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RESEARCH ARTICLE

Cu, Cd, As and Hg resistance levels in *Escherichia coli* isolated from Mediterranean mussel and sea snail in the Southeastern Black Sea

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ABSTRACT

Marine environment is exposed to various pollutants such as heavy metals, pesticides, and antibiotics. Bacterial resistance to these pollutants is a global problem all over the world. In this study, Mediterranean mussel (*Mytilus galloprovincialis*) and sea snail (*Rapana venosa*) were collected from 12 sampling points from Artvin, Rize, Trabzon, and Giresun Coasts of Black Sea, Turkey. A total of 54 *Escherichia coli* isolated from Mediterranean mussel and sea snail were tested for their ability to tolerate Cu, Cd, As, and Hg. For this purpose, minimum inhibitory concentration (MIC) tests for all isolates to the Cu, Cd, As, and Hg were done to determine tolerance or resistance using the broth dilution technique. MIC concentration for Cu, Cd, As, and Hg ranged between 100-400 µg/ml, 100-200 µg/ml, 25-400 µg/ml, and 3.125-25 µg/ml, respectively. All of the strains were determined as resistant to Cu, but sensitive to As. Resistance to Hg was determined as 7.4 %. The most common resistance gene in the bacteria was *nccA* and followed by *chrB* and *merA*. Tolerance or resistance of the bacteria to toxic pollutants including heavy metal(oid)s is of significant ecological importance. These bacteria could be used for monitoring environmental heavy metal(oid) pollution.

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Introduction

Mediterranean mussel (*Mytilus galloprovincialis* Lamarck, 1819) and sea snail (*Rapana venosa* Valenciennes, 1846) are commercially important marine species in the Southeastern Black Sea. The sea snail is known as native species to the Japan Sea, the East China Sea, and the Bohai Sea (Tsi et al., 1983). It is also known as one of the most invasive species in the world. It is reported that sea snails first entered the Black Sea in 1946 (Drapkin, 1963; Saglam et al., 2015). They have a carnivorous feeding feature and are usually fed with sessile aquatic organisms such as mussels and oysters (Bat and Öztekin, 2016).

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Another species that has an important place among shellfish is *M. galloprovincialis*, known as Mediterranean mussel or black mussel. Mediterranean mussels, which are widely consumed all over the world, are known as one of the most cultivated species with economic value and rich in organic matter (Fuentes et al., 2009).

Mediterranean mussels are filter-feeding organisms in the water, and they have the potential to accumulate heavy metals, pesticides, pathogens, and radioactive materials from aquatic environments (Bat and Öztekin, 2016; Kacar, 2011). That is why they are one of the most important organisms that indicate their aquatic environment status. The mussels contaminated with pollutants pose a risk factor for both environmental, animal, and human health (Avşar and Berber, 2014; Terzi and Isler, 2019).

The gram-negative bacteria *Escherichia coli* is an indicator organism used to monitor fecal pollution of aquatic environments and seafood. The occurrence of *E. coli* in aquatic environments indicates the area or organisms polluted with feces with animal or human origin (Avşar and Berber, 2014; Terzi, 2018a). It is also known to carry multi-resistance genes like heavy metal resistance, antibiotic resistance, disinfectant resistance genes (Ture et al., 2020; Yang et al., 2020).

This study aimed to determine Cu, Cd, As, and Hg resistance levels and some resistance genes in *E. coli* isolated from sea snail (*R. venosa*) and Mediterranean mussel (*M. galloprovincialis*) in the Southeastern Black Sea using minimum inhibitory concentration test and molecular methods. Differentiation of resistance levels and metal resistance genes in isolated bacteria were determined between locations. By determining the MIC values of the isolates, the contamination status of the aquatic environment of the Southeastern Black Sea was revealed.

Material and Methods

Study Area, Sampling, and Isolation of E. coli

Mediterranean mussel and sea snail samples were collected from 12 coastal points of the Eastern Black Sea, Turkey (Figure 1). A total of 54 *E. coli* strains were isolated from Mediterranean mussel (n = 35) and sea snail (n = 19). Detailed information on sample collection, bacterial isolation, and identification have been given in our previously published articles (Terzi, 2018a; Terzi and Isler, 2019).

Minimum Inhibitory Concentration Test

Minimum inhibitory concentration (MIC) tests of all isolated *E. coli* strains were performed by the broth dilution method of Clinical and Laboratory Standards Institute (CLSI, 2018). In the tests, analytical grade metal(oid) salts of $CuCl_2.2H_2O$, $CdCl_2.6H_2O$, $NaAsO_2$, and $HgCl_2$ were used for copper, cadmium, arsenic, and mercury, respectively. The final concentration of 0 (Control), 12.5, 25, 50, 100, 200, 400, and 800 µg/ml were used for copper, cadmium, and arsenic; and 0 (Control), 1.56, 3.12, 6.25, 12.5, 25, 50, and 100 µg/ml for mercury in Luria Bertani (LB) Broth. After the inoculation of each isolate to media containing different concentrations of metal(oid)s, it is recorded whether bacteria can grow or not after incubation at 35°C for 24-36 hours and the lowest concentration that visibly inhibits bacterial growth is recorded as MIC. To determine resistance status, the isolates that can grow in the concentrations of 10 mM (750 µg/ml) for arsenic, 1 mM (112 µg/ml) for cadmium, 1 mM (63.5 µg/ml) for copper and 0.1 mM (20 µg/ml) for mercury were recorded as resistant (Nieto et al., 1987).

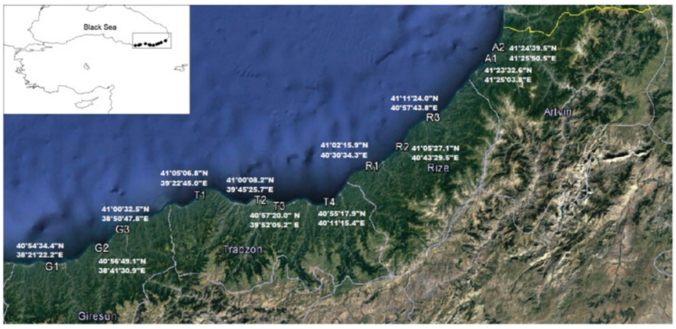

Figure 1. Sampling area

DNA Isolation and Detection of Resistance Genes by PCR

The boiling technique was used for the DNA extraction of the *E. coli* isolates for PCR assays (Boran et al., 2013; Capkin et al., 2015). The presence of *merA*, *nccA,* and *chrB* in the isolated bacterial strains was determined by PCR using the gene-specific primers shown in Table 1. Genomic DNA was used as template DNA in PCR assays.

PCR mixtures were prepared in 25 µl volumes (100 ng template DNA, 12.5 µl 2X Master Mix PCR mixture (NEB Master PCR Kit), 100 ng each of primer and sterile distilled water). Then, Thermal cycling was done with a Thermal Cycler (Biorad T100). The PCR amplification conditions consisted of initial denaturation at 95°C for 30 s; 35 cycles of denaturation at 95°C for 30 s, annealing at 55–59°C (see Table 1) for 45 s, and extension at 68°C for 45 s; and a final cycle of extension at 68°C for 90 s.

After the PCR, 10 µl of PCR mixture was subjected to electrophoresis system in 1% agarose gels prepared with 0.5 × Tris–Acetate– EDTA buffer and run at 100-110 V for 45-60 min. The size of resistance genes was estimated by 100 bp DNA

Table 1. Primers used in the PCR reactions

Target Gene	Sequencing (5′-3′)	PCR Product (bp)	Annealing Temperature (°C)	References
merA	FW"GAGATCTAAAGCACGCTAAGGC" R"GGAATCTTGACTGTGATCGGG"	1011	58	(Misra et al., 1984)
nccA	FW"ACGCCGGACATCACGAACAAG" R"CCAGCGCACCGAGACTCATCA"	450	59	(Nies et al., 1990)
chrB	FW"GTCGTTAGCTTGCCAACATC" R"CGGAAAGCAAGATGTCGATCG"	1141	55	(Abou-Shanab et al., 2007)

ladder (NEB). The gels were then stained with ethidium bromide and viewed by UV transillumination.

Results and Discussion

Metal(oid) Resistance/Sensitivity Test

A total of 54 *E. coli* strains were isolated and identified in our previous studies (Terzi, 2018a; Terzi and Isler, 2019). Of these, a total of 35 strains were isolated from Mediterranean mussels and 19 from the sea snails. The results of the MIC test showing the resistance/sensitivity levels of the isolated *E. coli* strains against Cu, Cd, Hg, and As were shown in Table 2. According to these test results, the MIC values of bacteria against copper, cadmium, arsenic and mercury ranged from 100-400 µg/ml, 100-200 µg/ml, 25-400 µg/ml and 3.125-25 µg/ml, respectively.

Table 2. Minimum inhibitory concentration results (µg/ml)

Location	Min-Max	Cu	Cd	As	Hg
Artvin	Max	400	200	200	12.5
	Min	200	100	100	3.125
Rize	Max	400	200	400	12.5
	Min	200	100	25	3.125
Trabzon	Max	400	200	400	25
	Min	100	100	50	3.125
Giresun	Max	400	200	400	25
	Min	100	100	50	3.125

According to the resistance levels of bacteria, it was determined that all the isolates were resistant to copper, followed by cadmium. No resistance to arsenic was detected in any of the bacteria. Mercury resistance (7.4%) was determined only in bacteria isolated from the stations in Trabzon and Giresun. Bacteria isolated from Rize and Artvin stations were determined to be sensitive to mercury (Table 3).

Distribution of Resistance Genes

The presence of *merA, nccA,* and *chrB* resistance genes in *E. coli* isolated from sea snail and Mediterranean mussel samples collected from the coast of Rize, Giresun, Artvin, and Trabzon provinces in the Eastern Black Sea were investigated by molecular methods. The presence of *merA, nccA,* and *chrB* genes in the isolates was shown in Table 4.

Table 3. Number of resistant (R) or susceptible (S) *Escherichia coli* strains to heavy metal(oid)s

Heavy Metal(oid)	Artvin		Rize		Trabzon		Giresun	
	R	S	R	S	R	S	R	S
Cu	4	0	13	0	18	0	19	0
Cd	1	3	5	8	8	10	11	8
As	0	4	0	13	0	18	0	19
Hg	0	4	0	13	2	16	2	17

Table 4. Distribution of resistance genes

Resistance Genes	Total (%)	Artvin (%)	Rize (%)	Trabzon (%)	Giresun (%)
N	54	4	13.0	18	19
merA	20.4	-	23.1	11.1	31.6
nccA	33.3	75	30.8	22.2	36.8
chrB	20.4	-	7.7	38.9	15.8

As a result of the research, *nccA* gene was the most common resistance gene in 33% of bacteria isolated from Mediterranean mussels and sea snails. While *nccA* gene was detected in 75% of bacteria isolated from Artvin stations, Giresun followed with 36.8%. *merA* gene responsible for mercury resistance was determined as 31.6%, 23.1%, and 11.1% at the stations in Giresun, Rize, and Trabzon, respectively and cannot be determined in Artvin station. *chrB* gene was detected the highest rate of 38.9% of *E. coli* in Trabzon. (Table 4).

Discussion

Resistance level differences against pollutants in the aquatic bacteria isolated from the different aquatic environments have been reported in various studies (Capkin et al., 2017; Terzi, 2018b). Sipahi et al. (2013) reported that all *Enterobacteriaceae* members they isolated were resistant to copper, also, almost all (99.9%) against manganese and 87.2% resistance to lead. Matyar et al. (2010) reported that all of the 356 bacteria isolated from three different stations of Iskenderun Bay (Turkey) were resistant to copper and cadmium. Similar to these studies, in this study, the MIC values of *E. coli* strains isolated from Mediterranean mussel and sea snail against copper, cadmium, arsenic, and mercury were determined as 100-400 µg / ml, 100-200 µg / ml, 25-400 µg, 3.125-25 µg/ml, respectively. All isolates were resistant to copper and this was followed by cadmium. Moreover, in this study, arsenic resistance status was not detected in any of the bacteria. Mercury resistance was determined only in bacteria isolated from the stations in Trabzon and Giresun with rates of approximately 10%. It has been determined that the bacteria isolated from the stations in Rize and Artvin are sensitive to mercury. The toxicity of some metals like zinc, copper, manganese was lower for the bacteria than mercury and arsenic. The low toxicity of zinc, copper, and manganese could play an important role in biochemical reactions of the cell as a trace element (Nies, 1999). Gedik (2018a) reported that the samples of Mediterranean mussels (*M. galloprovincialis*) collected from the same samling points had the highest metal concentrations (Cr, Cu, Mn, Pb, Zn) at the stations in Trabzon. Similarly, in this study, the resistance levels of bacteria isolated from Trabzon stations had the highest resistance compared to the other stations. This may show that coastal areas of Trabzon are more exposed to pollution than the other stations. Besides, Akçay and Moon (2004) reported that Trabzon has local pollution in the coastal areas due to the coastal mining areas and agricultural activities. Gedik (2018b) reported that heavy metal content was found higher in sea snails in the Trabzon region compared to Artvin, Giresun and Rize. Baltas et al. (2017) measured heavy metal concentrations in soft tissues of Mediterranean mussel and sea snail collected from Artvin, Giresun, Rize, and Trabzon regions. They found that Cu and Pb concentrations in sea snails had higher than mussel but lower for Zn.

Matyar et al. (2009) found that the tolerance to heavy metals in bacteria isolated from fish gills is Cd> Cu> Mn> Cr = Pb, but in bacteria isolated from intestines it is listed as Cd> Cu> Cr> Mn = Pb. Akinbowale et al. (2007) stated that this order was Cu = Pb> Mn> Cr> Zn> Co> Cd in the bacteria isolated from *Oncorhynchus mykiss*. Abou-Shanab et al. (2007) reported the resistance frequencies of the bacteria against various metal(oid)s such as Pb, Zn, Ni, Cu, Co, Cr, Cd, Hg, and As, as 100%, 100%, 100%, 98%, 93%, 53%, 42%, 29%, and 18%, respectively. Abskharon et al. (2008) found MIC values for *E.coli* bacteria against copper, cobalt, nickel, zinc, chromium, cadmium and lead metals as 1.57, 2.55, 1.7, 9.17, 0.48, 4.4, and 3.1 mM, respectively. Toroglu and Dincer (2009) reported that *E. coli* strains isolated from Aksu River showed different levels of resistance to Ni, Cd, Cu, and Cr and especially they were more resistant to high concentrations of cadmium. Similarly, in this study, we found that all of the bacteria were resistant to copper, followed by cadmium and sensitive to arsenic. Different levels of resistance to metals may be the temporal and spatial difference of bacterial isolations.

Sipahi et al. (2013) reported that the MIC values of Enterobacteriaceae members were 200, 1600, and 1600 µg/ml for copper, manganese, and lead, respectively. Gul-Seker and Mater (2009) showed that the bacterial strains isolated from the Marmara Sea show maximum resistance to Cd, Cu, and Cr, and from the Black Sea are similarly 100%, 92.3%, and 92.3%, respectively. Matyar et al. (2009) stated that bacteria in İskenderun Bay were resistant to copper and cadmium with a rate of 50.5% and 60.2%, respectively and the lowest resistance level was determined in the lead as6.5%. It can be said that the resistance levels detected in bacteria are caused by the fact that the location, life, environment, and time of the bacteria are isolated.

The presence of resistance genes, the genetic element responsible for resistance to contaminants such as heavy metal in bacteria, has been reported in several studies. Abou-Shanab et al. (2007) found that the *mer* and *ncc* metal resistance genes detected by PCR, and the bacteria are resistant to mercury and nickel. Moreover, the co-occurrence of antibiotic resistance genes and heavy metal resistance genes has been demonstrated that antibiotic resistance could correlate with heavy metal resistance. In this study, *nccA* gene was the most common resistance gene in 33% of bacteria isolated from Mediterranean mussel and sea snail. In our previous study, we determined 9 different antibiotic resistance genes in the same bacteria and it was found that 44.4% of bacteria contained plasmids (Terzi and Isler, 2019). In our both studies, the highest antibiotic resistance gene was fond as *ampC* (98 %) responsible for ampicillin resistance and the highest metal resistance gene was *nccA* (33 %) responsible for nickel, cobalt, and cadmium. The antibiotic resistance and heavy metal resistance genes of these bacteria may be found together on plasmids (Chapman, 2003).

Conclusion

Sub-lethal concentrations of metal(oid)s in the aquatic environment might induce the antibiotic resistance of bacteria (Li et al., 2019). Discharge of pollutants such as heavy metals into the aquatic environment may lead directly to heavy metal resistance, and directly or indirectly for other antimicrobial resistance such as antibiotic and disinfectant resistance. Tolerance or resistance of the bacteria to toxic pollutants including heavy metals is of significant ecological importance. These bacteria could be used for monitoring environmental metal pollution.

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Compliance with Ethical Standards

Authors' Contributions

ET designed the study and wrote the first draft of the article. ET and FC performed laboratory analyses. Both authors read and approved the final article.

Conflict of Interest

The authors declare that there is no conflict of interest.

Ethical Approval

For this type of study, formal consent is not required.

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