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Identification of *Escherichia Coli* and Heavy Metal Resistant Genes (*czc*A, *czc*D and *mer*A) in Cd and Pb Contaminated Surface Water from District Jhal Magsi, Balochistan

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Abstract

This study aimed to detect the concentrations of heavy metals and heavy metal resistance genes (HMRGs) in *Escherichia coli* (*E. coli*) isolated from running and standing surface drinking water of Jhal Magsi district, Balochistan. Heavy metal concentrations were determined using a flame atomic absorption spectrophotometer and identification of *E.coli* and heavy metal resistant genes were performed by Polymerase Chain Reaction (PCR). The concentrations of Cd and Pb ranged from 0.058±0.001 to 0.98±0.0003 mg/L and 1.01±0.0011 to 2.03±0.01155 mg/L, respectively in running water and in standing water it ranged from 0.098±0.001 to 0.23±0.0003 and 1.11±0.0011 to 2.97±0.0011 mg/L, respectively. The results showed that the concentration of Cd and Pb exceeded WHO and EPA safe limits. Out of 60 water samples, 39 positive samples of *E.coli* were confirmed through cultural, microscopic, biochemical and molecular techniques. All *E. coli* isolates were examined for the heavy metal-resistant genes and percentages of *czc*A, *czc*D, and *mer*A were recorded at 21/39 (32.81%), 23/39 (35.9%), and 20/39 (31.25%), respectively. It has been concluded that surface water is more contaminated with Cd and Pb as compared to groundwater, which played an important role in the development of HMRG in *E. coli*.

Keywords: Water contamination**,** Lead, Cadmium, *E. coli,* PCR, Heavy metals resistant genes

Introduction

Pakistan is a developing country in Southeast Asia, and drinking polluted water is a common issue due to a lack of drinking water treatment facilities and mismanagement of those that exist [1]. Area-wise Balochistan is the largest province of Pakistan and around 50-55% areas are irrigated from surface water and the remaining 45-50% are irrigated from groundwater [2]. Jhal Magsi district is one of the important districts of Balochistan province because of water reservoirs, minerals, and mines. The district's main surface water sources include rivers (Mula, Sukleji), canals, tube wells, and streams.

Water-related illnesses are the leading cause of human sickness in underdeveloped countries, due to unsanitary living conditions. The intake of untreated and polluted water exposes individuals to significant disorders such as hemorrhagic colitis, hemolytic uremic syndrome, and thrombotic thrombocytopenic purpura. Streams and rivers are contaminated as a result of contaminants from industry

mingling with sewage discharge. These contaminants may enter into lakes or reservoirs that contain potable water [3]. Heavy metals and microorganisms are two of the most important inorganic and biological pollutants in water (rivers, steam, and underground water) because they are non-degradable and frequently accumulate at the tropic level, creating a negative biological effect [4]. Heavy metals are found in water in trace amounts but are more hazardous to the human body. This study was necessary to examine the issue and propose solutions to reduce the danger of harmful heavy metals contaminating drinking water, especially in light of the hazardous nature of heavy metals pollution in water. Cd is exceedingly poisonous even at low doses, and it bio-accumulates in ecosystems and organisms, with a biological half-life ranging from 10 to 33 years in the human body. Long-term exposure to Cd and Pb causes kidney disease. As a result, Cd is regarded as a priority contaminant in most countries and international organizations [5].

Pb has the potential to replace calcium in bone, forming sites for long-term replacements. The central nervous system is the primary target organ for Pb damage in humans, and the developing brain is more sensitive to lead's neurotoxic effects than the adult brain [6,7]. Furthermore, the International Agency for Research on Cancer (IARC) has categorized Pb as probably carcinogenic to humans (Group 2A), with the suspected target organs being the lungs and stomach [8].

The kidney absorbs the maximum amount of Pb in the human body, followed by the liver and other soft tissues such as the heart and brain [9]. Pb poisoning is particularly dangerous to the neurological system. Early indications of Pb exposure on the central nervous system include headache, poor focus, spam, irritability, memory loss, and dullness [10,11].

The pathogenic bacteria *E. coli* can cause serious gastrointestinal disease in humans. This pathogen can be spread by a variety of means, including water and food. The presence of *E. coli* in aquatic habitats implies that the environment or organisms have been contaminated by fasces of animal or human origin [12,13]. It is also known to possess multi-resistance genes such as disinfection resistance, heavy metal resistance, and antibiotic resistance [14,15]. The purpose of this study was to investigate the pollution status of surface water by Cd and Pb, as well as the identification of respective resistance genes *czc*A, czcD, and *mer*A by molecular techniques, and the resistance levels of isolated *E.coli* from drinking water of district Jhal Magsi, Balochistan.

Materials and Methods

Chemicals

The materials used included hydrochloric acid (HCl) and Nitric acid (HNO_3) (Merck 100443), Luria Bertani (LB broth MILLER 110825), Eosin-methylene blue (EMB) and MacConkey (Merck 2.05284.05), API 20E (BioMerieux), PCR master mix (2X), primers (Lab Gentics), Agarose Jell (Thermo Fisher), DNA (Cat. No. 51304) and plasmids QIAprep Mini Kit (Cat. No. 12123).

Samples Collection

Water samples were collected from different areas of district Jhal Magsi (Latitude 28.3688° or 28° 22' 8" north and Longitude 67.543° or 67° 32' 35" east) Fig.1. The primary water reservoirs of the district are the Mula and Sukleji Rivers, as well as several minor canals and lakes that supply water to the whole district. To avoid sample contamination, polythene bottles were used for the collection of water samples.

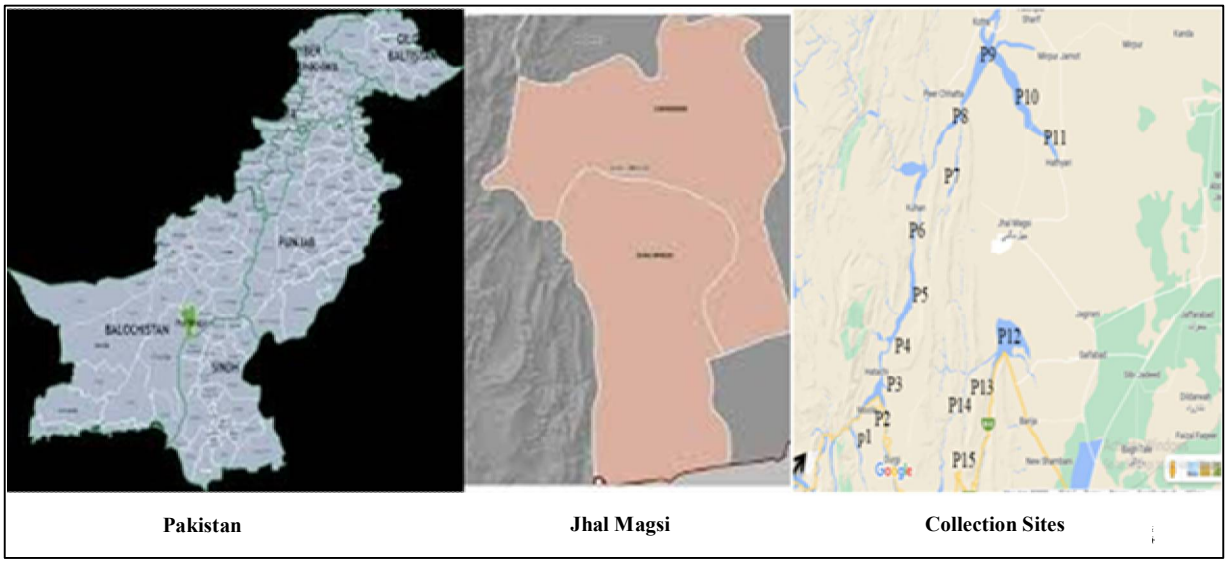

Figure 1. **Location map of the study area in district Jhal Magsi with Sampling Sites (P 1-15)**

Digestion of Water

Water samples were digested for heavy metals, following the Fong SS method [16,17]. Briefly, 5 mL of concentrated hydrochloric acid (HCl) and 10 mL of Nitric acid HNO_3 were added to the water samples. Samples were incubated for one hour at room temperature. Later sample suspension was heated on a hot plate until samples turned clear. After cooling samples were filtered and deionized water was added up to a volume of 100ml.Heavy metal detection was performed using atomic absorption spectrophotometer (AAS) Perkin Elmer AAnalyst 800. After starting the AAS blank solution (deionized water) was run first, and then a triplicate of standard solutions was used to obtain the calibration curves. After that, the stock solution (digested samples) was run and results were taken [18].

Microbial Analysis

All water samples were used for bacterial culture and isolation. 5 mL water was taken into falcon tubes and diluted with Luria Bertani (LB broth) (Merck), and incubated for 18-24 hours at 37 °C. Initial growth was performed on nutrient agar, which was followed by selective culture media Eosin-methylene blue (EMB) and MacConkey Agar for isolation of *E. coli.* Afterward, the bacteria were identified through Gram stain and biochemical tests [19, 20]. Microscopic examination was done by gram staining. The biochemical tests for the identification of *E. coli* were performed by using the API 20E strips.

Molecular Identification

For genomic identification, DNA was extracted through the QIAamp DNA mini kit and plasmids were extracted through the QIAprep Mini Kit (Qiagen) according to their given protocol and instructions.

The molecular base identification was performed by PCR. For amplification specific primers *Uid*A, *czc*A, *czc*D, and *mer*A were used for molecular identification of *E. coli* and metal-resistant genes respectively (Table 1). It was done in 20 µL reaction mixture, prepared by using 10 µL PCR master mix (2X), forward primer 1 µL, reverse primer 1 µL, DNA sample 3 µL and PCR grade water 5 µL.

Table 1. **Primers used in identification of *E.coli* and heavy metal resistant genes.**

Primers	Sequence(5`-3`)	Target length (bp)	Annealing Temp. (°C)	References
*Uid*A	FCCAAAAGCCAGACACGAGT RGCACAGCACATCAAAGAG	623	55	[21]
*czc*A	FGTTCACCTTGCTCTTCGCCATGTT RACAGGTTGCGGATGAAGGAGATCA	320	58	[22, 23]
czcD	FTTTAGATCTTTTACCACCATGGGCGCAGGTCACTCACACGACC RTTTCAGCTGAACATCATACCCTAGTTTCCTCTGCAGCAAGCGACTTC	1000	60	[24]
*mer*A	FGAGATCTAAAGCACGCTAAGGC RGGAATCTTGACTGTGATCGGG	1011	57	[24]

Amplifications were carried out with initial denaturation at 94 °C for 5 min and a final extension at 72 °C for 7 min for *E.coli* 30 cycles (94 °C for 30 s, 55 °C for 30 s, and 72 °C for 30 s) and for heavy metal resistant genes 35 cycles (94 °C for 35 s, annealing temperature for each gene were given in (Table 1) for 30 s each and 72 °C for 30 s) in an Eppendorf Master Cycler (Scilogex TC1000-S Thermal Cycler). Amplified icons were detected by gel electrophoresis on a 1.5% agarose gel and stained with ethidium bromide.

Results and Discussion

Heavy Metals

The concentrations of Cd in running surface water ranged from 0.058±0.001 to 0.98±0.0003 mg/L, and Cd in standing surface water ranged from 0.098±0.001 to 0.23±0.0003 mg/L. Pb was in the range from 1.01±0.0011 to 2.03±0.01155 mg/L in running surface water and Pb in standing water ranged from 1.11±0.0011 to 2.97±0.0011 mg/L. The results showed that the concentration of Cd and Pb in both running and standing surface water in all collected water samples exceeded EPA and WHO safe limits. As compared to running water, the samples of standing water were determined with higher concentrations of Cd and Pb (Table 2).

Table 2. **Concentration of heavy metals in surface water.**

Sites	Running surface water		Standing surface water	
	Cd (mg/L)	*Pb (mg/L)*	*Cd (mg/L)*	*Pb (mg/L)*
Sample 1	0.09± 0.0005	1.72.± 0.0005	0.19± 0.0005	2.22.± 0.0005
Sample 2	0.105± 0.001	1.64± 0.0011	0.17± 0.001	2.21± 0.0011
Sample 3	0.104± 0.001	1.97± 0.0011	0.11± 0.001	2.97± 0.0011
Sample 4	0.11± 0.0005	2.03± 0.01155	0.12± 0.0005	2.32± 0.01155
Sample 5	0.098± 0.001	1.98± 0.0003	0.18± 0.001	2.01± 0.0003
Sample 6	0.14± 0.001	1.878± 0.0011	0.16± 0.001	1.81± 0.0011
Sample 7	0.103± 0.001	1.988± 0.0011	0.21± 0.001	2.19± 0.0011
Sample 8	0.18± 0.0005	1.781± 0.0011	0.201± 0.0005	1.99± 0.0011
Sample 9	0.102± 0.001	1.88± 0.0006	0.22± 0.001	2.01± 0.0006
Sample 10	0.102± 0.0003	1.961± 0.0011	0.23± 0.0003	2.01± 0.0011
Sample 11	0.11± 0.0001	1.71± 0.0011	0.098± 0.001	1.80± 0.0001
Sample 12	0.088± 0.0011	1.29± 0.0006	0.101± 0.0011	1.98± 0.0003
Sample 13	0.107± 0.0011	1.01± 0.0011	0.21± 0.0003	1.21± 0.0011
Sample 14	0.98± 0.0003	1.31± 0.0011	0.201± 0.0011	2.01± 0.0011
Sample 15	0.16± 0.00011	1.06± 0.0011	0.17± 0.0011	1.11± 0.0011
EPA limits	0.01	<0.05	0.01	<0.05
WHO limits	0.003	0.01	0.003	0.01

*Concentration of metals (Mean±SD milligram per litre (mg/L) in running and standing water

In the current study 60 water samples were collected from different areas of district Jhal Magsi, Balochistan, each sampling site located at the distance of 40-50 km. Pesticides, herbicides, and other types of

chemical fertilizers increase heavy metal concentrations. As a result, when they are used in agricultural areas near aquatic environments, rainfall tends to drain their harmful elements into surface waterways, resulting in water pollution. Heavy metals above permitted levels sometimes have negative consequences for humans, other creatures, and the environment that ingest metal-polluted water [25]. Heavy metal acceptable limits in water and food samples are associated with negligible health concerns in humans [26]. In the current study, concentrations of Cd and Pb were examined in the surface water of district Jhal Magsi. Mustafa et al. [16] worked on the surface and groundwater of district Jhal Magsi and resulted in all water samples Cd and Pb being found high in concentration. In the current study, both metal Cd and Pb were found in higher concentrations than the safe limits recommended by WHO and EPA.

Abou-Shanab and Van Berkum, [27] researched on drinking water of the Indus River and northern areas of Pakistan, their result lined with the current study and found that Pb and Cd were reported in higher concentrations. Rashid, et al. [28] investigated the concentrations of Pb, Cd, Mn, and Fe in drinking water sources in primary schools of Sindh Province to quantify potential health risks among school children. The concentration of heavy metals in the drinking water exceeded the WHO permissible limits (67% of schools exceeded the Pb limit and 17% for Cd).

Heavy metals' toxicity in humans is determined by their dose, rate of emission, and duration of exposure. Heavy metals such as Hg, Cd, and Pb have received more attention in recent decades [29]. Several regulatory authorities classify Cd compounds as carcinogens for humans [30]. The International Agency for Research on Cancer (IARC) and the United States National Toxicology Program have decided that there is sufficient evidence that Cd is a carcinogen for humans [31]. This classification as a human carcinogen is based mostly on repeated observations of a link between occupational Cd exposure and lung cancer, as well as extremely strong rodent studies indicating the respiratory system as a target site [32].

Microbial identification

On MacConkey medium, *E. coli* bacteria were identified as a moderate colony with red brick colour, smooth and convex shape. The capacity of *E. coli* to digest lactose resulted in a reduction in pH, which increased neutral red absorption, making it easier to convert the colony into a red brick. *E. coli* were also identified on EMB agar media. Colonies grow with a metallic sheen with a dark center (Fig. 2). This is because Lactose-fermenting gram-negative bacteria acidify the medium, which reduces the pH, and the dye produces a dark purple complex usually associated with a green metallic sheen.

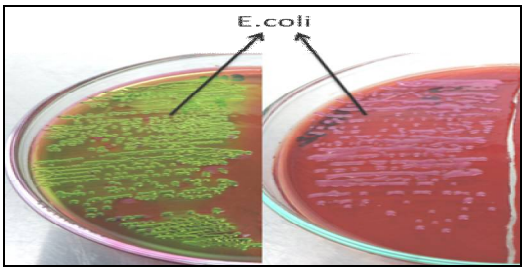

Figure 2. **Culture of *E.coli* on EMB and MacConkey agar**

Gram-negative *E.coli* was observed pink under a microscope. *E. coli* is a Gram-negative bacterial species (Fig. 3).

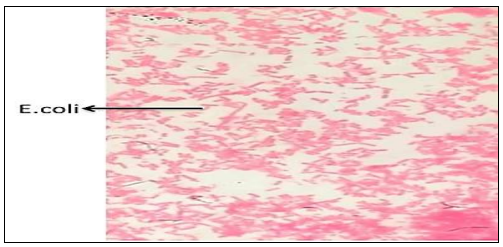

Figure 3. **Gram negative, pink colored, small rod shape *Escherichia coli* under a microscope**

Table 3. **API 20 E code result for the identification of *Escherichia coli.***

ONPG	ADH	LDC	ODC	CIT	H2S	URE	TDA	IND	VP	GEL	GLU	MAN	INO	SOR	RHA	SAC	MEL	AMY	ARA
+	−	+	+	−	−	−	−	+	−	−	+	+	−	+	+	−	+	−	+

API E20 strip was used for biochemical profiling. API 20 E strip is made up of 20 microtubes that contain dehydrated substrates. These microtubes were inoculated with a bacterial suspension made in API 20 E Medium, which only fills the tube section of the microtubes and does not fill the cupules. We filled the cupules with mineral oil to make the ADH and URE test anaerobic. Close the incubation box and incubate for 18-24 hours at 36 °C.

After the incubation time, added 1 drop of VP1 and VP2 reagents for the VP test, NIT1 and NIT2 reagents for the NIT test, and ZYM A and ZYM B reagents for the PAL test. To obtain the API 20 E Reading Scale, marked each test as positive or negative (Fig. 4) on the tray lid (color chart). The code 5144552 was confirmed through the Analytical Profile Index (Table 3) [33].

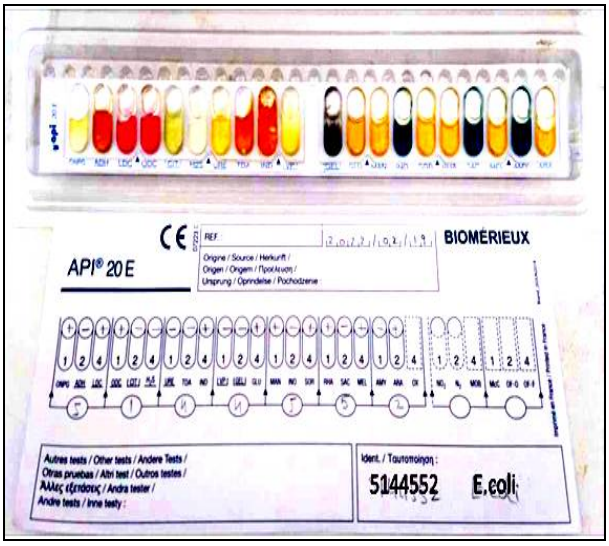

Figure 4. **Identification of *Escherichia coli* from API 20 E step**

Molecular identification of E.coli and HMRGs

Out of 60 water samples, in 39 samples *E.coli* were confirmed by *uid*A gene at 623bp through PCR. Results showed 65% (39/60) were positive for the target bacteria (Fig. 5).

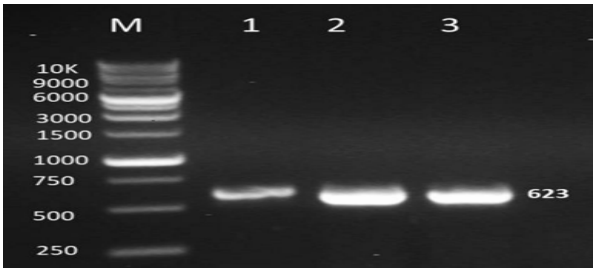

Figure 5. **Molecular identification of *Escherichia coli***

The amplified product (*uid*A gene) at 623bp was separated by gel electrophoresis on Agarose gel. DNA visualization was done by a gel documentation system. M: Ladder, 1-3: Positive sample for *Escherichia coli.*

Odonkor and Addo [34] Identified *E. coli* from different drinking water samples. Bonyadian et al. [35] cultured different bacteria (*E. coli*, *Salmonella* and *Vibrio cholera*) and detected them through a polymerase chain reaction, in tap water and bottled drinking water in Isfahan province, Iran. Osińska et al. [21] used the *uid*A gene primer for genetic characterization of *E.coli* from water samples as used same gene primer in the current study.

Heavy metal resistance genes (HMRG) were determined from *E.coli*. Previously published primers were applied for the amplification of various metal-resistant genes [36]. PCR results determined for Cd (*czc*A*)*,

(*czc*D), and Pb translocating ATPase (*mer*A) genes amplified at 320, 1000, and 1011bp (Fig.6-8), respectively.

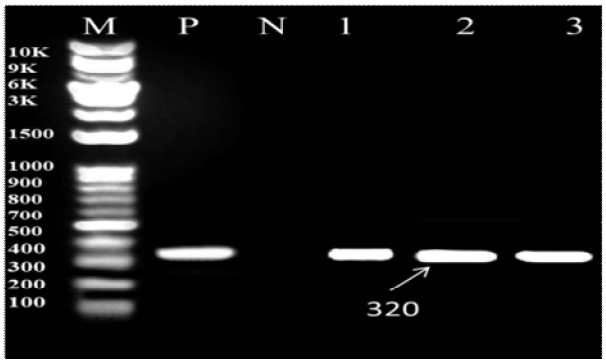

Figure 6. **PCR for *czc*A gene from *E. coli* isolates**

Heavy metal resistance Gene *czc*A (320bp) was amplified using specific gene primers from *Escherichia coli*. A size marker (DNA size marker 10k plus). M: ladder, P: positive control, N: Negative Control, 1-3: Positive sample for *czc*A gene.

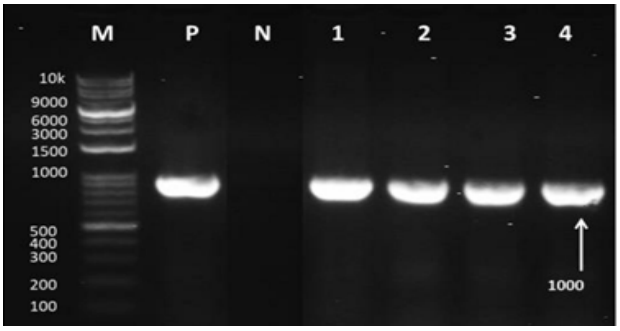

Figure 7. **PCR for*czc*D from *Escherichia coli* isolates**

Heavy metal resistance Gene *czc*D (1000bp) was amplified using specific gene primers from *Escherichia coli*. A size marker (DNA size marker 10k plus). M: ladder, P: positive control, N: Negative Control, 1-4: Positive sample for *czc*D metal resistant gene.

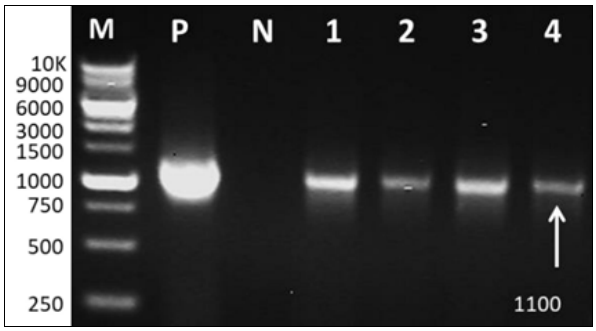

Figure 8. **PCR for *mer*A from *Escherichia coli* isolates**

Heavy metal resistance Gene *mer*A (1100bp) was amplified using specific gene primers from *Escherichia coli*. A size marker (1kb DNA ladder RTU; DM010-R500). M: ladder, P: positive control, N: Negative Control, 1-4: Positive sample for *merA* gene.

The findings of current research showed that standing water gets more contaminated than running water. Many microbiological pathogens, including bacteria (*salmonella* and *E.coli*) found there. Many of the contaminants found in standing water are organic, making them an ideal food source for bacteria [25]. In addition, stagnant water draws disease-carrying insects and rodents [37]. The results of the current study show that metal and microbial contamination was more frequent in standing surface water as compared to running surface water [38]. In the current study water samples contain a large number of *E.coli* as compared to other microbes. The result also recorded a large number of *E. coli* on standing surfaces as compared to running surface water.

Results showed that in 15 *E. coli* samples of running surface water, 9 samples showed positive results for *czc*A, 8 samples were positive for *czc*D and 7 isolates were positive for *mer*A. From standing surface water in 24 identified *E.coli* 12, 15, and 13 showed positive results for *czc*A, *czc*D, and *mer*A respectively.

Total percentage for *czc*A, 21/39 (32.81%), for *czc*D, 23/39 (35.9%), and 20/39 (31.25%) for *mer*A from both running and standing surface water samples (Table 4).

Table 4. **Percentage of heavy metal resistance genes of *E.coli* isolated from water samples.**

Heavy metal resistance genes	*czc*A	*czc*D	*mer*A
Running Surface Water	9/15	8/15	7/15
Standing Surface Water	12/24	15/24	13/24
Total no resistance genes	21/39	23/39	20/39
Percentages of genes	32.81	35.9	31.25

*Percentage (%) of heavy metal resistance genes isolated from *E.coli*

Several studies have found resistance genes, the genetic factor responsible for resistance to pollutants such as heavy metals in bacteria [26]. Abou-Shanab et al. [27]

discovered that the metal resistance genes mer and *ncc* were identified by PCR, and the bacteria are resistant to Hg and Ni. Abdelatey et al. [24] investigated heavy metals resistant genes *mer*A and *czc*D in bacteria to determine metal tolerance against Cd^{2+} and CO^{2+}. The *mer* and *czc* genes, which are important for heavy metal tolerance, were found in these bacteria using PCR. In the present study of Cd and Pb contaminated water, the considerable HMR genes *czc*A, *czc*D, and *mer*A were identified in *E.coli* from both types of water sources. The mutant bacteria can raise the risk of waterborne diseases in humans and other biotic masses. Many researchers investigate the different resistant genes in bacteria such as Malik et al. [39] studied heavy metals and their resistant genes *czc*A, *cop*A, and *ncc* from bacterial isolates in Pat Feeder Canal, district Jaffrabad, Balochistan. Rashid et al. [28] also identified several drug resistant genes in *S. aureus* isolated from a variety of fish in the Gwadar port of Balochistan. Ture et al. [22] identified heavy metals resistant genes czcA and nccA which were found in *E. coli* from the fish muscles. Several genes, including *mer*A, *ncc,* and *czc*A, were detected in a different bacterial isolate from mullet which tolerates different heavy metals [40].

Conclusion

In the current study sub-lethal metal concentrations of Pb and Cd from water, cause bacteria to develop their respective heavy metal resistant genes *czc*A, *czc*D, and *mer*A, which impacted aquatic ecosystems such as bioremediation methods. Surface water pollution, warns us to reduce the sources of metal pollutants and regular monitoring by the Environmental Protection Agency should be necessary to preserve water quality in this area. That can minimize the public health risks associated with polluted water consumption.

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Conflict of Interest

The authors declare that they have no conflicts of interest.

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