

Heavy metal tolerance in association with plasmid mediated multiple antibiotic resistances among clinical bacterial isolates

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ABSTRACT

The heavy metal tolerance in association with plasmid mediated antibiotic resistance among bacteria has been reported around the globe. This communication conducted an experiment to explore the co-existence of antibiotic resistance and heavy metal tolerance in clinical bacteria and the involvement of R-plasmid in such phenomenon. By disc diffusion method, 6 clinical bacteria: *Escherichia coli* (n=3), *Pseudomonas aeruginosa* (n=2) and *Proteus mirabilis* (n=1), utilized in the study, displayed resistance to multiple antibiotics with MAR (multiple antibiotic resistance) indices 0.15 – 0.77; such bacterial isolates showed tolerance to Hg²⁺, Cd²⁺, Cr⁶⁺ and Cu²⁺ at 3 – 37.5 µg/ml, 75 – 800 µg/ml, 100 – 400 µg/ml and 600 – 900 µg/ml, respectively. The SDS treatment induced the test bacteria to mislay their resistance property (following susceptibility test) with a parallel loss of single plasmid (following agarose gel electrophoretic analysis) contained in them. This study confirms the antibiotic co-resistance with heavy metal tolerance among human pathogenic bacteria, and underlines the regular vigilance of bacterial R-plasmid in order to combat the multiple antibiotic resistances of such bacteria as well as the infection caused by them.

KEY WORDS: HUMAN PATHOGENIC BACTERIA, R-PLASMID, HEAVY METAL TOLERANCE, ANTIBIOTIC RESISTANCE, MAR INDEX

INTRODUCTION

The antibiotics, which are still the gold standard therapeutics against a large number of bacterial infections, and the heavy metals, which are in use in various anthro-

pogenic activities, remain the two universal categories of environmental pollutants, and are unsafe to public health and biological safety (Zhu et al., 2013). Several anthropogenic processes cause contamination of environment with heavy metals leading to the selection and

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emergence of bacteria possessing the tolerance capacity to heavy metals in the niches (Nakahara *et al.*, 1977), and, as such, the heavy metal accumulation in the environment accounts for the bacterial antibiotic co-resistance (Baker-Austin *et al.*, 2006; Berg *et al.*, 2010; Das *et al.*, 2016). The imprudent use of antibiotics, on the other hand, results emergence of antibiotic resistant bacteria having the capacity to cause life-threatening infection to humans, around the world (Tenover, 2006; Mandal 2015). It has been reported that the exposure of heavy metals causes an effect in the co-selection of metal tolerant and antibiotic resistant bacteria (Filali *et al.*, 2000), and such co-resistances are plasmid mediated (Smith, 1967; Das *et al.*, 2018). Garhwal *et al.* (2014) observed a significant change in MAR (multiple antibiotic resistance) index in clinical bacterial isolates before and after lead (Pb^{2+}) exposure. Nakahara *et al.* (1977) studied the frequency of antibiotic and heavy metal resistance in clinical isolates of *Escherichia coli*, *Klebsiella pneumoniae*, *Pseudomonas aeruginosa*, and reported a similar as well as different heavy-metal resistance frequency, when compared to the antibiotic resistance frequency, among the isolates, and such resistances were proved to be plasmid mediated. The occurrence of R-plasmid (antibiotic resistance plasmid) conferring heavy metal tolerance in river water isolates of *E. coli* and *Ps. aeruginosa* has been documented earlier (Das *et al.*, 2016). The heavy metal induced antibiotic resistance in bacteria has also been reported (Chen *et al.*, 2015). A conjugative plasmid, approximately of 56.4 kb, encoding resistance to heavy metals (Hg^{2+} , Cu^{2+} , Pb^{2+} , Cd^{2+}) as well as antibiotics was detected among nosocomial isolates of *E. coli* and *K. pneumoniae* (Karbasizad *et al.*, 2003). Thus, an emerging concern, predominantly in the developing countries, for the treatment of infectious disease is the acquisition and dissemination of bacterial plasmid mediated resistance to multiple antibiotics. Hence, in order to evade the bacterial antibiotic resistance, by fixing an appropriate treatment 'to-do-list', precise and prompt detection of resistance phenotype is an emergent and imperative issue (Doddaiiah and Anjaneya, 2014), since bacterial antibiotic resistance has been marked as the global public health crisis (Martinez, 2008). Therefore, the current study has been undertaken to determine the association between antibiotic resistance and heavy metal tolerance among clinical bacterial isolates: *E. coli*, *Ps. aeruginosa* and *Pr. mirabilis*, West Bengal state, India.

MATERIAL AND METHODS

BACTERIAL STRAIN AND MEDIA

A total of 6 randomly selected clinical bacterial isolates: *Escherichia coli* (n=3), *Pseudomonas aeruginosa* (n=2)

and *Proteus mirabilis* (n=1), were considered for the current study. The tests, in the current study, were carried out by the utilization of nutrient broth (for subculturing, bacterial inocula preparation and plasmid DNA isolation) and nutrient agar (for performing antibiotic susceptibility and heavy metal tolerance test) media (Hi-Media, India).

ANTIBIOTIC SUSCEPTIBILITY TEST

The antibiotic susceptibility test for the bacterial isolates were determined following Kirby-Bauer disc diffusion (Bauer and Kirby, 1966), using tetracycline (Tc; 30- μ g), gentamicin (Gm; 10- μ g), cefotaxime (Ct; 30- μ g), cefpodoxime (Ce; 10- μ g), ampicillin (Am; 10- μ g), meropenem (Mp; 10- μ g), chloramphenicol (Cm: 10- μ g), ciprofloxacin (Cp; 10- μ g), ceftiofloxacin (Cx; 30- μ g), piperacillin (Pc; 100- μ g), piperacillin/tazobactam (PT; 100/10- μ g), amikacin (Ak; 30- μ g) and nalidixic acid (Nx; 30- μ g). The results, in terms of ZDI (zone diameter of inhibition) obtained around each of the antibiotic discs, for the test isolates were interpreted according to the CLSI criteria (CLSI, 2011).

MAXIMUM TOLERANCE CONCENTRATION OF HEAVY METAL

The MTC (maximum tolerance concentration) values, for the bacterial isolates, of heavy metals: using 4 salts, such as $HgCl_2$ (Hg^{2+}), $CdCl_2$ (Cd^{2+}), $K_2Cr_2O_7$ (Cr^{6+}), and $CuSO_4$ (Cu^{2+}) were determined by agar dilution method, using $\approx 10^4$ CFU/spot inocula, as described earlier (Das *et al.*, 2016). The concentrations of heavy metals utilized included: Hg^{2+} (3 - 50 μ g/ml), Cd^{2+} (25 - 1000 μ g/ml), Cr^{2+} (25 - 500 μ g/ml), Cu^{2+} (200 - 1000 μ g/ml). The obtained results were interpreted as described earlier (Das *et al.*, 2016). The bacterial isolates grown in presence of each of the heavy metals, at concentrations ≥ 3 μ g/ml, were considered as heavy metal tolerant.

PLASMID ANALYSIS

As mentioned earlier (Das *et al.*, 2016), the plasmid DNA from the test bacteria were isolated following the protocol of Kado and Liu (1981), and the agarose gel electrophoresis of the isolated plasmids were done following Maniatis *et al.* (1982). The plasmid DNA bands, in the gel after ethidium bromide staining, were visualized and documented using gel-doc system.

In order to investigate the loss of plasmid, the randomly selected bacterial isolates (*Pr. mirabilis* CSD1, *Ps. aeruginosa* CSD3, and *E. coli* CSD5) were subjected to plasmid curing with SDS, following the protocol of Anjanappa *et al.* (1993), as described elsewhere (Mandal *et al.*, 2008; Das *et al.*, 2016). The loss of antibiotic

resistance and heavy metal tolerance, along with the loss of plasmid, was determined based on the resistance patterns of the cured bacterial strains, and absence of plasmid in the gel following agarose gel electrophoresis for the cured bacterial strains.

RESULTS AND DISCUSSION

The antibiotic susceptibility test results, in terms of ZDI, are depicted in Table 1. The mounting use of antibiotics, not only in health care but also in agriculture and animal husbandry contribute to an emergent problem of antibiotic resistant bacteria (Dhanorkar and Tambekar, 2004). Pokhrel *et al.* (2018) reported, among the isolated environmental bacteria, 3-antibiotic resistance, 4 to 10-antibiotic resistances and more than 10-antibiotic resistance in 6.1%, 44.89% and 48.97% isolates, respectively. The fecal as well as soil isolates of *Klebsiella*, *Citrobacter*, *Shigella* and *Staphylococcus*, showed resistance to 6 – 10 antibiotics tested, and the MAR indices for the isolates ranged 6 – 10 (Ayandele *et al.*, 2018). The Mahananda river water bacterial isolates had resistance to multiple antibiotics, among Am, Cm, Ce, Cx and Tm, as per the report of the earlier study (Das *et al.*, 2016). In the current study, *Pr. mirabilis* (n=1) had 2-drug resistance “Cx-Pc”, *Ps. aeruginosa* isolates (n=2) had 8-drug resistance of two different patterns “Am-Ce-Cm-Ct-Cx-Nx-Pc-PT” and “Am-Ce-Cp-Ct-Cx-Nx-Pc-PT”, and the

E. coli isolates (n=3) showed 3 different patterns of resistance to antibiotics: 8-drug resistance “Am-Ce-Cm-Cp-Cx- Mp-Nx-Pc”, 9-drug resistance “Am-Ce-Cp-Ct-Cx-Mp-Nx-Pc-PT” and 10-drug resistance “Am-Ce-Cp-Ct-Cx-Mp- Nx-Pc-PT-Tc” (Table 2). As per the report of Malema *et al.* (2018), among the 100 pathogenic *E. coli* test isolates, 52% had multiple antibiotic resistance, of which 10 showed to 9 antibiotics, and 24 different MAR phenotypes have been identified.

The MAR indices for the human pathogenic bacteria are depicted in Figure 1. As has been reported by Sandhu *et al.* (2016), the majority of the clinical isolates of *Acinetobacter* had resistance to cotrimoxazole, Cp, Gm, Ak, A/S, cefepime, Im, Mp, with overall MAR indices of 0.3 – 1.0 for the isolates. Subramani *et al.* (2012) reported high MAR indices (0.64 - 0.74) among *Staphylococcus aureus* isolates from clinical settings demonstrating the origin of the bacteria from niches with high antibiotic exposure/contamination. The MAR indices of potential pathogenic bacteria, *E. coli* (MAR index: 0.44) and *Ps. aeruginosa* (MAR index: 0.43-0.57), were all > 0.2, indicating their origin from high risk source of antibiotic contaminated region (Okon *et al.*, 2016). In the previous communication, the MAR indices have been reported to be 0.47 in *Ps. aeruginosa* and zero to 0.2 in *E. coli* isolates from Mahananda river water, Malda (India) (Das *et al.*, 2016). In the current study, the MAR indices for the clinical bacteria were: 0.15 for *Pr. mirabilis* and 0.62 for *Ps. aeruginosa*, while the values ranged 0.62 – 0.77

Table 1. Antibiotic susceptibility test results for clinical bacterial isolates (ZDI; zone diameter of inhibition)

Antibiotic	ZDI (mm)					
	CSD1	CSD2	CSD3	CSD4	CSD5	CSD6
Tc	40	10	18	17	12	13
Gm	27	15	30	26	20	20
Ct	30	6	12	6	6	25
Ce	20	10	6	6	6	13
Am	20	6	6	6	6	6
Mp	30	12	33	34	14	18
Cm	18	22	8	22	25	8
Cp	40	8	46	15	10	6
Cx	14	6	6	6	6	11
Pc	6	6	6	6	6	6
PT	30	15	17	6	6	20
Ak	30	20	18	30	26	15
Nx	25	6	12	10	6	6

Ak: amikacin, Am: ampicillin, Ce: cefpodoxime, Cm: chloramphenicol, Cp: ciprofloxacin, Ct: cefotaxime, Cx: ceftiofur, Gm: gentamycin, Mp: meropenem, Nx: nalidixic acid, Pc: piperacillin, PT: piperacillin/tazobactam, Tc: tetracycline, CSD1: *Pr. mirabilis*; CSD2: *E. coli*; CSD3: *Ps. aeruginosa*; CSD4: *Ps. aeruginosa*; CSD5: *E. coli*; CSD6: *E. coli*.

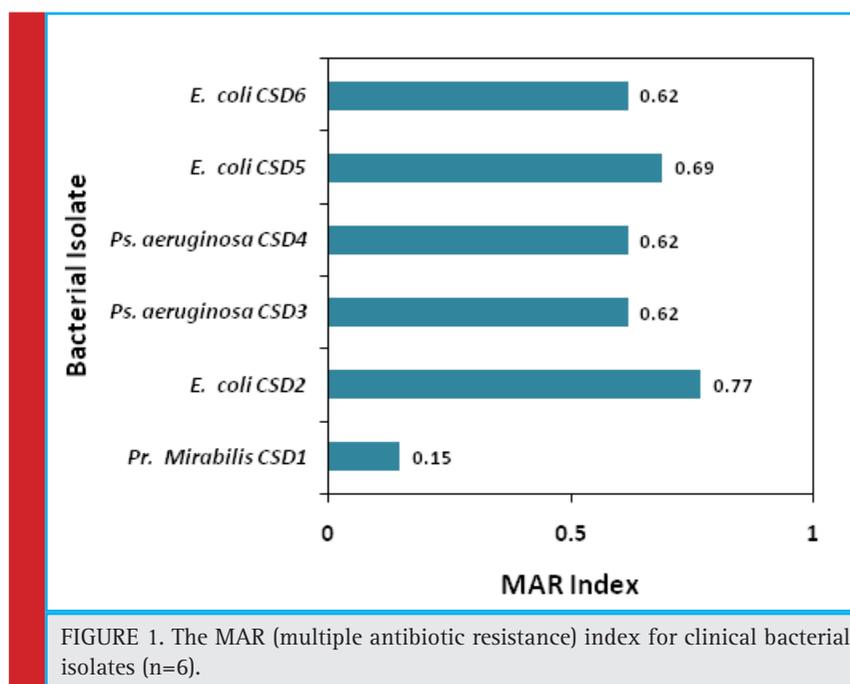
Table 2. Antibiotic resistance and heavy metal tolerance patterns of clinical bacterial isolates and their cured derivatives			
Bacterial isolates*	Resistance/tolerance patterns		Resistance patterns of cured bacteria
	Antibiotic resistance	Heavy metal tolerance	
<i>Pr. mirabilis</i> CSD1	Cx-Pc	Hg ²⁺ -Cd ²⁺ -Cr ⁶⁺ -Cu ²⁺	Pc
<i>E. coli</i> CSD2	Am-Ce-Cp-Ct-Cx-Mp-Nx-Pc-PT-Tc	Hg ²⁺ -Cd ²⁺ -Cr ⁶⁺ -Cu ²⁺	ND
<i>Ps. aeruginosa</i> CSD3	Am-Ce-Cm-Ct-Cx-Nx-Pc-PT	Hg ²⁺ -Cd ²⁺ -Cr ⁶⁺ -Cu ²⁺	Nx-Pc-PT
<i>Ps. aeruginosa</i> CSD4	Am-Ce-Cp-Ct-Cx-Nx-Pc-PT	Hg ²⁺ -Cd ²⁺ -Cr ⁶⁺ -Cu ²⁺	ND
<i>E. coli</i> CSD5	Am-Ce-Cp-Ct- Cx-Mp-Nx-Pc-PT	Hg ²⁺ -Cd ²⁺ -Cr ⁶⁺ -Cu ²⁺	Cp-Mp-Nx-Pc-PT
<i>E. coli</i> CSD6	Am-Ce-Cm- Cp-Cx- Mp-Nx-Pc	Hg ²⁺ -Cd ²⁺ -Cr ⁶⁺ -Cu ²⁺	ND

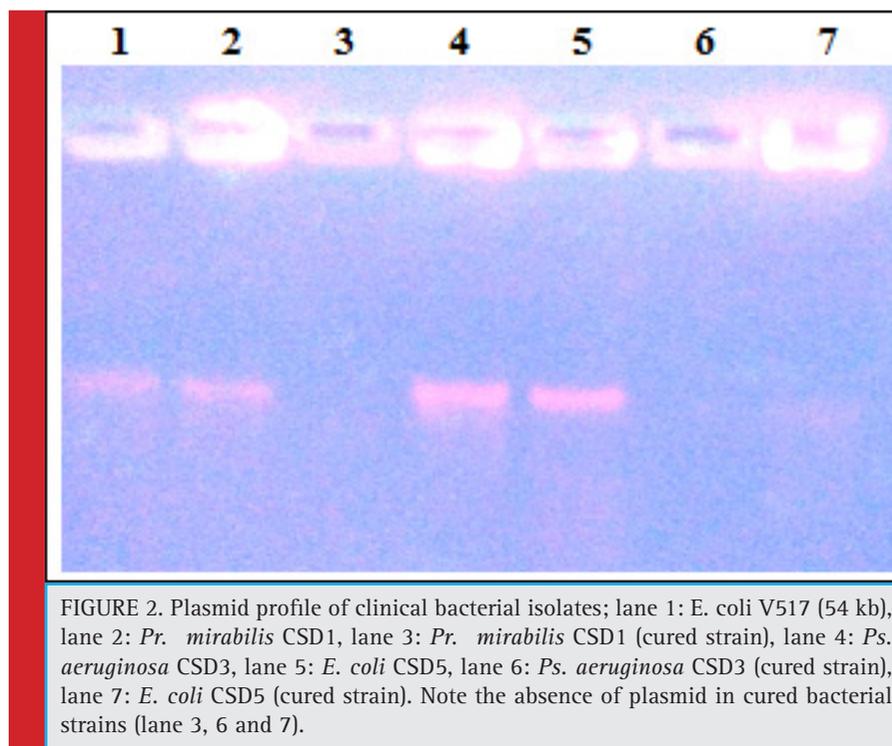
*The clinical bacterial isolates possessed a single plasmid of ≈54 kb, and the cured bacterial strains were plasmid-less. ND: curing not done.
Am: ampicillin, Ce: cefpodoxime, Cm: chloramphenicol, Cp: ciprofloxacin, Ct: cefotaxime, Cx: ceftiofur, Gm: gentamycin, Mp: meropenem, Nx: nalidixic acid, Pc: piperacillin, PT: piperacillin/tazobactam, Tc: tetracycline.

for *E. coli* isolates. Thus, considering the fact of origin of bacterial contamination from human-fecal sources, based on the MAR indices of >0.4 (Tambekar et al., 2005; Kaneene et al., 2007), and from high risk zone of contamination with antibiotics, based on the MAR indices of >0.2 (Krumperman, 1983), the currently studied clinical bacteria (*Ps. aeruginosa* and *E. coli*) might have been originated from niches with human-fecal contamination, due to antibiotic selection pressure.

The bacterial heavy metal tolerance has been depicted in Table 2. As has been reported by Mustapha and Halimoon (2015), the bacterial isolates from industrial effluents had tolerance to Cd²⁺, Cr⁶⁺, Pb²⁺ and Cu²⁺, at the concentration of 50 µg/ml, while one of the isolate

showed resistance to high level of Cu²⁺ (200 µg/ml), and for one isolate the Cd²⁺ MIC (minimum inhibitory concentration) was recorded as high as 200 µg/ml. Zhu et al. (2013) determined the MICs of Pb²⁺, Cu²⁺, Zn²⁺, Cr⁶⁺ and Hg²⁺ as 125, 100, 100, 100 and 25 µg/ml, respectively, for the livestock isolate of *Ps. fluorescens*, and recorded the occurrence of enhancement of bacterial resistance to antibiotics due to the presence of some heavy metals at certain concentrations. *Ps. aeruginosa*, *Ps. putida* and *Klebsiella pneumoniae* had Cd²⁺ MICs 300 – 950 µg/ml; such isolates had Zn²⁺ MICs of 1150, 1100 and 2000 µg/ml, respectively, and Hg²⁺ MICs of 20, 80 and 90 µg/ml, respectively (Yamina et al., 2014). *Ps. aeruginosa* and *E. coli*, isolated from Mahananda river water,





Malda (India) had resistance to Cd²⁺ and Hg²⁺ (Das et al., 2016). The continued usage of heavy metals since the ancient, in medicine and other anthropogenic purposes, select heavy metal resistant bacteria in polluted niches. For the bacteria utilized in the current study, the level of tolerance to Hg²⁺, Cd²⁺, Cr⁶⁺ and Cu²⁺ ranged 3 – 37.5 µg/ml, 75 – 800 µg/ml, 100 – 400 µg/ml and 600 – 900 µg/ml, respectively (Table 3). This communication is, for the first as we believe, to demonstrate the heavy metal tolerance among clinical bacterial isolates of *E. coli*, *Pr. mirabilis* and *Ps. aeruginosa* from our part of the globe (West Bengal state, India).

The plasmid profile of clinical bacterial isolates and the cured derivatives are represented in Figure 2. Apprehension grows in recent times in connection with the co-selection for antibiotic resistance among bacteria on

exposure to heavy metals, in several ecological niches (Wales and Davies, 2015). Because the heavy metal (pollution) acts discriminatorily as a selective agent in the emergence and propagation of antibiotic resistance among bacteria, wherein, along with the genes conferring antibiotic resistance, metal tolerance genes are also encoded in the same plasmids (Foster, 1983; Fang et al., 2016). The clinical isolates of *Pseudomonas* spp., as reported by Rajasekar and Mohankumar (2016), had resistance to multiple antibiotics and heavy metals, and the resistance properties were shown to be plasmid (10 kb) mediated. A conjugative plasmid (≈56.4 kb), carrying resistance to multiple heavy metals, such as, Hg²⁺, Cu²⁺, Pb²⁺, Cd²⁺, and also antibiotics was detected among the isolates of *E. coli* and *K. pneumoniae* causing nosocomial infections (Karbasizaed et al., 2003). Das et al.

Bacterial isolates	MTC of heavy metals (µg/ml)			
	HgCl ₂	CdCl ₂	K ₂ Cr ₂ O ₇	CuSo ₄
<i>Pr. mirabilis</i> CSD1	12.5	75	250	700
<i>E. coli</i> CSD2	37.5	500	250	600
<i>Ps. aeruginosa</i> CSD3	3	800	400	800
<i>Ps. aeruginosa</i> CSD4	9	100	200	900
<i>E. coli</i> CSD5	25	500	250	600
<i>E. coli</i> CSD6	9	100	100	800

MTC: maximum tolerance concentration

(2016) reported the occurrence of R-plasmid (antibiotic resistance plasmid) encoding heavy metal tolerance among *E. coli* and *Ps. aeruginosa*, isolated from river water. Herein, we demonstrated the involvement of ~54 kb plasmid (Figure 2) conferring heavy metal tolerance to Hg²⁺, Cd²⁺, Cr⁶⁺ and Cu²⁺, with associated multiple antibiotic resistances among the clinical bacterial isolates (Table 2).

The co-resistance to antibiotics and heavy metals has been reported among bacterial food pathogens (Wales and Davies, 2015). Wright *et al.* (2006) reported highest occurrence of heavy metal tolerance and antibiotic resistance among bacteria isolated from the contaminated most location, indicating the direct selection of heavy metal tolerant bacteria due to the exposure of heavy metals, thereby co-selecting bacterial antibiotic resistances. The *E. coli* isolates from urinary tract infection cases harbored copper/silver resistance genes, '*pco/sil*' with MIC of 500-µg/ml, presenting resistance to extended spectrum β-lactam antibiotics, too (Sutterlin *et al.*, 2018). Co-spread of antibiotic resistance (β-lactams: *bla*CTX-M; quinolones: *oqxAB*; aminoglycosides: *aac-Ib-cr*; amphenicols: *floR*; fosfomycin: *fosA3*) as well as the heavy metal resistance (Cu: *pco*; Ag: *sil*) genes, have been shown to be plasmid mediated (Zhu *et al.*, 2013). The Cd²⁺ resistant isolates of *Ps. aeruginosa* and *Ps. putida* showed multidrug resistance to kanamycin (Km), oxacillin (Oc), Nx and sulfonamids, while *K. pneumoniae* had resistance to Ct in addition to Km, Oc, Nx and sulfonamids resistances (Yamina *et al.*, 2014). In the earlier study, antibiotics (Am-Cm-Ce- Cx-Tm) and heavy metals (Cd²⁺-Hg²⁺) co-resistances have been reported among the river water bacteria (Das *et al.*, 2016). The co-resistance to heavy metals and antibiotics is, thus, a global concern, and the phenomenon among clinical bacteria, in our part of the globe, is not uncommon. The plasmid mediated resistance to the test heavy metals and to a number of antibiotics, as has been supported by SDS curing, approved the fact of heavy metal-antibiotic co-resistance in *Ps. aeruginosa*, *E. coli*, and *Pr. mirabilis* clinical isolates. The bacterial isolates displaying MAR indices of >0.4 have been regarded to be derived from human-fecal contaminated niches (Tambekar *et al.*, 2005; Kaneene *et al.*, 2007) and the bacterial isolates, with MAR indices of >0.2, deemed to be originated from highly antibiotic polluted regions (Krumperman, 1983). Still, the heavy metal inducing phenomenon of bacterial antibiotic resistances suggests that the emergence of multiple antibiotic resistant bacteria might be due to either the heavy metal or the antibiotic selection pressure, or both, and hence the bacterial high MAR index does also mean their (bacteria) origin from a region with high metal pollution, too.

CONCLUSION

The human pathogenic bacteria (*Ps. aeruginosa*, *E. coli*, and *Pr. mirabilis*) had resistance to two or more antibiotics, which in association with heavy metal resistance were found to be plasmid linked. This study endorses the dissemination of bacterial antibiotic resistance under the heavy metal as well as antibiotic selective pressure. Therefore, regular inspection of antibiotic resistance plasmid among human pathogenic bacteria, from our part of the globe, is urgently needed, in order to combat the bacterial multiple antibiotic resistance as well as the bacterial infection to humans.

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