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 gene, which encodes for an altered penicillin-binding protein (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 SCCmec [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.
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.

49
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, gentamyacin, amikacin, trimethoprim-sulamethoxazole, clindamycin, rifampin, cefoxitin, linezolid, teicoplanin and vancomycin. Antimicrobial susceptibility testing was confirmed with VITEK 2 Compact (Bio Merieux, 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 60°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 Perkin Elmer 9700 thermal cycler (Norwalk, CT, USA) with an initial activation step at 80°C for 5 minutes, followed by 45 cycles of denaturation at 94°C for 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’-ATGCACATTTGACATGC-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 1, 5’-GTGCAATCTATAAGCCTGAT-3’ (in the agrD gene)
2. **agr II**, 5′-GTATTACTAATTTGAAAAGTGCCATAGC-3′ (in the **agrC** gene)
3. **agr III**, 5′-CTGTTGAAAAAGTCACTAAAGGCTC-3′ (in the **agrD** gene)
4. **agr IV**, 5′-CGATAATGCCTAATACCCGG-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 of 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 **mecA** 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 μL of 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 35 μL PNM (biotin-labeled primers and dNTP), 5 μL polymerase incubation buffer, 2 μL 25 mM MgCl2, 1 μL 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 χ²-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 (χ²=70.586; df=10; p<0.001) (Figure 1A).

According to locations, in Zagreb the most common **spa**-type was to41 (64.3%), in Mostar to01 (64.7%) and in Heidelberg the most common type was to03 (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. (χ²-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<0.01). In CHC Zagreb, we have found exclusively **agr**-type II 29/29 (100%), in UCH Mostar
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**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).

**DISCUSSION**

*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 subotyping, 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
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-tying 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].

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

<table>
<thead>
<tr>
<th>Place</th>
<th>Sample</th>
<th>agr I</th>
<th>agr II</th>
<th>agr III</th>
<th>agr IV</th>
<th>agr I</th>
<th>agr II</th>
<th>agr III</th>
<th>agr IV</th>
<th>agr I</th>
<th>agr II</th>
<th>agr III</th>
<th>agr IV</th>
</tr>
</thead>
<tbody>
<tr>
<td>Zagreb, Croatia</td>
<td>BC</td>
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<td>1</td>
<td>0</td>
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<td>2</td>
<td>3</td>
<td>0</td>
<td>0</td>
<td>4</td>
<td>11</td>
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<tr>
<td></td>
<td>Wound</td>
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<td>0</td>
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<td>6</td>
<td>0</td>
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<td>1</td>
<td>3</td>
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<td>0</td>
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<td>0</td>
<td>0</td>
<td>11</td>
<td>35</td>
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**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

<table>
<thead>
<tr>
<th>Place</th>
<th>Number of isolates</th>
<th>agr-type</th>
<th>spa-type</th>
<th>mecA</th>
<th>PVL</th>
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<tbody>
<tr>
<td>Zagreb, Croatia</td>
<td>17</td>
<td>II</td>
<td>t041</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>7</td>
<td>II</td>
<td>t001</td>
<td>+</td>
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</tr>
<tr>
<td></td>
<td>5</td>
<td>II</td>
<td>t001</td>
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<td>-</td>
</tr>
<tr>
<td></td>
<td>1</td>
<td>II</td>
<td>t041</td>
<td>+</td>
<td>-</td>
</tr>
</tbody>
</table>
| Mostar, Bosnia-
| 11                 | II       | t001     | +    | -   |
| Herzegovina          | 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       | t1228    | +    | -   |
|                      | 1                  | II       | t535     | +    | -   |
|                      | 1                  | II       | t1083    | +    | -   |
|                      | 1                  | II       | t626     | +    | -   |
|                      | 1                  | I        | t024     | +    | -   |
|                      | 1                  | I        | t032     | +    | -   |
|                      | 1                  | I        | t001     | +    | -   |
|                      | 1                  | III      | +        | -    |     |
|                      | 1                  |         | +        | -    |     |

*agr* – Accessory gene regulator; *spa* – Staphylococcal protein A; *mecA* 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.](image)

We characterized MRSA strains by using different molecular typing tools. Using spa-tying 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.

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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 mecA in staphylococci. A various methods of mecA 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 mecA 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 susceptibility was associated with 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.

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