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First Report on PVL-Positive Methicillin-Resistant *Staphylococcus Aureus* of Sc*cmec* Type V, SPA Type T441 in Croatia

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A B S T R A C T

The aim of the study was to investigate the molecular epidemiology of MRSA in Primorsko-Goranska County of Croatia during a six-year period (2001–2007). In period from 2001 and 2007, 46 MRSA isolates were collected in Rijeka, strains were subjected to susceptibility testing according to CLSI guidelines, mecA gene detection and SCCmec typing as well as detection of PVL. Strains were typed by Pulse Field Gel Electrophoresis (PFGE) and spa typing. All isolates were susceptible to vancomycin, linezolid, mupirocin, nitrofurantoin, only one strain was resistant to fusidic acid and co-trimoxazole. Results of SCCmec typing showed the presence of SCCmec type IV in 26 MRSA strains, SCCmec type V in three strains, and 13 strains comprised SCCmec I. SCCmec type II and III were not observed. Four MRSA strains were non-typeable by applied SCCmec typing methods. PVL was detected in 4 strains, two SCCmec IV and two SCCmec V. PFGE analysis, grouped MRSA strains into six similarity groups and 18 singletons. Dominating spa types in this collection of strains were t015, with 15 strains, followed by t041 (N=7), t051, (N=2), t2850 (N=2), t008 (N=2) and single isolates t441, t002, t448, t018, t019, t355, t390, t026, t449, t148. We also detected two new spa types, t3510 and t3509, respectively. This is the first report on SCCmec type V in Croatia, and, to our knowledge, first report of PVL-positive mehicillin-resistant Staphylococcus aureus SCCmec type V and t441 (ST59-MRSA-V) in this part of Europe.

Key words: *MRSA, Croatia, SCCmec, spa type*

Introduction

Staphylococcus aureus, the most virulent *Staphylococcus* species, is also second most common isolate from patients in outpatient settings. *S. aureus* causes a wide range of syndromes, from minor skin and soft tissue infections to life-threatening pneumonia and toxinosis [1]. Rates of methicillin – resistance increased slowly, but progressively over decades. Increase in methicillin resistance worldwide was accompanied by isolation of MRSA isolates from community-acquired infections among previously healthy individuals with few or not traditional heathcare-associated risk factors[1].

The increase in the rate of CA-MRSA infections is a reason for public health concerns, and a challenge for infection control, since MRSA is found in nursing homes, kindergartens, and schools[2]. However, studies on the CA-MRSA prevalence are sporadic. HA-MRSA and CA-MRSA can be distinguished by their genetic background, the resistance determinant Staphylococcal Cassette Chromosome *mec* (SCC*mec*), and the presence of Panton-Valentine leukocidin (PVL)[3].

The aim of the present study was to investigate the molecular epidemiology of MRSA in Primorsko-Goranska County of Croatia during a six-year period (2001–2007).

Prevalence of MRSA in Croatia in blood culture isolates was 36,3 %, in 2009 as presented by European Resistance Surveillance System (EARSS) website[4].

Primorsko-Goranska County is situated in western part of Croatia, as seen at Figure 1., covering an area of 3.582 km². Largest city and county center is Rijeka, third largest city in Croatia. Public Health and Teaching Insti-

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tute in Rijeka is leading public health and teching institution in county with gravitating population of approximately 300 000 inhabitants (around 7% of Croatian population.). Department of microbiology mainly process specimens from outpatient population and ambulatory care.

Methods

In period from 2001 and 2007, all MRSA strains isolated (N=46) were collected and stored until further analysis. Strains originated from nasal swabs (N=20), 15 from wound swabs, and two or one isolate per following sites: skin infection sample, furuncullus, conjunctiva, tongue.

All isolates were identified based on colony morphology, catalase, coagulase and DNA-se presence. Isolates were screened for MRSA with oxacillin, cefoxitin disk and latex agglutination test.

All 46 MRSA strains were subjected to further analysis in Clinical Hospital Centre Zagreb, Department of Clinical and Molecular Microbiology and some non-typeable strains were submitted to University Hospital Maastricht, Mastricht Infection Center for additional typing.

Susceptibility testing was performed according to CLSI guidelines[5] for the following antibiotics: penicillin, oxacillin, cefoxitin, erythromycin, clindamycin, azythromycin, gentamycin, tetracycline, rifampicin, trimethoprim/sulfamethoxazole, linezolid, ciprofloxacin, vancomycin, nitrofurantoin, trimethoprim, except for fusidic acid and mupirocin, which were interpreted according to the criteria of the French Society of Microbioogy (CAFM)[6].

*mec*A gene detection and SCC*mec* typing was performed according to Oliveira et al.[7] with some modifications according to Deurenberg et al.[8] and for some strains, fluorescent PCR assay was used[9], partially based on Zhang multiplex PCR method[10]. Detection of PVL was performed as previously described[11].

Pulse Field Gel Electrophoresis (PFGE) was performed as previously described[12].

PFGE patterns were analyzed and compared with use of GelCompar III software (Applied Maths, Sint-Martens-Latem, Belgium). Spa typing was performed according to SeqNet protocol, spa types were assigned by using Ridom Spa software (Ridom, GmbH, Germany) and synchronization with Ridom server[13].

BURP alligment was used for spa CC alligment.

Results

All strains were resistant to penicillin, oxacillin and cefoxitin, 35% were resistant to erythromycin, 30% to clindamycin, 35% to azythromycin, 30% to gentamycin, 2% to tetracycline (17,4 % were intermediate resistant to tetracycline), 13% to rifampicin (4% intermediate resistant to rifampicin), 37 % to ciprofloxacin (2% intermediate resistant to ciprofloxacin), 4,3% to trimethoprim (2% intermediate resistant to trimethoprim). All isolates were susceptible to vancomycin, linezolid, mupirocin, nitrofurantoin, only one strain was resistant to fusidic acid and co-trimoxazole.

Typing results are shown in Figure 1.

All isolates were *mec*A positive. Results of SCC*mec* typing showed the presence of SCC*mec* type IV in 26 MRSA strains, SCC*mec* type V in three strains, and 13 strains comprised SCC*mec* I. SCC*mec* type II and III were not observed in study collection.

Four MRSA strains were non-typeable by applied SCC*mec* typing methods.

PVL was detected in 4 strains, two SCC*mec* IV and two SCC*mec* V.

PFGE analysis, using Tenover criteria, grouped MRSA strains into six similarity groups (A, B, C, D, E, F) containing 9, 9, 6, 2, and 2 isolates, respectively, and 18 singletons.

Dominating spa types in this collection of strains were t015, with 15 strains, followed by t041 (N=7), t051, (N=2), t2850 (N=2), t008 (N=2) and single isolates t441, t002, t448, t018, t019, t355, t390, t026, t449, t148. We also detected two new spa types, t3510 and t3509, respectively.

MRSA strains SCC*mec* type V are spa typed as t002 (assigned ST-5), t355 (assigned ST-377) , t441 (assigned ST-59).

Isolates were classified into 2 clusters: CC 1 with t008, t024 and t051, and other cluster, CC41 consisting of t002, t041 and t2027.

Discussion

With efforts to prevent the spread of MRSA in hospitals and raised awareness of possible routes of transmission, encouraged by hospital administration and, in some counties, media campaign and Government involvement, the MRSA rates decreased in some countries[14].

Worrying are reports and suggestions that CA MRSA is more and more present in hospitals. Children, young without risk factors are carriers and sometimes, hey develop clinical infection, ranging from mild skin infection to severe skin and soft-tissue infections, pneumonia[15,16] etc. CA- MRSA strains can cause infections with rapidly progressive, fatal diseased including necrotizing pneumonia, severe sepsis and necrotizing fasciitis[1].

There are few data on prevalence and structure of community-associated MRSA strains in Europe[17–19] although some report are showing increase in this distinct population of bacteria. In USA, majority of bloodstream infections[20] and especially skin and soft tissue infections[21] are caused by CA MRSA.

Genetic backgrounds of CA-MRSA isolates are distinct in different geographic locations[22,11] and many different backgrounds can exist within a narrow geographic area.

The most widely used molecular typing method for study of MRSA epidemiology is Pulse Field Gel Electrophoresis, PFGE, suitable method for the determination of clonal relationships but not for long term epidemiological

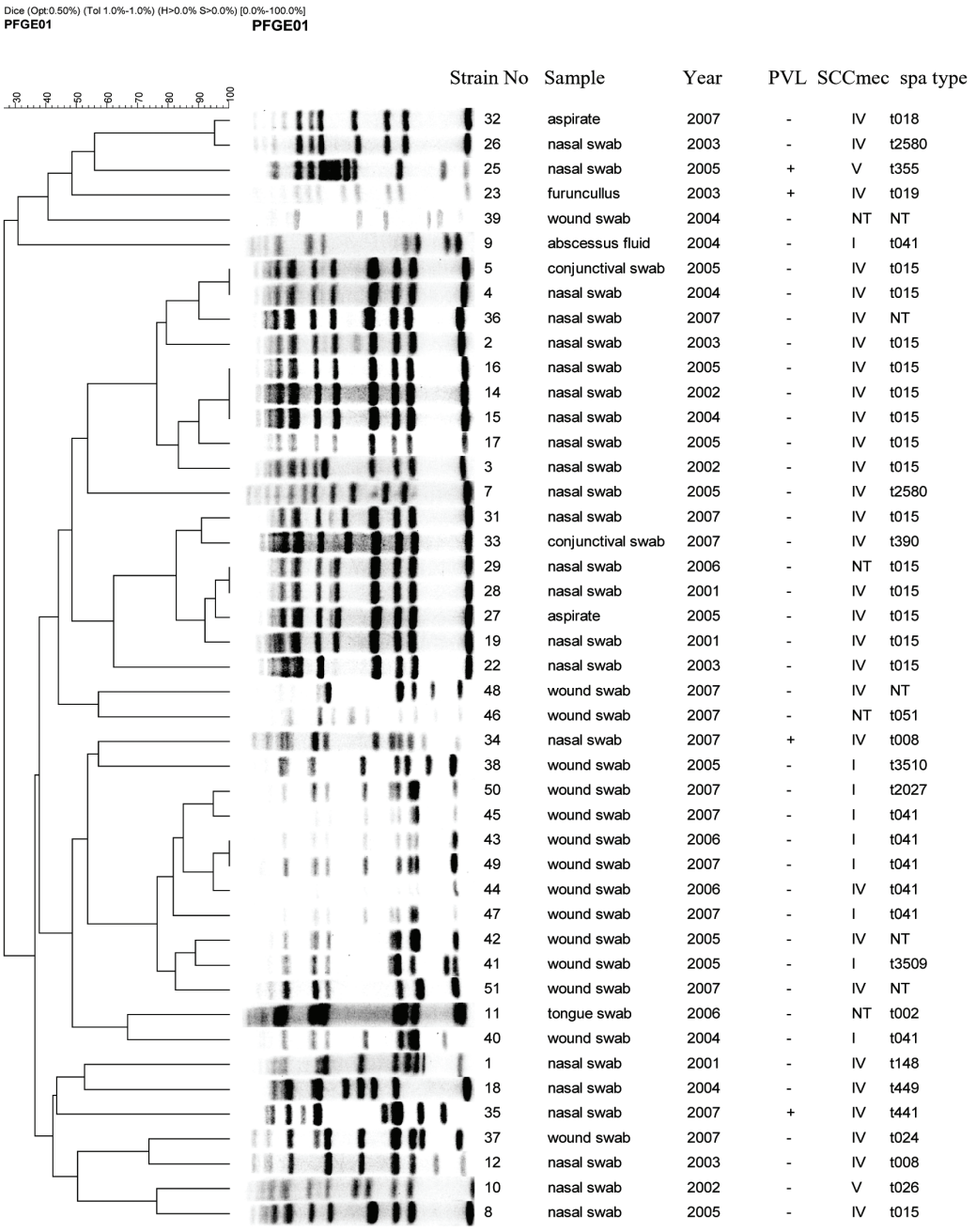

Fig. 1. Dendrogram of MRSA strains from Rijeka.

investigation. Spa typing is proposed as and accurate method for typing of *S. aureus*[23], and it has the advantages of speed, interpertation and interlaboratory comparison.

In this study, we combined sequnce-based, single locus-oriented spa typing method with whole-genome, restriction-based methods in order to compare isolates within larger time frame. For complete nomenclature of MRSA it is important to perform SCCmec typing as well.

Our previous reports suggested the dominance of ST-247/250 MRSA-I among MRSA strains described[24] and in collection of strains in 2004 (data not shown).

There were sporadic reports on community-associated MRSA in Croatia[25], isolated from non-infected body sites or skin and soft tissue infections, but this is the first report on SCC*mec* type V in Croatia, and, to our knowledge, in this part of Europe.

In this study we showed presence of PVL-positive strains, in four cases. Some epidemiologic and clinical

data provide compelling evidence that the high virulence potential of CA-MRSA is associated with genes lukS-PV and lukF-PV(pvl) encoding the subunits of the Panton-Valentine leukocidin.

Seems that clinical sequelae of pvl- positive infections are more severe than pvl-negative *S. aureus*[26].

The interesting finding is that half of PVL-positive isolates is characterized as MRSA SCC*mec* V, which is not an common finding, described in severe infection in Greece[27], but within different clonal lineage (ST-80).

SCC*mec* type V is typical for Australia but is usually PVL-negative. Strains causing infections with SCCmec V and PVL positive; ST 59 are observed in Taiwan, especially in children. This clone, observed in Taiwan is also present in hospitals in significant proportion[28].

This work is showing that there is a certain number of MRSA strains in the community of this area, with heterogenous background. This is in concordance with similar studies in some European countries[29,17,18], but some clonal lineages are desribed in this region (Southern Eastern Europe) for the first time.

Predominant spa type in this collection of strains, t015 is observed in Croatia, in eastern, western parts of country, and Istria, area which is situated close to Primorsko-goranska county.

It is obvious that some strains, although not having typical and direct hospital background, ended originated in hospitals, which is well known for spa type t041.

t041 is an frequently encountered spa type in Croatia, and characterizing typical hospital-associated MRSA together with SCC*mec* type I, also found in Italy [30].Two of predominating spa types are grouped in two major similarity groups, B, C and D, based on PFGE typing. There are some overlaps, and singletons are not grouped in between genetically related strains.

t002 is common spa type, isolated in Germany, Austria, Spain, Israel, Canada, USA, UK,

Switzerland etc[31–33,13,30].

spa type t355 is observed in Austria, Slovenia, Germany, Norway, Netherlands, Turkey, Israel, Sweden, Iceland, Finland, France[13,34].

Strains typed as t441 were previously observed in Sweden, Norway, Netherlands, Iceland, Taiwan[13].

In conclusion, CA-MRSA isolates have been emerging around the world in different *S. aureus* genetic lineages and are beginning to enter in healthcare environments where they could become endemic.

The strong epidemiologic link between PVL and CA-MRSA disease leaves little doubt that PVL must play an important role in the pathogenesis and course of disease; however, Croatian SCCmec IV MRSA isolates were mostly PVL-negative, and there were SCCmec V-PVL postive isolates which suggests that there is a lot of genetic exchange and variability among MRSA isolates from community.

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PRVI IZOLAT *STAPHYLOCOCCUS AUREUS* REZISTENTNOG NA METICILIN SCC*MEC* TIPA V, POZITIVAN NA PVL

S A Ž E T A K

Cilj studije je bio istražiti epidemiološke i molekulne karakteristika MRSA izolata iz Primorsko-Goranske županije tijekom šestogodišnjeg perioda. U periodu od 2001. Do 2007. godine, 46 MRSA izolata je prikupljeno, testirana je osjetljivost na antimikrobne lijekove prema CLSI smjernicama, također je provedena detekcija mecA gena, SCCmec tipizacija i detekcija PVL kodirajućih gena. Tipizacija sojeva dodatno je upotpunjena analizom elektroforeze u pulsirajućem polju (PFGE analizom) i tipizacijom spa lokusa. Svi MRSA izolati su osjetljivi na vankomicin, linezolid, mupirocin, nitrofurantoin, samo jedan soj je bio rezistentan na fucidinsku kiselinu i ko-trimoksazol. Rezultati SCC*mec* tipizacije pokazali su prisutnost SCC*mec* tipa IV u 26 MRSA sojeva, SCC*mec* tipa V kod tri izolata, a 13 sojeva je imalo SCC*mec* I. SCC*mec* tipovi II i III nisu nađeni. Četiri MRSA su bila netipabilna navedenom metodom. PVL je detektiran kod 4 soja, dva SCC*mec* IV I dva SCC*mec* V. PFGE analiza grupirala je MRSA sojeve u 6 grupa sličnosti 18 pojedinačnih, nesvrstanih sojeva. Dominantni spa tipovi u ovoj kolekciji sojeva su bili: t015, s 15 sojeva, te t041 (N=7), t051, (N=2), t2850 (N=2), t008 (N=2) i s po jednim izolatom tipiziranim kao: t441, t002, t448, t018, t019, t355, t390, t026, t449, t148. Otkrili smo i nove tipove, t3510 i t3509. Ovo je prvi spomen o prisutnosti SCC*mec* tipa V u Hrvatskoj, i, prema našem saznanju, prvo izvješće od PVL-pozitivnom MRSA s SCCmec tipom V i spa tipom t441 (ST59-MRSA-V) u ovom dijelu Europe.