

Phenotypic and genotypic investigation of the heavy metal resistance in *Escherichia coli* isolates recovered from cattle stool samples

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Abstract: The purpose of this study was to examine resistance against cadmium (Cd^{+2}), copper (Cu^{+2}), lead (Pb^{+2}), mercury (Hg^{+2}), and manganese (Mn^{+2}) in *Escherichia coli* isolates recovered from cattle stool using phenotypic (agar dilution) and genotypic [polymerase chain reaction (PCR)] methods. In addition, the isolates were genotyped via enterobacterial repetitive intergenic consensus polymerase chain reaction (ERIC-PCR). *Escherichia coli* was isolated and identified from the 100 stool samples that were examined in the study. It was determined phenotypically that all isolates were sensitive to mercury; 97% of them showed resistance to cadmium; 69% showed resistance to copper; 24% showed resistance to lead; and 20% showed resistance to manganese. In the isolates found resistant by phenotypic method, the presence of the *zntA* gene provided common resistance for Cd^{+2} and Pb^{+2} ; *pcoR* gene provided resistance for Cu^{+2} ; and *mntR* gene for Mn^{+2} in both genomic DNA and plasmid DNA. Since all isolates were sensitive to Hg^{+2} , the presence of *merA* gene was not examined. While the target genes that were examined for lead, cadmium, and manganese were detected in all isolates, the *pcoR* gene for copper was detected in 53.6% of the phenotypic-resistant isolates. As a result of the statistical analysis, it was determined that the phenotypic resistance rates of the isolates did not vary according to age group, county, or city at a significant level ($P > 0.05$). The high metal resistance detected in the present study led us to conclude that heavy metal contamination around cattle farms may be common. Metals are used as an additive substance in animal husbandry. Using the correct fertilizer to minimize contamination sources or limiting the use of materials that contain metals may be useful, and new legal rules may be required.

Key words: Cattle, *E. coli*, genotyping, heavy metal resistance

1. Introduction

Heavy metals are stable, nondisposable, and nondegradable environmental contaminants [1,2]. Industrial and agricultural applications create contamination sources [3]. Firstly, heavy metals are transmitted to animals through the food chain and then to people via environmental transformation. The ecological presence of heavy metals constitutes an important threat for all life forms [4]. Although some heavy metals are trace elements that are necessary for bacterial cell walls and several structural enzymes, they become toxic for bacteria at high concentrations [5–8]. Highly-developed organisms are influenced by the toxic effects of heavy metals at a significant level. However, similar influences are not observed in many microorganisms [9] since bacteria continue their lives with the help of resistance systems they develop in environments that include intense metal

contamination [10–12]. Although metal resistance has not received the same level of attention as antibiotic resistance in veterinary medicine, it is still a significant reality.

In one sense, heavy metal resistance is believed to be an indicator of environmental heavy metal contamination [13]. It is already well known that metals are used as additives in livestock feed, and the materials that contain metals cause resistance development in bacteria. In addition, antibiotic resistance is more easily formed in metal-resistant bacteria [4,7,10,14]. Natural bacterial ecosystems constitute a very important resource for the spread of various resistance systems among bacteria [7]. The fact that there is heavy metal resistance in isolates obtained from cattle led us to consider the potential for heavy metal contamination in cattle, as well as the products obtained from them, as a result of contamination in the environment where they are raised. The purpose of

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the present study was to examine resistance to cadmium (Cd^{+2}), copper (Cu^{+2}), lead (Pb^{+2}), mercury (Hg^{+2}), and manganese (Mn^{+2}) in isolates of *Escherichia coli* (*E. coli*), a common member of intestinal flora, recovered from cattle stool. In addition, all isolates were genotyped by ERIC-PCR.

2. Materials and methods

2.1. Samples

Stool samples were collected from farms located in İstanbul, Turkey and its surrounding cities at regular intervals. The samples were taken directly from the rectum. A total of 100 stool samples from 100 different animals were examined. The isolation and identification of *E. coli* from stool samples were carried out using conventional methods [15].

2.2. Phenotypic determination of heavy metal resistance

Phenotypic determination was performed according to the agar dilution method, modified by Akinbowale et al. [16]. Briefly, heavy metal salt solutions were prepared from 12.5 $\mu\text{g}/\text{mL}$ to 6400 $\mu\text{g}/\text{mL}$. Solutions that contained Cd^{+2} (Sigma 20899), Cu^{+2} (Sigma, 469130), Pb^{+2} (Sigma, 467790), Hg^{+2} (Sigma, 203777), and Mn^{+2} (Sigma, 105934) were added to Mueller–Hinton agar (MHA) medium (Merck). The test broth media were prepared; and 2 μL of the suspensions were added to broth media that contained heavy metals, as McFarland 0.5 in physiological saline, from the strains produced in pure form in nutrient agar. Isolates grown at concentrations higher than the minimal inhibition concentration (MIC) of *E. coli* K-12, which was used as the control organism, were considered resistant.

2.3. Genotypic determination of heavy metal resistance

The Thermo Scientific GeneJET Genomic DNA Purification (K0721) kit was used for genomic DNA extraction, while the Thermo Scientific GeneJET Plasmid Miniprep kit (K0502) was used for plasmid DNA extraction. The PCR amplification was carried out both for genomic and plasmid DNA. Target gene regions that were responsible for heavy metal resistance in isolates that showed phenotypic resistance to heavy metals were tested. For this purpose, *pcoR* (copper), *zntA* (lead and cadmium), and *mntR* (manganese) were examined [6,17–22]. Since all isolates were sensitive to Hg^{+2} , the presence of *merA* (mercury) gene was not examined. The primers used are given in Table 1.

For the PCR reaction, 1X PCR buffer, 1.5 mM MgCl_2 , 0.2 mM dNTP mix, 0.4 μM primers, 0.5 U taq polymerase (Thermo Scientific, EP0402), and 5 μL target DNA were used. Then, deionized distilled water was added until the total volume became 25 μL . For thermal cycling of *zntA*, analyses were made according to Becerra-Castro et al.

[21], and for *pcoR*, the analyses were made according to Chihomvu et al. [11]. As all the isolates were sensitive to mercury, the *merA* gene was not analyzed. For *mntR*, PCR optimization was carried out in the following context. DNA amplification was obtained with one cycle of predenaturation at 95 °C for 10 min, followed by 35 cycles of denaturation at 95 °C for 1 min, annealing at 5 min at 58 °C for 1 min, and extension at 72 °C for 90 s. The final extension was carried out at 72 °C for 8 min. *Escherichia coli* pMG101 (The University of Edinburgh, School of Biological Sciences), *E. coli* DH5- α (Ankara University, Faculty of Veterinary Medicine), and *E. coli* K-12 ATCC 53678 strains were used as positive controls.

2.4. Enterobacterial repetitive intergenic consensus polymerase chain reaction (ERIC-PCR)

For molecular typing of the isolates recovered from cattle stool samples, the ERIC primers 1R (5'-ATG TAA GCT CCT GGG GAT TCA C-3') and 2 (5'-AAG TAA GTG ACT GGG GTG AGC G-3') were used [23]. The evolutionary tree of the resistant isolates was constructed and evaluated by GelCompar II 6.6.11 gel electrophoresis software (Applied Maths), according to the ERIC-PCR gel image.

2.5. Statistical analysis

For the purpose of comparing the phenotypic resistance rates of the isolates age group, as well as the county and city in which isolations were carried out, were taken into consideration, and the chi-square (χ^2) test was applied using SPSS 13.0 package program (Table 2).

3. Results

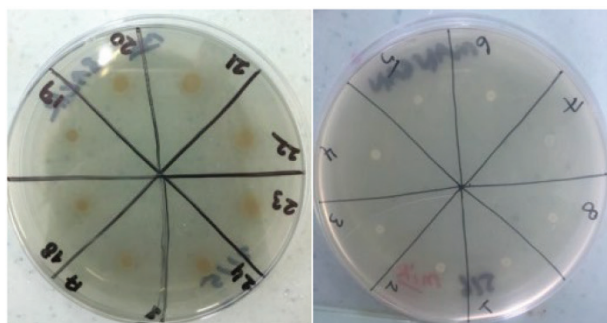
One hundred *E. coli* strains were isolated and identified from the 100 stool samples that were examined in the context of the study. One strain was isolated from each sample. All of the isolates showed resistance to mercury in phenotypical terms: 97% showed resistance to cadmium, 69% showed resistance to copper, 24% were sensitive to lead, and 20% were sensitive to manganese. Reproduction on the heavy metal test agar medium is shown in Figure 1. Since all isolates were sensitive to Hg^{+2} , *merA* gene was not examined in this study. The amplification process was only conducted on phenotype-resistance isolates. To determine the genotypic resistance, both genomic DNA and plasmid DNA were investigated in a separate manner, and it was determined that some isolates had the relevant gene only in the DNA or only in its plasmid (Table 3). When the positivity status was considered, while *zntA* and *mntR* genes were detected at a rate of 100% in all isolates that showed phenotypical resistance (in plasmids only, DNA only, or in both), the target gene *pcoR* was detected in only 53.6% of isolates that showed phenotypical resistance to Cu^{+2} (Figures 2–7).

Table 1. Oligonucleotide primers used in the study.

Target gen	Forward primer Reverse primer (sequence (5'-3'))	Size (bp)	Reference
<i>mntR</i>	TAAACACGCGCATACACCTCTTG	708	19
	GCGTGCGTAAAAAAGGCAGGCTC		
<i>zntA</i>	ATCGTCCGCTCGCTGTATCTCT	2375	20,21
	CCGCCTTTCCCTCACCTAACC		
<i>pcoR</i>	CAGGTCGTTACCTGCAGCAG	636	11,22
	CTCTGATCTCCAGGACATATC		

Table 2. The parameters used in statistical analyses.

Parameters	
Age	Group1 (age 0–2), group 2 (age 3–5), group 3 (age 6–8)
Town	Avçılar, Arnavutköy, Çatalca, Çerkezköy, Vize, Lüleburgaz
City	İstanbul, Kırklareli, Tekirdağ

**Figure 1.** Bacterial growth on MHA.

According to ERIC-PCR results, heterogeneity was detected between genomic DNA and plasmids. Dendrograms for the isolates are shown in Figures 8 and 9. At the end of the statistical analysis, it was determined that the differences among the phenotypic resistance rates of the isolates were not statistically significant according to the age group or the county and city where farms were located ($P > 0.05$).

4. Discussion

The resistance profiles of heavy metals vary among studies according to the heavy metal studied, bacteria, methodology, and geographical differences, etc.

In this study, 100% of the isolated bacteria were sensitive to mercury. The reason for this may be that mercury is more toxic than any other heavy metal, and its

presence as an environmental pollutant is less common. Nies [1] and Yavuz and Sarıgül [24] reported that the affinity of mercury in thiol groups was greater, when compared to cadmium; and for this reason, it was more toxic for *E. coli* cells.

In another study, Walsh and Caslake [25] identified 160 *E. coli* isolates that were sensitive to mercury from a lake in the United States and 145 isolates that were resistant to mercury. However, Moller et al. [26] reported only 71 among 791 bacteria isolates that were resistant to mercury. Balık-Tekin [27] examined heavy metal resistance profiles by isolating *E. coli* from chicken stools and reported that only 2.8% of isolates were resistant to mercury. The results reported in Tekin's study were parallel to the results in this study for mercury; however, there are no similarities for cadmium. The highest resistance reported in this study was against Cd^{+2} ions (97%). In the study conducted by Balık-Tekin [27], the samples were obtained from integrated facilities, and the hens had very low contact with the outer environment; this may account for the difference. Soils that are exposed to intense agriculture are subject to high heavy metal contamination levels. Phosphate fertilizers, in particular, are important contributors to cadmium contamination. Although many countries have imposed limitations on the Cd content of these fertilizers, there has been accumulation over many years, and the heavy metals in fertilizer granules are not degradable in nature [3,24,28]. In addition, Cd contamination is common because it is used to color plastic and ceramics, and rechargeable batteries contain Cd [24]. Deredjian et

Table 3. Resistance rates of isolates.

	Phenotype-resistant isolate count (n)	Genotype-resistant isolate count (n)			Phenotype resistance %	Genotype resistance %
		DNA	Plasmid	Total (n)		
Mn	20	20	20	20	20	100
Pb	24	23	24	24	24	100
Cu	69	22	32	37	69	53.6
Cd	97	92	89	97	97	100
Hg	0	-	-	-	0	-

-: not investigated



Figure 2. PCR results for *zntA* in plasmid DNA. M: Marker (100-3000 bp), 1: positive control, 2: negative control 3-14: samples

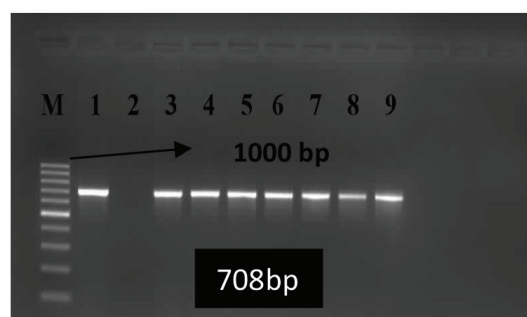


Figure 4. PCR results for *mntR* in genomic DNA. M: Marker (100-1000bp), 1: positive control, 2: negative control, 3-9: samples

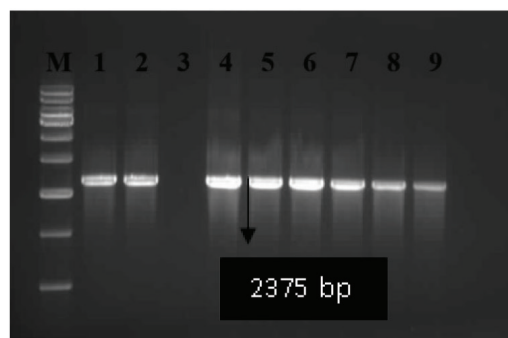


Figure 3. PCR results for *zntA* in genomic DNA. M: Marker (500-10000bp), 1-2: positive control, 3: negative control, 4-9: samples

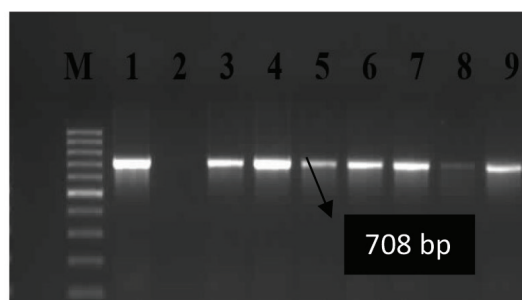


Figure 5. PCR results for *mntR* in plasmid DNA. M: Marker (100-1000bp), 1: positive control, 2: negative control 3-9: samples

al. [13] detected high cadmium resistance in bacteria that were isolated from external areas, and reported that the study team conducted qualitative and quantitative analyses on chemical contaminants in the environment.

In the studies conducted by Hacioglu and Tosunoglu [29] the resistance rate was 28.75% for manganese, which

supports the findings in the present study. Manganese toxicity is lower compared to resistance to other heavy metals. In resistance development, manganese contamination in the environment is observed at an extremely high level. The low manganese resistance rate may be due to this factor. However, manganese is an indispensable element in modern fertilizers and is used as an alloying element that increases steel durability;

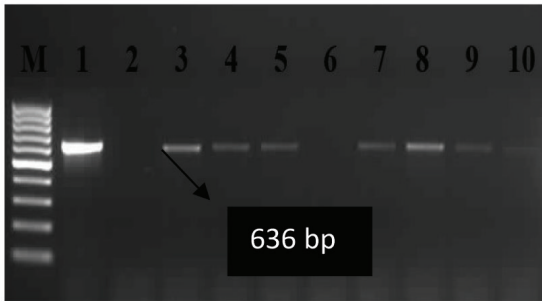


Figure 6. PCR results for *pcoR* in genomic DNA. M: Marker (100-1000bp), 1: positive control, 2: negative control 3-10: samples

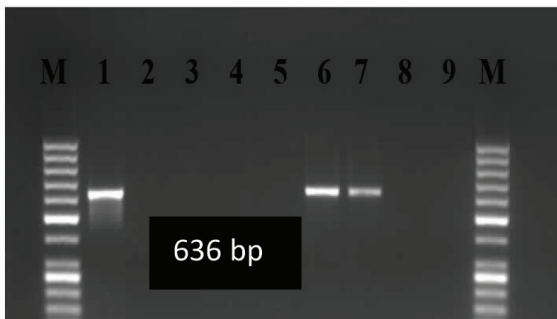


Figure 7. PCR results for *pcoR* in plasmid DNA. M: Marker (50-1000bp), 1: positive control, 2: negative control 3-9: samples

therefore, environmental pollution was encountered in some areas [30]. Seiler and Berendonk [4] reported that agricultural practices and the use of metals as additives in animal feeds causes pollution on a worldwide level, which explains the higher copper resistance (69%) found in this study. Compounds that contain copper and sulfate are used for disinfection in agriculture and animal breeding, and there is a high level of copper accumulation in nature [12]. Jacob et al. [31] reported that there were high resistance rates to copper in *E. coli* and *Enterococcus* spp. they isolated from cattle that were given feeds containing high levels of trace elements. Yazdankhah et al. [7] reported that enteric bacteria in farm animals were resistant to trace elements used as feed additives, and copper resistance was frequently observed in *E. coli*. Cavaco et al. [32] determined that isolates from pigs showed resistance to Cu^{+2} at a rate of 66%. In the current study, resistance to lead was 24%. Hölzel et al. [10] detected lead resistance in *E. coli* isolated from pig urine.

In the present study, 4 isolates were resistant to all metals (Cd, Cu, Mn, and Pb) except mercury, and 79% of these isolates were resistant to more than one metal. Resistance in the bacteria is caused by chromosomal, plasmid, and transposon-coded systems, and this may

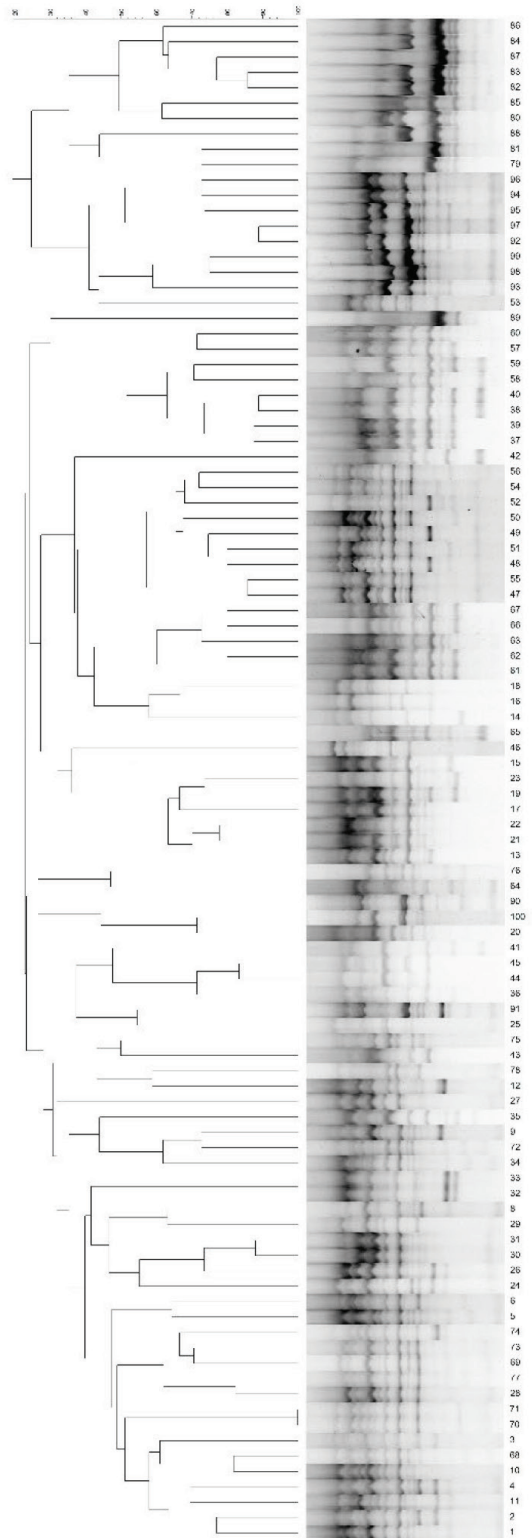


Figure 8. Dendrogram and genomic DNA fingerprinting profiles of isolates.

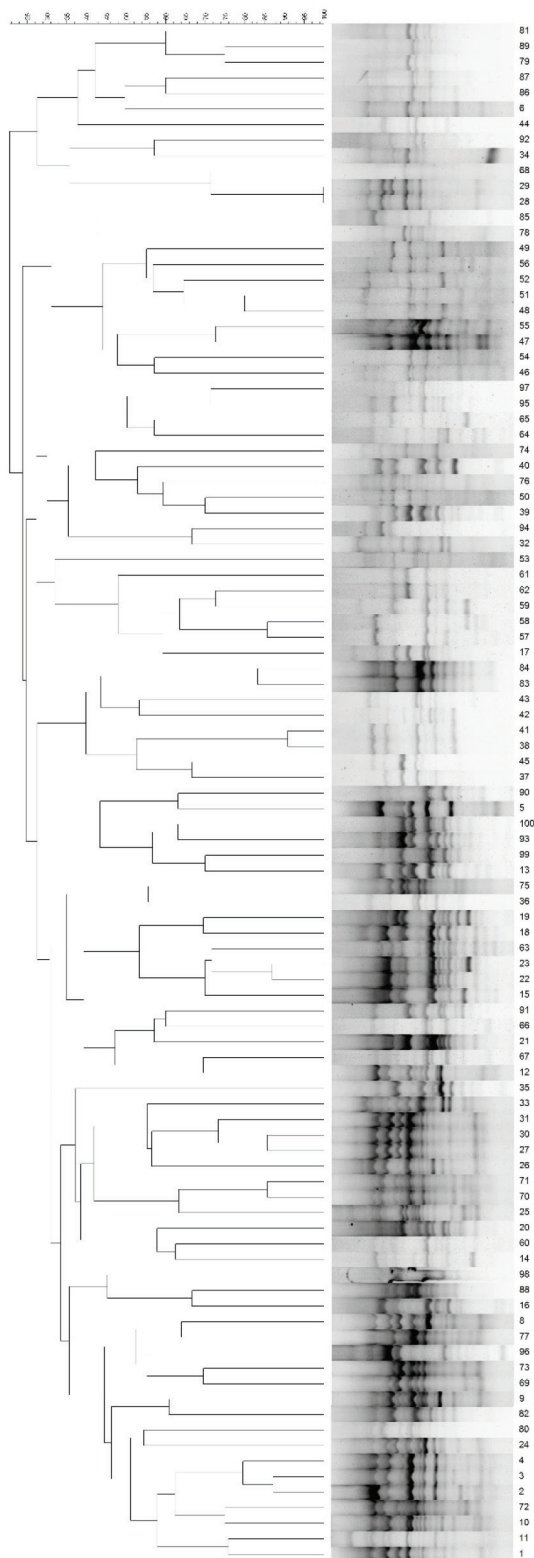


Figure 9. Dendrogram and plasmid DNA fingerprinting profiles of isolates.

account for the high levels of metal resistance. However, the high resistance rates that were determined in the present study show that the natural bacterial ecosystem is extremely significant in terms of the distribution of resistance systems among species. Yazdankhah et al. [7] emphasized that both ecosystems and the microbiota played a role in the horizontal transfer of some resistance genes that was extremely important.

The *zntA* gene, which provides a common cadmium-lead resistance in *E. coli*, was detected in 100% of the bacteria determined to have phenotypic resistance [17,20]. Becerra-Castro et al. [21] conducted a study and reported *zntA* gene in 34.3% of isolates. The *zntA* gene was detected in all resistant bacteria in the present study; however, the difference in phenotypic resistance observed for lead and cadmium stems from the fact that the N-terminal portion of the P-type ATPase protein which provides the efflux pump needs additional ligands [17].

The *mntR* gene was detected both as plasmid and as genomic DNA in all 20 bacteria that were phenotypically resistant to manganese. Waters et al. [6] reported that the *mntR* gene acted as the primary sensor and transducer of manganese excess in *E. coli*. Patzer and Hantke [19] identified the protein that regulated the metal for manganese that is encoded by the *mntR* gene in *E. coli*.

In the present study *pcoR*, which was examined as the target gene for copper, was not detected in all of the resistant isolates. There may be other genes responsible for resistance in bacteria that show phenotypic resistance and are missing the relevant gene. More than one operon and gene are responsible for copper resistance in bacteria [8,12]. Although Chihomvu et al. [11] detected *pcoR* gene in the *E. coli* isolates in an aquatic system of industrial pollutants, Dell'Amico et al. [5] detected phenotypic resistance to copper in bacteria isolated from soil. However, he could not detect the presence of the *pcoR* gene using the PCR method and reported that there might be other genes responsible for resistance. In studies conducted in recent years, 19 different genetic elements were detected for copper in one single bacterium [8].

The heterogeneity that was detected according to ERIC-PCR results in DNA and plasmids shows that the relevant genes have a wide distribution. In addition, there were no differences at statistically significant levels, because the *E. coli* strains examined in the study were all obtained from areas where intensive agriculture and industrial activities were carried out. There were no significant differences detected in the statistical analyses considering age groups, which shows that age is not an important variable for this study. And this may demonstrate that heavy metal exposure began in a much earlier period in the lives of the cattle.

Along with the metal resistance they develop, bacteria are beneficial as cleaners of soils contaminated with

metals; however, this also poses a potential risk for public health as it contributes to the development of antibiotic resistance. In addition, for the purposes of determining levels of ongoing heavy metal pollution, metal resistance has become the determining factor. For this reason, the high metal resistance detected in the present study suggests that heavy metal contamination around cattle farms might be common. The farms that provided cattle for the present study were located in areas that practiced intensive livestock, agricultural, and industrial activities. Materials containing heavy metals and used for disinfection purposes in animal husbandry cause environmental contamination and pose a risk to animal health [7,10,12,33,34]. However, the heavy metals that phosphorus fertilizers and feeds contain might also be the source of contamination, and, therefore, resistance [4,28]. Heavy metal resistance is very important because it provides information in multiple areas that concern public health. There is a need for future studies conducted for quantitative analysis of the heavy metals that exist in environments where cattle are kept. However, the fact that no Hg resistance was detected in any of the isolates was promising. The genotypic findings

obtained in the present study point to a contribution to the gene pool for heavy metal resistance in Turkey. Future studies conducted in genotypic fashion on bacteria that do not show phenotypic resistance will be important for filling this information gap.

The high heavy metal resistance that was detected in the present study has revealed a public health risk. In addition to raising awareness about environmental contamination, the purpose of the present study was to draw attention to the importance of using suitable fertilizers in agriculture, to the ongoing addition of metals as additives to feeds, and the use of materials that contain metals for various purposes in animal husbandry. In addition, metal resistance genes and mechanisms that are common among bacteria create the risk of antibiotic resistance. For this reason, new regulations governing the use of metals in animal husbandry are needed.

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