Pathogenesis of *Staphylococcus epidermidis* in prosthetic joint infections: Can the identification of virulence genes differentiate between invasive and commensal strains?

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ABSTRACT

Staphylococcus epidermidis is a commensal of cutaneous flora that has emerged as one of the most frequent causative microorganism of prosthetic joint infections (PJIs). The aim of this study was to elucidate possible antimicrobial resistance and virulence markers involved in the pathogenicity of *S. epidermidis* analyzing the possible differences between infecting and commensal strains. Fifty *S. epidermidis* strains from PJI patients, 50 from skin of healthy-individuals (HI) and 17 from the surgery-field during a primary arthroplasty (SF) were analyzed in regard to antimicrobial resistance, detection of resistance genes, PFGE, MLST, biofilm formation and detection of virulence genes The PJI strains were significantly more often resistant to antibiotics than HI and SF strains. PFGE defined 56 PFGE types and none was shared between PJI and SF groups. The ST2 was the predominant sequence type and was only present in PJI strains (44%), while HI and SF showed a large variety of STs. All strains were biofilm producers and significant differences were not detected. The genes *sdrF*, *bhp*, *icaA, icaB, icaD,* the complete *ica* operon and the *IS*256 were significantly predominant in PJI strains, whereas, *embp, hld,* ACME prevailed in HI strains. The SF strains behaved similarly to HI strains. In conclusion, there was no single marker to differentiate invasive from commensal *S. epidermidis* strains, but a combination of them were more common in strains from the PJI group than from the other groups including *ica* operon, *IS*256, *sdrF*, *bhp* and *mecA*, higher antimicrobial resistance and a predominance of the ST2.

INTRODUCTION

Prosthetic joint infections (PJIs) are considered serious complications since they are long-lasting and difficult to treat infections with high economic costs that can generate irreversible physical sequelae. (Becker, Heilmann, and Peters 2014b; Parvizi, Fassihi, and Enayatollahi 2016; Runner et al. 2019; Tsai et al. 2015).

The majority of PJI cases were caused by coagulase negative staphylococci (39.6%), with S. *epidermidis* as the more common etiological agent (23.3%) (Becker, Heilmann, and Peters 2014a; Benito et al. 2016) .

Staphylococcus epidermidis, is an important commensal specie in the nasal and cutaneous human microbiota, and it has a great clinical impact as an opportunistic pathogen implicated in device related infections. During the last years, *S. epidermidis* has been considered one of the principal causes of PJIs (Le, Park, and Otto 2018) after *S. aureus* (Post V, Harris LG, Morgenstern M, Mageiros L, Hitchings MD, Méric G, Pascoe B, Sheppard SK, Richards RG 2017). However, it is not always easy to establish if *S. epidermidis* isolated in periprosthetic clinical specimens represent true infection or only colonization/contamination. (Karsten Becker, Christine Heilmann, Georg Peters. Coagulase-Negative Staphylococci. Clin Microbiol Rev 2014; 27: 870 –926)

The production of a biofilm on surfaces of implanted materials is recognized as the main virulence factor in *S. epidermidis* (Bengt Hellmark, Soderquist, et al. 2013). During the biofilm formation a set of virulence genes (VG) are involved from the attachment to the surface that they colonize until the aggregation and the subsequent cellular detachment (Büttner et al. 2015). Some genes such as, *ica* operon, *IS*256, *bhp*, *aap* and *embp* among others, have been described as possible molecular markers that might differentiate invasive from commensal *S. epidermidis* strains (Gu et al. 2005;

Heilmann, Ziebuhr, and Becker 2018; B. Hellmark et al. 2013; Ortega-Peña et al. 2019). However, to date, no single marker has permitted distinguish both populations. The aim of this study was to elucidate possible genetic markers involved in the pathogenicity of *S. epidermidis* through molecular characterization, including epidemiological, resistance and virulence features of three populations of *S. epidermidis*: strains of PJIs, strains of skin or mucous membranes of healthy individuals and strains isolated from the surgery field during a primary arthroplasty (that did not be eventually involved in PJIs).

MATHERIALS AND METHODS

Bacterial strains

A total of 117 *S. epidermidis* strains from three populations were studied: 50 strains recovered from the collection of all microorganisms causing PJIs during 2013-2019 at the Microbiology Department, Hospital de la Santa Creu i Sant Pau, 50 strains isolated from nasal and skin swabs of healthy individuals (HI) not related with a clinical environment during 2017-2018, and 17 strains isolated from the surgery field (SF), during a primary arthroplasty performed during 2016-2019 in the Hospital de la Santa Creu i Sant Pau and the Hospital del Mar without causing infection (after at least one year follow-up) (Table 1).

The participants of the HI group did not consume antibiotics and were not hospitalized in the three months prior to sample collection; whereas patients from the SF group had received as surgical antimicrobial prophylaxis one dose of cefazolin orcefazolin plus gentamycin, (in penicillin-allergic patients, vancomycin or vancomycin plus gentamicin) before obtaining the samples Most patients with IPA did not received antibiotics prior to collect the diagnostic periprosthetic samples (by synovial fluid aspiration or during the surgical treatment).

One isolate per patient was included in the study except when the strains differed in the antibiogram.

Strains were isolated and identified according to the routine microbiological techniques and confirmed by MALDI TOF MS (Bruker).

The study was approved by the Hospital de la Santa Creu i Sant Pau ethics committee (IIBSP-STA-2016-81).

Antimicrobial susceptibility testing and resistance genes

All strains underwent antimicrobial susceptibility testing using the Sensititre ESTEN2F (ThermoFisher Scientific, Inc.). Results were interpreted according with the Clinical and Laboratory Standards Institute (CLSI 2017)(Clinical and Laboratory Standards Institute (CLSI) n.d.), except for fosfomycin and tigecycline that were interpreted according with the European Committee on Antimicrobial Susceptibility Testing (EUCAST 2017)

Total DNA of all strains was extracted using the GenElute-Bacterial Genomic DNA Kit (Sigma-Aldrich). Resistant strains were PCR-screened for a set of antimicrobial resistance genes (AGRs): to oxacillin (*mecA*), erythromycin (*ermA*, *ermB*, *ermC*, *msrA*, *mphC*), clindamycin (*lnuA*), cotrimoxazole (*dfrS*), linezolid (*cfr*, rRNA 23S, L3, L4, L22), aminoglycosides (*aacA-aphD* and *aadD)* (Table S1). PCR products were purified using EXOSAP (Isogen Life Science) and sequenced following Macrogen Inc guidelines. Sequences were analysed by BioNumerics v.7.6 (Applied Maths NV) and Basic Local Alignment Search Tool (BLAST). Staphylococcal cassette chromosome *mec* (SCC*mec*) typing was performed in all methicillin-resistant *S. epidermidis* (MRSE) strains by multiplex PCRs (M-PCR1 and M-PCR2) (Kondo et al. 2007) and http://www.sccmec.org (Table S1).

Molecular typing of the strains

Pulsed-Field Gel Electrophoresis (PFGE) methodology was applied to the analysis of strains from the PJI and SF groups. Strains from the HI group were excluded because the divergence of them (not clinically-epidemiologically related). *SmaI* enzyme (Sigma-Aldrich) was used for the genomic restriction of the strains following the procedure described (Goering and Fey 2014). Further analysis of the PFGE procedure was obtained with the Bionumerics software v.7.6 using the Dice similarity coefficient and the construction of a dendrogram with the UPGMA algorithm (Unweighted Pair Group Method using Arithmetic averages). The interpretation criteria was based on the previously established (Tenover et al. 1995) (M. Miragaia et al. 2008).

Multilocus sequence typing (MLST) was performed in all strains according to the published scheme of Thomas et al. (Thomas et al. 2007). Sequence types (STs) were assigned according to the *S. epidermidis* MLST data base (http://sepidermidis.mlst.net). Clonal complex was assigned by eBURST (http://eburst.mlst.net) and published reports (Feil EJ, Li BC, Aanensen DM, Hanage WP 2004; M. Miragaia et al. 2007, 2008).

The minimum spanning tree (MST) was constructed with the STs of all strains using Bionumerics software v.7.6.

Biofilm formation assay

S. epidermidis strains were growth in Tryptic Soy Broth supplemented with 0.5% glucose (TSBG, Oxoid). The bacterial suspensions adjusted to OD_{600} of 1.5 were diluted 1:100 with TSBG and inoculated (200 µL) in 96-well microtiter plates for 24 h at 37 ºC. Biofilm quantification was performed by 1% Crystal Violet (CV) stain following (Seyedi-Marghaki et al. 2019). The biofilm formation was calculated by the median absorbance of three replicates of the experiment. Strains were classified as strong (>2), moderate (1-2), weak (0.1-1) and non-biofilm producer (<0.1). The *S. epidermidis*

RP62A (ATCC 35984) and *S. epidermidis* ATCC 12228 strains were used as biofilm-forming and non-biofilm-forming controls, respectively.

Detection of virulence genes

Presence of virulence genes (VG) involved in the biofilm formation was determined by PCR of the following genes: *atlE, sdrG, sdrF, sesI, embp, ica* operon (*icaA, icaB, icaC, icaD, icaR*), *bhp, aap* and *hld.* In addition, the two *quorum sensing systems, agr* and *luxS* were analysed (Table S1).

The *agr* type was determined by the analysis of the deduced amino acid sequence (Bengt Hellmark, Söderquist, et al. 2013).

Additionally, the mobile elements *IS*256 and ACME were studied by PCR (Table S1). The ACME type was classified according with the *arc, opp3* and *kdp* genes presence: ACME-I (*arc* and *opp3*), ACME-II (*arc*), ACME-III (*opp3*), ACME-IV (*arc* and *kdp*), ACME-V (*arc, opp3* and *kdp*) and ACME-VI (*kdp)* (Maria Miragaia et al. 2009) (O'Connor et al., 2018).

Data analysis

The categorical variables were compared using Chi-square test ($\chi 2$ test) or Fisher´s exact test and the ordinal variables by Mann-Whitney or Kruskal-Wallis. All comparisons were performed with IBM SPSS Statistics v25.. A p <0.05 was considered statistically significant.

Results

Antimicrobial susceptibility testing and antimicrobial resistance genes

A total of 117 *S. epidermidis* strains were analysed (Table 1). The resistance rates to most of the antimicrobials tested were found significantly higher in PJI strains than in HI and SF strains (Table 2). All strains remained susceptible to teicoplanin, vancomycin, daptomycin and tigecycline. Fourty seven percent (55/117) of the strains were MRSE

and were predominant in PJI strains (37/50; 74%) versus SF (9/17; 53%) and HI (9/50; 18%) ($p<0.05$) (Table 2A). The *mecA* gene was detected in 53/55 (96.4%) of MRSE strains. Among erythromicin resistant strains (n=74), *mphC* and *msrA* genes and its combination were the predominant ones followed by the *ermC* and the combination of *ermC+mphC+msrA* genes (Table 2B). Gentamicin resistance was only present in PJI strains except for two SF strains. This resistance was mainly mediated by *aadD* (Table 2B). The same mutations were observed for both linezolid resistant strains: G2603T mutation in the 23S rRNA gene and Val154Leu amino acid alteration in L3.

Clonality of the strains

The PFGE analysis of 67 strains from PJI (n=50) and SF (n=17) showed a great diversity and were distributed into 56 PFGE types (Figure 1): 49 single-patterns (32 from PJI and 17 from SF), six PFGE types including two strains (4, 7, 26, 27, 31 and 42) and the PFGE type 24 that contained six strains recovered from patients P5, P6 and P7. PFGE types 31 and 42 harboured a pair of strains from patients P10 and P13, respectively (each pair of strains differed in the isolation date and/or antibiogram). A clinical epidemiological relationship suggesting cross-transmission between patients of PFGE types 4, 7, 24, 26 and 42 was not found. PFGE types were not shared between PJI and SF strains.

The MLST analysis of all strains determined a total of 66 different STs. The sequence type ST2 was the predominant in PJI strains (22/50; 44%) followed by the new ST640 and ST5 (6/50; 12% both). In HI and SF strains, a great variability of STs was observed, without a predominant sequence type. Twenty four new STs were described in this study (Figure 2 and S1).

196 The minimum spanning tree (MST) showed that ST35 and ST657 were shared between
197 the three populations; ST5 and ST57 were shared between PJI and HI strains, and,
198 ST17, ST32, ST88, ST89 and ST795 between HI and SF strains (Figure 2). Most of the
199 strains (67; 57.3%) belonged to the clonal complex 2 (CC2). The subgroup CC2-I was
200 predominant in PJI strains ($p<0.05$) (Table S2).

201 A SCC*mec* type was not possible to assign for any MRSE strain, showing a great
202 variability of combinations between *ccr* and *mec* complexes. The most frequent
203 combinations were: *ccr*1-*ccr*2-*mec* class B (19/55; 34.5%), *ccr*1-*ccr*2-*ccr*5-*mec* class B
204 and *ccr*3-*ccr*4-*ccr*5-*mec* class A and B (both 2; 3.6%), all only present in PJI strains.

205 **Biofilm formation and virulence genes**

206 All strains were biofilm producers and significant differences were not detected
207 between the three populations (Table 3). The majority of PJI and SF strains were
208 classified as strong or moderate biofilm producers (66% and 64.7%, respectively);
209 whereas, contrary to the expected, this percentage was even higher (76%) in HI strains.
210 Different virulence genes content was observed between all strains: *sdrF, bhp,* the
211 complete *ica* operon, *icaA, icaB, icaD* and the *IS*256 were significantly predominant in
212 PJI strains, whereas, *embp, hld* and ACME were more prevalent in HI strains. The
213 *quorum sensing luxS* and *agr* genes were present in all strains; however, *agr*-IV and
214 ACME-VI were statistically higher in PJI; *agr*-III, ACME-IV and ACME-V in HI; and
215 ACME-II in SF strains (Table 3).

216 Comparison of biofilm formation and the presence of VGs determined that *icaA*
217 positive strains were not associated with strong biofilm formation; only, *sdrF, embp* and
218 *hld* genes showed relation to strong biofilm production in HI strains (data not shown).

From gentamicin resistant strains, 82.1% (32/39) were positive for *IS*256 ($p<0.001$) and all of them belonged to the PJI group. Resistance to gentamicin were not detected in HI and only in two SF strains.

Finally, repetitive virulence gene content was observed in strains with specific STs, among them: all ST2 strains belong to PJI and was positive for *sesI, sdrF, ica* operon, *aap, hld*, *IS*256 and *agr*-I; ST35, present in the three populations, contained *embp, sesI*, *ica* operon, *aap, hld,* ACME and *agr*-I in all strains. In addition, some STs were associated with *agr* types, such as, ST89, ST35 and ST153 to *agr*-I; ST32 and ST59 to *agr*-II; and ST640 to *agr*-IV (Figure S2).

DISCUSSION

Because *S. epidermidis* is a predominant microorganism in the human skin microbiome, several studies have focused on the search for genetic markers that can differentiate commensal from invasive *S. epidermidis* strains in PJIs (B. Hellmark et al. 2013; Bengt Hellmark, Soderquist, et al. 2013; Méric et al. 2018; Ortega-Peña et al. 2019). Herein, we present a comparative study of *S. epidermidis* strains of patients with PJIs, healthy individuals and, as a novelty, a group of strains recovered in the surgery field during a primary arthroplasty that were not eventually involved in PJIs. We consider that the latter is a true control because, in addition to being commensal and not causing infection, these strains are present in a place that could be a route of entry for invasive strains.

Strains from PJI were significantly resistant to more antibiotics than HI and SF (Table 2), as has been already described (Soraya Cherifi et al. 2014; Ortega-Peña et al. 2019; Salih et al. 2018). (S. Cherifi et al. 2013; B. Hellmark et al. 2013; Rolo, de Lencastre, and Miragaia 2012). Despite SF strains have undergone antibiotic pressure due to

prophylaxis before the arthroplasty, they did not show elevate percentage of resistance than PJI strains.

Results determined the highly significance of MRSE (73.9%) in PJI strains than in commensals. In addition, MRSE rates remained similar to reported in other studies (Ortega-Peña et al. 2019; Salih et al. 2018)(B. Hellmark et al. 2013; Ortega-Peña et al. 2019; Rolo, de Lencastre, and Miragaia 2012). It highlights MRSE is an important feature to distinguish invasive from commensal strains (B. Hellmark et al. 2013).

The PFGE analysis showed heterogeneity of *S. epidermidis* strains (PJI and SF) that could be explained by the increment of horizontal gene transfer and mobile genetic elements during the adaptation to both, community and hospital environments (S. Cherifi et al. 2013; M. Miragaia et al. 2007). Although six PFGE types were found containing closely related strains, it was not possible to define cross transmission among them. Five of them belonged to ST2 and shared some VGs: *ica* operon, *IS*256, *aap*, *sdrF*, *sesI* and *hld*. PFGE type 27 belonged to ST640 and, in contrast, it was *ica* operon negative, therefore, biofilm formation seems to be mediated by other VGs such as, *bhp*, *sdrF* and *embp*.

Moreover, the great diversity of STs found, showed that commensal strains were more divergent than the invasive strains (Figure S1), similar to that found by Du et al. (Du et al. 2013). ST2 was the most frequent and only occurred in PJI strains; as is well known, it has been reported that ST2 in the most widespread hospital-associated ST worldwide (Soraya Cherifi et al. 2014; Du et al. 2013; M. Miragaia et al. 2007; Ortega-Peña et al. 2019; Salgueiro et al. 2017).

ST2 strains besides to being present in PJIs were *ica* operon, *IS*256, *mecA* positive and multirresistant. The combination of these pathogenicity determinants contributes to the

adaptation and survival of pathogenic clones, such as ST2, to specific physiological conditions during infection, the antibiotic barrier and the host defenses(Christensen and Brüggemann 2014; Du et al. 2013; Post et al. 2017, Meric 2018).

Instead, other STs such as ST5, ST35, ST57 and ST657 were shared between strains of different groups (Figure 2), which can be considered true opportunistic pathogens, where strains have the same ability to cause infection (meric 2018).

In this study, significant differences were not detected in biofilm production and the presence of *icaA*, among invasive and commensal strains; similar to other studies (Bengt Hellmark, Soderquist, et al. 2013; Méric et al. 2018; Salgueiro et al. n.d.). Although *icaA* positive strains have been related to strong biofilm producers (Soraya Cherifi et al. 2014), it was not observed in our study. Thus, from our point of view, biofilm formation is not a feature to discriminate between commensal and pathogenic strains.

Finally, *embp*, *hld* and ACME were statistically higher in HI. It could be explain due to the importance of these markers for bacterial survival and increase in the ability of *S. epidermidis* to colonize and spread in different environments (B. Hellmark et al. 2013; Bengt Hellmark, Soderquist, et al. 2013). Although *agr*-I has been previously associated with invasive strains (B. Hellmark et al. 2013) we did not observe this predominance.

The limitations of our study are those implicit to the sample size, mainly in SF and the absence of commensal strains from the skin of PJI patients, but the main strength is that includes a new group of strains to be compared. Until this study, clinical strains were always compared with those isolated from healthy skin, but never compared to strains that could reach the surgical field, but had not caused an infection. Development of the

infection depends on the strain, the inoculum of microorganisms that have reached the surgical field, the immune system of the patient and the prophylactic treatment.

Despite the low number of strains, our findings suggest that SF behaved similarly to HI strains. This new group of strains allowed it to be defined as a true control to discriminate between the commensal and PJI strains.

In conclusion, there was no single marker to differentiate invasive from commensal strains, but the combination of some markers allow to establish differences between populations: PJIs were caused by *S. epidermidis* strains that belonged more often to the ST2, were positive for *ica* operon, *IS*256, *sdrF*, *bhp* and *mecA*, whereas the presence of *embp*, *hld* and ACME was found significant in commensal strains. The development of rapid tests in the routine laboratory, such as a multiple PCR, which includes these pathogenicity determinants, could allow rapid differentiation of invasive and commensal strains into PJIs.