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ORIGINAL PAPER

Prevalence and molecular characterization of multidrug-resistant coagulase negative staphylococci from urban wastewater in Delhi-NCR, India

Sonali Rajput[1] · Sayani Mitra[1] · Aftab Hossain Mondal[1] · Himani Kumari[1] · Kasturi Mukhopadhyay[1]

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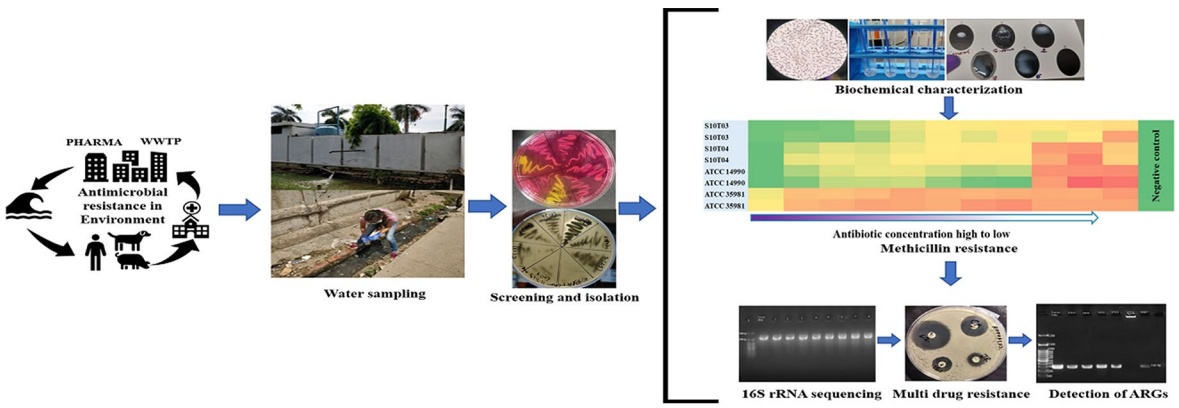
Abstract

Antimicrobial resistance (AMR) is global health concern escalating rapidly in both clinical settings and environment. The effluent from pharmaceuticals and hospitals may contain diverse antibiotics, exerting selective pressure to develop AMR. To study the aquatic prevalence of drug-resistant staphylococci, sampling was done from river Yamuna (3 sites) and wastewater (7 sites) near pharmaceutical industries in Delhi-NCR, India. 59.25% (224/378) were considered presumptive staphylococci while, methicillin resistance was noted in 25% (56/224) isolates. Further, 23 methicillin-resistant coagulase negative staphylococci (MR-CoNS) of 8 different species were identified via 16S rRNA gene sequencing. Multidrug resistance (MDR) was noted in 60.87% (14/23) isolates. PCR based detection of antibiotic resistance genes revealed the number of isolates containing *mecA* (7/23), *blaZ* (6/23), *msrA* (10/23), *aac(6')aph (2")* (2/23), *aph(3')-IIIa* (2/23), *ant(4')-Ia* (1/23), *dfrG* (4/23), *dfrA*(*drfS1*) (3/23), *tetK* (1/23) and *tetM* (1/23). The current research highlights the concerning prevalence of MDR-CoNS in aquatic environment in Delhi.

Graphical abstract



Keywords Antibiotic resistance gene · Genotype · Methicillin-resistant staphylococci · Multidrug resistance · Phenotype · Urban wastewater

Introduction

Staphylococci are a genus of great significance, as they are ubiquitous and affect the health of humans, animals, and the overall environment. Staphylococci fall into two primary groups: coagulase positive (CoPS) and coagulase negative staphylococci (CoNS). CoPS, spearheaded by

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✉ Kasturi Mukhopadhyay
kasturim@mail.jnu.ac.in

[1] Antimicrobial Research Laboratory, School of Environmental Sciences, Jawaharlal Nehru University, New Delhi 110067, India

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Staphylococcus aureus, is responsible for skin infections, pneumonia, septicemia, endocarditis and toxic shock syndrome. CoNS, like *Staphylococcus epidermidis*, are often associated with catheter and medical device-related infections, surgical site infections, neonatal sepsis, etc. In recent years, some species of CoNS, such as *Staphylococcus cohnii*, *Staphylococcus haemolyticus*, *Staphylococcus arlettae*, *Staphylococcus saprophyticus*, and *Staphylococcus sciuri*, have garnered attention due to their emerging pathogenic characteristics (Becker et al. 2014; Schoenfelder et al. 2017). *S. hemolyticus* also accounts for the largest clinical impacts related to meningitis, nosocomial bloodstream infections, endocarditis, urinary tract infection, surgical site infection, etc. (Spanu et al. 2003; Huang et al. 2005; Becker et al. 2014). Unlike *S. aureus*, CoNS species were considered out of the list of health care-associated pathogens until the early nineties. And then since the late nineties, methicillin resistance has been increasingly found in the CoNS species (Barbier et al. 2010). In comparing *S. epidermidis* and *S. haemolyticus*, publications from the 1980s reported high percentages of methicillin-resistant isolates from both of these species, but a higher prevalence of methicillin-resistant *S. haemolyticus* isolates was noted, where oxacillin was found to exhibit higher minimum inhibitory concentrations (MICs), thus stated the related pathogenicity (Eltwisy et al. 2022). In a recently published report, antimicrobial resistance (AMR) in the genus *Staphylococcus* represents a significant and evolving threat to public health worldwide (Antimicrobial Resistance Collaborators, 2023). Staphylococci, particularly *S. aureus*, have demonstrated an exceptional capacity to adapt and develop resistance mechanisms against a wide range of antibiotics. The emergence of methicillin-resistant *S. aureus* (MRSA) is perhaps the most notorious example, rendering many conventional antibiotics ineffective in treating infections caused by these bacteria (Uddin et al. 2021). MRSA has become endemic in India, with varying incidences ranging from 25% in western India to 50% in the southern part of the country (Patel et al. 2010; INSAR group. 2013). Antibiotic resistance in bacteria is often conferred by different antibiotic resistance genes (ARGs) present on transposons or plasmids which are transferred via transformation, transduction, or conjugation. One such gene responsible for the resistance to methicillin in staphylococci species is *mecA*, which was first transferred from an interspecies *Staphylococcus* to *S. aureus* via a mobile genetic element (Hiramatsu et al. 2014). This gene is considered one of the major ARGs that confer resistance to ß-lactams (Williams et al. 2020). In addition, the *blaZ* gene also has been reported to play an important role in ß-lactam resistance in *S. aureus* and various CoNS species (Zhang et al. 2022).

Methicillin-resistant coagulase negative staphylococci (MR-CoNS), which are frequently thought to cause hospital-associated infections, have also been linked to a variety of biological niches, including the community, wildlife, and environmental sources (Seng et al. 2017; Mkrtchyan et al. 2013). The quick spread of MR-CoNS in environment including rivers and wastewater is receiving more attention these days. Wastewater released as the pharmaceutical industry effluent is significant in this sense because it may serve as a reservoir for antibiotics as well as ARGs in the environment (Kotwani et al. 2021). This leads to the outspread of resistance in the wastewater environment through horizontal gene transfer (HGT). HGT initiates the intra and interspecies transfer of genes associated with antibiotic resistance (Calero-Cáceres et al. 2017). Additionally, AMR in CoNS species present in pharmaceutical wastewater may also be due to the selection pressure created by low loads of antibiotics present in the effluent (Rodriguez et al. 2020). Therefore, exploring the prevalence of drug-resistant species in urban aquatic environment containing antibiotics is highly noteworthy to get an idea of how wastewater systems pose environmental risks that may eventually lead to severe public health challenges. Globally, staphylococcal species have been found in wastewater sources by several studies (Börjesson et al. 2009; Goldstein et al. 2012; Gómez et al. 2016). Porrero and the research team reported the presence of *S. aureus* in a wastewater treatment plant (WWTP) in Madrid, Spain, while Faria et al. (2009) and Čuvalova et al. (2015) observed the prevalence of CoNS in treated effluents and drinking water from Portugal and the Slovak Republic, respectively (Porrero et al. 2016; Faria et al. 2009; Čuvalova et al. 2015). In particular, wastewater samples from Spain and Tunisia were found to include five CoNS species: *S. lentus*, *S. cohnii*, *S. sciuri*, *S. haemolyticus*, and *S. xylosus* (Gómez et al. 2016; Said et al. 2017). Additionally, *S. lentus*, *S. sciuri*, *S. cohnii*, and *S. haemolyticus* were noted in a Swedish municipal wastewater treatment facility (Börjesson et al. 2009). Few studies have reported the isolation of staphylococci from drinking water sources in India but reports on wastewater systems are very few till date. A study in Jalandhar city reported the occurrence of *S. aureus* from municipal wastewater and they claimed it as the first study ever done in India that is based on the sampling of municipal wastewater (Kumar et al. 2015). A recent report by Manisha Lamba and the group has shown how the wastewater system is being converted into the hub for antibiotic-resistant bacteria and genes due to the inefficacy of treatment plants in New Delhi (Lamba et al. 2017). This study has drawn our attention to this emerging problem of AMR in urban wastewater in Delhi, NCR, and the role of ARGs thereof. This study analyses the molecular characterization of MR-CoNS species, their phenotypic and genotypic aspects of

antibiotic resistance and the insight of complex pattern of AMR mechanism. To our best knowledge, no systematic study has been reported on the prevalence of MDR-CoNS in urban wastewater in Delhi NCR, India and their molecular basis of multidrug resistance. The results from this study will determine the AMR pattern of CoNS and related ARG markers. The current research is based on one of the key strategic objectives of the "One Health" (human, animal, and environment) priority research agenda for AMR, which is improving the understanding of AMR transmission and surveillance for action.

Materials and methods

Sample collection

Water samples were collected from seven urban wastewater sites adjacent to pharmaceutical industries among which three were located in New Delhi and four sites were in the Delhi-NCR area, Ghaziabad, and Faridabad, India (Fig. 1). Another three sites were chosen from the 22 km stretch of river Yamuna passing through Delhi, India. Upon entering Delhi, the river undergoes one of the most polluted stretches along its length, spanning from Wazirabad to Okhla. This segment constitutes less than 2% of the river's total length, yet it bears the brunt of the pollution burden, primarily stemming from sewage and industrial discharges (Mutiyar et al. 2018). Water samples were collected thrice during the year 2019–2021. Samples were taken in sterilized glass bottles transported to the laboratory in ice, and processed on the same day for further analysis. The temperature of the collected water samples was measured at the time of sampling and pH was measured in the laboratory (Table S1 in the Supplementary Information).

Isolation and characterization of bacteria

The collected water samples were serially diluted in sterilized saline (0.9% NaCl w/v solution) upto 10^5-fold and 100 µl from each dilution was spread on the agar plates in triplicate. Luria agar plates (LA) (Difco, India) were used to determine the total culturable bacterial load and mannitol salt agar plates (MSA) (Hi-media, India) were used for staphylococcal load (Silva et al. 2016; Boopathy 2017). All the plates were supplemented with 80 µg/mL (284.34 µM) cycloheximide to prevent the fungal contamination (Palumbo et al. 2021). Plates were then incubated at 37 °C

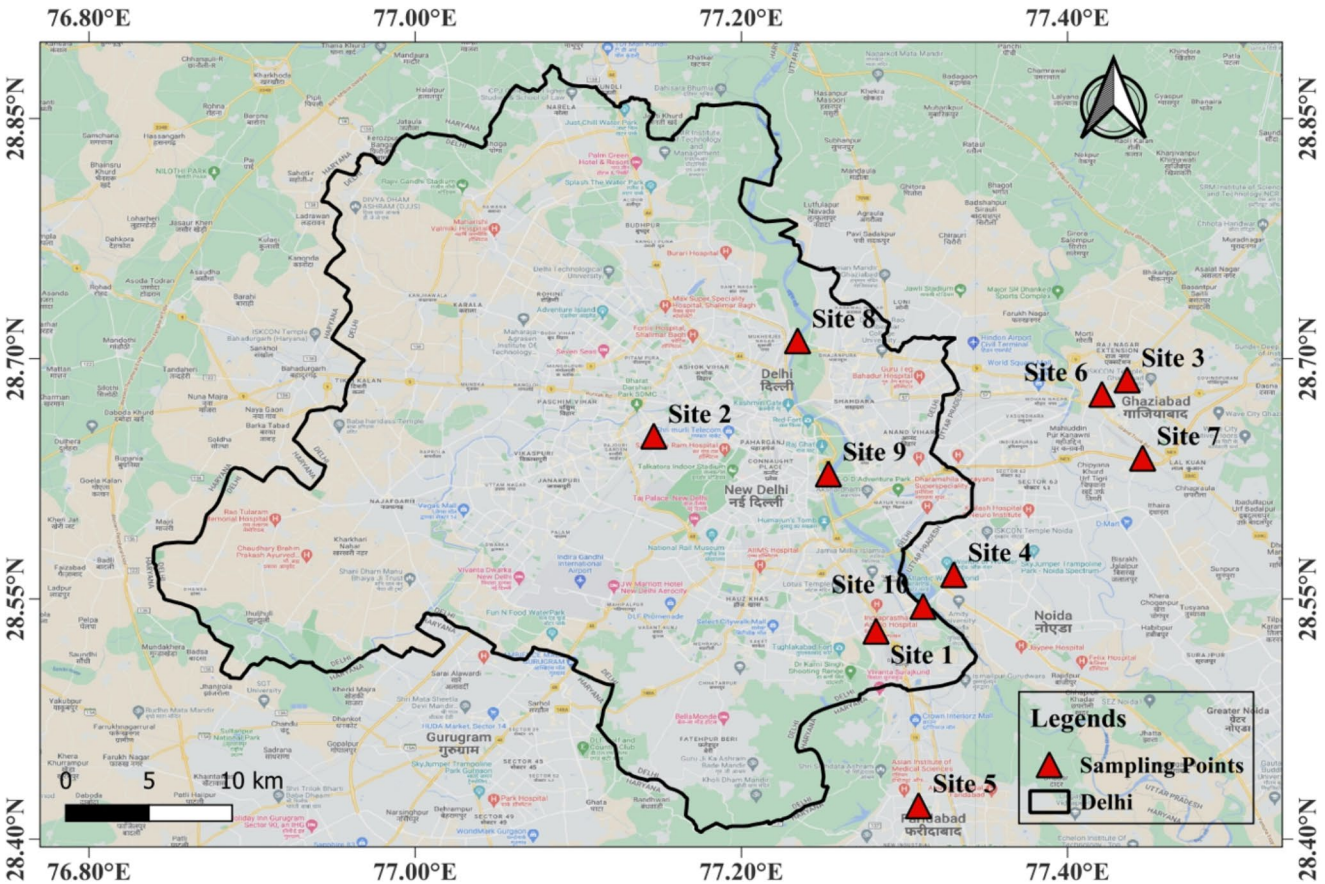

Fig. 1 Site map showing the study area (Delhi-NCR) and sampling sites of urban wastewater (site 1-site 7) and river Yamuna (site 8-site 10)

for 36 h. In our study, the first step screening was done on MSA plates from which, the pink and yellow colonies were further streaked on Baird Parker agar (BPA) plates for selective screening. The grey-black colonies from BPA were considered as suspected staphylococci and taken forward for biochemical characterization. The cell shape and Gram characteristics were examined using Gram staining. Afterward, biochemical tests including the catalase, coagulase, and Staphylo Monotec test were performed (Fluka, Sigma-Aldrich; Kumari et al. 2020). Biochemically positive isolates were considered as presumptive staphylococci (Table S2 in the Supplementary Information) and taken forward for screening of methicillin resistance.

Screening of methicillin resistance

To detect methicillin resistance, the presumptive staphylococci isolates were subjected to an agar dilution method against oxacillin, which is in the same class of drugs and is currently used widely for this purpose. Briefly, cation-adjusted Mueller Hinton agar (MHA) plates with oxacillin at 6 µg/mL (13.59 µM) for CoPS and 1 µg/mL (2.26 µM) for CoNS were prepared according to Clinical and Laboratory Standards Institute recommendations guidelines (CLSI 2020). All the presumptive isolates that grew on these plates were studied for MIC determination of oxacillin by broth micro-dilution, according to the CLSI guidelines (CLSI 2020). The minimum inhibitory concentration (MIC) was calculated using a final inoculum concentration of 10^5 CFU/mL of bacterial culture. MICs were calculated using *S. aureus* (ATCC 29213) and *S. epidermidis* (ATCC 14990) as reference strains.

Molecular identification of the methicillin-resistant isolates

The molecular identification of the methicillin-resistant isolates was conducted by 16S rRNA gene sequencing. Briefly, the universal primers, 27 F (5′-AGAGTTTGATCATGGCT CAG-3′) and 1492R (5′ TACGGTTACCTTGTTACGACT T-3′) were used to amplify the highly conserved ~1500 bp region of 16S rRNA gene following recent reports with brief modification (Morshdy et al. 2023). A preliminary denaturation stage of 95 °C for 5 min was followed by 25 cycles of 95 °C for 1 min, 45 °C for 30 s, and 72 °C for 2 min in the PCR process. The final extension step was carried out for 7 min at 72 °C. *S. aureus* (ATCC 29213) and *S. epidermidis* (ATCC 14990) were used as positive control in this experiment (Garcha et al. 2016; Gumaa et al. 2021). The 16S rRNA sequences were subjected to BLAST analysis (NCBI) and further deposited at GenBank.

Antimicrobial susceptibility testing

Antibiotic susceptibility patterns of the identified methicillin-resistant staphylococci (MRS) were studied against 12 different antibiotics belonging to 10 different classes by disc diffusion method as per CLSI guidelines (CLSI 2020). Antibiotics namely, CX: Cefoxitin (30 µg), AMP: Ampicillin (10 µg), GEN: Gentamicin (10 µg), RIF: Rifampicin (5 µg), TR: Trimethoprim (5 µg), TE: Tetracycline (10 µg), E: Erythromycin (15 µg), AZM: Azithromycin (15 µg), CD: Clindamycin (2 µg), LZ: Linezolid (30 µg), TEI: Teicoplanin (30 µg), CIP: Ciprofloxacin (5 µg) were used in this study. The multidrug resistance was defined as when the bacteria showed resistance to at least three different classes of antibiotics (Magiorakos et al. 2012).

Detection of antibiotic resistance genes (ARGs)

The MRS isolates were investigated for the presence of the ARGs related to different tested antibiotics. The occurrence of total thirteen ARGs namely, *mecA* and *blaZ* (β-lactam resistance); *aac* (6′) *aph* (2”), *aph* (3′)-IIIa and *ant*(4”)-Ia (gentamicin resistance); *ermA*, *ermC* and *msrA* (macrolide and clindamycin resistance); *dfrA(drfS1)*, *dfrG* and *dfrK* (trimethoprim resistance); *tetK* and *tetM* (tetracycline resistance) were screened by conventional PCR. All the information related to primer sequences and amplicon sizes is given in Table S3 in the Supplementary Information.

Results

Isolation and characterization of bacteria

The temperature and pH of the collected water samples are given in Table S1 in the Supplementary Information. The temperature ranged from 15 °C to 33 °C, while the pH of the samples was recorded mostly in the range of 6 to 9.25 among all sites. The maximum permissible pH value should be between 6.00 and 9.00 for the effluent to be discharged into the sea and environment, as directed by the Ministry of Environment, Forest and Climate Change, India in the Environment (Protection) Amendment Rules, 2015. The water samples taken in our study were mostly within the suggested limit and found to be neutral to slightly alkaline, a condition that is conducive to support bacterial growth (Oluseyi Osunmakinde et al. 2019). As shown in Fig. 2a, comparing all the sampling time, the total suspected staphylococci load was found to be maximum in wastewater Site 1 (4.15 ± 0.04 $\log_{10}$ CFU/mL, 4.46 ± 0.08 $\log_{10}$ CFU/mL and 3.89 ± 0.04 $\log_{10}$ CFU/mL respectively), while considerably lower load were noted in river Yamuna sites (2.13 ± 0.16 $\log_{10}$ CFU/mL

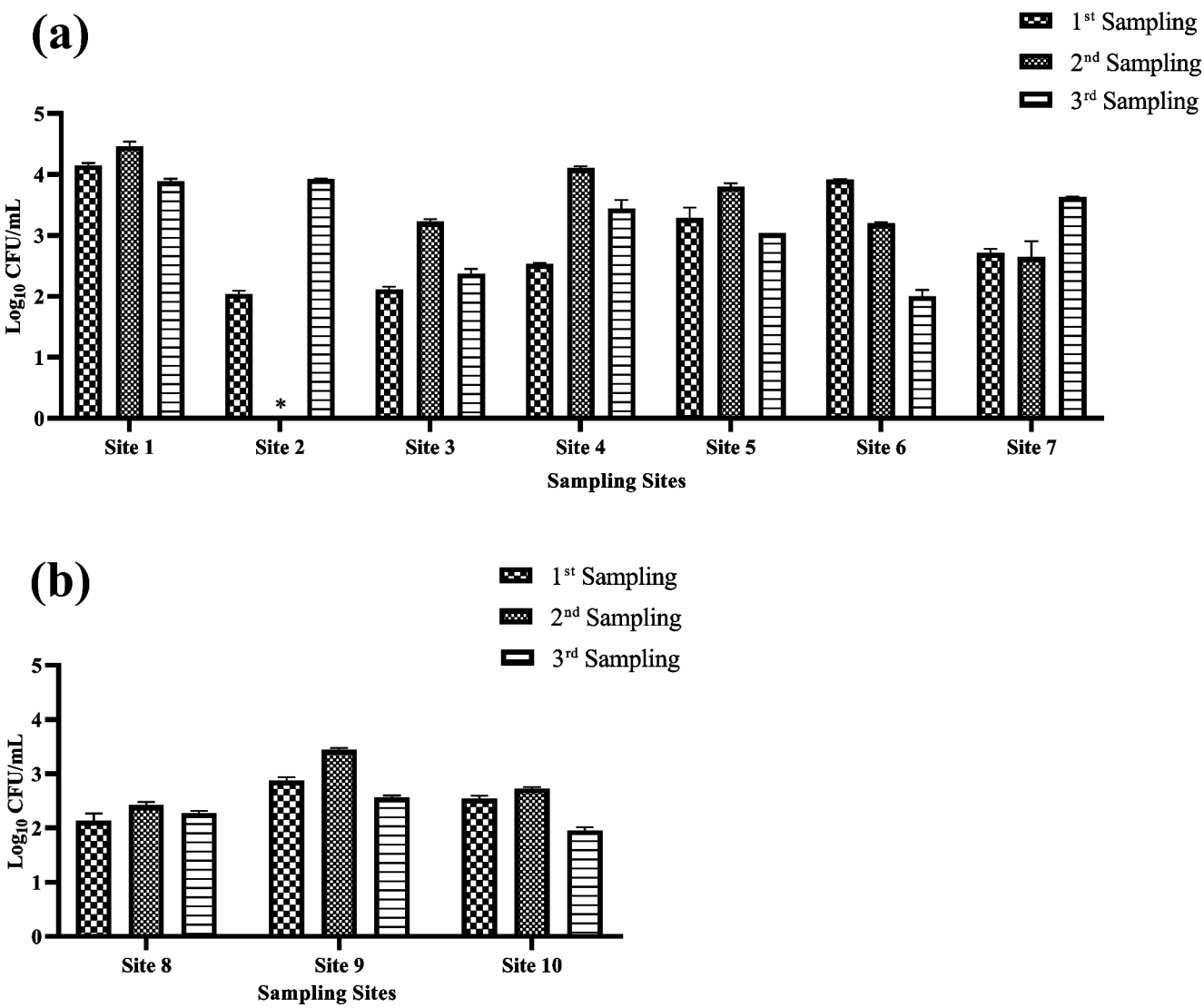

Fig. 2 The suspected staphylococci load in the collected water samples from (**a**) urban waste water sites and (**b**) river Yamuna. The samplings were done in three consecutive years from 2019–2021. The bacterial load is presented as Log_{10} CFU/mL. The data represents mean ± standard deviation (SD) *Due to some COVID-19 related issue, the 2nd time sampling at Site 2 could not be performed

and 2.42 ± 0.05 log_{10} CFU/mL in Site 8, 1.95 ± 0.06 log_{10} CFU/mL in Site 10 respectively) (Fig. 2b). Surprisingly, the wastewater sites, Site 2 (3.92 ± 0.01 log_{10} CFU/mL) and Site 7 (3.63 ± 0.00 log_{10} CFU/mL) showed remarkable rise in the staphylococci load in 3rd sampling compared to the others, indicating the possible increase in drug manufacturing by the pharmaceutical industries during COVID-19 pandemic that might result in the antibiotic pollution in the effluent. Conversely, it was noteworthy to observe the total culturable bacterial load higher during 2nd sampling, in most of the wastewater sampling sites. As depicted in Fig. S1a in the Supplementary Information, the range was noted from 6.20 ± 0.05 log_{10} CFU/mL in Site 4 to 4.36 ± 0.02 log_{10} CFU/mL in Site 5. Among the river Yamuna sites, the staphylococci load was maximum in midstream location, Site 9 during all the sampling (Fig. 2b). Correspondingly, the same midstream site was also found to be at upper limit for the total bacteria amid the river sites with a maximum load of 5.37 ± 0.06 log_{10} CFU/mL, implying the probable insufficient treatment of effluent by the industries in Delhi (Fig. S1b in the Supplementary Information). A total of 378 isolates were screened through biochemical tests, and out of these, 224 (208 coagulase negative and 16 coagulase positive) isolates were considered as presumptive staphylococci (Table S2 in the Supplementary Information).

Screening of methicillin resistance

According to CLSI guidelines, staphylococci that show resistance to oxacillin are reported as methicillin-resistant

(CLSI 2020). In our study, 56 out of 224 (25%) presumptive isolates were found to be methicillin-resistant, as analysed by agar dilution and MIC determination. As depicted in Fig. 3, the urban wastewater sites showed the highest number of resistant isolates in Site 5 with 28.57% (16/56) followed by Site 7 with 16.07% (9/56). Site 5 also revealed a maximum number of isolates with a higher range of MIC (16 µg/mL -128 µg/mL) (36.25 µM – 289.97 µM). Compared to the wastewater samples, the river Yamuna samples (Site 8- Site 10) were found to reveal a lesser number of resistant isolates (11/56) with a broad range of oxacillin MIC (1 µg/mL -64 µg/mL) (2.27 µM – 144.98 µM). None of the coagulase positive isolates were found resistant to methicillin. Therefore, only the methicillin-resistant coagulase negative isolates were further identified upto species level.

Molecular identification by 16S rRNA sequencing

Upon 16S rRNA sequencing and BLAST analysis, 41.07% (23/56) isolates were identified as staphylococci of different species in CoNS category; *S. haemolyticus* (4), *S. sciuri* (4),

Fig. 3 Minimum inhibitory concentration of oxacillin against 56 presumptive coagulase negative isolates (displayed on a color scale) *The nomenclature of the isolates are expressed as 'S' that stands for the Site number followed by the sampling time and isolate number. For example: S1F06, S1 Stands for Site 1, F stands for first sampling and 06 is the isolate number

S. hominis (5), *S. arlettae* (3), *S. cohnii* (3), *S. epidermidis* (1), *S. saprophyticus* (2) and *S. capitis* (1) (Table 1). The 16S rRNA sequences of all the isolates have been deposited in GenBank under the accession numbers given in Table 1. The MR-CoNS were detected from 8 out of 10 sites while both Site 1 and Site 5 were found to report a maximum number of isolates.

Antimicrobial susceptibility pattern of identified staphylococci

The antimicrobial susceptibility pattern of 23 MR-CoNS isolates against 12 antibiotics is presented in Fig. 4. The isolates were found to exhibit the highest percentage of resistance against macrolide and cephalosporin class, such as erythromycin 73.91% (17/23), azithromycin 60.86% (14/23) and cefoxitin 65.22% (15/23) (Fig. 5). Comparatively lesser percentage of resistance was observed against trimethoprim 47.82% (11/23), ampicillin 34.78% (8/23), ciprofloxacin 30.43% (7/23) and clindamycin 21.74% (5/23). Most of the isolates were noted to be susceptible to tetracycline and teicoplanin, where resistance was detected in only 8.6% (2/23) and 4.35% (1/23) of the isolates respectively. The same phenomenon was observed for both gentamicin and rifampicin with 17.39% (4/23) of resistance. Conceivably, it is to be hoped that 100% (23/23) susceptibility was noticed against linezolid, an oxazolidinone antimicrobial agent (Fig. 5), that is currently considered a promising candidate for the infections caused by MRS and vancomycin-resistant enterococci (VRE) (Zahedi Bialvaei et al. 2017). Moreover, it was noted that 60.86% (14/23) of the tested MR-CoNS isolates were multidrug-resistant (Fig. 4, Fig. S2 in the Supplementary Information). It was notable to observe all 5 isolates, *S. haemolyticus* (4) and *S. epidermidis* (1), obtained from Site 5 were found to be MDR. These species were considered as the most frequent aetiological agents of staphylococcal infections (Takeuchi et al. 2005). Additionally, *S. hominis* (4) frequent in Site 7, was found to be the second most occurring species exhibiting multidrug resistance (Fig. S2 in the Supplementary Information). Site 1 and Site 3 were the locations that accounted for three MDR isolates of *S. arlettae*, the species that are generally reported in the microbiota of animals (Karakulska et al. 2022). Overall, the study revealed the prevalence of MDR in wastewater sites, as 13 out of 14 MDR staphylococci were isolated thereof. This is concerning that the NCR area (Site 5 and Site 7) reported more MDR CoNS species than Delhi (Fig. 4).

Table 1 List of the methicillin-resistant coagulase negative staphylococci species identified by 16S rRNA sequencing with their GenBank accession numbers

Sites	Isolate ID	Identified species	Accession Number
Site 1	S1F06	*S. arlettae*	PP434793
	S1S01	*S. cohnii*	PP389921
	S1S02	*S. cohnii*	PP389922
	S1T03	*S. hominis*	PP389923
	S1T32	*S. arlettae*	PP389924
Site 3	S3S04	*S. sciuri*	PP389926
	S3S03	*S. sciuri*	PP389925
	S3T20	*S. arlettae*	PP389927
Site 4	S4S19	*S. saprophyticus*	PP389928
Site 5	S5F05	*S. haemolyticus*	PP389929
	S5F09	*S. haemolyticus*	PP434794
	S5F10	*S. haemolyticus*	PP390029
	S5F11	*S. haemolyticus*	PP390030
	S5T09	*S. epidermidis*	PP390031
Site 6	S6F06	*S. hominis*	PP390032
	S6S06	*S. sciuri*	PP406861
Site 7	S7S10	*S. sciuri*	PP406865
	S7T19	*S. hominis*	PP390034
	S7T12	*S. hominis*	PP390033
	S7T20	*S. hominis*	PP390035
Site 9	S9S07	*S. saprophyticus*	PP390036
Site 10	S10S12	*S. cohnii*	PP389877
	S10T04	*S. capitis*	PP389935

Detection of antibiotic resistance genes (ARGs) in identified staphylococci

The presence of antibiotic resistance genes (ARGs) was analyzed among the 23 identified MR-CoNS isolates. 30.43% (7/23) were found to be positive for *mecA* and 26.09% (6/23) for *blaZ* genes responsible for the resistance to the β-lactam class of antibiotics (Table 2). Related to macrolide resistance, none of the 17 resistant isolates were found to carry the *ermA* and *ermC* genes. However, 58.82% (10/17) of the isolates were detected to contain the *msrA* gene. Notably, the *msrA* gene is also responsible for conferring clindamycin resistance and was detected in 60% (3/5) of clindamycin-resistant isolates (Table 3). Phenotypic-genotypic disparities (phenotypic antibiotic resistance was detected without the related ARGs and vice-versa) were observed for *tetK* and *tetM* genes. The same phenomenon was also observed for 11 MR-CoNS isolates phenotypically resistant to trimethoprim, while only 6 of them were found to contain the related ARGs. Likewise, phenotypic disparity was also noted in 2 isolates from Site 5, where the gentamicin resistance genes were absent (Table 3).

Discussion

The current study investigated the presence of antibiotic-resistant bacteria particularly, MR-CoNS, their MDR pattern, and the assessment of their ARGs in the urban wastewater taken from the sites adjacent to hospitals,

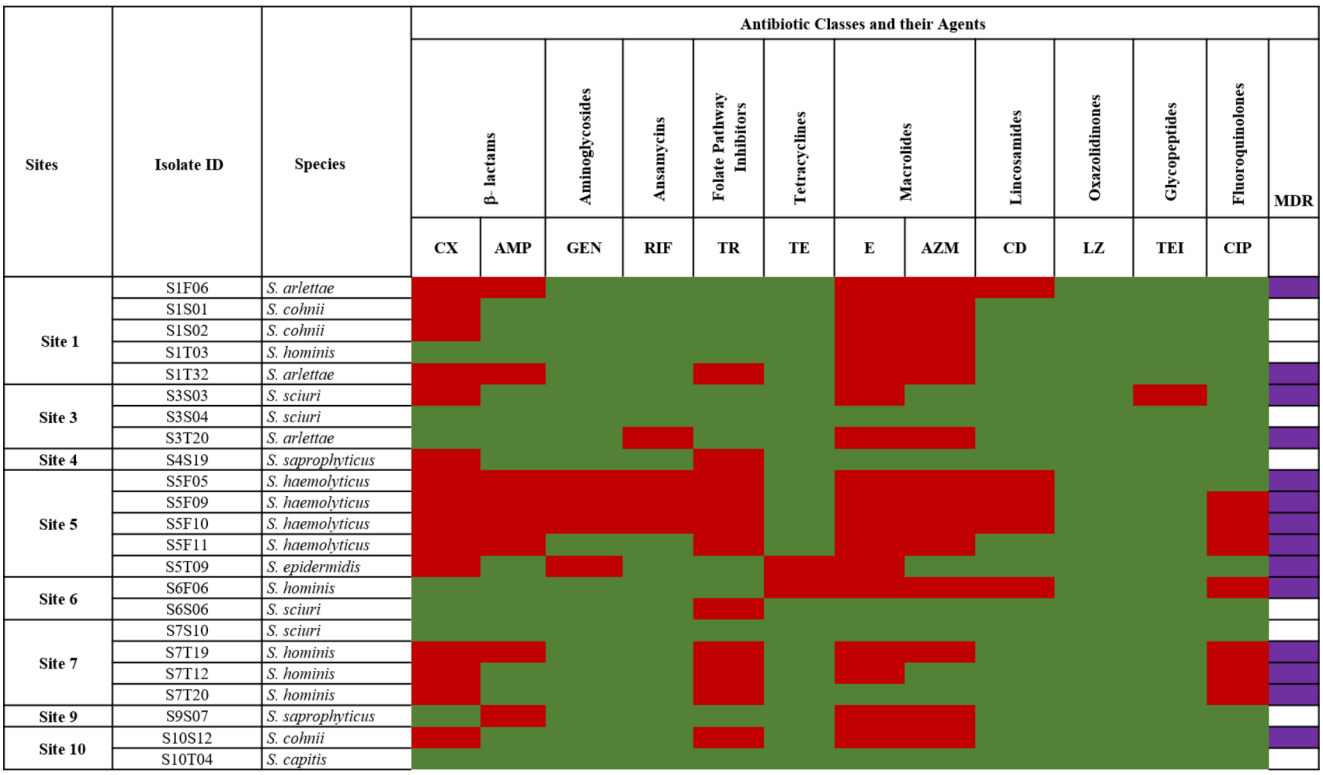

Fig. 4 Drug resistance profiles of 23 methicillin-resistant coagulase negative staphylococci collected in this study. Green, susceptible; Red, resistant; Purple, multidrug-resistant (≥ 3 classes of antibiotics). CX: Cefoxitin, AMP: Ampicillin, GEN: Gentamycin, RIF: Rifampicin, TR: Trimethoprim, TE: Tetracycline, E: Erythromycin, AZM: Azithromycin, CD: Clindamycin, LZ: Linezolid, TEI: Teicoplanin, CIP: Ciprofloxacin

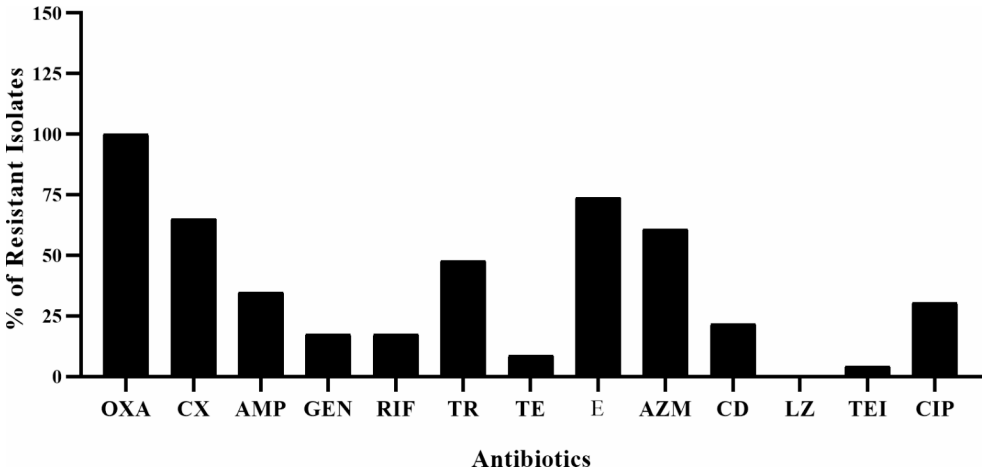

Fig. 5 Percentage of 23 identified coagulase negative staphylococci isolates resistant to different antibiotics; OXA: Oxacillin, CX: Cefoxitin, AMP: Ampicillin, GEN: Gentamycin, RIF: Rifampicin, TR: Trimethoprim, TE: Tetracycline, E: Erythromycin, CD: Clindamycin, LZ: Linezolid, TEI: Teicoplanin, AZM: Azithromycin, CIP: Ciprofloxacin

pharmaceutical industries, and river Yamuna in Delhi, NCR. The current research is based on one of the key strategic objectives of the "One Health" (human, animal, and environment) vision for AMR, which is improving the understanding of AMR transmission and surveillance required for understanding the human-environment association (Jin et al. 2022). Of note, the manifestation of COVID-19 (2019) has inflated antibiotic manufacturing by pharmaceuticals to combat secondary bacterial infections during the pandemic (WHO 2020). The manufacturing process is usually followed by the release of effluent via wastewater disposal that may retain the antibiotics and therefore, serve as the key factor for evolving drug-resistant pathogens through selective pressure (Rees 2020; UNEP 2017). To the best of our knowledge, no systematic study on the surveillance of AMR and molecular basis of MDR with reference to coagulase negative staphylococci species has been reported in the Indian aquatic scenario yet.

Table 2 Presence of *mecA* and *blaZ* genes in methicillin-resistant coagulase negative staphylococci isolates

Species	Isolate ID	Phenotypic resistance to β-lactam antibiotics [a](OXA, CX, AMP)	Genotypic resistance (*mecA* & *blaZ*)
S. haemolyticus	S5F05	OXA, CX, AMP	*mecA*, *blaZ*
	S5F09	OXA, CX, AMP	*mecA*, *blaZ*
	S5F10	OXA, CX, AMP	*mecA*, *blaZ*
	S5F11	OXA, CX, AMP	*mecA*, *blaZ*
S. epidermidis	S5T09	OXA, CX	*mecA*, *blaZ*
S. hominis	S7T19	OXA, CX, AMP	*mecA*, *blaZ*
S. saprophyticus	S9SO7	OXA, AMP	*mecA*

[a]Antibiotics; OXA: Oxacillin, CX: Cefoxitin, AMP: Ampicillin

Table 3 Antibiotic resistance genes in methicillin-resistant coagulase negative staphylococci isolates

Species	Isolate ID	Phenotypic antibiotic resistance	Antibiotic resistance genes (ARGs)
S. haemolyticus	S5F05	E, AZM, CD, [p]GEN, TR	*msrA*, *dfrG*
	S5F09	E, AZM, CD, GEN, [p]TR	*msrA*, *aac (6') aph (2")*
	S5F10	[p]E, [p]AZM, [p]CD, GEN, TR	*dfrG*, *aac (6') aph (2")*, *aph (3')-IIIa*
	S5F11	E, AZM, TR	*msrA*, *dfrG*
S. hominis	S1T03	[p]E, [p]AZM	[g]*ant(4')-Ia*
	S7T19	E, AZM, TR	*msrA*, *dfrA (drfS1)*
	S7T12	[p]E, [p]TR	--
	S7T20	[p]TR	--
	S6F06	[p]E, [p]AZM,[p]CD [p]TE	[g]*aph (3')-IIIa*
S. saprophyticus	S4S19	TR	*dfrA(dfrS1)*
	S9S07	[p]E, [p]AZM	--
S. cohnii	S1S01	E, AZM	*msrA*
	S1S02	E, AZM	*msrA*
	S10S12	E, AZM, [p]TR	*msrA*
S. arlettae	S1F06	E, AZM, CD	*msrA*,[g]*tetK*
	S3T20	E, AZM	*msrA*
	S1T32	E, AZM, TR	*msrA*, *dfrG*
S. epidermidis	S5T09	[p]E, [p]TE, [p]GEN	[g]*dfrA (drfS1)*
S. capitis	S10T04	---	[g]*tetM*
S. sciuri	S3S03	[p]E	--
	S3S04	--	--
	S6S06	[p]TR	--
	S7S10	--	--

[p]Phenotypic antibiotic resistance was detected without the related ARGs

[g]ARGs were detected without the related phenotypic antibiotic resistance

Antibiotics; GEN: Gentamycin, TR: Trimethoprim, TE: Tetracycline, E: Erythromycin, AZM: Azithromycin, CD: Clindamycin

In this study, it was noted from our study that, the suspected staphylococci load was higher in most of the urban wastewater sites, whereas, comparatively lower loads were eminent in the Yamuna sites. The reason may be because of the continuous flow of the river, moving from upstream to downstream in the 22 km stretch in Delhi, where abiotic chemical pollutants like drugs, antibiotics and biotic pollutants like microbes get carried along with the river flow, leading to their initial dilution. Following biochemical characterization, total 224 isolates were considered as presumptive of which, 56 MRS were taken forward for molecular identification. The biochemical tests designed for staphylococci may also give similar test results for *Micrococcaceae*, *Aerococcus urinae*, and other bacteria (Reiner 2010), suggesting the importance of 16S rRNA sequencing for reliable identification. Following the 16S rRNA sequencing, 41.07% (23/56) of the presumptive isolates were identified as MR-CoNS of 8 different species.

According to our data, the prevalence of MR-CoNS can be noticed in both wastewater and river water. Similar observations were reported previously in the study conducted in Spain by Gomez and the research team. They have found 16.67% of CoPS and 83.33% of CoNS including 12 different species from the superficial water (Gómez et al. 2017). Our study reported the two mostly occurring CoNS species, i.e. *S. haemolyticus* and *S. hominis* from Site 5 and Site 7 respectively. Both these species are known to cause bacteremia, septicaemia and several bloodstream infections (Eltwisy et al. 2022; Sorlozano et al. 2010). Importantly, in our study, the isolates of *S. haemolyticus* and *S. hominis* were highly resistant to oxacillin having MIC in the range of 16 µg/mL -128 µg/mL (36.25 µM- 289.97 µM). The highly resistant CoNS were also reported previously in the isolates from the clinical settings (Ahmed et al. 2021). The species like *S. cohnii* found in our study is corroborative of the results obtained earlier (Chen et al. 2015; Gomez et al. 2016). Further, other species like methicillin-resistant *S. sciuri*, *S. arlettae*, *S. saprophyticus*, and *S. capitis* were also found in the water samples.

Next, the MDR pattern of identified MR-CoNS isolates was thoroughly investigated. In our study, it was found that 60.86% (14/23) of the MR-CoNS isolates were MDR, as they were found to be resistant to at least three classes of antibiotics (Magiorakos et al. 2012). Moreover, we have observed that two isolates of *S. haemolyticus* from Site 5 were resistant to 10 out of 12 tested antibiotics. After β-lactam category (oxacillin, cefoxitin, and ampicillin), a maximum number of isolates were reported to be resistant to the macrolide class. In a recent study, a high concentration of macrolide class of antibiotics, particularly erythromycin was detected in the active form in river Yamuna, that may be the possible reason for this high percentage of

erythromycin resistance observed in our study (Akhter et al. 2023). Another study conducted in Portugal in 2009, also stated the high occurrence of erythromycin resistance in the CoNS species isolated from wastewater and drinking water (Faria et al. 2009). Additionally, 47.82% of the CoNS species showed resistance toward the antibiotic trimethoprim, that is of great concern. Isolates resistant to clindamycin, ciprofloxacin, rifampicin, and gentamicin were detected in the wastewater but not in river Yamuna samples. However, a low level of antibiotic resistance was noticed against teicoplanin and tetracycline, similar to the findings reported earlier (Stevoska et al. 2022). In the same line, the isolated MR-CoNS exhibited 100% sensitivity towards the oxazolidinone antibiotic linezolid. While resistance to linezolid is not as widespread as resistance to other antibiotics, it is essential to maintain proper stewardship of linezolid use to help preserve its effectiveness (Hashemian et al. 2018).

It is well known that staphylococci possess a variety of ARGs that provide resistance to distinct antibiotic classes. As previously indicated, the *mecA* gene produces a modified penicillin-binding protein (PBP2a) that has a lower binding affinity for β-lactam antibiotics, while *blaZ* also confers β-lactam resistance by producing β-lactamase enzyme, rendering these antibiotics ineffective (Shalaby et al. 2020). In our study, the presence of the *mecA* in 30.43% of isolates and *blaZ* in 26.09% of isolates were supportive of the high MIC of oxacillin in the range of 8 µg/mL -128 µg/mL (18.12 µM – 289.97 µM) against the isolates containing both genes. In this regard, it is worth to mention that all the *S. haemolyticus* species, obtained in the current study harbour those genes, and may be capable of interspecies transfer of resistance genes and pose a significant threat in the context of dwindling antibiotic efficacy (Czekaj et al. 2015). Next, the strain of *S. saprophyticus* (S9S07) against which oxacillin showed a comparatively lower MIC of 1 µg/mL (2.27 µM), was found to possess *mecA*, but not *blaZ*. Similarly, Zehra et al. (2017) obtained borderline oxacillin-resistant S. *aureus* (BORSA) negative for the *mecA* gene and found them as hyperproducers of β-lactamases. These studies suggest that genetic detection of *mecA* is not always enough for studying methicillin resistance in staphylococci. In several reports, variants of *mecA* (*mecC*) were found in MRSA as well as in CoNS justify the observation (Loncaric et al. 2019; Laurent et al. 2012; Paterson et al. 2014; Silva et al. 2021).

The absence of *ermC* and *ermA* genes in the 23 isolates suggests that macrolide resistance in these isolates is not mediated by these ARGs. However, the presence of the *msrA* gene in 43.47% of the isolates indicates that as a possible contributor to macrolide resistance in these CoNS (Duran et al. 2012). Specifically, the *erm* cluster is responsible for altering the ribosomal binding site alteration (by mutation within the 23S rRNA gene), whereas, the *msrA* gene encodes an ATP-dependent efflux pump of the ABC (ATP-binding cassette) family, responsible for conferring macrolide-lincosamide-streptogramin B (MLSb) resistance (Mišić et al. 2017). The fact that the *erm* and *msr* genes are also associated with clindamycin resistance highlights the potential of cross-resistance and inducible resistance that may lead to failure in therapy. Notably, the *msrA* gene was detected in 3 clindamycin-resistant isolates validating their phenotypic resistance to clindamycin in the current study. The results suggest that the tetracycline resistance conferred by the isolates devoid of *tetK* and *tetM* genes may be mediated by other classes of *tet* genes, like *tetL* and *tetO* (Trzcinski et al. 2000), but were not included in this study. Conversely, the presence of *tet* genes in phenotypically susceptible isolates was also supported by the recent study (Rasheed et al. 2023), which suggests that ARGs are not only the factors for conferring antibiotic resistance in staphylococci. Comparable results were also obtained from the study done in Iran where they found *tetK* in 30% and *tetM* in 75% of the susceptible isolates (Akya et al. 2020). These findings imply that certain ARGs that may remain dormant or inactive under specific conditions, can later become active when integrated into suitable genetic loci within the bacterial genome.

The presence of the *dfrG* and *dfrA(dfrS1)* genes in some isolates aligns with their phenotypic resistance to trimethoprim. According to our results, the presence of these ARGs in species like *S. haemolyticus*, *S. saprophyticus*, *S. hominis*, and *S. epidermidis* raises concerns about their potential role as reservoirs for these ARGs. The current study also revealed the resistance to aminoglycoside such as gentamicin in few CoNS isolates not possessing any of the three studied genes, *aac (6') aph (2")*, *aph (3')-IIIa*, *ant(4')-Ia*. This disparity was also observed earlier (Chandrakanth et al. 2008), where they found clinical isolates of aminoglycoside-resistant *S. aureus* lacking the ARGs and defined as aminoglycoside-modifying enzyme (AME) independent. The authors reported that these AME-deficit isolates underwent cell elongation with altered morphology and septa formation under sub-MIC concentration of aminoglycosides, thereby showing varied resistance. This phenomenon was termed adaptive resistance that was developed in the ARG-deficient strains under stress. Moreover, some CoNS isolates in our study were also found to harbor the resistance genes but showed susceptibility towards gentamicin. The results were in accordance with a study (Kime et al. 2019), where the authors explained the phenomenon as silencing of antibiotic resistance by mutation (SARM). Various mutations like frameshift, nucleotide deletions, or disruption of coding sequences in ARGs may suppress the gene functionality and strains show antibiotic susceptibility. However, SARM may be reversible under favourable

circumstances, thereby poses a possible threat to the clinical efficacy of the drug.

Overall, these results describe that multidrug resistance observed in wastewater, and the contamination of the Yamuna River may potentially have profound effects on public health. The presence of MDR-CoNS and ARGs in aquatic environment increases the risk of transmission to human through various pathways, such as direct contact with contaminated water or consumption of contaminated aquatic organisms. Effective waste management, water treatment strategies, and antibiotic stewardship are essential to mitigate the potential adverse impacts of multidrug resistance in these settings on human health.

Conclusion

The current investigation underscores the significance of antibiotic resistance pattern in staphylococci species. The study provides significant understanding on occurrence of MR-CoNS and their MDR pattern in Indian aquatic environment, thereby indicates the need of optimizing the effluent treatment strategy and implementing sustainable protocol for proper disposal into waterbodies, that would be beneficial for human health. To our best knowledge, this is the first study on the AMR pattern and molecular basis of MDR in staphylococci specifically CoNS, isolated from urban wastewater of industries particularly pharmaceuticals and river Yamuna in Delhi-NCR, India. The study not only investigates the phenotypic antibiotic resistance of the isolates but also delves into the genotypic aspects by analysing the presence of ARGs. This dual approach provides a comprehensive understanding of AMR mechanisms in CoNS, emphasizing the multifaceted nature of antibiotic resistance. Also, this study acknowledges the need for additional investigations to uncover the underlying mechanisms and implications of the observed resistance patterns. This suggests a commitment to ongoing research in the field and emphasizes the importance of addressing AMR in environmental settings. The findings contribute to the understanding of AMR in the context of industrial effluent pollution in India, providing a foundation for further research and potential interventions.

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Author contributions SR: Conceptualization, Methodology, Investigation, Writing – original draft, Visualization, Validation. SM: Writing – review & editing, Supervision, Visualization. AHM: Conceptualization, Methodology. HK: Methodology. KM: Writing – review & editing, supervision, conceptualization, resources.

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Data availability The datasets generated during and/or analysed during the current study are available from the corresponding author on reasonable request.

Declarations

Competing interests The authors declare no competing interests.

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