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Plasmid Mediated Antibiotic and Heavy Metal Co-Resistance in Bacterial Isolates from Mahananda River Water (Malda, India)

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Abstract

Aims: The current investigation stands for the determination of MAR (multiple antibiotic resistance) indices of antibiotic resistant and heavy metal (HM) tolerant bacterial isolates from Mahananda river water near Malda (West Bengal state, India) and profiling of R-plasmids of the isolated bacteria.

Methods: The water samples (n=5), collected from Mahananda river water, Malda town, (India), were checked for the presence of bacterial growth in nutrient broth cultures. The pure bacteria colonies, procured from each of the nutrient broth cultures, were identified by conventional methods. The susceptibilities to antibiotics and HMs of the isolated bacteria were determined by disc diffusion and agar dilution, respectively. Plasmid curing of the resistant bacteria was done with SDS treatment, and the isolated plasmids were screened through agarose gel electrophoresis. The MAR indices for the isolates were calculated.

Results: The isolated bacteria were identified as *Escherichia coli* (n=3), and *Pseudomonas aeruginosa* (n=2). The bacterial isolates showed various patterns of resistance to antibiotics and heavy metals. The MAR indices for the bacterial isolates ranged 0.0 – 0.2, for *E. coli*, and the value was 0.47, for both the *Ps. aeruginosa* isolates. The isolated bacteria harboured a single plasmid mediating co-resistance to antibiotics and HMs.

Conclusion: The current study demonstrates the occurrence of plasmid, encoding Am-Cm-Ce-Cx-Tm-Cd_{2+}-Hg^{2+} and Cm-Tm-Cd_{2+}-Hg^{2+} resistances, among the aquatic bacteria of Mahananda river that may potentially act as the source of dissemination of pathogenic bacteria and bacterial antibiotic resistances, requiring public awareness on the issues of misuse and/or overuse of antibiotics.

Keywords: Mahananda river water; Pathogenic bacteria; Antibiotic resistance; Heavy metal tolerance; Curing experiment; Plasmid

Introduction

The domestic, hospital, agriculture and aquaculture effluents act incessantly as the sources of pollution of various water bodies together with the rivers by means of various chemical agents, such as the toxic heavy metals (HMs such as Hg^{2+}, Cd^{2+}, Cu^{2+} and Zn^{2+}), and antibiotics, and microbial agents including pathogenic bacteria [1-3]. Due to various reasons mentioned earlier such bacterial strains at all times are in the process of acquiring resistance to antibiotics [4,5], and HMs [6]. The presence of multiple antibiotic resistant *Escherichia coli* in the river water of Mahanadi, Sambalpur of Odisha state, India, has been reported [7]. Upadhyay and Joshi [8] reported the presence of extended spectrum beta lactamase (ESBL) producing *E. coli* and *Pseudomonas aeruginosa* from river water and *Klebsiella pneumoniae* and *E. coli*, from sputum and stool samples, respectively, from Shillong, Meghalaya (India). It has been reported that the Obere river in Orile Igbon, Oyo state (Nigeria) has been polluted with pathogenic bacteria (*Pseudomonas* sp. and *Proteus* sp.), showing resistance to multiple antibiotics [9]. Determining the multiple antibiotic resistance (MAR) indices among the bacterial isolates has been reported to be a simple but important tool for health risk assessment of the environment, and the higher MAR indices of the isolates indicate their origin from high-risk sources of antibiotic contamination [7,10-12]. Low as well as high risk MAR indices have been demonstrated among the *E. coli* isolates from the Gomti River water samples indicating an adverse effect of antibiotic therapy of bacterial infection [13]. A varied MAR indices among the eye cosmetic isolates of *Ps. aeruginosa*, *Listeria monocytogenes* and *Bacillus cereus* have been reported previously from Malda, India [14].

Seiler and Berendonk [6] demonstrated the heavy metal driven co-selection of antibiotic resistance among bacterial populations. The *Ps. putida* isolate from marine milieu had resistance to Cd^{2+}, Cu^{2+}, Hg^{2+} and Pb^{2+}, in association with resistance to antibiotics: ampicillin (Am), kanamycin (Km), chloramphenicol (Cm), and tetracycline (Tc), ciprofloxacin (Cp), cotrimazole (Co), gentamycin (Gm), nalidixic acid (Nx) and streptomycin (Sm) [15,16]. The bacterial resistance to heavy metals (Cd^{2+}, Hg^{2+}, Cu^{2+}, Zn^{2+}) as well as antibiotics has been reported to be plasmid mediated [17, 18]. The earlier authors [19, 20] have reported the plasmid mediated antibiotic resistance and heavy metal tolerance among the clinical as well as environmental, including water, bacterial isolates. Thus, there has been an escalating concern in exploring the evolving of MAR in bacteria from hospital and domestic effluents as well as from the receiving riverine water in various part of the globe [21]. However, no systematic investigation has been made on

the plasmid mediated antibiotic resistance and HM tolerance of bacterial isolates from any water bodies including the river from the current study areas. Therefore, the present study has been undertaken to determine the MAR indices of antibiotic resistant and HM tolerant potential (human) pathogenic bacteria, isolated from Mahananda river water near Malda (West Bengal state, India) carrying R-plasmids.

Methods

Collection of water samples

A total of 5 water samples were collected from Mahananda river water, near Malda town of the West Bengal state, India, in sterilized plastic container and transported to the Laboratory of Microbiology and Experimental Medicine (Department of Zoology, University of Gour Banga) for microbiological processing.

Procurement of bacterial isolates and identification

The bacterial isolates from collected water samples were procured and stored as mentioned in the previous publication [14]. The colony morphology of the pure bacteria cultures was studied on blood agar, MacConkey agar, cetrimide agar, brilliant green bile agar and nutrient agar (Hi-Media, India), following streak dilution technique. The bacteria isolated were identified following the standard protocol [22,23], as described earlier [14].

Antibiotic susceptibility test

The antibiotic susceptibility of the bacteria procured from the Mahananda river water was determined by disc diffusion method [24] as mentioned elsewhere [14], using Mueller-Hinton agar (Hi-Media, India), and 15 antibiotic discs (µg/disc; Hi-Media, India): amikacin (Ak: 30); ampicillin (Am: 10); cefpodoxime (Ce: 10); ciprofloxacin (Cp: 5); chloramphenicol (Cm: 10); cefoxitin (Cx: 30); imipenem (Im: 10); gentamycin (Gm: 10); kanamycin (Km: 30); meropenem (Mp: 10); nalidixic acid (Nx: 30); piperacillin (Pi: 100); piperacillin-tazobactam (Pi-Tz: 100-10); tetracycline (Tc: 30); trimethoprim (Tm: 5). The test results, in terms of ZDI (zone diameter of inhibition) values from around the antibiotic discs against the isolates, were interpreted as per the Clinical Laboratory Standards Institute [25], to categorize the isolates as resistant, sensitive or intermediately susceptible.

Determination of MAR index

The MAR indices for the isolated bacterial strains were calculated following the formula as stated earlier [14]:

$$\text{MAR index} = \frac{\text{Number of antibiotics to which the isolate showed resistance}}{\text{Number of total antibiotics exposed to the isolate}}$$

, and interpreted according to the criteria mentioned earlier [12, 14]: MAR index ≤ 0.2 was considered low risk, and ≥ 0.2 indicated the high risk of antibiotic contamination.

Metal tolerance

The maximum tolerance concentration (MTC) value of the isolates to the metals was determined by agar dilution method, using inocula of ≈104 CFU/spot [5]. The metal salts, such as $HgCl_2$ (Hg^{2+}) and $CdCl^2$ (Cd^{2+}) were utilized in the study. The dilution of the test HM made to various concentrations, for the study, included: Cd^{2+} (125-325 µg/ml), Hg^{2+} (1.0-12.5 µg/ml) and were incorporated into Mueller-Hinton agar medium. The MTC was defined as the lowest dilution of the metal that did not inhibit the visible growth of the bacterial isolates on the medium, after 24 h incubation, at 35°C.

Curing experiment

The Mahananda river water isolates of *E. coli* and *Ps. aeruginosa* having resistance to one or more antibiotics and/or HMs was subjected to plasmid curing at 42°C to check the loss of resistance properties, following Anjanappa et al. [26], with slight modifications mentioned in our earlier publication [27]. In this study, the curing agent used was SDS (2.5 mg/ml).

Bacterial plasmid DNA isolation and agarose gel electrophoresis

The antibiotic and/or HM resistant isolates of *E. coli* and *Ps. aeruginosa* as well as the cured derivatives were subjected to plasmid DNA isolation following the protocol of Kado and Liu [28], with modification as mentioned elsewhere [29].

The agarose gel electrophoresis of the isolated plasmid DNAs was carried out in tris-borate buffer system [30], using 0.8% agarose, for 3 h at 50 volts. The gel was stained with ethidium bromide, and the results were documented using UV-transilluminator. The electrophoretic separation of the plasmids, by molecular weight and subsequent size estimations, were accomplished using the plasmid from *E. coli* V517 strain.

Results

A total of 5 gram-negative bacteria, one from each of the collected water samples were procured, of which 3 isolates (strain code: MC1, MC2 and MC3) were lactose fermenting, and 2 isolates (strain code: C1 and C2) were non-lactose fermenting (based upon TSI stab and MacConkey agar plate culture); the isolates designated as C1 and C2 were oxidase test positive, and produced characteristic pigments on cetrimide agar plate. Following cultural characteristics (colony morphology, and pigment production), gram-staining (cell shape), biochemical test results and sugar fermentation patterns, the isolated aquatic bacteria were identified as: *Ps. aeruginosa* (strain code: C1 and C2) and *E. coli* (strain code: MC1, MC2 and MC3).

The antibiotic susceptibility test results, in terms of DZIs, of the isolated bacteria are represented in Table 1. The *Ps. aeruginosa* C1 and *Ps. aeruginosa* C3 isolates showed resistance to Am, Cm, Ce, Tm, Cx, Km and Nx (ZDI: 6 – 15 mm), and sensitivity to the remaining antibiotics tested. Among the *E. coli* isolates, only *E. coli* MC3 was resistant to Cm, Nx and Tm (ZDI: 6 – 15 mm), and intermediately susceptibility to Pi and Pi-Tz. The *E. coli* MC1 had intermediately susceptibility to Ce, Km, Pi and Nx, while *E. coli* MC2 was intermediately susceptibility to Ce and Cx.

The HM tolerance of the isolated bacteria is shown in Figure 1. All the three *E. coli* isolates had Hg^{2+} MTC value of 9 µg/ml; for *Ps. aeruginosa* isolates the MTC value of Hg^{2+} was 3 µg/ml. The MTCs for the isolates of *E. coli* and *Ps. aeruginosa* ranged 250 - 300 µg/ml of Cd^{2+}.

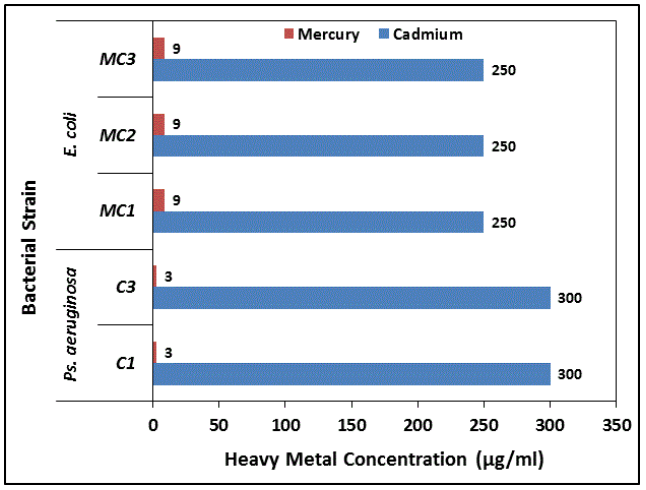

Figure 1: Maximum tolerance concentration (MTC) of the Mahananda riverine water bacteria to heavy metals; the values against the bars indicate the MTCs of the bacterial isolates.

The plasmid profile of the *E. coli* and *Ps. aeruginosa* isolates is shown in the Figure 2. All the isolated bacteria had a single plasmid band. The plasmid band isolated from *E. coli* MC3 isolate (resistance pattern: Cm-Tm-Nx-Cd^{2+}-Hg^{2+}), and *Ps. aeruginosa* C1 and *Ps. aeruginosa* C3 isolates, having common resistance pattern "Am-Cm-Ce-Tm-Cx-Km-Nx-Cd^{2+}-Hg^{2+}" co-migrated with *E. coli* V517 plasmid marker of ≈ 54 Kb; the plasmids (≈ 50 Kb) harboured within the antibiotic sensitive *E. coli* MC1 and MC2 isolates showing resistance to Cd^{2+} and Hg^{2+} migrated slightly faster than the *E. coli* V517 plasmid marker.

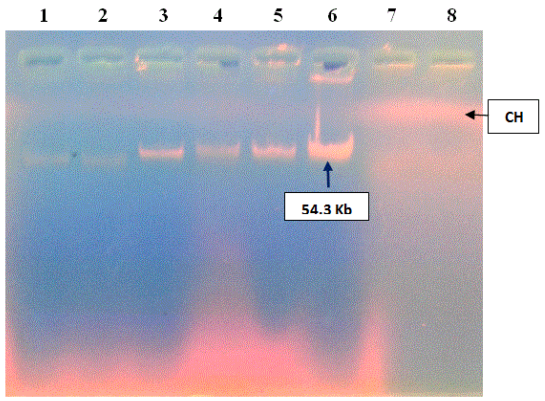

Figure 2: Plasmid profile of isolated riverine water bacteria; lane 1: *E. coli* MC1, lane 2: *E. coli* MC2, lane 3: *E. coli* MC3, lane 4: *Ps. aeruginosa* C1, lane 5: *Ps. aeruginosa* C2, lane 6: *E. coli* V517 (54 kb), lane 7: *E. coli* MC3 cured strain, lane 8: *Ps. aeruginosa* C1 cured strain. Note the absence of plasmid band in cured bacterial strains (lane 7 and 8). CH: chromosomal DNA.

Antibiotics	ZDI (mm) for bacterial isolates				
	E. coli			*Ps. aeruginosa*	
	MC1	MC2	MC3	C1	C3
Mp	30	30	29	35	34
Im	35	28	31	35	35
Tm	22	23	6	6	6
Cx	19	17	22	6	6
Km	17	19	20	10	6
Pi	20	21	20	38	26
Pi-Tz	22	21	20	40	30
Ak	18	19	20	34	25
Cp	26	21	25	44	46
Nx	17	20	6	14	13
Am	15	15	16	6	6
Cm	25	26	15	15	11
Gm	25	25	24	36	30
Tc	26	25	15	26	24
Ce	20	20	20	6	6

Table 1: Antibiotic susceptibility test results for the Mahananda riverine water bacteria. Ak: amikacin; Am: ampicillin; Ce: cefpodoxime; Cp: ciprofloxacin; Cm: chloramphenicol; Cx: cefoxitin; Im: imipenem; Gm: gentamycin; Km: kanamycin; Mp: meropenem; Nx: nalidixic acid; Pi: piperacillin; Pi-Tz: piperacillin-tazobactam; Tc: tetracycline; Tm: trimethoprim; ZDI: zone diameter of inhibition.

The results of curing experiments are represented in Table 2. The *E. coli* MC3 isolate that had Cm-Tm-Nx-Cd^{2+}-Hg^{2+} resistance pattern retained resistance only to Nx, after SDS treatment for 7 days, while both the *Ps. aeruginosa* isolates (C1 and C3) retained Nx-Km resistance pattern. Along with the loss of the above mentioned antibiotic as well as HM resistances, plasmid curing occurred in all the 3 bacterial isolates; the rate of curing was 100 %, in *E. coli* MC3, *Ps. aeruginosa* C1 and *Ps. aeruginosa* C3 isolates.

Discussion

In this investigation, five potentially human pathogenic bacteria belonging to two genera: *Ps. aeruginosa* (n=2) and *E. coli* (n=3), bearing varied resistance patterns, have been isolated from an unstudied riverine ecosystem (Mahananda River, India)) imminently receiving domestic, hospital and agricultural effluents from within and around Malda town, West Bengal state, India. The study with bacterial isolates from such aquatic body (Mahananda River, India) in MAR index issues remains a new one, though the MAR indices of individual bacterial strains from various sources have been reported globally. The MAR index in the clinical isolates of *E. coli* have been recorded high compare to the that of the *E. coli* isolates from drinking water, and thus, MAR index has been depicted as an indicator to distinguish the origin of the bacterial isolates, as has been reported by Kawane [31],

who demonstrated no considerable variation in HM tolerance of *E. coli* isolates from both types sources. The Staphylococcus aureus isolates from clinical settings had high MAR index (0.64-0.74), indicating their origin of niches having high antibiotic exposure [32]. The MAR indexing (0.64-0.68) of the isolates showed that all these strains originated from high risk source of contamination; *Escherichia coli* isolates isolated from Cochin estuary [33]. The MAR index of *Enterococcus* from Seine River, as has been reported by Servais et al. [34], was 0.24 for point source (hospital discharges) demonstrating high antibiotic usage, when compared to the MAR index, 0.078 for non-point source (agricultural discharges) and 0.168 for the river itself. Sani et al. [35] reported MAR index of the clinical *E. coli* isolates as > 0.2, indicating the significant level of antibiotic misuse within the study area. The MAR indices of the isolated bacterial strains: *E. coli* (0.44) and *Ps. aeruginosa* (0.43-0.57), were all > 0.2, which indicate high risk source of antibiotic contamination (Oko et al.) [36]. The *Pseudomonas* and *Klebsiella* isolates possessing multiple antibiotic resistances had MAR index of 0.4 [Osundiya et al. [37]. The MAR indices in the clinical isolates of *E. coli* have been recorded high compare to the MAR indices of *E. coli* from drinking water, and thus, MAR index has been depicted as the an indicator to distinguish the origin of the bacterial isolates, as has been reported by Kawane [31], who demonstrated no considerable variation in tolerance of *E. coli* isolates from both types of sources. As we have reported earlier, considering the MAR index values, the eye-cosmetic bacterial isolates have been categorized into different groups; the highest value was noted for *Ps. aeruginosa* (MAR index: 0.5) [14]. In this study, the MAR indices have been recorded as 0.47, for *Ps. aeruginosa*, and zero to 0.2, for *E. coli*.

The HM resistance has been reported to enhance the antibiotic resistance among the bacterial strains [38]. The genetic adjustment permits bacteria, in the environment, community, and in clinical settings, to attain resistance to antibiotics, and one way for such action is the acquisition of plasmid, mediating resistance to antibiotics, from HM antibiotic resistant strains possessing R-plasmid. Such R-plasmids might contain genes for HM tolerance along with the antibiotic resistance genes facilitating the co-resistance to HMs and antibiotics among the recipient bacterial strains. The concern of and antibiotic co-resistance in *Ps. aeruginosa* has been addressed by Perron et al. [39], who demonstrated that the isolates having exposed to Zn^{2+} had resistance to Cd^{2+} and Co^{2+} too, and to the antibiotics, such as Im, a carbapenem class of antibiotic. It has been reported that the HM - due to their non-degrading nature - stand for an obstinate selection pressure of environmental as well as clinical importance, potential to contribute in the emergence of antibiotic and HM co-resistance [40]. The metal-resistant profile of *Ps. aeruginosa*, from water sample of Alaro River (Lagos, Nigeria), which showed resistance to 18 antibiotics, was 10 mM for cadmium, 10 mM for cobalt, 15 mM for nickel, 12 mM for chromium, and 1.0 mM for mercury [41].

Bacteria	Original strain		Cured strain		% Curing
	Resistance pattern	Plasmid	Resistance pattern	Plasmid	
E. coli MC3	Cm-Tm-Nx-Cd_{2+}-Hg^{2+}	Present	Nx	Absent	100
Ps. aeruginosa C1	Am-Cm-Tm-Ce-Cx-Km-Nx-Cd_{2+}-Hg^{2+}	Present	Nx-Km	Absent	100
Ps. aeruginosa C3	Am-Cm-Tm-Ce-Cx-Km-Nx-Cd_{2+}-Hg^{2+}	Present	Nx-Km	Absent	100

Table 2: Results of curing experiments for the Mahananda riverine water bacteria; the abbreviation of the antibiotics are given in Table 1. Plasmid curing and loss of heavy metal resistance were not seen in the antibiotic sensitive isolates of *E. coli* MC1 and *E. coli* MC2 (not presented in the Table).

The plasmid encoded bacterial antibiotic resistance or HM tolerance, or antibiotic- HM co-resistance have been maintained due to the presence of such chemical contaminants/pollutants in various ecological niches, viz. water bodies, including rivers, receiving effluents from various sources. Gullberg et al. [42] demonstrated that an antibiotic or a HM, or combination of the compounds at very low concentrations might select for a plasmid encoding resistance to different HMs in addition to antibiotics of various classes. Sevgi et al. [43] reported multiple plasmids (size: 1.8 - 28 kb) in *Pseudomonas* spp. strains, with resistance to Cu^{2+}, Cr^{6+}, Zn^{2+} and Ni^{2+}, isolated from the industrial area in Kazanlı (Turkey). The resistance of *Pseudomonas* strains to Cu^{2+} and Ni^{2+} had been reported to be encoded in plasmid of 4.7 - 20.8 kb [44]. The multiple antibiotic resistances along with the Cr-tolerance were found to be plasmid mediated in the effluent isolates of *Ps. aeruginosa* and *E. coli* [45]. The plasmidic gene robA, in *E. coli*, has been shown to be responsible for increased resistance spectrum to different antibiotics and HMs such as Ag^{2+}, Cd^{2+} and Hg^{2+} along with Tc, Cm and novobiocin resistances [46]. In the current study, the isolated Mahananda river water bacteria *Ps. aeruginosa* (all isolates) and *E. coli* (MC3 isolate) had co-resistance to HMs and antibiotics, with resistance patterns "Am-Cm-Ce-Tm-Cx-Cd^{2+}-Hg2+" and "Cm-Tm-Cd^{2+}-Hg^{2+}", respectively, and the resistances were encoded with a plasmid of ≈ 54 kb; resistance to Nx and Km was not plasmid mediated. The concomitant loss of the resistance patterns (Am-Cm-Ce-Tm-Cx-Cd^{2+}-Hg^{2+}" and "Cm-Tm-Cd^{2+}-Hg^{2+}) along with the loss of plasmid DNAs among the isolates, in this investigation, supported the view. Thus, the rivers, which act as one of the major sources of water for human consumption, operate acquisition, maintenance and dissemination of bacterial antibiotic resistance and heavy metal tolerance [47]. The bacterial antibiotic resistance in the riverine ecosystem that might be achieved via transferable R-plasmids from clinical sources (viz. hospital effluents), has a great impact on human health. Because, being in regular use in irrigation and domestic purposes, the riverine antibiotic resistant bacteria plausibly spread, from riverine water, into drinking water constituting public health hazards [48,49].

The current findings, in addition to that from the other studies, showed the co-occurrence of HM tolerant and antibiotic resistant microorganisms, and directed the HM and/or antibiotics, even at very low concentrations in the water bodies, might be responsible in the maintenance and dissemination of antibiotic and HM resistance of bacteria in the environment possessing HM and antibiotic contamination [50]. Based upon the facts mentioned above it can be

concluded that the resistance to two or more antibiotics, from Am, Cm, Ce, Cx and Tm, and HMs (Cd^{2+} and Hg^{2+}) in the riverine water isolates of *E. coli* and *Ps. aeruginosa*, was mediated by plasmid, and the phenomenon of co-occurrence of antibiotic and HM resistance among the isolates prevails in the Mahananda river water.

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