Diagnostic Microbiology and Infectious Disease 79 (2014) 119–124

Contents lists available at ScienceDirect

Diagnostic Microbiology and Infectious Disease

journal homepage: www.elsevier.com/locate/diagmicrobio

Superantigen profiling of *Staphylococcus aureus* infective endocarditis isolates

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ARTICLE INFO

Article history:
Received 11 December 2013
Received in revised form 5 March 2014
Accepted 9 March 2014
Available online 17 March 2014

Keywords:
Superantigen
S. aureus
Infective endocarditis

ABSTRACT

The frequency of superantigen production among *Staphylococcus aureus* isolates associated with endocarditis is not well defined. We tested 154 *S. aureus* isolates from definite infective endocarditis cases for the presence of staphylococcal enterotoxins A-E, H, and TSST-1 by PCR, enzyme-linked immunosorbent assay, and using an HLA-DR3 transgenic mouse splenocyte proliferation assay. Sixty-three isolates (50.8%) tested positive for at least 1 superantigen gene, with 21 (16.9%) testing positive for more than 2. *tst* (28.6%) was most common, followed by *seb* (27%), *sea* (22.2%), *sed* (20.6%), *see* (17.5%), and *sec* (11.1%). Of 41 methicillin-resistant *S. aureus*, 21 had superantigen genes, with *sed* being more frequently detected in this group compared to methicillin-susceptible *S. aureus* ($P < 0.05$). Superantigen genes were not associated with mortality ($P = 0.81$). 75% of PCR-positive isolates induced robust splenocyte proliferation. Overall, more than half of *S. aureus* isolates causing endocarditis carry superantigen genes, of which most are functional.

1. Introduction

Staphylococcus aureus is a common cause of serious diseases including pneumonia, septicemia, infective endocarditis (IE), and toxic shock syndrome (Klevens et al., 2007). *S. aureus* produces many virulence factors, among which are the superantigens (Becker et al., 2003; Vojtov et al., 2002). To date, several superantigen (SAg) genes have been identified, and globally, SAg genes have been found in over 70% of *S. aureus* isolates (Becker et al., 2003; Varshney et al., 2009). SAgs trigger a massive release of pro-inflammatory cytokines and have been associated with septic shock and increased severity of infection (Bone et al., 1997; Dinges et al., 2000; Ferry et al., 2005). However, van Belkum et al. (2006) suggest that the impact of SAgs in septic shock and mortality is incompletely defined.

Salgado-Pabon et al. (2013) recently showed that staphylococcal enterotoxin C (SEC) plays a role in the pathogenesis of experimental rabbit *S. aureus* IE. Specifically, it was demonstrated that SEC production promotes initiation and establishment of vegetations and induces cytokine production by endothelial cells. Previous studies suggest that the ability of *S. aureus* to cause endocarditis is associated with genotype of the infecting strain (Fowler et al., 2007; Gill et al., 2011). Nienaber et al. (2011) reported that compared to *S. aureus* isolates associated with soft tissue infection, *S. aureus* isolates associated with IE were more likely to contain *tst*, *sea*, *sed*, *see*, and *sei*. They studied 114 methicillin-susceptible *S. aureus* (MSSA) IE isolates, of which 26 were from North America, showing that *tst*, *sea*, *seb*, *sec*, *sed*, *see*, *seg*, *seh*, *sei*, and *sej* were present in 94, 65, 4, 25, 21, 27, 70, 11, 90, and 4%, respectively. Aside from this study, which included only MSSA, a limited number of North American isolates, and did not examine SAg production, the prevalence of SAgs and their production among *S. aureus* associated with IE, particularly in the US, has not been well-defined. We analyzed the prevalence of SAg genes and their association with outcomes in patients with *S. aureus* IE. We also assessed for SAg production from *S. aureus* grown planktonically and in the biofilm state. Finally, we evaluated the biological activity of SAgs produced by IE isolates using an *in vitro* murine splenocyte proliferation bioassay.

2. Methods

2.1. Bacterial isolates and patient data

One hundred twenty-four clinical *S. aureus* isolates collected randomly between 1997 and 2011 from patients with definitive diagnosed endocarditis who were admitted to Mayo Clinic in

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http://dx.doi.org/10.1016/j.diagmicrobio.2014.03.009
0732-8893/

Rochester, MN, were studied. Demographic characteristics, clinical presentations, and outcomes were assessed by review of the medical records. Definitive *S. aureus* IE was defined according to the modified Duke Criteria (Li et al., 2000). Septicemia, systemic inflammatory response syndrome (SIRS), sepsis, severe sepsis, and septic shock were defined according to the criteria of American College of Chest Physicians and the Society of Critical Care Medicine Conference (Bone et al., 1992). Septicemia was defined by the presence of organism in blood without SIRS; SIRS was defined by the presence of 2 or more of the following: Body temperature, >38 or <36 °C; heart rate, >90 beats per minute; respiratory rate, >20 breaths per minute; $PaCO_2$ <32 mm Hg); and abnormal leukocyte count (i.e., >12,000 or <4000 cells per mm^3 or >10% of immature neutrophils). Sepsis was defined by the presence of SIRS associated with infection, with severe sepsis defined as sepsis associated with transient hypotension, organ dysfunction, or hypoperfusion. Septic shock was defined as sepsis-induced hypotension despite adequate fluid resuscitation, with hypoperfusion or organ dysfunction. This study was approved by the Institutional Review Board at to Mayo Clinic in Rochester, MN.

2.2. Preparation of genomic DNA

Bacteria were grown overnight on sheep blood agar. Five to six colonies were suspended in 180 μL of buffer ATL solution (DNeasy blood & tissue kit; Qiagen, Hilden, Germany), 20 μL of proteinase K added, and the suspension incubated at 56 °C for 30 minutes. DNA was extracted according to the manufacturer's instructions with DNA elution in 100-μL molecular grade water.

2.3. Typing of SAg genes

Genes for staphylococcal enterotoxins A, B, C, D, E, H, and TSST-1 were assayed using individual real-time PCR assays. The nucleotide sequences of the PCR primers are shown in Table 1. PCR was performed using the LightCycler® 1.0 instrument (Roche Applied Science, Indianapolis, IN, USA) with FastStart SYBR Green Master kits (Roche Applied Science). The presence of PCR product was determined by melting curve analysis, each product having a characteristic melting temperature of the target DNA.

2.4. Preparation of planktonic and biofilm supernatants with clinical endocarditis isolates

For planktonic cultures, *S. aureus* (10^8 CFU/mL) was grown at 37 °C in trypticase soy broth (TSB) for 24 hours. Biofilms were pre-established on 1-cm-diameter Teflon discs by incubating the discs overnight with 10^6 CFU *S. aureus*/mL in TSB at 37 °C. After 24 hours, the discs were rinsed in sterile saline (0.9% sodium chloride irrigation; Baxter Corp., Deerfield, IL, USA) to remove planktonic cells, placed into 2 mL TSB containing 4 μg/mL vancomycin (to inhibit planktonic growth) and incubated for an additional 24 hours. The fluid surrounding the discs was collected and centrifuged at 4000 g for 5 minutes. The supernatants were passed through a 0.22-μm syringe filter (MILLEX®GP; Millipore, MA, USA). To determine the bacterial biofilm colony count, the biofilm-coated discs were placed into 1-mL saline and sonicated as previously described (del Pozo et al., 2009); ~10^6–10^8 CFU *S. aureus*/mL were recovered from each disc. In addition to the IE isolates, we studied isogenic strains of *S. aureus* that either express only SEB or do not express any SAg, *S. aureus* RN6734 containing pRN5543::seb (pRN7114), and RN6734 containing a derivative of the plasmid with a large 3′ deletion in *seb*, pRN5543::seb(b.2) (pRN7116), respectively [generous gifts from Richard Novick, New York University Medical Center, New York, NY, USA (Vojtov et al., 2002)]. These strains were grown planktonically in TSB with 20 μg/mL chloramphenicol.

2.5. Enzyme-linked immunosorbent assay (ELISA)

The presence of staphylococcal enterotoxins A, B, C, D, and E in bacterial planktonic and biofilm supernatants was assessed using the TECRA™ Staph Enterotoxins ID kit (3M, St Paul, MN, USA) following the manufacturer's protocol. An OD_{405} <0.2 was considered negative.

2.6. T cells proliferation assay with HLA-DR3 transgenic mouse splenocytes

AE°.HLA-DR3 transgenic mice expressing functional HLA-DRA1*0101 and HLA-DRB1*0301 transgenes on an MHC class II–deficient background as well as mice devoid of all endogenous MHC class II molecules (AE°) were studied (Cheng et al., 2003; Rajagopalan et al., 2003; Tilahun et al., 2012). Naïve splenocytes from these mice

Table 1
Primer sequences, amplification product size, and cycling conditions for detection of superantigen genes by PCR.

Gene	Primer name	Oligonucleotide sequence (5′-3′)	Size of amplification product (base pairs)	PCR cycling[a] conditions	Reference
sea	SEA-1 SEA-2	GCA GGG AAC AGC TTT AGG C GTT CTG TAG AAG TAT GAA ACA CG	521	10 s, 95 °C; 5 s, 52 °C; 27 s, 72 °C	(Lovseth, Loncarevic, & Berdal, 2004)
seb	SEB-1 SEB-2	TCG CAT CAA ACT GAC AAA CG GCA GGT ACT CTA TAA GTG CC	478	10 s, 95 °C; 5 s,56 °C; 27 s, 72 °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	10 s, 95 °C; 10 s,50 °C; 15 s, 72 °C	(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	10 s, 95 °C; 5 s, 54 °C; 27 s, 72 °C	(Johnson, et al., 1991)
see	SA-U SEE-2	TGT ATG TAT GGA GGT GTA AC GCC AAA GCT GTC TGA G	213	10 s, 95 °C; 10 s, 50 °C; 15 s, 72 °C	(Letertre, et al., 2003)
seh	SA-U SEH-2	TGT ATG TAT GGA GGT GTA AC TCT CTA GGA GTT TTC ATA TC	245	10 s, 95 °C; 10 s, 48 °C; 10 s, 72 °C	(Letertre, et al., 2003)
tst	TSST-1 TSST-2	GCT TGC GAC AAC TGC TAC AG TGG ATC CGT CAT TCA TTG TTA T	559	10 s, 95 °C; 5 s, 52 °C; 27 s, 72 °C	(Lovseth, et al., 2004)

[a] Thirty cycles.