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Role of plasmid content in resistance of some enterobacteriaceae species to antibiotics, heavy metals, and biocides

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Abstract
Plasmids play an important role in the development of antimicrobial resistance by bacteria, particularly Enterobacteriaceae. This study was carried out to demonstrate the relationship of plasmid content to the resistance of some Enterobacteriaceae (*E. coli* O157.H7,*E. coli*, *K. pneumonia*, and *S. marcescens*) to antibiotics, heavy metals, and biocides. All isolates were subjected to plasmid profiling and curing as well as sensitivity testing towards antibiotics, heavy metals, and biocides before and after curing. The results showed that all the isolates were multi-drug resistant and showed high resistance against studied antibiotics, heavy metals, and biocides. all the isolates contained plasmids, where the largest share of *K. pneumoniae* was with three bands, with one band present in all remaining isolates, After curing, *K. pneumoniae* lost two plasmid bands, while other isolates lost their single plasmid band. the results showed a clear variation in the patterns of susceptibility to studied antimicrobials pre and post-curing, as this indicates the strong relationship of the plasmid to the their resistance, results also showed the presence of a combination of antibiotic, heavy metal, and biocidal resistance genes on plasmid of studied isolates especially for *K. pneumoniae*.

Keywords: enterobacteriaceae, plasmid, plasmid curing, antibiotics, heavy metals, biocides

Introduction

Antimicrobial resistance between bacteria belonging to the family Enterobacteriaceae has increased dramatically during the past several decades. which usually attained through the plasmid (Lynch III *et al.*, 2021) [12]. Due to a wide variety of plasmid-yielded tolerance genes and a high potential for mutation in the part of environmental pressure such as in the existence of antibiotics, numerous members of this bacterial group are clinically significant pathogens with a considerable capability to initiate antibiotic tolerance. Also, other factors that contribute to the formation and spread of multi-drug resistance genes, for example, heavy metals and biocides play a major role in the acquisition and development of antimicrobial resistance by exerting alternative selection pressure through co-selection. The combination of many tolerance mechanisms and the inadequate development of new medications, which renders these microbes resistant to nearly all current antibiotics, have made this bacterial group a severe hazard to public health. (Leinyuy *et al.*, 2022; Amarasekara *et al.*, 2023) [9, 1]. Plasmid is a genetic component that exists apart from chromosomal DNA and can replicate on its own. They differ from chromosomal DNA when they are small and harbor genes that are not necessary for cell survival, then their absence does not affect the bacteria but their presence provides cells with selective advantages. Its risk lies in carrying resistance and virulence genes (Helinski, 2022) [7]. Removal of plasmid will contribute to decrease antimicrobial resistance among bacteria, this occur through process called curing. Through curing, the plasmid of the bacteria that are resistant to a wide range of antimicrobials can be removed, leaving bacteria susceptible to them, then it is possible to prove the link between a genetic trait and the plasmid's carrying of a particular phenotype (Ekundayo, 2021) [4]. Curing may happen naturally or be forced. Application of several chemical and physical elements such as Ethidium bromide, Sodium dodecyl sulfate, and Acridine orange considerably increase it. Also, plasmid may potentially be destroyed by the use of UV, heavy metals, ionizing radiation, or growth at temperatures above optimal (Thabit *et al.*, 2020) [16].

This study aimed to demonstrate the role of plasmid content in resistance of some Enterobacteriaceae members towards antibiotics, heavy metals and biocides.

Materials and methods

The studied bacteria

Four multi-drug resistant enterobacteriaceae species previously diagnosed in the Biology Dept. College of Science, University of Mosul, were used in this study. They included *E. coli* O157.H7,*E. coli*, *K. pneumonia*, and *S. marcescens.*

Antibiotics sensitivity test

A test of bacterial sensitivity to antibiotics was carried out on all isolates using the disc diffusion method by the modified Kirby 1966 and Bauer method, A group of commercially antibiotic discs from (Bioanalyse / Turkey) Company was used, and following the recommendations of the Clinical Laboratory Standard Institute (CLSI, 2021).

Heavy metals and biocides sensitivity test

This test was conducted according to (Balouiri *et al.*, 2016) [2].

Filter paper discs with a diameter of 6 mm were prepared, and sterilized using an autoclave for 20 minutes, then sterilized filter paper discs were immersed in the used concentrations of metals and biocides for an hour an half, left to dry and placed on Muller-Hinton agar (provided by HiMedia/ India) plates inoculated previously with standardized bacterial suspension and the results were interpreted according to (Montagna *et al.*, 2019) [20].

Plasmid extraction

The plasmids were extracted from all studied bacteria with Promega Pure Yield plasmid Miniprep provided by Promega/ USA, following the manufacturer's instructions. The concentration and purity of the plasmids were determined by a Nanodrop device provided by the BioDrop/ English. The presence and size of plasmids in each sample were detected by electrophoresis in 0.9% agarose gel.

Plasmid curing

All the studied isolates were subjected to plasmid curing according to the method described by (Thabit *et al*., 2020) [16] [16], using two various agents, (Acridine orange at final concentration of 100µg/ml and Sodium dodecyl sulfate at final concentration of 10mg/ml.). Colonies that could still grow on nutrient agar after being treated with a curing agent were chosen at random and replicate-plated on nutrient agar plates containing the antibiotic discs that the wild isolate was resistant to. Colonies failed to grow were selected, performed plasmid extraction and ***0***.9% agarose gel electrophoresis on it once more.

Post-curing sensitivity test

A sensitivity test was conducted for colonies that were believed to have lost their plasmids again against antibiotics, heavy metals, and biocides that had previously shown resistance to them. By comparing isolates' pre- and post-curing resistance patterns, the presence of cured markers was identified. Removal of resistance markers revealed that they were most likely on plasmids rather than chromosomes.

Results and discussion

Antimicrobials Sensitivity Patterns

The sensitivity of the studied isolates was tested against 14 types of different classes of antibiotics, different concentrations of five types of heavy metals in the form of salts, and three types of biocides. The results showed that all the isolates were multi-drug resistant and there was a variation in the patterns of sensitivity and resistance toward the studied antimicrobials according to different bacterial species. It is noticed from (Tables 1, 2, and 3) that the highest level of resistance towards antibiotics and metals was in the case of isolate *E. coli* O157:H7, and *S. marcescens* and the lowest being in the case of *k. pneumoniae* and *E. coli* O157:H7 respectively. regarding biocides, all the isolates except *E. coli* O157:H7 showed complete resistance to the used biocides. Also, combinations between used antimicrobials resistance were observed in all isolates.

Table 1: Antibiotics sensitivity results of studied bacteria.

Antibiotics	Bacteria				Resistance %
	E.coli O157H7	*E.coli*	*K. pneumoniae*	*S. marcescens*	
OFX5	R	R	S	S	50%
AZM15	S	R	S	S	25%
CTX30	R	R	R	R	100%
CN10	R	S	R	R	75%
TOB10	R	S	R	R	75%
ATM30	R	R	R	R	100%
CFM5	R	R	R	R	100%
LEV5	R	R	S	S	50%
CIP10	R	R	S	S	50%
SXT25	R	R	S	R	75%
IPM10	S	S	S	S	0%
TMP10	R	R	S	R	75%

Table 2: Heavy metals sensitivity results of studied bacteria.

Heavy metals	Concet. mg/ml	Bacteria			
		E. coli O157:H7	*E. coli*	*K. pneumoniae*	*S. marcescens*
$ZnSO_4$	5	R	R	R	R
	20	R	R	R	R
	35	S	R	R	R
	50	S	R	R	R
$NiSO_4$	5	R	R	R	R
	20	R	R	R	R
	35	S	R	R	R
	50	S	R	R	R
Hg_2Cl_2	5	S	S	S	S
	20	S	S	S	S
	35	S	S	S	S
	50	S	S	S	S
$CoCl_2$	5	R	R	S	R
	20	S	R	S	R
	35	S	S	S	S
	50	S	S	S	S
$CdCl_2$	5	R	S	S	R
	20	S	S	S	R
	35	S	S	S	S
	50	S	S	S	S

Table 3: Biocides sensitivity results of studied bacteria.

Biocides	Bacteria			
	E. coli O157	***E. coli***	***K. pneumoniae***	***S. marcescens***
POVIDONE-IODINE 10%	R	R	R	R
ALPHA 15ml/L	S	R	R	R
ALPHA 10ml/L	S	R	R	R
ANIOS 10ml/L	S	R	R	R
ANIOS 5ml/L	S	R	R	R

Bacteria tend to develop AMR as a result of the stresses that cells are exposed to from the surrounding environment, whether inside or outside living bodies. Heavy metals and biocides cause elective pressure on bacteria, thus to survive they must alter their physiology and genetic makeup. Therefore, they may acquire and spread resistance genes, in addition to formation of structural changes in order to persist antimicrobials affectivity (Ture *et al.*, 2018; Li *et al*., 2022) [17, 11].

Besides the common presence of intestinal ARGs as a result of the unacceptable use of antibiotics, resistant bacteria are possible to move from the environment to humans via for example, the food chain, water, and animals where they settle in the intestines, the place that provides a suitable habitat for HGT, because of the presence of huge numbers of bacteria, its viscous nature, and abundance of MGEs especially conjugative plasmids that play the most important role in HGT and through which normal flora and opportunistic bacteria can acquiring resistance genes and transform to resistant ones (Tao *et al*., 2022) [15].

Several studies consistent with our findings regarding co-occurence of antibiotic, heavy metal, and biocidal resistance among bacteria, such as the study of (Thomas *et al*., 2020) [19] and (Amarasekara *et al*., 2023) [1].

Plasmid Profiling and Plasmid curing

All the studied isolates were subjected to a plasmid profiling process to find out their plasmid content. The results showed as shown in Table 4, that all the isolates contained plasmids by 100%, with a difference in the number of bands, where the largest share of *K. pneumoniae* was with three bands, with only one band present in all remaining isolates, (Figure 2) shows the number of plasmid bands in each isolate.

Table 4: Plasmid profiling of studied isolates before and after curing

NO.	Isolates	NO. of plasmid bands	
		B	A
1	*E.coli O157*	1	0
2	*E.coli (food)*	1	0
3	*K. pneumonia*	3	1
4	*S. marcescens*	1	0

Plasmids are extra-chromosomal pieces of DNA, small in size compared to a chromosome, carry genes that confer selective advantages on the cell, such as resistance genes. In addition, many plasmids carry genes (called *tra* gene) that allow them to self-transfer into other cells of the same or different species during conjugation,, the process that occurs through cell-to-cell contact and determined by several factors including the close distances between cells In the environment, presence of specific receptors that enable cells to bind each other, the closer the cells are genetically the more receptors they contain, so the human intestine is considered as a hotspot for genetic exchange to occur because it contains a large group of bacteria (physical distances between cells are closer) which often evolutionarily close to each other (Haudiqu et *et al*., 2022) [6]. This could explains the abundance of plasmids among enterobacteria.

Several studies agree with our findings regarding the prevalence and distribution of plasmids of different sizes among enterobacteriaceae, as a study of (Huang *et al*.) of the year (2012) confirmed that *K. pneumoniae and E. coli* contain different numbers of plasmid bands ranging from 1 to 4 bands per cell. While a study of (Nmesirionye *et al*.) for the year (2022) showed that most of the studied MDR *S. marcescens* possessed plasmids.

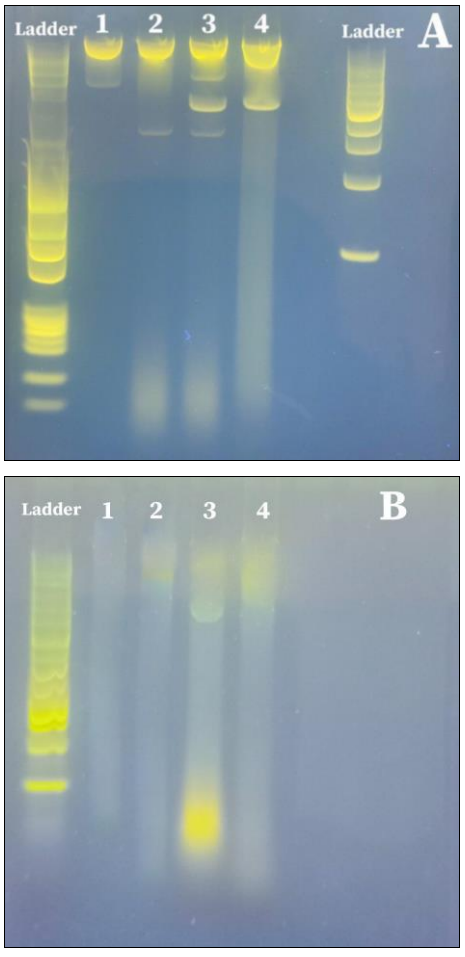

Fig 1: plasmid profile of studied isolates (A) before and (B) after curing. Lane 1 to 4 represent *E. coli* O157.H7, *E. coli, K. pneumoniae,* and *S. marcescens* respectively.

To find out the effect and relationship of the plasmid to the antimicrobial resistance of the studied bacteria, we tried to remove the plasmids from the isolates under study and keep the chromosome only. All isolates were treated with sub-inhibitory concentrations of (Acridine orange, and Sodium dodecyl sulfate) based on previous studies. Where AO showed its ability to remove the plasmids of *E. coli* O157.H7, *E. coli*, and *S. marcescens*., and SDS to cure *K. pneumoniae* plasmids, Table 4, and Figure 1.

EtBr and AO are DNA intercalating agents that relax the plasmids by making a break in the supercoiled form resulting in a straight or circular open strand of the plasmid and thus the inhibition of the replication process (Letchumanan *et al*., 2015) [10]. As for the curing effectiveness of SDS, it is through its ability to bind to the pili on the surface of the cells containing plasmid, and then prevent the process of conjugation and transfer of plasmids between cells (Okoye *et al*., 2022) [13].

Many studies showed the ability of these agents to remove plasmids from enterobacteriaceae, in a study conducted by (Faraj and Ghanima, 2010) showed the ability of SDS to remove plasmids from *K. pneumoniae* at higher rates than AO. While (Sulaiman *et al*., 2020) [14] were able to remove plasmids from *E. coli* isolates and (Nmesirionye *et al*., 2022) [18] from *S. marcescens* using AO.

Post-Curing Antimicrobials Sensitivity

After the curing process, plasmids containing resistance genes are removed from the cells, making them sensitive to antimicrobials if these genes are not present on the chromosome. In this case, the bacteria will continue to be resistant even after being cured as the chromosomal gene is not removed by curing. This allows us to know whether the resistance is encoded by the plasmid or the chromosome. Therefore, we conducted a sensitivity test(post-curing sensitivity test) for the antimicrobials to which the isolates showed resistance before curing. Where the results showed a great variation in the patterns of susceptibility pre and post-curing, as this indicates the strong relationship of the plasmid to the their resistance.

The results showed the close relationship of the plasmid with the resistance of the studied isolates towards antibiotics, heavy metals, and biocides. in addition to, the presence of a combination between ARGs, HMRGs, and BRGs on both plasmid and chromosome of the studied enterobacteriaceae, as shown in Table 6. Also, results confirm what was reached by previous studies concerning the selective pressure that metals and biocides exert on bacteria, which results in the acquisition and development of antibiotic resistance and the genes responsible for it, and also the association between ARGs and other resistance genes such as HMRGs and BRGs (Amarasekara *et al*., 2023) [1].

Table (4-9): number of studied antimicrobial agents the isolates lost its resistance to it after curing.

Bacteria	Types of agents whose sensitivity changed after curing		
	antibiotics	**Heavy metals**	**biocides**
E. coli O157:H7	CN10, ATM30, CFM5, LEV5, SXT25, TMP10	$ZnSO_4$, $NiSO_4$, $CdCl_2$	NF
E. coli	AZM15, CN10, ATM30, CFM5, LEV5, SXT25, TMP10, CIP10	$ZnSO_4$, $NiSO_4$, $CoCl_2$	NF
K. pneumoniae	CN10, TOB10, ATM30, CFM5	$ZnSO_4$	ALPHA, ANIOS
S. marcescens	CN10, TOB10, ATM30, CFM5, SXT25, TMP10	$ZnSO_4$, $NiSO_4$, $CdCl_2$, $CoCl_2$	NF

It has been observed through the results that bacteria have lost their resistance to antibiotics more than to metals and biocides. The reason for this may be since the majority of antibiotic resistance genes encode for only a single antibiotic type, while in contrast, most mechanisms of resistance to metals and biocides remove more than one type of these substances. This means that if for example, the *czcA* gene is present on the bacterial chromosome it will maintain resistance to cobalt, zinc, and cadmium even if other specific genes for the three metals are removed through curing (Li *et al*., 2022) [15].

However, despite the removal of the plasmid, the isolates maintained their resistance to some antibiotics, metals, and biocides used. This may be explained by the presence of resistance genes to these substances on the chromosome, or that the resistance is not linked to resistance genes, but rather results from the structural nature of the bacteria such as through the absence or modification of the drug's target site, the inability of the drug to reach its target as a result of decreased permeability of cell membrane which occurs mostly in gram-negative bacteria due to the presence of outer membrane (Edet *et al*., 2023).

References

1. Amarasekara NR, Mafiz AI, Qian X, Tiedje JM, Hao W, Zhang Y. Exploring the co-occurrence of antibiotic, metal, and biocide resistance genes in the urban agricultural environment. Journal of Agriculture and Food Research,2023:11:100474.
2. Balouiri M, Sadiki M, Ibnsouda SK. Methods for *in vitro* evaluating antimicrobial activity: A review. Journal of pharmaceutical analysis,2016:6(2):71-79.
3. Edet U, Bassey IU, Joseph A. Heavy Metal Co-Resistance with Antibiotics Amongst Bacteria Isolates from an Open Dumpsite Soil, 2023. Available at SSRN 3970491.
4. Ekundayo OK. Plasmid Profile and Plasmid Curing of Some Enterobacteriaceae Resistant to Ampicillin Isolated from Food, Human Stool, Chicken Stool in Ekiti State. Int.J.Curr.Microbiol.App.Sci,2021:10(01):2344-2362.
5. Faraj DN, Ghanima KK. Plasmid curing of local isolates of Klebsiella pneumoniae isolated from urinary tract infections and its role in multidrug resistance. Iraqi Journal of Science, 2010, 51(3).
6. Haudiquet M, de Sousa JM, Touchon M, Rocha EP. Selfish, promiscuous and sometimes useful: how mobile genetic elements drive horizontal gene transfer in microbial populations. Philosophical Transactions of the Royal Society B,2022:377(1861):20210234.
7. Helinski DR. A brief history of plasmids. *EcoSal Plus*,2022:10(1), eESP-0028.

8. Huang XZ, Frye JG, Chahine MA, Glenn LM, Ake JA, Su W, *et al*. Characteristics of plasmids in multi-drug-resistant Enterobacteriaceae isolated during prospective surveillance of a newly opened hospital in Iraq. *PloS one*,2012:7(7):e40360.
9. Leinyuy JF, Ali IM, Karimo O, Tume CB. Patterns of Antibiotic Resistance in Enterobacteriaceae Isolates from Broiler Chicken in the West Region of Cameroon: A Cross-Sectional Study. Canadian Journal of Infectious Diseases and Medical Microbiology, 2022.
10. Letchumanan V, Chan, KG, Lee LH. An insight of traditional plasmid curing in Vibrio species. Frontiers in microbiology,2015:6:735.
11. Li X, Rensing C, Vestergaard G, Arumugam M, Nesme J, Gupta S, Sørensen SJ. Metagenomic evidence for co-occurrence of antibiotic, biocide and metal resistance genes in pigs. Environment International,2022:158:106899.
12. Lynch III JP, Clark NM, Zhanel GG. Escalating antimicrobial resistance among Enterobacteriaceae: Focus on carbapenemases. Expert Opinion on Pharmacotherapy,2021:22(11):1455-1474.
13. Okoye EL, Kemakolam C, Ugwuoji ET, Ogbonna I. Multidrug Resistance Tracing by Plasmid Profile Analysis and the Curing of Bacteria from Different Clinical Specimens. Advanced Gut & Microbiome Research, 2022.
14. Sulaiman MA, Muhammad HS, Sani AM, Ibrahim A, Hussain IM, Anchau ZG. Evaluation of the impact of plasmid curing on antibiotic resistance on some clinical isolates of escherichia coli. Fudma journal of sciences,2020:4(3):323-327.
15. Tao S, Chen H, Li N, Wang T, Liang W. The spread of antibiotic resistance genes *in vivo* model. Canadian Journal of Infectious Diseases and Medical Microbiology, 2022.
16. Thabit AG, El-Sabour A, Nafie AMA, El-Mokhtar MA, Biomy YE. Detection of proteus species in diabetic wounds and their antibiotic resistance profile analysis. Bulletin of Pharmaceutical Sciences. Assiut,2020:43(1):1-10.
17. Ture M, Altinok I, Alp H. Effects of cage farming on antimicrobial and heavy metal resistance of Escherichia coli, Enterococcus faecium, and Lactococcus garvieae. Microbial Drug Resistance,2018:24(9):1422-1430.
18. Nmesirionye BU, Ugwu CT, Martins Nworie K. Antibiotic Susceptibility and Plasmid Profile of Multidrug resistant Uropathogenic Serratia marcescens. Journal of Microbiology & Infectious Diseases, 2022, 12(1).
19. Thomas IV JC, Oladeinde A, Kieran TJ, Finger Jr JW, Bayona-Vásquez NJ, Cartee JC, *et al*. Co-occurrence of antibiotic, biocide, and heavy metal resistance genes in bacteria from metal and radionuclide contaminated soils at the Savannah River Site. *Microbial biotechnology*,2020:*13*(4):1179-1200.
20. Montagna MT, Triggiano F, Barbuti G, Bartolomeo N, De Giglio O, Diella G, *et al*. Study on the *in vitro* activity of five disinfectants against nosocomial bacteria. International journal of environmental research and public health,2019:16(11):1895.