The human antibody response to *Staphylococcus aureus* in colonization and infection

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The Human Antibody Response to *Staphylococcus aureus* in Colonization and Infection

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tegen *Staphylococcus aureus*
tijdens kolonisatie en infectie

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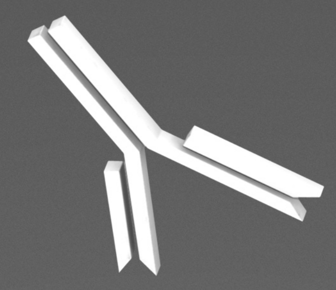
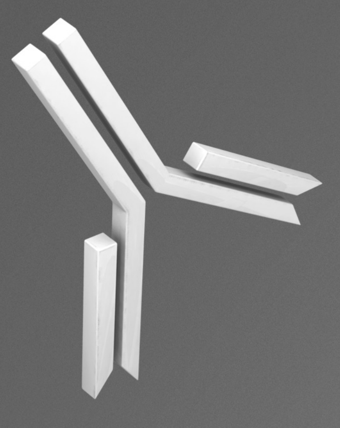
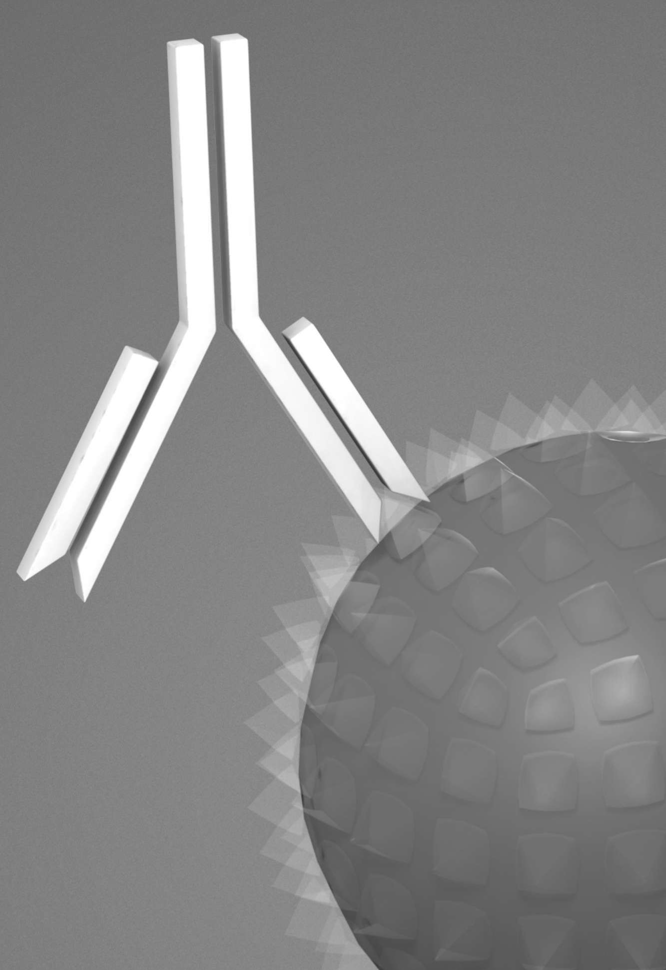


Chapter 1

General introduction, aim and outline of the thesis





GENERAL INTRODUCTION

Staphylococcus aureus is a bacterial species that was discovered in 1880 by the surgeon Sir Alexander Ogston [1]. He named the bacterium after its appearance: under the microscope bacterial cells appear round ('kókkos' in Greek) and form grape-like clusters ('staphyle' in Greek). A few years later, 'aureus', which means 'golden' in Latin, was added because of the yellow appearance of staphylococcal colonies on solid culture media. *S. aureus* colonizes the skin and mucosa of humans [2]. Although multiple body sites can be colonized, the nose is the most frequent carriage site [3]. *S. aureus* colonization is persistent in about 20-30% of the healthy human population [3], 70-80% of the human population carry *S. aureus* never or intermittently [4]. Nasal colonization is a significant risk for acquiring *S. aureus* infections in the community as well as in hospitals [3, 5, 6]. Infections include skin and soft-tissue infections such as furunculosis [7], but also life-threatening infections such as pneumonia, bacteremia and its severe complications endocarditis, septic arthritis and osteomyelitis [8]. In hospitalized patients, these infections extend the length of hospital stay, increase antibiotic usage, costs, morbidity and mortality [9-11].

Traditionally, *S. aureus* infections were treated with antibiotics derived from penicillin, such as methicillin. Unfortunately, some strains have become resistant to methicillin and other similar antibiotics. These strains are known as methicillin-resistant *S. aureus* (MRSA). MRSA was first noted in 1961, about two years after the antibiotic methicillin was initially used to treat *S. aureus* infections [12]. In the USA, Japan and many other countries, MRSA is now endemic and in the USA, the estimated number of MRSA-related hospitalizations more than doubled from 1999 to 2005 [13]. Vancomycin treatment is one of the last therapeutic options available to MRSA-infected patients. The emergence of vancomycin resistant strains is a great concern, though [14, 15]. Fortunately, prevention of *S. aureus* infections by eradication of *S. aureus* nasal carriage with the intranasal antibiotic ointment mupirocin has been shown to be effective [16-18]. Recently, a randomized-clinical trial showed a highly significant reduction of surgical-site infections after decolonization of nasal carriers with use of chlorhexidin and mupirocin [19]. However, this antibiotic treatment will only temporarily eliminate *S. aureus* carriage and repeated exposure may eventually also result in resistance [20, 21]. Therefore, the efficacy of other antistaphylococcal measures, including vaccines, is currently being explored [22, 23]. The *S. aureus* virulence factors that have been tested as potential vaccine candidates include a number of 'microbial surface components recognizing adhesive matrix molecules' (MSCRAMMs; proteins of *S. aureus* that bind to specific components of the human extracellular matrix), capsular polysaccharides (CPs) and staphylococcal toxins (Figure 1). Indeed, (passive) vaccination with (antibodies directed against) the MSCRAMMs iron-responsive surface determinant (Isd) A and B, clumping factor (Clf) A and B and serine-aspartate repeat protein (Sdr) D and E, CP5 and CP8 and the secreted pore-forming toxin α-hemolysin (or α-toxin), was shown to protect against colonization or infection in animal

models [24-29]. However, none of the antistaphylococcal vaccines successfully passed clinical trials in humans and, so far, it is still unknown which antigen or combination of antigens should be considered useful vaccine candidates [22, 30, 31]. In order to develop vaccines, understanding the bacterial and host-related determinants of carriage and understanding how humans respond to *S. aureus* exposure, is essential. Comparative analyses of the humoral immune responses in patients and healthy (non-)carriers will facilitate the identification of the antigens that are immunogenic and *in vivo* produced during colonization and infection and, therefore, will help to identify novel putative vaccine candidates.

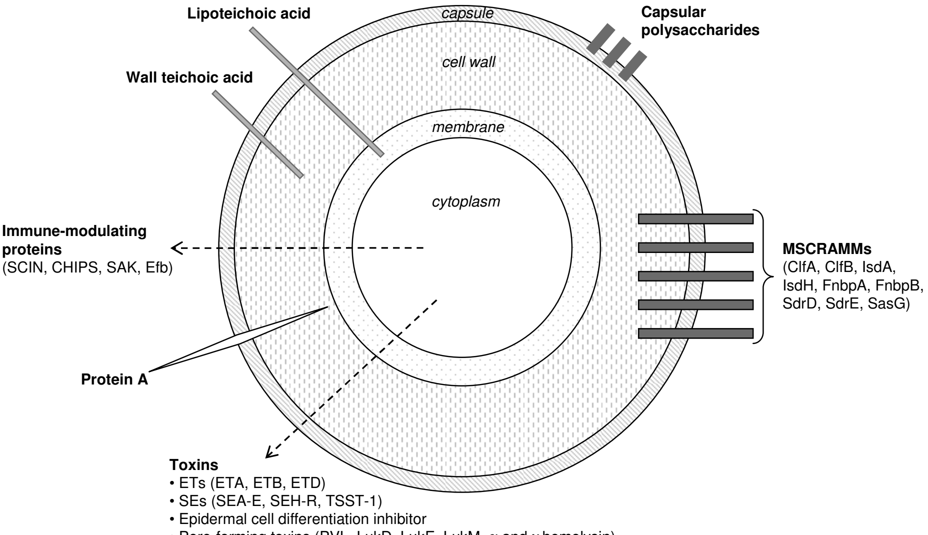


Figure 1. Schematic diagram of a single *Staphylococcus aureus* cell illustrating the localization of common *S. aureus* antigens. The outermost layer comprises a polysaccharide capsule (CP). The capsule is present in the majority of *S. aureus* strains, with serotype CP5 and CP8 being the most prevalent among human isolates. The cell wall consists mainly of peptidoglycan. The attachment of *S. aureus* to surfaces is mediated by several cell wall-associated proteins, the so-called 'microbial surface components recognizing adhesive matrix molecules' (MSCRAMMs). Well-characterized MSCRAMMs are clumping factor (Clf) A and B, iron-responsive surface determinant (Isd) A and H, fibronectin-binding protein (Fnbp) A and B, serine-aspartate repeat protein (Sdr) D and E and *S. aureus* surface protein G (SasG). Protein A is also cell wall-associated. Teichoic acids are anchored to either the cell wall or to the cytoplasmic membrane, which consists of a lipid bilayer with integral proteins. Immune-modulating proteins and toxins are secreted from the cytoplasm. CHIPS, chemotaxis inhibitory protein of *S. aureus*; Efb, extracellular fibrinogen-binding protein; ET, exfoliative toxin; Luk, leukocidin; PVL, Panton-Valentine leukocidin; SAK, staphylokinase; SCIN, staphylococcal complement inhibitor; SE, staphylococcal enterotoxin; TSST-1, toxic shock syndrome toxin-1. Note: merely the antigens that are frequently mentioned in the thesis are shown.

The antistaphylococcal humoral immune response – historical overview

The first manuscripts concerning the immune response to *S. aureus* were published a few years after its discovery in 1880. Metchnikoff's research on phagocytosis evolved into a theory suggesting that inflammation was a biological defense mechanism and that agents harmful to polynuclear leucocytes would thus be detrimental to the host [32, 33]. In both culture filtrates of *S. aureus* (at that time called *Staphylococcus pyogenes*) and in lung exudates, the

presence of such an agent, termed 'leukocidin', was demonstrated [33, 34]. By 1905, many of the toxic properties of staphylococcal filtrates had been explored. The production of hemolysin and leukocidin was described and the presence of anti-staphylolysin, anti-hemolysin and anti-leukocidin was noted in sera of humans [35, 36]. The term 'antibody' was not added at that time, since in the early 1900s the word 'antibody' (for the first time described by Ehrlich in 1891) was not generally accepted [37]. Wright revealed an important connection between humoral and cellular immunity and linked his opsonins to Metchnikoff's phagocytes [33, 38]. By 1935, many reports on toxoid (α-toxin) and anti-toxin were published [33]. Administration of toxoid had given encouraging results in the treatment of localized staphylococcal infections [39, 40]. Anti-toxin aided recovery in severe cases of acute, systemic infection [41]. It was unknown why and how this toxin and anti-toxin antibodies (the word 'antibody' was now used regularly) were effective. There was no further purpose in exploring anti-toxic immunity however, since in the early 1940s penicillin was introduced. After World War II, protein A was discovered and was found to precipitate human antibodies [42-45]. In addition, knowledge increased on anti-toxin and anti-staphylolysin antibodies [46, 47].

In the final decades of the previous century, literature on antistaphylococcal antibodies started to expand rapidly. In patients as well as healthy individuals the antistaphylococcal antibody responses directed to teichoic acid (TA), peptidoglycan (PG), CPs, a number of toxins, hemolysins, adhesins and extracellular enzymes were determined. Antibodies to TA were present healthy individuals, but also in patients suffering from *S. aureus* bacteremia, osteomyelitis, cystic fibrosis and deep tissue infections. Infective endocarditis patients posessed the highest levels of anti-TA antibodies [48-58]. An increase in the level of IgG to TA generally coincided with an increase in the level of IgG to PG [59]. Anti-PG antibody levels were higher in *S. aureus*-infected patients than in healthy individuals [59-62]. Furthermore, anti-staphylolysin and anti-α-hemolysin titers were higher in infected patients [52, 63]. Antibody levels to toxic shock syndrome toxin-1 (TSST-1) were higher in persons who carried toxin-producing strains of *S. aureus* than in non-carriers [64-66] and rheumatoid arthritis patients had higher anti-TSST-1 IgG and IgA levels than healthy controls [67]. Antibodies to CP5 and CP8 were detectable in healthy individuals, cystic fibrosis patients and endocarditis patients [68, 69]. The level of IgE directed to certain staphylococcal enterotoxins (SEs) was increased in patients with atopic dermatitis [70], nasal polyposis [71], chronic obstructive pulmonary disease [72] and allergic rhinitis [73]. In carriers, high titers of neutralizing antibodies specific for those superantigens that were expressed by their colonizing strain were found [74].

From the end of the 20th and the beginning of the 21st century onwards, manuscripts describing the antistaphylococcal antibody response directed to a number of MSCRAMMs were published. It was shown that antibody levels showed pronounced variability. Infected patients presented with higher antibody reactivity to certain MSCRAMMs than healthy individuals [24, 75, 76]. Furthermore, differences in anti-MSCRAMM antibody levels between healthy carriers and non-carriers were identified [24, 77]. Recently, a state-of-the art review

on the knowledge of the anti-*S. aureus* antibody response was published [31]. As stated previously, increasing our knowledge on the humoral immune response directed to *S. aureus* will help to identify novel putative vaccine candidates.

AIM OF THE THESIS

The primary aim of the research described in this thesis was to increase the current knowledge on the human antibody response to *S. aureus* proteins in colonization and infection and, thereby, to enhance knowledge on the immunogenicity and *in vivo* production of *S. aureus* proteins. An innovative high-throughput immunological assay was developed to simultaneously qualify and quantify antibody levels to multiple *S. aureus* antigens. A secondary aim was to enhance insights in the host-*S. aureus* interaction by performing artificial human colonization studies.

The high-throughput immunological assay

Antibodies can be measured by a variety of technologies which include immunodiffusion tests, Western blotting assays or enzyme-linked immunosorbent assays (ELISAs) [78]. Especially the latter technology is still often used. However, disadvantages of the ELISA tests are that relatively large amounts of serum (or other test liquids) are required, that it is time-consuming and that opportunities for multiplex testing are quite limited. This changed with the recent introduction of the so-called xMAP® Technology (Luminex Corporation). This innovative technology allows for simultaneous quantification of antibodies directed to different proteins in the same sample. We developed *S. aureus* multiplex assays by using this flow cytometry-based technique. Multiplexing is achieved by using 5.6 micron polystyrene beads, also called 'microspheres'. These beads are internally color-coded with 2 fluorescent dyes, red and infra-red. Through precise and balanced concentrations of these dyes, distinctly colored bead sets can be created, each of which can be coated with a specific protein. After coupling, a mix of the protein-coupled beads is introduced in the wells of 96-wells plate. Then, in each well, diluted serum is added. After a 35-minute incubation of the beads with serum and several washing steps, secondary antibodies labeled with a fluorescent dye are introduced in each well. After another 35-minute incubation and a washing procedure, the 96-wells plate is ready for analysis. The analyzer that is subsequently used resembles a flow cytometer with 2 lasers. The first (red) laser excites the internal dyes of the beads. The second (green) laser excites the fluorescent dye on the reporter molecule. Finally, high-speed digital processors identify each individual bead and quantify the level of antibodies, as based on fluorescent reporter signals. Results are reported in median fluorescence intensity (MFI) values [79].

Artificial human colonization studies

To enhance insights in the interaction between *S. aureus* and human beings, mainly *in vitro* models and animal models are used. However, a human model of colonization is probably the most appropriate. Therefore, in the Erasmus MC, a human artificial colonization model has been established [80, 81]. The study design is as follows. First, the carrier status of participating volunteers is determined on the basis of the culture results of 2 nasal swab samples. Then, volunteers are decolonized with the nasal ointment mupirocin and washes with chlorhexidin-containing soap. Five weeks after this treatment, their colonization status is assessed again. Thereafter, a single *S. aureus* strain or a mixture of strains is inoculated in the noses of the human volunteers. The volunteers are closely monitored by a physician and the elimination kinetics of *S. aureus* are studied by collecting serial nasal swab samples, culture of these swab samples and genotyping of the identified *S. aureus* strains. At the end of the study, patients receive a final medical check-up and decolonization therapy is repeated for participants still carrying the inoculated strain(s).

OUTLINE OF THE THESIS

In **Chapter 2**, we compare 2 bead types to determine which type is best used for the development of a multiplex assay involving His-tagged staphylococcal proteins: carboxylated beads or Penta-His beads. Carboxylated beads contain a surface layer of carboxyl groups for covalent attachment of proteins, Penta-His beads have Penta-His antibodies coated on their surface, which bind to the His-tags on recombinant proteins. We show that for carboxylated beads the specific signal intensity is higher and not dependent on the configuration of the protein. In addition, the non-specific binding is generally lower. Furthermore, carboxylated beads are stable over longer periods of time. Therefore, we developed an *S. aureus* multiplex assay using carboxylated beads. We describe this multiplex assay in **Chapter 3.** We coupled 19 staphylococcal proteins to carboxylated beads. The proteins include 9 MSCRAMMs, 7 SEs and 3 immune-modulating proteins. We validate the multiplex assay and we compare the level of IgG, IgA and IgM binding to the *S. aureus* proteins in serum of 40 healthy persistent, intermittent and non-carriers of *S. aureus*. Furthermore, we determine whether antistaphylococcal antibody levels are stable over time and detectable in nasal secretions. In **Chapter 4** we compare antistaphylococcal IgG, IgA and IgM levels in colonized versus non-colonized infants. In **Chapter 5** we describe the course of the antistaphylococcal humoral immune response in 15 bacteremic patients over time. We collected the *S. aureus* strains and in each strain, the genes encoding the 19 proteins included in the immunoassay were detected by PCR. In **Chapter 6** we describe the development and validation of an *S. aureus* toxin multiplex assay. Twenty toxins, including PVL and TSST-1, were coupled to carboxylated beads. The antistaphylococcal antibody levels in 206 hospitalized *S. aureus*-infected patients were

compared to antibody levels in 201 hospital-admitted controls. Furthermore, antibody levels were associated with PCR-defined presence of toxin genes in homologous *S. aureus* isolates. In **Chapter 7** we provide additional insight into the staphylococcal nasal carriage types by performing an artificial human inoculation study. In **Chapter 8** we explore the role of the immune evasion cluster (IEC) in human *S. aureus* nasal colonization. The IEC, located on β-hemolysin-converting bacteriophages (βC-Φ's), encodes the immune-modulating proteins chemotaxis inhibitory protein of *S. aureus*, staphylococcal complement inhibitor, staphylococcal enterotoxin A and staphylokinase. These proteins impair the human innate immune response. Because of the immune modulating function and the high prevalence of IEC-carrying βC-Φ's in nasal carriage isolates, we hypothesized that these bacteriophages play a role in *S. aureus* colonization. We performed an artificial inoculation study in human volunteers to confirm whether this hypothesis is correct. In **Chapter 9**, we summarize and discuss the results of our studies.

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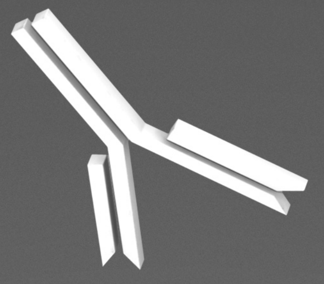
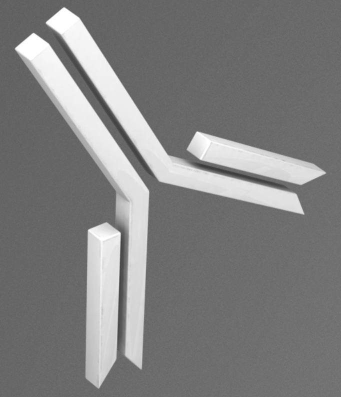


Chapter 2

Comparison of carboxylated and Penta-His microspheres for semi-quantitative measurement of antibody responses to His-tagged proteins



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ABSTRACT

The Luminex system is a flow cytometry based tool that permits the simultaneous measurement of many analytes from just a single serum sample. The technology uses microspheres, which are available in different colors and can be coated with different kinds of biomolecules. For the immobilization of His-tagged proteins, either chemically activated carboxylated beads or Penta-His beads, which have antibodies against His-tags on their surface, can be used. In this study, we compared these 2 bead types. For carboxylated as compared to Penta-His beads, the non-specific background is lower (median fluorescence intensity; MFI >250, 0% versus 15%), the specific signal intensity is higher and not dependent on the configuration of the protein. Above all, the protein-coupled carboxylated beads are useful over longer periods of time. Therefore, we conclude that for the development of a multiplex assay for semi-quantitative measurement of antibody responses against His-tagged proteins the best microspheres to use are carboxylated microspheres.

INTRODUCTION

Conventional immunological techniques such as enzyme-linked immunosorbent assays (ELISAs) allow for the detection of single antigens or antibodies at a time. The newer microsphere (bead)-based flow cytometry technique (xMAP®, Luminex Corporation) permits the simultaneous analysis of antibodies for up to 100 different antigens from a single serum sample in a single run [1]. Microspheres are internally color-coded with a pair of fluorescent dyes. Through precise and balanced concentrations of these dyes, 100 distinctly colored bead sets can be created, each of which can be coated with a specific reagent, usually a (poly)peptide or (poly)saccharide antigen. After the bead captures an analyte from a sample, a reporter molecule, labeled with a fluorescent dye, is introduced to complete the reaction on the surface of each microsphere. The analyzer that is subsequently used resembles a flow cytometer with two lasers. The first (red) laser identifies the microsphere by its internal fluorescence signature and hence determines which antigen is carried by that bead. The second (green) laser excites the fluorescent reporter dye and determines the qualitative and quantitative result of the assay. Studies have demonstrated the ability to effectively multiplex a range of assays including those for antibody detection and quantification [2].

We are interested in developing a multiplex assay to study antibody responses against His-tagged *Staphylococcus aureus* proteins. Besides the frequently used carboxylated microspheres, Penta-His beads can also be used for His-tagged protein coupling. Penta-His beads have Penta-His antibodies (mouse monoclonal IgG1) coated on the surface of the beads, which recognize and bind to the His-tags on recombinant proteins. At the bead surface, the antibodies are immobilized in a specific manner through a spacer that couples to the Fc

domain of the antibodies. In the present study we compared carboxylated beads and Penta-His beads to determine which bead type is best used for development of a multiplex assay involving His-tagged proteins.

MATERIALS AND METHODS

Antigens

The constructs to produce the recombinant *S. aureus* proteins were obtained from T. Foster [3]. The proteins were expressed in *Escherichia coli* XL1-Blue, purified under denaturing conditions with Ni-NTA agarose (Qiagen) recognizing the His-tag and quality controlled by SDS-PAGE and mass spectrometry (Ultraflex MALDI-ToF, Bruker Daltonics). The *S. aureus* recombinant proteins were iron-responsive surface determinant (Isd) A and H, fibronectin-binding protein B (FnbpB) and serine-aspartate repeat protein D (SdrD). They belong to the group of staphylococcal 'microbial surface components recognizing adhesive matrix molecules' (MSCRAMMs), proteins that are generally considered to be important for host colonization [4].

Serum samples

Venous blood samples were collected from 20 healthy volunteers and serum samples were stored at –80ºC until use. Volunteers had given informed consent and the local Medical Ethics Committee of the Erasmus Medical Center Rotterdam approved the sampling (MEC-2007-106).

Coupling methods

The 4 proteins were coupled to Penta-His beads (Qiagen) and SeroMAP beads (Luminex Corporation), a carboxylated bead type that is developed for serological applications. The coupling reactions were carried out according to the manufacturer's protocol. All centrifugation steps were carried out at 12.000*g* for 2 min at room temperature (RT).

Four µg protein per million beads was added to 2.5×10^5 Penta-His microspheres and mixed by vortexing for 30 sec at full speed. The microspheres were then incubated overnight at 4ºC in the dark. One mL PBS, 0.1% BSA was then added to the bead suspension after which they were centrifuged immediately. This step was repeated twice. The beads were resuspended in 400 µL blocking-storage buffer (PBS-BN; PBS, 1% bovine serum albumin, 0.05% sodium azide, pH 7.4). The suspension was adjusted to 125 beads/µL and stored at 4ºC in the dark. A coupling concentration of 12 µg protein per million beads was investigated also, but showed no increase in median fluorescence intensity (MFI) values (results not shown). Therefore, we used the former concentration.

The coupling procedure for carboxylated beads was as described before [2]. To 1.0×10^6 microspheres, 4 µg protein per million beads was added. As an activation buffer we used 100 mmol/L

monobasic sodium phosphate (pH 6.2). For activation of the carboxyl groups on the surface of the beads, 10 μL of 50 mg/mL of *N*-hydroxysulfosuccinimide (Sulfo-NHS) and 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC) was used (Pierce Biotechnology). The coupling buffer consisted of 50 mmol/L 2-(*N*-morpholino)-ethanesulfonic acid (MES; pH 5.0, Sigma-Aldrich). The final concentration of microspheres was adjusted to 500 beads/μL with blocking-storage buffer. The microspheres were protected from light and stored at 4ºC until use.

For uncoupled beads the coupling procedure for Penta-His or carboxylated beads was followed, only no *S. aureus* protein was added.

Assay for comparison of non-specific IgG microsphere binding

Twenty human serum samples were diluted 1:50 in PBS-BN. Fifty μL per diluted serum sample was incubated with 10 μL uncoupled Penta-His or 10 μL uncoupled carboxylated microspheres and 40 μL PBS-BN in separate wells of a 96-well filter microtiter plate (Millipore Corporation) for 30 minutes at RT on a thermomixer plate shaker (Eppendorf). The plate was washed twice with assay buffer (PBS, 1% bovine serum albumin, 0.05% sodium azide, pH 7.4) that was aspirated by vacuum manifold. The microspheres were resuspended in 50 μL assay buffer and 50 μL of a 1:200 dilution of R-phycoerythrin (RPE)-conjugated goat anti-human IgG (Fc_y fragment specific, Jackson Immuno Research) was added. The plate was incubated for 30 min at RT on the plate shaker and washed. The microspheres were resuspended in 100 μL of assay buffer. Measurements were performed on the Luminex 100 instrument (BMD) with Luminex IS software (version 2.2). The assay was performed in duplicate and the MFI values, reflecting semi-quantitative antibody levels, were averaged.

Assay for comparison of antigen-specific results

We used the 11 serum samples with low non-specific background in both types of beads. The procedure was the same as described above. In this assay, antigen-coupled microspheres were used. The samples were tested in 3 separate experiments with a short time interval of 2 weeks and a longer time interval of 3 months with the same batch of antigen-coupled Penta-His and carboxylated beads. The inter-assay variation was calculated and averaged per *S. aureus* protein. Intra-assay variation was determined from testing each of the 11 sera on 4 wells within a plate. The percentage coefficient of variation (CV) was calculated and averaged.

RESULTS

Non-specific IgG binding to microspheres

Some human sera contain antibodies that bind directly to the carboxylated surface of a microsphere, even in the absence of coupled antigen [5, 6]. We measured the non-specific background of Penta-His beads and carboxylated beads in 20 serum samples and compared

the MFI values. Diluent-only reactions resulted in MFI values of less then 10 for both groups of microspheres. Results presented in Table 1 show that the non-specific binding is higher in Penta-His beads than carboxylated beads. The Penta-His beads showed a background higher than 250 MFI in 3 of 20 (15%) serum samples, whereas carboxylated beads showed such a background in none of the samples.

Table 1. Comparison of non-specific IgG binding to Penta-His and carboxylated microspheres for 20 volunteers

	Carboxylated beads	Penta-His beads
Median	10	69
Mean	13	233
Standard deviation	9	619
Minimum	5	20
Maximum	32	2822
# of samples 0-75 MFI	20	11
# of samples 75-150 MFI	0	5
# of samples 150-250 MFI	0	1
# of samples > 250 MFI	0	3

Note. Results presented in the table are median fluorescence intensity (MFI) values

Antigen-specific results

Eleven serum samples were compared for specific signal intensity for the 4 different proteins. The MFI values obtained with IsdA, IsdH, FnbpB and SdrD coupled to carboxylated beads were higher than the MFI values obtained with these proteins coupled to Penta-His beads (Figure 1). Intra-assay reproducibility was high for both types of beads: the intra-assay percent CVs were 7.5%, 7.3% 9.6% and 10.2% for IsdA, IsdH, FnbpB and SdrD coupled to carboxylated beads and 6.6%, 10.9%, 6.9% and 10.9% for these proteins coupled to Penta-His beads. For carboxylated beads, the MFI values of first and second assay were comparable with differences in the range of the intra-assay variation. After 3 months, there was a small decline in MFI values. The inter-assay variation between the 3 experiments with a time interval of 3 months was 19.1%, 19.9%, 25.6% and 39.3%, respectively (Figure 1). Figure 2 shows the MFI values directed to IsdA, IsdH, FnbpB and SdrD per individual (values of the 3 experiments were averaged). The MFI values obtained with Penta-His beads declined between the first, second and third assay. The MFI values of the second assay declined to 53.1%, 40.9%, 42.5% and 62.6% of the original value. After 3 months, the MFI values for IsdH, FnbpB and SdrD declined below 200 for all serum samples. The inter-assay variation for IsdA was 56.7%. The inter-assay variation for IsdH, FnbpB and SdrD was 87.4%, 86.1% and 81.0%, respectively.

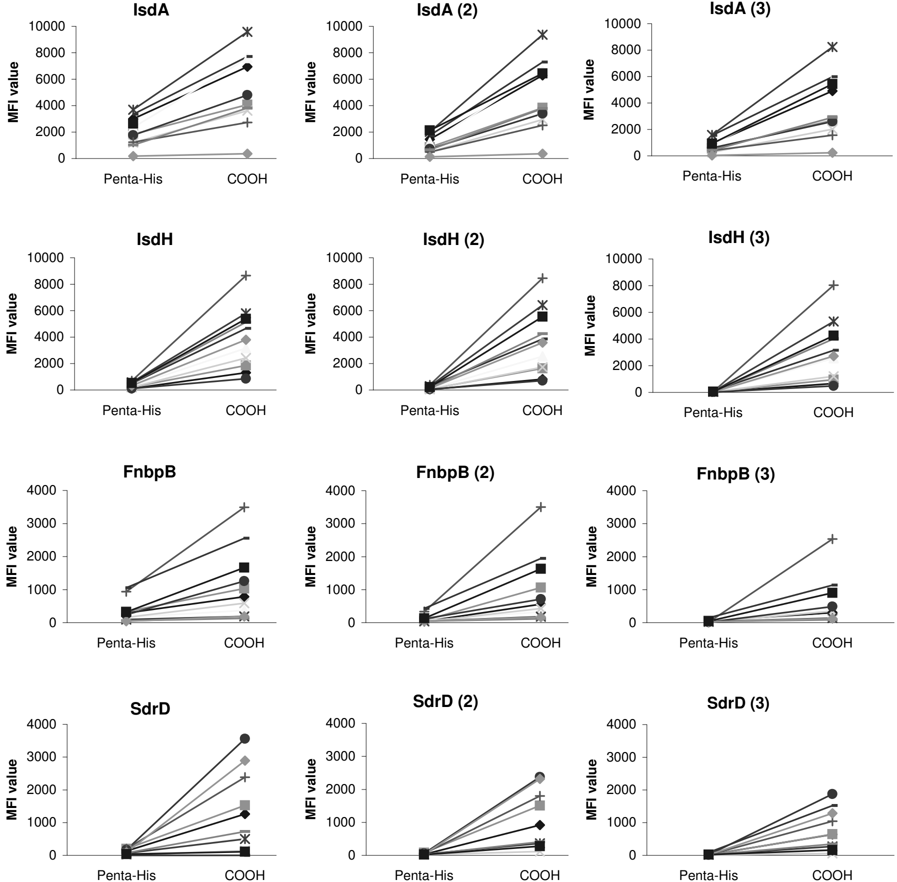

Figure 1. Median fluorescence intensity (MFI) values reflecting serum IgG levels in 11 volunteers for *S. aureus* proteins coupled to Penta-His or carboxylated (COOH) beads. Results of the first, second (2) and third assay (3) are shown separately. Each symbol represents one individual. FnbpB, fibronectin-binding protein B; Isd, iron-responsive surface determinant; SdrD, serine-aspartate repeat protein D.

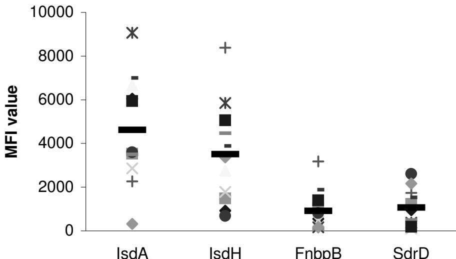

Figure 2. Median fluorescence intensity (MFI) values reflecting serum IgG levels in 11 volunteers for *S. aureus* proteins coupled to carboxylated beads (values of the 3 experiments averaged). Each symbol represents one individual. Black horizontal line represents the mean MFI value per protein. FnbpB, fibronectin-binding protein B; Isd, iron-responsive surface determinant; SdrD, serine-aspartate repeat protein D.

DISCUSSION

S. aureus is an important pathogen causing a variety of infections ranging from mild to life threatening. Carriers of *S. aureus,* ~20% of the healthy population, have an increased risk of developing *S. aureus* infection [7]. So far, little is known about the role of antigen-specific antibodies in (prevention of) infection. Therefore, we are interested in the development of a Luminex *S. aureus*-specific multiplex assay.

Because our recombinant *S. aureus* proteins are His-tagged, for the development of such a multiplex assay we were able to use either the most widely used carboxylated beads or Penta-His beads, which have Penta-His antibodies (mouse monoclonal IgG1) coated on their surface. First we tested for non-specific background. Waterboer et al. mentioned that non-specific background differs in different serum panels and appears to depend on the origin of the serum samples or may reflect sampling conditions [6]. We tested equal serum samples with both Penta-His and carboxylated beads and detected in 15% of the serum samples a non-specific background higher than 250 MFI with Penta-His beads but not with carboxylated beads. Thus, the non-specific background is also dependent on the type of bead applied. We hypothesized that the non-specific background was due to the mouse IgG1 coated on the bead surface, because it is known that human anti-mouse antibodies can cause interference in immunological assays [8]. An ELISA with the mouse monoclonal IgG1 coated on the plate surface could not confirm this hypothesis (data not shown). The higher non-specific background found with Penta-His beads is probably caused either by antibody binding to the spacer or binding of antibodies to the bead surface itself.

The specific signal intensities were higher when using carboxylated beads than Penta-His beads. Although a higher specific signal intensity is not necessarily better or more valid, it will give the assay a broader dynamic range. Otherwise, when the antigen-specific signal is low it could fall in the range of non-specific background and data become unusable. The lower signal obtained with Penta-His beads may be due to availability of epitopes. Penta-His antibodies enforce coordinated immobilization of His-tagged proteins. This means that all proteins are configured in the same way and not at random, in contrast to the carboxylated beads where randomized covalent coupling takes place. When an epitope is (partially) hidden and no antibodies in serum can bind to it, low MFI values will be found. There are alternative explanations for the lower MFI values found with Penta-His beads. A maximum of two His-tagged proteins can bind to the Penta-His antibody. Due to the relatively large Penta-His antibodies the amount of His-tagged protein on the surface of Penta-His beads is less than on carboxylated beads. The fact that a 3-fold increase in recombinant protein did not increase the signal underscores the suggestion that not enough Penta-His antibody molecules are coated on the bead surface to obtain optimal results. In addition, the Penta-His antibody used might not be the best antibody to couple His-tagged proteins. Individual His-tagged proteins are sometimes recognized better by Tetra-His than Penta-His antibodies

because of subtle differences in the exact conformation of the His-tag (Qia*express* Detection and Assay Handbook 2002).

We found that intra-assay variation was low and comparable for both types of beads. On the contrary, the inter-assay variation was not comparable. For carboxylated beads, the inter-assay variation for IsdA, IsdH, FnbpB and SdrD ranged from 19-39%. For Penta-His beads, the inter-assay variation ranged from 57-87%. The decline in MFI values observed in time was larger for Penta-His beads than for carboxylated beads. So, carboxylated beads are useful over a longer period of time than Penta-His beads. A possible explanation is that a covalent binding is more stable than an antibody-antigen interaction. Because of the decline in MFI values introducing an internal standard sample is important, though.

Overall, for carboxylated beads the non-specific background is lower, the specific signal intensity is higher and not dependent on the configuration of the proteins attached. Above all, the antigen-coupled carboxylated beads are useful over a longer period of time than Penta-His beads. Therefore, we conclude that for development of a multiplex assay for semi-quantitative measurement of antibody responses to His-tagged proteins the best microspheres to use are carboxylated microspheres.

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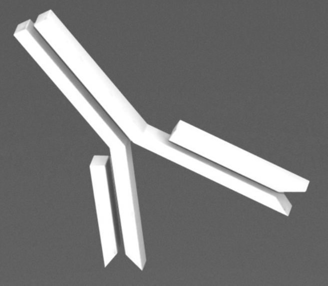

Chapter 3

Antistaphylococcal humoral immune response in persistent nasal carriers and non-carriers of *Staphylococcus aureus*

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ABSTRACT

Background. Persistent carriers have a higher risk of *Staphylococcus aureus* infections than non-carriers but a lower risk of bacteremia-related death. Here, the role played by antistaphylococcal antibodies was studied. **Methods.** Serum samples from 15 persistent carriers and 19 non-carriers were analyzed for immunoglobulin (Ig) G, IgA and IgM binding to 19 *S. aureus* antigens, by means of Luminex technology. Nasal secretions and serum samples obtained after 6 months were also analyzed. **Results.** Serum IgG levels were significantly higher in persistent carriers than in non-carriers for toxic shock syndrome toxin (TSST)–1 (median fluorescence intensity [MFI] value, 11554 vs. 4291; $P<0.001$) and staphylococcal enterotoxin (SE) A (742 vs. 218; $P<0.05$); IgA levels were higher for TSST-1 ($P<0.01$), SEA and clumping factor (Clf) A and B ($P<0.05$). The *in vitro* neutralizing capacity of anti–TSST-1 antibodies correlated with the MFI value ($R^2 = 0.93$) and was higher in persistent carriers (90.6% vs. 70.6%; $P<0.05$). Antibody levels were stable over time and correlated with levels in nasal secretions (for IgG, $R^2 = 0.87$; for IgA, $R^2 = 0.77$). **Conclusions.** Antibodies to TSST-1 have a neutralizing capacity and the levels of antibodies to TSST-1, SEA, ClfA and ClfB are higher in persistent carriers than in non-carriers. These antibodies might be associated with the differences in the risk and outcome of *S. aureus* infections between nasal carriers and non-carriers.

INTRODUCTION

Staphylococcus aureus is an important pathogen that causes superficial skin infections (furuncles and impetigo) as well as invasive infections such as endocarditis and bacteremia [1]. Persistent carriers of *S. aureus,* comprising ~20% of the healthy population [2, 3], have an increased risk of developing such infections [4-6], including a 3-fold higher risk of acquiring *S. aureus* bacteremia. Surprisingly, the risk of death in carriers with bacteremia is significantly lower than that in non-carriers with bacteremia [5, 7]. An explanation for this observation has not yet been provided, although a role for the immune system has been proposed. Genotyping has revealed that 80% of strains that cause bacteremia in persistent carriers are endogenous [5, 8]. Because of long-time exposure to their colonizing strain, carriers may have developed antibodies that protect them from bacteremia-related death. Otherwise, non-carriers may harbor antibodies that protect them from nasal colonization [9] and they therefore remain at lower risk of acquiring *S. aureus* bacteremia. Antistaphylolysin titers were found to be higher in carriers than in non-carriers [10], but the 2 groups had similar concentrations of antibodies directed to teichoic acid [11]. Recently, a higher level of IgG in non-carriers than in carriers was reported for α-hemolysin, major autolysin, iron-responsive surface determinant (Isd) A and H, immunodominant secretory antigen A (IsaA), major histocompatibility complex class II analogue protein w (Map-w) and clumping factor (Clf) B [9, 12]. These latter studies focused

mainly on antibodies to 'microbial surface components recognizing adhesive matrix molecules' (MSCRAMMs), proteins that are generally considered important for host colonization [13, 14]. At present, little is known about the humoral immune response to staphylococcal enterotoxins (SEs) and immune-modulating proteins in persistent carriers and non-carriers. SEs are superantigens and, therefore, potent pro-inflammatory agents [15]. They have been implicated in the pathogenesis of toxic shock [15, 16]. The immune-modulating proteins staphylococcal complement inhibitor (SCIN) and extracellular fibrinogen-binding protein (Efb) are potent complement inhibitors that lead to diminished phagocytosis and killing by human neutrophils [17, 18]. Chemotaxis inhibitory protein of *S. aureus* (CHIPS) impairs the response of neutrophils and monocytes to formylated peptides and C5a [19]. Consequently, both the SEs and the immune-modulating proteins might play a role in *S. aureus* carriage and disease.

In the present study we determined the levels of antibodies to 9 MSCRAMMs, 7 SEs and 3 immune-modulating proteins in serum samples and nasal secretions from well-defined persistent and non-carriers and measured the stability of antistaphylococcal antibody levels over time.

MATERIALS AND METHODS

Serum samples, nasal secretions and nasal swabs

At the beginning of the study, all volunteers completed a questionnaire on age, sex, weight, height, nationality, occupation, smoking and drinking habits, medication (including antibiotic usage) and medical history. Criteria for exclusion were diabetes mellitus, renal insufficiency, chronic obstructive pulmonary disease, heart disease, immunocompromised status, immunosuppressant use, antibiotic use in the last 4 weeks and skin diseases (such as impetigo and eczema). All 40 participants (median age 36.9 years; age range 21-60 years) fulfilled the inclusion criteria and did not suffer from apparent staphylococcal infections during the study period. Venous blood samples and at least 3 consecutive nasal swab samples (at 2-week intervals) were obtained for each of the 40 healthy volunteers. After 6 months, a second blood sample and 2 additional nasal swab samples were collected from 11 of these volunteers. Nasal swab samples were processed as described elsewhere [2]. Subjects were classified as persistent carriers when all nasal swab cultures were positive for *S. aureus*, as intermittent carriers when 1 or 2 nasal swab cultures were positive and as non-carriers when all nasal swab cultures were negative. Nasal secretions from 13 volunteers were collected at the beginning of the study by vacuum-aided suction without chemical stimulation and processed as described elsewhere [20, 21]. The collected fluid was sonicated in a water bath to disrupt the mucoprotein aggregates and to facilitate reproducible handling. The secretions and serum samples were stored at -80°C until use. Human pooled serum (HPS) from 36 healthy donors was used as a standard during Luminex experiments. Volunteers provided

written informed consent and the local Medical Ethics Committee of the Erasmus Medical Center Rotterdam approved the study (MEC-2007-106).

Antigens

The MSCRAMMs ClfA and ClfB, *S. aureus* surface protein (Sas) G, IsdA and IsdH, fibronectin-binding protein A and B and serine-aspartate repeat protein (Sdr) D and E were expressed with a His-tag in *Escherichia coli* XL1-Blue and purified under denaturing conditions with Ni-NTA agarose (Qiagen) recognizing the His-tag; quality control was done using SDS-PAGE and mass spectrometry (Ultraflex MALDI-ToF, Bruker Daltonics). Staphylococcal enterotoxin (SE) A was purchased from Sigma. The recombinant proteins SEB, SEI, SEM, SEO, SEQ and toxic shock syndrome toxin (TSST)-1 were provided by Dr. S. Holtfreter and D. Grumann (University of Greifswald) [16]. Dr. S. Rooijakkers (University Medical Center Utrecht) provided the recombinant proteins CHIPS and SCIN. Prof. J.I. Flock (Karolinska Institutet) supplied Efb [22-25].

Coupling methods

To quantify the level of antibodies directed against the 19 *S. aureus* proteins simultaneously, the recently introduced microsphere (bead)-based flow cytometry technique (xMAP®, Luminex Corporation) was applied. The purified proteins were coupled to SeroMAP beads, a carboxylated bead type developed for serological applications. The coupling procedure was performed as described elsewhere [26, 27]. In brief, 25 µg of protein was added to $5.0x10^6$ microspheres. This amount of protein was found to be optimal. As an activation buffer we used 100 mmol/L monobasic sodium phosphate (pH 6.2). To activate the carboxyl groups on the surface of the beads, 10 µL of 50 mg/mL of *N*-hydroxysulfosuccinimide and 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide was used (Pierce Biotechnology). The coupling buffer consisted of 50 mmol/L 2-(*N*-morpholino)-ethanesulfonic acid (pH 5.0, Sigma-Aldrich). The final concentration of microspheres was adjusted to 4000 beads/µL with blocking-storage buffer (PBS-BN; PBS, 1% bovine serum albumin, 0.05% sodium azide, pH 7.4). The microspheres were protected from light and stored at 4ºC until use. For control beads, the coupling procedure was done in the absence of *S. aureus* protein. In each experiment, control beads were included to determine non-specific binding. In case of non-specific binding, the median fluorescence intensity (MFI) values were subtracted from the antigen-specific results. As a negative control, PBS-BN was included.

Multiplex *S. aureus* antibody assay

The multiplex assay (serum incubated with the different fluorescence-colored antigen-coupled beads mixed in 1 well) was validated by comparing the MFI values for HPS obtained with this multiplex assay with the results for HPS obtained with singleplex assays (serum incubated with each different color of antigen-coupled beads in separate wells). After validation, the different antigen-coupled microspheres were mixed to a working concentration of

4000 beads per color per well. The procedure was the same as described elsewhere [26]. Serum samples were diluted 1:100 in PBS-BN for measurement of antigen-specific IgG and IgA and 1:25 for measurement of IgM. Fifty µL per diluted sample was incubated with the microspheres in a 96-well filter microtiter plate (Millipore) for 35 min at room temperature (RT) on a thermomixer plate shaker (Eppendorf). The plate was washed twice with assay buffer (PBS-BN) that was aspirated by vacuum manifold. The microspheres were resuspended in 50 µL of assay buffer. In separate wells, 50 µL of a 1:200 dilution of R-phycoerythrin (RPE)-conjugated goat anti-human IgG and IgA and 50 µL of a 1:50 dilution of RPE-conjugated donkey anti-human IgM (Jackson Immuno Research) were added. The plate was incubated for 35 min at RT on the plate shaker and washed. The microspheres were resuspended in 100 µL of assay buffer. Measurements were performed on the Luminex 100 instrument (BMD) using Luminex IS software (version 2.2). Tests were performed in triplicate and the MFI values, reflecting semi-quantitative antibody levels, were averaged. The coefficient of variation (CV) was calculated for each serum sample and averaged per protein and antibody isotype. For nasal secretions, the procedure was identical. Nasal secretions were diluted 1:20 and RPE-conjugated goat anti-human IgG and IgA were diluted 1:50.

TSST-1 neutralization assay

The *in vitro* TSST-1 neutralization assay was performed as described elsewhere [16, 28]. Initially, the concentration of recombinant TSST-1 that elicited submaximal T-cell proliferation was determined (10 pg/mL). Subsequently, 10 pg/mL TSST-1 was incubated with serial dilutions (1:50 to 1:6250) of heat-inactivated serum from the 40 healthy volunteers. At higher serum dilutions, maximal inhibition could no longer be obtained. As a control, TSST-1 was incubated with RPMI 1640 supplemented with 10% fetal bovine serum. After 20 minutes, $1x10^5$ peripheral blood mononuclear cells from healthy blood donors were added to test for TSST-1 neutralizing antibodies. T- cell proliferation was determined by the incorporation of [^{3}H]-thymidine after 72h, quantified by calculating the area under the proliferation curve (AUC) and expressed as a percentage of the control without human serum. All measurements were performed in triplicate and repeated in 2 independent experiments.

Statistical analysis

The Mann-Whitney *U* test was used to compare differences in antistaphylococcal antibody levels and neutralizing capacity of serum from persistent carriers and non-carriers. To compare the antibody levels in the first and second serum sample from an individual, paired *t* tests were used. Correlations between antigen-specific IgG and IgA in serum and nasal secretions were assessed using Pearson's correlation coefficient. Nonlinear regression was used to describe the relation between MFI value and neutralizing capacity. Differences were considered statistically significant when 2-sided *P*-values were ≤0.05.

RESULTS

Control of the multiplex assay and reproducibility

First, the multiplex assay was validated. The MFI values obtained for HPS with the multiplex assay were between 93% and 116% (median 100%) of those obtained with the singleplex assays, so it was valid to use the multiplex assay. Serum incubated with control beads (beads without protein coupled on their surface) resulted in median MFI values for IgG, IgA and IgM of 14 (range 6-82), 6 (range 3-22) and 75 (range 3-957), respectively. This indicates that there was low non-specific binding (with exception of IgM in 1 sample). The negative control (PBS-BN) incubated with protein-coupled beads resulted in low MFI values (<10 MFI).

Inter-assay variation was calculated from MFI values obtained from serum samples (n=40) run in 3 separate assays and was averaged per protein and antibody isotype. For IgG, the median CV was 15%; range 5% (CHIPS) to 25% (SEO). For IgA, the median CV was 20%; range 7% (Efb) to 25% (SdrD, SEB, SEI and SasG) and for IgM, the median CV was 16%; range 7% (ClfA) to 43% (SEO; high CV due to MFI values close to 0). Earlier studies found equal CVs for inter-assay variation [26, 29-31].

Differences in antistaphylococcal antibody levels in serum samples from persistent carriers and non-carriers

Nineteen volunteers were classified as non-carriers (48%), 6 as intermittent carriers (15%) and 15 as persistent carriers (38%). The MFI values reflecting serum antibody levels for each person and antibody isotype are shown in Figure 1. For most of the antigens there was no apparent quantitative difference in antibody level between persistent carriers and non-carriers. However, the serum levels of IgG directed against TSST-1 and SEA were significantly higher in persistent carriers than non-carriers (MFI 11554 vs. 4291; $P<0.001$ and 742 vs. 218; $P<0.05$ respectively). Additionally, the serum level of IgA was significantly higher in persistent carriers than non-carriers for TSST-1 (973 vs. 155; $P<0.01$), SEA (127 vs. 32; $P<0.05$), ClfA (1661 vs. 441; $P<0.05$) and ClfB (792 vs. 356; $P<0.05$).

Stability of antistaphylococcal antibody levels in serum

To study the stability of the level of *S. aureus* antigen-specific antibodies over time, a second serum sample and 2 more nasal swabs were collected after 6 months from 11 volunteers. None of these volunteers reported suffering from an apparent *S. aureus* infection between these time points. One of the volunteers (volunteer 2) was classified as an intermittent carrier instead of non-carrier because of a single positive nasal swab culture after 6 months. For all volunteers, the levels of IgG and IgA to the 19 *S. aureus* proteins did not change significantly during the 6-month period ($P>0.05$). Figure 2 shows representative results for the stability of IgG levels for 4 *S. aureus* proteins.

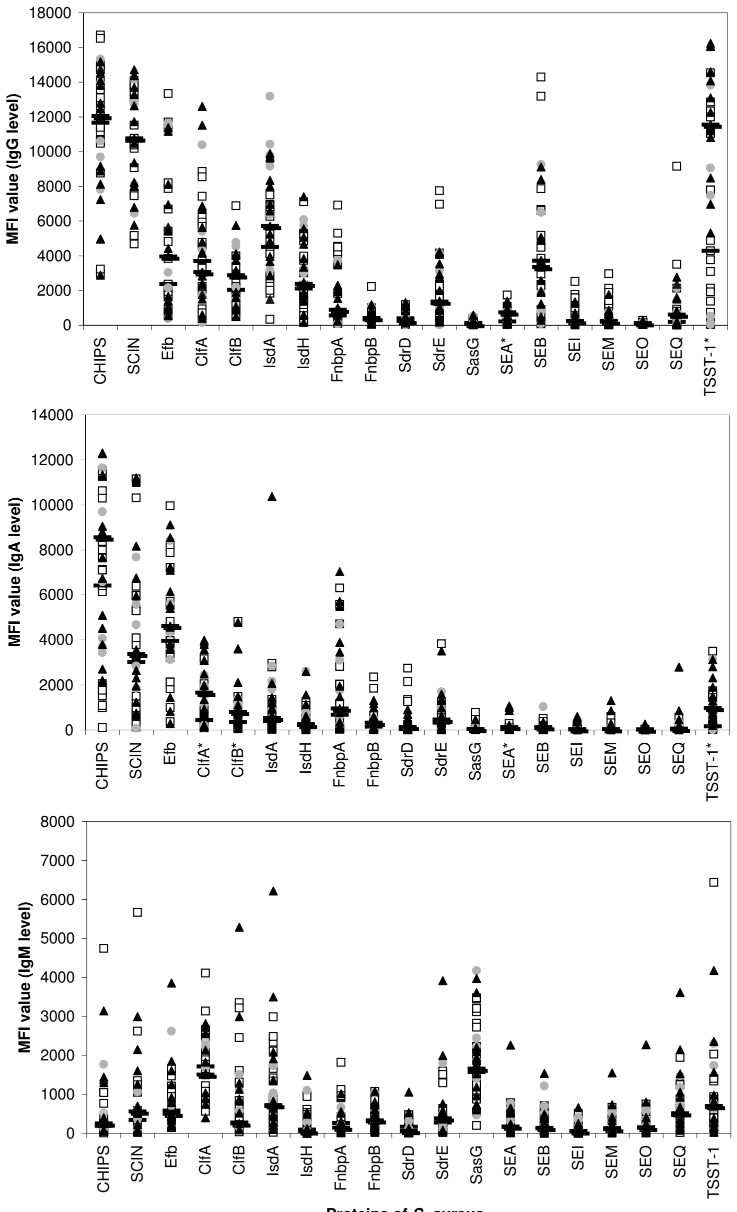

Figure 1. Median fluorescence intensity (MFI) values reflecting levels of antigen-specific IgG, IgA and IgM for 19 *S. aureus* antigens in 40 volunteers. Each symbol represents a single volunteer; black triangles represent persistent carriers, grey circles represent intermittent carriers and white squares represent non-carriers. Median levels of antistaphylococcal antibodies for persistent carriers and non-carriers are indicated by double and single horizontal lines, respectively. Statistically significant differences are indicated by asterisks ($P<0.05$, Mann-Whitney *U* test). CHIPS, chemotaxis inhibitory protein of *S. aureus*; Clf, clumping factor; Efb, extracellular fibrinogen-binding protein; Fnbp, fibronectin-binding protein; Isd, iron-responsive surface determinant; Sas, *S. aureus* surface protein; SCIN, staphylococcal complement inhibitor; Sdr, serine-aspartate repeat protein; SE, staphylococcal enterotoxin; TSST, toxic shock syndrome toxin.

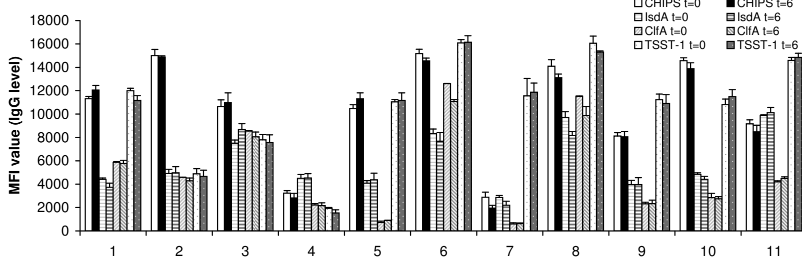

Figure 2. Stability of *S. aureus* protein-specific IgG levels, reflected by median fluorescence intensity (MFI) values, in serum samples from 11 healthy volunteers (1-11) at 0 and 6 months. Error bars represent the standard error of the mean. CHIPS, chemotaxis inhibitory protein of *S. aureus*; Clf, clumping factor; Isd, iron-responsive surface determinant; TSST, toxic shock syndrome toxin.

Correlation between antistaphylococcal antibody levels in serum and nasal secretion

To determine the correlation between the level of antistaphylococcal antibodies in serum and nasal secretions, these samples were collected simultaneously from 13 volunteers and the mean IgG and IgA levels (reflected by MFI values) in these samples were calculated for each protein. Antibody levels in serum correlated with antibody levels in nasal secretions (for IgG, $R^2 = 0.87$; for IgA, $R^2 = 0.77$, Figure 3).

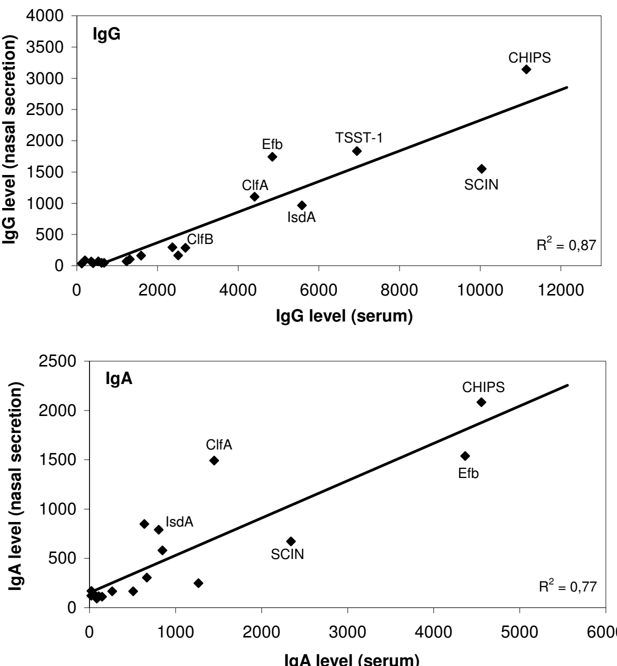

Figure 3. Correlation between IgG and IgA levels in serum and nasal secretions. Mean IgG and IgA levels in serum and nasal secretions, reflected by median fluorescence intensity values, were calculated for each protein. Pearson's correlation coefficient was used. CHIPS, chemotaxis inhibitory protein of *S. aureus*; Clf, clumping factor; Efb, extracellular fibrinogen-binding protein; Isd, iron-responsive surface determinant; SCIN, staphylococcal complement inhibitor; TSST, toxic shock syndrome toxin.

TSST-1 neutralization assay

The neutralizing capacity of TSST-1-specific antibodies in the 40 serum samples was determined. The neutralizing capacity was significantly higher in persistent carriers than in non-carriers (90.6% versus 70.6%; $P<0.05$, Figure 4A). The level of IgG binding to TSST-1 is highly related to the neutralizing capacity of the serum samples ($R^2 = 0.93$, Figure 4B).

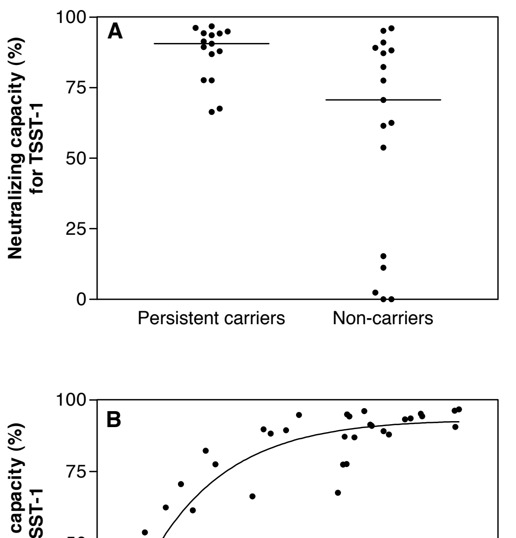

Figure 4. [A] Higher neutralizing capacity for toxic shock syndrome toxin (TSST)-1 in persistent carriers than in non-carriers (median 90.6% versus 70.6%, $P<0.05$, Mann-Whitney *U* test). Two non-carriers were excluded because of poor technical replicates. [B] High correlation between the level of IgG to TSST-1, reflected by median fluorescence intensity (MFI) values, and the neutralizing capacity of the serum samples (nonlinear regression, $R^2 = 0.93$).

DISCUSSION

We developed an *S. aureus* multiplex immunoassay that enables simultaneous quantification of antibodies to 19 antigens in small serum volumes. Therefore, this assay is more informative and less time- and serum consuming than the conventional ELISA technique. The method was used to determine the levels of antigen-specific IgG, IgA and IgM in serum samples from persistent carriers, intermittent carriers and non-carriers of *S. aureus*. An important message of our analyses is that antistaphylococcal antibody levels show extensive inter-individual

variability (Figure 1), probably owing to the variable number of previous encounters with different *S. aureus* strains of diverse antigenicity as well as inter-individual differences in the ability to mount an antigen-specific humoral immune response.

The most striking difference between persistent and non-carriers was the level of IgG to TSST-1 ($P<0.001$). An earlier study showed that individuals harboring TSST-1-producing strains had significantly higher levels of serum antibody to TSST-1 than did individuals who carried strains without TSST-1 or who did not carry *S. aureus* at all [32]. In our study, 5 (33%) of the 15 persistent carriers carried a TSST-1-positive strain (as determined by polymerase chain reaction; data not shown), which indicates that current carriage of a TSST-1-positive strain does not fully explain the higher antibody levels in persistent carriers. It is likely that the number of previous encounters with such strains also plays a role. We have shown that the level of anti-TSST-1 IgG is highly correlated with the neutralizing capacity of these antibodies ($R^2 = 0.93$, Figure 4B). This implies that these anti-TSST-1 antibodies are functional. It is known that humans with high anti-TSST-1-antibody levels do not develop toxic shock syndrome when they become infected with a TSST-1-expressing *S. aureus* strain [33]. As stated elsewhere, it is also known that carriers have a 3-fold higher risk of acquiring *S. aureus* bacteremia than do non-carriers, but a significantly lower risk of *S. aureus* bacteremia-related death [5]. Therefore, a possible explanation for this observation is that persistent carriers are protected from toxic shock syndrome because they have a high level of TSST-1-neutralizing antibodies, and consequently, a lower risk of death than non-carriers.

Other significant differences between persistent and non-carriers were found for IgG directed against SEA ($P<0.05$) and IgA directed against TSST-1 ($P<0.01$), SEA, ClfA or ClfB ($P<0.05$). These levels were found to be higher in persistent carriers than in non-carriers. Two other studies focusing on antistaphylococcal antibodies, showed higher levels of IgG to major autolysin, ClfB, IsdA, IsdH, IssA, Map-w, and α-hemolysin in non-carriers than in persistent carriers [9, 12]. Thus, the differences found between persistent and non-carriers differed from our data. One possible explanation for this discrepancy is that the carrier state was defined differently in these studies. Dryla et al. [12] defined persistent carriers and non-carriers as individuals who tested culture positive or negative at least twice, but they did not report whether the carrier state was based on nasal or pharyngeal swab samples (or both) or at what intervals these swab samples were collected. Clarke et al. [9] defined carriers and non-carriers as individuals who were culture positive or negative for *S. aureus* on the basis of just a single nasal swab sample. In the present study, we used at least 3 nasal swab samples collected at 2-week intervals to define the carrier state.

Antigen-specific IgG and IgA levels for all volunteers and to all 19 antigens were stable over a period of 6 months. Another study also showed antibody levels to 4 *S. aureus* proteins (IsdH, Map-w, SA0688 and SA2505) remaining stable over time [12]. Stability is the result of humoral memory. Humoral memory is assumed to rely on long-lived plasma cells, which even without antigenic contact will secrete antibodies for many years, and on memory B cells, which can

be (re)activated by antigen and/or by polyclonal stimuli [34]. Antistaphylococcal antibody levels in nasal secretions correlated with levels in serum, although for antigen-specific IgA in serum and nasal secretions the correlation was somewhat lower ($R^2 = 0.77$) than for IgG ($R^2 = 0.87$, Figure 3). There might be an explanation for this observation. In blood, IgA is found predominantly as a monomer and the ratio of IgA1 to IgA2 is ~4:1. In mucosal secretions IgA is almost exclusively produced as a dimer and the ratio of IgA1 to IgA2 is ~3:2 [35, 36]. Thus, whereas IgG simply diffuses from the vascular department into the tissues and similarly distributed antigen-specific IgG molecules are measured in blood and nasal secretions, for IgA this is not the case.

In the present study, we focused on nasal carriage. In the absence of nasal carriage, the likelihood of being a throat carrier is 12.6% [37], a rectal carrier 3.2% [38, 39] and an axilla carrier 2% [40]. In our study, this would mean that only a few of the intermittent and non-carriers would be reclassified into different *S. aureus* carriage types, which would not affect the results significantly. However, it does show the importance of reporting the culture sites when defining the *S. aureus* carriage state.

We have developed a novel high-throughput, low-volume method for detecting levels of antibodies to a wide range of staphylococcal proteins. We showed that antistaphylococcal antibody levels in serum are highly variable, are stable over time and correlate well with antibody levels in nasal secretions. Antibodies to TSST-1 have neutralizing capacity and levels of antibodies to TSST-1, SEA, ClfA and ClfB are higher in persistent carriers than in non-carriers. These antibodies might be associated with the risk of developing *S. aureus* infections and might be responsible for the lower risk of mortality observed in *S. aureus* carriers with bacteremia than in *S. aureus* non-carriers with bacteremia [5].

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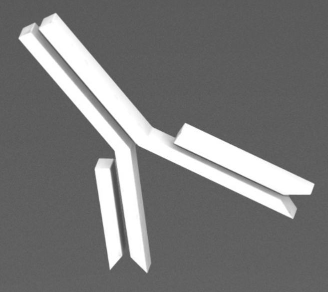

Chapter 4

Induction of antibodies by *Staphylococcus aureus* nasal colonization in young children

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ABSTRACT

In order to develop novel antistaphylococcal strategies, understanding the determinants of carriage and how humans respond to *Staphylococcus aureus* exposure is essential. Here, the primary *S. aureus*-specific humoral immune response and its association with nasal colonization was studied in young children. Sera from 57 colonized or non-colonized children, serially collected at birth and at 6, 14 and 24 months, were analyzed for IgG, IgA and IgM binding to 19 staphylococcal proteins using a flow cytometry-based technology. The antibody responses showed extensive inter-individual variability. On average, the levels of antistaphylococcal IgA and IgM increased from birth until the age of 2 years ($P<0.05$), whereas the levels of IgG decreased ($P<0.001$). Placentally transferred maternal IgG did not protect against nasal colonization. In colonized children, IgG and IgA levels for a number of proteins were higher than in non-colonized children. At both 14 and 24 months, the levels of IgG against chemotaxis inhibitory protein of *S. aureus* (CHIPS; at 24 months, median fluorescence intensity 4928 vs. 24, $P<0.05$), extracellular fibrinogen-binding protein (Efb; 987 vs. 604, $P<0.05$) and iron-responsive surface determinant H (IsdH; 62 vs. 5, $P<0.05$) were significantly higher in colonized children. The levels of IgA against CHIPS, IsdA and IsdH were higher ($P<0.05$). Therefore, CHIPS, Efb, IsdA and IsdH seem to play a role in nasal colonization of young children.

INTRODUCTION

Staphylococcus aureus efficiently colonizes human skin and, most frequently, nasal mucosa [1, 2]. Approximately 20-30% of adults carry *S. aureus* persistently and ~70-80% of adults carry *S. aureus* never or intermittently [2, 3]. *S. aureus* is carried by 10-35% of children [4]. During the first 2 months of life, the prevalence of colonization is 40-50%. Then, the prevalence rapidly decreases to ~20% by 6 months and to ~10% by 14 months [5, 6]. How nasal carriage is established and maintained is still largely unknown [2, 7], although the involvement of bacterial components such as teichoic acid, catalase, hydroperoxide reductase, iron-responsive surface determinant (Isd) A, *S. aureus* surface protein G (SasG) and clumping factor (Clf) B has been demonstrated [8-13].

Carriage of *S. aureus* can result in serious endogenous infections. Because of the increasing antibiotic-resistance of *S. aureus*, novel approaches concerning the prevention and therapy of staphylococcal disease are urgently needed. In order to develop such new strategies, understanding the determinants of carriage and understanding how humans respond to *S. aureus* exposure is essential. Here, we provide insights into the antistaphylococcal humoral immune response in young children. Studying their immune response will allow us to distinguish the bacterial factors that are expressed *in vivo* during early colonization. This may lead to the discovery of novel determinants of colonization.

MATERIALS AND METHODS

Study population

This project was performed with a subgroup of the Generation R Study, a population-based prospective cohort study of pregnant women and their children from fetal life onwards [14, 15]. The infants were presented at the Generation R research center at the ages of 1.5 months, 6 months, 14 months and 24 months. Research nurses obtained a nasal swab for *S. aureus* isolation from each infant at each visit whenever possible. The methods of nasal sampling and identification of *S. aureus* were as described previously [5]. Serum samples were collected from cord blood and through venapuncture at 6 months, 14 months and 24 months whenever possible. Included in this study were 57 healthy children, from each of whom 3 or 4 serial serum samples were collected. Of the 177 samples that were obtained, 54 (31%) were cord blood samples, 32 samples (18%) were obtained at 6 months, 46 (26%) at 14 months and 45 (25%) at 24 months. *S. aureus* colonization data were available at 1.5, 6, 14 and 24 months for 40 (70%), 49 (86%), 50 (88%) and 48 (84%) children, respectively. Children were classified as colonized if at least one of the nasal swab cultures was positive for *S. aureus*. Children were classified as non-colonized if all swab cultures were negative. Children with a culture moment missing at one time-point, and with the other nasal swab cultures negative, were classified as non-colonized as well. None of the children suffered from apparent staphylococcal infection. The Medical Ethics Committee of the Erasmus Medical Center Rotterdam approved the study. Written informed consent was obtained from the parents of all participating children.

Measurement of antistaphylococcal antibodies

The levels of antistaphylococcal antibodies directed against 3 important groups of *S. aureus* proteins, 'microbial surface components recognizing adhesive matrix molecules' (MSCRAMMs), staphylococcal enterotoxins (SEs) and immune-modulating proteins, were determined. The proteins have been described previously [16]. MSCRAMMs are generally considered to be important for host colonization [17]. The recombinant MSCRAMMs ClfA, ClfB, SasG, IsdA, IsdH, fibronectin-binding protein (Fnbp) A and B and serine-aspartate repeat protein (Sdr) D and E were used. SEs are superantigens and, therefore, potent pro-inflammatory agents [18]. The recombinant proteins SEA, SEB, SEI, SEM, SEO, SEQ and toxic shock syndrome toxin (TSST)-1 were used. In addition, the immune-modulating proteins staphylococcal complement inhibitor (SCIN), extracellular fibrinogen-binding protein (Efb) and chemotaxis inhibitory protein of *S. aureus* (CHIPS) were used. Efb and SCIN are complement inhibitors that lead to a reduction of bacterial phagocytosis and killing by human neutrophils [19, 20]. CHIPS impairs the response of neutrophils and monocytes to formylated peptides and complement factor C5a [21].

The levels of antigen-specific IgG, IgA and IgM were quantified using a bead-based flow cytometry technique (xMAP®, Luminex Corporation). The methods used were as described

previously [16, 22, 23]. Tests were performed as independent duplicates and the median fluorescence intensity (MFI) values, reflecting antibody levels semi-quantitatively, were averaged. In each experiment, control beads (no protein coupled) were included to determine non-specific antibody binding. In cases of non-specific binding, these non-specific MFI values were subtracted from the antigen-specific values. Human pooled serum was used as a standard.

Statistical analysis

Statistical analyses were performed with SPSS version 15.0. The Wilcoxon signed rank test was used to compare the antistaphylococcal antibody levels between different age groups. Mann-Whitney *U* tests were used to compare differences in antibody levels between colonized and non-colonized children. Binary logistic regression analysis was used to determine the relationship between maternal IgG levels and the dichotomous outcome colonization. A *P*-value ≤0.05 was considered statistically significant.

RESULTS

Dynamics of the antistaphylococcal antibody response

The changes in antistaphylococcal IgG, IgA and IgM levels during the first 2 years of life were determined (Figure 1, data shown for TSST-1, ClfB and Efb). The levels of antigen-specific IgG, IgA and IgM showed extensive inter-individual variability over time. For all *S. aureus* proteins tested, the level of antigen-specific IgG in cord blood was significantly higher than the antistaphylococcal IgG level at 6 months ($P<0.001$). This was due to the presence of maternal IgG at birth and catabolism of maternal IgG thereafter. In the time interval from 6 to 14 months, the levels of IgG directed to CHIPS, SCIN and SEB decreased further ($P<0.05$). As for the other proteins, no significant changes in the IgG levels were noted in this period.

Antistaphylococcal IgA and IgM levels in cord blood were low because maternal IgA and IgM are not transported across the placenta. In the first 2 years of life, IgA levels remained low, which is a well-known fact [24, 25]. However, for both IgA and IgM a significant increase from birth up to the age of 24 months was noted, for 18 of 19 *S. aureus* proteins in the case of IgA ($P<0.05$, with the exception of anti-SCIN IgA) and for all proteins in the case of IgM ($P<0.01$). It must be emphasized that not every infant developed an antigen-specific IgA or IgM response to each protein in the first 2 years of life. Within one individual, the level of IgG, IgA or IgM directed against one protein was not correlated with the level of IgG, IgA or IgM directed against another protein.

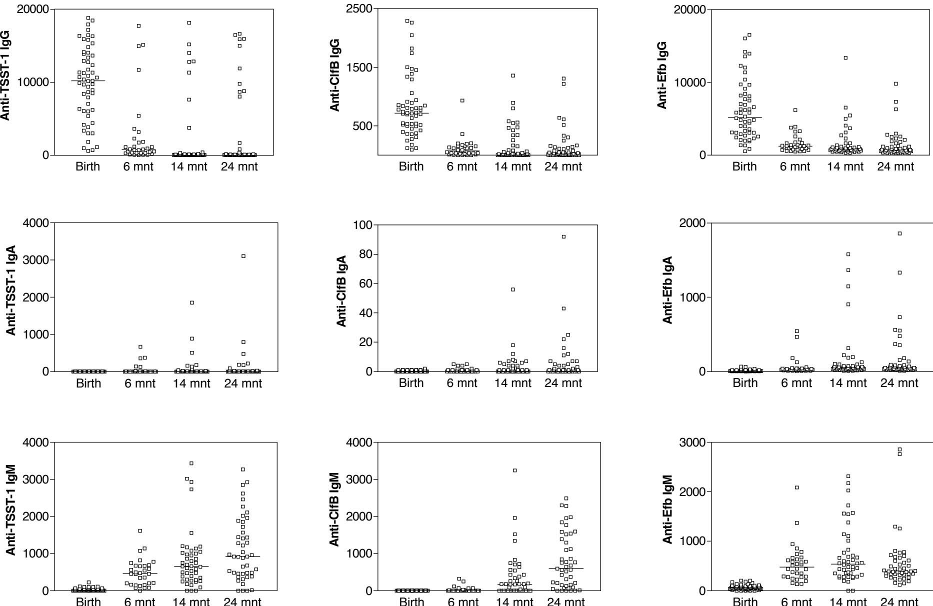

Figure 1. Levels of IgG, IgA and IgM directed against toxic shock syndrome toxin (TSST)-1, clumping factor B (ClfB) and extracellular fibrinogen-binding protein (Efb) in 57 children at birth, 6 months, 14 months and 24 months. Antibody levels are reflected by median fluorescence intensity values. Each dot represents a serum sample. Median values are indicated by horizontal lines.

Relationship between colonization and antistaphylococcal antibody levels

It was determined whether maternal antistaphylococcal IgG levels were predictive of the *S. aureus* colonization state of the infant and whether the colonization state determines the level of antistaphylococcal antibodies. Levels of *S. aureus*-specific IgG in cord blood were not predictive of the colonization state at 1.5 and 6 months ($P>0.05$). This implies that the large amounts of placentally transferred maternal IgG do not protect children from becoming nasally colonized with *S. aureus*.

For 45 of 46 (98%) children from whom serum samples were obtained at 14 months, the colonization status was known. For 1 child, the colonization status could not be determined, because 2 nasal swab cultures were missing. In the first year of life, 24 (53%) children were colonized at least once and 21 children (47%) were not colonized. Colonized children had significantly higher levels of IgG directed against CHIPS, Efb, ClfB, SdrD and IsdH than non-colonized children ($P<0.05$; Figure 2). In addition, their levels of IgA directed against CHIPS, IsdA and IsdH were higher ($P<0.05$). Incidentally, high levels of antibodies were detectable in non-colonized children, probably owing to exposure to *S. aureus* that was not recorded during this study. For 42 of 45 (93%) children from whom serum samples were obtained at 24 months, the colonization status was known. In the first 2 years of life, 24 (57%) children were colonized at least once and 18 (43%) were not colonized. Colonized children had higher levels of IgG directed against CHIPS, SCIN, Efb, IsdA, IsdH and SEB at 24 months than non-

colonized children ($P<0.05$; Figure 2). Their levels of IgA directed against CHIPS, IsdA and IsdH at 24 months were higher as well ($P<0.05$). The level of IgM did not differ significantly between colonized and non-colonized children ($P>0.05$).

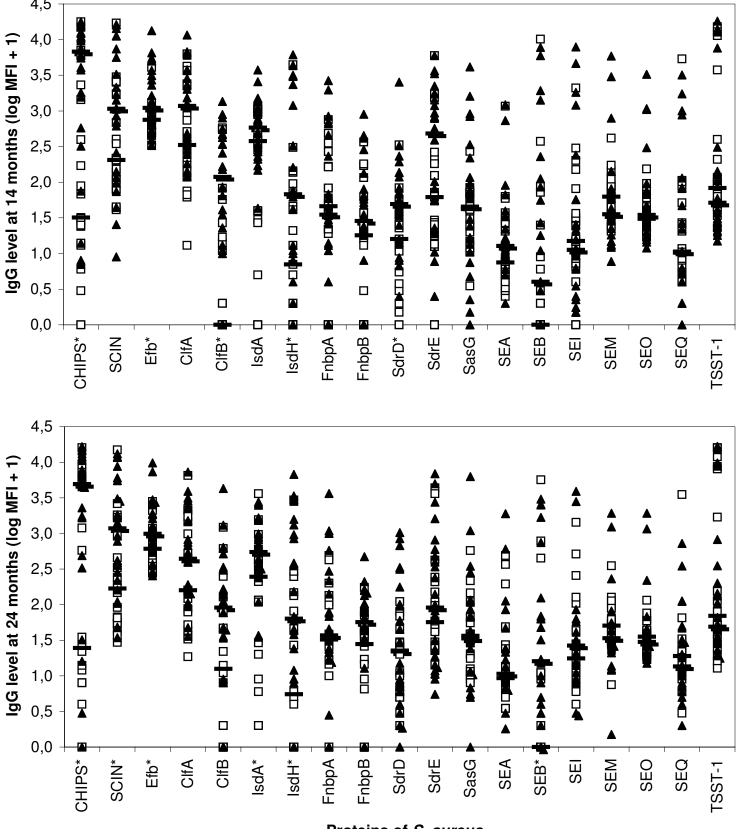

Figure 2. Relationship between *S. aureus* colonization in the first year or first 2 years of life and the levels of antistaphylococcal IgG, reflected by median fluorescence intensity (MFI) values, at 14 months or 24 months, respectively. Each symbol represents a single child. Black triangles represent colonized children and white squares represent non-colonized children. Median levels of antistaphylococcal antibodies for colonized and non-colonized children are indicated by double and single horizontal lines, respectively. Statistically significant differences are indicated by asterisks ($P<0.05$, Mann-Whitney *U* test). CHIPS, chemotaxis inhibitory protein of *S. aureus*; Clf, clumping factor; Efb, extracellular fibrinogen-binding protein; Fnbp, fibronectin-binding protein; Isd, iron-responsive surface determinant; Sas, *S. aureus* surface protein; SCIN, staphylococcal complement inhibitor; Sdr, serine-aspartate repeat protein; SE, staphylococcal enterotoxin; TSST, toxic shock syndrome toxin.

DISCUSSION

Understanding the determinants of carriage and how humans respond to *S. aureus* exposure is important for the development of novel antistaphylococcal measures. We show that, despite extensive inter-individual variability, the levels of IgG and IgA directed against a

number of *S. aureus* proteins were significantly higher in colonized, more exposed children than in non-colonized children. In both the first and second year of life anti-CHIPS, anti-Efb and anti-IsdH IgG levels were higher in colonized children. Furthermore, anti-CHIPS, anti-IsdA and anti-IsdH IgA levels were higher. This indicates that these proteins are expressed *in vivo* and that they might be determinants for colonization in early childhood. A potential role of IsdA in colonization was demonstrated in a previous study [10].

Furthermore, we show that maternally derived IgG antibodies specifically directed against a series of staphylococcal antigens do not seem to protect the young infant against *S. aureus* nasal colonization in the first months of life. Thus, although it is known that maternal IgG can cross epithelial barriers and can reach significant levels at the nasal mucosal surface [26, 27], these antibodies are not capable of preventing nasal colonization. In healthy adults, the considerable levels of antistaphylococcal antibodies that are found do not seem to protect against nasal colonization either. Carriers even have higher levels of antibodies than non-carriers [16]. These observations suggest that attempts to prevent mucosal colonization by *S. aureus* through passive immunization approaches are not likely to succeed. Whether this also applies to active immunization remains to be elucidated.

In summary, in healthy children, the antistaphylococcal IgG, IgA and IgM levels show extensive inter-individual variability. On average, the levels of antistaphylococcal IgA and IgM increase from birth until the age of 2 years, whereas the levels of antistaphylococcal (maternal) IgG decrease. Placentally transferred maternal IgG antibodies do not protect against nasal colonization. CHIPS, Efb, IsdA and IsdH are expressed *in vivo*, and therefore, seem to play a role in nasal colonization of young children.

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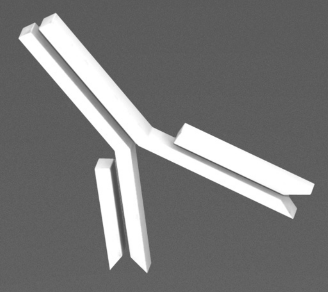

Chapter 5

Heterogeneity of the humoral immune response following *Staphylococcus aureus* bacteremia

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ABSTRACT

Background. Expanding knowledge on the humoral immune response in *Staphylococcus aureus*-infected patients is a mandatory step in the development of vaccines and immunotherapies. Here, we present novel insights into the antibody responses following *S. aureus* bacteremia. **Methods.** Fifteen bacteremic patients were followed extensively from diagnosis onwards (median 29 days, range 9-74 days). *S. aureus* strains (median 3, range 1-6) and serial serum samples (median 16, range 6-27) were collected. Strains were genotyped by pulsed-field gel electrophoresis (PFGE) and genes encoding 19 staphylococcal proteins were detected by polymerase chain reaction. The levels of IgG, IgA and IgM directed to these proteins were determined using a bead-based flow cytometry technique. **Results.** All strains isolated from individual patients were PFGE-identical. The genes encoding clumping factor (Clf) A, ClfB and iron-responsive surface determinant (Isd) A were detected in all isolates. Antigen-specific IgG levels increased more frequently than IgA or IgM levels. In individual patients, different proteins induced an immune response and the dynamics clearly differed. The anti-ClfB, anti-IsdH and anti-fibronectin-binding protein A IgG levels increased in 7 of 13 adult patients ($P<0.05$). The anti-IsdA IgG level increased in 12 patients (initial to peak level, 1.1-10.7-fold; $P<0.01$). Peak level was reached 7-37 days after diagnosis. In a bacteremic 5-day-old newborn, antistaphylococcal IgG levels declined from diagnosis onwards. **Conclusions.** Each bacteremic patient develops a unique immune response directed to different staphylococcal proteins. Therefore, vaccines should be based on multiple components. IsdA is immunogenic and, therefore, produced in nearly all bacteremic patients. This suggests that IsdA might be a useful component of a multivalent staphylococcal vaccine.

INTRODUCTION

Staphylococcus aureus is a leading cause of nosocomial bloodstream infections [1]. Risk factors for these invasive infections are intravascular catheters and nasal carriage [2]. Nasal carriers have a 3 to 4-fold increased risk of acquiring a nosocomial bacteremia as compared to non-carriers [3, 4]. Bacteremic patients can develop serious complications such as infective endocarditis, prosthetic device infection, septic arthritis, deep tissue abscesses and vertebral osteomyelitis [5-8]. *S. aureus* bloodstream infections extend the length of hospital stay and increase antibiotic usage, costs and mortality; approximately 20-30% of the patients die [1, 9, 10]. Worldwide, the increasing resistance of *S. aureus* isolates to various antibiotics complicates the treatment of bacteremia [11, 12]. Meanwhile, the number of new approved antimicrobial agents decreased over the last several years [13]. Therefore, alternative strategies to prevent and treat *S. aureus* bacteremia, such as vaccines and immunotherapy, are urgently required [14]. For the development of these alternative strategies, expanding knowledge

on the humoral immune response in *S. aureus*-infected patients is a mandatory step. In this study, we generate novel insights in the antibody responses following *S. aureus* bacteremia.

MATERIALS AND METHODS

Patients, definitions and setting

Fifteen patients (13 adults and 2 children, Table 1), admitted to the Erasmus Medical Center from March to June 2008, were followed from diagnosis *S. aureus* bacteremia until the end of hospital stay (range 9-74 days, median 29 days). Bacteremia was defined upon isolation of *S. aureus* from at least one blood culture set. From these patients, a total of 44 methicillin-susceptible *S. aureus* strains were collected for routine culture. The median number of collected strains per patient was 3 (range 1-6). The *S. aureus* strains were isolated from blood, sites of infection and, if applicable, other sites. Furthermore, serial serum samples were collected (leftover material). The median number of days between the first positive blood culture and

Table 1. Characteristics of the patients and their bacteremia

Pt no.	Sex	Age (y)	Underlying disease	Origin of *S. aureus* bacteremia*	Outcome	No. of samples
1	F	21	Kidney transplantation	Catheter related	Survived	27
2	M	71	B-cell Non-Hodgkin lymphoma	Catheter related	Survived	8
3	M	21	HIV positive	Pyomyositis	Survived	14
4	F	74	DM II, Cortisol-producing tumor of the adrenal gland	Abscess hand (catheter related)	Survived	26
5	M	60	DM II and cardiovascular disease	Unknown	Deceased **	9
6	M	75	DM II and cardiovascular disease	Diabetic foot	Survived	19
7	M	63	Cardiovascular disease	Osteomyelitis sternum (after CABG)	Survived	23
8	M	69	Esophageal carcinoma	Cellulitis knee	Survived	7
9	M	37	Myocarditis	Infected thrombus (catheter related)	Survived	18
10	M	64	Bronchus carcinoma	Unknown	Survived	22
11	M	63	None	Spondylodiscitis (dental origin)	Survived	8
12	F	64	Mamma carcinoma T-cell lymphoma	Catheter related	Survived	16
13	F	47	IV drug abuse Hepatitis C	Chronic osteomyelitis arm	Deceased (MOF caused by sepsis)	9
14	M	5 d	Congenital disorders	Infected wound head (catheter related)	Survived	20
15	M	2y 11m	Medulloblastoma	Catheter related	Deceased **	6

Note. d, days; DM, diabetes mellitus; F, female; M, male; MOF, multiple organ failure; m, month; Pt no., patient number; y, year.

* The origin of the bacteremia was recorded in the medical chart by the infectious diseases consultant

** Cause of death not related to bacteremia according to post-mortem examination

the first sampling of serum was 1 day (range 0-20 days). From each patient, at least 6 (median 16, range 6-27) serum samples were collected, leading to a total of 232 serum samples. The time between the first positive blood culture and the first negative (control) blood culture ranged from 1-6 days (median 2 days; excluding 1 patient for whom no additional blood culture was obtained).

Sera from 4 non-*S. aureus* bacteremic patients were used as controls. These 4 patients were diagnosed with a *Klebsiella pneumoniae*, *Proteus mirabilis*, coagulase-negative staphylococcus (CNS) or *Enterococcus faecium* bacteremia. The number of days between the first positive blood culture and the first sampling of serum ranged from 0-2 days. Per patient, 12-26 (median 16) serum samples were collected. Follow-up time ranged from 22-50 days (median 29 days).

Patients were treated with antibiotics according to hospital guidelines under supervision of infectious diseases consultants. Catheters were removed if they were the suspected origin of the bacteremia. The Medical Ethics Committee of the Erasmus Medical Center Rotterdam approved the study (MEC-2007-106, addendum 2).

S. aureus identification, detection of virulence genes and genotyping

S. aureus was identified on the basis of colony and microscopic morphology and Slidex Staph Plus agglutination testing (bioMérieux). The identification of *S. aureus* was confirmed by *spa* polymerase chain reaction (PCR) [15]. The isolates were screened for genes encoding important staphylococcal proteins: the 'microbial surface components recognizing adhesive matrix molecules' (MSCRAMMs), staphylococcal enterotoxins (SEs) and immune-modulating proteins [16-20]. Sequences specific for clumping factor (Clf) A and B, *S. aureus* surface protein G (SasG), iron-responsive surface determinant (Isd) A and H, fibronectin-binding protein (Fnbp) A and B, serine-aspartate repeat protein (Sdr) D and E, SEA, SEB, SEI, SEM, SEO, SEQ, toxic shock syndrome toxin (TSST)-1, staphylococcal complement inhibitor (SCIN), extracellular fibrinogen-binding protein (Efb) and chemotaxis inhibitory protein of *S. aureus* (CHIPS) were detected. Primers for *isdA* (Fw, CTGCGTCAGCTAATGTAGGA; Rv, TGGCTCTTCAGAGAAGTCAC), *isdH* (Fw, CTGCTGGTGGATACTGTTG; Rv, TGCCAGTGAGACTTGTATCG), *sasG* (Fw, GCCACTTG-GATGAGTTGGT; Rv, CGAAGAGCCAGTGGATGATG), *sdrD* (Fw, CGGAGCTGGTCAAGAAGTAT; Rv, TGCCATCTG-CGTCTGTTGTA) and *efb* (Fw, GAAGGATACGGTCCAAGAGA; Rv, TGTGGACGT-GCACCATATTC) were newly designed. The other genes were detected by PCR as described previously [21-26]. Furthermore, S. *aureus* strains were genotyped by pulsed-field gel electrophoresis (PFGE) [27].

Measurement of antistaphylococcal antibodies

The levels of IgG, IgA and IgM in the serum samples from the patients directed against the MSCRAMMs, SEs and immune-modulating proteins were quantified using a bead-based flow cytometry technique (xMAP®, Luminex Corporation). This technique allows for the quantifica-

tion of antibodies in small serum volumes to 19 antigens simultaneously. For IgG and IgA measurement, sera were diluted 1:100. For IgM measurement, sera were diluted 1:25. The methods were as described previously [16, 28, 29]. Tests were performed in independent duplicates and the median fluorescence intensity (MFI) values, reflecting semi-quantitative antibody levels, were averaged. In each experiment, control beads (no protein coupled) were included to determine non-specific antibody binding. In case of non-specific binding, these non-specific MFI values were subtracted from the antigen-specific results. Human pooled serum (HPS) was used as a standard.

Statistical analysis

Statistical analyses were performed with SPSS version 15.0. To compare the initial antibody level with the peak antibody level, the Wilcoxon matched pairs signed rank test was used. A *P*-value ≤0.05 was considered statistically significant.

RESULTS

PFGE analysis

PFGE analysis was performed for all *S. aureus* strains isolated. A dendogram of the PFGE data (Figure 1) shows the overall lack of relatedness among the strains from different patients, with exception of patient 4 and 5. There was no epidemiological relationship between these 2 patients. All *S. aureus* strains isolated from an individual patient were genotypically indiscriminate (>95% relatedness) and clustered in the dendogram; only in case of patient 9 the PFGE patterns of the strains were slightly different.

Detection of virulence genes

Based on PCR analyses, *clfA*, *clfB* and *isdA* were ubiquitous in all 44 isolates obtained from the 15 bacteremic patients. *scn*, *efb*, *fnbA* and *isdH* were detected in >90% of the isolates. *sdrD*, *sdrE*, *sasG*, *sei*, *seo*, *sem*, *chp* and *fnbB* were detected in 35 (80%), 33 (75%), 30 (68%), 28 (64%), 28 (64%), 23 (52%), 23 (52%) and 14 (32%) of the isolates, respectively. Less than 20% of the isolates harbored *sea*, *seb*, *seq* or *tst*. Overall, all strains isolated from a single patient (PFGE-identical) harbored the same virulence genes, with 2 exceptions. In patient 6, *scn* was detected only in the strain isolated from foot tissue, but not in the strains isolated from blood and the superficially cultured wound of the foot. In patient 15, *sasG* was detected only in the strain isolated from catheter-drawn blood, but not in the strain isolated from peripheral blood or bone marrow (Figure 1). An overview of the presence of virulence genes in *S. aureus* strains per patient is shown in Table 2.

Table 2. Fold increase from initial antigen-specific antibody level to peak antibody level in 13 adult patients suffering from an *S. aureus* bacteremia

Pt No.		CHIPS	SCIN	Efb	ClfA	ClfB	FnbpA	FnbpB	IsdA	IsdH	SasG	SdrD	SdrE	SEA	SEB	SEI	SEM	SEO	SEQ	TSST-1
1		+	+	+	+	+	+	-	+	+	-	+	+	-	-	+	+	+	-	-
	IgG	4.6	2.1	1.8	2.3	1.5	1.9		2.3	2.1		19.4								
	IgA	1.6		1.4		1.7														
	IgM	2.6	3.1		1.6		10.4	*16.4*	1.8		*6.8*		10.9							
2		+	+	-	+	+	+	-	+	-	-	+	+	-	-	+	+	+	-	-
	IgG				1.6	1.2	2.0		2.5			5.8	2.0			1.3	1.3			*1.3*
	IgA	1.2							2.8				2.0			2.2	2.0			
	IgM	1.6	1.4						2.6				3.4		*1.2*	3.7	1.7			*1.3*
3		+	+	+	+	+	+	+	+	+	+	+	+	+	-	-	-	-	-	-
	IgG			1.5	1.5	1.5	1.4		1.2					2.4						
	IgA		1.1																	
	IgM		5.7		2.2						2.6		2.9							*8.1*
4		+	+	+	+	+	+	-	+	+	-	-	-	-	-	+	-	+	-	+
	IgG	41.3	22.5		55.8	13.7			10.7	114.9										3.2
	IgA	13.3	14.1	9.2	9.1	10.4				23.4		*66.0*	*15.7*							
	IgM	14.5							4.1											21.0
5		+	+	+	+	+	+	-	+	+	-	-	-	+	-	+	-	+	-	+
	IgG			1.7			1.2	*1.4*	1.4						*1.4*					1.1
	IgA											*1.8*								1.3
	IgM				2.8				5.3						*5.2*					1.6
6		-	+	+	+	+	+	+	+	+	+	+	+	-	+	*-1.3*	-	-	+	-
	IgG	*1.5*	1.3			1.5	1.6		1.2		1.2				1.2		*1.2*			*1.6*
	IgA	*1.3*	1.3	1.3	1.3	1.7	1.4		1.7											
	IgM				1.7			5.9												
7		-	+	+	+	+	+	-	+	+	+	+	+	-	-	+	+	+	-	-
	IgG								6.9	5.8										
	IgA	*1.3*	1.6	1.3	1.6				7.5											
	IgM				1.6	1.4	2.4	*1.7*	20.0											

8		+	+	+	+	+	+	-	+	+	-	-	+	-	-	+	+	+	-	-
	IgG		1.3		1.1				1.1	1.5										
	IgA	1.8	1.7						2.8											
	IgM	23.9						*1.5*	1.4											
9		-	+	+	+	+	+	-	+	+	+	+	+	-	-	+	+	+	-	-
	IgG						1.6		2.9	5.1	64.7						3.1			
	IgA						1.9		1.9	5.8							8.2			
	IgM																			
10		+	+	+	+	+	-	-	+	+	-	+	+	-	-	-	-	-	-	-
	IgG			2.2					1.7	4.7		3.5	2.5							*2.7*
	IgA		1.5							2.3										
	IgM	1.2			4.6				3.6	3.4			9.8							
11		+	+	+	+	+	+	+	+	+	+	+	+	-	+	+	+	+	-	-
	IgG	1.1		1.5	2.2	1.7			1.5		2.3									
	IgA			1.2	1.8															*1.8*
	IgM																			
12		+	+	+	+	+	+	+	+	+	+	+	-	-	-	-	-	-	-	-
	IgG	1.2					2.0	1.8			2.0	2.0	*2.3*							
	IgA			1.6		1.4	1.6		1.3											
	IgM																			
13		-	+	+	+	+	-	-	+	+	-	+	+	-	-	-	-	-	-	-
	IgG		1.1	2.7		1.9			1.1	2.5		1.6	2.7							
	IgA		2.0	1.2	1.3	3.9			1.9	2.0										
	IgM																			

Note. CHIPS, chemotaxis inhibitory protein of *S. aureus*; Clf, clumping factor; Efb, extracellular fibrinogen-binding protein; Fnbp, fibronectin-binding protein; Isd, iron-responsive surface determinant; Sas, *S. aureus* surface protein; SCIN, staphylococcal complement inhibitor; Sdr, serine-aspartate repeat protein; SE, staphylococcal enterotoxin; TSST, toxic shock syndrome toxin. Empty cells indicate that there is no increase. If there was an increase in antibody level, but the corresponding gene was not detected in the *S. aureus* isolate, the fold increase is noted in italics. Peak IgG level was not reached for FnbpA in patient 2, FnbpA and IsdA in patient 5, Efb in patient 11 and Efb, ClfB, SdrD and SdrE in patient 13. Here, the fold increase from initial to highest IgG level is noted.

\+ The corresponding gene was detected in the *S. aureus* isolate

\- The corresponding gene was not detected in the *S. aureus* isolate

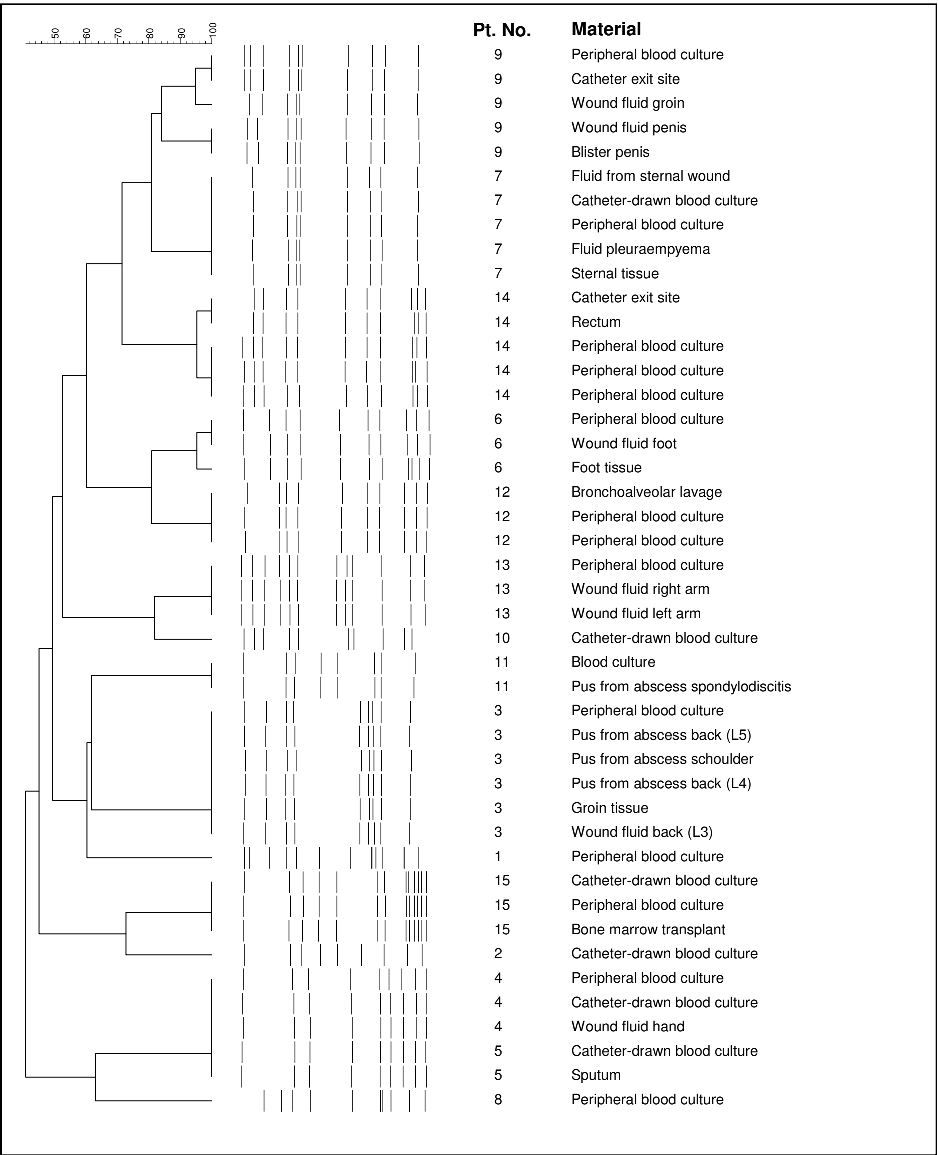

Figure 1. Dendogram of the pulsed-field gel electrophoresis data of 44 *S. aureus* strains isolated from 15 bacteremic patients.

Antistaphylococcal antibodies

The levels of IgG, IgA and IgM directed against 19 *S. aureus* proteins were measured from diagnosis bacteremia onwards. In Figure 2 the changes in antistaphylococcal IgG, IgA and IgM levels after diagnosis bacteremia are shown for one patient (patient 4). For all patients, the course of the antibody response was determined. If there was an increase in antibody level in the consecutive serum samples, the fold increase from the initial antibody level (as measured in the first obtained serum sample) to the peak level was calculated. An overview for the 13

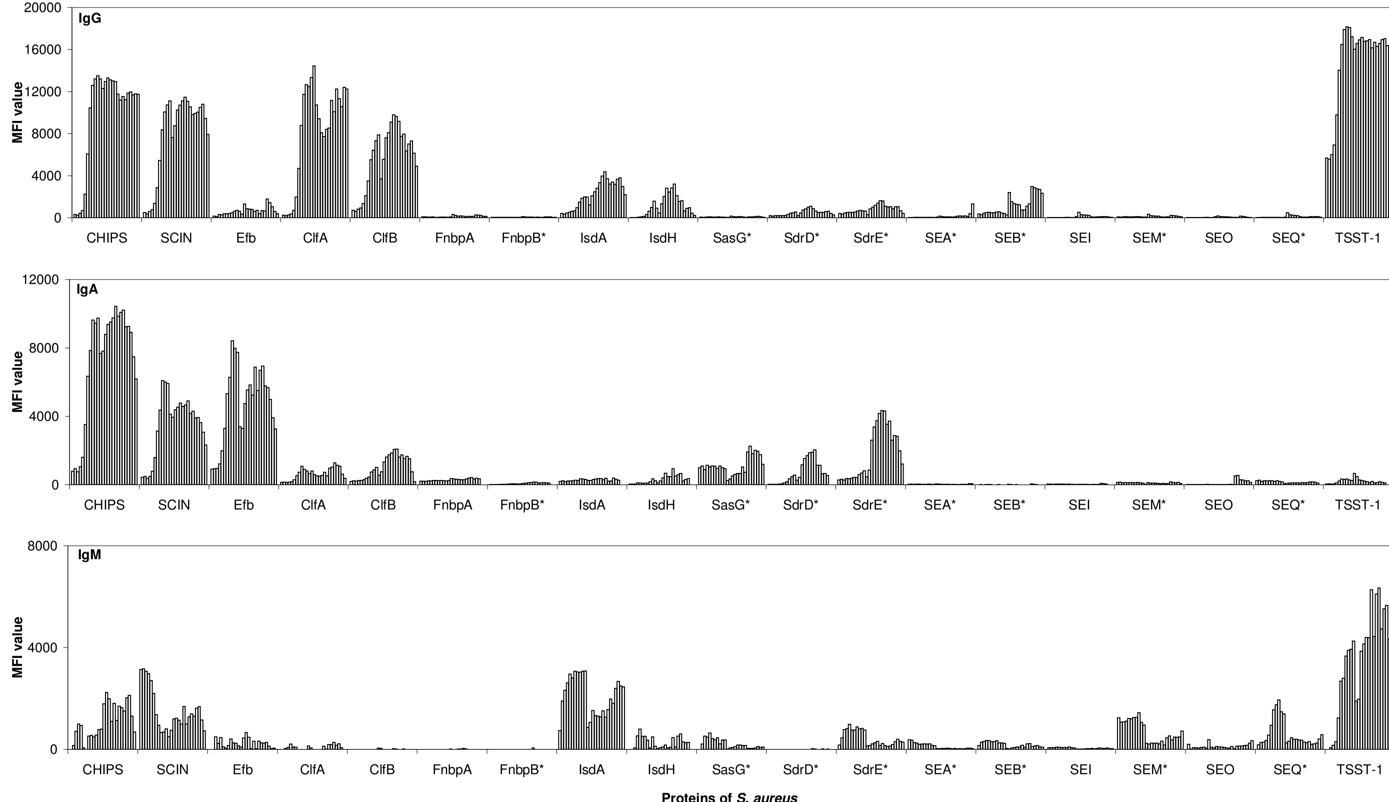

Figure 2. Course of the IgG, IgA and IgM levels directed to 19 *S. aureus* proteins following bacteremia. Results are shown for patient 4. Each bar represents a serum sample on a separate day. The time period between the first and last serum sample is 58 days. CHIPS, chemotaxis inhibitory protein of *S. aureus*; Clf, clumping factor; Efb, extracellular fibrinogen-binding protein; Fnbp, fibronectin-binding protein; Isd, iron-responsive surface determinant; Sas, *S. aureus* surface protein; SCIN, staphylococcal complement inhibitor; Sdr, serine-aspartate repeat protein; SE, staphylococcal enterotoxin; TSST, toxic shock syndrome toxin. * The corresponding gene was not detected in the *S. aureus* isolate.

adult patients is shown in Table 2. In each patient increases in antistaphylococcal antibody levels directed to different *S. aureus* proteins were observed. Antibody responses were directed mainly to the immune-modulating proteins and MSCRAMMs. An increase in anti-IsdA IgG level was detectable in 12 of 13 (92%) adult bacteremic patients (Figure 3; with the exception of patient 12). Some patients showed a small decline in anti-IsdA IgG level before the increase. The median increase from initial to peak level was 1.71-fold (range 1.13–10.72-fold, *P*<0.01). The number of days to reach anti-IsdA IgG peak levels ranged between 7 and 37 days (median 21 days). It appeared that patients with a site of infection other than catheter-related (patient 6, 7, 8, 11 and 13) reached higher levels, but the anti-IsdA IgG peak level was high in patient 2 as well (Figure 3). In Table 3, the dynamics of the IgG responses direct against all immune-modulating proteins and MSCRAMMs are summarized per *S. aureus* protein.

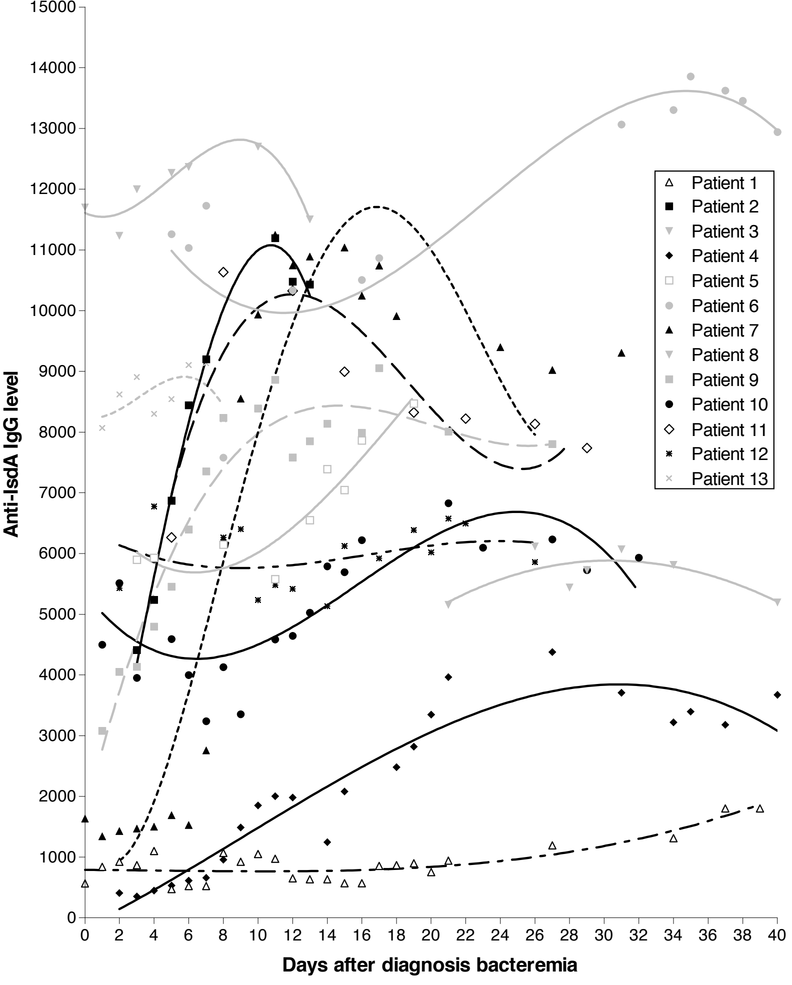

Figure 3. Course of the anti-iron-responsive surface determinant (Isd) A IgG levels following *S. aureus* bacteremia in 13 adult patients.

Table 3. Dynamics of the antistaphylococcal IgG response during bacteremia

Protein	No. of adult pt. with gene pos isolates (%)	No. of adult pt. with increase in IgG level (%)	Median fold increase from initial to peak level (range) #	Median no. of days to reach peak level (range) #	*P*-value *
CHIPS	9/13 (69%)	4/9 (44%)	2.9 (1.1-41.3)	25 (11-27)	-
SCIN	13/13 (100%)	5/13 (38%)	1.3 (1.1-22.5)	21 (5-42)	<0.05
Efb	12/13 (92%)	6/12 (50%)	1.7 (1.5-2.1)	24 (14-44)	<0.05
ClfA	13/13 (100%)	6/13 (46%)	1.9 (1.1-55.8)	19 (10-40)	<0.05
ClfB	13/13 (100%)	7/13 (54%)	1.5 (1.2-13.7)	28 (11-50)	<0.05
FnbpA	11/13 (85%)	7/11 (64%)	1.6 (1.4-2.0)	27 (11-42)	<0.05
FnbpB	4/13 (31%)	1/4 (25%)	1.8 ‡	23 †	-
IsdA	13/13 (100%)	12/13 (92%)	1.7 (1.1-10.7)	21 (7-35)	<0.01
IsdH	12/13 (92%)	7/12 (58%)	4.7 (1.5-114.9)	15 (7-34)	<0.05
SasG	6/13 (46%)	4/6 (67%)	2.1 (1.2-64.7)	23 (11-42)	-
SdrD	10/13 (77%)	5/10 (50%)	4.7 (2.0-19.4)	22 (11-27)	<0.05
SdrE	10/13 (77%)	3/10 (30%)	2.0, 2.5 ‡	11, 21 †	-

Note. CHIPS, chemotaxis inhibitory protein of *S. aureus*; Clf, clumping factor; Efb, extracellular fibrinogen-binding protein; Fnbp, fibronectin-binding protein; Isd, iron-responsive surface determinant; Sas, *S. aureus* surface protein; SCIN, staphylococcal complement inhibitor; Sdr, serine-aspartate repeat protein.

Peak IgG level was not reached for FnbpA in patient 2, FnbpA and IsdA in patient 5, Efb in patient 11 and Efb, ClfB, SdrD and SdrE in patient 13. Therefore, these data were excluded.

‡ Absolute fold increase in IgG level

† Absolute number of days until peak level

* *P*-values ≤0.05 (by the Wilcoxon matched pairs signed rank test) were considered statistically significant

An increase in antigen-specific IgA was detected less frequently than an increase in IgG. In 7 of 13 patients with a gene-positive isolate, the anti-IsdA, anti-SCIN and anti-Efb IgA level increased. Peak levels were reached between 6 and 34 days. In not a single patient was a rise in anti-FnbpB, SasG, SEA, SEB, SEO or SEQ IgA level detected. Increases in IgM were detected the least often out of the three antibody isotypes (Table 2). For patient 9 and 11, most antigen-specific IgM levels showed a decrease.

In the 2 children, the antibody responses were quite different from the antibody responses in adults. In the 5-day-old newborn (patient 14) a continuous decline in antigen-specific (maternal) IgG for 18 of 19 proteins was seen from the moment of diagnosis onwards. This could be due to extensive consumption of maternal antibodies in the absence of de novo antibody synthesis (Figure 4). The level of IgM increased for CHIPS (isolate *chp*-negative), SCIN, SasG, SdrD, SdrE, SEM, SEO and SEQ (*seq*-negative). The level of IgA was around zero. In the 2-year-old child (patient 15) an increase was noted for IgG directed to IsdA and CHIPS only. IgM increased for SCIN, ClfA, SdrD, SdrE (isolate *sdr*-negative), SEI, SEM, SEO and TSST-1 (*tst*-negative).

In the 4 control patients who did not suffer from an *S. aureus* bacteremia, the CNS-infected patient showed a 1.2-fold increase in anti-CHIPS IgG level (from MFI 13333 to MFI 16002) and the *K. pneumoniae* infected patient showed a 1.4-fold increase in anti-ClfB IgG level (from MFI 5647 to MFI 8058). Furthermore, no increases in antistaphylococcal antibody levels were noted. This shows that there is little inter-species cross-reactivity. However, in the patients

suffering from an *S. aureus* bacteremia a rise in antibody level while the corresponding gene was not present in the *S. aureus* isolate was observed in 23 of 179 increases (12.8%; Table 2).

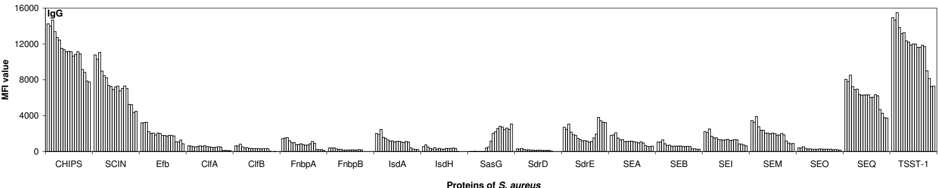

Figure 4. Course of the IgG levels directed to 19 *S. aureus* proteins in a 5-day-old bacteremic newborn (patient 14). Each bar represents a serum sample on a separate day. The time period between the first and last serum sample is 32 days. CHIPS, chemotaxis inhibitory protein of *S. aureus*; Clf, clumping factor; Efb, extracellular fibrinogen-binding protein; Fnbp, fibronectin-binding protein; Isd, iron-responsive surface determinant; Sas, *S. aureus* surface protein; SCIN, staphylococcal complement inhibitor; Sdr, serine-aspartate repeat protein; SE, staphylococcal enterotoxin; TSST, toxic shock syndrome toxin.

DISCUSSION

By using multiple, longitudinally collected serum samples, we were able to show that each bacteremic patient develops a unique *S. aureus*-specific immune response after infection. In each bacteremic patient, the *S. aureus* proteins to which the antibody response was directed differed. In addition, the height of the peak antibody level and number of days to reach peak antibody level differed. There are several possible explanations for the variability of the antistaphylococcal immune response in bacteremic patients. First, it may be the result of the genetic diversity of the S. *aureus* strains which caused the bacteremia [30]. Secondly, the variability might be due to differences in staphylococcal protein expression and/or selective protein recognition by the immune system in different patients. By way of illustration, although patient 4 and 5 were infected with PFGE-identical *S. aureus* strains with similar virulence genes, the antibody responses were very different (Figure 1 and Table 2). The observed variability in antibody response might also be caused by the difference in the time of onset of the bacteremia, which is difficult to determine accurately for patients with an origin of bacteremia other than catheter-related. Finally, colonization status, the number and severity of preceding infections, the level of immunity and the ability to mount an immune response probably also contribute to the development of the antistaphylococcal humoral immune response [31].

Although the antibody response after an *S. aureus* infection is a unique, personal characteristic, IsdA is immunogenic and, therefore, produced by *S. aureus* in nearly all bacteremic patients. IsdA interacts with and binds to fibrinogen and fibronectin of human cells [32] and is involved in the adherence of *S. aureus* to human desquamated nasal epithelial cells [33]. Furthermore, IsdA is required for nasal colonization in the cotton rat model [33]. In addition,

IsdA protects *S. aureus* against the bactericidal activity of apolactoferrin *in vitro* [34]. Since vaccines should contain multiple components that are expressed *in vivo* in many, if not all, patients, it seems that IsdA is an interesting target for inclusion in a multi-component vaccine [35].

In 23 of 179 (12.8%) observed increases, a rise in antistaphylococcal antibody level was found while the corresponding gene was not detected in the *S. aureus* isolate. Increases in anti-TSST-1, anti-SEB and anti-FnbpB antibody levels in the absence of *tst*, *seb* and *fnbB* genes were documented most frequently (Table 2). This might be the result of immunological cross-reactivity. In earlier studies, it was shown that anti-SEB cross-reacts with anti-SEC1 [36, 37]. Furthermore, it was shown that anti-TSST-1 also has cross-inhibitory activity against SEA and SEB [38, 39]. However, the relative binding affinity was 1000-fold lower for SEA than for TSST-1 and, therefore, this does not seem to be clinically relevant. Another explanation might be that the antibodies are secreted by plasma cells that are mobilized from their survival niche in the bone marrow by competition with newly generated plasma blasts [40]. Knowledge on non-specific antibody binding should be increased, however.

In summary, bacteremic patients usually carry PFGE-identical *S. aureus* strains at multiple sites. Each bacteremic patient develops a unique immune response directed against different *S. aureus* proteins. Therefore, a vaccine based on a single antigenic component is not likely to be effective. The surface protein IsdA is immunogenic and, therefore, produced by *S. aureus*, in nearly all bacteremic patients. This suggests that IsdA might be a useful component of a multivalent staphylococcal vaccine.

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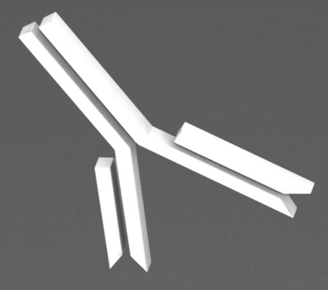
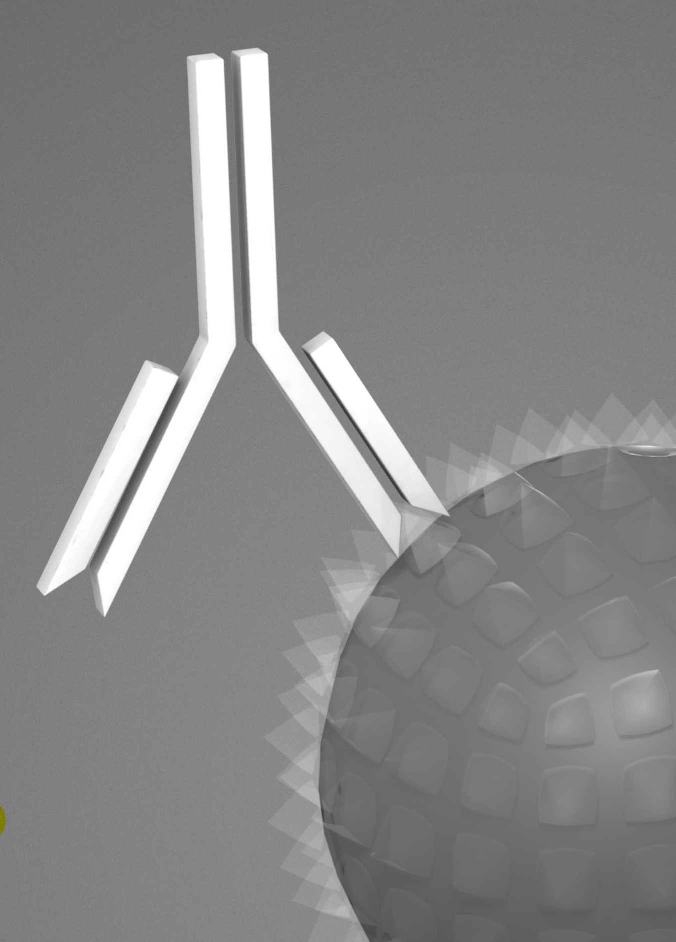


Chapter 6

Immunogenicity of toxins during *Staphylococcus aureus* infection



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ABSTRACT

Background. Toxins are important *Staphylococcus aureus* virulence factors, but little is known about their immunogenicity during infection. Here, additional insight is generated. **Methods**. Serum samples from 206 *S. aureus*-infected patients and 201 hospital-admitted control subjects were analyzed for IgG binding to 20 toxins, using a flow cytometry-based technology. Antibody levels were associated with polymerase chain reaction-defined presence of toxin genes in homologous *S. aureus* isolates. **Results.** IgG levels directed to exfoliative toxin (ET) A, ETB, γ hemolysin B, leukocidin (Luk) D, LukE, LukS, staphylococcal enterotoxin (SE) A, SEE, SEH, SEI and SEM were higher in *S. aureus*-infected patients than in control subjects (P<0.05). Furthermore, in the *S. aureus*-infected patient group, IgG levels were higher if genes encoding ETA, ETB, SEA, SEC, SEH, SEQ, toxic shock syndrome toxin (TSST)-1 or Panton-Valentine leukocidin (PVL) were present in the infectious isolate (P<0.05). Levels of anti-SEA IgG increased during infections with *sea*-positive (median fluorescence intensity [MFI] from 11555 to 12388, P<0.05), but not *sea*-negative strains. In addition, anti-LukS IgG levels increased during skin and soft-tissue infections (SSTI) with *luk-PV*-positive (MFI from 15231 to 15911, P<0.05), but not *luk-PV*-negative strains. Bacteremia was associated with *sea* (odds ratio [OR] 3.4, 95% confidence interval [CI] 1.2-10.0) and *tst* (OR 5.7, 95% CI 1.6-20.8). SSTI and bone and joint infections were associated with *luk-PV* (OR 2.5, 95% CI 1.2-5.2). **Conclusions.** Many toxins are expressed *in vivo* and recognized by the immune system during *S. aureus* infections, suggesting their involvement in pathogenesis.

INTRODUCTION

Staphylococcus aureus produces numerous virulence factors that contribute to its ability to cause infections [1, 2]. These include a variety of toxins that are known for their detrimental effects on cells of the immune system [3]. In addition, their toxinogenic activity is implicated in a broad range of *S. aureus* infections [4]. Staphylococcal toxins can be categorized in groups: pyrogenic toxin superantigens (PTSAgs), exfoliative toxins (ETs), leukocidins and other toxins. The family of PTSAgs includes staphylococcal enterotoxins (SEs) and toxic shock syndrome toxin (TSST)-1. Superantigens cross-link major histocompatibility complex class II molecules on antigen-presenting cells with T cell receptors, which leads to massive T-cell proliferation and cytokine release [5]. This disproportionate pro-inflammatory activity is implicated in the pathogenesis of food poisoning and toxic shock syndrome [3, 6]. ETs are responsible for staphylococcal scalded skin syndrome and bullous impetigo [7]. So far, 4 ETs are known and 3 of these (ETA, ETB and ETD) are linked to human infection [8]. Leukocidal toxins constitute a family of pore-forming toxins that are composed of 2 distinct components. The toxic effect depends on the synergistic action of both class S and F proteins on human neutrophils or

erythrocytes. Members of the leukotoxin family are LukD, LukE, LukM, γ hemolysin (Hlg) and Panton-Valentine leukocidin (PVL) [9, 10]. PVL is associated with necrotizing pneumonia, bone and joint infections, furunculosis and abscesses in humans [11-14]. Toxins of the epidermal cell differentiation inhibitor (EDIN) family inactivate GTPases and thereby block important immune cell functions, such as chemotaxis and phagocytosis [15].

Despite the fact that toxins are important staphylococcal virulence factors and that the prevalence of *S. aureus* infection is continuously increasing [16], little is known about the IgG response directed against PTSAgs, ETs and leukocidins during *S. aureus* infection in humans. By studying the immune response, important information can be collected concerning the antigenicity and *in vivo* expression of the toxins. This may increase knowledge on pathogenesis of *S. aureus* infection and might contribute to the development of new measures against staphylococcal disease. We studied the anti-toxin humoral immune response to 20 toxins in a large number of *S. aureus*-infected patients and hospital-admitted control subjects. Furthermore, we associated antibody levels with the presence of toxin genes in infectious *S. aureus* isolates and we related toxin gene presence to different types of staphylococcal infection.

MATERIALS AND METHODS

Collection of serum and *S. aureus* strains

S. aureus isolates and 2 serum samples were collected from 206 *S. aureus*-infected patients in the Mustapha Pacha hospital (Algiers, Algeria) during 2006-2007. The first serum sample was obtained 5 days (range 0-20 days) after strain identification. The second serum sample was collected 14 days (range 7-34 days) thereafter. Serum samples were stored at -80°C until use. Data on sex, age, hospital ward and type of infection were recorded. Isolates were considered to be community-acquired if a sample obtained within 48 hours after admission was culture-positive for *S. aureus*. Isolates obtained later were considered to be hospital-acquired. Furthermore, serum samples were collected for control patients (n=201). Control patients were admitted in the same period, but did not have an overt *S. aureus* infection. Patients with an immunocompromised status (e.g. HIV-positive patients, patients receiving corticosteroids or other immunosuppressive therapies) were excluded. Adult patients were defined as those aged ≥18 years. All patients provided written informed consent and the Medical Ethics Committee of the Mustapha Pacha hospital approved the study.

Bacterial identification and toxin gene detection

S. aureus was identified on the basis of colony and microscopic morphology and coagulase testing. The identification was confirmed by multiplex polymerase chain reaction (PCR) amplification of the accessory gene regulator (*agr*) [17] and by determining the *agr* allelic group. The isolates were PCR-screened for genes encoding methicillin resistance (*mec*A), SE

A, B, C, D, H, K, L, M, O, P, Q and R (*sea-d*, *seh*, *sek-m*, *seo-r*), TSST-1 (*tst*), ETA, ETB and ETD (*eta*, *etb*, *etd*), PVL (*luk-PV*), class F LukM leukocidin (*lukM)*, HlgB (*hlgb*) and EDIN (*edin*), as described elsewhere [17, 18].

Recombinant toxin production

The toxins SEC, SED, SEE, SEH, SEJ, SEN, SEQ, SER, ETA, ETB, LukF and LukS were produced as described elsewhere [19-21]. The *S. aureus* strains listed in Table 1 were used to produce the other toxins. Primers were designed following the identification of suitable hybridization sites in the toxin genes (Table 1). Chromosomal DNA of *S. aureus* was extracted and used as a template for PCR amplification as described previously [22]. PCR products were codigested with appropriate restriction enzymes (Promega), purified with the High Pure PCR Product Purification kit (Roche) and ligated using T4 DNA Ligase (Roche) in either the pQE-30 (Qiagen) or pIVEX 2.4d expression vector (Roche) digested with the restriction enzymes described in Table 1 [23]. The resulting pQE plasmids were transformed into *Escherichia coli* strain M15 (Qiagen). For toxin expression in pIVEX 2.4d, the vector was transformed into *E. coli* strain DH5α (Invitrogen) before transformation into *E. coli* strain BL21 pLys (Invitrogen). Open reading frame integrity was verified by sequencing the junctions between the plasmid and the insert.

Table 1. Bacterial strains and sequences of primers used for *S. aureus* toxin production

Toxin	*S. aureus* strains	*E. coli* strains and plasmids	Primers *	Restriction enzymes
SEA	A87 0502	*E. coli* M15	CTC AGG ATC CAA TGG TAG CGA GAA AAG CG	BamHI/PstI
		pQE30	CTT TCT GCA GTT AAC TTG TAT ATA AAT ATA TAT CAA TAT GCA TG	
SEG	A99 0372	*E. coli* M15	CAA TGG ATC CCC CGA TCT TAA ATT AGA CGA AC	BamHI/PstI
		pQE30	CGG ACT GCA GTC AGT GAG TAT TAA GAA ATA CTT CC	
SEI	A900322	*E. coli* M15	CTA TGG ATC CGG TGA TAT TGG TGT AGG TAA C	BamHI/PstI
		pQE30	CGG ACT GCA GTT AGT TAC TAT CTA CAT ATG ATA TTT CGA C	
SEM	A900322	*E. coli* M15	GCA GGA TCC GAT GTC GGA GTT TTG AAT CTT AG	BamHI/PstI
		pQE30	CGG ACT GCA GTC AAC TTT CGT CCT TAT AAG ATA TTT C	
TSST-1	N315	*E. coli* BL21 pLys	TGG TAC TGG CGG CCGCTC TAC AAA CGA TAA TAT AAA GGA TTT G	NotI/PstI
		pIVEX 2.4d	CGGACTGCAGT TAATTAATTT CTGCTTCTAT AGTTTTTATT TCATC	
HlgB	ATCC 49775	*E. coli* BL21 pLys	TGG TAC TGG CGG CCG CGA AGG TAA AAT AAC ACC AGT C	NotI/BamHI
		pIVEX 2.4d	CGG GAT CCC TAT TTA TTG TTT TCA GTT TCT TTT GTA TC	
LukD	A87 0555	*E. coli* BL21 pLys	ACC CTT AAT TAA AGC TCA AAA TAT CAC ACC TAA AAG	PacI/BamHI
		pIVEX 2.4d	ACG CGG ATC CTT ATA CTC CAG GAT TAG TTT CTT TAG	
LukE	RN4220	*E. coli* BL21 pLys	ACG CGG ATC CTT AAT TAT GTC CTT TCA CTT TAA TTT	BamHI/PacI
		pIVEX 2.4d	ACC CTT AAT TAA AAA TAC TAA TAT TGA AAA TAT TGG TGA TGG TGC	

Note. HlgB, γ hemolysin B; Luk, leukocidin; SE, staphylococcal enterotoxin; TSST-1, toxic shock syndrome toxin-1.

* Restriction sites are underlined

Protein purification

Transformed *E. coli* cells growing exponentially in Luria-Bertani (LB) medium supplemented with ampicillin 100 µg/mL were inoculated into 1 liter of fresh LB medium and incubated with continuous rotary shaking for 2-3 h at 37°C until OD_{600} 0.5-0.7. Then, the expression was

induced by adding isopropyl-β-D-thiogalactopyranoside (IPTG) to a final concentration of 1 mmol/L for 5 hours. The cultures were then centrifuged at 4.000*g* for 20 min at 4°C. The cell pellets were stored overnight at -80°C. The cell pellets were thawed for 15 min on ice and resuspended in lysis buffer (Qiagen). The lysates were sonicated on ice after adding lysozyme. Then, the lysates were centrifuged at 10.000*g* for 30 min at 4°C and the supernatants were collected. The His-tagged proteins were purified with Ni-NTA agarose (Qiagen) and dialyzed against phosphate-buffered saline. Protein concentrations were determined according to the Bradford method, using bovine serum albumin as the standard. The toxins were quality controlled by SDS-PAGE and mass spectrometry (Ultraflex MALDI-ToF, Bruker Daltonics).

Measurement of anti-toxin antibodies

The levels of IgG directed against the toxins ETA, ETB, HlgB, LukD, LukE, LukF, LukS, SEA, SEC, SED, SEE, SEG, SEH, SEI, SEJ, SEM, SEN, SEQ, SER and TSST-1 were quantified simultaneously using a bead-based flow cytometry technique (xMAP®, Luminex Corporation). Methods were as described before [24-26]. Tests were performed in independent duplicates and the median fluorescence intensity (MFI) values, reflecting semi-quantitative antibody levels, were averaged. In each experiment, control beads (no toxin coupled) were included to determine non-specific antibody binding. In the event of non-specific antibody binding, the non-specific MFI values were subtracted from the antigen-specific results. Human pooled serum (HPS) was used as a standard [25].

Statistical analysis

Statistical analyses were performed with SPSS version 15.0. Kruskal-Wallis and Mann-Whitney *U* tests were used to compare differences in anti-toxin antibody levels between patients and control subjects. The χ^2 test was applied for categorical variables and binary logistic regression to calculate odds ratio's (ORs). To compare antibody levels in the first and second serum sample of each patient, the Wilcoxon matched pairs signed rank test was used. $P \leq 0.05$ was considered statistically significant.

RESULTS

Patient characteristics

The group of 206 *S. aureus*-infected patients consisted of 183 adults and 23 children. Their median age was 40 years (range 0-84 years). Male-to-female ratio was 1.5:1. Of the 206 patients, 142 (69%) had skin and soft tissue infections (SSTIs; e.g. furunculosis, infected skin lesions or folliculitis), 18 (9%) had bacteremia, 22 (11%) had bone- or joint infections (e.g. arthritis or osteitis), 12 (6%) had respiratory infection (e.g. bronchitis), 3 (1%) had ocular infection (e.g. conjunctivitis or endophthalmitis), 2 (1%) had urinary tract infection, 4 (2%) had ear,

nose or throat infection (e.g. sinusitis) and 3 (1%) had central nervous system infection. The group of 201 control patients consisted of 143 adults and 58 children. Median age was 45 years (range 0-92 years) and male-to-female ratio was 1.1:1.

Validation and inter-assay variation of the toxin multiplex assay

The MFI values obtained for HPS with the multiplex assay (serum incubated with the differently fluorescence-colored antigen-coupled beads mixed in 1 well) were between 90% and 111% (median 96%) of the MFI values obtained with the individual assays (serum incubated with each individual color of antigen-coupled beads in separate wells). Therefore, the multiplex assay was considered reliable. Inter-assay variation was calculated from the MFI values obtained for HPS, which was included on each 96-wells plate. The median coefficient of variation was 11% (range 5% to 18%), which is comparable to what was found in previous studies [24, 27]

Differences in anti-toxin IgG levels between distinct types of *S. aureus* infection

The antibody levels to 20 *S. aureus* toxins were measured. The toxin-specific antibody levels showed extensive inter-individual variability. IgG levels directed to 11 of 20 toxins (ETA, ETB, HlgB, LukD, LukE, LukS, SEA, SEE, SEH, SEI and SEM) were significantly higher in adult *S. aureus*-infected patients than in adult control patients in both the first and second serum sample ($P<0.05$, Figure 1A). Adult patients with respiratory infections, as opposed to other *S. aureus* infections, had higher levels of IgG to SED (median MFI value 2824 vs. 1071; $P<0.01$), SEJ (5510 vs. 2815; $P<0.01$) and SER (461 vs. 133; $P<0.05$, Figure 1B). In adult patients with SSTI, the level of anti-ETA IgG was elevated (5657 vs. 2848; $P<0.05$). In the course of SSTI, respiratory and bone and joint infections, IgG levels showed no increase. In bacteremic patients, the levels of IgG directed to HlgB, LukD, LukE, LukF, LukS and SEE seemed to increase in the course of infection but this increase was not statistically significant (P=0.052–0.084; Figure 1B).

In *S. aureus*-infected children, IgG levels directed to 6 of 20 proteins (ETA, HlgB, LukD, LukE, LukF and LukS) were significantly higher ($P<0.05$, Figure 1A) than in children without *S. aureus* infections. Surprisingly, the level of anti-TSST-1 IgG was higher in young control patients (median MFI 4804 vs. 8195; $P<0.05$) than in *S. aureus*-infected patients. In the *S. aureus* patient group, IgG levels to 17 of 20 proteins were higher in adults than children ($P<0.05$). No difference was shown in anti-LukD, anti-LukF and anti-SER IgG. In the control group, IgG levels directed to all proteins were significantly higher in adults than children ($P<0.001$; Figure 1A).

Prevalence of genes encoding *S. aureus* toxins

The prevalence of genes encoding *S. aureus* toxins in the 206 infectious isolates is shown in Table 2. Of the 206 isolates, 182 (88%) harbored ≥1 toxin gene. The most prevalent genes were *edin*, *luk-PV* and *etd*, detected in 49%, 46% and 46% of isolates, respectively. Genes encoding SED, SER and LukM were found in none of the isolates. Bacteremia was associated with

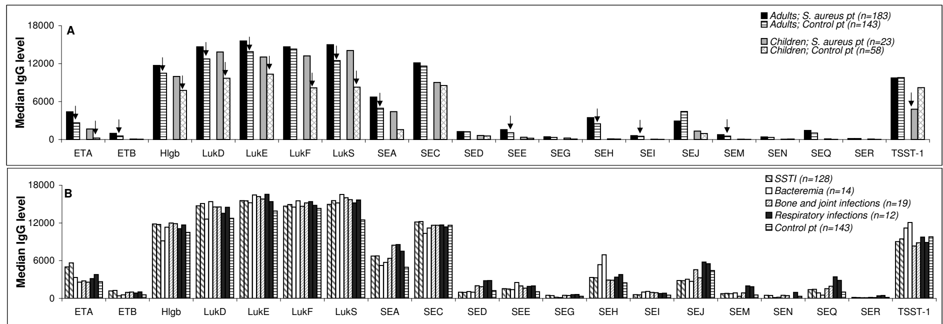

Figure 1. [A] Toxin-specific IgG levels in *S. aureus*-infected patients and control patients. IgG levels are reflected by median fluorescence intensity (MFI) values. Results are shown for the first serum sample and for adults and children separately. Statistically significant differences are indicated by black arrows (Mann-Whitney *U* test; $P<0.05$). [B] Differences in toxin-specific IgG levels between the first and second serum sample (first and second bar of the same pattern, respectively) and between different types of *S. aureus* infections. Results are shown for adult patients. ET, exfoliative toxin; HlgB, γ hemolysin B; Luk, leukocidin; SE, staphylococcal enterotoxin; TSST-1, toxic shock syndrome toxin-1.

Table 2. Presence of toxin genes in *S. aureus* strains isolated from patients with different types of *S. aureus* infection

	Number (%) of gene positive isolates							
	SSTI	Bacteremia	Bone infection	Respiratory infection	Other infection	Total	*P*-value	Odds ratio * (95% CI)
Toxin gene	n=142	n=18	n=22	n=12	n=12	n=206		
sea	20 (14%)	**6 (33%)**	1 (5%)	2 (17%)	1 (8%)	30 (15%)	0.024	3.4 (1.2-10.0)
seb	6 (4%)	0	2 (9%)	0	0	8 (4%)	NS	..
sec	4 (3%)	1 (6%)	1 (5%)	0	0	6 (3%)	NS	..
seh	9 (6%)	2 (11%)	0	0	0	11 (5%)	NS	..
sek	11 (8%)	2 (11%)	1 (5%)	1 (8%)	1 (8%)	16 (8%)	NS	..
sel	4 (3%)	1 (6%)	1 (5%)	0	0	6 (3%)	NS	..
sem	32 (23%)	6 (33%)	7 (32%)	3 (25%)	5 (42%)	53 (26%)	NS	..
seo	32 (23%)	6 (33%)	7 (32%)	3 (25%)	5 (42%)	53 (26%)	NS	..
sep	6 (4%)	0	1 (5%)	0	0	7 (3%)	NS	..
seq	11 (8%)	2 (11%)	1 (5%)	1 (8%)	1 (8%)	16 (8%)	NS	..
tst	6 (4%)	**4 (22%)**	0	1 (8%)	2 (17%)	13 (6%)	0.009	5.7 (1.6-20.8)
eta	2 (1%)	0	0	1 (8%)	0	3 (2%)	NS	..
etb	1 (1%)	0	0	0	0	1 (1%)	NS	..
etd	70 (49%)	6 (33%)	9 (41%)	4 (33%)	5 (42%)	94 (46%)	NS	..
luk-PV	**71 (50%)**	5 (28%)	**11 (50%)**	4 (33%)	3 (25%)	94 (46%)	0.015	2.5 (1.2-5.2)
hlgb	6 (4%)	3 (17%)	2 (9%)	0	1 (8%)	12 (6%)	NS	..
edin	73 (51%)	7 (39%)	11 (50%)	4 (33%)	6 (50%)	101 (49%)	NS	..

Note. Boldfaced fonts indicate genes that are significantly more prevalent in strains isolated from a particular type of infection.
CI, confidence interval; edin, epidermal cell differentiation inhibitor; et, exfoliative toxin; hlgb, γ hemolysin B; luk-PV, Panton-Valentine leukocidin; NS, not significant; se, staphylococcal enterotoxin; SSTI, skin and soft tissue infection; tst, toxic shock syndrome toxin-1.
* Odds ratio's were calculated by use of binary logistic regression

a higher prevalence of the *sea* and *tst* gene. Isolates recovered from 6 (33%) of 18 bacteremic patients versus isolates from 24 (13%) of 188 other patients were *sea*-positive. Isolates from 4 (22%) of 18 bacteremic patients versus isolates from 9 (5%) of 188 other patients were *tst*-positive ($P<0.05$ and $P<0.01$ respectively; Table 2). SSTI and bone and joint infections were

associated with a higher prevalence of *luk-PV* ($P<0.05$). Isolates recovered from 82 (50%) of 164 patients with SSTI or bone infection versus 12 (29%) of 42 other patients were *luk-PV*-positive ($P<0.05$; Table 2).

Of all infectious *S. aureus* isolates, 112 (54%) were methicillin susceptible (MSSA) and 94 (46%) methicillin resistant (MRSA). The prevalence of *sea*, *seb*, *sec*, *seh*, *sel*, *sem*, *seo*, *tst* and *hlgb* was higher among MSSA stains; *etd*, *luk-PV* and *edin* were more prevalent among MRSA strains ($P<0.05$, Table 3). Within the group of MSSA or MRSA infections, the prevalence of toxin genes was not significantly different between hospital-acquired and community-acquired infections (Table 3).

Table 3. Difference in presence of toxin genes between methicillin resistant *S. aureus* (MRSA) and methicillin susceptible *S. aureus* (MSSA) isolates

	Number (%) of gene positive isolates					
	MSSA	MRSA	HA-MSSA	CA-MSSA	HA-MRSA	CA-MRSA
Toxin gene	n=112	n=94	n=66	n=46	n=61	n=33
sea	28 (25%)	2 (2%) *	19 (29%)	9 (20%)	1 (2%)	1 (3%)
seb	8 (7%)	0 *	5 (8%)	3 (7%)	0	0
sec	6 (5%)	0 **	2 (3%)	4 (9%)	0	0
seh	11 (10%)	0 *	6 (9%)	5 (11%)	0	0
sek	10 (9%)	6 (6%)	6 (9%)	4 (9%)	5 (8%)	1 (3%)
sel	6 (5%)	0 **	2 (3%)	4 (9%)	0	0
sem	45 (40%)	8 (9%) *	23 (35%)	22 (48%)	7 (11%)	1 (3%)
seo	45 (40%)	8 (9%) *	23 (35%)	22 (48%)	7 (11%)	1 (3%)
sep	5 (5%)	2 (2%)	4 (6%)	1 (2%)	2 (3%)	0
seq	10 (9%)	6 (6%)	6 (9%)	4 (9%)	5 (8%)	1 (3%)
tst	13 (12%)	0 *	9 (14%)	4 (9%)	0	0
eta	3 (3%)	0	0	3 (7%)	0	0
etb	1 (1%)	0	1 (2%)	0	0	0
etd	16 (14%)	78 (83%) *	9 (14%)	7 (15%)	48 (79%)	30 (91%)
luk-PV	17 (15%)	77 (82%) *	7 (11%)	10 (22%)	47 (77%)	30 (91%)
edin	21 (19%)	80 (85%) *	13 (20%)	8 (17%)	50 (82%)	30 (91%)
hlgb	12 (11%)	0 *	8 (12%)	4 (9%)	0	0

Note. CA, community-acquired; edin, epidermal cell differentiation inhibitor; et, exfoliative toxin; HA, hospital-acquired; hlgb, γ hemolysin B; luk-PV, Panton-Valentine leukocidin; se, staphylococcal enterotoxin; tst, toxic shock syndrome toxin-1.
P-values ≤0.05 (by the χ^2 test) were considered statistically significant.
* $P\leq0.01$
** $P\leq0.05$

Association of anti-toxin IgG levels with presence of toxin genes in infectious *S. aureus* isolates

Data on both gene presence and antistaphylococcal antibody levels were available for 13 combinations (ETA, ETB, SEA, SEC, SED, SEH, SEM, SEQ, SER, TSST-1, HlgB, LukS and LukF). There were no *sed*- and *ser*-positive isolates. Antibody levels were elevated if the gene was present in 8 (*eta*, *etb*, *sea*, *sec*, *seh*, *seq*, *tst*, *luk-PV*) out of 11 combinations ($P<0.05$; Figure 2). Furthermore, in patients with *sea*-positive *S. aureus* infections, the anti-SEA IgG titers in-

creased significantly in the course of infection (median MFI from 11555 to 12388, *P*<0.05). In addition, in patients with a SSTI caused by a *luk-PV*-positive strain, the level of anti-LukS (not anti-LukF) IgG increased significantly (median MFI from 15231 to 15911, *P*<0.05). No increase in IgG level was seen in *sea*- and *luk-PV*-negative *S. aureus* infections.

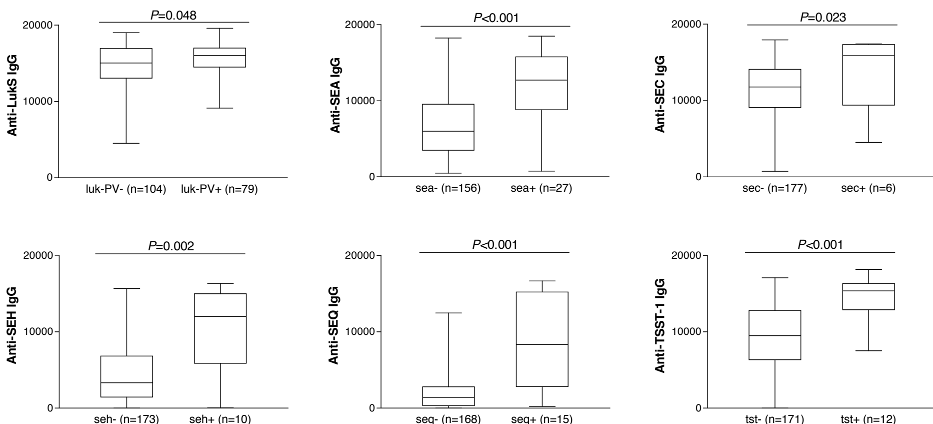

Figure 2. Box-and-Whisker plots showing the relationship between the anti-toxin IgG levels in serum and toxin gene presences in isolates collected from *S. aureus*-infected patients. Results for *eta* and *etb* are not shown because of the small number of gene positive isolates (n=3 and n=1, respectively). The box represents the 25th, 50th and 75th percentile, the whisker represents the lowest and highest obtained value. Luk, leukocidin; SE, staphylococcal enterotoxin; TSST-1, toxic shock syndrome toxin-1.

DISCUSSION

For the majority of toxins, antistaphylococcal IgG levels were higher in adults than in children and IgG levels were higher in *S. aureus*-infected patients than in hospital-admitted control subjects (Figure 1A and 1B). This suggests that the antistaphylococcal humoral immune state of an individual develops over the years and probably depends on the history of confrontations with *S. aureus*. IgG levels in serum samples from patients were higher if the toxin gene was present in their infectious isolates in 8 of 11 combinations (Figure 2). This indicates that *eta*, *etb*, *sea*, *sec*, *seh*, *seq*, *tst* and *luk-PV* are actively expressed and stimulate the humoral immune system during *S. aureus* infections. For PVL and SEA, there was additional support for expression *in vivo* during *S. aureus* infections, as was found earlier by Croze et al. for PVL [28]. The level of anti-SEA IgG increased in a time period of 2 weeks during infections caused by *sea*-positive, but not *sea*-negative strains. The level of anti-SEE IgG did not increase, although earlier studies observed cross-reactivity with anti-SEA antibodies [36]. Additionally, the level of anti-LukS IgG increased in the course of 2 weeks in patients suffering from SSTI caused by *luk-PV*-positive, but not *luk-PV*-negative strains (*P*<0.05). In contrast to LukS, IgG levels to LukF were not higher if isolates were *luk-PV*-positive and no increase anti-LukF IgG was detected in patients with a *luk-PV*-positive SSTI. In mice, a dominant anti-LukS IgG2a and 2b response

developed after immunizing mice subcutaneously with both components. Furthermore, intranasally-vaccinated mice generated anti-LukS IgA, but not anti-LukF IgA [29]. Therefore, it seems that LukS is the dominant antigenic protein subunit, in mice as well as in humans.

For *S. aureus* infections that were caused by strains other than strains positive for *sea* or *luk-PV*, the level of toxin-specific IgG showed no increase in the course of infection. This suggests that, in the case of SSTI, respiratory and bone and joint infections, these infections did not elicit a strong systemic humoral immune response. Alternatively, differences in antibody levels between the 2 samples were non-significant because of the high premorbid anti-toxin IgG levels. High preexisting IgG levels might be the result of the high incidence of *S. aureus* infections in this particular population. Subsequently, this would suggest that these antibodies do not protect against these types of staphylococcal infection. Preformed anti-toxin IgG might also explain the high anti-SED and anti-SER IgG levels that were found in patients with a respiratory infection, even though the causative *S. aureus* isolates were *sed*- and *ser*-negative.

Bacteremia was associated with a high prevalence of *sea* and *tst*, SSTI and bone and joint infections with a high prevalence of *luk-PV*, in agreement with earlier studies [12, 30-32]. The most prevalent genes in the 206 clinical *S. aureus* isolates of Algerian patients were *edin* (49%), *luk-PV* (46%) and *etd* (46%; Table 2). These genes were more prevalent among MRSA than among MSSA strains ($P<0.05$; Table 3). Likely, this is due to the frequent occurrence of the *luk-PV*-, *etd*- and *edin*-positive MRSA-ST80 clone that is predominant in Algeria [33, 34].

We determined IgG binding to toxins, but we do not have data on neutralizing capacity and cross-reactivity of these antibodies. In earlier studies, neutralizing capacity for anti-TSST-1, SEA, SEB, SEC and SEE antibodies was observed [5]. Cross-reactivity was shown between anti-SEA and anti-SEE antibodies and between anti-SEB and anti-SEC antibodies [35, 36]. For other enterotoxins, antibody titers specific for heterologous toxins were 10-fold lower than those directed against the toxin used for immunization, which argues against a strong cross-reactivity [5, 37]. Knowledge on cross-reactivity and functionality of the anti-toxin antibodies should be increased, though. Furthermore, we do not know the *S. aureus* carrier state of the patients. Because nasal carriers of *S. aureus* have an increased risk of infection and nearly 80% of the infections are endogenous [38, 39], their level of IgG might be influenced by colonization and/or previous infections with their colonizing strain. Therefore, including nasal swab cultures in future studies is important.

In conclusion, during *S. aureus* infection, the toxins ETA, ETB, SEA, SEC, SEH, SEQ, TSST-1 and Luk-PV are actively expressed and recognized by the humoral immune system. *S. aureus* bacteremia is associated with a high prevalence of *sea* and *tst* whereas SSTI and bone and joint infections are associated with presence of *luk-PV*. Significant increases in anti-SEA IgG and anti-Luk-PV IgG levels are observed during all types of infection and SSTI, respectively, suggesting their involvement in the pathogenesis of *S. aureus* infection.

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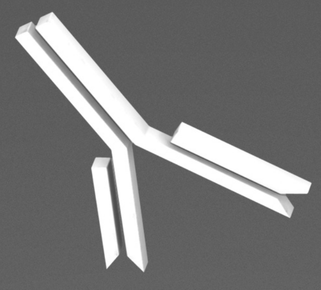
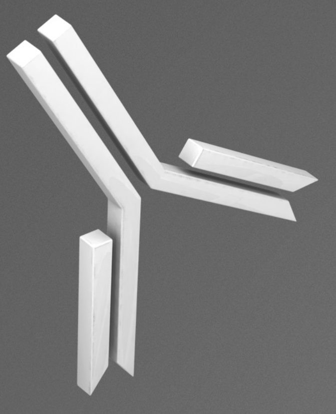


Chapter 7

Reclassification of *Staphylococcus aureus* nasal carriage types



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ABSTRACT

Background. Persistent nasal carriers have an increased risk of *Staphylococcus aureus* infection, whereas intermittent and non-carriers share the same low risk. This study was performed to provide additional insight into staphylococcal carriage types. **Methods.** Fifty-one volunteers who had been decolonized with mupirocin treatment and whose carriage state was known were colonized artificially with a mixture of *S. aureus* strains and intranasal survival of *S. aureus* was compared between carriage groups. Furthermore, antistaphylococcal antibody levels were compared among 83 carriage-classified volunteers. **Results.** Persistent carriers preferentially reselected their autologous strain from the inoculum mixture (P=0.02). They could be distinguished from intermittent carriers and non-carriers on the basis of the duration of post-inoculation carriage (154 vs. 14 and 4 days, respectively; P=0.017, by log-rank test). Cultures of swab samples from persistent carriers contained significantly more colony forming units per swab sample than did cultures of swab samples from intermittent and non-carriers (P=0.004) Analysis of serum samples showed that levels of IgG and IgA to 17 *S. aureus* antigens were equal in intermittent carriers and non-carriers, but not persistent carriers. **Conclusions.** Along with the previously described low risk of infection, intermittent carriers and non-carriers share similar *S. aureus* nasal elimination kinetics and antistaphylococcal antibody profiles. This implies a paradigm shift; apparently, there are only 2 types of nasal carriers: persistent carriers and others. This knowledge may increase our understanding of susceptibility to *S. aureus* infection.

INTRODUCTION

Nasal carriage of *Staphylococcus aureus* plays a key role in the epidemiology and pathogenesis of staphylococcal infection [1, 2]. Eradication of *S. aureus* from the nose has been proved to be effective in the reducing the incidence of staphylococcal infection [3-5]. This indicates that the anterior nasal region is a primary ecological reservoir of *S. aureus* [6, 7], although the throat and the perineum are important reservoirs as well [8]. However, nasal recolonization may occur within weeks to months in those who have successfully been decolonized [9, 10]. In light of the emergence of antibiotic resistance and the lack of long-term elimination strategies against *S. aureus* nasal carriage, new approaches are needed for the prevention of staphylococcal disease. To develop new strategies, it is important to acquire additional knowledge about the underlying mechanisms of *S. aureus* nasal carriage.

Historically, individuals have been assigned to 1 of 3 groups with regard to carriage of *S. aureus*: persistent carriers (~20% of individuals), intermittent carriers (~30%) and non-carriers (~50%) [1, 11, 12]. The prevalence of *S. aureus* nasal carriage varies, however, and is higher in young children [13], men [14], Caucasians [14], hospitalized patients [15] and a number of pa-

tient groups including patients with diabetes mellitus [16], those undergoing hemodialysis [17] or chronic ambulatory peritoneal dialysis [18], those with *S. aureus* skin infection [19] and HIV-infected patients [20]. It has been documented that certain individuals may carry their resident strain for extensive periods, sometimes even for years [21].

In addition to host factors, several bacterial carriage determinants have been studied. Cell wall teichoic acid, lipoteichoic acid and fibronectin-binding proteins have been shown to represent major ligands in the adherence of *S. aureus* to epithelial cells [2, 22]. Recently, such a role for the protein clumping factor (Clf) B was ascertained by use of human and animal artificial colonization models [23, 24]. Meanwhile, current knowledge still does not explain why some individuals are persistent carriers and others are intermittent carriers or non-carriers.

In this study, we provide additional insight into nasal carriage types. Differences in *S. aureus* survival in the human nose and differences in antistaphylococcal antibodies in healthy persistent carriers, intermittent carriers and non-carriers were analyzed. Also, the specificity of the interaction between bacterial strains and human individuals was determined.

MATERIALS AND METHODS

Study design: artificial nasal colonization

Study population Volunteers with any of the following features were excluded from the study: known history of recurrent skin infections, eczema, presence of skin lesions, allergy to β-lactam antibiotics, endocarditis, chronic pulmonary disease, chronic sinusitis, diabetes mellitus, dialysis, current use of antibiotics or immunosuppressive medication, pregnancy, expected contacts with patients at high risk of staphylococcal disease during the study period (e.g. patients in intensive care units or undergoing dialysis) and any other conditions that in the opinion of the investigators would incur a risk to the participant or their contacts. Fifty-one healthy volunteers aged 18–65 years were included. Written informed consent was obtained and participants were notified that an infectious diseases physician was on call for the entire study period. The study was approved by the Medical Ethics Committee of the Erasmus Medical Center Rotterdam (156.137/1996/186).

Screening The volunteers were screened for nasal carriage of *S. aureus* over a mean duration of 6 months. Nasal swab samples were collected on 5–10 separate occasions during this 6-month period; samples were collected ≥1 week apart, but the usual interval was 3–4 weeks. Participants were labeled as persistent carriers if ≥80% of the cultures were positive for *S. aureus;* allowing 1 of ≥5 swab samples to test negative minimizes misclassification of carriage state because of culture or laboratory errors. Participants were labeled as non-carriers if all nasal swab cultures were negative for *S. aureus.* All other participants were labeled as intermittent carriers [25]. Immediately before the start of mupirocin treatment, a venous blood

sample was obtained for measurement of antistaphylococcal antibody levels and baseline safety assessments (C-reactive protein levels and leukocyte counts).

Eradication After screening, participants self-administered mupirocin 2% nasal ointment (GlaxoSmithKline) twice daily for 5 days to eradicate resident *S. aureus* strains and other susceptible micro-organisms present in the vestibulum nasi. Both oral and written instructions were given to the volunteers and a visual presentation on the application of mupirocin ointment was given by the study staff. Nasal samples were obtained just before and 5 weeks after mupirocin treatment.

Inoculation On the day of artificial colonization, all inclusion and exclusion criteria were rechecked. All participants were inoculated with a mixture of *S. aureus* strains with nearly indistinguishable *in vitro* growth rates: strain 502a, 2 strains randomly collected from persistent carriers (P1 and P2), a strain collected from an intermittent carrier (I) and, in the case of persistent or intermittent carriers, the last cultured resident strain [26]. Inoculation was performed under medical supervision. Both anterior nares were inoculated once with $1x10^7$ cells per bacterial genotype.

Follow-up Nasal swab samples were collected and cultured 1, 2, 4, 8, 16 and 22 weeks after inoculation. Restriction fragment-length polymorphism (RFLP) of the coagulase and protein A genes was used to type the cultured *S. aureus* strains [26]. Pulsed-field gel electrophoresis (PFGE) was performed according to methods described elsewhere [8] to confirm the results of RFLP analysis. Participants received weekly medical examinations and were instructed to contact the investigator and the infectious diseases physician if their temperature would increase to >38.5°C and/or when other, putatively adverse reactions occurred. At the end of the study the safety assessments were repeated and participants underwent their last medical examination.

Microbiological procedures

Nasal swab samples were collected by streaking both anterior nares using a sterile cotton swab (Transwab; Medical Wire & Equipment). All swabs were processed within 24 hours. Nasal swabs were plated on a blood agar plate (Becton-Dickinson) and were submerged in phenol red mannitol enrichment broth [27]. Plates were read after 1 and 2 days of incubation and broth cultures after 7 days of incubation at 37°C. Broth cultures in which the color changed from red to orange-yellow were subcultured on blood agar plates. Identification of *S. aureus* was based on colony morphology, Gram stain, catalase test and latex-agglutination test (Staphaurex Plus, Murex).

All strains included in the mixture were tested by polymerase chain reaction as described elsewhere [28] for the absence of toxin production. Antibiotic susceptibility testing was performed for all strains and involved testing resistance to oxacillin, cefuroxim, vancomycin, rifampin, fusidic acid, ciprofloxacin and mupirocin. All strains included in the mixture had

clearly distinct PFGE patterns, which facilitated unequivocal strain identification in samples after the *in vivo* nasal inoculation.

Because multiple *S. aureus* strains were inoculated, 16 colonies were randomly selected from each nasal culture positive for *S. aureus* during follow-up. Colonies were stored separately in glycerol-containing medium at -20°C until genotyping. Final storage was at -80°C. The last strain found during the screening episode for persistent and intermittent carriers was typed by PFGE before it was added to the inoculation mixture. *S. aureus* DNA was obtained according to Boom et al. [29].

Measurement of antistaphylococcal antibodies

Serum samples from the 51 volunteers were analyzed, along with additional serum samples from 13 persistent carriers, 1 intermittent carrier and 18 non-carriers, as described elsewhere [30], to provide larger and more homogenous groups. Carrier state for the latter 32 volunteers was determined on the basis of ≥3 nasal swab samples obtained at 2-week intervals. Levels of IgG and IgA directed against the following *S. aureus* proteins were quantified: ClfA and ClfB, *S. aureus* surface protein G (SasG), iron-responsive surface determinant (Isd) A and H, fibronectin-binding protein (Fnbp) A and B, serine-aspartate repeat protein (Sdr) D and E, staphylococcal enterotoxin (SE) A, B, I, M, toxic shock syndrome toxin (TSST)-1, chemotaxis inhibitory protein of *S. aureus* (CHIPS), staphylococcal complement inhibitor (SCIN) and extracellular fibrinogen-binding protein (Efb). Antibodies were quantified simultaneously using a bead-based flow cytometry technique (xMAP®, Luminex Corporation). Methods have been described elsewhere [30-32]. Tests were performed in duplicate and median fluorescence intensity (MFI) values, reflecting semi-quantitative antibody levels, were determined.

Statistical analysis

The primary end point was survival of *S. aureus* in the nose after artificial colonization. Survival ended when results of ≥2 consecutive nasal swab cultures were negative for *S. aureus*. Kaplan-Meier curves and the log-rank test were used to compare *S. aureus* survival curves. Participants still carrying *S. aureus* in their noses at the end of follow-up were censored in the analysis. When data were missing, the last observation was carried forward. The secondary endpoint was the median number of colony forming units (cfu) over the course of 22 weeks. The χ^2 test (or Fisher's exact test) was used to compare proportions. For comparison of continuous data the Mann-Whitney *U* test or Kruskal-Wallis test was used when appropriate. *P*-values ≤0.05 were considered statistically significant.

RESULTS

Artificial nasal colonization

Screening Fifty-one volunteers were included in the artificial nasal colonization study. Fifteen volunteers (29%) were classified as non-carriers (median age 22 years; range 19-53 years). Twenty-four volunteers (47%) were classified as intermittent carriers (median age 25 years; range 20-50 years). The median percentage of positive cultures in this group was 25% (range 10%-78%). Twelve volunteers (24%) were classified as persistent carriers (median age 22 years; range 20-37 years). For 10 of 12 persistent carriers, all nasal swab cultures were positive for *S. aureus*. The percentages of positive culture results for the 2 other persistent carriers were 83% and 86%. In both of these volunteers, the result of a single nasal swab culture was negative.

Eradication All volunteers, including non-carriers, received mupirocin nasal ointment for *S. aureus* decolonization. All reported having applied the ointment according to instructions. Five weeks after mupirocin treatment and just before artificial inoculation, 8 volunteers, 4 classified as intermittent carriers and 4 as persistent carriers, still carried *S. aureus* in their noses. Bacterial counts were low in all except 1 participant (median 5 cfu per sample; range 1-4500 cfu).

Follow-up After artificial nasal colonization, *S. aureus* survival and the number of cfu per nasal swab sample were determined. The Kaplan-Meier curves in Figure 1 show the proportions of positive culture results during the follow-up period. Median *S. aureus* nasal survival was 4 days among non-carriers, 14 days among intermittent carriers and >154 days among persistent carriers (P=0.017). The nasal swab sample cultures from persistent carriers contained significantly more cfu *S. aureus* per swab sample than the nasal swab sample cultures from intermittent carriers or non-carriers. The median bacterial count during the 22 weeks after artificial inoculation was 0 (range 0-26.000) cfu per sample in non-carriers, 0 (range 0-17.000) cfu per sample in intermittent carriers and 69 (range 0-1.500.000) cfu per sample in persistent carriers (P=0.004). None of the volunteers experienced adverse events and all adhered to the study protocol for the duration of the study period.

Selection of strains Seven (58%) of the 12 persistent carriers selected their own resident strain from the inoculation mixture, compared with 4 (17%) of 24 intermittent carriers (P=0.02). None of the intermittent carriers but 3 of the persistent carriers who still carried *S. aureus* after mupirocin treatment selected their own strain from the inoculum mixture. For these 3 persistent carriers, the bacterial counts after mupirocin treatment were low (2, 4 and 4500 cfu per sample), compared with the bacterial counts 1 week after inoculation (600, 1100 and 37000 cfu per sample). Therefore, it was concluded that this finding indicated reacquisition of strains from the inoculum mixture, rather then regrowth of the resident strain. Strain 502A was selected in 1 (2%) participant, P1 was selected in 6 (12%) and P2 was selected in 4 (8%) participants. The intermittent carrier strain (I) was selected in 2 non-carriers and 1 intermit-

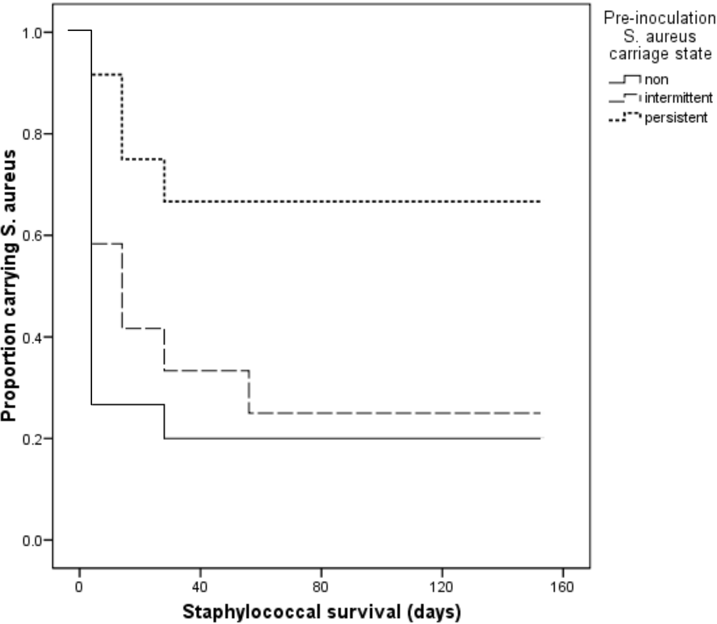

Figure 1. Kaplan-Meier survival curves showing proportions of individuals with culture-positive nasal swab samples after artificial inoculation of a mixture of *S. aureus* strains in the nasal cavities.

tent carrier; none of the persistent carriers selected this strain. Furthermore, after artificial colonization not a single culture result was positive for *S. aureus* in 1 persistent carrier (2%) and 13 (25%) intermittent carriers and non-carriers. *S. aureus* strains that were not part of the inoculation mixture were detected at various intervals after inoculation. Of the bacteria found in cultures of samples from non-carriers, 13% were of alien origin. For the (former) intermittent and persistent carriers, these percentages were 14% and 18%, respectively.

Antistaphylococcal antibodies

The levels of IgG and IgA directed against 17 *S. aureus* proteins were measured in serum samples from 25 persistent carriers (30%), 25 intermittent carriers (30%) and 33 non-carriers (40%). The MFI values reflecting the antistaphylococcal antibody levels for each individual are shown in Figure 2. For most of the antigens, there was no apparent quantitative difference in antibody level between persistent carriers, intermittent carriers and non-carriers. However, the levels of IgG directed against TSST-1 and SasG were different between these groups ($P<0.001$ and $P=0.019$, respectively, Table 1). The levels of IgA were higher in persistent carriers than in intermittent carriers or non-carriers for TSST-1 ($P<0.001$), SEA ($P=0.013$), ClfA ($P=0.005$) and CHIPS ($P=0.006$). The MFI values did not differ significantly between non-carriers and intermittent carriers (Table 1).

Table 1. Median fluorescence intensity (MFI) values reflecting *S. aureus* antigen-specific IgG and IgA levels in persistent carriers (PC), intermittent carriers (IC) and non-carriers (NC)

S. aureus protein	Antibody isotype	Carrier state	MFI value median (range)	*P*-values *			
				PC vs. NC	PC vs. IC	IC vs. NC	PC vs. IC and NC
TSST-1	IgG	PC	12123 (100 – 18322)	*P*=0.000	*P*=0.003	NS	*P*=0.000
		IC	5973 (57 – 18136)				
		NC	4714 (42 – 17053)				
	IgA	PC	754 (87 – 3130)	*P*=0.001	*P*=0.000	NS	*P*=0.000
		IC	134 (10 – 3139)				
		NC	110 (20 – 3506)				
SasG	IgG	PC	56 (29 – 485)	*P*=0.042	*P*=0.037	NS	*P*=0.019
		IC	127(32 – 556)				
		NC	110 (21 – 413)				
SEA	IgA	PC	48 (19 – 1070)	*P*=0.017	*P*=0.056	NS	*P*=0.013
		IC	31 (16 – 277)				
		NC	29 (17 – 284)				
ClfA	IgA	PC	1197 (108 -3997)	*P*=0.008	*P*=0.028	NS	*P*=0.005
		IC	524 (74 – 2430)				
		NC	441(87-3156)				
CHIPS	IgA	PC	5513 (1004 – 12309)	*P*=0.032	*P*=0.006	NS	*P*=0.006
		IC	3445 (156-11644)				
		NC	2525 (27 – 11532)				

Note. CHIPS, chemotaxis inhibitory protein of *S. aureus*; ClfA, clumping factor A; NS, not significant; SasG, *S. aureus* surface protein G; SEA, staphylococcal enterotoxin A; TSST-1, toxic shock syndrome toxin-1.

* *P*-values ≤0.05 (by the Mann-Whitney *U* test) were considered statistically significant

DISCUSSION

Although the amount of knowledge on host factors and bacterial determinants of *S. aureus* colonization is increasing, it remains unclear why certain individuals are persistent carriers, whereas others are intermittent carriers or non-carriers. This knowledge is important, because persistent carriers are at a higher risk for development of *S. aureus* infections [5, 33]. Although intermittent carriers also carry *S. aureus* in their noses at times, their risk of infection is similar to non-carriers [34]. Important questions are: what drives intermittent carriage and why do intermittent carriers have a lower risk of infection than persistent carriers? In the present study, we identified carriage features specific for the different carriage types by performing an artificial human nasal inoculation study. We compared the survival of artificially inoculated *S. aureus* strains in persistent carriers, intermittent carriers and non-carriers. Furthermore, we compared antistaphylococcal antibody profiles. The median *S. aureus* survival was 4 days among non-carriers and 14 days among intermittent carriers (not significantly different) but >154 days among persistent carriers. Furthermore, after artificial inoculation, the cultures of swab samples from persistent carriers contained significantly more cfu per sample than the cultures of samples from intermittent carriers and non-carriers. In addition, the antibody lev-

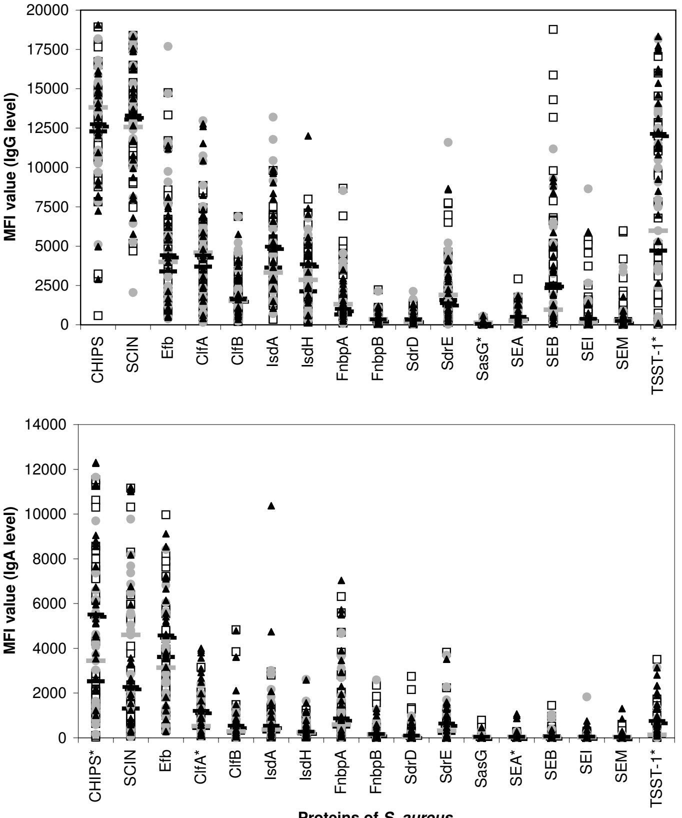

Figure 2. Median fluorescence intensity (MFI) values reflecting levels of antigen-specific IgG and IgA to 17 *S. aureus* antigens. Each symbol represents a single volunteer; black triangles represent persistent carriers, grey circles represent intermittent carriers and white squares represent non-carriers. Median levels of antistaphylococcal antibodies are indicated by double black, single grey and single black horizontal lines, respectively. Statistically significant differences are indicated by asterisks ($P \leq 0.05$, Mann-Whitney U test). CHIPS, chemotaxis inhibitory protein of *S. aureus*; Clf, clumping factor; Efb, extracellular fibrinogen-binding protein; Fnbp, fibronectin-binding protein; Isd, iron-responsive surface determinant; Sas, *S. aureus* surface protein; SCIN, staphylococcal complement inhibitor; Sdr, serine-aspartate repeat protein; SE, staphylococcal enterotoxin; TSST, toxic shock syndrome toxin.

els against 17 *S. aureus* proteins were not significantly different between intermittent carriers and non-carriers, whereas differences were detected with persistent carriers. Thus, persistent carriers stand out as a separate group whereas intermittent carriers and non-carriers seem to belong to a single group of individuals. This suggests that either non-carriage is incidental and most humans are actually intermittent *S. aureus* carriers or intermittent carriers might actually be non-carriers who carry *S. aureus* only under environmental pressure.

Seven of the 12 persistent carriers selected their own strain from the inoculation mixture. If the chances of selecting each strain from the inoculation mixture were equal, we would expect that only 2 of the 12 persistent carriers would select their own strain. Therefore, selection for the resident strain is not random in persistent carriers. When these data are combined

with those presented by Nouwen et al. [26] in a similar artificial colonization study, 11 of 19 persistent carriers selected for their own resident strain, where only 4 would be expected to do so if this effect was random. These cumulative data help to postulate that persistent carriage depends, at least in part, on an adequate biological match between human host and colonizing *S. aureus* strain.

We classified participants as persistent carriers, intermittent carriers or non-carriers on the basis of ≥5 nasal swab samples. We did not determine the *S. aureus* nasal carriage status of family members, pets and other close contacts. This would have provided valuable information, because *S. aureus* strains that were not part of the inoculation mixture were detected after inoculation in all 3 groups. This suggests that colonization may be incidentally acquired during contacts with other *S. aureus* carriers, contaminated environments, or (pet) animals. Furthermore, 8 volunteers still carried *S. aureus* 5 weeks after treatment with mupirocin. This finding might also be attributable to exposure to *S. aureus* strains from close contacts, but it may also be attributable to participants' compliance with mupirocin treatment or the efficacy of the treatment.

In the present study, we revealed that host-*S. aureus* interactions are highly specific because of the apparent reacquisition of the autologous strain in persistent carriers. Furthermore, intermittent carriers and non-carriers have similar *S. aureus* elimination kinetics and similar antistaphylococcal antibody patterns. Moreover, the median numbers of cfu yielded on cultures after artificial inoculation were clearly lower in these groups than in persistent carriers. It was also shown elsewhere that persistent nasal carriers have an increased risk of *S. aureus* infection, whereas intermittent nasal carriers share the same risk of infection as non-carriers [34]. This implies that a paradigm shift is required; apparently, there are only 2 human types of nasal *S. aureus* carriers: persistent carriers and others. This knowledge may increase our understanding of susceptibility to *S. aureus* infection.

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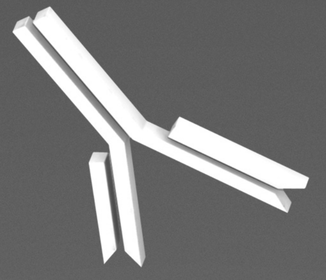
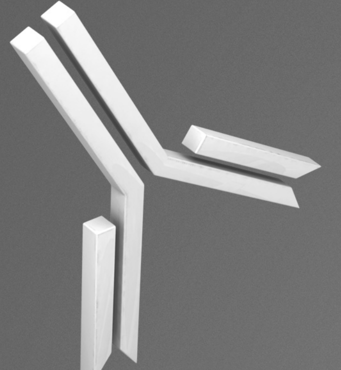
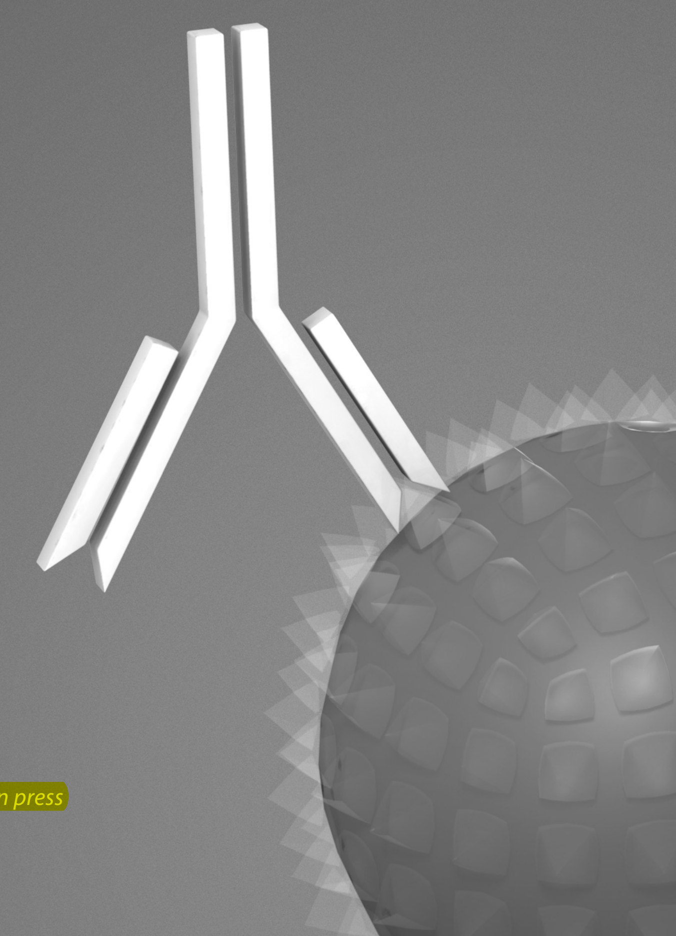


Chapter 8

Immune evasion cluster-positive bacteriophages are highly prevalent among human *Staphylococcus aureus* strains, but they are not essential in the first stages of nasal colonization





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ABSTRACT

The *Staphylococcus aureus* immune evasion cluster (IEC), located on β-hemolysin-converting bacteriophages (βC-Φ's), encodes the immune-modulating proteins chemotaxis inhibitory protein of *S. aureus*, staphylococcal complement inhibitor (SCIN), staphylococcal enterotoxin A and staphylokinase. Its precise role in *S. aureus* colonization is unclear. We studied the presence of the IEC-carrying bacteriophages in human and animal *S. aureus* isolates, using polymerase chain reaction for the gene encoding SCIN (*scn*). Human isolates were obtained by collecting serial nasal swab samples from 21 persistent carriers. *S. aureus* strains from 19 (90%) persistent carriers contained an IEC that was present and indistinguishable in 95% of cases at all 5 sampling moments over a 3-month period. Of the 77 infectious animal strains included in the study, only 26 strains (34%) were IEC-positive. Integration of these IEC-positive strains into an amplified fragment length polymorphism genotype database showed that 24 of 53 (45%) strains were human-associated and only 2 of 24 (8%) were 'true' animal isolates ($P<0.001$). The high prevalence and stability of IEC-carrying βC-Φ's in human strains suggested a role for these βC-Φ's in human nasal colonization. To test this hypothesis, 23 volunteers were colonized artificially with *S. aureus* strain NCTC 8325-4 with or without the IEC type B-carrying βC-Φ13. Intranasal survival was monitored for 28 days after inoculation. The strain with βC-Φ13 was eliminated significantly faster (median 4 days; range 1-14 days) than the strain without βC-Φ13 (median 14 days; range 2-28 days, $P=0.011$). In conclusion, although IEC-carrying βC-Φ's are highly prevalent among human colonizing *S. aureus* strains, they are not essential in the initial stages of *S. aureus* nasal colonization.

INTRODUCTION

Staphylococcus aureus is a human commensal, as well as an important pathogen that can cause infections ranging from mild to life-threatening [1]. *S. aureus* enters the body through breaches in the skin or mucous membranes, where it is immediately confronted by the innate immune system as the first line of defense. To counteract innate immunity, *S. aureus* expresses a number of immune-modulating proteins. One of the immune-modulating proteins is staphylococcal complement inhibitor (SCIN). It is an efficient complement inhibitor of the lectin, the classical and the alternative pathway. SCIN efficiently prevents opsonophagocytosis and killing of *S. aureus* by human neutrophils. Furthermore, SCIN prevents generation of C5a and neutrophil chemotaxis [2, 3]. Recently, the gene encoding SCIN (*scn*) was found to be part of a so-called 'immune evasion cluster' (IEC). Seven IEC variants have been identified [4]. All IEC variants carry *scn* and a different combination of *sea, sak* and *chp*. These genes encode the human-specific immune modulators staphylococcal enterotoxin A (SEA), staphylokinase (SAK) and chemotaxis inhibitory protein of *S. aureus* (CHIPS). SEA is a well known superanti-

gen [5] and is also involved in the down-regulation of chemokine receptors of monocytes [6]. SAK is a bacterial plasminogen activator. Plasmin formed by the conversion of plasminogen by SAK leads to removal of important opsonic molecules, such as IgG and C3b, by cleaving these molecules. Therefore, SAK can efficiently prevent phagocytosis of staphylococci by human neutrophils [7]. SAK also inhibits the bactericidal effect of antimicrobial peptides, the α-defensins [8]. CHIPS blocks neutrophil chemotaxis by binding the formylated peptide receptor and de C5a receptor on neutrophils [4, 9, 10]. IECs are located on bacteriophages. Bacteriophages are mobile genetic elements that can be transferred between strains. The IEC-carrying bacteriophages are incorporated in the gene encoding β-hemolysin (*hlb*). Therefore, β-hemolysin is not produced when the IEC-carrying bacteriophage is present. Consequently, these bacteriophages are called β-hemolysin-converting bacteriophages (βC-Φ's) [4]. It was shown that 90% of the human clinical *S. aureus* strains (isolated from blood, liquor, wounds, continuous ambulatory peritoneal dialysis fluid, pulmonary fluid, joint fluid and pericardial fluid) contain an IEC-carrying βC-Φ; this is an exceptionally high percentage compared to other mobile elements carrying virulence factors in human *S. aureus* strains [4].

Little is known about the prevalence of IEC-carrying βC-Φ´s in animal *S. aureus* strains. It was shown that only 13 of 290 (4.5%) *S. aureus* strains isolated from cows are IEC-positive [11]. Other studies showed that β-hemolysin was produced or *hlb* was present in 66%-92% of *S. aureus* strains isolated from cows [12-14]. This indicates that the prevalence of IEC-carrying βC-Φ's in animal *S. aureus* isolates is low in contrast to the prevalence of these bacteriophages in human infectious *S. aureus* isolates. Because *S. aureus* infections are often endogenous [15, 16], we hypothesized that IEC-encoded immune modulators play a role in colonization of *S. aureus* in humans, but not in animals. To test this hypothesis, we studied the presence and stability of IEC-carrying βC-Φ's in nasal *S. aureus* isolates collected from healthy volunteers. Furthermore, we studied the prevalence of IEC-carrying βC-Φ's in a well described collection of *S. aureus* animal isolates. Finally, we performed an artificial nasal inoculation study in human volunteers in which we compared survival between *S. aureus* strain NCTC 8325-4 with and without IEC-carrying βC-Φ.

MATERIALS AND METHODS

Distribution and stability of IEC-carrying βC-Φ's in human *S. aureus* strains

Human *S. aureus* strains were collected from nasal swab cultures from 21 healthy, adult persistent carriers who were positive for *S. aureus* at 5 culture moments over a time interval of 3 months. *S. aureus* was cultured quantitatively and identified as described previously [17]. For each culture, up to 3 colonies of each morphotype (colony morphology and hemolysis pattern) were stored at -80^{0}C. Single locus DNA-sequencing of the repeat region of the protein A gene (*spa*) was used for comparative genotyping of the human *S. aureus* strains [18]. In all

strains, *scn* was amplified to determine whether an IEC was present or absent [4]. Furthermore, *chp*, *sak*, *sea*, *sep* and *hlb* were amplified to determine the IEC type, using previously described PCR primers and amplification conditions [4]. Volunteers provided their written informed consent and the local Medical Ethics Committee of the Erasmus Medical Center Rotterdam approved the study (MEC-2007-106).

Prevalence of IEC-carrying βC-Φ's in veterinary *S. aureus* strains

Seventy-seven *S. aureus* strains isolated from different infection sites of a variety of animal species were used. All strains were genotyped by amplified fragment length polymorphism (AFLP) analysis previously [19]. The AFLP fingerprints of these animal strains were introduced in an AFLP database comprising data of 829 nonclinical carriage strains isolated from healthy human individuals [20]. 'Typical animal strains' were defined as strains that grouped in AFLP cluster IVa, as described by van Leeuwen et al. [19]. These strains show no clear AFLP integration with human carrier strains. 'Typical human strains' were defined as animal strains that show considerable integration with human carrier strains. They grouped in AFLP clusters I, II and III [19]. Chromosomal DNA was extracted from the animal strains and the *scn* gene was amplified [4].

Artificial nasal colonization with *S. aureus* strain NCTC 8325-4 with and without IEC-carrying βC-Φ

Study population Twenty-three healthy individuals were included (8 males and 15 females, median age 29 years, range 19-58 years). The volunteers provided their written informed consent and they were notified of the fact that an infectious diseases physician was on call for the entire study period. The study protocol was approved by the Medical Ethics Committee of the Erasmus Medical Center Rotterdam (MEC-2007-324).

S. aureus strains C. Wolz provided *S. aureus* strains NCTC 8325-4 and 8325-4 with βC-Φ13 [21]. This strain has a defect in the *SigB* locus. Thus, although a strain from a natural carrier would have been preferred, we used NCTC 8325-4 because this strain has been approved for artificial colonization, is susceptible to all common antibiotics (e.g. flucloxacillin and vancomycin) and is devoid of staphylococcal toxins [22, 23]. The volunteers were inoculated with either wild-type 8325-4 or 8325-4 with βC-Φ13 containing the IEC. We used βC-Φ13 because it has the most predominant IEC type (type B) in humans and this type has been completely sequenced [4, 24]. Bacterial growth in tryptic soy broth was equal for both 8325-4 and 8325-4 with βC-Φ13 (data not shown).

Artificial inoculation protocol The artificial inoculation protocol was as described previously [22, 25]. In brief, before inoculation, the carrier state of the participants was defined on the basis of 2 nasal swab samples. A volunteer was classified as a persistent carrier with 2 nasal swab cultures positive and as an intermittent or non-carrier with one or no swab culture positive. Furthermore, a serum sample was drawn for determination of the C-reactive protein (CRP)

level, leukocyte number and antistaphylococcal antibody levels. The antibody levels were determined using a bead-based flow cytometry technique (xMAP®, Luminex Corporation) as described elsewhere [26]. After the collection of the second swab sample, decolonization treatment was initiated for all participants (nasal mupirocin 2%, GlaxoSmithKline) twice daily for 5 days in combination with once daily washing with chlorhexidin-containing soap (SSL Healthcare). Five weeks after the treatment, nostrils were swabbed again to assess the colonization status and artificial inoculation was performed under medical supervision with either wild type 8325-4 or 8325-4 with βC-Φ13. Inoculation was performed in a blinded fashion to prevent bias in reading the microbiological culture results. In both the left and right nostril $1x10^7$ colony forming units (cfu) of the same strain were applied. Participants received hygiene advise and weekly medical examinations. Follow-up cultures were performed on days 1, 2, 3, 4, 7, 14, 21 and 28 after inoculation. At the end of the study, participants underwent their last medical examination and serum samples were drawn to again determine CRP levels, leukocyte numbers and antistaphylococcal antibody levels. Furthermore, nasal, throat and perineum swabs were collected. Decolonization therapy was repeated for participants still carrying the inoculated strain at the end of follow-up.

Nasal swabs cultures Nasal swab cultures were performed as described previously [25, 27]. Both the left and right anterior nares were swabbed. The swabs were cultured quantitatively at 37°C on blood agar plates (Becton-Dickinson) to visualize hemolysis pattern and were submerged in phenol red mannitol salt broth. For suspected colonies, a latex agglutination test (Slidex® Staph Plus, bioMérieux) was performed. Three colonies of each morphotype were stored at -80⁰C. For all isolates, a *scn* and *spa* PCR was performed and isolates were typed by pulsed-field gel electrophoresis (PFGE) for identification and discrimination between inoculated and autologous *S. aureus* strains [4, 28].

Statistical analysis

Statistical analyses were performed with SPSS software, version 15.0. The χ^2 test was used for comparing proportions or frequencies. After artificial colonization, the primary outcome was the survival time of *S. aureus* in the nose. The survival time was defined as the number of days until the final positive culture with the inoculated strain. Kaplan-Meier survival analysis (log-rank test) was used to compare survival between strain 8325-4 and 8325-4 with βC-Φ13. Participants still carrying *S. aureus* in their noses at the end of follow-up were censored in the analysis. To compare the median number of cfu and antibody levels between groups, the Mann-Whitney *U* test was used. $P \leq 0.05$ was considered statistically significant.

RESULTS

Distribution and stability of IEC-carrying βC-Φ's in human *S. aureus* strains

Spa-typing showed that each of the 21 participants carried the same *S. aureus* strain at all 5 culture moments. Two of them incidentally carried an additional *S. aureus* strain. Isolates from 19 (90%) persistent carriers contained an IEC-carrying βC-Φ, as demonstrated by the presence of *scn*. The IEC in strains isolated from each individual was identical at all 5 culture moments in 18 of 19 (95%) carriers. The predominant IEC variant was type B (*sak*-, *chp*- and *scn*-positive), present in strains from 11 (58%) of 19 volunteers. Variant A (*sea*-, *sak*-, *chp*- and *scn*-positive), C (*chp*- and *scn*-positive), D (*sea*-, *sak*- and *scn*-positive), E (*sak*- and *scn*-positive) and G (*sep*-, *sak*- and *scn*-positive) were present in strains isolated from 4 (21%), 2 (11%), 1 (5%), 0 and 1 (5%) of the 19 carriers, respectively.

Prevalence of IEC-carrying βC-Φ's in veterinary *S. aureus* strains

Twenty-six of the 77 (33.8%) veterinary isolates were *scn*-positive. Strains isolated from animals infected with a 'typical animal strain' were positive for *scn* in 8.3% (2 of 24 strains). By contrast, strains isolated from animals infected with a 'typical human strain' were *scn*-positive in 24 of 53 (45.3%) isolates (P<0.001). Animals infected with a 'typical human strain' were mostly (pet) animals in close contact with humans (cats, dogs, rabbits and horses). Human isolates (n=73), sharing similar genetic backgrounds with the isolates obtained from animals infected with a 'typical human strain' (both integrated in nearly the same position in the AFLP dendogram), were *scn*-positive in 83.6% of strains.

Artificial nasal colonization with *S. aureus* strain 8325-4 with and without IEC-carrying βC-Φ

Nine volunteers (39%) were classified as persistent carriers. Fourteen volunteers were classified as non-carriers (61%). Five persistent carriers and 7 non-carriers were inoculated with strain 8325-4. The others were inoculated with strain 8325-4 with IEC-carrying βC-Φ. According to PFGE analysis and *spa*-PCR, in 3 carriers, including 2 carriers who still carried their own strain after mupirocin treatment, the follow-up cultures after inoculation contained their autologous strain or strains other than 8325-4 only. In none of these cultures the inoculated strain was detected, suggesting colonization resistance as a result of the enduring presence of their own strain. Therefore, these participants (one inoculated with the phage-positive strain and 2 with the phage-negative strain) were excluded from the analysis. For the remaining 20 participants, *S. aureus* survival and the number of cfu per swab sample were determined. The Kaplan-Meier curves in Figure 1 show the proportion of positive cultures during follow-up. The strain containing the IEC-carrying βC-Φ was eliminated significantly faster (median 4 days; range 1-14 days) than the strain without IEC-carrying βC-Φ (median 14 days; range 2-28 days; P=0.011). Antibody measurement showed that this was not a result of higher pre-existing levels of anti-CHIPS or anti-SCIN IgG in the group of volunteers who were

inoculated with the IEC-positive strain (median level of anti-CHIPS IgG, median fluorescence intensity (MFI) 11604 versus 12603; median level of anti-SCIN IgG, MFI 8423 versus 11580; $P>0.05$). Although the bacterial count after inoculation appeared to be higher for the group of participants inoculated with the IEC-containing strain, this was not statistically significant (Figure 2). No significant difference in survival and bacterial count of inoculated *S. aureus* was shown when non-carriers were compared with persistent carriers (median survival 4 days for both groups, range 1-28 days and 2-21 days, respectively), although this might be a

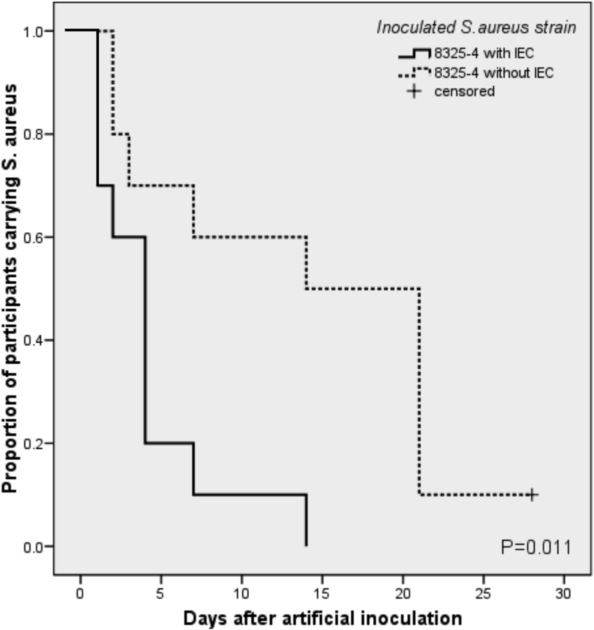

Figure 1. Kaplan-Meier survival curves showing proportions of individuals with culture-positive nasal swab samples after artificial nasal inoculation of *S. aureus* strain NCTC 8325-4 with or without immune evasion cluster (IEC)-carrying bacteriophage.

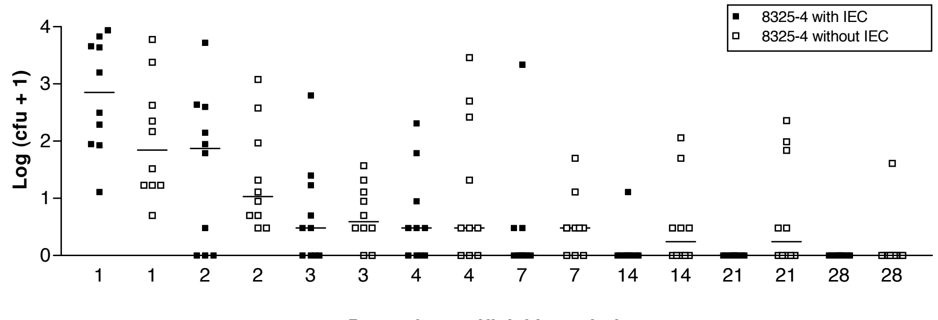

Figure 2. Bacterial counts after artificial inoculation. Each dot represents the amount of colony forming units (cfu) in a nasal swab culture of a volunteer collected 1, 2, 3, 4, 7, 14, 21 and 28 days after artificial nasal inoculation of *S. aureus* strain NCTC 8325-4 with or without immune evasion cluster (IEC)-carrying bacteriophage. Horizontal lines represent the median number of cfu.

result of the small number of persistent carriers included in the study. None of the volunteers experienced adverse events and all adhered to the study protocol. At the end of the study, all participants were in good physical condition. Laboratory values indicated no signs of infection (CRP <1-8 mg/L, leukocytes 4.5-9.5x10^9/L) and antistaphylococcal antibody levels showed no increase. All swabs were negative for the inoculated strain, except for the swabs from one volunteer who was therefore treated with mupirocin.

DISCUSSION

It is known that bacteriophages are intrinsically instable and that bacterial strains usually tend to lose their bacteriophages [29]. In the present study, we demonstrate that *S. aureus* strains from 90% of the persistent carriers contained an IEC-carrying bacteriophage that was present and indistinguishable in 95% of cases at all 5 culture moments over a 3-month period. This indicates that IEC-carrying bacteriophages are highly prevalent and stable over time in human *S. aureus* carriage isolates. The predominant IEC variant was type B, which is the predominant variant in human infectious isolates as well [4]. The prevalence of IEC-carrying βC-Φ's in the veterinary *S. aureus* isolates was much lower (33.8%). However, the percentage of IEC-carrying βC-Φ's in strains isolated from animals infected with human-related *S. aureus* strains (45.3%) was higher than in strains isolated from animals infected with animal-related strains (8.3%). Still, the percentage of IEC-carrying βC-Φ's in strains isolated from humans was much higher. This indicates that human-related *S. aureus* strains in animals appear to lose their bacteriophages, whereas *S. aureus* strains in humans do not. This apparent advantage of IEC-carrying βC-Φ's for human *S. aureus* strains, in combination with the high prevalence and stability, suggested a role for these bacteriophages in human *S. aureus* nasal colonization.

Using an artificial human inoculation study, we were unable to demonstrate an essential role for IEC-carrying bacteriophages in the initial stages of colonization, though. The latter is not a result of the lack of expression of the IEC-encoded proteins. Inhibition ELISA assays showed that, under *in vitro* conditions, both SCIN and CHIPS are produced in *S. aureus* strain 8325-4 with IEC-carrying βCΦ13. The production of SCIN and CHIPS in this strain is comparable to the production of these proteins in strains isolated from 8 of the persistent carriers included in the present study (data not shown). Perhaps, IEC-carrying βC-Φ's play a role in propagation and long-term survival, rather than adherence, of *S. aureus* in the nose. After all, these bacteriophages are present in almost all *S. aureus* carrier isolates and, in all individuals tested so far, significant levels of antibodies directed against CHIPS and SCIN are detected, indicating wide spread *in vivo* expression [26, 30]. Still, this does not explain why the phage-positive strain was cleared significantly faster than the phage-negative strain. A difference in growth rate *in vitro* was excluded. Furthermore, a difference in nose-picking behavior between the volunteers inoculated with the phage-positive strain and phage-negative strain

was excluded by analyzing the questionnaires that were filled in after inoculation. Perhaps, the introduction of IEC-carrying βC-Φ's altered the regulation and composition of surface and excreted components of 8325-4 that are normally involved in colonization and, therefore, the clearance of the phage-positive strain is faster. This question remains to be elucidated.

In conclusion, IEC-carrying βC-Φ's are highly prevalent among *S. aureus* strains, although these bacteriophages are not essential in the initial stages of nasal colonization. Further research into the role of IEC-carrying βC-Φ's in human nasal colonization is necessary.

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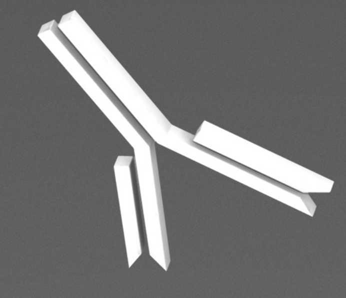
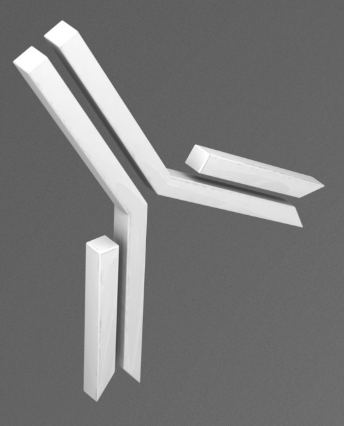
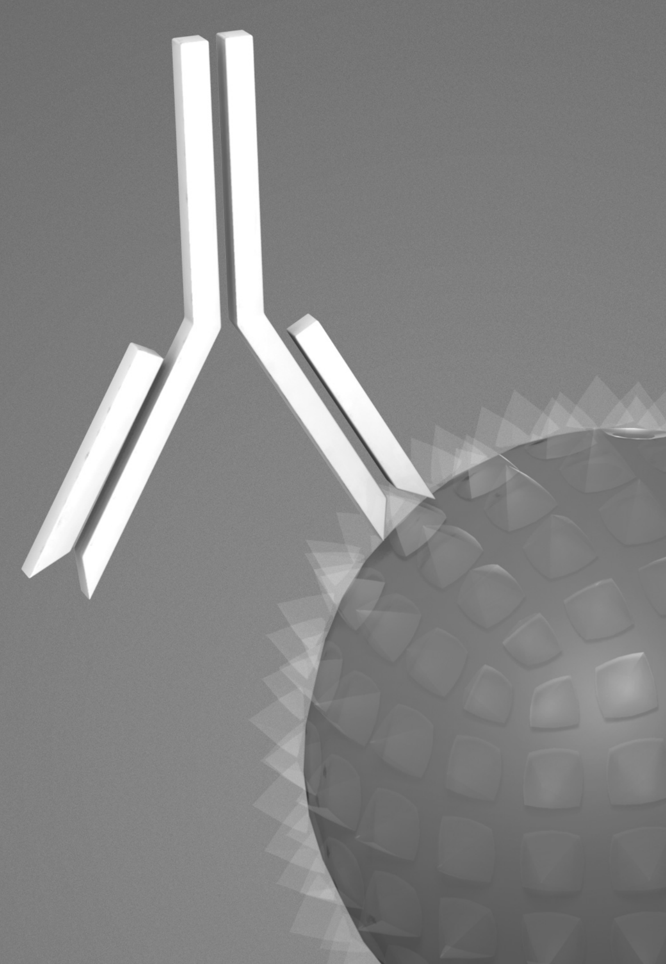


Chapter 9

Summary and discussion





SUMMARY AND DISCUSSION

Staphylococcus aureus is a human commensal, as well as an important pathogen that can cause infections ranging from mild (e.g. furunculosis) to life-threatening (e.g. bacteremia, osteomyelitis and endocarditis) [1]. Nasal carriage of *S. aureus* has been identified as a risk factor for *S. aureus* infection [1-3]. Consequently, eradication of *S. aureus* from the nasal cavities has been shown to prevent *S. aureus* infections [4-7]. However, decolonization may fail and does not protect against recolonization with *S. aureus* [8, 9]. Where *S. aureus* infections cannot be prevented, patients need treatment. Unfortunately, treatment options are becoming increasingly limited because of the emergence of antimicrobial resistance among *S. aureus* isolates [10, 11]. Consequently, novel approaches, including antistaphylococcal vaccines and immunotherapy, are urgently needed [12]. In order to develop such alternative strategies, understanding the determinants of carriage and understanding how humans respond to *S. aureus* exposure, is essential.

The primary aim of the research described in this thesis was to increase the current knowledge on the human antibody response to *S. aureus* proteins in colonization and infection and, thereby, to enhance knowledge on the immunogenicity and *in vivo* production of *S. aureus* proteins. An innovative high-throughput immunological assay (xMAP® Technology, Luminex Corporation) was developed to simultaneously quantify antibody levels to multiple *S. aureus* antigens in groups of healthy and diseased individuals. A secondary aim was to enhance insights in host-*S. aureus* interactions. An artificial human colonization model was used. In this model, volunteers are decolonized with the nasal ointment mupirocin and washes with chlorhexidin-containing soap. Five weeks thereafter, the noses of participants are inoculated with a single *S. aureus* strain or a mixture of strains. Then, the elimination kinetics of *S. aureus* are studied by collecting serial nasal swab samples, culture of these swab samples and genotyping of the identified *S. aureus* strains.

Main findings

In **Chapter 7** we showed, by using an artificial human colonization model, that persistent carriers tended to reselect their autologous strain from an inoculum mixture of different *S. aureus* strains. This indicates that host-*S. aureus* interactions are highly specific. Furthermore, we showed that persistent carriers stand out as a separate group of individuals in the population, distinct from both intermittent carriers and non-carriers. Intermittent carriers and non-carriers seem to belong to a single group of individuals who share the same risk of infection [13], the same nasal elimination kinetics and the same antistaphylococcal antibody profiles. In **Chapter 3** and **Chapter 7** we demonstrated that the antistaphylococcal antibody profiles in healthy individuals show extensive inter-individual variability. The levels of IgG directed to toxic shock syndrome toxin (TSST)-1 and the levels of IgA directed to TSST-1, staphylococcal enterotoxin (SE) A and clumping factor (Clf) A were significantly higher in persistent nasal

carriers than in intermittent or non-carriers, though. Furthermore, we showed that antibodies to TSST-1 had neutralizing capacity. The higher level of neutralizing antibodies might explain why bacteremic carriers have a lower risk of bacteremia-related death than non-carriers [14, 15]. However, from our data it cannot be concluded that these proteins are expressed during nasal colonization and that nasal colonization alone suffices to induce a humoral immune response. Since nasal colonization is a risk factor for infection, the level of antibodies in persistent carriers might also be higher due to preceding (minor) infections with *S. aureus*.

This may be different in healthy young children. Studying their immune response does allow us to distinguish the bacterial factors that are expressed *in vivo* during early colonization, since these children do not have an extensive medical history, do not use any medication and we suppose they experienced few, if any, *S. aureus* infections. In **Chapter 4** we showed that the IgG and IgA levels directed to a number of *S. aureus* proteins were significantly higher in colonized, hence more exposed, children as compared to non-colonized children. In both the first and second year of life, IgG levels directed to chemotaxis inhibitory protein of *S. aureus* (CHIPS), extracellular fibrinogen-binding protein (Efb) and iron-responsive surface determinant (Isd) H were significantly higher in colonized children. Furthermore, the anti-CHIPS, anti-IsdA and anti-IsdH IgA levels were higher. This indicates that CHIPS, Efb, IsdA and IsdH are expressed *in vivo* and might play a role in colonization during early childhood. Importantly, in **Chapter 4** it was also shown that maternal antistaphylococcal IgG antibodies do not protect the offspring against *S. aureus* nasal colonization.

CHIPS is an immune-modulating protein that blocks neutrophil chemotaxis by binding to the formylated peptide receptor and the C5a receptor on neutrophils [16-18]. The gene encoding CHIPS is located on a so-called 'immune evasion cluster' (IEC), which also encodes 3 other immune-modulating proteins: staphylococcal complement inhibitor (SCIN), staphylokinase (SAK) and SEA. IECs are located on β-hemolysin converting bacteriophages (βC-Φ's) [17]. Because of the immune-modulating function of the IEC-encoded proteins and the high prevalence of the IEC-carrying βC-Φ's in human carrier and infectious *S. aureus* isolates [17], a role for the IEC in human nasal colonization was hypothesized (**Chapter 8**). Using an artifical colonization study in which human volunteers were inoculated with *S. aureus* strain NCTC 8325-4 with or without IEC-carrying βC-Φ, we were not able to confirm our hypothesis and thus, to show an essential role for these bacteriophages in the initial stages of colonization. In contrast, the presence of IEC-carrying βC-Φ's seemed to expedite the elimination of *S. aureus*. Still, these bacteriophages are present in nearly all human *S. aureus* isolates and in all individuals tested so far significant levels of antibodies directed against CHIPS and SCIN are detected (**Chapter 3 and 4**), indicating wide spread *in vivo* expression [19, 20]. Perhaps, IEC-carrying βC-Φ's play a role in the long-term propagation of *S. aureus* in the nose, rather than in the initial adherence.

IsdA is one of the 'microbial surface components recognizing adhesive matrix molecules' (MSCRAMMs). IsdA interacts with and binds to fibrinogen and fibronectin of human cells [21].

It is involved in adherence of *S. aureus* to human desquamated nasal epithelial cells *in vitro* [22]. Furthermore, IsdA is required for nasal colonization in the cotton rat model [22] and IsdA protects *S. aureus* against the bactericidal activity of apolactoferrin *in vitro* [23]. In **Chapter 5** we followed 15 patients serologically from an early phase of bacteremia onwards. We were able to show that although each bacteremic patient develops a unique *S. aureus*-specific immune response after infection, the anti-IsdA IgG level increased in 12 of 13 patients (initial to peak level: 1.13-10.72 fold; $P<0.01$; days to reach peak level: 7-37). Thus, IsdA is clearly immunogenic in man and, therefore, actively produced by *S. aureus*, not only in young colonized children, but also in nearly all bacteremic patients.

In **Chapter 6** we showed that bacteremia caused by *S. aureus* is associated with presence of *sea* (OR 3.4, 95% CI 1.2-10.0) and *tst* (OR 5.7, 95% CI 1.6-20.8) in the infectious isolate. Furthermore, we demonstrated that staphylococcal toxins are immunogenic and, therefore, produced by *S. aureus* during bacteremia and other staphylococcal infections (e.g. bone infections and skin and soft-tissue infections). In 206 *S. aureus*-infected patients the IgG levels directed to exfoliative toxin (ET) A, ETB, γ hemolysin B (HlgB), leukocidin (Luk) D, LukE, LukS, SEA, SEE, SEH, SEI and SEM were higher than in 201 hospital-admitted controls ($P<0.05$). Furthermore, within the *S. aureus*-infected patient group, IgG levels were higher when genes encoding ETA, ETB, SEA, SEC, SEH, SEQ, TSST-1 or Panton-Valentine leukocidin (PVL) were present in the homologous isolate ($P<0.05$). Skin, soft-tissue, bone and joint infections were associated with presence of *luk-PV* in the infectious isolate.

Main conclusions

1. In patients as well as in healthy children and adults, the antistaphylococcal antibody profiles show extensive inter-individual variability. When groups are compared and significant differences are found, the level of antistaphylococcal antibodies is usually higher in the group that is more frequently exposed to *S. aureus*, either through colonization, infection or both.
2. The proteins CHIPS, Efb, IsdA and IsdH seem to play a role in *S. aureus* colonization in early childhood.
3. Placentally transferred maternal IgG antibodies specifically directed at a series of staphylococcal antigens do not protect the young infant against *S. aureus* nasal colonization in the first months of life.
4. The surface-protein IsdA is involved in the pathogenesis of *S. aureus* bacteremia. Furthermore, the toxins SEA and TSST-1 are involved in the pathogenesis of *S. aureus* bacteremia.
5. The pore-forming toxin PVL is involved in the pathogenesis of *S. aureus* skin, soft-tissue, bone and joint infections.
6. Intermittent and non-carriers of *S. aureus* seem to belong to a single group of individuals who share the same risk of infection, *S. aureus* nasal elimination kinetics and antistaphylococcal antibody profile. Persistent carriers form a separate group. Apparently, human

beings either tolerate *S. aureus* and become persistent carriers, or they do not tolerate this bacterial species and eliminate them from their epithelial surfaces.

7. IEC-carrying βC-Φ's are highly prevalent among human *S. aureus* carrier isolates, but they are not essential in the initial stages of nasal colonization.

Implications for vaccine development

In passive immunization, pre-synthesized antibodies directed against specific (parts of) micro-organisms are administered. Several observations from our studies suggest that, in the case of *S. aureus*, attempts to prevent or treat colonization or infection through passive immunization approaches are not likely to succeed. First, nasal colonization or minor (sub) clinical infections elicit a significant antibody response in persistent carriers, but this antibody response is not sufficient to eliminate *S. aureus* from the nose (**Chapter 3**). Secondly, maternally derived IgG antibodies specifically directed at staphylococcal antigens do not protect the young infant against *S. aureus* nasal colonization (**Chapter 4**). Thirdly, despite of the significant levels of antistaphylococcal antibodies in persistent carriers and *S. aureus*-infected patients, they are still susceptible to *S. aureus* infection **(Chapter 3, 5 and 6**). Furthermore, for a passive vaccine to work, the antigen(s) to which the vaccine is directed should be expressed *in vivo* in preferably all patients. However, in **Chapter 5** we showed that strains in different patients express different antigens. For example, the gene c*lfA* was present in all *S. aureus* strains isolated from bacteremic patients, but an antibody response directed against ClfA was detected in only 62% of them. If this is due to the lack of expression of ClfA *in vivo*, a hyperimmune vaccine preparation based on ClfA antibodies would have limited efficacy. This finding might also explain why Veronate, a passive vaccine partially based on ClfA antibodies, recently failed during Phase III clinical trials [24]. A role for passive immunization may be indicated in premature, critically ill infants and severely immunocompromised patients, though, [12, 25] and non-carriers with a low anti-TSST-1 titer (**Chapter 3**) might benefit from anti-TSST-1 antibodies, since it is known that humans with high titers against this toxin do not develop toxic shock syndrome [26].

In the case of active immunization, entire micro-organisms (e.g. inactivated *S. aureus*) or (combinations of) their subunits (e.g. CP, ClfA or PVL) are administered. Upon active vaccination, the body itself generates immunity against the target. This immunity comes from T-cells, B-cells and antibodies. Whether an active antistaphylococcal vaccine will succeed depends on the cellular immunity; our data does not support the concept that anti-*S. aureus* antibodies will be protective, as stated previously.

There are speculations about the optimal composition of an antistaphylococcal vaccine: it should be multi-component, the candidate antigens should be surface exposed and the candidate antigens should be expressed by the majority of clinical *S. aureus* isolates belonging to diverse lineages [12, 25, 27]. Our data suggest that IsdA would be an interesting target for inclusion in a multi-component vaccine: IsdA is surface exposed [21], *isdA* is present in all

S. aureus strains isolated from bacteremic patients **(Chapter 5)** and IsdA is expressed *in vivo* in nearly all bacteremic patients and colonized young children (**Chapter 4 and 5**). It remains to be elucidated whether staphylococcal toxins, involved in the pathogenesis of many *S. aureus* infections (**Chapter 6**) [28-31], should be included in an antistaphylococcal vaccine. On the one hand, the global vaccine potential of toxins seems restricted because most toxins are not ubiquitously present in *S. aureus* isolates (for example, only 20% of *S. aureus* strains carry TSST-1) [32]. On the other hand, toxin-containing vaccines might be useful in countries with a high prevalence of toxin producing *S. aureus* strains. Alpha-toxin seems an appropriate candidate: most *S. aureus* isolates possess *hla* (the structural gene for α-toxin) and α-toxin is one of the most potent bacterial toxins known [33]. By means of vaccination, biofilm formation and the hemolytic, cytotoxic, dermonecrotic and lethal effects caused by this pore-forming toxin might be reduced [33, 34].

Still, the feasibility of producing an effective antistaphylococcal vaccine is questionable, since *S. aureus* expresses several factors that can compromise the function of the human immune system. *S. aureus* secretes immune-modulating proteins that inhibit complement activation, neutrophil chemotaxis and neutralize antimicrobial defensin peptides (CHIPS, SCIN and SAK, **Chapter 8**) [17, 35]. Furthermore, *S. aureus* expresses several proteins that inhibit opsonisation by antibody and complement. Activated plasmin, formed by the conversion of plasminogen by SAK, removes important opsonic molecules, such as IgG and C3b, by cleaving these molecules [36, 37]. Protein A prevents opsonophagocytosis by binding the Fc region of IgG antibodies [38, 39]. *S. aureus* IgG-binding protein (Sbi) also interacts with the Fc part of IgG and acts as a complement inhibitor [40, 41]. Efb binds complement factor C3 and blocks its deposition on the bacterial cell surface. Complement activation beyond C3b attachment is prevented, thereby inhibiting opsonisation [42, 43]. Staphylococcal superantigen-like (SSL) proteins 7 and 10 bind IgA and IgG and inhibit complement activation [44, 45]. In addition, the capsular polysaccharide that most clinical isolates express, can compromise neutrophil access to complement and antibody bound on the cell wall [39, 46]. Finally, *S. aureus* can evade the immune system by intracellular persistence in endothelial cells, epithelial cells, fibroblasts, osteoblasts and keratinocytes for varying lengths of time [47].

In conclusion, antibodies directed against *S. aureus* cell wall-associated proteins, immune-modulating proteins and toxins proteins do not protect against nasal colonization or infection. Therefore, a vaccine aimed at the development of antibodies directed against these staphylococcal components is unlikely to succeed, especially when the immune evasive mechanisms of *S. aureus* are taken into consideration. Future research should focus on the vaccine potential of antigens that have not yet been studied in detail, in combination with the unravelling of the cellular immune responses directed against *S. aureus*. It is advisable to start with the determination of the cellular immune response directed against CHIPS, Efb, IsdA, IsdH, TSST-1, SEA and PVL, proteins that seem to play a role in *S. aureus* colonization or infection.

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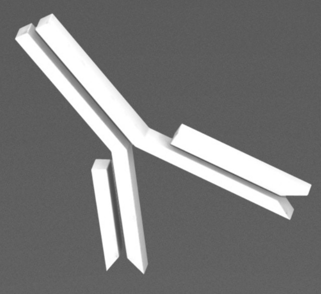
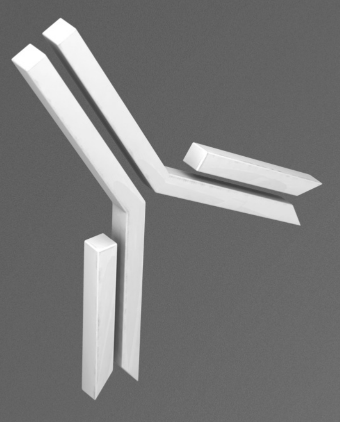
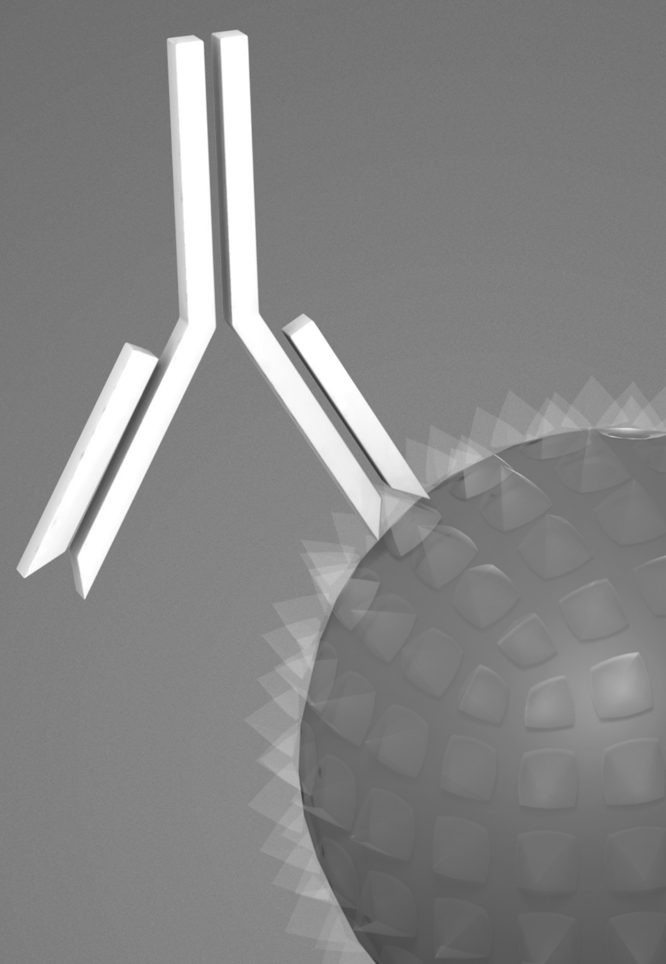


Chapter 10

Nederlandse samenvatting





NEDERLANDSE SAMENVATTING

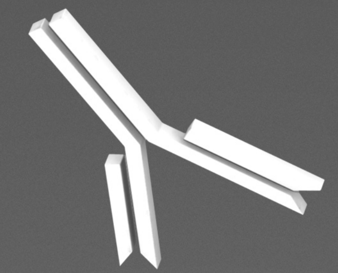
De bacterie *Staphylococcus aureus* koloniseert de neus van ongeveer 30% van de gezonde volwassenen permanent (persisterend dragers). Ongeveer 70% van de gezonde volwassenen zijn soms (intermitterend dragers) of nooit gekoloniseerd met *S. aureus* (niet-dragers). Mensen zijn vaak *S. aureus* drager zonder daar last van te hebben, maar dragerschap is wel een risicofactor voor het ontstaan van *S. aureus* infecties zoals furunculose (steenpuisten), bacteriëmie (infectie van de bloedbaan) en endocarditis (infectie van de hartkleppen). Helaas is *S. aureus* in de afgelopen jaren steeds minder gevoelig geworden voor diverse antibiotica. Daarom zijn andere methoden om *S. aureus* infecties te voorkomen of te behandelen, zoals vaccins of antilichaamtherapie, dringend nodig. Voor het ontwikkelen van deze methoden is het noodzakelijk om te weten hoe de mens reageert op *S. aureus* tijdens kolonisatie en infectie. Door middel van het onderzoek dat beschreven is in dit proefschrift hebben we de al bestaande kennis over dit onderwerp vergroot.

Eerst hebben we een systeem ontwikkeld op basis van flowcytometrie waarmee antilichamen (een deel van de menselijke afweer tegen bacteriën) tegen meerdere *S. aureus* eiwitten tegelijkertijd gemeten kunnen worden (**Hoofdstuk 2 en 3**). Met behulp van dit systeem zijn antilichamen tegen belangrijke *S. aureus* celwand-geassocieerde eiwitten, immuunmodulatoire eiwitten en toxines gemeten in gezonde volwassenen, kinderen en *S. aureus* geïnfecteerde patiënten. We tonen aan dat er grote verschillen bestaan tussen mensen in de antilichaamrespons gericht tegen *S. aureus*. Over het algemeen zijn de antilichaamniveaus hoger bij mensen die vaker aan *S. aureus* zijn blootgesteld. Dit betekent ook dat de *S. aureus* eiwitten waartegen de antilichamen gericht zijn, meer of vaker geproduceerd worden bij die groep mensen. Persisterend *S. aureus* dragers hebben hogere antilichaamniveaus tegen 'TSST-1' (toxic shock syndrome toxin-1), 'SEA' (staphylococcal enterotoxin A) en het celwand-geassocieerde eiwit 'ClfA' (clumping factor A) dan intermitterend dragers of niet-dragers (**Hoofdstuk 3 en 7**). Gekoloniseerde kinderen hebben hogere antilichaamniveaus tegen de immuunmodulatoire eiwitten 'CHIPS' (chemotaxis inhibitory protein of *S. aureus*) en 'Efb' (extracellular fibrinogen-binding protein) en tegen de celwand-geassocieerde eiwitten 'IsdA' (iron-responsive surface determinant A) en 'IsdH' dan niet-gekoloniseerde kinderen (**Hoofdstuk 4**). Patiënten met een *S. aureus* infectie hebben hogere antilichaamniveaus tegen de toxines 'ETA' (exfoliative toxin A), 'ETB', 'γ hemolysin B', 'LukD' (leukocidin D), 'LukE', 'LukS', 'SEA', 'SEE', 'SEH', 'SEI' en 'SEM' dan patiënten zonder een *S. aureus* infectie. Binnen de *S. aureus* patiëntengroep zijn de antilichaamniveaus hoger wanneer de genen die coderen voor 'ETA', 'ETB', 'SEA', 'SEC', 'SEH', 'SEQ', 'TSST-1' en 'PVL' (Panton-Valentine leukocidin), aanwezig zijn in de ziekmakende *S. aureus* stam. (**Hoofdstuk 6**). Bij patiënten met een *S. aureus* bloedbaaninfectie tonen we aan dat het antilichaamniveau tegen 'IsdA' bij 92% van de volwassen patiënten stijgt gedurende de infectie. (**Hoofdstuk 5**). 'IsdA' lijkt dus een rol te spelen in *S. aureus* bloedbaaninfecties.

Met behulp van zogenaamde 'artificiële humane inoculatiestudies' hebben we de inzichten in de interactie tussen de mens en *S. aureus* vergroot. In deze studies worden de neuzen van gezonde vrijwilligers kunstmatig gekoloniseerd ('de inoculatie') met één of meerdere *S. aureus* stammen en wordt bepaald hoe snel de aangebrachte stammen uit de neus verdwijnen. Met behulp van zo'n studie tonen we aan dat persisterend dragers na inoculatie hun eigen stam selecteren uit een mengsel van *S. aureus* stammen; de rest van de stammen verdwijnt uit de neus. Blijkbaar is persisterend dragerschap dus afhankelijk van een goede 'match' tussen *S. aureus* en de mens. Ook laten we zien dat intermitterend dragers en niet-dragers één groep vormen wat betreft de snelheid waarmee *S. aureus* uit de neus verdwijnt en wat betreft hun antilichaamniveau tegen *S. aureus* (**Hoofdstuk 7**). In **Hoofdstuk 8** hebben we de rol van 'immune evasion cluster (IEC)-positieve bacteriofagen' in *S. aureus* kolonisatie bestudeerd. Bacteriofagen zijn virussen die bacteriën kunnen infecteren. Het IEC codeert voor de eiwitten 'CHIPS', 'SEA', 'SCIN' (staphylococcal complement inhibitor) en 'SAK' (staphylokinase). Deze eiwitten remmen bepaalde delen van de aangeboren afweer. Ze zorgen er onder andere voor dat de witte bloedlichaampjes zich niet verplaatsen naar de plaats van infectie. Het is bekend dat bacteriofagen vaak voorkomen in *S. aureus* stammen die geïsoleerd zijn uit een *S. aureus* infectie (bijvoorbeeld uit een abces). In **Hoofdstuk 8** laten we zien dat IEC-positieve bacteriofagen ook vaak voorkomen in stammen geïsoleerd uit de neus van *S. aureus* dragers. Dit was eigenlijk geen verrassing: mensen krijgen het vaakst een infectie met de *S. aureus* stam die ze bij zich dragen. Met behulp van een artificiële inoculatie studie tonen we aan dat de IEC-positieve bacteriofagen geen rol spelen in de eerste fase van kolonisatie, de aanhechting van de bacterie aan de neus: de stam met een IEC-positieve bacteriofaag verdwijnt zelfs sneller uit de neus dan de stam zonder bacteriofaag.

In dit proefschrift laten we zien dat er grote verschillen tussen mensen bestaan wat betreft de antilichaamrespons tegen *S. aureus*. Over het algemeen zijn de antilichaamniveaus hoger bij mensen die vaker aan *S. aureus* zijn blootgesteld (bijvoorbeeld hoger bij persisterend dragers dan bij niet-dragers, hoger bij volwassenen dan bij kinderen, hoger bij geïnfecteerde patiënten dan bij gezonde mensen). Toch kunnen, ondanks de aanzienlijke aanwezigheid van antilichamen gericht tegen *S. aureus*, infecties bij persisterend dragers en patiënten (**Hoofdstuk 3, 5 en 6**), niet worden voorkomen. Ook kan kolonisatie bij pasgeboren kinderen niet voorkomen worden, ondanks de hoge antilichaamniveaus die zij van hun moeder via de placenta hebben gekregen (**Hoofdstuk 4)**. Het lijkt er dus op dat het toedienen van antilichamen ('passief vaccin') om *S. aureus* infecties te voorkomen of te behandelen niet zo veel zin zal hebben. Het is nog niet bekend of 'actieve vaccins' succesvol zouden kunnen zijn. Bij actieve vaccins moet het lichaam zelf de antistoffen en andere delen van de afweer (bijvoorbeeld T-cellen) aanmaken. Omdat het eiwit 'IsdA' geproduceerd wordt tijdens kolonisatie en infectie én aanwezig is in het overgrote deel van de *S. aureus* stammen, lijkt 'IsdA' in ieder geval een interessant eiwit voor inclusie in een vaccin. Toekomstig onderzoek zal zich moeten richten op het zoeken naar eiwitten die ook geïncludeerd zouden kunnen worden in een vaccin. Ook

zullen de andere delen van het afweersysteem, en de rol hiervan in de bescherming tegen *S. aureus* infecties, in kaart gebracht moeten worden.



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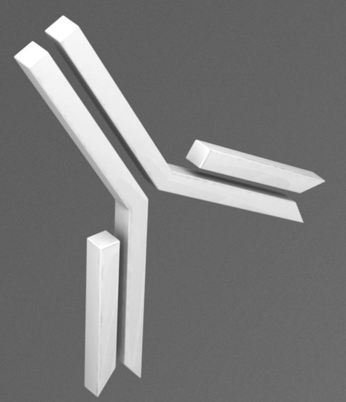

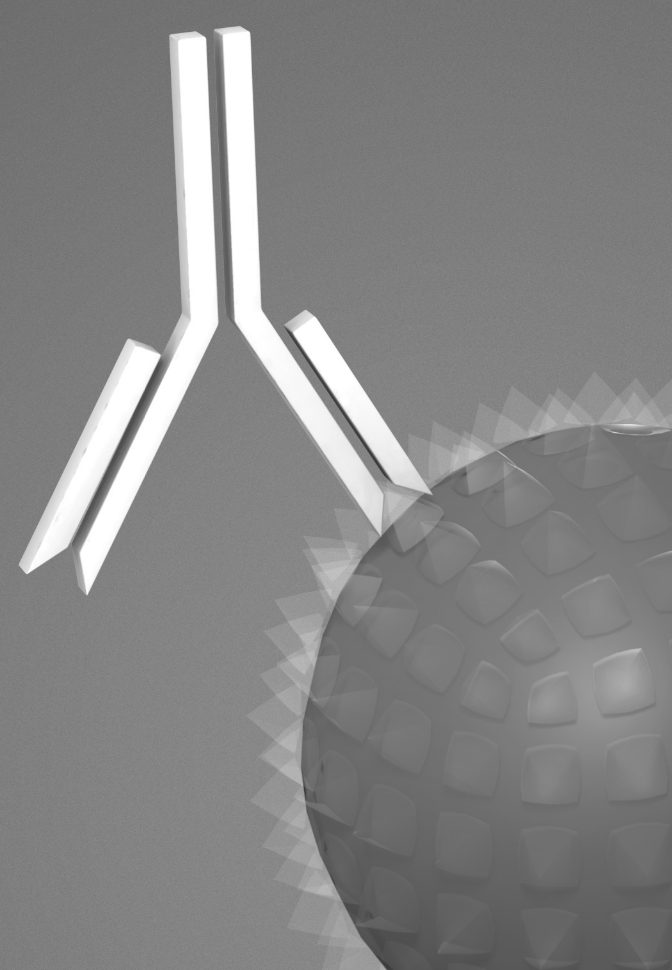

Dankwoord

Zonder de hulp van anderen kan geen proefschrift tot stand komen. Graag wil ik de mensen bedanken die op een of andere manier hebben bijgedragen aan dit proefschrift.

Prof. dr. dr. van Belkum, Alex, jij bent degene die mij motiveerde onderzoek te doen naar de antilichaamrespons tegen *Staphylococcus aureus*. Samen met Willem bedacht je dat ik een systeem moest opzetten om meerdere antigeen-specifieke antilichamen tegelijkertijd te kunnen meten. Dit was best een uitdaging, maar mede doordat je deur altijd open stond voor overleg is het in niet al te lange tijd toch gelukt. Ik heb je directheid als zeer prettig ervaren. Jouw snelheid van nakijken van manuscripten is ongeëvenaard! Dank voor het leuke en leerzame promotietraject.

Willem, jij leerde me de benodigde technieken in het laboratorium: gel-electroforese, transformatie van bacteriën, purificatie van eiwitten, etc, etc. Een flinke klus om dit te leren aan iemand zonder laboratorium ervaring! Na het experimentele werk kwam het schrijven van de manuscripten. Ook al waren we het niet altijd gelijk met elkaar eens, onze discussies hebben zeker bijgedragen aan de kwaliteit van dit proefschrift. Altijd was je beschikbaar om 'even' te overleggen. Ik wil je graag bedanken voor de goede, intensieve begeleiding.

Prof. dr. Verbrugh, u gaf mij de mogelijkheid te starten met het promotietraject. Uw kritische blik op de manuscripten en het proefschrift hebben het wetenschappelijke niveau ervan doen toenemen. Dank voor het plaatsnemen als secretaris in de kleine commissie.

Prof. dr. van Dijl, ik wil u bedanken voor het beoordelen van het proefschrift, uw mooie reactie hierop en het plaatsnemen in de kleine commissie.

Prof. dr. Hooijkaas, door het beschikbaar stellen van het apparaat op uw afdeling maakte u het mogelijk dat de geplande experimenten ook daadwerkelijk uitgevoerd konden worden. Ik heb veel van u geleerd over Immunologie. Dank voor het plaatsnemen in de kleine commissie en het beoordelen van het proefschrift. Diana en Claudia, bedankt voor jullie hulp bij het werken met het apparaat.

Eric, met veel enthousiasme leerde je mij omgaan met de techniek en mocht ik ook nog eens de eerste experimenten bij jou in het lab uitvoeren. Zonder jou was ik nu misschien nog steeds protocollen aan het lezen…

Corné, je begon als stagiair en nu ben je inmiddels al weer jaren 'echt' aan het werk. Hoeveel miljoenen beads zijn er al door je handen gegaan? Ik wil je bedanken voor je inzet tijdens alle experimenten. Ik heb prettig met je samengewerkt.

Hélène, we hebben samen zo'n 700 bloedagars beënt en bekeken. In je eentje vervolgens de resultaten analyseren, is iets dat je echt maar één keer doet... Verder heb je een aantal PCRs opgezet en uitgevoerd. Bedankt voor je bijdrage aan dit proefschrift.

Prof. dr. Etienne, Prof. dr. Vandenesch and Prof. dr. Lina, we visited Lyon to start a collaboration on the immunogenicity of toxins. This turned out to be a fruitful collaboration.

Prof. Etienne, thank you for being present at the defense. Hopefully, afterwards there will be plenty of time to buy some nice tulip bulbs…

Prof. dr. Bröker, when we visited Greifswald, you made us feel really welcome. The collaboration with your research group was pleasant and has lead to a nice publication. 'Vielen dank' for being present at the defense.

Dr. Vos, Prof. dr. van Strijp en Prof. dr. Rimmelzwaan, dank voor het plaatsnemen in de grote promotiecommissie.

Lonneke, mijn promotie- en opleidingstraject begon naast jou in kamer L-333. Je maakte me wegwijs op de afdeling en enkele keren zijn we samen in het buitenland geweest (ik zie onze koffers en badslippers nog drijven in de onder water gelopen kamer…). Bedankt dat je mij als paranimf terzijde wilt staan, daar ben ik heel blij mee! Frank, dank je wel voor het nakijken van de Nederlandse samenvatting.

Marjon, we studeerden allebei Biomedische Wetenschappen en Geneeskunde in Nijmegen, we verhuisden allebei naar Rotterdam, we volgen allebei een promotietraject, we zijn allebei in opleiding tot specialist. Ik jouw ceremoniemeester, jij mijn paranimf, logisch toch…?! Ik vind het erg fijn dat je naast me staat op deze dag.

Theo, dank voor het controleren of de *S. aureus* eiwitten die ik dacht opgezuiverd te hebben ook wel daadwerkelijk die eiwitten waren…

Mehri, jij hebt voor mij een flink aantal stammen gegenotypeerd. Ik wil je graag bedanken voor al je hulp.

Marianne, jij bent degene die het grootste aandeel heeft geleverd aan Hoofdstuk 8. Van vele vrijwilligers heb je neuskweken afgenomen en ook heb je veel PCR werk gedaan. Bedankt voor je bijdrage aan dit proefschrift.

Olivier, although we never met, our communication by e-mail went smoothly. Many excel files were sent from Lyon to Rotterdam and vice versa. You always responded very quickly. Thanks!

Silva, Dorothee and Julia, thank you for all the nice times we shared in Greifswald, Vilm, Cairns and Rotterdam. When will we eat knusperflocken again?

Prof. dr. Moll en Ankie, wij werkten samen aan een Generation R project. Ondanks enkele tegenslagen is er uit het onderzoek toch een mooi artikel ontstaan. Bedankt voor de samenwerking.

Heiman, Jan en Damian, jullie waren betrokken de inoculatiestudie(s). Bedankt voor het kritisch nakijken van de manuscripten en Jan, ook voor de rol als achterwacht.

Carla en Ger, bedankt voor doen van alle bestellingen en het maken van platen, buffers etc. Jullie ondersteuning is van grote waarde.

Marjan, jij deed onderzoek naar mastitis bij koeien. Ik heb hier deel van uit mogen maken. Jammer genoeg werk je niet meer bij ons. Ik wens je veel geluk met je verdere carrière.

Collega's van de Unit Diagnostiek, R&D en Infectiepreventie, steeds weer waren jullie bereid om belangeloos serum, neussnot en neuskweken af te staan. Ook heeft een aantal van jullie

zijn of haar neus ter beschikking van de wetenschap gesteld… Zonder jullie medewerking had ik het onderzoek niet kunnen uitvoeren. Dank hiervoor! Ook wil ik jullie graag bedanken voor de prettige samenwerking tijdens mijn promotie en opleiding.

Marian, Paula, Simone en Marja, ik kwam erg vaak bij jullie met een 'kort' vraagje. Ik wil jullie bedanken voor de secretariële ondersteuning (en ook voor de dropjes en gezelligheid…).

Collega arts-assistenten, samen maken we de leuke en minder leuke kanten van opleiding en promotie mee. Altijd fijn om alles weer even te bespreken tijdens een vrijdagmiddagborrel! Femke, het was gezellig om naast je te zitten tijdens het schrijven. Even bijpraten met een kop koffie was aan ons wel besteed... Leuk dat je binnenkort weer op de afdeling te vinden bent als internist-infectioloog in opleiding!

Stafleden Medische Microbiologie en Infectieziekten Erasmus MC en RdGG, bedankt voor jullie interesse in het onderzoek en voor de begeleiding tijdens mijn opleiding.

Suzanne, het onderzoek liep niet altijd even soepel… Fijn om dat met elkaar te kunnen bespreken. Heel veel succes met de laatste loodjes! Ook de andere mede-promovendi wil ik veel succes wensen met (de afronding van) het promotieonderzoek.

Collega's van de afdeling Klinische Chemie, ik heb bij jullie honderden serum monsters uit de serotheek gehaald. Bedankt voor jullie hulp bij het zoeken.

Lieve meiden, ik wil jullie graag bedanken voor de gezelligheid, afleiding en het fungeren als uitlaatklep. Dat er nog maar veel lunches, etentjes en borrels mogen volgen… Lieve andere vrienden en kennissen, bedankt voor jullie gezelligheid en belangstelling in het onderzoek.

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Lieve Niels, jij hebt als geen ander met me meegeleefd. Altijd weer kon ik mijn verhaal aan je kwijt en altijd weer wist je me gerust te stellen op momenten van stress. Je bent een lieverd!

Curriculum Vitae

Nelianne Jacomina Verkaik was born on July 23, 1980 in Oud-Beijerland, The Netherlands. She completed her secondary education at the Willem van Oranje in Oud-Beijerland in June 1998. The following four years she studied Biomedical Sciences, major Toxicology, at the Radboud University in Nijmegen. She wrote her term paper on 'Vascular pharmacology of adenosine-5'-triphosphate and dipyridamole' after a six month scientific training under supervision of Prof. dr. P. Smits and Dr. G. Rongen at the Department of Pharmacology and Toxicology. After obtaining her Master's degree in August 2002, she studied Medicine at the same University. In February 2004 she performed a clinical clerkship at the Department of Surgery, University Hospital Motol, Prague, Czech Republic, under supervision of Dr. J. Leffler. After receiving her Master's degree in Medicine in March 2006, she worked as a physician at the Department of Internal Medicine of the Medical Center Rotterdam Zuid. In January 2007 she started her PhD project on the humoral immune response to *Staphylococcus aureus* at the Department of Medical Microbiology and Infectious Diseases of the Erasmus Medical Center in Rotterdam, under supervision of Prof. dr. dr. A. van Belkum and Dr. W.J.B van Wamel. In January 2009 she initiated her speciality training in Medical Microbiology under supervision of Prof. dr. H.A. Verbrugh and Dr. R.W. Vreede.

List of publications

1. van Ginneken EE, Meijer P, Verkaik N, Smits P, Rongen GA. ATP-induced vasodilation in human skeletal muscle. Br J Pharmacol **2004**; 141:842-50.
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3. Verkaik NJ, de Vogel CP, Boelens HA, et al. Antistaphylococcal humoral immune response in persistent nasal carriers and noncarriers of *Staphylococcus aureus*. J Infect Dis **2009**; 199:625-32.
4. Tempelmans Plat-Sinnige MJ, Verkaik NJ, van Wamel WJ, de Groot N, Acton DS, van Belkum A. Induction of *Staphylococcus aureus*-specific IgA and agglutination potency in milk of cows by mucosal immunization. Vaccine **2009**; 27:4001-9.
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6. Verkaik NJ, Lebon A, de Vogel CP, et al. Induction of antibodies by *Staphylococcus aureus* nasal colonization in young children. Clin Microbiol Infect **2009**; in press.
7. Verkaik NJ, Dauwalder O, Antri K, et al. Immunogenicity of toxins during *Staphylococcus aureus* infection. Clin Infect Dis **2010**; 50:61-8.
8. Verkaik NJ, Boelens HA, de Vogel CP, et al. Heterogeneity of the humoral immune response following *Staphylococcus aureus* bacteremia. Eur J Clin Microbiol Infect Dis **2010**; 29:509-18.
9. Verkaik NJ, Benard M, Boelens HA, et al. Immune evasion cluster-positive bacteriophages are highly prevalent among human *Staphylococcus aureus* strains, but they are not essential in the first stages of nasal colonization. Clin Microbiol Infect **2010**; in press.

PhD Portfolio

Name PhD student:	Nelianne J. Verkaik
Erasmus MC department:	Medical Microbiology and Infectious Diseases
PhD period:	2007-2010
Research school:	Postgraduate School Molecular Medicine
Promotor:	Prof. dr. dr. A. van Belkum
Copromotor:	Dr. W.J.B. van Wamel

PHD TRAINING

General courses	**Year**	**ECTS**
Methodology of patient-related research	2007	0.3
Introduction to data analysis	2007	1
Desiderius School module 'Collaboration'	2009	0.3
Desiderius School module 'Communication'	2010	0.3
Specific courses		
Molecular Immunology	2007	2
Annual course Molecular Medicine	2008	1
Seminars and workshops		
Departmental Journal Clubs	2007-2010	2
Departmental Research Meetings	2007-2010	2
Research day Erasmus MC	2007	0.3
Scientific Spring Meeting NVMM	2007	1
MRSA symposium	2007	0.2
Symposium: 'Catheter-related bloodstream infections'	2008	0.2
TI Pharma Spring Meeting *(oral presentation)*	2008	1
2nd symposium Molecular Microbiology of Infectious Diseases	2008	0.3
Departmental Research day *(oral presentation)*	2008	1
Infectious Diseases symposium	2009	0.2
Scientific Meeting NVAMM	2009	0.3
Symposium: 'Success in research: learn from the expert'	2009	0.2
Scientific Spring Meeting NVMM	2009	1
Infectious Diseases symposium	2009	0.2
Scientific Fall Meeting NVMM/VIZ	2009	0.3
Departmental Research day *(oral presentation)*	2009	1

Scientific Spring Meeting NVMM	2010	1

International conferences

'International Symposium on Staphylococci and Staphylococcal Infections', Cairns, Australia *(poster presentation)*	2008	2
'Pathophysiology of Staphylococci', Bad Staffelstein, Germany *(poster presentation)*	2008	1
'Host pathogen interactions in generalized bacterial infections', Vilm, Germany *(oral presentation)*	2009	1
'International Symposium on Staphylococci and Staphylococcal Infections', Bath, United Kingdom *(poster presentation)*	2010	2

TEACHING

Lecturing

Wintercourse of the Infection and Immunity research master, Postgraduate School Molecular Medicine *(oral presentation)*	2010	1

Supervision of bachelor students

Supervision of a bachelor student Life Sciences	2007-2008	2

Supervision of medical students

Supervision of 2nd year medical students during practical sessions in the Theme 'Infectious Diseases and Immune Disorders'	2007-2010	5.4