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RESEARCH ARTICLE

Whole Genome Sequence Based Analysis of Heavy Metals Genes and Antibiotics Resistance Genes in *P. Aeruginosa* Isolated from Hospitals in Iraq

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ABSTRACT

Genes of resistance to heavy metals (HMRS) were detected in five Pseudomonas aeruginosa strains obtained from the hospitals under study, including environmental samples and clinical samples. Resistance isolates were assessed depending on their place of origin and possible exposure to contaminated heavy metals. The antibiotic resistance of these five isolates was examined using the Vitek-2 technology, as well as disinfectant resistance by spreading on dens, which revealed significant antibiotic resistance and insensitivity to disinfectants often used in hospitals. Metal resistance-encoding genes were identified by WGS using NGS technology. Metals resistance genes and antibiotic resistance genes were detected in all five isolates. The presence of resistance-encoding genes in contemporary isolates suggests a link to acquiring resistance features from external sources.

INTRODUCTION

P.aeruginosa is gram (-ve) bacilli which thrives in a large variety of different and complex environmental habitats causing opportunistic diseases to humans, and the natural environment serves as its reservoirs [1]which is a widespread bacterial genus in nature with high pathogenicity for humans, animals and plants, as it is found in humid and warm environments in particular, It can be isolated from several living sources, including humans, animals, plants and non-living sources, including soil, water and food [2], the reason for its wide spread in various environments and its ability to cause severe infections is due to its ability to survive at minimum nutritional needs and its resistance to various environmental conditions and antibiotics due to its diverse metabolic and enzymatic properties [3], And it is the pronounced diversity of metabolism that gives it such wide ecological success [4]. It is one of the most well-known opportunistic pathogens classified by (WHO) as a top priority in terms of importance [5]. p. aeruginosa has the ability to resist a wide and diverse range of antibiotics caused by the self-resistance that these bacteria possess, as it plays a major role in preventing the effect of antibiotics on the bacterial cell by reducing the permeability of the cell membrane, changing the location of the target, and having flow pumps, which is what made it among the most important pathogens and the most dangerous for humans [6] as well as the production of P.aeruginosa's beta-lactamase enzymes made it resistant to many β-lactam antagonists identified as virulence factors, which in turn further complicated the clinical treatment process [7]. Pseudomonas

aeruginosa has a large genome, which is the main contributor to the ability of P. aeruginosa to grow and adapt in various environments such as soil, water, hard surfaces and infect humans, animals and plants [8], it also has a very small primary chromosome of which only 1% has been discovered so far, and a large percentage of this genome consists of genes responsible for antibiotic resistance, and are in the form of plasmids, transposon mutating factors and insertion sequences [1]

Epidemiological assessment of P.aeruginosa bacteria at the level of molecular interpretation is important in detecting the potential danger related to the infection process, and helps to find epidemic strains in a certain number of patients and identify pathogenic strains from them [9]. Heavy metals have an adverse influence on microorganisms, particularly bacteria. Metals can cause cell membrane disintegration when concentrations above the minimum inhibitory concentration. They also interact with temperature and pH. [10]. Heavy metals in the cell, especially positively charged ions with large atomic weights such as cadmium and mercury, can attach to thiol groups in enzymes and proteins. In addition, they can bind to hydroxyl or phosphate groups in DNA, changing the structure or structure of the protein. One of its causes is DNA vulnerability . The nonspecific binding of cadmium to DNA also leads to the occurrence of single-stranded regions [11]. Metals ions can disrupt with alkali metals, causing destroy it. Alkali metals play crucial roles in bacterial cells by binding and replacing enzymes with Zn and Cd . Cd can substitute Mg and Ca ions, inhibiting enzyme activity [12] H.M can interfere with glutathione compound and prevent functioning properly. Heavy metals affect oxidative phosphorylation and permeability of membranes. [13]. Alternative therapies to standard antibiotics include heavy metals with antibacterial capabilities. Mutable studies has Confirmed heavy metal resistance genes (HMRGs) that drive distinct resistance paths. Understanding distribution of (HMRGs) among bacteria in wastewater is critical for controlling their development and using them as antimicrobial agents [14]. This study aims to evaluate the spread of HMRGs and ARGs in P. aeruginosa that contamented the hospitals in Iraq within Anbar province by whole genome sequencing analysis using NGS technology.

2-MATERIALS AND METHODS

2-1-Isolation of strain:

This study took almost three months from the start of the collection of samples at the beginning of February until the end of April 2023. It included two main stations, Al-Ramadi General Hospital and its subdivisions and Hit General Hospital and its subdivisions . The sample was taken from environmental and clinical places from the Departments of the mentioned hospitals. During the study, 200 isolates of Pseudomonas aeruginosa were collected and diagnosed by VITEK compact-2 methods, as these strains showed a variation in resistance to antibiotics and antiseptic used in hospitals, distributed between 26% non-resistant, 43% multi-resistant drug and 31% extensive-resistance drug, where five isolates of extensive-resistant strains were selected for the focus of this study .

2-2-Identification of P.aeruginosa using 16srRNA:

The genetic diagnosis of these isolates was made by means of the 16srRNA gene with a molecular size of 556bp and using the PCR sequencer , a specialized primers targeting the qualitative sequencing of The gene 16S rRNA was employed in the diagnosis of bacterial isolates., and after performing the reaction and transferring the multiplication product on the agarose gel at a concentration of 1.5% for one hour, the appearance of a single package was observed in the pits of the gel and at the same level for all isolates as noted in Figure (1), This demonstrates the link between the initiator primer and the related sequence in the bacterium's DNA strip. The molecular weights of the resulting beams were estimated based on the location of the beams in the agarose gel, which have known molecular weights through the M Path of the volumetric guide of 100 base pairs, as it appeared that the results were similar in molecular weight to the resulting beams, which were 556 base pairs relative to the molecular weight of the 16srRNA gene.

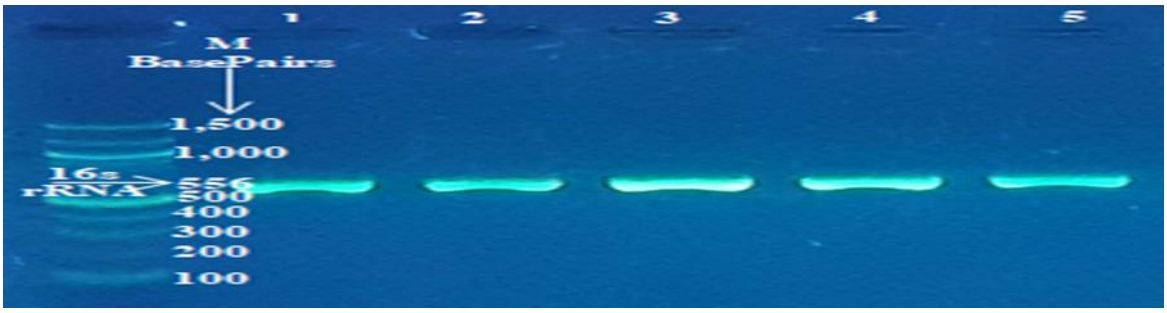

Fig 1: Electrophoresis of the results of the DNA chain polymerase reaction using the initiator 16srRNA on the agarose gel medium .

2-3-Determination of Biocide Effect on P.aeruginosa Isolates

The effectiveness of disinfectants commonly used in the aforementioned hospitals was tested on antibiotic-resistant bacterial isolates, which include five isolates classified as Extensive Drug Resistance(XDR), three types of disinfectants with their own composition were tested using Kirby Bauers diffusion method as shown in table (1) and figure (3) .

2-4Whole genome sequencing and Bioinformatics analysis

Before reviewing the results, it is necessary to clarify the mechanism by which the results were interpreted and what modern programs and techniques are used to analyze the results .

2-5- Empowering the Development of Genomic Expertise (EDGE):

EDGE bioinformatics was one of the platforms used to analyze the raw FASTQ files obtained from the Illumina miSeq sequencer because there's still no single algorithm or tool that can fit all cases in the bioinformatics field. It is a unique set of tools for different genomic analyses, from raw data to complete genome. This platform was developed by LosAlamos National Laboratories/ USA. The version used was 2.4.1 (as shown below in Figure 2).

Figure 2: The platform served the purpose of our study, for the vast type of analyses integrated for interpretation and visualization of the correlative statistical analyses. Giving the subject of study a real and adequate representation.

The Bioinformatic analysis approach in this study started with Data QC (both from management and storage). For each FASTQ file obtained, it ran through QC to evaluate the quality of each single file, and each single base- called. The quality of our files was very good even after trimming the bad ones.

3-RESULT AND DISCUSSION

3-1- Biocide Effectiveness: Pseudomonas aeruginosa resisted the action of antiseptic used in the hospitals under study in all its forms and the resistance was very sever(table1 ; fig 3) e. we believe that the cause of resistance is weak disinfection processes, which leads to the acquisition of bacteria resistance genes to disinfectants, and this is consistent with most recent international studies that have proven the transmission of resistance genes horizontally and vertically between generations of bacteria[15]. Contamination of hospitals with chemicals is another source of bacterial resistance to disinfectants [16] . Other studies have confirmed that some bacterial isolates that are foreign to the hospital as a result of external sources through imported devices and equipment can colonize

hospitals and cause acquired infections to those who are lying and working in them, and resistance genes may be transmitted to other types, and thus the strength of bacterial resistance and its spread will increase [17].

Table 1: The antiseptic and their combination that used in this study

No.	Antiseptic	combination
1	MPC surface disinfection and cleaning	8% Didecyl Dimethyl Ammonium Chloride 1% Benzalkonium Chloride Anti corrosive agent DI water
2	MPC spray disinfection	65% propan-2-ol 12.5% ethanol 0.2% Didecyl Dimethyl Ammonium Chloride Auxiliary substances
3	MPC Glutara disinfectant 2%	2% Glutaraldehyde Auxiliary substances DI water

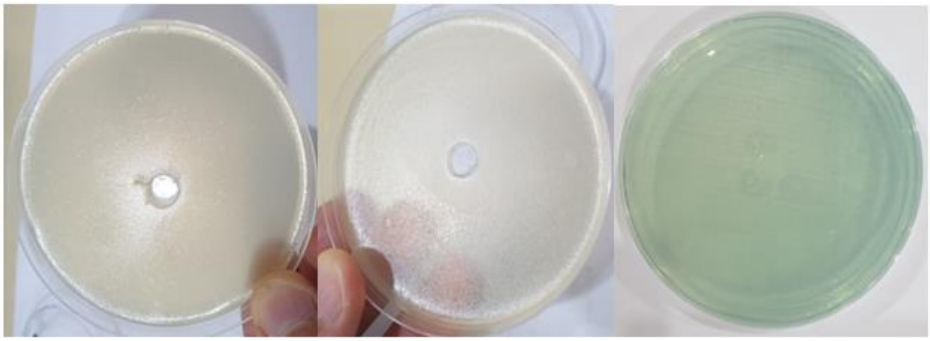

Figure 3 :The resistance of P.aeruginosa to Antiseptic using Kirby Bauers diffusion method (1) Resistance for biocide 1(MPC surface disinfection and cleaning) , (2)Resistance for biocide 2(MPC spray disinfection) , (3) Resistance for biocide 3 MPC Glutara disinfectant 2% .

3-2-MLST Analysis : After all the previous traditional and advanced analyses were applied to ensure the quality of data obtained by sequencing, other Bioinformatics tools were used for MLST.Five isolates of P. aeruginosa were diagnosed by MLST and through variation in the sequence of genes sites (acsA, aroE, guaA, mutL, nuoD, ppsA and trpE) , after comparison with data in pubmlst , it turned out that these isolates are new, which are previously unregistered Iraqi local isolates, which are resistant to antiseptics and antibiotics . These samples were registered at the British University of Oxford in the name of the researcher, and the bacterial strains were marked with their own ST and can be accessed via the following link (in this study):

https://pubmlst.org/bigsdb?page=profileInfo&db=pubmlst_paeruginosa_seqdef&scheme_id=1&profile_id=4426 ;
https://pubmlst.org/bigsdb?page=profileInfo&db=pubmlst_paeruginosa_seqdef&scheme_id=1&profile_id=4427 ;
https://pubmlst.org/bigsdb?page=profileInfo&db=pubmlst_paeruginosa_seqdef&scheme_id=1&profile_id=4436 ;
https://pubmlst.org/bigsdb?page=profileInfo&db=pubmlst_paeruginosa_seqdef&scheme_id=1&pr

ofile_id=4437 ;
https://pubmlst.org/bigsdb?page=profileInfo&db=pubmlst_paeruginosa_seqdef&scheme_id=1&profile_id=4438 .

3-3- Drug resistance genes: Sequences were applied to annotation after finishing all the q Annotation was performed against Pseudomonas strains. All the previous Bioinformatic analyses have been done through the EDGE platform to check the quality and integrity of the annotated sequences. One of the processes of imipenem resistance in clinical isolates is the deletion of the OprD porin. Changes in the oprD gene, (fig:4) such as substitutions, deletions, insertions, or mutations, might change the structure of OprD porin or suppress its presence, resulting in carbapenem resistance [18].

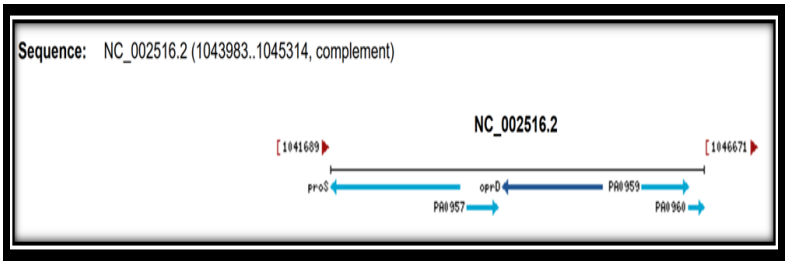

Figure 4:Genomic context (oprD) porin D of Pseudomonas aeruginosa

All of these isolates have a significant 8-bp mutation (GGCCAGCC) on nucleotide location 235 of the mexT regulatory gene.. using NCBI by (PGAP) .(fig:5).

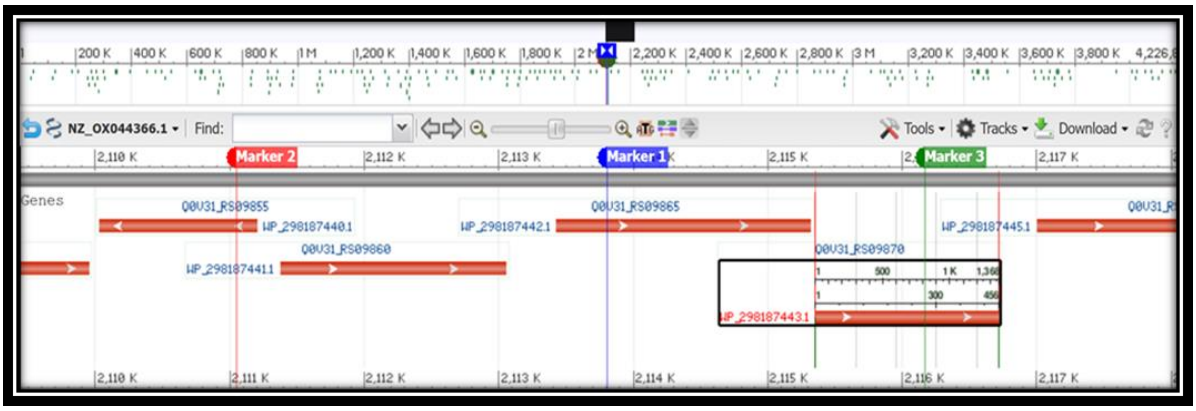

Figure 5: The annotation from the NCBI.(NCBI).

Following the previous protocol and through the ResFinder Program, the presence of antibiotic resistance genes was detected in the five isolates of P. aeruginosa and as shown in the following table:

Table 2 : Drug resistance gene in isolated (ST:4438) (NCBI).

Resistane gene	Identity	Alignmet Length/ Gene Length	Position in reference	Phenotype	Accession no.
msr(A)	98.7	1467/1467	1..1468	Streptogramin b erythromycin,azithromycin,telithromycin,quinupristin, pristinamycin,virginiamycin	X52085
fosB	99.77	429/429	1..430	fosfomycin	ACHE01000077
fosA	99.75	408/408	1..409	fosfomycin	ACWU01000146
tet(A)	97.17	1186/1200	15..1201	Tetracycline doxycycline,tetracycline	AY196695
msr(A)	98.7	1467/1467	1..1468	Macrolide erythromycin,azithromycin,telithromycin,quinupristin, pristinamycin,virginiamycin	X52085

crpP	98.48	198/198	1..199	Quinolone ciprofloxacin	HM560971
qnrVC1	100	657/657	1..658	ciprofloxacin	EU436855
dfrB2	100	237/237	1..238	Folate pathway antagonist trimethoprim	AY553333
dfrB5	100	237/237	1..238	trimethoprim	AY943084
sul1	100	840/840	1..841	sulfamethoxazole	U12338
aph(3')-IIb	99.63	807/807	1..808	Aminoglycoside unknown aminoglycoside	CP006832
ant(3'')-Ia	98.05	972/972	1..973	streptomycin	X02340
aac(3)-Id	99.79	477/477	1..478	gentamicin,astromicin,fortimicin	AB114632
aac(6')-Il	100	459/459	1..460	amikacin,tobramycin	U13880
ARR-2	100	453/453	1..454	Rifamycin rifampicin	HQ141279
blaPAO	96.15	1194/1194	1..1195	Beta-lactam amoxicillin,ampicillin, cefepime,ceftazidime	AY083592
mecA	98.06	1979/2007	29..2008	amoxicillin,amoxicillin+clavulanic acid,ampicillin, ampicillin+clavulanic acid,cefepime,cefixime, cefotaxime,cefoxitin,ceftazidime,ertapenem, imipenem,meropenem,piperacillin,piperacillin+tazobactam	NC_007168
blaVEB-1	100	900/900	1..901	amoxicillin, amoxicillin+clavulanic acid, ampicillin, ampicillin+clavulanic acid, cefotaxime,cefoxitin, cefepime, ceftazidime,piperacillin,piperacillin+tazobactam, ticarcillin, ticarcillin+clavulanic acid,aztreonam	HM370393
blaOXA-10	100	801/801	1..802	amoxicillin,ampicillin, aztreonam,piperacillin, piperacillin+tazobactam	J03427
blaOXA-50	99.37	789/789	1..790	amoxicillin,ampicillin	AY306130
fusB	100	642/642	1..643	Steroid antibacterial fusidic acid	AY373761
catB7	98.59	639/639	1..640	Amphenicol chloramphenicol	AF036933
qacE	91.59	319/333	1..320	Quaternary ammonium compound benzylkonium chloride,ethidium bromide,chlorhexidine,cetylpyridinium chloride	X68232
lnu(A)	99.38	486/486	1..487	Lincosamide lincomycin	M14039

3-4-Heavy metals genes:

The results of the genomic analysis of heavy metals showed the presence of five genes encoding heavy metals (fptA, recG,ruvB, ruvA,ruvC) (table 3) and when compared with the reference in the NCBI, it was found that there were variations of three genes from the original reference varied in their effects between a change in amino acid or not as shown in the (fig: 6)

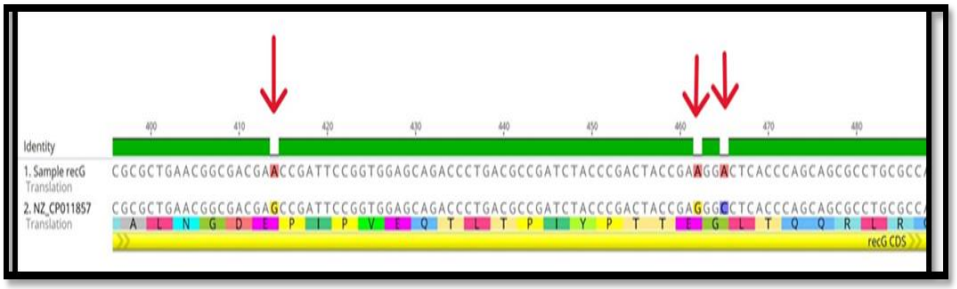

Figure 6: The recG gene mutation comparing with reference in NCBI.

Table 3: genes of heavy metals presence in p.aeruginosa(NCBI)

gene	location	Accession no.

recG	6579946-6582021	AAG08730.1
ruvC	4707405-4707929	AAG04354.1
ruvB	4705714-4706772	AAG04356.1
ruvA	4706783-4707388	AAG04355.1
fptA	781944-784106	AAG07609.1

For example, the recG gene found in the above-mentioned resistant isolates, when compared with the reference found in the NCBI, showed the presence of 19 mutations that changed only nucleotides without changing the amino acid (fig:6). Location of gene on Chromosome and resiste compounds Chromium, Tellurium, and Selenium. RecG is an ATP-dependent DNA helicase. It is responsible for repairing DNA damage produced by chromate or its metabolites. Can impart resistance to tellurite and selenite, but not arsenic, paraquat, or hydrogen peroxide. [19]. While in the fptA gene, there were two changes in nitrogenous bases, which led to a change in the resulting amino acid, where the amino acid at site 1055bp changed from threonine to methionine as a result of the change of the nucleotide from Cytosine to Thymine, which led to the change of the amino acid code from (ACG to ATG). Also, at position 412, the amino acid changed from threonine to alanine as a result of the change of the nucleotide from Adenine to Guanine, which led to the change of the amino acid code from(ACG to GCG). Location of gene on the Chromosome and resist compounds include Nickel, Cobalt, Iron, Gallium. Fe(3+)-pyochelin receptor FptA ; High-affinity outer membrane receptor required for the transport of Fe3+-pyochelin [20] .

Moving to the ruvB gene which include two variate on two sites the first one in 760 bp and the second in 289bp But these variations did not change the homocysteine and therefore have no effect on its protein composition . In the ruvA and ruvC genes, we did not observe any changes or mutations, so the results of their comparison matched the data in the NCBI.

3-5-CRISPR cas9 Results

The CRISPRcas9 results showed the presence of several regions eligible for cutting by the Cas 9 enzyme and according to the Doench 2016 classification (Fig: 7)

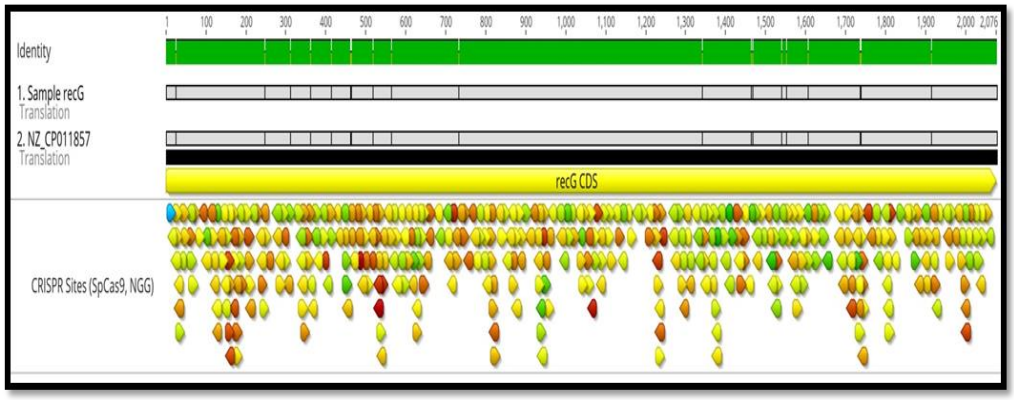

Figure 7: CRISPR cas9 sites in recG gene

Dark-colored areas represent the eligible sites for cutting, the darker the red-colored area, the more eligible it is for cutting than light-colored areas, which tend to be green, according to the Doench2016 classification.

The analyses showed the existence of active regions eligible for cutting by the Cas-9 enzyme in all genes with different locations, where the maximum Activity score reached 0.75 according to the Doench classification 2016, which is the upper value that is directly proportional to the probability of cutting . High concentrations of minerals in the soil are a direct result of the randome accumulation of metal-containing substances in the environment. This leads to the development of basic mechanisms of resistance of microbes to metals, and, consequently, to antibiotics and antiseptics. These mechanisms may eventually spread to other pathogenic microbes, raising the disease risks caused by antibiotic resistance. Antibiotic resistance is condition of bacteria that makes antibiotics ineffective against them. There are several reasons that lead to the emergence of antibiotic resistance in bacteria, including the overuse or misuse of antibiotics and their excessive availability in the environment . Therefore, the presence of chemical pollutants such as heavy elements in the environment may stimulate microbes to acquire resistance to several types of antibiotics, and this is a concern for Public Health and the surrounding environment [3]. According to multiple previous studies, environmental contamination such as metals, biocides and metallochemical compounds contribute to antibiotic resistance by increasing the spread of mobile genetic elements through bilateral transmission. , Bednors et al. Explain that contamination with the elements may stimulate antibiotic resistance. In addition, as the level of environmental contamination increases, the genes carrying this resistance increase. Heavy metals have been used as antimicrobial in some hospital antiseptic, feeders and materials, and long-term exposure to microorganisms in the same medium with high concentrations of heavy metals can lead to increased resistance to these metals and, as a result, to some other antimicrobials . The possibility of the spread of these heavy metals from contaminated environments to patients in hospitals, water and agricultural lands, as well as the movement of metal-resistant infectious microbes in these waters and their possible ingestion by animals and humans may lead to an increased risk of diseases that are difficult to treat due to resistance to these metals and antibiotics used [21]. H.M. resistance genes, AMRgene , and integrons increase in heavy metal contaminated environments, which means that high metal concentrations have a great potential to enhance metal and antibiotic resistance through bilateral gene transfer and affect bacterial colonies, which has produced bioresistance. In addition, Network analysis has been used to identify key host bacteria of various ARGs and ARGs that could be responsible for higher (MRGs) and (ARGs)levels in high polluted metal environments [22,23,24]. In addition to the genes mentioned above, several other genes have appeared to us from Annotation of genome P.aeruginosa, as shown in(Table3).

Table 3: Heavy metals genes from Annotation of genome P.aeruginosa

Gene	gene Id	Length(bp)	Accession no.
fpvA	PA2398	2448	AAG05786.1
fpvB	PA4168	2409	AAG07555.1
fpvI	PA2387	480	AAG05775.1
fpvR	PA2388	996	AAG05776.1
mexI	PA4207	3090	AAG07594.1
arsB	PA2278	1284	AAG05666.1
arsC	PA2279	471	AAG05667.1
arsR	PA2277	351	AAG05665.1

bfrA	PA4235	465	AAG07623.1
copR	PA2809	681	AAG06197.1
copS	PA2810	1332	AAG06198.1
corA	PA5268	981	AAG08653.1
czcA	PA2520	3156	AAG05908.1
czcB	PA2521	1455	AAG05909.1
czcC	PA2522	1287	AAG05910.1
dnaK	PA4761	1914	AAG08147.1
dsbA	PA5489	636	AAG08874.1
dsbB	PA0538	510	AAG03927.1
dsbC	PA3737	729	AAG07124.1
irlR	PA4885	690	AAG08270.1
mgtA	PA4825	2712	AAG08210.1
modB	PA1862	687	AAG05251.1
modC	PA1861	1086	AAG05250.1
pcoA	PA2065	1899	AAG05453.1
pcoB	PA2064	978	AAG05452.1

We conclude from the foregoing that the isolates of the aforementioned Pseudomonas aeruginosa bacteria isolated from the hospitals under study resisted antiseptics and antibiotics due to the presence of heavy metals and some trace elements that enter into the composition of antiseptics and antibiotics, in addition to possessing genes carried on their chromosome, which were described as heavy metal genes, and this confirms the ability of bacteria to resist heavy metals and their ability to develop other resistance mechanisms when they feel threatened or threatened . These mechanisms, which are possessed by bacteria, can threaten human lives in and deplete health institutions, cost the state a lot of money, not to mention the deaths that occur due to hospital-acquired infections .

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