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Monitoring of multiple drug-resistant pathogens in a selected stretch of Bay of Bengal, India

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Abstract The present work aims at identification of multiple drug-resistant pathogenic bacteria in a selected stretch, namely, Puri on the Bay of Bengal, India. Six stations at the coast of Puri were selected and samples of water and sediment were collected during the winter of 2008 and 2009 for this study. Thirty-eight pathogenic bacteria were isolated and identified from both the water and the sediment of 6 fixed stations (PU-1a, PU-1b, PU-2, PU-3, PU-4, and PU-5). The identified pathogens were *Escherichia coli*, *Vibrio cholerae*, *Vibrio parahaemolyticus*, *Klebsiella pneumoniae*, *Staphylococcus aureus*, *Enterococcus faecalis*, *Pseudomonas aeruginosa*, and *Proteus mirabilis*. Antibiotic sensitivity of the isolated bacteria was studied by using 12 selected antibiotics, commonly used for the medication of human beings and animals. The isolated pathogens from both the water and the sediment samples showed lowest resistance to chloramphenicol (C-30 μg) where as the pathogens showed highest level of resistance to ampicillin (10-μg) among the antibiotics used for the study. Among the isolated pathogens *E. faecalis* (PU-1a), *P. aeruginosa* (PU-2 and PU-3), *E. coli* (PU-3 and PU-4), and *K. pneumonia* (PU-4) showed resistance to more than four antibiotics. Out of the isolated species, 57.8% pathogens were multi-drug resistant. Antibiotic resistance indexes of all the stations were calculated and found to be in the range of 0.066 to 0.083.

Keywords Drug resistance · Antibiotics · Pathogen · Sensitivity

Introduction

Puri is a major attraction for tourists because of its religious importance and the sea beaches, and as a result, a huge amount of wastes of different forms are generated. Disposal of these wastes into the sea causes serious environmental hazards. Owing to this need, isolation, identification, enumeration, and drug resistance activity of pathogenic bacteria from selected stations at the coast (water and sediment) was necessary.

The amicable climate (17.1°C–31.9°C) attracts domestic and foreign visitors especially during the winter season; 4.1 million domestic and 28,000 foreign tourists visited Puri during 2004 (Puri City Development Plan 2006). According to the population study report of the Government of India, the urban population of Puri was 0.20 million; out of which urban slum dwellers contributed 0.15 million during 2001. Due to the

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overcrowded population and untreated urban sewage discharge, the microbial pollution in the coastal water and sediment is a matter of serious concern. Microbial studies have been conducted on Puri coastal water and higher coliforms, *Vibrios*, *Shigella*, *Pseudomonas aeruginosa*, *Proteus*, and *Klebsiella* were reported at the coast as compared to other transects of the coast of Orissa (Patra et al. 2009).

After the origin of antibiotics, various drugs have been developed to act against different disease-causing bacteria. Because of the regular application of antibiotics, these microorganisms developed resistance to them. Multiple drug-resistant pathogens are present in waste sites, dumping ground, and from those places, they reach different water bodies causing serious microbial pollution (Achudume and Olawale 2009; Toroglu and Toroglu 2008). In the coastal water, multi-antibiotic resistant bacteria prevail due to anthropogenic activities (Rodriguez and Zaidi 2007; Manjusha et al. 2005; Zbigniew 2005). Research work in this direction not only gives us information regarding antibiotic resistant microbes present in the environment of the sea but also it will present a significant indication about the abuse of medicine in different segments of the environment which is making more and more bacterial species resistant to these drugs and the subsequent failure of these medicines to act against pathogenic bacteria. Since the sea is the ultimate sink for many pollutants generated on terrestrial environment, its microbial characteristic may be considered as an indicator of microbial pollution on land. Therefore, the present case may be considered as an indicator of excess of medicinal use. Hence, the primary objective of this work is to analyze the level of resistance of different pathogens isolated from the coastal water and sediment of Puri to different antibiotics.

Materials and methods

Study area Figure 1 shows six selected stations in which the study was carried out. Two shore stations were selected and named as PU-1a and PU-1b. PU-1a has latitude 19° 47′ 55′ N and longitude 85° 50′ 24′ E, PU-1b has latitude 19° 47′ 36′ N and longitude 85° 49′ 23′ E. Four coastal stations were selected and named as PU-2, PU-3, PU-4 and PU-5. PU-2 has latitude 19° 47′ 00′ N and longitude 85° 50′ 03′ E, PU-3 has latitude 19° 46′ 11′ N and longitude 85° 50′ 23′ E, PU-4 has latitude 19° 45′ 00′ N and longitude 85° 50′ 48′ E and PU-5 has latitude 19° 42′ 42′ N and longitude 85° 51′ 42′ E. The sampling stations PU-1a and PU-1b are located at the shoreline. PU-2, PU-3, PU-4, and PU-5 are located in the sea with a distance of 1, 3, 5, and 10 km from the shoreline, respectively. Samples were collected during the winter of 2008 and 2009.

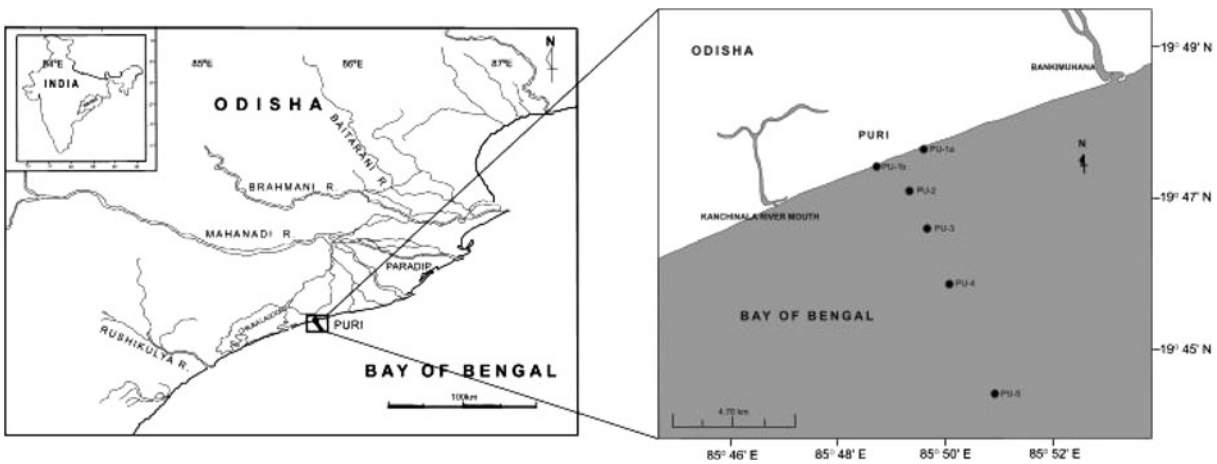

Fig. 1 Map of the location of study area and sampling sites

Surface water samples were collected aseptically using Niskin water sampler and were transferred to sterilized plastic bottles. Sediment samples were collected using Peterson grab sampler and were stored aseptically in sterilized plastic containers. The samples were aseptically carried to the laboratory at 4°C using ice gel packs. The water samples and sediment samples were serially diluted using sterile distilled water and plated on selective media. Enumeration and presumptive identification of desired pathogens were carried out by using Hichrome ECC agar for *Escherichia coli* and *P. aeruginosa*, Hichrome UTI agar for presumptive identification of *Enterococcus faecalis*,

Table 1 Antibiotic resistance index, population and sensitivity of the isolated pathogens from different stations to the studied antibiotics

						R			
Station	Bacterial strains	Source	Mean Population	*S*	*I*	≤2 Cluster 1	>2 ≤ 4 Cluster 2	>4 Cluster 3	ARI
PU1a	*P. aeruginosa*	Water	0.175×10^3 cfu/ml	6	4	2			0.0833
	V. parahaemolyticus	Water	0.630×10^3 cfu/ml	7	2		3		
	S. aureus	Water	0.350×10^3 cfu/ml	4	4		4		
	P. mirabilis	Water	0.565×10^3 cfu/ml	7	2		3		
	P. aeruginosa	Sediment	136.154×10^3 cfu/g	7	1		4		
	E. faecalis	Sediment	27.716×10^3 cfu/g	2	1			9	
PU1b	*V. cholerae*	Water	0.175×10^3 cfu/ml	6	4	2			0.0833
	S. aureus	Water	0.270×10^3 cfu/ml	10	2				
	P. aeruginosa	Water	0.165×10^3 cfu/ml	4	6	2			
	P. mirabilis	Sediment	15.441×10^3 cfu/g	8	1		3		
	P. aeruginosa	Sediment	63.235×10^3cfu/g	6	3		3		
PU-2	*P. aeruginosa*	Water	0.005×10^3 cfu/ml	5	2			5	0.074
	V. cholerae	Water	0.175×10^3 cfu/ml	7	3	2			
	P. mirabilis	Water	0.210×10^3 cfu/ml	5	4		3		
	E. faecalis	Water	0.100×10^3 cfu/ml	9	2	1			
	E. coli	Sediment	8.594×10^3cfu/g	3	5		4		
	K. pneumonae	Sediment	18.627×10^3cfu/g	6	3		3		
	P. aeruginosa	Sediment	4.688×10^3cfu/g	9	2	1			
	E. faecalis	Sediment	62.588×10^3cfu/g	8	3	1			
	S. aureus	Sediment	1.877×10^3cfu/g	9	3				
PU-3	*K. pneumoniae*	Water	0.230×10^3 cfu/ml	4	4		4		0.072
	V. parahaemolyticus	Water	0.215×10^3 cfu/ml	12					
	E. faecalis	Water	0.335×10^3 cfu/ml	8	1		3		
	P. mirabilis	Water	0.240×10^3 cfu/ml	10	1	1			
	S. aureus	Water	0.110×10^3 cfu/ml	4	4		4		
	P. aeruginosa	Water	0.060×10^3 cfu/ml	3	2			7	
	E. coli	Sediment	8.824×10^3 cfu/g	0	4			8	
	K. pneumonae	Sediment	5.882×10^3cfu/g	6	3		3		
PU-4	*S. aureus*	Water	0.080×10^3 cfu/ml	9	2	1			0.066
	K. pneumonae	Sediment	1.667×10^3cfu/g	3	4			5	
	E. coli	Sediment	3.333×10^3cfu/g	1	6			5	
	P. aeruginosa	Sediment	43.333×10^3cfu/g	7	3	2			
	S. aureus	Sediment	1.655×10^3cfu/g	10	2				
PU5	*P. aeruginosa*	Water	0.045×10^3 cfu/ml	6	3		3		0.066
	S. aureus	Water	0.060×10^3 cfu/ml	4	6	2			
	E. coli	Sediment	10.933×10^3 cfu/g	6	3		3		
	S. aureus	Sediment	6.857×10^3cfu/g	2	7		3		
	P. aeruginosa	Sediment	10.843×10^3cfu/g	8	4				

S susceptible, *I* intermediate, *R* resistance, *ARI* antibiotic resistance index

Klebsiella pneumoniae, *Proteus mirabilis* and *Staphylococcus aureus* and Hichrome thiosulfate citrate bile salt sucrose (TCBS) agar for presumptive identification of *Vibrio cholerae* and *V. parahaemolyticus*. Populations of respective organisms were quantified in each station for both water and sediment as presented in Table 1. Selected colonies were sub cultured and identification was confirmed by using biochemical tests for each organism according to Holt et al. (1994). Other biochemical identification processes for specific bacterium like *E. coli* (Trepeta and Edberg 1984), *V. cholerae* (Stravic and Buchanan 1995), *V. parahaemolyticus* (Shinoda et al. 1983), *K. pneumoniae* (Eguchi et al. 1987), *S. aureus* (Padhila et al. 2000), *E. faecalis* (Takahashi et al. 1999), *P. aeruginosa* (Garby and Hadley 1957) and *P. mirabilis* (Matsen et al. 1971) were also conducted.

Antibiotic sensitivity test Anti-microbial sensitivity test of each bacterium isolates was tested using the disc diffusion method (Bauer et al. 1966). The results were interpreted basing on the recommendations of National Committee for Clinical Laboratory Standards for antimicrobial susceptibility tests (Finegold and Martin 1982). The pure cultures of bacteria were transferred to tryptone soya broth and were incubated at 35°C for 2–8 h until it achieved light to moderate turbidity. The inoculum turbidity was then compared with that of a mixture obtained by mixing 0.5 ml of 1.175% barium chloride and 99.5 ml of 0.36 N sulfuric acid. The increase in turbidity was compared to the standard and was adjusted with sterile seawater and the decrease in turbidity was adjusted with longer incubation time. The standardized bacterial suspensions were then inoculated on to Muller Hinton Agar plates (Himedia laboratories, Mumbai) using sterile cotton swabs, which were then left to dry up for 10 min before placing the antimicrobial sensitivity discs. Antibiotic impregnated discs of 6-mm diameter were used for the test. Discs containing the following antibacterial agents: ampicillin (A-10 μg), azithromycin (At-15 μg), chloramphenicol (C-30 μg), ciprofloxacin (Cf-5 μg), erythromycin (E-15 μg), gentamycin (G-10 μg), nalidixic acid (Na-30 μg), neomycin (N-30 μg), norfloxacin (Nx-10 μg), polymixin-B (300 units), streptomycin (S-10 μg) and tetracycline (T-30 μg) were mounted on the plate and incubated overnight at 37°C and examined after 24 h as presented in Table 2. The diameter of the zone of inhibition was measured and compared with that of the zone diameter interpretative chart to determine the sensitivity of the isolates to the antibiotics. The procedure was intended for in vitro susceptibility testing of certain rapidly growing and fastidious bacterial pathogens.

Antibiotic resistance index Antibacterial resistance index (ARI) of each sampling site was determined using the formula

$$\mathrm{ARI} = a/ny,$$

where 'a' is the actual number of resistant determinants recorded in a population of size 'n' and 'y' is the total number of antibiotics tested in the sensitivity test. Basing on the occurrence of resistance to three or more than three antibiotics the isolates were considered as multiple antibiotic resistant (MAR) isolates (Manjusha et al. 2005).

Table 2 Antibiotic sensitivity of the pathogens isolated from water and sediment to the studied antibiotics

		Antibiotics studied											
		NA 30	C 30	CF 5	G 10	T 30	PB 300	A 10	N 30	S 10	NX 10	E 15	AT 15
Sediment	*S*	22.22	83.33	50.00	55.55	50.00	66.66	0.00	38.88	50.00	55.55	50.00	38.88
	I	50.00	11.11	27.77	22.22	22.22	5.50	33.33	22.22	16.66	38.88	38.88	33.33
	R	27.77	5.50	22.22	22.22	27.77	27.77	66.66	38.88	33.33	5.50	11.11	27.77
Water	*S*	45.00	85.00	70.00	65.00	50.00	65.00	5.00	55.00	65.00	80.00	35.00	35.00
	I	10.00	15.00	25.00	25.00	25.00	15.00	25.00	30.00	15.00	10.00	40.00	45.00
	R	45.00	0.00	5.00	10.00	25.00	20.00	70.00	15.00	20.00	10.00	25.00	20.00

S susceptible, *I* intermediate, *R* resistance. Abbreviations of the antibiotics are discussed in the Materials and Methods. Values are given in percentage

Table 3 Screening of the isolated pathogens

Isolated organisms								
Presumptive tests	*E. coli*	*V. cholerae*	*V. parahaemolyticus*	*K. pneumoniae*	*S. aureus*	*E. faecalis*	*P. aeruginosa*	*P. mirabilis*
Media and colony color	ECC (Purple)	TCBS (Yellow)	TCBS (Green)	UTI (Blue to purple)	UTI (Golden yellow)	UTI (Blue)	ECC (Straw)	UTI (Light brown)
Grams stain	-ve	-ve	-ve	-ve	+ve	+ve	-ve	-ve
Microscopic view	Rod shaped	Comma shaped	Curved rod shaped	Rod shaped	Cocci	Cocci	Rod shaped	Rod shaped

-ve negative, *+ve* positive

Results and Discussion

Thirty-eight strains were identified basing on different biochemical tests, i.e., 20 isolates from water and 18 isolates from the sediment of six stations. The presumptive and confirmative identifications of the isolated species are presented in Tables 3 and 4, respectively. The identified pathogens were *E. coli*, *V. cholerae*, *V. parahaemolyticus*, *K. pneumoniae*, *S. aureus E. faecalis*, *P. aeruginosa*, *P. mirabilis.* The microbial pollution at the coast of Puri is mainly due to untreated municipal sewage which includes medical wastes and fishermen slum having very poor sanitation as well as their fishing activity system adjacent to the station PU-1a. Hence, population enumerations were done at all the stations of the isolated strains and are given in Table 1. These microorganisms are well-known pathogens and are responsible for causing serious diseases in living beings like nausea, vomiting, diarrhea, and fever, etc., leading to death.

The studied antibiotics are commonly used as drugs for human beings, fishes, and in poultry. Resistance of pathogens to antibiotics studied is grouped into three clusters. Cluster 1 contains bacteria resistant to ≤ 2 antibiotics, cluster 2 contains bacteria resistant to $>2 \leq 4$ antibiotics. Cluster 3 contains microorganisms resistant to >4 antibiotics. The bacteria resistant to 3 or more than 3 antibiotics are known as MAR. The study shows that among the isolated pathogens 22 (57.8%) bacteria are MAR. Resistance percentage of all the isolated pathogens from water and sediment is represented in the Figs. 2 and 3, respectively. The pathogens isolated from water showed the lowest level of resistance to chloramphenicol (C-30 μg) followed by ciprofloxacin (Cf-5 μg) among the studied antibiotics. Similarly, pathogens isolated from sediment showed the lowest level of resistance to both chloramphenicol (C-30 μg) and norfloxacin (Nx-10 μg). Bacteria isolated from both the sources showed the highest resistance to ampicillin (A-10 μg) followed by nalidixic acid (Na-30 μg) in water and neomycin (N-30 μg) in sediment.

According to the antibiotic resistance index, PU-1a and PU-1b stations reflect the highest exposure to antibiotics having ARI of 0.083. PU-2,

Table 4 Biochemical tests for the identification of the pathogenic bacteria isolated from water and sediment of Puri coastal region

Biochemical tests	Isolated Pathogenic Strains							
	E. coli	*V. cholerae*	*V. parahaemolyticus*	*K. pneumoniae*	*S. aureus*	*E. faecalis*	*P. aeruginosa*	*P. mirabilis*
TSI	Glucose, sucrose, lactose (+ve), gas (+ve), acid (+ve)	Glucose, sucrose (+ve), gas, acid (-ve)	Glucose (+ve), sucrose (-ve), gas, acid (-ve)	Glucose, sucrose, lactose (+ve), gas (+ve), acid (+ve)	Glucose, sucrose, lactose (+ve), gas (-ve)	Glucose, sucrose, lactose (+ve), gas (-ve)	Glucose, sucrose, lactose (-ve)	Glucose (+ve), gas (+ve), acid (+ve)
H_2S	-ve	-ve	-ve	-ve	nd	-ve	nd	+ve
Motility	+ve	+ve	+ve	-ve	-ve	-ve	+ve	+ve
Citrate	-ve	+ve	+ve	+ve	nd	nd	+ve	+ve
Indole	+ve	+ve	+ve	-ve	nd	-ve	nd	-ve
Urease	-ve	nd	nd	+ve	+ve	nd	nd	+ve
Oxidase	-ve	+ve	+ve	-ve	nd	nd	+ve	-ve
Catalase	+ve	nd	nd	+ve	+ve	-ve	nd	nd
MR	+ve	V	nd	+ve	nd	nd	nd	+ve
VP	-ve	+ve	-ve	-ve	nd	+ve	nd	-ve
Mannitol	+ve	+ve	+ve	+ve	+ve	+ve	+ve	nd
Gelatin liquefaction	-ve	nd	nd	-ve	nd	nd	+ve	+ve
Nitrate reduction	+ve	+ve	+ve	nd	+ve	+ve	+ve	+ve
Coagulase	nd	nd	nd	nd	+ve	nd	nd	nd
Ornithine decarboxylase	nd	nd	nd	nd	nd	nd	nd	+ve

-ve negative reaction, *+ve* positive reaction, *V* variable, *nd* not done

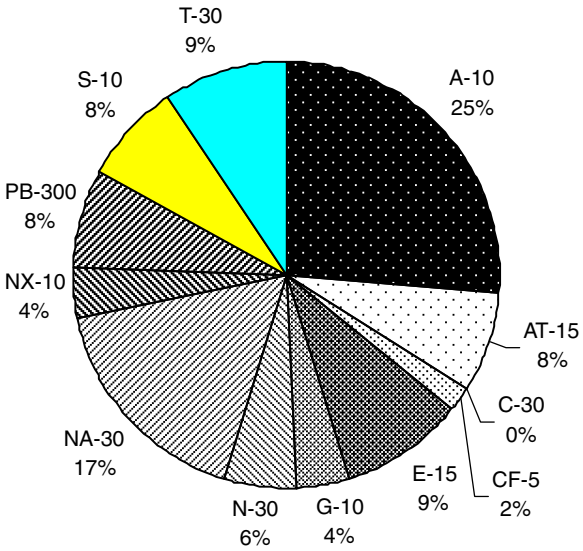

Fig. 2 Resistance of the pathogens isolated from water to the studied antibiotics (in percentage)

PU-3, PU-4, and PU-5 stations have ARI of 0.074, 0.072, 0.066, and 0.066, respectively. Microorganisms of the same species show different pattern of resistance to different antibiotics. This indicates that the pathogens isolated from the aforesaid stations are from different sources of contamination.

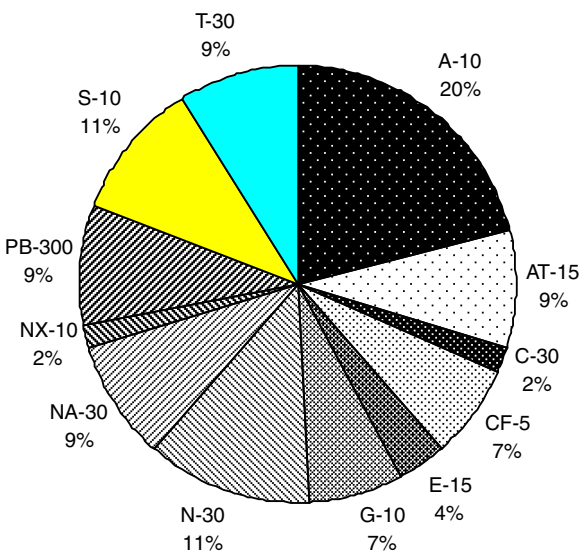

Fig. 3 Resistance of the pathogens isolated from sediment to the studied antibiotics (in percentage)

Conclusion

This study shows the occurrence of multi antibiotic resistant pathogens prevalent at the selected stretch at the coast of Puri. Thirty-eight pathogenic bacteria were isolated and identified from both water and sediment of six fixed stations. Antibiotic sensitivity of isolated bacteria was studied by using 12 selected antibiotics, commonly used for human beings and animal. Among the selected antibiotics chloramphenicol (C-30 μg) showed the lowest level of resistance by the isolated pathogens while ampicillin (10-μg) showed the highest level of resistance by the pathogens isolated from both sediment and water. Among the isolated pathogens *E. faecalis* (PU-1a), *P. aeruginosa* (PU-2and PU-3), *E. coli* (PU-3and PU-4) and *K. pneumonia* (PU-4) showed resistance to more than four antibiotics. Out of the isolated species, 57.8% pathogens were multi-drug resistant. ARI of all the stations were calculated and found to be in the range of 0.066 to 0.083.

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