Genotypic and phenotypic characteristics of Methicillin-resistant *Staphylococcus aureus* (MRSA) strains, isolated on three different geography locations

Maja Ostojić[1], Mirsada Hukić[2, 3*]

[1]Institute for Microbiology and Molecular Diagnostic, University Clinical Hospital Mostar, Bosnia and Herzegovina, [2] Academy of Science and Arts of Bosnia and Herzegovina, [3]International Burch University Sarajevo, Bosnia and Herzegovina

ABSTRACT

Staphylococcus aureus is a major cause of hospital-acquired infections worldwide. Increased frequency of methicillin-resistant ***Staphylococcus aureus*** (MRSA) in hospitalized patients and possibility of vancomycin resistance requires rapid and reliable characterization of isolates and control of MRSA spread in hospitals. Typing of isolates helps to understand the route of a hospital pathogen spread. The aim of this study was to investigate and compare genotypic and phenotypic characteristics of MRSA samples on three different geography locations. In addition, our aim was to evaluate three different methods of MRSA typing: ***spa***-typing, ***agr***-typing and GenoType MRSA. We included 104 samples of MRSA, isolated in 3 different geographical locations in clinical hospitals in Zagreb, Mostar, and Heidelberg, during the period of six months. Genotyping and phenotyping were done by ***spa***-typing, ***agr***-typing and dipstick assay GenoType MRSA. We failed to type all our samples by ***spa***-typing. The most common ***spa***-type in clinical hospital Zagreb was t041, in Mostar t001, and in Heidelberg t003.We analyzed 102/104 of our samples by ***agr***-typing method. We did not find any ***agr***-type IV in our locations. We analyzed all our samples by the dipstick assay GenoType MRSA. All isolates in our study were MRSA strains. In Zagreb there were no positive strains to ***PVL*** gene. In Mostar we have found 5/25 positive strains to ***PVL*** gene, in Heidelberg there was 1/49. ***PVL*** positive isolates were associated with ***spa***-type t008 and ***agr***-type I, thus, genetically, they were community-associated MRSA (CA-MRSA). Dipstick assay GenoType MRSA has demonstrated sufficient specificity, sensibility, simple performance and low cost, so we could introduce it to work in smaller laboratories. Using this method may expedite MRSA screening, thus preventing its spread in hospitals.

KEY WORDS: Methicillin resistant ***Staphylococcus aureus***; ***spa***-typing; ***agr***-typing; GenoType MRSA; infection control; MRSA
DOI: http://dx.doi.org/10.17305/bjbms.2015.402 *Bosn J Basic Med Sci. 2015;15(3):48-56. © 2015 ABMSFBIH*

INTRODUCTION

Staphylococci are among the most important causes of both hospital- and community-acquired infections worldwide. It is well known that methicillin-resistant ***Staphylococcus aureus*** (MRSA), like methicillin-sensitive ***Staphylococcus aureus*** (MSSA) could colonize or infect patients [1]. MRSA strains were not found to be more virulent than MSSA strains and to cause the same spectrum of infections. ***S. aureus*** causes superficial and deep skin and soft tissue infections, bacteremia, endocarditis, osteomyelitis, pneumonia, food poisoning, toxic-shock syndrome and staphylococcal scaled skin syndrome [1-5]. In the early 1950s, acquisition and spread of β-lactamase-producing plasmids decreased the effectiveness of penicillin for treating ***S.aureus*** infections. Methicillin, a modified penicillin, designed to resist the destructive action of the staphylococcal β-lactamase, became available for therapeutic use in 1959. However, MRSA was identified in 1960s. The resistance was a result of ***S. aureus***'s acquiring the ***mecA*** gen, which encodes for an altered penicillin-binding protein gen ***(PBP2a***). It was not blocked by methicillin and could replace the other ***PBPs***, thus allowing the survival of ***S.aureus*** in the presence of methicillin [6-9]. As opposed to the penicillinase gene, ***mecA*** does not reside on a plasmid but on the chromosome, embedded in a large mobile genetic element called ***Staphylococcal Chromosome Cassette mec*** or SCC***mec*** [10, 11]. The presence of ***PBP2a*** means MRSA is not only resistant to methicillin but also to all β-lactam antibiotics, including synthetic penicillins, cephalosporins and carbapenems. By the early 1960s, European hospitals were reporting outbreaks of MRSA

*Corresponding author: Mirsada Hukić,
International Burch University, Francuske revolucije bb, Ilidža, 71210 Sarajevo, Bosnia-Herzegovina, E-mail: mirsadahukic@yahoo.com

Submitted: 28 February 2015 / Accepted: 27 March 2015

infections. In Bosnia-Herzegovina, we noted an increment of MRSA infections in the early 1990s, with the beginning of the war [12]. Data from the European Antibiotic Resistance Surveillance System (EARSS) showed a rising trend of MRSA infections until 2005, with the proportion of MRSA infections varying from less than 1% in the northern to 50% in southern European countries [13]. This striking difference is probably due to differences in antibiotic use or in the implementation of measures to control MRSA spread in hospitals [14]. MRSA has been linked for many years- with hospital stay, homes for the elderly and infirm, and similar institutions. However, in 1990s, community-associated MRSA (CA-MRSA) has appeared, with a large number of characteristics different from previously known hospital acquired MRSA (HA-MRSA). HA-MRSA is mainly multi-resistant, and the choice of antibiotics for treating such infections is limited to glycopeptides and linezolid. Furthermore, HA-MRSA mainly causes serious infections in patients with weak immune system, after long-term hospitalization, long-term use of antibiotics, etc.[15]. In previous years, strains have emerged with an intermediate susceptibility or full resistance to vancomycin (VISA and VRSA, respectively), the antibiotic that for two decades represented the drug of choice for treating MRSA infections [16]. The multidrug-resistant phenotype of MRSA strains and their intrinsic β-lactam resistance make them difficult and costly to cure.

Controlling MRSA remains a primary focus of most hospital infections control programs [17-20]. Knowledge of the dissemination and the molecular epidemiology of MRSA strains are required to develop effective strategies to prevent the spread of MRSA. Various molecular typing techniques have been developed to investigate the spread and evolution of MRSA. Bacterial typing method should be highly discriminatory, reproducible, standardized, widely available and inexpensive. In bacterial strain typing, both phenotyping and genotyping procedures can be used.

The most common phenotyping methods are: biotyping, serotyping, antibiotic susceptibility testing and phagotyping. Disadvantage of these commonly applied techniques may be low discriminatory power. In recent years, the quality of microbiological assays has been increasing by using molecular biology techniques such as whole cell protein profile analysis and electrophoresis of multiple-locus bacterial enzymes [21].

Genotyping methods include the determination of plasmid profile, analysis of chromosomal DNA, Southern hybridization, pulsed-field gel electrophoresis (PFGE), polymerase chain reaction (PCR), SCC***mec*** typing and sequence-typing. The most commonly used techniques are PFGE, PCR, SCC***mec*** typing and sequence-typing [multilocus sequence typing (MLST) and ***spa***-typing] [15, 22-24].

Staphylococcal protein A (***spa***) typing is based on the characterization of the ***spa*** gene, which encodes for the ***S.aureus*** specific surface protein A. The ***spa*** gene consists of different functional regions including the Fc binding region and the X-region. The polymorphic X region of the ***spa*** gene is built of a variable number of 24-bp repeating fragments. Differences occur between the fragments due to deletion, point mutation and duplication of nucleotide groups. Different repeats can be assigned an alpha-numerical code, and the order of specific repeats defines the ***spa*** type. Two systems of nomenclature are in use for ***spa*** type determination. RidomStaphType is a software enabling straightforward sequence analysis and designation of ***spa*** types via synchronization to a central server [15, 25-27]. The discriminative power of ***spa*** typing lies between that of PFGE and MLST, and in contrast to MLST, ***spa*** typing can be used to investigate both the molecular evolution and hospital outbreaks of MRSA. The main advantage of ***spa*** typing is its simplicity, since it involves sequencing of only a single locus.

The accessory gene regulator ***(agr)*** of ***S. aureus*** is a global regulator of the staphylococcal virulence, which includes secreted virulence factors and surface proteins. The ***agr*** locus is important for virulence in a variety of animal models of infection, and has been assumed by inference to have a major role in human infection. ***S.aureus*** strains have been divided into four ***agr*** specificity groups.

The GenoType MRSA detects the ***mec***A gene and, in addition, a highly specific sequence for ***S. aureus*** by polymerase chain reaction (PCR) and reverse hybridization. Molecular genetic testing with the GenoType MRSA kit needs much less time than conventional microbiological methods. Therefore genetic testing provides not only a considerable advantage with respect to reliability but also to speed [28].

The aim of this study was to investigate and compare genotypic and phenotypic characteristics of MRSA strains in three different geography location and to evaluate the efficacy of three different methods of MRSA typing: ***spa***-typing, ***agr***-typing and GenoType MRSA.

MATERIALS AND METHODS

Samples

The study was carried out at 104 samples of MRSA, collected during the period of six months in three different locations in Europe: Clinical Hospital Center Zagreb, Croatia (30 samples), University Clinical Hospital Mostar, Bosnia-Herzegovina (25 samples), and University Clinical Heidelberg, Germany (49 samples). These isolates were taken from wound swabs, blood cultures, respiratory tract specimens, urine samples and surveillance cultures. MRSA isolates were collected and stored at –20°C until analyzed.

Methods

The strains were identified with current phenotypic methods. After 24 hours of incubation at 37°C the colonies of S.***aureus*** on 5% blood agar were 1 to 3 mm in diameter, pigmented yellow, smooth, and convex. Identification of S.***aureus*** was carried out by detection of DNase. All strains were DNase positive. The antibiotic susceptibility testing was performed by the disk-diffusion method, according to Clinical and Laboratory Standard Institute (CLSI) guidelines. We tested penicillin, erythromycin, azithromycin, gentamycin, amikacin, trimethoprim-sulfamethoxazole, clindamycin, rifampin, cefoxitin, linezolid, teicoplanin and vancomycin. Antimicrobial susceptibility testing was confirmed with VITEK 2 Compact (Bio Mérieux, France).

Spa-typing

DNA extraction

DNA was extracted from samples using the InstaGene Matrix (BioRad, Austria), according to the manufacturer´s protocol. Three colonies from overnight culture of S.***aureus*** were suspended in 500 µL of high performance liquid chromatography (HPLC) grade water and added to 100 µL InstaGene Matrix and vortexed, followed by heating at 56°C for 20 minutes. The samples were vortexed again and heated at 100°C for 8 minutes and then centrifuged to pellet the matrix. Aliquots of 80 µL were used as templates for PCR.

DNA amplification

The ***spa*** typing method is based on sequencing of the polymorphic X region of the protein A gene (***spa***), present in all strains of ***S. aureus.*** The X region is constituted of a variable number of 24-bp repeats flanked by well-conserved regions. This single-locus sequence-based typing method combines a number of technical advantages, such as rapidity, reproducibility, and portability. Moreover, due to its repeat structure, the ***spa*** locus simultaneously indexes micro- and macro variations, enabling the use of ***spa*** typing in both local and global epidemiological studies. These studies are facilitated by the establishment of standardized ***spa*** type nomenclature and Internet shared databases.

The X region of the spa gene was amplified by PCR with primers ***spa***-1113f (5´- TAA AGA CGA TCC TTC GGT GAG C – 3´) and ***spa***-1514r (5´- CAG CAG TAG TGC CGT TTGCTT – 3´). The PCR amplification was performed using Perking Elmer 9700 thermal cycler(Norwalk, CT, USA) with an initial activation step at 80°C for 5 minutes, followed by 30 cycles of denaturation at 94°Cfor 45 seconds, annealing at 60°C for 45 second extension at 72°C for 90 seconds, followed by final extension step at 72°C for 10 min.

Detection of products

The amplicons of PCR reactions were visualized using UV light box, after the electrophoresis on a 2% agarose gel with ethidium bromide.

DNA purification

For ***spa*** typing the amplified PCR products were purified using a common purification kit (New England Biolabs GmbH, Frankfurt-Hoechst, Germany and Amersham Pharmacia Biotech).Briefly, 5 µL of the PCR product was incubated with 1U of each enzyme Exonuclease I and Shrimp Alkaline Phosphatase, at 37°C for 30 minutes. Then the enzymes were inactivated at 80°C for 15 minutes and the PCR products were stored at 4°C.

PCR for sequencing

At least 30 ng of the above purified PCR product was used for PCR sequencing. ABI Big Dye Terminator Ready Reaction kit Version 3.1 (PE Applied Biosystems, CA, USA) was used under the following conditions: splitting chains at 95°C for 3-4 minutes, followed by 25 cycles of annealing at 60°C for 30 seconds, building of nucleotides at 60°C for 4 minutes, and splitting chains at 95°C for 30 seconds, and extension at 60°C for 7 minutes. Templates were purified for sequencing by Qiagen Spin Kit DyeEx (Qiagen GmBH, Hilden, Germany).

DNA sequencing

DNA sequences were obtained with an ABI 377 sequencer (Applied Biosystems, Foster City, Calif.).

Spa types were determined with the RidomStaphType software (Ridom GmbH, Wurzburg, Germany).

Agr-typing

DNA extraction

DNA extraction was made as described above.

DNA amplification

Agr specificity groups were identified by PCR amplification of the hypervariable domain of the ***agr*** locus using oligonucleotide primers specific for each of the four major specificity groups.A forward primer, pan-***agr*** (5'-ATGCACATGGTGCACATGC-3'), corresponding to conserved sequences from the ***agrB*** gene, was used in all reactions(primer sequences were obtained from GenBank accession numbers X52543, AF001782, AF001783, and AF288215). Four reverse primers, each specific for amplification of a single ***agr*** group based on ***agrD*** or ***agrC*** gene nucleotide polymorphism, were as follows:

1. ***agr*** I, 5'-GTCACAAGTACTATAAGCTGCGAT-3' (in the ***agrD*** gene)

2. ***agr*** II, 5'-GTATTACTAATTGAAAAGTGC CATAGC-3' (in the ***agrC*** gene)
3. ***agr*** III, 5'-CTGTTGAAAAAGTCAACTAA AAGCTC-3' (in the ***agrD*** gene)
4. ***agr*** IV, 5'-CGATAATGCCGTAATAC CCG-3' (in the ***agrC*** gene)

The PCR assay was performed in 50 μL of reaction mixture containing 5 μL KCl buffer, 8 μL MgCl2, 10 μL dNTP (dATP, dCTP, dGTP, dTTP), 2 μL of Taq polymerase, 20 pmol of each primer, and 10 μL of isolated DNA. The reaction mixtures were placed in a Perking Elmer thermal cycler (Norwalk, CT, USA). The thermal profile involved an initial denaturation step at 94°C for 5 min followed by 30 cycles of denaturation at 94°C for 1 min, annealing at 55°C for 1 min, and elongation at 72°C for 1 min. The cycling was followed by a final extension step at 72°C for 5 min. ***S. aureus*** strains RN6390 (***agr*** group I), RN6607 (***agr*** group II), RN8465 (***agr*** group III), RN4550 (***agr*** group IV), and RN6911 (***agr*** negative) were used as controls for ***agr*** group identification, and were kindly provided by Prof Wolfgang Witte PhD. Aliquots of amplified samples were analyzed by electrophoresis on a 1% agarose gel and stained with ethidium bromide.

GenoType MRSA

GenoType MRSA is newly available molecular genetic test kit (Hain Lifescience GmbH, Nehren, Germany), which detects the ***mec***A gene and, in addition, a highly specific sequence for ***S. aureus*** by polymerase chain reaction (PCR) and reverse hybridization [28]. We have analyzed all our strains by the dipstick assay GenoType MRSA.

DNA extraction

Five overnight ***S. aureus*** colonies were re-suspended in 150 μLof water. Bacterial DNA was released by incubation of the solution for 15 min at 95°C, followed by incubation in an ultrasonic bath for 15 min, and spun down for 5 min at maximum speed. Afterwards, we used 5 μL of supernatant for PCR.

DNA amplification

The PCR was performed using a hot start Taq polymerase (HotStartTaq, Quiagen, Germany). The amplification mix contained 35μL PNM (biotin-labeled primers and dNTP), 5 μL polymerase incubation buffer, 2 μL 25 mM $MgCl_2$, 1U polymerase, 2 μL distilled water and 5 μL DNA solution. The amplification was carried out in a Perking Elmer 9700 thermal cycler (Norwalk, CT, USA). The sensitivity of amplification and hybridization was monitored using an internal control.

Hybridisation

PCR products (20 μL) were mixed for 5 min with 20 μL of denaturing reagent (provided with the kit) at room temperature in separate troughs of a plastic tray. After addition of 1 ml of pre-warmed hybridization buffer, the membrane strips in the kit were added to every trough. Hybridization was at 45°C for 30 min, followed by two washing steps at 45°C for 30 min with 1 ml of pre-warmed stringent wash solution. For colorimetric detection of hybridized amplicons, streptavidin-conjugated alkaline phosphatase and the appropriate substrate were added. After final washing, the strips were air-dried and fixed on a data sheet.

Statistical methods

Statistical analysis of the data was performed using SPSS for Windows (version 13.0, SPSS inc, Chicago, Illinois, USA) and Microsoft Excel (version 11, Microsoft Corporation, Redmond, WA, USA). Fisher's exact test and χ^2 test, were used to compare categorical variables between groups. The p values < 0.05 were considered statistically significant.

RESULTS

Antimicrobial susceptibility

All MRSA isolates were resistant to the tested β-lactam antibiotics, i.e. penicillin, oxacillin and cefoxitin, and all isolates were susceptible to linezolid, teicoplanin and vancomycin.

Spa-typing

As described in introduction, the polymorphic X region of the ***spa*** gene is built of a variable number of 24-bp repeating fragments. According to RidomSpa Server, 45 of 104 analyzed strains of MRSA did not belong to any known ***spa***-type. Using ***spa***-typing method, we have successfully analyzed 59/104 (56.7%) of our samples. There were 11 different types. The most common ***spa***-type was t001(χ^2=70.586; df=10; ***p***<0.001)(Figure 1A).

According to locations, in Zagreb the most common ***spa***-type was t041 (64.3%), in Mostar t001 (64.7%) and in Heidelberg the most common type was t003 (53.6%) (Figure 1B).

Agr-typing

S. aureus strains have been divided into 4 ***agr*** specificity groups. By ***agr***-typing method, we have analyzed 102/104 (98%) of our samples. Of the 102 strains, the most common was ***agr***-type II 85/102 (83.3%), then ***agr***-type I 16/102 (15.7%) and 1/102 strain (1%) was ***agr***-type III. (χ^2 test=118.059; df=2; ***p***<0.001). We have not found any ***agr***-type IV on our locations (Figure 2A).

MRSA strain frequency were significantly different according to ***agr***-types, depending on the location of research (Monte Carlo 2-sided; ***p***=0.011). In CHC Zagreb, we have found exclusively ***agr***-type II 29/29 (100%), in UCH Mostar

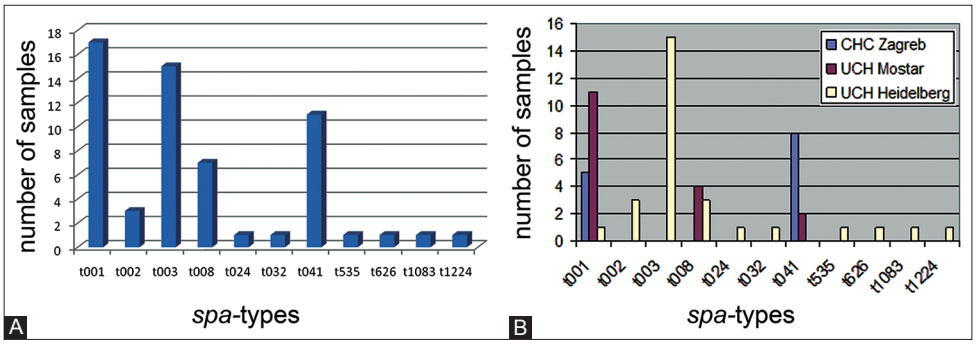

FIGURE 1. MRSA *spa*-types collected from Mostar (Bosnia and Herzegovina), Zagreb (Croatia) and Heilderberg (Germany) hospitals. (A) Total number of isolates according to MRSA strain. (B) Number of samples collected in each institution according to MRSA strain.

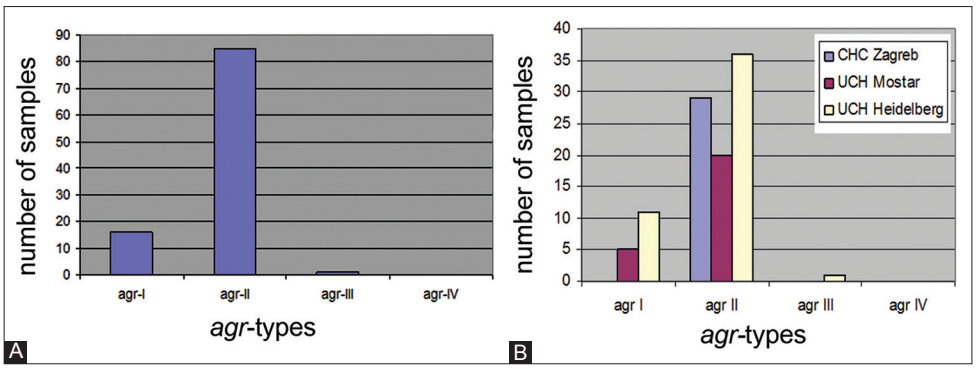

FIGURE 2. MRSA agr-types collected from Mostar (Bosnia and Herzegovina), Zagreb (Croatia) and Heilderberg (Germany) hospitals. (A) Total number of isolates according to MRSA strain. (B) Number of samples collected in each institution according to MRSA strain.

agr-type I and *agr*-type II and in UCH Heidelberg *agr*-type I, *agr*-type II and *agr*-type III (Figure 2B).

Distribution of *agr*-types according to type of sample

All isolates from clinical hospital Zagreb belonged to *agr*-type II. Most of the isolates were from the wound swab (Table 1). There was no significant difference in the *agr*-type depending of the source of the samples in the Mostar (Monte Carlo 2-sided; p=0.556) (Table 1).

There was no significant difference in the *agr*-type depending of the source of the samples in the Heidelberg (Monte Carlo 2-sided; p=0.381) (Table 1). Looking to the isolates from all 3 locations, we did not see any significant difference in the *agr*-types frequencies according to the source of the sample (Monte Carlo 2-sided; p=0.645). *Spa*-types varied significantly depending on the *agr*-types (Monte Carlo 2-sided; p<0.001). *spa*-type t008 (77.8%) predominated in *agr*-type I. *spa*-types t001 (35.4%) and t003 (31.3%)predominated in *agr*-type II.

GenoType MRSA

We analyzed all our samples by the dipstick assay GenoType MRSA. All isolates were positive to *mec*A gene. In clinical hospital Zagreb there were no positive strains to *PVL* gene, and in Heidelberg there was 1/49. In Mostar we have found 5/25 positive strains to *PVL* gene which is significantly more *PVL*-positive strains compared to the other two locations (Monte Carlo 2-sided; p=0.011) (Figure 3 and Table 2).

DISSCUSION

S. aureus is a major nosocomial pathogen that causes a range of diseases, including endocarditis, osteomyelitis, pneumonia, toxic-shock syndrome, food poisoning, carbuncles, boils and infection of surgical wounds. Increased frequency of MRSA or multidrug-resistant phenotype of MRSA strains and their intrinsic beta-lactam resistance, make them difficult and costly to treat.

Bacterial strain typing, or subtyping, has become an important clinical tool to investigate suspected outbreaks and to evaluate nosocomial transmission [29]. Numerous techniques are available to differentiate MRSA isolates. Historically, isolates were distinguished by phenotyping methods, however, many *S.aureus* isolates cannot be typed using this method. Genotyping methods have significant advantages [30].

In this study were examined 104 samples of MRSA, collected during the period of six months on three different locations in Europe. These isolates were taken from wound swabs, blood cultures, respiratory tract specimens, urine samples and surveillance cultures. The samples were obtained from patients in hospitals (97, 93.3%) and outpatient setting (7, 6.7%).

Sensitivity to antibiotics is a phenotypic method, which

TABLE 1. Agr-types according to samples from CHC Zagreb, UCH Mostar and UCH Heidelberg

Place	Zagreb				Mostar				Heidelberg			
Sample	agr I	agr II	agr III	agr IV	agr I	agr II	agr III	agr IV	agr I	agr II	agr III	agr IV
BC	0	1	0	0	2	3	0	0	4	11	0	0
Wound	0	18	0	0	2	6	0	0	1	3	1	0
BA	0	5	0	0	1	1	0	0	-	-	-	-
Sputum	0	1	0	0	0	1	0	0	-	-	-	-
BAL	0	1	0	0	-	-	-	-	-	-	-	-
Urine	-	-	-	-	0	2	0	0	0	4	0	0
Tube	-	-	-	-	0	6	0	0	-	-	-	-
Drain	0	3	0	0	0	1	0	0	-	-	-	-
SC	-	-	-	-	-	-	-	-	6	17	0	0
Total	0	29	0	0	5	20	0	0	11	35	1	0

BC-blood culture; BA-broncho-aspirate; BAL-bronchoalveolar lavage; SC-surveillance culture

TABLE 2. Genotypic characteristics of Methicillin-resistant *Staphylococcus aureus* (MRSA) strains, isolated on three different geography locations: Mostar, Zagreb and Heidelberg

Place	Number of isolates	*agr*-type	*spa*-type	*mec*A	PVL
Zagreb, Croatia	17	II		+	-
	7	II	t041	+	-
	5	II	t001	+	-
	1		t041	+	-
Mostar, Bosnia-Herzegovina	11	II	t001	+	-
	7	II		+	-
	4	I	t008	+	+
	2	II	t041	+	-
	1	I		+	+
Heidelberg, Germany	15	II	t003	+	-
	13	II		+	-
	6	I		+	-
	3	II	t002	+	-
	2	I	t008	+	-
	1	I	t008	+	+
	1	II	t1224	+	-
	1	II	t535	+	-
	1	II	t1083	+	-
	1	II	t626	+	-
	1	I	t024	+	-
	1	I	t032	+	-
	1	II	t001	+	-
	1	III		+	-
	1			+	-

agr – Accessory gene regulator; *spa* – Staphylococcal protein A; *mec*A gene - Absolute requirement for *S. aureus* to express methicillin resistance; PVL – Panton Valentin-leukocidin

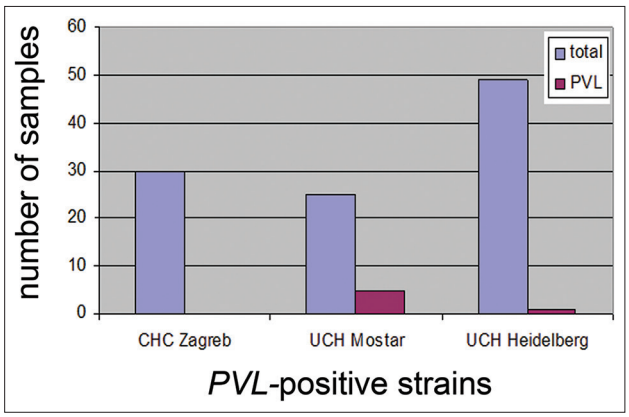

FIGURE 3. Total number and PVL-positive strains from all three locations.

is performed in all clinical microbiology laboratories. Disk-diffusion method is carefully standardized and reproducible, both within the laboratory and between different laboratories. But this method is limited in most of the epidemiological investigations, because genetically and epidemiologically unrelated strains can show the same pattern of sensitivity and resistance. However, despite these limitations, the routine preparation of susceptibility testing can detect antimicrobial resistance, which is very important and it is often early warning of a problem.

We characterized MRSA strains by using different molecular typing tools. Using ***spa***-typing method, developed by Frenay ***et al.*** [25], we successfully analyzed 56.7% of our samples. There were 11 different ***spa***-types with the most common being t001. In Zagreb the most common ***spa***-type was t041 (64.3%), in Mostar t001 (64.7%) and in Heidelberg the most common type was t003 (53.6%). According to RidomSpaServer the ***spa***-type t041, which was dominant in Zagreb (64.3%), is globally much less present (0.4%). This type is described as a Southern German MRSA. The same strain was found in Mostar in 11.8% of samples, while it was not found in Heidelberg.

Similarly, the t001 type dominant in Mostar (64.7%), was globally present with 0.9%. This type was described Southern German MRSA (prototype & subclone), Rhine Hesse MRSA (subclone), EMRSA-3, (New York clone).

In clinical hospital Heidelberg the most common type was t003 (53.6%). According to RidomSpaServer, it is globally the most represented type (10.9%). This strain was not found in Zagreb and Mostar. The high frequency of t041 in clinical hospital Zagreb and t001 in Mostar restricts the usefulness of ***spa***-typing for local investigations. According to Vindel ***et al.*** most common ***spa***-types in Spain, were t067 and t002, which is in contrast to relatively low frequency of these types in other European countries[31].

Most common agr-type was ***agr***-type II (83.3%), then ***agr***-type I (15.1%), ***agr***-type III (1%), while we did not find samples with ***agr***-type IV. In Zagreb, ***agr***-type II 29/29 (100%) was the only type found, in Mostar ***agr***-type I (20%) and ***agr***-type II (80%) and in Heidelberg ***agr***-type I (22.9%), ***agr***-type II (75%) and ***agr***-type III (2.1%). Our hypothesis was that we could classify the samples in 4 ***agr***-types, and to investigate their genetic background and possible relation between ***agr***-type and the capacity to induce a specific disease [32]. While our results do not show a direct role of the ***agr***-type in the type of human disease caused by MRSA, the higher prevalence of ***agr***-type II in our samples could suggest that ***agr***-type II is associated with nosocomial MRSA infections [33].

We notice correlation between ***agr***- and ***spa***-types. So, ***agr***-type I correlated with ***spa***-type t008 in 7/9 (77.8%) typed strains. ***Agr***-type II correlated with ***spa***-type t001 in 17/49 (34.7%) completely typed strains.

With the GenoType MRSA we expected to get sufficiently sensitive, specific, fast and low cost method for MRSA typing, which could be introduced to smaller microbiological laboratories. A PCR based test was developed for the detection of ***mec***A in staphylococci. A various methods of ***mec***A detections were described [34]. Some of them use gel-electrophoresis for visualization of amplifications products. However, these techniques are time consuming and require expensive equipment, and they are not acceptable in a smaller microbiological laboratories for daily use. All isolates in present study were positive to ***mec***A gene. In Zagreb there were no positive strains to ***PVL*** gene. In Mostar we have found 5/25 (20%) positive strains to ***PVL*** gene, and in Heidelberg there was 1/49 (2%).

According to definition of community acquired MRSA (CA-MRSA), i.e. strains isolated in an outpatient setting, or from patients within 48h of hospital admission [35-37] we found that neither of 5 ***PVL*** positive strains from Mostar were not CA-MRSA.

Described strains were resistant to penicillin, macrolide, and ciprofloxacin, and were susceptible to all other tested antibiotics. One of that strain was susceptible to ciprofloxacin. All five strains have belonged to ***spa***-type 008 and ***agr***-type I.

One ***PVL*** positive strain from Heidelberg, according to medical documentation, was CA-MRSA. This strain was resistant to penicillin, macrolides and clindamycin, but was susceptible to all other tested antibiotics. The strain belonged to ***spa***-type 008, and ***agr***-type I, the one described by Witte ***et al.*** as the first case of CA-MRSA in Germany 2005 [38]. Blanco ***et al***. conducted a study of PVL positive strains, which all belonged to ***spa***-type 008, and ***agr***-type I. Numerous studies show that the strains of CA-MRSA are expanded all over the world, although their prevalence varies from one area to another [39]. In the United States, CA-MRSA clone designated as USA 300 has become the most widespread [40]. This strain of recently started to cause the outbreaks in neonatal wards [41].

The prevalence of infections caused by CA-MRSA is a lower in Europe compared to USA, but recently and increasing trend is observed. Typical CA-MRSA is sensitive to many non-β-lactam antibiotics. However, increased use of these antimicrobials could lead to emergence of new multidrug resistant clones [42]. There are no sufficient data about the prevalence of CA-MRSA in Bosnia-Herzegovina. There is a wide range of methods for genotyping of MRSA strains for epidemiological research. Molecular testing will continue to be an essential tool and the testing has proven to be cost-effective and medically justified.

CONCLUSION

Antimicrobial susceptibility, as a phenotyping method, is a simple one to perform and interpret. However, many ***S.aureus*** strains cannot be typified using this method. Our results do not show a direct role for the ***agr***-type in the type of human disease caused by MRSA. Higher prevalence of ***agr***-type II in our samples could suggest that ***agr***-type II is associated with nosocomial MRSA infections. Dipstick assay GenoType MRSA has demonstrated sufficient specificity and sensibility, simplicity of performance and low cost, so it could be introduced into small microbiological laboratories for expediting the MRSA screening and preventing its spread.

DECLARATION OF INTERESTS

The authors declare no conflict of interests.

ACKNOWLEDGEMENTS

The authors would like to thank Prof Hans-Günther Sonntag PhD and the staff of Institut für Hygiene und Molekular Biologie, Heidelberg, Germany for performing the ***spa***- and ***agr***-typing.

REFERENCES

[1] Waldvogel FA. Staphylococcus aureus (including toxic shock syndrome) In: Mandell GL, Bennett JE, Dolin R eds. Principles and Practice of Infectious Diseases, 5th ed. London, Churchill Livingstone 2000; 2069-2089.

[2] Gram-positive cocci In: KonemanEW, Allen SD, Janda WM, Schreckenberger PC, Winn WC eds Color Atlas and Textbook of Diagnostic Microbiology 6th ed. Philadelphia: J.B. Lippincot Co., 2006; 623-662.

[3] Staphylococcus and related organisms In: Murray PR, Rosenthal KS, Pfaller MA. Medical Microbiology 5th ed. Philadelphia (Pe): Elsevier Mosby, 2005; 221-236.

[4] Ostojić M. Staphylococcus, Micrococcus and the other

catalasa-positive cocci In: Uzunović-Kamberović S, eds Medical microbiology, Zenica, Press Fojnica, 2009; 259-267.
[5] Hukić M. Gram-positive cocci. In: Hukić M et al. Bacteriology. Sarajevo: Jež d.o.o., 2005; 163-166.
[6] Reverdy ME1, Jarraud S, Bobin-Dubreux S, Burel E, Girardo P, Lina G, ***et al.*** Incidence of Staphylococcus aureus with reduced susceptibility to glycopeptides in two French hospitals. ClinMicrobiol Infect 2001; 7(5): 267-272. http://dx.doi.org/10.1046/j.1198-743x.2001.00256.x.
[7] Bradley SF. Methicillin-resistant Staphylococcus aureus infection. Infect Dis 1992; 8:853-868.
[8] Boyce J. Methicillin-resistant Staphylococcus aureus infections: a growing control problem. Infect Control 1980; 1:335-336.
[9] French GL. A short history of methicillin-resistant Staphylococcus aureus. Abstracts book: 2nd Croatian Symposium on Bacterial Resistance to Antibiotics. Croatian Academy of medical sciences, Zagreb; 1997:pp13-17.
[10] Katayama Y, Ito T, Hiramatsu K. A new class of genetic element, staphylococcus cassette chromosome mec, encodes methicillin resistance in Staphylococcus aureus. Antimicrob Agents Chemother 2000; 44: 1549–1555.http://dx.doi.org/10.1128/AAC.44.6.1549-1555.2000.
[11] Pantosti A, Venditti M. What is MRSA? EurRespir J 2009;34:1190–1196.http://dx.doi.org/10.1183/09031936.00007709.
[12] Ostojić M, Glibić I, Parčina M. Epidemiological typing of Methicillin-resistant Staphylococcus aureus (MRSA) by PFGE. Acta Medica Saliniana 2005; 34 (Supl 1): 1-98.
[13] European Antimicrobial Resistance Surveillance System. EARSS Annual Report 2007. Available from: www.rivm.nl/earss/Images/EARSS%202007_FINAL_tcm61-55933.pdf. 14th January, 2015.
[14] Tiemersma EW, Bronzwaer SL, Lyytikainen O, Degener JE, Schrijnemakers P, Bruinsma N, ***et al.*** Methicillin-resistant Staphylococcus aureus in Europe, 1999–2002. Emerg Infect Dis 2004; 10: 1627–1634. http://dx.doi.org/10.3201/eid1009.040069.
[15] Budimir A. Detection and typing methods of methicillin-resistant Staphylococcus aureus strains. Medical Sciences 37(2012): 73-88.
[16] Sievert DM, Rudrik JT, Patel JB, McDonald LC, Wilkins MJ, Hageman JC. Vancomycin-resistant Staphylococcus aureus in the United States, 2002–2006. Clin Infect Dis 2008;46:668–674. http://dx.doi.org/10.1086/527392
[17] IFIC, International Federation of Infection Control; Referral center for infections control of Republic Croatia. Infection control: Basic concept ant training. Merkur A.B.D. Zagreb, Croatia, 2004, pp 40-51.
[18] Ostojić M. Methicillin resistant ***Staphylococcus aureus*** (MRSA). In: Hukić M (ed). Prevention and control of infections in healthcare facilities, 1st ed. Sarajevo: TDP Sarajevo; 2010, pp. 291-295.
[19] Bischoff WE and Edmond MB. ***Staphylococcus aureus.*** In: Wenzel PR (ed). A guide to infection control in the Hospital. AGM d.o.o. B.C. Decker Inc Hamilton-London 1998; 173-176
[20] Damani N, Keyes J, Campbell H: Infection Control Manual, Greenwich Media Ltd, UK 2004; 6.1-6.7.
[21] Miljković-Selimović B, Kocić B, Babić T, RistićLj. Bacterial typing methods, Acta Fac Med Naiss 2009; 26: 225-233.
[22] De Sousa MA, De Lencastre H. Bridges from hospitals to the laboratory: genetic portraits od methicillin-resistant Staphylococcus aureus clones. FEMS Immunol Med Microbiol 40, 101 (2004). http://dx.doi.org/10.1016/S0928-8244(03)00370-5
[23] Deurenberg RH, Vink C, Kalenić S, Friedrich AW, Bruggeman CA and Stobberingh EE. The molecular evolution of methicillin-resistant Staphylococcus aureus. Clin Microbiol Infect 2007;13: 222–235.http://dx.doi.org/10.1111/j.1469-0691.2006.01573.x
[24] Stranden A, Frei R, Widmer AF. Molecular typing of methicillin-resistant Staphylococcus aureus; can PCR replace pulsed-field gel electrophoresis? J Clin Microbiol 2003: 41: 3181-6 http://dx.doi.org/10.1128/JCM.41.7.3181-3186.2003
[25] Frenay HM, Bunschoten AE, Schouls LM, Van Leeuwen WJ, Vanderbroucke-Grauls CM, Verhoef J, ***et al***. Molecular typing of methicillin-resistant Staphylococcus aureus on the basis of protein A gene polymorphism. Eur J ClinMicrobiol Infect Dis 1996; 15: 60–64.http://dx.doi.org/10.1007/BF01586186
[26] Stromenger B, Kettlitz C, Weniger T, Harmsen D, Friedrich A, Witte W. Assignment of Staphylococcus isolates to groups by spa typing, SmaImacrorestriction analysis, and multilocus sequence typing. J ClinMicrobiol. 2006; 44:2533-40. http://dx.doi.org/10.1128/JCM.00420-06
[27] Harmsen D, Claus H, Witte W, Rothgänger J, Turnwald D, Vogel U, ***et al.*** Typing of methicillin-resistant Staphylococcus aureus in a university hospital setting by using novel software for spa repeat determination and database management. J. Clin. Microbiol. 2003; 41:5442-5448.http://dx.doi.org/10.1128/JCM.41.12.5442-5448.2003
[28] Otte KM, Jenner S, Wulffen HV. Identification of methicillin-resistant Staphylococcus aureus (MRSA): Comparison of a new molecular genetic test kit (GenoType MRSA) with standard diagnostic methods. Clin Lab 2005; 51(7-8): 389-39.
[29] Mc Dougal LK, Steward CD, Killgore GE, Chaitram JM, Mc Allister SK, Tenover FC. Pulsed-field gel electrophoresis typing of oxacillin-resistant Staphylococcus aureus isolates from the United States: Establishing a national database. J Clin Microbiol 2003; 41: 5113-5120. http://dx.doi.org/10.1128/JCM.41.11.5113-5120.2003
[30] Ostojić M. Epidemiologic genotyping of methicillin-resistant Staphylococcus aureus (MRSA) by pulsed-field gel electrophoresis (PFGE). Bosn J Basic Med Sci 2008; 8(3):259-265
[31] Vindel A, Cuevas O, Cercenado E, Marcos C, Bautista V, Castellares C, ***et al.*** Methicillin-Resistant Staphylococcus aureus in Spain: molecular epidemiology and utility of different typing methods. J Clin Microbiol. 2009; 47(6): 1620-1627. http://dx.doi.org/10.1128/JCM.01579-08
[32] Jarraud S, Mougel C, Thioulouse J, Lina G, Meugnier H, Forey F ***et al.*** Relationships between Staphylococcus aureus genetic background, virulence factors, agr groups (alleles), and human disease. Infect Immun 2002; 70(2): 631-341. http://dx.doi.org/10.1128/IAI.70.2.631-641.2002
[33] Manago K, Nishi J, Wakimoto N, Miyanohara H, Jay Sarantuya MT, Tokuda K ***et al.*** Biofilm formation by and accessory gene regulator typing of methicillin-resistant Staphylococcus aureus strains recovered from patients with nosocomial infections. Infect Control Hosp Epidemiol. 2006;27:188–190. http://dx.doi.org/10.1086/500620
[34] Kearns AM, Seiders PR, Weeler J, Freeman E, and Steward M. Rapid detection of methicillin-resistant staphylococci by multiplex PCR. J. Hospital. Infection 1999; 43: 33-37. http://dx.doi.org/10.1053/jhin.1999.0631
[35] Deurenberg RH, Vink C, Kalenić S, Friedrich AW, Bruggeman CA and Stobberingh EE.: The molecular evolution of methicillin-resistant Staphylococcus aureus. ClinMicrobiolInfect 2007, 13: 222–235. http://dx.doi.org/10.1111/j.1469-0691.2006.01573.x
[36] David MZ, Daum RS. Community-associated methicillin-resistant Staphylococcus aureus: epidemiology and clinical consequences of an emerging epidemic. ClinMicrobiol Rev 2010;23:616-87. http://dx.doi.org/10.1128/CMR.00081-09
[37] Kalenić S, Payerl Pal M, Vlahović Palčevski V, Horvatić J, Meštrović T, Baršić B ***et al.*** Guidelines for preventing, control and treatment of methicillin-resistant Staphylococcus aureus (MRSA) associated infections. Electronic form only 2011. Available from: http://www.iskra.bfm.hr/hrv/GuidlinesArticle.aspx?id=58. Accessed 20 January 2015.
[38] Witte W, Strommenger B, Cuny C, Heuck D, Nuebel U. Methicillin-resistant Staphylococcus aureus containing the Panton-Valentine leucocidin gene in Germany in 2005 and 2006. J AntimicrobChemother 2007;60:1258-63. http://dx.doi.org/10.1093/jac/dkm384
[39] Blanco R, Tristan A, Ezpeleta G, Rhod Larsen A, Bes M, Etienne J ***et al.*** Molecular Epidemiology of Panton-Valentine Leukocidin-Positive Staphylococcus aureus in Spain: Emergence of the USA300 Clone in an Autochthonous Population. J Clin Microbiol. 2011;49(1):433-436. http://dx.doi.org/10.1128/JCM.02201-10
[40] Moran GJ, Krishnadasan A, Gorwitz RJ, Gregory E, McDougal LK, Carey RB ***et al.*** Methicillin-resistant Staphylococcus aureus infections among patients in the emergency department. N Engl J Med 2006;355:666–674. http://dx.doi.org/10.1056/NEJMoa055356

[41] Nguyen D, Bancroft E, Mascola L, Guevara R, Yasuda L. Risk factors for neonatal methicillin-resistant Staphylococcus aureus infection in a well-infant nursery. Infect Cont Hosp Epidemiol 2007;28:406–411. http://dx.doi.org/10.1086/513122

[42] Diep BA, Chambers HF, Graber CJ, Szumowski JD, Miller LG, Han LL, ***et al.*** Emergence of multidrug-resistant, community-associated, methicillin-resistant Staphylococcus aureus clone USA 300 in men who have sex with men. Ann Intern Med 2008; 148:249–257.