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Co-selection and Cross-resistance Mechanisms Promoting the Antibiotic Resistance in *E.Coli*

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Abstract

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. One hundred water samples were collected including 50 freshwater form Tigris River, were positive for bacterial growth. Besides, fifty samples from sewage were also positive for bacterial growth in Al-Kut, Wasit. Aliquots of the samples were used for selective isolation of *E coli*. Heavy metals and antibiotic resistance of 100 *E. coli* from sewage and fresh water were detected by polymerase chain reaction (PCR). Minimum inhibitory concentrations (MICs) of heavy metals were determined by the broth micro dilution method. Antibiotic discs used the frequency of different heavy metals resistance genes in *E. coli* ranged from 1 to 67 %. While prevalence of ESBL genes ranged from 5 to 17 % in freshwater isolates, and 54-96 % in swage isolates. Sewages isolates show a prevalence of ESBL resistance genes, in comparison with corresponding results of freshwater isolates. MICs of heavy metals for *E. coli* ranged from ≤ 8.0-1500 mg/L., heavy metal resistance genes (PbRT, cadD arsB, PcoA, czrc, and chrA) were found associated with one or more ARGs (blaOXA, blaTEM, blaSHV and blaCTX) ($P < 0.05$).

Keywords: Antibiotic Resistance, *E. Coli*, Freshwater.

1. Introduction

Antimicrobials elements, which are effective in striving pathogenic microorganisms, have been widely used in the prevention and treatment of diseases since their discovery in the 20th century [1]. The extensive use of antimicrobials led to leakage and residue in the environment [2]. Antimicrobials are discharged directly released into nature or through host metabolism, especially in aquaculture and agricultural applications [3]. Additionally,

metal-induced co-selection greatly expands the environmental resistance and increases the health risk of antibiotic resistance in environments [4]. These pollutants have influence on the enrichment and transfer of ARGs and the generation of multi-drug-resistant bacteria, these variable and uncontrollable factors may lead to more serious harm from ARGs and antimicrobials [5].

Concern over the possibility that a metal-contaminated environment could serve as a pool for the sequestration of antibiotic resistance genes in clinical isolates as well as environmental isolates is growing [6]. Comprehending the potentialities of these isolates will aid in identifying risk variables linked to pan-drug resistance and bolstering the deliberate creation of novel and efficacious antimicrobial medicines [7]. *E. coli* is receiving increased research attention based on observation that most commensal microorganisms display inherent resistance to specific antimicrobial agents [8].

The importance of these organisms to AMR risks are reinforced by recent findings showing that the use of broad-spectrum antibiotic treatments tends to select for resistant microorganisms within the human microbiome [9]. The accumulation and spread of resistance in both clinical and environmental *E. coli* highlight the urgent need for effective strategies against antimicrobial resistance, especially considering the challenges in developing new antimicrobial agents. The considerations and further investigations of co-selective agents and the efficacy of metal-based interventions to better manage and combat the global antibiotic resistance crisis within the one Health framework still need more work.

The project focus on investigation of the spread and emergence of environmental bacteria resistant to heavy metals in contaminated water. Also evaluate the impact of heavy metal contamination as a selective agent in the spreading of antibiotic resistance.

2. Materials and Methods

2.1 Samples Collection

A total of 100 freshwater, and sewage samples from December 2022 to February 2023 were collected in sterilized sample bottles in Al-Kut, Wasit Province, Iraq. Samples were collected aseptically in sterile 200 mL Duran Schott glass bottles from different sampling points by directly dipping the bottles into the surface of the water. Samples were labelled properly and transported on ice to the laboratory for analysis. Aliquots of the samples were used for selective isolation of *E coli*.

2.2 Identification

Isolates were identified by colony morphology (shape, structure, colour, pattern and size), and Gram's staining [10]. Biochemical tests of collected isolates were identified before performing molecular experiments to ensure their purity as they were stored for months in the refrigerator during the period of isolates collection and identification.

2.3 Antimicrobial Sensitivity Test

Disc diffusion method was applied according to the instructions of CLSI [11].

2.4 Molecular Detection

2.4.1 PCR-Based Detection of *E. coli*

Primers were supplied by the manufacturer (Integrated DNA Technologies, USA) as a lyophilized powder in Eppendorf tubes (1.5 ml).

2.4.2 DNA Extraction

DNA of isolates of *E. coli* were extracted depending on the instruction of Presto Mini gDNA bacteria Kit (Geneaid, Korea).

2.4.3 Detection Resistances Genes of *E. coli*

Resistance genes detection was performed by multiplex PCR resistance genes and divided into three fields as shown in (table 1).

Table 1: Resistances genes fields.

Resistance genes	Product size	Reaction Conditions (Steps)
PbrT	287	Initial denaturation at 95 ºC for 3 minutes. Then, 35 cycles of denaturation at 95 ºC for 30 seconds. Annealing at 55 ºC for 40 seconds, extension at 71 ºC for one minute, and final extension for 5 minutes at 72 ºC.
ChrA	830	
BlaOXA	643	Initial denaturation at 95 ºC for 3 minutes. Later, 35 cycles of denaturation at 95 ºC for 30 seconds. Annealing at 58 ºC for 30 seconds. Extension at 72 ºC for one minute, and final extension for 5 minutes at 72 ºC
blaCTX-M	860	

This was performed by multiplex PCR resistances genes were divided into three fields shown in (table 2).

Table 2: Components of 25 µL working reaction for detection of resistance genes.

Master mix	Primers	D.W	DNA
12.5 µL	Forward 1 µL (10 p mol) Revers 1 µL (10 p mol)	5.5 µL	5 µL

2.4.4 Agarose Gel Electrophoresis

Electrophoresis process accomplished to detect the amplified PCR products. Electrophoresis results were identified using a UV-Transilluminator system. DNA bands were measured

according to the ladder DNA. Positive results were distinguished when there was DNA band equal to the target product size.

2.4.5 Heavy Metal MIC Determination

Minimal inhibitory concentration (MIC) was determined according to method that was described by Wayne and co-workers in 2021 [11].

2.4.6 Statistical Analysis

Data of different results were analysed using Statistical Analysis System (SPSS- version 26) [12]. Percentages were compared by using the chi-squaer test. P-Value > 0.05 is considered statistically significant. Toward calculate the correlation between antibiotic resistance genes and heavy metal resistance genes. Pearson's correlation coefficient was adopted. The method could measure the strength and direction of the linear relationship between two variables.

3. Results and Discussion

Sampling was carried out, and a total of 100 samples were collected, 50 samples from freshwater, Tigris River and 50 sewage samples from four different locations were collected from west water of different locations through Al-Kut city.

3.1 Isolation and Identification of *E. coli* Cultural Characteristics

Cultural characteristics of 100 isolates on different media such as (MacConkey agar, EMB and Blood agar) results were summarized in (table 3), and isolates gave typical features of *E. coli*.

Table 3: *E. coli* Cultural Characteristics.

Culture media	Characteristics
MacConkey agar	Pink color with precipitation of bile salt around colonies
Eosin Methylene Blue Selective and differential media	Growth with green metallic sheen colonize which is characteristic feature for *E. coli* from other gram-negative pathogens
Blood ager	Growth with circular, moist colonies with gray color

3.2 *E. coli* Antibiotics Susceptibility Test

E. coli susceptibility tests were performed using a disk diffusion test to detect antibiotic resistance according to the standard method indicated by the Sewage and Laboratory Standards Institute [11]. All antimicrobials included in this study showed significant differences in resistance rates among sewage and environmental isolates, as shown in (table 4) for sewage isolates and (table 5) for environmental isolates.

Table 4: Antibiotic percentage of sewage isolates.

Antibiotics	R		I	
	N	%	N	%
Amx	71	70.3	14	13.9
Amc	16	15.8	15	14.9
CIP	66	65.3	1	1.0
CXT	57	56.4	31	30.7
CRX	55	54.5	4	4.0
CLX	56	55.4	2	2.0
CTX	54	53.5	7	6.9
IMP	4	4.0	1	1.0
FUR	7	6.9	6	5.9
AMK	7	6.9	0	0.0
CTZ	27	26.7	1	1.0
TMP_SMX	67	66.3	34	33.7
DOX	18	17.8	29	28.7
P-Value	< 0.001			

Where, Amoxilline (Amx), Amoxilline/Glavulinc acid (Amc), Ciprofloxacin (CIP), cefotaxamin (CTX), Ceftriaxone (CRX), Cephalexin (CLX), Cefoxitin (CXT) Imepenim (IMP), Nitrofurantoin (FUR), Ceftazidime (CTZ), Amokacin (AMK), Trimethoprim and Sulfamethoxazole (TMP_SMX), and Doxycyklin (DOX).

The study Results for antibiotic resistance in sewage isolates indicates a high resistance in comparison to 71 % to AMP. by Hussein [13]. Ojdana et al., [14] reported results showing a resistance rate for ampicillin was 97.5 %. In contrast the current study results, different result from Borujerdi et al., [15] in which ampicillin resistance was about 22 % only.

Regarding the rate of ampicillin resistance, it may be coming a direct result of frequent use of this antibiotic in hospitals and to personal use for a long time due to the low toxicity and being well known for most people. That eventually led to the emergence of resistant strains. However, ampicillin usage is being reduced in hospitals due to less effectively [16].

Table 5: Antibiotic percentage of freshwater isolates.

Antibiotics	R		I	
	N	%	N	%
Amx	54	53.5	21	19.8
Amc	7	6.9	12	11.8
CIP	20	10.6	1	1.0
CXT	16	15.8	7	6.9
CRX	8	7.9	0	0.0
CLX	17	16.8	1	1.0
CTX	8	7.9	2	2.0
IMP	0	0.0	1	1.0
FUR	3	2.9	3	2.9
AMK	0.0%	0.0	2	2.0
CTZ	27	26.7	1	1.0
TMP_SMX	11	10.7	8	7.9
DOX	2	2.0 %	0	0.0
P-Value	< 0.001			

Where, Amoxilline (Amx), Amoxilline/Glavulinc acid (Amc), Ciprofloxacin (CIP), cefotaxamin (CTX), Ceftriaxone (CRX), Cephalexin (CLX), Cefoxitin (CXT) Imepenim (IMP), Nitrofurantoin (FUR), Ceftazidime (CTZ), Amokacin (AMK), Trimethoprim and Sulfamethoxazole (TMP_SMX), and Doxycyklin (DOX).

The percentage of resistance to Nitrofurantoin and Amikacin was 6.9 % for both sewage and freshwater isolates. This result is considered the lowest rate recorded by Garba et al. [17], that showed 22 % of *E coli* isolates were resistant to Nitrofurantoin. While Amikacin showed about 30 % resistance [18]. Results showed that freshwater *E coli* isolates show no resistance to Imipenem. In this study Imipenem resistance was 3 %, in sewage isolates which spite being so low but it is considerably higher than the results obtained by many other studies in different parts of the world including Iraq [19].

However, Hasan et al. [20], Polse et al. [21], Hegazy et al., [22], and other study results obtained by Ramatla et al. [23] indicated urinary tract infections *E coli* samples were all sensitive for the Imipenem antibiotic. The result in this study for Ceftazidime resistant in the sewage isolates was 26.7 %. Several reports were in matching line with the present study [24]. Ciprofloxacin resistance in the study was 65.3 % regarding sewage and freshwater isolates.

That agrees with the study of Das et al. [25] 23 % and at 77 %. While these results were not in matching line with Ojdana et al., [14] study where the resistance rate was 97 %. *E. coli* isolates from sewage sources showed 66.3 % antibiotic resistance for trimethoprim-sulfamethoxazole (TMP_SMX).

The most accepted regarding the high rate of resistance is the frequent use of this antibiotic in hospitals for a long time especially for treating intestinal infection, which led to the emergence of strains resistant. These results were comparable with which were 84 %. Other studies by Namaei et al. [26] reported that *E. coli* isolates were sensitive to TMP_SMX (IPM). Resistance is probably due to drug abuse which could result in plasmid mediated antibiotic resistance that was found to be common in E. coli [27].

Results of antibiotic susceptibility testing for freshwater isolates gave a revelation that nearly all the isolates were phenotypically multidrug-resistant. Percentage of resistance to amoxicillin (53.5 %), Amoxicillin/Clavulanic acid (6.9 %), Ciprofloxacin (10.6 %), Ceftazidime (26.7 %). While third generation cephalosporin include, cefotaxime, ceftriaxone, cephalexin, and cefoxitin were 15.8 %, 7.9 %, 16.8 %, and 7.9% respectively. The lowest was to Imipenem (0 %). Freshwater isolates showed lower rates of resistance to all under study antibiotic, except Ceftazidime where the resistance was equal (26.7 %).

The difference in the percentage from my point of view is because the collected samples are not only from sewage

water for hospitals, as samples were collected from sewage water for residential areas, as well as sewage water collected from places close to industrial facilities. Similar results were obtained by results revealed high frequency of resistance to the *β*-lactam group, cefoxitin (53.5 %), amoxicillin/clavulanic acid combination (43.5 %), cefotaxime (22.7 %), aztreonam (21.3 %), cefpirome (19.2 %), ceftazidime (16.2 %), and to the non-lactam group, trimethoprim /sulfamethoxazol (21.1 %), tetracycline (18.2 %), followed by ciprofloxacin (14.1 %).

The hospital effluent showed the higher rates of resistance to all antibiotic, except two (chloramphenicol and gentamicin) [27].

Enterobacteriaceae family from wastewater sources in Dubai, UAE showed high resistance to cefpodoxime (39 %) followed by cefotaxime (33 %), ceftizoxime (32.5 %), and ceftazidime (29 %) [28]. The study differed from the study conducted by for fecal coliform isolated from different freshwater sources which showed 66.7% resistance to ampicillin [28]. Fadare and his colleagues [29] demonstrated the highest resistance rates against ampicillin (95 %), tetracycline and doxycycline (88 %), and trimethoprim-sulfamethoxazole (85 %) antibiotics were most prevalent of selected Enterobacteriaceae from Tsomo and Tyhume rivers of South Africa.

While resistance to cefotaxime, 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 the wastewater. Seven of these isolates were resistant to amekacin and ciprofloxacin (MICs > 32 µg/mL and > 4 µg/mL respectively and were classified as uropathogenic strains. Comparing resistance frequencies in Tigris and wastewater isolates, we observed significant differences in total resistance to antibiotic.

However, resistance to trimethoprim / sulfamethoxazole in isolates from the 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.

3.3. Prevalence of ESBL Genes

Genes responsible for ESBL production are associated with genetic elements that promote the spread of resistance [30]. Genes may also be responsible of heavy metal resistance.

3.3.1 blaOXA Gene

blaOXA gene encodes class D *β*-lactamases which hydrolyzing oxacillin, penicillins and inactivate the cephalosporins and carbapenems. A high frequency of blaOXA (96 %) was found among sewage isolates, on compare with corresponding freshwater isolated which present in very low frequency (5 %), as shown in figure (1).

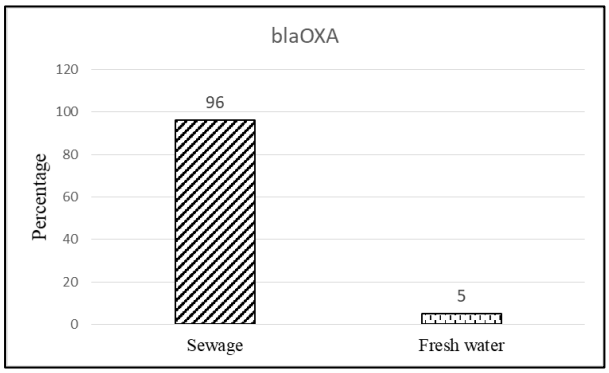

Figure 1: Prevalence of blaOXA among *E. coli* isolates from sewage and freshwater samples.

Different result with Ojdana et al., [31] study which shows 58 % of genes. The lack of proper use of this antibiotic and its provision to patients by doctors is the one reason for the emergence of this percentage, or a co-selection due to presence of heavy metals genes which may tend to persist together. Results of freshwater isolates were much less small blaOXA gene was 5 % only.

This may assist the previous conclusion of co-selection. Much like this result, are the results of a study carried out by Afsharnia et al. [32] which shows 91 % of blaOXA gene in heavy metal polluted areas.

3.3.2. Prevalence of blaCTX Gene

This gene is encoding cefotaxime resistance, the result of CTX gene in the sewage isolates was 54 %, as detailed in (figure 2). In comparison with the prevalence of CTX gene in freshwater isolates, results show no significant results since it's also abundant in these isolates too (37 %).

The high prevalence of CTX type was by other researchers In Al-Kut City, Iraq [33], revealed the occurrence of CTX in 50.8 %, (67.5 %) and (58.8 %) respectively. Among enteropathogenic *E. coli* isolates. Also, the high prevalence of CTX type was by (Harris et al., [34] reported the occurrence of blaCTX genes were 95 %, and 93.8 % of *E. coli*.

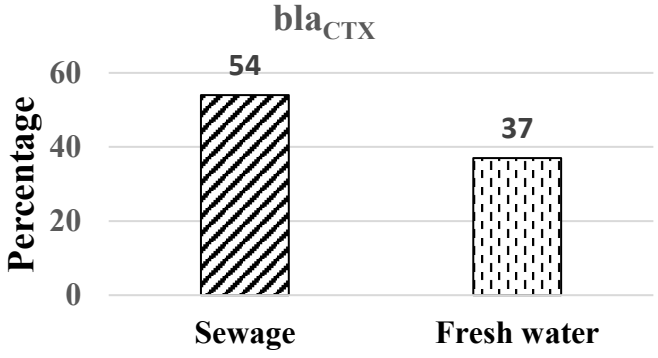

Figure 2: Prevalence of blaCTX among E coli isolates from sewage (n = 50) and freshwater samples (n = 50).

CTX genes are usually found on plasmids, and these plasmids are easily spread among microbial populations and carry resistance genes against other antibiotics such as aminoglycoside acetyltransferases and dihydropteroate synthases or other *β*-lactamases [35]. In general, the scientific and public health community must act now to address the growing threat of extended-spectrum beta-lactamase (ESBL)-producing organisms.

These resistant bacteria pose a significant challenge, as the window of opportunity for effective intervention is rapidly closing. Once certain critical thresholds are crossed, such as the absolute number of ESBL genes in the microbial world, their widespread dissemination across different bacterial species and clones, and their strong association with prevalent genetic platforms, controlling ESBL-producing bacteria. The increasing persistence of antibiotic-resistant bacterial pathogens is resulting in a worldwide increment of infections [36]. The loss of therapeutic efficiency of existing antibiotics, the constant decrease in the design and production of new antibiotics lead us to this problem.

The diversity of environmental bacteria is vast; the antibiotic resistance genes that cause problems in clinical settings are only the tip of the iceberg. Genes that are transferred from environmental strains to sewage pathogens go through a process of spread and diversification where they adjust to selection pressures unique to the sewage setting [37].

3.4 Determination of Heavy Metal Minimal Inhibitory Concentration (MIC)

Results revealed that most isolates exhibited robust growth at low levels of heavy metal concentrations, with their numbers gradually declining as the heavy metal ions concentration increased. In the case of zinc, 70 % of isolates could thrive at a concentration of 10 ppm whether it came from waste or freshwater.

On the contrary, swage isolates can resist copper ion up to 900 ppm on average, while freshwater isolates show lower resistance to that heavy metal (600 ppm). However, Cobalt proved to be the most toxic heavy metal, as no isolates were able to grow above 10 ppm. As for chromium, 28 % of all isolates demonstrated tolerance up to a concentration of 25ppm. Lead and chromium were identified as highly toxic heavy metals.

Since they successful inhibiting 82 % and 72 % of the isolates respectively at a concentration of (25 ppm for chromium and 100 ppm for lead), with no isolates able to grow beyond this threshold. On opposing,

isolates show a fair resistance to zinc and copper as 70 % of isolates can withstand a 25 ppm of zinc and 60 % of them can resist up to 900 ppm of copper. Cobalt heavy metal ions, from other hand show unavoidable toxicity to *E coil* sewage isolates, about on third (32 %) of them can pear up to 60 ppm cobalt.

Table 6: MIC measured in ppm for Heavy metal in sewage.

Element	Minimu. MIC	Maximum MIC	Average MIC	Isolates resist average concentration %
Ni	4	70	60	40
Zn	10	30	25	70
Cu	30	1800	900	60
Cr	8	40	25	28
Cd	10	45	20	36
Co	8	25	15	32
Pb	70	320	100	20

In comparison with the corresponding result of freshwater isolates, one can easily notice, not only they have a lower in minimum and maximum MICs but, a decrease of percentage of bacteria that can tolerate average MICs concentrations. For instance, Ni, Cd, and Pb in sewage isolates show 40 %, 36 % and 20 %. Can withstand the average MIC concentration while in compare with that of freshwater show incredibly lower percentages (35 %, 12 % and 17 % for the same heavy. Bacteria have evolved to tolerate elevated levels of heavy metals in different mechanisms.

This tolerance could depend upon several factors, namely type of metal ion deals with which transported into the cell, localization of metal resistance genes whether its harboured-on chromosome, plasmid or transposon, and the role of metal ion in the metabolisms [38]. However, bacterial develop evolutionary five mechanism to compromise the effect of heavy metal including: extracellular barrier; active transport of metal ions (efflux), extracellular sequestration, intracellular sequestration, and reduction of metal ions [39].

Table 7: MIC measured in ppm for Heavy metal in freshwater.

Element	Minimum. MIC	Maximum MIC	Average MIC	Isolate resist average concentration %
Ni	5	50	30	35
Zn	10	30	18	30
Cu	20	1200	600	26
Cr	4	20	10	8
Cd	4	20	10	12
Co	4	25	15	8
Pb	20	150	70	17
As	10	100	80	27

Where, several genes associated with heavy metal resistance were examined for their prevalence in both sewage and freshwater *E. coli* isolates.

3.4.1 PbrT Gene

Lead metal has diverse applications in both medicine and agriculture. The pbrT gene, which is part of Pb resistance operon, encodes a lead efflux pump that is responsible for transporting lead ions out of the bacterial cell, thereby reducing intracellular lead concentration and toxicity [40]. However, lead resistance is not exclusively attributed to that gene but also czrc gene involved in that process [25].

Results shown in (figure 3) showed that about 60 % (30/50) of sewage *E. coli* isolates against freshwater which has 6 % (3/50) were harbouring the gene which made them resistant to leads in pollutant water. Lead can disrupt normal functions by interfering with essential enzymes. It binds to the active sites of these enzymes or replaces important metal ions like calcium, zinc [41] or magnesium. This interference hampers vital metabolic processes, such as those needed for energy production and DNA synthesis [42].

The pbrT protein is one large family of proteins encoded in the widely bacteria, and they include PbrT, PbrA, PbrB, PbrC, PbrD and PbrR [43]. PbrT is a lead-specific efflux pump that transports lead ions out of the cell. This reduces the intracellular concentration of lead, thereby minimizing its toxic effects. Pollution with lead (Pb) will inevitably lead to the emergence of resistant bacterial strains. The presence of heavy metals, including lead, in the environment exerts selective pressure on microbial populations, favouring the survival and proliferation of those that have acquired or evolved mechanisms to tolerate these toxic substances.

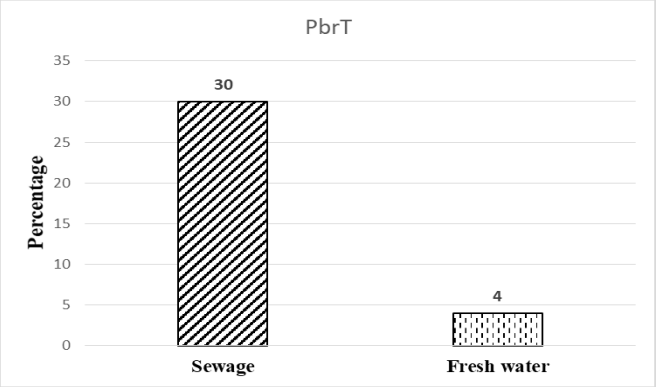

Figure 3: Prevalence of Pbrt gene in sewage and freshwater *E. coli* isolates.

4. Conclusion

Heavy metals resistance genes were widely present in *E. coli* isolated Tigris River and sewage water was significantly associated with antibiotic resistance genes. It is remarkably the coexistence of HMRGs and ARGs confer co-resistance to heavy metals, and antibiotics. Environmental impact in spreading of heavy metal resistance genes can lead to increased survival of bacteria in metal-contaminated environments, affecting microbial community dynamics and ecosystem functions.

Environmental impact in the spreading of heavy metal resistance genes can lead to

increased survival of bacteria in metal-contaminated environments, affecting microbial community dynamics and ecosystem functions. The co-location of copper resistance genes with antibiotic resistance genes on the same plasmids can lead to co-selection, where the use of heavy metal selects for antibiotic-resistant bacteria even in the absence of antibiotics. ESBL harbouring *E. coli* was found to be correlated with heavy metal resistance.

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