Antibiotic-resistant staphylococci from the wastewater treatment plant and grey-water samples in Obafemi Awolowo University, Ile-Ife, Nigeria

Tomiwa Olumide Adesoji, Beverly Egyir and Adebayo Osagie Shittu

ABSTRACT

This study examined the occurrence and molecular basis for antibiotic-resistant staphylococci from the wastewater treatment plant and grey-water samples in Obafemi Awolowo University, Nigeria. Standard microbiological techniques and molecular methods were utilized. The species identified (MALDI score >1.7) comprised *S. saprophyticus* (19), *S. cohnii* (8), *S. sciuri* (7), *S. aureus* (4), *S. epidermidis* (3), *S. warneri* (2), *S. equorum* (1), *S. haemolyticus* (1), *S. nepalensis* (1), *S. condimenti* (1), and *S. pasteuri* (1). Resistance to trimethoprim, tetracycline and cefoxitin were observed in 78.3% (47/60), 36.7% (22/60) and 25% (15/60) of the isolates, respectively. The rate of multidrug resistance was 53.3% (32/60) and observed in eight species from different sampling sites. Seven (*S. sciuri*; $n = 5$; *S. aureus*; $n = 1$; *S. warneri*; $n = 1$) of the 20 selected (representing the various staphylococcal species and antibiotypes) isolates were *mecA*-positive. Furthermore, the *tetK* gene was detected in nine isolates, six with *dfrA,* and four were positive for the *dfrG* gene. One *S. aureus* was *mecA*, *tetK* and *dfrG* gene positive. The study provides insights on antibiotic-resistant staphylococci from a non-clinical setting and highlights the need for active surveillance to understand the burden of antimicrobial resistance in Nigeria. This is key to improve synergy across the human, animal and environmental health sectors in Nigeria.

Key words | antimicrobial resistance, grey-water, staphylococci, wastewater treatment plant

HIGHLIGHTS

- The research provides information on the presence and species diversity of antibiotic-resistant staphylococci in wastewater treatment plant and grey-water in Ile-Ife, Nigeria.
- The molecular basis for antibiotic resistance in staphylococci to methicillin, tetracycline and trimethoprim was reported in the study.
- The study reports the development of a multiplex PCR procedure for the prompt detection of tetracycline and trimethoprim resistance genes in staphylococci.
- The study highlights the need for active antimicrobial resistance surveillance to understand the burden of antimicrobial resistance in Nigeria.
- In addition, the study highlights the need for synergistic approach between human, animal and environmental health in overcoming the fight against antimicrobial resistance in Nigeria using the 'One Health' approach.

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INTRODUCTION

Wastewater (including grey-water – household waste devoid of fecal matter) is an integral part of human activities. However, its indiscriminate discharge to various environmental receptors (stream/rivers, seas) has profound consequences on human health and ecosystems (Naidoo & Olaniran 2014). These adverse effects could be reduced by a combination of physicochemical and biological methods in wastewater treatment plants (WWTPs). These facilities are designed to mitigate the environmental and health hazards of polluted water, and make it suitable for various activities (Lood *et al.* 2017; Manaia *et al.* 2018). Despite these achievements, WWTPs are now widely regarded as hotspots for the development of antibiotic-resistant bacteria (ARB). This could be attributed to the presence of antibiotic residues at sub-lethal concentrations, the interaction of different bacterial species in the ecosystem, and horizontal gene transfer events (Manaia *et al.* 2018). ARB, including methicillin-resistant *Staphylococcus aureus* (MRSA), an important human and animal pathogen, have been recovered from WWTPs (Borjesson *et al.* 2010; Goldstein *et al.* 2012; Gómez *et al.* 2016). In addition, antibiotic-resistant coagulase-negative staphylococci (CoNS) have also been detected in wastewater and WWTPs (Faria *et al.* 2009; Gómez *et al.* 2016). ARB and residual antibiotics have been reported from wastewater in Nigeria (Lateef *et al.* 2007; Adelowo *et al.* 2008; Oyetibo *et al.* 2010). These studies identified ARB (Lateef *et al.* 2007), including those exhibiting co-resistance with antiseptics (Adelowo *et al.* 2008), and heavy metal, for example cadmium, cobalt, nickel and mercury (Oyetibo *et al.* 2010). Moreover, recent data on extended-spectrum beta-lactamase (ESBL) producing bacteria and associated genes from untreated hospital wastewater (Adelowo *et al.* 2018) provide evidence that wastewater could be a potential reservoir for ARB. This portends a risk to public health as ARB and resistance genes could be disseminated through this medium to humans, animals and the environment. There is a paucity of data on antibiotic-resistant staphylococci from environmental samples including wastewater or WWTPs in Nigeria. This study examined the occurrence of antibiotic-resistant staphylococci and their resistance genes from the WWTP and grey-water samples in Obafemi Awolowo University (OAU), Ile-Ife, Nigeria.

METHODS

Sample collection and identification of staphylococci

Wastewater samples were collected from the Obafemi Awolowo University oxidation pond (inlet, pond and outlet), while grey-water samples were obtained from effluent points in three student halls of residence (Fajuyi Hall, Moremi Hall, and Postgraduate Hall) on the university campus. Grab samples (250 mL) were collected in sterile 500 mL bottles and transported immediately to the laboratory (in an icebox). Samples were collected every fortnight for a period of six months (June–November 2017). A series of ten-fold dilutions of the samples were performed, and 100 μL of the sample was plated on mannitol salt agar (MSA) and incubated at 37 °C for 48 hours. Colonies (1–5 per sample) with typical morphology of staphylococci were selected from each MSA plate for subsequent investigations. Identification was carried out following standard microbiological techniques (Gram staining, catalase, coagulase, DNase and oxidase tests). This was followed by the matrix-assisted laser desorption/ionization-time of flight mass spectrometry (MALDI-TOF MS, Bruker, Dalton, Germany), using the identification threshold of 1.7 (Han *et al.* 2015). MALDI-TOF is a high throughput system that involves the generation of mass spectra from whole-cell material or extracted intracellular content which are then compared with a database reference (Angeletti 2016). The major advantages of MALDI-TOF MS compared with routine phenotypic methods include the quick and reliable identification of microbes.

Antibiotic susceptibility testing

Antibiotic susceptibility testing was carried out on staphylococcal isolates using the disk diffusion method. This was performed with 13 antibiotics: cefoxitin (30 μg),

chloramphenicol (30 μg), clindamycin (2 μg), ciprofloxacin (10 μg), erythromycin (15 μg), fusidic acid (10 μg), gentamicin (10 μg), mupirocin (5 and 200 μg), penicillin G (10 U), rifampicin (5 μg), tetracycline (30 μg), trimethoprim (5 μg), and trimethoprim-sulfamethoxazole (1.25/23.75 μg). The zones of inhibition were measured and interpreted using the guidelines of the Clinical and Laboratory Standard Institute (CLSI 2016). The breakpoint values of the British Standard for Antimicrobial Chemotherapy (BSAC 2011) were employed for fusidic acid and mupirocin. Multidrug resistance (MDR) was defined as resistance to at least three classes of antibiotics.

Detection of antibiotic resistance and virulence genes

The resistance genes for trimethoprim (*dfrA* and *dfrG*), methicillin (*mecA*), and tetracycline (*tetK*) were investigated by PCR based on the widespread use of these antibiotics (trimethoprim and tetracycline) and clinical importance (methicillin) in Nigeria. Twenty isolates were selected based on their antibiotypes and to represent the various staphylococcal species identified in the study. A multiplex PCR reaction was performed for the detection of staphylococcal protein A (*spa*), Panton–Valentine Leukocidin (PVL) and methicillin resistance (*mecA*) genes as previously described (Larsen *et al.* 2008). A uniplex PCR was performed for the detection of the *dfrA*, *dfrG*, and *tetK* genes (Ng *et al.* 2001; Argudín *et al.* 2011). Thereafter, a multiplex PCR was developed for the detection of the above-mentioned genes. The multiplex PCR conditions were as follows: initial denaturation at 94 °C for 5 minutes followed by 35 cycles of denaturation at 94 °C for 60 seconds, annealing at 54 °C for 60 seconds, and elongation at 72 °C for 60 seconds. The final extension was at 72 °C for 4 minutes. The primers for the detection of virulence and resistance genes are described in Table 1. The species confirmation (MALDI-TOF) and molecular characterization of the isolates were conducted at the Noguchi Memorial Institute for Medical Research, University of Ghana.

RESULTS

Of the 72 wastewater/grey-water samples obtained in the study, 153 isolates from 43 samples (wastewater: $n = 15$; grey-water: $n = 28$) were presumptively identified as staphylococci. For this baseline study, 60 isolates (WWTPs: $n = 15$, grey-water samples: Fajuyi Hall, $n = 15$; Moremi Hall, $n = 15$ and Post-Graduate Hall, $n = 15$) were selected for identification by MALDI-TOF MS to represent the various sampling sites. Overall, eleven staphylococcal species were noted with an identification score of >1.7. They comprised *S. saprophyticus* (19; 31.7%), *S. cohnii* (8; 13.3%), *S. sciuri* (7; 11.7%), *S. aureus* (4; 6.7%), *S. epidermidis* (3; 5%), *S. warneri* (2; 3.3%), *S. equorum* (1; 1.7%), *S. haemolyticus* (1; 1.7%), *S. nepalensis* (1; 1.7%), *S. condimenti* (1; 1.7%), and *S. pasteuri* (1; 1.7%). However, 12 (20%) of the 60 isolates gave an identification score of <1.7 (Supplementary material). Antibiotic susceptibility test results showed that

Table 1 | Primers used for the study

Genes	Description	Primers	Amplicon sizes	References
mecA	Methicillin resistance	Forward: TCCAGATTACAACTTCACCAGG Reverse: CCACTTCATATCTTGTAACG	162 bp	Larsen *et al.* (2008)
PVL	Panton-Valentine Leukocidin	Forward: GCTGGACAAAACTTCTTGGAATAT Reverse: GATAGGACACCAATAAATTCTGGATTG	80 bp	Larsen *et al.* (2008)
dfrA	Trimethoprim resistance	Forward: CACTTGTAATGGCACGGAAA Reverse: CGAATGTGTATGGTGGAAAG	270 bp	Argudín *et al.* (2011)
dfrG	Trimethoprim resistance	Forward: TGCTGCGATGGATAAGAA Reverse: TGGGCAAATACCTCATTCC	405 bp	Argudín *et al.* (2011)
tetK	Tetracycline resistance	Forward: TCGATAGGAACAGCAGTA Reverse: CAGCAGATCCTACTCCTT	169 bp	Ng *et al.* (2001)
Spa	Staphylococcal protein A	Forward : TAAAGACGATCCTTCGGTGAGC Reverse: CAGCAGTAGTGCCGTTTGCTT	Variable	Larsen *et al.* (2008)

all the 60 staphylococcal isolates were susceptible to mupirocin that is commonly used to treat staphylococcal skin infections. However, the proportion of isolates resistant to trimethoprim was >50% across all species except *S. nepalensis* and *S. condimenti* (Table 2). A total of 27 antibiotypes were identified and the two main groups consisted of the following: resistance to fusidic acid and trimethoprim ($n = 12$), and resistance to erythromycin, fusidic acid, cefoxitin, gentamicin, and trimethoprim ($n = 6$). MDR was recognized in eight species in various sampling sites (Supplementary material). They included *S. saprophyticus* ($n = 8$), *S. cohnii* ($n = 5$), *S. equorum* ($n = 5$), *S. sciuri* ($n = 5$), *S. haemolyticus* ($n = 3$), *S. epidermidis* ($n = 3$), *S. capitis* ($n = 2$), and *S. aureus* ($n = 1$). Overall, 15 staphylococcal isolates were resistant to cefoxitin of which 11 (73.3%) exhibited MDR. The major antibiotype, i.e. resistance to erythromycin, fusidic acid, cefoxitin, gentamicin, and trimethoprim, was noted in *S. sciuri* ($n = 5$) and *S. epidermidis* ($n = 1$).

Of the 20 selected staphylococci, seven (*S. sciuri* ($n = 5$), *S. aureus* ($n = 1$) and *S. warneri* ($n = 1$)) were *mecA* positive. One methicillin-susceptible *S. aureus* (MSSA) was PVL positive (Table 3). Based on the multiplex PCR, nine of the 10 tetracycline-resistant isolates were positive for the *tetK* gene. The trimethoprim resistance (dfrG and dfrA) genes were detected in four and six isolates, respectively (Figure 1 and Supplementary material).

DISCUSSION

Wastewater generation and treatment is an integral part of anthropogenic activities which could facilitate the development and spread of ARB. Wastewater usually ends up in other water bodies handled by lower stream end-users and could pose a threat to public health. In this study, a total of eleven staphylococcal species were identified from WWTP and grey-water samples in the University. Some members of the staphylococci recovered in this study (i.e. *S. aureus*, *S. epidermidis*, *S. saprophyticus*, *S. haemolyticus*, *S. sciuri* and *S. cohnii*) have been reported in hospital wastewater effluents and municipal WWTP in Nigeria (Oladipo *et al.* 2019) and Spain (Gómez *et al.* 2016), respectively. Furthermore, 53% (32/60) of the isolates in our study were MDR and detected across different sampling sites. This level of MDR is similar to recent reports from hospital wastewater in Ile-Ife (Oladipo *et al.* 2019) and Ibadan (Adekanmbi *et al.* 2019) in South-West Nigeria. These findings suggest that wastewater could be a potential reservoir for ARB in Nigeria. The proportion of methicillin-resistant staphylococci was 25% (15/60), and the *mecA* gene was detected in seven (including one MRSA) of the 20 isolates screened. Moreover, all the *S. sciuri* isolates investigated were *mecA* positive. This species has been described with carriage of the SCC*mec*-like *mecA* with a putative antibiotic resistance gene pool, with considerable ability to survive in various environments (Schoenfelder *et al.* 2017). The presence of MRSA in wastewater is a public health concern as it indicates that the non-clinical environment could play a role in its transmission to humans (Porrero *et al.* 2014). Although data on microbial exposure on WWTP workers are not available in Nigeria, the identification of a PVL-positive MSSA from WWTP in this study is also of concern, particularly for WWTP workers and individuals who could be exposed through inhalation or dermal exposure to reclaimed wastewater (Goldstein *et al.* 2012).

Trimethoprim and tetracycline are widely used in Nigeria due to their low cost and broad availability (Shittu *et al.* 2011). However, trimethoprim resistance in staphylococci has been reported to be as high as 85% in humans (Nurjadi *et al.* 2014; Ayepola *et al.* 2018). Furthermore, the prevalence of tetracycline-resistant staphylococci has been reported to be about 55% in human samples (Shittu *et al.* 2011). The molecular basis for trimethoprim resistance is attributed to these resistance (*dfrA*, *dfrB*, *dfrG* and *dfrK*) genes. Similarly, resistance to tetracycline is conferred by various mechanisms including efflux pump, enzymatic and ribosomal protections, but mainly through the *tetK* and *tetM* genes (Schwarz *et al.* 2014). In this study, resistance to trimethoprim and tetracycline was observed across different staphylococcal species. The multiplex PCR assay also provided some insights into their gene determinants. Resistance to trimethoprim was mediated by the *dfrA* and *dfrG* genes. This resistance determinant (*dfrG*) has been reported as predominant in clinical *S. aureus* isolates in Nigeria (Nurjadi *et al.* 2014). We observed that some isolates were resistant to trimethoprim but were *dfrA* and *dfrG* gene negative, and should be further investigated. In addition, the *tetK* gene was detected in most of the tetracycline-resistant

Table 2 | Antibiotic resistance of selected staphylococcal isolates obtained from wastewater and grey-water samples in Obafemi Awolowo University, Ile-Ife

	Number of staphylococcal isolates that exhibited resistance (%)												
Antibiotics (potency: μg)	***S. saprophyticus* (19)**	***S. cohnii* (8)**	***S. sciuri* (7)**	***S. aureus* (4)**	***S. epidermidis* (3)**	***S. warneri* (2)**	***S. equorum* (1)**	***S. nepalensis* (1)**	***S. haemolyticus* (1)**	***S. pasteuri* (1)**	***S. condimenti* (1)**	**Low identity score (12)**	**Total (60)**
C (30 μg)	5 (26.3)	2 (25)	0	0	0	0	0	1 (100)	0	0	0	1 (8.3)	9 (15)
CD (2 μg)	0	0	2 (28.6)	0	1 (33.3)	0	0	0	0	0	0	0	3 (5)
CIP (10 μg)	0	1 (12.5)	0	1 (25)	1 (33.3)	0	0	0	0	0	0	1 (8.3)	4 (6.7)
E (15 μg)	1 (5.3)	3 (37.5)	5 (71.4)	0	1 (33.3)	0	0	0	0	0	0	2 (16.7)	12 (20)
FD (10 μg)	19 (100)	8 (100)	7 (100)	0	1 (33.3)	1 (50)	1(100)	0	0	0	0	12 (100)	49 (81.7)
FOX (30 μg)	1 (5.3)	1 (12.5)	7 (100)	1 (25)	2 (66.7)	0	0	0	1 (100)	0	0	2 (16.7)	15 (25)
GM (10 μg)	0	0	5 (71.4)	1 (25)	2 (66.7)	0	0	0	0	0	0	0	8 (13.3)
MUP (5 μg)	0	0	0	0	0	0	0	0	0	0	0	0	0
MUP (200 μg)	0	0	0	0	0	0	0	0	0	0	0	0	0
PG (10 U)	5 (26.3)	2 (25)	6 (85.7)	2 (50)	3 (100)	0	0	0	1 (100)	0	0	9 (75)	28 (46.7)
RP (5 μg)	1 (5.3)	0	0	0	0	0	0	0	0	0	0	0	1 (1.6)
T (30 μg)	5 (26.3)	4 (50)	0	1 (25)	1 (33.3)	1 (50)	0	1 (100)	1 (100)	0	0	8 (88.9)	22 (36.7)
TM (5 μg)	18 (94.7)	7 (87.5)	5 (71.4)	4 (100)	2 (66.7)	1 (50)	1 (100)	0	1 (100)	1 (100)	0	7 (77.8)	47 (78.3)
TS (25 μg)	16 (84.2)	3 (37.5)	0	3 (75)	1 (33.3)	0	0	0	1 (100)	0	0	1 (8.3)	25 (41.7)

Key – C: Chloramphenicol; CD: Clindamycin; CIP: Ciprofloxacin; E: Erythromycin; FD: Fusidic acid; FOX: Cefoxitin; GM: Gentamicin; MUP: Mupirocin; PG: Penicillin; RP: Rifampicin; T: Tetracycline; TM: Trimethoprim; TS: Trimethoprim-sulfamethoxazole. Values in brackets are percentages.

Table 3 | Antibiotic resistance pattern and associated gene determinants in selected isolates

	Sample code	Sample site	Identity of Isolates	Antibiotic resistance pattern	Resistance genes	Virulence gene (PVL)
Isolates obtained from wastewater samples	OPI9E	Oxidation pond (inlet)	*S. aureus*	TM (TM)	*dfrG*	+
	OPI10C	Oxidation pond (inlet)	*S. aureus*	CIP-GM-FOX-T-TM (FOX-T-TM)	*dfrG*, *mecA*, *tetK*	–
	OPP3A	Oxidation pond (pond)	*S. sciuri*	E-FD-FOX-GM-TM (FOX-TM)	*mecA*	ND
	OPI4G	Oxidation pond (inlet)	*S. sciuri*	E-FD-FOX-GM-TM (FOX-TM)	*mecA*	ND
	OPI8A	Oxidation pond (inlet)	*S. sciuri*	E-FD-FOX-GM-TM (FOX-TM)	*mecA*	ND
	OPO5D	Oxidation pond (outlet)	*S. saprophyticus*	C-FD-T-TM (T-TM)	*dfrG*, *tetK*	ND
	OPI8H	Oxidation pond (inlet)	*S. pasteuri*	TM (TM)	*dfrA*	ND
	OPP8B	Oxidation pond (pond)	*S. condimenti*	Susceptible to all	–	ND
Isolates obtained from grey-water samples	WWPG8F	Postgraduate Hall	*S. sciuri*	E-FD-FOX-GM-TM (FOX-TM)	*mecA*	ND
	WWF8B	Fajuyi Hall	*S. sciuri*	E-FD-FOX-GM-TM (FOX-TM)	*mecA*	ND
	WWM1B	Moremi Hall	*S. saprophyticus*	C-FD-PG-RP-T-TM (T-TM)	*tetK*	ND
	WWF5D	Fajuyi Hall	*S. saprophyticus*	FD-T-TM (T-TM)	*dfrA*, *tetK*	ND
	WWM8I	Moremi Hall	*S. saprophyticus*	C-FD-T-TM (T-TM)	*dfrA*, *tetK*	ND
	WWPG8I	Postgraduate Hall	*S. cohnii*	FD-T (T)	*tetK*	ND
	WWPG16E	Postgraduate Hall	*S. cohnii*	FD-T-TM (T-TM)	*tetK*	ND
	WWF4F	Fajuyi Hall	*S. epidermidis*	E-FD-FOX-GM-TM (FOX-TM)	*dfrA*	ND
	WWF10G	Fajuyi Hall	*S. haemolyticus*	FOX-T-TM (FOX-T-TM)	*dfrG*	ND
	WWM4G	Moremi Hall	*S. nepalensis*	C-T (T)	*tetK*	ND
	WWF8D	Fajuyi Hall	*S. warneri*[a]	TM (TM)	*dfrA*, *mecA*	ND
	WWF5B	Fajuyi Hall	*S. equorum*	C- FD-FOX- T-TM- (FOX-T-TM)	*dfrA*, *tetK*	ND

Key – C: Chloramphenicol, CIP: Ciprofloxacin, E: Erythromycin, FD: Fusidic acid, FOX: Cefoxitin, GM: Gentamicin, PG: Penicillin G, RP: Rifampicin, T: Tetracycline, TM: Trimethoprim. ND: Not detected. WWM, WWF, and WWPG: grey-water samples from Moremi Hall, Fajuyi Hall and Postgraduate Hall, respectively. OPI, OPP, and OPO: wastewater samples from oxidation in-let, pond and outlet, respectively. Antibiotics in parenthesis were screened for their resistance genes using PCR.

[a]Phenotypically susceptible to cefoxitin (27 mm) but *mecA* positive.

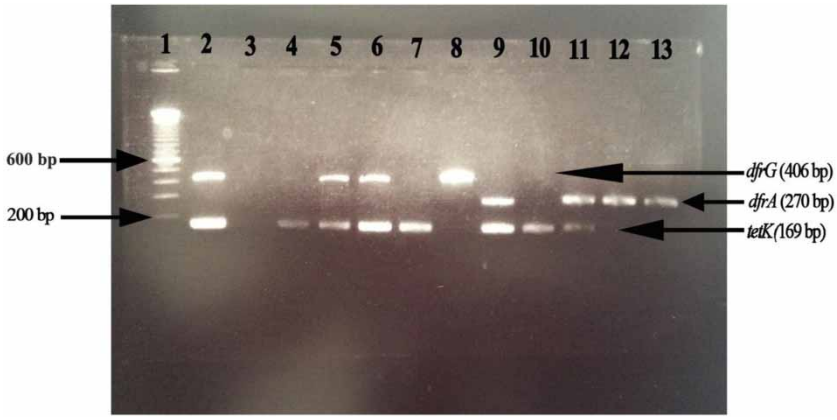

Figure 1 | Detection of the *dfrA*, *dfrG*, and *tetK* genes by multiplex PCR among staphylococcal isolates obtained from WWTP and grey-water samples.

isolates analyzed in our investigation. The multiplex PCR assay is a cost-effective method that could assist in the prompt characterization of staphylococcal isolates exhibiting resistance to these classes of antibiotics in Nigeria. The detection of these antibiotic resistance genes in a non-clinical environment warrants further investigation as potential reservoirs for dissemination to clinical settings.

The number of samples and isolates analyzed was a limitation in the study. Nevertheless, this investigation provides baseline information on antibiotic-resistant staphylococci from wastewater and the associated resistance genes from a non-clinical setting. This could be of public health significance as wastewater could be a medium for the dissemination of ARB in the environment. Moreover, innovative wastewater treatment strategies are needed to minimize the potential health risks associated with the spread of ARB. Furthermore, synergy across the human, animal and environmental health sectors is essential with the goal on the establishment of a national antimicrobial resistance (AMR) surveillance system using a 'One Health' approach in Nigeria.

CONCLUSIONS

The study has provided baseline information on antibiotic-resistant staphylococci in WWTP and grey-water samples in Ile-Ife, Nigeria. The detection of antibiotic resistance genes in staphylococcal isolates from the non-clinical environment could have a significant public health impact on humans. In addition, antibiotic resistance genes could be transferred to other pathogens through horizontal gene transfer. Further study is required to investigate the clonal nature (genetic relatedness) of the staphylococci. This would help to understand the evolution and epidemiology of the staphylococci in the wastewater environment.

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ETHICAL STATEMENT

Formal approval of the ethics committee was not required as environmental samples were analyzed.

DATA AVAILABILITY STATEMENT

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

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