Co-Resistance to Antibiotics and Heavy Metals in ESBL-Producing Gram-Negative Bacteria from Clinical Samples

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Abstract

There is growing concern about the combined impact of antimicrobial resistance in pathogenic bacteria and environmental contaminants, such as heavy metals, on the efficacy of routine antibiotics. The specific aim of this study is to evaluate the co-resistance of Extended Spectrum Beta-Lactamase (ESBL)-producing isolates to common heavy metal pollutants (Copper, Arsenic, and Lead) individually and in combination with antibiotics. The present study evaluated the effects of three heavy metals (arsenic, copper, and lead) on the antimicrobial susceptibility profiles of 30 ESBL-producing bacteria. A total of 200 clinical specimens were collected, from which 150 Gram-negative bacterial isolates were obtained and studied. This included Escherichia coli 60 (40%), Pseudomonas aeruginosa 35 (23%), Enterobacter aerogenes 27 (18%), Proteus mirabilis 15 (10%), and Klebsiella pneumoniae 13 (8.60%). The Antibiotics, Double Disc Synergy Test (DDST) test revealed that 30 samples were ESBL-producing bacteria: 15 E. coli, 5 Enterobacter aerogenes, 3 Proteus mirabilis, 5 Pseudomonas aeruginosa, and 2 Klebsiella pneumoniae. These samples were tested for heavy metal resistance; ESBL-producing bacteria grew densely in the presence of different concentrations of arsenic, copper, and lead and Growth was observed up to and including 0.5 mg/mL, but was inhibited at 0.75 mg/mL. When Antibiotic Susceptibility Test (AST) was performed in the presence of heavy metals, the susceptibility profile shifted from susceptible to resistant for meropenem. For the remaining antibiotics tested in combination with heavy metals, and antibiotics did not change the susceptibility profile, but heavy metals did affect the zone size. Heavy metal presence either reduced or increased the zone size, depending on the type of metal and antibiotic.

Keywords: Antibiotic Susceptibility Test, Antibiotics, Double Disc Synergy Test, Heavy Metals, Extended Spectrum Beta-Lactamase, Producing Bacteria, Antibiotic-resistant, Bacteria.

Introduction

The discovery of antibiotics and their use in medicine was a major step forward in healthcare during the 20th century (Øen et al., 2021; Umar

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et al., 2024). In addition to helping cure serious infections, antibiotics also made many advanced surgeries possible, like treating cancer, doing organ transplants and performing open-heart surgeries (Kan et al., 2021). The One Health approach aims to ensure the best health for both people and the environment. Antimicrobial resistance is seen as a challenge under One Health because bacteria and genes that carry resistance can rapidly spread around the world. To tackle this problem, global plans have been created with the cooperation of three major organizations: the World Health Organization, the Food and Agriculture Organization and World Organization for Animal Health (WOAH) (Regea, 2018; Ullah et al., 2023).

Antimicrobial resistance is recognized as a major and urgent challenge for treating infectious diseases and stands as a prominent public health concern in the 21st century (Kourkouta et al., 2018; Umar & Khan, 2025). Bacterial or other microbial strains resistant to treatment often exist at low levels within a population of hosts even before treatment starts. However, during treatment, these resistant strains can become more prevalent due to factors like antibiotic-induced hyper-mutation or the transfer of resistance genes via plasmids (Kofteridis et al., 2020). This dynamic is driven by two important characteristics. First, the strains that are resistant to antibiotics are typically less capable of survival compared to the non-resistant wild-type strains when there's no antibiotic treatment. Second, when antibiotics are present, the resistant strains tend to be better suited for survival compared to the original non-resistant strains. As a result, during treatment, the more resistant strains gain an advantage and become more prevalent than the initially sensitive strains (Serwecińska, 2020).

In the present-day scenario, there is a continuous evolution in drug sensitivity patterns, and regrettably, there is a rise in the prevalence of infectious diseases that are resistant to multiple drugs (Khan et al., 2024). This has led to a higher number of illnesses and fatalities. Multi-Drug Resistant (MDR) refers to bacteria that have developed resistance to resistance to at least one agent in three or more antimicrobial categories. On the other hand, XDR bacteria are non-susceptible to at least one agent in all but two or fewer antimicrobial categories(Catalano et al., 2022). Antibiotic susceptibility and identification of the causal agent implicated in the human body are critical for empirical treatment and the avoidance of resistant bacteria. Unfortunately, relatively little research on prevalent pathogens and their antimicrobial sensitivity profiles has been published in Pakistan (Bilal et al., 2021).

In Pakistan, infections are acquired from various sources, including local environments, agricultural activities, and healthcare

settings. The presence of heavy metals such as mercury and arsenic in aquatic environments might contribute to microbes acquiring genetic traits that provide resistance to these metals. These traits are found on the microbes' chromosomes or plasmids (Junaid et al., 2021). Heavy metals and antibiotics often exist together in various natural environments, such as the digestive systems of animals, animal waste, and poultry farms. For instance, arsenic and antibiotics are commonly used in the livestock industry to prevent infections and promote growth in animals, leading to the co-presence of arsenic and antimicrobials in the digestive systems of domesticated animals. Additionally, when poultry waste is used as fertilizer in soil, a common practice for recycling nutrients, native microorganisms are exposed to both arsenic and antimicrobial agents. While many antimicrobial agents break down relatively quickly, they are considered "pseudo-persistent" because they continue to be introduced into the ecosystem consistently over time (Gupta et al., 2022).

Enterobacteriaceae comprise a diverse group of microorganisms known for causing infections in both healthcare facilities and communities. Some examples of bacteria within the Enterobacteriaceae family include *Escherichia coli* (*E. coli*), *Klebsiella pneumoniae*, and various species of *Proteus* and *Enterobacter.* (Janda & Abbott, 2021). Antimicrobial agents often face newly developed defense methods called "resistive mechanisms". For instance, some types of Enterobacteriaceae can create Extended Spectrum Beta Lactamases (ESBLs). These ESBL enzymes can break apart and neutralize many commonly used antibiotics like penicillin and cephalosporin. As a result, these medicines become ineffective in treating infections caused by these bacteria (Onduru et al., 2021). Instead of taking antibiotics by mouth at home, patients with these infections might have to go to the hospital and get intravenous carbapenem therapy for treatment to work effectively. (Iseppi et al., 2020).

In the findings of McDonald et al. (2021), ESBLs were initially identified in 1985, originating from Germany and Britain. Over the past few decades, the proliferation of ESBLs has been markedly noteworthy. The prevalence of " ESBLs-producing *Klebsiella spp.*" varies across different countries. For instance, in a survey conducted in laboratories in the Netherlands, only 1.5% of *E. coli* and *K. pneumoniae* strains exhibited ESBLs. Conversely, in "France and Italy", resistance to ceftazidime was observed to reach up to 45% among various *K. pneumoniae* strains in the US, ranging from 0% to 30% with an average rate of around 3.5% in the general population. The Centers for Disease Control and Prevention reported a 50% increase in resistance to extended-spectrum cephalosporin in strains of *E. coli* found in US emergency room patients when comparing the rate in 1999 to the average rate from 1994 to 1998. Additionally, the

rate of resistance to extended-spectrum cephalosporin in "*Klebsiella pneumoniae* " isolates from US intensive care unit patients was found to be 12% in 1999 (Kaye et al., 2021).

ESBL is an enzyme produced by some bacteria that makes them resistant to routine β-lactam antibiotics. These ESBL-producing bacteria are becoming a challenge for healthcare professionals. Moreover, heavy metal resistance has been reported to be associated with antibiotic resistance due to shared mechanisms and may also contribute to the spread of antibiotic resistance. Widespread use of such chemicals has transformed many dangerous bacteria into superbugs. In this study, the co-resistance of heavy metals and antibiotics co-resistance of ESBL-producing bacteria was examined to confirm their association. The effects of heavy metals on antimicrobial susceptibility profiling were determined to understand their role in antibiotic resistance. The specific aim and objective is to evaluate the co-resistance of ESBL-producing isolates to common heavy metal pollutants (Copper, Arsenic, Lead) individually and in combination with antibiotics.

Materials and Methods

Sample Collection

From the 200 clinical specimens, 150 Gram-negative bacterial isolates were identified and selected for further analysis. ESBL-producing samples were collected from the Chughtai Labs and Neutro Pharma in Lahore.

Isolation and identification of Bacterial Strains

Samples were inoculated on Blood agar and MacConkey agar to grow the microbes in the samples, and then the bacteria by selecting and streaking on nutrient agar to get isolated bacterial strains.

Gram Staining

An inoculating loop was used to pick a colony from the culture and transfer it to the glass slide. A drop of distilled water was added to make a smear for Gram staining.

Biochemical Testing

VITEK Compact System

The VITEK Compact technique was performed by following the steps mentioned below: For an organism, a test tube was labelled with the Identification number. For inoculum preparation, 3 ml of normal saline

was added to a tube and an isolated colony with an inoculating loop mixed well. The density of the prepared inoculum was measured using a densitometer. The density range for bacteria must be 0.5-0.63. The reagent card was put in an inoculum tube and loaded in a Vitek compact cassette. After that, the cassettes were transferred into the incubator module, and the remaining steps were automatically handled by the machine. Result/Report comes after 5-7 hours.

Antibiogram Pattern

Kirby Bauer Methodology

Identical colonies of *E. col Single and same coloniesi* and *Proteus mirabilis, Klebsiella,* and *Pseudomonas aeruginosa* were picked and transferred into the McFarland. If the turbidity in the tube was less than the reference value of 0.5, an inoculum was added to the tube to maintain the turbidity. With this regard, the result of the lawn in plates became confluent. The sterilized swab was used to thoroughly spread the turbidity as a uniform layer on Muller-Hinton agar. After that, antibiotic discs were placed on the inoculated Muller Hinton agar plates using a sterile antimicrobial disc dispenser and forceps (where required). After that plates were incubated at 37 °C for 16 hours and results were observed.

Double Disc Synergy Test

The "combined-disc test" was performed on Mueller Hinton agar plates to describe the phenotypic attributes of (ESBL-producing bacteria). In both tests, (Cephalosporin and clavulanate antibiotics) were used. For the confirmation of ESBL-production, according to the guidelines of CLSI, the Double Disc Synergy Test (DDST) was performed with intersecting growth of the tested bacterial isolates on Mueller-Hinton agar plates, and two antibiotic discs were placed at distance 25 mm (center to center) that were ceftriaxone (30µg) and amoxicillin-Clavulanate (containing 10 µg of Clavulanate).

Stock Solution Preparation

For stock solution, 10 grams of heavy metal salt were dissolved in 100 ml autoclaved distilled water, which was 10% stock solution and was further diluted as required for the experimental procedures.

Heavy Metals

The study employed heavy metal salts such as Arsenic, Copper, and Lead. Each isolate was evaluated on nutrient agar plates containing

increasing concentrations of every heavy metal salt. The heavy metal concentrations were between 0.1 mg/mL to 1.75 mg/mL.

Media Preparation

Nutrient agar media with heavy metal was prepared by dissolving 20 grams of dehydrated media in 1 Litter of distilled water and autoclaved at 121 °C for 15 minutes and allowed to cool down up to 50-60 °C. Then 100 µl heavy metal from the prepared stock solution was added into 100 ml sterilized media (Nutrient agar) and 20 ml media in each Petri plate. This is 0.1 mg/µL concentration. The rest of the concentrations are mentioned in table 1 below. Allowed the plates to solidify. At the end, all plates were pre-incubated at 37 °C for 24 hours.

Table 1: Concentrations of heavy metals used in this study.

Concentrations	Preparation
0.2 mg/mL	200 µl heavy metal from stock solution was added in 100 ml sterilized media
0.3 mg/mL	300 µl heavy metal solution was added to 100 ml media
0.5 mg/mL	500 µl heavy metal solution was added in 100 ml media
0.75 mg/mL	750 µl heavy metal solution was added in 100 ml media
1 mg/mL	1 ml heavy metal solution was added to 100 ml media
1.25 mg/mL	1.25 ml heavy metal solution was added to 100 ml media
1.5 mg/mL	1.5 ml heavy metal solution was added to 100 ml media
1.75 mg/mL	1.75 ml heavy metal from stock solution was added to 100 ml sterilized media

Note: All the salts (Arsenic, Copper, and Lead) were prepared by the same method.

Inoculation of Bacteria on Heavy Metals Containing Plates

After pre-incubation of plates, required bacteria were streaked on those plates and again incubated for 24 hours at 35-37 °C. Results were observed after 24 hours.

Statistical Analysis

All data were analyzed by using SPSS software. Paired T-Test was applied to compare the means between groups. Results were expressed as mean ± standard deviation (SD). A *p*-value of less than 0.05 was considered statistically significant.

Results

Biochemical Reconfirmation of Bacteria by VITEK Compact System

GN-Reagent Card (Figure 1a) is a panel that shows Gram-negative bacteria with identification of testing organisms. Outcomes presented, Pseudomonas aeruginosa (98%), Enterobacter aerogenes (96%), *Proteus mirabilis* (98%), *Klebsiella pneumoniae* (97%) and *Escherichia coli* (99 %) shown in Figure 1b.

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bioMérieux Customer: Microbiology Chart Report Printed July 6, 2022 11:27:51 AM PKT

Lab ID: 409542 Isolate Number: 1

Organism Quantity: few

Selected Organism : Escherichia coli

Comments:	

Identification Information	**Analysis Time:**	4.05 hours	**Status:**	Final
Selected Organism	99% Probability	**Escherichia coli**		
	Bionumber:	0401410450406610		
ID Analysis Messages				

Biochemical Details																
2	APPA	-	3	ADO	-	4	PyrA	-	5	lARL	-	7	dCEL	-	9	BGAL	+
10	H2S	-	11	BNAG	-	12	AGLTp	-	13	dGLU	+	14	GGT	-	15	OFF	-
17	BGLU	-	18	dMAL	-	19	dMAN	+	20	dMNE	+	21	BXYL	-	22	BAlap	-
23	ProA	-	26	LIP	-	27	PLE	-	29	TyrA	-	31	URE	-	32	dSOR	+
33	SAC	+	34	dTAG	-	35	dTRE	+	36	CIT	-	37	MNT	-	39	5KG	-
40	lLATk	-	41	AGLU	-	42	SUCT	+	43	NAGA	-	44	AGAL	-	45	PHOS	-
46	GlyA	-	47	ODC	+	48	LDC	+	53	lHISa	-	56	CMT	+	57	BGUR	+
58	O129R	+	59	GGAA	-	61	lMLTa	-	62	ELLM	-	64	lLATa	-			

Figure 1: VITEK Compact System (a) GN-Reagent Card: Gram-negative fermenting and non-fermenting. (b) Report generated by Software run for the VITEK Compact system.

Prevalence

Out of 150 Gram-negative bacterial isolates, *Escherichia coli* was the most prevalent (60 isolates, 40.0%), followed by *Pseudomonas aeruginosa* (35 isolates, 23.3%), *Enterobacter aerogenes* (27 isolates, 18.0%), *Proteus mirabilis* (15 isolates, 10.0%), and *Klebsiella pneumoniae* (13 isolates, 8.7%), as shown in Figure 2.

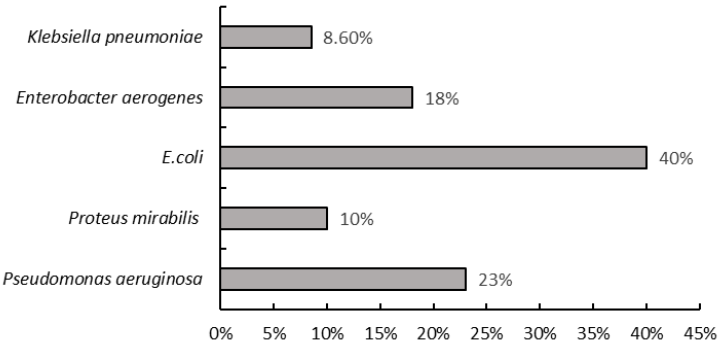

Figure 2: Prevalence of gram-negative bacteria in clinical samples of Chughtai lab.

Detection of ESBL bacteria and Non-ESBL Bacteria

All 150 Gram-negative isolates were tested for ESBL production. Of these, 30 isolates (20.0%) were ESBL producers and 120 isolates (80.0%) were non-ESBL producers. Among the 30 ESBL-positive isolates, the highest frequency was observed for *Escherichia coli* (15 isolates), followed by *Pseudomonas aeruginosa* (5 isolates), *Enterobacter aerogenes* (5 isolates), *Proteus mirabilis* (3 isolates), and *Klebsiella pneumoniae* (2 isolates), as shown in Figure 3.

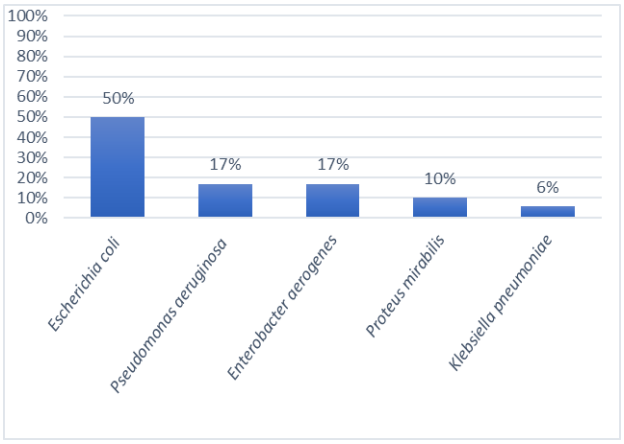

Figure 3: Prevalence of ESBL-producing bacteria among 5 test species.

Co-resistance of Common Heavy Metal Pollutants (Copper, Arsenic, and Lead) and Antibiotics on ESBL-producing Isolates

The Kirby-Bauer disc diffusion method was used for the combination of antibiotics such as Ceftriaxone (CRO),

Amoxicillin/Clavulanic acid (AMC), Piperacillin/Tazobactam (TZP), Amikacin (AK), Gentamicin (CN), Tobramycin (TOB), Ciprofloxacin (CIP), Meropenem (MEM), and Levofloxacin (LEV) and stress of Heavy metals such as (arsenic, copper, and lead) in Muller Hinton Agar to evaluate the antibiotic sensitivity and resistance pattern against *Enterobacter aerogenes*, *Escherichia coli*, *Klebsiella pneumoniae, Proteus mirabilis*, and *Pseudomonas aeruginosa*. According to the results given below, heavy metals reduce the effect of many antibiotics at a concentration of 1 mg/mL, but we also found an increasing effect in a few antibiotics.

Enterobacter Aerogenes

Antibiotic Susceptibility Profile of Arsenic (1 mg/mL Concentration) with Enterobacter aerogenes

Arsenic stress was concentrated as 1mg/mL in Muller-Hinton Agar and a combination of antibiotics to evaluate the sensitivity and resistance pattern of *Enterobacter aerogenes*. Results were that all 3 isolates with 7 antibiotics are resistant, i.e. Ceftriaxone (CRO), Amoxicillin/Clavulanic acid (AMC), Ciprofloxacin (CIP), Gentamicin (CN), Meropenem (MEM) and Tobramycin (TOB) was observed as 0 mm, and Levofloxacin (LEV) was observed as 8mm, 9mm, 10mm. Considering the permitted limits of CLSI 2 isolates with 1 antibiotic were observed as susceptible i.e. Piperacillin/Tazobactam (TZP) and zones of inhibition were 21mm and 21mm, while 3 isolates with 1 antibiotic were reported as sensitive drugs i.e. Amikacin (AK) and measured zones were 17mm, 18mm, and 18mm. Only 1 isolate with 1 antibiotic reported as intermediate, that is Piperacillin/Tazobactam (TZP) was observed as a 20 mm zone (Table 2). The paired T-test for antibiotics indicated that the difference between with and without Arsenic was highly significant statistically except for Ceftriaxone (CRO), Piperacillin/Tazobactam (TZP), and Ciprofloxacin (CIP).

Antibiotic Susceptibility Profile of Copper (1 mg/mL Concentration) with Enterobacter aerogenes

Copper stress was concentrated as 1mg/mL in Muller Hinton Agar and a combination of antibiotics to evaluate the sensitivity and resistance pattern of *Enterobacter aerogenes*. According to allowable CLSI limits result shows all 3 isolates with 7 antibiotics were resistant i.e. Ceftriaxone (CRO), Amoxicillin/Clavulanic acid (AMC), Ciprofloxacin (CIP), Gentamicin (CN), Meropenem (MEM), Tobramycin (TOB) and Levofloxacin (LEV) was observed as 0 mm zone. Out of the total isolates,

2 isolates with 1 antibiotic were reported as sensitive drugs i.e. Amikacin (AK), zones of inhibition were 18mm and 18mm. All 3 isolates with 1 antibiotic were intermediate i.e. Piperacillin/Tazobactam (TZP) observed as 20 mm, 20 mm, and 20 mm and 1 isolate with 1 antibiotic (Amikacin (AK) was observed as 16mm zone. The paired T-test for antibiotics indicated that the difference between with and without copper was highly significant statistically except for Ceftriaxone (CRO), Piperacillin/Tazobactam (TZP), and Ciprofloxacin (CIP).

Table 2: Resistance and sensitivity Pattern of Enterobacter aerogenes in a combination of antibiotics and 1 mg/mL concentration of Heavy metals.

		MEM mm		CRO mm		AK mm		TOB mm		TZP mm		AMC mm		CIP mm		LEV mm		CN mm	
		WOH	WH	WOH	WH	WOH	WH	WOH	WH	WOH	WH	WOH	WH	WOH	WH	WOH	WH	WOH	WH
Arsenic	Sample 9	29	0	4	0	21	18	0	0	22	21	15	0	0	0	0	8	0	0
	Sample 10	30	0	0	0	20	18	0	0	22	20	16	0	3	0	0	9	0	0
	Sample 11	29	0	2	0	19	17	0	0	21	21	15	0	0	0	0	10	0	0
Copper	Sample 9	29	0	4	0	21	16	0	0	22	20	15	0	0	0	0	0	0	0
	Sample 10	30	0	0	0	20	18	0	0	22	20	16	0	3	0	0	0	0	0
	Sample 11	29	0	2	0	19	18	0	0	21	20	15	0	0	0	0	0	0	0
Lead	Sample 9	29	0	4	0	21	0	0	0	22	20	15	0	0	08	0	13	0	9
	Sample 10	30	0	0	0	20	0	0	0	22	19	16	0	3	13	0	12	0	8
	Sample 11	29	0	2	0	19	0	0	0	21	17	15	0	0	10	0	10	0	9

*Without Heavy Metal (WOH), With Heavy Metal (WH)

Antibiotic Susceptibility Profile of Lead (1 mg/mL Concentration) with Enterobacter aerogenes

Lead stress was concentrated as 1mg/mL in Muller Hinton Agar and a combination of antibiotics to evaluate the sensitivity and resistance pattern of *Enterobacter aerogenes*. According to permitted CLSI limits all 3 isolates with 8 antibiotics were resistant i.e. Amikacin (AK), Amoxicillin/Clavulanic acid (AMC), Ceftriaxone (CRO), Tobramycin (TOB) and Meropenem (MEM) was observed 0mm zone, while Ciprofloxacin (CIP) was observed 8mm, 10 mm, and 13 mm zone, Gentamicin (CN) was observed 9mm, 8mm, and 9mm zone, Levofloxacin (LEV) was observed 10 mm, 12 mm, and 13 mm, and 1 isolate with 1 antibiotic that is Piperacillin/Tazobactam (TZP) shows 17 mm zone. Although 2 isolates with 1 antibiotic were intermediate, i.e. Piperacillin/Tazobactam (TZP) was observed at 19 mm and 20 mm. The paired T-test for antibiotics indicated that the difference between with and

without Lead was highly significant statistically except for Ceftriaxone (CRO).

Escherichia coli

Antibiotic Susceptibility Profile of Arsenic (1 mg/mL Concentration) with Escherichia coli

Arsenic stress was concentrated as 1mg/mL in Muller Hinton Agar and a combination of antibiotics to evaluate the sensitivity and resistance pattern of *Escherichia coli*. According to permitted CLSI limits all 3 isolates with 6 antibiotics are resistant that were Ceftriaxone (CRO), Amoxicillin/Clavulanic acid (AMC), Gentamicin (CN) and Tobramycin (TOB) were shows 0 mm zone of inhibition, Meropenem (MEM) was observed as 10mm, 9mm, and 8mm zone, and 2 isolates with Ciprofloxacin (CIP) were shows 20mm and 20 mm zone. While 3 isolates with 3 antibiotics reported as the sensitive drug that was Amikacin (AK) observed an 18mm, 18mm, and 18 mm zone, Piperacillin/Tazobactam (TZP) shown 22mm, 22mm, and 22mm zone, and Levofloxacin (LEV) was observed as 30mm, 30mm and 32mm zone of inhibition. Although 1 isolate with 1 antibiotic was reported as an intermediate that was Ciprofloxacin (CIP) observed a 22 mm zone of inhibition. Before and after heavy metal description with graphical representation shown in Figure 4. The paired T-test for antibiotics indicated that the difference between with and without Arsenic was highly significant statistically except for Amikacin (AK), Piperacillin/Tazobactam (TZP), and Gentamicin (CN).

Antibiotic Susceptibility Profile of Copper (1 mg/mL Concentration) with Escherichia coli

Copper stress was concentrated as 1mg/mL in Muller Hinton Agar and a combination of antibiotics to evaluate the sensitivity and resistance pattern of *Escherichia coli*. According to permitted CLSI limits, all 3 isolates with 7 antibiotics were resistant such as Ceftriaxone (CRO), Amoxicillin/Clavulanic acid (AMC), Gentamicin (CN), Tobramycin (TOB), Meropenem (MEM), Ciprofloxacin (CIP), Levofloxacin (LEV) were observed 0 mm zone of inhibition. 3 isolates with 1 antibiotic were reported as sensitive drugs that is Amikacin (AK) observed 22mm, 22mm, and 21 mm zones of inhibition. Although 3 isolates with 1 antibiotic reported intermediate that is Piperacillin/Tazobactam (TZP) observed as 20 mm, 20mm, and 20mm zone of inhibition. Before and after heavy metal description with graphical representation shown in Figure 4. The paired T-test for antibiotics indicated that the difference between with and without

copper was highly significant statistically except for Amikacin (AK), Piperacillin/Tazobactam (TZP) and Gentamicin (CN).

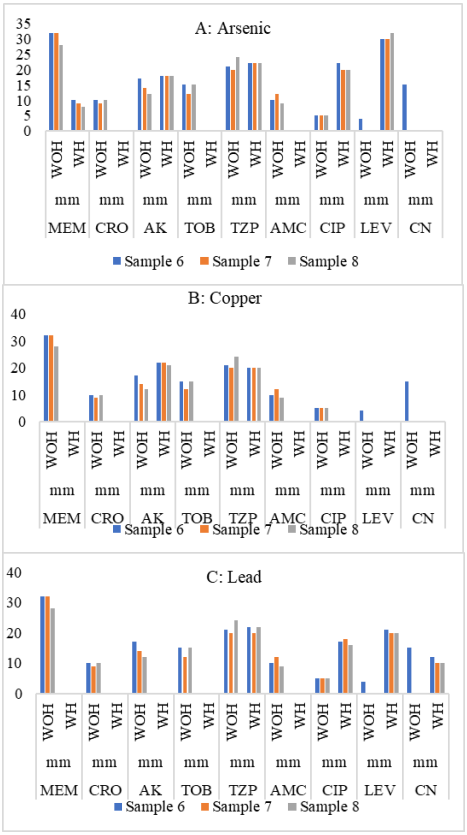

Figure 4: Graphical representation of resistance and sensitivity Pattern of Escherichia coli.

Antibiotic Susceptibility Profile of Lead (1 mg/mL Concentration) with Escherichia coli

Lead stress was concentrated as 1mg/mL in Muller Hinton Agar and a combination of antibiotics to evaluate the sensitivity and resistance pattern of *Escherichia coli*. According to permitted CLSI limits, all 3 isolates were resistant to 7 antibiotics such as Amikacin (AK), Amoxicillin/Clavulanic acid (AMC), Ceftriaxone (CRO), Tobramycin (TOB) and Meropenem (MEM) observed 0mm zone of inhibition, while Ciprofloxacin (CIP) was observed 17mm, 18 mm and 16mm zone, and Gentamicin (CN) was observed 12mm, 10mm and 10mm zone of inhibition. While 2 isolates with 1 antibiotic reported as sensitive drug i.e. Piperacillin/Tazobactam (TZP) observed 22 mm and 22 mm zones. Only

1 isolate with 1 antibiotic, i.e. Piperacillin/Tazobactam (TZP) was observed 20 mm zone and 3 isolates with 1 antibiotic i.e. Levofloxacin (LEV) observed in the 21 mm, 20 mm, and 20 mm zone of inhibition, were reported as intermediate. Before and after heavy metal description with graphical representation in Figure 4. The paired T-test for antibiotics indicated that the difference between with and without Lead was highly significant statistically except for Amikacin (AK), Piperacillin/Tazobactam (TZP) and Gentamicin (CN).

Klebsiella pneumoniae

Antibiotic Susceptibility Profile of Arsenic (1 mg/mL Concentration) with Klebsiella pneumoniae

According to permitted CLSI limits, all 3 isolates were resistant to 7 antibiotics such as Ceftriaxone (CRO), Amoxicillin/Clavulanic acid (AMC), Gentamicin (CN), Tobramycin (TOB), Ciprofloxacin (CIP), Meropenem (MEM) and Levofloxacin (LEV) observed 0mm zone. 2 isolates were resistant against Amikacin (AK) shows 10mm and 12mm zone of inhibition. While 2 isolates were susceptible to Piperacillin/Tazobactam (TZP) and showed 21mm and 22mm zones. Only 1 isolate was intermediate against Piperacillin/Tazobactam (TZP) showing a 20mm zone and 1 isolate was intermediate against Amikacin (AK) and observed a 15mm zone of inhibition. Before and after heavy metal description with graphical representation is explained in Figure 5. The paired T-test for antibiotics indicated that the difference between with and without Arsenic was highly significant statistically except for Amikacin (AK).

Antibiotic Susceptibility Profile of Copper (1 mg/mL Concentration) with Klebsiella pneumoniae

Copper stress was concentrated as 1mg/mL in Muller Hinton Agar and a combination of antibiotics to evaluate the sensitivity and resistance pattern of *Klebsiella pneumoniae*. According to permitted CLSI limits, all 3 isolates are resistant to 8 antibiotics such as Ceftriaxone (CRO), Amoxicillin/Clavulanic acid (AMC), Gentamicin (CN), Tobramycin (TOB), Ciprofloxacin (CIP), Meropenem (MEM), and Levofloxacin (LEV) observed 0mm zone and Piperacillin/Tazobactam (TZP) was observed as 12 mm, 12mm and 14mm zone of inhibition. 3 isolates were reported as susceptible against Amikacin (AK) which was observed as 22mm, 20mm, and 22mm zone of inhibition. Before and after heavy metal description with graphical representation is explained in Figure 5. The

paired T-test indicated for antibiotics that the difference between with and without copper was highly significant statistically.

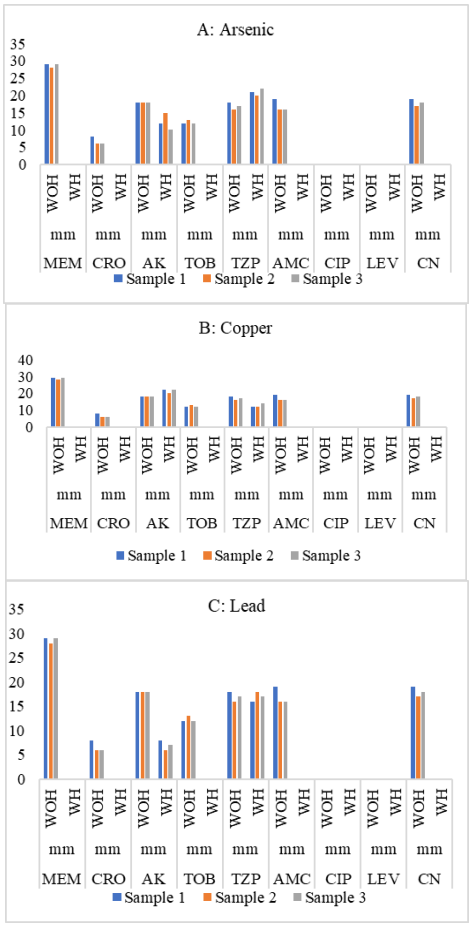

Figure 5: Graphical representation of resistance and sensitivity Pattern of Klebsiella Pneumoniae.

Antibiotic Susceptibility Profile of Lead (1 mg/mL Concentration) with Klebsiella pneumoniae

Lead stress was concentrated as 1mg/mL in Muller Hinton Agar and a combination of antibiotics to evaluate the sensitivity and resistance pattern of *Klebsiella pneumoniae*. According to permitted CLSI limits, all 3 isolates with 8 antibiotics such as Ceftriaxone (CRO), Amoxicillin/Clavulanic acid (AMC), Gentamicin (CN), Tobramycin (TOB), Ciprofloxacin (CIP), Meropenem (MEM), and Levofloxacin (LEV) observed 0mm zone and Amikacin (AK) was observed 8 mm, 6

mm, and 7 mm zone of inhibition. All 2 isolates were resistant against Piperacillin/Tazobactam (TZP) showing 16mm and 17 mm zone. While 1 isolate reported as intermediate against Piperacillin/Tazobactam (TZP) observed an 18 mm zone of inhibition. Before and after heavy metal description with graphical representation is shown in Figure 5. The paired T-test for antibiotics indicated that the difference between with and without Lead was highly significant statistically except for Piperacillin/Tazobactam (TZP).

Proteus mirabilis

Antibiotic Susceptibility Profile of Arsenic (1 mg/mL Concentration) with Proteus mirabilis

According to permitted CLSI limits, all 2 isolates were resistant to 8 antibiotics such as Ceftriaxone (CRO), Amoxicillin/Clavulanic acid (AMC), Gentamicin (CN), Tobramycin (TOB), Ciprofloxacin (CIP), Meropenem (MEM), and Levofloxacin (LEV) observed 0mm zone and Piperacillin/Tazobactam (TZP) was observed 16mm and 15mm zone of inhibition. 2 isolates were reported as susceptible to Amikacin (AK) observed 18mm and 18mm zone of inhibition. Before and after heavy metal description with graphical representation is explained in Figure 6. The paired T-test for antibiotics indicated that the difference between with and without Arsenic was highly significant statistically except Amikacin (AK) and Piperacillin/Tazobactam (TZP).

Antibiotic Susceptibility Profile of Copper (1 mg/mL Concentration) with Proteus mirabilis

Copper stress was concentrated as 1mg/mL in Muller Hinton Agar and combination of antibiotics to evaluated sensitivity and resistance pattern of *Proteus mirabilis*. According to permitted CLSI limits, all 2 isolates were resistant to 8 antibiotics i.e. Ceftriaxone (CRO), Amoxicillin/Clavulanic acid (AMC), Gentamicin (CN), Tobramycin (TOB), Ciprofloxacin (CIP), Meropenem (MEM), and Levofloxacin (LEV) were observed 0mm zone and 1 isolate was resistant to Piperacillin/Tazobactam (TZP) showed 17 mm zone of inhibition. While 1 isolate were reported as intermediate to Piperacillin/Tazobactam (TZP) observed 18mm zone and Amikacin (AK) showed 16mm zone of inhibition. Out of total 1 isolate was susceptible to 1 antibiotic i.e. Amikacin (AK) observed 17mm zone. Before and after heavy metal description with graphical representation shown in Figure 6.

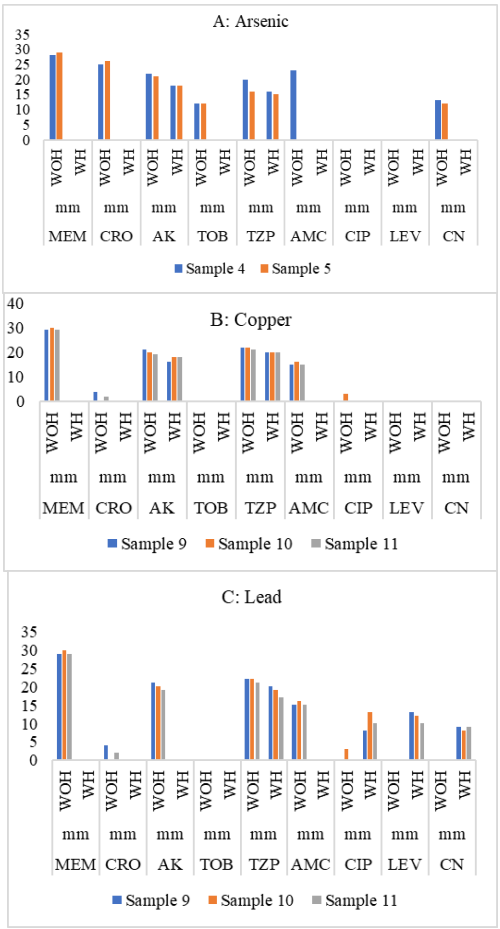

Figure 6: Graphical representation of resistance and sensitivity Pattern of Proteus mirabilis.

The paired T-test indicated for antibiotics that the difference between with and without copper was highly significant statistically except Amikacin (AK) and Piperacillin/Tazobactam (TZP).

Antibiotic Susceptibility Profile of Lead (1 mg/mL Concentration) with Proteus mirabilis

Lead stress was concentrated as 1mg/mL in Muller Hinton Agar and combination of antibiotics to evaluated sensitivity and resistance pattern of *Proteus mirabilis*. According to permitted CLSI limits 2 isolates were resistant to 8 antibiotics i.e. Ceftriaxone (CRO), Amoxicillin/Clavulanic acid (AMC), Gentamicin (CN), Tobramycin (TOB), Ciprofloxacin (CIP), Meropenem (MEM), and Levofloxacin

(LEV) observed 0mm zone and Amikacin (AK) was observed 11mm and 10mm zone of inhibition. While 2 isolates were reported intermediate to Piperacillin/Tazobactam (TZP) observed 20mm and 20mm zone of inhibition. Before and after heavy metal description with graphical representation shown in Figure 6. The paired T-test indicated for antibiotics that the difference between with and without Lead was highly significant statistically except Amikacin (AK) and Piperacillin/Tazobactam (TZP).

Antibiotic Susceptibility Profile of Arsenic (1 mg/mL Concentration) with Pseudomonas aeruginosa

Arsenic stress was concentrated as 1mg/mL in Muller Hinton Agar and combination of antibiotics to evaluated sensitivity and resistance pattern of *Pseudomonas aeruginosa*. According to the permitted CLSI limits 2 isolates were reported as resistant 7 antibiotics such as Ceftriaxone (CRO), Amoxicillin/Clavulanic acid (AMC), Ciprofloxacin (CIP), Gentamicin (CN), Meropenem (MEM), Tobramycin (TOB) and Levofloxacin (LEV) observed 0 mm zones of inhibition. All 2 isolates were intermediate to 2 antibiotics that were Amikacin (AK) observed 15 mm and 16 mm zones, and Piperacillin/Tazobactam (TZP) showed 17mm and 18mm zone of inhibition. Before and after heavy metal description with graphical representation shown in Figure 7. The paired T-test indicated for antibiotics that the difference between with and without Arsenic was significant statistically except Amikacin (AK), Piperacillin/Tazobactam (TZP) and Ciprofloxacin (CIP).

Antibiotic Susceptibility Profile of Copper (1 mg/mL Concentration) with Pseudomonas aeruginosa

Copper stress was concentrated as 1mg/mL in Muller Hinton Agar and combination of antibiotics to evaluated sensitivity and resistance pattern of *Pseudomonas aeruginosa*. According to the permitted CLSI limits all isolates were reported as resistant to 7 antibiotics such as Ceftriaxone (CRO), Amoxicillin/Clavulanic acid (AMC), Ciprofloxacin (CIP), Gentamicin (CN), Meropenem (MEM), Tobramycin (TOB) and Levofloxacin (LEV) observed 0 mm zones of inhibition. All isolates were susceptible to 2 antibiotics that are Amikacin (AK) observed 21 mm and 23 mm zones, and Piperacillin/Tazobactam (TZP) showed 22 mm and 22mm zones of inhibition. Before and after heavy metal description with graphical representation shown in Figure 7. The paired T-test indicated for antibiotic that the difference between with and without copper was significant statistically except Amikacin (AK), Piperacillin/Tazobactam (TZP) and Ciprofloxacin (CIP).

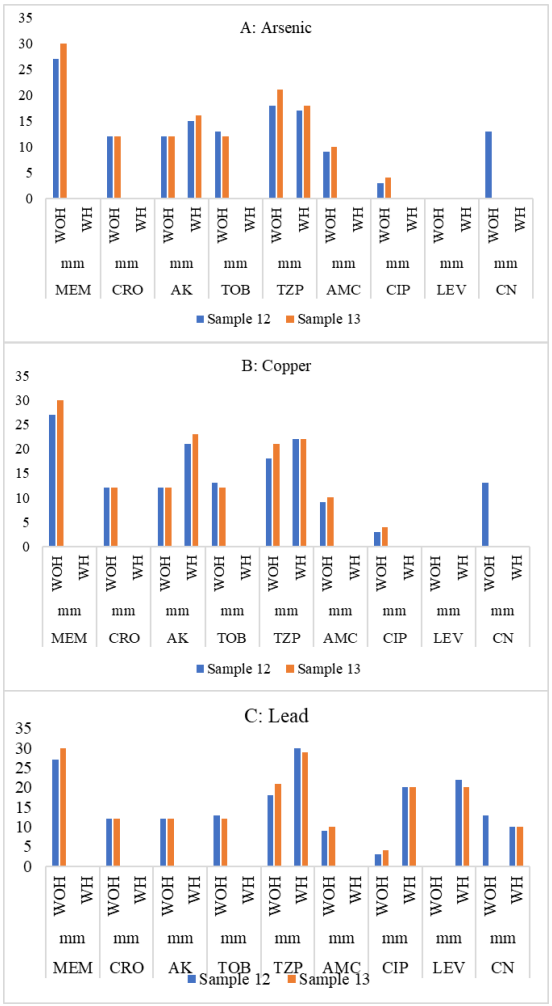

Figure 7: Graphical representation of resistance and sensitivity Pattern of Pseudomonas aeruginosa.

Antibiotic Susceptibility Profile of Lead (1 mg/mL Concentration) with Pseudomonas aeruginosa

Lead stress was concentrated as 1mg/mL in Muller Hinton Agar and combination of antibiotics to evaluated sensitivity and resistance pattern of *Pseudomonas aeruginosa*. According to the permitted CLSI limits 2 isolates were resistant to 6 antibiotics i.e. Amikacin (AK), Ceftriaxone (CRO), Amoxicillin/Clavulanic acid (AMC), Meropenem (MEM), Tobramycin (TOB) observed 0 mm zones of inhibition and Gentamicin (CN) showed 10 mm and 10 mm zones. Although 2 isolates

were susceptible to Piperacillin/Tazobactam (TZP) observed 30 mm and 29 mm zones and 1 isolate to 1 antibiotic i.e. Levofloxacin (LEV) showed 22mm zones of inhibition. All 2 isolates were intermediate to Ciprofloxacin (CIP) observed 20 mm and 20 mm zones and 1 isolate were intermediate to Levofloxacin (LEV) shows 20 mm zone of inhibitions. Before and after heavy metal description with graphical representation shown in Figure 7. The paired T-test indicated for antibiotics that the difference between with and without Lead was significant statistically except Amikacin (AK), Piperacillin/Tazobactam (TZP) and Ciprofloxacin (CIP).

Discussion

The study investigated the prevalence and co-resistance patterns of extended-spectrum β-lactamase (ESBL)-producing Gram-negative bacteria against antibiotics and heavy metals, specifically arsenic, copper, and lead. Of the 150 tested samples, *Escherichia coli* (40%) represented the most prevalent isolate, followed by *Pseudomonas aeruginosa* (23%), *Enterobacter aerogenes* (18%), *Proteus mirabilis* (10%), and *Klebsiella pneumoniae* (8.6%). Previous studies confirmed *E. coli* as the most prevalent ESBL-producing bacteria that operate in clinical settings (Carvalho et al., 2021). The Kirby-Bauer disc diffusion method served as the testing procedure to measure antibiotic response levels of heavy metals. Growth of *Enterobacter aerogenes* stopped responding to ceftriaxone, amoxicillin/clavulanic acid, ciprofloxacin, gentamicin, meropenem, and tobramycin when 1 mg/mL of arsenic was added to them. Tests with both copper and lead metals showed matching results regarding resistance development. Various research shows heavy metals have the ability to make bacteria develop co-resistance against antibiotic substances (Ajewole et al., 2021; Kaamoush et al., 2022; Aslam et al., 2025).

Microorganisms of *Escherichia coli* displayed multi-drug resistance during treatment with heavy metals. When bacteria were exposed to arsenic, they became resistant to ceftriaxone, amoxicillin/clavulanic acid, gentamicin, and tobramycin, which are all antibiotics. The presence of copper led to bacterial resistance against ceftriaxone, together with amoxicillin/clavulanic acid, as well as gentamicin, tobramycin, meropenem, ciprofloxacin, and levofloxacin (Basak & Chakraborty, 2023). Antibiotics like amikacin, amoxicillin/clavulanic acid, ceftriaxone, tobramycin, and meropenem no longer work on *E. coli* after it was exposed to lead. Studies confirm that heavy metals drive the growth of antibiotic-resistant bacteria, according to Jiang et al. (2021).

The heavy metal stress condition made *Klebsiella pneumoniae* isolates resistant to several tested antibiotics. *Klebsiella pneumoniae* became less sensitive to ceftriaxone, amoxicillin/clavulanic acid, gentamicin, tobramycin, ciprofloxacin, meropenem, and levofloxacin. It also became less sensitive to arsenic. Bacteria became resistant to eight antibiotics after being exposed to copper in large amounts (Dupont et al., 2011). These antibiotics are ceftriaxone, amoxicillin/clavulanic acid, gentamicin, tobramycin, ciprofloxacin, meropenem, levofloxacin, and piperacillin/tazobactam. After being exposed to lead, bacteria stopped responding to ceftriaxone and all antibiotic combinations, such as amoxicillin/clavulanic acid, gentamicin, tobramycin, ciprofloxacin, meropenem, levofloxacin, and amikacin. Heavy metal contamination selects antibiotic resistance in bacteria according to multiple studies (Nguyen et al., 2019; Vats et al., 2022).

Heavy metal stress caused *Proteus mirabilis* strains to develop resistance against different classes of antibiotics. Because they were exposed to arsenic, the bacterial strains became resistant to ceftriaxone, amoxicillin/clavulanic acid, gentamicin, tobramycin, and all other antibiotics, such as ciprofloxacin, meropenem, levofloxacin, and piperacillin/tazobactam. The bacteria became resistant to seven types of antibiotics after being exposed to copper. These are ceftriaxone, amoxicillin/clavulanic acid, gentamicin, tobramycin, ciprofloxacin, meropenem, levofloxacin, and piperacillin/tazobactam. When the strains were exposed to lead, they stopped responding to ceftriaxone, amoxicillin/clavulanic acid, gentamicin, tobramycin, ciprofloxacin, meropenem, levofloxacin, and finally amikacin. Numerous studies validate that contamination from heavy metals enables bacteria to develop resistance to antibiotic agents (Wales & Davies, 2015; Gillieatt & Coleman, 2024).

Under the influence of heavy metals, *Pseudomonas aeruginosa* isolates acquired resistance against various antibiotics. Bacteria developed resistance to ceftriaxone as well as amoxicillin/clavulanic acid and ciprofloxacin, gentamicin, meropenem, tobramycin, and levofloxacin when exposed to arsenic. Tobramycin, ciprofloxacin, amoxicillin/clavulanic acid, gentamicin, meropenem, tobramycin, and levofloxacin could no longer kill any P. aeruginosa isolates that were exposed to copper. Some medical isolates that were exposed to lead became resistant to amikacin, ceftriaxone, amoxicillin/clavulanic acid, meropenem, tobramycin, and gentamicin. The study matches previous research evidence demonstrating that heavy metals help bacteria exchange antibiotic resistance genes horizontally (Zhang et al., 2018; Wang et al., 2020). The researchers believe co-resistance occurs because mobile

genetic elements with both heavy metal resistance genes and antibiotic resistance genes exist. Bacteria populations keep and spread antibiotic resistance genes when heavy metals can act as co-selection pressure (Vats et al., 2022).

This study demonstrates why heavy metal pollution assessment needs to be performed in clinical environments and the environment because it enables the emergence and dispersal of antibiotic-resistant bacterial populations. The introduction of heavy metal reduction programs creates the potential to limit both antibiotic resistance gene co-selection processes and their spread (Murray et al., 2024). The research demonstrates that heavy metals, including arsenic together with copper and lead, substantially influence how ESBL-producing Gram-negative bacteria show resistance against antibiotics. Based on the co-resistance patterns they saw, researchers stress the need for multifaceted solutions to deal with heavy metal pollution in the environment and antibiotic resistance in healthcare settings (Vats et al., 2022; Murray et al., 2024).

Conclusion

This research was carried to find the antibiotic and heavy metal co-resistance of ESBL producing bacteria found in clinical samples. A total of 200 clinical specimens were collected from Chughtai Laboratories for a cross-sectional study. From a total of 200 clinical specimens, there were 150 Gram-negative bacteria and only 50 specimens were Gram-positive bacteria. These 150 gram-negative bacteria were tested for the detection of ESBL (Extended-spectrum beta-lactamase) producing bacteria and non-ESBL bacteria. The test revealed that only 30 bacterial isolates (20%) were ESBL producers and 108 bacterial isolates (80%) were non-ESBL producers. A significant frequency of ESBL-producing bacteria was discovered in clinical isolates, and these bacteria had a high ratio of resistant genes to chosen antibiotics. Bacteria showed no growth in the presence of 1.0 g/mL and above of arsenic, copper, and lead. In the presence of heavy metals, the susceptibility profile shifted from susceptible to resistant for meropenem, and rest of the test antibiotics did not change the susceptibility profile but reduced effectiveness. Heavy metal presence either reduced or increased the zone size, depending on the type of metal and antibiotic. The present study indicates that heavy metal presence in the environment can affect the resistance to routine antibiotics.

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