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RESEARCH ARTICLE/KLİNİK ÇALIŞMA

FLORA 2020;25(2):190-196 • doi: 10.5578/flora.68683

Phenotypic and Molecular Detection of Macrolide Lincosamide Streptogramin B Resistance in Clinical Isolates of Staphylococci

Klinik Örneklerden İzole Edilen Stafilokoklarda Makrolid Linkozamid Streptogramin B Direncinin Fenotipik ve Genotipik Olarak Araştırılması

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Cite this article as: *Uyar Güleç G, Öncü S, Bozdoğan B, Öztürk B, Ertuğrul B, Sakarya S. Phenotypic and molecular detection of macrolide lincosamide streptogramin B resistance in clinical isolates of staphylococci. FLORA 2020;25(2):190-6.*

ABSTRACT

Introduction: *Macrolide, lincosamide, and streptogramin B (MLS_B) antibiotics are commonly used in the treatment of staphylococcal infections. Some genes like erm, msr, and lnu confer resistance to these group antibiotics. Resistance phenotype can be one of the constitutive, inducible or efflux phenotypes. Resistance rates have been reported at varying rates from various countries. This study aimed to detect MLS_B resistance rates, phenotypes, and genotypes in clinical isolates of both Staphylococcus aureus and coagulase-negative staphylococci (CNS).*

Materials and Methods: *A total of 82 staphylococci strains comprising 65.8% (n= 54) S. aureus and 34.2% (n= 28) CNS were identified by conventional methods and 16S rRNA polymerase chain reaction (PCR). Antibiotic susceptibility testing for erythromycin, lincomycin, gentamicin, fusidic acid, levofloxacin, vancomycin, and linezolid was performed by minimum inhibitory concentration (MIC) agar dilution method. Double-disc diffusion test (D-test) was applied to investigate MLS_B resistance phenotypes. In erythromycin-resistant isolates, PCR was used to detect the presence of ermA, ermB, ermC, and msrA genes.*

Results: *Among 54 S. aureus strains, 51% (n= 28) were methicillin-resistant and 49% (n= 26) were methicillin-susceptible. Among 28 CNS, 50% (n= 14) were methicillin-resistant. Erythromycin, lincomycin, gentamicin, and levofloxacin resistance rates were higher in the methicillin-resistant group ($p< 0.05$). Erythromycin resistance rate was 54.9%. Rates of constitutive and inducible MLS_B and MS_B phenotypes were 30.5%, 18.3%, and 6.1%, respectively. The most prevalent resistance determinants were ermA (32.9%) and ermC (7.3%). In addition, gene combinations were detected.*

Conclusion: *Due to the wide geographic distribution of resistance phenotypes and genotypes, local statistics are of critical value for empiric therapy. Double-disc diffusion test is useful to guide interpretation of the susceptibility test.*

Key Words: *D-test; erm genes; MLS_B resistance; Staphylococci*

Received/Geliş Tarihi: 24/01/2019 - Accepted/Kabul Ediliş Tarihi: 17/09/2019

Available Online Date: 22.06.2020

ÖZ

Klinik Örneklerden İzole Edilen Stafilokoklarda Makrolid Linkozamid Streptogramin B Direncinin Fenotipik ve Genotipik Olarak Araştırılması

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Giriş: *Makrolid, linkozamid ve streptogramin B (MLS_B) grubu antibiyotikler stafilokokal infeksiyonların tedavisinde sıklıkla kullanılmaktadır. erm, msr, lnu gibi bazı genler bu grup antibiyotik direncinden sorumlu genlerdir. Direnç yapısal, indüklenebilir ya da efluks fenotipinde olabilir. Direnç oranları farklı ülkelerden farklı oranlarda bildirilmektedir. Bu çalışmada, klinik örneklerden izole edilen Staphylococcus aureus ve koagülaz-negatif stafilokok (KNS) suşlarında MLS_B direnç oranlarını, direncin fenotipik ve genotipik özelliklerini saptamayı amaçladık.*

Materyal ve Metod: *Toplam 82 stafilokok suşunun konvansiyonel yöntemler ve 16S rRNA polimeraz zincir reaksiyonu (PCR) yöntemi kullanılarak %65.8 (n= 54)'i S. aureus, %34.2 (n= 28)'si KNS olarak tanımlandı. Eritromisin, linkomisin, gentamisin, fusidik asit, levofloksasin, vankomisin ve linezolid için antibiyotik duyarlılıkları minimum inhibitör konsantrasyonu (MİK) agar dilüsyon yöntemi ile belirlendi. MLS_B direnç fenotiplerini belirlemek için çift disk testi (D-test) uygulandı. Eritromisine dirençli suşlarda ermA, ermB, ermC ve msrA genlerini saptamak için PCR kullanıldı.*

Bulgular: *Elli dört S. aureus suşunun %51 (n= 28)'i metisiline dirençli, %49 (n= 26)'u metisiline duyarlı idi. Yirmi sekiz KNS suşunun %50 (n= 14)'si de metisiline dirençli bulunmuştur. Eritromisin, linkomisin, gentamisin ve levofloksasin direnç oranları metisiline dirençli grupta daha yüksek tespit edilmiştir ($p< 0.05$). Eritromisin direnç oranı %54.9 olarak saptanmıştır. Yapısal, indüklenebilir ve efluks fenotip oranları %30.5, %18.3 ve %6.1 olarak bulunmuştur. En sık saptanan direnç genleri ermA (%32.9) ve ermC (%7.3) olmuştur. Ayrıca gen kombinasyonları da saptanmıştır.*

Sonuç: *Direnç fenotip ve genotipindeki geniş coğrafik dağılımdan dolayı ampirik tedavi seçimlerinde yerel istatistiklerin bilinmesi kritik değerdedir. Duyarlılık testinin yorumlanmasında çift disk difüzyon testi yol göstericidir.*

Anahtar Kelimeler: *D-test; erm genleri; MLS_B direnci; Stafilokok*

INTRODUCTION

Macrolide, lincosamide and streptogramin B (MLS_B) antibiotics are structurally distinct but affect protein synthesis by similar mechanisms[1]. During the elongation phase, they stimulate dissociation of the peptidyl-tRNA from the ribosomes which results in reversible inhibition of protein synthesis[2].

Staphylococcal infections in commmunity and hospital-settings are common. Although newer antibiotics have been developed, clindamycin with its excellent pharmacokinetic properties provides a good treatment option for methicillin-susceptible as well as resistant staphylococci[1]. Additionally, clindamycin is an alternative for penicillin-allergic patients[3].

In staphylococci, resistance to MLS_B antibiotics can occur one of the target site modification, efflux or drug inactivation mechanisms[4]. *erm* genes encode methylases that cause conformational modification of 23S rRNA and as a consequence of methylation binding of MLS_B antibiotics to their target is impaired[5]. Efflux which is controlled by the *msr*A gene (conferring resistance to macrolides and streptogramin B, MSB phenotype) and inactivation of lincosamides by a chemical modification which is mediated by *lnu*A gene are other mechanisms[6].

Expression of MLS_B resistance can be inducible ($iMLS_B$) or constitutive ($cMLS_B$)[7]. In $iMLS_B$ resistance, methylase is produced in the presence of an inducing agent, which causes resistance to 14-membered and 15-membered-ring macrolides. However, staphylococci, with an $iMLS_B$ resistance phenotype are susceptible to lincosamides, streptogramin B, and 16-membered macrolides[6].

Mutations in the 5' upstream sequences of the *erm* gene lead to continuous producing of the methylase, and bacteria are resistant to all macrolides, lincosamides and streptogramin B[6,8].

A double-disk test called D-test is performed for distinguishing inducible *erm* mediated resistance from *msr*A mediated efflux mechanism[7]. Erythromycin is an effective inducer. Erythromycin and clindamycin disks are placed on an agar plate. Erythromycin resistance and a flattening of the clindamycin inhibition zone proximal to the erythromycin disk produce letter D shape inhibition zone. This is considered a positive D-test, inducible MLS_B resistance. Both erythromycin and clindamycin resistant phenotypes are called constitutive MLS_B resistance. Erythromycin resistant but clindamycin susceptible isolates are considered to have an efflux phenotype (MS_B)[5,8,9].

Four major *erm* genes are detected in pathogenic microorganisms: *erm*A, *erm*B, *erm*C, and *erm*F, *erm*A and *erm*C are typical staphylococcal gene classes[4].

Since there was no previous study in our province, we aimed to investigate the macrolide resistance rates, the MLS_B resistance phenotypes and the genes responsible for resistance in staphylococci isolated in our hospital by polymerase chain reaction (PCR).

MATERIALS and METHODS

Bacterial Isolates

Staphylococci, which were accepted as pathogenic and isolated from various clinical specimens (wound, catheter, respiratory secretion, pus, intraabdominal, joint and pleural fluids), were collected from August 2008 to July 2009 in our Infectious Diseases and Clinical Microbiology laboratory. One isolate from each patient was used in the study. A total of 82 staphylococci were identified by conventional methods as *Staphylococcus aureus* and coagulase-negative staphylococci (CNS). All isolates were identified to the species level using 16s rRNA sequencing.

Antibiotic Susceptibility Test

Antibiotic susceptibility testing for erythromycin and lincomycin was performed by the minimum inhibitory concentration (MIC) agar dilution method. Also, gentamicin, fusidic acid, levofloxacin, vancomycin and linezolid susceptibilities were determined by the same method. Methicillin resistance was determined by disk diffusion method with cefoxitin. According to the Clinical and Laboratory Standards Institute (CLSI) breakpoints, resistance zone diameter was < 24 mm for CNS other than *Staphylococcus lugdunensis* and < 21 mm for *S. aureus* and *S. lugdunensis*[10]. Quality control was performed with *S. aureus* ATCC 25923.

Identification of MLS_B Phenotype by D-test

MLS_B resistance phenotypes were investigated using the D-test. A 0.5 McFarland suspension was prepared in sterile distilled water for each isolate and inoculated on the blood agar plate. 2 µg clindamycin and 15 µg erythromycin disks were placed 15-20 mm apart. Plates were read after 18 h of incubation at 35°C. Flattening of the clindamycin zone of inhibition proximal to the erythromycin disk indicated an inducible type, while resistance to both erythromycin and clindamycin indicated a constitutive MLS_B resistance. In erythromycin-resistant and clindamycin susceptible isolates, lack of a D shaped zone was considered as an MS_B efflux phenotype.

Detection of Resistance Mechanism in Erythromycin-Resistant Strains by PCR

In erythromycin-resistant isolates, PCR was used to detect the presence of *erm*A, *erm*B, *erm*C and *msr*A genes. DNAs were extracted from samples using the InstaGene matrix (Bio-Rad®) according to the manufacturer's protocol. Colonies from overnight grown plates were suspended in 100 µL of sterile distilled water and centrifugated at 10000-12000 rpm for 1 min. The supernatant was carefully removed and the pellet was suspended in 200 µL of InstaGene matrix and vortexed, followed by heating at 56°C for 15 min. The samples were vortexed again and heated at 100°C for 8 min and then centrifuged to pellet the matrix. Aliquots of 2 µL were used as templates for PCR.

The presence of the methylase genes *erm*A, *erm*B, and *erm*C and of the efflux gene *msr*A in the isolates studied was confirmed by PCR

Table 1. Primers specific for *erm*A, *erm*B, *erm*C, and *msr*A

Primer name	Primer sequence	Size of PCR product (bp)
*erm*A1	5′-TCT-AAA-AAG-CAT-GTA-AAA-GAA-3′	645
*erm*A2	5′-CTT-CGA-TAG-TTT-ATT-AAT-ATT-AGT-3′	
*erm*B1	5′-GAA-AAG-GTA-CTC-AAC-CAA-ATA-3′	639
*erm*B2	5′-AGT-AAC-GGT-ACT-TAA-ATT-GTT-TAC-3′	
*erm*C1	5′-TCA-AAA-CAT-AAT-ATA-GAT-AAA-3′	642
*erm*C2	5′-GCT-AAT-ATT-GTT-TAA-ATC-GTC-AAT-3′	
*msr*A1	5′-GCA-AAT-GGT-GTA-GGT-AAG-ACA-ACT-3′	399
*msr*A2	5′-ATC-ATG-TGA-TGT-AAA-CAA-AAT-3′	

PCR: Polymerase chain reaction.

as described previously by Sutcliffe et al.[11]. PCR amplification of *erm* and *msr* genes was performed with primers specific for *erm*A, *erm*B, *erm*C, and *msr*A (Table 1). PCR consisted of an initial denaturation at 94°C for 5 min; 35 cycles of denaturation (93°C, 30 min), annealing (50°C, 30 sec, for *erm*A and 52°C, 1 min for *erm*B, 50°C, 1 min, for *erm*C), and extension (72°C, 1 min); and a final extension at 72°C for 7 min. PCR products were separated on 1.5% agarose gels stained with ethidium bromide and visualized under UV light.

Statistical Analysis

Statistical analysis was performed using SPSS version 15.0 (SPSS Inc., USA). Counts and percentages were used to summarize the results. Nominal variables were compared using the Chi-Square test. When a p value was found less than 0.05, the result was considered as statistically significant and the null hypothesis was rejected.

RESULTS

Among the 82 isolates studied, 65.8% (n= 54) were *S. aureus*, 34.2% (n= 28) were CNS. CNS were *Staphylococcus epidermidis* (n= 23), *Staphylococcus haemolyticus* (n= 1), *Staphylococcus hominis* (n= 1), *Staphylococcus lugdunensis* (n= 1), *Staphylococcus simulans* (n= 1), and *Staphylococcus warneri* (n= 1). Among 54 *S. aureus* strains, 51% (n= 28) were methicillin-resistant and 49% (n= 26) were methicillin-susceptible. Among 28 CNS, 50% (n= 14) were methicillin-resistant.

According to MIC agar dilution results, 41.5% (n= 34) of the isolates were sensitive to either erythromycin or clindamycin. Erythromycin-sensitive and lincomycin-resistant isolates were 3.6% (n= 3). Resistance was detected to erythromycin as 54.9% (n= 45) and to lincomycin as 35.4% (n= 29). Resistance to erythromycin and lincomycin were higher in methicillin-resistant strains as compared to methicillin-sensitive strains. Of the 45 erythromycin-resistant isolates, 44.5% (n= 20) were methicillin-resistant *S. aureus* (MRSA), 13.4% (n= 6) were methicillin-sensitive *S. aureus* (MSSA), 24.5% (n= 11) were methicillin-resistant CNS (MRCNS) and 17.7% (n= 8) were methicillin-sensitive CNS (MSCNS). There was no statistically significant difference between CNS and *S. aureus* in terms of erythromycin resistance (p= 0.089).

18.3% (n= 15, 9 *S. aureus* and 6 CNS) of the erythromycin-resistant isolates exhibited inducible phenotype (the D-test positive), whereas 6.1% (n= 5, 1 *S. aureus* and 4 CNS) expressed MS_B phenotype (the D-test negative). Prevalence of $cMLS_B$ phenotype was 30.5% (n= 25, 16 *S. aureus* and 9 CNS) (Table 2).

The mechanism of resistance responsible for the MLS_B phenotype was determined by PCR. The prevalence of the resistance genes was detected as *erm*A 32.9% (n= 27, 20 *S. aureus* and 7 CNS), *erm*B 1.2% (n= 1, *S. aureus*), *erm*C 7.3% (n= 6, CNS), *msr*A 6.1% (n= 5, 1 *S. aureus* and 4 CNS). *erm*A in combination with *erm*C was present in 4.9% (n= 4, 3 *S. aureus* and 1 CNS), with *msr*A was detected in 1.2%

Table 2. Distribution of erythromycin-resistant strains according to phenotype, genotype and methicillin susceptibility

phenotype	MRSA			MSSA			MRCNS			MSCNS			
genotype	$cMLS_B$	$iMLS_B$	MS_B	$cMLS_B$	$iMLS_B$	MS_B	$cMLS_B$	$iMLS_B$	MS_B	$cMLS_B$	$iMLS_B$	MS_B	Total
ermA	8	7	-	3	2	-	2	2	-	2	1	-	27
ermB	1	-	-	-	-	-	-	-	-	-	-	-	1
ermC	-	-	-	-	-	-	3	1	-	1	1	-	6
msrA	-	-	1	-	-	-	-	-	2	-	-	2	5
ermA+C	3	-	-	-	-	-	1	-	-	-	-	-	4
ermA + msrA	-	-	-	-	-	-	-	-	-	-	1	-	1
No gene	-	-	-	1	-	-	-	-	-	-	-	-	1
Total	12	7	1	4	2	0	6	3	2	3	3	2	45
		20			6			11			8		

MRSA: Methicillin-resistant *Staphylococcus aureus*, MSSA: Methicillin-sensitive *Staphylococcus aureus*, MRCNS: Methicillin-resistant coagulase-negative staphylococci, MSCNS: Methicillin-sensitive coagulase-negative staphylococci, MLS_B: Macrolid-lincosamide-streptogramin B, c: Constitutive, i: Inducible.

Table 3. Susceptibility of antibiotics according to methicillin susceptibility

Methicillin susceptibility	Methicillin-sensitive (n= 40)	Methicillin-resistant, (n= 42)	p
Antibiotics (resistant strain numbers)			
Erythromycin	14	31	< 0.001
Lincomycin	9	19	0.030
Gentamycin	7	30	< 0.001
Fusidic acid	5	12	0.073
Levofloxacin	12	39	< 0.0001
Linezolide	0	0	
Vancomycine	0	0	

(n= 1, CNS) of the isolates. No resistance gene was detected in one isolate. The *erm*C gene was detected only in CNS strains (Table 2).

Of the 15 isolates with an $iMLS_B$ phenotype, *erm*A was present in 80% (n= 12), *erm*C was present 13.4% (n= 2). One *S. epidermidis* isolate contained *erm*A and *msr*A and showed an inducible MLS_B resistance phenotype (6.6%). Among the 25 isolates with constitutive phenotype, 60% (n= 15) had *erm*A, 16% (n= 4) had *erm*C, 16% (n= 4) had *erm*A+C and *erm*B was present only in 4% (n= 1) of the isolates. All isolates with MS_B phenotype (n= 5) contained the *msr*A gene (Table 2).

Distributions of erythromycin-resistant strains according to phenotype, genotype and methicillin-susceptibility are shown in Table 2.

MIC agar dilution results for other examined antibiotics in methicillin-sensitive and -resistant isolates are shown in Table 3. All isolates were susceptible to vancomycin and linezolid.

DISCUSSION

Inducible resistance to clindamycin is difficult to detect in routine laboratory when D-test is not applied. Strains with the $iMLS_B$ phenotype are susceptible to clindamycin in vitro. But it is described as clinically resistant to this antibiotic. In such cases, therapeutic failure with clindamycin may occur

since it can mutate into $cMLS_B$ during the treatment[11]. As a result of mutations occurring in vivo most often within the *erm*AR regulatory region, located upstream of the coding sequence of the *erm*A gene, the $cMLS_B$ phenotype occurs. Mutant strains are selected with clindamycin therapy[12]. Therefore, the use of clindamycin in the right indication is important to avoid unnecessary use.

However, MLS_B resistance rates vary between countries and even hospitals from the same country. In the present study, among the 82 staphylococci, erythromycin resistance rate was 54.9%. The most prevalent phenotype was $cMLS_B$, followed by $iMLS_B$ and MS_B.

In a study from neighboring province including CNS and *S. aureus* strains, the MS_B phenotype has been found most frequently (42%) followed by $iMLS_B$ (32%) and $cMLS_B$ (26%) respectively, not similar to our data[13]. D-test has been performed for 270 *S. aureus* in a study from Nepal and the overall prevalence of $iMLS_B$, $cMLS_B$, and MS_B phenotypes were 11.48% (31/270), 29.25% (79/270) and 13.7% (37/270) respectively. Both $iMLS_B$ and $cMLS_B$ phenotypes predominated in MRSA strains in accordance with our study[14]. Among 71 methicillin-resistant staphylococcal isolates, the $iMLS_B$ phenotype has been found in 16.6% of MRSA and 29.2% of MRCNS, similar to our study[15].

We performed PCR to detect the genes responsible for MLS_B resistance. *erm*A was the most prevalent gene with a rate of 32.9%. In another neighboring province, a study has been designed from clinical *S. aureus* isolates. Among 111 isolates showing $iMLS_B$ phenotype *erm*A gene has been found in 81.9% (83 MRSA, 8 MSSA), *erm*C gene in 10.8% (7 MRSA, 5 MSSA). Among 19 strains with $cMLS_B$ phenotype, *erm*A has been found in 57.9% (10 MRSA, 1 MSSA), *erm*C in 36.8% (6 MRSA, 1 MSSA) and *erm*B in 15.8% (3 MRSA)[16]. In contrast to our study, different distribution of MLS_B resistance phenotypes among *S. aureus* and CNS isolates in Serbia has been reported by Misic et al.[17]. They found that the most frequent phenotype was $iMLS_B$ (33.4%) and the second most prevalent was MS_B (17.6%). The most commonly detected MLS_B resistance genes were *msr*A/B, followed by *erm*C, *erm*B, and *erm*A[17]. In a study from Poland, the most frequent phenotypes and genes were MS_B and $cMLS_B$, and *erm*C among 75 erythromycin-resistant *S. epidermidis* isolates[18]. Our findings are in accordance with the studies of Vallianou et al.[19] from Greece and Sedhagat et al.[20] from Iran where $cMLS_B$ is the predominant resistance phenotype. But *erm*C was the most frequently found gene in their study[19,20]. A study from Thailand, among 125 erythromycin-resistant CNS, the prevalence of $cMLS_B$, $iMLS_B$ and MS_B resistance phenotypes was 72%, 13.60% and 14.40% respectively. These phenotypes were expressed in 80% of MRCNS strains. The *erm*C gene (79.20%) was found to be more prevalent than the *erm*A gene (22.40%), especially among MRCNS[21].

We also evaluated the susceptibilities of some other antibiotics for staphylococci. Considering methicillin susceptibility, other antibiotic resistance rates were found to be high in the methicillin-resistant group. This difference was statistically significant except for fusidic acid ($p= 0.073$). This situation can be explained by the fact that the genetic elements carrying the methicillin resistance genes carry other antibiotic resistance genes.

Due to the wide geographic distribution of resistance phenotypes and genotypes, local statistics are of critical value for empiric therapy. Double-disc diffusion test is useful to guide interpretation of the susceptibility test.

ACKNOWLEDGMENTS

This research was supported by Adnan Menderes University Research Fund. Project Number: TPF-09009. A special thanks to Can Hüseyin Hekimoğlu, MD for statistical analysis.

ETHICS COMMITTEE APPROVAL

Ethics committee approval is not required.

CONFLICT of INTEREST

The authors declare that they have no conflict of interest.

AUTHORSHIP CONTRIBUTIONS

Concept/Design: GUG, SÖ, BB

Analysis/Interpretation: GUG, BB

Data Acquisition: All of authors

Writting: GUG, SÖ, BB

Final Approval: All of authors

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