Antibiotic Profile and *czcA* Heavy Metal Resistance Gene of *Pseudomonas aeruginosa* Isolated from the Tanjaro River, a Heavy Metal-Contaminated Water Source in Sulaimaniyah City, Kurdistan Region, Iraq.

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Abstract:

Freshwater ecosystems are being increasingly threatened by human activities, particularly by the disposal of industrial waste and heavy metals. The Tanjaro River in Sulaymaniyah , Kurdistan Region, Iraq, serve as a prime case study and has been severely affected by rampant pollution due to uncontrolled urbanization and industrial activities. Water samples were collected from the Tanjaro River and sampling was conducted on October 1, 2024, from eight distinct locations. At each sampling point sterile 100 mL plastic container were used to collect water. Water samples were transported to the laboratory in cool box to maintain microbial viability. Directly inoculated to the Tryptic Soy Broth (TSB) and incubated at 37°C for 24 hours under aerobic conditions to enhance bacterial growth. In this research, *Pseudomonas aeruginosa* , a well studied multidrug resistant and metal-tolerant bacterium, was isolated and identified from this polluted water source. Sampling was conducted from eight distinct locations. Twenty *P. aeruginosa* strains were identified using cultural characteristics, microscopic features, and biochemical tests, and were further confirmed using the VITEK® 2 COMPACT system. Antibiotic susceptibility testing revealed that all isolates were susceptible to ciprofloxacin, levofloxacin, gentamicin, and meropenem, while showing lower sensitivity to aztreonam. Genomic DNA was extracted and specific primers were designed to target the *czcA* gene. PCR amplification showed the existence of the *czcA* gene that codes for the key component of the CzcCBA efflux pump system, able to release cobalt, zinc, and cadmium from bacterial cells. In addition to heavy metal contamination, there was no multidrug resistance, showing that the isolates remain treatable using regular antibiotics. This shows that *P. aeruginosa* of Tanjaro River is not only a bioindicator of environmental pollution but also as one candidate for bioremediation of heavy metal-contaminated sites. The findings underscore the necessity for immediate, increased environmental surveillance, controlled dumping, and synchronized public health responses to reduce the risks emanating from such contamination.

Keywords: Heavy metals, freshwater contamination, *Pseudomonas aeruginosa* , antibiotic resistance, Tanjaro river, *czcA* gene, bioremediation.

پوختە:

ئیکۆسیستەمی ئاوی شیرین زیاتر مەترسی لەسەرە بەهۆی چالاکییەکانی مرۆڤەوە، بەتایبەتی بەهۆی فڕێدانی پاشماوەی پیشەسازی و کانزا قورسەکانەوە. ڕووباری تانجارۆ لە شاری سلێمانی لە هەرێمی کوردستان لە عێراق، وەکو توێژینەوەیەکی سەرەکی کاردەکات و بەهۆی شارنشینی و چالاکییە پیشەسازییەکانی کۆنترۆڵنەکراوی پیسبوونی بەربڵاوەوە کاریگەرییەکی زۆری لەسەر بووە. نمونەی ئاو لە ڕووباری تانجارۆ وەرگیرا و لە ١ی تشرینی یەکەمی ٢٠٢٤، لە هەشت شوێنی جیاوازەوە نمونە وەرگرتن ئەنجامدرا. لە هەر خاڵێکی نمونەگرتندا ١٠٠ مل دەفری پلاستیکی تەعقیمکراو بەکارهێنرا بۆ کۆکردنەوەی ئاو. نمونەی ئاو لە سندوقێکی سارددا گواسترایەوە بۆ تاقیگە بۆ پاراستنی توانای میکرۆبی. ڕاستەوخۆ کوتێنراوە بۆ شۆربای سۆیا تریپتیک (TSB) و لە پلەی گەرمی ٣٧ پلەی سەدی بۆ ماوەی ٢٤ کاتژمێر لە ژێر بارودۆخی هەوایدا کەرەوز کراوە بۆ بەرزکردنەوەی گەشەی بەکتریا. لەم لێکۆڵینەوەیەدا، بەکتریایەکی جۆری Pseudomonas aeruginosa کە بە باشی لێکۆڵینەوەی لەسەر کراوە و بەرگری لە فرە دەرمان و بەرگەگرتنی کانزاکانە، جیاکراوەتەوە و لەم سەرچاوە ئاوە پیسەدا دەستنیشانکراوە. نمونەگرتن لە هەشت شوێنی جیاوازەوە ئەنجامدرا. بیست جۆری P. aeruginosa بە بەکارهێنانی تایبەتمەندییە کولتوورییەکان، تایبەتمەندییە وردبینییەکان، و تاقیکردنەوە بایۆکیمیاییەکان دەستنیشانکران، و زیاتر بە بەکارهێنانی سیستەمی VITEK® 2 COMPACT پشتڕاستکرانەوە. تاقیکردنەوەی ئامادەیی ئانتیبایۆتیک دەریخست کە هەموو جیاکراوەکان ئامادەن بۆ سیپرۆفلۆکساسین، لیڤۆفلۆکساسین، جێنتامایسین و مێرۆپینێم، لە کاتێکدا هەستیاری کەمتریان بۆ ئازتریۆنام نیشان دا DNA .جینۆمی دەرهێنرا و پرایمەری تایبەت دیزاین کرا بۆ ئامانجکردنی جینی .czcA گەورەکردنی PCR بوونی جینیczcA ی نیشان دا کە کۆدی پێکهاتەی سەرەکی سیستەمی پەمپە دەرچوونی CzcCBA دەدات، کە توانای دەردانی کۆبالت، زینک و کادمیۆمی هەیە لە خانەکانی بەکتریا. جگە لە پیسبوونی کانزا قورسەکان، هیچ بەرگرییەکی فرەدەرمان بەدی نەکرا، ئەمەش ئاماژەیە بۆ ئەوەی کە جیاکراوەکان هێشتا بە دژەزیندەیی ئاسایی چارەسەر دەکرێن. ئەمە ئاماژەیە بۆ ئەوەی کە P. aeruginosa لە ڕووباری تانجەرۆ تەنها نیشاندەرێکی زیندەیی پیسبوونی ژینگە نییە بەڵکو وەک کاندیدێکی پۆتانسێل بۆ چاککردنەوەی زیندەیی ژینگە پیسبووەکانی کانزا قورسەکان. توێژینەوەکە تیشک دەخاتە سەر پێویستی چاودێریکردنی ژینگەیی بەپەلە و چڕتر، بەڕێوەبردنی پاشماوەی کۆنترۆڵکراو و دەستێوەردانەکانی تەندروستی گشتی هەماهەنگ بۆ کەمکردنەوەی ئەو مەترسیانەی کە لەم جۆرە پیسبوونەدا سەرهەڵدەدەن.

وشەی سەرەکی: کانزا قورسەکان، پیسبوونی ئاوی شیرین، Pseudomonas aeruginosa، بەرگری دژەزیندەیی، ڕووباریTanjaro ، جینیczcA ، چاکسازی زیندەیی.

الملخص:

تتعرض النظم البيئية للمياه العذبة لتهديد متزايد بسبب الأنشطة البشرية، وخاصةً التخلص من النفايات الصناعية والمعادن الثقيلة. يُعد نهر تانجارو في السليمانية، إقليم كردستان العراق، دراسة حالة رئيسية، وقد تأثر بشدة بالتلوث المتفشي الناجم عن التوسع العمراني غير المنضبط والأنشطة الصناعية. جُمعت عينات من مياه نهر تانجارو، وأُجريت عملية أخذ العينات في ١ أكتوبر/تشرين الأول ٢٠٢٤، من ثمانية مواقع مختلفة. استُخدمت في كل نقطة أخذ عينات حاويات بلاستيكية معقمة سعة ١٠٠ مل لجمع المياه. نُقلت عينات المياه إلى المختبر في صندوق تبريد للحفاظ على حيوية الميكروبات. تم تطعيمهم مباشرة بمرق الصويا التربسين (TSB) وحضنهم عند ٣٧ درجة مئوية لمدة ٢٤ ساعة في ظل ظروف هوائية لتعزيز نمو البكتيريا. في هذا البحث، تم عزل وتحديد بكتيريا الزائفة الزنجارية، وهي بكتيريا مدروسة جيدًا مقاومة للأدوية المتعددة ومتحملة للمعادن، من مصدر المياه الملوث هذا. تم أخذ العينات من ثمانية مواقع مميزة. تم تحديد عشرين سلالة من الزائفة الزنجارية باستخدام الخصائص الثقافية والسمات المجهرية والاختبارات الكيميائية الحيوية، وتم تأكيدها بشكل أكبر باستخدام نظام VITEK® 2 COMPACT. كشف اختبار حساسية المضادات الحيوية أن جميع العزلات كانت حساسة للسيبروفلوكساسين والليفوفلوكساسين والجنتاميسين والميروبينيم، بينما أظهرت حساسية أقل للأزتريونام. تم استخراج الحمض النووي الجينومي وصُممت بادئات محددة لاستهداف جين czcA. كشف تضخيم تفاعل البوليميراز المتسلسل (PCR) عن وجود جين czcA الذي يُشفر المكون الرئيسي لنظام مضخة التدفقCzcCBA ، القادر على إطلاق الكوبالت والزنك والكادميوم من الخلايا البكتيرية. وإلى جانب التلوث بالمعادن الثقيلة، لم تُلاحظ أي مقاومة متعددة للأدوية، مما يُشير إلى أن العزلات لا تزال قابلة للعلاج بالمضادات الحيوية التقليدية. ويشير هذا إلى أن الزائفة الزنجارية (Pseudomonas aeruginosa) في نهر تانجارو لا تُمثل مؤشرًا حيويًا على التلوث البيئي فحسب، بل تُمثل أيضًا مرشحًا مُحتملًا للمعالجة الحيوية للبيئات الملوثة بالمعادن الثقيلة. وتُبرز الدراسة الحاجة إلى رصد بيئي عاجل ومكثف، وإدارة مُحكمة للنفايات، وتدخلات صحية عامة مُنسقة للتخفيف من المخاطر الناجمة عن هذا التلوث.

الكلمات المفتاحية: المعادن الثقيلة، تلوث المياه العذبة، الزائفة الزنجارية(Pseudomonas aeruginosa) ، مقاومة المضادات الحيوية، نهر تانجارو، جينczcA ، المعالجة الحيوية.

Introduction

Freshwater ecosystems have been significantly affected by human practices in this day and era, such as industrial effluents' release, agriculture, urbanization, and improper garbage disposal (Amoatey & Baawain, 2019). Of these, heavy metals' contamination represents an international issue bringing threats to ecosystems due to its persistence, toxicity, and biomagnification and bioaccumulation properties (Liu et al., 2022). For instance, the Tanjaro River in Sulaymaniyah , located in northern Iraq, serves as a pertinent case study, which has been heavily contaminated in the past decades, for which It is used as a source of water for drinking, irrigation, etc., but urbanization, industries, and uncontrolled waste treatment have caused excessive pollution there, impacting the environment, public health, and ecosystems as a whole (*Hamasalih et al., 2025*). These Pollutions, particularly the one by heavy metals like lead, cadmium, chromium, zinc, cobalt, and nickel, render the water unsuitable for human use and agriculture (*Nizar et al., 2012 ; Ferrini et al., 2024; Faque Salih et al., 2021*). Currently the available data shows us that the primary concern right now is presence of antibiotic resistant bacteria in Tanjaro River, almost 39 metal-resistant species, of which 17 (43.5%) were gram-positive and 22 (56.5%) gram-negative bacteria, were found in a particular study, all of these bacteria survived on heavy metal-containing LB agar, which was a sign of metal resistance (*Faque Salih et al., 2021*). Included in the list is one of the main isolated bacteria during that study was *Pseudomonas aeruginosa* , a widespread Gram-negative pathogen which is capable of metabolizing an assortment of organic and inorganic compounds.

The Tanjaro River in southern Sulaymaniyah , Kurdistan Region, Iraq, begins where the Qiliasan and Kani-Ban streams merge near Kani-Goma village and runs 58 km into Darbandikhan Dam (Nizar et al., 2012). The hilly region causes flooding there. Unplanned growth since 2003 has overwhelmed utilities like water, sanitation, health, education, and transport, with sewage flowing into the river (Hawrami, 2018). The 25-hectare Tanjaro dump area receives 500 tons of municipal, 60 tons of silt, and 5 tons of biomedical waste daily. Approximately 900 small-scale industries are located near it, with the majority situated in the Station area; however, most of these establishments operate without licenses, producing iron, oil, plastics, and asphalt (Hamasalih et al., 2025). Tanjaro, which was once a source of life to ancient settlements, was host to the Bronze Age city of Kunara. Since 2012, its excavations have unearthed connections with Mesopotamian civilisations, and it has grown into an industrial and modern hub (Tenu & Kepinski, 2016). Pollution of the Tanjaro River has devastated the environment, spread disease, harmed agriculture and aquaculture, and displaced some individuals from their residences. Aquatic life and biodiversity have been depleted, and cleanups are expensive (Hamasalih et al., 2025). Its industrial area is highly problematic, with waste disposal in its raw form discharging toxic metals such as lead, cadmium, and nickel. This pollution encourages antibiotic resistant pathogens, which increase health hazards (Nizar et al., 2012; Ferrini et al., 2024; Faque Salih et al., 2021).

Pseudomonas aeruginosa is the focus of our study, it is a Gram-negative, motile rod (0.5–1.0 µm by 1–5 µm) which aerobically or anaerobically respire with the assistance of nitrate (Diggle & Whiteley, 2020), it is a multidrug resistant microorganism causing acute or chronic infection in immunocompromised individuals, e.g., those with COPD, cystic fibrosis, cancer, burns, sepsis, VAP, and COVID-19 (Qin et al., 2022). The bacterium also produces pigments, pyoverdine, pyocyanin, pyorubrin, and pyomelanin that play an essential role in its pathogenicity and survival. The pigments assist in bacterial communication, virulence factor modulation, and the capture of iron, which is critical for the survival of the bacteria in the human body (Sarkheili et al., 2025). They are metabolically quite versatile with the capability of growth both aerobically and anaerobically on many different organic substances as a source of energy (Arai, 2011). For protection they form biofilms collection of cell defenses which shield it against stress as well as antibiotics to increase survival under polluted environments (Thi et al., 2020), but also presents a protein structure in which help the bacteria to survive under extreme conditions by withstanding toxic substances like antibiotics and heavy metals, through efflux pumps (Lorusso et al., 2022).

Efflux pumps are protein complexes in both bacteria and eukaryotes. Their function is to actively expel unwanted or harmful material, such as toxins, heavy metals, or antibiotics, from the cell and thus safeguard the organism (Blanco et al., 2016). In *Pseudomonas aeruginosa*, multiple efflux pumps are involved in resistance to both metals and antibiotics. One of the most studied cases involves the CzcCBA efflux pump, an RND (Resistance Nodulation Division) family transporter. This efflux pump, in particular, expels specific metals, including cadmium (Cd), zinc (Zn), and cobalt (Co) and represents the cell's initial protection mechanism in response to metal toxicity (Ducret et al., 2020). It consists structurally of three proteins: CzcA (the transporter), CzcB (the linker protein), and CzcC (the pore in the outer membrane) (Kim et al., 2011). The action of this pump is tightly regulated (Ducret et al., 2020). When elevated concentrations of Zn, Cd, or Co are sensed, CzcRS regulates it. CzcS perceives metals and activates CzcR, which in turn activates the genes for czcCBA. Ultimately, CzcR represses transcription of the oprD gene, encoding, under normal circumstances, a channel facilitating entry of Carbapenem antibiotics in to the cell (its role can, in fact, be inhibitory). Consequently, Carbapenem uptake is diminished. This regulatory connection, therefore, besides bringing about metal resistance, also lends support to resistance to antibiotics, notably to Carbapenems (Ducret et al., 2020).

In addition to this resistance to metals, *P. aeruginosa* also has resistance to antibiotics through various means. Efflux pumps in *P. aeruginosa* can remove not only metals but also antimicrobial compounds, thereby significantly minimizing the effectiveness of these medications (Muñoz-Cazalla et al., 2023). *P. aeruginosa* also generates enzymes in the form of β-lactamases to degrade antibiotics of the β-lactam class, and it generates biofilms, which serve as protective covers, such that antibiotics fail to reach bacterial cells (Liao et al., 2022).

This study was conducted for three major reasons. The first one was to investigate the antimicrobial resistance characteristics of *Pseudomonas aeruginosa* in order to understand its disease-causing capability, as this organism can re-enter into the human food supply through crop production and bioaccumulation in animals and therefore become harmful to human health. The second one was to investigate heavy metal resistance to understand whether the organism can tolerate highly contaminated waters and therefore act as an ideal candidate for bioremediation purposes. Third, as a secondary goal, the study explored whether the presence of heavy metal resistance genes is associated with resistance to antibiotics, particularly the carbapenem group. This study hypothesizes that *Pseudomonas aeruginosa* isolates obtained from the Tanjaro River harbor heavy metal resistance genes that not only contribute to their enhanced pathogenic potential and multidrug resistance, but also render them promising candidates for bioremediation applications.

Materials and Methods

Sample Collection and Preservation

Water samples were collected from the Tanjaro River, a site known for heavy metal pollution due to industrial and agricultural runoff. Sampling was conducted on October 1, 2024, from eight distinct locations referring to the map in (Figure 2.1.) At each sampling point sterile 100 mL plastic container were used to collect water and (100ml) water samples were transported to the laboratory in cool box to maintain microbial viability. Directly inoculated to the Tryptic Soy Broth (TSB) and incubated at 37°C for 24 hours under aerobic conditions to enhance bacterial growth.

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Bacterial Cultivation and Selective Isolation

Following growth enhancement, *P.aeruginosa* was isolated using cetrimide agar, a selective and differential medium that supports its growth while inhibiting other bacteria. Inoculated plates and uninoculated control plates of cetrimide agar incubated at 37 °C for 24 hours. Refer to Figure 2.2.

Figure 2.1. Map of the sample collection sites of Tanjaro River.
35.47970° N, 45.42548° E, 35.47964° N, 45.42524° E, 35.47970° N, 45.42609° E, 35.47975° N, 45.42659° E, 35.47940° N, 45.42789° E, 35.47943° N, 45.42791° E, 35.47927° N, 45.42823° E, 35.47937° N, 45.42830° E

Figure 2.2. Isolation of *Pseudomonas aeruginosa* from contaminated water using the spread plate technique on cetrimide agar.

Purification and Activation of *P. aeruginosa* Isolates

Single colonies from cetrimide agar plates were further purified using the quadrant streaking method on cetrimide agar to obtain isolate purified microbial colonies. Possible nineteen isolates of *P. aeruginosa* were identified based on their notable pigmentation, including: (pyocyanin), which is a blue-green pigment; (pyoverdine), a yellow-green fluorescent pigment; (pyorubin), known as red pigment; and (pyomelanin), a brown pigment (El-Fouly et al., 2020). As seen in Figure 2.3. *Pseudomonas aeruginosa (ATCC® 9027™)* was used as a control strain.

Identification of purified *Pseudomonas aeruginosa*

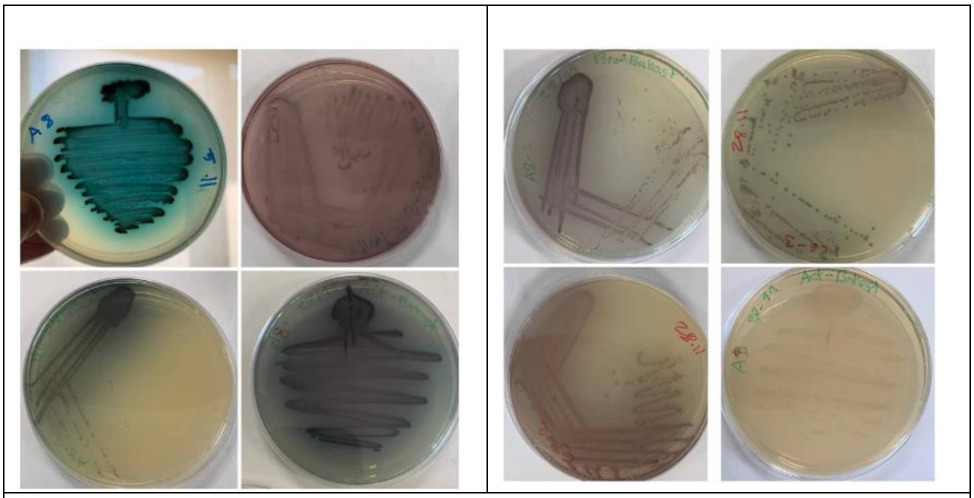

Figure 2.3. *Pseudomonas aeruginosa* colonies grown on cetrimide agar plates showing different characteristic pigmentation.

To confirm the identity of the purified isolates, biochemical and morphological tests were performed, including: Gram staining under the microscope, the stained bacteria appeared as Gram-negative rods, consistent with the expected morphology of *P. aeruginosa* . Oxidase and Catalase positive.

To further confirm the bacterial species, twenty isolates were analyzed using the VITEK® 2 COMPACT biochemical identification system. Prior to testing, the isolates were sub-cultured on nutrient agar medium and incubated for 24 hours. The VITEK® 2 system identifies microorganisms based on a panel of miniaturized biochemical assays, which assess characteristics such as carbohydrate utilization, enzyme activities, and resistance to inhibitory substances. For *Pseudomonas aeruginosa* , key reactions typically include oxidase positivity, the ability to utilize glucose oxidatively but not fermentatively, and production of enzymes such as arginine dihydrolase and gelatinase.

The results of the VITEK® 2 analysis confirmed that all tested isolates belonged to *P. aeruginosa* , with identification confidence percentages varying across samples. This biochemical confirmation provided strong support for the preliminary cultural and morphological observations.

Antimicrobial Susceptibility Testing (AST)

P. aeruginosa isolates were tested for antimicrobial susceptibility, using antibiotics with different modes of action, including Gentamicin 10 µg (CN; aminoglycoside), 10 µg Ciprofloxacin, Levofloxacin 5 µg (CIP; fluoroquinolone) Meropenem 10 µg (MEM; carbapenem), and Aztreonam 30 µg (ATM; monobactam).

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The antibiotic resistance profiles of the *P. aeruginosa* isolates suspensions prepared in sterile normal saline adjusted to a 0.5 McFarland Antibiotic susceptibility testing was performed on 20 isolates of *Pseudomonas aeruginosa* using the Kirby-Bauer disk diffusion method on Mueller Hinton agar plates incubated for 24 hours at 37°C. (Perilla et al, 2010).

The results were measured by reporting the inhibition zone (in millimeters) and interpreted according to Clinical and Laboratory Standards Institute documents (CLSI, 2025). After the incubation, the bacteria were classified as sensitive or resistant or intermediate to the antibiotics, based on the diameter of the zone of inhibition given in the standard antibiotic disc chart. Quality control measures were applied by including *Pseudomonas aeruginosa* *(ATCC® 9027™)*as reference strains to verify the accuracy and reliability of the susceptibility testing procedure.

Genomic DNA Extraction

Following AST, bacterial isolates were reactivated in 3 mL of Tryptic Soy Broth (TSB) and incubated at 37 °C for 24 hours prior to molecular analysis. Genomic DNA was extracted using the AddBio genomic DNA extraction kit according to the manufacturer's protocol. DNA concentration and purity were measured using a ThermoFisher C2000 NanoDrop spectrophotometer, and samples with an A260/A280 ratio between 1.8 and 2.0 were considered of sufficient purity for downstream applications.

To assess DNA integrity and confirm successful purification, 5 µL of each extracted DNA sample was analyzed by agarose gel electrophoresis (1% agarose, 100 V, 45 min) stained with safe gel stain dye, and high molecular weight genomic DNA bands without smearing were considered indicative of intact DNA. Extracted DNA samples were stored at −20 °C until use for PCR amplification.

Polymerase chain reaction (PCR) Amplification of the *czcA* Gene

PCR testing was performed to detect the heavy metal resistance *czcA* gene using specific primers. The primers designed explicitly for this study, which amplified a 485 bp region of the gene.

Forward Primer: 5' GAGTTCATTCCCAGCCTCAG 3'

Reverse Primer: 5' GTCTGCTCGACCTTCACCTC 3'

Primer specificity was validated in silico using NCBI BLAST to confirm exclusive targeting of the *czcA* sequence in *Pseudomonas aeruginosa* and avoid off-target amplification. Primer efficiency was assessed empirically through gradient PCR and optimization of annealing temperature, resulting in consistent amplification of the expected 485 bp product across all isolates. 57°C was selected as it yields the optimal specificity and produces a sharp, single PCR product with no visible non-specific bands. The PCR optimization gel results are shown in (Figure 2.8). The no template control (NTC) remained negative, confirming the absence of contamination.

PCR amplification of the *czcA* gene was carried out in a 20 µL reaction mixture containing 10 µL of 2X HotStart Master Mix, 1 µL of forward primer, 1 µL of reverse primer, 1 µL of DNA template, and 7 µL of nuclease-free water. The PCR protocol consisted of 35 cycles. As shown in Table 1. At first, primers were received in lyophilized form and prepared to a stock concentration of 100 pmol/µL using nuclease-free water. Working solutions of 10 pmol/µL were prepared from the stock for use in PCR reactions.

Table 1: PCR amplification for the *czcA* gene

Component/Steps	*czcA* gene (Heavy metal resistance gene)
Amplicon Size	485 bp
Primer Preparation	Stock: 100 pmol/µL (lyophilized), reconstitute in nuclease-free water Working: 10 pmol/µL
PCR Reaction Mixture (20 µL total)	10 µL 2X master mix 1 µL forward primer 1 µL reverse primer 1 µL template DNA 7 µL nuclease-free water
Thermal Cycling Conditions	- Initial denaturation at 95 °C for 10 min; - **35 cycles of:** 1- Denaturation: 95 °C for 30 s 2- Annealing: 57 °C for 30 s 3- Extension: 72 °C for 30 s - final extension at 72 °C for 5 min.

Gel Electrophoresis and Visualization of PCR Products

Gel electrophoresis was used to analyze the PCR products using 1% agarose gel. The gel was prepared by dissolving 1 g agarose in 100 mL 1X TBE buffer and staining with safe dye for DNA visualization under UV light. A 50 bp DNA ladder was used as a molecular size standard.

In addition, electrophoresis was run at 100 V for 45 minutes. DNA bands were visualized under UV light. The specificity of the reaction was confirmed by no bands in the negative control band.

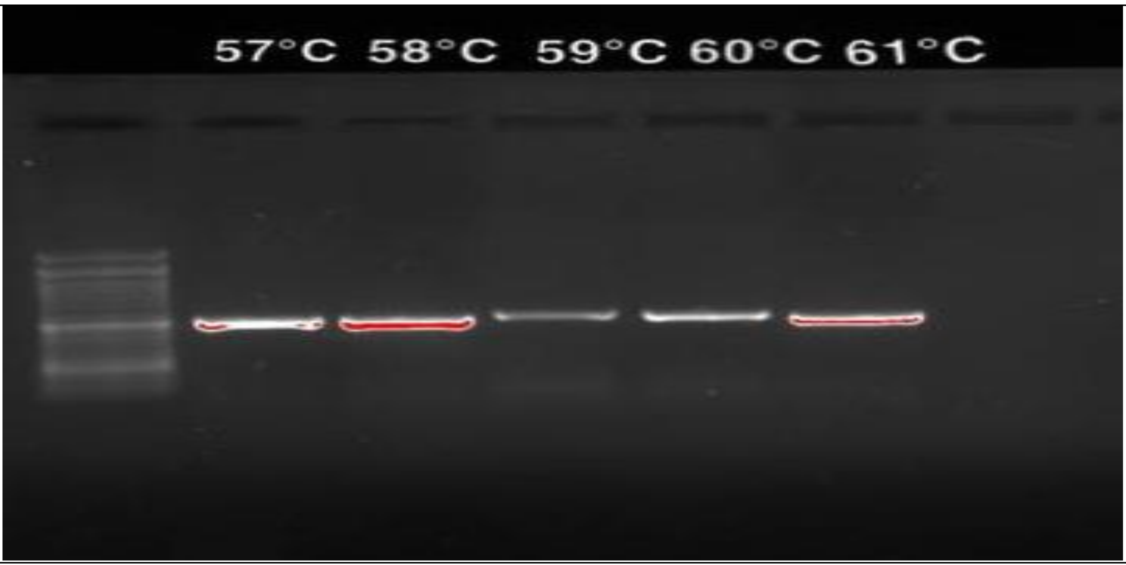

Figure 2.8. PCR temperature gradient used to find the best annealing temperature. 57°C was chosen as it gave a sharp, single PCR band without non-specific bands.

Results

Antibiotic Susceptibility Test (AST)

After 24 hours of incubation, all twenty *Pseudomonas aeruginosa* isolates were tested against five antibiotics using the disc diffusion method. Quantitative results are summarized in Table 2. All isolates (100%) were sensitive to ciprofloxacin (CIP) and levofloxacin (LEV), while sensitivity to meropenem (MEM) and gentamicin (CN) was 95% and 80%, respectively. Aztreonam (ATM) showed the lowest sensitivity at 45%, with the remaining 55% classified as intermediate. No isolates exhibited outright resistance to any of the tested antibiotics. Zone diameters varied modestly among isolates, ranging from 20–30 mm for ciprofloxacin and 18–28 mm for gentamicin, reflecting minor variability in susceptibility. These findings suggest strong overall antibiotic susceptibility in the sampled population; however, the limited sample size and single site collection may bias results and should be considered when interpreting resistance prevalence.

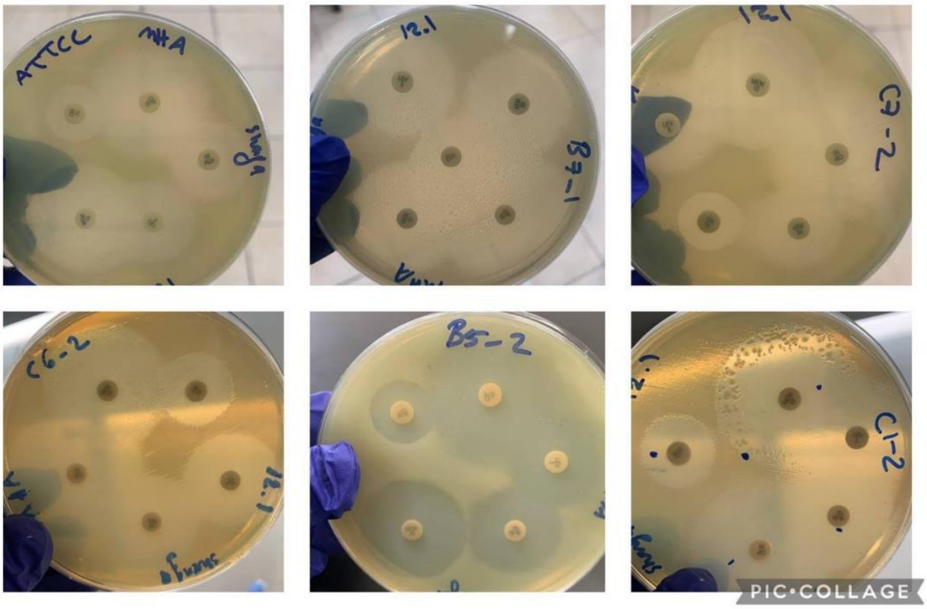

Figure 3.1. AST disk diffusion results for different antibiotic classes. Zone sizes reflect varying susceptibility patterns among some samples on Mueller Hinton agar plates.

Table 2: Antibiotic Sensitivity results

Antibiotic	Sensitive (%)	Intermediate (%)	Resistant (%)
ATM	45	55	0
CIP	100	0	0
CN	80	20	0
LEV	100	0	0
MEM	95	5	0

PCR Amplification of the *czcA* Gene

PCR analysis confirmed the presence of the ***czcA*** gene in all twenty isolates including the (ATCC® 9027™) control (sample 20), producing the expected 485 bp amplicon (Figure 3.2). The no-template control (NTC) remained negative, ruling out contamination. While the presence of *czcA* indicates genetic potential for resistance to cobalt, cadmium, and zinc, no expression analysis or functional assay was performed in this study to quantify efflux activity. Future work should examine gene expression levels under metal stress and perform functional assays to validate the effectiveness of the CzcCBA efflux system in metal removal.

Overall, these results demonstrate a strong correlation between *czcA* presence and potential metal resistance, alongside a generally high susceptibility to clinically relevant antibiotics, though additional sampling and molecular analyses are needed to confirm these trends.

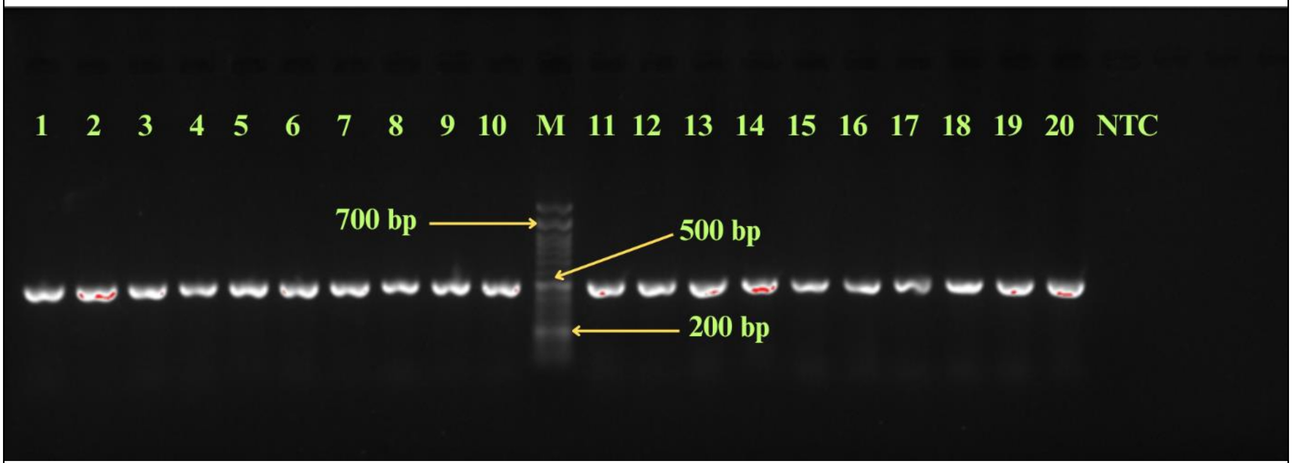

Figure 3.2. PCR products of ***the czcA* (485 bp)** from *P. aeruginosa* environmental isolates, separated on a 1% agarose gel in 1X TAE buffer. Safe DNA dye were used and visualized under UV light. A 50 bp DNA ladder was used as a molecular size marker.

Discussion

Findings from this study revealed that *Pseudomonas aeruginosa* recovered from Tanjaro River carried heavy metal resistance genes in particular, viz., czcA, but there was no regular association found in relation to multidrug resistance. The finding came in sharp contrast to earlier findings which most often showed strong links between heavy metal and antimicrobial resistance (Baker-Austin et al., 2006). However, recent studies provide an equally complex perspective, suggesting that interaction between both types of resistance is complex and mediated by diverse environmental and genetic forces.

Three core mechanisms responsible for concomitant heavy metal and antibiotic resistance were described by Murray et al. (2024) as follows: co-resistance, in which genes for both traits are found on the same mobile genetic element; cross-resistance, in which one mechanism, e.g., an efflux pump, provides resistance to both compounds; and co-regulation, in which heavy metal treatment activates genes of antibiotic resistance. The absence of common antibiotic resistance exhibited by *P. aeruginosa* isolates in this study suggests that, although present, it was not linked to determinants or regulatory systems of antibiotic resistance, limiting cross-resistance expression.

Environmental circumstances have an important role in defining these outcomes. Recent research shows that resistance gene expression often depends on local pollutant concentrations and co-stressor presence. For instance, interaction between heavy metals, microplastics, and other contaminants has been shown to affect resistance gene expression in aquatic ecosystems (Frontiers, 2025). Similarly, ASM (2024) reported that aqueous systems defined by high anthropogenic pollution levels will often have isolates showing marked co-resistance patterns, although there can be significant differences at different sites. The Tanjaro River, in spite of high pollution, can thus generate selective pressures leading to the retention of metal resistance, even without necessarily supporting co-selection for antibiotic resistance.

Local research provides more background to these findings. Alfarras (2022) described high resistance to heavy metals in Pseudomonas strains obtained from Iraqi sewage and sludges, citing extensive adaptation of this genus to polluted localities. At the same time, Alkhulaifi and Mohammed (2023) demonstrated increased levels of antibiotic resistance in both environmental and clinical strains of *P. aeruginosa* , citing possible separation of resistance profiles in clinical and environmental strains. The current research contributes to this collection of studies by demonstrating, in spite of genetic evidence for heavy metal resistance in environment strains of Tanjaro, their resistance to antibiotics does not reflect the high multidrug resistance characteristic of clinical cases.

Altogether, these findings demonstrate that cross-species correlation between heavy metals and antibiotic resistance in *P. aeruginosa* can not be made universally; rather, it depends on contextual elements, including gene location, regulating systems, and environmental parameters. This underlines the need to integrate localized ecological data and molecular assessments in order to holistically understand public health risks from antibiotic-resistant environmental bacteria.

Efflux systems like CzcCBA form the core of bacterial survival under heavy metal stress and offer promise in bioremediation. Future studies would be best spent in functional characterization of the czcA gene in varied environmental situations to bring forth an understanding of resistance regulation in natural ecosystems at the molecular level. On this foundation, genetic engineering strategies, including liposome-assisted systems through the paradigm of the AcrB-dR chimera (Kapoor & Wendell, 2013), can be assessed to boost *P. aeruginosa* or less toxic surrogate host organism potential to eliminate metals such as Cd^{2+}, Zn^{2+}, and Co^{2+} from poisoned water in an ecologically favorable process. These systems also offer potential for cost-effective recovery and reuse of antibiotics and other pollutants through vesicle solubilization. Equally important is the need to examine horizontal gene transfer, since resistance determinants linked to metals can also influence antibiotic resistance, posing clinical risks. Laboratory findings should then be validated under field-like conditions to test their effectiveness in complex microbial communities. Finally, an integrated risk–benefit assessment will be essential to ensure that strategies for enhancing metal efflux do not inadvertently accelerate the spread of antibiotic resistance.

Conclusion

In conclusion, the *czcA* gene was detected in all *Pseudomonas aeruginosa* isolates from the Tanjaro River, confirming their intrinsic ability to resist and potentially remove heavy metals such as cadmium, zinc, and cobalt. The isolates' sensitivity to antibiotics is a positive finding, indicating that any infections arising from environmental exposure could still be managed effectively.

However, this study has limitations, including a relatively small sample size, focus on only three heavy metals, and absence of functional assays to quantify metal removal efficiency. Expanding sampling across additional sites and metals, combined with molecular and biophysical analyses, would strengthen understanding of resistance mechanisms and bioremediation potential.

Practically, these findings support the potential use of local *P. aeruginosa* strains in controlled bioremediation strategies, such as bioaugmentation of contaminated water or development of liposome-based efflux systems, to reduce heavy metal loads in the environment. Policy interventions are also warranted: stricter regulations on industrial and hospital effluents, routine monitoring of irrigation water, and integration of environmental surveillance data with public health strategies could mitigate both heavy metal contamination and the spread of resistant bacteria. Finally, comparative studies of environmental and clinical *P. aeruginosa* isolates would help trace contamination sources and inform targeted mitigation efforts. This integrated approach combines scientific, regulatory, and public health perspectives, providing a framework for addressing heavy metal pollution while safeguarding community health.

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