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Original Research

Comparison of Heavy Metal and Disinfectant Resistance of *S. aureus* and *Enterococcus* Isolates with Antibiotic Resistance Profiles

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Abstract

Antimicrobial resistance is one of the most significant threats to our present and future. Recently, it has been suggested that antibiotic-resistant microorganisms also exhibit resistance to heavy metals and disinfectants, and these resistance profiles may interact with each other. Microorganisms can be exposed to heavy metals and disinfectants in different ways in various environments. They are especially likely to be exposed to quaternary ammonium compounds used in the food industry or heavy metals due to tap water. Thus, microorganisms can adapt to their environment and gain resistance thanks to horizontal gene transfer. In our research, the heavy metal and disinfectant resistance profiles of 42 isolates whose antibiotic resistance profiles were determined in previous studies revealed the connection between the antibiotic - heavy metal - disinfectant resistances of the isolates. The resistance of the isolates to various heavy metals and disinfectants was determined by

determining the minimum inhibition concentrations (MIC) using the broth microdilution method. MIC values obtained from the isolates were compared with those of the control well, and the resistance profiles of the isolates were determined. The isolates used in our research were found to be highly resistant to cobalt (83%), nickel (86%), and triclosan (81%). It has been observed that they are utterly resistant to zinc and lead. Based on the data obtained from the study, it is thought that the high rate of disinfectant and heavy metal resistance may be related to antibiotic resistance. However, more comprehensive studies are needed to understand this relationship fully.

Keywords
Heavy metal poisoning; antimicrobial agents; disinfectants; vancomycin-resistant enterococci

1. Introduction

According to the World Health Organization, the emergence and dissemination of antibiotic-resistant bacteria represent a significant threat to human health, jeopardizing modern medical practices and potentially becoming the leading cause of mortality by 2050 [1]. The loss of therapeutic efficacy of existing antibiotics, the continued decline in the production of new antibiotics, and the limited success of measures implemented to reduce the spread of antibiotic resistance highlight the need to reconsider our approach to the problem of antibiotic resistance [2, 3].

Antibiotic resistance arises from inherent resistance, spontaneous genetic mutations, the direct uptake of antibiotic-resistance genes through mobile genetic elements, and/or the spread of resistant strains driven by environmental pressures from antibiotics or other antimicrobial agents [4]. Bacteria encounter various antimicrobial agents like antibiotics, heavy metals, disinfectants, and organic solvents in their natural habitats and human environments such as the food industry. These antimicrobial agents exert pressure on bacteria, compelling them to evolve mechanisms to withstand and resist these stressors [5].

Microorganisms encounter elevated levels of heavy metals in their environment through wastewater, mining residues, and agricultural and industrial wastes. Some heavy metals are frequently used as additives in animal feed. Copper (Cu) and zinc (Zn), which are used as growth promoters in animal feed [6], and iron (Fe) and cobalt (Co), which are frequently used in livestock and fish feed, can be given as examples of these heavy metals [7]. On the other hand, disinfectants are extensively employed to manage infections and microbial contamination in food production facilities and environments. Resistance to these substances develops in bacteria regularly exposed to disinfectants at sublethal concentrations [8]. Among these, disinfectants from the quaternary ammonium compounds (QAC) class, such as benzalkonium chloride, cetylpyrinidium chloride, didecyldimethylammonium chloride (DDCA), N-alkyl dimethyl benzyl ammonium chloride, are frequently preferred in the food industry [9]. The widespread misuse of heavy metals and disinfectants in food environments exerts selective pressure on bacteria and contributes to the emergence of resistant bacteria [10].

It has been determined that the relationship between antibiotic resistance and microbial acquisition of heavy metal resistance is primarily governed by two main mechanisms: co-resistance and cross-resistance [11, 12]. Co-resistance happens when genes responsible for resistance traits are located on the same mobile genetic elements, such as plasmids, transposons, and integrons. Mobile genetic elements act as physical carriers, facilitating gene transfer between microorganisms during conjugation [11]. Upon cessation of necessity, mobile genetic elements may be excreted from the cell and enter other cells within the system. Furthermore, alongside antibiotic and heavy metal genes, numerous studies have demonstrated the presence of disinfectant resistance genes on these same mobile genetic elements [9, 13-15]. Cross-resistance occurs when different antimicrobial agents, such as heavy metals and disinfectants, affect the same cell and trigger common pathways that eventually lead to cell death [11].

While antibiotic, heavy metal, and disinfectant resistance mechanisms appear to operate independently, research has shown that these mechanisms can interplay and influence each other [4]. An efflux pump, a fundamental defense mechanism of the cell, can confer resistance to multiple antimicrobials through a single resistance mechanism [16]. These pumps are responsible for expelling a variety of toxic substances from the cell, including metals, disinfectants, and pesticides. For instance, the AcrAB efflux system, which mediates the excretion of numerous antibiotics, contributes to increased antibiotic resistance in the presence of Cu. Oxidative stress caused by copper causes the expression of proteins such as SoxS in *Escherichia coli*, which can lead to antibiotic resistance [17]. Likewise, class 1 integrons are frequently co-located with antibiotic-resistance gene cassettes and metal-resistance genes [7]. Class 1 integrons have also been reported to function in the presence of contaminants [18]. Similarly, numerous studies have demonstrated that resistance to disinfectants, particularly QACs, can trigger antibiotic resistance, analogous to metal resistance [9, 19, 20]. Understanding the resistance of *S. aureus*, *Enterococcus faecium* and *Enterococcus faecalis*, which can be assumed as indicators in the detection of bacteriological contamination in both the environment and food due to exposure to heavy metals and disinfectants, and the relationship between them will be important for both the environment, food safety and public health.

Based on the information above, our study aimed to detect heavy metal and disinfectant resistance in Methicillin-resistant *Staphylococcus aureus* (MRSA), Linezolid-resistant *S. aureus*, Vancomycin-resistant *S. aureus*, and Vancomycin-resistant *Enterococcus* isolates, which had not been previously investigated and were only beginning to gain attention in the literature. This study endeavored to contribute a novel and original research perspective to the existing body of knowledge. Furthermore, the results obtained from this study enhanced the understanding of the minimum inhibitory concentrations (MIC) for heavy metal and disinfectant resistance, potentially informing future international reports.

2. Materıals and Methods

2.1 Bacterial Strains

A total of 42 strains, isolated in previous studies and whose antibiotic resistance profiles were determined, were included in the study to investigate heavy metal and disinfectant resistance. Methicillin-resistant *S. aureus* (MRSA) (n = 28), Methicillin, vancomycin and linezolid-resistant *S. aureus* (n = 1), methicillin and vancomycin-resistant *S. aureus* (n = 2), methicillin and linezolid

resistant *S. aureus* (n = 3) isolates were isolated from animals with subclinical mastitis [21] *Enterococcus* isolates (*vanM*-type genotypic resistance, n = 8) were isolated from cattle and sheep feces [22]. The standard strain of *S. aureus* ATCC 25923 was used on a positive control well [19].

2.2 Determination of Heavy Metal MIC Values

The broth microdilution method, as recommended by the Clinical and Laboratory Standards Institute (CLSI), was utilized to determine the MIC of the isolates against the heavy metals cobalt ($CoCl_2$), zinc ($ZnCl_2$), cadmium ($CdCl_2$), copper ($CuCl_2 \cdot 2H_2O$), mercury ($HgCl_2$), nickel ($NiCl_2$), and lead ($PbCl_2$) [23]. Stock solutions were prepared by dissolving heavy metal salts in distilled water and subsequently sterilized by filtration through 0.22 µm Millipore filters. The concentrations of heavy metals used to determine the MIC values of heavy metals in the isolates were kept between 6.25 and 3200 µg/ml [24]. As an exception for mercury, the concentration range is 0.78 to 400 µg/ml [25].

The isolates were suspended in Mueller Hinton Broth overnight and incubated at 37°C. Final inoculations for each isolate were prepared according to the 0.5 McFarland standard (using the Biosan Den-1B McFarland Densitometer), resulting in a final inoculum of 5×10^5 CFU/ml [26]. 50 µl of the specified dilutions of heavy metal solutions and 50 µl of bacterial suspension were added to each well. Following inoculation, the plate was covered with a transparent film and incubated at 37°C for 24 hours. After incubation, it was ensured that there was proper growth in the positive control well and no growth in the negative control well, and the results were evaluated. The MIC value was recorded as the lowest concentration at which the heavy metal solution inhibited growth [27]. The experiment was performed in triplicate. Isolates with MIC values higher than standard strains were considered resistant.

2.3 Determination of Disinfectant MIC Values

The broth microdilution method assessed resistance to the disinfectants benzalkonium chloride, cetylpyridinium chloride, and triclosan [23]. The concentrations of disinfectants used to establish the MIC values for the isolates ranged from 0.125 to 1024 mg/L [14, 27]. The method was applied as described in the "Determination of heavy metal MIC values" section above. The experiment was performed in triplicate. MIC values obtained from the isolates were compared with the control wells and the resistance profiles of the isolates were determined. Isolates with MIC values higher than standard strains were considered resistant.

3. Results

Phenotypic resistance profiles of 42 *Enterococcus* and *S. aureus* isolates against various heavy metals and disinfectants were determined. Based on the identified phenotypic resistance profiles for heavy metals, all isolates exhibited resistance to zinc and lead, with MIC values of 3200 µg/mL. In addition, isolates exhibiting MIC values of 3200 µg/mL for cobalt and nickel were also identified. On the other hand, the isolates showed the lowest phenotypic resistance to mercury, with an MIC value of 6.25 µg/mL.

According to the phenotypic resistance profiles for disinfectants, the isolates showed the lowest phenotypic resistance to benzalkonium chloride, with MIC values of 2 µg/mL for 25 out of 42 (59.5%)

isolates, and MIC values ranged from 2 to 8 μg/mL. The highest phenotypic disinfectant resistance was seen in triclosan. Thirty-two (76.1%) isolates exhibited resistance to triclosan, and their MIC values ranged from 2 to 32 μg/mL. Additionally, 4 of these isolates' MIC values were determined to be 32 μg/mL.

Table 1 presents the isolates' MIC values (μg/ml). The isolates' phenotypical resistance to antibiotics is also included to compare with their heavy metal and disinfectant resistance. Table 2 and Table 3 give the number of isolates according to heavy metal and disinfectant MIC values (μg/ml).

Table 1 The phenotypical resistance to antibiotics and the MIC values of the isolates (μg/ml).

	Isolate number	M	V	L	Co	Zn	Cd	Cu	Hg	Ni	Pb	BC	Tri	CC
Staphylococcus aureus	8b	+	-	-	400	3200	100	800	12.5	800	3200	2	2	2
	10b	+	-	-	800	3200	100	800	12.5	1600	3200	4	8	2
	11a	+	-	-	1600	3200	100	800	12.5	1600	3200	4	8	32
	15a	+	-	-	1600	3200	400	1600	12.5	1600	3200	2	4	2
	16	+	-	-	800	3200	50	800	6.25	3200	3200	8	8	2
	17b	+	-	-	1600	3200	50	800	6.25	1600	3200	4	32	2
	19a	+	-	-	400	3200	50	800	12.5	1600	3200	4	4	2
	20a	+	-	-	1600	3200	12.5	800	50	1600	3200	4	32	16
	25b	+	+	+	800	3200	200	800	25	1600	3200	4	4	2
	26a	+	+	-	800	3200	100	800	25	1600	3200	2	8	2
	26b	+	-	-	1600	3200	100	1600	12.5	3200	3200	4	8	2
	29a	+	-	+	1600	3200	100	800	12.5	1600	3200	2	4	4
	29b	+	-	-	800	3200	200	800	25	1600	3200	4	8	2
	31a	+	-	-	800	3200	100	800	12.5	1600	3200	2	4	2
	31b	+	-	-	400	3200	25	800	50	800	3200	4	4	2
	32b	+	-	+	400	3200	100	800	25	800	3200	2	4	2
	38a	+	-	-	400	3200	100	800	12.5	800	3200	4	8	2
	39a	+	-	-	400	3200	100	800	12.5	1600	3200	2	8	8
	41a	+	-	-	3200	3200	100	800	12.5	800	3200	2	4	8
	46b	+	-	-	400	3200	100	800	12.5	800	3200	4	32	8
	48a	+	-	-	400	3200	100	800	12.5	1600	3200	4	4	2
	57	+	-	-	1600	3200	100	800	50	1600	3200	4	8	2
	62	+	-	-	1600	3200	100	800	12.5	1600	3200	2	8	4
	65	+	-	-	800	3200	200	800	25	1600	3200	2	8	2
	89b	+	-	-	1600	3200	200	800	12.5	3200	3200	4	8	2
	99	+	-	-	1600	3200	100	800	25	3200	3200	2	8	2
	100	+	+	-	800	3200	100	800	25	1600	3200	2	8	8
	101b	+	-	+	1600	3200	400	800	25	1600	3200	2	16	2
	102a	+	-	-	1600	3200	100	800	6.25	1600	3200	2	8	2
	104b	+	-	-	800	3200	200	800	12.5	3200	3200	2	16	2

Enterococcus	107a	+	-	-	1600	3200	200	800	12.5	3200	3200	2	16	4
	109a	+	-	-	1600	3200	100	800	12.5	3200	3200	2	32	2
	115b	+	-	-	1600	3200	50	800	6.25	1600	3200	2	16	2
	120b	+	-	-	1600	3200	50	800	25	3200	3200	2	16	4
	ATCC 25923				400	3200	100	800	50	800	3200	4	4	16
	K98b	-	-	-	1600	3200	200	800	12.5	1600	3200	8	16	2
	S1a	-	-	-	1600	3200	400	800	12.5	1600	3200	2	16	8
	S32	-	-	-	800	3200	200	800	25	3200	3200	2	8	2
	K73b	-	-	-	1600	3200	100	800	25	1600	3200	2	16	2
	S33a	-	-	-	1600	3200	50	800	25	1600	3200	2	8	2
	K14a	-	-	-	1600	3200	400	800	6.25	1600	3200	2	8	2
	K31a	-	-	-	800	3200	400	800	12.5	1600	3200	8	8	2
	K23a	-	-	-	1600	3200	50	800	12.5	1600	3200	2	8	2

M: Methicillin; V: Vancomycin; L: Linezolid; Co: Cobalt; Zn: Zinc; Cd: Cadmium; Cu; Copper; Hg: Mercury; Ni: Nickel; Pb: Lead; BC: Benzalkonium Chloride; Tri: Triclosan; CC: Cetylpyrinidium Chloride.

Table 2 Number of isolates according to heavy metal MIC values (µg/ml).

	1.56	3.12	6.25	12.5	25	50	100	200	400	800	1600	3200	n	%
Cobalt									8	11	22	1	34	81
Zinc												42	42	100
Cadmium				1	1	7	20	8	5				13	31
Copper										40	2		2	5
Mercury			5	22	12	3							0	0
Nickel										6	27	9	36	86
Lead												42	42	100

n: Number of the resistant isolates; %: Percentage of the resistant isolates.

Table 3 Number of isolates according to disinfectant MIC values (µg/ml).

	0.5	1	2	4	8	16	32	64	128	256	512	1024	n	%
Triclosan			1	9	20	8	4						32	76
BC			25	14	3								3	7
CC			31	4	5	1	1						1	2

BC: Benzalkonium Chloride; CC: Cetylpyridinium Chloride n: Number of the resistant isolates; %: Percentage of the resistant isolates.

4. Discussion

Within the scope of this study, phenotypic resistance profiles against various heavy metals and disinfectants were determined for 42 isolates using the broth microdilution method. According to the identified phenotypic resistance profiles to heavy metals, all isolates resist lead and zinc. Also, high resistance prevalence was observed with MIC values (3200 µg/mL) for cobalt (81%) and nickel (86%). Nickel is found in diets containing certain feed crops, such as corn, wheat, and barley, as well

as in hydrogenated vegetable oils [28]. Thus, exposure to nickel in livestock varies depending on the animal species and dietary habits. Average dietary exposure to nickel has been reported to be 61 µg/kg in broilers, 79 µg/kg in laying hens, and 21 µg/kg in dairy cows [29]. On the other hand, cobalt is an essential trace element used in the ration of dairy cows, playing a crucial role in synthesizing vitamin B12 (cobalamin) necessary for various physiological processes in cattle. It is reported to provide sufficient amounts of vitamin B12 to dairy cows, prevent infections, and enhance calf immunity [30]. Considering the exposures to cobalt and nickel, resistance to these heavy metals can be expected in *Enterococcus* and *S. aureus* isolates. At the genetic level, resistance to these metals may involve the activation of efflux pump systems like the *Czc* system, known for exporting toxic ions like cobalt and nickel from bacterial cells, thereby reducing intracellular concentrations [31]. Additionally, other resistance operons such as *pco* and *mer*, involved in copper and mercury resistance, might confer multi-metal resistance, including cobalt and nickel, due to overlapping regulatory pathways [32].

Determining MIC values for wild-type isolates is essential as it offers insights into resistance profiles within the study population [33]. Moreover, determining the MIC values of these isolates is essential for establishing effective antimicrobial concentrations in food-related environments. In this study, MIC values against two distinct QACs were determined for 42 isolates of *Enterococcus* and *S. aureus*, yielding MIC values ranging from 2-8 mg/L for BC and 2-32 mg/L for CC. Benzalkonium chloride (BC) is the most documented QAC among the tested disinfectants; however, there is limited data on resistance to other QACs. Compared with a study by Cufaoglu et al. [10] that determined MIC of QAC values in *E. coli* isolates, our findings suggest that *S. aureus* isolates exhibit higher MIC levels against QACs. This could be attributed to subclinical mastitis-related *S. aureus* isolates have been exposed to a greater variety of QAC preparations. Furthermore, the disinfectants examined in the study are commonly used in food-related environments in Turkey and globally [34]. Thus, these data provide significant insights into disinfectant resistance in Turkey.

This study observed that 76% (32/42) of the isolates exhibited resistance to triclosan, 7% to BC, and 2% to CC. Moreover, 86% of the isolates showed phenotypic resistance to nickel, 81% to cobalt, and 31% to cadmium. The isolates did not demonstrate phenotypic resistance to mercury. A study conducted in Turkey reported notably higher levels of heavy metal resistance among *E. coli* isolates isolated from cattle fecal samples compared to our findings [35]. Differences in heavy metal resistance among fecal-derived *E. coli* and milk-derived *S. aureus* bacteria may be due to species susceptibility and adaptation to environmental niches. Additionally, very low MIC values for heavy metals were observed in fecal-derived *Enterococcus* isolates used in our study. This *Enterococcus* isolates from fecal origins and *S. aureus* strains from subclinical mastitis occupy different ecological niches due to their origins. Variations in MIC values for heavy metals in these niches may influence the selection of specific resistance mechanisms indirectly.

In the study, it is noteworthy that isolates resistant to multiple antimicrobial agents (heavy metals and disinfectants) also exhibited resistance to various antibiotics (methicillin, vancomycin, and/or linezolid). Specifically, it was found that a *S. aureus* isolate resistant phenotypically to vancomycin and linezolid (25b) and another isolate resistant to linezolid and methicillin (101b) demonstrated resistance to 6 different heavy metals or disinfectants. Additionally, isolates of *S. aureus* resistant to vancomycin and linezolid (26a and 100), and to linezolid and methicillin (29a) were resistant to 3 different heavy metals or disinfectants. Remarkably, nickel, cobalt, and triclosan resistance were commonly observed in all isolates with multiple antimicrobial resistance. This

association is believed to be linked to the frequent use of nickel, cobalt, and triclosan in animal husbandry. Supporting this finding, it has been suggested that resistance developed against heavy metals and disinfectants can contribute to the development of antibiotic resistance through shared and cross-resistance mechanisms.[4] The use of metal-containing compounds in animal feed is also noted to contribute to the selection of MRSA [36] potentially.

Enterococcus isolates exhibited resistance to 5 or more different heavy metals or disinfectants. Given the presence of vancomycin-variable enterococci (VVE) isolates—Enterococci that display no phenotypic resistance to vancomycin despite possessing genotypic resistance—the data from this project could help address a gap in the literature. Significantly, *Enterococcus* isolates exhibited a higher prevalence of resistance to cobalt, cadmium, nickel, benzalkonium chloride, and triclosan than *S. aureus* isolates. On the other hand, it is noteworthy that only one *S. aureus* isolate exhibited resistance to cetylpyridinium chloride, and two isolates were resistant to copper, whereas all *Enterococcus* isolates were sensitive to cetylpyridinium chloride and copper. It was determined that all isolates were resistant to lead and zinc while being sensitive to mercury.

Numerous studies investigate the relationship between heavy metals, disinfectants, and antibiotics [19, 37-42]. It can be stated that within the survey, isolates exhibited higher resistance to heavy metals compared to disinfectants, with triclosan being particularly notable for its widespread resistance among the isolates. Triclosan is a disinfectant used to control or prevent bacterial contamination and animal infections. However, its use has raised concerns in public health due to potential environmental and health impacts. Studies have found that triclosan, although not an antibiotic, possesses antibacterial properties, and there is evidence suggesting that its use may contribute to the development of antibiotic resistance. Triclosan and certain antibiotics target specific enzymes involved in cell membrane synthesis in bacterial cells. The concern is that bacteria exposed to triclosan may develop resistance not only to triclosan itself but also to some antibiotics with similar mechanisms of action. Studies have shown that triclosan exposure can lead to cross-resistance to antibiotics in some bacteria. This implies that bacteria resistant to triclosan may also exhibit reduced susceptibility to certain antibiotics such as tetracycline, kanamycin, ampicillin, and chloramphenicol [43]. Triclosan is typically found in low environmental concentrations due to its widespread use. Continuous low-level exposure to triclosan in the environment may contribute to the selection and survival of bacteria with resistance mechanisms. Bacteria can adapt to environmental pressures, and exposure to antimicrobial agents like triclosan may provide a selective advantage to bacteria with resistance-conferring genetic mutations. Due to these concerns, regulatory actions have been taken in various regions to restrict or ban the use of triclosan in specific products. For instance, the US Food and Drug Administration (FDA) prohibited the use of triclosan in over-the-counter antiseptic wash products due to insufficient evidence of effectiveness and concerns regarding long-term safety. Similarly, the European Union has also restricted the use of triclosan in particular cosmetic and hygiene products [44-46].

As a result, *S. aureus* and *Enterococcus* spp. Isolates showed resistance to heavy metals and disinfectants, regardless of their antibiotic resistance status. Notably, significant levels of resistance to various heavy metals and disinfectants, which are not adequately addressed in the literature, were identified in Methicillin-resistant S. aureus (MRSA), Linezolid-resistant S. aureus, Vancomycin-resistant S. aureus, and Vancomycin-resistant Enterococcus isolates. Concerns have arisen regarding the potential of disinfectant use to lead to antibiotic resistance and subsequent treatment failures, given some similarities between antibiotic resistance and disinfectant tolerance

mechanisms and their specific uses in health, livestock, and food production contexts [47]. The notable prevalence of resistance to disinfectants and heavy metals observed in *S. aureus* and *Enterococcus* spp. Isolates in this study suggest the existence of a complex adaptive response at both cellular and genetic levels. These adaptations may involve efflux pump expression changes, membrane permeability alterations, or genetic mutations that enhance resistance [48]. However, further comprehensive research is essential to fully elucidate the cellular and genetic mechanisms underlying this resistance and their implications for antibiotic resistance.

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Author Contributions

Conceptualization: BOA; Design: BOA; Supervision: BOA; Materials: BOA, EK; Data Collection and/or Processing: BOA, HŞ; Analysis and/or Interpretation: BOA, HŞ; Literature Search: BOA, HŞ; Writing Manuscript: BOA, HŞ; Critical Review: BOA, HŞ.

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Competing Interests

The authors have declared that no competing interests exist.

Data Availability Statement

Researchers who provide a methodologically sound proposal.

Additional Materials

The following additional materials are uploaded at the page of this paper.

1. Table S1: Antibiotic, heavy metal, and disinfectant resistance profiles of the isolates used in the project.
2. Table S2: Number of isolates according to heavy metal MIC values (µg/ml).
3. Table S3: Number of isolates according to disinfectant MIC values (µg/ml).
4. Table S4: Number of isolates showing resistance to heavy metals.
5. Table S5: Number of isolates resistant to disinfectants.

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