Bacillus* species had been obtained from marine environment (Garabito *et al*., 1997; Ivanova *et al*., 1999; Zhuang *et al*., 2003) included *B. badius, Bsubtilis, B. cereus; B. licheniformis, B. pumilus, B. lentus* and marine derived species, *B. marinus, B. dispososauri* have been detected from marine environment. Ahmed and Yasmin (1988) had reported occurrence of 88% Gram positive bacteria in this region. The 32% of the isolates were Gram negative belonged to family pseudomonadaceae, enterobactereaceae, Moraxellaceae and Xanthomonadaceae of proteobacteria, a dominant group among marine bacteria (González and Moran, 1997).

Heavy metal resistance was found less common in Gram positive than Gram negative isolates which inhabit sea surface water in the region. Although Gram positive bacteria are known to possess strong metal binding properties at cell wall level (Beveridge *et al*., 1982). While the frequent resistance to Zinc, Nickel and chromium among the HM resistant isolates was due to abundance of Zinc, Nickel and Chromium metals in the coastal waters and alarming levels in sediments preferably in harbor and near harbor area the Clifton, near Manora channel and Paradise point (Beg *et al*., 1992; Saleem and Kazi, 1995, 1992). These heavy metals are the constituents of tannery and other industrial effluent disposed ultimately in the coastal waters of Karachi (Jamil and Ahmed, 2006).

We have isolated bacteria from sea surface water from various recreation beaches, Karachi harbor Kaemari, off the coast open sea area and sea weeds along the Karachi coast. These isolates were moderate halophiles and have shown moderate resistance towards HM and antimicrobials. Occurrence of resistant bacteria indicated that selected sites were predominantly polluted with HM and antimicrobials. Resistance profile of isolates revealed Clifton beach water was more polluted with antibiotics and heavy metals than other beaches. The study has also lead to short list few biotechnologically important isolates such as CMG505 (*Arthrobacter* sp), CMG527 (*Bacillus licheniformis*), CMG537 (*Enterobacter cloacae*), CMG541 (*Bacillus cereus*), CMG556 (*Pseudomonas* sp.), CMG571 (*Bacillus pumilus*). Successful isolation of plasmid

DNA in CMG537 (*Enterobacter cloacae*) and failure in rest of the 69 isolates indicate that the methods employed were probably not suitable for Plasmid DNA extraction from marine bacteria without suitable modifications, considering the expectations based on earlier reports (Hermansson *et al*., 1987; Silver and Misra, 1988; Sizemore and Colwell, 1997).

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