Detection of *mec*A gene in clinical *Staphylococcus aureus* isolates from Infectious Diseases Hospital, Iasi, Romania

Detectarea genei *mecA* la tulpini de *Staphylococcus aureus* izolate în Spitalul de Boli Infecţioase, Iaşi, România

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Abstract

Introduction. *Since it was first described in 1961, methicillin-resistant Staphylococcus aureus (MRSA) has been an increasingly important cause of health care-associated infections worldwide. During the 1990s, new strains of MRSA emerged and caused community-associated infections on multiple continents.* ***Methods.*** *We have investigated 151 clinically significant S. aureus strains isolated from blood cultures, cerebro-spinal fluid, pus, sputum samples collected from patients with infections admitted to the Infectious Diseases Hospital "Sf. Parascheva" Iaşi, Romania, between January 2008 and December 2009. We have performed real-time PCR for mecA gene detection, disk diffusion susceptibility testing and minimum inhibitory concentration determination to anti-staphylococcal antibiotics for all isolates.* ***Results.*** *The oxacillin resistance rate was 45.7%. All MRSA strains were susceptible to vancomycin, linezolid and teicoplanin; however, they exhibited high rates of resistance to erythromycin (70.5%), tetracycline (67.6%), gentamicin (48.5%) and ciprofloxacin (44.1%). Susceptibility rate to trimethoprim/sulfamethoxazole in MRSA strains was 89.7%. Almost a third of the tested strains had oxacillin MICs >256 µg/ml. For 90% of the strains vancomycin MICs was 2 µg/ml.* ***Conclusions.*** *Glycopeptides remain the first choice therapy for MRSA infections. To preserve their value, their use should be limited to those cases where they are clearly needed.*

Key words*: Staphylococcus aureus, mecA, resistance*

Rezumat

Introducere. *Staphylococcus aureus meticilino-rezistent (SARM) este o cauză cu importanţă crescândă a infecţiilor asociate îngrijirilor medicale în toată lumea. În cursul anilor 1990, noi tulpini de SARM au fost emergente şi au produs infecţii comunitare pe toate continentele.* ***Metode.*** *Am investigat 151 tulpini de S. aureus cu semnificaţie clinică de infectant, izolate din produse patologice (hemoculturi, lichid cefalo-rahidian, puroi, spută) de la pacienţi internaţi în Spitalul Clinic de Boli Infecţioase "Sf. Parascheva" din Iaşi, România, între 1.01.2008 şi 31.12.2009. Pentru toate izolatele am efectuat real-time PCR pentru detecţia genei mecA, antibiograma difuzimetrică şi determinarea concentraţiei minime inhibitorii (CMI) pentru antibiotice*

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antistafilococice. ***Rezultate.*** *45,7% dintre tulpini au fost rezistente la oxacilină. Toate tulpinile SARM au fost sensibile la vancomicină, linezolid şi teicoplanină; totuşi, au prezentat rate mari de rezistenţă la eritromicină (70,5%), tetraciclină (67,6%), gentamicină (48,5%) şi ciprofloxacin (44,1%). 89,7% dintre tulpinile SARM au fost sensibile la trimetoprim/sulfametoxazol. Aproape o treime din tulpinile testate au avut CMI pentru oxacilină >256 µg/ml. Pentru 90% dintre tulpini CMI pentru vancomicină au fost 2 µg/ml.* ***Concluzii.*** *Glicopeptidele rămân antibiotice de primă intenţie în infecţiile cu SARM. Pentru a le conserva valoarea, utilizarea lor trebuie limitată la cazurile în care sunt cu adevărat necesare.*

Cuvinte cheie: *Staphylococcus aureus, mec A, rezistenţă*

Introduction

Staphylococcus aureus is a major human pathogen causing a wide spectrum of diseases from local benign skin infections to severe life-threatening conditions (1, 2). There is enormous variation between strains of *S. aureus*. Acquisition and loss of mobile genetic elements often encode virulence and resistance genes. Selection of the fittest bacteria is likely being driven by human host factors and antibiotic use, and new strains of *S. aureus* are emerging that are increasingly virulent and resistant to antibiotics, causing novel healthcare issues.

Over the last decades, methicillin-resistant *S. aureus* (MRSA) strains have emerged as serious pathogens in the nosocomial and community setting. MRSA are endemic in most hospitals in many countries and they are considered the most serious hospital-acquired pathogen as they can cause large outbreaks that are frequently difficult to treat using antibiotics (3, 4). Endemic strains of MRSA carrying multiple resistance determinants have become a worldwide nosocomial problem only in the early 1980's, carrying a threefold attributable cost and a threefold excess length of hospital stay when compared with methicillin-susceptible *S. aureus* (MSSA) bacteraemia. Hospitalization costs associated with MRSA infections are substantially greater than those associated with MSSA infections, and MRSA has wider economic effects that involve indirect costs to the patient and to society. In addition, there is some evidence suggesting that MRSA infections increase morbidity and the risk of mortality (5).

The genetic backgrounds of community-acquired MRSA strains vary geographically but are distinct from those of MRSA strains that were established as health-care pathogens in the corresponding regions. This situation suggests that the new strains arose independently in different geographic areas via acquisition of methicillin resistance by strains of *S. aureus* circulating in the community (6). Despite their disparate genetic backgrounds, MRSA strains that emerged in community settings worldwide share some common characteristics. For example, they tend to be susceptible to most classes of antimicrobial agents other than beta-lactams, unlike previously described MRSA strains (7). This finding can be explained in part by differences in the mobile genetic element termed "staphylococcal cassette chromosome *mec*" (SCC*mec*), which contains the methicillin-resistance (*mec*A) gene (8).

Methicillin resistance in *S. aureus* is caused by the acquisition of the *mecA* gene encoding a beta-lactam low-affinity penicillin-binding protein (PBP), termed PBP2a. PBP2a substitutes for the essential functions of the high-affinity PBPs in the presence of beta-lactam antibiotics, hence rendering the bacteria resistant to this general and important class of antimicrobials (9).

Vancomycin and teicoplanin are glycopeptid antibiotics used in MRSA infections, but which are less active than oxacillin against staphylococci susceptible to methicillin. Efficacy of vancomycin against staphylococci is inversely correlated with MIC. MIC should be determined in severe infections and a seric concentration of vancomycin of 8 times the MIC should be reached as a target value (10). Unstable heteroresistance is common among clinical isolates of MRSA and may represent a cause of therapeutic failure (11).

Recent genetic advances have enabled identification and characterization of clinical iso-

Table 1. Primer and probe sequences (12)

Primer and probe	Sequence (5′→3′)	Reaction concentration (µM)
*mec*A For	GGCAATATTACCGCACCTCA	0.30
*mec*A Rev	GTCTGCCACTTTCTCCTTGT	0.30
*mec*A Probe	AGATCTTATGCAAACTTAATTGGCAAATCC	0.10

lates in real-time. Real-time PCR (RT-PCR) has established itself as a sensitive and specific qualitative and quantitative technique that has become important to all areas of microbiology. In RT-PCR, amplified products are detected by fluorescence at the moment they are generated. This technique is also attractive for understanding the epidemiology of MRSA and the relationship between genome content and virulence.

The objectives of this study were: 1. to evaluate, using a RT-PCR technique, the prevalence of *mecA* gene in *S. aureus* strains isolated from infections in patients from North East Romania hospitalized in Infectious Diseases Hospital "Sf. Parascheva" Iaşi, Romania; 2. to evaluate the antibiotic resistance rate in the same strains.

Material and methods

Clinical strains. We have investigated 151 non-duplicate clinically relevant *S. aureus* strains, isolated from blood-cultures, cerebrospinal fluid, pus or sputum specimens from patients admitted between January 2008 and December 2009 to the Infectious Diseases Hospital "Sf. Parascheva" Iaşi, Romania. This is a 300 beds teaching hospital caring for patients from North East Romania, including patients with infections acquired in other medical settings. Our hospital has an Intensive Care Unit (ICU) and a ward for HIV-infected patients. The strains were considered infectant based on microbiological criteria: isolation from normally sterile sites, association with inflammatory cells in microscopy and predominant isolation, in high amount.

Real-time PCR. We have retrospectively performed RT-PCR for *mec A* gene detection for all *S. aureus* isolates, using a single-target Taq-Man PCR assay. The RT-PCR assay was performed with genomic DNA extracts from broth culture lysates of the 151 clinical isolates and the reference strains *S. aureus* ATCC 25923 and *S. aureus* ATCC 33592, using *GenElute Bacterial Genomic DNA kit* (Sigma Aldrich, Germany).

The 20 µl PCR mixture contained: 10µl of 2× *HotStar Taq Master Mix kit* (Finnzymes, Finland), 4µl *mecA* specific primers, 0.2µl dual-labeled *mecA* probe with FAM as the 5′ reporter and 3′ TAMRA as the quencher, 2 µl of DNA sample, used as the target in the PCR and 3.8 µl PCR water. Primer and probe sequences for *mecA* gene are described in the *Table 1*. The set of primers was previously reported by McDonald *et al.* (12). Reactions were performed in triplicate, under the following thermal cycling conditions: 7 min at 95°C, followed by 40 cycles of 95°C for 15 s and 60°C for 60 s. Data for the PCR assay were collected using a Stratagene MX 3005P RT-PCR system (*Figure 1*).

The amplification conditions were optimized using reference strains *S. aureus* ATCC 25923 (MSSA) and *S. aureus* ATCC 33592 (MRSA).

Antimicrobial susceptibility testing. The strains were tested for antibiotic susceptibility using disk diffusion test according to CLSI guidelines (2008). *S. aureus* ATCC 25923 was used as control. Oxacillin susceptibility was assessed using cefoxitin disks (30 µg). Other tested antibiotics included: penicillin, trimethoprim/ sulfamethoxazole, erythromycin, clindamycin, gentamicin, tetracycline, doxycycline, ciprofloxacin, levofloxacin, rifampicin, chloramphenicol, linezolid, teicoplanin, vancomycin (Oxoid, UK). Inducible resistance to clindamycin was detected using double disk approximation test (D-test) at 15 mm disk separation.

E-test. Minimum inhibitory concentrations (MIC) to oxacillin and vancomycin were determined with E-test strips (bioMérieux, France). *S. aureus* ATCC 29213 was used as control.

Statistical analysis. Statistical analysis was performed using EPIINFO 2005. For data comparison chi square test was used; $p \leq 0.05$ was considered statistically significant.

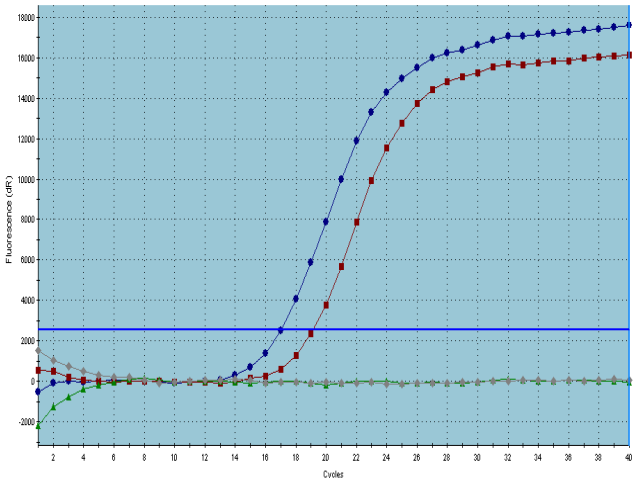

Figure 1. Real-time PCR for detection of *mecA* gene in strains isolated from blood-culture. Amplification plots for strain 113, Ct = 19 (red line); positive control, *S. aureus* ATCC 33592, Ct = 17 (blue line); negative control, *S. aureus* ATCC 25923 (grey line) and non template control, NTC (green line). Ct = cycle threshold.

Figure 2. Etest for vancomycin (MIC=1.5µg/ml) and oxacillin (MIC=8µg/ml, heteroresistance) for *S. aureus* strain 113 isolated from blood-culture.

Results

The 151 investigated strains were isolated from: blood-cultures (44), cerebrospinal fluid specimens (11), pus (74) and sputum (22). 72 strains were isolated in 2008 and 79 strains in 2009. There were 115 (76.1%) community-acquired *S. aureus* infections and 36 (23.8%) hospital-acquired ones. Some hospital-acquired infections were associated with surgery (6 cases), mostly brain surgery (5 cases) or indwelling devices (6 cases). Twenty-three (15.2%) patients have been hospitalized in ICU; only 2 (1.3%) of the patients were HIV-infected.

Detection of oxacillin resistance by disk diffusion test (*Figure 2*) yielded 1 false-positive and 1 false-negative result comparing with detection of *mecA* gene (sensitivity 98.5% and specificity 98.7%). There was 100% concordance between detection of *mecA* gene and phenotypic assessment of methicillin resistance by MIC detection. The oxacillin resistance rate in our study was 45.7% (69 of 151 *S. aureus* strains were *mecA* positive): 35/72 (48.6%) in 2008 and 34/79 (43.0%) in 2009; statistically insignificant difference, $p = 0.675$. The highest prevalence of MRSA strains was among sputum samples (81.8%) and the lowest among strains isolated from systemic infections (34.1%); this was a statistically significant difference, $p = 0.037$. There were 43 (37.4%) MRSA among community-acquired *S. aureus* strains and 26 (72.2%) MRSA among hospital-acquired ones (statistically significant difference, $p = 0.034$).

Oxacillin MICs varied between 0.06 and > 256 µg/ml. MIC_{50} was 1 µg/ml, and MIC_{90} > 256 µg/ml. We emphasize that 29.8% of the tested strains had oxacillin MICs > 256 µg/ml; 10% of the strains were heteroresistant to oxacillin.

Vancomycin MICs varied between 1-2 µg/ml for all types of clinical specimens. MIC_{50} was 1 µg/ml, and MIC_{90} 2 µg/ml. We

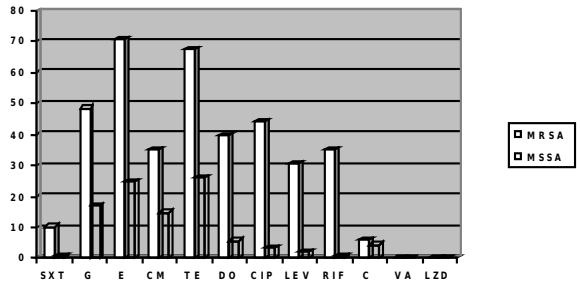

Figure 3. Antibiotic resistance (%) of MRSA (69 strains) versus MSSA (82 strains).
SXT = trimethoprim/sulfamethoxazole, G = gentamicin, E = erythromycin, CM = clindamycin, TE = tetracycline, DO = doxycycline, CIP = ciprofloxacin, LEV = levofloxacin, RIF = rifampicin, C = chloramphenicol, VA = vancomycin, LZD = linezolid.

haven't detected any resistant strains to vancomycin.

We have detected statistically significant differences ($p<0.05$) in susceptibility of MRSA *versus* MSSA strains to gentamicin, erythromycin, clindamycin, tetracycline, doxycycline, ciprofloxacin, levofloxacin, rifampicin (*Figure 3*).

Although resistance to clindamycin is more frequent among MRSA strains than MSSA ones (35.3% *versus* 14.7%, $p<0.05$), inducible resistance to clindamycin was detected more often in erythromycin-resistant MSSA strains (59.0%) than in erythromycin-resistant MRSA strains (50.0%).

Discussion

S. aureus is a well adapted human pathogen, capable of living freely in the inanimate environment and spreading from person to person, existing as a colonizer or commensal, hiding in intracellular compartments and, most importantly, inducing various forms of human disease. Infections caused by *S. aureus*, above all by antibiotic-resistant strains, have reached epidemic proportions globally. The overall burden of staphylococcal disease caused by antibiotic-resistant *S. aureus*, particularly by the methicillin-resistant strains, is increasing in Romania, in both healthcare and community settings.

From 2004 to 2005, 60%-72% of invasive *S. aureus* isolates from Romanian hospitals were MRSA, the highest frequency for any European nation (13). We found a significantly lower value for MRSA prevalence (34.1%) in strains isolated from systemic infections. There was a 38.4% (56/146) MRSA rate in *S. aureus* strains isolated during 2004-2005 in the County Hospital Braşov (Romania). All MRSA were resistant to tetracycline, but susceptible to vancomycin. Inducible clindamycin resistance was detected in 23/28 erythromycin-resistant isolates. Molecular typing identified 15 clonal backgrounds, only 4 of which were associated with MRSA (CC 1, 8/239, 30, and 80). Both hospital-associated (*SCCmec* type III) and community-associated (*SCCmec* type IV) elements were identified within MRSA strains, whereas Panton-Valentine leukocidin was detected in 10 MRSA and 12 methicillin-sensitive *S. aureus* strains (13).

During 2004-2005, 49.9% MRSA strains were detected among 423 strains from infected or colonized patients hospitalized in the intensive care and surgical units in a Romanian teaching hospital from Tg. Mureş. Most of them were multiresistant. One of the MRSA genotypes identified by PFGE was commonly recovered from patients treated in the intensive care unit (14). MRSA rate in Intensive Care Units in Timişoara was 50-60% during 2005-2007 (15). In the same study, data registered in ambulatory in the South-West part of Romania during 2006-2007 showed 26% MRSA strains.

The prevalence of MRSA in patients from Infectious Diseases Hospital Iaşi was 47.1% in 2006, with MICs ranging from 0.064 to >256 μg/ml. High resistance rates among *S. aureus* strains were found for erythromycin (81%), gentamicin (91%), and tetracycline (91%) (16).

The widespread use of antibiotics has undoubtedly accelerated the evolution of *S. aureus*, which, acquiring multiple resistance genes, has become able to survive almost all antibiotic families. An increasing proportion of *S. aureus* infections are caused by MRSA. Its prevalence found in our study (45.7%) was similar to other areas in Romania. We

have detected a 34.1% MRSA rate in systemic infections, higher than in most European countries. The lowest prevalence of invasive MRSA in Europe is found in Northern countries, less than 5%. Central European countries show methicillin resistance rates up to 25%, while in Mediterranean countries this is more than 25% (17). MRSA comprises 27.0% of all *S. aureus* isolates in Canadian hospitals; genotypically, 68.8% of MRSA were health care associated and 27.6% were community associated (18). Reiter *et al*. (19) have detected 35.0% methicillin resistance rate in *S. aureus* strains, but 44.5% in patients with cystic fibrosis.

Treatment of infections caused by this organism is challenging, since MRSA strains are associated with high prevalence of resistance to other antibiotics. In our study, the antibiotic susceptibility pattern showed increased levels of resistance to erythromycin, tetracycline, clindamycin, ciprofloxacin and gentamicin. Similar resistance levels were detected by other authors (13, 14, 20, 21). MRSA strains were more often associated with multiple resistances.

Vancomycin has become the standard therapeutic agent against MRSA strains, for which the choice of treatment is limited by the accumulation of a number of other antibiotic resistance markers acquired during recent evolution of the staphylococcal genome. In many countries the increasing spread of heteroresistant vancomycin-intermediate *S. aureus* (hVISA) and vancomycin intermediate (VISA) strains adds new problems, in terms of the treatment of severe infections sustained by these microorganisms (22, 23). In this study we haven't detected any strains with altered susceptibility to glycopeptides, but the standard vancomycin E-test that we have used has low sensitivity in detection of these strains, 57-75% (24, 25, 26). Vancomycin remains an excellent option for the treatment of MRSA infections. Newer drugs available for treatment of resistant Gram-positive bacterial infections include linezolid, daptomycin, tigecycline and telavancin (22).

Detection of *mecA* gene by RT-PCR allowed a more accurate assessment of methicillin resistance. Yet, price may be a limiting factor. High sensitivity and specificity of disk diffusion method recommends it for the detection of oxacillin resistance in clinical laboratories that cannot afford molecular techniques.

Conclusions

High prevalence of MRSA strains and ability of *S. aureus* to gain resistance to any antibiotic used in therapy impose monitoring of antibiotic susceptibility spectrum mainly for strains isolated from severe infections. Detection of methicillin resistance by RT-PCR yields more accurate results than disk diffusion method and allows better optimization of antibiotic therapy in hospitals. Further studies for the rapid direct detection of *mecA* gene in clinical specimens are needed. This method can also be used in the patient screening at hospital admission for MRSA colonization in order to prevent hospital transmission and decrease the rate of hospital-acquired *S. aureus* infections.

Glycopeptides remain the first choice therapy for MRSA infections. To preserve their value, their use should be limited to those cases where they are clearly needed. Other therapy options include linezolid and trimethoprim/sulfamethoxazole.

In order to prevent and control MRSA outbreaks successfully, restrictive antibiotic policy must be followed with strict infection prevention measures.

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Disclosure of Conflict of Interests. The authors declare that they have no conflict of interest.

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