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Detection of some Biological Parameters and Heavy Metals Presence in Tigris River Water by Novel Designed Bacterial Efflux Pump Genes

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Abstract

Enterobacteriaceae are Gram-negative bacteria that have many mechanisms to survive in contaminated environments. *Klebsiella pneumoniae* is one of them that express efflux pump genes in the presence of heavy metals, like *AcrAb* and *OqxA* genes. The purpose of this study is to investigate the relationship between the existence of efflux pump genes in *K. pneumoniae*, and heavy metal contamination in the Tigris River. By comparing important indicators to World Health Organization (WHO) criteria, this study aims to analyze the river's water quality. It focuses on detection of cadmium (Cd), lead (Pb), nickel (Ni), arsenic (As), mercury (Hg), iron (Fe), and cobalt (Co). Fifteen samples of the Tigris River near the Al-Dura project of water remediation were obtained and cultured on selective media, and the water quality parameters, like TSS, TDS, TH, pH, turbidity, EC, BOD, COD, OD, TOC, and heavy metals, were estimated. Bacterial DNA was extracted, then efflux genes were detected with the presence of heavy metals in the tested samples. Results illustrated that *K. pneumoniae* gave positive results in nine of the total fifteen samples, and the water quality parameters were within the normal ranges, except for elevated levels of DO and TOC, and decreased levels of BOD and COD, with a significant relationship among heavy metal levels with both AcrAB and OqxA genes at a significance level of $p \leq 0.05$.

1. Introduction

Heavy metals resistance is common in Enterobacteriaceae due to the multidrug resistance (MDR) of antibiotics and toxic substances via horizontal gene transfer (HGT) of plasmids [1]. *Klebsiella pneumoniae*, as a member of the coliform family, could be found in the soil, river sediments, waste water, and river water [2]. The efflux pump mechanism is one of the most potent ways to eliminate the effects of toxic materials or metals in the surrounding environment [3]. MDR and heavy metal resistance genes might share the same homology in sequences of the bacterial genome, which resembles the RND efflux family in coliforms to whom *AcrAB* and *OqxA* genes belong [4]. Recently, heavy metals were studied by many researchers as indicators of a multi-metal-contaminated environment, or they could be useful for remediation [5]. However, few studies have investigated correlations between water quality standards and heavy metals in river water, besides the estimation of genetic variations of aquatic microorganisms in lakes or rivers to minimize the overall costs and time of water quality approaches [6]. The purpose of this work is to determine whether the Tigris River and Enterobacteriaceae, especially *Klebsiella pneumoniae*, have efflux pump genes. By comparing important indicators to World Health Organization (WHO) criteria, this study aims to analyze the river's water quality. It focuses on heavy metal detection, specifically looking for lead, nickel, arsenic, mercury, iron, and cobalt. Furthermore, by identifying and analyzing the *AcrAb* and *OqxA* efflux pump genes in *K. pneumoniae* isolates, the study intends to investigate the genetic basis of heavy metal resistance in bacterial populations. The major objective is to improve knowledge of how pollution affects microbial resistance patterns in the environment and to aid in the creation of more efficient methods for treating water and reducing pollution in river ecosystems.

2. Experimental Procedure

2.1. Collection of Samples and water Quality Assessment

Fifteen samples of Tigris river water (from the Al-Dura project of water remediation) were collected in sterile containers to evaluate water quality parameters, which were: Total soluble substances (TSS), total dissolved substances (TDS), total hardness (TH), concentration of hydrogen ion (pH), turbidity, electrical conductivity (EC), biological oxygen demand (BOD), chemical oxygen demand (COD), oxygen demand (OD), and total organic carbon (TOC) were all detected by standard laboratory techniques like the titrimetric method or digital meters for each parameter. Some heavy metals (Cadmium (Cd), Lead (Pb), Nickel (Ni), Arsenic (As), Mercury (Hg), Iron (Fe), and Cobalt (Co)) were measured by an atomic absorption flame emission spectrophotometer apparatus, model AA-6200 (Shimaduz/Japan) by using special standards for each of them, then their values were compared with the normal ranges of the World Health Organization (WHO) for water quality parameters [7].

2.2. Cultures of Water Samples

All water samples were aseptically collected and cultured on selective media (MacConkey and blood agar) as aseptically as possible, and the positive cultures were confirmed by morphological and biochemical tests of 64 biochemical tests of the VITEK-2 system (Biomereux/France). *K. pneumoniae* isolates were selected because they own RND efflux pump genes that are specific to pump heavy metals like lead and silver out of the bacterial cells.

2.3. Genetic Studies and DNA Sequencing

Genomic DNA of *K. pneumoniae* was extracted by the Wizard Kit (Promega, USA), and the PCR Master Mix Kit (Promega, USA) was also used for amplifying the approach of selected genes. The sequences of the primers regarding both the *AcrAb* gene and the *OqxA* gene were obtained and tested in many references [8]. As mentioned in Table (1), the forward and reverse primers of each gene with the optimal conditions and precise gene sizes are clarified according to optimization approach in the laboratory until obtaining PCR amplicons of each genes [9]. The agarose electrophoresis technique at 1% concentration of agarose was applied by using 100 volts for 45 minutes for the *AcrAb* gene and 30 minutes for the *OqxA* gene, regarding the demanding time to settle at their specific bands of DNA amplified fragments. The DNA sequence of positive PCR mixtures for both efflux genes was performed by Microgen Company/South Korea by the Sanger method [10]. Alignment was done by comparison with standard strains of the NCBI database using the pairwise alignment tool (BLAST).

2.4. Statistical Analysis

The least significant difference (LSD) ($p \leq 0.05$) was used to detect differences among water samples in their content of heavy metals. Chi square was used to find the correlation between each efflux pump distribution and heavy metal samples in positive culture media of *K. pneumoniae* only.

Table (1): Optimal conditions for studied efflux pump genes (optimization approach).

Efflux gene	**Sequence of the gene**	**Annealing temperature (°C)**	**Duration of electrophores is (min.) at 100 volt**	**Size of the gene (bp)**
AcrAb	F: 5'ATCAGCGGCCGGATTGGTAAA3' R: 5'CGGGTTCGGGAAAATAGCGCG3'	58.5	45	312
OqxA	F: 5' GGCAACAGCCAAAACGCAGG3' R: 5' GGGGCGGTCACTTTGGTGAA3'	62.5	30	200

3. Results and Discussion

The water quality in all samples was within acceptable limits for both TSS, TDS, TH, turbidity, EC, and pH. Biological oxygen demand (BOD) was higher than the requirements of microorganisms in river samples, accompanied by low dissolved oxygen (DO) level in water and a low carbon (COD) level much lower than the required levels in river water, like in Table (2). The obtained results are similar to those of [10] and [11]. The quality of water is very important for all living organisms for their growth and respiration systems [12]. Both CO_2 and O_2 were lower than the required limits in the studied water samples, which might be a sign to pay more attention to the biological demands of dissolved gases that are essential to living aquatic organisms [13].

Table (2): Water quality parameters with the accepted WHO limits.

Parameter	**Value and unit (mg/ L)**	**Method**	**WHO accepted parameters**
pH	7.6	pH meter	7
TSS	600	Standard gravimetric method	Below 1000
TDS	319.8		Below 1000
EC	533	Digital conductivity meter	200-600
Turbidity	15	Turbidity meter	Below 25
TH	320	Standard titrimetric method	Below 500
COD	**90**	Standard titrimetric method	Above 150
BOD	8.8	Closed bottle test	Below 3
DO	**3.4**	Digital D.O meter	Above 6
TOC	**11**	TIC-NPOC analysis	Below 0.5

In the current study, there were markedly high values of TOC. The TOC level might be a good detector of carcinogenetic substances and total carbon amounts in water and aqueous systems [14]. It is also considered in water quality standards in relation to both COD and BOD for the assessment of pollution and safety of water for specific organizations and needs [15]. Our result regarding TOC is close to those of [16] and [17]. TOC was referring to the sources of carbon based on water contamination due to unconventional fuel usage, anthropological habits, and improper ventilation in surrounding areas [18].

Heavy metals were higher than the normal range of river-contaminated levels for Cd, Pb, As, and Fe, which recorded higher levels than acceptable. The Hg element was absent in all collected water samples, while Ni and Co levels were both within the acceptable WHO values as shown in Table (3). These results are close to the findings of [19] and [20] regarding river water samples.

Table (3): Heavy metal levels in tested river water with accepter WHO limits regarding efflux genes.

Heavy metal	Value by ppm	Method	WHO parameters (ppm)
Cadmium (Cd)	**0.00745**	Atomic absorption flame emission spectrophotometer apparatus	0.003
Lead (Pb)	**0.08**		0.01
Nickel (Ni)	2.0		2.1
Arsenic (As)	**3.5**		0.05
Mercury (Hg)	Nil		0.06
Iron (Fe)	**0.7**		0.3
Cobalt (Co)	0.05		0.065

It was recorded that the same water quality parameters as in the current study as the high levels of such heavy metals might affect the food chain, health, and fish in such ecosystems [21]. There was a significant difference in levels of certain metals compared to the others in the study [22]. This might reflect the effect of the surrounding environment and temperature on the heavy metal content in contaminated fresh water [23]. It was also mentioned that bioaccumulation and water consumption take place in the human body. The relationship between bad water quality and the elimination of some river organisms and the contamination of water by heavy metals might be as a result of human uncontrolled activities near aquatic ecosystems [24]. *K. pneumonia* was isolated from nine river samples, and the positive cultures were assured by the VITEK-2 system after giving pink mucoid, viscous colonies on MacConkey agar with no blood hemolysis on blood agar. This result is similar to [25] and [26], as *K. pneumoniae* has many mechanisms to tolerate heavy metals at high levels, which implies RND efflux gene expression, and their presence is very critical as metalo-tolerant organisms with such metal resistance genes [27].

Efflux genes were detected in PCR mixtures as five positive results for the *AcrAb* gene and four positive results for the *OqxA* gene. The isolates that owned both studied efflux genes were three of nine PCR-positive mixtures. The following figures resemble the presence of agarose positive bands of two studied efflux genes with pairwise identity with standard strains of (LR025-92) for *AcrAb* and (CP047700) as in Figure (1) and *OqxA* as in Figure (2) for their sequence similarity, which reflects the homology with high drug- and heavy metal-resistant *K. pneumoniae*, representing a pathway of community-acquired infections and transmission of the resistance bacterial genes through environmental and aquatic reservoirs [28].

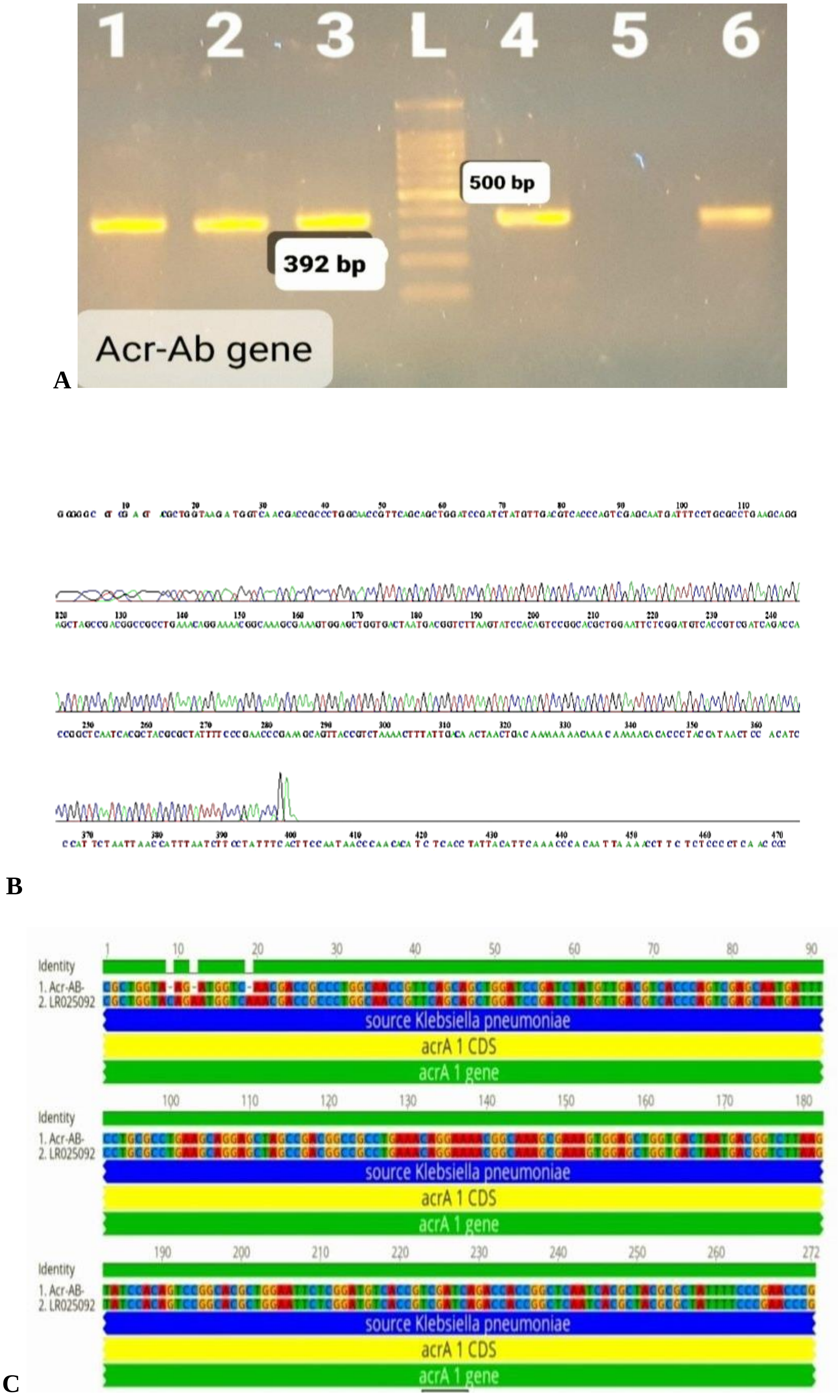
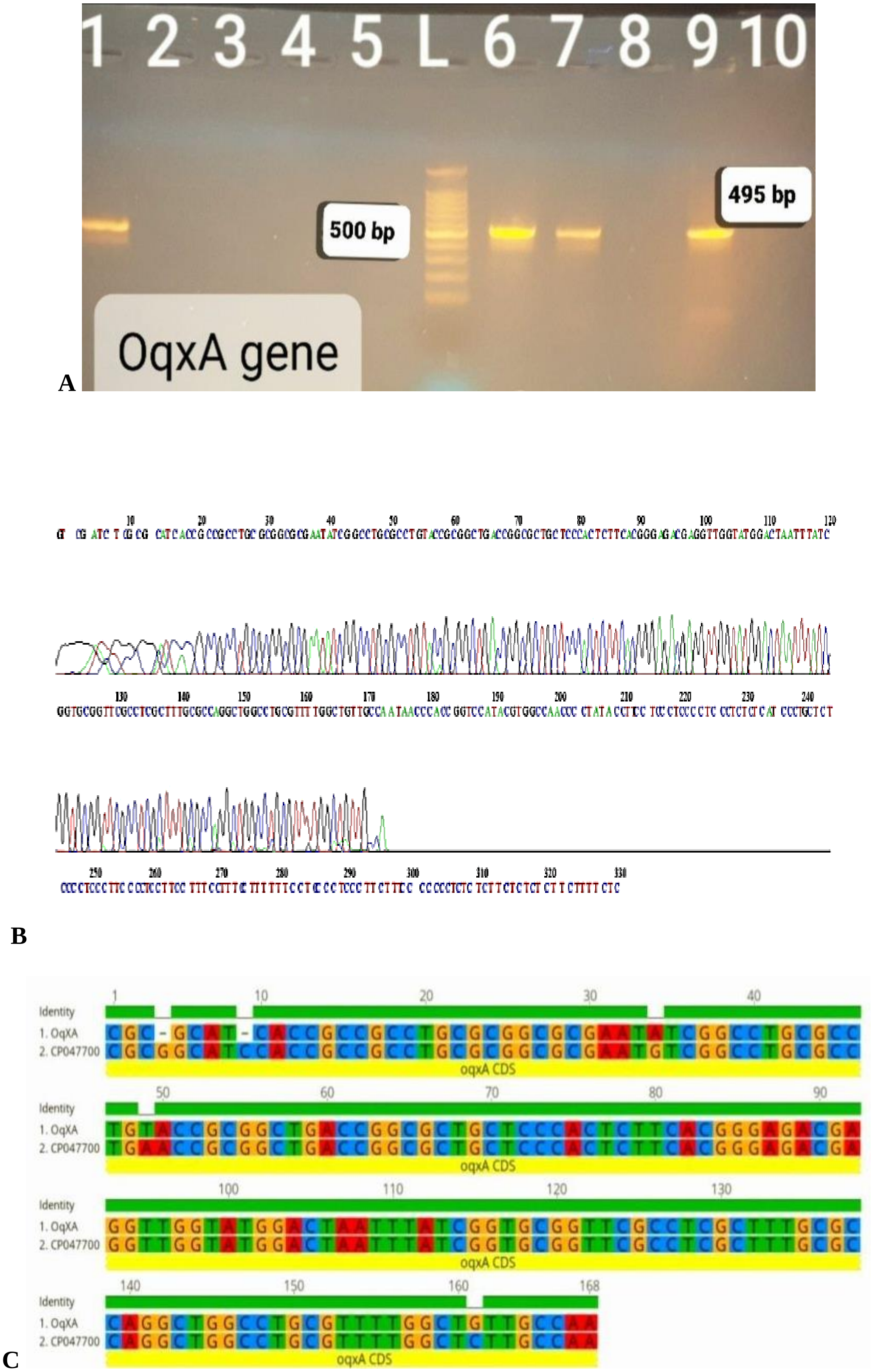


Figure (1): Gel electrophoresis bands of *AcrAb* genes at 392 bp. B: sequencing of *AcrAb* amplicons. C: Pairwise alignment of the Sanger results of the *AcrAb* sequence.



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         1        10        20        30        40
1. OqXA  CGC-GCAT-CACCGCCGCCTGCGCGGCGCGAATATCGGCCTGCGCC
2. CP047700 CGCGGCATCCACCGCCGCCTGCGCGGCGCGAATGTCGGCCTGCGCC

           50        60        70        80        90
1. OqXA  TGTACCGCGGCTGACCGGCGCTGCTCCCACTCTTCACGGGAGACGA
2. CP047700 TGAACCGCGGCTGACCGGCGCTGCTCCCACTCTTCACGGGAGACGA

             100       110       120       130
1. OqXA  GGTTGGTATGGACTAATTTATCGGTGCGGTTCGCCTCGCTTTGCGC
2. CP047700 GGTTGGTATGGACTAATTTATCGGTGCGGTTCGCCTCGCTTTGCGC

          140       150       160     168
1. OqXA  CAGGCTGGCCTGCGTTTTGGCTGTTGCCAA
2. CP047700 CAGGCTGGCCTGCGTTTTGGCTCTTGCCAA
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Figure (2): Gel electrophoresis bands of *OqxA* genes at 392 bp. B: sequencing of *OqxA* amplicons. C: Pairwise alignment of the Sanger results of the *AcrAb* sequence.

The differences in heavy metal concentrations in these nine river samples were calculated by the LSD test, and their correlation with the presence of RND efflux genes was estimated by the Chi-square correlation test, which showed that the LSD among water river samples of measured heavy metals was 0.084 at significant levels of ($p \leq 0.05$), while the chi-square indicated a significant correlation of both *AcrAb* and *OqxA* genes as they were 0.003 and 0.021, respectively, at ($p \leq 0.05$).

Many studies have documented the positive results of efflux pump genes in *Enterobacteriaceae* with heavy metal tolerance in aquatic systems [29] and [30]. There is a strong relationship between the expression of these pump genes and elevated levels of heavy metals in the studies of [31] and [32] due to the selective pressure of heavy metals in river water, just similar to our results, while there was no relatedness among RND efflux pump genes of *K. pneumoniae* tolerance to heavy metals in other studies like [33] and [34]. This might be related to the seasonal changes and climate in such origins, besides their locations in distance to factories, electricity generators, or treatment stations near rivers [35, 36].

4. Conclusions

Although most measures fall within permissible ranges, this study on the water quality of the Tigris River reveals significant heavy metal contamination (Cd, Pb, As, and Fe) exceeding WHO criteria. Heavy metal resistance was indicated by the presence of the efflux pump genes *AcrAb* and *OqxA* in *K. pneumoniae* isolates from the water, as revealed by analysis. These genes' statistical associations with higher heavy metal concentrations demonstrate how the environment affects microbial resistance patterns. The results highlight the necessity of ongoing surveillance and cutting edge investigation into the relationship between heavy metal contamination and bacterial genes. This is essential for creating efficient water treatment plans and guaranteeing the safety of the environment and public health in riverine areas affected by human and industrial activity.

Conflict of Interest: The authors declare that there are no conflicts of interest associated with this research project. We have no financial or personal relationships that could potentially bias our work or influence the interpretation of the results.

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