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# Antibiotic Susceptibility and Methicillin Resistance in Staphylococcal Isolates from Water Samples in Dharwad and Surrounding Districts of North Karnataka

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**Incidences of methicillin-resistant *Staphylococcus aureus* (MRSA) and multidrug-resistant (MDR) staphylococci are rising globally, prompting the need to understand human exposure routes, including water sources. This study aimed to analyse water samples from different sources and locations in North Karnataka over a six-month period (November 2021 to April 2022) to determine the prevalence and antibiotic susceptibility of staphylococcal isolates. Water samples were collected using standard microbiological techniques and analysed. Staphylococci were detected in 35.5% (55/155) of the samples and identified using phenotypic tests such as Gram stain, catalase test, and growth and fermentation on mannitol salt media. Methicillin resistance was assessed via cefoxitin disk diffusion, and the presence of the *mecA* gene was tested by PCR. Among the 55 isolates, 23.6% were resistant to cefoxitin and considered methicillin-resistant, while 36.4% carried the *mecA* gene. Antibiogram results showed the highest susceptibility to gentamicin (100%) and the lowest to penicillin (12.7%) and oxacillin (1.8%). The *nuc* gene, encoding thermonuclease, was present in 20% (11/55) of the isolates. Multiple antibiotic resistance (MAR) indices ranged from 0.08 to 0.67, with most staphylococci resistant to at least two antibiotics, and 54.55% (30/55) were MDR. These findings highlight the importance of water, sanitation, and hygiene (WASH) initiatives in reducing the spread of antimicrobial resistance in environmental bacteria.**

**Keywords:** CA-MRSA, MAR index, *mecA, nuc,* Staphylococci.

*Staphylococcus* is a common bacterium found as a commensal in humans and livestock.[1,2] Often opportunistic, it usually causes infections of the skin and soft tissue and, in extreme cases, species such as *Staphylococcus aureus*, can cause endocarditis, osteomyelitis, pneumonia, and septicemia.[3] *S. aureus* is also the primary pathogen for mastitis in cattle[1]. It can survive adverse environmental conditions such as sunlight and dehydration.[1] Drug-resistant variants of this Gram-positive bacterium have emerged as major pathogens among clinical isolates worldwide, posing significant challenges in patient treatment. Based on the global morbidity and mortality rates due to methicillin-resistant *S. aureus* (MRSA), the World Health Organization (WHO) has categorized it as a 'serious threat' pathogen on its 2024 Bacterial Priority Pathogens List.[4] MRSA strains

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are categorized as HA-MRSA, LA-MRSA, and CA-MRSA based on their origin and association (hospital, livestock, or community).

Formerly mostly found in hospitals, MRSA has recently spread significantly among individuals without risk factors or healthcare exposure and has become endemic in India.[5] Globally, CA-MRSA strains have proliferated among the general public, affecting both those with and without hospital exposure, and are found in environmental samples such as water, animals, and foods of animal origins. These strains can cause even fatal illnesses, and the transmission of resistance genes from environmental bacteria to human pathogens is a major threat.[6] The incidence of CA-MRSA isolates is reportedly on the rise in India.[7] The inefficiency of treatment plants is turning the wastewater system into a hotspot for antibiotic-resistant bacteria and genes.[5]

In staphylococci, methicillin resistance is due to methicillin-hydrolyzing β-lactamase and an altered penicillin-binding protein, PBP2a.[8] This 76 kDa protein, encoded by the *mecA* gene, is situated on the staphylococcal cassette chromosome *mec* (SCCmec). The altered protein has reduced affinity towards beta-lactam antibiotics in methicillin-resistant strains of staphylococci.[9] The acquisition of this gene by sensitive variants of staphylococci leads to the spread of resistance.[8]

Some strains of the staphylococci produce an extracellular thermonuclease enzyme coded by the *nuc* gene.[10] This 17 kDa protein is an endonuclease that degrades both DNA and RNA, and its enzymatic activity can resist 100$^{o}$C for at least 1 hour.[11] This protein is one of the important virulence markers and adds to the pathogenicity of the bacteria.[12]

One of the major global threats to the public health is antibiotic resistance, and the emergence of multidrug-resistant (MDR) pathogens poses serious problems in the effective treatment of infectious diseases, causing more deaths than cancer and road accidents combined.[13] The alarming rise in antibiotic resistance in India is driven by several factors, including a large, dense population, significant infectious disease burden, high rates of antibiotic consumption due to overprescribing, easy availability of over-the-counter antibiotics, environmental contamination from pharmaceutical industrial effluents, and widespread use of antibiotics in veterinary and agricultural practices.[14,15] India leads the global market in antibiotic consumption for human use, with an annual consumption of 12.9 x $10^9$ units.[16] Surveillance is vital for detecting MDR organisms, tracking epidemiologic trends, and assessing treatment effectiveness.

*Staphylococcus* has the ability to adapt and develop resistance to several antibiotics and has been extensively studied in clinical settings.[17] However, the prevalence and resistance patterns of *Staphylococcus* in non-clinical environmental sources, such as water bodies, have not been thoroughly investigated, particularly in North Karnataka, India. While *S. aureus* is a major pathogen among staphylococci species, recent findings, indicate that the coagulase-negative staphylococci (CoNS), are emerging as stronger pathogens, exhibiting methicillin resistance nearly twice that of *S. aureus*.[18] Therefore, this study examines the prevalence and antibiogram profiles of staphylococcal isolates from water samples collected in and around Dharwad city, North Karnataka.

## MATERIALS AND METHODS

### Sampling sites and sample collection

The sampling sites were selected using a random sampling method. Water samples were collected from sources such as sewage, ponds, lakes, and rivers over a six-month period (November 2021 to April 2022) from different locations in Dharwad and the surrounding districts of North Karnataka. A total of 155 water samples were collected and screened for staphylococci. Samples were collected in sterile falcon tubes and brought to the laboratory the same day for further processing. For distant locations, samples were inoculated in mannitol salt broth (MSB) at the time of collection and brought to the laboratory within 24 - 48 hours.[19]

### Isolation of staphylococcal isolates

Water samples were enriched before isolating staphylococci. For the enrichment, 1 ml of water sample was added to 10 ml of MSB and incubated overnight at 37$^{o}$C. The enriched broth was streaked onto mannitol salt agar (MSA) plates, followed by overnight incubation at 37$^{o}$C. Mannitol-fermenting colonies (yellow) from the

MSA plates were transferred to a fresh MSA plate and incubated overnight at 37°C. Round golden yellow colonies with smooth, shining surface and 2-3 mm in diameter were further subjected to Gram staining and catalase testing.[19] Mannitol-fermenting, catalase-positive, and Gram-positive cocci present in clusters were identified as staphylococci.[20] Purified isolates were stored as stabs in nutrient agar media at 4°C for further characterization and at -20°C in 70% glycerol for long-term storage.

**Antibiotic susceptibility testing**

This was conducted using the standard Kirby-Bauer disk diffusion method, with results compared to CLSI breakpoints.[21] The isolates were tested against 12 different antibiotics (Hi-Media Laboratories Pvt. Ltd.): penicillin (1 U), oxacillin (1 µg), cefoxitin (30 µg), ciprofloxacin (5 µg), erythromycin (15 µg), clindamycin (2 µg), gentamicin (10 µg), linezolid (30 µg), tetracycline (30 µg), rifampicin (5 µg), teicoplanin (30 µg), and chloramphenicol (15 µg). This panel used in routine sensitivity testing is supported by the CLSI guidelines.[21] Briefly, an inoculum was prepared by dispersing 2-3 identical colonies followed by incubation until a turbidity of 0.5 McFarland was reached. Using a cotton swab, the inoculum was spread uniformly over MHA plates. Antibiotic discs were placed on the plates, followed by overnight incubation at 37°C. The zones of inhibition were measured, and the results were interpreted according to CLSI guidelines as sensitive, intermediate and resistant.[21] Isolates rated as intermediate in antibiotic susceptibility were grouped with the sensitive category, as they can still be treated with elevated doses of antibiotics.[22] The Multiple Antibiotic Resistance (MAR) indices were calculated by dividing the number of antibiotics the isolate is resistant to by the total number of antibiotics tested.[23]

**DNA extraction**

Bacterial DNA was isolated using the boil lysis method.[24] Briefly, 3-4 similar isolated colonies were mixed and boiled in 100 µl of nanopure water at 95°C for 10 minutes. The mixture was cooled and centrifuged at 10,000 rpm for 5 minutes. The supernatant was transferred to a fresh tube and used as a template for gene amplification.

**Polymerase chain reaction (PCR)**

DNA from the isolates was subjected to multiplex PCR for detection of the thermonuclease (*nuc*) gene and methicillin resistance (*mecA*) gene using published primers. Primer sequences were: *nuc*F (5'GGC ATA TGT ATG GCA ATT GTT T3'), *nuc*R (5' ATA CGC TAA GCC ACG TCC AT3')[12]; *mecA*F (5'GTA GAA ATG ACT GAA CGT CCG ATA A3'), *mecA*R (5'CCA ATT CCA CAT TGT TTC GGT CTA A3')[25]. Primers were synthesized at Eurofins Genomics India Pvt. Ltd. The 25 µl of PCR reaction mixture consisted of 2.5 µl of PCR amplification buffer (10X), 2.5 µl $MgCl_2$ (25 mM), 2.0 µl dNTPs (2 mM), 0.2 µl Taq DNA Polymerase (5U/µl), 1.25 µl of each primer (10 mM), 1.25 µl lysate and 11.55 µl of PCR-grade nanopure water.

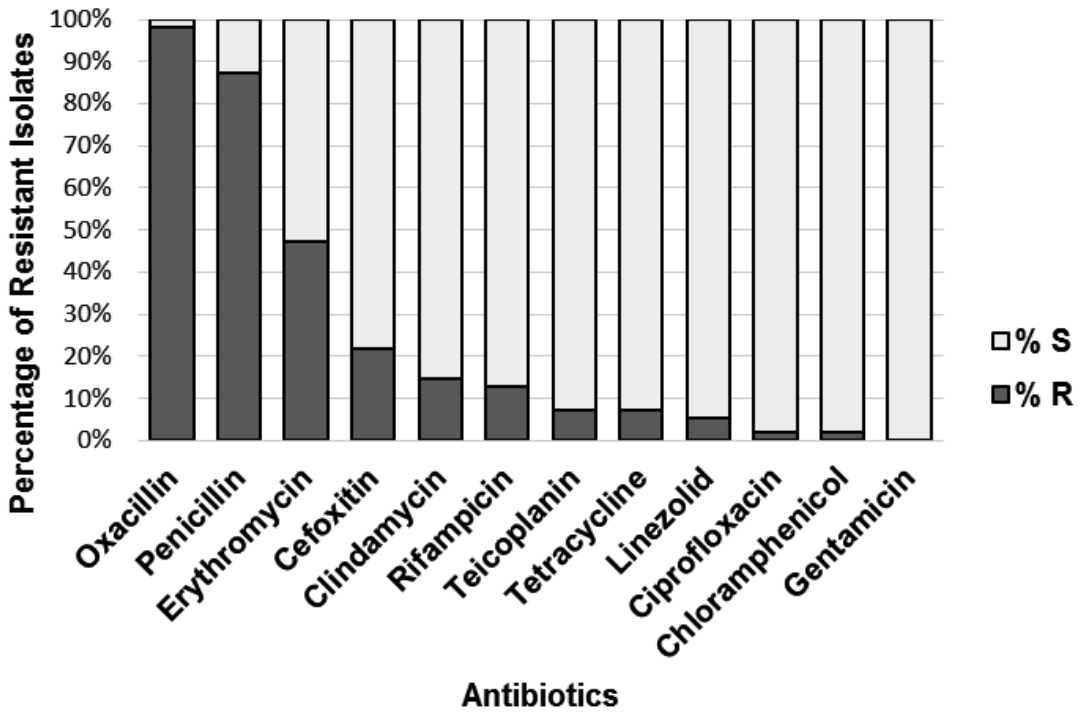
PCR amplification was performed using an automated thermal cycler (Eppendorf MC nexus gradient, Flexlid) with the following conditions: initial denaturation at 95°C for 2 minutes; 35 cycles of 95°C for 30 seconds, 62°C for 30 seconds and 72°C for 45 seconds; final extension at 72°C for 10 minutes. *Staphylococcus aureus* NCIM 5719 (equivalent ATCC BAA-44) was used as a positive control for detecting *mecA* and *nuc* genes. To visualize, 5 µl of the PCR product was electrophoresed in 1.5 % agarose, and bands were observed using a UV transilluminator. Amplicons of approximately 489 bp and 310 bp correspond to the presence of *nuc* and *mecA* genes, respectively.

The PCR amplified products were sequenced at Biokart, Bengaluru, and analysed using the NCBI BLAST for confirmation. The *nuc* gene and *mecA* gene amplicons showed sequence similarities of over 98% and 99%, respectively, with the NCBI database sequences. Additionally, the *nuc* gene sequence was observed to be distinctive to *S. aureus* in the database.

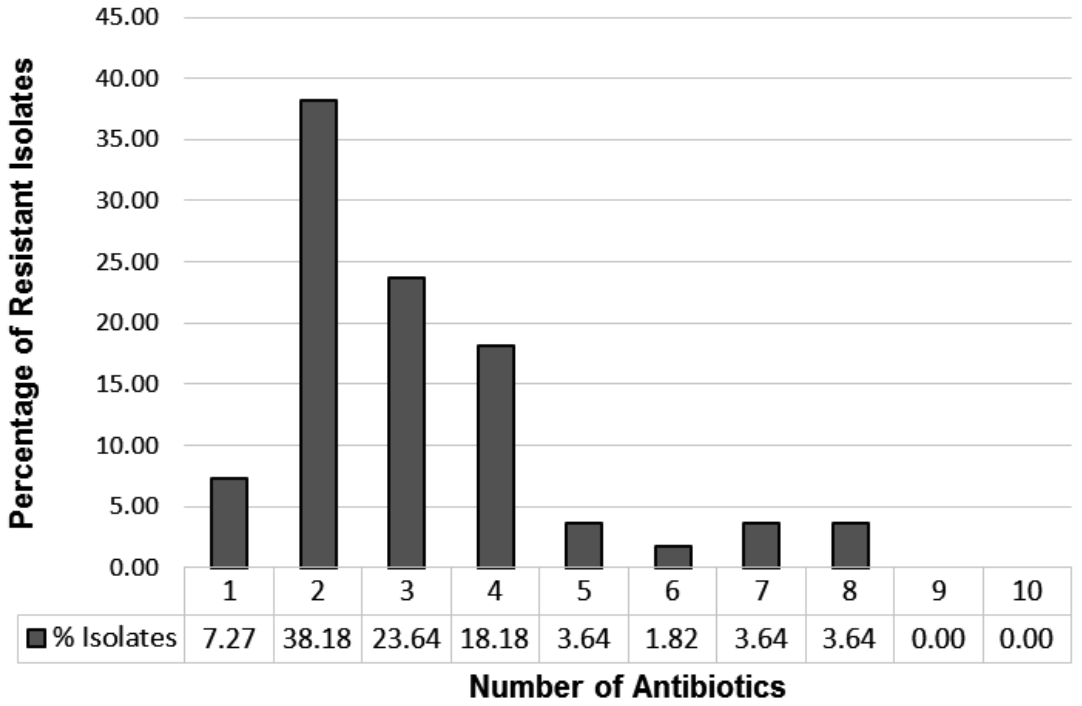
## RESULTS

In this study, 155 water samples collected and screened from different locations in North Karnataka. From these samples, 55 isolates of staphylococci were recovered, yielding an overall staphylococcal incidence of 35.48%. For convenience, samples from larger water bodies were grouped together. Of the 55 isolates, 18 (32.7%) were from sewage, 23 (41.8%) from lakes and rivers, and 14 (25.5%) from ponds.

The highest antibiotic resistance among the isolates was observed for oxacillin (54/55,




**Graph 1.** Antibiotic resistance profile of the environmental isolates




**Graph 2.** Percentage of resistant isolates of staphylococci for the number of antibiotics

**Table 1.** Frequency of methicillin-resistant staphylococci by phenotypic and genotypic tests

| Test | Methicillin Resistance | Percentage |
|---|---|---|
| Cefoxitin Disc diffusion | 13 | (23.6%) |
| *mecA* gene (PCR) | 20 | (36.4%) |

98.2%) followed by penicillin (48/55, 87.3%), erythromycin (26/55, 47.3%), cefoxitin (13/55, 23.6%), clindamycin (8/55, 14.5%), rifampicin (7/55, 12.7%), teicoplanin (4/55, 7.3%), tetracycline (4/55, 7.3%) and linezolid (3/55, 5.5%). Antibiotic resistance was lowest for ciprofloxacin (1/55, 1.8%) and chloramphenicol (1/55, 1.8%), with no resistance observed for gentamicin. The antibiotic susceptibility pattern is represented in Fig. 2.

Out of the 55 isolates, 30 (54.55%) were considered as multi- drug resistant (MDR) according to the criteria stated by Magiorakos et al.[23] However, none exhibited extensive drug resistance (XDR) or pan-drug resistance (PDR) according to the Centers for Disease Control and Prevention (CDC) definitions. The MAR indices ranged from 0.08 and 0.67, with a mean of 0.26, and both median and modal values were 0.16, indicating that most isolates were resistant to at least two antibiotics. Fig. 3 represents the frequency of resistant staphylococci for the number of antibiotics.

A one-way analysis of variance (ANOVA) was conducted to determine if there were significant differences in the mean MAR values among the four groups (Lake, Pond, River, and Sewage). The analysis revealed no statistically significant differences in the mean MAR values across the four groups at the 0.05 significance level, [F= 1.850, F crit = 2.798, p = 0.1508], indicating all samples contained MDR isolates without any significant difference.

Out of the 55 isolates, 13 (23.6%) were identified as methicillin-resistant using cefoxitin sensitivity testing. The *mecA* gene PCR was positive in 20 (36.4%) isolates, while 35 isolates were negative for *mecA*. Among the cefoxitin-resistant isolates, four were negative for the *mecA gene*, while 11 isolates with the *mecA* gene were sensitive to cefoxitin. Given that the cefoxitin disc diffusion method is the standard practice in most laboratories, the prevalence of methicillin resistance in our study was 13 out of 55 (23.6%). Table 1 presents MRSA detection results by phenotypic and genotypic methods.

In this study, we explored the *nuc* gene as an indicator of *S. aureus* pathogenicity and included it in our species confirmation tests. Our findings showed that 11 out of 55 staphylococcal isolates (20%) carried the *nuc* gene. The presence of thermonuclease is indicative of their potential pathogenic nature.[11]

## DISCUSSION

This study investigated the dissemination of antibiotic resistance among staphylococci isolated from various surface water sources in and around Dharwad city, North Karnataka, India. We recorded antibiotic susceptibility and conducted comparative analysis for methicillin resistance using both phenotypic and genotypic tests. Similar studies in India and other countries have reported MDR staphylococci in diverse water samples.[1,5,17,26]

The incidence of water-related diseases in India is high, with nearly 3.7 million cases reported annually.[27] This is due to poor waste management, human and animal interventions, and inadequate wastewater treatment, leading to pollution of water bodies and becoming pathogen breeding grounds.[17] Hospital effluents, disposal of unused medicines, and untreated wastewater from slaughterhouses contribute to MDR bacterial populations in the environment.[6] A 2018 study found that about 30% of the Indian population lacks proper latrine facilities, leading to open defecation.[28] Climatic changes and heavy rains cause the mixing up of sewage water, thereby increasing health risks.[27]

The detection of staphylococci in water sources is linked to human activities and the presence of domestic animals and livestock.[2] Infected animals serve as reservoirs of MRSA.[1] The prophylactic use of antimicrobials in agriculture, aquaculture, animal husbandry, poultry, and pig farming has led to a rise in MDR strains of staphylococci in the environment.[29]

Some researchers found a strong association between *S. aureus* and the use of the *nuc* gene for its rapid identification. However, it is not

highly specific, as other species of staphylococci, streptococci, and possibly other bacteria may mimic the enzymatic activity produced by the thermonucleases.[11] Our study reported the presence of *nuc* gene in 11 out of 55 (20%) staphylococcal isolates, indicating greater thermal stability and pathogenicity.[11] Another study conducted by our team during the same period, involving 250 clinical isolates of staphylococci found the *nuc* gene in 60.8% of *S. aureus* and 17.2% of the CoNS isolates.[30] Other researchers reported its presence in 80.2%[31] and 50%[12] of *S. aureus* isolates, respectively.

The prevalence of methicillin resistance in our study was found to be 23.6%. This finding aligns with other research conducted in India, which reported methicillin resistance prevalence of 25% in Delhi NCR,[5] 22.62% in Jalandhar, Punjab,[32] and 19.09% in Assam,[33] in studies focused on antimicrobial resistance (AMR) in water samples for staphylococcal isolates. Internationally, methicillin resistance prevalence has been reported as follows: 36.7% in Brazil,[17] 16.7% in Nigeria,[34] 97.5% in South Africa,[35] and 27% in USA.[36]

According to the Indian Council of Medical Research's 2023 antimicrobial resistance surveillance report, the prevalence of MRSA in clinical isolates across India varied significantly, ranging from 25% to 85% among the different regional centers involved in the study.[30,37] Between 2015 and 2019, the mean frequency of MRSA was as follows in various regions of India: North: 41%, East: 43%, West: 33%, South: 34%, Central: 36%, and Northeast: 40%.[38] Internationally, MRSA has the highest prevalence (60-80%) in the Middle East and North African countries, while in sub-Saharan Africa and several European countries have reported the least prevalence (< 5%).[39]

Presently, most staphylococci show high resistance to penicillin and ampicillin.[1] Our study observed similar resistance patterns, with high resistance to erythromycin (47.3%). This can be attributed to selection pressure due to the excessive use of these commonly used antibiotics not just by human and veterinary clinical practitioners but also in poultry and agriculture as a prophylactic treatment. Interestingly, low resistance to chloramphenicol and ciprofloxacin, and no resistance to gentamicin, suggests these antibiotics remain effective against majority of the environmental isolates. This finding is in agreement with other studies.[29,40] This is particularly relevant considering the global concern over the decreasing effectiveness of numerous antibiotics.

In our study, 98.2% of isolates were resistant to oxacillin. Some researchers have even reported 100% oxacillin resistance in environmental isolates.[26] Due to high specificity and sensitivity, the CLSI have recommended cefoxitin over oxacillin in the phenotypic detection of methicillin resistance in staphylococci.[21,41] However, phenotypic sensitivity might not accurately reflect the true nature of the organism, as it can be influenced by external factors such as inoculum concentration, salt concentration in the media, incubation time, and temperature, among others.[42] OS-MRS (oxacillin-susceptible methicillin resistant staphylococci) is a subpopulation of MRS that, despite carrying the *mecA* gene, appears susceptible to routine phenotypic tests like cefoxitin disc diffusion or oxacillin screen agar, potentially leading to its misidentification as methicillin susceptible staphylococci[43] this could be due to frameshift mutation, gene regulation, modification of cell wall, heterogenous resistance.[31,44] In our current study, we identified 11 (20%) instances of OS-MRS. Previous studies have reported rates of 2%,[43] 7.09%,[18] and 7.2%.[30]

Isolates phenotypically detected as methicillin-resistant but lacking the *mecA* gene by PCR might result from altered expression of penicillin-binding proteins (PBPs) due to gene mutation, β-lactamase overproduction leading to borderline oxacillin resistance staphylococci (BORS), or the potential involvement of alternative resistance mechanisms, like efflux pumps or target site modifications.[21,29] Recently, the *mecC* gene has been identified as another factor contributing to methicillin resistance, with a 60% homology to the *mecA* gene.[43] However, we did not test for the presence of the *mecC* gene in our isolates. The incidence of BORS/ORS (oxacillin-resistant staphylococci) in our study was 7.2%, other researchers have reported rates of 2.4%,[18] 6%,[45] 12.8%,[30] and 25.5%.[46]

The global spread of MDR staphylococci is concerning. An important observation from this study is the slight elevation in MAR indices, with a value of 0.26. MAR indices above 0.25 suggest higher and recurrent antibiotic exposure

environments.[23] Other studies have reported MAR value ranging from 0.38–0.52,[32] 0.435.[34] Rajput et al.[5] reported 60.86% MDR strains of staphylococci in their study.

In our clinical isolates study, 86.4% showed methicillin resistance by cefoxitin, and 80.8% by the presence of *mecA*. MAR indices ranged from 0.12 to 0.65 in *S. aureus* and 0.06 to 0.71 in CoNS. The average MAR values in *S. aureus* and coagulase-negative staphylococci (CoNS) were 0.42 and 0.44, indicating resistance to seven or more antibiotics.[30] This highlights higher and more frequent antibiotic resistance in clinical isolates compared to environmental isolates. However, MAR values above 0.25 still indicate a rising AMR threat among environmental isolates. This issue requires timely attention and adequate action to curb the rise of AMR in environmental isolates.

The broader implications of our findings underscore the increasing prevalence of AMR globally, driven by antibiotics misuse in human and animal healthcare, inadequate wastewater treatment, and the improper pharmaceutical disposal. The emergence of 'superbugs' and the corresponding rise in healthcare costs and treatment complexities represent a substantial burden on public health systems worldwide.[47] The situation is further complicated by the lack of strong surveillance data in many regions, including India, where environmental AMR remains underreported.

To address the escalating threat of antimicrobial resistance, global initiatives such as the World Health Organization's 'One Health' approach, Global Action Plan, Antibiotic Stewardship Programme, and the Global Antimicrobial Resistance and Use Surveillance System (GLASS) play a crucial role.[13] These initiatives advocate for coordinated efforts across human, animal, and environmental health sectors to tackle AMR, monitor the spread of drug resistance, and improve patient care by reducing irrational antibiotic use.

In India, the National Action Plan for AMR has been introduced to regulate the use of antimicrobials, including prohibiting the use of last-resort antibiotics in poultry feed and farming. The National Centre for Disease Control and the Indian Council of Medical Research (ICMR) monitor AMR through the AMR Surveillance and Research Network (AMRSN). This network combats antibiotic misuse in hospitals and monitors AMR status in clinical isolates through regular surveillance of 30 tertiary care hospitals selected across the nation by ICMR's Antimicrobial Stewardship Program (AMSP).[15] ICMR raises awareness through initiatives in educational institutes and conducts workshops for microbiologists and healthcare workers. Emphasis is laid on all stakeholders, including clinicians, farmers, public, and drug companies, to jointly address antibiotic resistance and curb its growing menace.[48]

Additionally, the WHO has introduced the WASH program in India and other countries, aiming to provide clean drinking water, better sanitation, and improved hygiene practices. Key initiatives under this program in India include the Swachh Bharat Mission and the Jal Jeevan Mission.[28]

## CONCLUSION

The present study was driven by the scarcity of data on antimicrobial resistance in environmental isolates of staphylococci. Our study contributes to understanding the AMR burden in these isolates and emphasizes the need to address this complex challenge by adopting integrated approaches. These approaches include raising public awareness about the rational use and disposal of pharmaceutical products and ensuring adequate wastewater treatment to avoid the contamination of freshwater and other water bodies. Further research is required to inspect the spread of drug resistance in other environmental samples, such as food and meat, and to develop effective interventions to prevent the dissemination of resistant pathogens in the environment. Such initiative can significantly prolong the efficacy of antibiotics in clinical applications.

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**Conflict of interest**

The authors have no financial and non-financial conflicts of interest to disclose.

**Data Availability Statement**

This statement does not apply to this article

**Ethics Statement**

This research did not involve human participants, animal subjects, or any material that requires ethical approval.

**Informed Consent Statement**

This study did not involve human participants, and therefore, informed consent was not required.

**Clinical Trial Registration**

This research does not involve any clinical trials.

**Permission to reproduce material from other sources**

Not Applicable

**Author Contributions**

Shafa Contractor: Data collection, Methodology, Analysis, Funding Acquisition, Writing- Original Draft & Editing. Suresh Arakera: Conceptualization, Supervision, Writing-Review & Finalized the manuscript. Both the authors mentioned have significantly and directly contributed intellectually to the project and have given their approval for its publication.

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