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Prevalence of MDR Strains in the Dumping Region Near Vellar Estuary

KEYWORDS: Antibiotics; Resistance; Bacteria;Municipal waste; Extracellular proteins

*** Pingal Kumari**
Centre of Advanced Study in Marine Biology, Faculty of Marine Sciences, Annamalai University, Parangipettai – 608502. * correspondig author

A. Divya
School of Chemical and Biotechnology, Shanmugha Arts, Science, Technology and Research Academy, SASTRA University, Thanjavur-613402

Arun Kumar
Centre of Advanced Study in Marine Biology, Faculty of Marine Sciences, Annamalai University, Parangipettai – 608502

S. Thirugnanasambandan Somasundaram
Centre of Advanced Study in Marine Biology, Faculty of Marine Sciences, Annamalai University, Parangipettai – 608502

ABSTRACT *Antibiotics and their metabolites are discharged in various amounts into the environment as a result of indiscriminate use of those organic compounds in medical, veterinary, agriculture, animal husbandry and aquaculture practices. The present study revealed that the microbial community of the municipal waste has wide incidence of antibiotic resistance strains. High incidences of resistance to bacitracin, penicillin and tetracycline whereas most sensitivity to chloramphenicol were noticed from the isolated bacterial strains in this study. Most virulence factors are either found on the bacterial cell surface or secreted into their immediate environment. Secreted/Extracellular proteins are of particular importance for vaccine development because they are often immunogenic and have the potential to be recognized early in infection. In this study, different strains showed varied concentrations of extracellular protein levels with increasing concentrations of ampicillin such as 50,100,200, and 300µg/100ml. In all the cases, the resistant strains yielded higher percentage of protein whereas the control strains (without ampicillin) found to produce lesser concentration of protein. It can be concluded that the concentration of secreted proteins is directly proportional to the increasing concentration of antibiotic. The higher protein content of PDS2 provides possible indication of increased production of protein like metabolites. There is thus a need to know the kind of proteins expressed by resistant strains so as to provide a basis for developing an interference. Further work is being done to characterize the proteins and to investigate their potential for use as vaccine target through antigenicity tests.*

INTRODUCTION :- 1

The incidence of antibiotic-resistant bacteria in environment has increased dramatically as consequence of the widespread use of antibiotics by humans. The high incidence of resistant bacteria has been documented for chronically polluted waters (Cooke, 1975). Such bacteria also occur in sewage (Cooke, 1976), rivers and marine waters (Sizemore and Colwell, 1977).

In general, resistance is acquired by mutational change or by the acquisition of resistance-encoding genetic material. The most frequent type of resistance is acquired and transmitted horizontally via the conjugation of a plasmid (Alfonso J. Alanis, 2005). It is known that bacteria can transfer resistance plasmids in situ to indigenous microflora(Mach and Grimes, 1982). Interspecies and intergeneric transfer of R plasmids has also been shown to occur (Datta and Hedges, 1972).

Antibiotics and their metabolites are discharged in various amounts into the environment as a result of indiscriminate use of those organic compounds in medical, veterinary, agriculture, animal husbandry and aquaculture practices (Alpay-Karaoglu *et al.,* 2007). Selective pressure resulting from antimicrobial administration can lead to the growth of previously susceptible strains that have acquired resistance or to the overgrowth of strains that are intrinsically resistant. This is why many studies have been recently carried out to determine the distribution of antibiotic-resistant bacteria in freshwater basins, estuaries, municipal drinking water and sewage waters (Herwig*et al*. 1997; Ash *et al*, 2002; Smith, 2006; Dotson, 2008).

Municipal wastes contain all types of terrestrial discharges. The discharges may contain medical and microbial contaminated wastes along with lots of nutrients which will adversely increase the existing microbial load. The drug resistant strains may mingle with the local microbial communities resulting in the rise of resistant varieties. So it becomes necessary to study the prevalence of antibiotic resistant strains, which may subsequently enter and disrupt the food chain. The present study was conducted in the coastline of the Parangipettai region and adjoining area of the Vellar estuary to evaluate the occurrence of such resistant strains.

2. MATERIALS AND METHODS

2.1. Sample Collection

The MDR bacteria included in this study where isolated from dumping region near Vellar estuary, Parangipettai, Tamil Nadu, India (Lat 11° 29´ N Long 79° 46´ E). The sediment samples collected in sterile polythene bag was brought immediately to the laboratory for further studies.

2.2. Isolation of Antibiotic Resistant Bacterial Strain

The collected samples were serially diluted upto10^{-5} dilutions in 50% sea water and the same was used throughout the study. An aliquot of 0.1ml of serially diluted samples was inoculated on the sterile plates containing nutrient media (Himedia) along with 50 µg/100ml different concentrations of about ampicillin. Then the plates were incubated at 30°C for 18-24 hours and colonies so obtained were taken for further analysis.

2.3. Screening for Multi Drug Resistant Strains

Morphologically different strains were selected and swabbed over the nutrient media along with ampicillin, on which the octodiscs were placed and kept for incubation at 30°C for about 24 hours.

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Susceptibility testing of the isolates was carried out by disk diffusion technique in accordance with CLSI (*Clinical Laboratory and Standards Institute)* criteria using antibiotic impregnated octodiscs (HiMediaOD038R) with concentrations quoted in parenthesis bacitracin (10 units), chloramphenicol (30mcg), co-trimoxazole (25mcg), penicillin-G(10 units), polymixin-B (300 units), gentamycin (10mcg), neomycin (30mcg), tetracyclin (30mcg). Interpretation of data was based on the standard CLSI chart as updated.

2.4. Protein Extraction

The proteins secreted by each bacterial isolates into culture medium during their growth were analyzed according to the protocol given by Ngwai *et al*. 2005. The broth culture was provided with antibiotic (ampicillin) at different concentrations such as 50µg, 100µg, 200µg and 300µg/100ml. To 50 ml of nutrient broth the potent strain was inoculated from 18hr old culture and incubated at 30°C for 24 h with shaking. Culture supernatant collected after centrifugation at 4000g for 20 min in 4°C was filtered through 0.4 µm cellulose acetate filter to avoid the bacterial cells. The protein in cell free culture supernatant was precipitated on ice for 45 min with 10% trichloroacetic acid, washed three times with ice-cold 100% acetone, and air-dried.

2.5. Protein Estimation

The proteins content was determined according to Lowry *et al*. (1951)with BSA as standard. Readings were taken at 660 nm. The amount of protein present in the sample was calculated from the standard graph. The amount of protein was expressed as mg/ml of sample.

3. RESULTS

3.1 Bacterial Isolation

About 184 resistant strains of bacteria were isolated from the dumping site samples. Based on the diverse colony morphology after culture on nutrient medium, they were grouped and isolated subsequently. Around 30 individual bacterial strains were segregated and exposed to increasing concentration of antibiotic (ampicillin) from 50µg, 100µg, and 200µg and 300µg/100 ml. Among them only four strains showed resistance to all the concentrations (50µg-300µg). The strains were preserved at 4°C in nutrient slants for further analysis. For identification purposes, the studied strains were labeled as PDS2, PDS4, PDS6 and PDS8.

3.2.Screening for Multi Drug Resistant Bacteria

Among the four strains tested, the bacterial strains, PDS6 & PDS4 showed resistance against seven antibiotics and the other two strains PDS2 & PDS8 showed resistance against Six antibiotics including ampicillin. The zone of inhibition was measured and the observations obtained were used to calculate the Antibiotic Resistance Index (ARI) for bacteria (Jones et al., 1986). The results are presented in Table 1.

3.3. Protein Estimation

The protein secreted by the four strains showing maximum drug resistance (PDS2, PDS4, PDS6 and PDS8) was estimated according to the method of Lowry *et al*., (1951).It was that found that the increase in the concentration of ampicillin led to increase in the secretion of extracellular protein after exposure (Table 2-5). Different strains showed varied concentrations of extracellular protein levels with increasing concentrations of ampicillin such as 50µg, 100µg, 200µg, and 300µg/100ml. In all the cases, the resistant strains yielded higher percentage of protein whereas the control strains (without ampicillin) found to produce lesser concentration of protein. So it can be concluded that the concentration of secreted proteins is directly proportional to the increasing concentration of antibiotics (ampicillin).

4.DISCUSSION

Media supplemented with the antibiotic (ampicillin) were used to evaluate the antibiotic resistant bacterial load. The observation correlates with the significant variations noted among the percentages of bacterial resistance to different antibiotics (Sudeshna Ghosh and Timothy, 2007).High incidences of resistance to ampicillin, as well as, most sensitivity to chloramphenicol were noticed from the isolated bacterial strains. Environmental bacteria may play an important role as reservoirs of antibiotic resistance; resistance genes are exchanged by bacteria from different freshwater and marine ecosystems (Dang *et al*., 2008).There was a positive, (though not statistically significant) correlation between antibiotic prescription quantity and residue levels in hospital effluent as the report suggested by Diwan *et al*. 2009.

Different strains showed varied concentrations of extracellular protein levels and in all the cases, the resistant strains yielded higher percentage of protein whereas the control strains (without ampicillin) found to produce lesser concentration of protein. It can be concluded that the concentration of secreted proteins is directly proportional to the increasing concentration of antibiotic. The higher protein content of PDS2 provides possible indication of increased production of protein like metabolites.As work done by, Marina NoemiTorrero (2003) secreted proteins represent a distinct group of proteins with respect to their structure and function and contribution to virulence. They are of particular importance for vaccine development because they are often immunogenic and have the potential to be recognized early in infection.

The cell surface is important to virulence, as most virulence factors are either found on the surface or secreted into their immediate environment (Finlay and Falkow, 1997). Detailed analysis of inner membrane and outer membrane preparations from strains may allow identification of further components of the efflux system which may serve as the resistant factor of the bacterial isolate. Further work is being done to characterize the proteins and to investigate their potential for use as vaccine target through antigenicity tests.

Table 1. Kirby-Bauer screening for MDR strains

ANTIBIOTICS	STRAINS ISOLATED			
	PDS2	PDS4	PDS6	PDS8
Bacitracin	R	R	R	R
Chloramphenicol	S	S	R	S
Co-Trimoxazole	S	R	R	R
Penicillin-G	R	R	R	R
Polymyxin-B	R	R	S	S
Gentamicin	I	R	I	R
Neomycin	R	I	R	S
Tetracyclin	R	R	R	R

Table 1 exhibits the resistance patterns of the four selected strains. 'S' indicates the strains' sensitivity towards specific antibiotic whereas 'R' represents their resistance towards specific antibiotic and I denotes the intermediate strains.

Table 2. Protein Value estimated for strain PDS2

Ampicillin concentration(mg/ml)	0	0.05	0.1	0.2	0.3
Protein concentration (mg/ml)	2.40	2.80	3.30	5.20	5.80

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Table 3.Protein Value estimated for strain PDS4

Ampicillin concentration(mg/ml)	0	0.05	0.1	0.2	0.3
Protein concentration (mg/ml)	2.65	2.99	3.19	3.38	5.07

Table 4. Protein Value estimated for strain PDS6

Ampicillin concentration mg/ml	0	0.05	0.1	0.2	0.3
Protein concentration mg/ml	1.20	2.10	2.20	2.30	4.00

Table 5. Protein Value estimated for strain PDS8

Ampicillin concentration(mg/ml)	0	0.05	0.1	0.2	0.3
Protein concentration (mg/ml)	1.34	1.78	2.08	2.18	3.06

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