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Antibody responses in patients with invasive infections

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Title: Antibody Responses in Patients with Invasive Staphylococcus aureus infections

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Abstract: Purpose

Correlation between antibody response and clinical outcome in *S. aureus* bacteremia has yielded conflicting results. Immunization schedules have failed in clinical trials. Is the humoral response toward *S. aureus* of protective nature?

Methods

A prospective study was performed in patients with Invasive Staphylococcus aureus infections (ISA) during 2003-2005. The antibody levels were determined at the beginning and at the end of treatment, and one month later (n=96, n=71, n=51). As controls, 115 healthy individuals were used. A quantitative ELISA against eight purified antigens was performed. Bacterial isolates were grouped as to production of alpha-toxin, agr-type and PFGE-type.

Results

Great variations were seen in the antibody levels. Levels in the second sample were the highest. A correlation between agr-group, PFGE-group, alpha-toxin production and initial antibody levels was observed. Patients with fatal outcome displayed lower initial antibody levels to all antigens and significantly to teichoic acid, lipase, enterotoxin A, and scalded skin syndrome toxin. In episodes with complicated bacteremia, initial significantly low levels to teichoic acid and lipase were registered.

Conclusions

Low initial antibody levels against several antigens were associated with increased mortality and complicated bacteremia in invasive *S. aureus* infections. Bacterial properties, strain and toxin-production, affected the antibody response.

Response to Reviewers: Skövde

2010-01-18

Alex van Belkum
Editor-in-Chief
European Journal of Clinical Microbiology & Infectious Diseases

Dear Editor.

Thank you for very valuable comments on our manuscript!
We enclose a new, revised manuscript of EJCMIID-D-09-00433 Antibody responses in Patients with Invasive Staphylococcus aureus infections.

Reviewers' comments:

Version 2 is improved from version 1. However, there were still many areas where the language was very shaky. If the authors can clean it up I recommend the manuscript be accepted for publication. Below is a list of some of the areas that need modification.

Thank you for the opportunity to serve in the review process.

Sincerely,
Eric Brown

Line 7-8 reads:

Staphylococcus aureus continues to cause medical problems in the community and in all kinds of medical care.

Should read:

Staphylococcus aureus continues to cause medical problems in community and healthcare settings.

Has been changed accordingly

Lines 15-23 read:

A microbiological enigma is the immunologic response in man towards this bacterium. Almost everybody is now and then colonized with this S. aureus; the population has serum antibodies to most antigens tested for, while rises in serum antibody levels during invasive infections vary greatly ?4?.

Should read:

A microbiological enigma is the immunologic response in humans towards this bacterium since most individuals are exposed to S. aureus and can be classified as carriers, intermittent carriers or non-carriers of the bacteria. High levels of exposure to this organism (among humans) is supported by the observations of human populations describing serum antibodies to most antigens tested for, while rises in serum antibody levels during invasive Infection types vary greatly ?4?.

Has been changed accordingly

Line 22 reads:

Data from experimental animal studies ?5, 6??indicate.

Should read:

Data from experimental animal studies ?5, 6??indicated.

Has been changed accordingly

Page 2. Line 40-45 reads:

S. aureus pathogenicity is multifactorial, and this fact is related to the experience that immunity to S. aureus infections is difficult to achieve and that proper diagnostic serological tools are scarce.

Should read:

S. aureus pathogenicity is multifactorial and related to the array of virulence factors among different S. aureus isolates and the inability of
Has been changed accordingly

humans to develop protective immunity against this pathogen.

[Not sure what you mean by diagnostic tools. The tools are there; therapeutics are not.]

Page 2. Line 50-60 reads:

The use of S. aureus serology is of limited clinical value. For diagnostic purposes serology is dependent upon accurate quantitative test methods and a proper combination of antigens, however, there is no evidence that any serological assay or combination of assays can distinguish between complicated and uncomplicated S. aureus bacteraemia [11]. Instead, there is a mounting interest in the human humoral response to S. aureus in perspective of future passive and active vaccine development [10, 12]. This involvement is hampered by a lack of knowledge about the production of possible specific, protective antibodies in humans during infection, especially in large clinical surveys.

Should read:

Because the use of S. aureus serology is of limited clinical value for diagnostic purposes (there is no evidence that any serological assay or combination of assays can distinguish between complicated and uncomplicated S. aureus bacteraemia [11]) there is a mounting interest in the human humoral response to S. aureus in the context of developing future passive and active vaccination strategies [10, 12]. However, the development of these formulations has been hampered by a lack of knowledge regarding the nature of the specific, protective antibodies in humans during infection, especially in large clinical surveys.

Has been changed to: The use of S. aureus serology is of limited clinical value for diagnostic purposes (there is no evidence that any serological assay or combination of assays can distinguish between complicated and uncomplicated S. aureus bacteraemia [11]). However, there is a mounting interest in the human humoral response to S. aureus in the context of developing future passive and active vaccine strategies [10, 12]. The development of these formulations has been hampered by a lack of knowledge regarding the nature of the specific, protective antibodies in humans during infection, especially in large clinical surveys

Materials section:

Study population

Should read:

A prospective study was performed in consecutive patients with ISA over a two year period between 2003-2005, in western Sweden using healthy individuals as controls [13,14]. The antibody response in patients was determined by taking three serum samples; one at the time of diagnosis (n=96), one following completion of antibiotic treatment (n=71) and the last sample collected during convalescence i.e, one month after the end of treatment (n=51). From the 115 healthy control individuals one serum sample was obtained. Consent was obtained from the patients or their relatives after written and oral information.

Has been changed accordingly

Definitions

Should read:

Severe sepsis and complicated bacteraemia are described in [14]. For this study, all patients presenting with either hypotension, hypoperfusion or organ dysfunction were diagnosed with severe sepsis. Complicated bacteraemia was defined as bacteraemia presenting with a secondary focus, such as endocarditis, spondylitis, osteomyelitis, arthritis, as a deep-seated abscess or pneumonia. Line-associated infections, urinary tract infections nor soft-tissue infections with no metastatic seeding or bacteraemia without focus were not defined as complicated bacteraemia.

Has been changed accordingly

ELISA

Should read: two-fold not twofold.

Also the authors stated that Clf had been changed to CflA. It Still reads Clf in this section.

Has been changed accordingly

Statistics

Should read: 'geometric means' not 'geometric mean'. Also should read 'using the Chi-square test' not 'with Chi-square test'.

Has been changed accordingly

Page 8. Line 7 reads:

PFGE group 1 was also correlated with tst carriage ($p < 0.001$) among the strains and strains.

I suggest it be changed to 'correlated with the presence of tst among.' since the word 'carriage' in the context of *S. aureus* is used specifically to define the colonization status of humans.

Has been changed accordingly

Page 8. Line 8 reads:

'belonging to PFGE group 1 significantly correlate'

Should read:

'belonging to PFGE group 1 significantly correlated'

Has been changed accordingly

Page 9. Lines 24-25 read:

Patient with osteomyelitis (n=13) showed a significantly high proportion of increasing levels of Clf A, and.

Should read:

Patients with osteomyelitis (n=13) showed a significantly high proportion of increasing levels of ClfA, and.

Has been changed accordingly

Page 9. Line 39-41 reads:

(83% had a first high response compared to 31%).

Not sure what you mean by 'first high response'.

Has been changed to: (83% had high antibody level at presentation as compared to 31% in patients with no previous history of ISA).

Page 10. Lines 36-39 reads:

Clf A in sample 1 and 3, and the antibody levels for alpha-toxin in sample 1 and 3.

Should read:

ClfA in sample 1 and 3 and the antibody levels to alpha-toxin in samples 1 and 3.

Has been changed accordingly

Page 10. Line 41 reads:

to Efb in sample 1 and 3, and to the antibody levels to Clf A in sample 1, 2 and 3.

Should read:

to Efb in samples 1 and 3 and to the antibody levels against ClfA in samples 1, 2 and 3.

Has been changed accordingly

Page 10. Line 54 reads:

'Dryla ???reported of a pronounced heterogeneity of antibody.'

Should read:

Dryla ???reported a pronounced heterogeneity in antibody.'

Has been changed accordingly

Page 11. Lines 5-10.

Our cohort was more homogenous, with only invasive infections, and still great variations in antibody levels were seen. The timing of the samples was not the reason for this, as there was no major correlation between time from start of symptoms to sampling and antibody levels.

Should read:

'Our cohort was more homogenous consisting of only invasive infections and great variations in antibody levels were still observed. The timing of when serum samples were examined for anti-S. aureus antibodies was not the reason for this, as there was no major.'

Has been changed accordingly

Don't know what you mean by 'correlation between time from start of symptoms to sampling and antibody levels.'

Answer:

There was no correlation between antibody levels and symptom time, i.e. the time from start of symptoms and to serum sampling

Page 11. Lines 11-17 reads:

'develop a dysregulation of the humoral immunity affecting quality and quantity of the antibody response. They display lower levels against several antigens and notably towards the superantigens included in this study'

Should read:

'develop a dysregulation of humoral immune responses that can affect the quality and quantity of the antibody response. They displayed lower antibody levels against several antigens; notably towards the superantigens examined in this study.'

Has been changed accordingly

Page 11. Lines 34-42 reads:

'In fact, it is seen in Figure 2B that about 70 % of the patients had a high (H) level against lipase in one of the samples and 95 % of all patients showed a "positive serology (PS)" against this antigen alone. This finding indicates that patients in general do react to S. aureus antigens, although to varying extent.'

Should read:

'In fact, as seen in Figure 2B, about 70% of patients had high (H) antibody levels against lipase in one of the samples and 95% of all patients showed "positive serology (PS)" against this antigen alone. This finding indicated that patients in general do react to S. aureus antigens, although to varying extents.'

Has been changed accordingly

Page 11. Line 51 reads:

'rise in antibody level in.'

Should read:

'rise in antibody levels in.'

Has been changed accordingly

Page 11 line 59 and page 12 line 1 reads:

'immune response, as opposed to other risk groups as patients with diabetes mellitus.'

Should read:

'immune response, compared to other risk groups, for example, patients with diabetes mellitus.'

Has been changed accordingly

Page 12. Lines 1-2 read:

'In order to implement successful strategies as immunoglobulin therapy or vaccination it is important to know the patients' immune status.'

Should read:

'In order to implement successful strategies such as immunoglobulin therapies or vaccinations, it will be important to know the patient's immune status.'

Has been changed accordingly

Page 11. Line 24-25 reads:

'Still, we do not believe this is the cause why we did not register more diagnose-specific differences.'

Should read:

'Still, we do not believe this is the reason why we did not observe more diagnosis-specific differences.'

Has been changed accordingly

Page 11. Line 27 reads:

'which invasive infection, a polyclonal B-cells response is generated.'

Should read:

'which type of invasive infection, a polyclonal B-cells response is generated.'

Has been changed accordingly

Page 11. Line 37.

Should read 'in antibody levels' not 'of antibody levels.'

Has been changed accordingly

Page 11 and 12. Last line of Page 11 and lines 1-5 page 12 read:

'The significance of this observation could be argued. We can not conclude if the observed correlation between low initial antibody levels and a fatal outcome (Table 3) is of causative art or due to a general weak immune response.'

Should read:

'The significance of this observation could be argued since we cannot conclude if the observed correlation between low initial antibody levels and a fatal outcome (Table 3) was the cause of the infection or if it resulted from generally weak immune responses.'

Has been changed to: The significance of this observation could be argued since we cannot conclude whether the observed correlation between low initial antibody levels and a fatal outcome (Table 3) was caused by a lack of specific immune responses or if it resulted from general weak immune responses

Page 12. Lines 17-22 read:

'The innate immunity in old patients with concurrent diseases could be a major denominator of clinical outcome. A lack of specific immune response to other antigens than tested in our study is possible.'

Should read:

'The innate immune response in older patients presenting with concurrent infections could be a major denominator of clinical outcome. A lack of specific immune response to other antigens than those tested in this study is also likely.'

Has been changed to: The innate immune response in older patients presenting with concurrent diseases could be a major denominator of clinical outcome. A lack of specific immune response to other antigens than those tested in this study is also possible.

Page 12. Line 32.

Is ASTA defined?

Has been defined: ASTA (Anti-staphylolysin assay)

Yours sincerely

Gunnar Jacobsson

**Antibody Responses in Patients with Invasive Staphylococcus aureus
infections**

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Introduction

Staphylococcus aureus continues to cause medical problems in the community and healthcare settings. The emergence of multidrug-resistant strains [1-3] has heightened the need for non-antimicrobial approaches.

A microbiological enigma is the immunologic response in humans towards this bacterium since most individuals are exposed to *S. aureus* and can be classified as carriers, intermittent carriers or non-carriers of the bacteria. High levels of exposure to this organism (among humans) is supported by the observation of human populations describing serum antibodies to most antigens tested for, while rises in serum antibody levels during invasive infections vary greatly [4]. Data from experimental animal studies [5, 6] indicated that this mainly polyclonal B-cell response with antibodies of low affinity is not protective. The vast majority, 60 to 85%, of antibodies in healthy individuals are induced by lipoteichoic acid, which fails to promote opsonisation [7, 8]. An individual who has suffered from a *S. aureus* infection is usually not protected from a subsequent infection and to date in clinical trials the development of protective active immunization schedules has failed [9, 10].

S. aureus pathogenicity is multifactorial and related to the array of virulence factors among different *S. aureus* isolates and the inability of humans to develop protective immunity against this pathogen.

The use of *S. aureus* serology is of limited clinical value for diagnostic purposes (there is no evidence that any serological assay or combination of assays can distinguish between complicated and uncomplicated *S. aureus* bacteraemia [11]). However, there is a mounting

1 interest in the human humoral response to *S. aureus* in the context of developing future
2 passive and active vaccine strategies [10, 12]. The development of these formulations has
3
4 been hampered by a lack of knowledge regarding the nature of the specific, protective
5 antibodies in humans during infection, especially in large clinical surveys.
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12 The objective of this study was to describe the humoral immune response to *S. aureus* in a
13 well-characterized patient cohort with Invasive *S. aureus* infections (ISA).
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17 18 19 **Materials and methods**

20 21 **Study population**

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27 A prospective study was performed in consecutive patients with ISA over a two year period
28 between 2003-2005, in western Sweden using healthy individuals as controls [13,14]. The
29 antibody response in patients was determined by taking three samples: one at the time of
30 diagnosis (n=96), one following completion of antibiotic treatment (n=71), and the last
31 sample collected during convalescence i.e. one month after the end of treatment (n=51). From
32 115 healthy individuals one serum sample was obtained.
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42 Consent was obtained from the patients or their relatives after written and oral information.
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46 47 **Definitions**

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51 Severe sepsis and complicated bacteraemia are described in [14].
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54 For this study, all patients presenting with either hypotension, hypoperfusion or organ
55 dysfunction, were diagnosed with severe sepsis. Complicated bacteraemia was defined as
56 bacteraemia presented with a secondary focus, such as endocarditis, spondylitis,
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1 osteomyelitis, arthritis, as a deep-seated abscess or pneumonia. Line-associated infections,
2 urinary tract infection nor soft-tissue infection with no metastatic seeding or bacteraemia
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4 without focus were not defined as complicated bacteraemia.
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9 **Antigens**

10 Ribitol teichoic acid (TA) and Alpha-toxin (AT) were purchased from PhPlate AB
11
12 (Stockholm, Sweden). Enterotoxin A (SEA), Toxic Shock Syndrome Toxin 1 (TST), and
13
14 Scalded Skin Syndrome toxin (SSS) were purchased from Toxin Technology (Florida, USA).
15
16 Lipase was kindly supplied by S. Tyski (Medical University of Warzaw, Poland), Clumping
17
18 factor A (ClfA) and Extracellular fibrinogen binding protein (Efb) by J.I. Flock (Karolinska
19
20 Institute, Stockholm).
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29 **ELISA**

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31 The enzyme-linked immunosorbent assay (ELISA) to determine serum IgG antibodies has
32
33 been described earlier [15]. The antigen coating concentration was 1 µg/ml for TA, 3.5 µg/ml
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35 for AT, 2 µg/ml for ClfA, 0.6 µg/ml for Efb, and 1 µg/ml for TST, SEA and SSS. Serum
36
37 samples were diluted two-fold in PBS-T (1/250 to 1/2000) in AT, ClfA, Efb, TST and SSS
38
39 assays and (1/2500 to 1/20000) in TA and lipase assays. The reference serum consisted of
40
41 pooled sera from six patients with confirmed sepsis [15]. The four dilutions, controls and
42
43 reference serum were applied and incubated for 1 h at 37°C. Thereafter alkaline phosphatase
44
45 conjugated to goat anti-human antibodies (γ-chain) (Sigma Chemical Co., St. Louis, Mo.)
46
47 diluted 1/3000 in PBS-T was added, and incubation was continued for 2 h at 37°C. Finally,
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49 the enzymatic reaction was measured at 405 nm in a Titertek Multiscan microplate reader
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Interpretations of ELISA results.

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2 The absorbance values were transformed into arbitrary units by using the reference line units
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4 calculation method [16]. The dilution curve of each sample was drawn up in parallel to that of
5
6 the reference serum, after which the two curves were compared. The reference serum was
7
8 given the value of 1,000 (arbitrary) units for all antigens and the patient serum antibody levels
9
10 were expressed in these units. Thus the unit levels obtained for each antigen cannot be
11
12 directly compared others, since the absolute values are contingent on those of the reference
13
14 serum.
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19 The upper limits for “normal antibody levels” were established as the upper 95th percentiles
20
21 of the levels in the healthy individuals. Levels above this cut-off point were designated as
22
23 high levels (H). A significant increase in antibodies was defined as a 50% increase between
24
25 two samples (I) and a significant decrease of antibodies was defined as a 50% decrease (D).
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27
28 Positive serology (PS) was considered to exist when a high level was noted in at least one
29
30 serum sample from a patient with at least two samples and/or a significant increase/decrease
31
32 of antibody levels. Some sera were designated as low levels (L). The limits for these levels
33
34 varied from 50 to 150 for the different antigens as determined in terms of the reproducibility
35
36 of each respective test. The actual limit values for high and low levels are plotted in Figure 1.
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Typing of isolated *S.aureus* strains

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45 *Agr* group typing was performed as previously described [14].
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49 Genome typing by pulse field gel electrophoresis was performed in principle as described by
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51 Tenover [17]. Visual interpretation of the banding pattern according to cited criteria were
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53 made, resulting in a cut-off level of 75% in the dendrograms generated, above which strains
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55 were considered to be closely related.
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Production of alpha-haemolysin

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2 Screening for alpha-haemolysin production was carried out on nutrient agar plates (8 mm
3
4 high) containing rabbit erythrocytes. The diameter of the bacterial colonies on the agar plates
5
6 after incubation at 37°C overnight was < 3 mm and the diameter of the zone of haemolysis
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8
9 (mm), was determined.
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Statistics

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18 Data were analyzed with SPSS (version 15.0) and Excel 97. Antibody levels were presented
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20 as arithmetic/geometric means, and comparisons between groups regarding quantitative
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22 variables were analyzed using the Mann-Whitney U nonparametric test regarding. Category
23
24 variables were analyzed using the Chi-square test.
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Results

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33 The median age was 68 y (mean 64 y) in the patient group as compared with 70 y (mean 61 y)
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35 among the controls. Fifty-six percent of the infected patients were men, while the
36
37 corresponding figure for the healthy individuals was 59%.
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Antibody levels in all patients

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41
42 The antibody levels of all patients were distributed as depicted in Figure 1 for the various
43
44 antigens. It is evident that antibody levels against teichoic acid, α -toxin and lipase were higher
45
46 than those of healthy individuals of corresponding ages. Great interindividual variation in
47
48 antibody levels was seen, ranging from 50 to 5000 units against the same antigen in the same
49
50 sample category. As expected, the levels achieved in the second sample (T2), at the end of
51
52 treatment, were generally the highest, while the median levels varied relatively little between
53
54 the three sample occasions. Exceptions to this were lipase and α -toxin.
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60 High levels (H) were, concordantly, most common for teichoic acid, α -toxin and lipase, where
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1 25 to 42% of the samples were positive in the second sample (Figure 2A). In the third sample,
2 drawn at least on month after finished treatment, the levels against alpha-toxin and lipase
3 remained high.
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7 The kinetics of the antibody levels are shown in Figure 2B, which shows the frequencies at
8 which the patients appeared to react to the different antigens. With the exception of SSS, 40 to
9 95% of the patients reacted to a staphylococcal antigen, where lipase stimulated the highest
10 number of patients, regardless of kinetic parameter measured. Sixty per cent of the patients
11 showed an increase in antibody levels against this antigen, and almost 40% showed a
12 decrease. This latter value was probably related to the high levels of antibodies in the first two
13 samples (Figures 1 and 2).
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27 **Bacterial properties**

28 Bacterial strains belonging to *agr* groups I and II produce significantly larger zones of
29 hemolysis as compared with strains belonging to *agr* group III (Table 1), indicating a
30 correlation between *agr* group and production of alpha-toxin. The zones of hemolysis were
31 significantly correlated with levels of antibodies against alpha-toxin at presentation (Table 1).
32 We also found a significant correlation between *agr* group III and a low initial anti-alpha-
33 toxin antibody level ($p < 0.001$) as determined Chi-square analysis (Table 2).
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44 Screening for *tst* among the strains revealed that 17% of the strains were positive for the *tst*
45 gene with 90% of the *tst*-positive isolates belonging to *agr* group III ($p < 0.001$). A correlation
46 between *tst* among the strains and absolute anti TST antibody levels (sample 1 $p = 0.002$ and 2
47 $p < 0.001$) was found among the patients. As seen in Table 2, *agr*-group III correlates to higher
48 TST antibody levels.
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56 A significant correlation between *agr* groups and PFGE groups was found in agreement with
57 earlier studies. Interestingly, we also found that PFGE group 1, which was correlated with
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1 small zones of hemolysis (Table 1), was correlated with low levels of anti alpha-toxin (Table
2). PFGE group 1 was also correlated with the presence of *tst* ($p<0.001$) among the strains
3 and strains belonging to PFGE group 1 correlated significantly with high levels of anti TST
4 antibody among the patients (Table 2).
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9 Surprisingly, levels of antibodies to teichoic acid, a cell wall constituent expressed in all
10 strains, was significantly correlated to *agr*-group. Strains belonging to *agr*-group 1 showed a
11 positive serology response in 45% of the patients, as compared with 78% of strains belonging
12 to *agr*- group 3 (Table 2). This pattern was even more pronounced in the PFGE groups, as
13 seen in Table 2.
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21 We did not screen for the gene for Scalded Skin Syndrome toxin, SSS. However, in
22 accordance with the antibody response against the other superantigen TST, differences were
23 found between the various *agr*-groups and PFGE-groups. PGFE-group 2 and *agr*-group 1
24 were correlated with initial low levels of antibodies, as compared with *agr*-group 2 which
25 correlated with higher initial antibody levels (Table 2).
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36 **Clinical outcome**

37 Patients with a fatal outcome only displayed significantly lower initial antibody levels to 4
38 antigens (Table 3). The same trend was noted for 5 antigens in patients with complicated
39 bacteraemia, but not for TST, SEA and SSS. The mean age was significantly higher in the
40 patients with a fatal outcome (79y vs. 63y, $p=0.014$). Patients with severe sepsis were also
41 significantly older (75 y vs. 60 y, $p<0.001$), but no difference in mean age was registered in
42 patients with complicated bacteraemia (65 y vs. 64 y). We did not detect any significant
43 differences in the time from start of symptoms to inclusion in the study, or to the first blood
44 sample, in any group, although patients with a fatal outcome tended to have shorter duration
45 of symptoms.
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2 The antibody levels in sample 2 and 3 did not differ significantly between patients with
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4 different clinical outcome, such as in patients with bacteraemia (n=84), complicated
5
6 bacteraemia (n=32), severe sepsis (n=28), or in patients suffering from a sequelae (n=37).
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11 No major differences in the antibody production related to the tissue involved were noted.
12
13 However, patients with endovascular infections (n=7) tended to have initial lower antibody
14
15 levels towards all antigens. Patients with bacteraemia without focus (n=19) tended to have
16
17 higher antibody levels towards several antigens. Patients with osteomyelitis (n=13) showed a
18
19 significantly high proportion of increasing levels of ClfA, and a significantly higher frequency
20
21 of positive serology, than patients without osteomyelitis.
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29 Patients with a previous history of ISA (n=14) had a trend toward lower risk of both a fatal
30
31 outcome and complicated bacteraemia. They were also significantly less prone to developing
32
33 severe sepsis, and their initial antibody response to alpha-toxin tended to be higher (83% had
34
35 high antibody level at presentation as compared to 31% in patients with no previous history of
36
37 ISA). No endovascular infections were registered in patients with a previous history of ISA.
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43 Figure 4 shows the antibody levels in patients with collagenosis (n=14), a group at risk of
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45 developing ISA [6]. Low antibody levels as compared with patients without collagenosis were
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47 noted. Patients with collagenosis were not significantly older (69 y vs. 66 y), had the same
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49 duration of symptoms (4 days), but received longer antibiotic treatment (94 days vs. 46 days),
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51 and tended to have longer periods of hospitalization (31 days vs. 18 days). Other patients at
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53 risk of developing ISA, such as patients with diabetes mellitus (n=27), and patients with
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55 kidney disease (n=13) showed no major differences in antibody levels. Patients with liver
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1 disease (n=13) showed (as opposed to collagenosis patients) significantly higher antibody
2 levels against several antigens.
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7 Older people (>65 y) displayed significantly lower proportion of positive serology against the
8 superantigens TST, SEA and SSS (Table 4). The mean antibody levels in older patients were
9 also lower than in younger patients against all the antigens except Efb in the first sample,
10 while they were significantly lower (Mann-Whitney) only against SSS at the end of antibiotic
11 therapy and one month later (data not shown).
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21 We did not discern any major differences in the antibody response of men and women.
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26 The duration of symptoms correlated positively only to the antibody level to lipase in sample
27 2, but correlated negatively to lipase in sample 1. We noted a positive correlation between
28 duration of hospitalization and the antibody levels to ClfA in samples 1 and 3, as well as the
29 antibody levels for alpha-toxin in samples 1 and 3. The length of antibiotic treatment was
30 positively correlated with the antibody levels against Efb in sample 1 and 3, and to the
31 antibody levels against ClfA in samples 1, 2 and 3.
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43 **Discussion**

44 This is one of few studies comparing the humoral immune response in a cohort of well-
45 characterized patients with ISA with healthy individuals. Dryla [7] reported a pronounced
46 heterogeneity in antibody levels against 19 *S. aureus* antigens among both healthy individuals
47 and patients with an *S. aureus* infection. However, the patient group was not uniform,
48 consisting of 17 patients with wound infection, 15 subjects with bacteraemia and sepsis, and
49 10 patients with miscellaneous infections.
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1 Our cohort was more homogenous consisting of only invasive infections and great variations
2 in antibody levels were still observed. The timing of when serum samples were examined for
3 anti-*S.aureus* antibodies was not the reason for this, as there was no major correlation
4 between time from start of symptoms to sampling and antibody levels. One obvious
5 explanation is that older people (>65 y) develop a dysregulation of humoral immunity
6 responses that can affect the quality and quantity of the antibody response. They displayed
7 lower antibody levels against several antigens: notably towards the superantigens examined in
8 this study (Table 4). Aging is associated with a shift in antibody isotype from IgG to IgM, and
9 a decline in the activity of lymphocytes [18, 19]. Staphylococcal serology is further
10 complicated by the findings of immunomodulating activities in *S. aureus*, which may be
11 exerted by antigens such as EAP, lipoteichoic acid and peptidoglycan [20-22].

12 In spite of weak antibody response in general, Figures 1 and 2 show elevated levels of
13 antibodies, as compared with the healthy control group, especially against alpha-toxin and
14 lipase. In fact, as seen in Figure 2B, about 70 % of patients had high (H) antibody levels
15 against lipase in one of the samples and 95 % of all patients showed a “positive serology
16 (PS)” against this antigen alone. This finding indicated that patients in general do react to *S.*
17 *aureus* antigens, although to varying extent. Analysis of serum IgG with reactivity against
18 teichoic acid and alpha-toxin identified 80 % of patients with “positive serology”. These
19 contradictory findings may be explained by the fact that, in spite of relatively low absolute
20 levels, the immune system is activated during disease and increases and/or decreases in
21 antibody levels can therefore be detected. There was a rise in antibody levels in more than 40
22 % of the patients even against the TST antigen (Figure 2B).

23 Different groups, displaying variable antibody levels, are at higher risk of ISA [13]. Patients
24 with rheumatic disorders develop weak immune responses, compared to other risk groups, for
25 example, patients with diabetes mellitus. In order to implement successful strategies such as

1 immunoglobulin therapies or vaccinations, it will be important to know the patient's immune
2 status. We did not demonstrate significantly lower antibody levels among haemodialysis
3 patients. Trials with an anti-capsular vaccine in these patients [9,10] have been unsuccessful
4 in eliciting a protective immune response. One proposed explanation is their weak immune
5 response.
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11 Invasive infections include infections with variable tissue-tropism, for example, endovascular
12 infections, bone infections, and deep soft tissue infections. Surprisingly, we detected only
13 minor differences in antibody levels related to clinical diagnosis. In some cases it is
14 questionable whether the clinical diagnosis was correct. Only 40 % of the patients were
15 examined with ultrasound examination as part of the diagnosis of endocarditis [14], so we
16 may have underestimated the rate of endocarditis. Still, we do not believe this is the reason
17 why we did not observe more diagnosis-specific differences. Regardless of which type of
18 invasive infection, a polyclonal B-cells response is generated [5, 6,23] and may mask a
19 specific, tissue-orientated reaction. However, patients with very high antibody levels were
20 tested for antibody levels against non-related bacterial antigens such as *E. coli* endotoxin and
21 *S. pneumoniae* pneumolysin (data not shown), and there was no sign of a polyclonal increase
22 in antibody levels.
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44 There was a tendency, in some instances significantly, towards a lower antibody response
45 against several antigens in patients with complicated bacteraemia as compared to patients
46 with uncomplicated bacteraemia (Table 3). We have shown this previously for alpha-toxin,
47 teichoic acid and lipase [4]. Others, however, have not made the same observation [24, 25].
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56 To our knowledge, this is the first time an association has been found between a fatal outcome
57 and initial low antibody response in ISA, with the exception of TST [26-28]. The significance
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1 of this observation could be argued since we cannot conclude whether the observed
2 correlation between low initial antibody levels and a fatal outcome (Table 3) was caused by a
3 lack of specific immune responses or if it resulted from general weak immune responses.
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9 The potential of improved treatment including immunoglobulin therapy has not yet been
10 properly evaluated. A clinical trial with a pooled human immunoglobulin preparation with
11 high antibody titers against ClfA failed, as compared with placebo, to reduce sepsis and
12 mortality [29]. The patient group consisted of infants; interpretation for other age groups is
13 unclear.
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21 The innate immune response in older patients presenting with concurrent diseases could be a
22 major denominator of clinical outcome. A lack of specific immune response to other antigens
23 than those tested in this study is also possible.
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28 However, we cannot rule out the possibility that among the examined antibodies, there may
29 be a protective response embedded in the polyclonal reaction. Teichoic acid has been shown
30 to be an essential factor for colonization [30]. In our study, patients with endovascular
31 infections tended to have lower initial antibody levels to all antigens. Ruotsalainen [31] found
32 an elevated initial ASTA (Anti-staphylolysin assay) titer significantly more often among
33 injection drug users without endocarditis than among individuals with endocarditis (44% vs.
34 6%). There were no endovascular infections among our patients with recurrent ISA infections,
35 and they displayed higher initial levels of antibodies against alpha-toxin. Vaccination with
36 alpha-toxin in an animal model has been found to protect against *S. aureus* pneumonia [32].
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54 Dryla [7] showed that high-titer antistaphylococcal antibodies remain stable for years in
55 healthy individuals, providing evidence of these antibodies being functional on the basis of
56 opsonophagocytic and neutralizing activity. In a study of paediatric patients infected with
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1 community-acquired *S. aureus* isolates [33], it has been demonstrated that patients infected
2 with Panton-Valentin leucocidin (PVL)-positive strains developed a dominant IgG anti-PVL
3 antibody response. This response was attributable to a specific humoral response against the
4 antigens Lukf and LukS at the expense of antibody response against other virulence factors.
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6 Holtfreter [34] reported that carriers neutralize superantigens with antibodies specific for their
7 colonizing strain which may explain the observation [35] that carriers who develop sepsis
8 have a better prognosis than to non-carriers. Animal studies have also demonstrated that it is
9 possible to elicit a protective response to various bacterial components including
10 MSCRAMMs (e.g. ClfA, FnBp and collagen binding protein) [36-39], surface
11 polysaccharides (type 5 and 8) [40] as well as secreted toxins [41-43].
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27 We found an association between both the PFGE group and the *agr* group and the antibody
28 level to different virulence factors, such as cell wall constituent (teichoic acid), exotoxins
29 (alpha-toxin), and superantigens (TST and SSS) (Table 2). The immune response against
30 alpha-toxin was related both to bacterial factors based on typing groups (*agr* and PFGE), and
31 to TST based on gene-occurrence (*tst*). A correlation between antibody response to teichoic
32 acid and SSS based on typing groups was also seen in Table 2.
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44 In conclusion, the antibody response varied among both patients and antigens. Patients with
45 collagenosis showed a lower response and patients with a fatal outcome had lower initial
46 levels of antibodies. A relationship between antibody levels and the nature of the infecting
47 *Staphylococcus aureus* strain was seen. Serological analysis of antibody profiles to the
48 respective virulence factors in different patient groups presenting with diseases caused by
49 different *S. aureus* isolates may help us identify patient groups of patients who could benefit
50 from adaptive immunotherapeutic interventions.
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Fig1

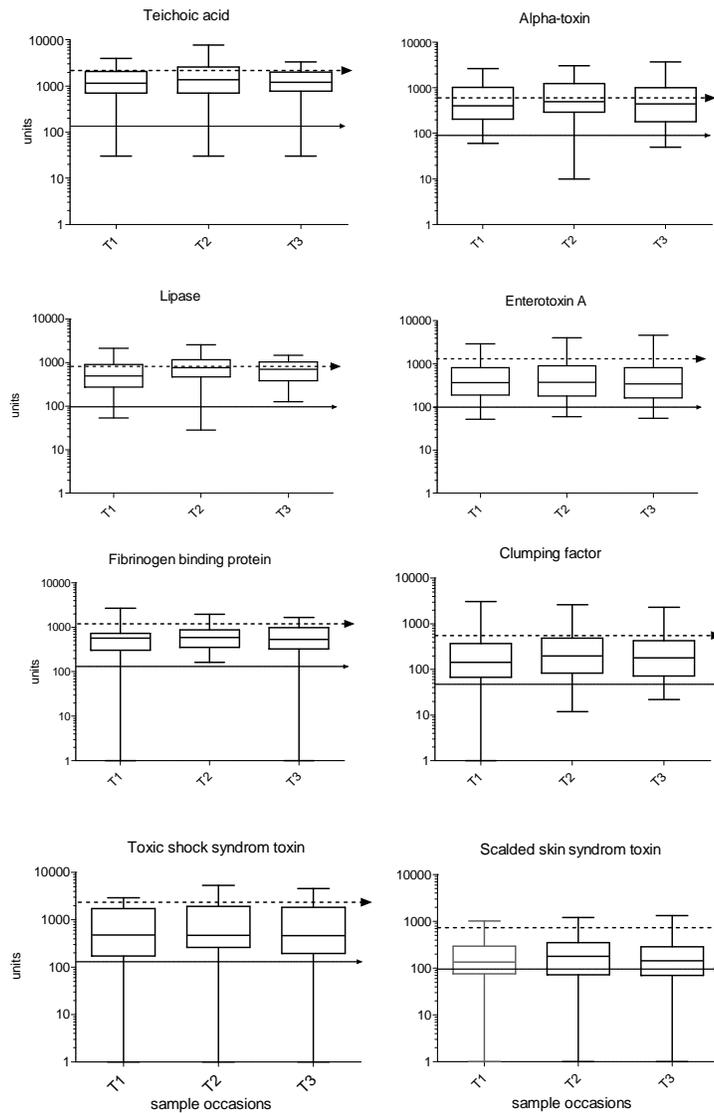


Figure 1.

Antibody levels against 8 different antigens in patients with invasive *S. aureus* infection. For each antigen 3 box plots present the antibody levels achieved at each of the three sampling occasions; at the time of diagnosis (T1), after completed antibiotic treatment (T2) and one month after the end of treatment (T3). In each box-plot we see a horizontal line representing the median level and a vertical line indicating the total range. The dotted lines crossing the 3 box plots indicate the upper 95th percentile obtained in a healthy control population which define high antibody levels(H); the fine horizontal lines indicate limits defining low levels (L) (see M & M).

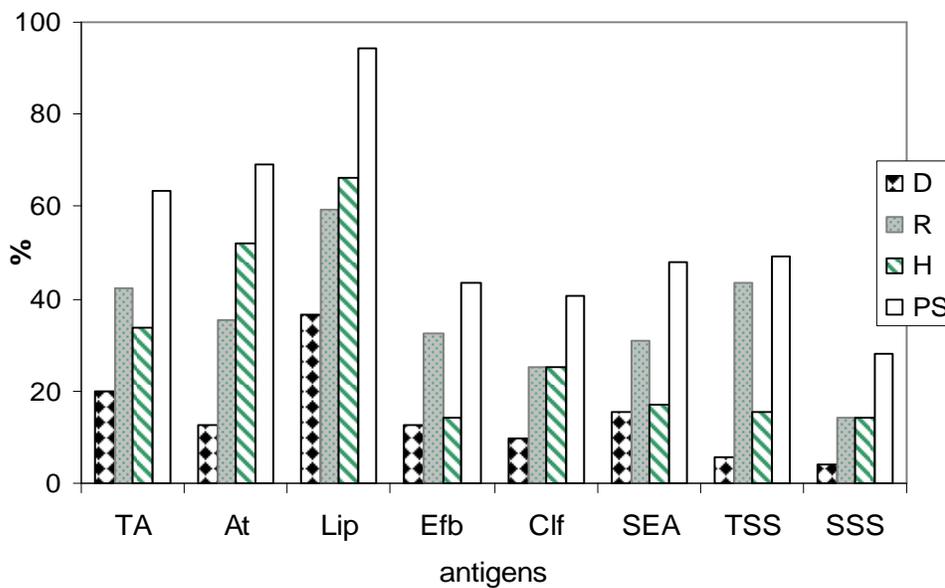
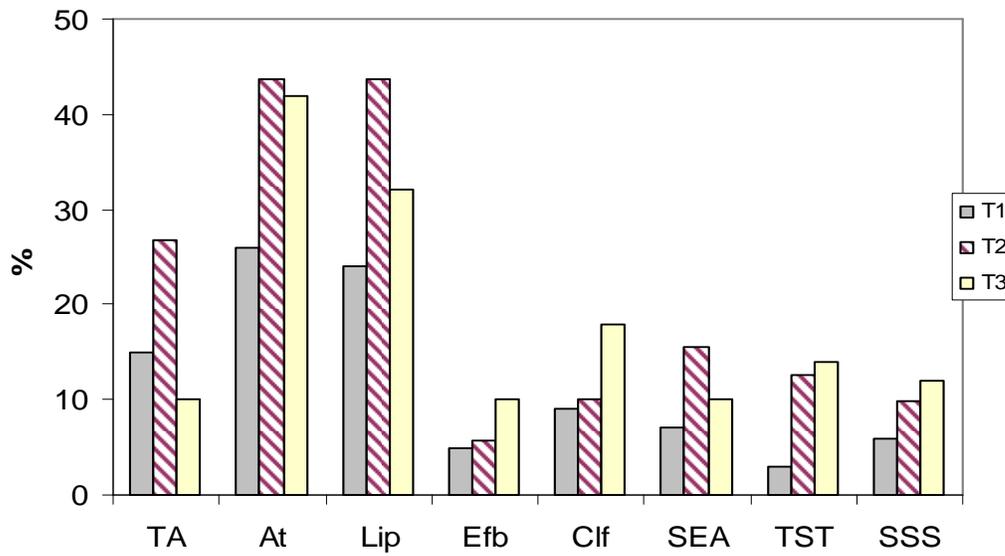


Figure 2A. Percent patients showing high antibody levels (H) at different sampling occasions (T1,T2 and T3).

Figure 2B. Percent patients that shows an antibody response to various antigens. Bars represent the percentage of patients showing decrease of levels (D), rise of levels between any pair of samples (R), high level at any sample occasion (H), positive serology i.e. high and/or rise and /or decrease in antibody levels achieved at any of the previous reactions.(PS).

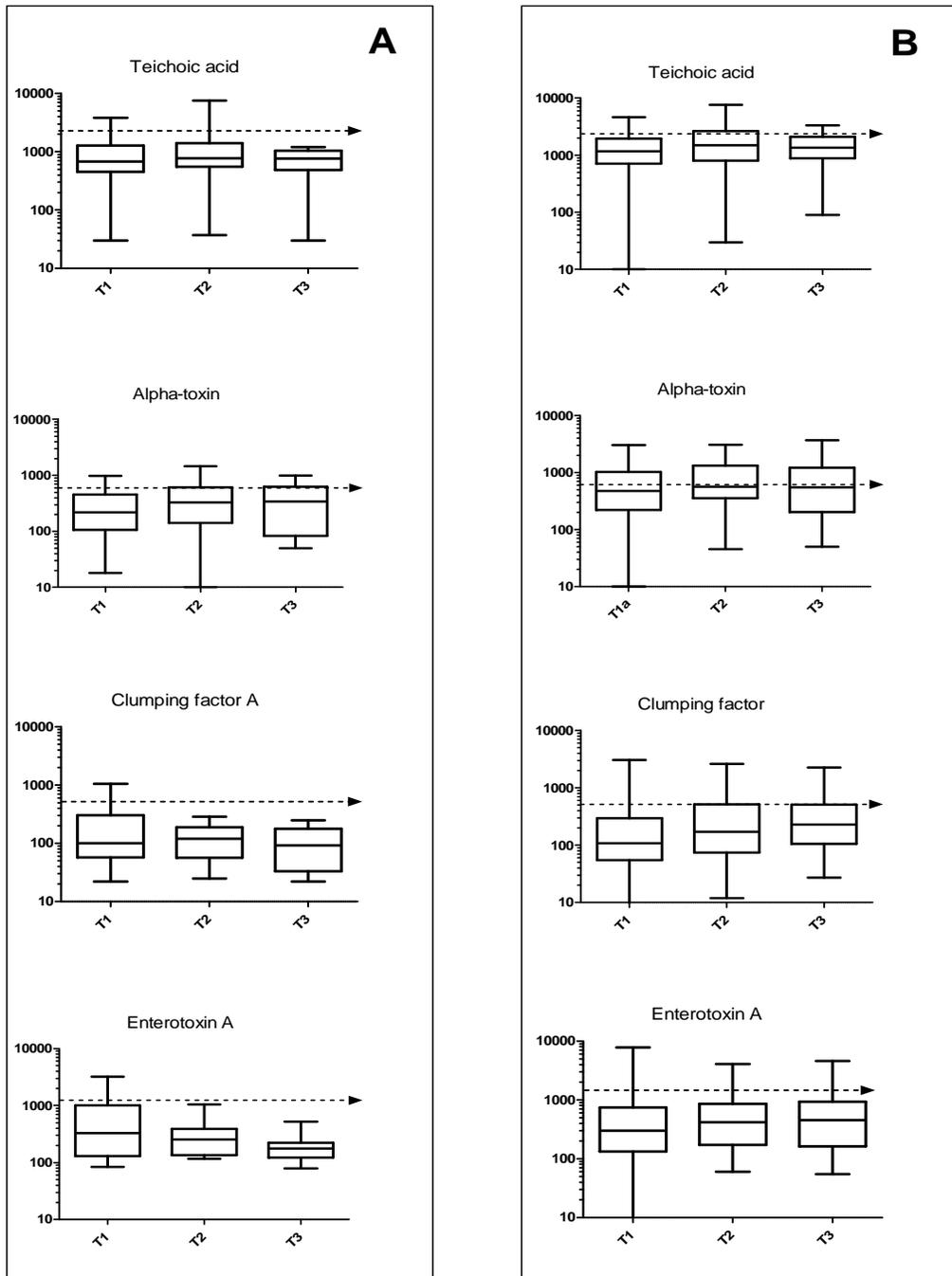


Figure 3.

Antibody levels against four different antigens in patients with and without collagenosis.

A: patients with collagenosis

B: patients without collagenosis

Symbols as in Figure 1.

Table 1. Production of α -toxin in vitro by *S. aureus* isolates in relation to clinical outcome, PFGE typing group, *agr*-group and patient antibody levels.

Variable Isolates from	α -toxin production hemolysin zon median value, mm (range)	p-value
All patients n=150	8 (0-15)	
Severe sepsis n=46	9 (4-15)	n.s.
Complicated bacteraemia n=48	10 (0-15)	0.024
Mortality 28-days n=26	5 (0-13)	n.s.
Isolates PFGE typing group		
Type 1 n=33	3 (0-13)	<0.001
Type 2 n=37	10 (0-14)	<0.001
Type 3 n=9	7 (0-14)	n.s.
Type 4 n=7	7 (0-14)	n.s.
Isolates <i>agr</i> system		
<i>agr</i> 1 n=57	10 (0-14)	0.018
<i>agr</i> 2 n=34	10 (3-14)	<0.001
<i>agr</i> 3 n=51	3 (0-15)	<0.001
<i>agr</i> 4 n=8	11 (7-14)	n.s.
Patients antibody levels		
HTAT n=31	9 (0-14)	0.014
LTAT n=17	3 (0-14)	0.005

HTAT=High levels of antibodies against α -toxin at presentation (T1);

LTAT=Low levels at presentation;

Significant level: <0.05, Mann-Whitney U.

Table 2. Antibody level to *S. aureus* in relation to *agr*-group and PFGE typing group.
4 antigens, 3 *agr*-groups, 2 PFGE groups shown.

Antigen	Antibody level	All	<i>agr</i> 1	<i>agr</i> 2	<i>agr</i> 3	PFGE 1	PFGE 2
		patients L,H n=96 PS n=71 (%)	L,H n=36 PS n=29 (%)	L,H n=23 PS n=14 (%)	L,H n=32 PS n=23 (%)	L,H n=21 PS n=16 (%)	L,H n=23PS n=18 (%)
Teichoic acid	Low	10	6	9	16	10	13
	High	24	22	22	28	33	9
	Pos. serology	61	45*	71	78*	88*	28**
Alphatoxin	Low	21	11	4*	44**	43*	22
	High	50	61	61	31*	24*	48
	Pos. serology	70	72	86	61	50	67
TST	Low	25	19	30	25	14	26
	High	13	6	9	25*	33*	4*
	Pos.serology	51	34*	71	61	75*	28*
SSS	Low	40	53*	22*	41	38	56*
	High	12	11	13	9	5	9
	Pos. serology	29	34	29	22	19	22

* = p<0.05

** = p<0.001, Chi-square

L = Low = low first level, sample drawn 0-14 days within start of infection,
see M&M for definition

H = High = any high level, value over 95th percentile among controls

PS = Positive serology = either high, increase or decrease (increase = 50%, decrease = 50%)

Table 3. The initial antibody levels presented as Relative Risk for different clinical outcomes.
Five antigens are shown.

Antigen	Ab level	All patients n = 96		28 days mortality n = 10			Severe sepsis n = 28			Compl. bacteremia n = 32		
		Yes ¹	No ²	Pos ³	Neg ⁴	RR ⁵ (95% CI)	Pos	Neg	RR (95%) CI	Pos	Neg	RR (95% CI)
TA	High	15	81	0	10	-	4	24	0.9	5	27	1.0
	Low	9	87	3	7	4.1 (1.3-13.3)*	4	24	1.6	6	26	2.2 (1.3-3.9)
AT	High	33	63	2	8	0.5	12	16	1.4	11	21	1.0
	Low	21	75	3	7	1.5	4	24	0.6	10	22	1.6
Lipase	High	24	72	1	9	0.3	6	22	0.8	6	26	0.7
	Low	13	83	4	6	4.3 (1.3-13.1)*	4	24	1.1	8	24	2.1 (1.2-3.7)*
SEA	High	7	89	1	9	1.4	2	26	1.0	3	29	1.3
	Low	28	68	6	4	3.6 (1.1-11.9)*	9	19	1.2	8	24	0.8
SSS	High	7	89	0	10	-	2	26	1.0	3	29	1.3
	Low	39	57	9	1	13.2 (1.7-99.7)*	15	13	1.7	12	20	0.9
Any of 8 antigens	High	61	35	3	7	0.2 (0.1-0.9)*	17	11	0.9	20	12	1.0
	Low	71	24	9	1	3.0	28	8	0.8	25	7	1.2

*) p < 0.05

¹ Number of patients with high/low levels of antibodies at presentation

² Number of patients that did not present high/low levels at presentation

³ Number of patients with a high/low level at presentation that belong to the respective clinical outcome group

⁴ Number of patients that did not present a high/low level at presentation and that belong to the respective clinical outcome group

⁵ Relative risk for a patient with high/low level of antibodies at presentation to develop the respective clinical outcome, i.e. the quotient between the percent positive⁽³⁾ and the percent negative⁽⁴⁾, plus the 95 % confidential interval

Table 4. Antibody levels in relation to age.

Four antigens are shown.

Antigen	Antibody levels	All patients	Age < 65y	Age > 65 y
		L, H n=96 I, D, PS n=71 (%)	L, H n=45 I, D, PS n=34 (%)	L, H n=51 I, D, PS n=37 (%)
Lipase	Low	14	13	14
	High	53	58	49
	Increase	59	59	60
	Diminish	37	50*	24*
	Pos serology	94	97	92
TST	Low	26	24	28
	High	12	22*	4*
	Increase	44	62*	27*
	Diminish	6	9	3
	Pos serology	51	71*	32*
SEA	Low	29	24	33
	High	16	24*	8*
	Increase	31	50*	14*
	Diminish	14	12	16
	Pos serology	48	62*	35*
SSS	Low	41	29*	51*
	High	12	18	6
	Increase	14	21	8
	Diminish	4	9	0
	Pos serology	28	44*	14*

*Chi-square, p<0.05

L= Low= low first level, sample drawn 0-14 days within start of infection, see M&M for definition

H= High= any high level, value over 95th percentile among controls

I= Increase= any increase 50%

D= Decrease= any decrease 50%

PS= Positive serology= either high, increase or decrease