

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 noncarriers. SEs are superantigens and, therefore, potent proinflammatory agents [15]. They have been implicated in the pathogenesis of toxic shock [15, 16]. The immune modulators 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 levels of antibodies to 9 MSCRAMMs, 7 SEs, and 3 immune-modulating proteins in serum samples and nasal secretions from well-defined persistent carriers and noncarriers and measured the stability of anti-staphylococcal antibody levels over time.

METHODS

Serum samples, nasal secretions, and nasal swab samples. 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 noncarriers 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 facilitate reproducible handling. The secretions and serum samples were stored at -80°C until use. Human pooled serum (HPS) from 36 healthy donors of unknown *S. aureus* nasal carriage state 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 dipeptide repeat proteins (Sdr) D and E were expressed with a His tag in *Escherichia coli* XL1-blue strain and purified under denaturing conditions with nickel-nitrilotriacetic acid 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. 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. Rooijackers (University Medical Center Utrecht) provided the recombinant proteins CHIPS and SCIN. Prof. J. I. Flock (Karolinska Institutet) supplied the Efb [22–25].

Coupling methods. To quantify antibodies directed against the 19 *S. aureus* proteins simultaneously, the recently introduced microsphere (bead)–based flow cytometry technique (xMAP; Luminex) 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 5.0×10^6 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 *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, and 0.05% sodium azide [pH 7.4]). The microspheres were protected from light and stored at 4°C 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 nonspecific binding. In case of nonspecific 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 de-

scribed 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 microliters per diluted sample was incubated with the microspheres in a 96-well filter microtiter plate (Millipore) for 35 min at room temperature 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 μ L of assay buffer. In separate wells, 50 μ L of a 1:200 dilution of R-phycoerythrin (RPE)-conjugated AffiniPure goat anti-human IgG and IgA and 50 μ L 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 room temperature on the plate shaker and washed. The microspheres were resuspended in 100 μ L 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 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 min, 1×10^5 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 72 h, quantified by calculating the area under the proliferation curve, 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 median differences in anti-staphylococcal antibody levels and the median neutralizing capacities of serum from persistent carriers and noncarriers. To compare the antibody levels in the first and second serum samples 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 $<.05$.

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 nonspecific binding (with the exception of IgM in 1 sample). The negative control (PBS-BN) incubated with protein-coupled beads resulted in low MFI values (<10).

Interassay 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%, and the range was 5% (CHIPS) to 25% (SEO); for IgA, the median CV was 20%, and the range was 7% (Efb) to 25% (SdrD, SEB, SEI, and SasG); and for IgM, the median CV was 16% and the range was 7% (ClfA) to 43% (SEO; relatively high CV due to MFI values close to 0). Earlier studies found equal CVs for interassay variation [26, 29–31].

Differences in antigen-specific antibodies in serum from persistent carriers and noncarriers. Nineteen volunteers were classified as noncarriers (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 noncarriers. However, the median serum levels of IgG directed against TSST-1 and SEA were significantly higher in persistent carriers than in noncarriers (MFI value, 11,554 vs. 4291 [$P < .001$] and 742 vs. 218 [$P < .05$], respectively). Additionally, the median IgA serum level was significantly higher in persistent carriers than in noncarriers for TSST-1 (973 vs. 155; $P < .01$), SEA (127 vs. 32; $P < .05$), ClfA (1661 vs. 441; $P < .05$), and ClfB (792 vs. 356; $P < .05$). The MFI values reflecting IgG levels were highest for CHIPS, SCIN, and TSST-1; those for IgA were highest for CHIPS, SCIN, and Efb; and those for IgM the were highest for ClfA and SasG.

Stability of anti-staphylococcal 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 swab samples 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 a noncarrier 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 ($P > .05$). Figure 2 shows representative results for the stability of IgG levels for 4 *S. aureus* proteins.

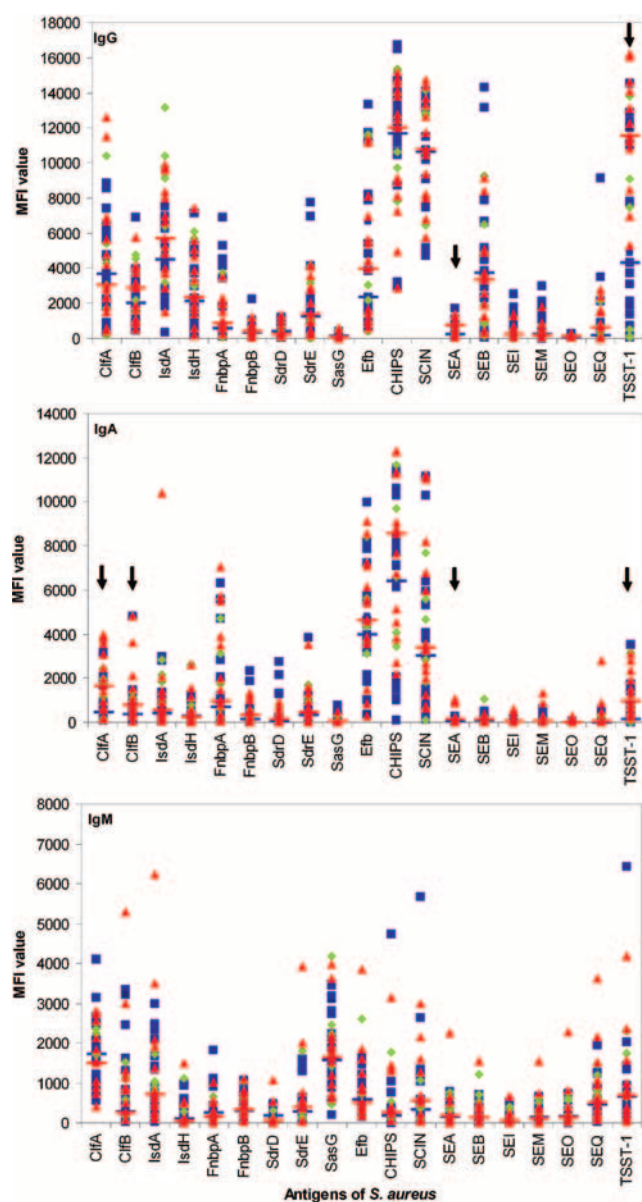


Figure 1. Median fluorescence intensity (MFI) values reflecting levels of antigen-specific IgG, IgA, and IgM for 19 *Staphylococcus aureus* antigens in 40 volunteers. Each symbol represents a single volunteer; red triangles represent persistent carriers, green diamonds represent intermittent carriers, and blue squares represent noncarriers. Horizontal lines indicate median levels of anti-staphylococcal antibodies for persistent carriers and noncarriers, and arrows indicate statistically significant differences in median values (persistent carriers vs. noncarriers, Mann-Whitney *U* test). For IgG, differences were significant for toxic shock syndrome toxin (TSST)-1 (11,554 vs. 4291; $P < .001$) and staphylococcal enterotoxin (SE) A (742 vs. 218; $P < .05$); for IgA, differences were significant for TSST-1 (973 vs. 155; $P < .01$), SEA (127 vs. 32; $P < .05$), and clumping factor (Clf) A and B (for ClfA, 1661 vs. 441; for ClfB, 792 vs. 356; $P < .05$). CHIPS, chemotaxis inhibitory protein of *S. aureus*; Efb, extracellular fibrinogen-binding protein; Fnbp, fibronectin-binding protein; Isd, iron-responsive surface determinants; Sas, *S. aureus* surface protein; SCIN, staphylococcal complement inhibitor; Sdr, serine-aspartate dipeptide repeat protein.

Correlation between anti-staphylococcal antibody levels in serum and nasal secretion. To determine the correlation between anti-staphylococcal 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. The correlation coefficient for the comparison between serum and nasal secretions was 0.87 for IgG and correlation 0.77 for IgA (figure 3).

TSST-1 neutralization assay. The neutralizing capacity of TSST-1-specific antibodies in the 40 human samples was determined. The median neutralizing capacity was significantly higher in persistent carriers than in noncarriers (90.6% vs. 70.6%; $P < .05$) (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).

DISCUSSION

We developed an *S. aureus* multiplex immunoassay that enables simultaneous quantification of antibodies to 19 antigens in small serum volumes. This assay is therefore more informative and less time- and serum-consuming than the conventional ELISA technique. The methods was used to determine the levels of antigen-specific IgG, IgA, and IgM in serum samples from persistent carriers, intermittent carriers, and noncarriers of *S. aureus*. An important message of our analyses is that anti-staphylococcal antibody levels showed extensive interindividual variability (figure 1), probably owing to the variable number of previous encounters with different *S. aureus* strains of diverse antigenicity as well as interindividual differences in the ability to mount an antigen-specific humoral immune response. In the group of persistent carriers, differences in carrier strain type (as determined by pulsed-field gel electrophoresis [PFGE]; data not shown) might also contribute to the diversity in antibody levels. Thirteen different PFGE types were found; only 3 of 15 persistent carriers carried the same strain.

The most striking difference between persistent carriers and noncarriers was the median level of IgG to TSST-1 ($P < .001$). 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

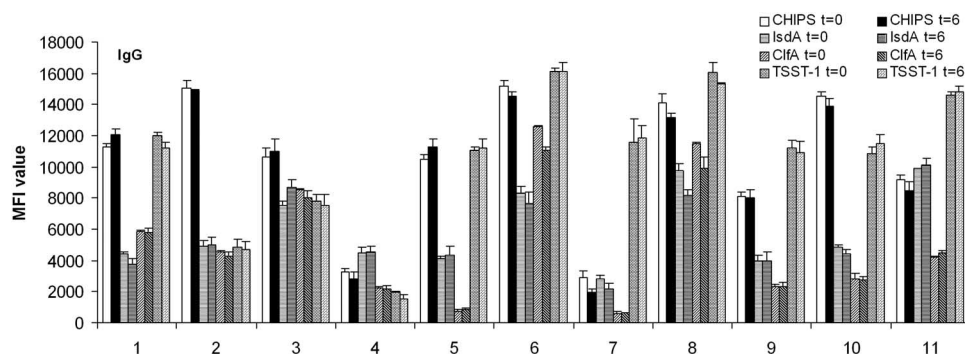


Figure 2. Stability of IgG levels, reflected by median fluorescence intensity (MFI) values for *Staphylococcus aureus* proteins in serum samples from 11 healthy volunteers (1–11) at 0 and 6 months ($t = 0$ and $t = 6$). Error bars represent SEs. CHIPS, chemotaxis inhibitory protein of *S. aureus*; Clf,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

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 noncarriers 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 noncarriers. These observations should be verified by studying persistent carriers and noncarriers with bacteremia; their anti-TSST-1 antibody levels should be determined and correlated with the outcomes of infection (work in progress).

Other significant differences between persistent carriers and noncarriers were found for IgG directed against SEA ($P < .05$) and IgA directed against TSST-1 ($P < .01$), SEA, ClfA, or ClfB ($P < .05$). These levels were found to be higher in persistent carriers than in noncarriers. Two other studies focusing on anti-staphylococcal antibodies showed higher IgG levels for major autolysin, ClfB, IsdA, IsdH, IsaA, Map-w, and α -hemolysin in noncarriers than in persistent carriers [9, 12]. Although these authors did not measure anti-enterotoxin antibodies or antibodies to immune-modulating proteins, the differences found between persistent carriers and noncarriers differed from our data. One possible explanation for this apparent discrepancy is that the carrier state was less well defined in these studies. Dryla et al. [12] defined persistent carriers and noncarriers 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 noncarriers as individuals who were culture positive or negative for *S. aureus* on the basis of just a single nasal swab sample, which cannot reliably distinguish between the different carrier states. Thus, the differences in anti-staphylococcal antibody levels observed in these studies might be explained by the fact that carriage was not defined according to a precise and validated “culture rule,” which is based on 2 nasal swab samples and quantitative culture data [2]. In the present study, we used at least 3 nasal swab samples collected at 2-week intervals to define the carrier state.

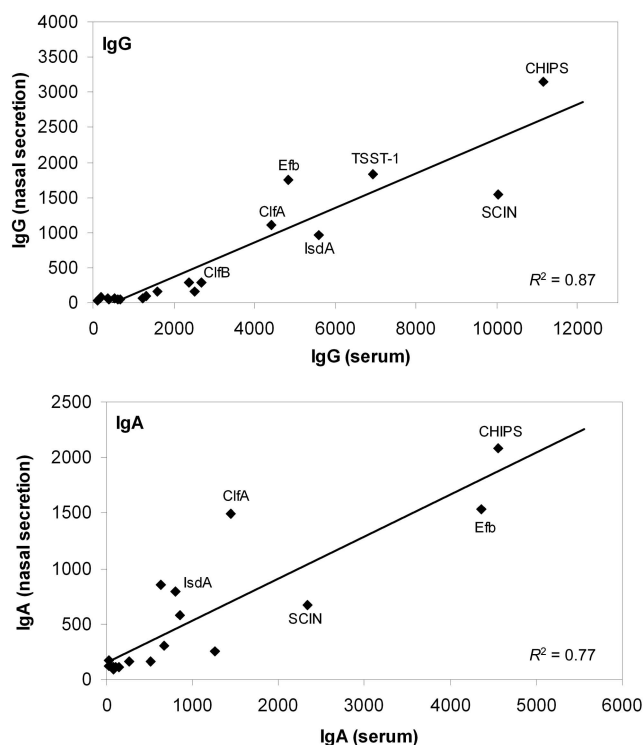


Figure 3. Correlation between IgG and IgA levels in serum and nasal secretions. Mean IgG and IgA levels in serum and nasal secretions, reflected by median fluorescence intensity values, were calculated for each protein. Pearson's correlation coefficient was used. CHIPS, chemotaxis inhibitory protein 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.

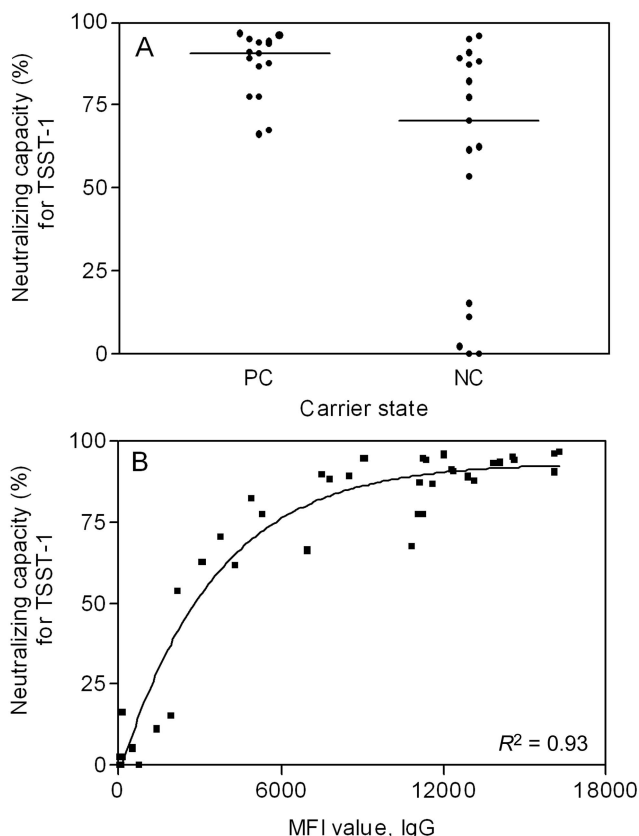


Figure 4. A, Higher neutralizing capacity for toxic shock syndrome toxin (TSST)–1 in persistent carriers (PC) than in noncarriers (NC) (median, 90.6% vs. 70.6%; $P < .05$, Mann-Whitney U test). Two noncarriers were excluded because of poor technical replicates. B, High correlation between the level of IgG antibodies to TSST-1, reflected by median fluorescence intensity (MFI) values, and the neutralizing capacity of the serum samples (nonlinear regression, $R^2 = 0.93$).

The observed IgG and IgA MFI values were highest for CHIPS and SCIN, indicating that these staphylococcal proteins are quite immunogenic. The IgM values were highest for SasG, ClfA, and IsdA. This can be due to a primary immune response to recent exposure to these antigens or to so-called natural IgM antibodies that appear in the absence of stimulation by specific antigens and that are secreted by long-lived, self-renewing B cells belonging to the B1 subset [34]. These natural antibodies are commonly polyspecific and play an important role in the antimicrobial response in humans [35]. Even though the measured IgM antibodies may not be antigen specific, they were directed mostly to MSCRAMMs. This implies that MSCRAMMs are structures that are recognized in an early phase by the immune system and that natural IgM antibodies recognize antigens in a very economic way [34]. 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 [36]. Stability is the result of humoral memory. Humoral mem-

ory is assumed to rely on long-lived plasma cells, which even without antigenic contact will secrete antibodies for many years, and memory B cells, which can be (re)activated by antigen and/or polyclonal stimuli [37].

Components of nasal secretions that complement the innate host defense include IgG and IgA [21, 38]. Therefore, antigen-specific IgG and IgA levels in nasal secretions were determined. IgG and IgA values were highest for CHIPS. Anti-staphylococcal 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 that 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 $\sim 4:1$. In mucosal secretions, IgA is produced almost exclusively as a dimer, and the ratio of IgA1 to IgA2 is $\sim 3:2$ [39, 40]. Therefore, although 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% [41]; a rectal carrier, 3.2% [42, 43]; and an axilla carrier, 2% [44]. In our study, this would mean that only a few of the intermittent and noncarriers 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.

Although our study was focused exclusively on antibodies directed against *S. aureus* proteins, it should be noted that cell-wall components (such as capsular polysaccharide 5 and 8 [45], peptidoglycan [46] and lipoteichoic acid [47]) are also immunogenic. Therefore, including these cell-wall components in future studies is important; this is the topic of our current methodological investigations.

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 anti-staphylococcal 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 a neutralizing capacity, and median levels of antibodies to TSST-1, SEA, ClfA, and ClfB are higher in persistent carriers than in noncarriers. 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* noncarriers with bacteremia [5].

Acknowledgments

We thank Claudia Brandt-Hagens and Diana Dufour–van den Goorbergh of the Department of Immunology, Erasmus Medical Center, for their help

with the Luminex system. We thank Suzan Rooijackers and Jan-Ingmar Flock for kindly supplying the *S. aureus* proteins. Special thanks go to Silva Holtfreter for providing the *S. aureus* toxins and for her help with the assay.

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