

Molecular Characterization of Methicillin-Susceptible And Methicillin-Resistant *Staphylococcus aureus* in Food Handlers in Bosnia and Herzegovina

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Abstract: *Objectives:* To determine the prevalence and genetic background of methicillin-sensitive (MSSA) and methicillin-resistant *Staphylococcus aureus* (MRSA) obtained from healthy food handlers admitted to the Cantonal Public Health Institute of Zenica, Bosnia and Herzegovina, during 2007-2009.

Methods: *S. aureus* were isolated and identified using standard microbiological methods including coagulase and catalase tests. Antibiotic susceptibility testing by disc diffusion method was performed according to the CLSI guidelines. Methicillin resistance was confirmed by the presence of the *mecA* gene by PCR. The genetic characterization was performed using *spa*-typing and BURP algorithm.

Results: A total of 189 non-duplicated *S. aureus* isolates were collected from 13 690 nasal swabs (1.4%), of which three were MRSA (1.6%). Among 173 MSSA analyzed, 66 *spa* types were clustered into nine *spa*-CCs, four no founders, and singletons. The MSSA *spa*-CC015 associated with MLST CC45 was predominant, having 41 (24%) strains. All three MRSA were associated with MLST 152 (*spa*-CC 355/595) which was not found in MSSA isolates. MRSA-related background had 60% MSSA isolates. There were 127 (71%) MSSA and one MRSA sensitive to all antibiotic tested (the beta-lactam compounds excepted); multi-drug resistance was found in 13 (7.3%) of MSSA.

Conclusion: Very low prevalence of *S. aureus*, as well as MRSA was noted. MSSA were more heterogeneous than MRSA. Although the number of MSSA with a genetic background common to MRSA clones was high, the prevalence of MRSA was low, and MLST CC152 of MRSA was not found among MSSA isolates suggesting that MRSA did not arise from predominant MSSA clones.

Keywords: Antimicrobial susceptibility, BURP, food handlers, MRSA, MSSA, *spa*-typing.

INTRODUCTION

Staphylococcus aureus is a very important human pathogen causing localized as well as invasive infections [1, 2]. When penicillin G was initially introduced in the early 1940s, over 85% of *S. aureus* isolates were susceptible to <0.1 mg/L of penicillin. However, penicillin-resistant staphylococci appeared and rose to 80% by 1957 [1].

Methicillin resistant *S. aureus* (MRSA) has traditionally been considered a hospital-associated (HA-MRSA) pathogen in patients with established risk factors [3, 4]. However, MRSA infections have been described in community dwellers too, without established risk factors for the acquisition of MRSA [5-7]. Yet, spreading of community-associated methicillin resistant *S. aureus* (CA-MRSA) in hospitals, causing nosocomial infections has been reported [8].

Active surveillance for MRSA detecting nasal carriage in a person without clinical symptoms of infection is a very important step in the infection control strategies in hospitals, because undetected MRSA carriers may serve as a source of the transmission [9, 10]. It is well documented that environment has been identified as a source of MRSA, environment could also serve as a vehicle of transmission [10-13]. Therefore, cleaning of all surfaces by which hospital care workers may transmit MRSA to patients *via* their hands is of great importance [11]. Staphylococcal food poisoning resulting from the growth of enterotoxigenic staphylococci in foods is the most common food illness existent in almost all parts of the world [14-16]. Accordingly, nasal and hand carriage of *S. aureus* in food handlers is an important source of staphylococcal food contamination [14-16].

It has been suggested that MRSA originated through the transfer of SCC*mec* element into methicillin-susceptible *S. aureus* (MSSA) and that genetic background determines the stability of the new MRSA clone [17, 18]. *S. aureus* has a high clonal population structure, and several typing methods have been developed for the investigation of population structure diversity of *S. aureus* [9]. It has been previously

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shown that the *spa* typing, as well as *spa* typing along with the algorithm based upon repeat pattern (BURP), which is in accordance with typing results obtained by multilocus sequence typing (MLST), represents a very good tool for global epidemiology as well as the evolutionary history of bacterial species [19, 20].

Nasal colonization of *S. aureus* in general as well as in specific population groups has been the object of a great number of studies [7, 9, 21- 23], but the diversity and population structure of *S. aureus* isolates from healthy food handlers have rarely been investigated [9, 14, 23]. Most of the studies on *S. aureus* associated with food poisoning have focused only on the screening of the isolates for enterotoxins, and/or the carriage and antimicrobial resistance among *S. aureus* obtained from food handlers [15, 16, 24].

In Bosnia and Herzegovina, there are limited data about the prevalence of *S. aureus* obtained from hospitalized/outpatient as well as from healthy food handlers [21], and there is no information about the MSSA/MRSA population structure and accordingly about the relation between MSSA/MRSA lineages. Therefore, in the present study, molecular characterization of *S. aureus* isolated from food handlers employed in the public objects dealing with food, in Zenica Doboj Canton, Bosnia and Herzegovina (B&H) has been investigated. Nowadays, investigation of *S. aureus* colonization, particularly in a specific population, as well as in outpatient settings, is very important for understanding the basic biology of *S. aureus* as well as providing a basis for the studies in hospital settings.

MATERIALS AND METHODS

Settings, Bacterial Isolates and Study Design

Laboratory for Sanitary and Clinical Microbiology of the Cantonal Public Health Institute of Zenica, Bosnia and Herzegovina (Laboratory) performs microbiological analyses of nasal swabs of food handling persons for the presence of *Staphylococcus aureus* (colonization). According to the law in B&H, for all persons dealing with food (employed in food industry or in distribution/preparation of food) it is mandatory to perform analyses of nose, throat and stool samples twice per year.

In this laboratory-based study all consecutive, non-duplicate *S. aureus* strains isolated from nasal swabs of food handling person admitted to the Laboratory in the period September 2007 - December 2009 were included. Institutional Review Board's approval was obtained before the initiation of the study.

For nasal sampling (one swab for both nares) sterile culture swabs (Stuart transport medium, Eurotubo, Espana) were used. Each swab was plated onto sheep blood (5%) agar plate (Columbia agar base, Oxoid, Basingstoke, UK). All plates were incubated at 35°C ambient air for 24h. *S. aureus* isolates were identified according to standard microbiological methods [25]. Isolates suspected of being *S. aureus* from sheep blood agar were first checked for catalase and Gram stain if it was necessary. All *S. aureus* isolates were confirmed by coagulase latex agglutination test (Oxoid). All *S. aureus* isolates were tested for oxacillin and cefoxitin sensitivity/resistance by disk-diffusion method at Mueller-Hinton (MH) agar (Oxoid, Basingstoke, UK) (growth zone inhibition around 1 µg and 30 µg oxacillin and

cefoxitin disk, respectively) in accordance to CLSI (Clinical Laboratory Standards Institute) [26].

The disc diffusion method using Mueller-Hinton agar (Oxoid, Basingstoke, UK) was used to test against 12 antimicrobials (Oxoid). The applied susceptibility criteria were according to CLSI [26]. *Staphylococcus aureus* ATCC 25923 control strain were used.

The oxacillin- and cefoxitin resistant or intermediate isolates were analyzed for the presence of the *S. aureus*-specific *femA* gene as well as the MRSA-specific *mecA* gene using a multiplex real-time PCR assay [5].

The genetic characterization of *S. aureus* isolates was performed at the department of Medical Microbiology of the Maastricht University Medical Centre (MUMC) (one isolate from each patient).

Information record for study patients admitted to the Laboratory included protocol number, gender, place of residence, agent isolated (MSSA or MSA). Age groups among healthy population were not defined, all were 18-65 years of age employed in the public objects dealing with food (food handlers).

Typing of the *spa* Locus

Real-time amplification of the *spa* locus, followed by sequencing, was performed as described before [2]. The *spa* types were clustered into *spa* CCs using the algorithm based upon repeat pattern (BURP) with the Ridom StaphType, version 1.5, software package (<http://www.ridom.de>) [19]. The default settings recommended by the manufacturer were used. Since it has been shown that *spa* typing, together with the algorithm BURP, yields results consistent with typing results obtained by multi-locus sequence typing (MLST) [19, 27-29], the associated MLST CCs, as determined with MLST, were allocated through the Ridom SpaServer (<http://spaserver.ridom.de>).

RESULTS

In the 2007 - 2009 period, a total of 189 non-duplicated *S. aureus* isolates (seven, 49 and 133, respectively) were collected out of 13,690 nasal swabs of food handling persons admitted to the Laboratory for Clinical and Sanitary Microbiology of Cantonal Public Health Institute Zenica, resulting in average year prevalence of 1.4% (1.1%, 0.9% and 1.8%, respectively). Only three (out of 189 *S. aureus*, 1.6%) MRSA were isolated, all during 2009.

Thirteen out of 189 isolates were contaminated, leaving 176 (173 MSSA and 3 MRSA) isolates for *spa* typing.

Among 173 MSSA isolated 130 (75.1%) were clustered into nine *spa*-clonal complexes (*spa*-CCs), nine (5.2%) strains into four no founder *spa*-CCs, and 19 (11.0%) strains were singletons. Fifteen (8.7%) strains were excluded from the BURP analysis because they belonged to *spa*-types smaller than five repeats (Table 1).

The main MSSA *spa*-CC was *spa*-CC015 associated with MLST CC45, which harbored 41 (23.7%) of the isolates, followed by *spa*-CC192 (26, 15.0%), *spa*-CC091 (14, 8.1%) which was associated with MLST CC22 and CC7, respectively (Table 1).

Table 1. Distribution of *spa* Types and *spa*-CCs Among Colonising MSSA*

<i>spa</i> -CC Type	Associated MLST CCs	No (%) of Strains	No (%) of <i>spa</i> -Types	<i>spa</i> -Types [†]
<i>spa</i> -CC015	45	41(23.7)	12 (18.2)	t015 (6), t050 (1), t505 (1), t 550 (2), t589 (1), t630 (1), t728 (24), t1469 (1), t1510 (1), t2223 (1), <u>t7135</u> (1), <u>t7157</u> (1)
<i>Spa</i> -CC192	22	26 (15.0)	9 (13.6)	t005 (15), t192 (1), t449 (1), <u>t6717</u> (1), <u>t7131</u> (3), <u>t7141</u> (2), <u>t7142</u> (1), <u>t7153</u> (1), <u>t7155</u> (1)
<i>Spa</i> -CC159	121	8 (4.6)	4 (6.1)	t159 (5), t435 (1), t645 (1), t1425 (1)
<i>Spa</i> -CC002	5	13 (7.5)	4 (6.1)	t002 (4), t010 (5), t088 (3), t1107 (1)
<i>Spa</i> -CC008/024	8	9 (5.2)	3 (4.5)	t008 (6), t024 (2), t701 (1)
<i>Spa</i> -CC084	15	4 (2.3)	3 (4.5)	t084 (1), t085 (2), t774 (1)
<i>Spa</i> -CC021/012	30	13 (7.5)	4 (6.1)	t012 (5), t017 (2), t019 (1), t021 (5)
<i>Spa</i> -CC1211		2 (1.2)	1 (1.5)	t1211 (2)
<i>Spa</i> -CC091	7	14 (8.1)	3 (4.5)	t091 (12), t289 (1), t796 (1)
2322/4373 (No founder)		2 (1.2)	2 (3.0)	t2322 (1), t4373 (1)
688/7144 (No founder)	5	1 (0.6)	1 (1.5)	<u>t7144</u> (1)
267/521 (No founder)	1	5 (2.9)	1 (1.5)	t267 (5)
3277/3589 (No founder)		1 (0.6)	1 (1.5)	t3277 (1)
Singletons	3	19 (11.0)	13 (19.7)	t089 (2), t177 (2), t375 (1), t488 (2), t845 (3), t1361 (2), t2526 (1), t2930 (1), t5890 (1), t6011 (1), <u>t7112</u> (1), <u>t7118</u> (1), <u>t7137</u> (1)
Excluded [‡]		15 (8.6)	5 (7.7)	t026 (11), t282 (1), t779 (1), t2736 (1), t7159 (1)
Total		173 (100.0)	66 (100.0)	

*CC, clonal complex as determined with MLST; MSSA, methicillin-sensitive *Staphylococcus aureus*; †underline *spa* types are new *spa* types; ‡*spa* types smaller than five *spa* repeats.

Among 173 MSSA isolates 66 *spa*-types were observed. The most common *spa*-types were t728 (24, 36.4%), t005 (15, 22.7%), t091 (12, 18.2%), t015 and t008 (six in each, 9.1%), t159, t012, t021, t267 and t010 (five in each, 7.6%) and each of the remaining 91 (52.6%) isolates were within 57 *spa*-types accounted for between 6.1% and 1.5% each (Table 1).

The three MRSA isolates belonged to *spa*-CC 355/595 (two strains were t355 and one t595 *spa*-types) associated with MLST CC152 (Table 2).

Fifteen (8.7%) isolates within 12 (out of 66, 18.2%) new *spa*-types among 173 MSSA were observed. All but two new *spa*-types contained one isolate; *spa*-type marked as t7131 contained three strains (two of which were isolated during year 2008 and one during year 2009), and t7141 contained two strains (Table 1).

No resistance to vancomycin, mupirocin and rifampicin was detected in MSSA or MRSA isolates. There were 127 (70.9%) MSSA and one (of the three) MRSA isolates sensitive to all antibiotics was tested (excluding of beta-lactam antibiotics). Prevalence of resistance to amikacin, erythromycin and ciprofloxacin of 22.0%, 7.3% and 12.5%,

respectively was noted in MSSA, but none in MRSA isolates. Much higher prevalence of resistance to gentamicin was noted in MRSA isolates, 66.7%, than in MSSA isolates, 6.9%. Thirteen (7.3%) MSSA isolates showed resistance to more than three antibiotics, but none of the MRSA isolates showed such resistance (Table 3).

DISCUSSION

This laboratory-based investigation describes a prevalence and population structure of the colonizing *S. aureus* (MSSA/MRSA) isolates using *spa*-typing and the algorithm BURP in Bosnia and Herzegovina.

Prevalence rates of MSSA and MRSA in the carriers (food-handlers) in this study were far lower than that in some studies investigating community carriers, 25% and 0.4%, respectively [7], military population (20.0% for MSSA), or children attending day care centres (31.3% and 1.2%, respectively) [22, 23]. Much higher *S. aureus* prevalence among food-handlers, of 44.6%, 53.2% and 23.1% was noted in Botswana, Kuwait, and South-eastern Anatolia, respectively [16, 24, 30] than in this investigation. However, there are also data about similar (lower) *S. aureus* prevalence

Table 2. Distribution of *spa* Types and *spa*-CCs Among Colonising MRSA*

<i>spa</i> -CC Type	Associated MLST CCs	No (%) of Strains	No of <i>spat</i> ypes	<i>spa</i> -Types
355/595 (No founder)	152	3 (100.0)	2	t355 (2), t595 (1)

*CC, clonal complex as determined with MLST; MRSA, methicillin-resistant *Staphylococcus aureus*.

Table 3. Antimicrobial Resistance of *S. aureus* Isolates Obtained from Healthy Food Handlers

Period	MSSA/MRSA	Percentages of Resistant Strains to Antimicrobial Agent*											
		MUP	IMP	ERY	VAN	GEN	AMK	TET	CIP	CLI	SXT	CHL	RIF
2007	MSSA (7)	0	0	0	0	0	28.6	14.3	0	0	0	0	0
2008	MSSA (49)	0	0	2.6	0	7.0	7.0	7.0	0	0	0	4.7	0
2009	MSSA (130)	0	0.8	1.5	0	9.2	26.9	5.4	17.8	2.9	5.0	5.0	0
2007-2009	MSSA (186)	0	0.5	1.9	0	6.9	22.4	5.9	12.6	1.7	4.0	5.2	0
	MRSA† (3)	0	0	0	0	66.7	0	0	0	0	0	0	0
	Total (189)	0	0.6	1.6	0	7.9	22.0	5.8	12.5	1.9	4.0	5.1	0

*MUP, mupirocin (5 µg); IMP, imipenem (10 µg); ERY, erythromycin; VAN, vancomycin (30 µg); GEN, gentamicin (10 µg); AMK, amikacin (30 µg); TET, tetracycline (30 µg); CIP, ciprofloxacin (5 µg); CLI, clindamycin (2 µg); SXT, trimethoprim/sulfamethoxazole (25 µg); CHL, chloramphenicol (30 µg); RIF, rifampicin (5 µg);
†MRSA isolated during 2009.

in carriers in Turkey (0.7%) in comparison with the results of this study [31]. Moreover, in the study from Turkey, none of *S. aureus* isolates were MRSA [31]. Comparing to previous investigations from this region [21] prevalence of MRSA among *S. aureus* isolates has remained the same (1.6%). However, overall prevalence of *S. aureus* (among nose samples analyzed) was much lower in this investigation as compared to the previous one from this region (1.4% vs 7.3%, respectively) [21]. Only three carriers were found to be colonized with MRSA, similarly to the results of other studies describing no risk population, like hospital personnel [9], as well as food-handlers according to the study from Kuwait [24]. However, the finding of MRSA among food-handlers is always significant because food handlers, as well as hospital personnel carrying MRSA had initiated outbreaks in hospitals in The Netherlands [32], USA [33, 34] and Brazil [15]. Furthermore, in case of household contacts, it was established that MRSA carriers had first contaminated the environment which served as MRSA source for further transmission [7, 35].

Genetic background of MSSA isolates observed in this study was heterogeneous and showed distinct clusters [9, 18]. The same MLST CCs found in this study were also found in other studies investigating MSSA colonizing humans [8, 9]. However, number of *spa*-types obtained in our population (66 *spa*-types among 173 MSSA isolates, 38.2%) was higher than in some other studies which found 37 and 49 *spa* types among 133 (27.8%) and 179 (27.3%) isolates, respectively [9, 18].

Some of bacterial genotypes found in this study, like MLST CC45 and CC22 were more successful in colonizing the investigated population than others [9]. This clone is widely spread both as MSSA and MRSA in Western Europe, Scandinavia, Canada, the United States, in hospital as well as ambulatory patients [9, 36- 38].

Although the number of MSSA with a genetic background common to MRSA (CC45, CC05, CC08, CC22, and CC30) in our carriers was high, the prevalence of MRSA was low. The presence of MLST CCs related to MRSA among MSSA isolates was found in other countries too, in hospitalized and outpatients, as well as in carriers [2, 4, 9].

No epidemiological connection between MRSA carriers was found, all of them were from distinct municipalities (all MRSA were MLST CC152). All three MRSA isolates were

associated with MLST CC152, which was not found in MSSA isolates. This finding might suggest that the MRSA isolates in our region have not originated from the introduction of SCC*mec* into dominant MSSA backgrounds presented in the investigated population, e.g. community environment, as it has been previously observed [6]. The presence of MSSA strains with MRSA genetic background is not a sole factor sufficient for the stable integration of SEC*mec* element and probably other factors (e.g. an environment, adaptation to different niches, carrier's characteristics) play an important role in the appearance and maintenance of the clusters [9, 17, 37].

Among our MSSA isolates several MSSA lineages were found that were not associated with MRSA background, CC7, CC12, CC15, CC25, and CC121, the observation previously described in other countries [2].

There was a large number of MSSA, and all (three) MRSA sensitive to all antibiotics tested (excluding beta-lactams), and small proportion of MSSA and none of MRSA isolates were multidrug resistant. Multidrug resistance characterizes HA-MRSA isolated from patients with identified risk, while antibiotic resistance in CA-MRSA is usually limited to beta-lactam antibiotics [1, 7, 8]. The results obtained from this study are largely different from the results obtained from studies in which far higher number of resistant *S. aureus* isolates among food handlers was found [16, 24].

The variations in the prevalence rates of *S. aureus* among food handlers between regions clearly reflect differences in personal and environmental hygienic measures. Staphylococcal food poisoning outbreaks are ranked highly (fifth place, 43 outbreaks with 1535 cases, which is 1.5% of all bacterial outbreaks) [34]. *S. aureus* strains were found in the specimens from the patients, and a hand lesion of a food handler, suggesting that the source of contamination for the outbreak most likely originated from a food handler [14]. Evaluation of the epidemiology of *S. aureus* colonization and risk factors may be useful for the development of effective prevention strategies aimed to control the spread of MSSA and MRSA [22].

The presented data have certain limitations that are inherent to all laboratory-based surveys, thus the number of sampled population in this study was probably not representative of food-handler population as a whole in

Zenica-Doboj Canton (the exact data do not exist) and might influence the prevalence. Secondly, about 50% of samples were sampled by staff in other Epidemiology Departments from the Zenica-Doboj Canton, so we are not able to control adequacy of nasal sampling method. Moreover, *S. aureus* strains from our collection belonged to one specific population (food handlers) from the three-year period only. For the estimation of spreading success mode of some genotypes (colonization of previously non-colonized carriers or displacement of other genotypes) a longitudinal study should be conducted, where the same cohort of carriers would be sampled over a longer period of time [9].

CONFLICT OF INTEREST

The authors confirm that this article content has no conflict of interest.

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