Original Article

Molecular typing of *Staphylococcus aureus* collected from a Major Hospital in Amman, Jordan

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Abstract
Introduction: Over the past decade methicillin-resistant *Staphylococcus aureus* (MRSA) has been recognized as a major cause of healthcare associated infections. Recently, however, epidemiology of this pathogen has changed drastically with the emergence of new clones in the community. Efficient epidemiological typing methods are essential to monitor and limit the occurrence and spread of epidemic clones.
Methodology: A total of sixty *S. aureus* isolates were collected from the Jordan University hospital in Amman-Jordan. All isolates were characterized using *Staphylococcus* protein A (*spa*) typing and pulsed-field gel electrophoresis (PFGE). Samples were tested for their susceptibility patterns against seven antimicrobial agents and for their potential to form biofilms.
Results: *spa* typing showed that *spa* type t044 was the most common representing 28% of the isolates studied and 38% of the MRSA population. PFGE revealed forty-six pulsotypes among the sixty tested isolates clustering similar *spa* types together. The predominant resistance was detected against levofloxacin, chloramphenicol and clindamycin. One MSSA isolate typed as *spa* t955 showed biofilm formation potential through protein deposition..
Conclusion: The study results are based on one hospital, but the findings of this and other studies conducted in the region indicate that there is an urgent need for standardized surveillances combined with the application of well-validated typing methods to assess the occurrence of MRSA and to control its spread.

Introduction

Staphylococcus aureus is the most commonly isolated human bacterial pathogen and is an important cause of skin and soft tissue infections (SSTIs) [1]. Colonization is an important step in the pathogenesis of *S. aureus* infection and is contributory for acquiring nosocomial infections [2]. Diseases ranging from superficial skin infections to severe endocarditis, pneumonia and bacteremia may result from an infection caused by *S. aureus* [3]. Several virulence factors contribute to the pathogenicity of *S. aureus* including Panton-Valentine leukocidin (PVL), protein A, toxic shock syndrome toxin 1 (TSST-1), enterotoxins and exfoliative toxins [1]. The induction of biofilm formation, by the adhesion of bacteria to a surface, followed by accumulation in multilayered cell clusters through intercellular adherence, is an additional vital infectious component [4].

Treatment of *S. aureus* infections is complicated due to the ability of this bacterial species to become resistant to antibiotics [5]. Methicillin resistant *S.*

aureus (MRSA) emergence and dissemination are of global significance in both health care and community settings [6]. MRSA has been reported to cause serious invasive and life-threatening infections in young children [7]. In addition, children may act as vectors helping *S. aureus* and specifically MRSA to spread in both community and hospital environments [6].

Efficient epidemiological typing methods are essential to monitor and limit the occurrence and spread of epidemic clones between hospitals; typing systems may facilitate the discrimination between unrelated isolates and the recognition of isolates that belong to the same clonal lineages. Different molecular approaches such as *spa* typing, multilocus sequence typing (MLST), SCC*mec* typing, and pulsed-field gel electrophoresis (PFGE) can be used to determine the genetic relatedness between isolates of clinical relevance [3].

In previous studies conducted in Jordan nasal carriage of MRSA was detected in 550 hospital staff members of four hospitals in north Jordan [8] and in

40% of the Jordanian healthy young adult population. MRSA represented 19% and 57% of the nasal and clinical isolates, respectively [9]. Furthermore, a retrospective study conducted on positive blood cultures taken from 378 children younger than 15 years of age, showed that *S. aureus* was the most common isolate in bacteremic children [10]. Finally, fourty-one MRSA and sixty-two MSSA (methicillin-sensitive) isolates were collected from children in Jordan. Genotyping based on *spa* and MLST revealed fourty-eight different *spa* types and identified distinct allelic profiles or STs, with the majority belonging to ST80. PFGE of fifteen different *spa* types revealed eight different pulsotypes, while SCC*mec* showed the predominance (53%) of subtype IV [11]. Taking into consideration the changing epidemiology of *S. aureus*, this study was conducted to determine the clinical, epidemiological, and molecular features of currently circulating MSSA and MRSA strains. Understanding the epidemiology of MSSA and MRSA infection, particularly in developing countries where the knowledge of *S. aureus* transmission dynamics is probably limited, is fundamental for devising effective prevention and control strategies.

Methodology

Isolates

A total of sixty *S. aureus* isolates were obtained from clinical specimens in the period between 2009 and 2010. These isolates were kindly provided by Dr. Asem Shehabi from the Jordan University hospital in Amman-Jordan (JUH). The JUH provides a specialized and high quality health care for about half a million patients a year from all over the country, with 600-beds inpatient capacity. The isolates were recovered from different sites of infection including mainly blood, wounds, catheters, burns, and nasal and breast swabs. Patients were distributed along a wide age range (one day up to 43 years). Isolates included both MRSA and MSSA. The samples were streaked on Mannitol Salt Agar (MSA) to be further purified and stored in Cryobanks at -20°C and -80°C. The isolates were designated with letters JS D, followed by the assigned lab number.

DNA extraction

Isolates were grown overnight on MSA at 37°C. DNA extraction was carried out using Nucleospin Tissue (Macherey-Nagel, Düren, Germany) and BIORAD InstaGene Matrix (Bio-Rad, Hercules, USA) according to the manufacturer's instructions. The extracted DNA was stored at -20°C until needed.

Multiplex PCR for detection of 16S rRNA, PVL, and mecA genes

Amplification of the 16S rRNA, PVL and *mec*A genes was done as previously described by McClure *et al.* [12]. PVL negative MRSA (N315) and PVL positive MSSA (ATCC 49775) were used to determine assay sensitivity. PCR products (7 µl) were resolved in a 1.5% Seakem gold agarose (Lonza, Rockland, USA) gel in 0.5% Tris-borate-EDTA buffer (Bio-Rad, Hercules, USA) at 6V/cm for one hour and were visualized with ethidium bromide.

spa typing Based Upon Repeat Pattern (BURP) analysis

The polymorphic X region of protein A gene (*spa*) was amplified as described previously [3, 13]. By applying the BURP algorithm implemented by the software, *spa* types with more than five repeats were clustered into different groups, with the calculated cost between the members of the group being ≤ 6 [14]. The *spa* type was assigned using the Ridom StaphType software version 2.2.1 (Ridom GmbH, Wüzburg, Germany).

Pulsed-field gel electrophoresis (PFGE)

All the sixty isolates were typed using PFGE technique to reveal a banding pattern on the gel. The protocol was adapted from Georing and Winters 1992 [15]. A bacteriophage lambda ladder PFG marker (New England BioLabs, Hitchin, UK) was included in each gel. Cluster analysis was performed with Gel-Compar II software version 6.5 (Applied Maths, Sint-Martens-Latem, Belgium) using dice coefficient and visualized as a dendrogram by the unweighted pair group method (UPGMA).

Antibiotic susceptibility testing

All tested *S. aureus* samples were tested against susceptibility for seven antibiotics using Kirby-Baüer disc diffusion assay following the recommendations of the Clinical and Laboratory Standards Institute (CLSI, 2010) [16]. Isolates were streaked on Müeller-Hinton agar (Oxoid, Basingstoke, UK) and incubated at 37˚C for 24 hours with the following antibiotic: levofloxacin (5 µg), chloramphenicol (30 µg), clindamycin (2 µg), erythromycin (15 µg), gentamicin (10 µg), rifampicin (5 µg) and oxacillin (1 µg). All discs were obtained from Oxoid (Basingstoke, UK) and *S. aureus* ATCC 29213 was used as a quality control strain.

Table 1: Determination of the OD$_{590\,nm}$ using ELISA reader for the studied isolates before and after the addition of one of the following: DNase I, proteinase K, and sodium metaperiodate

	Isolate								
	JS D 1	**JS D 2**	**JS D 3**	**JS D 9**	**JS D 10**	**JS D 51**	**ATCC 35984**	**TM 300**	**Blank**
Before enzymatic addition	0.176	0.176	0.216	0.193	0.174	0.893	0.812	0.201	0.166
DNase I	0.163	0.204	0.150	0.261	0.241	0.733	0.924	0.209	0.207
Proteinase K	0.162	0.150	0.158	0.145	0.199	0.167	0.975	0.188	0.180
Sodium metaperiodate	0.251	0.278	0.291	0.214	0.199	0.880	0.213	0.309	0.212

Biofilm assay

All isolates were grown in 96 well plates (Corning, New York, USA) according to the protocol adapted from Izano *et al.* [4]. Biofilm stability was then tested against DNase I, proteinase K, and sodium metaperiodate (Sigma Aldrich, Saint Louis, USA) enzymes, which were then added to separate wells for the same isolate leaving the fourth as a control (BHI + 0.25% glucose). Optical density OD$_{590nm}$ was read with ELISA reader (Multiskan FC Microplate reader, Thermo Fisher Scientific, Waltham, USA). *Staphylococcus epidermidis* RP62A (ATCC 35984) and TM300, kindly donated by Dr. Barbara Kahl from University of Münster, Germany, were used as positive and negative controls, respectively.

Results

Results showed that 68% (41 samples) of the isolates were MRSA, with 70% (43 samples) being PVL negative. Out of the eighteen PVL positive isolates, fifteen were MRSA (83%). All sixty isolates were typeable by s*pa* typing, which showed the presence of twenty-five different *spa* types; the most common being t044 representing 28% of the isolates followed by t037, t1149 and t318, t159 (Figure 1). Within MRSA, 38% were from *spa* type t044 and 12% *spa* type t037. Using BURP algorithm, the twenty-five *spa* types were clustered so that each *spa* type belonged to one cluster and was related to at least one other *spa* type within the cluster. There were two major *spa*-CCs, eight singletons, two clusters with no founder, and one s*pa* type (t386) was excluded.

All sixty isolates were genotyped by PFGE after *Sma*I enzyme digestion of chromosomal DNA. Forty-six pulsotypes were identified using 80% similarity as a cutoff value, and seven groups had more than one isolate. It is noteworthy that most isolates belonging to the same *spa* type such as t044, t386, and t037, were clustered together (Figure 2).
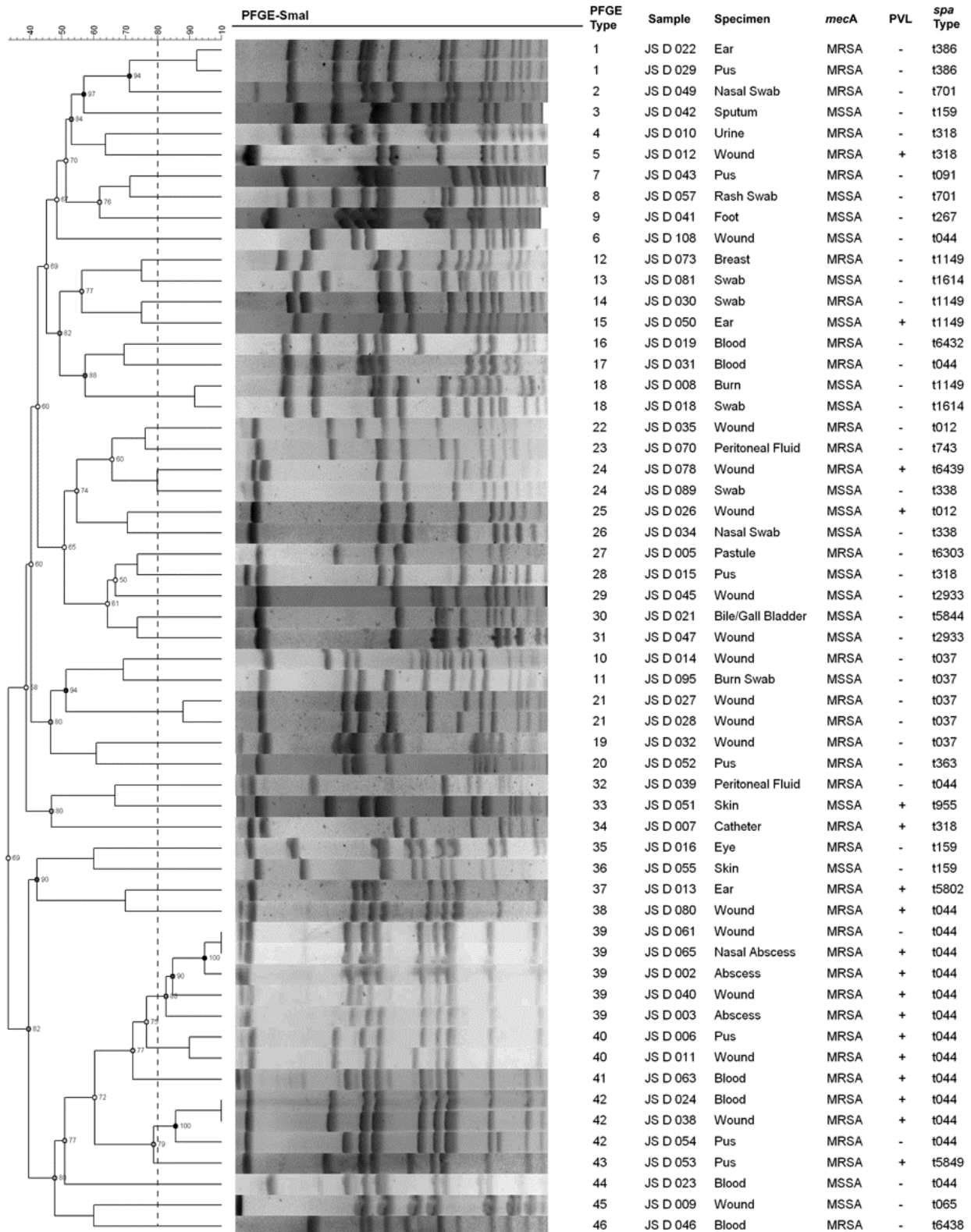
S. epidermidis RP62A (ATCC 35984) showed an OD of 0.812 in the control well at λ$_{590nm}$. This value did not vary significantly upon the addition of DNase I

and proteinase K. On the other hand, addition of sodium metaperiodate changed the OD significantly to 0.213. Tested *S. aureus* isolates showed an OD value between 0.160 and 0.216, similar to that of the blank, in the control well. JS D51, an MSSA with *spa* type t955, showed an absorbance value of 0.893 similar to that of the positive control. The OD however, dropped to 0.167 upon proteinase K addition, indicating the potential ability of this isolate to form biofilm through protein deposition (Table 1).

Antibiotic susceptibility was tested against seven antimicrobial categories including: aminoglycosides, ansamycins, penicillin, fluroquinolones, lincosamides, macrolides, and phenicols. According to the definitions proposed by Magiorakos *et al.* [17], 25% of all tested isolates were multidrug-resistant (MDR) and 67% were extensively drug resistant (XDR). MRSA and MSSA isolates exhibited resistance patterns to levofloxacin, chloramphenicol, clindamycin, erythromycin and gentamicin. Figure 3 demonstrates the percentage resistance to the tested antimicrobial agents for MRSA and MSSA, and the percentage of strains being MDR among MRSA and MSSA.

Figure 1. Percentage distribution of the recovered *spa* types in this study.
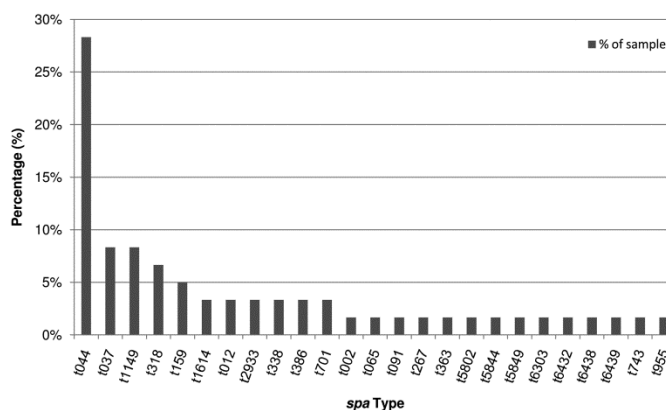
Figure 2. Dendrogram of all 60 *S. aureus* isolates following digestion with *Sma*I restriction enzyme, using GelCompar with percent similarity calculated by Dice coefficient (tolerance: 1%, optimization: 0.5%) and represented by unweighted-pair group method (UPGMA) settings. Lambda ladder PFG marker (New England BioLabs, UK) was included in each lab for alignment of bands. Similarity cutoff of 80% is represented by a vertical line and PFGE groups are numbered from 1 to 46. Specimen origin, presence of *mec*A and PVL genes as well as *spa* type for each sample is also included. The scale bar shows percentage similarity.

PFGE Type	Sample	Specimen	*mec*A	PVL	*spa* Type
1	JS D 022	Ear	MRSA	-	t386
1	JS D 029	Pus	MRSA	-	t386
2	JS D 049	Nasal Swab	MRSA	-	t701
3	JS D 042	Sputum	MSSA	-	t159
4	JS D 010	Urine	MRSA	-	t318
5	JS D 012	Wound	MRSA	+	t318
7	JS D 043	Pus	MRSA	-	t091
8	JS D 057	Rash Swab	MSSA	-	t701
9	JS D 041	Foot	MSSA	-	t267
6	JS D 108	Wound	MSSA	-	t044
12	JS D 073	Breast	MRSA	-	t1149
13	JS D 081	Swab	MSSA	-	t1614
14	JS D 030	Swab	MRSA	-	t1149
15	JS D 050	Ear	MSSA	+	t1149
16	JS D 019	Blood	MRSA	-	t6432
17	JS D 031	Blood	MRSA	-	t044
18	JS D 008	Burn	MSSA	-	t1149
18	JS D 018	Swab	MSSA	-	t1614
22	JS D 035	Wound	MRSA	-	t012
23	JS D 070	Peritoneal Fluid	MRSA	-	t743
24	JS D 078	Wound	MRSA	+	t6439
24	JS D 089	Swab	MSSA	-	t338
25	JS D 026	Wound	MSSA	+	t012
26	JS D 034	Nasal Swab	MSSA	-	t338
27	JS D 005	Pastule	MRSA	-	t6303
28	JS D 015	Pus	MSSA	-	t318
29	JS D 045	Wound	MSSA	-	t2933
30	JS D 021	Bile/Gall Bladder	MSSA	-	t5844
31	JS D 047	Wound	MSSA	-	t2933
10	JS D 014	Wound	MRSA	-	t037
11	JS D 095	Burn Swab	MSSA	-	t037
21	JS D 027	Wound	MRSA	-	t037
21	JS D 028	Wound	MRSA	-	t037
19	JS D 032	Wound	MRSA	-	t037
20	JS D 052	Pus	MRSA	-	t363
32	JS D 039	Peritoneal Fluid	MRSA	-	t044
33	JS D 051	Skin	MSSA	+	t955
34	JS D 007	Catheter	MRSA	+	t318
35	JS D 016	Eye	MRSA	-	t159
36	JS D 055	Skin	MSSA	-	t159
37	JS D 013	Ear	MRSA	+	t5802
38	JS D 080	Wound	MRSA	+	t044
39	JS D 061	Wound	MRSA	-	t044
39	JS D 065	Nasal Abscess	MRSA	+	t044
39	JS D 002	Abscess	MRSA	+	t044
39	JS D 040	Wound	MRSA	+	t044
39	JS D 003	Abscess	MRSA	+	t044
40	JS D 006	Pus	MRSA	+	t044
40	JS D 011	Wound	MRSA	+	t044
41	JS D 063	Blood	MRSA	+	t044
42	JS D 024	Blood	MRSA	+	t044
42	JS D 038	Wound	MRSA	+	t044
42	JS D 054	Pus	MRSA	-	t044
43	JS D 053	Pus	MRSA	+	t5849
44	JS D 023	Blood	MSSA	-	t044
45	JS D 009	Wound	MSSA	-	t065
46	JS D 046	Blood	MRSA	-	t6438

Figure 3. Percentage of *S. aureus* isolates that are resistant to the tested antimicrobial agents for MRSA and MSSA, along with the percentage of strains that were MDR and being MRSA or MSSA. Levofloxacin (LEV), Chloramphenicol (C), Clindamycin (DA), Erythromycin (ERY), and Gentamicin (GEN).
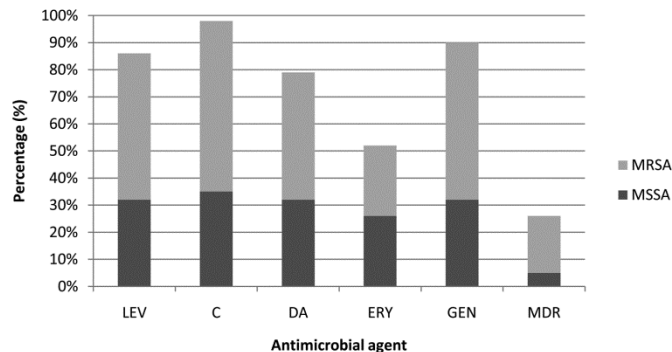
Discussion

Infections due to MRSA are becoming a major health issue in the Middle East, yet questions on the epidemiology and infection remain unanswered. This study focuses on *spa* typing and PFGE of *S. aureus* samples obtained from one hospital in Amman, Jordan. Although the results of this study cannot be generalized to the whole country, they revealed the distribution of fourty-one MRSA isolates over eighteen *spa* types, as opposed to fourteen *spa* types for the nineteen MSSA isolates. This reflects a higher diversity in the genetic profile of MSSA compared to MRSA, which was an observation previously reported also by Grundmann *et al.* [18]. The most commonly occurring *spa* types were t044 and t037. The high prevalence of *spa* type t044 was in concordance with several other studies including: Vindel *et al.* [19] who showed that this type was widely disseminated in Europe, Tokajian *et al.* [20] reporting that 38% of *S. aureus* isolates belonged to *spa* type t044 in Lebanon, and Khalil *et al.* [11] revealing that 18% of MRSA recovered from stool and nose specimens from children in Jordan were of *spa* type t044. *spa* type t044 belongs to the one of the recently emerging PVL-positive MRSA strains (ST80-MRSA-IV), known to occur and predominate in certain regions in Europe and in the Middle East [21].

Using BURP algorithm, all *spa* types were clustered into two *spa*-CCs, eight singletons, and three groups with no founders. Both *spa*-CC 044 and 037 contained more than one *spa* type being both MSSA and MRSA. These overlapping clonal complexes, which included both MRSA and MSSA, could be indicative of the extension of MRSA from MSSA through the acquisition of the SCC*mec* [22].

The sixty isolates were analyzed by PFGE, and fourty-six pulsotypes were obtained when 80%

similarity cutoff value was used. PFGE has clustered similar *spa* types together showing the high discriminatory power of PFGE as previously described by Cookson *et al.* and Bosch *et al.* [23, 24].

The ability to form biofilm was determined through measuring the absorbance values of all of the sixty isolates and comparing them to that of *S. epidermidis* (positive control). Only one MSSA isolate belonging to *spa* t955 and which clustered as a singleton, showed an initial absorbance of 0.893, which was comparable to the positive control. This value decreased to 0.167 upon proteinase K addition and remained high in the presence of sodium metaperiodate and DNase. Since sodium metaperiodate, proteinase K, and DNase indicated the presence of polysaccharide intercellular adhesion (PIA), proteinaceous factors, and genomic eDNA, respectively, we could infer that this isolate was capable of forming biofilm through protein deposition [25]. The ability of *S. aureus* to form more pronounced biofilms was previously correlated to the site of infection. The potential biofilm former obtained in this study was isolated from the skin, which was in harmony with the finding that *S. aureus* isolated from skin has a better ability to form completely recognized biofilms compared to those taken from other sites of the body [26]. This might be explained by the tendency of *S. aureus* to use the biofilm formation as an advantage to occupy the unstable environment of the human skin, placing the host at risk if the skin is breached [27]. Bacterial biofilms are structured communities and the formation of these sessile communities and their inherent resistance to antibiotics and immune attack are at the root of many persistent chronic bacterial infections [28].

Conclusion

Given the great diversity observed within the MSSA and MRSA clinical isolates, it seems likely that many different strains circulate into the hospital and in the region. To obtain a better view of what is circulating in the region, and what the dimensions of the problem are, it is recommended that surveillance programs combined with dedicated typing methods should be performed.

University Hospital from Amman, Jordan as well as Dr. Barbara Kahl from University of Münster, Germany, for providing the reference strains used in the biofilm protocol.

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