Molecular Detection of Macrolide-Induced Clindamycin Resistance Among Clinical Isolates of Staphylococcus aureus from Selected Hospitals in Katsina Metropolis


INTRODUCTION

Macrolides, including erythromycin, clarithromycin, and azithromycin, lincosamides such as clindamycin, and streptogramin B antibiotics like quinupristin, belong to a distinct group of antibiotics that possess unique structures but share a similar mechanism of action. These antibiotics bind to the 23S ribosomal RNA within the 50S ribosomal subunit, thereby impeding the process of protein synthesis (Moosavian et al., 2014; Heyar et al., 2020). Clindamycin, in particular, offers several advantages. It is available in both parenteral and oral formulations, has high bioavailability and good permeability in the skin and soft tissues, inhibits toxin production, is cost-effective, and has fewer side effects (Baral and Khanal, 2017; Spížek and Řezanka, 2017; Heyar et al., 2020). As a result, clindamycin is the most commonly prescribed antibiotic among the MLSB group (Moosavian et al., 2014; Khoshnood et al., 2019). Unfortunately, the improper use and inappropriate utilization of MLSB antibiotics have contributed to the proliferation of MLSB-resistant strains of S. aureus. This misuse has created selective pressure, allowing for the survival and spread of bacteria that possess mechanisms to evade the effects of these antibiotics. As a result, the effectiveness of MLSB antibiotics in treating S. aureus infections has been compromised (Harkins et al., 2017; Heyar et al., 2020). Mechanisms of MLSB...
resistance include the activity of macrolide efflux pumps, modifications of the target site, and enzymatic inactivation of the antibiotics (Leclercq, 2002). The major resistance mechanism involves modifications or mutations in the ribosomal target, preventing the binding of the antibiotic. The erythromycin ribosome methylases (erm) gene, primarily ermA and ermC, in S. aureus, is responsible for mediating this mechanism (Leclercq, 2002; Cetin et al., 2010).

Macrolides, lincosamides, and streptogramin B resistance can manifest in two primary forms: inducible and constitutive, which are not dependent on the type of erm gene (Leclercq, 2002; Moosavian et al., 2014). Constitutive MLSB-resistant (cMLSb) phenotypes continuously produce functional methylase mRNA regardless of the presence of an inducer (Saderi et al., 2011; Medugu et al., 2021). In contrast, inducible MLSB-resistant (iMLSb) phenotypes produce non-functional mRNA that does not encode methylase under normal conditions but can become activated in the presence of an inducing agent.

These resistance phenotypes pose a challenge in clinical use, as they may not be detected by routine susceptibility testing methods, leading to treatment failure and prolonged illness in affected patients. Standard disk diffusion antibiotic susceptibility testing effectively detects constitutive MLSB-resistant phenotypes exhibiting resistance to erythromycin and clindamycin. However, to identify iMLSB-resistant phenotypes, D-test is employed. The D-test, also known as the Double-disk diffusion test, serves as a method for identifying macrolide-inducible clindamycin resistance. By performing this simple test, healthcare providers can determine the appropriate treatment options for infections caused by S. aureus (CLSI, 2023).

Understanding the prevalence and mechanisms of inducible clindamycin resistance in S. aureus infections is crucial for guiding treatment and infection control measures. Limited data exist on this resistance in Katsina Metropolis, Nigeria, emphasizing the need for local epidemiological insights.

This study assesses the prevalence and characterizes the phenotypic and genotypic aspects of inducible clindamycin resistance among clinical isolates of S. aureus obtained from Katsina Metropolis.

MATERIALS AND METHODS

The study was conducted in three hospitals in the Katsina metropolis: General Hospital Katsina, General Amadi Rimi Specialist Hospital, and Turai Umar Musa Yar’adua Women and Children Specialist Hospital. The study utilized a convenient sampling method, where S. aureus previously isolated from diverse clinical specimens like urogenital swabs, aspirates, urine, blood, and sputum were collected.

The sample size for this study was determined using the following formulae, considering a previous prevalence reported by (Medugu et al., 2021) conducted in Nigeria:

\[ n = \frac{Z^2pq}{d^2} \]

Where:

- \( n \) = Sample size
- \( Z \) = Standard normal deviate at 95% (1.96) \[ Z = 3.8416 \]
- \( p \) = Prevalence, which is 7.0% \[ p = 0.07 \]
- \( q \) = Complement of \( p \) (1 - 0.07 = 0.93) \[ q = 0.07 \]
- \( d \) = Precision 5% (0.05) \[ d = 0.05 \]

\[ n = \frac{3.8416 \times 0.07 \times 0.93}{0.05^2} = 0.0025 \]

In accordance with the dictates of ethics in medical research, approval for the research was sought from the Katsina State Ministry of Health (MOH/ADM/SUB/1152/1/530). Each isolate was inoculated onto quadrant plates of blood agar and mannitol salt agar and incubated at 37°C for 24 hours. S. aureus colonies on Mannitol salt agar showed a yellow halo, indicating mannitol fermentation, while on blood agar, colonies appeared greyish or golden yellow, ranged in size from medium to large (0.5-1.5 µm), and exhibited smooth texture, well-defined edges, and slightly raised elevation. Most colonies were pigmented creamy yellow and displayed beta-hemolysis. All predominant S. aureus isolates were further identified in accordance with Bannerman, 2003.

Gram staining was performed on recently cultured isolates to maintain staining capacity. Air-dried fixed smears were stained using the Gram staining technique. A catalase test was also conducted on colonies grown on Mannitol Salt Agar (MSA) by preparing a clean slide with 3% hydrogen peroxide. Subsequently, a coagulase production test was performed, starting with the rapid slide coagulase test. If clumping occurred, no further investigations
were required; however, if the result was negative, further testing with the tube coagulase test was conducted to confirm coagulase activity, considering the limitations of the slide coagulase test.

The susceptibility tests were conducted in accordance with CLSI (2023) guidelines, utilizing a turbidity standard of barium sulfate (BaSO₄) equivalent to a 0.5 McFarland standard to ensure consistent inoculum density. For isolates showing resistance to erythromycin and sensitivity to clindamycin, the D-test was performed to detect the iMLSB phenotype. This involved placing 15μg erythromycin and 2μg clindamycin disks on the agar surface, with a 15-26 mm distance between them. After incubation at 35°C for 18 hours, a flattened zone of inhibition adjacent to the erythromycin disk (D-zone) indicated inducible clindamycin resistance and a positive test.

**Polymerase Chain Reaction**

The genomic DNA of bacterial isolates was extracted using the Phenol-Chloroform DNA extraction method as described by Javadi et al. in 2014. Primers were adopted from Ghanbari et al. (2016), and Adeiza et al. (2019) were obtained from Inqaba Biotechnical Industries (Pty) Ltd. The primers’ characteristics are shown in Table 1. For each isolate, three separate PCR reactions were performed using the BIO-RAD IQTM5 Multicolour Real-Time PCR Detection System, with 1μL of each forward and reverse primer, 15μL of master mix, 4μL of molecular-grade water, and 4μL of DNA template. Negative controls (no template controls) and positive controls from known (erm positive) S. aureus isolates were included.

**Table 1: Primers used in Polymerase Chain Reaction for Amplification of erm genes**

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence (5'→3')</th>
<th>Product (bp)</th>
<th>Temp. (°C)</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>ermA F</td>
<td>GCCTGACTTTCAAGGTAATTC</td>
<td>249</td>
<td>57.1</td>
<td>Ghanbari et al., 2016; Adeiza et al., 2019</td>
</tr>
<tr>
<td>R</td>
<td>TCGGATCCAGAAAAGGACAT</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>ermB F</td>
<td>GCCATGCGTCTGACATCTAT</td>
<td>192</td>
<td>58.7</td>
<td>-</td>
</tr>
<tr>
<td>R</td>
<td>CTGTGCGTATGGCGGTAAATG</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>ermC F</td>
<td>ATCTTTGAAATCGGCTCAGG</td>
<td>294</td>
<td>59.3</td>
<td>-</td>
</tr>
<tr>
<td>R</td>
<td>CAAACCCGTATTCCACGATT</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

Following the PCR amplifications, a 5μl aliquot of each PCR product was extracted and prepared for agarose gel electrophoresis. A 1% agarose gel, prepared in 1× Tris-borate-EDTA (TBE) buffer, was used for the electrophoresis. The gel was run at 100V for approximately 40 minutes. Midori Green Advance dye (Nippon Genetics) was added to the gel to facilitate the visualization of DNA bands during electrophoresis. To estimate the sizes of the PCR products, a 100-bp standard DNA ladder designed by Bioneer, which was ready-to-use, was employed as a size reference. The PCR products, along with the DNA ladder, were loaded onto the agarose gel for electrophoresis. Following electrophoresis, the gel was visualized using a gel documentation system, specifically the BIO-RAD Gel Doc 2000, allowing for the visualization and documentation of the DNA bands. The approximate sizes of the amplicons were determined by comparing the migration of the PCR products with the known sizes of the ladder bands.

**RESULTS**

During the 6 months study, 100 distinct clinical isolates of S. aureus were collected. Following standard bacteriological techniques, 49 (49%) were confirmed to be S. aureus. Table 2 illustrates the antibiogram profile of the utilized S. aureus isolates. Chloramphenicol, clindamycin, tetracycline, and gentamycin demonstrated the most effective outcomes among the tested antibiotics, with resistance recorded in only 8.2%, 6.1%, 30.6%, and 28.6% of cases, respectively (Figure 1). All bacterial isolates resistant to cefoxitin also exhibited resistance to erythromycin.

The findings from the PCR analysis, as presented in Table 3, indicate that only the ermC gene was identified among the three samples analyzed, as depicted in Figure 2. Conversely, the ermA and ermB genes were undetected in any isolates.
Table 2: Antibiogram Pattern of S. aureus Isolates

<table>
<thead>
<tr>
<th>Antibiotics</th>
<th>Contents (μg)</th>
<th>No. (%) of isolates and Susceptibility pattern</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Sensitive</td>
</tr>
<tr>
<td>Clindamycin</td>
<td>2</td>
<td>39 (79.6)</td>
</tr>
<tr>
<td>Erythromycin</td>
<td>15</td>
<td>23 (46.9)</td>
</tr>
<tr>
<td>Gentamicin</td>
<td>10</td>
<td>29 (59.2)</td>
</tr>
<tr>
<td>Tetracycline</td>
<td>30</td>
<td>34 (69.4)</td>
</tr>
<tr>
<td>Trimethoprim-sulfamethoxazole</td>
<td>1.25/23.75</td>
<td>15 (30.6)</td>
</tr>
<tr>
<td>Ciprofloxacin</td>
<td>5</td>
<td>25 (51.0)</td>
</tr>
<tr>
<td>Chloramphenicol</td>
<td>30</td>
<td>43 (87.8)</td>
</tr>
<tr>
<td>Azithromycin</td>
<td>15</td>
<td>33 (67.3)</td>
</tr>
<tr>
<td>Cefoxitin</td>
<td>30</td>
<td>27 (55.1)</td>
</tr>
</tbody>
</table>

Table 3: Distribution of erm Genes Expression among the iMLS \(_g\) Phenotypes

<table>
<thead>
<tr>
<th>Isolates</th>
<th>D-Test</th>
<th>ermA</th>
<th>ermB</th>
<th>ermC</th>
</tr>
</thead>
<tbody>
<tr>
<td>03</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>08</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>12</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>+</td>
</tr>
</tbody>
</table>

Key: (+) = detected, and (-) = not detected
DISCUSSION
The antimicrobial susceptibility test revealed a moderate level of resistance among the isolates, notably to erythromycin (30.6%), cefoxitin (44.9%), ciprofloxacin (42.9%), trimethoprim-sulfamethoxazole (40.8%), and tetracycline (30.6%). These drugs, readily available and often misused empirically, are commonly used in Nigeria for treating various infections such as the common cold, typhoid fever, peptic ulcer, and sexually transmitted infections. The high resistance observed may indicate prior exposure of the isolates to these antibiotics, potentially contributing to the development of resistance mechanisms.

The isolates displayed the highest resistance to cefoxitin, commonly used as a phenotypic screening test for methicillin-resistant S. aureus (MRSA). This suggests that all isolates resistant to cefoxitin are likely MRSA, as cefoxitin resistance is a reliable indicator of methicillin resistance (CLSI, 2023). The presence of MRSA emphasizes the severity of antimicrobial resistance in the tested population, which is to be expected given that MRSA often carries additional drug-resistance genes, as documented in previous studies (Li et al., 2018; Gan et al., 2021).

Chloramphenicol has a reported resistance rate of 14.2% in Nigeria, according to a study by Medugu et al. (2021). However, compared to other antibiotics, chloramphenicol still maintains a relatively low resistance rate in this study, with only 8.2%. Despite its availability and cost-effectiveness, physicians in Nigeria do not commonly prescribe chloramphenicol due to concerns about potential side effects, such as the risk of inducing aplastic anaemia (Wolters et al., 2020). These concerns may contribute to the reduced use and abuse of chloramphenicol in clinical practice.

The findings indicate that the ermC gene was the predominant gene detected among all the inducible macrolide-lincosamide-streptogramin B (iMLSB) isolates. This observation is consistent with a study conducted in Punjab (Heyar et al., 2020), as well as other studies that have also reported ermC as the most prevalent gene (Ghanbari et al., 2016; Mahfouz et al., 2023). However, it is worth noting that some studies...
have reported ermA as the most prevalent gene instead (Cetin et al., 2010; Saderi et al., 2011; Khoshnood et al., 2019). The variation in the frequency of erm resistance genes observed in different studies can be attributed to two possible factors: the complex and diverse nature of erythromycin resistance and the possibility of small plasmids carrying erm genes being lost during bacterial replication or under the selective pressure of antibiotics. Even though the erm genes were amplified in our study, it’s important to note that no additional molecular analysis or DNA sequencing of the amplicons was done.

CONCLUSION
Based on the available data, the study represents the first investigation of the frequency of erm genes among clinical isolates of S. aureus in Katsina. Detecting the erm gene further substantiates the sufficiency of the D-test in monitoring and assessing potential clindamycin treatment failures. However, chloramphenicol showed the highest efficacy against the S. aureus isolates.

RECOMMENDATIONS
The erm genes amplified should be further sequenced to determine their specific genetic sequences. Additionally, we suggest examining species other than S. aureus for the presence of the erm gene in future research studies.

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