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Incidence of methicillin-resistant *Staphylococcus aureus* in wastewater and its survival after discharge from two hospitals in Akure, Nigeria

TOLULOPE EMORUWA♥, OLUFUMILOLA OMOYA
Federal University of Technology Akure. P.M.B. 704 Akure, Ondo State, Nigeria. Tel.: +234-906 670-7545, ♥email: emoruwatolu09@gmail.com

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Abstract. *Emoruwa T, Omoya O. 2024. Incidence of methicillin-resistant* Staphylococcus aureus *in wastewater and its survival after discharge from two hospitals in Akure, Nigeria. Nusantara Bioscience 16: 119-129.* The prevalence of Methicillin-Resistant *Staphylococcus aureus* (MRSA), a silent infection-causing bacteria that is resistant to several antibiotics is rising in the population, increasing morbidity and mortality rates. The goal of this study was to find MRSA in hospital wastewater from the University of Medical Science Teaching Hospital and University Health Center, The Federal University of Technology Akure, Nigeria. Wastewater were collected from outlets in different wards, and pipe-borne water was collected as a control. The wastewater underwent bacteriological analysis using membrane filtration, identifying all the bacteria isolates. Zones of inhibition were interpreted to screen *S. aureus* isolates for antibiotic susceptibility. The *mecA* gene was molecularly identified in *S. aureus* isolates using bacterial DNA extraction and polymerase chain reaction. The plasmid profile and MRSA survivability at various pH, temperature, and salt concentrations were examined as well. The total bacterial counts in wastewater collected from UNIMEDTH and FUTA Health Center ranged from 49.72±0.86 CFU/100 mL (pipe-borne water) to 877.91±1.55 CFU/100 mL (Accident and Emergency ward) and 73.71±0.72 CFU/100 mL (pipe-borne water) to 422.05±1.55 CFU/100 mL (Wound treatment ward) respectively, while the total staphylococcal counts in UNIMEDTH and FUTA Health Center ranged from 0.00±0.00 CFU/100 mL (pipe-borne water) to 220.14±1.06 CFU/100 mL (Medical Laboratory Science Laboratory) and 1.02±0.11 CFU/100ml (pipe-borne water) to 60.11±0.11 CFU/100 mL (doctors' station) respectively. Isolates of *S. aureus* were more resistant to ampiclox 10 (62.50%), oxacillin 7 (43.75%), zinnacef 10 (62.50%), and amoxicillin 8 (50.00%). The incidence of MRSA in hospital wastewater and its survival under different environmental conditions could present a public health challenge as the discharge of untreated wastewater could contaminate different water bodies.

Keywords: Antimicrobial resistance, *Staphylococcus aureus*, wastewater

INTRODUCTION

Staphylococcus aureus (*S. aureus*) is a Gram-positive bacterium that can be normal flora in the upper respiratory tract and the skin. It has been implicated in causing infection of the skin and various tissues as well as toxin-mediated diseases like food poisoning and toxic shock syndrome (Tong et al. 2015). It can cause both community- and hospital-acquired infections, and it can easily acquire antibiotic-resistant genes by horizontal gene transfer (Haaber et al. 2017).

Water is necessary for hospital operations and hygiene. Due to the nature and significance of the compounds they contain, hospital wastewater discharge poses threats to human health and the environment. Because there aren't enough wastewater treatment and purification facilities in developing nations, managing hospital wastewater is a serious issue.

Hospitals are one of the sectors in the world with the greatest pollution emissions (Achak et al. 2021). Reusing treated water presents a health risk to the public since wastewater treatment plants, and hospital wastewater treatment plants in particular, are believed to be hotspots for the emergence of antibiotic resistance (Rizzo et al. 2013; Yuan and Pia 2023).

Previous investigations have detected significant quantities of drugs and residual microorganisms in Hospital Wastewater (HWW). These results may exert a selective pressure on the growth of microorganisms resistant to antibiotics (Rowe et al. 2017). Because of this, HWWs have a higher chance of spreading Antibiotic Resistant Genes (ARGs) than other wastewater systems, like urban wastewater systems (Verlicchi et al. 2015; Zheng et al. 2018). Hospitals utilize antibiotics such as carbapenems, glycopeptides, and others more often than in other settings.

This divergence raises the possibility of an increase in ARGs associated with hospitals. Since the 1980s, restrictions for sludge and wastewater emission limitations have been put into place globally to reduce the harm caused by post-discharge effluent (Meng et al. 2016).

Only a few nations, like France and Italy, have raised legal concerns about pre-release HWW therapy (Verlicchi et al. 2010; Al Aukidy et al. 2017; Mehanni et al. 2023). Antibiotic resistance levels in hospital wastewater may differ from those in other aquatic ecosystems due to variations in antibiotic application patterns. Hospitals are the primary settings for the use of certain antibiotics, including cefotiam, piperacillin, and vancomycin (Mehanni et al. 2023).

The ability of *S. aureus* to outwit the immune system, above and beyond its Multidrug-Resistance (MDR) phenotype, makes it one of the most intractable pathogenic bacteria in the history of antibiotic chemotherapy (Rowe et al. 2017). The spread of methicillin-resistant *S. aureus* (MRSA) has become a significant concern for both animal and human health worldwide (Rowe et al. 2017; Zheng et al. 2018; Mehanni et al. 2023). MRSA is predominantly mediated by the expression of the *mecA* gene, which is located on a mobile genetic element; the Staphylococcal Cassette Chromosome mec (SCCmec), encoding an altered Penicillin-Binding Protein (PBP2a) with an exceedingly low susceptibility to beta-lactam antibiotics. Thus, *S. aureus* will be practically resistant to most beta-lactam antibiotics (Al Aukidy et al. 2017; Mehanni et al. 2023). On the other hand, resistance to vancomycin is accomplished by horizontal transfer of a plasmid-born transposon carrying the *vanA* gene from vancomycin-resistant Enterococcus to *S. aureus* across the genus barrier (Mehanni et al. 2023).

The *S. aureus* has been used as an indicator microorganism in wastewater and river water (López et al. 2019). Antibiotic-resistant *S. aureus* itself is considered an opportunistic organism, but it is spreading to a wider spectrum of society due to the difficulty of treating it with antimicrobials and disinfectants in medical facilities (Garcia et al. 2017). Therefore, this research aimed to clarify the status of *S. aureus* in hospital wastewater in two selected hospitals in Akure, Nigeria.

The objectives of this study are to determine the antibiotic susceptibility profile of *S. aureus* isolated from hospital wastewater in Akure. Investigate the presence of Methicillin-Resistant *S. aureus* (MRSA) in hospital wastewater. Evaluate the survival of MRSA isolates in hospital wastewater under different environmental conditions. The plasmid profile of Methicillin-Resistant *S. aureus* (MRSA) isolated from hospital wastewater in Akure.

MATERIALS AND METHODS

Study design and area

This case-control study was carried out in selected hospitals in Akure, and microbiological analysis was conducted at the Department of Microbiology, The Federal University of Technology, Akure (FUTA), Nigeria. Wastewater (200 mL) was collected from the University of Medical Science (UNIMED) teaching hospital and University Health Center FUTA. These two hospitals were selected because they are the only hospitals affiliated with tertiary institutions in Akure, the state capital. A letter of introduction (Ethical approval) was collected from the Head of the Department of Microbiology, FUTA, and was used as a valid means of identification at the hospitals where samples were collected in Akure.

Isolation of bacteria from wastewater

Samples were randomly collected from twelve (12) different locations at UNIMED and seven (7) different locations from FUTA two times daily for three weeks. Microbiological examinations of wastewater were carried out using the membrane filtration method as described by WHO (2016), on nutrient agar, some selective and differential media (Salmonella Shigella agar, Eosin Methylene Blue agar, MacConkey agar and Mannitol Salt agar). One hundred (100 mL) milliliters of wastewater was gently shaken and filtered with a 0.45 µm membrane filter and the filter was aseptically placed on molten agar and incubated at 37°C for 24 hours. A colony count was performed and the average of all the tests for each sample location was considered as the colony-forming unit per 100 milliliters (CFU/100 mL) of hospital wastewater. Morphological and biochemical characterization of bacterial isolates were used for identification (Fawole and Oso 2004; Cheesbrough 2014). Bacterial colonies showing typical characteristics of *S. aureus* including golden yellow color colonies on MSA were subjected to gram staining, catalase test, and DNase test (Olutiola et al. 2018).

Antimicrobial susceptibility testing

CLSI (5th edition) guidelines recommend the Kirby–Bauer disk diffusion method, which involves using Muller–Hinton Agar (MHA) to inoculate bacterial suspensions (CLSI 2017). The method is standardized and incubated at 37°C for 18 hours. After incubation, the diameter of clear zones around the disk was measured in millimeters and recorded as the zones of inhibition and then compared with Clinical and Laboratory Standards Institute (CLSI) standard interpretative charts for their sensitivity, intermediate, or resistance. The *S. aureus* ATCC strain 25923 was used for quality control. The isolates were defined as Multidrug-Resistance (MDR) strains by lack of susceptibility to at least three classes of antibiotics (Akya et al. 2020).

Determination of physicochemical parameters of water

The American Public Health Association (APHA, 5th edition) standard procedures were used to determine several physicochemical properties of hospital wastewater samples that were collected in their raw state. According to Oladipo et al. (2019), these factors include pH, dissolved oxygen, Chemical Oxygen Demand (COD), and Biochemical Oxygen Demand (BOD). Detection of the *mecA* Gene in Multiple Antibiotic Resistance *S. Aureus*.

Genomic DNA extraction

Multidrug-resistance bacterial broth culture of 1.5 mL was taken in the centrifuge tube, centrifuged at 10,000 rpm for 2 minutes, and the supernatant was discarded. To the pellet, 1 mL of distilled water was added, which dissolved the pellet completely. Again, after centrifuging at 10,000 rpm for 2 minutes, the procedure was repeated two times. The supernatant was discarded, and to the pellet, 100 µL of Tris-EDTA buffer was added, which dissolved the pellet completely in the buffer. The supernatants containing the DNA were transferred to another tube and stored at -20°C. The concentration and purity of the extracted DNA were estimated using a Nanodrop spectrophotometer (Model 752) (Natàlia et al. 2019).

PCR amplification of the *mecA* gene in multidrug-resistance *S. aureus*

The *mecA* gene, which in *S. aureus* species encodes for methicillin resistance, was amplified by PCR. For every PCR reaction, the following ingredients were added: 12.5 µL of 2x PCR Master Mix (Thermos Scientific Technologies, Waltham, MA, USA), 50 ng of DNA template, 5 µM of both forward and reverse primers, and 25 µL of nuclease-free water. Table 4 provides specific information on the primers and PCR conditions. The C1000 thermocycler (Bio-Rad, Hercules, USA) was used to amplify DNA. GelRed staining was used to visualize the amplicons after they were electrophoresed on 1% w/v agarose gel under a UV transilluminator.

Sequencing of amplified 16S rRNA Gene

The BigDye terminator V. 3.1 cycle sequencing kit (Applied Biosystems, Warrington, UK) was used to sequence the purified PCR products on an Applied Biosystems/Hitachi 3130 genetic analyzer (Tokyo, Japan). After utilizing Finch TV for inspection, the produced sequence electropherograms were manually modified. MUSCLE, which was integrated into MEGA V. 7.0, was used to perform multiple sequence alignment (Kumar et al. 2017). The Neighbor-Joining tree method was utilized to generate phylogenetic sequence dendrograms from closely related sequences found in GenBank, utilizing the substitution model.

Plasmid DNA extraction and profiling of MRSA

Plasmid DNA extraction was carried out as stated by Zyppy™ Plasmid Miniprep Kit Catalog Nos. D4019's manufacturer's procedure. Overnight growth of bacteria in broth culture was used for the plasmid isolation using Zyppy™ Plasmid Miniprep Kit Catalog Nos. D4019.

A 600 µL of bacterial culture grown in LB medium was added to a 1.5 mL microcentrifuge tube and centrifuged for 30 seconds at 14,000 rpm. The supernatant was discarded, and 100 µL of 7X Lysis Buffer (Blue) 1 was added and mixed by inverting the tube 4-6 times. After the addition of 7X Lysis Buffer, the solution changed from opaque to clear blue, indicating complete lysis. Then 350 µL of cold Neutralization Buffer (Yellow) was added and mixed thoroughly. The sample then turned yellow with a yellowish precipitate. Centrifuge at 11,000-16,000 x g for 2-4 minutes and transfer the supernatant (~900 µL) into the provided Zymo-Spin™ IIN column. The flow-through in the column was discarded after centrifugation for 15 seconds. After which, 200 µL of Endo-Wash Buffer was added and centrifuged for 30 seconds. Elution Buffer 2 was added directly to the column matrix, left for one minute at room temperature, and centrifuged for 30 seconds to elute the plasmid DNA. The extracted plasmid was examined on 0.8% agarose gel, 1 kbp DNA ladder (NEB) was used as control, 1 and 5 µL of loading dye (bromophenol blue) and plasmid DNA respectively were mixed and loaded on solidified agarose gel and 1X TAE buffer was used for the electrophoresis.

Survival of MR *Staphylococcus aureus* Isolates

The survival of MR *S. aureus* subjected to different environmental conditions (pH, temperature, and salt concentration) was examined as described by Marwan et al. (2014). For the influence of pH on the survival of MRSA, each isolate was inoculated into sterile test tubes with 9 mL of Nutrient broth (with the following pH adjusted to 3, 4, 5, 6, 7, 8, 9, 10, and 11) and incubated at 37°C for 24 hours, the influence of temperature on the survival of MRSA was carried out by inoculating the tubes with 9 mL of nutrient broth, and the tubes were incubated at different temperatures (ranging from 4 to 40°C) for 24 hours. Also, salt concentrations ranging from 0 to 30% v/v were prepared in test tubes and inoculated at 37°C for 24 hours to determine the survival of MRSA at different salt concentrations. The microbial growth was observed after 24 hours using a spectrophotometer at an absorbance of 600 nm, and 0.1 mL of each preparation was poured on nutrient agar and incubated at 37°C for 24 hours, after which the colonies were counted.

Statistical analysis of data

Data obtained was expressed as mean ± standard error of mean. The new Duncan Multiple Range test was used to compare means. A p-value of < 0.05 was considered statistically significant

RESULTS AND DISCUSSION

Bacterial counts of wastewater collected from selected hospitals in Akure

Bacterial counts of hospital wastewater are shown in Table 1 (UNIMED) and Table 2 (FUTA). All the wastewater samples were contaminated with bacteria, and the total viable bacterial counts of the tap water (source) used as a control were significantly ($p<0.05$) lower than those in other water sources. All the wastewater samples were also contaminated with *Staphylococcus*, except those from the community clinic, blood bank, and water source at UNIMED that had zero staphylococcal counts. The highest staphylococcal counts were observed in wastewater samples collected from the MLS laboratory (220.14±1.06 CFU/100 mL) and the doctor's station (60.11±0.11 CFU/100 mL) at UNIMED and FUTA, respectively.

Physicochemical parameters of hospital wastewaters

The physicochemical parameters of the wastewaters are shown in Table 5. The pH, DO, COD, and BOD of the wastewaters ranged from 5.31±0.62 (postnatal ward, H) to 8.93±0.74 (Children's ward, N), 2.01±0.02 mg/L (UNIMED Laundry, K) to 8.31±0.11 mg/L (UNIMED Water Source, L), 5.11±0.05 mg/L (UNIMED Water Source, L) to 931.44±5.06 mg/L (UNIMED Laundry, K) and 3.68±0.07 mg/L (FUTA Water Source, T) to 11.73±0.93 mg/L (UNIMED Laundry, K), respectively. The ratio of BOD and COD in some samples was less than 0.03.

Antibiotic susceptibility profiles of *Staphylococcus aureus* isolated from hospital wastewater

Antibiotic susceptibility profiles of all the *S. aureus* isolates are shown in Figures 1 and 2. In wastewater collected from UNIMED (Fiure 1), the isolates from all sample locations were more susceptible to gentamicin (3.06±0.21 to 20.41±0.03 mm), pefloxacin (7.06±0.02 to 20.11±0.01 mm), ciprofloxacin (20.06±0.11 to 25.72±0.04 mm), streptomycin (11.42±0.10 to 20.21±0.22 mm), septrin (10.41±0.04 to 18.21±0.31 mm) and erythromycin (12.01±0.05 to 20.02±0.21 mm). On the other hand, *Staphylococcus aureus* isolates from wastewater collected in FUTA (Figure 2) were more susceptible to ciprofloxacin (18.21±0.10 to 25.03±0.06 mm) and streptomycin (12.03±0.04 to 23.00±0.05 mm), while those from the wound treatment ward showed lesser susceptibility to all the antibiotics tested, and those isolated from water sources were not susceptible to ampiclox, zinnacef, and amoxicillin. Also, those isolated from children's wards and doctor's stations were not susceptible to amoxicillin.

Multiple antibiotic resistant patterns of *Staphylococcus aureus* isolated from wastewater

Multiple antibiotic resistance patterns of isolated *S. aureus* are shown in Table 6. Generally, the *S. aureus* isolates showed varying proportions of resistance to ampiclox (62.50%), amoxicillin (50%), oxacillin (43.75%), rocephin (31.25%), gentamicin (31.25%), pefloxacin (18.75%), erythromycin (12.50%), and septrin (6.25%). The multiple antibiotic resistant index (MARi) of the isolates from FUTA had a MARi of greater than 0.3 except for the water source and laundry While in UNIMED, isolates from the eye clinic, postnatal, and laundry have a MARi greater than 0.

Molecular detection of the *MecA* gene in antibiotic resistant *Staphylococcus aureus* from hospital wastewaters in Akure

Molecular detection of the *mecA* gene in the isolate of *S. aureus* is shown in Figure 3. It was noted that of all thirteen (13) multidrug-resistance isolates examined, six (6) were positive for the *mecA* gene, which was amplified at approximately 300 bp. The isolates were from the eye clinic, postnatal ward, nurses' station, children's ward, doctor's station, and wound treatment ward.

Effects of temperature on MRSA isolated from different wastewater sources in selected hospitals in Akure

The effects of temperature on MRSA from hospital wastewater are shown in Figure 4. All the MRSA isolates were able to survive in the temperature range of 4°C to 40°C except those that were isolated from postnatal ward and survived the temperature range 30°C to 40°C. Generally, there were variations in the staphylococcal counts at different temperatures, with 35°C being the optimum temperature for growth.

Effects of pH on MRSA isolated from different wastewater sources in selected hospitals in Akure

The effects of pH on MRSA isolated from different wastewater sources in selected hospitals in Akure are shown in Figure 5. The result showed that MRSA isolates survived a wide range of pH; the isolates from the children's ward and eye clinic survived in the pH range of 3 to 11, while others survived in the pH range of 4 to 11, with optimum growth pH at 7.0.

Effects of salt concentration on MRSA isolated from different wastewater sources in selected hospitals in Akure

The effects of salt concentration on MRSA isolated from different wastewater sources in selected hospitals in Akure are shown in Figure 6. It was noted that all the MRSA survived the salt concentrations between 0 and 30%; however, the isolates from the eye clinic survived better than other isolates within these ranges of salt concentrations.

Table 1. Bacterial Counts of wastewater collected from UNIMED Teaching Hospital Akure, Nigeria

Wastewater sampling points	Total bacterial counts (cfu/ 100 mL)	Total staphylococcal counts (cfu/100 mL)
Chemical Laboratory	421.08±1.41^{c}	1.65±0.10^{b}
Microbiology Laboratory	873.08±1.32^{f}	10.02±0.43^{c}
Eye Clinic	672.08±1.19^{e}	21.04±0.59^{d}
Community Clinic	471.38±0.55^{d}	0.00±0.00^{a}
Blood bank	743.09±0.08^{e}	0.00±0.00^{a}
MLS Laboratory	743.44±0.60^{e}	220.14±1.06^{g}
Antenatal	721.07±0.42^{e}	20.07±0.21^{d}
Post natal	801.47±1.30^{ef}	39.22±0.62^{e}
Accident and Emergency	877.91±1.55^{ef}	77.61±0.50^{f}
Pharmacy	516.03±0.70^{d}	11.14±0.31^{c}
Laundry	293.06±1.09^{b}	11.06±0.51^{c}
Water source	49.72±0.86^{a}	0.00±0.00^{a}

Note: Values are presented as mean ± standard error, values in the same column carrying the same superscript are not significantly different at $p<0.05$ using the new Duncan Multiple Range test

Table 2. Bacterial counts of wastewater collected from FUTA Health Center Akure, Nigeria

Wastewater sampling points	Total bacterial counts (cfu/ 100 mL)	Total staphylococcal counts (cfu/100 mL)
Nurses' station	81.66±0.08^{b}	7.14±0.08^{c}
Children's ward	94.43±1.31^{c}	17.05±0.50^{d}
Doctor's station	241.58+1.44^{d}	60.11±0.11^{e}
Laundry	62.11±1.58^{a}	15.041±0.55^{d}
Health center entrance	291.17±1.83^{e}	4.53±0.14^{b}
Wound treatment ward	422.05±1.55^{f}	1.23±0.07^{a}
Water source	73.71±0.72^{a}	1.02±0.11^{a}

Note: Values are presented as mean ± standard error, values in the same column carrying the same superscript are not significantly different at $p<0.05$ using the new Duncan Multiple Range test

Table 3. Occurrence of Bacteria in Wastewater from UNIMED Teaching Hospital and FUTA Health Center Akure, Nigeria

Wastewater Sampling Points	*Aeromonas hydrophila*	*Bacillus cereus*	*Bacillus subtilis*	*Citrobacter freundii*	*Enterobacter aerogenes*	*Escherichia coli*	*Klebsiella pneumonia*	*Proteus mirabilis*	*Pseudomonas aeruginosa*	*Salmonella typhi*	*Staphylococcus aureus*
UNIMED											
Chemical Laboratory	-	+	-	-	-	+	-	-	+	-	+
Microbiology Laboratory	-	+	-	+	-	+	-	+	-	+	+
Eye clinic	-	+	+	-	-	+	+	-	-	-	+
Community clinic	-	-	+	+	-	+	+	-	-	-	+
Blood bank	-	-	+	+	-	-	-	-	-	-	-
MLS laboratory	-	+	-	-	+	+	+	-	-	-	+
Antenatal	-	+	+	+	-	+	-	-	-	-	+
Post natal	-	+	-	-	-	+	-	+	+	+	+
Accident and Emergency	-	-	+	-	-	+	-	-	-	-	+
Pharmacy	-	-	-	-	+	-	+	-	-	-	+
Laundry	-	-	-	-	-	+	-	-	-	-	+
Water source	-	-	-	-	-	+	-	-	-	-	-
Total = 47	0(0)	6(12.77)	5(10.64)	4(8.51)	2(4.26)	10(21.28)	4(8.51)	2(4.26)	2(4.26)	2(4.26)	10(21.28)
FUTA Health Center`											
Nurses' station	-	-	-	+	-	+	-	-	-	-	+
Children's ward	-	-	-	-	-	-	-	-	+	-	+
Doctor's station	-	-	-	-	+	-	-	-	-	-	+
Laundry	-	-	-	+	-	-	-	-	-	-	+
Health center entrance	-	+	-	-	+	-	-	-	-	-	-
Wound treatment ward	+	+	+	-	-	-	-	-	-	-	+
Water source	-	-	-	-	-	+	-	-	+	-	+
Total = 18	1(5.56)	2(11.11)	1(5.56)	2(11.11)	2(11.11)	2(11.11)	0(0)	0(0)	2(11.11)	0(0)	6(33.33)

Note: +: Present in the sample, -: Absent in the sample

Table 4. Primers used for the identification of *Staphylococcus* species and the detection of antibiotic resistant marker genes

Primers	Primer Sequence (5′–3′)	PCR Conditions	Size (bp)
Staphylococcus spp.	27F - 5′GAGTTTGATCATGGCTCAG3′ 1492R - 5′GGTTACCTTGTTACGACTT3′	1 cycle of 2min at 95°C; 35 cycles of 30 sec at 94°C; 30sec at 53°C for, 1 min at 72°C; 1 cycle 10 min at 72°C	1500
mecA gene	F - 5′AACGATTGTGACACGATAGCC3′ R - 5′GGGATCATAGCGTCATTATC3	1 cycle of 5min at 94°C; 30 cycles of 30s at 94°C; 30sec at 55oC; 1 min at 72°C; 1 cycle of 10 min at 72°C	527

Table 5. Physicochemical Parameters of Wastewater from UNIMED Teaching Hospital and FUTA Health Center Akure, Nigeria

Sample source	pH	DO (mg/L)	COD (mg/L)	BOD (mg/L)	BOD:COD
A	5.41±0.41^{ab}	5.13±0.43^{b}	280.03±2.32^{d}	8.92±0.82^{b}	0.03
B	7.38±0.31^{b}	6.04±1.83^{bc}	170.11±3.07^{d}	4.86±0.11^{a}	0.03
C	8.42±0.03^{b}	4.02±0.04^{b}	123.31±3.41^{d}	9.07±0.08^{b}	0.07
D	5.53±0.46^{ab}	3.10±0.55^{a}	143.06±1.03^{d}	11.06±0.26^{b}	0.08
E	8.26±0.12^{b}	5.06±0.07^{b}	219.04±5.07^{d}	9.02±0.33^{b}	0.04
F	8.42±0.22^{b}	8.11±0.64^{d}	280.33±4.03^{d}	5.01±0.39^{a}	0.02
G	7.11±0.05^{b}	6.82±1.32^{c}	7.86±0.82^{a}	4.29±0.06^{a}	0.55
H	5.31±0.62^{a}	7.53±0.32^{d}	8.31±1.02^{a}	4.88±0.05^{a}	0.59
I	6.48±0.01^{b}	4.16±0.07^{b}	155.39±4.06^{d}	8.63±0.51^{b}	0.06
J	6.77±0.01^{b}	5.62±0.41^{b}	293.06±2.22^{d}	7.03±0.04^{b}	0.02
K	7.03±0.03^{b}	2.01±0.02^{a}	931.44±5.06^{e}	11.73±0.93^{b}	0.01
L	7.41±0.48^{b}	8.31±0.11^{d}	5.11±0.05^{a}	4.81±0.22^{a}	0.94
M	8.29±1.32^{b}	6.54±0.71^{c}	9.42±0.31^{b}	5.22±1.28^{a}	0.55
N	8.93±0.74^{b}	6.93±0.07^{c}	6.17±0.71^{a}	4.82±0.93^{a}	0.78
O	7.16±0.86^{b}	7.32±0.61^{d}	10.32±0.42^{b}	5.02±0.11^{a}	0.49
P	5.93±0.12^{b}	6.34±0.73^{c}	11.63±1.06^{b}	5.38±0.08^{a}	0.46
Q	9.82±1.33^{c}	2.04±0.61^{a}	34.16±2.65^{b}	14.57±0.55^{c}	0.43
R	6.83±0.06^{b}	5.72±0.54^{b}	43.26±1.32^{bc}	10.31±0.03^{b}	0.24
S	6.59±0.05^{b}	5.32±1.03^{b}	721.05±0.63^{e}	9.42±0.52^{b}	0.01
T	7.31±0.22^{b}	8.22±0.55^{d}	5.32±0.07^{a}	3.68±0.07^{a}	0.69
EPA	7.0 - 8.5	6 - 9.5	3.0 - 900	<5.0	

Note: Values are means ± SE for samples. Values in the same column carrying the same superscript are not significantly different at ($p \leq 0.05$) using the Duncan's New Multiple Range test. A: Chemical Laboratory, B: Microbiology Laboratory C: Eye clinic, D: Community clinic E: Blood bank, F: MLS laboratory, G: Antenatal, H: Post natal, I: Accident and Emergency, J: Pharmacy, K: Laundry (UNIMED), L: Water source (UNIMD), M: Nurses' station, N: Children's ward, O: Doctor's station, P: Oda Road, Q: Laundry (FUTA), R: Health center entrance, S: Wound treatment ward, T: Water source (FUTA), EPA: environmental protection agency Standards. DO: Dissolved Oxygen, COD: Chemical Oxygen Demand, BOD: Biochemical Oxygen Demand

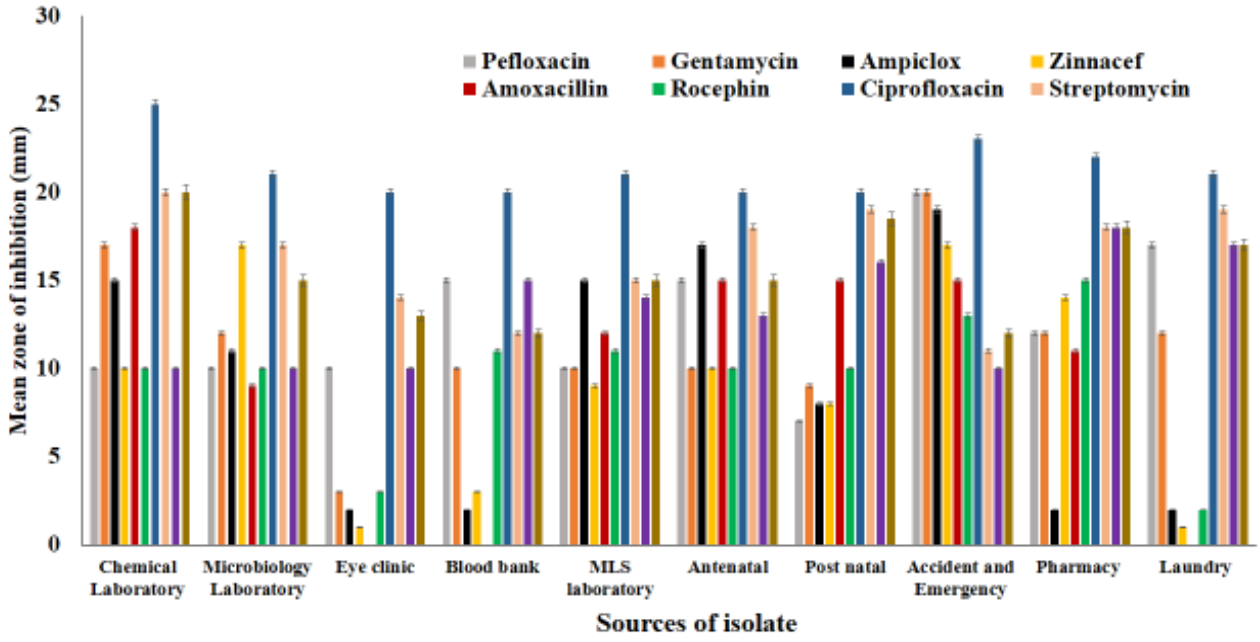

Figure 1. Antibiotic susceptibility profiles of *Staphylococcus aureus* isolated from wastewater in UNIMED Teaching Hospital Akure, Nigeria

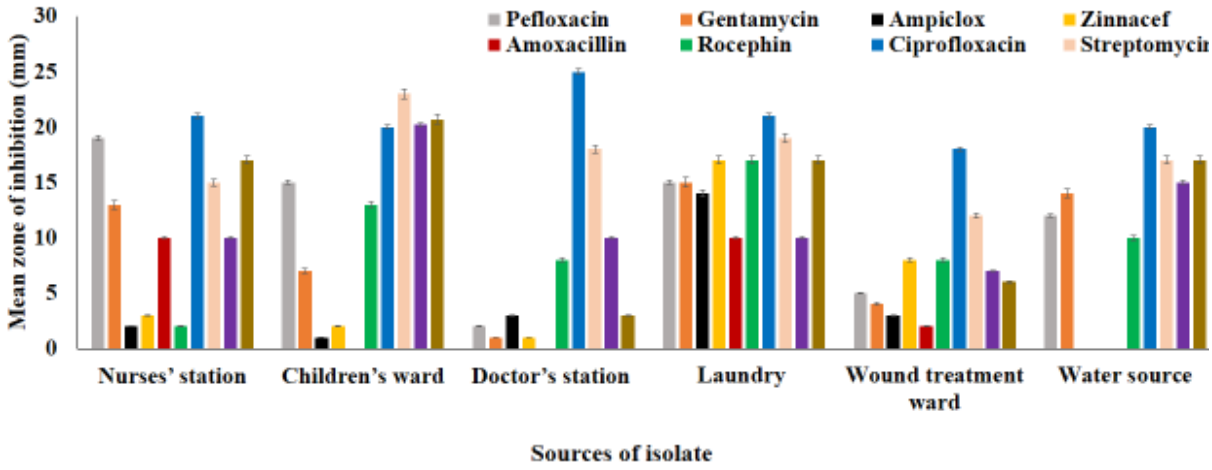

Figure 2. Antibiotic susceptibility profiles of *Staphylococcus aureus* isolated from wastewater in FUTA Health Center Akure, Nigeria

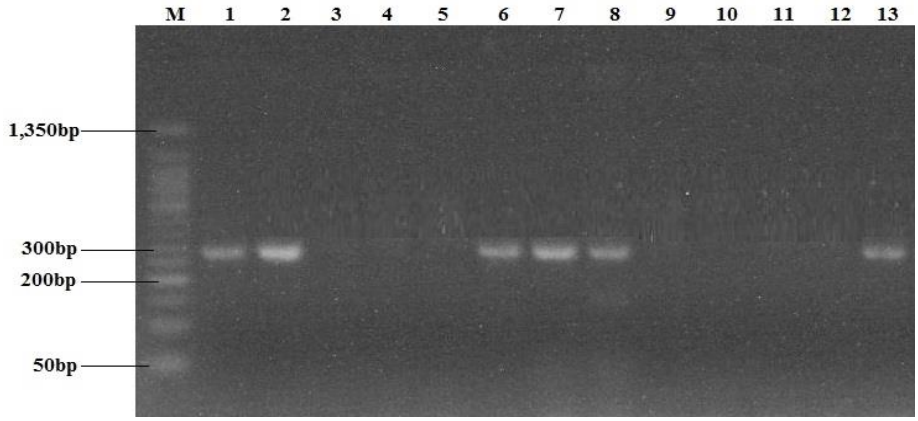

Figure 3. Agarose gel electrophoresis of amplified *MecA* gene (300 bp) in antibiotic resistant *Staphylococus aureus* from hospital wastewaters in Akure, Nigeria. M: 50 bp ladder, Wells 1 (Eye clinic), 2 (Post natal), 6 (Nurses' station), 7 (Children's ward), 8 (Doctor's station) and 13 (Wound treatment ward) showed positive amplification of *MecA* gene

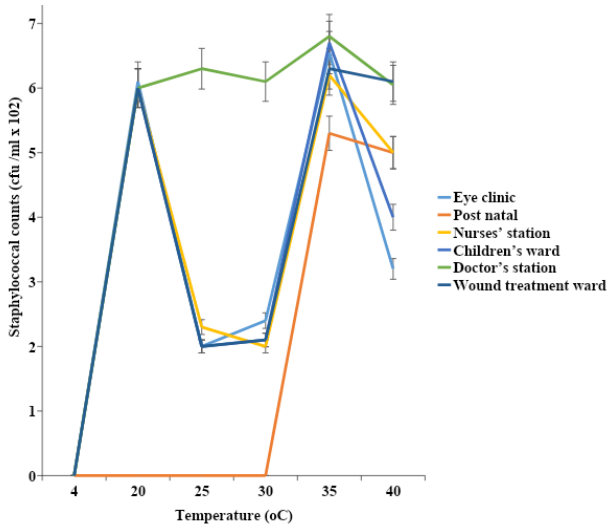

Figure 4. Effects of temperature on MRSA isolated from different wastewater sources in selected hospitals in Akure, Nigeria

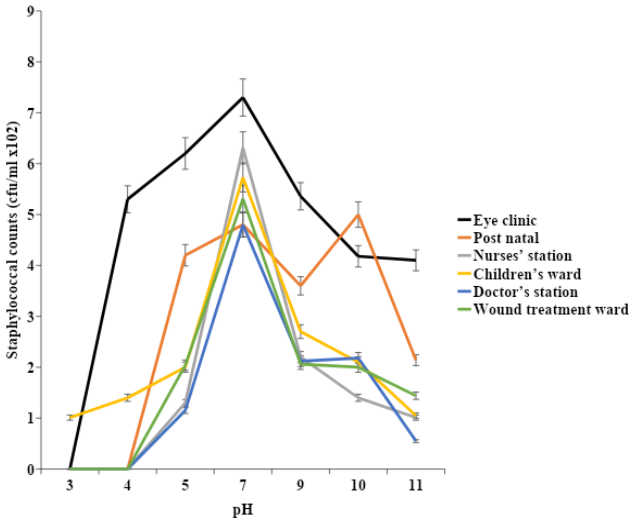

Figure 5. Effects of pH on MRSA isolated from different wastewater sources in selected hospitals in Akure, Nigeria

Agarose gel electrophoresis of plasmids in MRSA isolated from different wastewater sources in selected hospitals in Akure, Nigeria

Agarose gel electrophoresis of plasmids in MRSA isolated from different wastewater sources in selected hospitals in Akure is shown in Figure 7. It was noted that all the MRSA isolates had one (1) to three (3) plasmids of different molecular weights. Plasmids with the highest molecular weight were observed in the children's ward (80 kbp), while those with the lowest molecular weight were observed in the eye clinic (3 kbp).

The plasmid size and post plasmid curing resistance patterns of MRSA isolated from different wastewater sources in selected hospitals in Akure

The plasmid size and post-plasmid curing resistance patterns of MRSA isolated from different wastewater sources in selected hospitals in Akure are shown in Table 7. The number of plasmids did not correlate to the phenotypic antibiotic-resistant patterns; the resistance observed in isolates from the postnatal ward with plasmid size 4.5 kbp was plasmid-mediated; also, after plasmid curing, it was noted that the resistance to oxacillin, gentamicin, ampiclox, amoxicillin, pefloxacin, erythromycin, and septrin by MRSA was plasmid-mediated.

Table 6. Multiple antibiotic resistant patterns of *Staphylococcus aureus* isolated from wastewater in UNIMED Teaching Hospital and FUTA Health Center Akure, Nigeria

Wastewater sampling points	Antibiotics Used											Multiple Antibiotic-Resistant Index
	Oxacillin	Septrin	Erythromycin	Perfloxzcin	Gentamicin	Ampiclox	Zinnacef	Amoxicillin	Rocephin	Ciprofloxacin	Streptomycin	
UNIMED												
Chemical Laboratory	S	I	S	I	S	S	I	S	I	I	S	-
Microbiology Laboratory	I	I	I	I	I	I	I	R	I	S	I	0.10
Eye clinic	R	I	I	I	R	R	R	R	R	S	I	0.55
Blood bank	I	S	S	S	S	R	R	R	S	S	S	0.27
MLS laboratory	I	S	S	I	S	S	R	S	I	S	S	0.10
Antenatal	S	I	S	S	I	S	I	S	I	S	S	-
Post natal	S	S	S	R	R	R	R	I	S	S	S	0.36
Accident and Emergency	S	I	I	S	S	S	S	S	I	S	I	-
Pharmacy	R	S	S	I	I	R	I	I	I	S	S	0.18
Laundry	R	S	S	S	I	R	R	R	R	S	S	0.45
FUTA Health Center												
Nurses' station	R	I	S	S	I	R	R	I	R	S	S	0.36
Children's ward	R	S	S	S	R	R	R	R	S	S	S	0.45
Doctor's station	R	I	R	R	R	R	R	R	R	S	S	0.73
Laundry	S	I	S	S	S	S	S	I	S	S	S	-
Wound treatment ward	R	R	R	R	R	R	R	R	R	S	I	0.82
Water source	I	S	S	I	S	R	R	R	I	S	S	0.27
Percentage resistance	7 (43.75)	1 (6.25)	2 (12.50)	3 (18.75)	5 (31.25)	10 (62.50)	10 (62.50)	8 (50.00)	5 (31.25)	0	0	

Note: R: Resistance, I: Intermediate, S: Susceptible

Table 7. The plasmid size and post plasmid curing resistance patterns of MRSA isolated from different wastewater sources in selected hospitals in Akure, Nigeria

Bacterial strain	Plasmid size (kbp)	Phenotypic resistance patterns	
		Resistance patterns before plasmid curing	Post plasmid curing resistance patterns
Staphylococcus aureus	3, 10, 50	OX GEN AMP AMX Z R	Z R
Staphylococcus aureus	4.5	PFX GEN AMP Z	-
Staphylococcus aureus	25	OX AMP Z R	Z R
Staphylococcus aureus	50, 80	OX GEN AMP Z AMX	GEN Z
Staphylococcus aureus	4	OX ERY PFX GEN AMP Z AMX R	OX AMP AMX R
Staphylococcus aureus	25	OX S ERY PFX GEN AMP Z AMX R	S ERY Z R

Note: 1**.** Eye clinic, 2. Post natal, 3. Nurses' station, 4. Children's ward, 5. Doctor's station, and 6. Wound treatment ward**,** OX: Oxacillin, GEN: Gentamicin, AMP: Ampiclox, AMX: Amoxicillin, R: Rocephin, Z: Zinnacef, PFX: Pefloxacin, ERY: Erythromycin, S: Septrin, kbp: kilobase pair

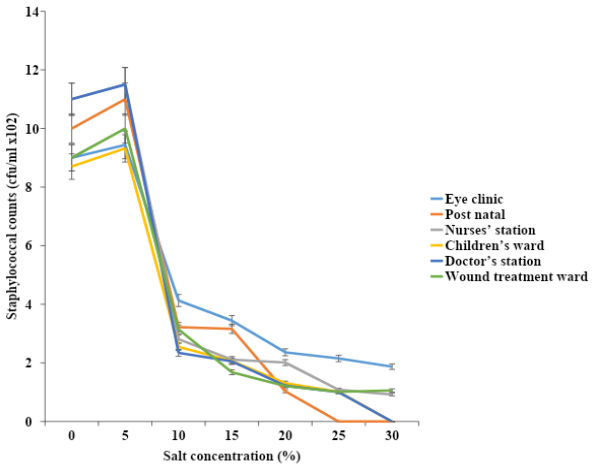

Figure 6. Effects of salt concentration on MRSA isolated from different wastewater sources in selected hospitals in Akure, NIgeria

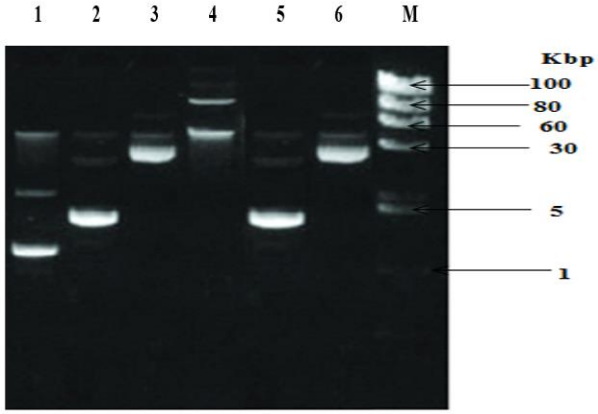

Figure 7. Agarose gel electrophoresis of plasmid in MRSA isolated from different wastewater sources in selected hospitals in Akure, Nigeria. M: 100 bp ladder, Wells 1. Eye clinic, 2. Post natal, 3. Nurses' station, 4. Children's ward, 5. Doctor's station, and 6. Wound treatment ward showed the plasmid

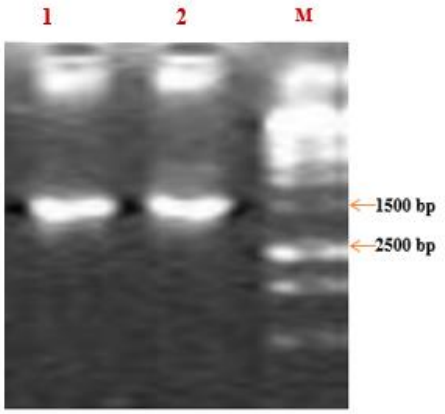

Figure 8. Agarose gel electropherogram of 16S rRNA gene of *Staphylococcus aureus* isolated from wastewater in UNIMED Teaching Hospital and FUTA Health Center Akure, NIgeria. Note: Line 1-2: Bacterial isolates, M: Molecular marker 1kb DNA ladder

Molecular detection of *Staphylococcus aureus* isolated from wastewater in UNIMED Teaching Hospital and FUTA Health Center Akure

The agarose gel electrophoretogram of the 16S rRNA gene of *Staphylococcus aureus* isolated from wastewater at UNIMED teaching hospital and FUTA health center Akure is shown in Figure 8. The 16S rRNA of MRSA was amplified at 1500 bp.

Discussion

To evaluate the risk to human health, it is imperative to ascertain the present state of *S. aureus* in the aquatic environment. Wastewaters, including the tap water used as a control, that was collected in this study from different locations in hospitals were contaminated with bacteria. The presence of bacteria in hospital wastewater was reported in previous research, stating that there is a presence of residual bacteria in hospital wastewater (Rowe et al. 2017; Mehanni et al. 2023). There was a presence of *S. aureus* in hospital wastewater, and higher staphylococcal counts were observed in samples collected from the MLS laboratory and doctor's station at UNIMED and FUTA, respectively. Although the counts were less than what was reported by Rice et al. (2012), who reported bacterial counts of 67 x 10^7 CFU in the untreated wastewater outlet pipe of Beni-Suef University Hospital, The presence of bacteria, especially *S. aureus*, in hospital wastewater justified the statement that hospital wastewaters are hot spots for the dissemination of bacteria that could pose a threat to public health (Rizzo et al. 2013; Yuan and Pia 2023).

Different bacterial species were present in the wastewater examined in this study, *Bacillus subtilis*, *Escherichia coli*, and *S. aureus* dominated in UNIMEDTH wastewater while *S. aureus* dominated the wastewater from FUTA (Table 3) and this could be as a result of the source of the water used at both hospitals.

In this study, the MLS laboratory and doctor's station could be the major hotspots for the dissemination of bacteria into the environment. The differences in microbial diversity of these wastewaters could be due to the degree of pollutant, type of pharmaceutical, chemical disinfectant, hospital general practices, and antibiotic-resistant patterns of the bacteria in the wastewater (Kumar et al. 2017; Azuma et al. 2022). Also, differences in the bacterial composition of wastewater have been reported to vary from region to region and country to country (Adachi et al. 2016; Mackul'ak et al. 2021; Azuma et al. 2022).

The findings of this study support the need for further research by considering different hospital practices and chemical or pharmaceutical pollutants that are present in the wastewater. In most hospitals, the BOD and COD concentrations of wastewater are almost equal to domestic wastewater values. In another study, the averages of BOD and COD in the wastewater of Tehran hospitals were 444.3 mg/L and 792 mg/L, respectively (Tchobanoglous et al. 2004). In this study, the pH, DO, COD, and BOD of the wastewaters ranged from 5.31±0.62 to 8.93±0.74, 2.01±0.02 mg/L to 8.31±0.11 mg/L, 5.11±0.05 mg/L to 931.44±5.06 mg/L and 3.68±0.07 mg/L to 11.73±0.93 mg/L respectively. The ratio of BOD and COD in some

samples was less than 0.03. The lower BOD-to-COD ratio seen in some wastewater samples could be because the sample contains non-biodegradable substances. The high biodegradability of organic matter is very desirable from the viewpoint of wastewater treatment and promotes the efficiency of wastewater treatment plants (Mesdaghinia et al. 2009). Hospital wastewater effluents contain pathogenic microorganisms, partially metabolized pharmaceuticals, radioactive elements, heavy metals, and toxic chemicals (Mehanni et al. 2023), which may have influenced the physicochemical parameters of the wastewater. The *S. aureus* isolates showed varying proportions of resistance to ampiclox, amoxicillin, oxacillin, rocephin, gentamicin, pefloxacin, erythromycin, and septrin. The abundance of antimicrobial-resistant *S. aureus* in hospital wastewater has been reported (Azuma et al. 2022; Mehanni et al. 2023); their resistance to these antibiotics could be because the antibiotics were mostly used in these hospital settings. Antibiotic-Resistant *S. aureus* (ARSA) is classified as a high-priority bacterium in hospital wastewater; however, all ARSA should be screened for the presence of the methicillin-resistant gene (Azuma et al. 2022). Therefore, there is a need for further study to determine if the *S. aureus* isolates are Methicillin-resistant.

The majority of the antibiotic-resistant *S. aureus* isolates from the two hospitals had a Multiple Antibiotic Resistance index (MARi) greater than 0.3. MARi greater than 0.3 has been attributed to the overuse of antibiotics (Sharkir et al. 2021). Therefore, the resistance shown by the *S. aureus* isolates in this study could be a result of the use of antibiotics in hospital environments. In this study, 37.5% of *S. aureus* isolates harbored the *mecA* gene. The presence of *mecA* genes in *S. aureus* indicates the resistance of the isolates to methicillin. A higher presence of MRSA has been reported by Hiramatsu et al. (2002), in wastewater generated from industry, hospitals, and domestic activities, respectively.

In this study, MRSA isolates were able to survive different ranges of temperature, pH, and salt concentrations. The staphylococcal cell membrane is rich in Fatty Acids (FAs) and lipid content, which are essential to its adaptive functions and acclimatization to environmental fluctuations. The survival of MRSA isolates in different environmental conditions is a public health challenge, as this could aid the fast dissemination of the isolates beyond the hospital environment. All the MRSA isolates in this study had plasmids. Possession of a plasmid by *Staphylococcus* could enhance its virulence and antibiotic-resistant ability (Akindolire et al. 2015). Also, resistance to oxacillin, gentamicin, ampiclox, amoxicillin, pefloxacin, erythromycin, and septrin by MRSA was plasmid-mediated, and this could pose an additional serious threat to public health as the plasmid could transfer the resistance to other non-antibiotic-resistant *S. aureus* in the environment.

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