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Antimicrobial susceptibility, inducible macrolide-lincosamide-streptogramin B, and clonal diversity patterns of nosocomial methicillin-resistant *Staphylococcus aureus* strains isolated in Hacettepe University adult hospital

Aim: Nosocomial infections due to methicillin resistant *Staphylococcus aureus* (MRSA) are an important problem with limited therapeutic options. The aim of this study was to determine the vancomycin, teicoplanin, linezolid, tigecycline, erythromycin, gentamicin, ciprofloxacin, rifampin, trimethoprim/sulfamethoxazole, and clindamycin susceptibility, inducible macrolide-lincosamide-streptogramin B ($iMLS_B$) resistance, and clonal diversity patterns of 109 *mecA* positive *Staphylococcus aureus* strains isolated from patients with nosocomial infections in Hacettepe University Adult Hospital between 2002 and 2004.

Materials and methods: The nosocomial isolates of S. aureus from various clinical samples (58 blood, 45 pus, 6 catheter) were identified by Sceptor (Becton Dickinson, USA) automated system. Polymerase chain reaction (PCR) was performed for the presence of *mecA* gene of the MRSA isolates. The susceptibility to vancomycin, teicoplanin, linezolid, and tigecycline was determined by Etest (AB Biodisk, Sweden) and to the other antibiotics by disk diffusion methods according to the CLSI recommendations. Inducible macrolide-lincosamide-streptogramin B ($iMLS_B$) resistance phenotypes were determined by the double disk method. Pulse field gel electrophoresis (PFGE) was performed to examine the clonal diversity.

Results: All MRSA strains were susceptible to vancomycin, teicoplanin, linezolid, and tigecycline with MIC_{90} (μg/mL) values of 2, 2, 2, and 0.25, respectively. The isolates were highly resistant (≥90%) to gentamicin, ciprofloxacin, and rifampin, whereas the susceptibility to trimethoprim/sulfamethoxazole was 89%, to clindamycin was 62%, and to erythromycin was 46%. $IMLS_B$ resistance was determined among 13% of the MRSA strains. Thirteen different clones were shown by PFGE, whereas 80% of the isolates were in a dominant clone.

Conclusion: Vancomycin, teicoplanin, linezolid, and tigecycline were highly active against nosocomial isolates of MRSA in our hospital. Although these are effective therapeutic options for MRSA, the high rate of cross-contamination of the patients is a matter of concern. We should pay more attention to infection control practices.

Key words: Methicillin-resistant Staphylococcus aureus (MRSA), antibiotic susceptibility, inducible macrolide-lincosamide-streptogramin B (iMLSB), pulse field gel electrophoresis (PFGE)

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Hacettepe Üniversitesi erişkin hastanesi'nde izole edilen hastane enfeksiyonu etkeni metisiline dirençli *Staphylococcus aureus* suşlarının antimikrobiyallere duyarlılık, indüklenebilir makrolid-linkozamid-streptogramin B direnç ve klonal benzerlik durumları

Amaç: Metisiline dirençli *Staphylococcus aureus* (MRSA)'a bağlı gelişen nozokomiyal enfeksiyonlar tedavide az sayıda seçenek olması nedeniyle önemli bir sorun oluşturmaktadır. Bu çalışmada Hacettepe Üniversitesi Erişkin Hastanesi'nde 2002-2004 yılları arasında hastane enfeksiyonu etkeni olarak izole edilen *mecA* geni pozitif 109 *Staphylococcus aureus* suşunun vankomisin, teikoplanin, linezolid, tigesiklin, eritromisin, gentamisin, siprofloksasin, rifampin, trimethoprim-sulfametoksazol

ve klindamisine karşı duyarlılık, indüklenebilir makrolid-linkozamid-streptogramin B ($iMLS_B$) direnç ve klonal benzerlik durumlarının belirlenmesi amaçlanmıştır.

Yöntem ve gereçler: Farklı klinik örneklerden (58 kan, 45 püy, 6 kateter) izole edilen *S. aureus* suşlarının tür düzeyindeki tanımlaması Sceptor (Becton Dickinson, ABD) otomatize sistemi ile yapılmıştır. Otomatize sistemden metisiline dirençli *S. aureus* (MRSA) olarak rapor edilen izolatların *mecA* geni varlığı PZR yöntemiyle gösterilmiştir. Vankomisin, teikoplanin, linezolid ve tigesiklin için duyarlılık testleri Etest (AB Biodisk, İsveç) ile üretici firma önerilerine uygun olarak gerçekleştirilmiştir. Diğer antibiyotiklere karşı duyarlılık disk difüzyon yöntemi ile belirlenip, sonuçlar CLSI kriterlerine göre yorumlanmıştır. $İMLS_B$ direnci çift disk difüzyon, suşlar arasındaki klonal benzerlik 'pulse field gel electrophoresis (PFGE)' yöntemi ile belirlenmiştir.

Bulgular: MRSA izolatlarının vankomisin, teikoplanin, linezolid ve tigesiklin $MİK_{90}$ değerleri sırasıyla 2, 2, 2 ve 0,25 µg/ml ve tümü bu antibiyotiklere duyarlı olarak saptanmıştır. Bununla birlikte tüm izolatlar gentamisin, siprofloksasin ve rifampisine yüksek oranda (≥% 90) dirençli; trimetoprim/sülfametaksazole % 89, klindamisine % 62 ve eritromisine % 46 oranında duyarlı bulunmuştur. MRSA izolatlarının % 13'ünde $iMLS_B$ direnci saptanmıştır. PFGE analizi sonucunda 13 farklı klon saptanmış, izolatların % 80'inin belli bir tek klonda yer aldığı gözlenmiştir.

Sonuç: Vankomisin, teikoplanin, linezolid ve tigesiklin hastanemizde izole edilen MRSA suşlarına karşı yüksek düzeyde aktivite göstermektedir. MRSA enfeksiyonlarının tedavisi için etkin seçeneklerimiz olmasına rağmen, klonal analiz sonuçlarına göre hastalar arasında yüksek oranda çapraz bulaş saptanması endişe uyandırmaktadır. Bu durum enfeksiyon kontrol önlemlerine daha fazla dikkat göstermemiz gerektiğini göstermektedir.

Anahtar sözcükler: Metisiline Dirençli *Staphylococcus aureus* (MRSA), antibiyotiklere duyarlılık, makrolid-linkozamid-streptogramin direnci ($iMLS_B$), pulse field gel electrophoresis (PFGE)

Introduction

Methicillin-resistant *Staphylococcus aureus* (MRSA) is one of the most important causes of nosocomial infections worldwide. It is known to be encoded by the *mecA* gene and is mostly multi-resistant to a wide range of antibiotics (1).

The glycopeptides vancomycin, teicoplanin and an oxazolidinone linezolid have been regarded as the drug of choice for the treatment of MRSA infections (2). However, vancomycin-intermediate (VISA) and -resistant isolates of *S. aureus* (VRSA) have been described in some countries (3). Recently, tigecycline represents an exciting new class of glycylcycline antimicrobial agents for the treatment of multi-drug resistant gram-positive bacteria (4).

This study was conducted to determine the vancomycin, teicoplanin, linezolid, tigecycline, as well as erythromycin, gentamicin, ciprofloxacin, rifampin, trimethoprim/sulfamethoxazole, and clindamycin susceptibility, inducible macrolide-lincosamide-streptogramin B ($iMLS_B$) resistance, and clonal diversity patterns of *mecA* positive *S. aureus* strains isolated from patients with nosocomial infections in Hacettepe University Adult Hospital between 2002 and 2004.

Methods

Strains

All MRSA isolates from clinical samples of inpatients with nosocomial infections at Hacettepe University Adult Hospital between 2002 and 2004 were included in the study. Only one isolate from each patient was included in the study. The identification of the isolates was made by Sceptor automated system (Becton Dickinson, USA) and methicillin resistance was confirmed by the presence of the *mecA* gene by polymerase chain reaction (PCR), which is considered the 'gold standard' in the determination of heterogeneous resistance. Pulse field gel electrophoresis (PFGE), which is the 'gold standard' as a genotypic typing method, was performed to examine the clonal diversity. The isolates were stored at -80 °C until studied. *S. aureus* ATCC 29213, *S. aureus* 27R, and *S. aureus* 8328 were included as control strains.

Antibiotic susceptibility tests

Susceptibility testing against erythromycin (15 µg), gentamicin (10 µg), ciprofloxacin (5 µg), rifampin (5 µg), trimethoprim/sulfamethoxazole (TMP-SMX, 1.25/23.75 µg), and clindamycin (2 µg) disks (Oxoid, UK) was performed on Mueller Hinton agar (MHA) (Oxoid, UK) incubated at 35 °C for 12-16 h. In addition, Etest strips (AB Biodisk, Sweden) were used to test the susceptibility against vancomycin, teicoplanin, linezolid, and tigecycline with an inoculum of McFarland 2.0 onto brain heart infusion agar (BHIA) (Oxoid, UK) according to the manufacturer's recommendations. The results were interpreted according to CLSI criteria (5). Moreover, inducible and constitutive macrolide-lincosamide-streptogramin B ($iMLS_B$) resistance phenotypes were determined by double disk method with erythromycin and clindamycin disks placed at a distance of 15-20 mm. After 18 h of incubation at 37 °C, blunting of the clindamycin zone of inhibition near the erythromycin disk indicated $iMLS_B$ resistance and resistance to both erythromycin and clindamycin indicated constitutive MLS_B resistance (6). For tigecycline, interpretive criteria from the US Food and Drug Administration packaging insert were applied as susceptible ≤0.5 µg/mL (7).

PCR

The isolation of bacterial DNA was performed as described previously (8). Amplification was performed in a mixture consisting of 50 mM Tris (pH 8.3), 50 mM KCl, 2.5 mM $MgCl_2$, 0.2 mM of deoxyribonucleotide triphosphates (dATP, dGTP, dCTP, and dTTP), 50 pmol of each primer, 0.5 U Taq DNA Polymerase (MBI Fermentas, USA), and 5 mL of staphylococcal DNA extract. The sequences of the primers *mecA*1 and *mecA*2 were 5' GTT GTA GTTB GTC GGG TTT GG 3' and 5' CCA CCC AAT TTG TCT GCC AGT TTC 3', respectively. The amplification was performed in a Flexigene (Techne, Cambridge, UK) thermalcycler by a program consisting of an initial denaturation step at 94 °C for 4 min; this was followed by 30 cycles of 94 °C for 30 s, 55 °C 30 s, 72 °C 2 min, and 72 °C for 5 min (9). PCR products were then separated by electrophoresis in 1.5% agarose gels with 1× Tris-acetate EDTA (TAE) running buffer (40 mM Tris-acetate, 1 mM EDTA) and visualized on a UV transilluminator and photographed. The molecular weight standard was ϕx174 (MBI Fermentas, USA). Isolates with the *mecA* genotype showed a 1817 bp band.

PFGE

MRSA DNA embedded in agarose blocks was prepared as described by Lencastre (10). The DNA fixed in the agarose disk was incubated for 20 h in 45 µL of restriction buffer and Sma I (20 U). The reaction was stopped with 5 µL of loading buffer. Gels with 1.1% agarose (Genexis Spech bach, Germany) were prepared in 0.5 × TBE buffer (50 mM boric acid, 0.2 mM EDTA). PFGE was carried out by General Navigator, Pharmacia (Uppsala, Sweden). The running conditions were performed for 22 h at 14 °C at 150 V. Pulse times were 20 s for 15 h, 35 s for 7 h, 50 s for 15 h, and 90 s for the last 3 h.

Results

A total of 109 MRSA isolates from different clinical samples (58 blood, 45 pus, 6 catheter) were included in the study. The methicillin resistance of all *S. aureus* isolates included in the study was confirmed by detection of the *mecA* gene. All isolates were multi-drug resistant (≥3 groups of antibiotics). They were highly resistant (≥90%) to the antimicrobials gentamicin, ciprofloxacin, and rifampin, whereas the susceptibility to TMP-SMX was 89%, to clindamycin was 62%, and to erythromycin was 46%. Of the 68 clindamycin-susceptible strains, 14 had $iMLS_B$ resistance. Overall 13% of the isolates had $iMLS_B$ resistance. All MRSA strains were susceptible to vancomycin, teicoplanin, linezolid, and tigecycline with MIC_{90} (µg/mL) values of 2, 2, 2, and 0.25 respectively (Table).

PFGE analysis of genomic DNA from 109 isolates revealed 13 different clones (Figure). One epidemic clone (clone A) was identified for 87 isolates (79.8%). Six other clones contained 5, 3, 2, 2, 2, and 2 isolates, respectively. There were also 6 different sporadic clones each contained a single isolate.

Discussion

MRSA is one of the leading causes of nosocomial infections worldwide. In a multicenter study, the prevalence of MRSA infections was reported to be

Table. Antimicrobial resistance of 109 nosocomial MRSA strains isolated in Hacettepe University Adult Hospital between 2002 and 2004.

Antimicrobial agent	Resistance (%)
Clindamycin	38*
Erythromycin	54
TMP-SXT	11
Rifampin	90
Gentamicin	93
Ciprofloxacin	95
Vancomycin	-
Teicoplanin	-
Linezolid	-
Tigecycline	-

* Although 62% of the strains were susceptible to clindamycin, 13% had $iMLS_B$ resistance

>40% in southern and western European hospitals (11). The prevalence of methicillin resistance among nosocomial *S. aureus* isolates was 70% in Hacettepe University Adult Hospital between 2000 and 2004 (12, Unpublished data). According to PFGE analysis, a dominant clone was prevalent in our hospital, which is a serious concern for an effective infection control program. Contact isolation is performed for patients infected with MRSA in our hospital. All intensive care units are visited daily by an infection control nurse. There are reminder posters about the infection control measures on the walls and cautionary cards are placed at the bedsides of the patients who are infected with MRSA. Alcohol-based hand rinses are available at the bedsides of patients infected with MRSA in wards and regardless of infection with MRSA at the ICU. Since MRSA colonization often precedes infection there is great interest in decolonizing persons who harbor these bacteria. The most extensive research in MRSA decolonization has been conducted with mupirocin, which is applied to the anterior nares 2-3 times/day for 5 days. Routine decolonization is not prudent unless MRSA colonization is confirmed in the nares or other site. Screening for MRSA colonization is not a routine infection control practice in our hospital and we do not use mupirocin for decolonization. The compliance to infection control practices such as hand hygiene and isolation precautions is poor in our hospital, mainly due to the overcrowding of patients and the inadequate number of medical staff.

The high rates of resistance to ciprofloxacin, rifampin, and gentamicin were significant. Similar results had been reported from a different center in Turkey, recently (13). The susceptibility rate of MRSA strains to TMP-SMX is 89% in this study. Moreover, Samra et al. reported the TMP-SMX susceptibility of nosocomial MRSA strains as 86% (14).

Although 62% of the strains were susceptible to clindamycin, the high rate of erythromycin resistance raised the question of inducible resistance and $iMLS_B$ resistance was determined in 13% of MRSA isolates. Different resistance rates have been reported from

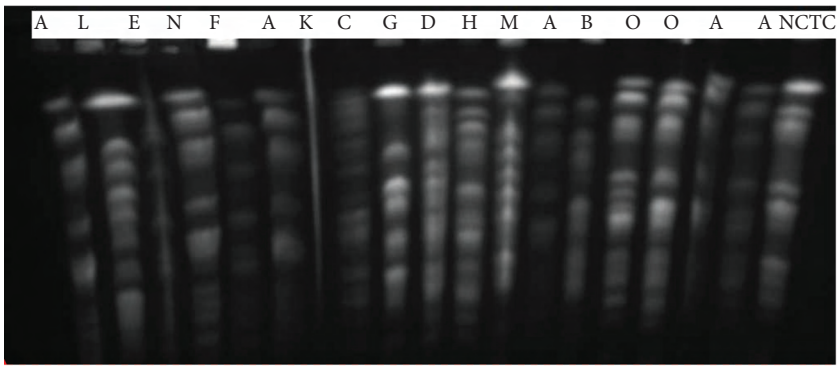

Figure. PFGE analysis of different clonal patterns of MRSA isolates.
Lanes 1, 6, 13, 17, 18 pattern A, lane 2 pattern L, lane 3 pattern E, lane 4 pattern N, lane 5 pattern F, lane 7 pattern K, lane 8 pattern C, lane 9 pattern G, lane 10 pattern D, lane 11 pattern H, lane 12 pattern M, lane 16 pattern B, lanes 15, 16 pattern O, lane 19 NCTC isolate.

different geographical regions for $iMLS_B$ resistance. Delialioglu et al. reported 5.4% inducible phenotype among 128 MRSA strains from Turkey (15). Inducible clindamycin resistance has been reported in 5.6% of 108 MRSA isolates from Başkent University Hospital, Turkey (16). However, in a study from Maryland, USA, that examined 161 clinical MRSA isolates for $iMLS_B$ resistance, 56% of the strains harbored $iMLS_B$ resistance (17), and similar high rates were also reported from India (18). These results clearly indicate that, based on institution and geographic region, the prevalence rates of $iMLS_B$ phenotypes vary. The reason for this is not clear and it is a good subject for further research. Vancomycin, teicoplanin, linezolid, and tigecycline had very good activity against MRSA isolates in this study. Glycopeptides are recommended therapeutic options for MRSA infections, but acquired resistance to vancomycin in staphylococci has become known in recent years and many clinicians are concerned about the significance of acquired resistance that could show a rapid progress in clinical use (19-24). Furthermore, teicoplanin resistant *S. aureus* has been reported from the Mediterranean region (25,26). There has been no VRSA report in Turkey, but there was a report of VISA strains isolated from various clinical samples in 2005 (27). Amongst 256 MRSA isolates 46 had reduced susceptibility to vancomycin. Twelve of the 46 patients with hetero-VISA had a history of previously treatment with vancomycin or teicoplanin. In another study, 1 (1.2%) out of 81 *S. aureus* isolates had intermediate resistance to teicoplanin (MIC 16 mg/L), but VISA was not detected. Six strains of *Staphylococcus haemolyticus* (13%) out of 54 coagulase negative staphylococci (CoNS) were detected to have heterogeneously reducing susceptibility to vancomycin (MICs ranged between 5 and 8 mg/L) (28). In this study we did not examine the heterogeneous resistance to glycopeptides. Besides resistance, the vancomycin MIC may have important consequences on the efficacy of this antibiotic and on mortality in patients with bacteremia due to MRSA. Mortality associated with MRSA bacteremia was significantly higher when vancomycin was empirically used for treatment of infection with strains with a high vancomycin MIC (>1 mg/mL) (29).

Gram-positive cocci are rarely resistant to linezolid. A recent study examined the linezolid susceptibility of 1930 isolates of MRSA collected from the different regions of the United States; 99.9% were susceptible to linezolid (30). Two clinical isolates of MRSA resistant to linezolid have been reported from different parts of the world (31,32). There are 2 other reports about linezolid susceptibility of MRSA strains in Turkey. The first study includes 127 MRSA strains and the second includes 38, and all are susceptible to linezolid (33,34). Clinical outcomes are significantly better with linezolid than with vancomycin in several indications (35,36). The efficacy and safety of linezolid and vancomycin were compared for the treatment of nosocomial pneumonia, complicated skin and soft-tissue infections, or sepsis caused by MRSA infections in Japan, recently. One hundred patients received linezolid and 51 received vancomycin with outcomes evaluated at the end of therapy (EOT). At EOT, clinical success rates in the MRSA microbiologically evaluable population were 62.9% and 50.0% for the linezolid and vancomycin groups, respectively; and microbiological eradication rates were 79.0% and 30.0% in the 2 groups, respectively ($P \leq 0.0001$). Reversible anemia (13%) and thrombocytopenia (19%) were reported more frequently in linezolid patients (37).

Tigecycline, a new glycylcycline antibiotic, has shown promising in vitro activity against many common pathogens, including MRSA. The MIC90 of tigecycline against *S. aureus* was 0.25 μg/mL in a study performed at 40 study centers in 11 countries (38). Tigecycline was shown to be non-inferior to combination vancomycin-aztreonam regimens and exhibited high clinical success rates for complicated soft tissue infections. MIC90 values for tigecycline were uniformly low for both susceptible and resistant pathogens. Adverse events were similar in incidence for both patient populations, with nausea and vomiting reported more frequently with tigecycline treated patients while rash and elevated liver transaminases were most commonly observed in the vancomycin-aztreonam treatment group (39). A randomized (3:1), double-blind, multicentre, phase 3 study compared the safety and efficacy of tigecycline with vancomycin in hospitalized patients with MRSA.

Clinical cure rates in the microbiologically evaluable population (n = 117) were 81.4% (70 of 86 patients) with tigecycline and 83.9% (26 of 31 patients) with vancomycin (40).

In conclusion, glycopeptides and linezolid are effective drugs for MRSA infections in our hospital. Moreover, tigecycline is a promising alternative for the treatment of MRSA infections. Even though there are effective therapeutic options, surveillance of resistance should guide the selection of empirical therapy and continuous attention should be paid to infection control measures.

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