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Exploration of Erythromycin Ribosomal Methylase Genotypes Among D+ Methicillin-resistant *Staphylococcus aureus* Strains in Sokoto, Nigeria

Sokoto, Nijerya'da D+ Metisiline Dirençli *Staphylococcus aureus* Kökenlerinde Eritromisin Ribozomal Metilaz Genotiplerinin Araştırılması

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Abstract

Introduction: Antibiotics are lifesaving compounds that have been successful for decades. However, many pathogenic bacteria are becoming resistant to them. Cross-resistance of the macrolide-lincosamide-streptogramin B (MLSB) antibiotic classes is a major cause of increased morbidity. This study appraises the phenotypic and genotypic distribution of inducible clindamycin resistance among methicillin-resistant *Staphylococcus aureus* (MRSA) isolates.

Materials and Methods: Erythromycin-induced resistance to clindamycin antibiotics among MRSA isolates was verified phenotypically using the Double-disk diffusion test (D-test) and genotypically by the polymerase chain reaction.

Results: All MRSA isolates were resistant to erythromycin. The prevalence of iMLSB (iMLSB: inducible macrolide-lincosamide-streptogramin B) phenotype was 23.7% (9/38), macrolide streptogramin (MS) phenotype 47.4% (18/38), and cMLSB (cMLS: constitutive macrolide-lincosamide-streptogramin) phenotype 28.9% (11/38) of the isolates. The nine isolates with the iMLSB phenotype were tested for the presence of the erythromycin ribosomal methylase (*erm*) gene. The *ermA* gene was detected in five (55.6%) isolates, the *ermB* gene in two (22.2%) isolates, and the *ermC* gene in two (22.2%) isolates.

Conclusion: The *erm*-positive isolates expressed the iMLSB phenotype, and the *ermA* gene was predominant. We showed that the cMLSB phenotype was prevalent among the MRSA isolates, signifying the possibility of achieving a good therapeutic outcome when clindamycin is used. The observed distribution of the *erm* gene explored here gives credence to the adequacy of the D-test in monitoring and testing for potential clindamycin treatment failures.

Keywords: ORSA, cefoxitine-resistant *Staphylococcus aureus*, linezolid, molecular epidemiology, oxacillin-resistant *Staphylococcus aureus*

Öz

Giriş: Antibiyotikler, onlarca yıldır hayat kurtaran önemli bileşiklerdir. Ancak pek çok patojenik bakteri antibiyotiklere direnç kazanmaktadır. Makrolid-linkozamid-streptogramin B (MLSB) antibiyotik sınıflarında çapraz direnç morbidite artışına neden olabilmektedir. Bu çalışmada, metisilin-dirençli *Staphylococcus aureus* (MRSA) izolatlarında indüklenebilir klindamisin direncinin fenotipik ve genotipik dağılımı değerlendirilmektedir.

Gereç ve Yöntem: MRSA kökenlerinde eritromisin ile indüklenen klindamisin direnci, fenotipik olarak D-testi yoluyla ve genotipik olarak polimeraz zincir reaksiyonu ile doğrulandı.

Bulgular: Tüm MRSA izolatları eritromisine dirençliydi. İzolatlarda indüklenebilir iMLSB fenotipi (iMLSB: indüklenebilir makrolid, linkozamid streptogramin B) prevalansı %23,7 (9/38), makrolid streptogramin (MS) fenotipi prevalansı %47,4 (18/38) ve cMLSB (cMLSB: yapısal (constitutive)

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makrolid linkozamid streptogramin B) fenotipi prevalansı %28,9 (11/38) idi. iMLSB fenotipi gösteren dokuz köken, *erm* genleri açısından test edildi. *ermA* geni 5 (%55,6) izolatta, *ermB* geni 2 (%22,2) izolatta ve *ermC* geni 2 (%22,2) izolatta tespit edildi.
Sonuç: *erm* pozitif izolatlar iMLSB fenotipi göstermekteydi ve *ermA* geni baskındı. MRSA kökenlerinde cMLSB fenotipinin yaygın olduğunu ve klindamisin ile tedavide başarı sağlanabileceğini gösterdik. Bu çalışmada araştırılan *erm* genlerinin dağılımı, klindamisin tedavisinin başarısız olduğu olguların izlenmesinde D-testinin yeterliliğini göstermektedir.
Anahtar Kelimeler: ORSA, sefoksitin dirençli *Staphylococcus aureus*, linezolid, moleküler epidemiyoloji, oksasiline dirençli *Staphylococcus aureus*

Introduction

Antibiotics are lifesaving compounds that have been successful for decades. However, almost all pathogenic bacteria are becoming resistant to them[1]. The frequency of infections due to antibiotic-resistant bacterial strains is on the rise[2]. *Staphylococcus aureus* is among the most prevalent nosocomial pathogens[3]. There is documentation on the treatment of methicillin-resistant *Staphylococcus aureus* (MRSA) skin infections with macrolide-lincosamide-streptogramin B (MLSB) antibiotics[4]. Sometimes resistance to MRSA therapy with erythromycin (macrolide) may extend to clindamycin (lincosamide) and streptogramin B drug classes. Earlier literature reported induced resistance with erythromycin ribosomal methylase (*erm*) via the modification of the common drug binding sites of the three drug classes on the *23S rRNA*[5]. The *erm* gene codes for the enzyme that alters the binding site for MLSB antibiotics causing cross-resistance to multiple antibiotic classes and thus increases the risk of treatment failure[6]. The *ermA* gene is located on transposon Tn554/Tn6133, *ermB* on Tn917/Tn551, and *ermC* on smaller plasmids[7]. There are ten different *erm (A, B, C, F, G, Q, T, Y,* 33 and 43) genotypes proficient with erythromycin resistance (ER) but the *erm A, B,* and *C* genotypes confer resistance to erythromycin in 94% to 98% of ER staphylococcal strains[7,8]. MLSB resistance can be inducible (erythromycin-resistant and clindamycin susceptible) or constitutive (resistant to all MLSB antibiotics)[9]. The Double-disk diffusion test (D-test) is used to detect erythromycin-inducible clindamycin resistance. Improper screening for clindamycin resistance in iMLSB MRSA phenotypes increases the risk of treatment failure, which may result in increased morbidity and mortality[10]. This study appraises the phenotypic and genotypic distribution of inducible clindamycin resistance among MRSA isolates.

Materials and Methods

This study was carried out in Sokoto State, at the extreme north-west of Nigeria, between longitudes 4°8[1] and 6°54[1] and latitude 12°N and 13°58[1]N. The population of the state is about 5.4 million[11]. It shares a border with the Republic of Niger and covers a land area of 32,000 km^2. The Hausa and Fulani ethnic groups are the principal inhabitants of the state[11].

During the six-month study, from February to July 2018, 38 phenotypically confirmed MRSA strains that were isolated from the nasal swabs of consenting participants (eight inpatients, 11 outpatients, and 19 healthcare workers) after receipt of ethical approval from the review board of Sokoto State ministry of health under approval number SMH/1580/V.IV. Resistance to methicillin by the isolates was confirmed by the oxacillin resistance screening agar base test[12,13].

Double-disk diffusion/approximation test (D-test) was used to determine resistant phenotypes of the MRSA isolates to MLSB as specified by the Clinical and Laboratory Standards Institute[14]; erythromycin (15 µg) and clindamycin (2 µg) disks (Oxoid™, Basingstoke, UK) were placed side by side at a distance of 12 mm on a pre-inoculated (0.5 McFarland standard) Mueller Hinton agar plates. The plates were incubated at 35 °C for 16 to 18 hours. Isolates that were resistant to erythromycin, clindamycin sensitive, and formed a D-shaped zone of inhibition around clindamycin were noted as inducible phenotypes of MLS resistance (iMLS). Furthermore, the presence of the constitutive type of MLS resistance (cMLSB) was evidenced by isolates that showed resistance to erythromycin and clindamycin[15]. Finally, MRSA isolates that displayed resistance to erythromycin and sensitivity to clindamycin with no D-shaped zone were considered to have the MS (macrolide streptogramin) phenotype[16].

The standard disk diffusion method was used to determine the resistant profiles of D+ isolates according to the criteria of the Clinical Laboratory Standard Institute[14] using WHONET 2018 software. The antibiotics used were clindamycin (2 µg), erythromycin (15 µg), ceftazidime (30 µg), trimethoprim/sulfamethoxazole (1.25/23.75 µg), linezolid (30 µg), tetracycline (30 µg), cefoxitin (30 µg), and levofloxacin (5 µg) (Oxoid™, Basingstoke, UK).

The genomic DNA of bacterial isolates was extracted using Qiagen™ Kit (Hilden, Germany) as described by the manufacturer[17]. The primer sequences used in this study were calculated using Primer3Plus®[18] on a matrix of oligonucleotide sequences obtained from GenBank/NCBI and sent for production (Inqaba Biotec™, South Africa). The primer sequences used are shown in Table 1.

The products of polymerase chain reaction (PCR) amplification were electrophoresed on a 1.5% agarose gel pre-stained with ethidium bromide and visualized in a Bio-Rad™ gel (Bio-rad laboratories, Milan, Italy) documentation device using an UV trans-illuminator.

A multiplex PCR assay targeting the *erm* (inducible clindamycin resistance) gene was performed under the following conditions: The PCR cocktail contained 1 µl of each primer pair (3 µl), 3 µl molecular grade water, 4 µl of DNA template, 12.5 µl of Qiagen master mix, and 2.5 µl of Q-reagent totaling a 25 µl reaction mixture. The PCR process (Applied BioSystems 9700 thermocycler) was started with an initial denaturation step (94 °C for 5 min) after which it was trailed by another denaturation process (for 30 cycles at 94 °C for 30 s), followed by an annealing step (55 °C for 30 s), an extension step (72 °C for 30 s), and a final extension (72 °C for 7 min)[19].

Statistical Analysis

Statistical analysis was conducted on the results obtained using SAS® software, version 9.4 (SAS Institute Inc., Cary, North Carolina). Descriptive statistics were performed on the presence or absence of *erm A, B,* and *C* genes.

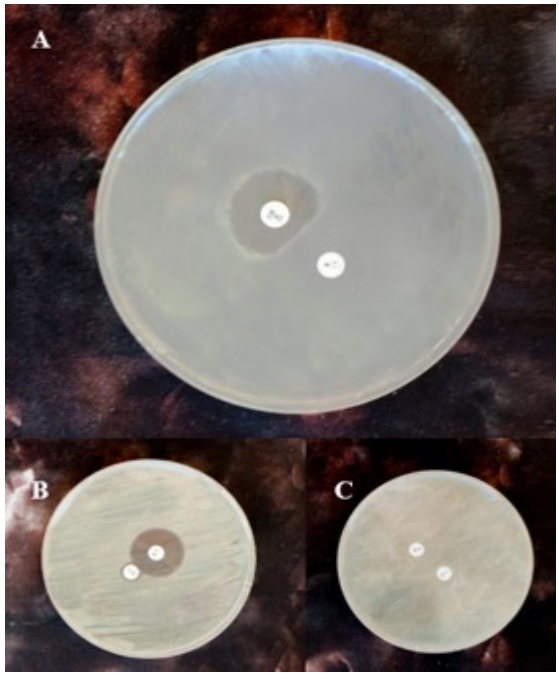

Figure 1. Mueller Hinton agar plates demonstrating (A) inducible macrolide-lincosamide-streptogramin B phenotype, (B) macrolide streptogramin-MS phenotype, and (C) constitutive macrolide-lincosamide-streptogramin phenotype

Results

In this study, MRSA isolates showed 100% resistance to cefoxitin, ceftazidime, and erythromycin. They were also resistant to tetracycline (84.2%), levofloxacin (55.3%), clindamycin (52.6%), linezolid (44.7%), and trimethoprim/sulfamethoxazole (39.5%).

Figure 1 depicts the D-test demonstrating: (A) The formation of the D-zone around clindamycin due to erythromycin resistance, (B) the MS phenotype, and (C) the cMLSB phenotype. All MRSA isolates were resistant to erythromycin. The prevalence of the iMLSB phenotype (Figure 2A) was 23.7% (9/38), the MS phenotype 47.4% (18/38), and the cMLSB phenotype 28.9% (11/38) among the study isolates. Nine isolates with the iMLSB phenotype were tested for the presence of the *erm* gene (Figures 2B and 3). The *ermA* gene was detected in five (55.6%) isolates, the *ermB* gene in two (22.2%) isolates, and the *ermC* gene in two (22.2%) isolates.

Figure 4 displays the resistance profiles of iMLSB positive isolates. The isolates were profiled as being predominantly resistant to

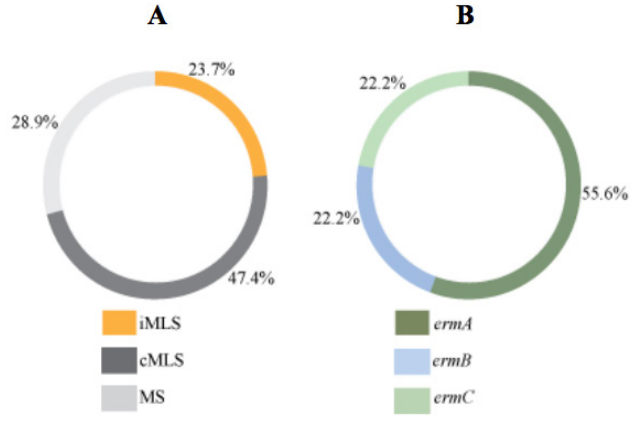

Figure 2. Percentage distribution of (A) clindamycin resistance phenotypes and (B) genotypes among methicillin-resistant *Staphylococcus aureus* isolates

iMLS: inducible macrolide-lincosamide-streptogramin B phenotype, MS:macrolide streptogramin phenotype cMLS: constitutive macrolide-lincosamide-streptogramin phenotype *erm*: erythromycin resistance methylase

Table 1. Nucleotide sequences of primer used in multiplex polymerase chain reaction of *erm* genotypes

Primer		Sequence (5'-->3')	Product	Temp (°C)	Accession
ermA	F	GCCTGACTTTCAAAGGTAATTC	421bp	57.1 °C	AF466412
	R	TCGGATCAGGAAAAGGACAT	-	-	-
ermB	F	GCCATGCGTCTGACATCTAT	359bp	58.7 °C	KP823590
	R	CTGTGGTATGGCGGGTAAGT	-	-	-
ermC	F	ATCTTTGAAATCGGCTCAGG	572bp	59.3 °C	AF466407
	R	CAAACCCGTATTCCACGATT	-	-	-

erm: erythromycin resistance methylase

clindamycin, erythromycin, cefoxitin, ceftazidime, tetracycline, and levofloxacin. However, 40% (2/5) of isolates with *ermA* genotypes were linezolid-resistant phenotypically. Resistance to trimethoprim/sulfamethoxazole was also evidenced in only 2/9 isolates.

Discussion

The present study showed that erythromycin, cefoxitin, and ceftazidime were the antimicrobial agents with the highest antibiotic resistance rates. This finding is similar to reports by others[13,20,21]. Erythromycin is an inexpensive and available antimicrobial agent in Nigeria[15]. Its prophylactic and therapeutic use in healthcare and community settings may establish a persistent selective pressure favoring the evolution of resistant strains[22]. Further, widespread availability of antibiotics favors mutational events that confer resistance with little or no fitness cost like compensatory mutations, genetic co-selection, genetic plasticity, and others[22,23].

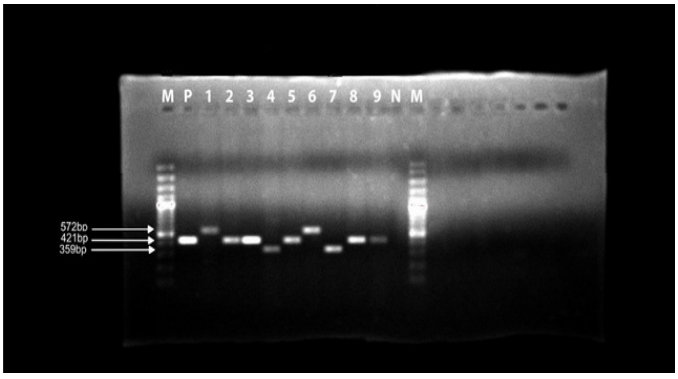

Figure 3. Electrophoretogram of multiplex polymerase chain reaction for erythromycin resistance methylase *(erm)* genes for nine inducible macrolide-lincosamide-streptogramin B (iMLSB) positive isolates. In the Figure: *ermA* positive (421 bp), *ermB* positive (359 bp), *ermC* positive (572 bp). M is 100 bp+ DNA ladder, P and N positive and negative controls. Lane 1 and 13=100 bp+ DNA ladder, Lane 2=*S. aureus* ATCC 25923 (positive control), Lane 3-11=MLSB positive isolates, and Lane 12=negative control (nuclease-free water)

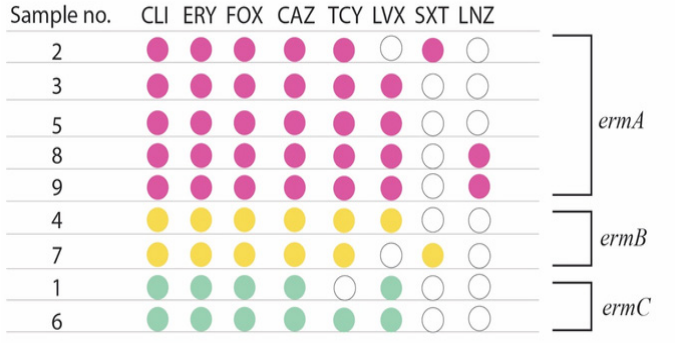

Figure 4. Resistance profile of inducible macrolide-lincosamide-streptogramin B positive isolates and their corresponding genotypes. The antibiotic resistance profiles for the *erm* genotypes are displayed in pink (*ermA*), yellow (*ermB*), and green (*ermC*).

CLI: Clindamycin, CAZ: Ceftazidime, SXT: Trimethoprim/sulfamethoxazole, LNZ: Linezolid, CAZ: Cefoxitin (30 µg), LVX: Levofloxacin, TCY: Tetracycline, ERY: Erythromycin, *erm*: erythromycin resistance methylase

Clindamycin is a bacteriostatic antimicrobial with good tissue penetration. Nevertheless, reports of the rise in MRSA resistance has limited its clinical application. Experts use clindamycin monotherapy in the management of mild to moderate MRSA infections[24] or in combination therapy with rifampicin[24], vancomycin[25], and ceftaroline[26] when the induction of resistance is suspected. Our study revealed that the prevalence of inducible clindamycin resistance among 38 MRSA isolates was 23.7%, which is less than the 28.6% (2/7 isolates) and 75% (3/4 isolates) extracted from the data buried within the source files[27,15]. Another study[28] in Iran reported a lower rate of iMLSB of 10.7% (9/84). The MS phenotype of 28.9% found in this study is also higher compared with the 14.3% reported by others[27]. We found the constitutive (cMLSB) resistance phenotype in this study to be 47.9%, which is higher than the 35.7% (30/84 isolates) reported by Goudarzi et al.[28], and the 28.6% (2/7 isolates) reported by Gadekar et al.[27]. Our result is not surprising, as many studies have associated a large percentage of cMLSB with MRSA isolates than MSSA isolates[29]. The majority of the MRSA isolates in this study were D-test negative, suggesting that if a large percentage (47.9%) of our tested isolates were tested with clindamycin, then treatment would be effective. The large difference in resistance rates observed in this study might be because of various factors, including differences in sample populations, phenotypic methods, geographical distributions, and the diversity of the circulating MRSA strains[30].

Similar with other studies[31], the *ermA* gene was the most prevalent (55.6%) among iMLSB isolates in the current study. None of the studied MRSA strains harbored the double *erm* gene. The differences in *ermA* prevalence relative to other studies may be because of geographic inconsistency among the iMLSB resistance phenotypes[19].

The MLSB profile observed in this study may have occurred due to the modification (methylation) of the common drug binding sites of the three drug classes on the *23S rRNA* encoded by the *erm* gene[32]. This resistance mechanism provokes drug binding to translational attenuator sequences upstream, which subsequently leads to a change in the mRNA secondary structure. It is also possible for spontaneous mutations to occur and transform iMLSB strains to the cMLSB phenotype without a genetic inducer[20].

All nine D+ MRSA isolates in this study were multidrug-resistant (MDR) besides being methicillin-resistant, indicating

the presence of other genes not evaluated in this study. The documented MDR profile may have arisen because *erm* is a multidrug resistance gene that mediates co-resistance to multiple classes of antibiotics[33]. Based on this premise, the *erm*-carrying plasmid may recombine or integrate with another multi-resistance plasmid like the tetracycline resistance gene (*tetK*) carrying plasmid resulting in plasmid modifications that may broaden the MDR spectrum when expressed[7].

A major limitation of this study was that we did not perform further molecular analysis and DNA sequencing of the amplicons to verify the relationship between the resistance mechanisms and the antibiotic susceptibility test results (e.g., linezolid resistance) further by comparative genome analysis.

Conclusion

In our study, the *erm*-positive isolates expressed the iMLSB phenotype, and the *ermA* gene was predominant. We showed that the cMLSB phenotype was prevalent among the MRSA isolates, signifying the possibility of achieving a good therapeutic outcome when clindamycin is used. The observed distribution of the *erm* gene explored here gives credence to the adequacy of the D-test in monitoring and testing for potential clindamycin treatment failures.

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Ethics

Ethics Committee Approval: The study protocol was approved by the Ethical Review Board of Sokoto State Ministry of Health (reference number: SMH/1580/V.IV.).

Informed Consent: Informed consent was obtained from each study participant after assurance of anonymity.

Peer-review: Externally and internally peer-reviewed.

Authorship Contributions

Concept: J.A.O., B.O.O., Design: J.A.O., B.O.O., S.S.A., Data Collection or Processing: S.S.A., Analysis or Interpretation: S.S.A., Literature Search: S.S.A., Writing: S.S.A., J.A.O., B.O.O.

Conflict of Interest: No conflict of interest was declared by the authors.

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