Archives of Razi Institute, Vol. 77, No. 3 (2022) 1041-1047

DOI: 10.22092/ARI.2022.357399.2036

Original Article

Heavy Metal Resistance Ability of *Pseudomonas* Species Isolated from Sludge and Sewage in Iraq

Fawwaz Alfarras, A[1], Hamid AL-Fahdawi, M[1], Albayaty, M. K[2]*

1. Department of Medical Laboratory Techniques, College of Medical Technology, Al-Farahidi University, Baghdad, Iraq
2. Department of Forensic Evidence Techniques, College of Medical Technology, Al-Farahidi University, Baghdad, Iraq

Received 6 January 2022; Accepted 1 April 2022
Corresponding Author: mustafaalbayaty42@gmail.com

Abstract

It has been well documented that one of the best ways to remediate water and soil heavy metal pollution would be the use of microorganisms able to absorb heavy metals. The ability to resist toxic and heavy metals has been developed in some bacteria and microorganisms. This study, therefore, aimed to test the resistance ability of *Pseudomonas* species (spp.) isolated from sludge and sewage in Iraq against heavy metals, including mercury (Hg), copper (Cu), nickel (Ni), and cadmium (Cd) with a minimal concentration of 50 µg/ml for each. Water and soil samples were collected from different locations in Iraq. To test the tubes, 1 ml of each water sample, 1 gm of each soil sample, and 9 ml of sterilized distilled water were added and mixed thoroughly, followed by serial dilutions for each test tube separately. A total of 100 µl of aliquots from the appropriate dilution (10^{-2}) were also cultured on nutrient agar plates and then incubated at 37°C for 18 h. Different colonies from both water and soil samples were selected and grown on king A and king B media plates to confirm that these types of bacteria belong to the *Pseudomonas* genus. The isolates were identified based on their staining ability, shape, color, size, production of pigments, transparency, and mucoid properties of colonies growing on nutrient agar plates. In addition, some other biochemical tests were conducted. Several colonies were obtained and selected from the cultured samples and consequently, cultured and purified as a single colony. The preliminary observation and biochemical identification of these isolates indicated that two of them belonged to *Pseudomonas* spp.: Ps-1(M9) and Ps-2(M19). The screening of the bacteria isolates for resistance against Cu (II), Hg (II), Cd (II), and Ni (II) was performed by the use of Minimum Inhibitory Concentration. During the experiment and screening, different metal levels were evaluated to choose the best bacterial isolates with the ability of normal growth and resistance against heavy metal toxicity. The recorded data showed that two *Pseudomonas* isolates could tolerate heavy metal concentrations ranging from 50 to 180 µg/ml. Additionally, the two resistant *Pseudomonas* isolates also showed resistance to some antibiotics.

Keywords: Heavy metal, Isolation, *Pseudomonas* spp., Resistance

1. Introduction

In animal and human physiology, several metal ions, such as trace elements, play a pivotal role in body functions. However, when concentrations of these elements increase beyond a certain level, they could be harmful to the living animals' health. Heavy metals are one of the major components of industrial discharges that go into the environment untreated (1).

The well-known sources of heavy metals in the environment are water, soil, and rocks. As mentioned by Chi Fru, Rodriguez (2), heavy metals have also been present in the Earth's crust since 4.5 billion years ago. Rock erosion, soil erosion, petroleum, as well as coal burning, industrial operations, and finally, volcanic eruptions are the main sources and reasons for the continuous introduction of heavy metals into the ecosystem. In addition to the above-mentioned sources

of heavy metals, some other activities, such as agricultural and farming procedures, including applying pesticides, as well as fungicides, could be other sources of heavy metal pollution.

Heavy metals have several useful applications in the field of medicine as well. They have been used as antiparasitic, antimicrobial agents for treating some skin disorders. One of the best examples in this regard would be the application of heavy metals in the treatment of leishmaniasis. In addition, heavy metals have been utilized as anti-inflammatory agents for treating itchiness and in cancer chemotherapy (3).

Heavy metals present in the industrial wastewater and sewage sludge have been considered the main sources of environmental pollution, which have had permanent toxic impacts on humans and living animals' health, as well as the environment (4). The accumulation of heavy metals in human organs and tissues has led to many dangerous diseases, such as kidney failure, as well as cardiovascular and nervous system disorders (5). Several studies have also proved the accumulation of heavy metals in water, rice, vegetables (6, 7), and fish (8). Reactive Oxygen Species (ROS) have been known as the main source of cellular damage *in vivo*. The toxicity impacts of heavy metals are due to an increment in ROS production and the accumulation of this harmful oxidant consequently leads to the destruction of essential biomolecules and sub-cellular organelles. It is well documented that heavy metals, such as lead (Pb), cadmium (Cd), copper (Cu), arsenic (As), silver (Ag), and zinc (Zn), can accumulate in bacterial cells. Therefore, they induce the production of free radicals which in turn lead to severe DNA damage and disruption in the bacterial membrane through lipid peroxidation (9).

The World Health Organization has listed Cd, mercury (Hg), Pb, and As as toxic heavy metals which are a major public concern. In addition to the above-mentioned list, other elements, such as manganese (Mn), chromium (Cr), cobalt (Co), nickel (Ni), copper (Cu), zinc (Zn), selenium (Se), silver (Ag), antimony (Sb), and thallium (Tl), belong to the category of toxic heavy metals. All of these heavy metals are non-degradable; therefore, a limited range of concentration is allowed to be discharged into the environment (10). However, industrial wastes contain high levels of heavy metals, which are the most important causative reasons for plants, animals, aquatic life, and human health hazards and problems. They also lead to increasing pressures on living organisms and the ecosystem (11). Elements, such as Cu, Cr, Co, and Ni, are known to be the most commonly-used heavy metals and the most widespread contaminants in the environment. Traces of these heavy metals are necessary as they are cofactors of enzymatic reactions; however, high levels of them may cause extreme toxicity to living organisms due to the inhibition of metabolic reactions. Each heavy metal has unique biofunctions or biotoxicities. As a case in point, Cu can enhance microbial growth at low concentrations but represses growth at high concentrations, and Cd has high toxicity even at low concentrations (12). Some other heavy metals have no biological role and are harmful to organisms even at very low concentrations (13).

In recent years, several efforts have been made to decrease the number of toxic elements, such as heavy metals, in the environment by applying green and biomedical methods (14). One of these methods could be the use of microorganisms for the elimination of heavy metal contamination from polluted sources, such as water and waste streams, as well as soil and sediments. On the other hand, these microorganisms could be used to solubilize metals and facilitate their extraction. One of the pivotal ways to remediate polluted water and soil would be the use of bacteria with an interesting ability of metal absorption. The ability of toxic metal resistance has been developed by different bacteria and higher microorganisms. These unique features make them appropriate biological tools to make heavy metals innocuous. Due to the ease of access and use, these microorganisms are mostly acceptable and applicable for fighting toxic metal pollution in the ecosystem (15).

Therefore, the present study was designed to investigate the heavy metal resistance ability of *Pseudomonas* spp. isolated from sludge and sewage in Iraq.

2. Materials and Methods

2.1. Sample Collection and Preparation

Water and soil samples were collected from different locations in Iraq. To test the tubes, 1 ml of each water sample, 1 gm of each soil sample, and 9 ml of sterilized distilled water were added and mixed thoroughly, followed by serial dilutions for each test tube separately. A total of 100 µl of aliquots from the appropriate dilution (10^{-2}) were cultured on nutrient agar plates and then incubated at 37°C for 18 h.

2.2. Identification of *Pseudomonas* Isolates

Different colonies from both water and soil samples were selected and grown on king A and king B media plates to confirm that these types of bacteria belong to the *Pseudomonas* genus. The isolates were identified according to their staining ability, shape, color, size, production of pigments, transparency, and mucoid properties of colonies growing on nutrient agar plates. In addition, some other biochemical tests were conducted.

2.3. Screening of the Metal Resistant *Pseudomonas* Species

The obtained colonies were plated on nutrient agar plates containing 50 µg/ml of Ni, sulfate, cadmium nitrate, copper sulfate, and mercury nitrate as sources of different metal ions. The colonies were sub-cultured on the nutrient agar medium, and their culture purity was checked. The isolated strains were tested individually by culturing them on the nutrient agar medium containing different concentrations of each heavy metal ranging from 60 to 180 µg/ml. Growth was quantified concerning control plates containing no heavy metals. The minimum inhibition concentration (MIC) and maximum tolerable concentration (MTC) methods were used to evaluate the resistance level of isolated strains. The MTC is the highest concentration of metal, which does not affect the growth of the resistant strain.

2.4. Determination of Minimum Inhibitory Concentration

The MIC of the heavy metal resistant *Pseudomonas* spp. grown on heavy metals incorporated media against the respective heavy metal was determined by gradually increasing the concentration of the heavy metal, 10 µg/ml each time on the nutrient agar plate until the strains failed to give colonies on the plate. The starting concentration was 50 µg/ml. The culture growing on the last concentration was transferred to the plate containing a higher concentration of the heavy metal by streaking on the plate. When the isolates failed to grow on the plate, the MIC was assessed according to the standard protocol of the European food safety authority (Parma, Italy, 2012). (16).

2.5. Determination of Antibiotic Sensitivity and Resistance Pattern

An antibiotic sensitivity test was performed by an agar dilution method, and 0.1 ml of the mid-log phase culture (O.D600 = 0.6) of the two *Pseudomonas* isolates were spread on Brain Heart Infusion agar. The plates were left at room temperature to allow the absorption of excess moisture; then, antibiotic discs were placed on the inoculated agar medium and incubated at 37°C for 24 h. After the incubation, organisms were classified as sensitive or resistant to an antibiotic, based on the diameter of the inhibition zone given in the standard antibiotic disc chart.

3. Results and Discussion

3.1. Isolation of Heavy Metal Resistant *Pseudomonas* Species

Sludge and sewage samples were collected from different locations in Iraq, which were contaminated by heavy metals, to isolate heavy metal resistant *Pseudomonas* spp. This study resulted in the isolation and purification of 18 bacterial isolates originating from 50-150 colonies found in collected samples. After the characterization of these isolates, only two were identified to belong to the *Pseudomonas* genius, which were named Ps-1(M9) and Ps-2(M19). These two isolates were identified according to the cultural, morphological, and biochemical tests. It was found that both Ps-1(M9) and Ps-2(M19) isolates could resist and accumulate one or more toxic heavy metals [Cd (II),

Hg (II), Ni (II), and Cu (II)]. This was in accordance with a previous study by Clausen finding that environmental samples containing elevated concentrations of heavy metals were potential sources for toxic metal tolerant bacteria. Such environments might foster the adaptation and selection for heavy metal resistance (17). Both Ps-1(M9) and Ps-2(M19) isolates were highly fluorescent, which further confirms that they belong to the *Pseudomonas* genus. This result was compatible with the findings of Koedam, Wittouck (18) revealing that *Pseudomonas* isolates were highly fluorescent.

3.2. Identification of Bacterial Isolates

Colonies that were suspected to belong to the *Pseudomonas* genus were grown on the nutrient agar (Figure 1) and characterized by mucoidal properties. Most of the isolates produced pyocyanin and had a grapelike odor. The colonies showed a fried egg shape, with smooth, flat edges, and an elevated center. The isolates were whitish or creamy in color and had a fruity odor. These results are in accordance with the findings demonstrated by Gill, Arora (19). The microscopic examination of the two isolates showed that they were rod-shaped, non-spore-forming, and Gram-negative. These findings were also compatible with the microscopic findings of Holt, Kreig (20).These two isolates were also subjected to some biochemical tests, the results of which, as shown in table 1, indicate that these isolates belong to *Pseudomonas* spp., according to the Bergey's Manual of Systematic Bacteriology (21).

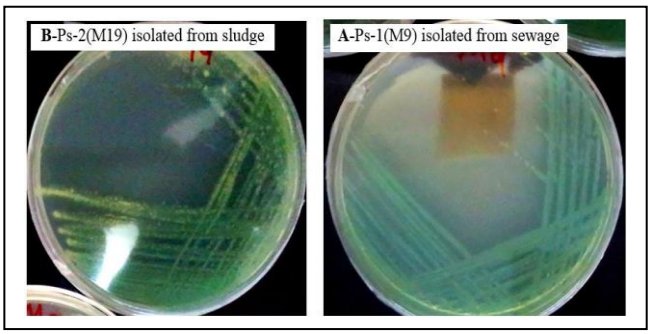

Figure 1. Growth of *Pseudomonas* species on the nutrient agar. A: Ps-1(M9) isolated from sewage, and B: Ps-2(M19) isolated from sludge

Table 1. Morphological, Physiological, and Biochemical characteristics of the isolated *Pseudomonas* species

Test	Result
Colony color	Green
Cell shape	Rod
Gram stain	Negative
Catalase production	Positive
Growth on king A	Positive
Growth on king B	Positive

3.3. Heavy Metal Resistance

The two isolates [Ps-1(M9) and Ps-2(M19)] were screened for their metal resistance ability on the nutrient agar media containing different metal concentrations of Ni, Cd, Cu, and Hg (Figure 2). The MICs test results for each heavy metal are presented in table 2. The Ps-1(M9) showed a different tolerance ability with maximum resistance against Cu at a concentration of 180 µg/ml. According to the MICs, the order of resistance in a decreasing manner was Cu> Cd and Ni>Hg. The Ps-2 (M19) showed a tolerance ability ranging from 120 to 140 µg/ml for Hg, Cu, and Cd with maximum resistance against Ni at a concentration of 160 µg/ml. The order of resistance for Ps-2(M19) was Ni> Cd and Cu > Hg, in a descending order.

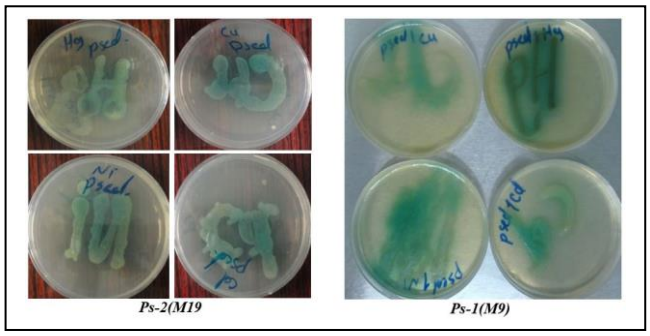

Figure 2. Growth of Ps-1(M9) and Ps-2(M19) on the nutrient agar media containing 50 µg/ml of cadmium, nickel, mercury, and copper

Table 2. Minimum Inhibitory Concentration of Ni, Cd, Cu, and Hg for both Ps-1(M9) and Ps-2(M19) isolates

Bacteria	Resistant to	MIC
Ps-1(M9)	Ni	140 µg/ml
	Cd	140 µg/ml
	Cu	180 µg/ml
	Hg	60 µg/ml
Ps-2(M19)	Ni	160 µg/ml
	Cd	140 µg/ml
	Cu	140 µg/ml
	Hg	120 µg/ml

Ni: Nickel
Cd: Cadmium
Cu: Copper
Hg: Mercury
MIC: Minimum Inhibitory Concentration

3.4. Antibiotic Sensitivity of Heavy Metal Resistant Isolates

The inhibition zone measurements shown in figure 3 were used to evaluate the antibiotic sensitivity of the two isolates [Ps-1(M9) and Ps-2(M19)]. The results in table 3 show that both bacterial isolates, Ps-1(M9) and Ps-2(M19), were resistant to Ampicillin but sensitive to Chloramphenicol and Erythromycin. In addition, Ps-1(M9) showed resistance to Nalidixic acid, while Ps-2(M19) was found to be sensitive to it. There is evidence showing that metal tolerance and antibiotic resistance are often found together in many clinical isolates and that metal and antibiotic resistance are closely associated. The very broad resistance of *Pseudomonas* spp. against antibiotics may mean that it is rich in plasmids that contain simultaneous antibiotic and metal resistance genes (13).Microorganisms resistant to antibiotics and tolerant toward metals appear to be the result of exposure to a metal-contaminated environment that causes coincidental selection for resistance factors for heavy metals and antibiotics. The ability of microbial strains to grow in the presence of heavy metals would be helpful in wastewater treatment where microorganisms are directly involved in the decomposition of organic matter in biological processes. The reason is that the inhibitory effect of heavy metals is often a common phenomenon that occurs in the biological treatment of wastewater and sewage. Although the conditions in which bacteria grow actually differ from laboratory to natural environment, it can be concluded from the present study that *Pseudomonas* spp. could be used in the bioremediation of metal-contaminated wastewater (13).

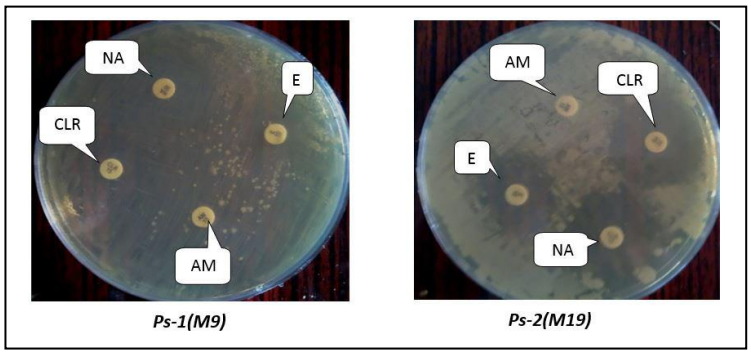

Figure 3. Antibiotic susceptibility of the two isolates Ps-1(M9) and Ps-2(M19) growing on the nutrient agar medium where AM=Ampicillin, CLR= Chloramphenicol, E= Erythromycin, and NA=Nalidixic acid.

Table 3. Antimicrobial Susceptibility test profile for heavy metal resistant Ps-1(M9) and Ps-2(M19) isolates

Name	Concentration	Abbreviation	Ps-1(M9)	Ps-2(M19)	Diameter of inhibition zone Ps-1/Ps-2
Ampicillin	10 mcg	AM	R	R	--------/-------
Chloramphenicol	15 mcg	CLR	S	S	14mm/24mm
Erythromycin	10 mcg	E	S	S	9mm/18mm
Nalidixic acid	30 mcg	NA	R	S	-------/19mm

4. Conclusion

Iraq sewage and sludge can be used for the isolation of heavy metal-resistant bacteria. The cultural, morphological, and biochemical tests conducted in the present study showed that the culture isolates belonged to the *Pseudomonas* genus. The two isolates [Ps-1(M9) and Ps-2(M19)] showed resistance against different heavy metal concentrations, such as Cu, Ni, Hg, and Cd. In addition, Ps-1(M9) and Ps-2(M19) isolates showed multidrug resistance. In conclusion, *Pseudomonas spp.* could be usefully applied in the bioremediation of metal-contaminated wastewater.

Authors' Contribution

Study concept and design: M. K. A.
Acquisition of data: A. F. A.
Analysis and interpretation of data: A. F. A.
Drafting of the manuscript: M. H. A.
Critical revision of the manuscript for important intellectual content: M. K. A.
Statistical analysis: M. H. A.
Administrative, technical, and material support: M. K. A.

Conflict of Interest

The authors declare that they have no conflict of interest.

References

1. Singh P, Purakayastha TJ, Mitra S, Bhowmik A, Tsang DCW. River water irrigation with heavy metal load influences soil biological activities and risk factors. J Environ Manage. 2020;270:110517.
2. Chi Fru E, Rodriguez NP, Partin CA, Lalonde SV, Andersson P, Weiss DJ, et al. Cu isotopes in marine black shales record the Great Oxidation Event. Proc Natl Acad Sci U S A. 2016;113(18):4941-6.
3. Pal C, Asiani K, Arya S, Rensing C, Stekel DJ, Larsson DGJ, et al. Metal Resistance and Its Association With Antibiotic Resistance. Adv Microb Physiol. 2017;70:261-313.
4. Khallef M, Benouareth DE, Konuk M, Liman R, Bouchelaghem S, Hazzem S, et al. The effect of silver nanoparticles on the mutagenic and the genotoxic properties of the urban wastewater liquid sludges. Environ Sci Pollut Res Int. 2019;26(18):18403-10.
5. Marshall TM, Colvin KL, Nevin R, Macrellis J, Dardia GP. Neurotoxicity Associated with Traumatic Brain Injury, Blast, Chemical, Heavy Metal and Quinoline Drug Exposure. Altern Ther Health Med.2019;25(1).
6. Mao C, Song Y, Chen L, Ji J, Li J, Yuan X, et al. Human health risks of heavy metals in paddy rice based on transfer characteristics of heavy metals from soil to rice. CATENA. 2019;175:339-48.
7. Gemeda FT, Guta DD, Wakjira FS, Gebresenbet G. Occurrence of heavy metal in water, soil, and plants in fields irrigated with industrial wastewater in Sabata town, Ethiopia. Environ Sci Pollut Res Int. 2021;28(10):12382-96.
8. Liu Q, Xu X, Zeng J, Shi X, Liao Y, Du P, et al. Heavy metal concentrations in commercial marine organisms from Xiangshan Bay, China, and the potential health risks. Mar Pollut Bull. 2019;141:215-26.
9. Williams CL, Neu HM, Gilbreath JJ, Michel SL, Zurawski DV, Merrell DS. Copper Resistance of the Emerging Pathogen Acinetobacter baumannii. Appl Environ Microbiol. 2016;82(20):6174-88.
10. Rahman Z, Singh VP. The relative impact of toxic heavy metals (THMs) (arsenic (As), cadmium (Cd), chromium (Cr)(VI), mercury (Hg), and lead (Pb)) on the total environment: an overview. Environ Monit Assess. 2019;191(7):419.
11. Noreen U, Ahmed Z, Khalid A, Di Serafino A, Habiba U, Ali F, et al. Water pollution and occupational health hazards caused by the marble industries in district Mardan, Pakistan. Environ Technol Innov. 2019;16:100470.
12. Cardoso P, Nunes T, Pinto R, Sa C, Matos D, Figueira E. Rhizobium response to sole and combined exposure to cadmium and the phytocompounds alpha-pinene and quercetin. Ecotoxicology. 2020;29(4):444-58.
13. Bhuiyan MAR, Khan T, Huq SM. Cadmium and lead tolerant bacteria isolated from industrial waste water. Dhaka Univ J Biol Sci. 2017;26:29-38.
14. Sher S, Rehman A. Use of heavy metals resistant bacteria-a strategy for arsenic bioremediation. Appl Microbiol Biotechnol. 2019;103(15):6007-21.
15. Medfu Tarekegn M, Zewdu Salilih F, Ishetu AI. Microbes used as a tool for bioremediation of heavy metal from the environment. Cogent food agric. 2020;6(1):1783174.

16. Marzan LW, Hossain M, Mina SA, Akter Y, Chowdhury AMMA. Isolation and biochemical characterization of heavy-metal resistant bacteria from tannery effluent in Chittagong city, Bangladesh: Bioremediation viewpoint. Egypt J Aquat Res. 2017;43(1):65-74.
17. Xing D, Magdouli S, Zhang J, Koubaa A. Microbial remediation for the removal of inorganic contaminants from treated wood: Recent trends and challenges. Chemosphere. 2020;258:127429.
18. Koedam N, Wittouck E, Gaballa A, Gillis A, Hofte M, Cornelis P. Detection and differentiation of microbial siderophores by isoelectric focusing and chrome azurol S overlay. Biometals. 1994;7(4):287-91.
19. Gill JS, Arora S, Khanna SP, Kumar KH. Prevalence of Multidrug-resistant, Extensively Drug-resistant, and Pandrug-resistant Pseudomonas aeruginosa from a Tertiary Level Intensive Care Unit. J Glob Infect Dis. 2016;8(4):155-9.
20. Holt JG, Kreig NR, Williams ST. Bergyy's Manual of Determination bacteriology. 9th ed: Williams and Wilkins; 199.
21. Garrity G, Bell J, Lilburn T. Taxonomic Outline of the Prokaryotic Genera. Bergey's Manual of Systematic Bacteriology.2003. 397 p.