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Role of heavy metals pollution on emergence of antibiotic co-resistance in *E. coli* isolates

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Abstract

Background: Heavy metal pollution in water is one of the most serious environmental problems. These findings have prompted the idea that metal-exposed bacteria may have altered resistance to antibiotics. This study was performed to investigate the presence of heavy metal resistance genes (HMRGs) in *Escherichia coli*.

Methods: HMRGs and antibiotic resistance of 100 *E. coli* from sewage and fresh water were detected by PCR. Minimum inhibitory concentrations (MICs) of heavy metals were determined by the broth micro dilution method. Antibiotic discs used to detect multidrug resistance, was recovered and assembled using third-generation sequencing.

Results: The frequency of different HMRGs in *E. coli* ranged from 1–67%., while prevalence of ESBL genes ranged 5-17% in freshwater isolates, and 54- 96% in sewage isolates. MICs of heavy metals for *E. coli* ranged widely from ⩽8.0-800 mg/L. Moreover, HMRGs (PbRT, cadD arsB, PcoA, czrc, and chrA) were found to be significantly associated with one or more ARGs (tetA, blaTEM, blaSHV and blaCTX) ($P < 0.05$).

Conclusion: In conclusion, HMRGs were widely present in *E. coli* isolated Dujla river and sewage water were significantly associated with drug resistance genes (DRGs). It is remarkable that the coexistence of HMRGs, DRGs and ARGs confer co-resistance to heavy metals, and antibiotics.

Introduction

Globally, the number of diseases brought on by bacteria resistant to antibiotics is increasing [1]. Some publications have already begun to describe the start of the post-antibiotic age, with the consequence of increasing difficulties in treating infectious diseases, due to this key development related with the loss of antibiotics' therapeutic ability. Reduced use of antibiotics does not always stop the development and upkeep of antibiotic resistance in natural and clinical settings [2]. Consequently, we must devise new strategies to halt this concerning trend [3]. Because of this, it's critical to comprehend the mechanisms underlying the emergence and spread of antibiotic resistance, including its triggers that drive the evolution and spread of antibiotic resistance. According to [4], bacteria are typically the first microorganisms exposed to heavy metals found in the environment. Extended exposure of bacterial populations to environments contaminated with heavy metals creates a selection pressure that eventually results in the establishment of resistant variants [5]. In response to heavy metals, bacteria develop resistance mechanisms that include intracellular sequestration of lower molecular weight, cysteine rich proteins; precipitation of metals as phosphates, carbonates, and sulfides; metal volatilization via methyl or ethyl group addition; physical exclusion by electronegative components in membranes and exopolymers; and energy-dependent metal efflux systems [6,7].

These are frequently plasmid-borne and can disperse by lateral gene transfer across the bacterial community [8]. Scientists have discovered that when bacteria are exposed to these pollutants, they can become resistant not only to antibiotics but also to metals, creating a complex web of resistance mechanisms [9,10]. Penicillin, cephalosporins, and the monobactam aztreonam are among the majority of β-lactam antibiotics that are resistant to extended-spectrum β-lactamases (ESBLs). ESBL-producing organism infections have been linked to unfavorable results. Six Multidrug-resistant (MDR) and ESBL-producing *Escherichia coli*, which can result in potentially fatal infections, is a significant example of antibiotic resistance [11]. The accumulation and spread of resistance in both clinical and environmental *E. coli* highlights the urgent need for effective strategies against antimicrobial resistance, especially considering the challenges in developing new antimicrobial agents [12].

Methods

Isolation of bacterial strains

Water samples of Dujla River were collected approximately 200m, while sewage samples from the discharge point of effluent of waste pipes. Air samples were collected using the MAS-100 Eco (Merck) on Petri dishes filled with CCA ES medium (Chromo Coliform Agar Enhanced Selectivity, Merck), according to [13]. Samples were cultured by direct streaking method on Eosin Methylene Blue and MacConkey agars, and then incubated for 18–24 h at 37°C [10]. The tests were carried out on triple sugar iron agar media, urea, and indole, methyl-red, Voges–Proskauer, and citrate (IMViC) media. Bacterial incubation in the triple sugar iron agar and urea media was carried out at 37°C for 18–24 h. IMViC test was carried out at 37°C for 48 h. Test results referring to *E. coli* were then stained with Gram stain to determine bacterial cell morphology.

Antibiotic Susceptibility Testing (AST)

The AST of bacterial isolated was done using Kirby Bauer disk diffusion methods as per the standard protocol from clinical laboratory standard institute (CLSI) guidelines 2024 [14].

Determination of the minimum inhibitory concentrations (MICs) of heavy metals

The broth micro dilution method was used to determine the MICs of heavy metals as described elsewhere [15]. In brief, all 100 samples of *E. coli* were cultured in tryptic soy agar medium overnight at 37 C. The bacterial suspension was diluted to a 0.5 McFarland standard with sterilized saline water. Seven heavy metals were selected, including cobalt (Co, $CoCl_2$), zinc (Zn, $ZnSO_4$), manganese (Mn, $MnCl_2$), chromium (Cr, $CrCl_36H_2O$), cadmium (Cd, $CdCl_2$), copper (Cu, $CuCl_22H_2O$) and lead ($PbCl_2$). The compounds were purchased from Sigma Aldrich (USA). MICs were determined as the lowest concentration of heavy metal that completely inhibited the growth of a strain after 18–20 h of cultivation at 35 C.

PCR amplification of HMRGs and ARGs genes

According to the manufacturer's instructions, the PrestoTM Min. gDNA bacteria kit (Geneaid) was used to extract bacterial DNA. PCR reactions were prepared in 25 µL volumes containing 4 µL of template, 2 µl forward primer (20 µM), 2 µl reverse primer (20 µM), 12 µl MyTaqTM Red Mix (2×), and added ddH2O to 25 µl. Primer used in the study were listed in Table (1). Thermal Cycler T100TM (Bio-Rad) was used to carry out PCR amplification. Pre-denaturation was carried out at 95°C for 1 min. Amplification of 30 cycles consisted of denaturation at 95°C for 15 sec, annealing at 58°C for 15 sec, and extension.

Gene	Primer sequence	Ta	Size	Reference
copA	F: GAAATAGCTCATTGCCGAGGCGTT R: CGGTCTCTACGAATACCGCTTCAA	55	475	[16]
cadD	F: AATTGCAAGTTGTGGTGCAG R: CCCACACCAGGAATTCTAGC	57	155	[17]
czrC	F: TAGCCACGATCATAGTCATG R: ATCCTTGTTTTCCTTAGTGACTT	47	654	
chrA	F: TGGCTCTCGCTGTTCTTTGT R: TAAGTGCGACAAGGGCAACT	53	520	[18]
pcoA	F: CGTCTCGACGAACTTTCCTG R: GGACTTCACGAAACATTCCC	61	1791	[19]
pbrT	F: AGCGCGCCCAGGAGCGCAGCG TCTT R: GGC TCG AAG CCG TCG AGR TA	57	448	[18]
bla_{SHV}	F: AGCCGCTTGAGCAAATTAAAC R: ATCCCGCAGATAAATCACCAC	58	713	[20]
bla_{CTX}	F: CGTTAACGGCACGATGAC R: CGATATCGTTGGTGGTRCCAT	56	404	
bla_{OXA}	F: CATTTCCGTGTCGCCCTTATTC R: CGTTCATCCATAGTTGCCTGAC	57	800	
bla_{TEM}	F: GATCTCAACAGCGGTAAG R: CAGTGAGGCACCTATCTC	55	786	

Table 1: Primer sequences Ta and product size, used to detect antibiotic resistance genes in *E. coli* isolates.

Methods

Antibiotic susceptibility profile

The resistance phenotypes of *E. coli* isolated from the fresh water Dujla river (n = 50), and wastewater (n = 50) were determined by measuring MICs to the 13 antibiotics listed in Table (2). Ninety-six percent of isolates from either river were resistant to one or more of the antibiotics tested. Seventy-one percent of all isolates were resistant to three or more antibiotics. Resistance to amoxicillin, trimethoprim/ sulfamethoxazole and ciprofloxacin was most frequent (71, 67, and 66% respectively of all wastewater isolates.

While resistance to cefotaxamin, Ceftriaxone, Cephalexin acid, and Cefoxitin was less frequent but still relatively high (54-57% of all isolates). Ciprofloxacin resistance (MIC > 4 µg/ml) was observed in 20 isolates of fresh water and 66 from wastewater. Seven of these isolates were resistant to amikacin and ciprofloxacin (MICs > 32 µg/ml and > 4 µg/ml, respectively) and were classified as uropathogenic strains. By comparing resistance frequencies in Dujla and wastewater isolates, we observed significant differences in total resistance to antibiotic. However, resistance to trimethoprim / sulfamethoxazole in isolates from wastewater was about eight times higher than that in wastewater isolates. In general, data show a high prevalence of antibiotic resistance in *E. coli* isolates from both sources. This is consistent with our previous studies of *E. coli*.

MIC of heavy metals

Sewage water *E. coli* isolates show high difference (p value <0.05) in MIC for both copper and lead (1600 mg/L, 320 mg/L Respectively) while other heavy metal shows no significant differences, either in sewage or in fresh water, Table (3). Generally, the MICs of heavy metals for swage water *E. coli* isolates ranged from ⩽30 mg/L to 800 mg/L. The MIC ranges for the seven selected heavy metals were as follows: Pb, ⩽8–450 mg/L; Cr, 40–150 mg/L; Zn, 30–800 mg/L; Co, 25–100 mg/L; Cu, 80- 400 mg/L; and Cd, 10–45 mg/L.

For freshwater *E. coli* isolates, the lowest MIC found was ⩽8 mg/L for Pb and the highest MIC was 300 mg/L for Cu. Table (3).

Sewage isolates					Fresh water isolates			
Antibiotics	R		I		R		I	
	n	%	N	%	n	%	N	%
Amx	71	70.3%	14	13.9%	54	53.5%	21	19.8%
Amc	16	15.8%	15	14.9%	7	6.9%	12	11.8%
CIP	66	65.3%	1	1.0%	20	10.6%	1	1.0%
CXT	57	56.4%	31	30.7%	16	15.8%	7	6.9%
CRX	55	54.5%	4	4.0%	8	7.9%	0	0.0%
CLX	56	55.4%	2	2.0%	17	16.8%	1	1.0%
CTX	54	53.5%	7	6.9%	8	7.9%	2	2.0%
IMP	4	4.0%	1	1.0%	0	0.0%	1	1.0%
FUR	7	6.9%	6	5.9%	3	2.9%	3	2.9%
AMK	7	6.9%	0	0.0%	0.0%	0.0%	2	2.0%
CTZ	27	26.7%	1	1.0%	27	26.7%	1	1.0%
TMP_SMX	67	66.3%	34	33.7%	11	10.7%	8	7.9%
DOX	18	17.8%	29	28.7%	2	2.0%	0	0.0%
P-Value	< 0.001							

Amoxicillin (Amx), Amoxicillin/Clavulanic acid (Amc), Ciprofloxacin (CIP), cefotaxamin (CTX), Ceftriaxone (CRX), Cephalexin (CLX), Cefoxitin (CXT) Imipenem (IMP), Nitrofurantoin (FUR), Ceftazidime (CTZ), Amikacin (AMK), Trimethoprim and Sulfamethoxazole (TMP_SMX) and Doxycycline (DOX).

Table 2: Frequency of swage isolates antibiotic resistance (R) and sensitive (S) and intermediate (I).

	MIC (mg/liter) for study sewage isolate n= 50 *E. coli*		MIC (mg/liter) for study isolates fresh water n= 50 *E. coli*	
Compound	Min.	Max.	Min.	Max.
Nickel	20	100	20	60
Zinc	30	800	30	250
Copper	80	400	80	300
Chromium	40	150	8	80
Cadmium	10	45	10	25
Cobalt	25	100	15	40
Lead	8	450	8	90

Table 3: MIC of heavy metal tested for minimum and maximum.

Prevalence of heavy metal resistance genes

Six HMRGs, including chrA, pcoA, arsB, pbrT, *cad*D and *czrc*, were detected in *E. coli* isolates Figure (1). In sewage, the *chrA* gene was most widespread 67%, followed by arsb (48), pcoA (45%) then by nearly the same percentage of both pbrT and cadD genes (30 and 28% respectively), whilst the frequency of *czrc* was lowest (6 %). In fresh water, the frequency of *arsB* was the highest 17% and the frequency of czrc was lowest 1%. In general, frequencies of HMRDs in sewage water are much higher than corresponding ones of fresh water, Figure (1).

Prevalence of ESBL genes

Four ESBL genes, including CTX, CTX-M, bla_{SHV}, OXA, were detected in *E. coli* isolates Figure (2). In sewages samples, *E. coli* isolates, showing prevalence ESBL genes ranged from 96- 23, and as follows: OXA (96.1%) > CTX-M (75%) > bla_{SHV} (70%) > CTX (54%). On the other hand, freshwater *E. coli* isolates, showing lesser

percentages and as follows: CTX (54%) > bla_{SHV} (70%) > CTX-M (75%) > OXA (96.1%).

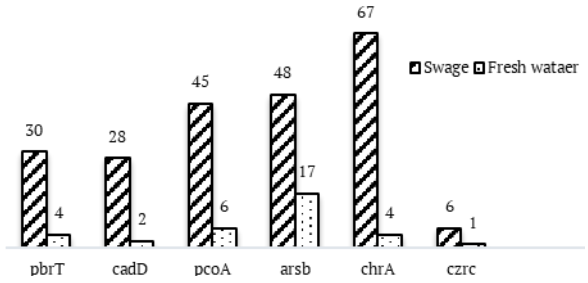

Figure 1: Prevalence of heavy metal resistance genes in *E. coli.* pbrT confers resistance to lead (pb); cadD confer resistance to cadmium; pcoA confers resistance to copper (Cu); czrc confers resistance to zinc (Zn) and nickel (Ni); and chrA confers resistance to chromium (Cr).

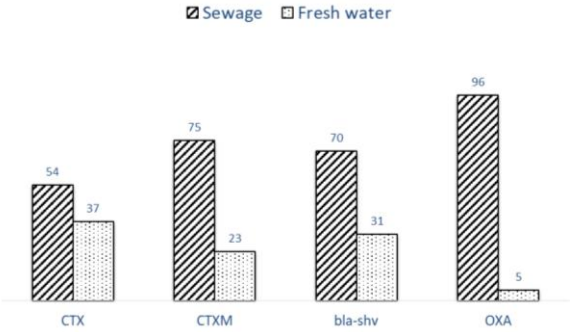

Figure 2: Prevalence of ESBL genes in *E. coli* isolates.

Correlation between HMRGs and ARGs

As shown in Table (4), cadD showed significantly positive correlation with CTX (r=0.96), bla_{SHV} (r=0.947). pbrT shows a correlation with CTX-M (r=0.95) and with bla_{SHV}. arsB gene is correlated with both CTX-M and bla_{SHV} (r=0.94, and 0.95 respectively). pcoA gene has strong correlation with CTX-M (r=0.991) (r=0.980), finally, chrA gene shows no relation with any Args tested (p value greater than 0.05).

Antibiotic resistance genes	Heavy metal resistance gens				
	chrA	pcoA	arsB	pbrT	cadD
CTX	0.542	0.764	0.87	-0.09	0.96*
CTX-M	0.212	0.991**	0.94**	0.95*	0.874
blaSHV	0.185	0.432	0.95*	0.991**	0.947*
OXA	0.643	0.947*	0.825	0.264	0.721

Table 4: Correlation between HMRGs and ARGs (* Significant at P < 0.05, * * Significant at P < 0.01).

Discussion

The unsupervised consumption of antibiotics, excessive intake, and improper independent use are the major contributors for the emergence of antibiotic resistance bacteria which are already freely lived in our environment. Heavy metal pollutants could help persist with these bacteria and emerging new ones. In the study, we examined the presence of heavy metal resistance genes among bacterial strains harboring ESBL genes collected from sources, sewage and fresh water. The most important finding of this work was co-occurrence of antibiotic and heavy metal resistance elements among bacterial strains and their co-transfer to the host strain. The 100 tested isolates had an elevated level of β-lactam antibiotics resistance.

The rise in antibiotic resistance could stem from changes in efflux or metal sequestration processes, likely due to a coordinated response triggered by both metal and antibiotic stress. Various genetic responses at the transcriptional and translational levels can be interconnected to create a unified reaction to these dual stresses [21]. However, heavy metal resistance capability when coinciding with antibiotic resistance can lead to an increase in antibiotic resistance and enhance their environmental stability. Antimicrobial-resistant *E. coli* showed high resistance to beta-lactams. Correlation study showed a strong correlation between HMRGs and ARGs, indicating co-resistance, Wastewater likely contributes to this resistance due to the overuse of these antibiotics, resulting in antibiotic residues and the development of bacterial resistance.

ESBL-harboring *E. coli* was found to be correlated with HMRGs. ESBL positive bacteria, found in open aquatic environments affected by human activities, have been globally reported. The genes responsible for ESBL production are associated with genetic elements that promote the spread of resistance [22-25], it may also be responsible for HMRGs too.

The origins of metals like mercury, cadmium, copper, and zinc vary, stemming from sources such as solid waste disposal [26,27]. These metals, as they accumulate in the environment, can reach critical levels that trigger a co-selection mechanism alongside antibiotic resistance [28]. Interestingly, the prevalence of heavy metal resistance genes exhibited a positive correlation with cadmium levels, indicating a significant role of this metal in selecting such genes. Our research findings indicate that at the highest concentrations, 10% of the samples exhibited resistance to cadmium, while 20% showed resistance to lead.

E. coli isolate shows resistant to both antibiotics and heavy metals, which is a major concern for public health. These bacteria can survive in conditions with heavy metal levels way above what's considered safe. What's more concerning is that these bacteria are the same types that often cause infections in humans. The ones that are resistant to multiple drugs can also withstand high levels of heavy metals. It's like they've co-evolved to survive under stress of antibiotic and heavy metals at the same time. This suggests that pollutants like heavy metals might be helping these bacteria become even more resistant to drugs. It is also

noticed that the heavier metals there were, the more resistant these bacteria became.

Author Contributions

Assel R. Kadhim conducted the laboratory work.
Melad K. Mohammed designed the experiments.
Ahmed D. Jabbar wrote the manuscript and data analysis

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Conflict of Interest

The author declare that there is no conflict of interest regarding the publication of this paper.

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