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grlA AND *gyrA* MUTATIONS AND ANTIMICROBIAL SUSCEPTIBILITY IN CLINICAL ISOLATES OF CIPROFLOXACIN- METHICILLIN-RESISTANT STAPHYLOCOCCUS AUREUS

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Abstract

Objective: To determine *grlA* and *gyrA* mutations in ciprofloxacin- methicillin-resistant *Staphylococcus aureus* isolates and their susceptibility to current antimicrobials, including a newer fluoroquinolone gatifloxacin, glycopeptides vancomycin, teicoplanin and oxazolidinone linezolid.

Methods: A total of 56 methicillin-resistant *S. aureus* (MRSA) isolates were collected during 2003-2006 from inpatients of Süleyman Demirel University Hospital. The isolates were confirmed to be MRSA by the production of coagulase, showing resistance against cefoxitin and having the *mecA* gene and tested by disk diffusion for susceptibility to vancomycin, teicoplanin and linezolid. The minimum inhibitory concentrations (MICs) of ciprofloxacin and gatifloxacin were measured using the E-test. The quinolone resistance determining regions (QRDRs) of isolates were amplified by PCR and mutations in *grlA* and *gyrA* genes were identified by direct sequencing.

Results: Sequencing data revealed that 96% of our isolates had mutations in both *grlA* and *gyrA* genes. Among these, the *grlA* mutation of Ser-80→Phe or Tyr and the *gyrA* mutation of Ser-84→Leu were the most dominant ones being detected in 50 (89%) and 40 (71%) isolates, respectively. Although 96% of isolates were highly resistant to ciprofloxacin (MIC, ≥ 32 mg/l), only 54% of ciprofloxacin-resistant MRSA isolates were resistant to gatifloxacin and exhibited lower-level resistance (MIC, ≤ 6 mg/l). Of the isolates tested, 46% were found to be susceptible to gatifloxacin (MIC, ≤ 0.5 mg/l). Full susceptibility was observed for vancomycin, teicoplanin and linezolid.

Conclusions: This study provided information on *grlA* and *gyrA* mutations and current antimicrobial susceptibility in clinical MRSA isolates. The results indicated that gatifloxacin is still effective against MRSA isolates and might be useful for treatment of less serious MRSA infections but careful monitoring of susceptibility is required.

Key words: MRSA; antimicrobial susceptibility; *grlA* and *gyrA* mutations; gatifloxacin

INTRODUCTION

MRSA continues to be a major human pathogen responsible for nosocomial and community-acquired infections worldwide. Antibiotic resistance of MRSA is a significant problem with severe consequences including increased medical cost, morbidity and mortality of patients. Recently, MRSA infections have been treated with parenteral glycopeptides (vancomycin and teicoplanin) because of their excellent activity against MRSA. The glycopeptides prevent the transglycosylation and transpeptidation reactions, resulting in the inhibition of cell-wall sythesis in Gram-positive bacteria (Hiramatsu 2001). The oxazolidinones, a class of synthetic compounds, inhibit bacterial protein synthesis and are highly active against MRSA. Linezolid, the first licensed oxazolidinone antibiotic, provides an oral therapeutic option for patients who failed on conventional therapy (Zurenko et al. 1996). The fluoroquinolones (FQ) are not new antibiotics but many studies are being conducted especially to assess the use of newly developed FQs. Important features of FQs include good bioavailability after oral administration and activity against many Gram-positive bacteria. FQs exert their antimicrobial effects by inhibiting two bacterial topoisomerase enzymes, DNA gyrase and topoisomerase IV, that are essential for bacterial DNA synthesis (Drlica and Zhao 1997; Levine et al. 1998). Mutations in the "quinolone-resistance-determining-regions" (QRDR) of the *gyrA*, *gyrB*, *grlA* or *grlB* genes of DNA gyrase and topoisomerase IV were shown to be responsible for quinolone resistance. Studies have also demonstrated that mutations in *gyrA* and *grlA* are primarily responsible for FQ resistance, while mutations in *gyrB* and *grlB* contribute minimally (Hooper 1999). Additionally, topoisomerase IV appears to be the principal target for most FQs in *S. aureus* (Hooper, 2000). Ciprofloxacin resistance in strains of MRSA is common worldwide, and most of these strains have at least two resistance mutations, usually in *grlA* and *gyrA*, rendering them highly resistant to ciprofloxacin (Bell 2002). Resistance of MRSA strains to different FQs varies and emergence of resistance was found to be slower to levofloxacin, ofloxacin and sparfloxacin than to ciprofloxacin (Evans and Titlow 1998; Limoncu et al. 2003). Recently, some newer FQs with enhanced antimicrobial activity have been developed including gatifloxacin and moxifloxacin (Blondeau 2001; Noguchi et al. 2005).

To our knowledge, there is no report from Turkey regarding the mutations in QRDRs of *grlA* and *gyrA* genes in clinical MRSA isolates and their effect on

FQ-resistance. Therefore, we investigated *grlA* and *gyrA* mutations in 56 clinical ciprofloxacin-resistant MRSA isolates, and determined their antibiotic susceptibilities to current antimicrobials gatifloxacin, vancomycin, teicoplanin and linezolid.

Materials and Methods

Bacterial Strains and Antibacterial Agents

Fifty-six non-repeat clinical isolates of MRSA were obtained from patients of Süleyman Demirel University Hospital. The isolates were collected within a period of tree years (2003-2006), and confirmed to be MRSA by the production of coagulase, showing resistance against cefoxitin and finally by the detection of the *mecA* gene by PCR. In our study, ATCC33591 (MRSA) and ATCC29213 (MSSA) were included as reference strains. All isolates were maintained and cultivated as described by the Clinical and Laboratory Standards Institute (CLSI, formerly NCCLS) (2005). Antibiotic disks (Oxoid, Basingstoke, UK) of cefoxitin, vancomycin, teicoplanin and linezolid, and E-tests (AB-Biodisk) of ciprofloxacin and gatifloxacin were purchased from Tan-Med Inc. (Turkey).

Antimicrobial Susceptibility Testing

Disk diffusion susceptibility testing was performed for cefoxitin, vancomycin, teicoplanin and linezolid by following the method recommended by the CLSI (2005). Briefly, a bacterial suspension of 0.5 McFarland density from each of the 56 isolates was inoculated onto Mueller-Hinton agar (Difco, Detroit, MI, USA) plate and following the placement of antibiotic disks on the surface of agar, the plates were incubated at 35 °C for 16-18 h. The minimum inhibitory concentrations (MICs) of ciprofloxacin and gatifloxacin were measured using the E-test method, following the instructions of the manufacturer (AB-Biodisk, Solna, Sweden). Briefly, each Mueller-Hinton agar plate was inoculated with bacterial suspension of 0.5 McFarland density and then E-test strips were aseptically placed onto the surface of plate. Following the incubation at 35 °C for 16-18 h, MICs were read on the basis of the intersection of the elliptical zone of growth inhibition with the MIC scale on the E-test strip. Both agents were tested at concentrations ranging 0.002 to 32 mg/l and breakpoints were those defined by CLSI (2005).

PCR Amplifications of *mecA*, *grlA* and *gyrA* Genes

In the study, colony PCR approach was used to amplify *mecA* gene and also the QRDRs of *grlA* and *gyrA* genes. PCR reactions were carried out independently using the primers 5'AAAATCGATGGTAAAGGTT GGC3' and 5'AGTTCTGCAGTACCGGATTTGC3' for *mecA*, (Wichelhaus et al. 1999) and, 5'CAGTCGG TGATGTTATTGGT3', 5'CCTTGAATAATACCACC AGT3' for *grlA* and 5'ATGGCTGAATTACCTCAA TC3', 5'GTGTGATTTTAGTCATACGC3' for *gyrA*, (Sierra et al. 2002). Briefly, three bacterial colonies were obtained from a fresh culture and suspended in 50 µl of distilled steril water. The suspension was boiled at 100 °C for 30 min and after centrifugation at 14.000 rpm for 5 min, supernatant containing the bacterial DNA was used as a template. Each 50 µl reaction mixture (20mM Tris-HCl (pH 8.0), 100 mM KCl, 20 mM $MgSO_4$) contained 3 µl DNA template, 2.5 units of *Taq* polymerase (BioBasic Inc., Canada), 250 µM dNTPs (each), and 250 nM of each primers. DNA amplification was carried out as follows: initial denaturation at 94 °C for 5 min, followed by 30 cycles of amplification (denaturation at 94°C for 45 s, annealing at 50 °C for 45 s, extension at 72 °C for 1 min), ending with a final extension at 72 °C for 5 min. PCR products were analyzed on a 1% agarose gel.

Sequencing of Quinolone Resistance-determining Regions (QRDRs) of *grlA* and *gyrA* Genes

The amplified PCR products (398 bp fragment of *gyrA* from base 1 to 397 and 469 bp fragment of *grlA* from position 197 to 665) were recovered by using a DNA gel extraction kit according to the manufacturer's instructions (BioBasic Inc., Canada). The amplimers were analyzed by direct sequencing to detect mutations. For this purpose, the purified samples were sent to the IONTEK Company (Istanbul, Turkey) where sequencing was performed by using the DYEnamic ET Terminator Cycle Sequencing kit (Amersham) and analyzed in an automatic DNA sequencer (Abi Prism 310, Perkin Elmer). Mutations in these genes were identified by comparison with the published sequences of *grlA* and *gyrA* (Yamagishi et al. 1996; Ito et al. 1994, respectively)

Results

Fifty six MRSA isolates included in our study were investigated phenotypically using the cefoxitin disk and inhibition zones for all isolates were ≤19, indicating that all isolates were resistant to methicillin. PCR confirmed the results of disk diffusion since all isolates had the predicted 533 bp product of *mecA* gene (Fig. 1A).

Activities of ciprofloxacin and gatifloxacin against 56 isolates were determined by the E-test. Although, 56 isolates (100%) were resistant to ciprofloxacin, 26 isolates (46%) were susceptible to gatifloxacin. The range of MICs for ciprofloxacin was 6 to ≥32 mg/l and for gatifloxacin was 0.1 to 6 mg/l (Table 1).

To identify the mutations causing FQ resistance, the quinolone resistance-determining regions (QRDRs) of *grlA* and *gyrA* genes of 56 MRSA isolates were amplified, following the agarose gel electrophoresis, the expected 469 bp *grlA* and 398 bp *gyrA* amplimers were visualized (Fig. 1B and Fig. 1C). DNA fragments were then recovered and sequenced for identification of point mutations. As shown in Table 1, we observed three types of single-point mutations in *grlA* gene of 56 MRSA isolates (100%). Among these mutations, Ser-80→Phe or Tyr single-point mutation was the principle one, and detected in 46 isolates (82%). The Glu-84→Lys mutation was found in 6 isolates (11%). Of the isolates tested, 54 MRSA (96%) contained two

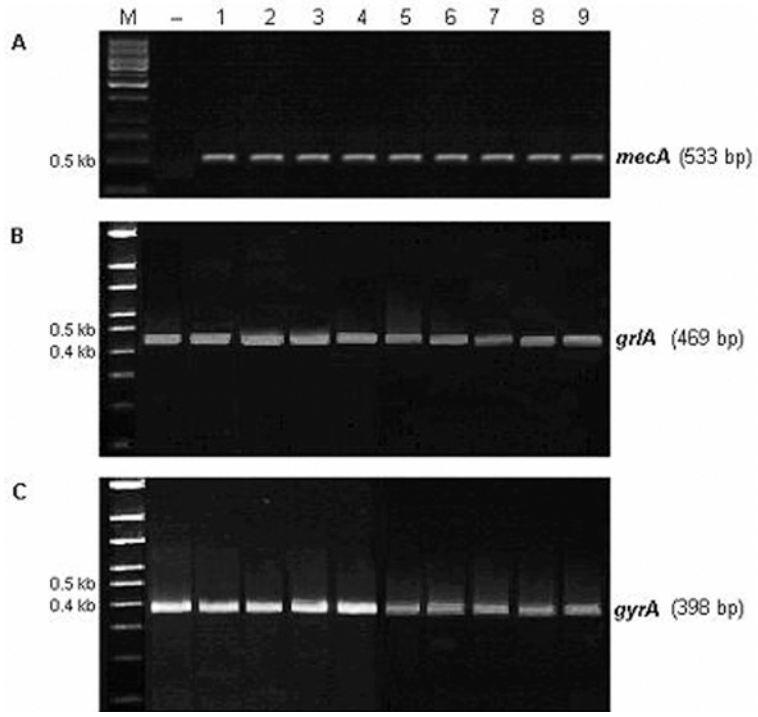

Fig. 1. Agarose gel pictures of the PCR products. A, *mecA* gene; B, QRDR of the *grlA* gene; C, QRDR of the *gyrA* gene; M, molecular size marker; –, MSSA strain; 1-9, test isolates.

types of single-point mutations and one type of double mutation in *gyrA* gene. The single-point mutation of Ser-84→Leu was the most frequent one found in 40 isolates (71%). The Glu-88→Lys mutation was observed in 14 isolates (25%). Additionally the double mutation of Ser84→Leu and Gly106→Asp was detected in 2 isolates.

Susceptibility testing of vancomycin, teicoplanin and linezolid revealed that all ciprofloxacin-resistant MRSA isolates were susceptible to these agents.

Discussion

Nosocomial MRSA infections are challenging current therapeutic options. The prevalence of MRSA differs widely among different countries and there are limited choices of antimicrobial agents to treat infections caused by MRSA (Voss and Doebbeling 1995). To determine the characteristics of nosocomial MRSA in our region, 56 MRSA isolates were collected from inpatients of Süleyman Demirel University Hospital in Turkey during 2003-2006. The sources of the samples included pus, bronchial secretions, blood, sputum and catheters. In order to exclude multiple isolates of MRSA included in the study, the isolates were obtained from different patients and at different times. All of the isolates were characterized as MRSA based on cefoxitin resistance and production of 533 bp PCR fragment of *mecA* gene.

Although the efficiency of old generation FQs in the management of MRSA infections has decreased by time, the newly developed FQs have been reported to exhibit very good activity against Gram-positive cocci, including MRSA. In our study, susceptibility of 56 MRSA isolates to ciprofloxacin and gatifloxacin were determined by E-test and MIC distribution is shown in Table 1. Our results showed that while 100% of MRSA isolates were resistant to the old FQ ciprofloxacin (MIC, 6-≥ 32 mg/l), only 54% of isolates were resistant to the new FQ gatifloxacin (MIC, ≥ 0.5-6 mg/l).

Table 1. Mutations of *grlA* and *gyrA* and the corresponding MICs in MRSA isolates.

Mutations		Total no. of isolates	MIC range (mg/l)		No. of GFLX sensitive isolates
grlA	*gyrA*		CPFX	GFLX	
–	–	1[a]	0.25	0.094	
Ser80Phe	–	2	6–12	0.3– 0.5	2
Ser80Phe	Ser84Leu	22	≥ 32	0.1– 6	4
Ser80Tyr	Ser84Leu	10	≥ 32	0.1– 6	3
Ser80Phe	Glu88Lys	14	≥ 32	0.2– 0.5	14
Glu84Lys	Ser84Leu	6	≥ 32	0.3– 6	2
Ser80Phe	Ser84Leu + Gly106Asp	2	≥ 32	0.3– 4	1

[a]MSSA reference strain (ATCC29213).

Sequencing analysis showed that mutations in the QRDRs of *grlA* and *gyrA* were caused by single-nucleotide changes, conferring variable levels of resistance against two FQs tested (Table 1). In this study, we observed three types of single-point mutations in *grlA* genes of 56 MRSA isolates (100%). Among these mutations, Ser-80→Phe or Tyr single-point mutation was detected in 46 isolates (82%) and Glu-84→Lys mutation was found in 6 isolates (11%). Of the isolates tested, 54 MRSA (96%) contained two types of single-point mutations and one type of double mutation in *gyrA* gene. The single-point mutation of Ser-84→Leu was found in 40 isolates (71%) and Glu-88→Lys mutation was also observed in 14 isolates (25%). The double mutation of Ser84→Leu and Gly106→Asp was detected in 2 isolates. To date, Gly106→Asp mutation has been observed in clinical isolates of *S. aureus* only twice in two studies reported previously (Schmitz et al. 1998; Hooper 1999), but it has not yet been shown to contribute to resistance directly. All mutations detected in this study were identical to those reported for clinical MRSA isolates previously (Schmitz et al. 1998; Wang et al. 1998;Hooper 1999; Sierra et al. 2002; Noguchi et al. 2005). There was no isolate that possessed an amino acid change in *gyrA* in the absence of *grlA* mutation. This observation supported the notion that DNA topoisomerase IV is the primary target of FQs in *S. aureus* (Ng et al. 1996; Drlica and Zhao 1997).

Ciprofloxacin resistance in strains of MRSA is common worldwide, and most of these strains have single mutations in both the *grlA* and *gyrA* genes, rendering them highly resistant to ciprofloxacin (Schmitz et al. 1998; Bell 2002; Noguchi et al. 2005). With respect to ciprofloxacin susceptibility, our MRSA isolates with combined mutations at codons 80/84, 80/88 and 84/84 (in *grlA*/in *gyrA*) had MIC of ≥ 32 mg/l. Our data thus agrees well with the previous findings. In consistent with this observation, two isolates contained the Ser-80→Phe alteration in *grlA*, but no mutation in *gyrA* showed lower-level ciprofloxacin resistance (MICs, 6 mg/l and 12 mg/l).

In comparison to older FQs, new generation fluoroquinolones such as gatifloxacin and moxifloxacin have been reported to have enhanced activity (lower MIC) against Gram-positive pathogens including MRSA. In a study reported by Schmitz et al. (1998), 44.6% of 457 MRSA isolates collected from 13 different European countries including Turkey were susceptible to gatifloxacin. Recently, susceptibility to gatifloxacin was found to be 53% in 100 MRSA isolates from a university hospital from Japan (Noguchi et al. 2005). These susceptibility rates were close to the rate we observed for gatifloxacin. In our study, gatifloxacin was found to be active against 26 of 56 isolates (46%) with mutations at codon 80 or 84 of *grlA* in the presence or absence of concomitant mutations at codons 84 or 88 in *gyrA* (MIC, ≤ 0.5 mg/l). On the other hand, we observed resistance to gatifloxacin, remaining at a comparatively low level (MIC, ≤ 6 mg/l) in 30 gatifloxacin- resistant MRSA isolates (54%) with 80 or 84 alterations in *grlA* and 84 alteration in *gyrA*. The observed diversity of the FQ MICs for isolates with the same mutation combination might be associated with the mutations in the remaining regions of *grlA* and *gyrA*, the alterations in *grlB* and *gyrB*, and the quinolone efflux sytem.

Currently, MRSA infections are treated with vancomycin, teicoplanin or linezolid. In the present study, all MRSA isolates were found to be susceptible to these agents. Similarly, isolates reported from Poland (Matynia et al. 2005) Austria (Presterl et al. 2001), Israel (Samra et al. 2005) and Kuwait (Al Sweih et al. 2005) were also fully susceptible to these antimicrobials.

Among MRSA isolated during 2003-2006, 46% was susceptible to gatifoloxacin. The result suggests that gatifloxacin is still active and might be used to treat MRSA infections that are not life-treatening. However, prior to the application, the use of quantitative methods of susceptibility determination (MIC) is of importance for the appropriate use of gatifloxacin in treatment of MRSA infections and for prevention of further increases in resistance.

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