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HIGH PREVALENCE AND RESISTANCE PATTERNS OF COMMUNITY-ASSOCIATED METHICILLIN-RESISTANT *STAPHYLOCOCCUS AUREUS* IN THE POMORAVLJE REGION, SERBIA

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With a view to estimating the prevalence and resistance patterns of CA-MRSA in one region of Serbia, we performed an analysis of MRSA isolates from healthy people and hospitalised patients. The detection of CA-MRSA was carried out by SCC*mec* typing. In MRSA isolates from hospitalised patients SCC*mec* types IV and V were found in 76% of the strains. Similar percentage (80%) of CA-MRSA genotypes was present in healthy people. SCC*mec* type V harbouring MRSA was the most successful clone. Higher prevalence of type V in hospitalised patients to that in healthy people (70% vs 54%) may indicate nosocomial transmissions in at least some hospital units. All MRSA strains from hospitalised patients were resistant to one or more non-β-lactam antibiotics while 52% were multi-resistant. In isolates from healthy people, 16% were sensitive to all non-β-lactam antibiotics and 40% were multi-resistant. Similar percentage of multi-resistant CA- and HA-genotypes occurred in a particular environment (53% vs 50% in hospitalised patients, and 37.5% vs 37.5% in healthy people) indicating selective pressure of antibiotics as a leading force conferring antibiotic resistance. High prevalence of CA-MRSA and high resistance rate both in hospitals and the community suggest that this pathogen has been present in the Pomoravlje Region, central Serbia for years.

Keywords: CA-MRSA, SCC*mec* typing, resistance patterns, healthy people, hospitalised patients

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Introduction

Methicillin-resistant *Staphylococcus aureus* (MRSA) is highly prevalent human pathogen that causes nosocomial infections worldwide. Initially, MRSA strains were isolated only from hospitalised patients (healthcare-associated MRSA, HA-MRSA). In the last decade of the 20th century, epidemiologically and genetically distinct strains known as community-associated MRSA (CA-MRSA) occurred in patients without previous contact with healthcare institutions, most often in children [1]. Since then, outbreaks of CA-MRSA have been reported on all continents, including areas with otherwise low prevalence of MRSA, such as Scandinavian countries [2, 3]. A trend of increase in the number of MRSA infections and asymptomatic carriage has been demonstrated. According to the data published in 2013, the highest prevalence of MRSA in healthy patients across nine European countries was reported in Belgium, 2.1% [3]. In Asia, the rate of MRSA in all community-associated *S. aureus* infections ranges from 2.5% to 39% [4]. The consequences of CA-MRSA infections are often severe, including prolonged hospitalisation and high mortality rates [1, 5, 6].

MecA gene, which encodes PBP2a and provides the resistance to β-lactams, is located on the "Staphylococcal Cassette Chromosome *mec*" (SCC*mec*) genetic element. Most CA-MRSA strains possess SCC*mec* types IV or V. These elements are smaller than those found in HA-MRSA, and are more easily transferred to other strains of *S. aureus* [7]. SCC*mec* types IV and V were found in different genomic backgrounds [8], confirming the apparent movement of these genetic elements. HA-MRSA strains may carry *mec*A in many types of SCC*mec* elements with types I, II or III as the most common ones. Apart from *mecA* gene and *ccr* gene that encode recombinases, SCC*mec* types II and III may carry genes located on transposons or plasmids, conferring resistance to multiple non-β-lactam antistaphylococcal antibiotics [9].

In recent years CA-MRSA has spread to healthcare settings and acquired additional antimicrobial resistance determinants under selective pressure of antibiotics. Changes in epidemiology made CA-MRSA recognition confusing, and for that reason they can be differentiated from HA-MRSA only by means of molecular methods. Commonly used typing techniques are pulsed-field gel electrophoresis (PFGE), multilocus sequence typing (MLST), typing of SCC*mec* element, *spa* typing, and detection of genes encoding Panton-Valentine leukocidin [4].

The aim of this study was to estimate the prevalence and susceptibility of CA-MRSA strains isolated from hospitalised patients and healthy people in the Pomoravlje Region of Serbia.

Materials and Methods

Bacterial isolates

MRSA strains were isolated from nasal swabs of 50 healthy adults and from different clinical samples from 50 hospitalised patients in the Pomoravlje Region, Serbia, during the period between 2011 and 2013. The specimens were cultured on blood agar (bioMérieux) and incubated for 24 hours aerobically at 37 °C. MRSA isolates were identified by tube coagulase test with rabbit plasma (Torlak, Belgrade) and PBP2a agglutination test "Slidex MRSA Detection" (bioMérieux). The identity of isolates was confirmed by PCR for *nuc* gene [10] and *mec*A gene [11].

MRSA isolates were stored in dextrose broth at –20 °C, and re-cultivated on blood agar for further examination.

Antimicrobial susceptibility test

Antimicrobial susceptibility was determined by the automated Vitek2 system (bioMérieux) in accordance with the European Committee on Antimicrobial Susceptibility Testing (EUCAST) recommendation (http://www.eucast.org). The Vitek2 Gram-positive susceptibility card AST-P592 was used.

Minimal inhibitory concentrations (MICs) of cefoxitin were determined by E test (bioMérieux, France), as per the manufacturers recommendation.

SCCmec typing

Typing of SCC*mec* was performed in separate PCR amplifications using the primers and PCR conditions described by Milheiriço et al. [12].

Results

MRSA rates

A total of 168 *S. aureus* isolates were collected from hospitalised patients. MRSA was identified in 77 (45.8%) of them. In further investigation, 50 randomised MRSA isolates were analysed.

Table I. Resistance of MRSA isolates to non-β-lactam antibiotics

No of antibiotics	Source of MRSA isolates	
	Hospitalised patients	Healthy people
0	0	8 (16%)
1	3 (6%)	7 (14%)
2	7 (14%)	6 (12%)
3	14 (28%)	9 (18%)
≥4[a]	26 (52%)	20 (40%)
Total	50 (100%)	50 (100%)

[a]Multi-drug resistance

Among 1362 healthy adult *S. aureus* carriers that were neither hospitalised nor treated with antibiotics in the course of the previous one-year period, MRSA was confirmed in 52 (3.8%) cases. Two MRSA isolates were excluded from further study.

Susceptibility to non-β-lactam antimicrobials

MRSA strains harboured resistance to non-β-lactam antistaphylococcal antibiotics. All MRSA isolates from hospitalised patients were resistant to at least one or more antibiotics: three out of 50 isolates (6%) were resistant to only one antibiotic, 7 (14%) were resistant to two antibiotics, 14 (28%) to three, and remaining 26 (52%) were multi-resistant (resistant to more than 3 non-β-lactam antibiotics) (Table I). The largest number of MRSA strains were resistant to ciprofloxacin (62%), erythromycin (60%), clindamycin and gentamicin (58% each), and tetracycline (56%).

Antimicrobial resistance of MRSA isolates from healthy people was as follows: eight out of 50 isolates (16%) were sensitive to all tested non-β-lactam antibiotics, seven (14%) were resistant to one, six (12%) to two, nine (18%) isolates were resistant to three antibiotics, and 20 isolates (40%) were multi-resistant (Table I). In this group, 58% of the isolates were resistant to erythromycin and clindamycin, 54% to gentamicin, and 52% to tetracycline.

Table II. SCC*mec* typing of MRSA strains from hospitalised and healthy people

Source of strains	SCC*mec* types (%)								
	HA-MRSA types				CA-MRSA types			Non-typable	Total
	I	II	III	Total I–III	IV	V	Total IV–V		
Hospitalised patients	7 (14)	1 (2)	4 (8)	12 (24)	3 (6)	35 (70)	38 (76)	0	50 (100)
Healthy people	2 (4)	1 (2)	5 (10)	8 (16)	13 (26)	27 (54)	40 (80)	2 (4)	50 (100)

*SCC*mec *types in MRSA isolates from hospitalised and healthy people*

In the group of strains isolated from hospitalised patients, patterns characteristic for HA-MRSA were found in 12 (24%) out of 50 strains: seven had SCC*mec* type I, one had type II, and four belonged to type III (Table II). In the remaining 38 (76%) strains SCC*mec* types characteristic for CA-MRSA were confirmed: type IV in three (6%) strains, and type V in 35 (70%) strains.

Analysis of MRSA isolated from healthy people showed that the majority of SCC*mec* elements (80%) were of CA-MRSA types IV and V. Other SCC*mec* types were also found: type I in two strains, type II in one strain, and type III in five strains. Two strains did not give any amplification with primers used for SCC*mec* typing.

*SCC*mec *types in multi-resistant and sensitive MRSA strains*

In hospitalised patients six out of 12 MRSA isolates (50%) that contained SCC*mec* types I-III were multi-resistant, and 20 out of 38 isolates (53%) with SCC*mec* types IV and V (Table III).

In the group of MRSA isolates from healthy people, the percentage of multi-resistant strains that harboured SCC*mec* types I–III was 37.5% (3/8). The same percentage (37.5% or 15/40) of multi-resistant strains possessed SCC*mec* types IV and V.

All eight isolates sensitive to tested non-β-lactam antibiotics contained SCC*mec* type IV (five isolates) or type V (three isolates).

Table III. SCC*mec* types of multi-resistant MRSA strains from hospitalised and healthy people

Source of strains	Multi-resistant strains (%)		Total (%)
	SCC*mec* type		
	I–III	IV–V	
Hospitalised patients (n = 50)	6/12 (50)	20/38 (53)	26/50 (52)
Healthy people (n = 48)	3/8 (37.5)	15/40 (37.5)	18/48 (37.5)

Discussion

CA-MRSA appeared recently, when SCC genetic element containing *mec* gene was horizontally transferred into methicillin-sensitive *S. aureus* (MSSA) strains established in the community [13]. First CA-MRSA strains were isolated from persons with no previous health problems or HA-MRSA risk factors (stay in hospital or in a long-term care facility, kidney dialysis, antibiotic treatment, and so on). A few years after their appearance, CA-MRSA strains spread to hospitals and other healthcare facilities. In this study, we discovered that CA-MRSA strains were more prevalent compared to HA-MRSA, both in healthy people with MRSA colonisation and in hospitalised patients. Specifically, we identified CA-MRSA in 80% (vs HA-MRSA in 20%) of healthy people, and similar proportions of CA- vs HA-MRSA in hospitalised patients (76% vs 24%).

CA-MRSA emerged as more virulent strains than HA-MRSA, so their spreading to hospital settings was expected, but the opposite direction of spreading was less probable. Some authors deemed that methicillin resistance can reduce the virulence of HA-MRSA strains [14], as a consequence of cell wall alterations in MRSA strains that influence the Agr quorum-sensing system. The result is the reduction in the expression and secretion of cytolitic toxins [14]. According to these authors, this is the reason why some strains of HA-MRSA only scarcely spread across the community. On the other hand, the rate of expression of *mecA* gene in CA-MRSA strain is typically lower and the virulence is not decreased. In contrast, Queck et al. [15] found that *psm* gene, encoding a cytolytic toxin, phenol-soluble modulin, can in some strains of MRSA be located inside the SCC*mec* types II and III. In these strains the expression of *psm* gene is unaffected, leaving the HA-MRSA more virulent.

The first detected CA-MRSA strains were commonly susceptible to non-β-lactam antibiotics. In hospital environments, under the selective pressure of antibiotics, they adopted resistance genes to antistaphylococcal antibiotics. In our

study, although eight CA-MRSA isolates from healthy people were susceptible to all tested non-β-lactam antibiotics, all other CA-MRSA isolates from healthy people and patients approached resistance pattern and multi-resistance of HA-MRSA strains. In hospitalised patients, multi-resistance was present in 53% of CA-MRSA and 50% of HA-MRSA isolates. Slightly lower percentage of multi-resistant strains was observed in isolates from healthy people: 37.5% multi-resistant CA-MRSA and 37.5% multi-resistant HA-MRSA.

Occurrence of antibiotic-resistance determinants in bacteria, by mutations or horizontal gene transfer, is associated with the decrease in their fitness [16]. If fitness cost is present, resistant and multi-resistant CA-MRSA strains will not be widely spread in the environments with very low or no antibiotic selective pressure, e.g. community environment. However, CA-MRSA strains are becoming more resistant to non-β-lactam antibiotics, and at the same time the frequency of CA-MRSA is increasing both in hospitals and the community. These facts lead to the conclusion that at least some resistance mechanisms are cost-free. Experimental evidence confirmed that antibiotic resistance can persist in bacterial population living in environments without antibiotic selective pressure, owing to other mechanisms: stabilisation mechanisms in which compensatory mutations can reduce fitness cost without the loss of resistance, and co-selection between resistance genes and virulence genes, because of their genetic linkage [17]. Horváth et al. [18] presented that fluoroquinolones were marked as an antibiotic group associated with high fitness cost in CA-MRSA. In our study, ciprofloxacin resistant CA-MRSA from hospitalised patients was more abundant than sensitive strains (52% vs 26%, respectively). In the community population, the ratio was quite opposite: 22% ciprofloxacin resistant strains to 58% ciprofloxacin sensitive strains.

High prevalence of multi-resistant CA-MRSA in healthy people of the Pomoravlje District, which is identical to prevalence of multi-resistant HA-MRSA circulating in the community, may be a consequence of the use of antibiotics without medical supervision. For this reason and because of intensive use of antibiotics in agriculture, CA-MRSA population is exposed to antibiotic selective pressure even in natural environments. Some authors concluded that antibiotic concentrations far below MIC were sufficient for selecting resistance in bacteria [17].

Virulence and resistance genes are not the only factors influencing successful spread of pathogens. The whole genetic background contributes to epidemiological success. Only five main MRSA clonal complexes, specified by MLST, are present in hospitals around the world [19]. A retrospective study has shown that MSSA isolates from the 1960s belonged to the same clonal complexes as MRSA, suggesting that these genetic lineages, carrying genes contributing to

superior epidemicity, were present in hospitals before the acquisition of SCC*mec* element [19]. Similarly, successful clones circulating in the community are ST1 and ST8, mostly reported in the USA and Canada, ST80 in Europe, ST59 in the Asia-Pacific, Taiwan and Australia, and ST30 in the USA, Europe, western Pacific, Japan, and other countries worldwide [4]. The Balkan region has been designated as a reservoir for the ST152 clone as this type was isolated among immigrants from former Yugoslavia [20]. A national surveillance study of MRSA in Serbia demonstrated the presence of ST80 and ST152, but they were not prevalent [20].

The analysis of genetic diversity of MRSA among patients and healthcare workers (HCW) in a major referral hospital in Belgrade, Serbia, revealed that genotype CC5-MRSA-SCC*mec*I was predominant in patients (86.9%) [21]. CA-MRSA genotypes were also observed, but primarily among HCW. A study was conducted on MRSA isolated in 2008 and 2009, when CA-MRSA had just appeared in this hospital and had slowly been spreading in the environment with a high antibiotic selective pressure. Our study showed that a few years later in another part of Serbia the results were completely different. High prevalence of CA-MRSA and multi-resistant CA-MRSA in hospitals suggest that they have existed in hospitals of the Pomoravlje District for a few years. CA-MRSA adapted to hospital environment, acquired antibiotic resistance to non-β-lactam antibiotics without loss of fitness, and became twice as abundant in hospitals as HA-MRSA. SCC*mec* type V-harbouring MRSA is the most successful clone. Its higher percentage (70%) in hospitals vs 54% in the community, may indicate clonal spread in at least some hospital units. The main external factor influencing this clonal spread may be inadequate hygiene measures.

Fast detection of resistance and virulence genes and rapid typing of MRSA are crucial for effective infection control [22]. As a result of the implementation of infection-preventing strategies in healthcare settings and the community in the USA, the incidence of invasive MRSA infections in hospitals dropped by 54% between 2005 and 2011 [23]. In the same period CA-MRSA infections were reduced by only 5%.

In conclusion, we detected high prevalence of CA-MRSA and high resistance rate to non-β-lactam antistaphylococcal antibiotics, both in hospitals and the community, implying that this pathogen has been present in the Pomoravlje District, Serbia for years. CA-MRSA type V, the most frequent genotype, is significantly more common in hospitalised patients than type IV, and twice more common in healthy people. The multi-resistance rate was similar for both types of MRSA (CA- or HA-) in a particular environment, indicating that selective pressure of antibiotics in environments is a leading force conferring antibiotic resistance and multi-resistance. Effective infection control measures guided by

genotyping results must be implemented on both sources of MRSA strains to prevent infections and epidemics with devastating results.

Conflict of Interest

The authors declare that there is no conflict of interests regarding the publication of this paper.

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