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Occurrence of *Staphylococcus* spp. in the wastewaters from Iran: Diversity, antimicrobial resistance, and virulence potential

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ABSTRACT

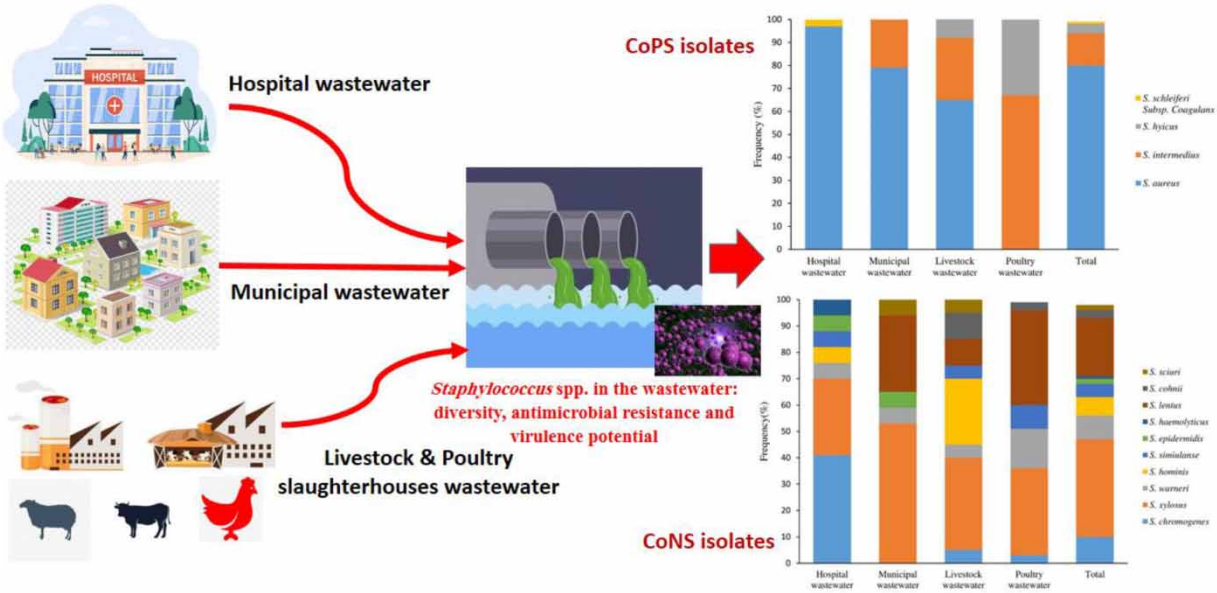
The prevalence, antibiotic resistance, and virulence characteristics of *Staphylococci* from hospitals, livestock, municipals, and poultry wastewaters were investigated in Ardabil, Iran. From 155 staphylococcal isolates, 44.5% were coagulase-positive *Staphylococcus* (CoPS) and 55.5% were coagulase-negative *Staphylococcus* (CoNS) spp. Both CoPS and CoNS species were mainly found in hospital and poultry wastewater samples. The most prominent CoPS and CoNS species were *Staphylococcus aureus* at 80% and *Staphylococcus xylosus* at 37%. Methicillin resistance was found in 2% of *S. aureus* isolates. Overall, 49.2% of CoPS and 47.6% of CoNS isolates exhibited multidrug resistance phenotypes. CoPS isolates were the most resistant to penicillin (89%) and erythromycin (62%) and CoNS isolates exhibited the highest resistance to erythromycin (55%) and tetracycline (49%). Inducible clindamycin resistance was detected in 11% of *S. aureus* isolates. The *ermC* and *aac* genes were detected as the most common macrolide–lincosamide–streptogramin B and aminoglycoside-resistance encoding genes in 82.5 and 22.5% of *S. aureus* isolates, respectively. Most of the *S. aureus* isolates were positive for multiple virulence factors. The methicillin-resistant *S. aureus* isolates belonged to SCC*mec* type V. A new spa type t19215 was also identified. The occurrence of multidrug-resistant *S. aureus* with diverse genetic resistance and virulence background in wastewater is of great health concern.

Key words: coagulase-negative *Staphylococci*, coagulase-positive *Staphylococci*, drug resistance, pathogenic factors, sewage, slaughterhouse

HIGHLIGHTS

- Ten coagulase-negative and four coagulase-positive *Staphylococcus* spp. were identified in wastewaters in Iran.
- 50% of both coagulase-negative and coagulase-positive *Staphylococcus* spp. isolates were multidrug-resistant.
- Occurrence of methicillin-resistant *S. aureus* was rare in wastewater samples in Iran.
- *S. aureus* isolates with high virulence potentials were common.
- A new spa type t19215 was identified in *S. aureus* isolates.

GRAPHICAL ABSTRACT



1. INTRODUCTION

Wastewater is a complex ecological environment hosting different types of microorganisms (Börjesson *et al.* 2010). Pathogenic bacteria enter the wastewater from hospitals, or from any diseased people or healthy carriers. Animal wastes often originate from farms, slaughterhouses, meat processing industries, and from rodents found around sewages (Dweba *et al.* 2018). When untreated wastewater reaches water used for drinking or irrigation of vegetable farms there can be significant public health risks. Pathogens in wastewater are transmissible through ingesting drinking water or crops contaminated with sewage or as a result of contact with the animal, human, or insect carriers (Wagner & Loy 2002). The wastewater is an environmental reservoir which plays a significant role in the development and spread of antimicrobial resistance (Martinez-Huitle & Ferro 2006; Börjesson *et al.* 2010). Several studies showed the residues of many antibiotics in wastewaters around the world (Watkinson *et al.* 2009). Residual antibiotics are capable of selecting resistant bacteria through inhibiting or killing the susceptible organisms. Resistant bacteria survive in the presence of antibiotics and act as vectors for the dissemination of antibiotic-resistance genes (Kruse *et al.* 1999). Excessive and uncontrolled application of antibiotics in human medicine, veterinary, and as growth promoting agents in food-producing animals lead to an increment in antibiotic resistance and in the spread of bacteria carrying resistance genes (Iversen *et al.* 2002; Dweba *et al.* 2018). The major risk threatening general health is the environmental bacteria transferring resistance encoding genes to human pathogens (Moges *et al.* 2014). The majority of studies regarding the characterization of resistant bacteria in wastewater have emphasized the fecal pollution indicator bacteria (Zaatout *et al.* 2021). However, other antibiotic-resistant clinically significant pathogens have been reported in wastewater as well (Nishiyama *et al.* 2021).

Staphylococcus spp. are normal flora, found in mucus membranes and skin of human and other mammals (Gómez *et al.* 2016) which are capable to enter hospital, municipal, livestock, and poultry wastewater. Hence, they have been frequently isolated from wastewater of various sources (Faria *et al.* 2009; Börjesson *et al.* 2010; Goldstein *et al.* 2012; Heß & Gallert 2014; Kumar *et al.* 2015). *Staphylococcus* genus comprises several species classified into coagulase-positive *Staphylococci* (CoPS) and coagulase-negative *Staphylococci* (CoNS) groups. Most staphylococcal infections are caused by CoPS. So far, seven CoPS species have been recognized: *Staphylococcus intermedius, Staphylococcus aureus*, *Staphylococcus schleiferi* subsp. *coagulanse, Staphylococcus delphini, Staphylococcus hyicus, Staphylococcus lutrae,* and *Staphylococcus pseudintermedius. S. aureus* is the most common and highly virulent species in the genus responsible for a diverse array of life-threatening nosocomial and community-acquired infections (Sasaki *et al.* 2010; Holmes *et al.* 2014; Kumar *et al.* 2015). Instead, CoNS established themselves as opportunistic pathogens which are less virulent than CoPS. However, they can cause clinically significant infections in immunocompromised people and in those with implanted foreign materials

such as catheters, shunts, and prosthetic joints (Hitzenbichler *et al.* 2017). There are currently over 40 identified species in the CoNS group, in which *Staphylococcus haemolyticus, Staphylococcus epidermidis*, and *Staphylococcus saprophyticus* are mostly responsible for infection in human beings (Hitzenbichler *et al.* 2017). Other species, such as *Staphylococcus hominis*, *Staphylococcus warneri*, *Staphylococcus capitis*, *Staphylococcus simulans*, *Staphylococcus cohnii*, *Staphylococcus xylosus*, *Staphylococcus saccharolyticus*, and *Staphylococcus lugdunensis* are sometimes collected from clinical specimens (Garza-González *et al.* 2010). The most striking situation regarding *Staphylococci* is emerging methicillin-resistant strains. Methicillin is the first semisynthetic penicillinase-stable penicillins including oxacillin, cloxacillin, naficillin, and dicloxacillin. *Staphylococcus* spp. resistant to this group are historically termed Methicillin-Resistant Staphylococci (MRS). What sets it apart is that MRS isolates are resistant to all other currently available β-lactam antimicrobial agents, with the exception of ceftaroline (CLSI 2017). Additionally, MRS isolates are commonly resistant to several other classes of antibiotics such as aminoglycosides, chloramphenicol, quinolones, macrolides, and tetracycline (Kumar *et al.* 2015). The resistance against methicillin is mainly mediated by *mec*A gene. Staphylococcal cassette chromosome *mec* (SCC *mec*) has been reported as the only vector for the *mec* A gene (Zhang *et al.* 2005).

Studies showed large differences in *Staphylococci* population structure among wastewaters. The structure of the bacterial community is likely influenced by the operating parameters and composition of the effluents (Börjesson *et al.* 2010). Some studies have shown that common disinfecting agents such as chlorine and ultra-violet irradiation could not efficiently reduce the antibiotic-resistant bacteria and antibiotic resistance genes in the wastewater disinfecting process (Munir *et al.* 2011). So, understanding the microbial communities is essential for the implementation of effective disinfection approaches in wastewater treatment facilities (Gonzalez-Martinez *et al.* 2018).

The goals of the current study are (i) to assess the occurrence of *Staphylococcus* spp. in hospitals, slaughterhouses (poultry and livestock), and municipal wastewater sources in Iran; (ii) to evaluate the antimicrobial resistance profile of the isolates against common antibiotics; (iii) to investigate the occurrence of Methicillin-Resistant *S. aureus* (MRSA) isolates; (iv) to identify the SCC*mec*- and spa type of *S. aureus* isolates; (v) to study the genetic background responsible for aminoglycoside and macrolide resistance in *S. aureus* isolates; and (vi) to identify the virulence determinants of *S. aureus* isolates.

2. MATERIALS AND METHODS

2.1. Sampling

Sampling was done from wastewaters from municipal, hospitals, and slaughterhouses (poultry and livestock) in Ardabil, northwestern Iran. From August 2017 to June 2018, a total of 40 non-treated wastewater samples were taken twice a month on the 1st and 15th of each month. Specimens were collected in sterile glass bottles (500 mL), and transferred to the microbiology laboratory in ice cold containers, and were analyzed in less than 6 h after collection (Rahimi & Bouzari 2015; Gómez *et al.* 2016). This study was approved by the regional ethics committee of Islamic Azad University of Shiraz (IR.IAU.SHIRAZ.REC.1399.014).

2.2. Isolation and identification of *Staphylococcus* spp.

Samples (250 mL) were diluted 10-fold with 0.9% NaCl (normal saline) and filtered through a 0.45 μm pore membrane (Millipore Corporation, Bedford, MA, USA). For enrichment of *Staphylococcus* isolates captured on filters, the filters were placed in tubs containing 40 mL M *Staphylococcus* broth (Becton, Dickinson and Company, Franklin Lakes, NJ, USA), shaken and incubated at 37 °C for 24 h. A 10-μL aliquot of overnight bacterial culture was transferred on Mannitol salt agar (BioMaxima, Lublin, Poland) for the isolation of total *Staphylococcus* spp. Then, the plates were incubated at 37 °C for 24 h (Goldstein *et al.* 2012). Up to eight colonies per wastewater specimen with morphology resembling *Staphylococci* were cultured on Brain Heart Infusion (BHI) agar (BioMaxima, Poland) to provide a richer environment for bacterial growth, and then initially identified by conventional microbiological tests [Catalase, Gram stain, DNase (Merck, Darmstadt, Germany) and tube coagulase tests]. CoNS were identified using a commercial kit (Microgen™ Staph-ID system, UK). This system employs 12 standardized biochemical tests to characterize important species of the *Staphylococci. S. aureus* isolates were identified by PCR targeting the thermonuclease gene (*nuc*) with the primers and cycling conditions shown in Supplementary material, Table S1 (Murakami *et al.* 1991). PCR was carried out in a total volume of 25 μL using 12.5 μL of Premix Taq® mix (CinnaGen, Tehran, Iran), 1 μL of template DNA (5 μg), l μL (10 pmol) of each forward and reverse primers, and 9.5 μL of nuclease-free water. PCR products were analyzed via electrophoresis at 100 V for 1 h in a 1% agarose gel (Sinaclon, Tehran, Iran),

stained with DNA-safe stain (Sinaclon, Tehran, Iran) and DNA bands were visualized using UV illumination (Uvi Tec, Cambridge, UK). *S. aureus* ATCC 33591 was used as the positive control and the negative control was nuclease-free distilled water.

After identification of the isolates, two purified colonies grown on BHI agar were transferred into 10 mL of Trypticase soy broth (TSB) (Merck, Germany) and incubated overnight at 37 °C. Then, the cultures were aliquoted in 1.5 mL of cryovials including 15% glycerol (Merck, Germany), and stored at −80 °C until further use.

2.3. Antimicrobial susceptibility analysis

Antimicrobial susceptibility analysis was performed with the disk diffusion approach on Muller–Hinton agar (BioMaxima, Poland) according to the recommendations of the Clinical and Laboratory Standards Institute (CLSI) guidelines (CLSI 2017). The evaluated antibiotics (Padtan Teb, Iran) were chloramphenicol (30 μg), co-amoxiclav (30 μg), tetracycline (30 μg), penicillin (10 μg), erythromycin (15 μg), ciprofloxacin (10 μg), cefazolin (30 μg), clindamycin (2 μg), imipenem (10 μg), ceftriaxone (100 μg), rifampicin (30 μg), mupirocin (5 μg), trimethoprim sulfamethoxazole (25 μg), azithromycin (15 μg), gentamicin (10 μg), and amikacin (30 μg).

Methicillin resistance was investigated using the cefoxitin disk diffusion test (inhibition zone diameter ≤21 mm indicated MRSA) and oxacillin minimum inhibitory concentration (MIC) (≥4 μg/mL indicated MRSA) were determined using the agar dilution method (concentration range: 0.125–512 μg/mL) (CLSI 2017).

MICs of erythromycin, kanamycin, and tobramycin were assessed using a standard agar dilution technique (0.125–512 μg/mL). The isolates with MICs of ≥8 (μg/mL), ≥64 (μg/mL) and ≥16 (μg/mL) were defined as erythromycin-, kanamycin- and tobramycin-resistant, respectively (CLSI 2017).

BHI agar including 6 μg/mL vancomycin (Bio Basic, Canada) was used to screen isolates resistant to vancomycin. The MICs for isolates growing on BHI–vancomycin screening agar were determined by agar dilution technique (0.125–512 μg/mL). Resistance against vancomycin was considered as MIC ≥ 16 (μg/mL). The susceptibility testing was carried out and interpreted based on the CLSI's guidelines. *S. aureus* ATCC33591 was used as a quality control strain (CLSI 2017).

The *D*-test was carried out to identify inducible clindamycin resistance in clindamycin-susceptible staphylococcal isolates resistant to erythromycin. The clindamycin disks (2 μg) were located 25 mm apart (center to center) from erythromycin disks (15 μg). Following incubation at 37 °C for 18 h, the D phenotype was indicated by bacterial lawn exhibiting flattening of the inhibition zone (D formation) surrounding clindamycin disks near the erythromycin disks (Steward *et al.* 2005; Heß & Gallert 2014; CLSI 2017).

2.4. Detection of antimicrobial resistance genes

Erythromycin-resistant genes (*erm* A, *erm* C, *erm* B, *erm* TR, *msr* A), aminoglycoside-resistant genes (*ant, aac, aph(2)-Ib, aph (2)-Ic*, *aph(2)-Id*), and methicillin-resistant gene (*mec* A) were detected through PCR using specific primers (Supplementary material, Table S1) (Choi *et al.* 2003; O'Sullivan *et al.* 2006; Leelaporn *et al.* 2008; Dibah *et al.* 2014). DNA from previously identified isolates containing target genes was used as a positive control in the PCR experiment (Omid *et al.* 2021).

2.5. Detection of virulence genes

PCR (Supplementary material, Table S1) was used to detect the genes encoding enterotoxins (*sea, seb, sec,* and *sed*), exfoliative toxins (*eta* and *etb*), hemolysin toxins (*hla* and *hld*), Panton-Valentine leucocidin (PVL,*lukF/S*), and the toxic-shock syndrome toxin (*tst*) (Omid *et al.* 2021). DNA from previously identified isolates carrying the corresponding virulence genes was used as a positive control in PCR experiments (Omid *et al.* 2021).

2.6. Molecular typing

2.6.1. SCC *mec* typing

SCC*mec* type was determined on all *mec*A-positive isolates. Two series of multiples PCR assays were used to detect SCC*mec* types and subtypes I, II, III, IV_a, IV_b, IV_c, IV_d, and V according to the conditions in Supplementary material, Table S1 with some changes in the PCR cycling conditions and annealing temperature (Omid *et al.* 2021). Amplicons were analyzed as introduced previously in this paper. DNA harvested from previously identified isolates with the known SCC*mec* types was used as a positive control in PCR testing (Omid *et al.* 2021).

2.6.2. Spa typing based on repeat pattern analysis

The *S. aureus* strains' genetic profiles were generated using protein A (spa) typing. The *spa* gene polymorphic X region was amplified via primers and PCR conditions reported in Supplementary material, Table S1 as introduced by Harmsen *et al.* (2003). Both strands of the amplified fragments were sequenced by Macrogen (South Korea). The spa types were identified using the spa typing website available online at http://www.spaserver.ridom.de.

3. RESULTS

A total of 155 Staphylococcal isolates were collected from four wastewater sources. Overall, 69 (44.5%) and 86 (55.5%) isolates were found to be CoPS and CoNS, respectively. Figures 1 and 2 show the distribution of the isolates based on species type. *S. aureus* ($n = 55$; 80%) and *S. xylosus* ($n = 32$; 37%) were the most prominent CoPS and CoNS species isolated from wastewater samples, respectively. In total, most of CoPS (53.6%) were isolated from hospital wastewater samples and most of CoNS (38.4%) species were isolated from poultry wastewater samples. *S. aureus*, the most clinically important CoPS, comprised 97% ($n = 37/38$), 78.5% ($n = 11/14$), and 64% ($n = 7/11$) of the CoPS isolates from hospitals, municipal, and livestock wastewater samples, respectively, and *S. intermedius* ($n = 4/6$; 67%) were the most common species isolated from poultry wastewater samples (Figure 1). In CoNS, *S. lentus* ($n = 12/33$; 36%), *S. xylosus* ($n = 9/17$; 53%), and ($n = 7/20$; 35%), *S. chromogenes* ($n = 7/17$; 41%) were the majority of isolates from poultry, municipal, livestock, and hospital wastewater samples, respectively (Figure 2).

The susceptibility profile of staphylococcal isolates, evaluated through the disk diffusion assay, is provided in Tables 1 and 2. In general, imipenem, rifampicin, mupirocin, vancomycin (for each, $n = 0/69$; 0%), and penicillin ($n = 49/69$; 89%) were the antibiotics with the highest and the lowest activity against CoPS isolates, respectively. CoNS isolates were the most resistant to erythromycin ($n = 47/86$; 55%) followed by tetracycline ($n = 42/86$; 49%) and azithromycin ($n = 29/86$; 34%), respectively.

As shown in Supplementary material, Table S2, totally, 49% ($n = 34/69$) CoPS isolates were multidrug-resistant (MDR) (resistant against three or more classes of antibiotics). Among CoPS species, *S. aureus* ($n = 28/55$; 51%), *S. intermedius* ($n = 4/10$; 40%), and *S. hyicus* ($n = 2/3$; 67%) showed an MDR phenotype, respectively.

Antibiotic resistance patterns of CoNS isolates are shown in Supplementary material, Table S3. Results showed that in total, 46.5% ($n = 40/86$) of CoNS isolates were MDR. Among CoNS species, 55.5% ($n = 5/9$), 58% ($n = 11/19$), 75% ($n = 3/4$), 62.5% ($n = 5/8$), 44% ($n = 14/32$), 17% ($n = 1/6$), and 33% ($n = 1/3$) of *S. chromogenes*, *S. lentus*, *S. simulanse*, *S. warneri*, *S. xylosus*, *S. hominis*, and *S. cohnii subsp. urealyticum* showed an MDR phenotype, respectively.

According to oxacillin MIC (Supplementary material, Table S4) and *mec*A PCR testing, 2% ($n = 1/55$) of *S. aureus* isolates was identified as MRSA. This isolate belonged to SCC*mec* type V.

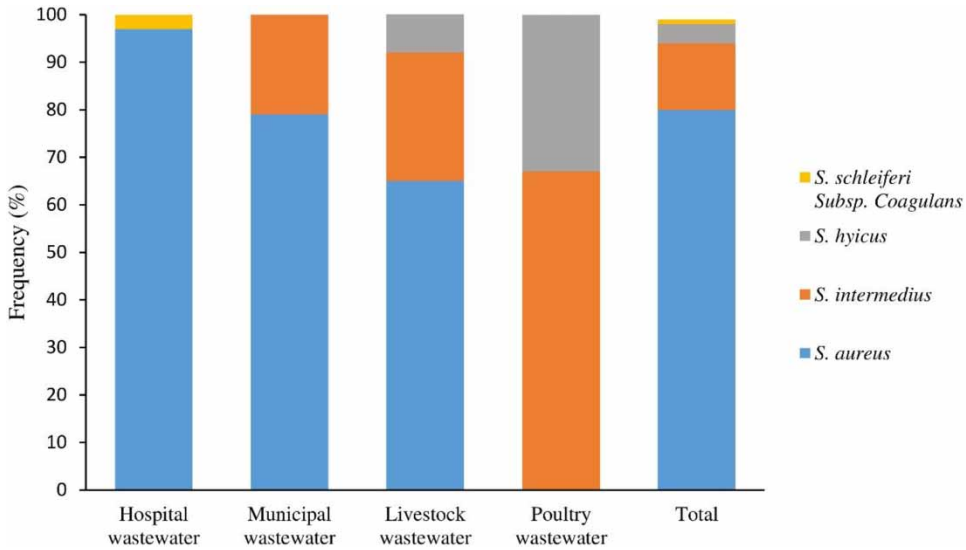


Figure 1 | Frequency distribution of CoPS isolates collected from wastewater in Ardabil, northwestern Iran.

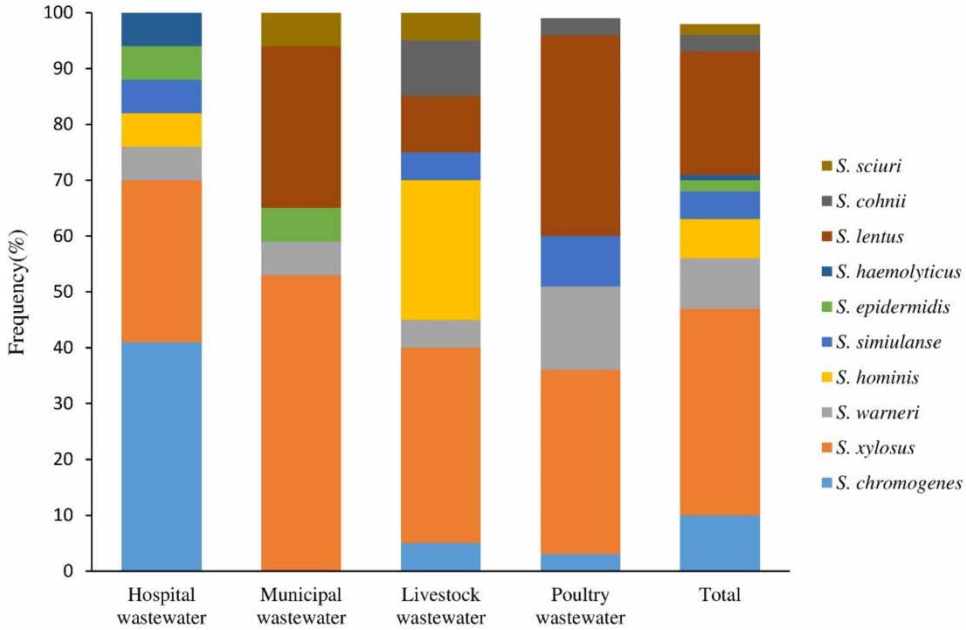


Figure 2 | Frequency distribution of CoNS isolates collected from wastewater in Ardabil, northwestern Iran.

Results of MIC testing showed that 64% ($n = 35/55$) of *S. aureus* isolates were erythromycin-resistant (Supplementary material, Table S4). Overall, 11% ($n = 6/55$) of erythromycin-resistant and clindamycin-susceptible isolates were found to be D-test positive. All erythromycin-resistant *S. aureus* isolates were assessed for the existence of the prominent MLS_B resistance genes (*erm* A, *erm* B, *erm* C, *erm* TR, and *msrA*). The most common MLS_B resistance gene *erm* C was found in 82.5% ($n = 33/40$) of these isolates, while *erm* A gene was not found in our isolates (Figure 3). A total of 10 different genetic profiles were obtained for macrolide resistance encoding genes with profile *erm B, erm C, msrA* having the highest (25%) frequency (Supplementary material, Table S5).

According to MIC testing, resistance to aminoglycoside antibiotics was observed in 11% ($n = 6/55$), 4% ($n = 2/55$), and 2% ($n = 1/55$) of *S. aureus* isolates against tobramycin, kanamycin, and gentamycin, respectively. The resistance gene *aac* was detected in 75% ($n = 9/12$) and the *ant* and *aphc* genes were identified in 33% (for each, $n = 4/12$) of aminoglycoside-resistant isolates. Other aminoglycoside resistance-encoding genes were not identified in our *S. aureus* isolates (Figure 3). A total of four different genetic profiles (*aac, ant, aac/aphc, aac/ant/aphc*) were obtained for aminoglycoside resistance-encoding genes with profile only *aac* having the highest (42%) frequency (Supplementary material, Table S5).

The results of PCR testing revealed 89% ($n = 49$), 5.4% ($n = 3$), 49% ($n = 27$), 14.5% ($n = 8$), 33% ($n = 18$), 94.5% ($n = 52$), 96% ($n = 53$), 84% ($n = 47$), and 31% ($n = 17$) of the S. *aureus* isolates ($n = 55$) were positive for *sea, seb, sec, sed, eta, hla, hld, tst,* and *pvl* virulence encoding genes, respectively. *hld* was the most common virulence gene and *etb* gene was not observed in this study (Figure 4). Regarding the simultaneous presence of virulence encoding genes, 23 different combined profiles were identified among *S. aureus* isolates (Supplementary material, Table S6). The profile, *sea + hld + hla + tst* with ($n = 7$; 13%) isolates showed the highest frequency.

In total, among eight *S. aureus* isolates subjected to *spa* typing, six different spa types were identified and one (12.5%) isolate was not typeable. *spa* type t346 was detected in 25% ($n = 2$) and types t026, t14870, t937, t19215, and t17068 were detected in 12.5% (for each, $n = 1$) of *S. aureus* isolates, respectively. The t19215 is a new spa type identified in this study (spa.ridom.de/194598, 2020, MRSA).

4. DISCUSSION

Despite the extensive investigation on *Staphylococcus* spp. in clinical settings, systematic assessments of their role in the aquatic environment and sewage samples are limited in most parts of the world. We found that the dominant species of *Staphylococcus* spp. was CoNS (55.5%) which itself is dominated by in wastewater samples. Similar results were reported

Table 1 | Antibiotic susceptibility patterns of CoPS isolated from wastewater by disk diffusion assay in Iran

CoPS															
	S. aureus $n = 55$ *n* (%)			*S. intermedius* $n = 10$ *n* (%)			*S. hyicus* $n = 3$ *n* (%)			*S. schleiferi Subsp. Coagulans* $n = 1$ *n* (%)			Total $n = 69$ *n* (%)		
Antibiotic	**S**[a]	**I**[b]	**R**[c]	**S**	**I**	**R**	**S**	**I**	**R**	**S**	**I**	**R**	**R**	**I**	**S**
P	6 (11)	0	49 (89)	8 (80)	0	2 (20)	3 (100)	0	0	1 (100)	0	0	49 (89)	0	18 (26)
AMC	45 (82)	0	10 (18)	10 (100)	0	0	3 (100)	0	0	1 (100)	0	0	0	0	59 (85.5)
C	41 (74.5)	0	14 (25)	8 (80)	0	2 (20)	1 (33.3)	0	2 (66.6)	1 (100)	0	0	18 (26)	0	51 (74)
TE	35 (64)	0	20 (36)	4 (40)	0	6 (60)	1 (33.3)	0	2 (66.6)	1 (100)	0	0	28 (40.5)	0	41 (59.4)
CP	51 (93)	0	4 (7)	9 (90)	0	1 (10)	2 (66.6)	0	1 (33.3)	1 (100)	0	0	6 (9)	0	63 (91.3)
CRO	54 (98)	1 (2)	0	10 (100)	0	0	3 (100)	0	0	1 (100)	0	0	0	1 (2)	68 (98.5)
CZ	54 (98)	1 (2)	0	9 (90)	0	1 (10)	3 (100)	0	0	1 (100)	0	0	1 (2)	1 (2)	67 (97)
CC	39 (71)	0	16 (29)	7 (70)	0	3 (30)	1 (33.3)	0	2 (66.6)	1 (100)	0	0	21 (30)	0	48 (69.5)
IPM	55 (100)	0	0	10 (100)	0	0	3 (100)	0	0	1 (100)	0	0	0	0	69 (100)
RA	55 (100)	0	0	10 (100)	0	0	3 (100)	0	0	1 (100)	0	0	0	0	69 (100)
GM[d]	49 (89)	5 (9)	1 (2)	10 (100)	0	0	3 (100)	0	0	1 (100)	0	0	1 (2)	5 (9)	63 (91.3)
MUP	55 (100)	0	0	10 (100)	0	0	3 (100)	0	0	1 (100)	0	0	0	0	69 (100)
E[d]	0	20 (36)	35 (64)	5 (50)	0	5 (50)	1 (33.3)	0	2 (66.6)	0	0	1 (100)	43 (62)	20 (36)	0
FOX	54 (98)	0	1 (2)	nd	nd	nd	nd	nd	nd	nd	nd	nd	1 (2)	0	54 (98)
SXT	51 (93)	0	4 (7)	7 (70)	0	3 (30)	2 (66.6)	0	1 (33.3)	1 (100)	0	0	8 (11.6)	0	61 (88.4)
K[d]	49 (89)	4 (7)	2 (3.6)	10 (100)	0	0	3 (100)	0	0	1 (100)	0	0	2 (3.6)	4 (7)	63 (91.3)
TOB[d]	47 (85.4)	2 (3.6)	6 (11)	10 (100)	0	0	3 (100)	0	0	1 (100)	0	0	6 (11)	2 (3.6)	61 (88.4)
AN	54 (98)	0	1 (2)	10 (100)	0	0	3 (100)	0	0	1 (100)	0	0	1 (2)	0	68 (98.5)
AZM	27 (49)	0	28 (51)	8 (80)	0	2 (20)	2 (66.6)	0	1 (33.3)	0	0	1 (100)	32 (46.4)	0	37 (54)
V	55 (100)	0	0	9 (90)	1 (10)	0	2 (66.6)	1 (33.3)	0	1 (100)	0	0	0	2 (3)	67 (97)

Note: Antibiotics: P, penicillin G; FOX, cefoxitin; E, erythromycin; MUP, mupirocin; TE, tetracycline; AMC, amoxiclav; TOB, tobramycin; K, kanamycin; CC, clindamycin; CZ, cefazolin; RA, rifampicin; GM, gentamicin; CRO, ceftriaxone; SXT, trimethoprim sulfamethoxazole; CP, ciprofloxacin; AN, amikacin; AZM, azithromycin; V, vancomycin; IPM, imipenem; C, chloramphenicol.

CoPS, coagulase-positive *Staphylococcus* spp.; nd, not determined.

[a]Susceptible.

[b]Intermediate resistant.

[c]Resistant.

[d]Susceptibility profile was determined using the agar dilution method.

Table 2 | Antibiotic susceptibility patterns of CoNS isolated from wastewaters using disk diffusion assay in Iran

	CoNS *n* = 86 *n* (%)		
Antibiotic	**S[a]**	**I[b]**	**R[c]**
P	62 (72)	0	24 (28)
AMC	86 (100)	0	0
C	66 (77)	0	20 (23)
TE	44 (51)	0	42 (49)
CP	79 (92)	0	7 (8)
CRO	69 (80)	11 (13)	6 (7)
CZ	85 (99)	0	1 (1)
CC	60 (70)	3 (3)	23 (27)
IPM	86 (100)	0	0
RA	82 (95)	0	4 (5)
E	39 (45)	0	47 (55)
SXT	65 (75.6)	0	21 (24.4)
AZM	56 (65)	1 (1)	29 (34)

Note: Antibiotics: P, penicillin G; E, erythromycin; TE, tetracycline; AMC, amoxiclav; CC, clindamycin; CZ, cefazolin; RA, rifampicin; CRO, ceftriaxone; SXT, trimethoprim sulfamethoxazole; CP, ciprofloxacin; AZM, azithromycin; IPM, imipenem; C, chloramphenicol; CoNS, coagulase-negative *Staphylococcus* spp.
[a]Susceptible.
[b]Intermediate resistant.
[c]Resistant.

from Spain (75%), Germany (57.3%), and Portugal (78.7%) previously (Faria *et al.* 2009; Heß & Gallert 2014; Gómez *et al.* 2016). CoNS species was dominated by *S. xylosus* (37%) among 10 different types identified in our study, while *S. saprophyticus* was reported as a dominant CoNS species type in wastewater in Europe (Faria *et al.* 2009; Heß & Gallert 2014). *S. saprophyticus* has been associated with urinary tract infections in young women (Jordan *et al.* 1980). Other CoNS species, with low frequencies, found in the current study were *S. epidermidis, S. hominis,* and *S. haemolyticus,* which are common pathogens responsible for human and veterinary diseases (Nagase *et al.* 2002). Similar results were reported

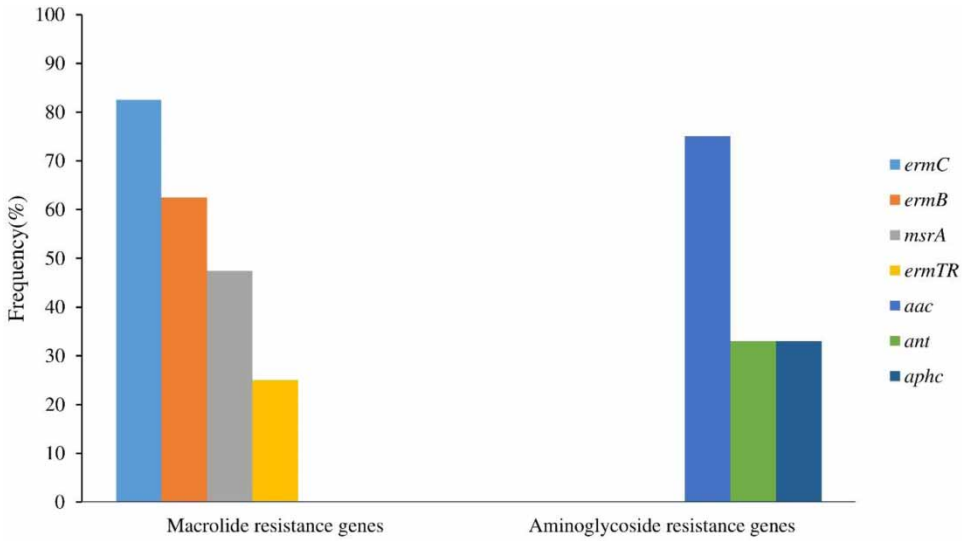


Figure 3 | Frequency distribution of macrolide and aminoglycoside resistance genes in *Staphylococcus aureus* isolates collected from wastewater in Ardabil, northwestern Iran.

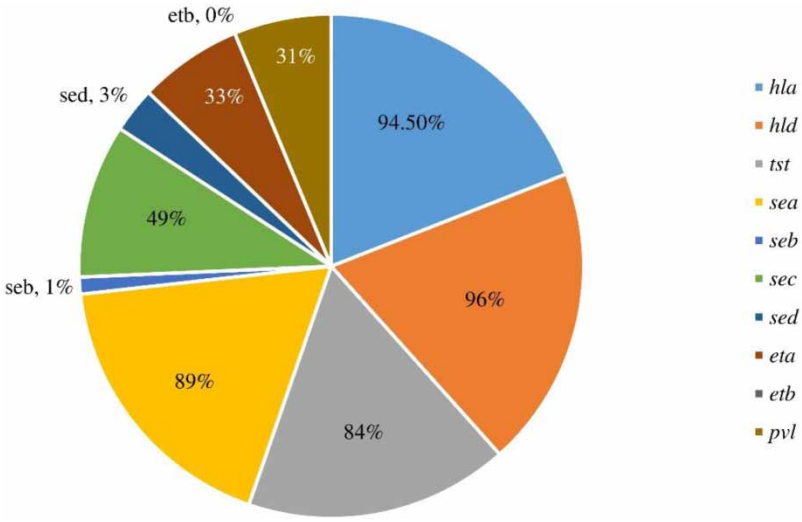


Figure 4 | Frequency distribution of genes encoding virulence factors in *Staphylococcus aureus* isolates collected from wastewater in Ardabil, northwestern Iran.

around the world on the low occurrence of these species in wastewater. The prevalence was estimated to be ≤6.2% for *S. hominis* and *S. haemolyticus* and 23% for *S. epidermidis* in wastewater (Faria *et al.* 2009; Heß & Gallert 2014; Gómez *et al.* 2016).

CoNS are among the animals and human normal microbiota (Nagase *et al.* 2002) entering sewage through animal and human excrements (Aarestrup 2001; Werckenthin *et al.* 2001; Heß & Gallert 2014). Therefore, the variation in the occurrence of CoNS in wastewaters may arise from the variation in the abundance of microorganisms in animal and human host bodies at different regions.

In the present study, 44.5% of the isolates were characterized as CoPS, majorly belonging to *S. aureus* (80%). Most of the *S. aureus* isolates (67%) were found in hospital wastewater. Our findings are in contrast with earlier studies indicating that *S. aureus* is absent or less prevalent (0.4–20%) in wastewater (Schwartz *et al.* 2003; Volkmann *et al.* 2004; Shannon *et al.* 2007; Faria *et al.* 2009; Börjesson *et al.* 2010; Heß & Gallert 2014; Moges *et al.* 2014; Gómez *et al.* 2016). Only 2% of isolates were MRSA strains. Albeit the low occurrence of MRSA was reported in urban wastewater in Spain (Gómez *et al.* 2016) but most of the studies showed higher occurrence of MRSA strains, as in Iran (15.3%), Australia (50–55%), USA (68%), and Sweden (100%). Most of these studies have been on municipal and hospital wastewater (Börjesson *et al.* 2010; Goldstein *et al.* 2012; Thompson *et al.* 2013; Rahimi & Bouzari 2015).

Similar to some other reports around the world (Igimi *et al.* 1990; Abraham *et al.* 2007; Faria *et al.* 2009; Sasaki *et al.* 2010; Heß & Gallert 2014), *S. intermedius* (14.5%), *S. hyicus* (4.3%), and *S. schleiferi subsp. coagulans* (1.4%) were the other CoPS species identified in wastewater samples in our study. Most of them were isolated from livestock and poultry wastewater. This is in accordance with the fact that these species mainly colonize animal hosts and are responsible for infections such as pyoderma, otitis externa and genitourinary tract diseases in animals. However, they also cause opportunistic infections in humans (Sasaki *et al.* 2007, 2010).

Unless clinics, the antibiotic resistance in aquatic environments was less studied (Sharma *et al.* 2016). Extensive usage of antibiotics in hospitals, home therapy, veterinary and other areas hence continues the release of their residues into wastewater develops drug-resistant organisms (Heß & Gallert 2014). MDR *Staphylococci* (*S. aureus* and CoNS) has been detected in various environmental sources (Abulreesh 2011). In the present study, overall 50% of *S. aureus* isolates and 47% of CoNS isolates were MDR.

We found that *S. aureus* isolates were mostly penicillin resistance (89%). Similarly, penicillin resistance frequency was reported as 100% in *S. aureus* isolates collected from poultry and municipal wastewaters in Iran (Rahimi & Bouzari 2015; Rahimi & Karimi 2015). As well as the frequency of resistance against erythromycin, azithromycin and tetracycline were high in *S. aureus* and CoNS isolated from wastewaters. This finding is in agreement with other studies all over the world (Nawaz *et al.* 2000; Schwartz *et al.* 2003; Faria *et al.* 2009; Heß & Gallert 2014; Kumar *et al.* 2015).

Higher prevalence of macrolides, penicillin and tetracycline resistance among *Staphylococci* could be attributed to the expanded utilization of these agents in human and outside human medicine. In a systematic review performed in 2021, it was revealed that the median of antibiotic prescribing accounted for 45.25 and 68.2% of outpatient and inpatient settings in Iran, respectively. β-lactams (e.g., penicillins, cephalosporins, and carbapenems) and macrolides were the most commonly prescribed antibiotic classes (Nabovati *et al.* 2021). According to World Health Organization (WHO), about half of the world-wide-produced antibiotics are sold for use by outside humans (World Health Organization 2002). Medically important antibiotics were commonly used for the prophylactic purpose, growth promotion in food-producing animals and veterinary. In line with the global trends, tetracycline, penicillin and macrolides are among the most common antibiotics used in live-stock and poultry farms in Iran (Alipour *et al.* 2014). Widespread usage of tetracycline, penicillin and macrolides both in humans and outside of human medicine could explain the high prevalence of resistance to these antibiotics in the current study.

Erythromycin-resistant *Staphylococcus* spp. isolates may display cross-resistance to clindamycin. About 11% of *S. aureus* isolates showed inducible clindamycin resistance in our study. Inducible resistance to clindamycin was reported as 19 and 32% in Germany and France, respectively (Lina *et al.* 1999; Heß & Gallert 2014). *erm* A, *erm* B, *erm* C, and *erm* TR genes are involved in erythromycin resistance in *S. aureus* by expressing methylase enzyme (Heß & Gallert 2014). While *erm* B gene is prominent in isolates from animal sources (Lina *et al.* 1999), *erm* C and *erm* A are dominant in clinical isolates (Fiebelkorn *et al.* 2003; Gherardi *et al.* 2009). Similarly, the frequency of *erm C* gene was high in our study (82.5%) and may be due to sources of our isolates mainly collected from hospital sewage. Also the *erm* B, *erm* TR, and *msr* A genes were found in our *S. aureus* isolates. Another mechanism to develop erythromycin resistance is to express *msr*A, a drug efflux pump-encoding gene (Heß & Gallert 2014). The *msr A* gene was found in nearly half of our isolates. It can be concluded that drug efflux along erm genes causes erythromycin resistance in current *S. aureus* isolates.

Resistance to aminoglycoside antibiotics tobramycin, kanamycin and gentamycin were identified in 11, 4 and 2% of *S. aureus* isolates, respectively. Aminoglycoside-resistant isolates carried *aac (*22.5%), ant *(1%,)* and *aphc* (10%) genes. Some of the previous studies reported that *aac* gene has the highest frequency among other genes encoding aminoglycoside resistance (Börjesson *et al.* 2010; Goldstein *et al.* 2012; Gómez *et al.* 2016).

Toxins play a vital role in the pathogenesis of staphylococcal infections. High variation in frequency of toxin encoding genes in *S. aureus* has been documented worldwide (Shallcross *et al.* 2013; Bhowmik *et al.* 2021). Wastewater pathogenic microbial population originates from human or animal excretions. Therefore, variations in the abundance of virulence genes in wastewater isolates in different regions may arise from the differences in virulence traits of the normal flora of the hosts. This can be approved by the fact that the pattern of virulence genes among our isolates is similar to the previous isolates collected in Ardabil from patients (unpublished data) and healthy people (Omid *et al.* 2021). It is clear that changing the sampling places can lead to differences in the virulence genes frequency.

In this study, the most prominent toxin genes were *α*-hemolysin encoding, *hld* (96%) and *hla* (94.5%) in *S. aureus* isolates. According to results reported by some previous studies, the *hla* and *hld* gene prevalence is 70–98% in *Staphylococci* isolates in clinical and non-clinical settings (Sharma-Kuinkel *et al.* 2015; Rossato 2018; Verdú-Expósito *et al.* 2020; Zieliński *et al.* 2020). *α*-hemolysin damages host cells by creating channels and disrupting the membrane (Tang *et al.* 2019). This toxin affects innate immune cells, disrupts epithelial and endothelial barriers, and stimulates a hyperinflammatory response following staphylococcal pneumonia (Tabor *et al.* 2016).

Several enterotoxins are responsible for food poisoning *by S. aureus*. Type A enterotoxin is the most common cause of food poisoning in humans (Argudín *et al.* 2010). In this study, *sea,* encoding type A staphylococcal enterotoxin was the predominant gene (87%) followed by *sec* (47%) encoding type C, *sed* (14.5%) encoding type D and *seb* (5.4%) encoding and B enterotoxins.

Toxic shock syndrome (TSS) is a deadly staphylococcal disease typically caused by the toxic shock syndrome toxin 1 (TSST-1) encoded by the *tst* gene (Parrish *et al.* 2019). We found *tst* gene in 84% of *S. aureus* isolates which is higher than other reports ranging 2–28.5% (Robert *et al.* 2011; Zieliński *et al.* 2020; Bhowmik *et al.* 2021).

The exfoliative toxins (encoded by *eta* and *etb*) cause the staphylococcal scaled-skin syndrome (SSSS). *eta* gene was carried by 33% of *S. aureus* isolates, while we could not detect *etb* gene. Previous studies have shown a higher prevalence (34–61%) of both of these genes in clinical and colonizing *S. aureus* isolates (Shukla *et al.* 2010; Champion *et al.* 2014).

Another studied gene was pvl encoding a two components toxin named Panton-Valentine leukocidin (PVL). PVL damages leukocytes membrane by creating pores. *pvl* was detected in 31% of isolates which locates in the range of earlier reported

values (4.4–100%) (Shukla *et al.* 2010; Alfatemi *et al.* 2014; Papadimitriou-Olivgeris *et al.* 2017; Wang *et al.* 2017; Darboe *et al.* 2019; Veloso *et al.* 2019).

In the present study, eight isolates of *S. aureus* were assessed by spa typing approach in which six various types were identified including t346, t026, t14870, t937, t17068, and a new type t19215. Among them just t19215 belongs to MRSA strain. This finding is in agreement with our previous investigation on spa types in isolates from healthy people (Omid *et al.* 2021). Surveys on *S. aureus* isolates from wastewater in Spain and United States (Sauer *et al.* 2008; Friese *et al.* 2013) identified 15 spa types and just type t346 was identical to our result.

5. CONCLUSION

In the current study, multiple CoNS and CoPS species with veterinary and clinical significance were identified in the sewages from different sources. The most common CoPS and CoNS species were *S. aureus* and *S. xylosus*, respectively. A significant portion of both CoNS and CoPS isolates were resistant to multiple antibiotics. *S. aureus* isolates carried multiple virulence-encoding genes. The occurrence of these superbugs in wastewater is of great health concern indicating the importance of water treatment to eliminate antibiotic-resistant bacteria. We suggest using chemicals with high antimicrobial activity in wastewater processing in order to remove antibiotic-resistant bacteria and antibiotic resistance-encoding genes efficiently.

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AUTHORS' CONTRIBUTION

M.R.O. performed the experiments, contributed to the analysis of the results and drafted the manuscript. H.J. helped supervise the project and worked on the manuscript. F.K. helped in interpreting the results and worked on the manuscript. A.B. contributed to the final version of the manuscript. M.A. supervised the project, designed the study, conceived and planned the experiments, contributed to the analysis of the results, and took the lead in writing the manuscript.

DATA AVAILABILITY STATEMENT

All relevant data are included in the paper or its Supplementary Information.

CONFLICT OF INTEREST

The authors declare there is no conflict.

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