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Biofilm formation and its relationship with the molecular characteristics of food-related methicillin-resistant *Staphylococcus aureus* (MRSA)

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Abstract

The capability to produce biofilm is an important persistence and dissemination mechanism of some foodborne bacteria. This paper investigates the relationship between some molecular characteristics (SCC*mec*, ST, *spa*-type, *agr*-type, *cna*, *sar*A, *ica*A, *ica*D, *clf*A, *fnb*A, *fnb*B, *hla*, *hlb*) of 22 food-related methicillin-resistant *Staphylococcus aureus* (MRSA) strains and their ability to form biofilm on stainless steel and polystyrene. Five (22.7%, 5/22) strains were able to synthesize biofilm on polystyrene, and one of these (4.5%, 1/22) strains was also able to synthesize biofilm on stainless steel. The largest amount of biofilm was formed on polystyrene by 2 MRSA strains isolated from cows' milk, thus raising concern about the dairy industry. The majority of MRSA biofilm producers carried SCC*mec* type IVa, suggesting that the presence of SCC*mec*IVa and/or *agr* type III could be related to the ability to form biofilm. In conclusion, in order to achieve an acceptable level of food safety, Good Hygiene Practices should be strictly implemented along the food chain to reduce the risk of colonization and dissemination of MRSA biofilm-producing strains in the food industry.

Keywords: *agr*-type, biofilm formation, food safety, *icaA*, *icaD*, MRSA, SEM

Practical Application

In this study, some assayed isolates of food-related MRSA demonstrated the capacity to form biofilm. Biofilm formation differed according to surface characteristics and MRSA strains. A

relationship was observed between some molecular characteristics and the ability to form biofilms. Few studies have investigated the ability of MRSA to form biofilms, and the majority of these studies have investigated clinical aspects. This work was performed to investigate whether or not there is a difference between MRSA food isolates and MRSA clinical isolates in their ability to form biofilm. These initial findings could provide information that will contribute to a better understanding of these aspects.

Introduction

Methicillin-resistant *Staphylococcus aureus* (MRSA) is the most commonly identified antimicrobial-resistant pathogen in many parts of the world (Taylor 2013). While it has long been recognized as a hospital-related infection (Virgin and others 2009), MRSA epidemiology has changed in recent years with the emergence of community-acquired MRSA (Jones and others 2002). At present, new evidence suggests that domestic animals, including food animals, are capable of acting as reservoirs and MRSA shedders, and that transmission may be possible between host species (de Boer and others 2009). The emergence of MRSA in food-producing animals has caused great concern for the presence of MRSA in animal-derived foodstuffs; MRSA has been isolated from various foods of animal origin, giving cause for concern about possible dissemination throughout the food production chain (Crago and others 2012; Hiroi and others 2012; Caballero G´omez and others 2013; Normanno and others 2015; Parisi and others 2016). Both methicillin-sensitive *S. aureus* (MSSA) and MRSA can form biofilms on various surfaces (Rode and others 2007; Scott and others 2008; Mirani and others 2013; Di Ciccio and others 2015). Biofilm is defined as a community of organisms encased in a protective and adhesive matrix that is a prevalent mode of growth for microorganisms (Mandlik and others 2008). Furthermore, staphylococcal biofilms allow MRSA strains to adhere to surfaces, including surgical implants and materials used in the food industry; Scott and others (2008) reported MRSA found on various household surfaces such as sponges/cloths, dish drainers, and work surfaces.

Biofilm formation in *S. aureus* is mediated by the intercellular adhesion operon (*ica*) formed by the genes *ica*A, *ica*B, *ica*C, and *ica*D and a regulator gene, *ica*R, which encodes the ICAA, ICAB, ICAC, and ICAD proteins (Gad and others 2009), but *agr*-locus and other genes have also been implicated in biofilm formation by *S. aureus* (Tsang and others 2008). Although the exact mechanisms and process of biofilm formation in MRSA are poorly understood, 2 studies performed by the same research group suggested that PBP2a is also an important factor in biofilm accumulation (Pozzi and others

2012; Rudkin and others 2012). The contribution of the contaminated environment to the spread of antimicrobial-resistant microorganisms is not well understood; to date, few studies have investigated the ability of MRSA to form biofilms, and the majority of these studies have investigated clinical aspects (Kwon and others 2008; Atshan and others 2013; Cha and others 2013). Here we studied the biofilm-forming ability of food-related MRSA strains and the relationship between some molecular characteristics of MRSA and their ability to form biofilm.

Materials and Methods

Bacterial strains

The experiment was conducted on 22 MRSA strains isolated from milk and meat (*n* = 9 strains from cows' milk and *n* = 13 strains from pork). The strains were identified by phenotyping methods. Stock cultures were stored at −80 °C, and the strains were incubated for 24 h at 37 °C in Tryptone Soy Broth (TSB; Oxoid S.p.A., Milan, Italy) before testing.

MRSA characterization. All isolates were confirmed as MRSA by the detection of *mec*A and *nuc* genes, and were characterized by SCC*mec* typing, *spa*-typing, and multilocus sequence typing (MLST).

DNA extraction. The Genomic Prep DNA isolation Kit (Amersham Pharmacia Biotech, N.Y., New York, U.S.A.) was used to extract bacterial DNA from 1 mL of each culture broth, following the manufacturer's instructions. The extracts were tested for the detection of methicillin resistance *mec*A, thermostable nuclease *nuc* genes, and for some virulence factors, as reported below.

Detection of nuc and mecA genes. The DNA extracts were subjected to a duplex-PCR protocol for the detection of methicillin resistance *mec*A and thermostable nuclease *nuc* genes (Virgin and others 2009). A methicillin-susceptible *S. aureus* strain (ATCC 29213) was used as a negative control and an MRSA strain (ATCC 33591) as a positive control.

SCC-mec typing. Staphylococcal cassette chromosome *mec* element (SCC-*mec*) typing was carried out as described by Zhang and others (2005).

Spa-typing. The x region of the *spa* gene was amplified by PCR using primers reported in Table 1. DNA sequences were obtained using an ABI 3130 Genetic Analyzer (Applied Biosystems, Foster City, Calif., U.S.A.) and BigDye 3.1 Ready reaction mix (Applied Biosystems) according to the manufacturer's instructions. BioNumerics 7.1 (Applied Maths, Bruxelles, Belgium) software was used to determine *spa* types.

MLST. Alleles at the 7 loci, *arc*, *aro*E, *glp*F, *gmk*, *pta*, *tpi*, and *yqi*L, were assigned by comparing the sequences at each locus with those of the known alleles in the *S. aureus* MLST database. The allele numbers at each of the 7 loci define the allelic profile of each isolate, and an allelic profile is defined as a sequence type (ST). The eBURST program was used to determine the group of each ST based on the MLST database. Grouping was carried out using an analysis panel that selects 6 minimum numbers of identical loci out of 7 loci for group definition and 3 minimum single locus variant contents for subgroup definition (Kwon and others 2005).

Detection of virulence factors. The 22 food-related MRSA strains and 3 reference strains (*S. aureus* ATCC 35556, *S. aureus* ATCC 12600, *S*. *epidermidis* 12228) were tested for the detection of some virulence determinants. Genotyping of the *S. aureus* accessory gene regulator (*agr)* was conducted by multiplex PCR amplification of the hypervariable domain of the *agr* locus using a single forward primer and 4 reverse primers specific for each of the 4 major specificity groups (*agr I to IV*), according to Shopsin and others (2003). PCR assays were conducted on genes encoding for intercellular adhesion (*icaA* and *icaD*), the encoding collagen binding protein (*cna*), the encoding clumping factor A (*clfA*), the encoding fibronectin binding proteins A and B (*fnbA* and *fnbB*), the *α* and *β* hemolysins (*hla* and *hlb*), and the regulator protein (*sarA*). Primers used in this work were synthesized by Integrated DNA Technologies (IDT, Coralville, Iowa, U.S.A.), and are reported in Table 1. Gene amplification was performed as described by Graber and others (2009), with some modifications. Briefly, the PCR reaction mix (total volume of 25 μL) for amplification of the *icaA*, *icaD*, *cna*, *fnbA*, *fnbB*, *clfA*, *hla*, *hlb*, and *sarA* genes contained 1X Maxima Hot Start PCR Master Mix (Thermo Fisher Scientific, Waltham, Mass., U.S.A.), 1 μM of each primer, and 5 μL of the lysate nucleic acids. For the *agr* genotyping, multiplex PCR assays were conducted using 0.3 μM of each primer. The PCR profile for *agr* genotyping was 95 °C for 5 min, followed by 35 cycles comprising 95 °C for 40 s, 50 °C for 40 s, and 72 °C for 40 s. The final elongation step was performed at 72 °C for 10 min. Then *icaA*, *icaD, cna, fnbA, hla*, and *sarA* were amplified using the following PCR cycle: 95 °C for 5 min, followed by 40 cycles comprising a denaturation step at 95 °C for 40 s, followed by the annealing step at 50 °C for 40 s and extension at 72 °C for 40 s. The final elongation step was performed at 72 °C for 10 min. The PCR conditions for *clfA*, *fnbB*, and *hlb* amplification were 95 °C for 5 min followed by 35 cycles of 95 °C for 35 s, 50 °C for 35 s, and 72 °C for 1 min. The final elongation step was performed at 72 °C for 10 min. Negative and positive controls were included in every run for all the different PCRs. Nucleic acid of *S. epidermidis* ATCC 12228 DNA was used for the negative control, and *S. aureus* ATTC 35556 DNA (Gene Bank; NCBI Reference Sequence: NC_007795.1) as the positive control. All

PCR reactions for the detection of virulence genes were performed using a Techne TC- 412 thermal cycler (Bibby Scientific Limited, Staffordshire, U.K.). PCR assays were visualized by agarose electrophoresis (1% agarose gel in Tris-acetate-EDTA buffer) and GelRed (Biotium, Hayward, Calif., U.S.A.) staining.

Biofilm production assay

The 22 food-related MRSA strains were tested for biofilm production. For this purpose, the 2 *S. aureus* and the *S. epidermidis* reference strains (*S. aureus* ATCC 35556, *S. aureus* ATCC 12600, *S. epidermidis* 12228) were used as controls to classify the MRSA studied into different categories. Biofilm formation, expressed as Biofilm Production Index (BPI), was compared with reference strains: *S. aureus* ATCC 35556—strong biofilm producer (Cramton and others 1999; Seidl and others 2008) as positive control (BPIPC); *S. aureus* ATCC 12600—moderate biofilm producer (Di Ciccio and others 2015) (BPI12600); *S. epidermidis* 12228— negative biofilm producer (Atshan and others 2012; Lee and others 2014) as negative control (BPINC) for each isolate (Table 2). The cutoff point for biofilm production was the BPI value obtained by negative control on polystyrene (BPINC = 0.294) and stainless steel (BPINC = 0.149). MRSA biofilm-producing strains were classified as weak (BPINC ≤ MRSA BPI < BPI12600), moderate (BPI12600 ≤ MRSA BPI < BPIPC), or strong (MRSA BPI ≥ BPIPC). Before the experiments, all MRSA strains were activated by culturing twice in 10 mL TSB (Oxoid S.p.A.) at 37 °C for 24 h. A previously described method was used (Di Ciccio and others 2015). Polystyrene tissue culture plates (961 mm^2) and AISI 304 stainless steel chips (530 mm^2) were used for biofilm formation assays at 37 °C. Stainless steel chips were degreased before use by overnight immersion in ethanol, then rinsed thoroughly in distilled water and autoclaved for 15 min at 121 °C. *S. aureus* cultures were grown overnight on Tryptone Soy Agar (TSA, Oxoid, S.p.A.), and then incubated in TSB at the selected temperature of 37 °C. Cultures were then washed 3 times with phosphate buffered saline (PBS; pH 7.3) (Sigma-Aldrich S.r.l., Milan, Italy) and diluted with fresh TSB to reach a concentration of about 10^8 CFU/mL by reading the optical density (OD) level at 550 nm (UV Mini-1240—Shimadsu, New York, U.S.A.). We then added 3 mL of the standardized *inocula* to polystyrene tissue culture plates (35 mm dia) and stainless steel chips. Samples were then incubated at 37 °C. After 24 h incubation, nonadherent cells were removed by dipping each sample 3 times in sterile PBS. Samples were fixed at 60 °C for 1 h and stained with 3 mL of 2% crystal violet solution in 95% ethanol for 15 min. After staining, samples were washed 3 times with distilled water. Negative controls underwent the same treatment, without inoculation. Quantitative analysis of biofilm

production was performed by adding 3 ml of 33% acetic acid to destain the samples. Then 200 μL of each sample was transferred to a microtiter plate and the OD level of the crystal violet present in the destaining solution was measured at 492 nm (Varian SII Scan Cary 100, New York, U.S.A.). Considering the different growth areas of the tested surfaces (polystyrene = 961 mm2 and stainless steel = 530 mm2), results were normalized by calculating the BPI as follows: BPI = [ODmean biofilm surface $(mm^2)^{-1}$] × 1000. Two independent sets of all experiments were performed in triplicate. Biofilm formation, expressed as BPI, was compared with reference strains for each isolate. Finally, all isolates were classified into different categories on the basis of their BPI values.

Scanning electron microscopy (SEM) of MRSA biofilms

Biofilm formation was further confirmed by SEM. For SEM analysis, we selected one biofilm positive strain (MRSA 4) that was categorized as a strong biofilm producer on polystyrene. Biofilms were prepared as described above. The microbial cells were grown at 37 °C for 24 h on polystyrene tissue plates and then washed by dipping 3 times in sterile PBS to remove non-adherent cells. Samples were dehydrated in ethanol–water mixtures with increasing ethanol concentrations (65%, 75%, 85%, 95%, and 100%), and finally air-dried overnight.

Statistical analysis

Hierarchical cluster analysis was performed by the single Linkage method, in order to segment the microbial strains by using their ability to produce biofilm on polystyrene and stainless steel (STATISTICA ver. 10, StatSoft Inc., Tulsa, Okla., U.S.A.). BPI values of 0.294 and 0.149 were defined as lower limits for considering the sample as able to produce biofilms on polystyrene and stainless steel, respectively.

Results and Discussion

MRSA characterization

All the strains were confirmed to be MRSA harboring the *nuc* and *mec*A genes; the molecular characteristics of the strains are reported in Table 3. Of the 22 strains analyzed, 13 *spa* types and 8 STs were detected, with a prevalence of ST 398 (12/22; 54.5%). All strains carried the SSC*mec* type IVa (36.4%) or V (50%) and showed the presence of the *ica*A, *ica*D, *fnb*A, and *hla* genes. All MRSA strains were found to be *fnbB* negative. The distribution of virulence-associated genes (adhesin

encoding, toxin encoding, and gene regulators) detected in the 22 MRSA strains studied are shown in Table 3. With a range of over 90%, most isolates had a similar distribution of adhesion genes (*icaA, icaD, cna, fnbA*, and *fnbB*), toxin genes (*hla* and *hlb*), and staphylococcal regulators (*agr* and *sarA*).

Biofilm production assay

In this study, polystyrene and stainless steel were selected because they are the most widely used materials in medical devices and food processing equipment. Biofilm formation was observed in 5 (22.7%, 5/22) isolates that were able to synthesize biofilm on polystyrene; of these, one (4.5%, 1/22) strain was also able to synthesize biofilm on stainless steel (weak producer). Of the biofilm producers on polystyrene, one (20%, 1/5) strain produced moderate/strong biofilm, one (20%, 1/22) moderate biofilm, and 3 (60%, 3/5) strains were classified as weak biofilm producers (Figure 1). The highest amount of biofilm was formed on polystyrene by 2 MRSA strains (MRSA 7 – BPI = 0.61; MRSA 4 – BPI = 0.71) isolated from cows' milk, although they were either weak biofilm producers (MRSA 4) or nonproducers (MRSA 7) (MRSA 7 – BPI = 0.12; MRSA 4 – BPI = 0.15) on stainless steel (Figure 1). In agreement with the results of Pagedar and others (2010) and Di Ciccio and others (2015), our data demonstrated that biofilm formation occurred more on polystyrene (22.7 %) than on stainless steel (4.5 %). In particular, the strain (MRSA 4) that produced biofilm on stainless steel (weak producer) was also able to synthesize biofilm on polystyrene (moderate/strong producer).

Relationship between molecular characteristics and biofilm production

Of the biofilm-forming isolates, 4/5 (80%) showed SCC*mec* type IVa. Biofilm-forming isolates had the following genetic profiles: ST1/t174/IVa; ST1/t127/IVa; ST5/t688/V; ST8/t unknown/ IVa; ST398/t011/IVa. The majority (11/17; 64.7%) of nonbiofilm-forming isolates contained ST398 clones. The majority (3/5, 60%) of biofilm positive isolates were found to carry *agr* type III; 1 isolate carried *agr* type I and 1 carried *agr* IV. Fourteen out of 17 (82.3%) of the biofilm-negative isolates were found to carry *agr* type I, and 3/17 (17.6%) carried *agr* type III. Of the biofilm-forming isolates, 1/5 (20 %) were *cna* negative. The production of the *ica*ADBC operon encoded PIA by *S. aureus* is one of the most studied mechanisms of biofilm formation (O'Gara 2007; Joo and Otto 2012). However, biofilm formation independent of the *ica* operon has also been described in *S. aureus* (Geoghegan and others 2010). Expression of biofilm-associated genes is very complex and is influenced by a variety of factors, such as environmental conditions. In this study, all MRSA isolates

(both biofilm positive or negative) were found to carry the *ica*A gene. However, as shown in the present study, and also reported elsewhere, individual strains are often found that are both *ica* positive and biofilm negative (Cha and others 2013). The *fnb*B gene was not found among biofilm-producing MRSA strains and nonproducing MRSA strains. This means that the *fnb*B gene may not be correlated with biofilm-forming ability, although a previous study suggests that *fnb*B mediated biofilm development is common (O'Neill and others 2008). Regarding the relationship between genetic characteristics (virulence factors and the accessory-gene-regulator) and the ability to form biofilm on both polystyrene and stainless steel of our strains, no clear relationship was observed between these strains and their genetic characteristics. However, considering only the biofilm-forming strains, SCC*mec*-typing performed in this study found that 4 (80%) of the 5 biofilm-positive MRSA strains belong to SCC*mec* type IVa, suggesting that MRSA strains carrying SCC*mec* type IVa are more likely to form biofilm than those with SCC*mec* type V. Our results on polystyrene showed that the majority of MRSA biofilm-producers carried SCC*mec* type IVa; in contrast, the most common SCC*mec* type in non-biofilm-forming strains (17) was SCC*mec* typeV (11/17, 64.7%) (Table 3). Similarly, the results regarding stainless steel showed that one (4.5%) biofilm-producing strain (MRSA 4) carried SCC*mec*IVa (Table 3). This corroborates the hypothesis that the presence of SCC*mec*IVa may be related to the ability to form biofilm. Furthermore, a cluster analysis on the 5 biofilm-producing strains gave 2 groups. The 1st contained strains MRSA 7 and MRSA 4, and the 2nd contained strains MRSA 10, MRSA 11, and MRSA 3. These findings, limited to SCC*mec*-typing, agree with the results ofMirani and others (2013) that all the biofilm-positive isolates belonged to SCC*mec* type Iva, and the majority (91.8%) carried *agr* type II. Biofilm-negative isolates, on the other hand, belonged to SCC*mec* type V (Mirani and others 2013). Kwon and others (2013) also supported this finding, and reported that MRSA strains with SCC*mec* type IV are more likely to form biofilm than other types of SCC*mec.* Other authors also reported that strong biofilm-producing strains belong to SCC*mec* type IV and *agr*-type II; these authors suggested that SCC*mec* type IV and *agr* type II are a good combination for biofilm formation in foodborne MRSA isolates (Manago and others 2006; Cafiso and others 2007). In contrast, our study showed that of the moderate/strong biofilm producers, one MRSA strain contained ST8/SCC*mec*IVa/*agr*III (MRSA 4) and one contained ST5/SCC*mec*V/*agr*IV (MRSA 7) genotypes, whereas the 2 weak biofilm producers contained ST1/SCC*mec*IVa/*agr*III genotype (MRSA 3, MRSA 11), and one weak biofilm producer contained ST398/SCC*mec*IVa/*agr*I genotype (MRSA 10). However, further studies involving a larger number of food-related MRSA strains are needed in order to confirm a relationship between the *SCCmec* ST

and the ability to form biofilms. Interestingly, the highest amount of biofilm was formed on polystyrene by 2 MRSA strains (MRSA 7: ST5/t688/SCC*mec*V/*agr*IV and MRSA 4 ST8/t-unknown/SCC*mec*IVa/*agr*III) isolated from cows' milk (Table 3). It is well known that MRSA detected from milk and dairy products can be staphylococcal enterotoxin(s) (SEs) producers (Parisi and others 2016). *S. aureus* has been described as forming biofilm on various materials commonly used in food processing plants (Lee and others 2014). The biofilm-forming ability of MRSA that are potentially SEs producers should be of concern for food safety, since they may colonize and spread in food-producing plants and cause food contamination.

Statistical analysis

In order to classify the MRSA strains according to their ability to produce biofilm, a hierarchical dendogram was obtained by considering all the data from both polystyrene and stainless steel. The similarities among the strains were evaluated using the Euclidean distances. Although some similarities were highlighted, such as a cluster consisting of strains MRSA 3, MRSA 10, and MRSA 11 and a cluster consisting of strains MRSA 7 andMRSA 4, no common genetic characteristic was observed among these strains. A 2nd series of cluster analysis was performed to separate the ability to produce biofilm on polystyrene and stainless steel. The results on polystyrene showed that 5 (22.7%) strains out of a total of 22 may be considered as biofilm-forming isolates. Moreover, 80% of these showed SCC*mec* type IVa, while the most common SCC*mec* type in the nonbiofilm-forming isolates was V. Similarly, the results regarding stainless steel showed that one (4.5%) strain (MRSA 4, SCC*mec* type IVa) of the 22 may be considered as biofilm forming. SEM of MRSA biofilms SEM analysis allows observation of bacteria/surface interaction and may be used as a semiquantitative technique; in our study, SEM proved to be a useful technique for confirming the presence of an extracellular polysaccharide and glycoprotein network layer, and also for better understanding of biofilm structures. As shown in Figure 2, an MRSA isolate (MRSA 4) showed an extracellular product surrounding the cell aggregate on polystyrene tissue plates. In more detail, after 24 h of incubation, meshwork-like structures were observed around the cells at 37 °C and the surface tested was covered with dense cell clusters.

Conclusion

As far as we know, few researchers have investigated the relationship of molecular characteristics to the ability of MRSA to form biofilm, and the majority of these studies have investigated biofilm

formation by MRSA of clinical origin. Few works on biofilm have focused on MRSA isolated from food. In order to provide data on biofilm formation by food-related MRSA, we monitored the molecular characteristics, the presence of some genetic virulence markers, and of the accessory-gene-regulator *agr* and *sarA* among biofilm-forming and nonbiofilm-forming MRSA isolates from cows' milk and pork. We also investigated whether the presence of these genes affects biofilm formation. Briefly, we attempted to investigate the biofilm formation of food-related MRSA strains when they are exposed to conditions simulating those in food processing plants. The majority of biofilm studies, in fact, used microtiter plates in the 96 wells format, whereas in the present study we used microtiter plates in the 6 wells format. This system overcomes the limitation of the basic microtiter plate assay (96 wells format) concerning possible nutrient limitation and the inability to observe biofilm structure by direct microscopy. With regard to this, in order to show the presence of biofilm matrix, expressed as BPI, the SEM analysis was performed on a selected MRSA strain (MRSA 4 classified as a moderate/strong biofilm producer). Our findings have shown that genotypically different isolates of MRSA from food (milk and pork) have different abilities to produce biofilms on the materials commonly used for food processing equipment. As reported in literature, PBP2a protein is an important factor in biofilm accumulation (Pozzi and others 2012; Rudkinet and others 2012). However, in this survey there was no difference between the biofilm formation by food-related MRSA (expressed as BPI) compared to the biofilm formation (BPI values) by MSSA strains isolated from the food sector in a previous work (Di Ciccio and others 2015). Among the biofilm-forming strains it seems that SCC*mec*IVa could be a characteristic that better contributes to the ability of MRSA isolated from the food sector to produce biofilm on polystyrene. In conclusion, our findings confirm for food isolates of MRSA what has been found previously for clinical MRSA. Further studies are needed in order to acquire better understanding as to whether the presence of SCC*mec* IVa is also related to the ability to form biofilm.

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Authors do not have any conflict of interest.

Author Contributions

A. Vergara designed the study and drafted the paper. P. Di Ciccio carried out experiments in the lab and SEM imaging, and participated in drafting the paper. G. Normanno performed the statistical analysis and analyzed and interpreted the results. F. Pedonese and R. Nuvoloni carried out the phenotypic biofilm assays. A. Parisi and G. Santagada collected MRSA strains and performed molecular characterization studies. A. Colagiorgi carried out the detection of virulence factors. E. Zanardi and S. Ghidini participated in drafting the article. A. Ianieri helped in revising the paper and gave final approval of the last version.

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Table 1

Table 1–Oligonucleotide primers used in this study.

Target gene	Forward primer (5'-3')	Reverse primer (5'-3')	Amplicon size (bp)	References
nuc	GCGATTGATGGTGAT ACGGTT	AGCCAAGCCTTGACGA ACTAAAGC	270	Brakstad and others (1992)
mecA	AACAGGTGAATTATTA GCACTTGTAAG	ATTGCTGTTAATATTTT TTGAGTTGAA	174	Martineau and others (2000)
agrI	ATGCACATGGTGCAC ATGC	GTCACAAGTACTATAAG CTGCGAT	441	Shopsin and others (2003)
agrII	ATGCACATGGTGCA CATGC	TATTACTAATTGAAAAGT GGCCATAGC	575	Shopsin and others (2003)
agrIII	ATGCACATGGTGCACA TGC	GTAATGTAATAGCTTGTA TAATAATACCCAG	323	Shopsin and others (2003)
agrIV	ATGCACATGGTGCAC ATGC	CGATAATGCCGTAAT ACCCG	659	Shopsin and others (2003)
icaA	ACACTTGCTGGCGCA GTCAA	TCTGGAACCAACATC CAACA	188	Kouidhi and others (2010)
icaD	ATGGTCAAGCCCAGA CAGAG	AGTATTTTCAAT GTTTAAAGCAA	198	Kouidhi and others (2010)
cna	AAAGCGTTGCCTAGT GGAGA	AGTGCCTTCCCAAAC CTTTT	192	Montanaro and others (1999)
fnbA	GATACAAACCCAGG TGGTGG	TGTGCTTGACCATGC TCTTC	191	Arciola and others (2005)
fnbB	GGAGAAGGAATTAA GGCG	GCCGTCGCCTTGA GCGT	811	Booth and others (2001)
clfA	CCGGATCCGTAGCTGC AGATGCACC	GCTCTAGATCACTCATC AGGTTGTTCAGG	1000	McDevitt and others (1995)
hla	CTGGCCTTCAGCCTTT AAGG	CTGTAGCGAAGTCTGG TGAAA	455	Ando and others (2004)
hlb	GCCAAAGCCGAATC TAAG	CGCATATACATCCCA TGGC	845	Ando and others (2004)
sarA	TTAGCTTTGAAGAATTC GCTGT	TTCAATTTCGTTGTTT GCTTC	275	Padmapriya and others (2003)
spa	TAAAGACGATCCTTC GGTGAGC	CAGCAGTAGTGCCGT TTGCTT		Strommenger and others (2006)

471

472 Table 2

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Table 2–Biofilm formation, expressed as BPI_s, by *S. aureus* and *S. epidermidis* ATCC on polystyrene and stainless steel a 37 °C.

Reference strains	$OD_{mean\ biofilm}$ Polystyrene[a]	BPI Polystyrene	$OD_{mean\ biofilm}$ Stainless steel[a]	BPI Stainless steel
S. aureus ATCC 35556 (positive control)	0.728 ± 0.15	0.758	0.424 ± 0.05	0.801
S. aureus ATCC 12600	0.389 ± 0.07	0.405	0.258 ± 0.02	0.486
S. epidermdis ATCC 12228 (negative control)	0.283 ± 0.05	0.294	0,079 ± 0,00	0.149

[a]Values are expressed as OD mean ± standard deviation.

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Table 3

Table 3–Results from genotyping of the 22 MRSA strains used in the study.

Strain nr.	*spa* type	ST	SCC*mec*	*agr* type	*nuc*	*icaA*	*icaD*	*cna*	*fnbA*	*fnbB*	*clfA*	*hla*	*hlb*	*sarA*	Source
MRSA 1	t1255	398	V	1	+	+	+	+	+	–	+	+	+	+	Bovine milk
MRSA 2	t127	1	IVa	3	+	+	+	+	+	–	+	+	+	+	Bovine milk
MRSA 3	t174	1	IVa	3	+	+	+	+	+	–	+	+	+	+	Bovine milk
MRSA 4	new	8	IVa	3	+	+	+	+	+	–	+	+	+	+	Bovine milk
MRSA 5	t524	71	V	1	+	+	+	+	+	–	+	+	+	+	Bovine milk
MRSA 6	t899	398	IVa	1	+	+	+	+	+	–	+	+	+	+	Bovine milk
MRSA 7	t688	5	V	4	+	+	+	–	+	–	+	+	+	+	Bovine milk
MRSA 8	t786	88	IVa	3	+	+	+	–	+	–	+	+	+	+	Bovine milk
MRSA 9	t1730	2781	V	1	+	+	+	–	+	–	+	+	+	+	Bovine milk
MRSA 10	t011	398	IVa	1	+	+	+	+	+	–	+	+	+	+	Pork
MRSA 11	t127	1	IVa	3	+	+	+	+	+	–	+	+	+	+	Pork
MRSA 12	t899	398	V	1	+	+	+	+	+	–	+	+	+	+	Pork
MRSA 13	t1255	398	V	1	+	+	+	+	+	–	+	+	+	+	Pork
MRSA 14	t9301	398	ND	1	+	+	+	–	+	–	+	+	+	+	Pork
MRSA 15	t034	398	V	1	+	+	+	+	+	–	+	+	–	+	Pork
MRSA 16	t4474	9	ND	1	+	+	+	+	+	–	+	+	–	+	Pork
MRSA 17	t899	398	V	1	+	+	+	+	+	–	+	+	–	–	Pork
MRSA 18	t127	1	IVa	3	+	+	+	+	+	–	+	+	–	+	Pork
MRSA 19	t011	398	V	1	+	+	+	+	+	–	+	+	+	+	Pork
MRSA 20	t034	398	V	1	+	+	+	+	+	–	+	+	+	+	Pork
MRSA 21	t1255	398	V	1	+	+	+	+	+	–	+	+	+	+	Pork
MRSA 22	t899	398	V	1	+	+	+	+	+	–	–	+	+	+	Pork

Figure 1

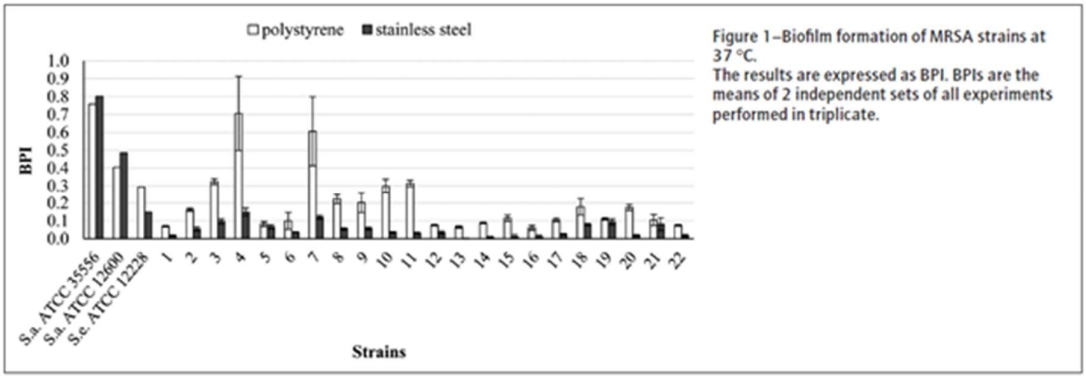

Figure 1–Biofilm formation of MRSA strains at 37 °C.
The results are expressed as BPI. BPIs are the means of 2 independent sets of all experiments performed in triplicate.

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491 Figure 2

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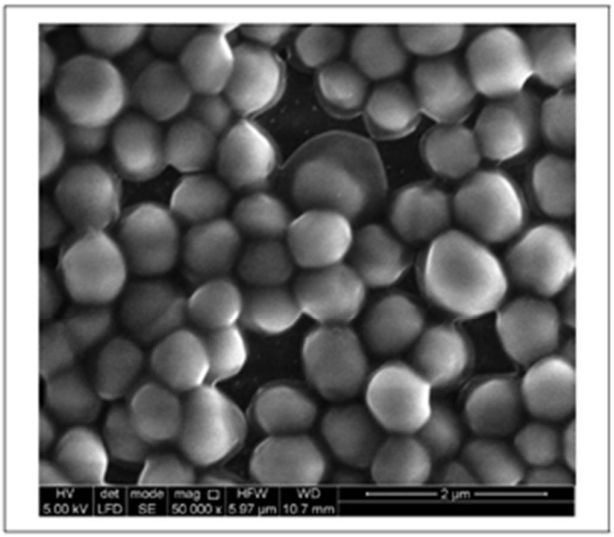

Figure 2–Scanning electron microscopy image of biofilm formed by an MRSA strain (MRSA 4) at 37 °C on polystyrene (BPI: 0.76). Magnification 50000 x. After 24 h of incubation at 37 °C, the surface tested was covered with dense cell clusters.

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