ORIGINAL RESEARCH

Antimicrobial Resistance of *Staphylococcus aureus* Isolated from Hospital Wastewater in Kermanshah, Iran

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Introduction: Hospital wastewater contains highly resistant and virulent bacteria that can spread into the environment. This study was conducted to investigate the antimicrobial resistance of methicillin-resistant *Staphylococcus aureus* (MRSA) and methicillin-sensitive *S. aureus* (MSSA) in raw and treated hospital wastewater.

Methods: During a three-month period, 40 sewage samples were collected from the hospital sewage (Kermanshah, Iran), and *S. aureus* were identified using culture and biochemical tests. MRSA was detected by resistance to cefoxitin. Antibiotic resistance (AR) was determined using disk diffusion according to the Clinical and Laboratory Standards Institute (CLSI) in 20 MSSA (10 raw and 10 treated sewage) and 40 MRSA isolates (20 raw and 20 treated sewage). The antimicrobial resistance genes (ARGs) were determined by PCR.

Results: Eleven and eight percent of the isolates were MRSA in raw and treated sewage samples, respectively. Out of 60 isolates, 59 (98%) were multidrug resistant (MDR). The most common ARGs were mecA (raw=100%, treated=100%), aacA-D (raw=100%, treated=85%) and tetK (raw =95%, treated =45%) in MRSA, while the tetM gene was the most abundant gene (raw=50%, treated=80%) in MSSA. None of isolates (n=60) was positive for the vanB gene. MSSR (n=20) had the highest level of resistance against penicillin (100%), clindamycin (raw=90%, treated=90%), azithromycin (raw=80%, treated=90%). All MRSA isolates (n=40,100%) in both raw and treated sewage samples were non-susceptible to penicillin, oxacillin and azithromycin. There was no significant difference in the frequency AR and ARGs between raw and treated sewage samples (p>0.05).

Conclusion: The results indicated a high frequency of MDR and ARGs in both raw and treated sewage isolates which could be released into the environment through sewage system and pose a serious threat to public health. Hospital wastewater treatment processes should be improved in order to prevent the dissemination of the most resistant strains of *S. aureus*.

Keywords: antibiotic resistance, antibiotic resistance genes, hospital wastewater, *Staphylococcus aureus*

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Introduction

Staphylococcus aureus (*S. aureus*) is a Gram-positive bacterium that can act as both a commensal (in the upper respiratory tract and on the skin) and a major pathogen (causing infections in skin and various tissues as well as toxin-mediated diseases, eg, food poisoning and toxic shock syndrome).[1] It can cause both community-acquired and hospital-acquired infections. *S. aureus* can easily acquire genes for resistance to antibiotics by horizontal gene transfer.[2] Recently methicillin-resistant *S. aureus*

(MRSA) has been reported as an important agent in the development of severe infections in health-care facilities, community, and farm environments.[3] Methicillin resistance is due to the expression of the mecA gene, which encodes the penicillin-binding protein 2a (PBP2a), a protein that is essential to bacterial cell wall synthesis and confers resistance to most β-lactam antibiotics such as methicillin, nafcillin, oxacillin, and cephalosporins.[4,5]

The widespread use of antimicrobials for prophylaxis or treatment of human infections in hospitals results in the selection of resistant bacterial strains. About 10–90% of antibiotics used are excreted in the urine and/or feces of patients as unchanged or metabolites.[6] The concentrations of antibiotics are much higher in the hospital wastewater than in the household effluent due to their widespread use in hospitals.[7,8] Given the mixed population of various bacteria, antimicrobial agents, and nutrients in the sewage environment, it provides a selective pressure for the selection of antibiotic resistance strains or new resistant mutations in bacteria.[9] Furthermore, the resistance genes can be transferred to other bacteria in the sewage. Despite advances in the wastewater treatment technology, resistant microorganisms are not completely removed from the hospital sewage during the wastewater treatment process.[10,11] Thus, the hospital wastewater is a particular public health concern. Several studies have reported the presence of MRSA in hospital wastewater using both cultivation and molecular methods.[12,13] There are also reports of the presence of *Staphylococcus* spp. in 80% of urban wastewater treatment plants in Spain.[14]

According to the Economic Co-operation and Development (OECD) report, Iran is among countries with high-level use of antibiotics.[15] A study of the prevalence of MRSA strains in hospitalized patients in intensive care units (ICU) in Iran showed 93.3% MRSA in *S. aureus* isolates.[3] Very few studies have evaluated the antibiotic susceptibility of *S. aureus* from hospital wastewater systems in Iran. It is pivotal to understand the prevalence of *S. aureus* in hospital wastewater to find appropriate strategies to prevent the dissemination of resistant strains in the environment. The aim of this study was to determine the antibiotic susceptibility of *S. aureus* isolated from the raw and treated hospital sewage.

Materials and Methods
Sample Collection
During 3 months from October 2017, sampling was done for 20 times and 40 samples were collected from the sewage treatment plant of Imam Reza Hospital, as the largest general and referral center in the west of Iran. With a few days interval between sampling, it was tried to cover any fluctuation in the bacterial load of the sewage. The pH and wastewater temperature were measured in situ. The samples were collected from the raw and treated sewage (20 samples for each sampling site). The samples were taken in 50 mL sterile bottles, transferred to the Microbiology Laboratory of Kermanshah University of Medical Sciences under cold conditions, and stored at 4 °C until tested. The wastewater treatment plant of this hospital used the extended aeration activated sludge system.

Bacterial Load of Sewage
To evaluate the bacterial load of the hospital sewage, six samples were randomly selected, three from raw and three from treated sewage samples, and tested within 3 hours after sampling. One mL of wastewater samples was added to the sterile test tube containing 9 mL of 0.85% NaCl solution and mixed properly. Three serial dilutions of wastewater samples, 0.001, 0.0001 and 0.00001 mL were then prepared; then, 15 mL of Muller-Hinton agar was poured in a plate containing 100 microliter of each sample and mixed thoroughly and uniformly with the agar medium. The plates were incubated at 37 °C for 24 hours. Colony count was performed and the average of three tests for each set was considered as the colony-forming unit per milliliter (CFU/mL) of hospital sewage.

Isolates Identification
The wastewater samples were cultured on Mannitol salt agar (Merck, Germany) plates. The plates were incubated at 37ºC for 48 h. Gram-positive cocci isolates that formed yellow colonies with a yellow halo on Mannitol salt agar were considered as presumptive *S. aureus* isolates. A few colonies per each wastewater sample with presumptive *Staphylococci* morphology were chosen and *S. aureus* isolates were identified using conventional microbiological methods (catalase, coagulase, deoxyribonuclease, hyaluronidase, hemolysis on sheep blood agar). To determine the MRSA frequency, 100 *S. aureus* colonies were picked up by toothpicks and cultured on Mannitol salt agar plates supplemented with cefoxitin (4 mg/L). Three sets of these cultures were curried out to determine the mean frequency of MRSA. Finally, 60 isolates included 20 MRSA and 10 MSSA isolates from raw sewage and the same sets from the treated sewage were randomly selected for further analysis.

Antibiotic Susceptibility Testing

The susceptibility of *S. aureus* isolates was tested against several antibiotic classes including β-lactam (penicillin, 10 μg and oxacillin, 1 μg), β-lactam+betalactamase inhibitor (amoxicillin+clavulanic, 30 μg), aminoglycosides (gentamicin, 10 μg), macrolide (azithromycin, 15 μg), tetracyclines (tetracycline, 30 μg), lincosamide (clindamycin, 2 μg) and ansamycins (rifampin, 5 μg) using the disc diffusion method according to the Clinical and Laboratory Standards Institute (CLSI) guidelines. After incubation at 37 °C for 16–18 h, the diameter of growth inhibition zones was measured. According to the inhibition zone diameter, the isolates were classified as resistant, intermediate and susceptible. *S. aureus* ATCC strain 25,923 was used for quality control. The isolates were defined as non-susceptible if they were resistant or intermediate to an antibiotic. Multidrug resistant (MDR) strains were determined by lack of susceptibility to at least three classes of antibiotics.[16]

DNA Extraction and Detection of Antibiotic Resistance Genes

Seven antimicrobial resistance genes including 1 β-lactam resistance gene (mecA), 2 tetracycline resistance genes (tetK, tetM), 3 glycopeptide resistance genes (vanA, vanB, vanC) and 1 aminoglycosides resistance gene (aacA-D) were detected by conventional PCR using specific primers (Table 1). The bacterial genome was extracted from the pure cultures using the High Pure PCR Template Preparation Kit (SinaClon, Iran) according to the manufacturer's guidelines. The DNA concentration was assessed using a spectrophotometer. A number of strain collections from our laboratory were used as positive and negative controls for the genes in each PCR reaction. The PCR reactions were performed in a total volume of 25 μL. The PCR cycle was performed in a Thermal Cycler (Eppendorf, Germany) with an initial 5 min denaturation at 95 °C, followed by 30 amplification cycles (denaturation at 94 °C for 30 s, 30 s annealing at temperatures specified for each gene (Table 1) and elongation at 72 °C for 145 s) followed by a final extension step at 72 °C for 5 min.

Statistical Analysis

Statistical analysis was performed using the SPSS software (version 16, SPSS Inc., Chicago, IL, USA). For continuous variables, mean were calculated. Categorical variables were expressed as frequencies and percentages. The *S. aureus* load, pH and temperature in different sewage samples were compared using the independent-sample *t*-test. The relationship between antibiotic resistance and ARG of isolates in raw and treated sewage was analyzed using the chi-square or Pearson's test. The p≤ 0.05 was considered significant.

Results

The mean bacterial load of raw and treated hospital sewage samples was 7.5×10^6 and 6.1×10^6, respectively (p=0.45). As shown in Table 2, there was no significant difference in pH and temperature between raw and treated sewage (p>0.05).

Frequency of MRSA Strains

The results showed that 11% and 8% of *S. aureus* isolates were MRSA in raw and treated hospital sewage on

Table 1 Primers Used for PCR Amplification of Antibiotic Resistance Genes.[17]

Gene Target	Sequence (5′→3′)	Amplicon Size (bp)	Annealing Temperature (°C)
mecA-F	AAAATCGATGGTAAAGGTTGGC	532	56
mecA-R	AGTTCTGCAGTACCGGATTTGC		
aacA-D -F	TAATCCAAGAGCAATAAGGGC	227	56
aacA-D -R	GCCACACTATCATAACCACTA		
tetK-F	GTAGCGACAATAGGTAATAGT	360	54
tetK-R	GTAGTGACAATAAACCTCCTA		
tetM-F	AGTGGAGCGATTACAGAA	158	54
tetM-R	CATATGTCCTGGCGTGTCTA		
VatA-F	TGGTCCCGGAACAACATTTAT	268	56
VanA-R	TCCACCGACAATAGAATAGGG		
VanB-F	GCTGCGAATTCAGTTGTTACA	136	55
VanB-R	CTGACCAATCCCACCATTTTA		
VanC-F	AAGGCCCCAATCCAGAAGAA	467	56
VanC-R	TCAACGTTCTTTGTCACAACC		

Table 2 Values of pH, Temperature, and Bacterial Load in Raw and Treated Sewage Samples

Parameter	Raw Sewage	Treated Sewage	p-value
Temperature, °C (mean)	21.8	19.6	0.002
pH (mean)	7.71	7.39	0.003
Bacterial load, CFU/mL (mean)	7.5×10^6	6.1×10^6	0.45

average, respectively. However, the difference was not statistically significant (p=0.411).

Antimicrobial Susceptibility

In order to determine the antibiotic resistance patterns, 20 MSSA (10 raw and 10 treated sewage) and 40 (20 raw and 20 treated sewage) MRSA isolates were evaluated.

The MSSA isolates (n=20) showed resistance to penicillin (100%), clindamycin (90%), azithromycin (85%), tetracycline (80%), amoxicillin/clavulanic acid (75%), gentamicin (70%), oxacillin (40%) and rifampin (20%). The frequency of MSSA isolates that were not susceptible to amoxicillin/clavulanic acid, azithromycin, gentamicin and rifampin was higher in treated sewage, but it was statistically significant only for rifampin (p=0.025). However, the frequency of MSSA isolates that were not susceptible to penicillin, tetracycline, oxacillin and clindamycin did not show marked changes after sewage treatment (Table 3).

The MRSA isolates (n=40) showed resistance to penicillin (100%), oxacillin (100%), azithromycin (100%), gentamicin (90%), clindamycin (85%), amoxicillin/clavulanic acid (80%), tetracycline (45%) and rifampin (32.5%). For MRSA isolates, non-susceptibility to rifampin was higher after sewage treatment, non-susceptibility to amoxicillin/clavulanic acid, penicillin, azithromycin and oxacillin did not change following sewage treatment, and non-susceptibility to gentamicin, tetracycline and clindamycin was lower. The changes in the non-susceptibility of MRSA isolates to antibiotics were not statistically significant (p>0.05).

For the all *S. aureus* isolates, the highest rate of non-susceptibility was to penicillin (100% of raw and 100% of treated sewage isolates) followed by azithromycin (93% of raw and 96% of treated sewage isolates) and clindamycin (90% of raw and 83% of treated sewage isolates) (Figure 1).

As shown in Figure 2, 11 (18.33%) isolates were resistant to all antibiotics tested, of which 5 (16.66%) and 6 (20%) were isolated from raw and treated hospital sewage, respectively. In addition, 98% (59/60) of the isolates were MDR (Figure 2).

Antibiotic Resistance Genes of Isolates

Table 4 presents the frequency of antibiotic resistance genes in MSSA and MRSA isolated from raw and treated sewage. Of the 40 MRSA isolates tested, the mecA gene was detected in 40 (100%), tetK in 28 (70%), tetM in 14 (35%), vanC in 8 (20%) and aacA-D gene in 37 (92.5%) isolates. None of these isolates was positive for vanB and vanA genes.

Regarding 20 MSSA isolates, mecA was detected in 2 (10%), tetK in 6 (30%), tetM in 13 (75%), vatA in 1 (5%), vatC in 6 (30%) and aacA-D gene in 6 (30%) isolates.

Table 3 Antimicrobial Susceptibility of MSSA and MRSA Isolates in Raw and Treated Sewage Samples

| Antibiotic	MSSA				P value	MRSA				P value
	Raw sewage (n=10)		Treated Sewage (n=10)			Raw Sewage (n=20)		Treated Sewage (n=20)		
	S	N	S	N		S	N	S	N	
Amoxicillin/Clavulanic acid	3(30)	7(70)	2(20)	8(80)	0.606	4(20)	16(80)	4(20)	16(80)	1
Azithromycin	2(20)	8(80)	1(10)	9(90)	0.531	0	20(100)	0	20(100)	–
Penicillin	0	10(100)	0	10(100)	–	0	20(100)	0	20(100)	–
Rifampin	10(100)	0	6(60)	4(40)	0.025	14(70)	6(30)	13(65)	7(35)	0.736
Tetracycline	2(20)	8(80)	2(20)	8(80)	1	8(40)	12(60)	14(70)	6(30)	0.057
Gentamicin	4(40)	6(60)	2(20)	8(80)	0.329	1(5)	19(95)	3(15)	17(85)	0.292
Oxacillin	6(60)	4(40)	6(60)	4(40)	1	0	20(100)	0	20(100)	–
Clindamycin	1(10)	9(90)	1(10)	9(90)	1	2(10)	18(90)	4(20)	16(80)	0.376

Note: Values have been expressed as number (%).
Abbreviations: N, non-susceptible; S, susceptible.
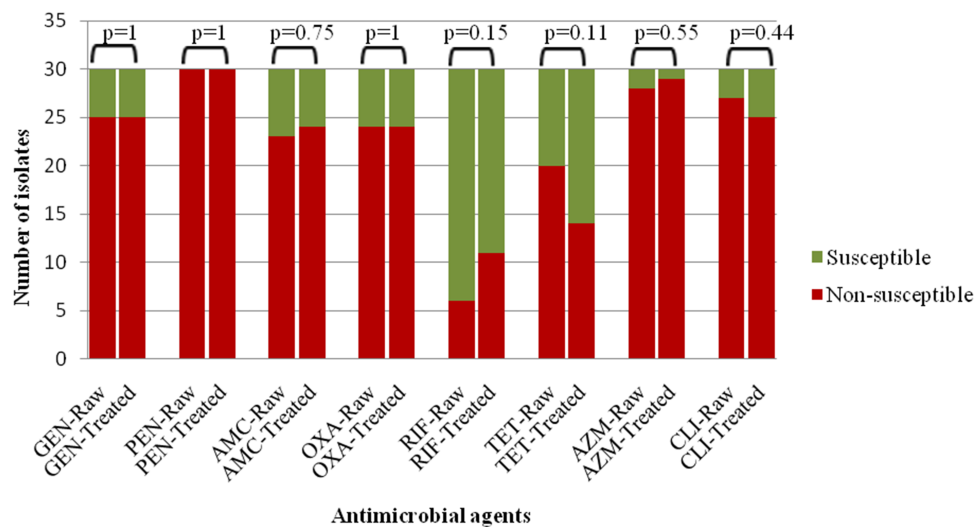
Figure 1 Comparison the antimicrobial susceptibility of *S. aureus* (MRSA and MSSA) isolates in raw (n=30) and treated (n=30) sewage samples.
Abbreviations: GEN, gentamicin; PEN, penicillin; AMC, amoxicillin/clavulanic acid; OXA, oxacillin; RIF, rifampicin; TET, tetracycline; AZM, azithromycin; CLI, clindamycin.
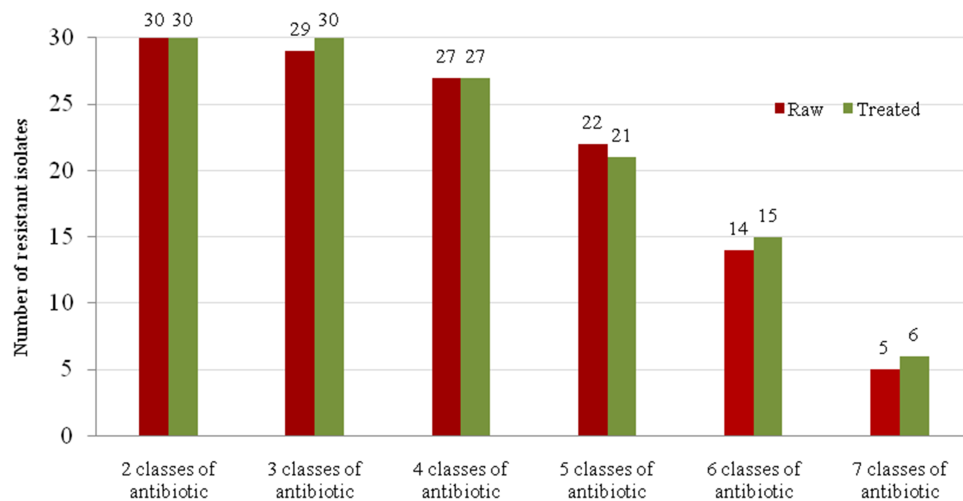
Figure 2 Frequency of multidrug-resistant *S. aureus* (MRSA and MSSA) isolates in raw (n=30) and treated (n=30) sewage samples.

None of isolates was positive for vatB gene. There was no significant difference in the frequency of antibiotic resistance genes between raw and treated samples (p>0.05).

Of the 60 MSSA and MRSA isolates examined, the most frequent ARGs were mecA (70% of raw and treated sewage isolates) followed by aacA-D (70% of raw and 73% of treated sewage isolates) and tetK (73% of raw and 40% of treated sewage isolates). There was no significant difference in the frequency of antibiotic resistance genes between raw and treated samples (p>0.05).

Discussion

Hospital sewage can be a dangerous source for spreading resistant bacterial strains including most pathogenic

S. aureus. Research shows that the frequency of antibiotic resistant bacteria in the hospital wastewater could be an easy way to monitor the antibiotic resistance of the bacteria isolated from clinical specimens.[18] It has been reported that the size of the hospitals could also contribute to the number of MRSA in the wastewater and wastewater treatment could reduce the number of MRSA isolates.[13] Studies in Iran showed an increase in the prevalence of MRSA isolates among clinical specimens. For instance, 46.3%, 30.38%, and 25% of clinical isolates in Ardabil, Sanandaj, and Hamadan were MRSA.[19–21] A study conducted in the USA found a decline in the MRSA isolates in treated wastewater.[22] These data are consistent with the findings

Table 4 Antibiotic Resistance Genes of MSSA and MRSA Isolates in Raw and Treated Sewage Samples

Resistant Genes	MSSA				MRSA		
	Positive Number [n(%)]				Positive Number [n(%)]		
	Raw Sewage (n=10)	Treated Sewage (n=10)	p value		Raw Sewage (n=20)	Treated Sewage (n=20)	p value
mecA	1(10)	1(10)	1		20(100)	20(100)	1
vanA	1(10)	0	0.305		0	0	–
vanB	0	0	–		0	0	–
vanC	1(10)	0	0.305		3(15)	5(25)	0.429
aacA-D	1(10)	5(50)	0.051		20(100)	17(85)	0.072
tetK	3(30)	3(30)	1		19(95)	9(45)	0.001
tetM	5(50)	8(80)	0.160		9(45)	5(25)	0.185

of the present study indicating a small decline in the MRSA frequency after hospital sewage treatment.

Rifampin, a semisynthetic antibiotic that inhibits DNA-dependent RNA polymerase, is used for treatment of *S. aureus* infections.[23] In our study, the rate of isolates that were not susceptible to rifampin was low. It has been reported that *S. aureus* resistant to rifampin comprises 8–17% of the isolates in Iran.[24] This low rate of resistance may be explained by the low prescription of this drug in Iran and regional distribution of resistant isolates.

The rate of non-susceptibility to penicillin (100%), clindamycin (90%), and azithromycin (85%) was higher in MSSA isolates (n=20). However, all MRSA isolates were non-susceptible to penicillin, oxacillin, and azithromycin, indicating higher resistance of MRSA to conventional antibiotics. In Iran, β-lactam and macrolides/lincosamides/streptogramins are the most frequently used antibiotic groups.[25] The high prescription rate of these antibiotics can accelerate the development of resistance among bacterial population. In a study in South Africa in 2019, MRSA isolates from treated wastewater effluent and surface water showed a high frequency of resistance to oxacillin, ampicillin, and penicillin, which was consistent with our results.[26]

The genes that encode resistance to tetracyclines belong to four categories based on their resistance mechanisms (efflux genes, ribosomal protection genes, enzymatic and other genes).[27] The ribosome protection (tetM) and efflux (tetK) genes were evaluated in our study. We found that the tetK gene was more prevalence than the tetM (56.6% vs 45%) gene in all MSSA and MRSA isolates (n=60). This is consistent with the results of a previous study that found that efflux genes were more common in the environmental isolates compared to the ribosomal protection genes.[28] Moreover, in our study, only

half of the isolates with tetracycline resistance genes showed a non-susceptible phenotype to tetracycline, which is similar to the findings of a previous study.[27] These data suggest that some antibiotic resistance genes remain silent or inactive in the bacteria under certain circumstances. These silent ARGs may still be a threat since they can be expressed when they integrate into the appropriate genetic locations in the bacterial genome or mobile genetic elements.

In the present study, the aminoglycoside resistance gene, aacA-D, was found in 100% and 85% of MRSA isolates from raw and treated sewage, respectively. The low frequency of the aacA-D gene in the MRSA isolates of treated wastewater was previously reported.[26] On the other hand, some authors found an increase in some antibiotic resistance genes after conventional wastewater treatment indicating that wastewater treatment may have different effects on various ARGs.[29]

Vancomycin is a glycopeptide usually used as the last choice for the treatment of MRSA infections. A systematic review showed that the overall prevalence of vancomycin intermediate *S. aureus* strains was 0.09% in Iran.[30] We evaluated the presence of three genes (vanA, vanB and vanC) involved in resistance to vancomycin in *S. aureus* isolates. The overall frequency of these genes was low and none of the isolates harbored the vanB gene. This finding is supported with research results reporting lack of vanB in Staphylococcal isolates.[31]

The absolute majority of isolates in this study showed resistance to three different antibiotic classes and were designated as MDR as defined previously,[32] which was similar to the results of a study investigating treated wastewater in South Africa in which 100% of the isolates were MDR.[26] The present study found no significant difference in the frequency of MDR between raw and the treated sewage isolates. However, a study showed an increase in multidrug

resistance after wastewater treatment.[33] The treatment of infections caused by these MDR strains is challenging, particularly in vulnerable populations such as children, elderly subjects, and immunocompromised patients.

Conclusion

The findings of this study underlined an important public health concern regarding the high frequency of antibiotic resistant strains of *S. aureus* and related antibiotic resistance genes, not only in raw but also in treated hospital sewage. Given the fact that most of the antibiotic resistance genes are located on the mobile genetic elements in *S. aureus*, it is reasonable to assume that these genes can transfer to other pathogenic bacteria in the wastewater environment. It seems that hospital wastewater treatment should be improved to prevent dissemination of potential antibiotic resistant strains and their related genes.

Data Sharing Statement

The data sets used and/or analyzed during this study are available from the corresponding author on reasonable request and were received permission for use by the Kermanshah University of Medical Sciences Ethics Committee.

Ethics Approval and Consent to Participate

The protocol was approved by the Kermanshah University of Medical Sciences Ethics Committee (IR.KUMS. REC.1396.338).

Acknowledgments

Disclosure

The authors declared no conflicts of interest in this work.

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