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Research Article

DETECTION OF MERCURY AND CADMIUM RESISTANCE AMONG MULTIPLE ANTIBIOTIC RESISTANT ENTERIC BACTERIA FROM MUNICIPAL SEWAGE WATER IN MALDA, INDIA

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ABSTRACT

The plasmid mediated co-resistance to antibiotics and heavy metals among environmental as well as clinical bacteria have been reported from various parts of the globe. In the current study, the sewage water samples (n=9), collected within from Malda town (West Bengal state, India), were processed microbiologically for the isolation of bacteria (n=12), which were identified, following conventional methods, as *Pseudomonas aeruginosa* (n=3), *Proteus vulgaris* (n=2), *Escherichia coli* (n=5) and *Klebsiella pneumoniae* (n=2). The isolated bacteria had resistance to ≥4 antibiotics among ampicillin (Am), cholramphenicol (Cm), trimethoprim (Tm), tetracycline (Tc), ciprofloxacin (Cp) and gentamicin (Gm), and to heavy metals (Cd^{2+} and Hg^{2+}) tested, and contained a single plasmid conferring such resistances. The highest resistance was shown among *E. coli* isolates, having the resistance pattern of 'Am-Cm-Tm-Tc-Cp-Gm'. The multiple antibiotic resistance indices for the isolated sewage-water bacteria ranged 0.4 – 0.6. Thus, the current study further authenticated the association between antibiotic resistance and heavy metal tolerance among sewage-water bacteria (from our part of the world), the sources of origin for which were the niches with high antibiotic pollution and/or human-fecal contamination.

Keywords: Sewage water bacteria, plasmid, multiple antibiotic resistance, heavy metal tolerance, MAR index

INTRODUCTION

The synthetic chemicals such as antibiotics and heavy metal salts have been in use in industries, agricultures, and in clinical practices, leading to an extensive environmental contamination and the emergence of bacterial resistance to antibiotics and heavy metals (in such niches); the effect of such phenomena on human health is of global concern[1]. The bacterial populations, pathogenic as well as non-pathogenic, are exposed continuously to various chemicals including antibiotics and heavy metal salts in sewage systems receiving hospital, agriculture and domestic effluents, and acquire resistance to antibiotics as well as heavy metals, either through chromosomal gene mutation (intrinsic way) or through the acquisition of R- plasmid from resistant bacterial strains (extrinsic way)[2,3]. It has been documented that a low level of metal in aquatic body might be associated with co-selection of resistance to antibiotic(s) in bacterial isolates, and the increase of MAR (multiple antibiotic resistance) indices, on exposure of heavy metal (lead: Pb), among the bacteria tested[4]. Report has been made to show the development of resistance to multiple antibiotics, including third- and fourth-generation cephalosporins, gentamicin (Gm), ciprofloxacin (Cp), co-trimoxazole and tetracycline (Tc) among bacteria belonging to the family Enterobacteriaceae, on lead exposure, while in *Pseudomonas* spp., colistin, levofloxacin and cefepime resistances were developed[5]. The event of co-occurrence of plasmid encoding antibiotic resistance and heavy metal tolerance of bacterial isolates (*E. coli* and *Ps. aeruginosa*) from the Mahananda river water, near Malda town, has been documented scientifically[6]. Conversely, no report has been made from the current study area on the occurrence of association between plasmid conferring antibiotic resistance and heavy metal tolerance of sewage-water bacteria. Thus, the present study aims to determine the plasmid mediated antibiotic resistance and heavy metal tolerance among potential (human) pathogenic bacteria isolated from municipal sewage-water bacteria within from Malda (West Bengal state, India).

MATERIALS AND METHODS

Bacteriological analysis of sewage water

A total of 9 sewage-water samples (n=9) were collected aseptically in sterilized collecting bottles (Hi-Media, India), from within and around Malda town, West Bengal state, India, and transported immediately to the laboratory for microbiological study.

The sewage water samples were inoculated into nutrient broth and incubated for 24 h at 35 ^{0}C, for bacterial growth enrichment. The pure bacteria cultures were obtained, from the collected sewage-water samples, by streak-plate dilution technique, as mentioned earlier[7], using nutrient agar (Hi-Media, India), and were subjected to bacterial colony morphology study on MacConkey agar, cetrimide agar, blood agar, and TCBS agar (all procured from Hi-Media, India). The bacteria isolated were identified following the standard protocol of Halt[8] and Forbes et al.[9], as described earlier[7].

Bacterial antibiotic susceptibility

The isolated sewage-water bacteria were tested for antibiotic susceptibility, following disc diffusion method[10]; incubation was done at 37 ^{0}C, for 24 h. The antibiotic discs used in the study were: amikacin (Ak: 30 µg/disc), ampicillin (Am: 10 µg/disc), chloramphenicol (Cm: 30 µg/disc), ciprofloxacin (Cp: 5 µg/disc), gentamicin (Gm: 10 µg/disc), imipenem (Im: 10 µg/disc), meropenem (Mp: 10 µg/disc), streptomycin (Sm: 10 µg/disc), tetracycline (Tc: 30 µg/disc), and trimethoprim (Tm: 5 µg/disc). The nearest whole zone diameter of inhibition (ZDI) was measured, and the isolates were categorized as resistant (R), intermediately susceptible (IS), or sensitive (S), following the criteria of the Clinical Laboratory Standards Institute[11].

The MAR indices of the isolated bacteria were calculated, using the formula, stated earlier and interpreted according to the previous publication[6,7,12].

Heavy metal tolerance

Tolerance of the isolated bacteria to heavy metals, such as Cd^{2+} and Hg^{2+} (using $CdCl_2$ and $HgCl_2$ salts), was determined by agar dilution method and the results were interpreted as described before[6].

Agarose gel electrophoretic analysis of bacterial plasmid

The isolated sewage-water bacteria were subjected to plasmid DNA isolation following Kado and Liu[13], with little modification[14]. Agarose gel electrophoresis of plasmid DNAs isolated from sewage-water bacteria was carried out in tris-borate buffer system[15], using 0.8% agarose, for 2 h at 50 volts. After ethidium bromide staining, the gel was examined in gel-doc system for plasmid DNA bands and photographed. The molecular weight and subsequent size assessment of the bacterial plasmids were consummated with plasmids from *E. coli* V517 reference strain.

Curing experiment

Plasmid curing was done with SDS treatment (1.25 mg/ml) for 2 – 7 days at 37^{0}C, to check the loss of resistance properties, following Anjanappa et al.[16], with slight modifications mentioned earlier[6]. The plasmid DNA, from randomly selected SDS treated bacterial colonies, was isolated in order to check the plasmid loss through agarose gel electrophoresis, and thereafter, the antibiotic susceptibility as well as heavy metal tolerance of the plasmid-cured strains was tested by disc diffusion, in order to confirm the loss of resistance property along with the plasmid.

RESULTS AND DISCUSSION

A total of 12 bacterial isolates were obtained from sewage water samples; all the isolated bacteria were gram-negative rod. Among the isolated bacteria, 7 were lactose fermenting (strain code: SW6 to SW12), as per the characteristics of colonies developed on MacConkey agar plates and the bacterial growth patterns in TSI agar; 2 such isolates (strain code: SW4, SW5) showed negative test results for gelatin hydrolysis and were H_2S producing, while the rests of the isolates had the capacity to liquefy gelatin. The biochemical test results and sugar fermentation patterns of the isolated bacteria are represented in Table 1 and Table 2, respectively. Therefore, 3 isolates (strain code: SW1, SW2, SW3) were designated as *Ps. aeruginosa*, 2 isolates (strain code: SW4, SW5) were identified as *P. vulgaris*, 5 isolates (strain code: SW6, SW7, SW9, SW11, SW12) as *E. coli* and the remaining 2 (strain code: SW8, SW10) as *K. pneumoniae* (Table 3). The water bodies are the most important niches, providing bacterial habitation on the earth, and thus represented as the important source of spreading of bacteria and also the significant reservoir of bacterial antibiotic resistances around the globe[17-19].

The disc diffusion test results in terms of ZDIs are represented in Table 4. The patterns of antibiotic resistance and heavy metal tolerance of the sewage-water bacteria are represented in Table 3. The heavy metals are non-biodegradable elements because of their higher stability and persistence capacity in a given environment, such as aquatic bodies like river, causing long term selection pressure, leading to the development of metal resistances among bacteria[20-22]. It has also been suggested that bacterial revelation to the heavy metals in various ecological niches might be an explicit dynamic factor for the acquisition and/or development of antibiotic resistance too, among the bacteria exposed[20,23]. As per the report of Sair and Khan[20], prevalence of antibiotic and heavy metal resistant bacteria was seen in the river downstream, Lahore (Pakistan), and in connection with this phenomenon bacterial co-resistance to antibiotic and heavy metals, such as Cd^{2+} and Hg^{2+}, were recorded. In the instant case, the sewage bacterial isolates were multiple antibiotic resistant, showing resistances to ≥4 antibiotics, with different resistance patterns: *Ps. aeruginosa* (Am-Cm-Tm-Tc-Cp-Gm), *Pr. vulgaris* (Am-Cm-Tm-Tc), *E. coli* (Am-Cm-Tm-Tc-CP), and *K. pneumoniae* (Am-Tm-Tc-Gm). Earlier, we have reported the co-resistance to antibiotics (Am-Cm-Ce- Cx-Tm) and heavy metals (Cd^{2+}-Hg^{2+}), among the bacteria isolated from Mahananda river water, Malda, India[6]. The all sewage bacteria, procured and characterized in the current study, had resistance to Cd^{2+} and Hg^{2+}, with the tolerance level of 3 – 9 µg/ml and 200 – 300 µg/ml, for Hg^{2+} and Cd^{2+} respectively (Figure 1). The bacterial isolates: *E. coli* and *Ps. aeruginosa* had almost equal level of HM tolerance compared to the previously reported Mahananda river water bacteria[6]. The *Pseudomonas* sp. isolated from sewage of industrial effluents had resistance to a number of heavy metals and to multiple antibiotics, with MICs of heavy metals ranging from 50 µg/ml to 350 µg/ml[24].

The MAR indices of the bacterial isolates ranged 0.4 – 0.6 (Table 3). As has been reported by Chitanand et al.[25], the multiple antibiotic resistance has notably been screened among faecal- and total- coliforms at the downstream sites of the river Godavari (MAR index: 0.43) while it was stumpy among the coliform bacteria at the upstream sites (MAR index: 0.15), and thus, providing MAR index as a tool in distinguishing high risk contamination sites from the low/moderate risk areas in aquatic environment. The MAR index has also been utilized as an important tool to trace the source of bacterial isolates as well to distinguish between human-source and nonhuman-source of fecal bacteria[26,27]. It has been reported earlier that the MAR index for *E. coli* was 0.0 – 0.2, while for *Ps. aeruginosa* isolates, from Mahananda river water samples, the value ranged upto 0.47[6]; therefore, the MAR index for sewage water bacteria: *E. coli* and *Ps. aeruginosa* found higher when compared to the river water bacteria. The bacteria isolated from water sources exhibiting MAR indices of >0.4 have usually been regarded to be originated from human-fecal sources, while MAR indices of <0.4 indicate the nonhuman-fecal contamination of water sources[28,29]. The bacterial isolates with MAR index indices of >0.2 have been reported to be isolated from sources with high risk of contamination with antibiotics[12]. The MAR indices of ≥ 0.4, such as 0.4 for *K. pneumoniae* and *P. vulgaris*, 0.5 for *E. coli* and 0.6 for *Ps. aeruginosa*, in the current study, entailed the association of such bacterial strains with high level of antibiotic contaminated niches indicating their (bacterial isolates) human fecal-source of origin. The calculated MAR indices of bacteria: *E. coli* (0.4 –

0.51), *Ps. aeruginosa* (0.73 – 0.79) and *P. vulgaris* (0.59 – 0.6), isolated from main drains in the River Nile at Rosetta branch (Egypt), classified the area as potentially health risk environment[30]. The MAR indices among bacteria (*E. coli*, *Pseudomonas* spp., *P. vulgaris* and *Klebsiella* spp.) isolated from water samples of five rivers, in Pakistan, ranged from <0.2 to 0.6[20]. The MAR indices have been shown to be increased among clinical bacteria, such as, *E. coli* (from 0.4 to 0.5), *Pseudomonas* spp. (from 0.6 to 0.7), *Proteus* spp. (from 0.3 to 0.6) and *Klebsiella* spp.(from 0.4 to 0.7), on exposure of the heavy metal, lead, as per the report of Kawane[22]. Therefore, the development and emergence of multiple antibiotic resistant bacteria with high values of MAR indices is a global concern requiring regular monitoring of bacterial antibiotic resistances in order to combat the infection to humans around the world.

Among the isolated sewage-water bacteria a single plasmid of ≈ 54 Kb, co-migrated with each other, was detected (Figure 2). The high MAR indices of point source (waste water and surface water) isolates of *E. coli* have been reported to be due to a high range of antibiotic usage in human therapy than in agriculture, and the potential exchange of resistance elements, such as plasmids, as well[31]. The gram-negative bacterium, *E. coli*, has been recognized as an opportunistic pathogen fit to survive in aquatic niches, and play a great role in horizontal gene transfer, thus serving as the vector for the dissemination of bacterial antibiotic resistances[32,33]. The bacterial isolates belonging to the genera *Enterobacter, Escherichia, Serratia, Acinetobacter, Pseudomonas, Kiebsiella* and *Proteus*, isolated from sewage samples, were tested resistant to heavy metals (Pb^{2+} and Hg^{2+}) as well as antibiotics (ampicillin, ampiclox, streptomycin, lincocin and chloramphenicol), and such resistances were proved to be plasmid mediated by SDS curing studies[34]. Osman et al.[35] isolated a plasmid bearing aquatic bacterium conferring resistance to antibiotics as well as heavy metals. The *Pseudomonas* sp. showing high resistance to Pb^{2+} displayed resistance to multiple antibiotics, as has been reported by Trevors et al.[36]. In the current study, the multiple antibiotic resistances were found to be mediated with plasmid encoding $Cd^{2+}$and Hg^{2+} resistances among the isolated sewage bacteria; the loss of plasmid along with the concomitant loss of antibiotic and heavy metal resistance properties following curing experiments supported this view.

Table 1: Biochemical characteristics of sewage-water bacterial isolates

Bacterial Isolate	TSI				Ind	MR	VP	Cit	Oxe	Cat	Ure
	Butt	Slant	H_2S	Gas							
SW1	P	P	–	–	–	–	–	+	+	+	+
SW2	P	P	–	–	–	–	–	+	+	+	+
SW3	P	P	–	–	–	–	–	+	+	+	+
SW4	Y	P	+	+	–	+	–	–	–	–	+
SW5	Y	P	+	+	–	+	–	–	–	–	+
SW6	Y	Y	–	+	+	+	–	–	–	+	–
SW7	Y	Y	–	+	+	+	–	–	–	+	–
SW8	Y	Y	–	+	–	–	+	+	–	+	+
SW9	Y	Y	–	+	+	+	–	–	–	+	–
SW10	Y	Y	–	+	–	–	+	+	–	+	+
SW11	Y	Y	–	+	+	+	–	–	–	+	–
SW12	Y	Y	–	+	+	+	–	–	–	+	–

Cat: Catalase; Cit: Citrate; Ind: Indole; MR: methyl red; Oxe: Oxidase; TSI: triple sugar iron; VP: Voges-Proskauer; Ure: urease; '+': positive; '–': negative; P: pink; Y: yellow.

Table 2: Sugar fermentation and gelatin hydrolysis test results for sewage-water bacteria

Sugar	Bacterial Isolate											
	SW1	SW2	SW3	SW4	SW5	SW6	SW7	SW8	SW9	SW10	SW11	SW12
Mannitol	–	–	–	–	–	+	+	+	+	+	+	+
Xylose	–	–	–	+	+	+	+	+	+	+	+	+
Sorbitol	–	–	–	–	–	+	+	+	+	+	+	+
Mannose	–	–	–	–	–	+	+	+	+	+	+	+
Cellobiose	+	+	+	–	–	–	–	+	–	+	–	–
Rhamnose	–	–	–	–	–	–	–	–	–	–	–	–
Inositol	–	–	–	–	–	+	+	+	+	+	+	+
Arabinose	+	+	+	–	–	+	+	+	+	+	+	+
Melibiose	–	–	–	–	–	+	+	–	+	–	+	+
Gelatin hydrolysis	+	+	+	–	–	+	+	+	+	+	+	+

'+': positive; '–': negative.

Table 3: Antibiotic resistance patterns of sewage water bacteria and the cured strains and MAR indices of wild strains

Bacterial isolates	Strain code	Resistance pattern of wild strain		Cured strain	MAR index
		Antibiotic	Heavy metal		
Ps. aeruginosa (n=3)	SW1, SW2, SW3	Am-Cm-Tm-Tc-Cp-Gm	$Cd^{2+}Hg^{2+}$	Cp-Gm	0.6
Pr. vulgaris (n=2)	SW-4, SW-5	Am-Cm-Tm-Tc	$Cd^{2+}Hg^{2+}$	–	0.4
E. coli (n=5)	SW-6, SW-7, SW-9, SW-11, SW-12	Am-Cm-Tm-Tc-Cp	$Cd^{2+}Hg^{2+}$	Cp	0.5
K. pneumoniae (n=2)	SW-8, SW-10	Am-Tm-Tc-Gm	$Cd^{2+}Hg^{2+}$	Gm	0.4

Am: ampicillin; Cm: chloramphenicol; Cp: ciprofloxacin; Gm: gentamicin; Tc: tetracycline; Tm: trimethoprim: MAR: multiple antibiotic resistance

Table 4: Disc diffusion susceptibility test results for sewage water bacterial isolates against ten antibiotics

Antibiotic	ZDI (mm)											
	Ps. aeruginosa			*Pr. vulgaris*		*E. coli*					*K. pneumonia*	
	SW1	SW2	SW3	SW4	SW5	SW6	SW7	SW9	SW11	SW12	SW8	SW10
Ak	18	18	19	30	29	26	26	25	25	26	20	20
Am	10	11	10	8	7	6	7	6	6	6	7	6
Mp	25	25	26	26	26	27	27	27	27	27	26	26
Cm	11	10	11	10	11	10	9	9	9	10	22	22
Tm	6	7	6	6	6	7	6	6	6	6	6	6
Sm	21	21	20	20	20	21	22	21	20	22	21	22
Im	26	26	27	25	25	27	27	27	27	27	26	26
Tc	10	11	10	10	10	11	10	9	9	9	8	8
Cp	12	11	12	23	23	11	10	10	10	11	24	24
Gm	10	10	10	27	26	20	21	21	20	20	10	10

Ak: amikacin; Am: ampicillin; Cm: chloramphenicol; Cp: ciprofloxacin; Gm: gentamicin; Im: imipenem; Mp: meropenem; Sm: streptomycin; Tc: tetracycline; Tm: trimethoprim: ZDI: Zone diameter of inhibition

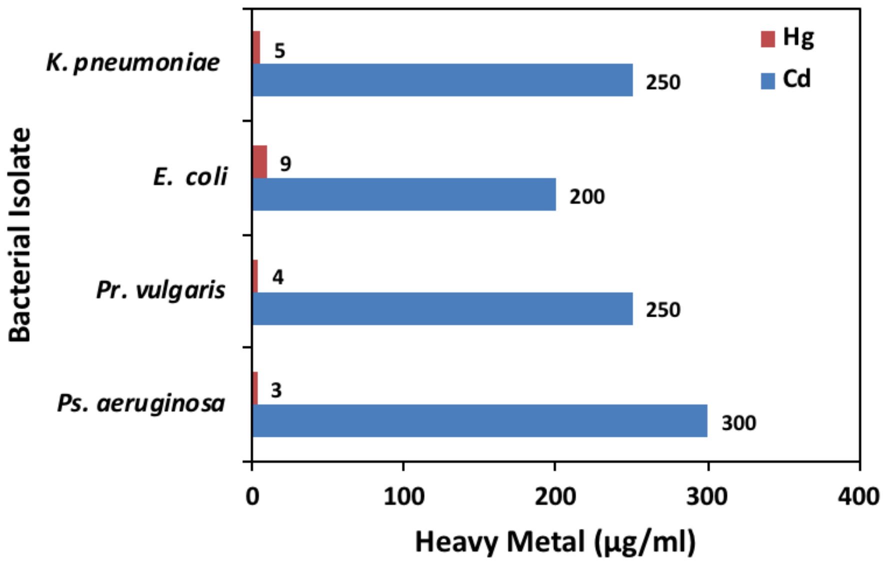

Figure 1: Heavy metal tolerance level for bacterial isolates from sewage water samples. Hg: mercuric chloride (Hg^{2+}), Cd: cadmium chloride (Cd^{2+})

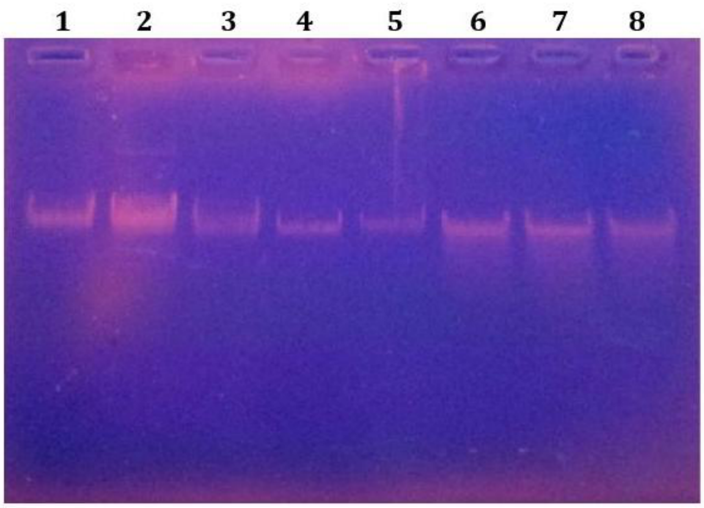

Figure 2: Plasmid profile of isolated sewage water bacteria; lane 1: *Ps. aeruginosa* SW1, lane 2: *Ps. aeruginosa* SW2, lane 3: *Pr. Vulgaris* SW4, lane 4: *Pr. vulgaris* SW5, lane 5: *E. coli* SW6, lane 6: *E. coli* SW10, lane 7: *K. pneumonia* SW12, lane 8: E. coli V517 (54 kb plasmid).

CONCLUSION

In the current study, the type and number of the isolated sewage-water bacteria were *Ps. aeruginosa* (n = 3), *Pr. vulgaris* (n = 2), *E. coli* (n = 5) and *K. pneumoniae* (n = 2) that contained a single plasmid encoding antibiotic and heavy metal resistances. Also, this study further emphasized that the bacterial MAR indices might be of help to identify the source of fecal contamination as well as to categorize the health risk contaminated niches, and such environmentally isolated bacteria might be used as important tools in detecting and monitoring heavy metal and antibiotic pollution in studied areas.

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