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Detection of rifampicin resistance in methicillin-resistant *Staphylococcus aureus* isolates from the medical centers at Ahmadu Bello University

Maryloveth Oluchukwu Akpudo[1], Dominic Agbo Oche[1,2]✉, Peter Adikwu[2], Augustine Ameh Agbo[2], Cecilia Towobola Atolagbe[1], Olanrewaju Jimoh[3], Busayo Olalekan Olayinka[1]

[1]*Department of Pharmaceutical Microbiology, Faculty of Pharmacy, Ahmadu Bello University Zaria, Kaduna State, Nigeria*

[2]*Department of Microbiology, Faculty of Sciences, Federal University of Health Sciences Otukpo, Benue State, Nigeria*

[3]*Department of Medical Microbiology, Ahmadu Bello University Teaching Hospital, Kaduna State, Nigeria*

ABSTRACT

Objective: The aim of this study was to evaluate the prevalence of rifampicin resistance among methicillin-resistant *Staphylococcus* (*S.*) *aureus* (MRSA) isolates obtained from patients in two health facilities at Ahmadu Bello University, Zaria.

Methods: A total of 200 non-duplicate presumptive Staphylococcal isolates were collected from the health facilities. The isolates were confirmed using mannitol salt agar, and biochemical tests were performed to identify *S. aureus*. The confirmed *S. aureus* isolates underwent antibiotic susceptibility tests, and the results were interpreted according to the guidelines provided by the European Committee on Antimicrobial Susceptibility Testing (EUCAST). Polymerase chain reaction (PCR) was used to investigate virulence and antibiotic resistance determinant, such as *Spa*, *mecA*, and *rpoB*.

Results: This study revealed a *S. aureus* isolation rate of 22 (11.0%), and 18.2% of these *S. aureus* isolates were methicillin-resistant, with the highest prevalence of resistance (9.1%) found in wound swab. The results further showed that 75.0% of the MRSA isolates were resistant to rifampicin. The molecular analysis showed that all the isolates carried the virulence gene (*Spa*), with a prevalence of 10.0% for *mecA* and 20.0% for *rpoB* genes, respectively.

Conclusions: This study underscores the prevalence of rifampicin resistance among MRSA isolates in medical centers, advocating for the development of alternative strategies to combat the current trends in antibiotic resistance and control of rifampicin-resistant MRSA.

KEYWORDS: Methicillin-resistant *S. aureus*; Rifampicin; *Spa*; *mecA*; *rpoB*

1. Introduction

Rifampicin, a first-line drug for treating tuberculosis, has long been believed to improve outcomes in serious *Staphylococcus* (*S.*) *aureus* infections[1]. The study by Scheper *et al.*[2] provided fresh insights into the antimicrobial treatment for patients with Staphylococcal prosthetic joint infection (PJI), establishing that short-term postoperative treatment with rifampicin led to high recovery rates among patients with Staphylococcal hip PJI.

Significance

Methicillin-resistant *Staphylococcus aureus* (MRSA) is a widespread issue in developing countries. Studies have demonstrated the efficacy of rifampicin against MRSA, particularly when used in conjunction with fluoroquinolones. In our study, we examined the effectiveness of rifampicin against MRSA and identified the *rpoB* gene in the rifampicin resistant MRSA isolates. Our study exposed the misdiagnosis of *Staphylococcus aureus* infections and highlighted the risk associated with treating MRSA infections with rifampicin in the area of this study.

✉To whom correspondence may be addressed. E-mail: ochedominic1@gmail.com

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Adjunctive rifampicin remained the standard treatment for *S. aureus* prosthetic valve, endocarditis, and PJI[3]. The most effective combination partners for rifampicin are fluoroquinolones, and the use of rifampicin as monotherapy result in treatment failure, reoccurrence of infection, and the emergence of resistance[4].

Worldwide efforts have been put forth to tackle the growing issue of antibiotic resistance, including a well-designed quantitative system for the surveillance of the spread of antimicrobial resistance, which has proven to be a fundamental strategy in the control of antimicrobial resistance[5]. The prolonged and singular use of rifampicin has been linked to the development of rifampicin resistance in *S. aureus*[1]. The re-emergence of resistance to rifampicin was caused by mutations in the beta-subunit of the RNA polymerase gene *rpoB*, as reported by Albert *et al*., who demonstrated the presence of rifampicin resistance among MRSA isolated from horses and clinic workers in an equine clinic[6]. High-level resistance to rifampicin was attributed to multiple mutations, including H481N, indicating a step-by-step mechanism in resistance development, probably due to exposure to rifampicin therapy[7]. A study by Huang *et al*.[8] revealed that using rifampicin-containing regimens for tuberculosis could eliminate *S. aureus* nasal carriage, potentially extending to MRSA colonizing the nasal cavity. Simultaneously, patients could be colonized with both rifampicin-resistant *S. aureus* and MRSA during treatment. Most mutations in the *rpoB* gene were found within the 81 bp rifampicin-resistance determining region[9]. A study by Akanbi *et al*.[10], recorded 80.0% rifampicin-resistant *S. aureus* and 45.8% expressed *rpoB* gene in the Eastern Cape Province of South Africa. Research in Ghana reported a 7.0% prevalence of rifampicin-resistant *S. aureus* isolated from a hospital[11]. In Nigeria (Kano), Kumuraya *et al*.[12] reported a rifampicin resistance rate 33.3% among MRSA.

Rifampicin exerts its effect by inhibiting bacterial DNA-dependent RNA polymerase β-subunit, encoded by the *rpoB* gene, and 95.0% of rifampicin-resistant mutations are in the *rpoB* gene. Thus, the rifampicin resistance mutation gene (*rpoB*) acquired during rifampicin treatment is accompanied by persistent infection, promoting cross-resistance to vancomycin and daptomycin[13]. The mutation in *rpoB* that emerged *in vivo* after daptomycin treatment was found to result in a variety of phenotypes, including increased cell wall thickness, reduced expression of virulence traits, and induced expression of the slow growth in addition to decreased susceptibility to daptomycin, vancomycin, beta-lactams, and rifampicin[14].

We believe that obtaining antimicrobial resistance data is an essential approach to defining the extent of the resistance problem, developing interventions that improve the appropriate use of the antimicrobial agents, and reducing resistance selection pressure[15]. This work aims to investigate and characterize rifampicin-resistant MRSA isolates taken from two health facilities at Ahmadu Bello University Zaria.

2. Methods

2.1. Scope of the study

For this study, we obtained isolates that were presumptively identified as Staphylococcal (based on morphology and without biochemical tests) from samples submitted to the Medical Microbiology unit of Ahmadu Bello University Medical Center (ABUMC), Samaru, and Ahmadu Bello University Teaching Hospital (ABUTH), Shika, over three months. These isolates were analyzed in the Microbiology Laboratory, Department of Pharmaceutical Microbiology, Ahmadu Bello University, Zaria, Nigeria.

2.2. Ethical consideration

To conduct this research, ethical approval (ABUTHZ/HREC/W35/2021) was obtained from the Ethical Committee of Ahmadu Bello University Teaching Hospital, Zaria for the use of human subjects for research.

2.3. Sample collection

Using a prevalence (P) of 14.6%[16], and the formula (Figure 1)[17], where '*n*' represents the quantity of samples, 'Za' signifies standard normal deviation at 95% confidence interval, equalling 1.96, 'Z1-P' equals 1 – 0.146 and 'L' denotes the permission error of 5% equivalent to 0.05, we calculated our sample size to be 192, which was then rounded up to 200 samples. A total of 200 consecutive and non-duplicated presumptive Staphylococcal isolates were collected from September to December, 2019, using aseptic techniques on Nutrient Agar slant. The isolates were transported to Microbiology Laboratory, Department of Pharmaceutical Microbiology, Faculty of Pharmaceutical Sciences, Ahmadu Bello University, Zaria for identification and confirmation.

$$n = \frac{Z_a^{\,2} \times P \times Z_{1-p}}{L^2}$$

Figure 1. Samples size caculation formula.

2.4. Identification and confirmation of Staphylococcus aureus isolates

All the presumptive Staphylococcal isolates were inoculated on mannitol salt agar (MSA) (Oxoid Ltd., Basingstoke, Hampshire, England) and incubated at 37 ℃ for 24 hours. Colonies with distinct features of small yellow were sub-cultured on Nutrient Agar to obtain pure colonies. Preliminary identification of the isolates was performed to identify and isolate *S. aureus*.

To differentiate the isolates into Gram-positive and Gram-negative isolates, we performed the Gram reaction test on all of them, and the catalase test was further performed on the Gram-positive isolate, according to the protocol of Happy *et al*.[18].

To further differentiate the isolates into coagulase-positive and coagulase-negative isolates, a coagulase enzyme detection test was performed for the Gram-positive and catalase-positive isolates from the preceding tests using the Staphytech Plus Test Kit (Oxoid Ltd., Basingstoke, Hampshire, England) to observe surface antigens of the *S. aureus* isolates. The coagulase-positive Staphylococci were subjected to further biochemical tests using a Microgen Staph ID kit (Oxoid Ltd., Basingstoke, Hampshire, England) that employs 12 standardized biochemical substrates in micro-wells to identify *S. aureus* isolates.

2.5. Determination of antibiotic susceptibility of Staphylococcus aureus isolates

The modified Kirby-Bauer disc diffusion method was used to determine the antibiotic susceptibility pattern of the *S. aureus* isolates to cefoxitin (30 µg) and rifampicin (5 µg), using cefoxitin as a marker for phenotypic expression of methicillin resistance.

Discrete colonies of *S. aureus* isolate from overnight MSA plates were emulsified in 5 mL of sterile physiological saline to the turbidity of 0.5 McFarland standard (approximately a cell density of 1.5×10^8 CFU/mL). The standardized suspension was inoculated on Mueller Hinton agar using a sterile swab stick to spread the inoculum over the surface of the agar medium. The antibiotic discs were aseptically placed using a sterile antibiotic disc dispenser (Oxoid Ltd, Basingstoke, Hampshire, England) on the dried inoculated agar surface, and incubated at 37 ℃ for 18 hours. After this, the diameters of zones of inhibition were measured, recorded, and the results were interpreted according to EUCAST[19].

2.6. Molecular detection of Spa, mecA, and rpoB genes in the Staphylococcus aureus isolates

Molecular detection of virulence and antibiotic-resistant genes, including *Spa*, *mecA*, and *rpoB*, was performed on phenotypical MRSA isolates as well as randomly selected *S. aureus* isolates from the methicillin-susceptible *S. aureus* (MSSA), which served as a negative control for the resistant genes' tests (Table 1).

MRSA isolates that were also resistant to rifampicin were subjected to a polymerase chain reaction (PCR) according to the procedure described by[20] to determine the presence of *Spa*, *mecA*, and *rpoB* genes respectively in the isolates. Pure colonies from an overnight culture of isolate on an MSA plate were inoculated into 5 mL Luria and Bertani broth (Oxoid Ltd., Basingstoke, Hampshire, England) medium and incubated overnight at 37 ℃ for 24 h. The bacterial cells were harvested by centrifugation at 4 ℃, 8000 rpm (6800 x g) in a refrigerated micro-centrifuge for 2 min in an Eppendorf tube. The supernatants were decanted and cells were harvested.

Following the protocol described by Tiisetso Elizabeth Lephoto[20], genomic DNA was extracted, and the targeted genes were amplified using PCR after external optimization of the reaction. For each isolate and every single reaction, thin-walled PCR tubes were marked, and a master mix of 50 µL was prepared as follows: 25 µL of Dream TaqTM PCR master mix was added in the PCR tube, 1.0 µL of forward primer, 1.0 µL of reverse primer, 7.0 µL of template DNA (genomic DNA), and 16 µL of nuclease-free water. The sample was spun down while performing PCR using the thermal cycling conditions stated by Zymo Research UK[20]. After each gene was amplified, the PCR amplicon was isolated on 1.0% agarose gel after complete staining procedure with ethidium bromide and subjected to electrophoresis. The gel was removed from the buffer and observed using a short-wave ultraviolet trans-illuminator.

Table 1. Primers used for the study.

Gene	Primer description	Sequence	Amplicon size (bp)	Reference
rpoB	Forward	5'- TCAATTCGTTCTTTGGAAC -3'	538	[21]
	Reverse	3'- GCATCATGTTAGATCCCAT -5'		
mecA	Forward	5'-AAAATCGATGGTAAAGGTTGGC-3'	533	[22]
	Reverse	3'-AGTTCTGCAGTACCGGATTTGC-5'		
Spa	Forward	5'-TAAAGACGATCCTTCGGTGAGC-3'	180	[23]
	Reverse	3'-CAGCAGTAGTGCCGTTTGCTT-5'		

2.7. Statistical analysis

Statistical analysis was presented as mean ± standard deviation (SD) when necessary, while descriptive statistics were used to analyze the data using Microsoft Excel 2013.

3. Results

3.1. Distribution of samples collected

Two hundred non-duplicated suspected Staphylococcal clinical isolates were collected from the Medical Microbiology Department Laboratory of the two hospitals for this study. Samples obtained were 41 (20.5%) from ABUMC and 159 (79.5%) from ABUTH. While 115 (57.0%) of the samples were collected from female patients, 85 (42.5%) were from male patients. The samples were from different sources which include urine 38 (19.0%), blood 49 (24.5%), sputum 15 (7.5%), urethra 8 (4.0%), wound 38 (19.0%), skin 1 (0.5%), ear swab 6 (3.0%), high vaginal swab 28 (14.0%), endocervical swab 6 (3.0%), throat swab 7 (3.5%), eye swab 1 (0.5%), stool 3 (1.5%).

3.2. Isolation and identification of Staphylococcus aureus

The presumptive Staphylococcal isolates were subjected to biochemical tests for identification, and 22 (11.0%) were confirmed to be *S. aureus* isolates as shown in figure 2. The distribution of the *S. aureus* isolates according to gender and sample source showed that the female and wound samples had the highest prevalence of 63.6% and 54.5%, respectively. Detailed distribution of the *S. aureus* isolates by age and gender are shown in Table 2, while Figure 3 shows the distribution of the *S. aureus* isolates by sample source.

3.3. Detection and distribution of methicillin resistance Staphylococcus aureus

The isolation rate of MRSA is 18.2%. The distribution of the MRSA isolates by sample sources is shown in Table 3.

3.4. Rifampicin resistance among methicillin–resistant Staphylococcus aureus isolates

The antibiotic susceptibility test result for the MRSA isolates to Rifampicin (5 µg) is shown in Table 4, with 3 (75.0%) of the MRSA isolates showing resistance to rifampicin.

3.5. Prevalence of virulence and resistance gene in the Staphylococcus aureus isolates

In the *S. aureus* isolates analyzed, the antimicrobial-resistant genes detected included *rpoB* (20.0%), and *mecA* (10.0%) respectively (Figure 4A and 4B). In terms of the virulence factors, the gene encoding *Spa* was detected across all isolates, with a prevalance rate of 100% (Figure 4C).

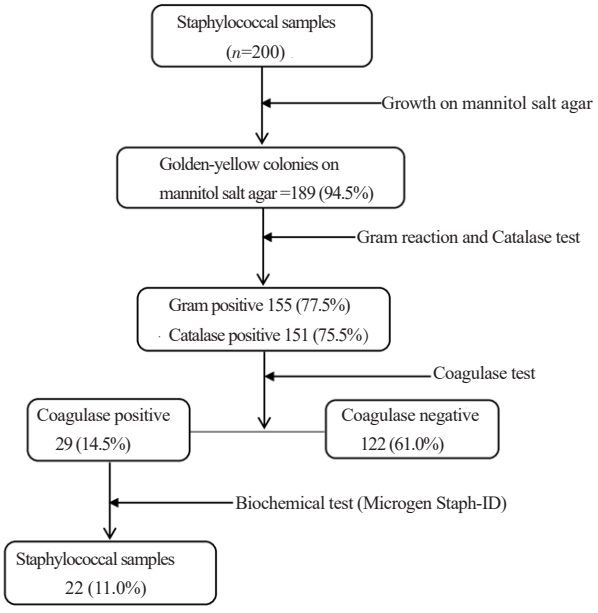

Figure 2. Flow-chart of the isolation and identification process of *Staphylococcus aureus*.

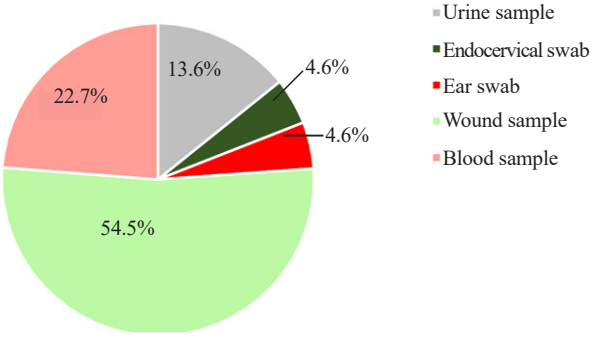

Figure 3. Distribution of *Staphylococcus aureus* isolates according to specimen types.

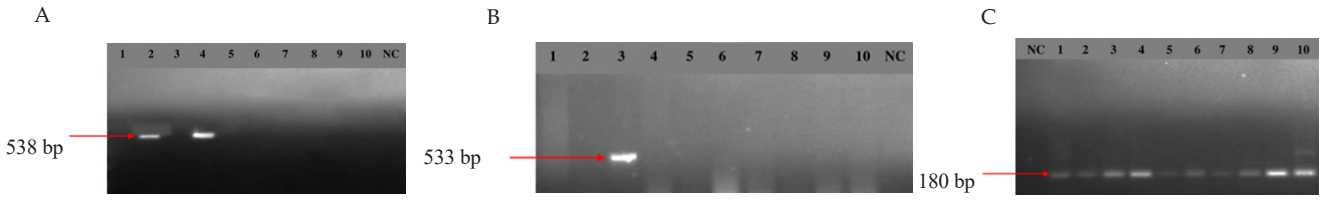

Figure 4. (A) Electrophoretograph of amplified *rpoB gene* in MRSA and *S. aureus* isolates; (B) Electrophoretograph of amplified *mecA gene* in MRSA and *S. aureus* isolates; (C) Electrophoretograph of amplified *Spa gene* in MRSA and *S. aureus* isolates.

Note: NC: Negative control; bp: Base pair.

Table 2. Percentage distribution of *S. aureus* isolates among age-group and gender [*n* (%)].

Age (years)	Male	Female	All samples
≤ 17	4 (18.2)	4 (18.2)	8 (36.4)
18 – 40	4 (18.2)	8 (36.4)	12 (54.5)
≥ 41	0 (0)	2 (9.1)	2 (9.1)
Total	8 (36.4)	14 (63.6)	22 (100)

Table 3. Percentage distribution of methicillin-resistant and methicillin-susceptible *S. aureus* isolates [*n* (%)].

Sample type	MRSA	MSSA
Urine	0	3 (13.6)
Endocervical swab	0	1 (4.5)
Wound	2 (9.1)	10 (45.5)
Blood	1 (4.5)	4 (18.2)
Ear swab	1 (4.5)	0
Total	4 (18.2)	18 (81.8)

Table 4. Distribution of rifampicin resistance methicillin-resistant *S. aureus* isolates [*n* (%)].

Sample type	Resistance	Susceptibility
Urine	0	0
Endocervical swab	0	0
Wound	2 (50)	0
Blood	1 (25)	0
Ear swab	0	1 (25)
Total	3 (75)	1 (25)

4. Discussion

This study identified a *S. aureus* prevalence of 11.0% (*n*=22). This is significant as *S. aureus* known to cause numerous life-threatening infections, leading to extended hospital stays and the use of antibiotics. Furthermore, *S. aureus* has been associated with increased morbidity, mortality, and health care costs[24]. The prevalence of *S. aureus* identifiedin this study was lower than the 18.6% *S. aureus* prevalence reported by Oche *et al*.[25]. Although these studies were conducted in different states in the Northwestern part of Nigeria, they both showed high isolation rates (50.0% and 46.1%, respectively) of *S. aureus* from wound samples. This may be attributed to the hemolytic nature of *S. aureus*[26]. Therefore, they were found in wounds where there was a supply of blood.

S. aureus isolates often exhibit resistance to frequently prescribed antibiotics. This study revealed a prevalence rate of 18.2% for phenotypically determined MRSA. Previous clinical studies have reported a 13.0% MRSA isolation rate among hospital workers in Pakistan[27], and a significantly higher rate of 67.9% in Kano-Nigeria[28].The high percentage of resistance to cefoxitin recorded in this study may be attributed to one or more of the following reasons: 1) misdiagnosis, which could have resulted in the misuse and/or overuse of antibiotics by both healthcare personnel and patients in the study area without any clinical indication; 2) poor surveillance of drug-resistant infections; 3) the easy availability of antibiotics[29]. Hospital-associated risk factors such as prolonged hospital stays, immunocompromised patients, hospital settings and equipment, and continuous or excessive antibiotic use[30] may also play a role. These factors could lead to treatment failure, extended illnesses, increased healthcare costs, and in severe cases, increased mortality risk. However, the *S. aureus* isolates in this study showed above-average susceptibility to cefoxitin (81.8%), suggesting that methicillin

resistance among the isolates is relatively low. Therefore, cefoxitin may still be effective and could be utilized in treating *S. aureus* infections in this region.

The antibiotic rifampicin is a clinically reserved antibiotic used as a last-line drug for the treatment of multi-drug resistant infections including those caused by *M. tuberculosis*, *S. aureus* and MRSA[31]. However, resistance to rifampicin by isolates derived from the clinical specimens is rapidly increasing. In this study, in addition to methicillin resistance among the *S. aureus* isolates, 3 (75.0%) of the MRSA isolates were phenotypically resistant to rifampicin, leaving only 1 MRSA isolates (25.0%) as susceptible to rifampicin. The rifampicin resistance reserved in this study, along with others such as the 33.3% rifampicin resistance reported among MRSA clinical isolates from Kano[12] and the 2017 Malaysian National Surveillance on Antimicrobial Resistance showing a 3.9% prevalence of rifampicin resistance among MRSA isolates, underscores the global challenge of rifampicin resistance. This result was similar to the prevalence of 20.0% reported in Hospital Universiti Sains Malaysia (HUSM) from 2002 to 2007[32]. These study outcomes necessitate global attention, as the bactericidal efficacy of rifampicin, which are attributed to its ability to inhibit the DNA-dependent RNA polymerase transcription initiation complex, is being compromised by MRSA.

From the PCR done on the isolates, three genes including *Spa*, *mecA*, and *rpoB* were tested in the 4 MRSA and 6 randomly selected MSSA isolates, making ten *S. aureus* isolates. The *Spa* gene encodes a surface coat protein known as protein A, which is preserved among *S. aureus* as a virulence factor and has polymorphism properties that can be used for strain typing[33]. The surface IgG-binding protein A (*Spa*) is a virulence factor of *S. aureus*, which is typically and purposefully made to seize the FC region of immunoglobulin of the bacterial cells with the host immune system[34]. In this study, the *Spa* gene was detected in all the tested *S. aureus*, indicating the strong resistance against the host cell's natural immune response. This agrees with the findings of Al-Muhanna *et al*.[34] which reported a high prevalence of the *Spa* gene (63.3%) among *S. aureus* clinical isolates.

Methicillin resistance is conferred by the *mecA* or *mecC* gene, which is positioned on the Staphylococcal chromosomal cassette (SCCmec) and encodes penicillin-binding protein 2A (PBP2A) [35]. In this study, the *mecA* gene was detected in 1 (10.0%) of the 10 tested *S. aureus* isolates, and it turns out that the isolate possessing the *mecA* gene was also phenotypically identified as MRSA. Among the 10 isolates tested for the presence of *mecA*, 4 were phenotypically MRSA isolates, while 6 others were randomly selected MSSA isolates. Therefore, the methicillin resistance observed in the other 3 phenotypic MRSA isolates may be due to other factors that were not tested in this study. The study reported that 10.0% of the MRSA possess the *mecA* gene, and this result of the presence of *mecA* in this study is contrary to the findings of Okiki *et al*.[36], in which they reported that 30.0% of their MRSA isolates possessed the *mecA* gene.

The resistance of *S. aureus* to rifampicin is accompanied by alterations in the main region (cluster I and II) of the *rpoB* gene that encodes the beta subunit of RNA polymerase in the rifampicin resistance determining region[37]. Among the 10 *S. aureus* isolates analyzed for the *rpoB* gene in this study, 3 were phenotypically resistant to rifampicin and belonged to the phenotypical MRSA, while 7 were susceptible to rifampicin phenotypically, including 1 phenotypic MRSA and the randomly selected 6 MSSA isolates. The *rpoB* gene was detected in 2 (20.0%) of the *S. aureus* isolates tested, and these 2 isolates were phenotypically resistant to rifampicin and were also among the phenotypic MRSA isolates. However, 1 of the *rpoB* gene was detected in a phenotypical MRSA isolate that does not possess the *mecA* gene. Studies across the world have reported the presence of the *rpoB* gene in clinical isolates[38, 39], indicating that rifampicin resistance is becoming not only a national healthcare challenge in Nigeria but also a global challenge in the healthcare system.

5. Conclusions

Although most of the *S. aureus* isolates in this study were susceptible to cefoxitin (*i.e.* methicillin-susceptible), the majority of the MRSA isolates reported in this study exhibited high resistance to rifampicin, which is known as a last resort antibiotic against severe infection, including *S. aureus* infections. The detection of *Spa*, *mecA*, and *rpoB* genes in the isolates is concerning, especially given the unexpected trend in antibiotic resistance suggested by the presence of the *rpoB* gene in the phenotypically MRSA isolates. This situation necessitates urgent attention and enhanced surveillance of antibiotic resistance. Consequently, it is recommended to develop new antibiotics to combat the current trends of antibiotic resistance.

Conflict of interest statement

The authors declare that there is no conflict of interest.

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This study received no extramural funding.

Data Availability Statement

The data supporting the findings of this study are available from the corresponding author upon request.

Authors' contributions

Akpudo MO and Olayinka BO conceptualized and designed the experiment. Akpudo MO and Oche DA analyzed, interpreted the data. Oche DA wrote the first draft. All authors contributed to the series of reviewing, editing of the manuscript drafts and final approved version.

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