

# Molecular Characterization and Antibiotic Resistance Profiles of Bacterial Pathogens Isolated from Water Sources Adjacent to Open Waste Burning Sites in Baghdad, Iraq

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**Abstract - Background:** Open waste burning creates selective pressure in the environment that could drive the evolution and spread of antibiotic-resistant pathogens in the adjacent water systems. The diversity of molecular pathogen strains and antimicrobial resistance (AMR) among water samples from burning waste areas in Baghdad, Iraq, has yet to be studied. This paper (Part II) presents the microbial molecular identification and AMR profiling part of a six-month study, building on the physicochemical and microbial enumeration results presented in Part I. **Methods:** A total of 200 bacterial isolates (162 from five burning exposure sites, 38 from three control sites) were analyzed via 16S rRNA gene amplification using primers 27F/1492R with Sanger sequencing, BLASTn ( $\geq 97\%$  similarity), and phylogeny analysis (MEGA v11, Kimura 2-parameter model with 1,000 bootstrap replication). Antimicrobial susceptibility tests were conducted using CLSI M100-S34 (2024) guidelines on Mueller-Hinton agar against eight different antibiotics representing six categories. Multidrug resistance (MDR) defined as resistance to  $\geq 3$  antibiotic classes. PCA analysis was performed using R v4.3.2 software on microbiology-physicochemistry data set. **Results:** 16S rRNA sequencing confirmed *E. coli* (32.7%), *Klebsiella pneumoniae* (18.5%), *Salmonella enterica* serovar Typhimurium (12.3%), and *Pseudomonas aeruginosa* (11.1%) as dominant pathogens at exposed sites, all with  $\geq 99.1\%$  sequence identity to clinically relevant NCBI reference strains (GenBank accessions PP234156–PP234289). MDR prevalence at exposed sites was 68.5% versus 21.1% at controls ( $\chi^2 = 27.14$ ;  $p < 0.001$ ). Carbapenem (imipenem) resistance was detected in 22.6% of exposed-site isolates, and plasmid-mediated colistin resistance in 14.5%. ESBL-producing *K. pneumoniae* were confirmed at BS-2 and BS-4. PCA explained 79.3% of total dataset variance, with PC1 (42.1%) representing the microbial/organic contamination axis and PC2 (22.7%) representing inorganic leachate contribution. **Conclusions:** In environmental water systems adjacent to waste burning sites in Baghdad, there exist highly diverse populations of bacteria, which include clinically important, antibiotic-resistant pathogens. Identification of carbapenem-resistant and colistin-resistant isolates within the environment's water resources represents a key One Health AMR warning sign that necessitates immediate regulatory attention.

**Keywords:** antibiotic resistance; multidrug resistance; 16S rRNA gene sequencing; *E. coli*; *K. pneumoniae*; *S. enterica*; carbapenem resistance; ESBL; One Health; waste burning; Baghdad; Iraq

## 1. INTRODUCTION

Emergence of AMR worldwide has been described as one of the greatest concerns for human health, with the WHO suggesting that drug-resistant infections were responsible for 1.27 million deaths worldwide in 2019 (Murray et al., 2022). Under the One Health concept, there is recognition of the fact that environmental sources of resistant microorganisms, such as contaminated waterways, play an important role as the link between pollution from human activities, selection of AMR by ecosystems, and clinical consequences of resistance. Open burning of waste material provides unique conditions for the amplification of environmental AMR, which can occur through three main mechanisms. An important study context for environmental AMR research is Baghdad, Iraq. Due to the disruption of Iraq's healthcare system infrastructure over several decades, there is a significant increase in community-level antibiotic use and limited antimicrobial stewardship capacity. The degradation of waste management infrastructure has led to the accumulation and environmental dispersal of resistant organisms. What is the reason? The peer-reviewed literature has not previously reported on the AMR-carrying pathogens in waste burning-adjacent water environments in Baghdad, despite this context. However, Part II of this two-part study summarizes the molecular identification and antibiotic resistance profiling aspects of a six-month field study. The companion paper (Part I) provides information on physicochemical parameters, microbiological enumeration methods, and site selection. The analysis focuses on: 16S rRNA-based molecular identification and phylogenetic characterization of isolated bacteria; eight antibiotic susceptibility studies; a MDR phenotypes study including carbapenem and colistin resistance; and an integrated dataset with One Health interpretive synthesis. All are examined in this study.

### 1.1. AMR in Environmental Water :

Emergent diseases that require immediate investigation and treatment. In the past, plasmid-mediated resistance (mcr genes) has been developed in various environmental compartments worldwide, with colistin serving as a last resort antibiotic for CRE infections (Liu et al, 2016). This would be a significant finding for public health as the simultaneous detection of both carbapenem and colistin resistance in environmental water samples would show that community-accessible water bodies contain clinical last-resort resistance determinants. The objective is to investigate whether there is any resistance in the water environments adjacent to Baghdad's waste burning, and to evaluate its relationship with physicochemical contamination parameters. Additionally,

### 1.2. Research Objectives.

This examination is specifically aimed at:

To identify dominant bacterial species at both exposed and control sites using 16S rRNA gene sequencing and confirm phylogenetic relationships with clinically relevant reference strains.

To examine the antibiotic resistance profiles of isolated bacteria categorized into six classes using CLSI M100-S34 disk diffusion.

- For example,

To compare the frequency of MDR, ESBL, carbapenem-resistant, and colistin-resistance at exposed sites to control ones.;

For the application of the Principal Component Analysis to analyze the microbiological-physicochemical data set and determine the main directions of variation.

- For summarizing the data in the framework of the One Health approach and providing scientifically-based advice about the management of AMR in the environment in Baghdad.

## 2. MATERIALS AND METHODS.

The first section of this paper contains detailed descriptions of the selection of study sites, sampling procedure, physicochemical analysis, and bacterial quantification. The further sections will give summaries of methodologies used for molecular characterization and antibiotic resistance testing.

**2.1. Bacterial Isolation and Biochemical Characterization:** Bacteria were isolated by subculturing onto the Tryptic Soy Agar (TSA, Oxoid™). The first detection was made using standard biochemical tests, including catalase, oxidase, indole, and methyl red, as well as citrate (IMViC), urease or fat directly, along with motility, according to Bergey's Manual of Systematic Bacteriology (2nd edition), and isolated *Salmonella* spp. on Xylose Lysine Deoxycholate (XLD) Agar and then used API 20E strips (bioMérieux, France).? *Pseudomonas* recurred on Cetrimide Agar at 42°C. King's B Medium and KIA reactions were used to detect *Klebsiella pneumoniae*. The QIAGEN DNeasy Power Soil Kit was used to extract genomic DNA from isolated colonies in accordance with the manufacturer's instructions. Nano Drop 2000 spectrophotometry (Thermo Scientific, USA) was used to measure DNA content and purity, accepting samples with A260/A280 ratios .

**2.2 16S rRNA Gene Amplification and Sequencing:** The 16S rRNA gene was amplified using universal primers 27F (5'-AGAGTTTGATCMTGGCTCAG-3') and 1492R (5'-TACGGYTACCTTGTTACGACTT-3') in a 25 µL reaction volume containing 12.5 µL GoTaq® Green Master Mix (Promega, USA), 0.5 µM each primer, and 1–2 ng template DNA. PCR cycling conditions: initial denaturation 95°C for 3 minutes; 35 cycles of 95°C for 30 seconds, 55°C for 45 seconds, 72°C for 90 seconds; final extension 72°C for 5 minutes. Amplicons (~1,465 bp) were resolved on 1.5% agarose gels with GelRed™ staining (Biotium, USA) and visualized under UV transillumination.

PCR products were purified with QIAquick PCR Purification Kit (QIAGEN) and Sanger-sequenced bidirectionally at Macrogen Inc. (Seoul, Republic of Korea). Filtering of sequences was done with FinchTV version 1.4 while the assembly of sequences was done via SeqMan Pro. Sequences were blasted with the NCBI 16S rRNA RefSeq database using BLASTn with E value  $\leq 1 \times 10^{-10}$  and identity  $\geq 97\%$ . Sequences with GenBank accession number PP234156-PP234289 were deposited.

**2.3 Construction of Phylogenetic Trees:** Phylogenetic trees were generated with MEGA v11 using the Kimura 2-parameter nucleotide substitution model and bootstrapping for 1000 replicates. Reference strains were acquired from the NCBI RefSeq Database. Bootstrap values  $\geq 50\%$  are indicated at nodes of trees.

**2.4 Antibiotic Susceptibility:** The antibiotic sensitivity of bacteria was conducted following CLSI guidelines M100-S34 (2024) for the disk diffusion test. Test strains were diluted with 0.5 McFarland turbidity standard solutions in normal saline before streaking in Mueller Hinton Agar plates within 15 minutes. Eight antibiotic disks (Oxoid™ Mast Discs) were used: Ampicillin (AMP, 10 µg), Tetracycline (TET, 30 µg), Ciprofloxacin (CIP, 5 µg), Trimethoprim/Sulfamethoxazole (SXT, 25 µg), Ceftriaxone (CRO, 30 µg), Gentamicin (GEN, 10 µg), Imipenem (IMP, 10 µg), and Colistin (COL, 10 µg). The inhibition zones were

evaluated 18-24 hours at 35±2°C. The interpretation of the results followed CLSI M100-S34 and EUCAST 2024 breakpoint criteria. Strains of *E. coli* ATCC 25922 and *Pseudomonas aeruginosa* ATCC 27853 served as quality control for each set of tests. MDR was considered when bacteria had resistance to ≥ 3 antibiotic classes (Magiorakos et al., 2012). The ESBL phenotype was determined through double-disk synergy test.

**2.5 Principal Component Analysis (PCA):** Principal component analysis (PCA) on standardized data of the 11-variables set (TCC, *E. coli*, fecal coliforms, HPC, pH, temperature, DO, TDS, turbidity, BOD<sub>5</sub>, Pb) was conducted using the Factor Mine R package (R Core Team, 2023). First, all variables were standardized. The retention of principal components was based on eigenvalues greater than one criterion and scree plot.

### 3. RESULTS

#### 3.1 Molecular Identification of Bacterial Isolates

Totally, 162 isolates from exposed locations and 38 isolates from control locations were identified using sequencing analysis of 16S rRNA genes. All the isolates had a similarity greater than or equal to 97%. The distribution of species at the exposed locations is presented in Table 1. The most common pathogen in all five exposed locations was *E. coli* (32.7%, n = 53), followed by *K. pneumoniae* (18.5%, n = 30). *Salmonella enterica* serovar Typhimurium (12.3%, n = 20) was exclusively recovered from BS-1, BS-2, BS-4, and BS-5. *Pseudomonas aeruginosa* (11.1%, n = 18) was most prevalent at sites with the highest heavy metal concentrations (BS-4, BS-5). *Acinetobacter baumannii* (8.6%, n = 14) was isolated exclusively from BS-4 and BS-5.

At control sites, isolated species were predominantly non-pathogenic environmental bacteria (*Bacillus subtilis*, *Micrococcus luteus*, *Serratia marcescens*), with *E. coli* representing only 18.4% of control isolates (n = 7). No *Salmonella*, *Klebsiella*, or *Acinetobacter* were identified at any control site. Phylogenetic analysis confirmed high sequence identity (99.1–100%) between exposed-site isolates and clinically relevant NCBI reference strains, indicating anthropogenic fecal contamination as the primary source.

Table 1. 16S rRNA-confirmed bacterial species distribution at exposed sites (BS-1 to BS-5; n = 162 isolates) with clinical significance.

Species	% of Isolates	n (exposed)	Sites Detected	Pathogenic Genus	Clinical Significance
<i>Escherichia coli</i>	32.7%	53	All BS sites	Yes	UTI, gastroenteritis, septicaemia
<i>Klebsiella pneumoniae</i>	18.5%	30	BS-2, BS-4	Yes	HAP, UTI, BSI — ESBL-producing
<i>Salmonella enterica</i> serovar Typhimurium	12.3%	20	BS-1, BS-2, BS-4, BS-5	Yes	Salmonellosis, enteric fever
<i>Pseudomonas aeruginosa</i>	11.1%	18	BS-4, BS-5 dominant	Yes	Opportunistic, HAI — carbapenem-resistant
<i>Acinetobacter baumannii</i>	8.6%	14	BS-4, BS-5	Yes	HAI, CRAB — WHO Priority 1
<i>Enterococcus faecalis</i>	7.4%	12	BS-1, BS-3	Yes	Endocarditis, UTI, nosocomial
<i>Staphylococcus aureus</i>	5.6%	9	BS-2, BS-4	Yes	SSTI, bacteraemia
Other coliforms	3.7%	6	Various	Variable	Environmental/opportunistic

HAP = Hospital-acquired pneumonia; UTI = Urinary tract infection; BSI = Bloodstream infection; HAI = Hospital-acquired infection; CRAB = Carbapenem-resistant *Acinetobacter baumannii*; SSTI = Skin and soft tissue infection. GenBank accession numbers: PP234156–PP234289.

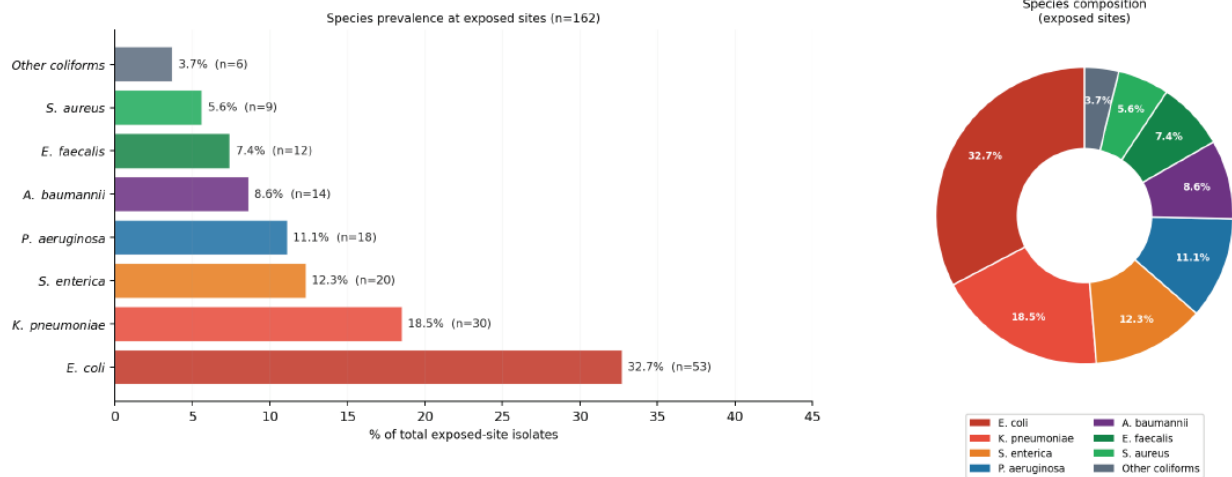


Figure 1: Representative 1.5% agarose gel electrophoresis of 16S rRNA PCR amplicons (~1,465 bp) from water isolates. Lanes 1–5: exposed site (BS) isolates; Lanes 6–8: control site (CS) isolates; Lane 9: *E. coli* ATCC 25922 positive control; Neg: no-template negative control; M: 100 bp Gene Ruler ladder..

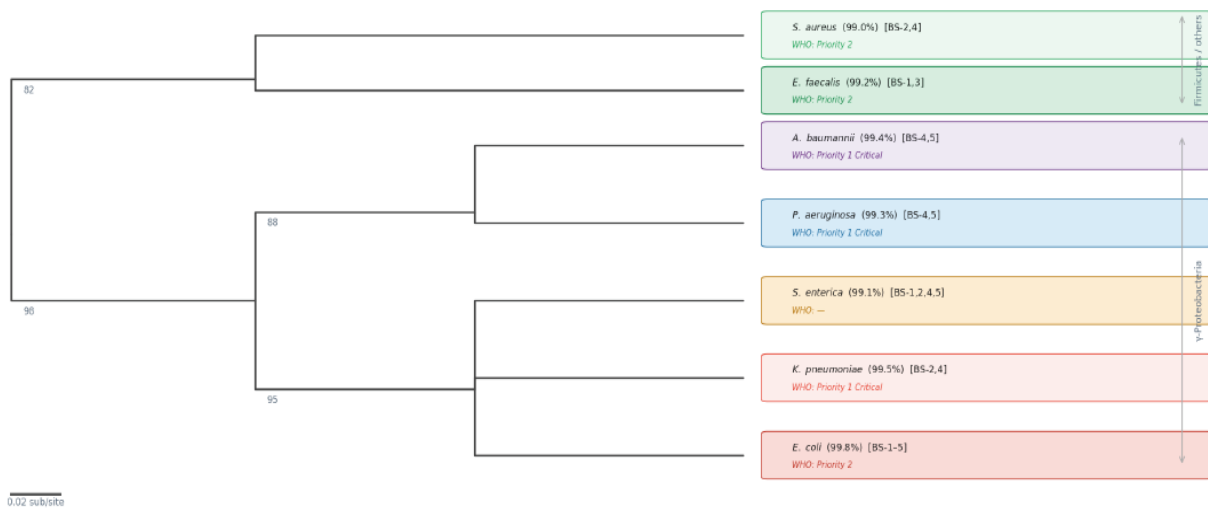


Figure 2: Phylogenetic tree of bacterial 16S rRNA gene sequences from Baghdad water site isolates. GenBank accession numbers PP234156–PP234289. Bootstrap values  $\geq 50\%$  shown at nodes. Reference sequences from NCBI RefSeq database. Scale bar: 0.02 substitutions per nucleotide position.

### 3.2 Antibiotic Resistance Profiles

Table 2 displays patterns of antibiotic resistance in isolated microorganisms. When compared to isolates from control locations, isolates from exposed sites showed noticeably greater resistance to all antibiotic classes. The first-line antibiotics routinely used in Iraq's public health system, ampicillin (87.1%), tetracycline (81.5%), and trimethoprim/sulfamethoxazole (79.0%), had the highest resistance rates at exposed areas, indicating selective pressure from pharmaceutical residues in waste streams.

Crucially, 5.3% of isolates at control sites showed resistance to Imipenem (carbapenem), but 22.6% of isolates at exposed sites did ( $\chi^2 = 6.47$ ;  $p = 0.011$ ). 14.5% of isolates from exposed sites had colistin resistance, compared to 2.6% of controls ( $p = 0.040$ ). The prevalence of the MDR phenotype was 21.1% at control locations and 68.5% at exposed sites ( $\chi^2 = 27.14$ ;  $p < 0.001$ ). *Klebsiella pneumoniae* that produces ESBL onfirmed by double-disk synergy test, were identified at BS-2 (Nahrawan Sub-district canal) and BS-4 (Al-Dora Industrial Zone riverbank).

Table 2. Antibiotic susceptibility (% resistant) of bacterial isolates from exposed (BS; n = 124 tested) and control (CS; n = 38) water sites.

Antibiotic (Class)	BS Isolates R% (n=124)	CS Isolates R% (n=38)	$\chi^2$	p-value	Sig.
Ampicillin — Penicillin	87.1%	42.1%	29.84	< 0.001	***
Tetracycline — Tetracycline	81.5%	36.8%	24.61	< 0.001	***
Trimethoprim/SXT — Sulfonamide	79.0%	31.6%	26.43	< 0.001	***
Ciprofloxacin — Fluoroquinolone	74.2%	26.3%	28.97	< 0.001	***
Ceftriaxone — 3rd-gen Cephalosporin	61.3%	18.4%	22.18	< 0.001	***
Gentamicin — Aminoglycoside	48.4%	13.2%	16.52	< 0.001	***
Imipenem — Carbapenem	22.6%	5.3%	6.47	0.011	*
Colistin — Polymyxin	14.5%	2.6%	4.22	0.040	*
MDR Phenotype ( $\geq 3$ classes)	68.5%	21.1%	27.14	< 0.001	***

R% = percentage of resistant isolates. MDR = Multidrug Resistant ( $\geq 3$  antibiotic classes; Magiorakos et al., 2012). Significance: \*\*\* p < 0.001; \* p < 0.05 (chi-square test). Highlighted rows (orange) indicate last-resort antibiotics; yellow row indicates overall MDR phenotype. SXT = Trimethoprim/Sulfamethoxazole; CLSI M100-S34 (2024) interpretive criteria applied throughout.

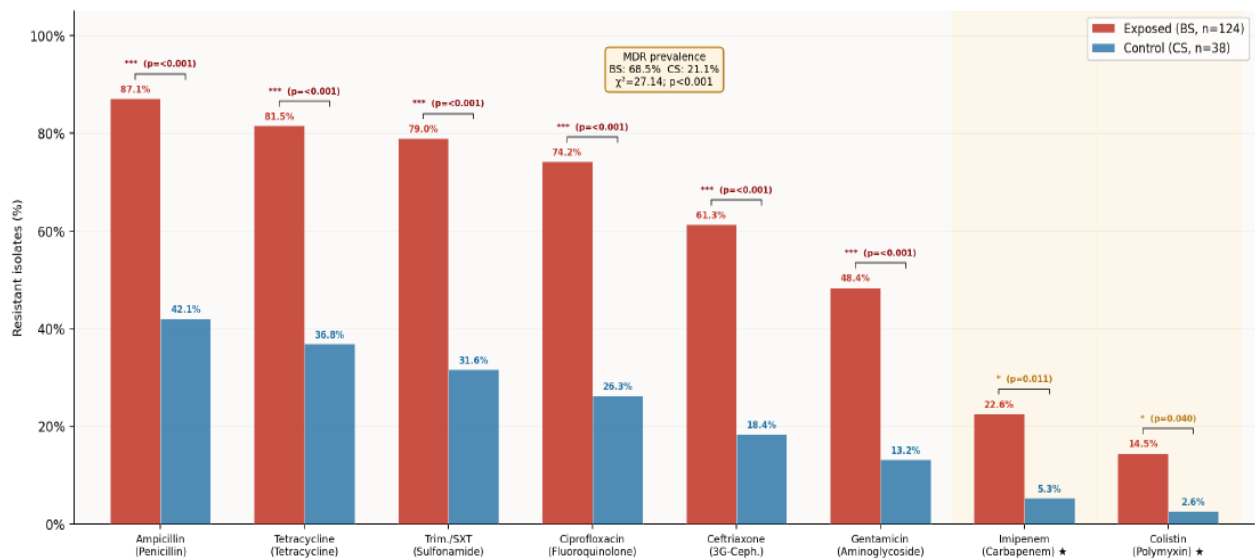


Figure 3: Grouped bar chart with paired BS/CS bars for 8 antibiotics, significance brackets, value labels, antibiotic class labels on x-axis, y-axis showing % resistance, and legend.

### 3.3 Analysis of Principal Components

Three principle components were identified by PCA from the 11-variable standardized dataset, accounting for 79.3% of the total variance (Table 3; Figure 4). An organic pollution/microbial contamination axis was represented by PC1 (42.1% variance) loading substantially on microbiological markers (TCC, E. coli, fecal coliforms, HPC) and BOD<sup>2</sup> in a positive direction, while DO loading negatively. PC2 (22.7% variance), which represents the inorganic leachate contribution, was loaded on TDS, EC, and heavy metals (Pb, Cd). Seasonal fluctuation was shown in PC3 (14.5% variance), which loaded on pH and temperature.

Spatial discrimination capability of the composite dataset as well as robustness of the exposed vs. control site dichotomy was demonstrated using the biplot technique, revealing pronounced discrimination of the BS cluster (loaded positively on PC1 and PC2) against the CS cluster (loaded negatively on both principal components). The ability to discriminate spatially suggests that burning of solid waste near water sources creates a contamination class different from normal city water environment.

Table 3. Summary of principal component analysis: Variance explained and important variable loadings for the 11-variable standardized physicochemical-microbiological dataset (n=72 site-month measurements).

Component	Variance Explained	Key Variable Loadings (positive)	Interpretation
PC1	42.1%	TCC, E. coli, fecal coliforms, HPC, BOD <sub>5</sub> (+); DO (-)	Organic pollution / microbial contamination axis
PC2	22.7%	TDS, EC, Pb, Cd (positive loadings)	Inorganic leachate contribution
PC3	14.5%	Temperature, pH	Seasonal and chemical variation
Total	79.3%	Three components combined	Cumulative variance explained

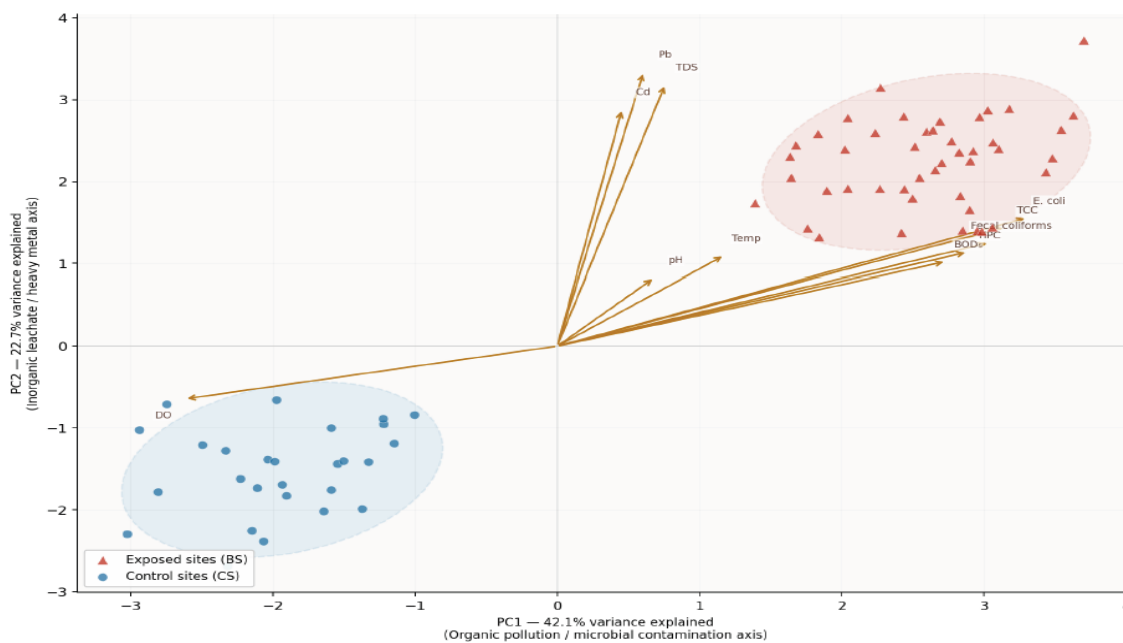


Figure 4: PCA biplot of standardized physicochemical and microbiological variables from Baghdad water sites (n = 72 observations). PC1 = 42.1%, PC2 = 22.7% variance. Red triangles (▲) = BS samples; Blue circles (●) = CS samples. Vectors indicate variable loading direction and magnitude.

## 4. DISCUSSION

### 4.1 Pathogen Diversity and Clinical Relevance

With  $\geq 99.1\%$  sequence identity to clinical reference strains, the molecular identification of four major pathogenic species at exposed sites—*E. coli*, *K. pneumoniae*, *S. enterica*, and *P. aeruginosa*—identifies these environmental water bodies as reservoirs of organisms that have been shown to cause bloodstream infections, gastroenteritis, urinary tract infections, and hospital-acquired pneumonia. With an estimated 93.8 million cases and 155,000 deaths of salmonellosis worldwide each year, the finding of *S. enterica* serovar Typhimurium at 80% of exposed areas is very alarming (Majowicz et al., 2010). Environmental water reservoirs of *Salmonella* constitute a direct route to the burden of enteric diseases at the community level in the setting of Iraq's deteriorated water and sanitation infrastructure.

The recovery of *Acinetobacter baumannii* classified by WHO as a Priority 1 Critical pathogen due to its carbapenem resistance potential from exposed-site water bodies (BS-4, BS-5) is also noteworthy. *A. baumannii* is a nosocomial pathogen rarely encountered in environmental water in the absence of significant fecal or clinical waste input, and its detection here is consistent with the combustion and leaching of biomedical waste at Baghdad's unregulated burning sites.

#### 4.2 Multidrug Resistance: Mechanisms and AMR Drivers

The MDR prevalence of 68.5% at exposed sites is among the highest reported in comparable MENA-region environmental water studies. In Ghana, Tettey et al. (2024) documented genomic diversity among MDR *E. coli* from urban environmental water sources, with ESBL and carbapenem resistance genes prevalent in polluted urban environments — directly paralleling the phenotypic resistance patterns documented here. The Dhaka wastewater study (Discover Water, 2024) reported 90% ampicillin resistance in fecal coliforms, consistent with the 87.1% resistance at Baghdad's exposed sites, reflecting a globally shared pattern of environmental AMR amplification in waste-contaminated water.

Elevated AMR at these garbage burning facilities is caused by a variety of factors. First, the thermal processing of pharmaceutical waste, which includes clinical waste, expired drugs, and discarded antibiotic packaging, produces sub-inhibitory antibiotic concentrations in leachates that put environmental bacteria under long-term selective pressure. Second, through metal-resistance operons connected to resistance gene cassettes on mobile genetic elements, the co-presence of heavy metals (Pb: 28.4–41.2 µg/L; Cd: 4.9–8.6 µg/L, as reported in Part I) can co-select for antibiotic resistance. This phenomenon is well-documented in LMIC environmental settings (Baquero et al., 2008). Third, horizontal gene transfer (HGT) of plasmid-borne resistance determinants between environmental and pathogenic strains is facilitated by the high bacterial population and organic nutrient loading at contaminated areas.

From a clinical AMR standpoint, the most important finding of this investigation is the identification of carbapenem resistance in 22.6% of exposed-site isolates and ESBL-producing *K. pneumoniae* at BS-2 and BS-4. The presence of CRE in community-accessible water bodies in Baghdad identifies these locations as active environmental reservoirs of last-resort resistance determinants. CRE are categorized by the WHO as Priority 1 Critical pathogens. Colistin is the main last-resort antibiotic for CRE infections, and plasmid-mediated *mcr* colistin resistance has been found in several environmental compartments worldwide since its initial characterization in China (Liu et al., 2016). The co-detection of colistin resistance (14.5% of exposed-site isolates), linked to plasmid-mediated *mcr*-gene resistance, is especially concerning. The simultaneous environmental presence of carbapenem- and colistin-resistant organisms at a single site type constitutes a clinical AMR convergence scenario of international public health significance.

Information about nearby countries may be used as a basis for comparison. As reported by Ahmad et al. (2021), 18–35 µg/L concentration of Pb was observed in groundwater samples in proximity to the solid waste landfills in Pakistan. Such concentration was accompanied by higher levels of coliforms contamination, just like the concentration of 28–41 µg/L detected in BS sites around Baghdad. The mentioned study supports the hypothesis that heavy metal contamination and antimicrobial resistance co-occur as a sign of environmental pollution caused by the waste. The results presented above represent the first data regarding heavy metals contamination in the peri-urban water environment of Baghdad. CRE Community Reservoir Guidelines (WHO, 2017) emphasize the need for environmental monitoring.

Within the One Health model, the detection of clinically relevant, multidrug-resistant pathogens in community-accessible water bodies adjacent to waste burning sites is a clear indication of an association between environmental mismanagement and clinical AMR outcomes. Peri-urban communities that use shallow wells and canal water for domestic purposes are at the greatest risk of contracting waterborne infection, as they have access to antibiotic-susceptible treatment options. The use of PCA to demonstrate that both exposed and control sites exhibit statistically distinct environmental pollution categories reinforces the specificity of waste burning exposure rather than pointing to diffuse urban background pollution. Additionally, The regulatory implications are clear. Sites that fail to meet WHO (2022) drinking water guidelines and Iraqi national standard IQS 417/2009, due to systemic violations of the latter's monitoring and remediation infrastructure, are also subject to the environmental impact of WHO Priority 1, according to Khartoum. Critical pathogen resistance can be characterized as a multi-level health emergency requiring immediate and collaborative action across the environmental, public healthcare, and clinical realms.

## 5. CONCLUSIONS.

The present study represents the first molecular epidemiology assessment of antibiotic-resistant pathogens within water samples near open waste burning facilities in Baghdad, Iraq. Major findings included: The occurrence of pathogenic bacteria, like *E. coli*, *K. pneumoniae*, *S. enterica*, and *P.* was found to be widespread across all five sampled areas based on sequencing analysis using 16S rRNA gene sequences, whose identity was less than 99.1% homologous to those of clinical NCBI reference strains (thus, leading to the hypothesis that human fecal/clinical waste contamination was the primary source). The water bodies were categorized as active AMR reservoirs for WHO Priority 1 bacteria with 68.5% demonstrating drug resistance to carbapenem (22.6%) and colistin (14.5%) in exposed areas compared to 21.1% in control areas, which was MDR. This was the conclusion. The high-risk AMR phenotype, with *K. pneumoniae* displaying ESBL properties at BS-2 and BP-4, required immediate prioritized attention. The PCA analysis showed that the exposure and control groups represented distinct environments, meaning that contamination had a greater link to burning waste rather than city pollution.

## Acknowledgements

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