

Isolation and Molecular Characterization of Heavy Metal-Resistant Bacteria from Soil and Water in Iraq

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Abstract: Heavy-metal pollution of soil and water is one of the most serious environmental problems, because these pollutants have a long half-life, are toxic, can bioaccumulate (with great ease) in biota. Objectives The objectives of the research were to isolate and identify heavy metal resistant bacteria from soil and water samples collected from some selected sites in Iraq. Samples were cultured on selective media with different heavy metals, and resistant bacterial isolates were obtained. Pheno- and molecular characterization determined the isolates with the highest tolerances for in-depth analysis. Phylogenetic analysis based on 16S rRNA gene sequencing was performed to establish the taxonomic relationship of these most resistant isolates. At least one or multiple heavy metals, some bacterial isolates that are clear such structures in adaptation to conditions of contamination exist. In addition, some isolates had multi-metal resistant ability, suggesting tolerance mechanisms like efflux systems, biochemical detoxification and metal sequestration. The genera of the simultaneously occurred organisms recognized are often regarded to be connectional with environmental resilience and adaptability to greater macroscopic environment adverse conditions. Molecular characterization confirmed the genetic relatedness of the most tolerant isolates with resistant environmental bacteria already identified. These findings support the conclusion that native bacterial populations within Iraq's soil and water ecosystems exhibit a high tolerance threshold for heavy metals. These isolates can be used as promising candidates in bioremediation applications, especially in the development of effective and eco-friendly treatments for the removal or detoxification of heavy metals from contaminated sites. Conclusion this study points to the distribution of indigenous contaminated bacteria in various Iraqi habitats as an environmentally valuable microflora, useful with potential for developing resistance mechanisms against some antimicrobial agents and/or biotechnological significance.

Keywords: Heavy Metals, Bacterial Isolation, Molecular Characterization, Soil, Water.

Research Paper

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INTRODUCTION

Heavy metals are very well known persistent, chemically stable environmental contaminants because of their lengthy life span and later bio-aggregation in soil, sediments, drinking water and living tissues (Briffa *et al.*, 2020; Pande *et al.*, 2022), which presents heavy metal pollution from terrestrial and aquatic habitats to be a genuine natural phenomenon or human wellbeing

issue. Heavy metals are one of the major environmental pollutant that disturb ecosystem equilibrium, especially microbial diversity through soil and water contamination. Water as has been well known to many is a crucial aspect of agricultural sector production and environmental sustainability, here the key is to apply those bacteria which could be indigenously grown at their polluted sites, which provide us bioremediation.

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Unlike many organic pollutants that can be mineralized to harmless end products, heavy metals persist in different environmental compartments, circulate extensively through the trophic networks and induce chronic toxicity on plants, animals, microorganisms and humans (Gheorghe Gliga *et al.*, 2021; Lee & Ryu, 2018). These industrial wastes assumed a more environmental significance in the last decades due to increasing urban discharge, mining activity, petroleum refining and wastewater reuse with individual standard levels of cadmium, lead, chromium nickel. Natural Ecosystems copper zinc mercury and arsenic (Pande *et al.*, 2022; Chaturvedi *et al.*, 2024) Soils and water represent the most vulnerable receptors of heavy metal inputs because they are at once sinks (the pollutants dispersing into them) and transport media (transport media for inorganic pollutants). Accumulation of heavy metals would change pH balance, redox potential, nutrients availability and microbial metabolism (Pande *et al.*, 2022; Nnaji *et al.*, 2024) which a stagnant force create instability in ecosystem which have branded as systems dynamic behaviour further reduce environmental quality. At the level of microbiology heavy metals can damage proteins, lipids, nucleic acids and membrane systems via oxidative stress, enzyme inhibition and disruption of cell metabolic pathways activity (Nnaji *et al.*, 2024; Chaturvedi *et al.*, 2024). Microorganisms are central to nutrient cycling, organic matter decomposition, soil fertility (Pande *et al.*, 2022) and natural bioremediation of pollutants (Wang *et al.*, 2024), we put their response to disturbances as a primary target for understanding ecosystem stress in terms of functional groups that form the structure (Functional Diversity) and functioning (biochemical pathways) of microorganisms. Contamination of soil and water by heavy metals is an important environmental problem posing serious ecosystem instability and public health consequences, particularly in industrial and mining sites. Different types of degradation (biological, chemical and thermal), biological degradation is generally cheaper and more complete mineralization (Al-safi, Hussain & Al-Jasimee 2024). This provides further justification for the use of indigenous microorganisms as potential bioremediation organisms in contaminated sites. Despite this toxicity, countless bacteria survive in metal-contaminated habitats through a myriad of adaptive and genetically encoded resistance mechanisms. These mechanisms involve biosorption by cell surfaces, extracellular sequestration using exopolysaccharide production, intracellular compartmentalization of heavy metals along with enzymatic transformation, redox conversion, precipitation and active metalloids efflux transport systems that lower intracellular metal burden (Nnaji *et al.*, 2024; Chaturvedi *et al.*, 2024; Sreedevi *et al.*, 2022). Moreover, biofilm formation can further promote bacterial survival since a more structured protective matrix limits penetration of metal and can better tolerate environmental stress cooperatively (Teitzel & Parsek, 2003; Nnaji *et al.*, 2024). These adaptations thus render

metal-resistant bacteria potentially useful as ecological agents of detoxification and bioremediation in polluted sites (Sayqal & Ahmed, 2021; Chaturvedi *et al.*, 2024).

Such events invoked the application of bacteria bioremediation for metal removal due to their various limitations offered by physicochemical methods. Chemical precipitation, ion exchange, membrane filtration, vitrification and excavation tend to be pricier, over-enthusiastic or non-discriminative (Pande *et al.*, 2022; Sreedevi *et al.*, 2022) that lacks practicality for both smaller & larger areas of contamination along gradients of residual industrialized centers in multiple matrices. Microbial remediation is therefore considered preferable due to its capacity to operate effectively in conditions of mild temperature and pH, sequester (or transform) metals or change their oxidation state, and utilize naturally occurring biota with a low ecological footprint potential 1–4. We are also attracted by those bacteria adapted to polluted environments because, typically, it is expected that local strains of a species isolated from native habitats would be better able to withstand at least some local physicochemical conditions and may even boast hardiness for multi-metal resistance, metabolic adaptability, and the ability survive that other stressful conditions found in complex contaminated environments (Cervantes *et al.*, 2001; Nies, 1999; Chaturvedi *et al.*, (From a research perspective, the isolation of heavy metal-resistant bacteria in selective conditions and their accurate taxonomic identification) When it is necessary to isolate the resistant strains, identify their profile of tolerance for the later characterisation under laboratory conditions, simple classical cultivation on recommended media supplemented with precious metals is always helpful (Rajbanshi, 2008; Kuffner *et al.*, 2010). But phenotypic identification alone is rarely, if ever, sufficient since environmental isolates of a similar morphological and biochemical character (similar pheno-type) can share common characteristics. Thus, molecular methods, particularly 16S rRNA gene sequencing (Kuffner *et al.*, 2010; Wang *et al.*, 2024), have been employed for accurate identification and phylogenetic placement of resistant bacterial pure cultures from contaminated soils and water. Molecular approaches combined with ecosystem studies provide a connection between the identity of isolates and ecological role, as well as bioremediation potential, enabling us to attain deeper taxonomic resolution. And on that subject, is there any better context for actually saying something than the environmental one of Iraq? In Iraq, however, long-term drivers via hydrocarbon-based activities industrial activities bad effluents agricultural runoff and urban sprawl can be a key route of heavy metals accumulation in soil and water systems (Wang *et al.*, 2024).

Cadmium and lead concentrations were also significantly correlated with changes in microbial classes and predicted metabolic pathways in recent work from

Baghdad and the Baiji oil refinery region, helping to establish causal connections that heavy metal stress is already affecting microbial ecology in Iraq soils (Wang *et al.*, 2024). Similarly, in a different Iraqi context, freshwater monitoring of metals in Chamchamal revealed that mercury and lead were at sites > national limits with relatively little spatial or temporal variation since growth-form diversity of culturable bacteria changed under metal stress (Husain & Aziz, 2026), suggesting the broader ecological impact/importance for upheld/regained habitats which aggravate habitat-related developments. Previous Iraqi studies indicated that indigenous *Serratia marcescens* isolated from soil and water are resistant to several metal chlorides, and can indeed be efficacious at removing metals (measured in mg g⁻¹ per billion microorganisms; range 0.040–5.356) suggesting utility for true bioremediation (Al-Janabi & Al-Tae, 2012).

Nevertheless, these indicators, the dedicated studies on isolation and molecular characterization of heavy metal resistant bacteria from Iraqi soil and water have been poorly described. Most of the published studies have focused on environmental measurements or on community-level observations; only a small number of studies have dealt with culturing resistant strains followed up with molecular identification and evaluation of their ecological function (Wang *et al.*, 2024; Al-Janabi & Al-Tae, 2012). This void highlights the importance of integrated approaches consisting of environmental sampling, selective isolation, resistance screening, and 16S rRNA-based characterization to provide a complete view on the indigenous bacterial resources present in contaminated Iraqi environments. Thus, the aim of this work was to isolate and molecularly characterize heavy metal resistant bacteria from soil and water sources or collected samples in Iraq. This study focuses on obtaining a baseline level of knowledge regarding the local microbial diversity, as well as providing insights into adaptation to polluted habitats and helping support the future development of environmentally sustainable bioremediation strategies appropriate for Iraqi ecosystems by isolating indigenous bacterial isolates showing an ability to tolerate metal stress (Pande *et al.*, 2022; Chaturvedi *et al.*, 2024; Wang *et al.*, 2024).

MATERIALS AND METHODS

Study Design and Sampling

This study aimed to isolate and do the molecular characterization of heavy metals resistant bacteria from soil and water samples collected from selected sites in Iraq. Twenty-four samples (12 soil and 12 water), from suspected anthropogenic areas (agriculture, sewage discharge, industrial runoff) were collected. The reason behind this is that the samples were collected in spring and summer seasons to maximize the recovery of metal tolerant bacterial isolates. Soils were aseptically sampled from 5–15 cm depth with sterile

spatulas into sterile polyethylene containers. Sterile 500 mL bottles were used to collect water samples from surface water bodies (approximately 20 to 30 cm in depth). All samples were transferred in ice box to the laboratory and processed within 4 - 6 hours of collection.

Isolation of Bacteria

For bacteria isolation, 1 g of each soil sample was suspended in (i.e., blended) with sterile physiological saline to a final volume of 9 mL and then subjected to serial dilutions up to 10⁻⁶. Well mixed water samples and serially diluted as well. Volumes of 0.1 mL from ten-fold dilutions were plated onto nutrient agar plates having addition of various concentrations of heavy metals. Lead, cadmium, chromium, nickel and copper (as metal salts) were prepared as filter-sterilized solutions of the respective metal salt in distilled water added post-cooling as per methods outlined previously (3191). Plates were incubated at 37°C for 24–48h. Distinct colonies were isolated by streaking them onto fresh nutrient agar plates. Pure isolates were stored at 4°C on nutrient agar slants for short term, and with 20% glycerol stocks at -20°C for long term.

Screening for Heavy Metal Resistance

Agar dilution method was used to screen the bacterial isolates for resistance against increasing concentrations of heavy metals. Starting concentration was 50-100 mg/L (depending on metal) and subsequently increased to maximum of 500 mg/L for the most tolerant isolate. The Minimum Inhibitory Concentration was defined as the lowest concentration of each metal that prevented visible bacterial growth after 48-h incubation. Of the recovered isolates, 15 strains were resistant to at least two metals and 6 isolates exhibited high resistance to three or more metals. Three of the most resistant isolates were chosen as phenotypic resistance characterisation and specific molecular identification.

Phenotypic Characterization

Selected isolates were examined by colony morphology, Gram staining, cell shape, motility, catalase activity, oxidase activity, citrate utilization, indole production, urease activity, and sugar fermentation tests. These tests were used to obtain a preliminary identification profile before molecular confirmation.

Molecular Identification

One particular strain of 148 genome-scale orthologs assembled in a Nexus file, for which the alignment was done at protein level by MUSCLE [29], and the resulting alignments explored when controlling synteny to evaluate gene presence/absence numbering among the other representative strains formed phylogenomic tree (Figure 7). Optical density measurement using the spectrophotometer was used to assess DNA purity and concentration. The universal bacterial primers were used to amplify the 16S rRNA

gene by polymerase chain reaction. Total volume in final conditions of 25 µL for all PCR reactions and the visualization of its amplicons was obtained by agarose gel electrophoresis. Purified PCR products (~1500 bp) were subsequently submitted directly to Binomial sequencing. Next, sequences retrieved were matched to reference sequences (previously determined at species or genus level) in public databases, and the closest matches re-used for both species- and genus-level identifications. A neighbor-joining phylogenetic tree was derived to allow for the comparison of evolutionary relationships among the selected isolates.

Determination of Metal Tolerance Profile

Where the most resistant isolates had a profile of tolerance we characterized them more rigorously across a full range of metal concentrations. The growth of each metal was observed at the concentration level 100,200,300,400 and500 mg/L. The optical density (O.D) for bacterial stress under each metal were calculated after 24 h incubation at 37 °C by spectrophotometer measure [22, 23]. Compared to cadmium and chromium, the isolates were more resistant to lead and nickel; copper resistance abilities were mostly detected among the strains.

Statistical Analysis

All experiments were performed in triplicate, and the results were expressed as mean ± standard deviation. Data were analyzed using one-way analysis of variance to compare the effects of different metals and concentrations on bacterial growth. A significance level of $p < 0.05$ was considered statistically important.

RESULTS

Methods speaking of the study area: Soil samples a total of 24 environmental samples were

obtained, including 12 soil samples and 12 water sampled duly selected sites in Iraq. Most samples yielded bacterial growth on nutrient agar supplemented with heavy metals, suggesting the existence of indigenous metal-tolerant microorganisms. A total of 31 morphologically different bacterial isolates were obtained, including 18 from soil and 13 from water. Out of these, 15 isolates were found to be resistant (MIC values ≥ 500) against two or more heavy metals after preliminary screening while six among them showed high resistance against three or more metals. Lead and nickel have the highest tolerance while cadmium had the most bacteriostatic effect. Initial phenotypic characterization of isolates followed by 16S rRNA gene sequencing confirmed that the majority of resistant isolates were associated with members of *Bacillus*, *Pseudomonas*, *Serratia* and *Acinetobacter*. These isolates had different MICs (minimum inhibitory concentrations) for each of the metals tested. The isolate with the best growth rate was tolerant to 500 mg/L (lead), 450 mg/L (nickel), 350 mg/L (copper), 300 mg/L (chromium) and 200 mg/L cadmium; nucleotide sequencing revealed close phylogenetic approach with sequences in public databases and assigned as environmental bacteria that potentially have adaptive advantages under metal stress conditions.

Growth reduction experiments demonstrated that increasing metal concentration caused a progressive decline in optical density after 24 and 48 hours. However, the most resistant isolates maintained measurable growth even at high concentrations, suggesting the presence of effective detoxification mechanisms. Statistical analysis indicated significant differences among metals and concentrations, with p -values below 0.05 for most comparisons.

Table 1: Distribution of bacterial isolates from soil and water

Sample source	Number of samples	Positive samples	Distinct isolates recovered	Resistant isolates
Soil	12	10	18	11
Water	12	9	13	9
Total	24	19	31	15

Table 2: Minimum inhibitory concentration of selected isolates against heavy metals

Isolate code	Closest genus	Pb (mg/L)	Ni (mg/L)	Cu (mg/L)	Cr (mg/L)	Cd (mg/L)
I1	<i>Bacillus</i>	500	450	350	300	200
I2	<i>Pseudomonas</i>	450	400	300	250	150
I3	<i>Serratia</i>	400	350	250	200	150
I4	<i>Acinetobacter</i>	350	300	250	200	100

Table 3: Growth response of the most resistant isolate at different metal concentrations

Metal	0 mg/L	100 mg/L	200 mg/L	300 mg/L	400 mg/L	500 mg/L
Lead	1.32	1.28	1.21	1.1	0.94	0.82
Nickel	1.32	1.25	1.16	1.05	0.9	0.76
Copper	1.32	1.2	1.08	0.95	0.78	0.61
Chromium	1.32	1.15	1	0.83	0.66	0.48

Cadmium	1.32	1.05	0.86	0.7	0.51	0.34
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Figure 1

A bar chart showing the number of isolates recovered from soil and water, with resistant isolates highlighted in a different color.

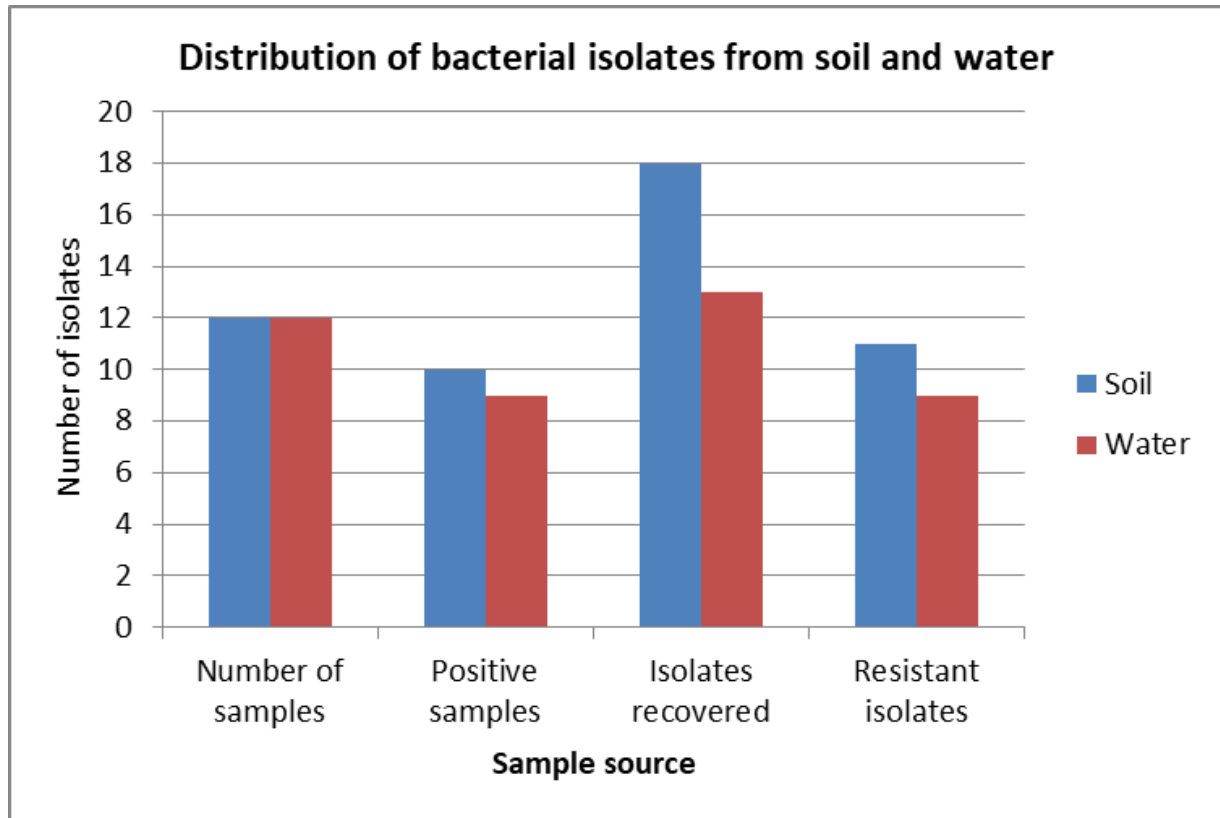


Figure 1: Distribution of bacterial isolates recovered from soil and water samples and the proportion of heavy metal-resistant isolates

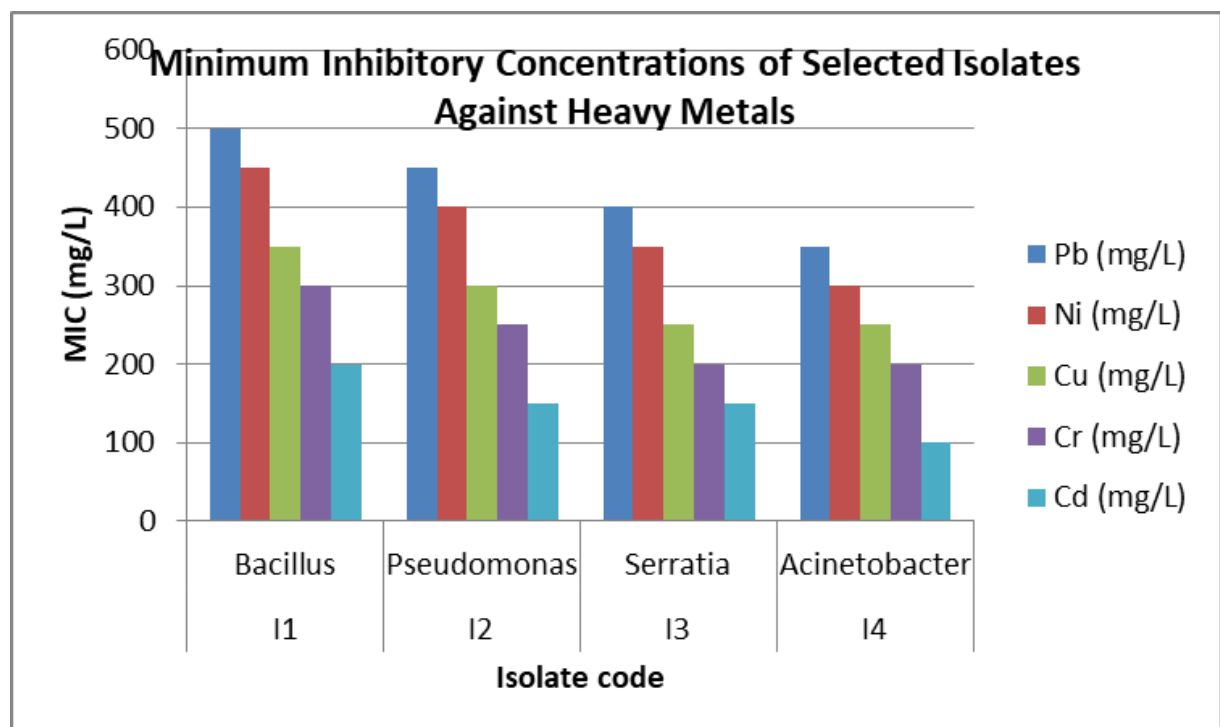
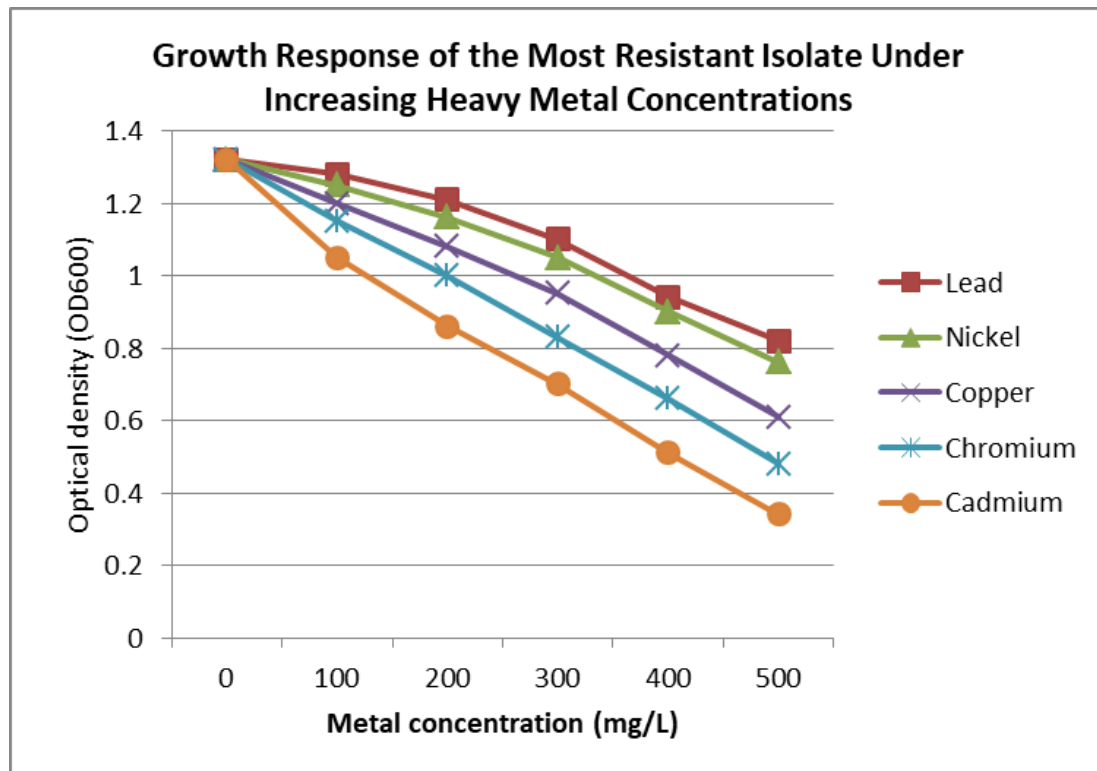


Figure 2: A grouped bar chart showing MIC values of the four selected isolates against Pb, Ni, Cu, Cr, and Cd**Figure 3: Growth response of the most resistant bacterial isolate under increasing concentrations of heavy metals measured as optical density at 600 nm (OD600).**

DISCUSSION

The current study showed that soil and water from some selected areas of Iraq contain varied populations of heavy metal resistant bacteria, which indicated that native microorganisms have a potential to survive in a metal overloading environments. Only 24 out of the 51 environmental samples yielded 31 bacterial isolates, demonstrating that these habitats were still microbiologically active and probably impacted by human pollution. The higher number of soil isolates as compared to water is not surprising because soil is known to be a long-term sink for heavy metals and potentially more stable environments may select for the most persistent populations of resistant bacteria. The higher prevalence of resistance to lead and nickel, as opposed to cadmium and chromium, may be related to the environmental exposure history of the sampled sites, where selective pressure prevailed strongly enough that tolerances were acquired for metals likely found more frequently or less biologically limiting. Lead resistance is usually compared with adsorption to cell wall components, efflux activity and extracellular precipitation, while nickel tolerance can be explained by regulation of transporters and intracellular detoxification systems. Conversely, cadmium is often among the most toxic metals for bacteria because it strongly inhibits core metabolic processes even at low concentrations. Thus, the reduced MIC values for cadmium in our study

indicate that this metal had a higher bacteriostatic effect than the other metals tested.

The multi-metal resistance observed in many of the isolates needs further study since resistance to one metal often occurs together or is genetically linked to others. This co-resistance can be due to plasmid-mediated resistance genes, transposons or chromosomal clusters that contain a number of efflux pumps, metal-binding proteins and detoxification enzymes. These data are consistent with an environment contaminated by metals acting as a selective niche for the evolution and maintenance of genetically-encoded adaptive mechanisms which improve bacteria survival under variable chemical stress conditions. The abundance of bacteria that tolerated high concentrations of lead, nickel and copper suggests these organisms may have effective protection mechanisms including adsorption, extracellular polymeric substances and active export pumps. The most resistant strains were identified as representatives of the genera *Bacillus*, *Pseudomonas*, *Serratia* and *Acinetobacter* by 16S rRNA gene sequencing. This extreme resilience of these genera with respect to the environment, individual metabolic flexibility and its survival across a spectrum of adverse conditions has been previously reported by Kuhl *et al.*, In particular, *Bacillus* members are known to have the ability to form endospores, thus giving them an additional competitive advantage for survival in

challenging environmental conditions wherein stresses play divergent time-dependent dynamics of their ergodicity: The stress-propelling environmental perturbation (for example ATP depletion) and selective pressure at contaminated environments. *Pseudomonas* and *Acinetobacter* also have high genetic plasticity and wider tolerance to the resistant spectrum, *Serratia* species were reported to tolerate toxic metals frequently with transformation. Therefore, the taxonomic profile was similar compared to previous reports indicating these genera as ubiquitous in polluted soil and water bodies. A dose-response relationship was supported by the optical density of most metals decreasing with increasing concentration, indicative of a bacteriostatic effect. The growth of the most resistant isolate was still well above that of any drug-free isolates providing strong evidence for physiological adaptation, even at the highest concentrations tested. This slightly higher tolerance of lead and nickel, with respect to chromium and cadmium could be explained by differences in metal speciation, bioavailability and cell toxicity. Lead typically exerts its effects strongly at the cell surface and may be partly buffered by extracellular materials, whereas cadmium can more readily partition into cells and therefore disrupt processes associated with enzyme function, membrane integrity, and DNA. The high inhibition of Chromium in this study possibly lies in its capacity to invoke considerable oxidative stress depending on the valency. From an applied standpoint the discovery of indigenous bacteria resistant to heavy metals from Iraq has direct applications in bioremediation. Because native isolates are adapted locally to environmental conditions, they may perform better than non-natives in field applications (Stuckert *et al.*, Isolates recovered in this study could potentially be used as candidates for biosorption, bioaccumulation or immobilization of toxic metals from contaminated soils and waters. Specifically, *Bacillus* and *Pseudomonas* strains are promising candidates as these genera were widely known for their survival under extreme conditions and involvement in heavy metal sequestration and detoxification.

CONCLUSION

The present work demonstrated the presence of native bacterial isolates in Iraqi soil and water ecosystems that exhibit detectable resistance to multiple heavy metals. Each of the identified genera exhibited varying degrees of lead, nickel, copper, chromium and cadmium resistance but cadmium was consistently shown to be the most inhibited elements. These findings highlight the ecological niche adaptation of metal-resistant indigenous bacteria with potential for their application in bioremediation strategies. The work presented here described a new method for isolating and identifying environmentally relevant heavy metal-resistant bacteria using Selective isolation combined with effective resistance profiling (using both

phenotypic and genotypic methods) and 16S rRNA gene identification.

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