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Superantigens in *Staphylococcus aureus* isolated from prosthetic joint infection

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Abstract

Staphylococcus aureus is a common cause of prosthetic joint infection (PJI). The prevalence of superantigens (SAgs) among PJI-associated *S. aureus* is unknown. Eighty-four *S. aureus* isolates associated with PJI isolated between 1999 and 2006 were studied. SAg genes, *sea, seb, sec, sed, see, seg, seh, sei* and *tst*, were assayed by PCR. Seventy-eight (92.9%) isolates carried at least one SAg gene studied, with 61 (72.6%) harboring more than one. *seg* was most commonly (70.2%) and *seh* was least frequently (4.8%) detected. *tst*-positive isolates were associated with early infection and increased ESR at diagnosis ($P = 0.006$ and $P = 0.021$, respectively). *seg* and *sei* were associated with methicillin resistance ($P = 0.008$ and 0.002, respectively). SAg genes are prevalent in *S. aureus* causing PJI; a majority of PJI-associated isolates produce biologically active SAgs in both planktonic and biofilm growth modes.

Keywords

S. aureus; superantigen; prosthetic joint infection

1. Introduction

With the increasing numbers of primary arthroplasty surgeries being performed, complications associated with prosthetic joints are becoming increasingly frequent (Kim,

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2008). Of the post-operative complications associated with prosthetic joints, prosthetic joint infection (PJI) is the most detrimental (Harris & Sledge, 1990). PJI is often caused by staphylococci, including coagulase-negative staphylococci (CNS) and *Staphylococcus aureus* (Zimmerli, Trampuz, & Ochsner, 2004). PJI-associated bacteria grow as biofilms on prosthetic joints (Gallo, Kolar, Novotny, Rihakova, & Ticha, 2003), and as a result, management of PJI requires a combination of antimicrobial agents and surgery.

Clinical features and outcomes of staphylococcal PJI, including associated symptomatology, chronicity, tendency to relapse and even mortality, may be influenced by virulence factors, such as exotoxins, produced by the infecting organisms (Cunningham, Cockayne, & Humphreys, 1996). Among the exotoxins of *S. aureus*, staphylococcal enterotoxins (SEs) and toxic shock syndrome toxin (TSST-1) are well known superantigens (SAgs). They cause robust activation of T-cells expressing certain T cell receptor β chain variable region fragments (TCR Vβ), irrespective of antigenic specificity. SAgs may also contribute to establishing *S. aureus* infection by causing immune evasion or immune subversion (Gaus, Miethke, Wagner, & Heeg, 1994; Kawabe & Ochi, 1990; Llewelyn & Cohen, 2002; Novick, 2003; O'Hehir & Lamb, 1990; Taylor & Llewelyn, 2010).

The prevalence of SAgs have been mostly investigated among *S. aureus* strains isolated from diseases such as septic shock (Ferry, et al., 2005), infective endocarditis (Nienaber, et al., 2011) and toxic shock syndrome (DeVries, et al., 2011), in which clinical features reflect immune activation. To our knowledge, the prevalence of SAgs in *S. aureus* associated with PJI has not been investigated. Staphylococcal SAgs may help in establishment of PJI and contribute to its clinical features. We investigated the prevalence of SAgs in *S. aureus* associated with PJI and related the presence or absence of SAgs to clinical findings. We also examined whether *S. aureus* in *in vitro* biofilms produces functional SAgs and correlated the presence of SAgs with methicillin resistance.

2. Materials and Methods

2.1 Collection of bacterial isolates

A collection of 84 *S. aureus* isolates from patients diagnosed with PJI at Mayo Clinic (Rochester, MN) from 1999 to 2006 were studied. *S. aureus* were isolated from periprosthetic tissues, synovial fluid or the explanted prosthetic joints themselves (Trampuz, et al., 2007). Medical records of corresponding subjects were retrospectively reviewed for demographic characteristics, clinical course and outcome. This study was approved by the Mayo Clinic Institutional Review Board.

2.2 Clinical definitions

PJI was defined using diagnostic criteria outlined by the Infectious Diseases Society of America (Osmon, et al., 2013). Timing of infection was classified according to time since the most recent prosthesis implantation, defined as early (<3 months), delayed (3–12 months) and late (>12 months). Duration of symptoms before admission was categorized by one month intervals. Treatment strategies were categorized as chronic suppression, débridement and implant retention, resection and reimplantation, permanent resection, and

disarticulation. Diagnosis of recurrence was confirmed by re-isolation of *S. aureus* from the same joint after a treatment strategy had been applied.

2.3 Preparation of genomic DNA and PCR

S. aureus was grown on sheep blood agar, and genomic DNA extracted using the DNeasy blood & tissue kit (Qiagen, Hilden, Germany). Genes for staphylococcal enterotoxins A, B, C, D, E, G, H, and I and TSST-1 were assayed by PCR using a Veriti® Thermal Cycler (Applied Biosystems, CA). Primers were synthesized by Integrated DNA Technologies®, (IA); primer sequences and PCR conditions are shown in Table 1 (Blaiotta, et al., 2004; Johnson, et al., 1991; Letertre, Perelle, Dilasser, & Fach, 2003; Lovseth, Loncarevic, & Berdal, 2004).

2.4 Preparation of culture supernatants and quantitation of planktonic and biofilm cultures

For planktonic cultures, *S. aureus* was grown to 10^8 CFU/ml in trypticase soy broth (TSB) for 24 hours. After centrifugation at 4000 rpm for 5 min, supernatants were collected, filtered through a 0.22 μm syringe filter (MILLEX®GP; Millipore, MA) and stored at −80ºC until further analysis. Biofilms were grown on Teflon® discs in 2 stages. During the first stage, the discs were placed in 24-well flat bottom plates with 2 ml of TSB containing a 1×10^6 CFU/ml inoculum. After 24 hours of incubation, each disc was removed, rinsed with sterile saline to remove planktonic cells and transferred to new 24-well flat bottom plates, with each well containing 2 ml of TSB containing 4 μg/ml of vancomycin (to inhibit the planktonic growth). After incubation for an additional 24 hours, culture media was collected, filtered through 0.22 μm syringe filters and frozen at −80ºC until further testing was performed. Quantitative cultures of biofilms on the discs were performed in duplicate after the initial incubation stage without vancomycin and following incubation with vancomycin. Prior to culture, biofilms were dislodged and disaggregated using vortexing and sonication, as previously described (del Pozo, et al., 2009). Quantitative culture results were expressed as the average biofilm density ($\log_{10}$CFU/cm^2) or, for planktonic cells, $\log_{10}$CFU/ml for comparison of the biologic activity of supernatants from biofilm and planktonic cultures, respectively. To specifically investigate the correlation between methicillin susceptibility and biofilm growth in the presence of vancomycin, a convenience sample of 8 MSSA and 7 MRSA isolates was first grown on Teflon discs as described above. Then, during the second stage, the discs were cultured in TSB with and without vancomycin. After 24 hours of reincubation, the colony counts were determined.

2.5 T-cell proliferation assay with HLA-DR3 transgenic mouse splenocytes

To measure the biological function of SAgs produced by *S. aureus* grown in planktonic as well as biofilm states, a splenocyte proliferation assay was performed using HLA-DR3 transgenic mice. HLA-DR3 mice expressing functional HLA-DRA1*0101 and HLA-DRB1*0301 transgenes on a MHC class II-deficient background (AE°) have been previously described (Rajagopalan, et al., 2003). Splenocytes harvested from naive HLA-DR3 mice were cultured with 100 μl of serial twofold dilutions of the culture supernatant (from 1:2 to 1:256) prepared as above. Supernatants from isogenic *S. aureus* strains, RN6734 containing pRN5543::seb (pRN7114) and RN6734 containing pRN5543::seb(b.2)

(pRN7116), which produce SEB only or no SAgs (generous gifts from Richard Novick, New York University Medical Center, New York, NY), were used as positive and negative controls, respectively (Vojtov, Ross, & Novick, 2002). Splenocytes were cultured at 10^5 cells per well in 100 μl of HEPES-buffered RPMI 1640 containing 5% fetal calf serum, serum supplement, and streptomycin and penicillin. After 48 hours of incubation, 1 μg of tritiated [^{3}H] thymidine was added to the splenocytes and incubated for an additional 17 hours. The extent of proliferation was determined by measuring incorporated radioactivity using standard procedures. The dilution concentration that induced maximal proliferation among the varying concentrations was defined and data associated with that dilution concentration taken to mitogenic activity for comparative purposes. Mean radioactivity counts per minute (CPM) with standard deviation was recorded in triplicate wells. Supernatants from planktonic and biofilm cultures of a convenience sample of 38 isolates were tested by this method. Among those 38 isolates, 15 isolates were further tested using splenocytes from AE° (MHC class II-deficient) mice to determine whether there was nonspecific mitogenic activity.

2.6 Statistical analysis

Statistical analysis was performed using SPSS software (version 21; SPSS, Chicago, IL, USA). Continuous scaled data were compared using the Student's t test or Mann-Whitney U test and categorical variables were compared using Fisher's exact test. Probability of recurrence was calculated by Kaplan-Meier analysis and compared by log rank test. All tests were 2 sided; *p*-values less than 0.05 were considered statistically significant.

3. Results

3.1 Clinical characteristics

Of the 84 PJI isolates, medical records from 83 corresponding subjects were available for review. Demographics and clinical characteristics are shown in Table 2. Of the 83 subjects, 50 had a knee prosthesis, 31 had a hip prosthesis and 2 had a shoulder prosthesis. All had localized symptoms involving the prosthetic joint, with only 20 having fever at the time of diagnosis. The most common treatment strategy was resection and reimplantation, which was done following a two-stage procedure in 41 subjects. Another 22 subjects underwent resection arthroplasty, but without prosthesis reimplantation (i.e., permanent resection). Chronic suppression, débridement and implant retention, and disarticulation were performed in 2, 14 and 4 subjects, respectively. The mean follow up period after diagnosis of PJI was 23 months. Ten subjects had recurrence at an average of 8.5 months after intervention, with *S. aureus* alone re-isolated in nine and multiple organisms, including *S. aureus*, isolated in the tenth.

3.2 Distribution and prevalence of superantigen genes by PCR

Of the 84 *S. aureus* isolates tested by PCR, 78 (92.9%) had at least one SAg gene and 61 (72.6%) had more than one (Table 3). *seg* was most frequently found (70.2%), followed by *sei* (47.6%), *sea* (27.4%), *tst* (17.9%), *seb* (9.5%), *sec* (9.5%), *sed* (9.5%), *see* (7.1%) and *seh* (4.8%). The most common combination of genes were *seg* and *sei* (20.2%) followed by *sea* and *seg* (8.3%). Four isolates harbored four SAg genes. Among the 61 isolates with

either *seg* or *sei*, 37 (60.7%) isolates had both together and of the remaining 24 isolates which had *seg* or *sei* but not both, 22 had *seg* only and 2 *sei* only.

3.3 Association of superantigen genes with clinical characteristics

Timing of infection and systemic symptoms were similar among subjects irrespective of their SAg status (data not shown). Surrogate markers of inflammation, including WBC, ESR and CRP, were similar in both groups (data not shown). However, *tst*-positive isolates were associated with earlier infection compared to *tst*-negative isolates (7/15 vs 12/68, $P = 0.006$), and subjects with *tst*-positive isolates were more likely to have an elevated ESR than were those with *tst*-negative isolates (mean 76.4 vs 54.6 mm/hr, $P = 0.021$). The presence of SAgs was not associated with duration of symptoms or recurrence (data not shown). Fever was more prevalent in subjects with *sed*-positive than those with *sed*-negative isolates (5/8 vs 15/75, $P = 0.018$). However, the clinical significance of this result is unclear since the total number of *sed*-positive isolates was small.

3.4 Association of superantigen genes and biofilm growth

Biofilms were grown on coupons following the two-stage protocol described in the methods section. There was no difference in the biofilm density ($\log_{10}$CFU/cm^2) between the SAg gene-positive and -negative isolates during the first stage of biofilm growth (data not shown). The average biofilm density of all isolates was higher during the second compared to first stage [6.56±0.77 (mean±SD) at the end of first stage and 7.26±0.77 (mean±SD) at the end of second stage following 24-hour reincubation in 4 μg/ml of vancomycin ($P < 0.001$)]. We next analyzed the association between the presence of SAg genes and heightened growth during the second stage in the presence of vancomycin. Overall, the colony counts of SAg-positive, but not SAg-negative, isolates were greater in the second stage biofilm culture ($P < 0.001$ and $P = 0.439$ for SAg-positive and -negative isolates, respectively, Figure 1A). In a subgroup analysis, *sec*-, *sed*-, *seg*-, *sei*- and *tst*-positive isolates grew more abundantly in the second stage, and *seb*-, *see*- and *seh*-positive isolates did not (Figure 1A). For example, the average biofilm densities of *seg*-positive (7.39±0.75) and *sei*-positive (7.59±0.69) isolates were higher than *seg*-negative (6.95±0.72) and *sei*-negative (6.96±0.74) isolates on discs reincubated with vancomycin ($P = 0.017$ and $P < 0.001$, respectively, Figure 1B). In contrast, *sea*-positivity (n = 23) was negatively correlated with biofilm density ($P = 0.001$, Figure 1B).

3.5 Association of superantigen genes with methicillin susceptibility

All 84 isolates were vancomycin-susceptible; 24 were methicillin-resistant *S. aureus* (MRSA) and 60 were methicillin-susceptible *S. aureus* (MSSA), as determined by oxacillin susceptibility testing using agar dilution. While *sed, seg* and *sei* positive isolates had a statistically significant association with methicillin resistance ($P = 0.039$, $P = 0.008$ and $P = 0.002$, respectively), the other SAgs were not associated with methicillin susceptibility (Figure 2A).

3.6 Association of biofilm growth with methicillin resistance and vancomycin exposure

Both MSSA and MRSA showed enhanced biofilm growth in the presence of vancomycin. However, enhanced biofilm growth in the presence of vancomycin was more pronounced for MRSA than MSSA (P <0.001). For MRSA (n = 24), the biofilm density ($\log_{10}$CFU/cm^2) was 6.52±0.63 (mean±SD) in the first stage and 7.83±0.64 (mean±SD) in the second stage (P <0.001), whereas for MSSA it was 6.57±0.82 in the first stage and 7.03±0.69 (P <0.001, Figure 2B). We also investigated the association between the most prevalent SAg genes, *seg* and *sei*, and enhanced biofilm growth in the presence of vancomycin. As shown in Fig 2A, these SAgs were also strongly associated with methicillin resistance. Therefore, to investigate the association between the presence of these SAgs and enhanced growth with vancomycin independent of methicillin resistance, we analyzed only the 60 MSSA isolates. While *seg*-positive (7.15±0.73) isolates had a tendency toward having a higher biofilm density than *seg*-negative (6.85±0.62) isolates (P = 0.106), *sei*-positive (7.29±0.60) isolates grew biofilms with significantly more cells than did *sei*-negative (6.88±0.71) isolates (P=0.025) (Figure 2C).

In all the biofilm culture experiments described above, vancomycin was always included in the second stage of culture (to prevent planktonic growth). Therefore, we next investigated whether vancomycin augments biofilm growth. For this, 15 isolates comprising of both MRSA (n = 7) and MSSA (n = 8), were grown without vancomycin during the first stage as usual. However, during the second stage, they were grown either in the presence or absence of vancomycin. Overall, the biofilm density of reincubated discs was paradoxically higher with 4 μg/ml of vancomycin (7.57±0.70) than without vancomycin (7.18±0.50) (P = 0.031). This effect was pronounced in the MRSA group (7.87±0.70 vs 7.14±0.65, with and without vancomycin, respectively, P = 0.005), but not MSSA group (7.31±0.64 vs 7.22±0.37, with and without vancomycin, respectively, P = 0.680).

3.7 T-cell proliferation assay

We next tested whether SAg-positive *S. aureus* PJI isolates produced functional SAgs in planktonic and biofilm growth modes. Culture supernatants from the SAg gene-positive isolates (which included 14 single SAg gene-positive isolates and 18 multiple SAg gene-positive isolates) induced robust proliferation of splenocytes from HLA-DR3 transgenic mice but not from AE° mice, which lack MHC class II molecules and hence cannot present SAgs (Figure 3). The supernatants from the six SAg gene-negative isolates tested, except IDRL-5999, had mitogenic activity similar to the supernatants from the SAg-negative control strain. The supernatants of IDRL-5999 had similar mitogenic activity to the SAg gene-positive isolates tested. IDRL-5999 may produce a SAg not included in the PCR assays studied. The *tst* only-positive isolates, IDRL-5964, IDRL-6018, IDRL-6091 and IDRL-6101 exhibited weaker mitogenic activities than other single SAg gene-positive isolates in both planktonic (P = 0.004) and biofilm (P = 0.01) culture. Overall, mitogenic activities were similar between planktonic and biofilm cultures for the 32 SAg gene-positive isolates (mean 75,432 vs 69,148 CPM, P = 0.506) tested.

4. Discussion

S. aureus is a common cause of PJI. It elaborates several exotoxins including SAgs; these toxins may contribute to its pathogenicity and virulence. Given their immune stimulating properties, associations between SAgs and acute diseases such as sepsis, toxic shock, endocarditis and pneumonia have been explored. Since *S. aureus* can produce SAgs in the biofilm state (Chung, et al., 2014), we hypothesized that *S. aureus* biofilms growing on prosthetic joints might elicit an inflammatory response through SAgs, impacting the pathogenesis of PJI. Therefore, we investigated the prevalence of SAg-producing *S. aureus* in PJI and correlated our findings with clinical features.

We found a 92.9% prevalence of SAgs in *S. aureus* associated with PJI isolates, which is similar to or higher than that reported in isolates from other disease states such as septic shock (Ferry, et al., 2005), infective endocarditis (Nienaber, et al., 2011) and diabetic foot ulcer (Vu, et al., 2014). While *seg* was the most common staphylococcal SAg gene associated with PJI (70.2%), close to two-thirds of the isolates tested had more than one SAg gene. The most common SAg gene combination was *seg* and *sei*, which coexist in enterotoxin gene cluster (*egc*) (Jarraud, et al., 2001). *egc* normally includes *seg*, *sei*, *sem*, *sen*, and *seo* together, but they are not always present due to *egc* polymorphisms (Blaiotta, Fusco, von Eiff, Villani, & Becker, 2006) and varying *egc* types (Collery, Smyth, Tumilty, Twohig, & Smyth, 2009).

In the present study, we found that subjects with *tst*-positive isolates had earlier onset of infection (P= 0.006), and elevated ESR (P = 0.021), a marker for inflammation, than those with *tst*-negative isolates. This suggests that TSST-1 may be actively produced and systemically absorbed in the early stages of PJI. However, the presence of other SAg genes was not associated with clinical findings of inflammation such as fever (except *sed* which was associated with fever, P=0.018). The lack of association between the presence of SAg genes and systemic findings of inflammation may be attributed to the small sample size studied. Also, even though the isolates studied had SAg genes, the actual production of SAgs may be repressed under the anaerobic conditions of PJI (Yarwood & Schlievert, 2000). Further, even if SAgs are produced, they may be confined to the fibrous zone in the setting of chronic PJI (Gristina, 1994). Finally, SAg activity may be limited by neutralizing anti-SAg antibodies (Holtfreter, et al., 2004; Parsonnet, et al., 2008).

There have been variable reports of an association between methicillin resistance and SAgs (Hu, et al., 2008; Yu, et al., 2012). Given that certain SAgs exist on mobile genetic elements, SAg genes can be transferred horizontally among the *S. aureus* strains irrespective of methicillin resistance. The association of *sed* and other SAg genes of the *egc* cluster with methicillin resistance in our study may be due to clonality among the MRSA isolates tested. An interesting observation is that *S. aureus* biofilms continued to grow in the presence of 4 μg/ml of vancomycin even though all of the isolates were vancomycin-susceptible. Vancomycin has been shown to promote biofilm formation in some strains of *S. aureus*, including MRSA, through several mechanisms (Abdelhady, et al., 2014; Abdelhady, et al., 2013; Boles & Horswill, 2008; Hsu, et al., 2011; Pozzi, et al., 2012; Sakoulas, et al., 2003). From a clinical viewpoint, it is important to recognize that after standard dosing, the

concentration of vancomycin in periprosthetic tissues would be similar to or slightly higher than the 4 μg/ml used to inhibit planktonic growth in our study (Luzzati, et al., 2000). Another interesting finding of our study is the relationship between SAgs and biofilm growth in general. *seg*- and *sei*-positive isolates showed high biofilm densities. Therefore, *egc* may influence biofilm growth; there may be a clonal association between *egc* and the accessory gene regulator (*agr*) phenotype, impacting biofilm growth (Cafiso, et al., 2007; Vuong, Saenz, Gotz, & Otto, 2000). Overall, our finding that vancomycin paradoxically enhances biofilm growth of *S. aureus*, especially MRSA and that this is associated with the presence of certain SAgs may have clinical significance. Given that SAgs can also cause repression of other exotoxins (Vojtov, et al., 2002), SAgs might influence the growth of *S. aureus* through bacteria-intrinsic, non-immune mechanisms.

In the setting of prosthetic joints, wherein nutrition and essential elements for growth are depleted, *S. aureus* likely grows as biofilms rather than in the planktonic growth mode. Therefore, the biofilm growth conditions used in the present study may be a model of PJI. In addition to demonstrating the presence of SAg genes, we performed biological assays to demonstrate their functionality, which is a major strength of our study. However, there are some limitations. First, we did not test for the presence of all reported staphylococcal SAgs (Xu & McCormick, 2012). Nonetheless, the supernatants from most SAg gene-negative isolates had no mitogenic activity in our HLA-DR3 splenocyte proliferation assay. Second, we reviewed medical records retrospectively, which may impose bias in analyzing clinical characteristics. Third, we did not investigate the clonality of the *S. aureus* isolates studies, which may have resulted in sampling error in reporting prevalence and showing an epidemiologic linkage of MRSA with *egc*. Fourth, we used splenocytes of transgenic HLA-DR3 mice instead of human T-cells for functional assay of SAgs. Even though splenocytes from HLA class II transgenic mice respond robustly to SAgs, they may still be less responsive to SAgs than are human T cells due to differences in the repertoire of T cells expressing SAg-reactive T cell receptors (as shown by the low proliferative responses to TSST-1 in our study).

In conclusion, we showed a high prevalence of SAgs in *S. aureus* associated with PJI. A prospective study involving large number of subjects and an in-depth investigation into various systemic and local immunological/inflammatory markers is needed to determine whether there is an association between the presence of SAgs and PJI outcome.

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Highlights

- *Staphylococcus aureus* is a frequent cause of prosthetic joint infection (PJI).
- The prevalence of superantigens (SAgs) among PJI-associated *S. aureus* is unknown.
- Close to 90% of *S. aureus* PJI isolates carried at least one SAg gene.
- *seg* and *seh* were the most and least common superantigens, respectively.
- Most SAg-carrying isolates produced biologically active SAgs in biofilms *in vitro*.

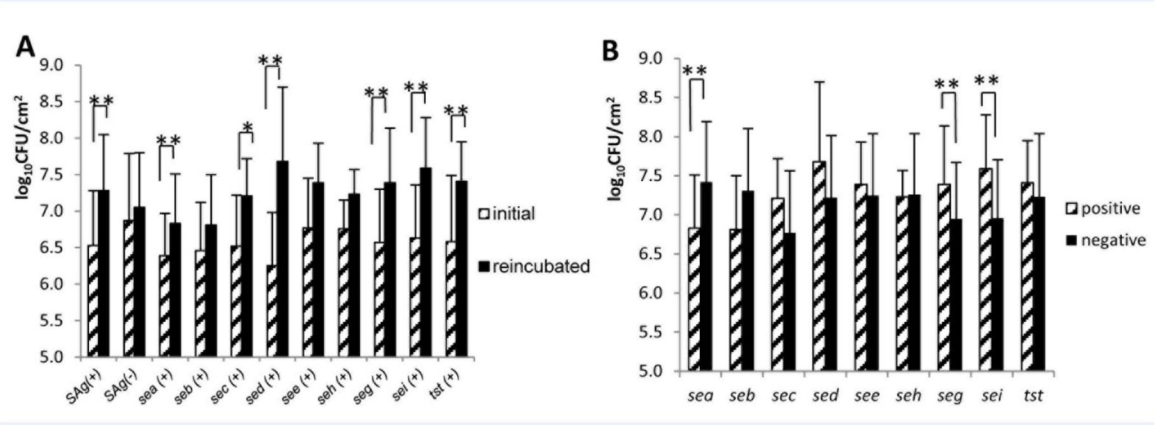

Figure 1. Correlation between SAg genes and biofilm growth

Teflon discs were grown in trypticase soy broth for 24 hours (initial biofilm), rinsed in normal saline and reincubated in trypticase soy broth with 4 μg/ml of vancomycin for 24 hours (reincubated biofilm). Panel A shows the changes in bacterial biofilm quantities between initial and reincubated biofilms for each of SAg gene-positive isolates. *P*-values were <0.001 for any SAg gene-positive, 0.430 for all SAg gene-negative, 0.010 for *sea*, 0.190 for *seb*, 0.022 for *sec*, 0.005 for *sed*, 0.183 for *see*, 0.145 for *seh*, <0.001 for *seg*, <0.001 for *sei* and 0.003 for *tst.* Panel B shows bacterial biofilm quantities after 24 hours incubation in 4 μg/ml vancomycin between each of SAg gene-positive and -negative isolates. *P*-values for the difference of biofilm densities are as follows: 0.001 for *sea*, 0.090 for *seb*, 0.768 for *sec*, 0.243 for *sed*, 0.593 for *see*, 0.868 for *seh*, 0.017 for *seg*, <0.001 for *sei* and 0.451 for *tst.* Error bars depict the standard deviation of means. **P* <0.05 and ** *P* <0.01, by Student t-test.

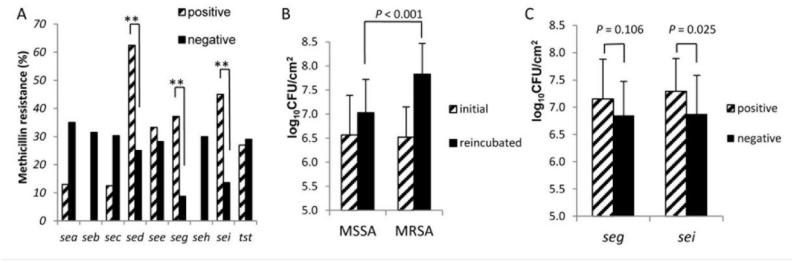

Figure 2. Relationship between bacterial biofilm quantity, methicillin resistance and enterotoxin gene cluster (*seg, sei*)

All 84 PJI isolates were tested for methicillin susceptibility by agar dilution. Panel A shows the distribution of methicillin resistance among each of SAg gene-positive and -negative isolates. *P* -values for the difference of rates of methicillin resistance are as follows: 0.062 for *sea*, 0.098 for *seb*, 0.429 for *sec*, 0.039 for *sed*, 1.000 for *see*, 0.008 for *seg*, 0.321 for *seh*, 0.002 for *sei* and 1.000 for *tst*. Panel B shows the change in bacterial biofilm quantity after reincubation in 4 μg/ml vancomycin comparing MRSA (n = 24) and MSSA (n = 60) (*P* <0.001). Panel C shows the differences in reincubated biofilm density between *seg* and *sei*-positive (n = 37 & 22, respectively)and -negative (n = 23 & 38, respectively) isolates limited to the MSSA group (n = 60). **P* <0.05 and ***P* <0.01, by Fischer's exact test and Student t-test. Error bars denote the standard deviation of means.

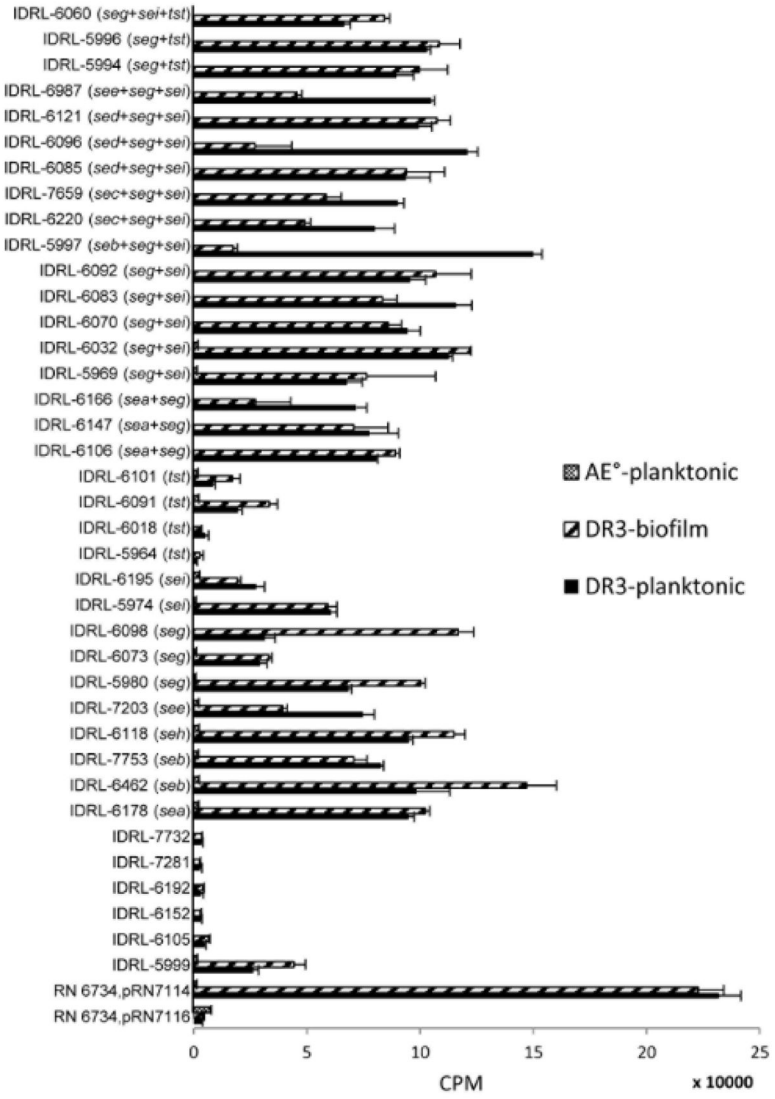

Figure 3. Bioassay to test the mitogenicity of SAg produced by PJI isolates

Culture supernatants from 38 PJI isolates were tested by T cell proliferation assay using splenocytes of transgenic HLA DR3 mice. Overall, there was no difference in mitogenic activity between the supernatants of planktonic and biofilm cultures. SAg genes present in each of isolates are noted in parentheses after the name of isolate. IDRL-5999, IDRL-6105, IDRL-6152, IDRL-6192, IDRL-7281 and IDRL-7732 were negative for all SAg genes tested. RN6734, pRN7116 was used as negative control; it is known to be negative for all SAg genes. RN6734, pRN7114, which produces SEB, was used as a positive control. A convenience sample subset of 14 planktonic supernatants from the 38 isolates was further tested using splenocytes of AE° mice (deficient in MHC class II molecules). CPM, counts per minute. Error bars; standard deviations.

Table 1

Nucleotide sequences of primers and references

Gene	Primer name	Oligonucleotide sequence (5′-3′)	Amplicon size (base pair)	Ta(°C)[a]	Reference strain (control)	References
sea	SEA-1 SEA-2	GCA GGG AAC AGC TTT AGG C GTT CTG TAG AAG TAT GAA ACA CG	521	55	IDRL-7971[b]	(Lovseth, Loncarevic, & Berdal, 2004)
seb	SEB-1 SEB-2	TCG CAT CAA ACT GAC AAA CG GCA GGT ACT CTA TAA GTG CC	478	55	RN6734, pRN7114[c]	(Johnson, et al., 1991)
sec	SA-U SEC-2	TGT ATG TAT GGA GGT GTA AC AAT TGT GTT TCT TTT ATT TTC ATA A	102	55	ATCC 19095[d]	(Letertre, Perelle, Dilasser, & Fach, 2003)
sed	SED-1 SED-2	CTA GTT TGG TAA TAT CTC CT TAA TGC TAT ATC TTA TAG GG	317	45	ATCC 23235	(Johnson, et al., 1991)
see	SA-U SEE-2	TGT ATG TAT GGA GGT GTA AC GCC AAA GCT GTC TGA G	213	50	ATCC 27664	(Letertre, Perelle, Dilasser, & Fach, 2003)
seg	SEG-1 SEG-2	TGC TAT CGA CAC ACT ACA ACC CCA GAT TCA AAT GCA GAA CC	704	53	ATCC 19095[d]	(Blaiotta, et al., 2004)
seh	SA-U SEH-2	TGT ATG TAT GGA GGT GTA AC TCT CTA GGA GTT TTC ATA TC	245	53	ATCC 51811	(Letertre, Perelle, Dilasser, & Fach, 2003)
sei	SEI-1 SEI-2	GAC AAC AAA ACT GTC GAA ACT G CCA TAT TCT TTG CCT TTA CCA G	630	52	ATCC 19095[d]	(Blaiotta, et al., 2004)
tst	TSST-1 TSST-2	GCT TGC GAC AAC TGC TAC AG TGG ATC CGT CAT TCA TTG TTA T	559	55	ATCC 51651	(Lovseth, Loncarevic, & Berdal, 2004)

[a] PCR was performed using a total volume of 20 μl and 35 to 40 cycles: denaturation for 2 min at 94°C, annealing for 2 min, and primer extension for 1 min at 72°C. Platinum® PCR SuperMix (Invitrogen, NY, USA) was used. Ta represents annealing temperature.

[b] *Staphylococcus aureus* IDRL-7971, isolated from human nares, was confirmed by ELISA to produce staphylococcal enterotoxin A (SEA) and staphylococcal enterotoxin B (SEB).

[c] RN6734, pRN7114 is a generous gift from Richard Novick (New York Medical Center, NY), and is known to produce only SEB.

[d] *S. aureus* ATCC 19095 is known to have both *sec* and the enterotoxin gene cluster (*egc*).

Table 2

Characteristics of 83 subjects with *Staphylococcus aureus* prosthetic joint infection

Characteristics		**N = 83 (%)**
Age	Range (mean)	32–98 (68)
Gender		
	Male	48 (57.8)
	Female	35 (42.2)
Infection site		
	Hip	31 (37.3)
	Knee	50 (60.2)
	Shoulder	2 (2.4)
Timing of infection		
	Early (<3 months)	19 (22.9)
	Delayed (3–12 months)	8 (9.6)
	Late (>1 year)	56 (67.4)
Duration of symptoms		
	<1 month	40 (48.2)
	≥1 month	43 (51.8)
Clinical presentation		
	Fever	20 (24.1)
	Local symptoms only	63 (75.9)
Treatment		
	Chronic suppression	2 (2.4)
	Débridement and implant retention	14 (16.9)
	Resection and reimplantation	41 (49.4)
	Permanent resection	22 (26.5)
	Disarticulation	4 (4.8)
Recurrence of infection		
	Recurrence	10 (12.0)
	No recurrence	73 (88.0)
Laboratory finding (mean)[a]		
	WBC (/mm^3)	10639
	ESR (mm/hour)	38
	CRP (mg/dL)	32

[a] WBC, ESR and CRP denote white blood cell, erythrocyte sedimentation rate and C-reactive proteins, respectively. The reference ranges of WBC, ESR and CRP are 3500~10,500/mm^3, 0~22 mm/hour and 0.02–0.8 mg/dL, respectively.

Table 3

Distribution of superantigen genes in 84 *Staphylococcusaureus* prosthetic joint infection isolates

Superantigen genes	N = 84 (%)
Overall	
at least 1 gene	78 (92.9)
more than 1 genes	61 (72.6)
sea	23 (27.4)
seb	8 (9.5)
sec	8 (9.5)
sed	8 (9.5)
see	6 (7.1)
seg	59 (70.2)
seh	4 (4.8)
sei	40 (47.6)
tst	15 (17.9)
Profile of combination [a]	
seg+sei	17 (20.2)
sea+seg	7 (8.3)
sed+seg+sei	5 (6.0)
sea+seg+sei	4 (4.8)
seg+tst	4 (4.8)
seg+sei+tst	3 (3.6)
sea+sec	2 (2.4)
sec+seg+sei	2 (2.4)
sed+seg+sei+tst	2 (2.4)
sea+sec+seg	2 (2.4)
see+seg+sei+tst	2 (2.4)

[a] Profile of combination is listed in order of frequency; combinations identified in single isolates are not shown.