

USE OF MICROBES FROM SEABIRD FAECES TO EVALUATE HEAVY METAL CONTAMINATION IN ANTARCTIC REGION

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Abstract. This study evaluates the levels of heavy metal pollution in Antarctic marine ecosystem by monitoring gut microbial flora of seabird skua (*Catharacta* spp). Aerobic and anaerobic bacteria isolated from fresh faecal sample of skua were tested for their heavy metal tolerance and antibiotic resistance pattern. More than 50% isolates were able to tolerate 200 g/ml concentration of lead (Pb), chromium (Cr), zinc (Zn) and nickel (Ni) and >80% isolates showed resistance towards Bacitracin. More than 50% isolates were resistance to multiple antibiotics (MAR) and the traits were found to be plasmid-borne in nature. The elevated rate of tolerance to the heavy metals reflects an adaptive response to the toxic metals present in marine environment.

Keywords: *Antarctica, skua, gut microflora, heavy metals*

Introduction

The largest and diverse ecosystem on the globe, Antarctica is considered as a major biotic province. Antarctica ecosystems are comprised of marine, freshwater, ice and terrestrial habitats. Besides microbial population, a number of bird species also inhabit Antarctica, which includes polar skuas (*Catharacta* spp). Pollutant level determination in polar skuas (*Catharacta* spp) are interesting, as this species has one of the longest migration of any birds, taking it through polluted wastes of the Northern Hemisphere (Devillers, 1977) as far north as the Grand Banks of Newfoundland (Veit, 1978) and Greenland (Fullager, 1976). Seabirds, which are top predators, are predominantly studied for monitoring changes in lower trophic levels of the marine food chain (Bourne, 1976; Furness and Monaghan; 1987). Because seabirds are conspicuous creatures, they are a suitable choice to play a role as sentinel organisms. Seabirds can range over huge areas of the ocean when feeding or migrating, and many accumulate high concentrations of anthropogenic pollutants while foraging during these flights (Risebrough & Carmignani, 1972; Bull et al, 1977; Furness and Hutton, 1979, 1980; Gardner et al, 1985). Some pollutants are characteristically lipid-soluble but have low water solubility such as organo-chlorines and organo-metallic compounds. Thus, seabirds are appropriate as monitors of food chain exposure to lipid soluble pollutants (Furness, 1993).

The seabirds, skua (*Catharacta* spp.) are scavengers in nature but now they have made their foster home in Antarctica. These birds visit Antarctica during summer season and migrate towards Arctic Ocean during winters. *Catharacta* spp are top predators feeding on fishes and other seabirds. They make their nests around places of human habitation. Being migratory and top predators in marine ecosystem, skuas are ideal species through which to monitor trends in oceanic pollution (Furness, 1987). Since microbial flora of any habitat indicates the environmental quality status and the exploitation or pollution levels of that particular area this study was attempted with a

view to assess the pollution levels of Antarctica marine ecosystems by analysing the gut microbial flora of an Antarctic seabird skua (*Catharacta* spp).

Materials and methods

Sampling

Sampling was performed during the mid –summer period (during 18th Indian Expedition to Antarctica, January, 1999). Stool sample from Antarctic skua was collected from the vicinity of Indian station “Maitri” of the Schirmacher Oasis (Queen Maud Land) which is situated in the geographical coordinates of 70° 45' 12" S and 11° 46' E.

Flying individual was tracked using 10x42 binoculars and fresh fecal matter of the skua delivered on a rock was collected aseptically in a sterile container and subjected for bacteriological analysis immediately

Microbiological analysis

Faecal sample was homogenized thoroughly with sterile phosphate buffer saline (pH 7.0) for the isolation of bacteria. Sample was appropriately diluted and plated by the standard spread plate method (APHA, 1992) on plate count agar and anaerobic agar (Hi-Media Ind. Pvt. Ltd.) plates. The inoculated plates were incubated aerobically and anaerobically respectively at room temperature (24°C) for 24 hours. After appropriate incubation, clones representing different colony types were purified on Mac Conkey agar plates.

Characterization of bacterial isolates

Twenty-two colonies purified on the basis of morphological differences were subjected to different morphological and biochemical tests such as gram staining, oxidase, catalase, TSI, etc and were characterized according to Cowan and Steel (1974).

Evaluation of heavy metal tolerance

Tolerance of faecal bacteria to various heavy metals was determined by agar dilution method (Cervantes et al., 1986). In agar dilution method, plates were prepared freshly by supplementing agar medium with metal salts to give a final cationic concentration in µg/ml of 50, 100, 200, & 400 for Cd²⁺, Cu²⁺, Zn²⁺, Ni²⁺, Pb²⁺, As²⁺, Cr²⁺ and 50 & 100 for Hg²⁺. The compounds used were Cd(NO₃)₂, CuCl₃, ZnCl₂, Ni(NO₃)₂, (CH₃COO)₂Pb, As₂O₃, K₂Cr₂O₇, and HgCl₂. Plate were inoculated and incubated aerobically & anaerobically at room temperature (24 ± 2°C) for 24 hours. The minimum concentration of metals inhibiting complete growth was taken as minimal inhibitory concentration (MIC).

Determination of antibiotic resistance

Susceptibility to different antibiotics was determined by Disc Diffusion method (Bauer et al., 1966). For determining antibiotic resistance, antibiotic impregnated discs were plated on Mueller-Hinton agar (Hi-Media Ind. Pvt. Ltd) plates that had been seeded with culture. The plates were incubated aerobically and anaerobically at room temperature ($(24 \pm 2^{\circ}\text{C})$) for 3-4 days. The isolates were classified as resistant or susceptible following the standard disc sensitivity testing method (Difco, 1984). Discs containing the following antibiotics $\mu\text{g}/\text{discs}$ were tested; ampicillin (10), gentamycin (10); vancomycin (30), Tetracycline (30), Polymixin-B (50); chloramphenicol (30); Bacitracin (10); Streptomycin (25); Kanamycin (30), Neomycin (30), Carbenicillin (100); Nalidixic acid (30); cephaloridine (30), Co-trimoxazole (25) and Colistin (25).

Plasmid curing: Spontaneous loss

A total of eight isolates- six aerobic and two anaerobic, resistant to multiple antibiotics were selected for observing plasmid curing and spontaneous loss of resistance. Test organisms to be cured were inoculated in peptone water and were brought up to their logarithmic growth phase. Plasmid curing was observed by the application of curing agent, acridine orange. Tubes containing 10 ml peptone water were supplemented with $20\mu\text{g}/\text{ml}$ acridine orange and were inoculated with 0.1 ml of over night grown broth culture and incubated at 37°C for 24 hours. Loop-full of inoculum was streaked on Mac Conkey agar plates to obtain single colony isolates having stress due to curing agent. After overnight incubation at 37°C , resulting colonies were again tested for loss of heavy metals and antibiotic resistance for which the wild strain was found to be resistant. To determine spontaneous loss of plasmid, cultures were grown in peptone water tubes and transferred at regular intervals and the procedure described above was repeated.

Results

Isolation and characterization results

Isolation of bacteria yielded a total of twenty-two (22) pure colonies, among which six colonies were anaerobic in nature. The isolated organisms were found to be the members of family Enterobacteriaceae. Among the aerobic bacteria, 87.5% of the isolates were found to be *Escherichia coli* (S-1, S-2, S-3, S-4, S-5, S-7, S-8, S-9, S-10, S-11, S-12, S-13, & S-16) while 12.5% isolates were characterised as *Citrobacter* (S-15, S-17). Thus altogether *E. coli* was the predominant genera (77.46%) among the total number of isolates followed by *Citrobacter* and *Providencia*, both representing 9% of the total population while *Klebsiella* (4.54%) were the least reported species (Fig. 1). The anaerobic bacterial population represented 50% of the isolates as *E. coli* (SA-2, SA-3, & SA-6) followed by *Providencia* (33.33%, SA-4, SA-5) and *Klebsiella* (SA-1) as 16.67% of the total anaerobes (Fig. 1).

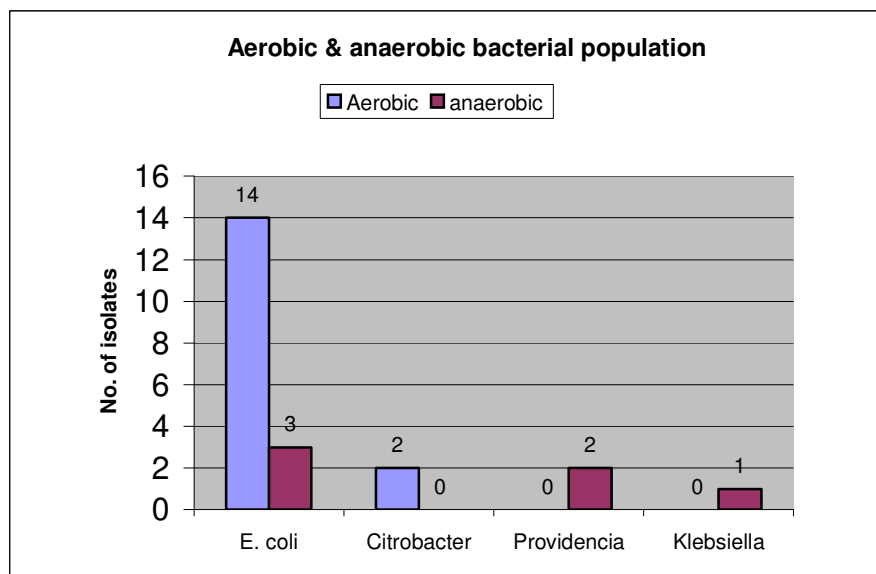


Figure 1. Types of bacterial strains from skua faeces

Heavy metal tolerance

The isolated organisms showed various levels of tolerance towards different heavy metals (Fig. 2). All the isolates, aerobic as well as anaerobic, were able to tolerate 50 µg/ml concentration of all the heavy metals tested except for mercury (Hg) for which only 68.1% of the isolates showed tolerance.

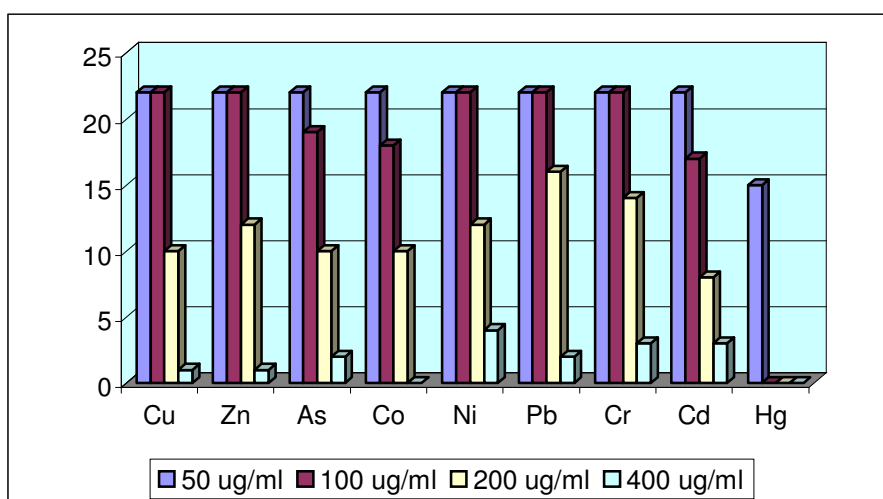


Figure 2. Heavy metal tolerance among bacterial isolates from skua faeces

Further, at 100µg/ml concentration of Hg, none isolate was found to be tolerant. However, a tolerance level at this concentration (100 µg/ml) was shown by maximum number of isolates (86.3%) for Arsenic (As), followed by Cobalt (81.8%) and Cadmium (77.2%) isolates. At 200 µg/ml concentrations of heavy metals, maximum tolerance was observed for lead (Pb) shown by 72.7% of the isolates followed by Chromium

(63.6%), Zinc and Nickel (54.5%), Copper, Arsenic & cobalt (46.4%) and the least for Cadmium (Cd) shown by 36.3% isolates. At the maximum concentration of heavy metal tested i.e.

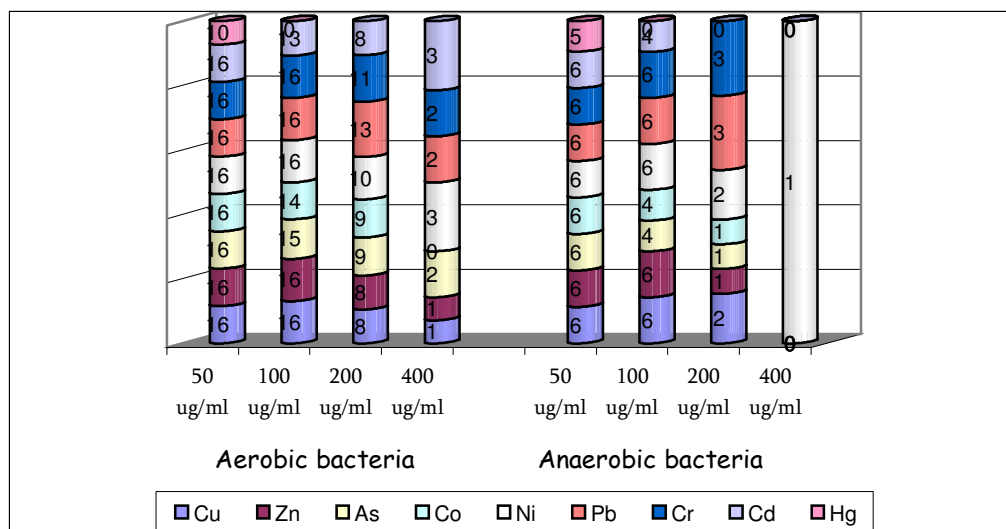


Figure 3. Comparative analysis of tolerance as shown by aerobic and anaerobic isolates for various heavy metals

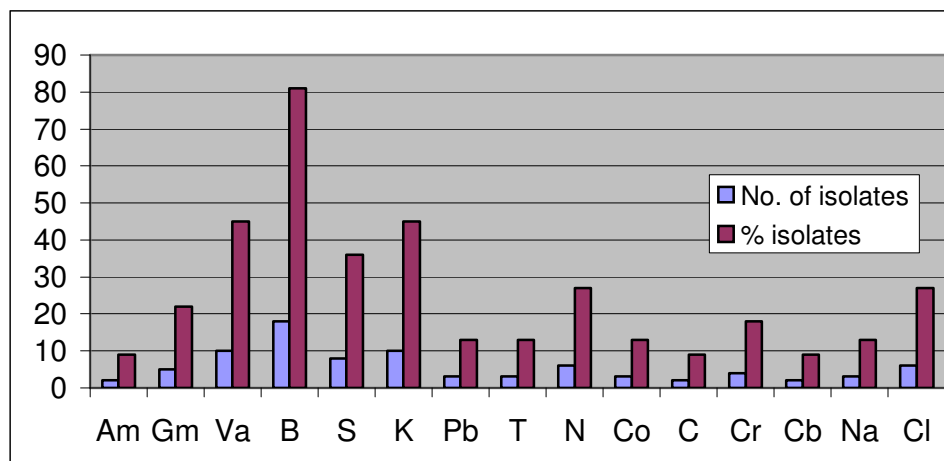
400 µg/ml, highest tolerance was showed by 18.18% of the isolates for Nickel while 13.6% isolates showed tolerance for Chromium and Cadmium respectively. Tolerance for Arsenic and Lead was observed in 9.09% isolates and least tolerance was shown by 4.54% isolates for copper and zinc respectively. None isolate was found tolerant at this level of concentrations for cobalt.

Comparing the tolerance levels of these isolates on their aerobic and anaerobic nature, it was found that aerobic bacterial isolates were more tolerant towards heavy metals as compared to their anaerobic counterparts. A comparative analysis of tolerance as shown by aerobic and anaerobic isolates for various heavy metals is depicted in Fig. 3. Tolerance at higher concentration (400 µg/ml) was observed by 16.67% anaerobic isolates only for Nickel while no isolate was tolerant to rest of the heavy metals tested at this concentration.

Among aerobic bacteria none of the isolate showed tolerance for cobalt at 400 µg/ml concentration of heavy metals while maximum tolerance was revealed for Nickel and Cadmium by 18.75% isolates.

Resistance to antibiotic

The microbial isolates from Skua faeces showed a varied pattern of resistance towards different antibiotic (Fig. 4). Predominant resistance was observed for bacitracin showed by 81.8% of the isolates. 45% isolates were found to be resistant to both vancomycin and kanamycin while for streptomycin 36.3% of the isolates showed resistance.



Am: ampicillin, Gm: gentamycin, Va: vancomycin, B: bacitracin, S: streptomycin, K: kanamycin, Pb: polymixin-B, T: tetracycline, N: neomycin, Co: Co-trimaxazole, C: Chloramphenicol, Cr: cephaloridine, Cb: Carbenicillin, Na: Nalidixic acid, Cl: Colistin

Figure 4. Antibiotic resistance among bacteria from skua faeces

Resistance for three antibiotics Gentamycin, Neomycin and Colistin was observed in 27.2% of the isolates. 18.1% isolates showed resistance for cephaloridine and 13.6% isolates were found to be resistant for polymixin-B, Tetracycline, Co-trimaxazole and Nalidixic acid. Least resistance was observed for Ampicillin, shown by 9.09% isolates.

As a comparative study for resistance among aerobic and anaerobic isolates aerobic strain were found to be more resistant to various antibiotics (Fig. 5).

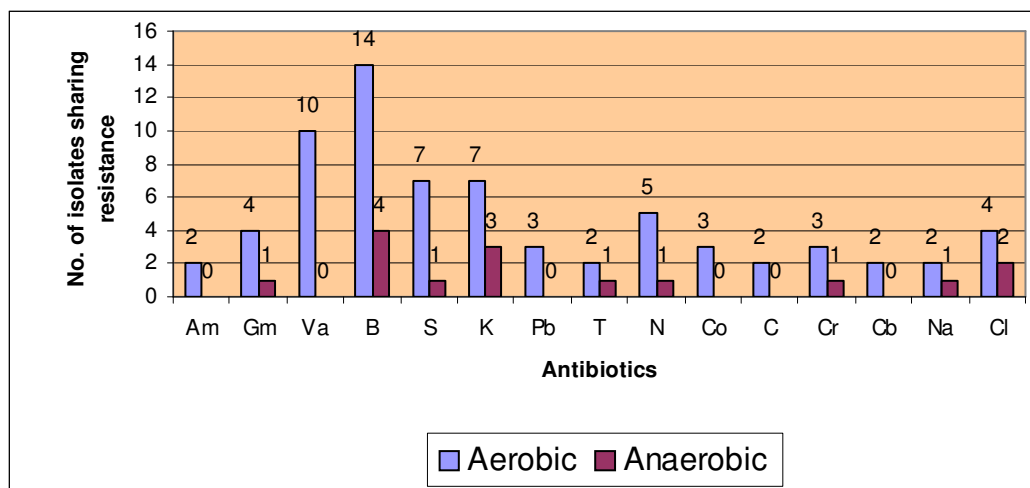


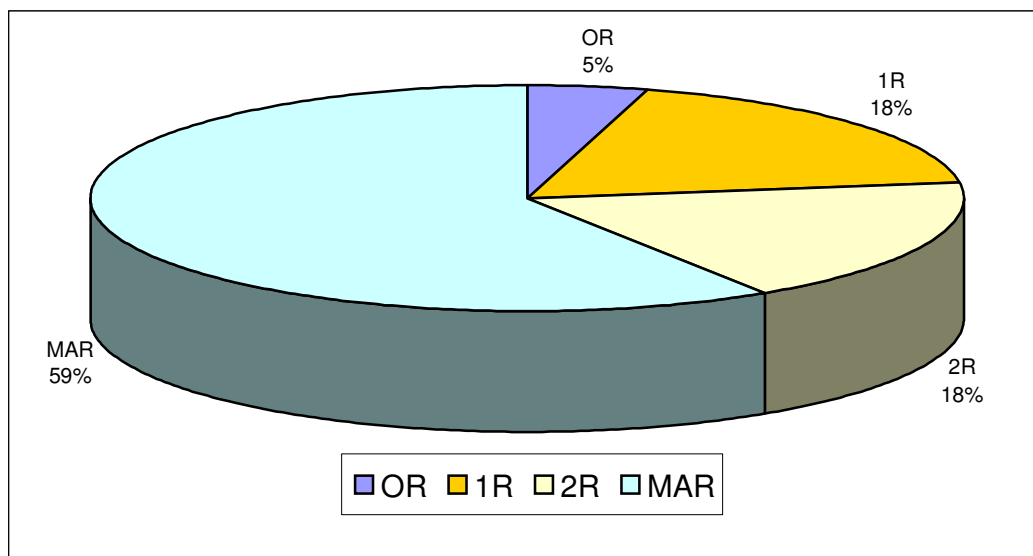
Figure 5. Comparison between aerobic versus anaerobic isolates sharing antibiotic resistance

None of the anaerobic isolate was found to be resistant against antibiotics- Ampicillin, vancomycin, Polymixin-B, co-Trimaxazole, chloramphenicol and carbenicillin. Maximum resistance among anaerobic isolates was shown by 66.67% of the isolates for Gentamycin, streptomycin, tetracycline, Neomycin, Cephaloridine and Nalidixic acid.

Among aerobic isolates, predominant resistance was observed by 90% isolates for bacitracin, followed by vancomycin (65%) and streptomycin and Kanamycin (both 45%

isolates). Least resistance was shown by 12.5% isolates for a number of antibiotics (ampicillin, Tetracycline, chloramphenicol, carbenicillin and Nalidixic acid).

A graphical analysis between the number of isolates resistant to the number of antibiotics is shown in *Fig. 6* which reveals that 59% of the isolates were resistant to multiple antibiotics (MAR) while one aerobic (S-12) isolate was fully susceptible to all the antibiotics tested; not showing resistance towards any antibiotic.



OR: susceptible to all antibiotics, 1R: resistant to one antibiotic, 2R: resistant to two antibiotics, MAR: resistant to multiple antibiotics

Figure 6. Antibiotic determinants among isolates from skua faeces

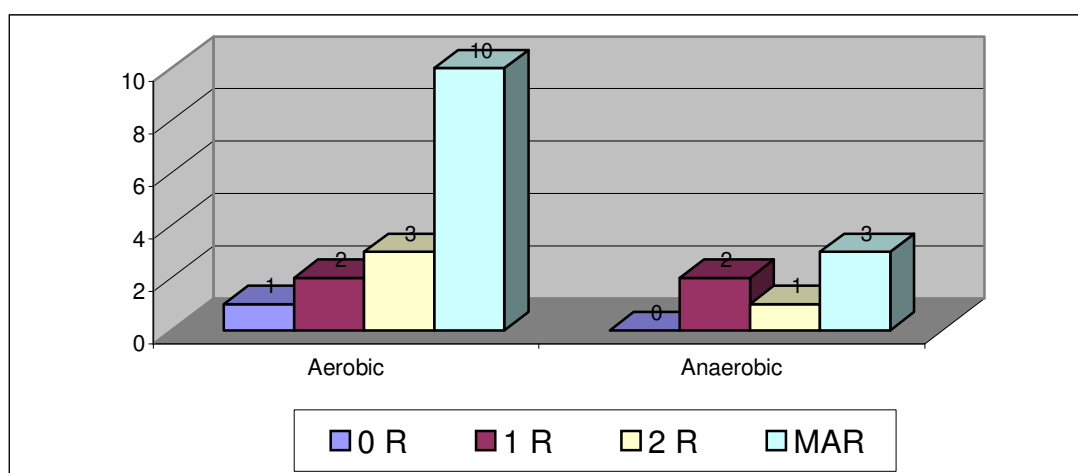


Figure 7. Comparative graphical analysis of aerobic and anaerobic isolates number resistant to the number of antibiotics.

Spontaneous loss of resistance and curing of plasmids

Results of plasmid curing among isolates is depicted in *Table 1*. Most of the colonies showed no loss of resistance due to the presence of curing agents for Cadmium and Nickel. Cent percent curing was observed in one aerobic *E. coli* (S-10) for chromium while one anaerobic isolate of *Providencia* (SA-4) showed 100% loss of tolerance towards Cobalt. Two aerobic isolates of *E. coli* (S-2 and S-10) showed 90-100% curing for copper.

Table 1. Loss of resistance in various isolates due to stress by curing agent

Heavy metals	Resistance %							
	<i>E. coli</i> (S-2)	<i>E. coli</i> (S-9)	<i>E. coli</i> (S-10)	<i>E. coli</i> (S-11)	<i>E. coli</i> (S-13)	<i>Citrobacter</i> (S-15)	<i>Klebsiella</i> (SA-1)	<i>Providencia</i> (SA-4)
Chromium	30	0	100	78.3	85	0	0	70
Cadmium	20	0	0	0	0	0	0	0
Copper	90	76.6	96.6	0	0	0	0	0
Nickel	61.6	0	43.3	0	0	0	0	0
Cobalt	78.3	71.6	81.6	76.6	73.3	70	88.3	100
Antibiotics								
Streptomycin	88.3	0	33.3	0	0	6.6	23.3	90
Kanamycin	95	88.3	85	66.6	73.3	6.	90	0
Ampicillin	13.3	0	20	0	0	0	0	85
Tetracycline	0	0	43.3	0	0	18.3	60	0
Cephaloridine	35	0	23.3	0	0	0	30	0
Bacitracin	16.6	0	0	0	0	0	0	0
Carbenicillin	36.6	0	0	76.6	0	0	0	0
Nalidixic acid	28.3	0	46.6	83.3	63.3	0	0	0

Among antibiotics 85-95% curing was observed in three aerobic *E. coli* isolates (S-2, S-9, S-10) and one anaerobic *Providencia* isolate (SA-4) for kanamycin while one aerobic *E. coli* (S-2) and one anaerobic *Providencia* (SA-4) showed 85-90% loss of resistance towards streptomycin. The anaerobic isolate, *Providencia* (SA-4) also showed 85% curing for ampicillin while none other isolate was found to be cured for ampicillin. One of the aerobic *E. coli* isolate (S-11), not showing curing for most of the antibiotics tested was found to lose 83.3% of resistance after curing towards Nalidixic acid. Spontaneous loss of resistance was also observed in the same isolates for kanamycin and chromium.

Discussion

Bacterial resistance may be due to the presence of R-plasmid containing genes for both antibiotics & heavy metals (Timoney et al, 1978; Calomiris et al, 1984). Metal tolerance may also be related to the production of capsular polysaccharides usually by Enterobacter group of organisms, which can combine with metals to protect themselves from the toxicity of metals (Bitton & Freihofer, 1978). More often the resistance phenomenon is plasmid borne and transferable in nature resulting its spread among the sensitive aquatic bacteria including coliforms.

The detection of elevated rate of tolerance to heavy metals in the present study as is depicted in *Fig 3* may also reflect an adaptive response to the presence of toxic elements

in marine environment in addition to the gut-intestine of Skua itself. However, the tolerance to heavy metal toxicity can also be influenced by several environmental factors like constituents of ecosystem, ion interactions, pH, the form and availability of metals to the microbes. Thus, bacterial tolerance to metal toxicity is a significant environmental phenomenon (Sterritt and Lester, 1980; Jones et al, 1986).

The results show 59% bacterial isolates from skua faeces are resistant to more than one antibiotic (MAR) (Fig. 7), which may be related to the process of bacterial survival and adaptation, and/or to the inconstancy of selective pressure in the environment. A number of reports confirm the presence of antibiotic resistant bacteria present in the soil samples from Antarctica (Shivaji et al, 1988, 1989a, 1989b).

Plasmid borne nature of resistance is evident by their spontaneous loss and curing by curative agents. Among antibiotics, resistance to Kanamycin was stable in most of the isolates while single isolate reported 80-90% curing for streptomycin and Nalidixic acid respectively indicating their plasmid-linked inheritance. Chromium and cobalt heavy metals also indicate their plasmid-mediated inheritance as 70-100% curing was observed by most of the isolates for these two heavy metals.

Ray et al, 1991 have isolated antibiotic resistant plasmid from Antarctic soil bacteria. Such bacteria may have evolved due to ecological interaction among the antibiotic producing strains and other strains lacking resistance. The bacteria carrying R-plasmids have a greater chance of survival and propagation in natural ecosystems than that of the strains lacking plasmids (Grabow et al. 1975,). Such plasmid carrying bacteria from Antarctica need detailed investigation. As the enzymatic machinery of these microbes is always under stress due to extreme cold condition, they seem to acquire enzymes, which are cold active, heat-labile and freeze-thaw resistant (Shivaji et al, 1994). But, as yet, the exact mechanism by which these microorganisms sense low temperature and adapt to these conditions is unknown. Whether these enzymes linked genes are plasmid-mediated or not will reflect for a new avenue for survival among psychrotrophs through psychrophilic enzymatic machinery.

The incidence of plasmids in bacteria is reported to be greater in polluted compared to clearer sites (Hada & Sizemore, 1981). Hence, in Antarctica, especially in those regions free of human intervention, the frequency of plasmids should be low. Our studies detected plasmids in eight and of twenty-two bacterial isolates indicating the lower artificial selection pressure due to human interference in Antarctica.

Earlier research on heavy metal and organochlorine pollution in Antarctic wildlife identified the brown skua (*Catharacta lonnbergi*) and the south polar skua (*Catharacta maccormicki*) as having some of the highest pollutant levels of all birds sampled (Norheim et al, 1982; Risebrongh and Carmignani, 1972; Gardner et al, 1985). Hence, it is possible that these species may be the marine equivalents of certain raptors, which show light pollutant loads through exposure to polluted environments when migrating and on wintering grounds (Cade and Fyfe, 1970; Peakall et al., 1976).

The present study sheds light on the pollution level in the Antarctic marine as well as terrestrial ecosystem and through the isolation of plasmid-borne characters provides better biotechnological prospects for survival in the fluctuating extreme external environment.

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