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Research Article

Accumulation of heavy metals and detection of resistant-associated genes in *Pseudomonas aeruginosa* in an edible catfish (*Wallago attu*) from Pat Feeder Canal, Pakistan

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Abstract

Seafoods are the main source of animal protein in our daily diet and their consumption has been increased due to its high health benefits over red meats. This study aimed to evaluate the heavy metals accumulation in a freshwater catfish muscle (*Wallago attu*) and the detection of heavy metal resistance genes (HMRGs) in *Pseudomonas aeruginosa* isolated from the fish intestine. *W. attu* (n = 60) was collected from four different sites (Qabula Shakh, Magsi Shakh, Umrani Shakh, and Jamali Shakh) of Pat Feeder Canal, Balochistan. The heavy metals and HMRGs were detected using atomic absorption spectrophotometer and polymerase chain reaction. The concentrations of Cd (0.27±0.001 mg/L), Fe (1.23±0.001 mg/L), and Pb (1.23±0.0005 mg/L) were found to be above the permissible limits of WHO in the samples from Jamali Shakh. Moreover, a strong Pearson's correlation of the metal Cd was observed with Zn, Fe, Cr, Cu, Pb, and Ni. However, Zn has a strong correlation with Fe and Cr; Fe with Cu, Ni, and Cu. Cr with Pb and Cu; Cu with Ni and Pb; while Ni has a strong correlation with Pb and Mn. *P. aeruginosa* was also identified from 41 species out of all fish intestine specimens (68%). Similarly, different heavy metal resistance genes (MRGs) including *czc*A 4 (36.3%), *ncc* 4 (36.3%), *chr*R 2 (18.1%), and *cop*A 1 (9%) were confirmed using PCR. In conclusion, Cd, Fe, and Pb concentrations were higher than the WHO permissible limits. However, other heavy metals (Ni, Zn, Cr, Cu, and Mn) were permissible limits in the fish muscle. The results of this study have shown a correlation between the buildup of heavy metals and the presence of MRGs.

Keywords: Food Safety, Atomic Absorption Spectrometer, Metal Resistance, PCR, Bioaccumulation

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Introduction

There are many different sources of environmental pollutants and heavy metals, such as industrial and agricultural processes, acid rain, and human and animal waste (Facchinelli *et al.,* 2001). Each metal has a minimum threshold of toxicity, which is dangerous to life, and the environment. The heavy metals accumulate in fish body through absorption by the gills, and skin, and become part of the food chain (Alkan *et al.,* 2012).

The fish get contaminated by heavy metals (HMs) that accumulated in the aquatic environment due to the food chain and water contamination (Łuczyńska *et al.,* 2018). However, some of them remain accumulated in fish tissues, while others are eliminated through feces discharge. Fish are directly linked to the aquatic food chain, which can transfer heavy metals contamination to human beings (Araújo *et al.,* 2022). The most common environmental contaminants include mercury (Hg), cadmium (Cd), chromium (Cr), cobalt (Co), lead (Pb), nickel (Ni), copper (Cu), and arsenic (As) (Jaiswal *et al.,* 2018).

The freshwater catfish species, *Wallago attu*, is an important fish used as human food. It consumes leftovers and other organic matters, so it can easily be grown in a normal environment without any special care (Bano *et al.,* 2018). These fish have significant attention because of their economics, nutritional health, quick development, and delicious flavor (Araújo *et al.,* 2022).

Bacteria develop

A tolerance for different amounts of hea -vy metal contaminants. Researchers are paying close attention to metal resistant bacteria in metal-contaminated environments (Pirela *et al.,* 2014). *Pseudomonas aeruginosa* is a normal part of the fish microbiome, but under stressed conditions such as hunger and overpopulation, the bacteria have become extremely opportunistic and harmful, causing severe diseases such as hemorrhagic septicemia, gill necrosis, abdominal distension, splenomegaly, friable liver and congested kidney (Ardura *et al.,* 2013; Dian Fitria *et al.*, 2021).

Balochistan is the largest province of Pakistan and Jaffarabad is one of the urbanized districts of the province, where the Pat Feeder Canal is the primary source of irrigation in Jaffarabad. The canal water is used by the residents for a variety of purposes, including agriculture, drinking, household use, and fish farming. The people of the area catch fish for their food and finance, hence, the contamination of aquatic ecosystems by heavy metals may pose a serious threat to consumers. The purpose of this study was to analyze the relationship between heavy metals contamination in fish and the frequency of heavy metals resistance genes in bacteria living in the fish. Furthermore, this study highlights that metal-resistant bacteria have a high potential in the remediation of metal-contaminated sites, with biosorption being the most promising process.

Material and methods

Sample collection

A total of 60 fish specimens were collected aseptically from the Pat Feeder Canal, Balochistan for heavy metal accumulation and bacterial isolation (Table 1).

Table 1: Biometry of *Wallago attu.*

Sex	Length (cm)	Weight (g)	Mean Length	Mean weight
Male (28)	50-110	1500-4000	89.87	3300
Female (32)	50-130	2000-4500	101	3900
Combine(60)	50-130	1500-4500	95.435	3600

The samples were collected from different active aquaculture sites (Qabula Shakh, Magsi Shakh, Umrani Shakh, and Jamali Shakh) of the district Jafarabad respectively. The collected samples were properly labeled and transported in the cold chain to the Centre for Advanced Studies in Vaccinology and the Biotechnology University of Balochistan and were stored at -4^{o}C for further analysis.

Area Description

Jaffarabad is the second most populated district of Balochistan with 0.64 million population. The district Coordinates are 28°25′N 68°10′E / 28.417°N 68.167°E. The district consists of three tehsils (Dera Allah Yar (Jhat Pat), Ustaa Muhammad and Gandakha. Most of the land is irrigated with Canals because of low annual rainfall which is 50 to 150 mm. The description of the sampling sites along with the longitudinal and latitude coordinates are mentioned in Table 2 and Figure 1.

Table 2: Description of Pat Feeder Canal characteristics for each sampling site

S. No	Sites	Description
1	Qabula Shakh	Located at coordinates: 28.637437, 68.396480 large size canal approximately 3-6 m wide, medium flowing water. Bottom substrate consists of sand mud and silt continuously turbid water.
2	Magsi Shakh	Coordinates: 28.644876, 68.405836 main canal, large size canal approximately 5-8 m wide, medium flowing water. Bottom substrate consists of sand, mud, silt and waste of animals and human continuously turbid water.
3	Umrani Shakh	Coordinates: 28.650619, 68.413432 large canal approximately 6-9 m wide, medium flowing water. Bottom substrate consists of sand, mud, silt, animals, human and agriculture wastes continuously turbid water.
4	Jamali Shakh	Coordinates: 28.660599, 68.427487 large size canal approximately 7-10 m. Medium flowing water. Bottom substrate consists of sand, mud, silt, animals, human and agriculture wastes continuously turbid water.

Heavy metal Analysis

For heavy metals detection, the fish samples were dried and ground to powder form. The dried fish powder (~1g) was suspended in 10ml of Nitric acid (67%), and incubated in a fume hood for 24 hours. Later 4ml Per-Chloric acid ($HClO_4$) was added and heated on a hot plate, cooled and diluted with deionized water to make the volume up to 100ml as proposed by Raka *et al*. (2020). Heavy metal detection was performed by using atomic absorption spectrophotometer (Perkin- Analyst 800 JAPAN) (Malik *et al.*, 2017).

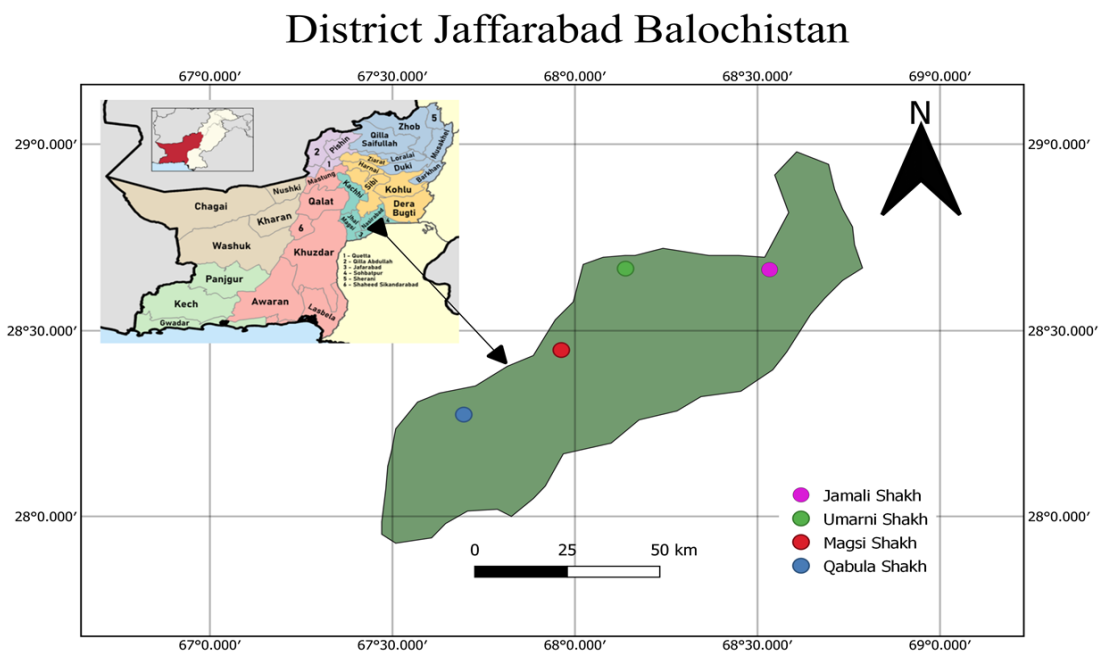

Figure 1: Map showing the location of the study area in district Jafarabad with Sampling Sites.

Microbial analysis

All fish samples were subjected to bacterial culturing and target bacteria isolation. The intestinal content of fish was aseptically removed, diluted with Luria Bertani (LB; Merck) broth, and incubated for 18 hours at 37°C. Initial growth was taken on nutrient agar followed by Cetrimide agar (Merck 1.05284.0500).

Molecular identification

DNA extraction

For genomic identification DNA was extracted through QIAamp (Cat. No. 51304 DNA mini kit) according to the given protocol and instructions.

PCR amplification

A molecular base identification was performed using PCR amplification by targeting 16sRNA, PA-SS-F, and PA-SS-R primers for molecular identification of *Pseudomonas aeruginosa* (Table 3). In total 20 µL PCR reaction mixture 10 µL PCR master mix, 1 µL forward and reverse primer and 5 µL molecular grade water and 3 µL DNA sample was added following (Sukri *et al*., 2021). Similarly, initial

denaturizing was done at 94°C for 5 minutes while amplifications were carried out for 30 cycles (94°C for 30s, 55°C for 30s and 72°C for 30 s) and a final extension at 72°C for 7 min (SCILOGEX TC1000-G Master Cycler). Amplicons were detected by electrophoresis on 1.5% agarose gel, stained with ethidium bromide and visualized under a gel documentation system.

Table 3: Primers used in identification of *Pseudomonas aeruginosa* heavy metal resistant genes.

Primer	Sequence(5`-3`)	Target length (bp)	Annealing temp (ºC)	References
16sRNA-F 16sRNA-R	AGAGTTTGATCCTGGCTCAG TACGGYTACCTTGTTACGACTT	1500	58ºC	Al-Jailawi *et al*., 2014
PA-SS-F PA-SS-R	GGGGGATCTTCGGACCTCA TCCTTAGAGTGCCCACCCG	956	60.9ºC	Al-Jailawi *et al*., 2014
ncc-F *ncc*-R	ACGCCGGACATCACGAACAAG CCAGCGCACCGAGACTCATCA	457	54ºC	Ture *et al.,* 2018
*czc*A-F *czc*A-R	GTTCACCTTGCTCTTCGCCATGTT ACAGGTTGCGGATGAAGGAGATCA	320	58ºC	Kaci *et al*., 2014
*cop*A-F *cop*A-R	CGGTCTCTACGAATACCGCTTCAA GAAATAGCTCATTGCCGAGGCGTT	1,300	58ºC	Bouskill *et al.,* 2007
*chr*R-F *chr*R-R	ATGTCTGATACGTTGAAAGTTGTTA CAGGCCTTCACCCGCTTA	350	55ºC	Ture *et al*., 2018

Identification of heavy metal resistant genes (HMRGs)

Isolation/extraction of plasmid

Pseudomonas aeruginosa isolated from 60 fish samples, were examined for the presence of HMRGs. For this purpose, QIAGEN Plasmid Mini Kit (Cat. No. 12123) was used to extract the plasmids by following the manufacturer's instructions. PCR was done in 20 µL reaction mixture using 10 µL PCR master mix (2X), 1 µL of each of the forward and reverse primer 3 µL plasmid DNA, and 5 µL PCR grade water.

Amplification of HMRGs and Visualization

To amplify genes PCR was carried out as previously mentioned. For each target gene, the primers and annealing temperature are listed in Table 3. Distilled water was used as a negative control (Ture *et al.,* 2018). The final product was visualized in 1.5 percent (w/v) agarose gel.

Statistical analysis

The determined heavy metals values were tabulated and analyzed using SPSS Version 20. Pearson's correlation was used to evaluate the correlation among these metals.

Results

A total of 60 *W. attu* freshwater species were used for the detection of different heavy metals, Ni, Zn, Cr, Pb, Cu, Mn, and Cd from muscles. All three sites (Site 1-3) of Pat Feeder Canal showed significant values for all the selected

heavy metals (Fe, Zn, Cd, Pb, Cu, Ni, Mn, and Cr), under WHO permissible limits. An interesting result was observed in site 4 (Jamali Shakh), where the 3 metals concentrations were found significantly higher for Cd (0.27±0.001 mg/L), Fe (1.23±0.001 mg/L), and Pb (1.23±0.0005 mg/L). However, Zn (3.01±0.0004 mg/L), Cr (0.39±0.0002 mg/L), Cu (0.45±0.0002 mg/L), Ni (0.49±0.001 mg/L), Mn (0.78±0.001 mg/L) were found under WHO permissible limit and person Correlation were found (Fig. 2; Table 4).

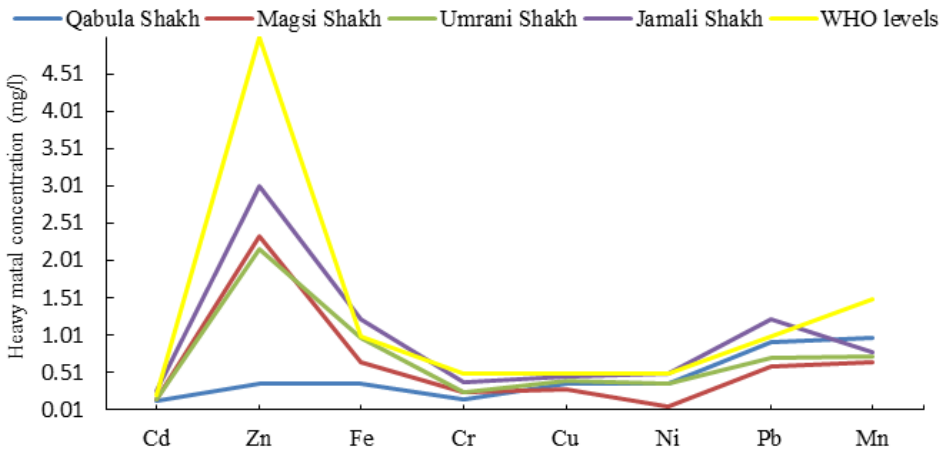

Figure 2: Concentration of different heavy metals in four different sites of Pat Feeder Canal.

Table 4: Mean Concentration (±SD) of heavy metals in *Wallago attu* collected from Pat Feeder Canal

Metals	Cd	Zn	Fe	Cr	Cu	Ni	Pb	Mn
Qabula Shakh	0.13±0.001	0.36±0.0004	0.37±0.002	0.16±0.001	0.36±0.0003	0.36±0.001	0.92±0.0005	0.98±0.002
Magsi Shakh	0.15±0.0004	2.34±0.001	0.64±0.001	0.25±0.001	0.29±0.0006	0.06±0.0004	0.59±0.001	0.64±0.001
Umrani Shakh	0.15±0.0003	2.17±0.0003	0.97±0.0009	0.25±0.0003	0.40±0.001	0.36±0.002	0.71±0.002	0.72±0.001
Jamali Shakh	0.27±0.001	3.01±0.0004	1.23±0.001	0.39±0.0002	0.45±0.002	0.49±0.001	1.23±0.0005	0.78±0.001
WHO	0.2	5	1	0.5	0.5	0.5	1	1.5

Concentration of metals (Mean ± SD microgram/gram (mg/L) in *Wallago attu.*

Samples (60) were processed for heavy metals detection, the data was considered normal according to the central limit theorem. So, Pearson's correlation was used. The results of correlation analysis showed that, Cd has a strong correlation with Zn, Fe, Cr, Cu, Pb and Ni; Zn has a strong correlation with Fe and Cr; Fe has a strong correlation with Cu, Ni and Cu; Cr has a strong correlation with Pb and Cu; Cu has a strong correlation with Ni and Pb; Ni has a strong correlation with Pb and Mn (p<0.01) (Table 5).

Molecular identification of P. aeruginosa and heavy metals resistant genes

All the bacteria, isolated from intestine of fish were confirmed through 16sRNA universal primers amplification with 1500bp (Fig. 3) and using specific gene primers PA-SS-F and PA-SS-R with

956bp (Fig. 4). Out of 60 fish samples, *P. aeruginosa* was confirmed in 41(68%).

Similarly, targeting *czc*A (320), *ncc* (457), *chr*R (350), and *cop*A (1300 bp), respectively to conform the HMRGs in the isolates (Figs. 5-8; Table 6). The *czc*A, *ncc*, *chr*R, and *copA* genes were found in 36.3, 36.3, 18.1, and 9 %, respectively.

Table 5: Correlation of different heavy metals in *Wallago attu* collected from Pat Feeder Canal.

	Cd	Zn	Fe	Cr	Cu	Ni	Pb	Mn
Cd	1							
Zn	0.721**	1						
Fe	0.830**	0.872**	1					
Cr	0.951**	0.900**	0.922**	1				
Cu	0.716**	0.330*	0.742**	0.615**	1			
Ni	0.567**	0.039	0.507**	0.391*	0.954**	1		
Pb	0.797**	0.166	0.477*	0.579**	0.810**	0.845**	1	
Mn	-0.143	-0.782	-0.451	-0.435	0.251	0.529**	0.482*	1

** Correlation is significant at the 0.01 level. * Correlation is significant at the 0.05 level.

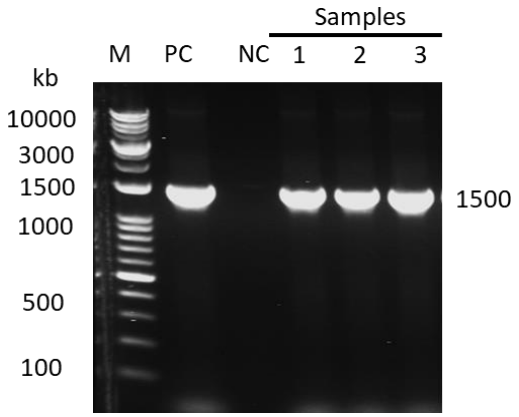

Figure 3: Agarose gel electrophoresis of 16sRNA PCR products, (M) 10 kb ladder: (PC) positive control for *P. aeruginosa*; (NC) negative control; lines 1-3: samples. English.

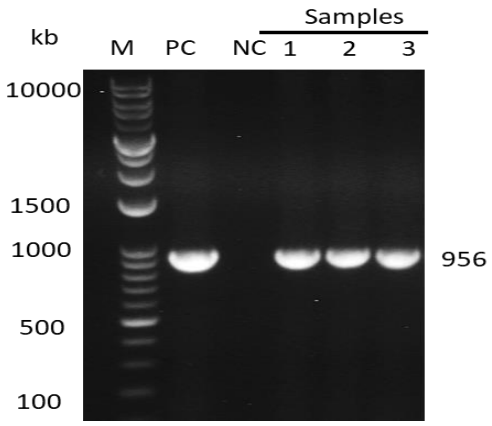

Figure 4: Agarose gel electrophoresis of *PA-SS-F* and *PA-SS-R* PCR products (M) 10 kb ladder: (PC) positive control; (NC) negative control; lines 1-3: samples of *P. aeruginosa.*

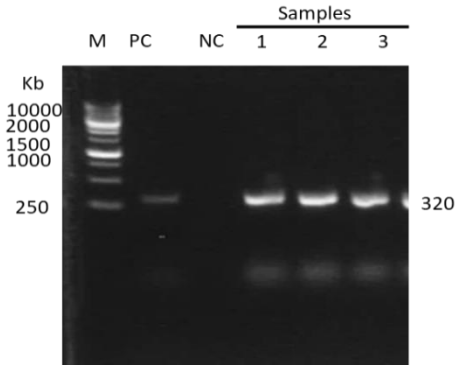

Figure 5: Agarose gel electrophoresis of *czcA* PCR products, (M) 10 kb ladder: (PC) positive control; (NC) negative control; lines 1-3: samples of *czc*A.

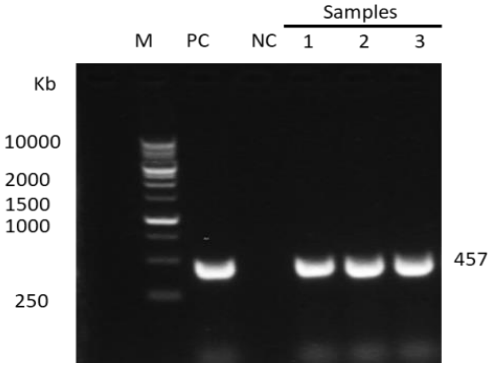

Figure 6: Agarose gel electrophoresis of *ncc* PCR products, (M) 10 kb ladder: (PC) positive control; (NC) negative control; lines 1-3: samples of *ncc.*

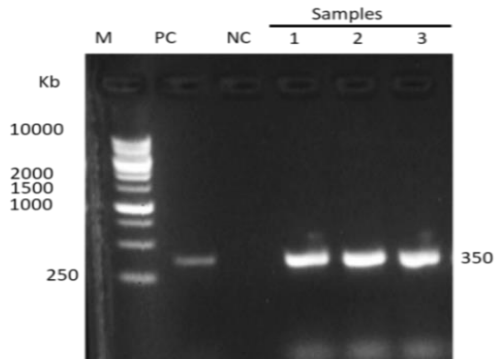

Figure 7: Agarose gel electrophoresis of *chrR* PCR products, (M) kb ladder: (PC) positive control; (NC) negative control; lines 1-3: samples of *chrR*

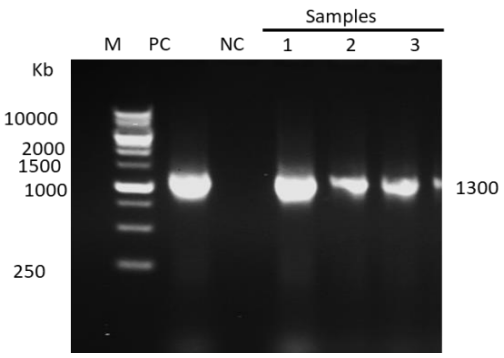

Figure 8: Agarose gel electrophoresis of *cop*A PCR products, (M) kb ladder: (PC) positive control; (NC) negative control; lines 1-3: samples of *cop*A.

Table 6: Heavy metal resistance genes of *Pseudomonas aeruginosa* isolated from *Wallago attu*

Heavy metal resistance gene (%)		***ncc***	***czc*A**	***chrR***	***cop*A**
No of genes		04	04	02	01
Total number of genes	11				
Percentages of genes found		36.3%	36.3%	18%	9%

Percentage (%) of heavy metal resistance genes isolate from *P. aeruginosa.*

Discussion

Freshwater catfish are important due to their commercial value. They are readily available and cheap freshwater food resources and eventually, they are largely consumed by the local and poor communities of the area.

In this study, significantly higher concentrations of Cd (0.27±0.001 mg/L), Fe (1.23±0.001 mg/L), and Pb (1.23±0.0005 mg/L) were found in the fish muscles collected from Jamali Shakh of Pat Feeder Canal. Similar results have been reported by Mahdi *et al*. (2020) from Iran, who reported the high concentrations of Cd, Cu, and Hg in the fish muscles caught from the Oman Sea. Dhaneesh *et al*. (2012) also found the higher concentrations of Cd, Co, and Pb in the fish muscles samples taken from the coastal water of Agatti Island. The outcomes of Mahboob *et al*. (2016) align with the present findings, as they elevated levels of heavy metals (Fe, Cu, Cd, and Pb) in two fish species, *C. carpio* and *W. attu*, collected from the Indus River in Pakistan.

Many researchers reported significant levels of different metals accumulation in the fish tissues (Bawuro *et al*., 2018). The reason behind this phenomenon is the activation of heavy metals through the chelation process involving trace elements and the mixing of sewage water (contaminants) along with the widespread use of pesticides and other chemicals in the area. Many researchers reported that a positive correlation was present among the heavy metals originating from atmospheric changes and anthropogenic activities (Wei *et al*., 2015), seasonal effect (Bawuro *et al.*, 2018), water or soil of the area and trace element (Mahdi *et al.*, 2020). Tariq *et al*. (1994) results are similar to our study; they reported trace elements (Mg^+, K^+, Ca^+, and Na^+) in the fish, and their habitat water and

sediment. This phenomenon can be applied to the current situation of our study area. Many studies investigate that the development of heavy metal resistance in fish which is associated with different bacteria like *E. coli* and Coliforms, *Aeromona* spp, *Vibrio parahaemolyticus* and, *Staphylococcus* spp, because of their prolong exposure to the metals dense habitats (He *et al.*, 2016; Liu *et al.*, 2019; Ture *et al.*, 2021). This bacterial resistance is transferred to other bacteria through vertical and horizontal gene transfers (Liu *et al*., 2016). *Pseudomonas* being prominent resistant bacteria showed resistance, to the diversity of heavy metals, that is why it has been extensively studied under the metals stress conditions due to the presence of HMRGs (Cerdeira *et al*., 2020).

Metal resistances are usually associated with plasmid-borne genes and the transformation of metal resistance genes (HMRGs) from the environments (Wei *et al.*, 2015). Several genes, including *mer*A, *ncc* and *czc*A, sustain the bacteria to tolerate different heavy metals (Ture *et al.*, 2021). In the current study, sixty (60) samples were evaluated, and results showed that *Pseudomonas aeruginosa* contains the majority of heavy metal resistant genes. We found *czc*A (36.3%), *ncc* (36.3%), *chr*R (18%) and *cop*A (9%) gene in *P. aeruginosa*. Bouskill *et al*. (2007) in a similar study confirmed the presence of resistances genes *Cop*A and *ncc*A which are in agreement with the current study. Rashid *et al*. (2021) identified drug resistance in *S. aureus* isolated from a variety of fish in the Gwadar port of Balochistan.

The current study showed a modest presence ratio of *czc*A and *ncc* genes, which is in accordance with the findings of Ture *et al*. (2021). We found *chrR* (18%) and *cop*A (9%) genes, these results concur with those of Chen *et al*. (2019) and Ture *et al*. (2021). Many bacteria are suitable for heavy metal biosorption, as they include a large number of genes related to different metals tolerance (Sukri *et al.*, 2021). Various tolerance mechanisms have been identified in heavy metal resistance genes (Chen *et al.*, 2019).

In conclusion, a significant higher impermissible limit of Cd, Fe, and Pb was found in the muscles of catfish *W. attu* collected from Jamali Shakh of Pat Feeder Canal. However, other metals Ni, Zn, Cr, Cu, and Mn were within permissible limits. Moreover, different HMGs were identified in *P. aeruginosa* isolated from the intestine of the fish. The presence of heavy metals in the fish and the resistant genes are most probably because of the leaching of heavy metals from the surrounding environment to the canal water. Sources identification of this pollution needs a comprehensive study to be conducted on targeted goals. The situation warns of the careful human consumption and regular monitoring for heavy metal concentration in the fish from the area. That can minimize the public health risk associated with the consumption of contaminated fish.

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