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ORIGINAL PAPER

Macrolide-lincosamide-streptogramin B Resistant Phenotypes and Genotypes for Methicillin-resistant *Staphylococcus aureus* in Turkey, from 2003 to 2006

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Abstract

Methicillin-resistant *Staphylococcus aureus* (MRSA) strains with inducible macrolide-lincosamide-streptogramin B ($iMLS_B$) resistance phenotype may lead to clinical failure during clindamycin (CLI) therapy. The aim of this study was to determine the incidence of MLS_B phenotypes by using D-test method and genotypes by using multiplex real-time PCR method in MRSA strains. A total of 265 MRSA strains were obtained from clinical samples from hospitalized and outpatients. Of the MRSA isolates, 225 (84.9%) were resistant to erythromycin (ERT), and 170 (64.1%) to CLI. Among the 225 ERT-resistant MRSA strains, the constitutive MLS_B ($cMLS_B$) rate was found in 49.3%, $iMLS_B$ in 39.1% and the M phenotype in 11.5%. Overall, *ermA*, *ermC*, *ermA+ermC*, *msrA*, *ermC+msrA*, and *ermA+ermC+msrA* genes were detected in 85 (37.7%), 60 (26.6%), 42 (18.6%), 26 (11.5%), 11 (4.8%), and 1 (0.4%) isolates, respectively. Most prevalent resistance determinant in MRSA strains was *ermA*, which was detected in 37.7% of the isolates. The 26 MRSA strains with M phenotype harboured only *msrA* gene. In conclusion, due to aware of the potential of CLI treatment failure, D-test should be performed and reported in MRSA strains in clinical laboratories. The multiplex real-time PCR method is easy to perform, fast and reliable method for the detection of MLS_B resistance genotypes.

Key words: *Staphylococcus aureus*, MLS_B, MRSA, multiplex real-time PCR

Introduction

Methicillin-resistant *Staphylococcus aureus* (MRSA) is one of the most important pathogens as a world-wide health problem now (Deresinski, 2005). MRSA became increasingly prevalent in the hospital and community settings in the United States, Asia, and some parts of European countries (Robinson and Enright, 2003). MRSA has also been increasingly found in the Turkish hospitals as an important problem (Oztop *et al*., 2004).

Although macrolide, lincosamide and streptogramin B antimicrobial agents (MLS_B) are chemically distinct, they have similar mode of action (Leclercq and Courvalin, 1991). Erythromycin (ERT) and clindamycin (CLI) inhibit protein synthesis in a wide range of bacteria by binding to a single site the large ribosomal subunit located near the entrance to the growth of the polypeptide chain in bacterial ribosome. They can also prevent the formation of the 50S ribosomal subunit in growing bacteria (Garza-Ramos *et al*., 2001). Streptogramin B affects the 50S large subunit in a similar way as the ERT and competes for the same binding site (Harms *et al*., 2004).

Since MLS_B antimicrobial agents are widely used in the treatment of *S. aureus* and other Gram-positive organism infections, resistance rate of this strain has been rapidly increased (Khan *et al*., 1999). Mainly, two different mechanisms are liable for most of the acquired bacterial resistance to MLS_B antibiotics in staphylococci: through efflux of the antibiotic and through target-site modification by methylation. In the first mechanism, antibiotic efflux is typically mediated by ATP-dependent efflux pump encoded the *msrA* gene (Lerlercq, 2002). The efflux mechanism yields resistance to macrolides and type B streptogramins but not to lincosamides, the so-called M phenotype. In the second mechanisms, modification of the drug-binding site is mediated by controlling the methylation of the 23S rRNA binding site of adenosine

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2058 (A2058) encoded by ERT ribosomal methylase (*erm*) genes. The *ermA* and *ermC* are most frequently found in staphylococci. This mechanism confers cross-resistance to MLS_B antibiotics, the so-called MLS_B phenotype. Expression of MLS_B resistance can be either constitutive ($cMLS_B$) or inducible ($iMLS_B$) (Volokhov *et al*., 2003). Although strains with $iMLS_B$ resistance display *in vitro* susceptiblity to lincosamides and type B streptogramin which are not inducer, they show *in vitro* resistance to macrolides which are inducer. Since $iMLS_B$ resistance can not be determined by using standard susceptibility test methods such as broth-based or agar dilution test, the double-disk diffusion agar inhibitory assay or D-test methods are used for demonstration of this phenotype in isolates that are susceptible to CLI and resistant to ERT (Chavez-Bueno *et al*., 2005).

The objective of this study was to determine the existence of $iMLS_B$ phenotype among MRSA strains by using D-test and occurrence of the ERT-resistant genes by using a new multiplex real-time PCR in MRSA strains collected from Gulhane Military Medical Academy Hospital (GMMAH) Clinical Microbiology Laboratory during a four year study period. To our knowledge, this is the first multiplex real-time PCR study to detect ERT-resistant genes in MRSA strains.

Experimental

Materials and Methods

Bacterial strains. GMMAH is a teaching hospital with more than 1500 beds in Ankara, the capital of Turkey. A total of 265 MRSA strains were obtained from clinical samples from hospitalized and outpatients at the GMMAH from 2003 through 2006. These strains were isolated from skin, soft tissue, abscess (114; 43.1%), blood stream (89; 33.5%), respiratory (25; 9.4%), and other clinical samples (37; 13.9%). Of the patients included, 89.88% were adults, 10.2% were child, 71.5% were male, and 28.5% were female. Only the first isolate from each patient during the study period was included to avoid overrepresentation. Colony morphology, Gram-staining, catalase, tube-coagulase, DNase, and mannitol tests were used for identification of all *S. aureus* isolates. Final identification was performed with the Phoenix, an automated bacteriology system that performs bacterial identification (Becton Dickinson Diagnostic Systems, Sparks, MD, USA) (Layer *et al*., 2006). The isolates were kept at –70°C in trypticase soy broth (Merck, Darmstadt, Germany) supplemented with 15% glycerol before being tested.

Antimicrobial susceptibility testing. The susceptibility of the isolates was determined by a disk diffusion method in accordance with Clinical and Laboratory Standards Institute standards (CLSI, 2006) against thirteen antimicrobial agents (Oxoid, Hampshire, England). The antibiotics tested were amoxicillin-clavulanate, cefazolin, CLI, ERT, gentamicin, levofloxacin, minocycline, penicillin, rifampin, trimethoprim/sulfamethoxazole (SXT), quinupristin-dalfopristin, linezolid, and vancomycin. Inducible CLI resistance was identified as a D-shaped inhibition zone by the CLI-ERT double-disk test. This test was performed by placing a 2 μg CLI disk from 15 mm to 26 mm away from the edge of a 15 μg ERT disk as part of the normal disk diffusion procedure on Mueller-Hinton Agar. After 16–18 h incubation, organisms that do not show flattening of the CLI zone recorded as CLI susceptible. Organisms that show flattening of the CLI zone adjacent to the ERT disk indicated iMLSB phonotype (Seybold *et al*., 2006). Penicillin Binding Protein 2' Latex Agglutination Test (PBP2') (Oxoid Limited, Basingstoke, England) also was also performed for the confirmation of *mecA*-positive *S. aureus* as recommended by the manufacturers. *S. aureus* ATCC 29213 and ATCC 25923 were used as control strains.

DNA extraction and multiplex real-time PCR. DNA was prepared as described previously by Kilic *et al.* (2004). A real-time TaqMan PCR method was performed on the 7500 ABI Prism Sequence Detector (Applied Biosystems, Foster City, Calif., USA). The primers and fluorophore TaqMan probes for *ermA*,

Table I
Oligonucleotide sequences of the primers and probes used in this study

Target genes		Oligonucleotide sequence (5'–3')	GenBank accession no
ermA	Forward Reverse Probe	ggatcaggaaaaggacattttac ttatatccatctccaccattaatagtaa fam-tagtcaaaatgagtcgatcagttactgcta-bhq-1	AF466413.1
ermC	Forward Reverse Probe	gctcaggaaaagggcatttta gctaatattgtttaaatcgtcaattc vic-attagtacaaaggtgtaatttcgtaactgctat-bhq-1	Y09003.1
msrA	Forward Reverse Probe	gcacaataagagtgtttaaaggta atgattggataattattatggatatcata texas red-acagtatcaaaatcaatatgaacaagaacag-bhq-2	EF0922840.1

ermC and *msrA* genes were designed with reference to the sequences deposited in GenBank under accession numbers AF466413.1, Y09003.1, and EF0922840.1, respectively (Table I). In brief, 1 µl of the extracted nucleic acid was added to 24 µl of reaction mixture containing 0.8 µM of each primer and 0.4 µM fluorophore probe (final concentration), and mixed with 25 µl of TaqMan Universal PCR Master Mix (Applied Biosystems). The TaqMan cycling conditions were a 2 min degradation of the pre-amplified templates at 95°C and then 40 cycles of denaturation at 95°C for 15 s and annealing and extension at 58°C for 60 s (Kilic *et al*., 2006). *S. aureus* strains containing *ermA* (*S. aureus* RN1551), *ermC* (*S. aureus* FPR3757) and *msrA* (*S. aureus* 15114) genes were kindly provided from Fred C. Tenover at Centers for Disease Control and Prevention, Atlanta, GA, USA.

Statistical analysis. Statistical comparisons were performed using SPSS 15.0 for Windows (SPSS Inc., Chicago, Illinois, USA). Associations between $cMLS_B$ phenotype and $iMLS_B$ phenotype for antibiotic susceptibilities were analyzed using the χ^2 test or the Student t test. P values of ≤0.05 were considered statistically significant.

Results

A total of 265 MRSA isolates collected during a four year period between 2003 and 2006 from the Clinical Microbiology Laboratory at GMMAH were tested. Of the MRSA isolates, 225 (84.9%) were resistant to ERT, and 170 (64.1%) to CLI. Among 225 ERT-resistant MRSA strain, *ermA*, *ermC*, *ermA+ermC*, *msrA*, *ermC+msrA*, and *ermA+ermC+msrA* genes were detected in 85 (37.7%), 60 (26.6%), 42 (18.6%), 26 (11.5%), 11 (4.8%), and 1 (0.4%) isolates, respectively (Table II). The *ermA* gene was predominant in these ERT-resistant MRSA strains. The 88 (39.1%) isolates had $iMLS_B$ phenotype which showed a blunted edge but an otherwise clear zone of inhibition around the CLI disk. In these isolates, *ermA* (55; 62.5%), *ermC* (23; 26.1%), and *ermA+ermC* (10; 11.3%) genes were detected. In the 111 (49.3%) strains, growth was observed around both disks, the so-called $cMLS_B$ phenotype. They had *ermC* (37; 33.3%), *ermA+ermC* (32; 28.8%), *ermA* (30; 27.1%), *ermC+msrA* (11; 9.9%), *ermA+ermC+msrA* (1; 0,9%) genes. The 26 (11.5%) isolates showed CLI susceptible zone diameter with no blunting of the zone, the so-called M phenotype. In these 26 strains, only *msrA* gene was found. Antibiotic susceptibility for amoxicillin-clavulanate, cefazolin, gentamicin, levofloxacin, minocycline, penicillin, rifampin, trimethoprim/sulfamethoxazole (SXT), quinupristin-dalfopristin, linezolid, and vancomycin was also determined and their resistance rates between the $cMLS_B$ phenotype and $iMLS_B$ phenotype were compared in Table III. The strains with $cMLS_B$ phenotype were more resistant than the strains with $iMLS_B$ phenotype to SXT (χ^2=11.903, p = 0.001). No isolates were resistant to linezolid or vancomycin in both groups.

Discussion

Since its first isolation by Barber in 1961, MRSA is an increasingly important nosocomial pathogen in the worldwide (Manian *et al*., 2003). In Turkey, MRSA prevalence rates vary from 9% to 40% in studies (Karadenizli, 2002). Since MRSA strains are resistant to a variety of antimicrobial agents, therapy of

Table II
Distribution of macrolide-lincosamide-streptogramin B resistant genotypes and phenotypes according to year among methicillin-resistant *Staphylococcus aureus*

Variable		No (%) of strains (n = 225)		
		Constitutive MLS_B phenotype (n = 111)	Inducible MLS_B phenotype (n = 88)	M phenotype (n = 26)
Year	2003	40 (54.1)	27 (36.4)	7 (9.4)
	2004	16 (37.2)	23 (53.4)	4 (9.3)
	2005	31 (56.3)	17 (30.9)	7 (12.7)
	2006	24 (45.2)	21 (39.6)	8 (15.1)
Genes	*ermA*	30 (27.1)	55 (62.5)	0
	ermC	37 (33.3)	23 (26.1)	0
	ermA+ermC	32 (28.8)	10 (11.3)	0
	msrA	0	0	26 (11.5)
	ermC+msrA	11 (9.9)	0	0
	ermA+ermC+msrA	1 (0.9)	0	0

MLS_B: Macrolide-lincosamide-streptogramin B

Table III
Antibiotic resistance profiles between constitutive MLS_B phenotype and inducible MLS_B phenotype in MRSA strains recovered from patients at Gulhane Military Medical Academy Hospital between 2003 and 2006

Antibiotics	No (%) of strains			
	Constitutive MLS_B phenotype (n = 111)	Inducible MLS_B phenotype (n = 88)	chi square	*p*
Amoxicillin Clavulanate	102 (91.8)	83 (94.3)	0.442	0.506
Penicillin	111 (100.0)	87 (98.8)	1.268	0.260
Cefazolin	101 (90.9)	81 (92.1)	0.070	0.792
Gentamicin	102 (91.8)	79 (89.7)	0.268	0.605
Levofloxacin	88 (79.2)	67 (76.1)	0.282	0.596
Minocycline	4 (3.6)	0 (0.0)	3.236	0.072
Rifampin	99 (89.1)	81 (92.2)	0.464	0.496
Trimethoprim/sulfamethoxazole	39 (35.1)	12 (9.2)	11.903	0.001
Quinupristin-Dalfopristin	3 (2.7)	0 (0.0)	2.415	0.120
Linezolid	0 (0.0)	0 (0.0)	NA	
Vancomycin	0 (0.0)	0 (0.0)	NA	

MLS_B: Macrolide-lincosamide-streptogramin B

infections caused by these strains is difficult and leads to a high mortality rate (Ardic *et al*., 2005). Because of the good oral absorption and remarkable distribution into skin and skin structures, CLI is considered an alternative drug of the treatment of MRSA infection in outpatient and inpatient. However, reliable antibiotic susceptibility results are required for appropriate therapy decision (Angel *et al*., 2008). Gadepalli *et al.* (2006) reported ERT and CLI resistance in 63% and 79% of MRSA strains, respectively. In Taiwan, ERT resistance rate was more than 90% and CLI resistance was between 70% and 90% in MRSA strains were reported (Janapatla *et al*., 2007). Otsuka *et al.* (2007) found 97% resistance to ERT and 59.5% to CLI in MRSA strains. Schmitz *et al.* (1999) detected 94% resistance to ERT and 89.1% to CLI in 342 MRSA strains collected from 20 European university hospitals. Azap *et al*. (2005) reported that in Turkey ERT and CLI resistance rates were 69.6% and 63.8% in MRSA strains. In our study, ERT and CLI resistance were found in 225 (73.7%) and 170 (55.7%) MRSA strains, respectively as less than other studies.

CLI resistance can develop in staphylococcal isolates either constitutively or inducible. If the strains have $iMLS_B$, they may appear susceptible to CLI by the microdilution and disk diffusion method as a false *in vitro* result. The iMLSB, however, can be expressed during a double disk diffusion test (D-test). Therefore, 2005 CLSI guideline has recommended to routine test detecting staphylococci strains with $iMLS_B$ (Park *et al*., 2007). In our study, the cMLSB, iMLSB and M resistance phenotype were examined. Eighty-eight (39.1%) isolates were found to be the $iMLS_B$ phenotype, 111 (49.3%) the $cMLS_B$ phenotype and 26 (11.5%) the M phenotype among ERT-resistant MRSA. In Turkey, the cMLSb, iMLSB and M phenotype resistance rates were found to vary from 43.7%, 5.4% and 0% to 64%, 24.4%, 18%, respectively in MRSA strains (Azap *et al*., 2007; Delialioglu *et al*., 2005; Yilmaz *et al*., 2007; Aktas *et al*., 2007). The results of this study demonstrated that MLS_B resistance phenotype rate was consistent with previous studies reported from Turkey. In the other countries such as France, Greece, Taiwan, India, The United States, Japan, these rates were reported vary from 38%, 4%, and 0% to 83%, 38.7%, 12%, respectively (Lina *et al.* 1999; Schreckenberger *et al*., 2004; Fokas *et al*. 2005; Gadepalli *et al*. 2006; Janapatla *et al*. 2007; Otsuka *et al*. 2007). The $cMLS_B$ phenotype was found predominant over the $iMLS_B$ phenotype in MRSA strains in previously published studies consistent with our study. This high rate of iMLSB resistance phenotype among our hospital suggests that CLI should be used cautiously. If CLI is used for treatment of infection caused by MRSA strains with $iMLS_B$ phenotype, the patients should be closely monitored during infection progress for failure of treatment. CLI only may be used safely treating of infection caused by MRSA strains with M phenotype if appropriate.

The use of PCR and multiplex PCR for the detection of antibiotic resistance genes has been described in many studies for MRSA strains previously (Lina *et al*., 1999; Martineau *et al*., 2000; Lim *et al*., 2002; Strommenger *et al*., 2003; Sekiguchi *et al*., 2003). To our knowledge, this is the first investigation of ERT-resistant genes in MRSA isolates by using multiplex real-time PCR. When MLSB genotypes were examined by using real-time PCR in this study, the *ermA* gene was detected 85 (37.7%) of these isolates, *ermC* in 60 (26.6%), *ermA*+*ermC* in 42 (18.6%), *msrA* in 26 (11.5%), *ermC*+*msrA* in 11 (4.8%), and *ermA*+*ermC*+*msrA* in 1 (0.4%). The *ermA* gene was more prevalent

in MRSA strains with $iMLS_B$ phenotype, while the *ermC* gene was more common in $cMLS_B$ phenotype strains. Fourty-two isolates contained both *ermA* and *ermC* gene, while one (0.4%) isolate had all three genes (*ermA*+*ermC*+*msrA*). In two studies from Turkey, Aktas *et al*. (2007) found the *ermC* gene was more prevalent in MRSA strains as 63.6%, while Ardic *et al*. (2005) reported the *ermA* gene is more common in their MRSA strains as 71.4%. Schmitz *et al*. (2000) reported from 24 countries in an European study, the *ermA* gene was more common in MRSA strains expressing a cMLSB phenotype. Janapatla *et al*. (2007) detected the *ermA* gene in 93% and the *ermC* gene in 7% of MRSA strains from Taiwan. Distinctly, Spiliopoulou *et al*. (2004) from Greece in MRSA strains reported most of MRSA strains in their study carried the *ermC* gene (96.5%), mainly in strains with cMLSB phenotype. Lina *et al*. (1999) reported the *ermA* gene was more common in MRSA strains (57.6%), mainly in strains with cMLSB phenotype from France. To our knowledge, this is the first investigation of ERT-resistant genes in MRSA isolates by using multiplex real-time PCR. The multiplex real-time PCR described in this study detects three relevant resistance genes in a reaction.

In conclusion, the D-test should be performed to avoid treatment failure in ERT-resistant, CLI susceptible MRSA strains. The multiplex real-time PCR method should be used to determine the MLS_B genotypes in MRSA strains. Our study has revealed that the *ermA* gene and $cMLS_B$ phenotype were predominant in MRSA strains in Ankara, capital of Turkey. This study suggested that MLS_B resistance phenotypes and genotypes in MRSA strains should be monitored in every region and country.

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