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Resistance to Macrolide, Lincosamide and Streptogramin Antibiotics in *Staphylococci* Isolated in Istanbul, Turkey

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The purpose of this study was to investigate the prevalence and genetic mechanisms of erythromycin resistance in staphylococci. A total of 102 erythromycin resistant non-duplicate clinical isolates of staphylococci [78 coagulase negative stapylococci (CNS), 24 *Staphylococcus aureus*] were collected between October 2003 and August 2004 in Istanbul Faculty of Medicine in Turkey. The majority of the isolates were from blood and urine specimens. Antimicrobial susceptibilities were determined by the agar dilution procedure and the resistance phenotypes by the double disk induction test. A multiplex PCR was performed, using primers specific for *erm*(A), *erm*(B), *erm*(C), and *msr*A genes. Among the 78 CNS isolates, 57.8% expressed the MLS_B-constitutive, 20.6% the MLS_B-inducible, and 21.6% the MS_B phenotypes. By PCR, 78.2% of these isolates harbored the *erm*(C) gene, 8.9% *erm*(A), 6.4% *erm*(B), and 11.5% *msr*A genes. In *S. aureus*, the constitutive MLS_B (58.3%) was more common than the inducible phenotype (20.8%). *erm*(A) was detected in 50% and *erm*(C) in 62.5% of the isolates, while 37.5% contained both *erm*(A) and *erm*(C). *erm*(C)-associated macrolide resistance was the most prevalent in CNS, while *erm*(C) and *erm*(A, C) was the most prevalent in *S. aureus*.

Keywords: *Staphylococcus*, MLS_B, *erm*(A,B,C), *msr*A

Resistance to macrolides in staphylococci may be due to target-site modification, active efflux (encoded by *msr*A) of the antibiotic, and by drug inactivation. The *erm* (*e*rythromycin *r*ibosome *m*ethylase) genes are responsible for target-site modification, by methylation or mutation of the 23S rRNA, that prevents the binding of the antibiotic to its ribosomal target. This is the most widespread mechanism of resistance to macrolides and lincosamides, and leads to cross-resistance between macrolides, lincosamides, and streptogramin B, giving way to the well known MLS_B phenotype. Among the four major classes of the *erm* genes [*erm*(A), *erm*(B), *erm*(C) and *erm*(F)], *erm*(A) and *erm*(C) are frequently responsible for the MLS_B resistance in staphylococci, which can be either constitutive or inducible (Weisblum, 1995; Leclercq, 2002; Fiebelkorn *et al.*, 2003). In inducible resistance, inactive mRNA is produced that is unable to encode methylase. The mRNA becomes active only after exposure to a macrolide inducer. Even though the strains with an inducible *erm* gene are resistant to the inducers and remain susceptible to non-inducer macrolides and lincosamides in general, different patterns of MLS_B inducible resistance may be observed changing with the *erm* gene or its expression. In constitutive resistance, active methylase mRNA is produced even in the absence of an inducer and a single characteristic phenotype with high-level cross-resistance to the MLS_B drugs is conferred (Leclercq, 2002). While strains with constitutive resistance are resistant to all 14-(clarithromycin, dirithromycin, erythromycin, and roxithromycin), 15-(azithromycin) and 16-membered macrolides (josamycin and spiramycin), lincosamides, and streptogramin B, inducible lincosamide resistant strains are seemed to be resistant only to 14- and 15-membered macrolides *in vitro*. For erythromycin resistant ones, a D-shaped zone around clindamycin, through induction of methylase production by erythromycin, can be observed for the inducible type of resistance when erythromycin disk is placed near a clindamycin disk in disk diffusion tests (Fiebelkorn *et al.*, 2003). The presence of a D-shaped zone would mean that *in vivo* selection of constitutive mutants are highly probable. Erythromycin susceptible strains do not possess inducible clindamycin resistance.

The *msr*A gene is responsible for the efflux mechanism in staphylococci, which is activated after exposure to a macrolide and which pumps out 14- and 15-membered macrolides and streptogramin B (Allignet *et al.*, 1992; Leclercq, 2002). Therefore, the strains are resistant to erythromycin, but remain susceptible to clindamycin since it is not an inducer or a substrate for the pump. Against streptogramin B, they are susceptible until and resistant after an exposure to a macrolide. This so-called M or MS_B efflux phenotype can be differentiated from the MLS_B-inducible phenotype by use of the double disk test, which shows abscence of interaction between erythromycin and clindamycin in this case (no D-shaped zone). Efflux mediated macrolide resistance is common among coagulase-negative staphylococci (CNS) and is increasingly found in methicillin-susceptible *Staphylococcus aureus* (MSSA), with a reported incidence of 13% in a recent European study (Schmitz *et al.*, 2000).

Even though CNS and *S. aureus* are recognized as causes

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of nosocomial and community-acquired infections, data concerning the prevalence and mechanisms of macrolide resistance have not been reported previously in the isolates from our hospital. For this reason, the aim of this study was to investigate the prevalence of macrolide resistance and of *erm*(A), *erm*(B), *erm*(C), and *msr*A genes in clinical *S. aureus* and CNS isolates from our hospital.

Materials and Methods

Bacterial strains

A total of 102 erythromycin-resistant non-duplicate consecutive clinical staphylococcal isolates, including 62 methicillin-resistant (MRCNS) and 16 methicillin-susceptible coagulase negative staphylococci (MSCNS); 22 methicillin-resistant (MRSA) and 2 methicillin-susceptible *S. aureus* (MSSA) strains, were collected between October 2003 and August 2004 in 1750-bed University Hospital of Istanbul Faculty of Medicine in Istanbul, Turkey. The strains were isolated from adult or pediatric, in- or out-patients who applied to the emergency unit. The majority of the isolates were from blood (n=47), urine (n=27), and abscess (n=8), as well as from pleural fluid, sputum, and tracheal aspirate specimens. Isolates were characterized by Gram stain, growth on mannitol salt agar (BBL, Becton Dickinson, France), catalase and coagulase production (Slidex Staph Plus, Biomérieux, France).

Antibiotics

The test antibiotics were as follows: clarithromycin (Abbott Laboratories), azithromycin (Pfizer), oxacillin (Sigma), erythromycin (Kocak, Turkey), clindamycin, gentamycin (Bilim, Turkey), and quinupristin-dalfopristin (Rhone-Poulenc Rorer, France). Disks were purchased from Oxoid.

Antimicrobial susceptibility

Antimicrobial susceptibilities were determined by the agar dilution methods according to the recommendations of the Clinical and Laboratory Standards Institute (CLSI, formerly NCCLS). *S. aureus* ATCC 29213, *S. aureus* ATCC 25923 and *Enterococcus faecalis* ATCC 29212 were used for quality control purposes in susceptibility testing.

Determination of resistance phenotypes

The resistance phenotypes were determined by the double disk test with erythromycin (15 μg) and clindamycin (2 μg) disks applied 20 mm apart. After 18 h incubation at 35°C in ambient air, blunting of the clindamycin zone of inhibition proximal to the erythromycin disk indicated an inducible type (D-shaped zone) of MLS$_B$ resistance (IR), while resistance to both erythromycin and clindamycin indicated a constitutive type (CR). Lack of a D-shaped zone in erythromycin-resistant and clindamycin-susceptible isolates was interpreted as M/MS$_B$ efflux phenotype (Leclercq, 2002).

Multiplex PCR

PCR amplification of *erm* and *msr* genes was performed with primers specific for *erm*(A), *erm*(B), *erm*(C), and *msr*A (Martineau *et al.*, 2000) (Table 1). Multiplex PCR assays were all performed directly from a bacterial suspension, turbitidy of which was adjusted to that of a 0.5 McFarland standard, corresponding to approximately 1.5×10^8 bacteria/ml. One microliter of the standardized bacterial suspension was transferred directly to PCR mixture. PCR was always carried out in a 20 μl volume with 50 mM KCl, 10 mM Tris-HCl (pH 9.0), 0.1% Triton X-100, 0.4 μM of each primer, 200 μM deoxynucleoside triphosphates, 2.5 mM $MgCl_2$, and 0.5 U *Taq* DNA polymerase. The PCR mixtures were subjected to thermal cycling; initial denaturation at 95°C for 3 min; denaturation at 95°C for 30 sec, annealing at 55°C for 30 sec, and extention at 72°C for 45 sec, repeated for 35 cycles; and a final extention at 72°C for 7 min. The PCR products were separated in 1.5% agarose gels stained with ethidium bromide and visualized under UV light. The ϕX174 replicative-form DNA *Hae*III fragments were used to assess the PCR product size (Martineau *et al.*, 2000).

The expected PCR products for *ermA* and *ermB* were 139 and 142 bp, respectively, and the bands on the stained gel were hard to discriminate. Therefore, after confirmation of the presence of an *erm* gene, single PCRs were performed in order to verify the class of the *erm* gene, either *erm*(A) or *erm*(B).

Results

The MIC ranges, MIC_{50}, and MIC_{90} for all isolates were displayed in Table 2. The MIC_{90} for erythromycin, clarithromycin, azithromycin, clindamycin, and quinupristin-dalfopristin were >128, >128, >128, 128, and 2 mg/L, respectively.

Among the erythromycin-resistant 78 CNS isolates, the

Table 1. Primers used in this study

Target gene		Primer sequence (5'→3')	GenBank accession number	Amplicon size (bp)
Erm(A)	*ermA*-1	TATCTTATCGTTGAGAAGGGATT	KO2987	139
	ermA-2	CTACACTTGGCTTAGGATGAAA		
erm(B)	*ermB*-1	CTATCTGATTGTTGAAGAAGGATT	U35228	142
	ermB-2	TTTACTCTTGGTTTAGGATGAAA		
erm(C)	*ermC*-1	CTTGTTGATCACGATAATTTCC	M17990	190
	ermC-2	ATCTTTTAGCAAACCCGTATTC		
*msr*A	*msr*A-1	TCCAATCATTGCACAAAATC	X52085	163
	*msr*A-2	AATTCCCTCTATTTGGTGGT		

Table 2. Antimicrobial susceptibitlity of CNS (n=78) and *S. aureus* isolates (n=24)

Antimicrobials	Antimicrobial susceptibility (%)			MIC (mg/L)		
	S	I	R	Range	MIC_{50}	MIC_{90}
Erythromycin	-	3.9	96.1	0.03->128	>128	>128
Clindamycin	42.1	1.9	55.9	0.03-128	128	>128
Azithromycin	2.9	1.0	96	0.5->128	>128	>128
Clarithromycin	1.9	2.9	95	0.06->128	>128	>128
Quinupristin/dalfopristin	80.4	12.7	6.9	0.03-8	0.5	2

S, susceptible; I, intermediate; R, resistant

Table 3. Phenotypic distribution of 102 CNS and *S. aureus* isolates by disk induction test

Phenotype	MRCNS (n=62)	MSCNS (n=16)	MRSA (n=22)	MSSA (n=2)	Total (n=102)
$cMLS_B$	38 (62%)	7 (44%)	14 (63%)	-	59 (57.8%)
$iMLS_B$	12 (19%)	4 (25%)	4 (18%)	1	21 (20.6%)
M/MS_B	12 (19%)	5 (31%)	4 (18%)	1	22 (21.6%)

CNS, coagulase-negative staphylococci; MRCNS, methicillin-resistant coagulase-negative staphylococci; MSCNS, methicillin-susceptible coagulase-negative staphylococci; MRSA, methicillin-resistant *S. aureus*; MSSA, methicillin-susceptible *S. aureus*

Table 4. Distribution of the *erm*(A,B,C) genes and the *msr*A gene among 102 staphylococcal isolates

Genes	No. (%) in MRCNS (n=62)	No. (%) in MSCNS (n=16)	No. (%) in total CNS (n=78)	No. (%) in MRSA (n=22)	No. (%) in MSSA (n=2)	No. (%) in total *S. aureus* (n=24)	No. (%) in total staphylococci (n=102)
erm(A)	5 (8)	2 (12.5)	7 (8.9)	12 (54.5)	0	12 (50)	19 (18.6)
erm(B)	5 (8)	0	5 (6.4)	2 (9)	0	2 (8.3)	7 (6.8)
erm(C)	47 (75.8)	14 (87.5)	61 (78.2)	14 (63.6)	1 (50)	15 (62.5)	76 (74.5)
*msr*A	9 (14.5)	0	9 (11.5)	3 (13.6)	0	3 (12.5)	12 (11.7)
erm(A)+*erm*(C)	2 (3.2)	1 (6.3)	3 (3.8)	9 (40.9)	0	9 (37.5)	12 (11.7)
erm(B)+*erm*(C)	1 (1.6)	0	1 (1.2)	0	0	0	1 (0.9)
erm(C)+*msr*A	3 (4.8)	0	3 (3.8)	0	0	0	3 (2.9)
erm(A)+*erm*(B)+ *erm*(C)+*msr*A	0	0	0	1 (4.5)	0	1 (4.1)	1 (0.9)
PCR (-)	2 (3.2)	1 (6.3)	3 (3.8)	3 (13.6)	1 (50)	4 (16.6)	7 (6.8)

MRCNS, methicillin-resistant coagulase-negative staphylococci; MSCNS, methicillin-susceptible coagulase-negative staphylococci; MRSA, methicillin-resistant *S. aureus*; MSSA, methicillin-susceptible *S. aureus*.

constitutive MLS_B resistance was found in 57.7% (45/78), MLS_B-inducible resistance in 20.5% (16/78), and the MS_B phenotype in 21.8% (17/78) (Table 3). The *erm*(C) gene was detected in 78.2% (61/78) of the these isolates, *erm*(A) in 8.9% (7/78), *erm*(B) in 6.4% (5/78), and *msr*A in 11.5% (9/78). Co-existence of *erm*(C) and *msr*A genes in three isolates and of *erm*(A) and *erm*(C) in another three were observed, while a single isolate co-harbored *erm*(B) and *erm*(C) (Table 4).

Among the 24 erythromycin-resistant *S. aureus* strains, 14 (58.3%) exhibited the MLS_B-constitutive, five (20.8%) the MLS_B-inducible, and five (20.8%) the MS_B-phenotypes (Table 3). *erm*(C) was positive in 62.5% (15/24), *erm*(A) in 50% (12/24), *erm*(B) in 8.3% (2/24), *msr*A in 12.5% (3/24) isolates, and out of these, *erm*(A) and *erm*(C) were co-present in 37.5% (9/24). One MRSA isolate carried all of the *erm*(A,B,C) and *msr*A genes (Table 4).

Discussion

Clindamycin is frequently used to treat skin, soft tissue, and bone infections because of its tolerability, cost, oral form, and good tissue penetration. There have been a number of reports on clindamycin or lincomycin therapy failures in serious infections due to staphylococci with inducible MLS_B resistance (Levin *et al.*, 2005). D-test was developed to identify potential clindamycin resistance so that possible ineffective therapy is not started even when conventional

tests show the susceptible range (≤0.5 μg/ml) of MICs in clindamycin (Thakker-Varia *et al.*, 1987). The use of clindamycin for the treatment of an infection due to an inducible-resistant strain of *S. aureus* is not devoid of risk. Constitutive mutants can be selected *in vitro* at frequencies of 10^{-7} CFU in the presence of these antibiotics. Bacterial inocula exceeding 10^7 CFU can be found in mediastinitis and in certain lower respiratory tract infections. The risk to patients is illustrated by reports of selection of constitutive mutants during the course of clindamycin therapy for severe infections due to inducible-erythromycin-resistant *S. aureus* strains (Watanakunakorn, 1976; Drinkovic *et al.*, 2001). If the staphylococcal inoculum at the site of the infection is higher, the risk for selection of a constitutive mutant would be higher. Clinical isolates that are constitutively resistant to MLS$_B$ antibiotics are widespread, particularly among the methicillin-resistant strains (Leclercq, 2002). In our study, methicilllin resistance was found as 44.2% of *S. aureus* and 50.4% of CNS isolates during the study period. Among the methicillin-resistant ones, constitutive MLS$_B$ resistance was found in 61.3% (38/62) of CNS isolates and 63.6% (14/22) of MRSA isolates.

In this study, *erm*(C) was the most prevalent gene found as 69.2% (54/78) alone and 78.2% (61/78) together with the other genes in CNS isolates. The predominant genes associated with macrolide resistance were the *erm*(C) (20.8%, 5/24) and *erm*(A) and (C) (62.5%, 15/24) for *S. aureus*. Similar findings were also reported from Denmark, showing a higher incidence for *erm*(A) and *erm*(C) genes in 98% of the 428 erythromycin-resistant *S. aureus* isolates (Westh *et al.*, 1995). The incidence of constitutive and inducible MLS$_B$ resistance varies by geographic region and even from hospital to hospital, or patient group. These variations may be associated with variable use of erythromycin in each country and/or may depend on the source of the strains such as nosocomially or community acquired, patient age, and sample origin. Although scarce detailed study on the distribution of these resistance mechanisms has so far been performed in our country, Ozyurt *et al.* (2004) reported an incidence of 64% for *erm*(C) in methicillin-resistant coagulase negative and methicillin-resistant coagulase positive staphylococci, and 71% in MRSA. Lim *et al.* (2002) have also reported a high incidence of CNS strains carrying *erm*(C) (47.2%). It is important to distinguish the inducible MLS$_B$ strains from those that contain the *msr*A gene to encode an efflux pump that affects only macrolides, not clindamycin. Out of the 102 isolates in our study, it was interesting that the *msr*A gene was present in 12 (11.7%), all of which were methicillin-resistant. A multicenter study in 24 European university hospitals revealed that in *S. aureus*, the *erm*(A) gene was more common among MRSA isolates (88%) than in MSSA isolates (38%), and that it occurred mainly in strains with constitutive MLS$_B$ expression. In contrast, *erm*(C) was more common in MSSA (47%) than in MRSA (5%), occurring mainly in strains with inducible expression. It has been reported that macrolide resistance due to efflux by the *msr*A/*msr*B genes has been detected only in MSSA isolates (13%) and the most prevalent resistance gene in *S. aureus* was *erm*(A) (67%), followed by *erm*(C) (23%) (Schmitz *et al.*, 2000).

In conclusion, constitutive MLS$_B$ resistance was the most frequently encountered phenotypic pattern, *erm*(C) was the most prevalent gene, and *erm*(A,C) was the most frequent gene combination. *erm*(C)-associated macrolide resistance was the most prevalent in CNS isolates, while *erm*(C) and *erm*(A,C)-related resistance was the most prevalent in *S. aureus* isolates. Constitutive resistance was predominant particularly among MRSA. *msr*A was observed alone and in combination with the *erm* genes only in methicillin-resistant staphylococci, including MRSA.

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