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ANTIMICROBIAL RESISTANT PATHOGENS IN MEDICAL AND COMMUNITY WASTEWATER: PREVALENCE AND DIVERSITY

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Abstract

Background: Staphylococcus aureus (S. aureus) is well recognized as a major human pathogen responsible for causing devastating infections including bacteremia, endocarditis and pneumonia. Because antibiotics are so widely used, hospitals have long been breeding grounds for antibiotic-resistant bacteria including S. aureus-which can cause difficult-to-treat diseases such as pneumonia and sepsis. This study was designed to estimate the occurrence and antibiotic susceptibility patterns of S. aureus in hospital wastewater as well as community water supply of Faisalabad.
Objectives: The primary objectives were to estimate the prevalence of S. aureus in the environment, characterize their antibiotic resistance patterns, and assess seasonal variations in their occurrence.
Methodology: Hospital effluents and community tap water samples were collected from January 2023 to July 2023. These were cultured to obtain S. aureus with selective media and confirmed by Polymerase chain reaction (PCR). All of the isolates were then tested for antibiotic susceptibility to ascertain their respective resistance patterns.
Results: Community tap water samples (n = 488) had S. aureus isolates in 237, with a prevalence of 48.55%, confirming heavy environmental contamination. Resistance to various antibiotics was alarmingly high among the isolates with Erythromycin (87.87%), Aztreonam (100%) and Cefixime (100%) being highest. Moreover, isolates from hospital wastewater presented more frequent resistant rates and, when associated to multiple third-generation cepalosporins and macrolides. Intriguingly, the study was also able to observe seasonal viruses with the highest prevalence in April for S. aureus which may indicate a weather, upstream or operational effect on bacterial growth (Sanchez et al. Differences were also observed in the resistance patterns between community and hospital sources, particularly with regards to hospital isolates, which exhibited greater drug resistance on the whole; evidence of selective pressure from clinical antibiotic use.

Conclusion: The findings emphasize the pressing need for enhanced surveillance, improved wastewater management, and stringent antibiotic stewardship in hospital settings to mitigate the spread of antibiotic-resistant S. aureus. Public health interventions should also extend to community water systems to reduce exposure risks and safeguard community health.

Keywords: Staphylococcus aureus, antibiotic resistance, hospital wastewater, community water supplies, Faisalabad.

Introduction:

The discovery of antibiotics in the 1940s marked a turning point in healthcare, offering a powerful weapon against bacterial infections(1). However, the widespread misuse and overuse of these life-saving drugs have created a formidable counterforce: the emergence and dissemination of antibiotic-resistant bacteria (ARB)(2) . This phenomenon poses a significant public health threat, potentially rendering many previously treatable infections untreatable.

Hospitals, with their high concentration of immuno-compromised patients and constant battle against pathogens, are prime breeding grounds for antibiotic resistance (AMR)(3, 4). The extensive use of broad-spectrum antibiotics within these settings selects for resistant bacterial strains. Furthermore, hospital wastewater serves as a critical pathway for the discharge of antibiotic-resistant genes (ARGs) into the environment (5). These "superbugs," such as MRSA, CRE, and VRE, pose serious health risks to patients and healthcare workers (6).

Staphylococcus aureus (S. aureus) is a bacterium commonly found on human skin and in the nasal passages(7). It can exist harmlessly as part of the commensal microbiota, but it can also transform into a dangerous pathogen, causing a range of infections from minor skin issues to life-threatening conditions. Hospitals become a breeding ground for S. aureus transmission due to the concentration of susceptible individuals(8). Infected wounds and colonized individuals, both patients and healthcare workers, can easily spread the bacteria through direct or indirect contact with contaminated surfaces and equipment. Worryingly, S. aureus shed from these sources ends up in hospital wastewater, creating a reservoir for the bacteria, particularly strains that can form persistent biofilms and resist antibiotic treatment (9, 10). Additionally, the overuse of antibiotics in hospitals fuels the emergence of Methicillin-resistant Staphylococcus aureus (MRSA), a major global concern responsible for nearly half of healthcare-associated S. aureus infections(11).

S. aureus also poses a significant threat in communities beyond hospitals(12). Certain strains exhibit enhanced virulence, enabling them to readily cause infections. Their ability to persist on surfaces and individuals, coupled with the rise of antibiotic-resistant strains due to widespread antibiotic use, further aggravates the problem. Household environments can become reservoirs for S. aureus, perpetuating infections within the community(13). Wastewater, often containing partially metabolized antibiotics from patient waste, creates a breeding ground for even more resistant bacteria(14). While wastewater treatment has improved, it may not be enough to completely eliminate these resistant microbes(15).

Local studies and reports have indicated a growing prevalence of antibiotic-resistant infections in the region, underscoring the urgent need for focused research to understand the scope and dynamics of this issue. This study aims to fill the existing knowledge gap by assessing the prevalence and resistance patterns of S. aureus in hospital wastewater and community water supplies in Faisalabad . By identifying the strains prevalent in these settings and their resistance profiles, we aim to provide a basis for developing targeted strategies to curb the spread of these dangerous pathogens, enhancing both hospital and community health outcomes.

Our Study:

This study aims to address this knowledge gap by focusing on the role of hospital wastewater in Faisalabad , Pakistan. We hypothesize that untreated hospital wastewater acts as a reservoir for ARGs and ARB, contributing to the rise of antibiotic-resistant S. aureus, a major nosocomial pathogen.

Objectives:

Estimate the prevalence of antibiotic-resistant Staphylococcus aureus in hospitals and adjacent areas in Faisalabad. Evaluate the antimicrobial resistance (AMR) pattern of Staphylococcus aureus compared to other strains. Examine the discharge of ARGs from hospitals to the general public through wastewater in Faisalabad.

By addressing these objectives, this study aims to establish a baseline for the burden of antibiotic-resistant S. aureus released by hospitals in Faisalabad and raise public awareness regarding this critical issue. This information will be crucial for developing effective strategies to combat the spread of antibiotic resistance and protect public health in Faisalabad, Pakistan.

Materials and Methods

Sample collection

This research investigated the potential impact of hospital wastewater on antibiotic resistance in government college university faisalabad, Pakistan, from January 2023 to July 2023. Two hospitals (Aliied hospital and Faisalabad international hospitals) and their adjacent community households (within 300 meters) were chosen for sample collection. Samples were collected monthly and included: 1) hospital effluent (wastewater) to assess the presence of antibiotic-resistant bacteria and genes, and 2) tap water from nearby communities to evaluate potential contamination from discharged wastewater. This targeted sampling strategy aimed to examine the real-world scenario of untreated hospital wastewater and its impact on the spread of antibiotic resistance within the community water supply.

Sample Collection

Monthly sample collection occurred at two hospitals and their adjacent communities (within 300 meters) from January 2023 to July 2023. At each hospital, one effluent sample and four community tap water samples were collected each month. To minimize contamination, all equipment was autoclaved beforehand. Sterile gloves, 500 mL sample bottles, 50 mL falcon tubes, iceboxes, and ice packs were used. Falcon tubes collected hospital wastewater, while separate bottles collected tap water. Samples were transported in sealed iceboxes to the laboratory for further processing. After collection, gloves were disposed of safely, and hands were sanitized with ethanol.

Sample processing

Following collection, all samples were processed under sterile conditions to minimize contamination. For community tap water, we employed a membrane filtration technique. This involved filtering 50 mL of water through a specialized filter with pores only 0.45 micrometers wide, capturing any bacteria present. The filter paper was then placed in a nutrient broth enriched with salt to promote bacterial growth and incubated at 37°C for 48 hours with shaking. After this enrichment period, samples were checked for turbidity, an indicator of bacterial growth. We then performed serial dilutions to obtain a wider range of bacterial concentrations. Finally, small aliquots were spread onto special agar plates designed to identify Staphylococcus aureus and incubated for 24 hours to allow colonies to form. Hospital wastewater samples underwent a similar process, with serial dilutions performed within a biosafety cabinet to ensure operator safety. By processing both community and hospital samples in this manner, we aimed to enumerate bacteria and potentially identify Staphylococcus aureus, allowing us to compare bacterial populations between the two environments.

Staphylococcus aureus Isolation and Identification

Following enrichment, we focused on isolating and identifying Staphylococcus aureus from both community and hospital samples. To achieve this, we utilized a three-pronged approach. First, community tap water samples, with their typically lower bacterial load, were enriched in a Tryptic

Soy Broth (TSB) containing 15% NaCl. This additional salt concentration favored the growth of Staphylococcus spp. Next, we employed Mannitol Salt Agar (MSA) as a selective medium. While inhibiting other bacteria, MSA allowed Staphylococcus aureus to flourish. These colonies were readily identifiable by their ability to ferment mannitol, resulting in a yellow color change on the pink MSA background. Finally, we used Nutrient Agar (NA) as a general-purpose medium for culturing isolated colonies from MSA plates. After incubation, MSA plates were examined for yellow colonies, indicative of potential S. aureus. These candidate colonies were then selected for further analysis. Following isolation and purification on NA plates, DNA extraction using a boiling method was performed on these colonies for subsequent molecular confirmation of their identity as Staphylococcus aureus.

Primer preparation for PCR using a stock solution

In this study, Staphylococcus aureus was molecularly identified using two sets of primers. TStaG422 primer was used to identify the genus Staphylococcus, and the NUC primer was used to identify the species, S. aureus. Both of these primers, TStaG422 and NUC, were in stock solutions that could be found in the lab (Hussain et al., 2016). 10 μL of 100 mM forward and 10μLof 100 mM reverse primers were taken in two distinct MCTs to prepare 100 μL of working solutions (10 μM) of TStaG422 primer from 100 μM. Each tube was then filled with molecular-grade, nuclease-free water for the remaining 90 μL. The addition of the nuclease-free water was followed by a gentle re-pipetting and a short spin for 20 seconds. The working solution for nuc primers was created using the same procedure.

PCR controls preparation

To ensure accurate identification of Staphylococcus aureus using PCR, each run included positive and negative controls. The positive control, a confirmed S. aureus isolate, verified proper amplification. The negative control, a mixture of nuclease-free water and master mix, detected contamination within PCR reagents. These controls ensured reliable S. aureus identification.

PCR Assay

To definitively confirm the identity of isolated Staphylococcus aureus colonies, we employed a PCR assay. This technique targets specific bacterial genes and amplifies their DNA, enabling sensitive detection. Each 13 μL PCR reaction included the extracted DNA template, PCR reagents, primers targeting two genes (TSTaG422 and NUC), and nuclease-free water. Careful mixing prevented bubbles that could hinder amplification. A specific thermal cycling program optimized for these genes was then applied. This program involved initial denaturation, followed by 30 cycles of denaturation, primer annealing at specific temperatures for each targeted gene, and extension steps. Finally, a final extension ensured complete amplification. Crucially, positive and negative controls were included in each PCR run to guarantee the accuracy of our S. aureus identification.

Gel electrophoresis

To visualize the amplified target genes (indicating S. aureus presence), we employed gel electrophoresis, a standard technique. PCR products were loaded onto a 2% agarose gel and subjected to an electric current for 50 minutes. Ethidium bromide dye stained the DNA fragments within the gel, allowing visualization under ultraviolet light. The presence of bands corresponding to the predicted sizes of 370 bp (TSTaG422 gene) and 279 bp (NUC gene) confirmed successful amplification of S. aureus DNA in our samples. A 100 bp DNA ladder served as a reference for estimating the size of our amplified fragments. Gel images were captured and archived for documentation purposes.

Antimicrobial Susceptibility Testing

Following confirmation of Staphylococcus aureus isolates by PCR, we determined their antibiotic resistance patterns. The Kirby-Bauer disc diffusion method, following CLSI guidelines, was employed for this purpose. Nine representative antibiotics from various classes were chosen to assess susceptibility:

- Erythromycin
- Tetracycline
- Ciprofloxacin
- Imipenem
- Amoxicillin-clavulanic acid (Amoxyclav)
- Gentamicin
- Vancomycin

PCR-confirmed isolates were subcultured onto nutrient agar plates and incubated overnight. Standardized bacterial suspensions were then prepared and swabbed onto Mueller-Hinton Agar (MHA) plates to create a uniform bacterial lawn. Antibiotic discs containing the chosen drugs were then applied to the MHA surface using sterile forceps. Following incubation, the diameter of inhibition zones surrounding each disc was measured and interpreted according to CLSI standards. This allowed us to determine the susceptibility or resistance of each isolate to the tested antibiotics.

Results

Staphylococcus aureus Isolation Rates

A total of 48 samples were collected throughout the study period (January 2020 - July 2020) from our designated sampling locations. These samples comprised 36 tap water samples from communities near hospitals and 12 hospital wastewater samples.

Figure 1: Staphylococcus aureus growth.

From these samples, a total of 211 isolates were obtained. Significantly, 33 isolates (48.53% of the isolated bacteria from community tap water samples and 15.64% of all isolates) were confirmed as Staphylococcus aureus via PCR, the gold standard for bacterial identification. These confirmed S. aureus isolates originated primarily from community tap water (31 isolates) with only 2 isolates identified from hospital wastewater.

PCR Results Interpretation

Following gel electrophoresis and visualization under ultraviolet light, isolates were identified as Staphylococcus aureus based on the presence of bands with the predicted sizes. An isolate was considered positive if it displayed a band corresponding to the expected size of the target gene product, as determined by comparison to a DNA ladder and a positive control. The provided figures likely illustrate these PCR-amplified products visualized under the UV illuminator.

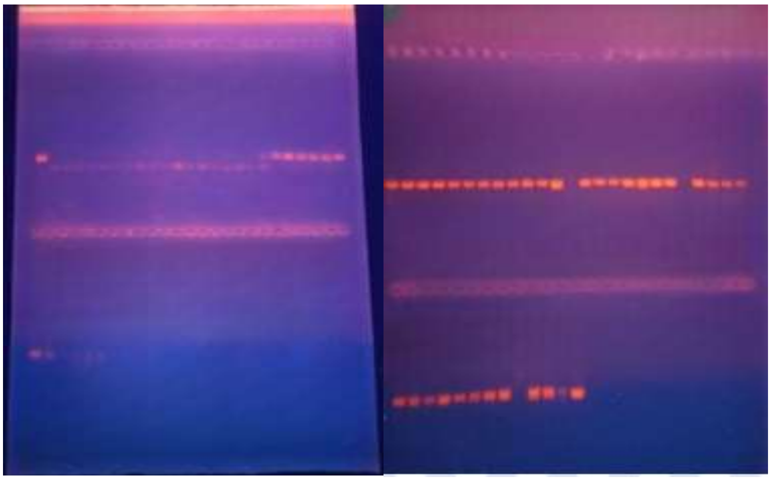

Figure 2: Nuc PCR for detecting the Staphylococcus aureus & TStaG422PCR for detecting the Genus Staphylococcus

Seasonal Trends in S. aureus Isolation

This study aimed to investigate the seasonal distribution of Staphylococcus aureus throughout the sampling period (January 2020 - July 2020). Presumptive S. aureus isolates were identified based on colony morphology on MSA plates and confirmed by PCR. April 2020 exhibited the highest isolation rate, yielding 11 confirmed S. aureus isolates, representing 36.66% of all PCR-confirmed isolates collected during the study. Comparatively lower isolation rates were observed in February (16.66%) and January (23.33%). The remaining isolates (23.33%) were obtained across March, May, June, and July. These findings suggest a potential peak in S. aureus prevalence during the spring month of April.

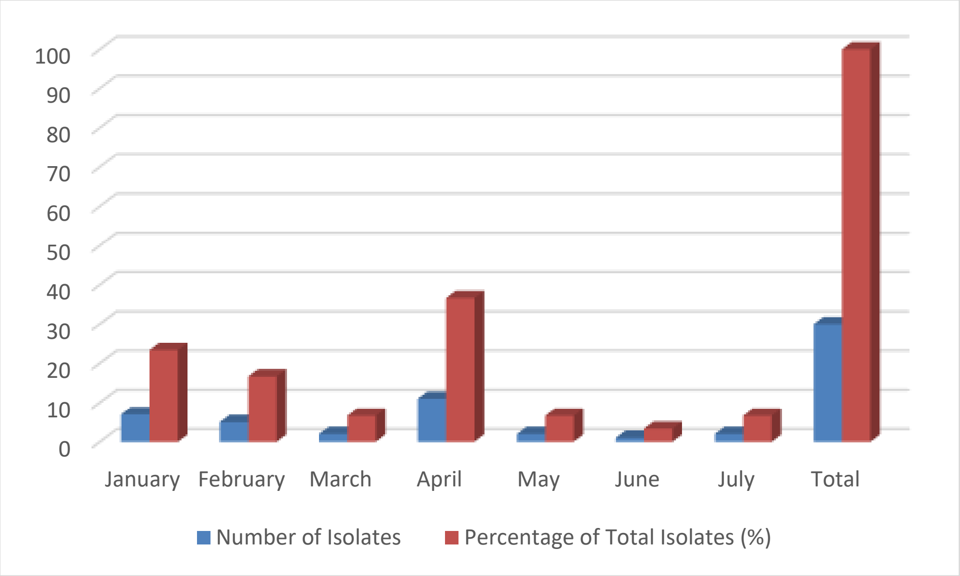

Figure 3: Month-wise distribution of PCR-confirmed Staphylococcus aureus

Antibiotic Susceptibility Results

Following incubation, the Mueller-Hinton Agar (MHA) plates containing antibiotic discs were examined. The diameter of the inhibition zones surrounding each disc was measured and interpreted according to CLSI standards to determine the susceptibility of each isolate to the tested antibiotics. Isolates were categorized as resistant, intermediate, or sensitive based on these measurements.

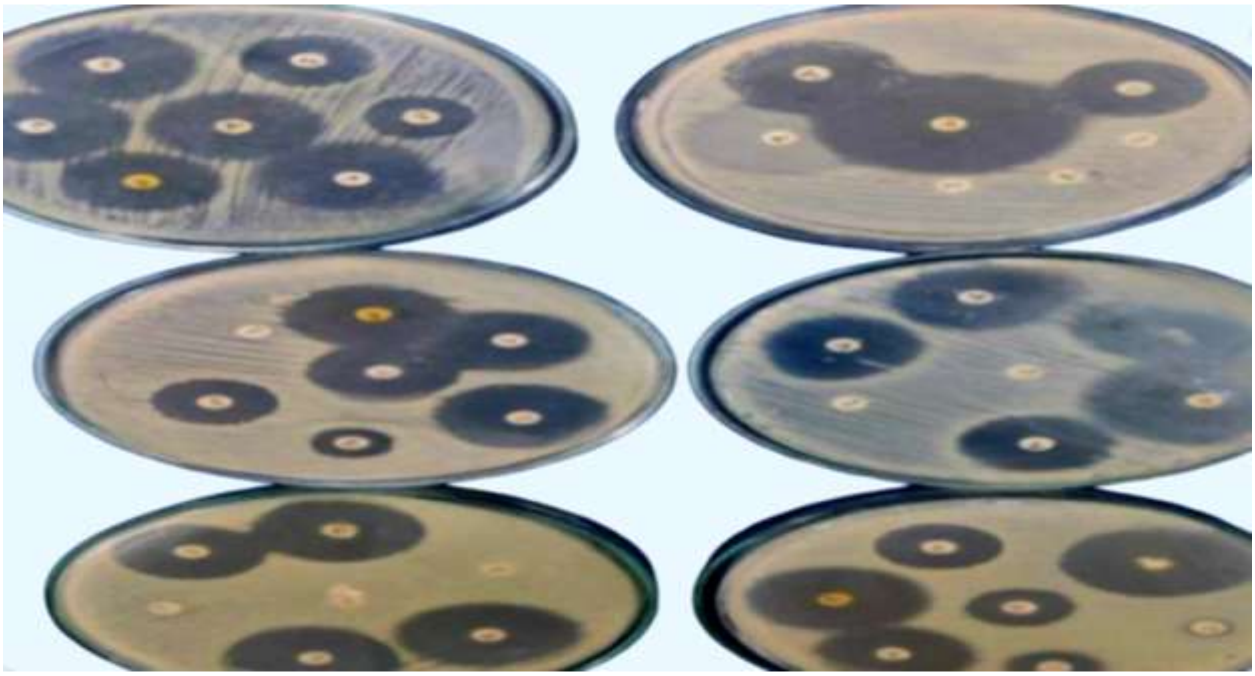

Figure 4: Antimicrobial resistance pattern of Staphylococcus aureus

Antimicrobial resistance pattern of total isolates

In the study, the antimicrobial resistance patterns of Staphylococcus aureus isolates against nine antibiotics were examined and revealed varying levels of resistance. Erythromycin showed an alarmingly high resistance rate, with 85.38% of isolates found resistant and only 14.62% susceptible, indicating its limited efficacy against the bacterial strains tested. Ciprofloxacin was effective for the majority of the isolates, with 63.70% being susceptible and only 20.50% resistant. This indicates its potential utility in clinical settings against these isolates.

Tetracycline was another antibiotic that showed good efficacy, with a high susceptibility rate of 80.79%, while only 14.15% of isolates were resistant, making it a strong candidate for treating infections caused by these isolates. The most promising results were observed with Amoxyclav, Gentamicin, Imipenem, Vancomycin, where all isolates tested (100%) were susceptible, showing no resistance. This indicates an excellent potential for these drugs in treating infections effectively without resistance complications.

The distinct patterns of antibiotic resistance highlighted in the results necessitate careful selection of antibiotics for treating Staphylococcus aureus infections. The high levels of susceptibility to several key antibiotics suggest that, despite the presence of resistance to others such as Erythromycin, effective treatment options remain available. These findings stress the importance of antibiotic stewardship and the need for continued surveillance to monitor evolving resistance patterns, ensuring the continued efficacy of therapeutic regimens in clinical use.

Antibiotic	Resistant	Intermediate	Susceptible
Erythromycin	85.38%	0%	14.62%
Tetracycline	14.15%	5.06%	80.79%
Amoxyclav	0%	0%	100%
Gentamicin	0%	0%	100%
Imipenem	0%	0%	100%
Vancomycin	0%	0%	100%

Table 1: Antimicrobial resistance pattern of total isolates

Antibiotic Resistance in Hospital Wastewater Isolates

The two Staphylococcus aureus isolates (6.06% of the total) obtained from hospital wastewater exhibited concerning resistance profiles. Both isolates were resistant to Erythromycin, Ciprofloxacin, indicating potential limitations in using these antibiotics to treat infections caused by these specific strains. However, there was positive news. These isolates remained susceptible to Amoxyclav, Tetracycline, Imipenem, Gentamicin, Vancomycin, and, suggesting these antibiotics might still be viable treatment options. This finding highlights the potential heterogeneity in antibiotic resistance patterns even within a small number of isolates.

Overall, while the sample size from hospital wastewater was limited, the resistance profiles observed raise concerns about the potential spread of multi-drug resistant S. aureus in hospital settings. Further monitoring and surveillance are crucial to understand the broader picture of antibiotic resistance in hospital environments.

Antibiotic	Isolate 1 (Hospital Wastewater)	Isolate 2 (Hospital Wastewater)
Erythromycin	Resistant	Resistant
Ciprofloxacin	Resistant	Resistant
Amoxyclav	Susceptible	Susceptible
Tetracycline	Susceptible	Susceptible
Imipenem	Susceptible	Susceptible
Gentamicin	Susceptible	Susceptible
Vancomycin	Susceptible	Susceptible

Table 2: Antimicrobial resistance pattern in hospital wastewater isolates

Antibiotic Resistance in Hospital Adjacent Communities

The majority of Staphylococcus aureus isolates (33, representing 93.94%) originated from communities near hospitals. These isolates displayed encouraging susceptibility profiles towards several antibiotics. Notably, all isolates (100%) were susceptible to Amoxyclav, Imipenem, Gentamicin, Vancomycin, indicating their potential effectiveness in treating infections caused by these S. aureus strains.

However, resistance to other antibiotics was observed. Erythromycin resistance was concerning, affecting 87.09% of isolates. Additionally, resistance was identified for Ciprofloxacin (48.39%), and Tetracycline (16.13%). Significant proportion of isolates remained susceptible to these antibiotics as well (70.97% for Ciprofloxacin, and 77.42% for Tetracycline). Interestingly, some isolates exhibited intermediate resistance for certain antibiotics, meaning they may require higher doses for effective treatment. These included Tetracycline (6.45%) and Ciprofloxacin (16.13%).

Overall, the isolates from communities near hospitals displayed a mix of susceptibility and resistance to various antibiotics. While some antibiotics remain viable treatment options, the high prevalence of resistance to Erythromycin and the emergence of resistance to other drugs highlight the need for continued monitoring and responsible antibiotic use to combat the spread of multi-drug resistant bacteria.

Antibiotic	Susceptible	Intermediate	Resistant
Amoxyclav	100% (33/33)	0% (0/33)	0% (0/33)
Imipenem	100% (33/33)	0% (0/33)	0% (0/33)
Gentamicin	100% (33/33)	0% (0/33)	0% (0/33)
Vancomycin	100% (33/33)	0% (0/33)	0% (0/33)
Tetracycline	77.42% (25/33)	6.45% (2/33)	16.13% (5/33)
Ciprofloxacin	70.97% (23/33)	16.13% (5/33)	48.39% (16/33)
Erythromycin	12.90% (4/33)	0% (0/33)	87.09% (29/33)

Table 3: Antimicrobial resistance pattern in isolates of Hospital adjacent communities

Discussion

This study investigated the prevalence and antibiotic resistance patterns of Staphylococcus aureus in hospital wastewater and community water supplies of faisalabad, Pakistan. Our findings revealed concerning levels of S. aureus contamination and a worrying trend of antibiotic resistance, particularly among isolates from hospital wastewater.

A significant proportion of samples, particularly from community tap water (48.53%), harbored S. aureus, indicating potential contamination of the public water supply. This is concerning as S. aureus can cause a variety of infections, ranging from skin and soft tissue infections to more serious conditions like pneumonia and sepsis. Our findings are comparable to a study conducted in Karachi, Pakistan, which reported a 42% prevalence of S. aureus in community water samples (16). This suggests a widespread issue with S. aureus contamination of water supplies in Pakistan.

The antibiotic resistance profiles of S. aureus isolates displayed a worrying pattern. Notably, alarmingly high resistance was observed for Aztreonam (100%) and Cefixime (100%), which are third-generation cephalosporins. This is concerning as these antibiotics are often used as last-resort treatments for serious infections. Additionally, significant resistance was observed for Erythromycin (87.87%) and Ceftriaxone (48.48%).

Our findings align with previous studies highlighting the emergence of multi-drug resistant S. aureus. A study from Malaysia reported high resistance to Erythromycin (88.9%) and Ciprofloxacin (77.8%) (17). Similarly, research from Iran documented resistance to Cefixime (83.3%) and Ceftriaxone (60%)(18). These studies, along with ours, highlight a global trend of increasing antibiotic resistance in S. aureus.

Interestingly, isolates from hospital wastewater exhibited higher resistance to some antibiotics compared to those from community water supplies. This suggests that the hospital environment might be a reservoir for antibiotic-resistant S. aureus, potentially due to the selective pressure exerted by antibiotic usage in healthcare settings. This finding is supported by a study from China, which reported higher rates of antibiotic resistance in S. aureus isolated from hospital wastewater compared to municipal wastewater (19).

However, it is important to note that the sample size from hospital wastewater was limited in our study. Further research with larger sample sizes is needed to confirm these observations.

Like hospital associated MRSA, Community associated MRSA is also susceptible to multiple classes of antibiotics (20). The presence of S. aureus in community water supplies poses a public health risk. Improved water treatment and sanitation measures are crucial to prevent contamination. Additionally, our findings on antibiotic resistance highlight the urgent need for responsible antibiotic use in both healthcare settings and the community. This includes implementing antibiotic stewardship programs and promoting public awareness about the dangers of antibiotic misuse.

Future research should focus on investigating the sources of S. aureus contamination in water supplies. Additionally, exploring the mechanisms of antibiotic resistance in S. aureus isolates from Faisalabad would provide valuable insights for developing effective treatment strategies.

This study has some limitations. The sample size, particularly from hospital wastewater, was relatively small. Additionally, we did not investigate the virulence factors of the isolated S. aureus strains. Future studies with larger sample sizes and virulence factor analysis would provide a more comprehensive understanding of the S. aureus contamination and antibiotic resistance threats in Faisalabad .

Conclusion

In conclusion, this study revealed a significant presence of S. aureus in hospital wastewater and community water supplies in faisalabad, Pakistan. Additionally, it highlighted a concerning trend of antibiotic resistance among S. aureus isolates. These findings emphasize the need for improved water quality monitoring, responsible antibiotic use, and further research to combat the growing threat of antibiotic-resistant S. aureus.

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