EGYPTIAN ACADEMIC JOURNAL OF
BIOLOGICAL SCIENCES
MICROBIOLOGY

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ISSN
2090-0872

WWW.EAJBS.EG.NET

Vol. 14 No. 1 (2022)

Citation: *Egypt. Acad. J. Biolog. Sci. (G.Microbiolog) Vol.14 (1) pp.171- 187 (2022)*
DOI: 10.21608/EAJBSG.2022.238167

Egypt. Acad. J. Biolog. Sci., 14(1):171-187 (2022)

Egyptian Academic Journal of Biological Sciences
G. Microbiology

ISSN: 2090-0872
https://eajbsg.journals.ekb.eg/

Heavy Metal and Antibiotic Resistance in Bacteria from a Wastewater Treatment Plant

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ARTICLE INFO

Article History
Received: 7/3/2022
Accepted:2/5//2022
Available:4/5/2022

***Keywords*:**
Antibiotic resistance, heavy metal resistance, wastewater effluents, plasmid, bacteria

ABSTRACT

Bacterial resistance to heavy metals and antibiotics is a global concern to public health, animals, and ecosystems. This study was conducted to determine bacteria with multiple heavy metals and antibiotic resistance from the effluent of a wastewater treatment plant. Heavy metal concentrations in the effluents were analyzed using Atomic Absorption Spectrophotometer (AAS). Selective isolation of heavy metal-resistant bacteria, metal tolerance concentration, Minimum Inhibitory Concentration (MIC), and Minimum Bactericidal Concentration (MBC) was done using heavy metals salts at different concentrations (25-750 ppm) incorporated in broth and Mueller Hinton agar (MHA). The isolates were identified based on their phenotypic and 16 rRNA analysis. The antibiotic resistance was determined by the disk diffusion method. The plasmid DNA of the bacterial isolates was analyzed on agarose gel electrophoresis. Four bacteria exhibited resistance to heavy metal concentrations between 200 -750 ppm. The isolates were identified as *Alcaligenes, Paenalcaligenes, Providencia* and *Klebsiella* species. The bacteria were resistant to 2 or more antibiotics with Multiple Antibiotic Resistance (MAR) index between 0.25 - 0.75. All the isolates possessed plasmids, and the resistance in the bacteria was either chromosome or plasmid-borne. The bacterial isolates obtained could serve as potential candidates for the bioremediation of heavy metal contaminated effluents. However, the multiple antibiotic resistance could result in bacteria proliferation, contributing to the maintenance and spread of antibiotic-resistant disease-causing bacteria.

INTRODUCTION

Wastewater is a term that encompasses effluent from domestic, commercial, industrial, agricultural, and faecal sludge (UN-Water, 2017). Effluent flowing into wastewater treatment plants contains antibiotics, heavy metals, antibiotic-resistant bacteria, and antibiotic resistance genes (ARGs) (Charlesworth *et al.*, 2011; Chen *et al.*, 2019; Osińska *et al.*, 2020; Hubeny *et al.*, 2021**)**. In highly industrialized areas, wastewater is composed of municipal, industrial, and hospital wastewater. In contrast, industrial wastewater has a smaller share of the sewage mix in less industrialized regions that reaches wastewater treatment plants (Hubeny *et al.*, 2021**)**.

As a result, wastewater from industrialized areas is characterized by higher concentrations of pollutants than sewage generated in less industrialized regions (Cheng *et al*., 2014; Di Cesare *et al*., 2016a; Hubeny *et al.,* 2021). Some of the heavy metals from wastewater effluents are essential micronutrients for several cellular functions and components of biological macromolecules (Seiler and Berendonk, 2012) but can also be toxic when accumulated to a particular concentration (Fosmire, 1990; Chen *et al.,* 2019).

Heavy metals are generally more persistent and stable than organic contaminants such as pesticides or petroleum byproducts and are non-biodegradable in the environment (Zieliński *et al.,* 2021). Unlike many other pollutants, heavy metals are difficult to remove from the environment (Ren *et al*., 2009). They may impose selection pressures on microorganisms present (Berendonk *et al*., 2015) and even change the diversity of microbial communities (Epelde *et al*., 2015). Heavy metal pollution causes environmental diseases, which often manifest as cancer, chronic lung disease, kidney disease, liver disease, and neurodegeneration (Gupta *et al.,* 2015). Heavy metal toxicity can result in high morbidity, and mortality rates inappropriately treated cases (Adal and Wiener, 2020). For instance, in 2010, Nigerian health officials reported the death of more than 100 children from lead poisoning due to illegal mining of gold in Zamfara state, North West Nigeria (WHO, 2010; Dooyema *et al.,* 2012).

Wastewater treatment plants are hotspots of antibiotic resistance (Che *et al.,* 2019, Mukherjee *et al*., 2021). They are regarded as direct sources of the spread of antibiotic resistance in the environment (Di Cesare *et al*., 2016b). Antibiotic resistance has become one of the most significant problems threatening populations globally. The World Health Organization (2014) reported antibiotic-resistant genes (ARGs) as a new pollutant because of their emerging prevalence and wide distribution (Chen *et al.,* 2019). The increasing antibiotic-resistant genes have been recognized as a consequence of the massive use of antibiotics in therapeutics and agriculture (Huerta *et al*., 2013). This has resulted in the loss of efficacy of newly developed antibiotics against many bacterial infections within a few years after their introduction (Davies and Davies 2010).

Heavy metal resistance and antibiotic resistance can be selected simultaneously in a heavy metal contaminated ecosystem (Timoney *et al.,* 1978). These phenomena can be interpreted as co-selection (selecting two or more genetically linked resistance genes when one of the genes is selected) (Seiler and Berendonk, 2012). These co-selection mechanisms include co-resistance (different resistance determinants present on the same genetic element) and cross-resistance (the same genetic factor responsible for resistance to antibiotics and metals) (Baker-Austin *et al.,* 2006). Several studies have revealed positive correlations between antibiotic-resistant genes and heavy metal levels in environments exposed to anthropogenic pressure (Pal *et al*., 2015) and free of antimicrobials (Barancheshme and Munir, 2018).

Selective pressure promotes the exchange of antibiotic resistance genes through horizontal gene transfer between commensals and environmental species to pathogenic species (Qian *et al*., 2016; von Wintersdorff *et al.,* 2016; Zieliński *et al.,* 2021). The dissemination of antibiotic resistance genes via horizontal gene transfer is most often linked with the presence of mobile genetic elements such as plasmids which play an essential role in the transfer of antibiotic-resistant genes through conjugation (Hall *et al.,* 2017; Lerminiaux and Cameron, 2019; Osińska *et*

al., 2020). According to many authors, integrons are also significantly implicated in the spread of antibiotic resistance (Deng *et al*., 2015; Osińska *et al.,* 2020).

The development of antibiotic-resistant bacteria is often linked with the type of antibiotic and the bacterial species (Kolár *et al.,* 2001; Barancheshme and Munir, 2018). Similarly, several bacterial species belonging to the Proteobacteria have been shown to tolerate heavy metal stress, including toxic salts of noble metals (Johnson *et al.,* 2019). As a result, identifying bacteria with multiple resistance to heavy metals and antibiotics in wastewater treatment plant effluent and assessing the heavy metal tolerance of bacteria strains for potential use as bioremediation agents is essential.

MATERIALS AND METHODS

Sample Source:

Wastewater was collected from a university community central sewage treatment plant located at the precinct of the Lagos lagoon (06º25'N 03º27'E). Effluent samples from the septic tank and raw sewage sludge were collected in sterilized glass bottles aseptically within 8-9 am and transported to the laboratory in an ice bucket for analysis within 6 h of collection.

Physicochemical Analysis:

The physicochemical parameters of the effluent from the wastewater and sludge were assayed using the American Public Health Association (APHA) (1998) procedures. These parameters include Biological Oxygen Demand (BOD), Chemical Oxygen Demand (COD), Total Hardness (TH), phosphate, nitrate, sulphate, Dissolved Oxygen (DO), pH, conductivity, Total Dissolved Solids (TDS), Total Suspended Solids (TSS), Total Solids (TS), oil grease and heavy metal analysis.

Media Preparation:

The media used include Nutrient Agar (NA) (Oxoid), Muller- Hinton Agar (MHA) (Rapid labs), Luria Bertani medium (LB), Muller- Hinton Broth (MHB) (Rapid labs), and Nutrient Broth (NB) (Oxoid). These were prepared according to the manufacturer's specifications.

Preparation of Metal Stock Solution:

The stock solutions of chromium (Cr), copper (Cu), cadmium (Cd), and lead (Pb) were prepared in deionized water and stored at 4°C. The salts used were chromium (III) trioxonitrate (V) ($Cr(NO_3)_3.9H_20$), copper (II) sulfate pentahydrate ($CuSO_4.5H_2O$), Cadmium tetraoxosulphate (VI) octahydrate ($3CdSO_4.8H_20$) and lead nitrate $(PbNO_3)_2$ (Table 1). All working concentrations were obtained by diluting the stock solution with sterile deionized water, and the pH was adjusted to the desired values using 1M HCl and 1M NaOH solutions (Bahig *et al.,* 2008).

Table 1: Heavy metal salts and properties

Heavy Metal	Salts	The molecular weight of salts	The atomic weight of the metal	The concentration of stock solution (ppm)
Chromium (Cr)	$Cr(NO_3)_3.9H_20$	400.15	51.996	1000
Cadmium (Cd)	$3CdSO_4.8H_20$	769.56	112.41	1000
Copper (Cu)	$Cu_2SO_4.5H_20$	249.68	63.546	1000
Lead (Pb)	$Pb(NO_3)_2$	331.20	207.20	1000

Isolation Of Heavy Metal-Resistant Bacteria:

Bacterial strains were isolated from the effluent samples using the serial dilution and pour plate method. The selective isolation of heavy metal-resistant bacteria was done using heavy metals incorporated media. An aliquot of 1 ml from different dilutions was aseptically transferred into sterile Mueller Hinton agar plates incorporated with 25 ppm concentrations of various heavy metals of Cr^{3+}, Cu^{2+}. Pb^{2+} and Cd^{3+} by standard pour plate method. Plates were incubated at 37ºC for 24 - 48 h. The developed colonies were sub-cultured on sterile Mueller Hinton agar

incorporated with 25 ppm of the heavy metals to obtain pure cultures.

All the strains were maintained on slants of Mueller Hinton agar medium containing 25 ppm of heavy metal stored in the refrigerator at approximately 4°C. These were sub-cultured at about 45-60 days intervals.

Screening Of Heavy Metal Resistant Bacteria:

Pure cultures of the bacteria were directly streaked on sterile Mueller Hinton agar incorporated with different concentrations of heavy metals ranging from 25 ppm to 750 ppm to determine the heavy metals Maximum Tolerance Concentrations (MTC) of the organisms. The pure cultures of colonies that survived at high concentrations were identified based on their morphology and biochemical characteristics according to Bergey's manual of Systematic bacteriology, Microbact GNB 24E kit, and 16 rRNA analysis.

Determination of Minimal Inhibitory Concentration (Mic):

Resistance of the bacterial isolates to varying concentrations of heavy metals (Pb, Cu, Cr, Cd) was determined by the broth dilution method (Luli *et al*. 1983). Sterilized Mueller Hinton broth medium was spiked with different concentrations of heavy metal salts. Colonies of overnight bacterial culture were used to prepare 0.5 MacFarland standard, and 0.1 ml was inoculated into the test tubes containing heavy metal salts and broth (5 ml). Positive controls consisted of metal-deficient medium inoculated with bacterial cultures, and negative ones consisted of metal-supplemented medium without bacterial cultures. The tubes were incubated aerobically at 37ºC for 24 h. The minimum concentrations of heavy metals that completely inhibited growth when compared with the controls and optical density measurement was considered the MIC. The optical density (OD) was measured at 620 nm using a colorimeter (AE-11D Digital photoelectric colorimeter).

The cultures were streaked onto Mueller Hinton agar containing metal salts using sterile loops and then incubated at 37ºC for 24 - 48 h. The plates were checked for bacterial growth.

Determination of Minimum Bactericidal Concentration (Mbc):

Minimum bactericidal concentration is the lowest heavy metal concentration that kills at least 99.9% of the test organism. This was determined by streaking Mueller Hinton agar plates with the content from the MIC test tubes consisting of different concentrations of heavy metal salts. After 24 h incubation at 37ºC, plates without growth were considered Minimum Bactericidal Concentration.

Antibiotic Susceptibility Test:

Antibiotic susceptibility of the heavy metal resistant bacteria was determined by the Kirby-Bauer disc diffusion method (Bauer *et al.,* 1966). The antibiotics discs (Rapid labs) were placed on Mueller Hinton agar plates uniformly swabbed with cell suspension with a turbidity of 0.5 McFarland standards. The plates were incubated at 37º C for 24 h and observed for inhibition zones. The diameter of the inhibition zones around the discs was measured in mm. Antibiotic resistance or sensitivity was determined by comparing the diameter of the inhibition zone around each antibiotic disc with the zone size interpretive chart supplied by the clinical laboratory standard institute (CLSI, 2013).

The MAR index was calculated as the ratio (*a*/*b*) between the number of antibiotics to which the isolate was resistant (*a*) and the total number of antibiotics tested (*b*). A MAR index value >0.2 is observed when the isolates are exposed to high-risk human or animal contamination sources, where antibiotics use is common; in contrast, a MAR index value ≤0.2 is observed when antibiotics are seldom or never used (Krumperman, 1983).

Identification of Bacterial Strains:

The pure cultures were identified using the Microbact GNB 24E and 16 rDNA analysis based on their morphology and biochemical characters. The Gram stain, catalase test, oxidase test, and motility test were done as described by Bisen *et al.* (2012).

Molecular Characterization:

A modified Moore *et al.* (2004) protocol was used to extract the total genomic DNA. The 16S rDNA genes were amplified with bacterial universal primers 27F (5'- AGAGTTTGATCMTGGCTCAG -3') and reverse primer 1525r (5'- AAGGAGGTGWTCCARCCGCA -3'). The PCR amplification of the DNA started with an initial denaturation at 94 °C for 5 min, followed by 36 cycles of denaturation at 94 °C for 30 s, annealing at 56 °C for 30 s, extension at 72 °C for 45 s. and final extension step was at 72 °C for 7 min. The PCR products were analyzed by 1.5% agarose gel electrophoresis stained with ethidium bromide and visualized under a UV transilluminator. The PCR product was sequenced by ABI3730xl Genetic Analyzer. Sequences were matched with previously published bacterial 16S rRNA sequence in the NCBI database using Standard Nucleotide BLAST (www.ncbi.nlm.nih.gov/BLAST) (Altschul *et al*., 1997).

Isolation of Plasmid:

Plasmid DNA extraction from the bacterial cells was done by an amended version of the procedure of Birnboim and Doly (1979). These were electrophoretically separated on 0.8% (w/v) agarose gels at 60 V/cm using 0.5X concentration of Tris-Borate-EDTA (TBE) buffer (Adeyemo and Onilude, 2015).

Plasmid Curing:

The plasmids were cured by treatment with acridine orange according to the method of Brown (2000) and as described by Adeyemo and Onilude (2015). The nutrient broth was prepared and supplemented with 0.1 mg/ml acridine orange. An aliquot of 20 µl of an overnight culture of the bacteria was sub-cultured into 5 ml of the nutrient broth containing acridine orange. The samples were incubated at 37°C for 72 h. After 72 hours of incubation, the isolates were sub-cultured onto Mueller Hinton agar, and plasmid extraction was repeated for the organisms to verify if the plasmid was successfully cured. Growth of isolates on heavy metals and Antibiotic susceptibility was carried out again.

PCR Amplification of Chromium and Cadmium Resistant Genes:

The metal tolerance ability of the isolates to chromium and cadmium was determined by amplification of the *ChrB* (Kamika and Momba, 2013) and *czc* (Chiboub *et al.*, 2016) genes, which encode for chromium and cobalt-zinc-cadmium resistance, respectively. The specific primers used for *chrB* were 5'- GTCGTTAGCTTGCCAACATC -3' (forward primer) and 5'- CGGAAAGCAAGATGTCGATCG -3' (reverse primer) (Kamika and Momba, 2013). That of czc was 5'- AACCAG ATCTCGCGCGAGAAC -3' (forward primer) and 5'- CGGCAACACCAGT AGGGTCAG -3' (reverse primer) (Chiboub *et al.*, 2016). The conditions of PCR amplification were, denaturation of template DNA at 94°C for 2 min, followed by 30 cycles of denaturation at 94°C for 1 min, annealing of template DNA for 30 s at 57ºC (*ChrB*) and 55ºC (*czc*) and an extension time of 1 min at 72°C for the primers. After the last cycle, the samples were kept at 72°C for 10 min to complete the synthesis of all the strands, and a cooling temperature of 4°C was applied. The PCR products were analyzed by electrophoresis using 1% (m v^{-1}) agarose gel stained with ethidium bromide and visualized under a UV transilluminator.

Statistical Analysis:

Plotting of graphs was done by Microsoft Excel 2019.

RESULTS

Physicochemical Analysis of Samples:

The physicochemical properties of the freshly collected water samples are shown in Table 2. The pH of the sampling points was within an acceptable limit. The conductivity reduced down the treatment line, with raw sewage sludge having a value of 1460 s/cm and a septic tank of 1058 s/cm. The total dissolved solids, total solids, and total suspended solids were more significant than the acceptable limits of NESREA (2009). The values for the Biochemical oxygen demand, 83.95ppm and 69.70 ppm for both samples, were higher than the permissible limit (Table 2). In comparison, the values for chemical oxygen demand (38.10 ppm 25.80 ppm) were within the acceptable limit (Table 2). The concentrations of nickel, manganese, cadmium, chromium, copper, zinc, and iron were all within acceptable limits for both samples except for lead.

Table 2: Physicochemical analysis of the septic tank and raw sewage sludge from a wastewater treatment plant

Parameters	Sampling Points		Limits
	Raw sewage sludge	Septic tank	NESREA
pH	6.99	6.90	6-9
Conductivity	1460	1058	NS
TDS (ppm)	700	520	500
TSS (ppm)	256	110	25
TS (ppm)	970	630	NS
Nitrate (ppm)	97.70	91.50	10
Phosphate (ppm)	209.8	150.54	NS
BOD (ppm)	83.95	69.70	30; 50
COD (ppm)	38.10	25.80	60; 90
DO (ppm)	ND	ND	NS
TH (ppm)	7.5	8.5	NS
Sulphate	3.10	ND	250
Appearance	Not Clear	Not Clear	Clear
Odour	Objectionable	Objectionable	Odourless
Temperature (^oC)	27.2	26.9	40
Pb (ppm)	0.082	0.056	0.05
Ni (ppm)	0.019	0.012	0.05
Mn (ppm)	0.044	0.057	0.2
Cd (ppm)	ND	0.980	1.0
Cr (ppm)	0.011	0.208	1.0
Cu (ppm)	0.060	0.043	0.5
Zn (ppm)	0.019	0.100	2.0
Fe (ppm)	0.974	0.755	2.0
Oil/Grease (m g^{-1})	3.40	3.75	0.5

TDS, Total Dissolved Solids; TSS, Total Suspended Solids; TS, Total Solids; BOD, Biochemical Oxygen Demand; COD, Chemical Oxygen Demand; TH, Total Hardness; Pb, Lead; Ni, Nickel; Mn, Manganese; Cd, Cadmium; Cr, Chromium; Cu, Copper; Zn, Zinc; Fe, Iron; ND, Not Detected; NS, Not Stated; NESREA, National Environmental Standards and Regulations Enforcement Agency (2009).

Isolation and Identification of Heavy Metal Resistant Bacteria:

After screening the isolates from the wastewater treatment plant, four bacteria that tolerated over 200 ppm concentration of the four heavy metals were selected for further studies (Table 3). The isolates were molecularly and phenotypically identified as *Alcaligenes faecaclis* (*Burkholderia. pseudomallei*),

Klebsiella ornithinolytica, Paenalcaligenes hominis (*Enterobacter cloacae*) and *Providencia vermicola* (*E. cloacae*). *Alcaligenes faecaclis* tolerated > 400 ppm concentrations of the metals on agar. All the isolates tolerated 700 ppm concentrations of chromium. The isolates were less tolerant to copper (300-400 ppm).

Table 3: Screening and identification of heavy metal resistant bacteria in wastewater on solid media

Phenotypic identification	Blast result (molecular identification)	Heavy metals (ppm)			
		Copper	Lead	Chromium	Cadmium
Burkholderia pseudomallei	*Alcaligenes faecalis*	400	750	700	600
Klebsiella ornithinolytica	Unidentified	300	600	700	300
Enterobacter cloacae	*Paenalcaligenes hominis*	200	700	700	350
Enterobacter cloacae	*Providencia vermicola*	300	600	700	250

Minimum Inhibitory Concentration (Mic):

The minimum inhibitory concentration (MIC) of the metals in Figure 1 revealed, that the bacterial isolates had a high level of resistance to lead (>500 ppm) except for *Providencia vermicola* (150 ppm). Among the isolates, *Providencia vermicola* had the least resistance to the heavy metals used (copper 60 ppm, lead 150 ppm cadmium 20 ppm).

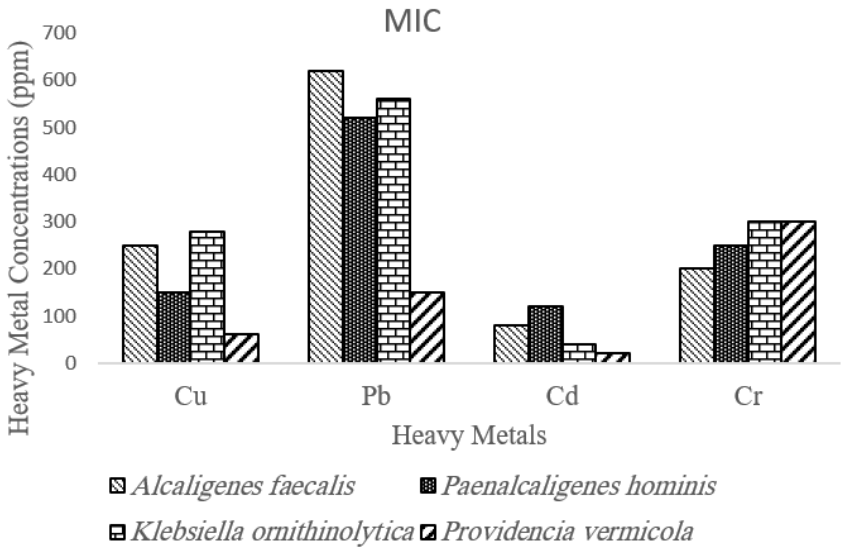


Fig. 1. Minimum Inhibitory Concentrations (ppm) of heavy metals on bacterial strains from wastewater.

Minimum Bactericidal Concentration (Mbc):

The minimum bactericidal concentrations of the heavy metal in Figure 2 showed that cultures of *Alcaligenes faecalis, Paenalcaligenes hominis,* and *Klebsiella ornithinolytica* were no longer viable at concentrations of 750 ppm,700 ppm, and 600 ppm of lead, respectively. The bacteria also had a high tolerance level to chromium before losing their viability between 320 – 450 ppm concentrations. Cadmium and copper were more toxic to the bacteria strains. However, *Alcaligenes faecalis* tolerated the metals more than the other strains before losing their viability at concentrations of 380 ppm and 400 ppm to cadmium and copper respectively. *Providencia vermicola* was the most sensitive to heavy metals.

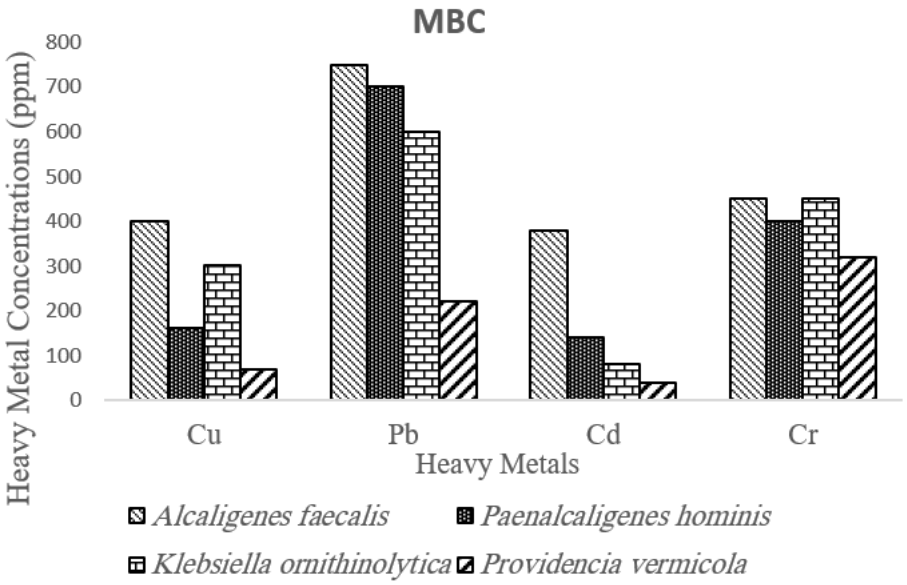


Fig. 2. Minimum Bactericidal Concentrations (ppm) of heavy metals on bacterial strains from wastewater.

Antibiotic Sensitivity of Heavy Metals Resistant Isolates:

The bacteria all exhibited multidrug resistance to 2 or more antibiotics, which include ampicillin (10 µg), ceftazidime (30 µg), and augmentin (30 µg) (Table 4). However, they were all susceptible to ciprofloxacin (5 µg). The highest incidence of multiple drug resistance to six antibiotics was observed in *Klebsiella ornithinolytica*. Multiple antibiotic resistance indices ranged from 0.25 to 0.75.

Table 4: Antibiotic sensitivity of heavy metals resistant bacteria

Organism	**GEN 10 µg**	**AMP 10 µg**	**CAZ 30 µg**	**OFX 5 µg**	**AUG 30 µg**	**CXM 30 µg**	**CIP 5 µg**	**NIT 300 µg**	**MAR Index**
	Zone of inhibition (mm)								
Alcaligenes faecalis	S (22)	R (0)	R (0)	S (28)	R (0)	R (0)	S (28)	S (17)	0.5
Klebsiella ornithinolytica	R (10)	R (0)	R (0)	R (14)	R (0)	S (26)	S (26)	R (0)	0.75
Paenalcaligenes hominis	S (20)	R (0)	R (0)	S (19)	R (0)	R (0)	S (24)	S (17)	0.5
Providencia vermicola	S (19)	R (0)	R (14)	S (28)	I (14)	I (18)	S (28)	S (17)	0.25

GEN, gentamycin (10 µg); AMP, ampicillin (10 µg); CAZ, ceftazidime (30 µg); OFX, ofloxacin (5 µg); AUG, augmentin; CXM, cefuroxime (30 µg); CIP, ciprofloxacin (5 µg); NIT, nitrofurantoin (300 µg); S, Sensitivity; R, Resistant; I, Intermediate; MAR, Multiple Antibiotic Resistance.

Plasmid Curing Activity on Heavy Metals and Antibiotics:

All the bacteria had a single plasmid ≥10 Kbp in size (Table 5). After curing the bacteria plasmids, the metal tolerance test revealed that all the bacteria were able to grow (resistant) in the presence of Cu and Pb at 300 ppm concentrations. However, they were sensitive to the presence of Cr and Cd in the growth medium. The resistance of the bacteria to antibiotics was not affected by plasmid curing (Table 6). The PCR amplification of *ChrB* (Chromium) and *czc* (cobalt-zinc-cadmium) resistant genes in the bacterial strains produced no bands for all the strains (Fig. 3).

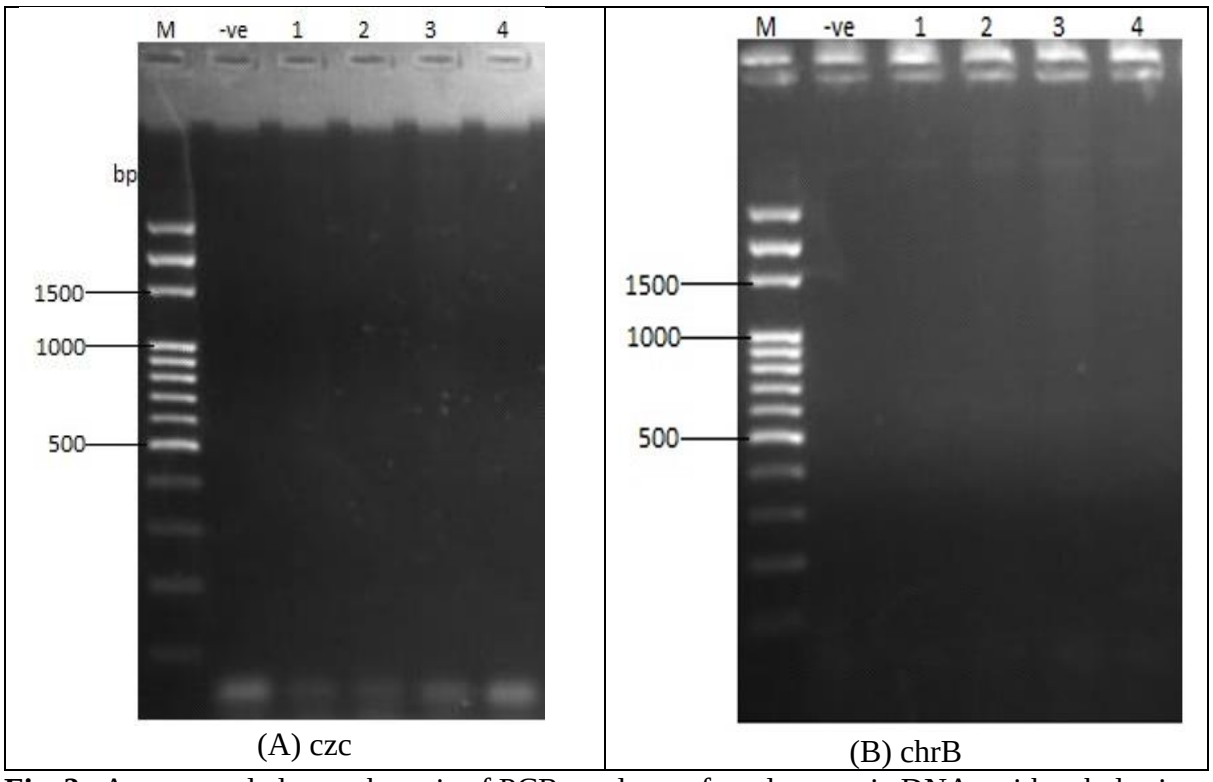
Table 5: Plasmid curing effect on heavy metal resistance in bacteria

Isolates	Plasmid (kbp)	Heavy metals (300 ppm)							
		Cr		Cu		Cd		Pb	
		Before	After	Before	After	Before	After	Before	After
Alcaligenes faecalis	12.5	700	-	400	+	600	-	750	+
Klebsiella ornithinolytica	10.0	700	-	300	+	300	-	600	+
Paenalcaligenes hominis	10	700	-	200	+	350	-	700	+
Providencia vermicola	11.1	700	-	300	+	250	-	600	+

Table 6: Plasmid curing effect on antibiotic resistance in bacteria

Antibiotics	Bacterial Strain and Curing Activity							
	Alcaligenes faecalis		*Klebsiella ornithinolytica*		*Paenalcaligenes hominis*		*Providencia vermicola*	
	Before	After	Before	After	Before	After	Before	After
CAZ (30µg)	R	R	R	S	R	R	R	I
CXM (30µg)	R	R	S	S	R	R	I	S
GEN (10µg)	S	S	R	I	S	S	S	S
CIP (5µg)	S	S	S	I	S	S	S	S
OFX (5µg)	S	S	R	R	S	S	S	I
AUG (30µg)	R	R	R	I	R	R	I	I
NIT (300µg)	S	I	R	R	S	S	S	I
AMP (10µg)	R	R	R	R	R	R	R	R

GEN, gentamycin (10 µg); AMP, ampicillin (10 µg); CAZ, ceftazidime (30 µg); OFX, ofloxacin (5 µg); AUG, augmentin; CXM, cefuroxime (30 µg); CIP, ciprofloxacin (5 µg); NIT, nitrofurantoin (300 µg); S, Sensitivity; R, Resistant; I, Intermediate.



(A) czc (B) chrB

Fig. 3. Agarose gel electrophoresis of PCR products of total genomic DNAs with cobalt-zinc-cadmium (*czc*) and chromium (*chrB*) resistance genes. Lanes: M, DNA ladder (Marker); +ve, Negative (Non-template DNA); 1 to 4, amplified PCR products of *Providencia vermicola* (1), *Alcaligenes faecalis* (2), *Klebsiella ornithinolytic* (3) and *Paenalcaligenes hominis* (4).

DISCUSSION

The bacteria isolated demonstrated multiple tolerance to the Cd, Cr, Pb and Cu evaluated in this investigation. Multiple metal tolerance occurs because metals do not occur singly in the environment. For instance, Cd is often accompanied by Zn (Ugwuja *et al.,* 2015), while Cr is accompanied by Co and Ni (Poznanović Spahić *et al.,* 2018). Apart from the multiple heavy metal resistance observed in the bacteria from the wastewater treatment plant, multiple antibiotic resistance was also exhibited. This reinforces the fact that wastewater is a hotspot for developing pollutants such as antibiotics, heavy metals, antibiotic resistance genes, and heavy metal resistant genes (Barancheshme and Munir, 2019).

Numerous studies have looked at the isolation and characterization of heavy metal, and antibiotic-resistant bacteria in wastewater from places like the dumpsites (Sanuth and Adekanmbi, 2016), printeries (Adekanmbi *et al.*, 2019), bight sediments (Timoney *et al.*, 1978), lakes and rivers (Matyar *et al.*, 2014), and many genera of bacteria have been discovered, including those isolated in this study. Multiple heavy metal and antibiotic resistance in *Alcaligenes faecalis* and *Paenalcaligenes hominis* from wastewater was reported by Abo-Amer *et al*. (2015), Sanuth and Adekanmbi (2016), Adekanmbi *et al.* (2019); Ayyal Al-Gburi (2020), Olowo-okere *et al.* (2020) and Ibrahim *et al.* (2021). According to a comparative analysis of *Paenalcaligenes hominis* 16S rRNA gene sequences, this bacterium shares <95 % similarity with all reported species of the Alcaligenaceae genera (Kämpfer *et al.,* 2010). *Alcaligenes faecalis* usually cause opportunistic infections in humans and are often difficult to treat due to their increased resistance to several antibiotics (Huang, 2020). The bacterium *Providencia vermicola* in particular displayed a 100% resistance to amoxicillin, clavulanic acid, and ampicillin\sulbactam. The heavy metal resistance gene (*ChrB)* is present in this bacterium by Adekanmbi *et al.* (2019). Resistance to antibiotics, heavy metals, and genes resistant to both are of ecological importance because bacteria may transfer their resistance to previously non-resistant bacteria by horizontal gene transfer.

The minimal bactericidal concentration and tolerance of the bacteria to heavy metals were generally higher on Mueller Hinton agar than in broth. For example, *Alcaligenes faecalis* had MIC value of 620 ppm and MTC value of 750 ppm in lead. Hassen *et al.* (1998) had similar observations. They explained that toxicity testing in a liquid medium allows a good evaluation of metal toxicity in polluted environments, such as industrial effluents and sewage sludge leachates. They attributed this difference between liquid and a solid medium to the differences in the conditions of diffusion, complexation, and availability of metals in the media. The MIC values for this study are consistent with that observed by other studies where the concentrations of heavy metals (Cu^{2+}, Cr^{6+}, Cd^{2+}, Hg^{2+}, Zn^{2+}, Pb^{2+}, Cd^{2+}, Al^{3+}, Cu^{2+}, Ag^{2+}, and Sn^{2+}) in bacteria from wastewater ranged from 100 to 1400 mg/L (Shakoori and Muneer, 2002; Abo-Amer *et al*., 2015; Adekanmbi *et al.*, 2019).

The resistance of the bacteria from the wastewater to antibiotics confirms Bhattacherjee *et al.* (1988) observation that multiple antibiotic-resistant bacteria occur in polluted water. This was reinforced by the bacteria's multiple antibiotic resistance indices, which were>2, suggesting exposure of the isolates to antibiotic contamination (Matyar *et al.,* 2014). Similarly, the bacteria resistance to both heavy metals and antibiotics in the wastewater confirms that resistance factors for heavy metals and antibiotics are co-selected in heavy metal-contaminated systems or habitats exposed to anthropogenic pressure (Timoney *et al.,*

1978; Bhattacherjee *et al.,* 1988; Baker-Austin *et al.,* 2006; Heydari *et al.,* 2022).

Plasmids greater than 10 kb were found in the bacterial isolates. The plasmid of this size was similarly reported in bacteria from printeries effluent by Adekanmbi *et al.* (2019). On the genetic level, bacterial resistance to heavy metals or antibiotics can be chromosomal and/or in mobile elements (e.g transposons and plasmids) (Gupta *et al*.,1999; Legatzki *et al.,* 2003; Gutiérrez-Barranquero *et al.,* 2013; Di Cesare *et al*., 2016b). Resistance to Cu and Pb remained active after plasmid curing, implying that the resistance genes are chromosome-mediated. However, after curing, the bacteria were sensitive to Cd and Cr, suggesting that the genes controlling resistance to these metals in the bacteria were mobile element genetic element mediated. The PCR amplification of the *czc* and *chrB* genes for Cd and Cr resistance tested negative. This suggests that the bacteria lacked the Cd resistance genes *czcCBA.* Cadmium resistance is coded for by the *czcCBA genes* located on a plasmid pMOL30, and the *cadA* and *zntA genes* located on the chromosome (Legatzki *et al.,* 2003). Several works have reported plasmid pMOL30 mediating resistance to Co^{2+}, Zn^{2+}, and Cd^{2} in *A. eutrophus* (Grosse *et al.,* 1999), *Ralstonia metallidurans* (Legatzki *et al.,* 2003) and *Cupriavidus metallidurans* (Scherer and Nies, 2009). The Cr resistance genes *ChrB* and *ChrA* can be carried on transposable elements either on a plasmid or chromosomally integrated. For example, bacteria with mobile genetic elements in plasmids containing Cr resistance genes *ChrB* and *ChrA* were demonstrated in strains *Acinetobacter* spp. (Mindlin *et al.,* 2018) from the environment. A transposon carrying a chromate resistance determinant was reported in a plasmid pB4 from an uncultured bacterium (Tauch *et al.,* 2003), and a chromosomally integrated chromate-inducible *chrBACF* operon from the transposable element Tn*OtChr* which confers resistance to Chromium (VI) and Superoxide was described in *Ochrobactrum tritici* 5bvl1 (Branco *et al.,* 2008).

To prevent the spread of antibiotic-resistant bacteria in the environment, wastewater must be adequately treated before being discharged into water bodies. In the case of heavy metals, wastewater effluents will be suitable sources of possible bacteria candidates with multiple metal resistance abilities for bioremediation of metal-contaminated sites.

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