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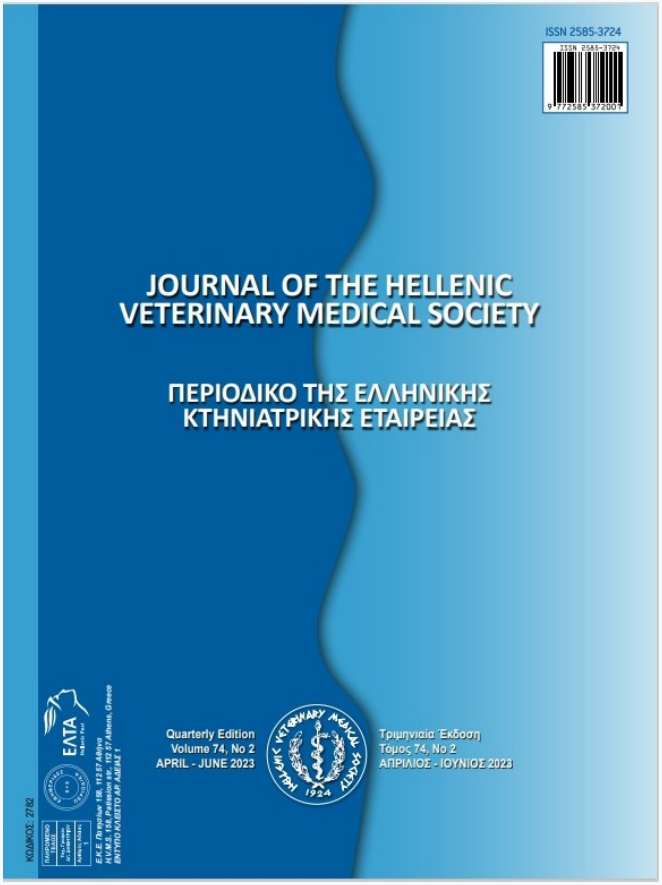

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Research article
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Investigation of Some Heavy Metal Resistance Genes in *E.coli* Isolated from Shrimp and Mussels

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ABSTRACT: One of the biggest problems in the modern world is environmental pollution, which affects all organisms, albeit at different levels. An important contributor to pollution comes in the form of stable and non-degradable heavy metal compounds produced from the use of heavy metals in a wide range of environments. Heavy metal pollution, especially in the seas, is bioaccumulated in aquatic life and the food chain, causing heavy metal-related diseases. The most critical sign of heavy metal pollution in water is the resistance formed against heavy metals in bacteria living in these waters. Heavy metals resistance in *E. coli* isolated from 18 mussels and 16 shrimps was genotypically investigated in both plasmid and genomic DNA. Out of 34 *E. coli* isolates examined, 31 (1.17%) harbored heavy metal resistance genes. The presence of the *pcoR* gene was found in 2 isolates (5.88%), the *merA* gene in only one isolate (2.94%), and the *mntR* gene in 8 isolates (23.52%). While *pcoR* and *merA* were not observed together in any isolate, *pcoR* and *mntR* were detected together in the genetic material of 10 isolates (29.41%) and *mntR* and *merA* were detected together in the genetic material of seven isolates (20.58%). In three samples, all of the resistance genes against heavy metals were detected (8.82%).

In this study, approximately 90% of the isolates carried one or more of the heavy metal resistance genes. This study shows the widespread distribution of heavy metal resistance genes in the isolates examined and reports that the extent to which this situation affects antibiotic resistance should be evaluated.

Keywords: copper, mercury, manganese, *E. coli*, resistance

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INTRODUCTION

Heavy metals are elements that make up the earth's structure and are essential to organisms. However, the stable and undegradable compounds of heavy metals, which are increasing in the environment as a result of agricultural and industrial applications, pose a significant threat to all life forms (Jaishankar et al., 2014). These metal compounds have become common pollutants due to the use of disinfectants containing mercury and silver in clinics, the use of heavy-metal-containing preparations with copper, manganese, and zinc in veterinary medicine, the treatment of diarrhea, and feed supplements, as well as industrial activities such as mining, textile, and paint (Chakraborty et al., 2019). However, when industrial, medical, and domestic wastes end up in aquatic masses, they cause heavy metal contamination and thus the emergence of heavy metal resistance in bacteria (Di Cesare et al., 2016; Uncumusaoğlu et al., 2016). Heavy metals can easily show cumulative properties in aquatic ecosystems, accumulating in living systems and reaching the organism at the top of the food chain (Sipahi et al., 2019). While advanced organisms are known to be highly affected by the toxic effects of heavy metals, similar effects are observed in many microorganisms (Martins et al., 2014; Xavier et al., 2019). Bacteria have developed various resistance mechanisms against the increased amount of heavy metals present in soil and aquatic ecosystems. These resistance mechanisms are similar to those described in antibiotic resistance. The fact that common genes mediate resistance is seen as an important health problem that requires further research (Argudin et al., 2019).

Heavy metal resistance in bacteria is an indicator of contamination, which is frequently encountered. In many microorganisms, the genes encoding these resistances are located in the chromosome, extrachromosomal genetic elements, and integrons found in genetic areas (Seiler and Berendonk, 2012; Staehlin et al., 2016). Studies show that metal resistance genes spread between bacteria through horizontal and vertical gene transfer to adapt to the environment (Hernández Ramírez et al., 2018; Ture et al., 2018; Argudin, 2019). In addition, the proven positive correlations of heavy metal resistance with antibiotic resistance are of great concern in terms of public health (Dweba et al., 2018). However, the cellular activities of bacteria to cope with metal toxicity also allow bioremediation. This activity is vital from an ecological point of view. Bacteria play an essential role in restoring the environment by establishing both synergistic and antagonistic interactions with heavy metals in contaminated water (Mishra et al., 2019). For this reason, determining the distribution of metal resistance genes in bacteria can make an important contribution to sustainable living studies or draw attention to metal pollution (Aljerf and Almasri, 2018). This study aimed to investigate the resistance to copper, manganese, and mercury in *E. coli* isolated from aquatic organisms.

MATERIALS AND METHOD

Bacterial Strains and Cultures

The isolates used in the study were obtained from the culture collection of Istanbul University, Cerrahpaşa Faculty of Veterinary Medicine, Department of Microbiology. Genomic and plasmid DNA extracts of 34 *E. coli* strains isolated and identified from aquatic organisms (18 mussels and 16 shrimps) in the collection were used to detect the presence of heavy metal resistance. Tryptic soy broth (TSB, Merck) and tryptic soy agar (TSA, Merck) were used to revive the isolates for DNA extraction.

Genomic DNA and Plasmid DNA Extraction

Investigation of resistance genes was performed

Table 1. Target genes and amplification temperatures

Target Gene	Chain	TM °C	Amp (bp)	References
mntR	5'-TAAACACGCGCATACACCTCTTG-3' 5'-GCGTGCGTAAAAAAGGCAGGCTC-3'	58	708	Patzer and Hantke, 2001
merA	5'-GAGATCTAAAGCACGCTAAGGC-3' 5'-GGAATCTTGACTGTGATCGGG-3'	62	1011	Misra et al., 1984 Abou-Shanab et al., 2007
pcoR	5'-CAGGTCGTTACCTGCAGCAG-3' 5'-CTCTGATCTCCAGGACATATC-3'	57	636	Trajanovska et al., 1997 Chihomvu et al., 2015

on genomic DNA and plasmid DNA for each isolate separately. Thermo Scientific GeneJET Genomic DNA Purification (K0721-USA) was used for genomic DNA extraction, and Thermo Scientific GeneJET Plasmid Miniprep Kit (K0502) was used for plasmid DNA extraction.

PCR Amplification of Heavy-Metal-Resistant Genes

The genes *merA* were investigated for mercury resistance, *pcoR* for copper resistance, and *mntR* for manganese resistance. The primers used are given in Table 1. PCR commercial mix (Thermo Fisher K0171-USA) was used for amplification.

RESULTS

Both genomic DNA and plasmid DNA were analyzed for the presence of heavy metals resistance genes, including *merA* for mercury, *pcoR* for copper, and *mntR* for manganese in 34 *E. coli* isolated and identified from 18 mussel and 16 shrimp samples (Table 2).

The results obtained in this study show that the genomic DNA of 5 mussel and 6 shrimp isolates har-

Table 2. Isolates and results

Isolate No	Species	PLASMID			DNA		
		merA	*pcoR*	*mntR*	*merA*	*pcoR*	*mntR*
1	Mussel	-	+	+	-	-	-
2	Shrimp	-	-	-	-	-	-
3	Mussel	-	-	+	-	-	+
4	Mussel	-	+	-	+	+	+
5	Mussel	-	+	+	-	-	-
6	Shrimp	-	+	+	-	-	+
7	Mussel	-	+	-	-	-	+
8	Mussel	-	-	+	-	-	-
9	Shrimp	-	+	-	-	-	+
10	Mussel	-	-	-	-	-	+
11	Mussel	-	+	+	-	+	-
12	Mussel	-	+	-	-	+	-
13	Mussel	-	-	-	-	-	+
14	Shrimp	-	+	-	-	-	+
15	Mussel	-	-	-	+	-	-
16	Shrimp	-	-	+	+	-	-
17	Shrimp	-	-	+	+	-	-
18	Shrimp	-	-	-	-	-	+
19	Mussel	-	-	-	+	-	+
20	Mussel	-	-	+	-	-	-
21	Shrimp	-	-	+	+	-	-
22	Shrimp	-	-	-	+	-	+
23	Shrimp	-	-	-	-	-	-
24	Shrimp	-	+	+	+	-	+
25	Mussel	-	-	-	+	-	+
26	Mussel	-	+	+	-	-	+
27	Mussel	-	-	+	+	-	-
28	Shrimp	-	+	+	+	-	+
29	Mussel	-	+	+	-	-	+
30	Shrimp	-	+	-	-	-	-
31	Mussel	-	-	-	-	-	-
32	Shrimp	-	-	-	-	-	+
33	Shrimp	-	+	+	-	-	-
34	Shrimp	-	-	-	-	-	+

bored the *merA* gene. However, this gene was not found in any of the plasmid DNAs. When the results are evaluated for *pcoR* gene, it is found that the genomic DNA of three mussel isolates harbored *pcoR* gene, no *pcoR* gene was detected in the genomic DNA of any of the shrimp isolates. *pcoR* gene was positive in the plasmid DNA of eight mussel and seven shrimp isolates. For *mntR* gene, the genomic DNA of nine samples from mussels and nine samples from shrimps were positive for *mntR*. The *mntR* gene was detected in plasmid DNA from nine and seven mussel and shrimp isolates, respectively.

Eleven *E. coli* isolates were found positive for *merA*. Three mussel isolates positive for *pcoR* also contained the gene as a plasmid. A total of 15 isolates were positive for *pcoR*. When we looked at *mntR*, three mussel and three shrimp isolates were positive in genomic and plasmid DNA. A total of 28 isolates were positive for *mntR* (Figure 1). Resistance genes to heavy metals were not detected in three (8.82%) of the 34 *E. coli* isolates, while the number of isolates containing resistance genes to all three heavy metals was detected at the same rate.

While one isolate was found to be resistant to each mercury and copper separately (5.5%), only five isolates were found to be resistant to manganese (27.7%) in mussel isolates. Resistance was not detected in one mussel isolate (5.5%).

Resistance genes to three heavy metals were detected in shrimp isolates at 12.5%. The resistance of copper and manganese was found to be 25%, and the resistance of manganese and mercury was 25%. Copper and mercury resistance were not found in common. Only copper was found at 6.25%, while only manganese was found at 18.75%. Not "only mercury-resistant isolates" were detected. Two of the 16 shrimp isolates (12.5%) showed no heavy metal resistance.

DISCUSSION

The presence of heavy metals in the environment in different forms creates substantial stress, especially on bacteria, significantly changing their activities (Di Cesare et al., 2016). Two points make the ensuing heavy metal resistance significant. One of them is that it indicates environmental heavy metal contamination. For bacteria to develop resistance to heavy metals, they must be exposed to that heavy metal or acquire the relevant gene, which provides resistance

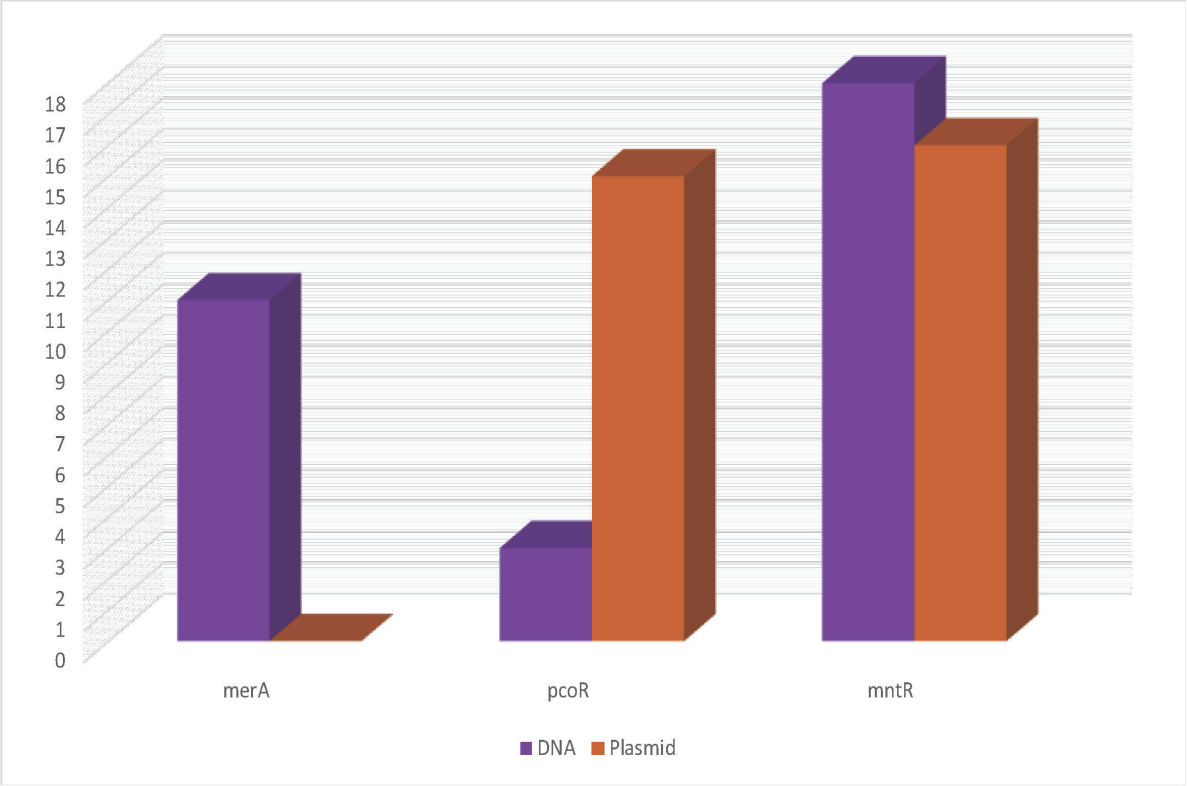

Figure 1. Plasmid and DNA Resistance Gene Counts

development by transferring genetic material between bacteria. Secondly, the potential for antibiotic resistance is high in metal-resistant bacteria (Eggers et al., 2018). Metal resistance was reported long before antibiotic resistance, and many studies emphasize that it triggers antibiotic resistance (Seiler and Berendonk, 2012).

Although the exposure of bacteria to heavy metals causes them to decrease in number, after a while, the bacteria adapt to this environment and form resistant subpopulations. Since this situation has a healing effect in the context of environmental pollution, it is crucial to determine the distribution of related genes in various ecosystems. On the other hand, considering the studies showing that some operons related to heavy metal resistance are also responsible for antibiotic resistance, it is believed that this situation poses a threat to human and animal health (Rasmussen et al., 2008; Li et al., 2020). Because common mechanisms that provide resistance development and common genetic elements contribute to both processes (Baker-Austin et al., 2006), these mechanisms are provided physiologically (cross-resistance) and genetically (co-resistance). Cross-resistance can be defined as mechanisms that provide tolerance to multiple antimicrobial agents, such as antibiotics and heavy metals (Chapman, 2003). For example, it is known that an effective efflux mechanism for more than one drug is mediated by rapid extrusion of toxins from the cell, reducing sensitivity to antibiotics and heavy metals (Martinez et al., 2009). Co-resistance is defined as two or more genetically linked resistance genes, meaning that two or more genes responsible for resistance are close together on a mobile genetic element (Chapman, 2003). Holzel et al. (2012) detected various heavy metals in pig urine, reporting high resistance to β-lactams in environments containing copper and zinc in *E. coli*, which they recovered from the same urine. Seiler and Berendonk (2012) showed that heavy metals used for disinfection on farms support the spread of antibiotic resistance along with common resistance. Yazdankhah et al. (2014) reported that enteric bacteria are resistant to trace elements used as additives in farm animal feeds, along with increased multi-antibiotic resistance in *E. coli*. Martins et al. (2014) observed copper and mercury resistance in tetracycline-resistant *Pseudomonas aeruginosa* strains. They found that genes responsible for tetracycline and heavy metal resistance were on the same conjugative plasmid. These genes could be transferred together in conjugation experiments. Walsh and Caslake (2016) showed a significant relationship between mercury and multi-antibiotic resistance in *E. coli* isolated from the aquatic environment. Another study reported that there is cross-resistance between various antibiotics and silver, copper, and mercury in bacteria isolated from polluted water (Miloud et al., 2021).

This study observed that 11 of 34 *E. coli* isolates carried *merA*, 15 carried *pcoR*, and 28 carried *mntR*. Almost all isolates contain single or multiple heavy metal resistance genes. This result shows that the distribution of genetic material for heavy metals is relatively high. Today, the fact that industrial, medical, and domestic wastes somehow end up in aquatic masses may be the main reason for this finding (Di Cesare et al., 2016). In addition, many factors such as mining activities, wastewater/sludge applications, air pollution, agriculture, and animal husbandry cause widespread metal pollution in soils and aquatic ecosystems, threatening animal and human health (Jensen et al., 2018). Here, bacteria develop various mechanisms to cope with this metal contamination, ensuring interspecies spread through plasmids (Martins et al., 2014). In this study, *mntR* was detected in the plasmid DNA of 16 isolates, and *pcoR* was detected in 15 isolates. *mntR* is an essential gene that regulates manganese, which is commonly detected in bacteria (Peng et al., 2021). Studies have reported that it provides manganese homeostasis and is protective against intoxication in environments containing high amounts of manganese (Paruthiyil et al., 2019; Sipahi et al., 2019).

Similarly, copper, a trace element like manganese, is expected to be toxic to bacteria in high amounts. The *cus*, *cop*, and *pco* operons, which regulate the accumulation of copper in the cell, are frequently encountered in bacteria. *pcoR*, found in the *pco* operon, is a gene that is commonly isolated from *E. coli* that can grow in environments containing dense copper (Hao et al., 2015; Adekanmbi et al., 2019). The genetic elements responsible for the resistance detected, especially in *E. coli*, are essential because *E. coli* has a wide host range and can deliver its plasmids, which determine many factors, to large bacterial populations in different ecosystems (Staehlin et al., 2016; Bukowski et al., 2019).

The *merA* gene, responsible for mercury resistance, was not found in the plasmid DNA of any of the 34 isolates tested in the study. This is a surprising result, because *merA*, which encodes the mercury reductase enzyme (the common root cause of mercu-

ry resistance), is mainly associated with plasmids. A study showed that bacteria in polar regions reduce mercury toxicity with the plasmid-mediated *merA* (Binish et al., 2021). Another study determined that the *mer* operon, in which the *merA* gene is located, is located in the plasmid of *E. coli* strains isolated from water sources (Azam et al., 2018). This study detected *merA* positivity originating from genomic DNA in 11 of 34 isolates. The least common heavy metal resistance gene in the isolates was *merA*.

Similarly, in Yang et al.'s study (2020), it was found that *merA* was detected at a rate of 11.4% in *Salmonella* strains and 2.5% in *E. coli* strains. Moller et al. (2014) reported *merA* in strains with phenotypic mercury resistance. In another study, mercury-resistant (7.4%) *E. coli* strains were isolated from mussels and sea snails, and the presence of *merA* was reported (Terzi and Civelek, 2021).

Many studies report heavy metal contamination in aquatic organisms such as mussels, scallops, and oysters, and heavy metal resistance in isolated bacteria (Sforzini et al., 2018; Dahanayake et al., 2019; Wang et al., 2020). In this study, there were 18 mussel-derived isolates, 17 of which were found to have one or more of the heavy metal resistance genes. Mussels mostly choose regions with different pollution levels as their habitat (Bayne, 2009). Therefore, the findings in the study were quite expected. Similarly, shrimps live on contaminated shorelines and make up 15% of the aquaculture industry. However, it is frequently reported that shrimps are stored under poor hygienic conditions.

For this reason, shrimp is believed to be a good source of antibiotic and heavy metal resistance genes (De Silva et al., 2018; Jiang et al., 2020). In this study, two of the three isolates that did not contain any resistance genes were from shrimp, and one from mussels. Considering their habitats, although it is possible to detect a higher rate of resistance genes in bacteria isolated from mussels, there was no significant difference between the sampled species, since there were not enough isolates in this study. However, when the findings of the current study are evaluated, the results support this idea.

CONCLUSIONS

We observed that approximately 90% of the isolates in this study carried one or more of the heavy metal resistance genes. Although the gene frequency in question suggests that aquatic organisms are highly exposed to heavy metals, heavy metal contamination in living tissues should be determined by further studies. This study shows the widespread distribution of heavy metal resistance genes in the isolates examined and reports that the extent to which this situation affects antibiotic resistance should be evaluated.

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