Invasive *Staphylococcus aureus* infections

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”Men de verkliga resenärerna är de som reser utan mål”
Charles Baudelaire

Abstract

Staphylococcus aureus is a leading cause of septicaemia-related death.
The aims of this thesis were to describe the epidemiology of invasive *Staphylococcus aureus* infections (ISA), the clinical course, and serological response in ISA in a prospective, population-based study. The antibody response was compared with the serological findings in healthy individuals.

During two years 170 episodes of ISA were registered, with an incidence of 33.9 cases/100,000/year. Haemodialysis (relative risk 291) and peritoneal dialysis (relative risk 204) patients were at the highest risk. Soft tissue infections, bacteraemia without focus, infections of intravenous lines, and joint/bone infections were the most common diagnoses. The spectrum of signs and symptoms was wide, with nearly a quarter of the patients being afebrile.

The mortality rate was 19.1% (28-day mortality), with an annual population mortality of 5.9/100,000. Patients with complicated bacteraemia (32% of all episodes) had a mortality rate of 32%, and patients with severe sepsis (30% of all episodes) 54%. Patients with bacteraemia without focus, patients with respiratory infections, and patients with endovascular infections had the highest mortality figures.

Only severe sepsis and low systolic blood pressure were independent factors for mortality in a multivariable regression model. We found a relapse rate of 9.3%, and a rate of remaining symptoms after the antibiotic treatment had ended of 34%. Sequelae were seen among 60% of the patients with arthritis.
The frequency of different *agr,* accessory gene regulator, groups within the bacterium, was not correlated to the disease presentation.

The antibody response in ISA showed a great variability. Patients with a fatal outcome produced lower amounts of antibodies to all antigens, and significantly to four antigens (teichoic acid, lipase, enterotoxin A, and scalded skin syndrome toxin). The same trend was noted for patients with a complicated course of infection.

Healthy carriers of S. aureus in the nares had higher levels of antibodies to all eleven tested antigens, and significantly to five (teichoic acid, lipase, enterotoxin A, toxic shock toxin-1, and extracellular adherence protein) than non-carriers. Ages over 65y showed only slightly lower levels.

Keywords: *Staphylococcus aureus*, epidemiology, risk factors, clinical presentation, mortality, recurrence, sequelae, *agr*, serology, colonization.

List of papers

This thesis is based on the following papers, referred to in the text by their Roman numerals:

I. Jacobsson G, Dashti S, Wahlberg T, Andersson, R.
The epidemiology of and risk factors for invasive *Staphylococcus aureus* infections in western Sweden.
Scand J Infect Dis 2007;39(1):6-13.
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II. Jacobsson G, Gustafsson E, Andersson R.
Outcome for invasive *Staphylococcus aureus* infections.
Eur J Clin Microbiol Infect Dis 2008;27(9):839-848.
With permission from the publisher.

III. Jacobsson G, Colque-Navarro P, Gustafsson E, Andersson R, Möllby R.
Antibody responses in patients with invasive *Staphylococcus aureus* infections.
Submitted 2009.

IV. Colque-Navarro P, Jacobsson G, Andersson R, Flock JI, Möllby R.
Antibody levels against eleven *Staphylococcus aureus* antigens in a healthy population.
In manuscript.

Contents

Abbreviations

agr	accessory gene regulator
AFLP	Amplified Fragment Length Polymorphism
ASTA	Antistapholysin assay
AT	Alpha toxin
Bsp	Bone sialoprotein binding protein
CA MRSA	Community-acquired methicillin resistant *Staphylococcus aureus*
CA MSSA	Community-acquired methicillin sensitive *Staphylococcus aureus*
ClfA	Clumping factor A
ClfB	Clumping factor B
CP	Capsular polysaccharides
Eap	Extracellular adherence protein
EARSS	European Antibiotic Resistance Surveillance System
Efb	Extracellular fibrinogen binding protein
IgG	Immunoglobulin G
ISA	Invasive *Staphylococcus aureus* infections
Isd	Iron-responsive surface determinant
MLST	Multi Locus Sequence Typing
MSCRAMM	Microbial Surface Components Recognizing Adhesive Matrix Molecules
PCR	Polymerase chain reaction
PFGE	Pulsed-field gel electrophoresis
PVL	Panton-Valentin leucocidin
SAB	*Staphylococcus aureus* bacteraemia
Sag	Superantigens
SCC*mec*	Staphylococcal chromosomal cassette *mec*
SCV	Small colony variants
SEA	Staphylococcal enterotoxin A
spa	Staphylococcal protein A
SSS	Staphylococcal scalded skin toxin
TA	Teichoic acid
TST	Toxic Shock Toxin-1

Introduction

S. aureus

Staphylococcus aureus is a unique microorganism as compared with other clinically relevant bacteria in three respects. The organism expresses a variety of virulence factors. The bacterium continues to demonstrate the ability to develop resistance to include a broad array of antimicrobial classes, and S. aureus is a prominent pathogen in both hospital and the community settings.

The number of serious infections with *Staphylococcus aureus* is increasing; this is true for both community-acquired (CA) and nosocomial infections. S. aureus was diagnosed in 1% of all discharge diagnoses in hospitals in the USA in 2003, with 3 times the length of hospital stay, 3 times the total charges, and 5 times the risk of in-hospital death as compared with stays without this diagnosis in 200/2001 (Noskin, Rubin et al. 2005; Noskin, Rubin et al. 2007). Different medical disciplines face complicated S. aureus infections in different patient categories, from newborns to the elderly, and from immunocompetent to immunosuppressed patients. In spite of advances in diagnosis and treatment, mortality and complication rates are still high, with reports of in-hospital mortality of more than 20% (Seifert, Wisplinghoff et al. 2008).

There is no vaccine available, and the role of passive immunprophylaxis is unclear. The population at risk increases with more elderly people and more patients receiving imunosuppression or having indwelling catheters and other foreign materials.

The numbers of resistant bacteria, MRSA (methicillin resistant S. aureus) are rising. Within a year after the introduction of semi-synthetic penicillins such as methicillin, there were reports of resistant isolates in 1961 (Jevons 1961). MRSA was initially confined to hospitals, with increasing reports of outbreaks all over the world, and with the search-and-destroy-strategy the epidemic was controlled. During the 1990s the situation changed, with endemic clones in hospitals being established, and a sharp increase in the rate of MRSA-bacteraemia and postoperative infections (Diekema, Pfaller et al. 2001; Wisplinghoff, Bischoff et al. 2004). In the last decade, CA MRSA infections have become prevalent in many locations around the world (Fridkin, Hageman et al. 2005; Tenover 2006; Tristan, Ferry et al. 2007). Circulating CA strains of MRSA are genetically distinct from those traditionally detected in health care-associated infections, and often carry the genes encoding Panton-Valentine leukocidin (Orscheln, Hunstad et al. 2009). This exotoxin causes severe necrosis in skin, soft-tissue and lungs, resulting in serious infections, with a fatal outcome in some cases.

The entire genome of S. aureus was sequenced in 2001 (Kuroda, Ohta et al. 2001) and ongoing molecular and genetic dissection of S. aureus has revealed a large number of surface adhesins, which mediate adherence to and colonization of target tissue, and secreted enzymes and toxins that make invasion possible. S. aureus harbour a number of mobilizable exogenous DNA stretches, including insertion sequences, transposons, bacteriophages, and pathogenicity islands (also referred to as genomic islands) (Ruzin, Lindsay et al. 2001; Baba, Takeuchi et al. 2002; Novick 2003), which contain specific determinants responsible for disease and antibiotic resistance. These exogenous elements explain the high capacity of S. aureus to undergo horizontal gene transfer and to exchange genetic elements with other organisms, including both staphylococcal and nonstaphylococcal genera. Because gene exchange is a key player in evolution, this genetic plasticity is a probable explanation for the success of S. aureus as both a colonizer and a disease-producing microbe.

Epidemiology of S. aureus infections

S. aureus was the most prevalent species isolated from inpatient isolates, irrespective of origin, (18.7% of all bacterial isolates) and the second most prevalent (14.7%) from outpatient isolates in a laboratory-based surveillance in USA from 1998 to 2005 (Styers, Sheehan et al. 2006). The most common organisms causing nosocomial bacteraemia were coagulase negative staphylococci (31% of isolates) and S. aureus (20%) in a nationwide hospital surveillance study in the USA from 1995 to 2002 (Wisplinghoff, Bischoff et al. 2004). S. aureus was second to E.coli in a populations-based study of blood stream infections, both among all isolates and among CA infections (Uslan, Crane et al. 2007). The incidence of S. aureus bacteraemia (SAB) has increased in recent years in the United States and in Europe (Shorr 2007) (EARSS 2008). The incidence of SAB doubled in England between 1993 and 2002, mostly owing to a huge increase in MRSA infections, but also due to an increase in MSSA (methicillin sensitive S. aureus) bacteraemia (Johnson, Pearson et al. 2005).

The incidence of SAB is not known in Sweden since this is not a mandatory reportable disease, but approximations based on data reported to EARSS (European Antibiotic Resistance Surveillance System) claims a figure of 2,195 cases per year (Melander, Burman et al. 2007), i.e. 24.3/100,000 population.

The epidemiology of invasive Staphylococcus aureus infections (ISA), i.e. not only bacteraemia, has only been defined in few studies of population-based study design. Other studies have been limited either by inclusion of only selected patients with ISA or by failure to include clinical information (Jensen, Wachmann et al. 1999; Morin and Hadler 2001). Laupland conducted a population-based surveillance of all invasive S. aureus infections occurring in the Calgary Health Region in Canada from 1999-2001, and estimated an incidence of 28.4

cases/100,000 population (17.9/100,000 population, for bacteraemia), of which 46% were classified as nosocomial (Laupland, Church et al. 2003).

S. aureus is responsible for 25% to 68% of cases of native endocarditis in adults, and the proportion is increasing (Miro, Anguera et al. 2005).

Bone and joint infections attributable to S. aureus are also increasing in number. Between 2000 and 2004 the incidence of acute osteoarticular infections in children rose from 2.6/1,000 admissions to 6/1,000 in a university hospital in USA, with MSSA being responsible for 10%-13% and the proportion of MRSA increasing from 4% to 40% (Arnold, Elias et al. 2006). Laupland (Laupland, Church et al. 2003) reported an annual rate of arthritis of 2.4/100,000 population.

Risk factors for ISA

Several conditions have been identified as associated with ISA, including diabetes, alcohol abuse, immunosuppression, nasal colonization by S. aureus, admission to hospital or intensive care unit, intravenous drug abuse, haemodialysis, HIV infection, older age, newborn, male, use of intravenous cannulas, ot the presence of a foreign body (Lowy 1998; Steinberg, Heling et al. 1999). Laupland (Laupland, Church et al. 2003) quantified risk factors, and found the highest risk for haemodialysis patients (relative risk 257), peritoneal dialysis (RR 150), HIV-infection (RR 24), and solid organ transplantation (RR 21). The distribution and magnitude of comorbidity risk factors for acquiring MRSA and MSSA are comparable (Laupland, Ross et al. 2008).

Individuals lacking antibodies against TST (Toxic Shock Syndrome Toxin-1) are at risk of developing toxic shock (Parsonnet, Hansmann et al. 2005) (Lappin and Ferguson 2009). It has been hypothezid that mutations in the innate immune system, Toll-Like Receptor 2 Gene, may predispose to ISA (Mullaly and Kubes 2006).

Clinical presentation

ISA has a broad clinical presentation from bacteraemia with unknown focus to bacteraemia with a primary focus such as skin and soft tissue infection, arthritis, skeletal infection, deep abscesses from various organs, respiratory infection, and urinary tract infection. S. aureus can also present as a toxin-mediated disease without bacteraemia or a focal infection as toxic shock syndrome, scalded-skin syndrome, neonatal toxic shock syndrome-like exanthematous disease, or food poisoning.

The risk of a secondary or a metastatic focus such as endocarditis or other endovascular focus, arthritis, skeletal infection, CNS-infection, abscesses in various

organs is characteristic. Presence of a secondary focus defines complicated infections, and it is crucial to successful management of ISA to separate uncomplicated from complicated infections owing to the need for different therapies and follow up. This clinical decision-making may, in theory, be simple and straightforward but in clinical practice it is difficult to perform. For example, in a report of 260 patients with SAB owing to endocarditis, the diagnosis of endocarditis was not clinically suspected and was first detected at autopsy in 32% of patients (Roder, Wandall et al. 1999). Risk factors for complicated infections have been evaluated by many researchers (Fowler, Olsen et al. 2003; Troidle, Eisen et al. 2007). According to Fowler the strongest predictor for complicated SAB was a positive follow-up blood culture at 48 to 96 hours, and a scoring system based on the presence or absence of 4 risk factors (community acquisition, skin examination findings suggesting acute systemic infection, persistent fever at 72 hours, and positive follow-up blood culture results at 48-96 hours) accurately identified complicated SAB.

In the ongoing worldwide epidemic of CA-MRSA it has been proposed that patients presenting to hospitals with risk factors for MRSA should receive empirical therapy covering MRSA. Unfortunately, clinical and epidemiological characteristics cannot distinguish CA-MRSA and CA-MSSA, as Miller (Miller, Perdreau-Remington et al. 2007) showed in a prospective investigation. MRSA infection was associated with younger age, skin/soft-tissue infection, snorting illegal drugs, recent incarceration, lower comorbidity index, more frequent visits to bars, rave parties, and clubs, but the sensitivity, specificity and predictive values were low.

Wang found, in a study concerning CA-SAB, that independent risk factors for MRSA were cutaneous abscess (OR 5.46), and necrotizing pneumonia (OR 24.81), and an independent risk factor for MSSA was endovascular infection (Wang, Chen et al. 2008).

Could virulence factors influence clinical presentation? In toxin-mediated diseases this is obvious, but among other ISA infections it is not clear. Hogevik (Hogevik, Soderquist et al. 1998) found the same virulence factors in isolates from endocarditis patients as in isolates from superficial skin infections. It has been claimed that virulence gene regulators, such as *agr* (accessory gene regulator), are associated with certain disease presentations (Jarraud, Mougel et al. 2002; Ben Ayed S 2006).

Elderly patients have different clinical presentation than younger patients. McClelland (McClelland, Fowler et al. 1999) showed that it is more common to find afebrile elderly patients with SAB, patients aged 66-90y, but the white blood cell count did not differ compared to younger patients. The rate of pacemakers and prostheses was higher, but not the rate of unknown bacteraemia.

Mortality

During the last two decades, mortality has declined for S. aureus bacteraemia. Benfield (Benfield, Espersen et al. 2007) reported a decrease in overall in-hospital mortality rate from 34% to 22% in the period 1981 to 2000 in Denmark. This decrease was more pronounced in nosocomial bacteraemia (from 36% to 21%) than in community-acquired bacteraemia (34% to 26%). In recent years no further decrease has been observed. Laupland (Laupland, Ross et al. 2008) noted unchanged annual mortality from 2000-2006, with a higher overall case fatality rate for patients with MRSA (39%) than for patients with MSSA (24%). This latter finding is in accordance with proof of the efficacy of early antibiotic therapy. Lodise (Lodise, McKinnon et al. 2003) demonstrated that delaying therapy for 45 h substantially increase the risk of infection-related mortality in patients with hospital-acquired S. aureus bacteraemia.

Numerous studies have demonstrated higher mortality rates for MRSA-infections than for MSSA (Whitby, McLaws et al. 2001; Cosgrove, Sakoulas et al. 2003; Daskalaki, Otero et al. 2007). The unresolved question is whether or not this mortality difference is attributable to increased virulence for MRSA. Daskalki found no higher mortality for MRSA when adjusting the two groups for factors such as age, respiratory focus, and inappropriate antibiotic therapy. Nosocomial MRSA is still more common than CA-MRSA bacteraemia, although the latter is on the rise. Wang (Wang, Chen et al. 2008) registered the same mortality for CA-MSSA bacteraemia as for CA-MRSA bacteraemia, with a hazard ratio for MRSA 1.01, 95% CI 0.3-3.39. This was in spite of the fact that most patients with MRSA did not receive empirical glycopeptide treatment.

Several predictive factors for mortality, such as acute severity of illness, respiratory focus, unknown focus, endovascular focus, meningitis, age, MRSA, presence of shock, inadequate treatment, underlying disease status, lack of source control, mode of acquisition, and infectious disease consultant, have been registered in varying degrees (Mylotte and Tayara 2000; Hill, Birch et al. 2001; Jensen, Wachmann et al. 2002; Chang, Peacock et al. 2003; Kaech, Elzi et al. 2006). McClelland (McClelland, Fowler et al. 1999) reported an odds ratio for overall mortality in SAB for patients aged 66-90y of 2.21 as compared with patients aged 18-60y, adjusted for confounding factors, and an OR of 2.30 concerning death attributable to SAB. Nickerson concluded (Nickerson, Wuthiekanun et al. 2009), in a study on ISA in a developing country, that simple clinical measures such as drainage of pus and timely antibiotic therapy are key to the successful management of S. aureus infections, and that defining the presence of genes encoding PVL, for example, provides no practical bedside information and detracts attention away from identifying verified clinical risk factors and interventions that can save lives.

Endocarditis is the complicated infection with the highest mortality, with figures ranging from 20% to 65% (Murray 2005), and with higher mortality for left-sided

(38%) than right-sided (17%) endocarditis. S. aureus has been identified as an independent risk factor for mortality in large prospective studies of infective endocarditis of all causes (Cabell, Jollis et al. 2002).

The mortality rate for osteomyelitis, not only caused by S. aureus, was 2.8% in a study by Tice (Tice, Hoaglund et al. 2003), and Shirtliff (Shirtliff and Mader 2002) reviewed the literature for acute septic arthritis and found mortality figures ranging from 5% to 20% irrespective of causative organism.

Recurrence

Recurrence is common, but incidence and risk factors for recurrence are uncertain. Chang (Chang, Peacock et al. 2003) noted a recurrence rate of 9.4% following SAB. Most were relapses with the same pulsed-field gel electrophoresis (PGFE) pattern. Duration of treatment was not found to be correlated to relapses. In contrast, Verhagen (Verhagen, van der Meer et al. 2003) demonstrated relatively high relapse rate in patients receiving less than 10 days of iv therapy for SAB, 18%.

β-lactam antibiotics were found to be superior to vancomycin in efficacy in MSSA-patients in the study by Chang. Failure to remove infected intravascular devices/catheters is common in patients experiencing multiple relapses (Chang, Peacock et al. 2003; Walker, Bowler et al. 2009).

Small colony variants, SCV, of S. aureus have slow growth rates, persist inctracellularly, and are less susceptible to antibiotics. They are associated with persistent or relapsing osteomyelitis and device-related infections (Proctor, von Eiff et al. 2006). SCVs are often recovered from S. aureus strains that have been exposed to gentamicin or other aminoglyosides.

Sequelae

Sequelae or functional impairment have not been reported in large, retrospective or prospective surveys of both SAB and ISA (Laupland, Church et al. 2003; Fätkenheuer, Preuss et al. 2004). Fätkenheuer registered a crude mortality in SAB after 1 year of 37.6%, but provided no information on surviving patients. Zeylemaker (Zeylemaker, Jaspers et al. 2001) reported, in a study of catheter-related SAB, favourable outcome in 20 of 49 patients. Also 17% of the patients developed a complication more than 3 months after treatment. The study was undertaken in a tertiary teaching hospital with selected high-risk patients. Davies (Davis 2005) reviewed the literature on bone and joint infections and concluded that 40-50% of patients have residual joint dysfunction after septic arthritis.

O'Daly (O'Daly, Morris et al. 2008) described an adverse outcome in 66% of patients with pyogenic spinal infection, irrespective of causative organism, at a

median follow up of 61 months, 17% with neurologic deficits, and 17% with acute sepsis-related death. Delay in diagnosis of spinal infection and neurologic impairment at diagnosis were significant predictors of neurologic deficit at follow up.

Accessory gene regulator

Virulence of S. aureus is multifactorial, and attributable to the combined action of virulence determinants such as cell-surface proteins, secreted toxins, and enzymes. The expression of virulence factors is generally regulated in a growth phase dependent manner governed by the accessory gene regulator (*agr*) system (Janzon and Arvidson 1990) and by several DNA binding proteins including *sar*A homologues (Arvidson and Tegmark 2001). *Agr* functions via an auto-inducing peptide, AIP, and is activated in late and post-exponential growth. *Agr* mutants display reduced virulence in several animal models, including studies of arthritis, subcutaneous abscess, mastitis, endocarditis, and osteomyelitis, reviewed by Collins (Collins V.L. and Tarkowski 2000). S. aureus strains can be divided into four groups depending on the variants of the *agr* locus sequence, and strains belonging to different *agr* groups express different patterns of secreted virulence factors (Ji, Beavis et al. 1997). Strains belonging to the same *agr* group can cross-activate each other via AIP, whereas cross-inhibition occurs between different *agr* groups, and also between strains from S. aureus and other staphylococci species such as *Staphylococcus epidermidis*. Simultaneous inoculation of inhibitory AIP with virulent S. aureus suppressed the lesions in a mouse subcutaneous abscess model (Mayville, Ji et al. 1999).

It has been proposed that type of disease correlates with *agr* group. For example, Jarruad (Jarraud, Mougel et al. 2002) linked endocarditis with *agr* groups I and II, and toxic shock syndrome with *agr* group III. Ben Ayed (Ben Ayed S 2006) reported a relationship between *agr* group III and non-invasive infections, and between *agr* group I and invasive infections. Sakoulas (Sakoulas, Eliopoulos et al. 2003) demonstrated a connection between *agr* group II and glycopeptide resistance.

Antibody response

Diagnostic use

The diagnosis of ISA is based on cultures from normally sterile body sites, most often blood. Sometimes there is a clinical suspicion of ISA but cultures are negative or impossible to obtain, e.g. from deep abscesses. In patients with bacteraemia it is necessary to differentiate between patients with complicated infections from those with an uncomplicated infection. In these settings serology against various S. aureus antigens has been tried.

Healthy adults have detectable antibody levels against most S. aureus antigens (Espersen and Schiotz 1981). These antibodies develop during childhood, and adult antibody levels are generally reached by the age of 15 years (Granstrom, Julander et al. 1983; Julander, Granstrom et al. 1983; Christensson, Fehrenbach et al. 1985; Dryla, Prustomersky et al. 2005). The humoral immune response varies greatly during invasive infections (Colque-Navarro, Soderquist et al. 1998). Hence, the clinical value of diagnostic S. aureus serology is low. This is because of varying sensitivity, specificity, and insufficient predictive value of the tests or combinations of tests used. It is believed that complicated infections generate a higher antibody response than uncomplicated ones. Ryding (Ryding 2001) concluded in his thesis on S. aureus serology, however, that there is no evidence that any serological assay or combination of assays can distinguish between complicated and uncomplicated S. aureus infections. Sensitivity, defined as percentage of patients with S. aureus endocarditis or complicated bacteraemia with a positive outcome, has been found to vary from 36% to 100%. Specificity, defined as percentage of patients with uncomplicated S. aureus bacteraemia with a negative outcome, has been found to vary from 34% to 100% (Julander, Granstrom et al. 1983; Christensson, Espersen et al. 1985; Christensson 1986; Verbrugh, Peters et al. 1986; Ryding 2001). However, the reliability of the diagnosis of, for example, endocarditis in older studies can be questioned, because of the low use of echocardiography (no use of transeosophageal examination). The time of sampling also differs in different studies. In some studies samples in the first week after the start of illness is not considered, and in others the maximum titer is compared. In fact, it has been reported (Colque-Navarro, Soderquist et al. 1998) lower levels of antibodies against several antigens in patients with complicated bacteraemia as compared with patients with uncomplicated bacteraemia.

Toxic shock syndrome attributable to TST producing S. aureus can be diagnosed serologically and by determination of specific toxin production from a patient's isolate.

Protective immune activation

Is the antibody response in human beings of a protective nature? S. aureus is mainly an extracellular pathogen, and host defence consequently relies mostly on innate immunological mechanisms supported by antistaphylococcal adaptive humoral responses. Individuals deficient in antibodies (hypo- and agammaglobunemies) and/or neutrophil function suffer from an increased frequency of staphylococcal infections (Liese, Jendrossek et al. 1996).

However, an individual who has had a S. aureus infection is usually not protected from a subsequent infection in spite of detectable antibodies against various antigens. A critical step in the elimination of S. aureus in the humans is complement-mediated opsonisation (Cunnion, Zhang et al. 2003). It has been claimed that the vast majority of antibodies in healthy individuals, 60 to 85%, are induced by lipoteichoic acid, which fails to promote opsonisation (Dryla, Prustomersky et al. 2005; Peterson, Wilkinson et al. 1978).

Gjertsson (Gjertsson, Hultgren et al. 2000) found no difference in outcome between mice deficient in B lymphocytes, i.e. with no IgG production, and congenic controls in a haematogenous arthritis model. Later, Gjertsson (Gjertsson, Kleinau et al. 2002) also showed that mice deficient in FcγII-receptor on B-lymphocytes had higher survival rates, and showed elevated serum levels of IgG antibodies against ClfA, increased levels of IL-10, and enhanced phagocytic capacity. FcγII-receptor mediates inhibitory signals, and activation of this receptor results in decreased B-cell activation and proliferation. These results suggest that protective antibodies are produced during a S. aureus infection but to a relatively low extent, and that the massive increase in IgG production early on during the course of infection is of no protective value. Gjertsson's (Gjertsson 2003) explanation for these assumptions is intriguing (Figure 1). The extensive early IgG-production is a result of the mitogenic and superantigenic stimulation of B cells provided by the bacterium, as well as the B cell response to thymus-independent antigens (i.e. no memory cells are produced). The antibodies are mainly of low affinity and have broad specificity, some with specificities for non-staphylococcal epitopes, including self-epitope. These antibodies may down-regulate the subsequent B cell response towards a thymus-dependent antigen by cross-linking the B-cell receptor (mediating stimulation) to a FcγII receptor. The antibodies formed early may also mask the protective epitopes, rendering them invisible to the B cells and thereby preventing further response.

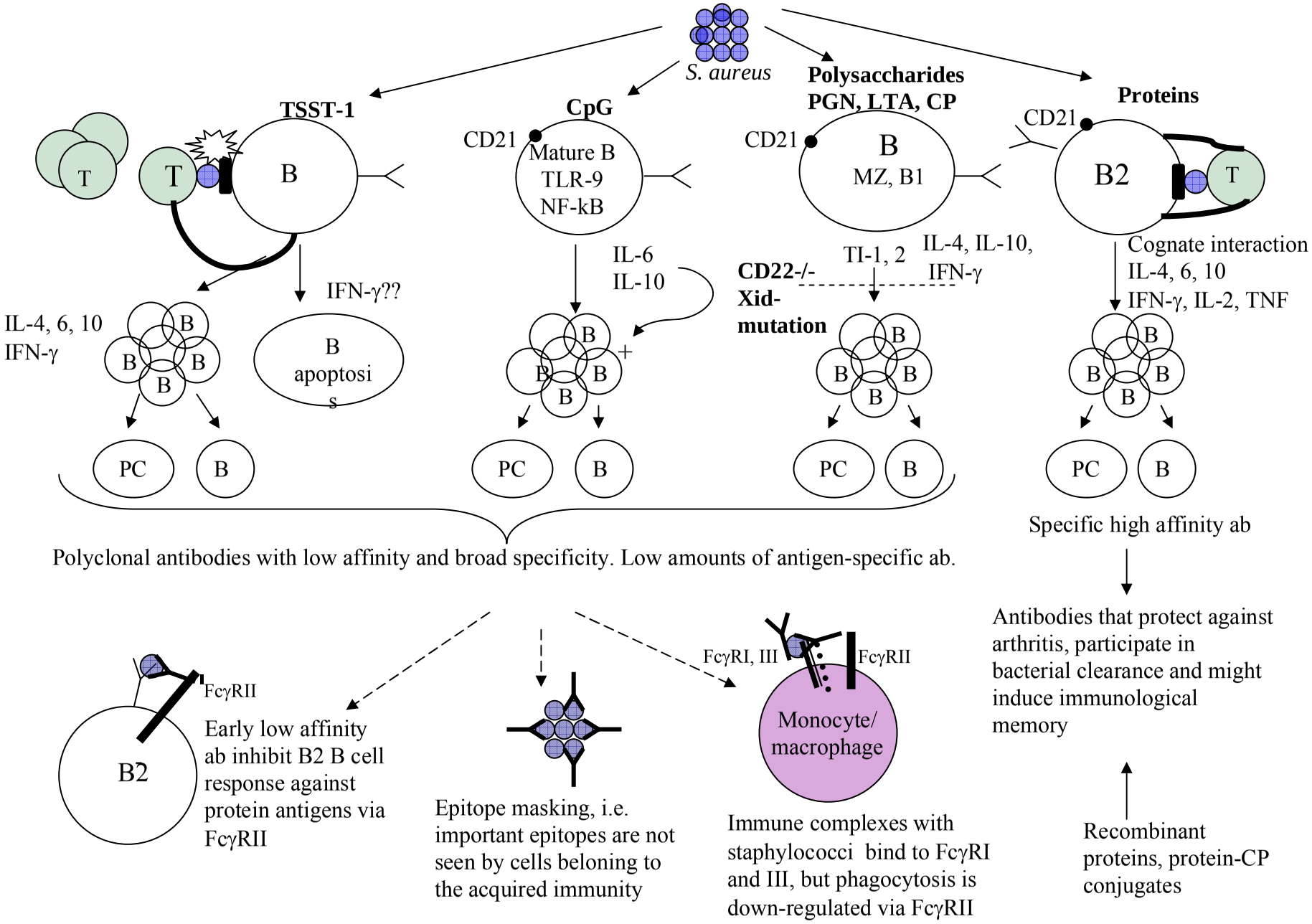

Figure 1. Early polyclonal production of antibodies in response to TI-antigens, B cell mitogens and superantigens produced by *S. aureus* inhibits further protective and specific antibody responses by the acquired immunity. Dotted lines represent inhibiting events. Plasma cell (PC), marginal zone (MZ), peptidoglycan (PGN), lipoteichoic acid (LTA), capsule polysaccharide (CP), antibodies (ab).
With permission from Gjertsson I. The B lymphocyte in Staphylococcus aureus arthritis. Rheumatology and Inflammation research. Thesis Göteborg, (2003) University of Göteborg.

Numerous experimental studies on animals have shown that it is possible to elicit a protective response to several different bacterial components, such as surface adhesin (ClfA, FnBp and collagen binding protein) (Flock 1999; Josefsson, Hartford et al. 2001; Rennermalm, Li et al. 2001; Hall, Domanski et al. 2003), surface polysaccharides (type 5 and 8) (Fattom, Sarwar et al. 1996) and secreted toxins (Nilsson, Verdrengh et al. 1999; LeClaire, Hunt et al. 2002; Hu, Omoe et al. 2003). The present dogma in immune protection against S. aureus is that a protective immune response is possible when a recombinant antigen is used, but during natural infection no protective antibody response is generated. This view can be challenged. Dryla (Dryla, Prustomersky et al. 2005) showed that high-titer antistaphylococcal antibodies are stable for years in healthy individuals, and provided evidence that these antibodies are functional on the basis of opsonophagocytic and neutralizing activity. In a study of paediatric patients infected with CA S. aureus (Brown, Bowden et al. 2009), it was demonstrated that patients infected with Panton-Valentin leucocidin (PVL)-positive strains developed a dominant IgG anti-PVL antibody response. This response was attributable to a specific humoral response against the antigen, and it was at the expense of antibody response to other virulence factors.

Active immunization in clinical trials

Most S. aureus strains are encapsulated, with capsular polysaccharides serotype 5 (CP5) or serotype 8 (CP8) being the most common among 11 serotypes. Fattom (Fattom, Schneerson et al. 1993) conjugated CP5 and CP8 to protein (recombinant Pseudomonas aeruginosa exotoxoid A). The vaccines were highly immunogenic in mice and humans, and antibodies elicited by immunization opsonised encapsulated S. aureus for phagocytosis.

A combined bivalent vaccine, both CP5 and CP8, was tested in haemodialysis patients to prevent bacteraemia 2002 (Shinefield, Black et al. 2002). At week 54 after vaccination the vaccine efficacy was only 26%, which was not statistically significant, but when earlier time periods were analyzed, the vaccine was found to significantly reduce the incidence of bacteraemia between weeks 3 and 40. A subsequent confirmatory clinical trial in 3,600 haemodialysis patients did not repeat the positive results, and the further development of this vaccine ceased (Schaffer and Lee 2009). The failure of the vaccine may, in part, have been due to the immunocompromised status of the patients.

Passive immunization in clinical trials

S aureus adheres to host molecules through surface protein adhesins, also referred to as microbial surface components recognizing adhesive matrix molecules, or MSCRAMMs. Clumping factor A (ClfA) mediates binding to fibrinogen (McDevitt, Francois et al. 1994), biomaterial surfaces (Vaudaux, Francois et al.

1995), blood clots, damaged endothelial surfaces (Moreillon, Entenza et al. 1995), and platelets (Sullam, Bayer et al. 1996). Protective antibodies were demonstrated in experimental models (Josefsson, Hartford et al. 2001), and the Inhibitex company developed a pooled immunoglobulin preparation from donors with high antibody titers against staphylococcal adhesins that bind fibrinogen and fibrin. A phase 3 double-blind, placebo-controlled study was conducted in 1,983 infants, aiming to reduce bacteraemia (DeJonge, Burchfield et al. 2007). There were no differences in the two groups, 6% bacteraemia in the study group and 5% in the control group. The results were disappointing, because the immunoglobulin product, although selected for its antibodies to Clf A, probably contained antibodies to many other staphylococcal antigens, and so could be considered multicomponent passive immunotherapy. The product was not elicited by immunization but by natural exposure to S. aureus, and therefor the antibodies might have recognized the wrong Clf A epitopes, or may have been of low affinity.

Antigens

Numerous virulence factors are described in S. aureus, cellwallbound factors, toxins and enzymes (Table 1).

Table 1. *Staphylococcus aureus* Extracellular Factors Involved in Pathogenesis, and Response to Global Regulatory Elements during Bacterial Growth

					Action of Regulatory Genes[†]			
Gene	***Location***	***Product***	***Activity/Function***	***Timing****	***agr***	***sae***	***rot***	***sarA***
Surface Proteins								
spa	Chromosome	Protein A	Anti-immune, antiphagocytosis	exp		≠	+	
cna	PT islet[§]	Collagen BP	Collagen binding	p-exp	0			
fnbA	Chromosome	Fibronectin BPA	Fibronectin binding	exp				+
fnbB	Chromosome	Fibronectin BPB	Fibronectin binding	exp				+
clfA	Chromosome	Clumping factor A	Fibrinogen binding	exp	0			
clfB	Chromosome	Clumping factor B Lactoferrin BP	Fibrinogen binding Lactoferrin binding	exp	0		+	0
Capsular Polysaccharides								
cap5	Chromosome	CP5	Antiphagocytosis?	p-exp	+			+
cap8	Chromosome	CP8	Antiphagocytosis?	p-exp	+			
Cytotoxins								
hla	Chromosome	α-Hemolysin	Hemolysin, cytotoxin	p-exp	+	+	+	≠
hlb	Chromosome	β-Hemolysin	Hemolysin, cytotoxin	p-exp	+	+	+	≠
hld	Chromosome	δ-Hemolysin	Hemolysin, cytotoxin	xp	+	0		+
hlg	Chromosome	γ- Hemolysin	Hemolysin, cytotoxin	p-exp	+		+	≠
lukS/F	PVL phage	PVL leukocidin	Leukolysin	p-exp	+		+	
Superantigens								
sea	Bacteriophage	Enterotoxin A	Food poisoning, TSS	xp	0			
seb	SaPI3[¶]	Enterotoxin B	Food poisoning, TSS	p-exp	+			≠
sec	SaPI4[¶]	Enterotoxin C	Food poisoning, TSS	p-exp	+			
sed	Plasmid	Enterotoxin D	Food poisoning, TSS	p-exp	+			
eta	ETA phage	Exfoliatin A	Scalded skin syndrome	p-exp	+			
etb	Plasmid	Exfoliatin B	Scalded skin syndrome	p-exp	+			
tst	SaPI1, 2, bov1	Toxic shock toxin-1	Toxic shock syndrome	p-exp	+			+
Enzymes								
spIA-F	Chromosome	Serine protease-like	Putative protease		+		+	
ssp	Chromosome	V8 protease	Spreading factor	p-exp	+	0		+
aur		Metalloprotease (aureolysin)	Processing enzyme?	p-exp	+			+
sspB		Cysteine protease	Processing enzyme?		+		+	
scp		Staphopain (protease II)	Spreading, nutrition	p-exp	+			+
geh	Chromosome	Glycerol ester hydrolase	Spreading, nutrition	p-exp	+	0	+	≠
lip		Lipase (butyryl esterase)	Spreading, nutrition	p-exp	+	0		≠
fme	Chromosome	FAME	Fatty acid esterification	p-exp	+			≠
plc		PI-phospholipase C		p-exp	+			
nuc	Chromosome	Nuclease	Nutrition	p-exp	+	+		
hys	Chromosome	Hyaluronidase	Spreading factor	xp	≠			
coa	Chromosome	Coagulase	Clotting, clot digestion	exp		+	+	+
sak	Bacteriophage	Staphylokinase	Plasminogen activator	p-exp	+	0		

[†]*agr,* accessory gene regulator; *sae, Staphylococcus aureus* exoproteins; *rot,* repressor of toxins; *sarA, Staphylococcus* accessory regulator.

*Timing: xp, throughout exponential phase; exp, early exponential phase only; p-exp, postexponential phase; 0, no effect of gene on expression;+, upregulated; -, downregulated.

≠Controversial.

[§]PT islet, pathogenic islet.

[¶]SaPI, *Staphylococcus aureus* pathogenic island.

BP, binding protein; ETA, exfoliative toxin A; PVL, Panton-Valentine; TSS, toxic shock syndrome.

Adapted from Cheung AL, Projan SJ, Gresham H. The genomic aspect of virulence, sepsis, and resistance to killing mechanisms in *Staphylococcus aureus.* Curr Infect Dis Rep. 2002;4:400-410, and Novick RP. Autoinduction and signal transduction in the regulation of staphylococcal virulence. Mol Microbiol. 2003; 48:1429-1449.

Teichoic acid TA

Teichoic acids are cellwall components which are covalently linked to peptidoglycan. They consist of ribitol residues joined through phoshpodiester linkages and substituted with glucose and/or N-acetylamino sugar, and/or sometimes D-alanine. The antibody test is the most thoroughly investigated serological test, and probably the most frequently used one. Antibody rise is seen in serious staphylococcal infections (Julander, Granstrom et al. 1983). Weidenmaier (Weidenmaier, Kokai-Kun et al. 2004) showed that teichoic acid plays a role in the nasal colonization of. S aureus.

Alpha toxin AT

AT is a pore-forming, secreted toxin with haemolytic activity and broad specificity. Most clinical isolates are reported to produce AT. Already more than a century ago, antibodies against staphylolysin were demonstrated in healthy individuals and in patients with staphylococcal infections (Neisser 1901). As with teichoic acid, antibody rise is seen in serious staphylococcal infections (Julander, Granstrom et al. 1983). In an animal model vaccination against AT protected against pneumonia (Bubeck Wardenburg and Schneewind 2008). Ruotsalainen (Ruotsalainen, Karden-Lilja et al. 2008) found an elevated initial ASTA titer significantly more often among injection drug users without endocarditis than those with endocarditis (44% vs. 6%). A toxoid prepared from AT, but also from other toxins, has been in use in Russia for several decades for production of a hyperimmune plasma product (Kelly 2000). Healthy individuals were vaccinated with the toxoid and 2-3 L of immune plasma was collected from each donor. The product was used in many clinical settings, but there exist no controlled studies and the scientific literature are almost entirely in the Russian language.

Lipase

S. aureus is one of the few bacterial species that produces an extracellular lipase. Strains isolated from deep or subcutaneous infections show a higher lipase production than strains isolated from superficial locations, and in addition to the nutritive function of the enzyme, it is believed to be an invasive factor (Rollof, Hedstrom et al. 1987). Antibody response is seen in bacteraemia (Christensson, Fehrenbach et al. 1985; Colque-Navarro, Soderquist et al. 1998).

Extracellular fibrinogen binding protein Efb

Extracellular fibrinogen binding protein is one of several fibrinogen binding proteins produced by S. aureus. Efb binds to both fibrinogen and platelets and inhibits platelet function both in vitro and in vivo, as well as contributing to virulence in wound infections. An antibody response is seen in patients with bacteraemia (Colque-Navarro, Palma et al. 2000). Efb-negative mutants have

shown less virulence in a murine model of wound infection (Palma, Nozohoor et al. 1996). Antibodies generated in response to immunization with Efb protected against the effects of Efb in a foreign-body-associated wound infection model in mice (Shannon, Uekotter et al. 2006). In the same study, human IgG against Efb was prepared from commercial IgG pools; however the enriched monospecific human anti-Efb was unable to neutralize Efb.

Clumping factor A ClfA

Clumping factor A is a cellbound fibrogen-binding protein, and the gene for CflA is found in the vast majority of S. aureus strains (Peacock, Moore et al. 2002). The biological role of ClfA has been evaluated in experimental animal models of septic arthritis (Josefsson, Hartford et al. 2001) and endocarditis (Moreillon, Entenza et al. 1995), with mutants exhibiting reduced virulence. A monoclonal antibody has shown protective properties in a murine sepsis model (Hall, Domanski et al. 2003). As with Efb, an antibody response is documented in patient with bacteraemia (Colque-Navarro, Palma et al. 2000).

Clumping factor B ClfB

Clumping factor B is a fibrinogen-binding protein, but it also binds to type I cytokeratin of squamous epithelial cells in animals and humans (Wertheim, Walsh et al. 2008). There are conflicting results concerning the antibody responses: Wertheim reported higher titers in carriers of S. aureus in the nose, than in non-carriers, Dryla (Dryla, Prustomersky et al. 2005) demonstrated the opposite.

Bone sialoprotein binding protein Bsp

Bone sialoprotein-binding protein is a cellwall glycoprotein that binds bone sialoprotein. It has been shown in several studies that patients with S. aureus caused osteomyelitis mount a specific antibody response to Bsp (Ryden, Yacoub et al. 1989; Persson, Johansson et al. 2009).

Extracellular adherence protein Eap

This protein, also called the major histocompatibility class II analogue protein, Map, mediates adherence to a variety of extracellular matrix components such as vitronectin, fibronectin, fibrinogen, and collagen, and enhances internalisation into eukaryotic cells (Joost, Blass et al. 2009). It reduces neutrophil recruitment and T cell proliferation and response. S. aureus mutants have shown reduced virulence in abscess and wound models (Joost, Blass et al. 2009). Human anti-Eap antibodies prepared from polyspecific immunoglobulin G (IgG) were found to block the immunomodulatory effects of Eap (Haggar, Shannon et al. 2005).

Staphylococcal Enterotoxin A SEA

Staphylococcal enterotoxin A belong to a family of heat-resistent toxins, also resistant to acid and basic pH. SEA was the first toxin identified in staphylococcal food poisoning (Chu, Thadhani et al. 1966). It also has superantigeinic properties as a potent T cell mitogen. Most staphylococcal superantigens are encoded by accessory genetic elements, and these mobile elements are not uniformly distributed among clinical isolates.

Toxic Shock Toxin TST

TST belongs to the same family of pyrogenic toxin superantigens, as staphylococcal enterotoxins (SE). TST is unique in its ability to cross mucosal surfaces. The toxin is a potent T-cell activator and is found in patients with Toxic Shock Syndrome (TSS). Patients with recurrent TSS are unable to produce antibodies against TST. In patients with bacteraemia, an antibody response was noted in 92% of patients infected with a S. aureus isolate producing TST or SEA-SED (Kanclerski, Soderquist et al. 1996). However, 10% of patients infected with isolates with no toxin production reacted with an antibody response.

TST has been implicated in arthritis (Schwab, Brown et al. 1993), and a potential role is suggested in Kawasaki disease (Matsubara and Fukaya 2007).

Staphylococcal Scalded Skin Toxin SSS

SSS toxin consists of two serotypes, exfoliatin A and B, which cause bullous impetigo, and the generalized counterpart, scalded skin syndrome. These skin diseases mainly affect children, but also immunocompromised adults. One proposed explanation is immunologic immaturity with low antibodies titers (Ladhani 2003). It has been a controversy if the toxin possesses superantigenic properties. Current evidence suggests that the exfoliate toxins exert superantigenic activity because they induce selective polyclonal expansion of T-cells restricted to certain Vβs (variable part of the β-chain in the T-cell receptor).

Carriers of S. aureus

Healthy individuals can be carriers of S. aureus in the anterior nasal region. The throat and the perineum are also important reservoirs. It is believed that approximately 20% of the population are persistent carriers, 30% are intermittent carriers, and 50% are non-carriers (Wertheim 2005). The prevalence of nasal carriage varies, however, and is higher in young children, men, white people, hospitalized patients, and a number of patient groups, including patients with diabetes mellitus, patients undergoing haemodialysis, or chronic ambulatory peritoneal dialysis, patients with S. aureus skin infection, and HIV-infected patients (van Belkum, Melles et al. 2009). Several bacterial carriage determinants have been studied, such as teichoic acid, lipoteichoic acid, fibronectin-binding protein, and clumping factor B. Eradication of S. aureus from the nose has proved to be effective in reducing the incidence of staphylococcal infection (Kalmeijer, Coertjens et al. 2002; Perl, Cullen et al. 2002). Carriers have increased risk of nosocomial bacteraemia as compared with non-carriers, but their outcome is better (Wertheim, Vos et al. 2004). Holtfreter (Holtfreter, Roschack et al. 2006) reported that carriers neutralize superantigens by antibodies specific to their colonizing strain. Persistent carriers have higher antibody titers than non-carriers against TST, SEA, ClfA and ClfB (Verkaik, de Vogel et al. 2009). Recently, van Belkum (van Belkum, Verkaik et al. 2009) showed that there is no difference between non-carriers and intermittent carriers regarding nasal elimination kinetics and antibody profile, and proposed just two categories, carriers and non-carriers.

Aims

Our investigation was undertaken with the following aims:

- To describe, prospectively, the epidemiology of ISA in a well-defined population with low frequency of MRSA colonization and to characterize and quantitative estimate the risk factors, and to characterize the clinical presentation of ISA.

- To determine the mortality, recurrence rate, and residuals symptoms of ISA in a prospective, population-based manner.

- To examine the impact of virulence regulator *agr* on disease presentation.

- To investigate the humoral immune response towards S. aureus in a well-characterized patient cohort with ISA.

- To evaluate the humoral immune response towards S. aureus in a cohort of healthy individuals, and to estimate the impact of carrier-state of S. aureus on antibody production.

Materials and Methods

Patients

Every individual, irrespective of age, who resided in the health care region of Skaraborg Hospital and who had an ISA between 1 March 2003 and 28 February 2005 were prospectively included in the study. The health care region of Skaraborg Hospital consists mainly of small towns and rural areas. Patients requiring transplantation and thoracic/neurosurgical surgery are transferred out of the region and were therefore not included, unless diagnosed in the county. Patients with recurrences were included several times. Relapses in the follow-up period until 1 September 2005, were also considered (1 episode).

During the two-year study, 168 patients had at least one ISA. Eight patients were excluded. Five were considered as insignificant culture results (contaminants) and 3 were excluded because they lived outside the catchment area. Three patients were missed for inclusion. A total of 170 episodes were registered, all episodes were included in the analysis. The median age of the patients was 72 years (range newborn to 97 years), and the mean age was 65 years. Men dominated, 58%, versus 42% women. Thirteen children (age $\leq$ 18 years) were registered with ISA. The median duration of symptoms before admission was two days (range 0-193 days).

The antibody response was determined in three samples, at the time of diagnosis (n=96), after completed antibiotic treatment (n=71), and in convalescence, one month after the end of treatment (n=51). The median time from start of symptoms to sample one was 6 days, with the mode time 4 days.

Controls

Healthy individuals attending an outpatient vaccination clinic at Skaraborg Hospital were asked to join the study group. They had to fill in a questionnaire, determining that they had no chronic illnesses, had not been cared for in hospital the last 12 months, and did not have a joint prosthesis. From 115 healthy individuals one serum sample was obtained. They were screened once for nasal carriage of S. aureus, and 25 (22%) were positive. The median age was 70 y (mean 61 y), with 56% men.

In paper IV, 36 samples of younger blood donors were added, in order to compensate the skewed age distribution of the individuals.
Among the compound group, the gender distribution was 90 men and 60 women (missing information about sex in one individual), with age averages 56 and 50 y.

Study protocol

The Clinical Microbiological Laboratory, Unilabs Skövde, which handles all the routine bacterial specimens from the area, carried out surveillance for ISA. A total of 197 invasive isolates of Staphylococcus aureus were obtained from the 157 patients with a total of 170 episodes. All isolates were of MSSA. In 141 episodes the isolates were from blood.

Other isolates were from synovial fluid in 19 episodes, diverse sites (deep-seated abscesses, bone, and pleural fluid) in 15 episodes and from cerebrospinal fluid in 2 episodes. There were no outbreaks of S. aureus infections reported during the study period.

The study physician in charge was notified and included the patients who fulfilled the inclusion criteria. Consent was obtained from the patients or their relatives after written and oral information. The Ethics Committee at Sahlgrenska Academy of Gothenburg University approved of the study. In the protocol we registered medical history, clinical findings and laboratory results at the time of diagnosis. A blood sample was drawn for serology. At the end of antibiotic therapy we conducted a clinical examination if the patient was still in hospital or was able to attend the outpatient clinic, drew a blood sample for serology, and reviewed clinical data. We repeated the procedure 1 month after the end of antibiotic therapy.

Definitions

ISA

ISA was defined by the isolation of S. aureus from an otherwise sterile site, i.e. blood, synovial fluid, cerebrospinal fluid, pleural fluid, bronchoalveolar lavage, or from a sterile taken deep-seated abscess. Bacteraemia was defined as the presence of at least one positive blood culture for SA. All positive cultures were categorized as true or contaminant by evaluating the clinical history, physical findings, clinical course, and response to treatment. Community-acquired infections were defined as those associated with the first positive culture within 48 h of admission. Health care-related infections were defined as those occurring in patients residing in a nursing home or receiving health care at home. If the first positive culture was obtained later then 48 h after admission, the case was classified as nosocomial. A diagnosis was assigned on the basis of clinical, radiological and microbiological information.

Endocarditis

Endocarditis was diagnosed using the Duke criteria (Durack, Lukes et al. 1994). Ten patients were diagnosed with endocarditis (7% of the bacteraemia cases, 6 definitive and 4 possible).

Severe sepsis

Severe sepsis was defined according to the 2001 SCCM/ESICM/ACCP/ATS/SIS International Sepsis Definitions Conference (Levy, Fink et al. 2003). All patients with sepsis, together with hypotension, hypoperfusion or organ dysfunction, were classified as severe sepsis.

In 51 (30%) of the episodes, severe sepsis occurred.

Complicated bacteraemia

Complicated bacteraemia was considered if bacteraemia presented with a secondary focus, such as endocarditis, spondylitis, osteomyelitis, arthritis, deep-seated abscess or pneumonia. Line-associated infections were not registered as complicated bacteraemia, nor was urinary tract infection, soft-tissue infection with no metastatic seeding or bacteraemia without focus.

In 55 (32%) episodes, complicated bacteraemia occurred, representing 40% of the bacteraemia cases.

Recurrence

Recurrence was defined as a new episode of ISA occurring more than 4 weeks after the time of the first diagnosis and after the antibiotic therapy was complete. Twelve patients had a second episode of illness. One patient suffered two recurrences, i.e. a total of 13 relapses. The median time for recurrences was 76 days, with a minimum of 29 days, a maximum of 422 days, and an interquartile range of 42-274 days. The median follow-up time was 347 days.

Residual symptoms

Residual symptoms were evaluated one month after the antibiotic therapy was completed, either by clinical examination in the hospital or at the outpatient clinic, or by a telephone call with the patient or an appropriate caregiver. Forty-four (34%) patients reported sequelae.

Adequate empirical antibiotic therapy

Adequate empirical antibiotic therapy was defined in cases of bacteremia as parenteral antibiotics to which the bacterium was susceptible. In non-bacteremia cases adequate empirical therapy was defined as parenteral or oral antibiotic to which the bacteria was susceptible. Examples of non-adequate antibiotics were ceftazidim, penicillin G, ciprofloxacin, trimethoprim/sulfamethoxazol.

Fifteen (10%) patients received non-adequate empirical antibiotic therapy, and 18 (13%) were not treated with antibiotics within 24 h of admission or start of illness in hospital.

Antigens

Ribitol teichoic acid (TA) and Alpha-toxin (AT) were purchased from PhPlate AB (Stockholm, Sweden). Enterotoxin A (SEA), Toxic Shock Syndrome Toxin 1 (TST), and Scalded Skin Syndrome toxin (SSS) were purchased from Toxin Technology (Florida, USA). Lipase was kindly supplied by S. Tyski (Medical University of Warzaw, Poland). Clumping factor A (Clf A), Clumping factor B (Clf B), Extracellular fibrinogen binding protein (Efb), and Extracellular adherence protein (Eap) by J.I. Flock (Karolinska Institute, Stockholm). Bone sialoprotein protein (Bbp) by C. Rydén (Uppsala University).

ELISA

The antigen coating concentration was 0.6 μg/ml for Efb, 1 μg/ml for TA, ClfA, ClfB, SEA, TST, SSS, and Eap, 2 μg/ml for Clf, 3.5 μg/ml for AT, and 4 μg/ml for Bbp. Serum samples were diluted twofold in PBS-T (1/250 to 1/2000) in AT, Clf, Efb, TST and SSS assays and (1/2500 to 1/20000) in TA and lipase assays. The reference serum consisted of pooled sera from six patients with confirmed sepsis (Colque-Navarro, Palma et al. 2000). The four dilutions, controls and reference serum were applied and incubated for 1 h at 37°C. Thereafter alkaline phosphatase conjugated to goat anti-human antibodies (Sigma Chemical Co., St. Louis, Mo.) diluted 1/3000 in PBS-T was added, and incubation was continued for 2 h at 37°C. Finally, the enzymatic reaction was measured at 405 nm in a Titertek Multiscan microplate reader (Flow Laboratories, Irvine, Scotland) after approximately 20 min incubation.

Interpretations of ELISA results

The absorbance values were transformed into arbitrary units by using the reference line units calculation method (Reizenstein, Hallander et al. 1995). The dilution curve of each sample was made parallel to that of the reference serum, after which the two curves were compared. The reference serum was given the value of 1,000 (arbitrary) units for all antigens and the patient serum antibody levels were

expressed in these units. This means that the unit levels obtained cannot be directly compared between antigens, since the absolute values are dependent upon those of the reference serum.

The upper limits for "normal antibody levels" were established as the upper 95th percentiles of the levels found in the healthy individuals. Levels above this cut-off level were designated as high levels. A significant rise of antibodies was defined as a 50% increase between two samples and a significant decrease of antibodies was defined as a 50% decrease. A patient was considered to show positive serology when a high level in at least one serum sample from the patients with at least two samples and/or a significant increase/decrease of antibody levels was noted. Some sera were designated as low levels. The limits for these levels varied from 50 to 150 units for the different antigens as determined by the reproducibility of the respective test.

Typing of *agr* groups by PCR

To screen the isolates for *agr* groups I, II, II, and IV, primers of Peacock (Peacock, Moore et al. 2002) were used with Polymerase Chain reaction (PCR). Each strain was analysed using all primer pairs. Sequenced strains, used as positive controls, were included in each run. The final concentration of the PCR mixture was 5 ng DNA template, 1x reaction buffer, 2 mM Cl2, 100 pmol of forward and reverse primers, 0.2 mM deoxynucleoside triphosphate mix and 2.5 U of Taq polymerase (New England BioLabs). The PCR thermal cycling program was initial denaturation of 94°C for 5min, 35 cycles of 94° for 1 min, 55.8°C for 1min, and 72°C for 1min, and a final extension of 72°C for 10 min.

Sixty-two (37%) of the episodes belonged to *agr* group I (one isolate in one episode could not be typed), 42 (25%) belonged to *agr* II, 56 (33%) to *agr* III, and 9 (5%) to *agr* IV.

PCR *tst* gene

The presence of the *tst1* gene in *S. aureus* strains were analyzed by PCR using the following primers (5'→3'): *tst1* forward: ATC GTA AGC CCT TTG TTG CTT G; and *tst1* reverse: CTT TGA TAT GTG GAT CCG TCA TTC. The final concentration of the PCR mixture was same as for *agr* group typing except that 20 pmol of forward and reverse primers were used in each PCR reaction. The PCR thermal cycling program was an initial denaturation of 94°C for 5 min, 35 cycles of 94°C for 1 min, 63°C for 1 min, and 72°C for 1 min, and a final extension of 72°C for 10 min.

One hundred sixty-seven isolates were evaluated, 29 (17%) were positive for the *tst* gene.

Typing by PFGE

Genome typing by pulse field gel electrophoresis was performed in principle as described by Tenover (Tenover, Arbeit et al. 1994).Visual interpretation of the banding pattern according to cited criteria was made, resulting in a cut-off of 75% in the dendrograms generated, above which strains were considered to be closely related. Eighty-six isolates could be evaluated with PFGE, 33 (38%) belonged to group 1, 37 (43%) to group 2, 9 (10%) to group 3, and 7 (8%) to group 4.

Production of alpha-haemolysin

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

Data sources

Regional data that account for all patients in the health care region of Skaraborg were used, and the study population was 255,109 on 31 December 2004. The Swedish Register of Uraemia Care provided the number of haemodialysis and peritoneal dialysis patients, 108 and 22 respectively, on 1 March 2004. The expected number of patients with rheumatoid arthritis aged over 18 years, 997, was estimated from a survey in Sweden (Simonsson, Bergman et al. 1999). The regional Diabetes Register provided the number of patients with diabetes mellitus: 9,912, on 1 January 2004. The number of cancer patients, i.e. the prevalence of patients living with a cancer diagnosis diagnosed during the last 15 years, 8,554, was supplied by the regional Oncology Centre.

Statistics

Data were analysed using SPSS (version 11.5 and version 15.0), Excel 97, GraphPad Prism, and PhPWin. Medians with range were used to describe age; means and medians were used to describe duration. Differences in proportions were compared using Fisher's exact test or Pearson Chi square test, and quantitative variables were analysed with Student's t-test. The non-parametric Mann-Whitney test was used for variables that were not normally distributed.

Antibody levels were presented as by descriptive statistics as medians and quartiles and illustrated by box-plots. Comparisons between groups were analyzed with Mann-Whitney U nonparametric test regarding quantitative variables. Comparisons with obtained and expected numbers of individuals showing high or low antibody levels were performed with Chi square test. For comparisons of levels between individuals the unit values were normalized into quotients of their respective mean values, upon which pairwise correlation coefficients and clustering was performed according to the UPGMA method by the PhPWin software.

Statisticians Anders Odén and Salmir Nasic were consulted when calculating 95% confidence interval for relative risk. Confidence intervals for odds ratio, OR, were calculated by use of logistic regression. Variables found to be significant in a univariate analysis of mortality were included in the multivariable logistic regression analysis. Two variables (At least one previous episode and Age) were included for other reasons than the significant contribution to the prediction of death within 28 days. One variable (Bacteraemia) found to be significant in the univariate analysis was not included in the multivariable logistic regression analysis for technical reasons (0 deaths in a category variable). Based on the significant variables in the logistic regression analysis plus the variable Age, a compound quantitative predictor of probability of death was constructed and evaluated in a 1-Specificity -Sensitivity curve (ROC (Relative Operating Characteristics)-curve).

Results and Discussion

Incidence, risk factors for acquisition, clinical presentation (Paper I, II)

Hitherto, the incidence of ISA or S. aureus bacteraemia has not been described in Sweden The annual incidence of ISA in our study among residents of Skaraborg was 31.4 cases/100,000 population (not including relapses), 33.9 cases with relapses included.. The incidence of ISA is higher than that of pneumococcal disease with 10.3 cases/100,000/y reported in a retrospective study from a nearby region by Dahl (Dahl, Trollfors et al. 2001). Invasive pneumococcal disease is a mandatory reportable disease in Sweden, and the Swedish Institute for Disease Control (SMI) report an incidence of 19.3/100,000 for y 2008 (SMI 2009). Dahl (Dahl, Tessin et al. 2003) reported an incidence of invasive group B streptococcal infection to be 2.4/ 100,000/y. The incidence of ISA is 5-20 fold higher than that of invasive group A streptococcal or group B streptococcal infections as reported in studies from other regions (Davies, McGeer et al. 1996; Farley, Harvey et al. 1993). Our reported incidence is slightly higher than that in the prospective, population-based study of ISA in Canada by (Laupland, Church et al. 2003) with an incidence of 28.4 cases/100,000/y, recurrences not included in the analysis.

We found only 13 children with ISA, giving an incidence of 10.4 cases/100,000/y. Hill (Hill, Wong et al. 2001) reported a higher figure (16.9/100,000/y) for bacteraemia in children.

Morin 2001 (Morin and Hadler 2001) reported, in a population-based study although with a retrospective design, an incidence of 35.4 cases/100,000/y, only including bacteraemia cases. Our incidence of S. aureus bacteraemia cases was 27.6/100,000/y, and presumably the rates would be higher in other areas with higher prevalence of risk groups. Approximations based on data reported to EARSS claim a figure of 2,195 cases of bacteraemia (Melander, Burman et al. 2007) i.e. 24.3/100,000/y in Sweden. Laupland (Laupland, Ross et al. 2008) reported an annual rate of S. aureus bacteraemia on 19.7/100,000. Jensen (Jensen, Wachmann et al. 1999) estimated an incidence of 30/100,000/y, but this was a case-control study of S. aureus bacteraemia acquired in hospitals.

We documented 25 episodes of arthritis, with an annual rate of 4.7/100,000. Weitoft (Weitoft and Makitalo 1999) reported an incidence of 4.1/100,000/y of all bacterial arthritis in another region of Sweden, where children were not included. Kaandorp (Kaandorp, Krijnen et al. 1997) observed a rate of 2.5/100,000/y for septic arthritis attributable to S. aureus.

In summary, our results show that S. aureus is a leading bacterial pathogen in invasive diseases, and that figures based on non-mandatory surveillance are not reliable.

We identified known risk groups for ISA, but in a quantitative manner, with haemodialysis and peritoneal dialysis patients as the groups with highest relative risk. Laupland (Laupland, Church et al. 2003) estimated the quantitative risk for ISA, but our prevalence data for risk groups seem to be more reliable.

The spectrum of clinical presentation of ISA is wide, and a small proportion, 18% of the episodes, were initially cared for at the Department of Infectious Diseases. One quarter of the patients had no history of fever at presentation, even among bacteraemia cases. Several cases of bacteraemia showed a modest inflammatory response in relation to several parameters: 31% with a normal white blood cell count ($<10x10^9$ /l), and 29% with a CRP(C-reactive protein) value below 100mg/l. In non-bacteraemia episodes, only 45% reported a history of fever. This prompts a high degree of clinical suspicion of ISA, not to fail to detect a substantial number of infections. Children had significantly higher fever at presentation than adults.

The most common diagnoses, irrespective of primary or secondary focus diagnosis, were soft tissue infection, bacteraemia without focus, infections of intravenous lines, and joint/bone infections (Table 2), as reported by others (Laupland, Church et al. 2003). Thirty-two percent of the episodes were classified as complicated bacteraemia, and 30% were classified as severe sepsis. In children, the most common diagnosis was line-associated infection and osteomyelitis, the same as observed by (Hill, Wong et al. 2001).

Table 2.

Primary diagnosis in 170 episodes with invasive *Staphylcoccus aureus*

Diagnosis	n	% (n=170)	Annual incidence (per 100 000)	Age Median (range) years	Community-acquired infections n (%)	Health-related infections n (%)	Nosocomial infections n (%)	p[b]
Soft tissue infection	47	27	9.2	74 (4-93)	25 (53)	10 (21)	11 (23.4)	n.s
Bacteraemia without focus	32	19	6.5	75 (36-97)	14 (44)	6 (19)	12 (36.4)	n.s
Arthritis	25	15	4.7	67 (15-90)	19 (76)	2 (8)	4 (16.7)	0.004
Line associated infection	24	14	4.9	68 (0-89)	3 (12)	5 (21)	16 (64)	0.001
Osteomyelitis other than vertebral	16	9	2.9	59 (10-89)	12 (75)	3 (19)	1 (6.7)	0.031
Endovascular infection	12	7	2.4	78 (38-95)	6 (50)	2 (17)	4 (33.3)	n.s.
Urinary tract infection	10	6	1.9	70 (37-89)	6 (60)	2 (20)	2 (22.2)	n.s.
Respiratory infection	9	5	1.9	73 (0-94)	2 (22)	2 (22)	5 (55.6)	n.s.
Vertebral osteomyelitis	8	5	1.6	70 (53-95)	7 (88)	1 (12)	0 (0)	0.027
Intraabdominal infection	7	4	1.4	68 (18-87)	4 (57)	1 (14)	2 (28.6)	n.s.
Meningitis	2	1	0.4	53 (50-55)	1 (50)	1 (50)	0 (0)	n.s.
Other (epidural abscess)	1	1	0.4	60	1 (100)	0 (0)	1 (100)	n.s.
Sum	193[a]							

[a] Patients could have several diagnosis

[b] Community-acquired infections versus health-related infections and nosocomial infections

Mortality (Paper II)

In the course of the first 28 days, 30 (19.1%) patients died, and during the total follow-up time, 41 patients (26.1%) died, giving an annual population mortality due to ISA of 5.9/100,000. This is a higher figure than Laupland (Laupland, Ross et al. 2008) described from the Calgary Health Region in Canada: he reported an annual mortality during 2000-2006 of between 4 and 5/100,000, with the exception of 2005, when reaching nearly 6/100,000. This was in spite of the substantial number of cases with MRSA with known higher mortality, and with a seemingly higher prevalence of risk groups. It is important not to translate incidence- and mortality figures for ISA from different catchement areas uncritically.

Among specific diagnoses we found the highest mortality in patients with bacteraemia without known source, patients with respiratory infections, and patients with endovascular infections, which is in agreement with the findings of others (Mylotte and Tayara 2000). Only 40 of the patients in our study were examined with either transthoracic or transoesophageal echocardiography (34% of the bacteraemia cases), so we may have underestimated the rate of endocarditis. Higher rates of endocarditis than ours, 6%, have been reported by Chang (Chang, MacDonald et al. 2003), 13%, and echocardiography has been proposed as a standard examination method in the early evaluation of patients with SAB (Fowler, Li et al. 1997; Rosen, Fowler et al. 1999).

We could not discern any difference in mortality between cases with community-acquired infections, cases with health care-associated infections, and cases with nosocomial infections. Age over 65 years and concomitant heart disease are variables associated with increased mortality (Table 3), in accordance with other studies (Laupland, Church et al. 2003). We could not find any treatment characteristic that affects mortality, apart from the surprising result that patients with adequate empirical antibiotic therapy had higher mortality. Laupland found that patients treated with empirical antibiotics within 8 hours had a higher mortality rate. The explanation may be that patients with a poorer prognosis present with more severe clinical findings and are more likely to be treated empirically with broad-spectrum antibiotics. Several studies have documented the beneficial effects of early antibiotic therapy (Leibovici, Shraga et al. 1998; Lodise, McKinnon et al. 2003). Lodise demonstrated that delaying therapy for >45 h substantially increased the risk of infection-related mortality in patients with hospital-acquired SAB. Like ours, others studies (Kaech, Elzi et al. 2006) have failed to demonstrate the effects of antibiotic therapy within 24 hours on mortality. Roghmann (Roghmann 2000) did not to find any relationship between survival and the administration of effective antibiotic therapy within 48 h after SAB onset.

Ammerlaan (Ammerlaan, Seifert et al. 2009) conducted a retrospective study of SAB in 9 European countries. Inadequate therapy was common (28% of the patients), but the investigators found no association between inadequate treatment and 30-day mortality.

Table 3.
Predictive factors for death (28 day) among patients with invasive *Staphylococcus aureus* infection

Factor	Fatality rate			p
	With factor(%)	Without factor(%)	OR (95% CI)	
Patient characteristic				
Male sex	19/92(21)	11/65(17)	1.28(0.56-2.91)	0.710
Age >65 years	25/92(27)	5/65(8)	4.48(1.1-12.43)	0.003
Heart vitium	8/15(54)	21/136(15)	6.26(2.05-19.10)	0.004
Coronary disease	12/34(35)	18/105(14)	2.64(1.11-6.28)	0.052
Alcohol abuse	5/11(46)	25/135(18)	3.67(1.04-12.98)	0.098
Infection characteristic				
Community-acquired infection	11/80(14)	19/77(25)	0.49(0.21-1.11)	0.134
Healthcare-associated infection	7/28(25)	23/129(18)	1.54(0.58-4.04)	0.529
Nosocomial infection	12/49(24)	18/108(17)	1.62(0.71-3.70)	0.348
Bacteraemia	30/131(23)	0/26(0)		0.005
Severe sepsis	27/50(54)	3/107(3)	40.7(11.4-145.7)	<0.001
Complicated bacteraemia	17/53(32)	13/104(12)	3.29(1.46-7.50)	0.007
Bacteraemia without focus	10/32(31)	20/125(16)	2.39(0.98-5.80)	0.096
Arthritis	0/23(0)	30/134(22)		0.010
Endovascular infection	6/11(54)	24/146(16)	6.10(1.72-21.61)	0.014
Osteomyelitis other than spondylitis	0/13(0)	30/144(21)		0.070
Respiratory infection	4/8(50)	26/149(17)	4.73(1.11-20.15)	0.036
Treatment characteristic				
Adequate empirical antibiotic therapy	28/132(21)	0/15(0)		0.070
Antibiotic therapy within 24 hours	23/116(20)	3/18(17)	1.24(0.33-4.63)	>0.900

Recurrences were not included in the analysis (i.e. the first episode was not included if a patient had several episodes).

The prevalence of resistant isolates in our area is low, as we had no MRSA cases. We could not evaluate how treatment with a betalactame antibiotic or a glycopeptide antibiotic affects outcome because we had so few patients with the latter regime. It is a well-known fact that vancomycin therapy clears bacteraemia less effectively than betalactames (Khatib, Johnson et al. 2006).

Patients with bacteraemia, complicated bacteraemia and severe sepsis all had high mortality rates. Only severe sepsis and systolic blood pressure were independent factors for mortality in the multivariable regression model. This highlights the importance of the initial assessment and early care of ISA patients.

Recurrence (Paper II)

We observed 13 recurrences, i.e. a recurrence rate of 9.3%, the same as reported by Chang (Chang, Peacock et al. 2003) for SAB. Patients with line-associated infections have a high relapse rate if the IV line is not replaced. In 3 of 5 of our patients with recurrent line-associated infections the IV line was not replaced. Some researchers report higher relapse rates with shorter antibiotic therapy (Verhagen, van der Meer et al. 2003) others not (Chang, Peacock et al. 2003; Johnson, Almoujahed et al. 2003). We did not find any differences in relapse rate among patients with shorter antibiotic therapy than 14 days as compared with patients with therapy exceeding 14 days. The issue concerning the length of antibiotic treatment may distract attention from the most important factor: identifying and eradicating the focus/source of infection. There was no difference among bacteraemia cases versus non-bacteraemia cases regarding the relapse rates in our study. There is no previous study on relapse rate among ISA patients.

Sequelae (Paper II)

At follow-up one month after the antibiotic therapy ended, forty-four patients had not experienced full recovery. Patients with osteomyelitis other than vertebral suffered sequelae in 69% of the cases, and 60% of arthritis cases reported remaining symptoms in this short-term follow-up. Davis (Davis 2005) summarized that 40-50% of patients had residual joint dysfunction with highest risk in the older population. These unacceptably high rates of failure demand measures for identifying factors important to improvement in management. This is a joint responsibility of various medical specialties.

agr (Paper II)

The distribution of *agr* groups was not different in different disease entities. Jarraud (Jarraud, Mougel et al. 2002) found an association between type of disease and *agr* group for toxin-mediated diseases and endocarditis. However, the relevant factor was the phylogenetic group, as determined with AFLP (Amplified Fragment Length Polymorphism), in the type of human disease, which is in accordance with our results. Fowler (Fowler, Nelson et al. 2007) found an association between bacterial clonality, based on MLST, multilocus sequence typing, and hematogenous complications. Isolates within these clonal complexes were also implicated by use of *spa*, staphylococcal protein A, and SCC*mec*, staphylococcal chromosomal cassette, typing.

The humoral immune response in patients with ISA (Paper III)

Antibody levels in all patients

Great interindividual variations in antibody levels were seen, from 50 to 5,000 units against the same antigen in the same sample category, as reported previously (Colque-Navarro, Palma et al. 2000; Dryla, Prustomersky et al. 2005). As expected, the levels achieved in the second sample at the end of treatment were generally the highest, but the median levels varied relatively little between the three sampling occasions. With the exception of SSS, 40% to 95% of the patients reacted to a staphylococcal antigen, where lipase stimulated the highest number of patients, regardless of kinetic parameter measured.

Antibody response and bacterial properties

Bacterial strains belonging to *agr* groups I and II, produced significantly larger zones of haemolysis as compared with strains belonging to *agr* group III, indicating that there is a correlation between *agr* group and production of alpha-toxin. The zones of haemolysis were significantly correlated with levels of antibodies against alpha-toxin at presentation. We also found a significant correlation between *agr* group III and a low initial anti alpha-toxin antibody level ($p<0.001$). Söderquist (1993) reported that strains from patients with a positive antibody response to alpha-toxin showed a higher production of alpha-toxin. Most strains produce alpha-toxin in vitro (Möllby 1983), while less is known about production in vivo. In the study by Söderquist (1993) alpha-toxin was detected in 8/41 patients with S. aureus septicaemia. *Staphylococcus aureus* global regulators *agr*, *sar*A, and *sae* coordinatedly control alpha toxin gene (*hla*) expression in vitro. Xiong (Xiong, Willard et al. 2006) demonstrated that *sae* appears to play a crucial role.

Screening for *tst* among the strains revealed that 17% of the strains carried the *tst* gene with 90% of the *tst*-positive isolates belonging to *agr* group III ($p<0.001$). A correlation between occurrence of *tst* among the strains and absolute TST antibody levels (samples 1 and 2, $p=0.002$ and $p<0.001$ respectively) among the patients was found. *agr*-group III correlated to higher TST antibody levels. The proportion of *tst*-positive strains from clinical isolates varies between <20% and 90%, according to country and clinical background (Nagao, Okamoto et al. 2009). Nagao found 75% *tst*-positive strains among MRSA isolates, mostly belonging to *agr* group II. The amounts of TST varied, and no correlation was seen with mutation within the *agr*-locus, so there must also be other factors determining the production of the toxin.

We did not screen for the gene for SSS. However, in accordance with the antibody response to TST, differences were found between different strains. PGFE-group 2 and *agr*-group I correlated with initial low levels of antibodies, as compared with *agr*-group II, which correlated with higher initial antibody levels.

The immune response to alpha-toxin, TST, and SSS is specific in the respect that it is correlated with strain specificity.

Surprisingly, levels of antibodies to teichoic acid, a cellwall constituent expressed in all strains, correlated significantly with *agr*-group. Strains belonging to *agr*-group I showed a positive serology response in 45% of the patients as compared with 78% of strains belonging to *agr*-group III. We did not find any correlation between disease presentation and *agr*-group, but 4/8 patients with respiratory infections were infected with strains belonging to *agr*-group III.

Clinical outcome

Patients with a fatal outcome displayed lower initial antibody levels for all antigens, and significantly for 4 antigens (Table 4). The same trend was noted in patients with complicated bacteraemia for 5 antigens, but not for TST, SEA and SSS. The mean age was significantly higher in the patients who died (79y vs. 63y, p=0.014). Patients with severe sepsis were also significantly older (75y vs. 60y, p<0.001), but no difference in mean age was registered in patients with complicated vs. uncomplicated bacteraemia (65y vs. 64y). We could not detect any significant differences in the time from start of symptoms to inclusion in the study, or to the first blood sample, in any group, although patients who died tended to have had shorter duration of symptoms.

Table 4.
The initial antibody levels presented as Relative Risk for different clinical outcomes
Five antigens are shown, n=96

		28 day mortality n = 10	Severe sepsis n = 28	Compl.bacteraemia n = 32
Antigen	Ab level	RR (95% CI)	RR	RR (95% CI)
TA	Low	4.1(1.3-13.3)	1.6	2.2 (1.3-3.9)
AT	Low	1.5	0.6	1.6
Lipase	Low	4.3 (1.3-13.1)	1.1	2.1(1.2-3.7)
SEA	Low	3.6 (1.1-11.9)	1.2	0.8
SSS	Low	13.2 (1.7-99.7)	1.7	0.9
ANY of 8	High	0.2 (0.1-0.9)	0.9	1.0
antigens	Low	3.0	0.8	1.2

One obvious explanation for the observation of lower antibody levels among patients with a fatal outcome is the age. Older people (>65y) develop a dysregulation of the humoral immunity, affecting quality and quantity of antibody response. They display lower levels of several antigens, notably the superantigens TST, SEA and SSS. Aging is associated with a shift in antibody isotype from IgG to IgM, and a decline in the activity of lymphocytes (Weksler 2000).

To our knowledge, this is the first time an association has been found between fatal outcome and initial low antibody response in ISA, except for TST (Todd, Fishaut et

al. 1978; Vergeront, Stolz et al. 1983; Andrews, Parent et al. 2001). The significance of this observation is debatable.
We cannot draw any conclusions concerning whether the observed correlation between low initial antibody levels and fatal outcome is of causative art or attributable to a general weak immune response.

Colque-Navarro (Colque-Navarro, Soderquist et al. 1998) showed, in a study of S. aureus septicaemia that patients with a complicated course displayed lower antibody levels against alpha-toxin, teichoic acid, and lipase, not only at admission, but also during the first month. Verbrugh (Verbrugh, Peters et al. 1986) demonstrated the opposite, patients with a complicated course were more frequently positive in serology against alpha-toxin, teichoic acid, and peptidoglycan than patients with an uncomplicated course, and they displayed higher peak values. However, this was not shown for the initial antibody response. Several studies relate higher antibody response with a complicated course (Julander, Granstrom et al. 1983; Christensson, Espersen et al. 1985), based on repeated samples and peak values.

Patients with a previous history of ISA (n=14) had a trend toward a lower risk of a fatal outcome and complicated bacteraemia. They were significantly less prone to developing severe sepsis, and their initial antibody response to alpha-toxin tended to be higher (83% had an initial high response compared to 31% in patients with no previous ISA). There was no bacteraemic endovascular infection registered among patients with a previous history of ISA.
This is in accordance with Ruotsalainen (Ruotsalainen, Karden-Lilja et al. 2008) who found an elevated initial ASTA titer significantly more often among injecting drug users without endocarditis than with endocarditis in a study of SAB (44% vs. 6%).

Antibody response in healthy individuals (Paper IV)

Antibody levels in relation to age

The median value was lower at a higher age; significant differences were only found for Clumping factor B. An exception was seen for antibodies against TST, where the median levels were twice as high in individuals above 65 y compared with individuals below 65 y, although there were no significant changes due to the great variation. Several studies have shown that antibody levels in the healthy elderly decrease with age (Granstrom, Julander et al. 1983; Julander, Granstrom et al. 1983; Dryla, Prustomersky et al. 2005).

Antibody levels in relation to sex

There were no differences seen in the antibody levels of men as compared with women.

Antibody levels in relation to colonization of the nares

Individuals carrying S. aureus in the nose displayed higher antibody levels against all antigens except Efb (Table 5). For five of the analysed antigens, the difference was statistically significant, e.g. the antibody levels against Extracellular adherence protein were three times higher in healthy individuals colonized with S. aureus than in individuals not colonized.

Table 5
Antibody levels in colonized individuals versus non-colonized (median values)

Antigen	Colonized n=26	Non colonized n=89	p
Surface antigens			
Teichoic acid	912	422	0.01
ClfA	170	134	0.24
ClfB	191	163	0.09
Bsp	233	161	0.07
Extracellular proteins			
Alpha toxin	325	165	0.06
Lipase	364	176	0.01
SEA	483	271	0.01
TST	1043	378	0.02
SSS	161	115	0.28
Efb	394	438	0.40
Eap	268	85	0.01

None of the individuals showing low antibody levels to more than four antigens were colonized (p=0.04), and six out of eleven of the individuals with high antibody levels against 4-5 antigens were colonized with S. aureus (p=0.02).

It has been demonstrated that carriers show higher levels of antibodies against TST, SEA, ClfA and ClfB (Wertheim, Walsh et al. 2008; Verkaik, de Vogel et al. 2009). The immune response does not protect from colonization. However, Clark (Clarke, Brummell et al. 2006) found higher levels of reactive IgG to iron-responsive surface determinant (Isd) A and Isd H from non-carriers, and he also showed beneficial effects of vaccination with Isd A or H in an animal model against nasal carriage. So colonization could be the result of a failing humoral immune response, or the humoral immune response could be a consequence of the colonization. The work of Cole 2001 (Cole, Tahk et al. 2001) is in favour of the latter hypothesis, demonstrating that colonized individuals had defects in their local innate immunity towards S. aureus.

We showed higher antibody levels in carriers for all three superantigens (Sag), TST, SEA and SSS, although not significant for the latter. This is demonstrated repetitively in studies concerning antibody response in correlation to carrier-state. What are the Sag doing for the bacterium in the context of nasal colonization? It is certainly not to induce systemic toxic shock in the host. The most likely role of Sag is during the early stages of infection when the very low levels of Sag activate only local T cells. One possible advantage of T-cell activation at the infection site might be to produce cytokines such as IL-2, IFN-γ, and TFN-α that suppress local inflammation (Fraser and Proft 2008). Vojtov (Vojtov, Ross et al. 2002) revealed a remarkable difference in local inflammation in hairless mice subcutaneously injected with a TST producing strain of S. aureus compared with an isogenic knockout strain. After 4 days, mice injected with Sag producing strain produced no significant inflammatory lesion, while the Sag knockout strain produced a purulent open abscess characteristic of a subcutaneous S. aureus infection. The authors suggested that this outcome was due to a global regulatory effect of TST on the expression of other staphylococcal exoproteins, but alternatively the lack of an inflammatory response may have been caused by T-cell activation and suppression of innate mechanisms.

Carriers have better prognosis than non-carriers in nosocomial bacteraemia (Wertheim, Vos et al. 2004), and Holtfreter (Holtfreter, Roschack et al. 2006) have explained the improved prognosis in terms of the increased levels of preformed antibodies. For toxins such as TST and SEA the existence of antibody levels are clearly protective (Holtfreter, Roschack et al. 2006; Verkaik, de Vogel et al. 2009).

High or low levels of antibodies

Dryla (Dryla, Prustomersky et al. 2005) stated that most of the antibodies were produced against surface antigens. In our study the protein surface antigens used did not appear to produce particularly high levels, since ClfA antibodies had to be tested for at an initial dilution of 1/250 and ClfB and Bsp antibodies at the lowest initial dilution of 1/125.

Some individuals showed low antibody levels against several antigens. None of the individuals showing low antibody levels to more than four antigens were colonized ($p=0.04$). Similarly, there was a skewed distribution of the number of antigens against which certain individuals showed high levels of antibodies.

Antibodies against the extracellular proteins alpha-toxin, lipase, enterotoxin A and extracellular adhesive protein more often were found in the same individuals, most often together with the surface bound teichoic acid.

General summary

The magnitude of the clinical problem caused by *Staphylococcus aureus* is not fully recognised. Incidence and prognosis are unknown in Sweden. Melander (Melander, Burman et al. 2007) tried to describe the consequences of rising MRSA prevalence in terms of costs, both medical expenses and human suffering. However, she could not rely on adequate incidence figures. Hence her estimation of bacteraemia attributable to S. aureus was a rough extrapolation.

The intention of our studies was to describe the epidemiology of serious infections of S. aureus in a prospective population-based study, to describe mortality, recurrence, and functional impairment, and to detect humoral immune response conferring protection/susceptibility to infection and outcome.

The incidence of ISA of 33.9/100,000 inhabitants is higher than that for pneumococcal disease, 19.3/100,000 population 2008 (SMI 2009). *Streptococcus pneumoniae* is a pathogen subject to mandatory reporting. Out of the cases of ISA, 49% were classified as community-acquired, 32% as nosocomial and 19% as health care-associated.

Haemo and peritoneal dialysis patients showed a several hundred-fold relative risk of acquisition, which highlights the need for individual risk assessment and preventive measures. It was also clear that the clinical presentation is a challenge to the clinician, with nearly one quarter of the patients having no fever.

In spite of improvement in diagnosis and treatment of ISA, the mortality figures are still high, and study number two demonstrated an overall mortality of 19.1% (28-day mortality), with an annual population mortality of 5.9/100,000. Laupland (Laupland, Ross et al. 2008) reported an annual mortality in Canada in bacteraemia due to S. aureus during 2000-2006 of between 4 and 5/100,000, with exception of 2005, when it reached nearly 6/100,000. This was in spite of a substantial number of cases with MRSA with known higher mortality, and with seemingly a higher prevalence of risk groups. These findings underscore the importance of not translating incidence and mortality figures for serious S. aureus infections from one area to another uncritically. Perhaps, for a key pathogen like S. aureus, each region should actively monitor incidence and mortality data.

The most common diagnoses, irrespective of primary or secondary focus diagnosis, were soft tissue infection, bacteraemia without focus, infections of intravenous lines, and joint/bone infections, as reported by others (Laupland, Church et al. 2003). Patients with complicated bacteraemia (32%) had mortality rates of 32%, and patients with severe sepsis (30%) of 54%.

Patients with bacteraemia without known source, patients with respiratory infections, and patients with endovascular infections had the highest mortality which is in agreement with the findings of others (Mylotte and Tayara 2000).
Only severe sepsis and systolic blood pressure were independent risk factors for mortality in a multivariable regression model. This underlines the importance of early recognition and of the initial assessment and care of ISA patients.

A signum of S. aureus is its capacity to cause metastatic infections and relapses. We demonstrated a relapse rate of 9.3%, and a rate of remaining symptoms after the conclusion of antibiotic treatment of 34%. In some diagnoses, e.g. arthritis, 60% of the patients reported sequelae, indicating a potential for improvement. This is a joint responsibility for various medical disciplines.

The frequency of different *agr* (accessory gene regulator), groups was not correlated to clinical disease. *agr* is a key regulator of virulence in S. aureus, and Jarraud (Jarraud, Mougel et al. 2002) found an association between type of disease and *agr* group for toxin-mediated diseases and endocarditis.

It is generally believed that the antibody response toward S. aureus is not protective. An individual could suffer from repeated infections in spite of demonstrable antibody levels. There is no vaccine available, and passive immunprophylaxis has not been successful.

The present study on the humoral immune response in ISA showed a great variability in antibody levels among patients. People over 65 y react to a lesser extent and those with concurrent illnesses, e.g. patients with rheumatoid arthritis, displayed lower levels. Patients with fatal outcome produced lower amounts of antibodies to all tested antigens, and significantly to 4 antigens. The same trend was noted for patients with a complicated course of infection in 5 antigens in the initial serum sample. Others (Verbrugh, Peters et al. 1986) have reported higher antibody levels among patients with complicated bacteraemia, but the initial serum answer was not considered.

The significance of these findings is not clear. Possibly this is part of a weakened polyclonal reaction owing to age and concurrent diseases, or a specific response to some antigens may be missing in some individuals.

Healthy individuals display variable antibody levels, and individuals colonized with S. aureus (carriers) in the nares show higher antibody levels than non-carriers toward some antigens (Verkaik, de Vogel et al. 2009). Being a carrier of S. aureus is a well-known risk factor for ISA, but carriers have a better outcome than non-carriers with ISA (Wertheim, Vos et al. 2004), which may be explained in terms of preformed antibodies (Holtfreter, Roschack et al. 2006).

It was demonstrated in the study of healthy individuals that carriers had higher levels to all 11 antigens tested, and significantly to 5. Some individuals displayed high antibody levels to several antigens, and some showed low antibody levels to several antigens. Identifying protective immune response in clinical studies is important in order to develop vaccine candidates.

Conclusions

- The annual incidence of invasive Staphylococcus aureus infections was 33.9 cases/100,000 population.

- Haemodialysis (relative risk 291), and peritoneal dialysis (relative risk 204) were the predisposing conditions with highest risk.

- Soft tissue infection, bacteraemia without focus, arthritis, and line-associated infection were the most common clinical presentations, and the spectrum of signs and symptoms were wide, with one quarter of the patients reporting no fever.

- During the first 28 days, 30 patients (19.1%) died, with mortality rates for complicated bacteraemia and severe sepsis of 32% and 54% respectively.

- Patients with bacteraemia without focus, patients with respiratory infections, and patients with endovascular infections had the highest mortality.

- Recurrence and residual symptoms were common, 9.3% and 34% respectively.

- The frequency of different *agr,* accessory gene regulator, groups was not different in different disease presentations.

- The antibody response showed great variability in relation to age, concurrent illness, strain properties and severity of infection. Patients with fatal outcome and complicated bacteraemia had lower antibody levels at the time of diagnosis.

- Healthy individuals colonized with S. aureus in the nares (carriers) displayed higher antibody levels than non-carriers.

Future perspectives

S. aureus is a leading, possible *the* leading, pathogen in bacteraemia. It is unsatisfactory not to know the incidence and complication rates in a given region. Mandatory reports on occurrence in invasive diseases are warranted.

Risk groups with high rates of infection could be identified and headed prophylaxis such as nasal decolonization should be tried (von Eiff, Becker et al. 2001).

We showed that the only independent predictors for mortality were severe sepsis and low systolic blood pressure. This highlights the importance of early recognition and treatment of serious infections. Several campaigns for improving survival in sepsis have been launched in recent years (Townsend, Schorr et al. 2008), but implementation in clinical practice is still deficient.

Algorithms for early recognition of sepsis patients stress the use of physiological parameters such as respiratory rate, percutaneus saturation, and blood pressure. Patients with invasive infection due to S. aureus are often elderly and with concurrent diseases. This complicates the evaluation of physiological parameters, and opens up for misinterpretation. There is a clear need for better diagnostic tools in identifying patients with invasive infections.

Bacteraemic patients make up the majority of cases with invasive infections. Blood culture is time-consuming. Faster methods with PCR in detection on bacteraemia are under development (Dierkes, Ehrenstein et al. 2009). Small colony variants constitute a subpopulation of bacteria with distinctive phenotypic and pathogenic traits. They survive intracellular and in biofilms, and thus responding poorly to antibiotic therapy. Microbiological laboratories need to be more attentive diagnosing those strains (Proctor, von Eiff et al. 2006).

In our study only 18% of the patients were cared for initially at the Department for Infectious Diseases. Many of the patients were first seen by doctors not committed to infectious disease practice. Outcome of S. aureus bacteraemia may be improved by consultation with an infectious disease consultant (Rieg, Peyerl-Hoffmann et al. 2009), and this practice must be encouraged. We also demonstrated the unacceptably high rate of sequelae, e.g. 60% of the arthritis patients. This stresses the joint responsibility and the need for cooperation between different specialists (Stenmark 2009).

S. aureus has a propensity to cause metastatic infections, especially endocarditis. Echocardiography should be part of the standard care (Rosen, Fowler et al. 1999). Vos (Vos 2009) has shown the value of PET (Positron Emissions Tomography)

screening in detection of metastatic foci in S. aureus bacteraemia. Patients undergoing PET examination had both lower relapse rates and lower mortality rates. Clinical examination is not enough, since up to one third of metastatic foci have no localizing signs or symptoms.

Focus identification and eradication are of key importance in management of S. aureus bacteraemia, as is timely and adequate antibiotic treatment. Beta-lactam antibiotics, especially penicillins, are regarded as first line therapy. However, it is known that cellwall active antibiotics have a slow killing time of large inoculates of bacteria.

The repeated demonstration that vancomycin (the current "gold standard" for the treatment of MRSA infection) is associated with a significantly worse outcome when used in MSSA infection (Fowler, Sanders et al. 1998; Chang, Peacock et al. 2003; Stryjewski, Szczech et al. 2007) emphasizes the need for drugs that are equally effective for the treatment of MSSA and MRSA infections.

Combined anti-inflammatory and antibiotic treatment down-regulates the severity of septic arthritis (Sakiniene, Bremell et al. 1996). Addition of corticosteroids to antibiotic treatment in staphylococcal arthritis should be evaluated.

The resistance potential of S. aureus not only call for new antibiotic classes but also non-antibiotic treatment. In vitro studies on interfering with the quorum-sensing control in S. aureus suggest this possibility (Otto 2004). There is a renewed interest in bacteriophage therapy in various clinical settings (Deresinski 2009). Bacteriophages were used for the treatment of patients with bacterial infections mainly in the former Soviet republics for more than nine decades, but no adequate clinical trials of the safety and efficacy of the treatment have been reported. This also applies to the Russian experience with antistaphylococcal hyperimmune plasma and immunoglobin (Kelly 2000).

There is no commercial vaccine available. A pentavalent vaccine (PentaStaph™) is undergoing clinical trials. This vaccine is based on capsular polysaccharides types 5 and 8, the cell wall antigen type 336, Panton-Valentine Leukocidin, and alpha toxin. This is a promising vaccine candidate, especially as compared with StaphVAX, which was based only on capsular polysaccharides type 5 and 8. Pressure to develop vaccines against staphylococci will be driven by the increasing spread of antibiotic resistance.

The humeral immune response is still not well described in large cohorts of patients with S. aureus infections. Such surveys are now in progress (Wertheim 2009).

Svensk sammanfattning

Gula stafylokocker, Staphylococcus aureus, är en vanlig orsak till blodförgiftning, bakteremi. Bakterien har utvecklat resistens mot ett flertal antibiotikagrupper och i många länder ökar såväl kostnader för som dödlighet i svåra stafylokockinfektioner. Än så länge är problemet med resistenta stafylokocker litet i Sverige. Hur stort problemet är med icke-resistenta stafylokocker vet vi inte, bakterien är inte anmälningspliktig och statistik över antal bakteremier och död till följd av detta finns inte.

Förutom att orsaka bakteremi har bakterien egenskapen att orsaka nedslag av infektionen i olika delar av kroppen. Mest fruktat är nedslag på hjärtklaffarna, med hög dödlighet som följd. Andra organ som kan drabbas är leder, skelett och inre organ. Återkommande infektioner förekommer och resttillstånd med bestående handikapp är inte ovanligt.

Det finns inget vaccin. Mot andra bakterier, som t ex pneumokocker, finns effektiva vacciner med antikroppssvar som skyddar mot allvarlig sjukdom. Så gott som alla människor har påvisbara nivåer av antikroppar mot stafylokocker vid provtagning. Trots detta skyddar inte dessa antikroppar mot infektion, oklart varför.

Syftet med avhandlingen var att ta reda på hur vanligt det är med allvarliga stafylokockinfektioner och att hitta människor som har högre risk att drabbas. Ytterligare avsikt var att beskriva dödlighet, återfall och resttillstånd, samt att jämföra antikroppssvar hos patienter med stafylokockinfektion och friska.

I delstudie I visade vi att ca 34 fall av allvarlig stafylokockinfektion inträffar per 100 000 invånare och år. Detta är betydligt högre än t ex allvarliga pneumokockinfektioner som drabbar färre än 20 per 100 000 och år. Vissa patientkategorier, som dialyspatienter, hade nästan en 300 ggr högre risk än normalbefolkningen att insjukna.

I delstudie 2 visade vi att dödligheten under den första månaden efter infektion var knappt 20%, dvs. 1 av 5 dör. Patienter med hjärtklaffsinfektion, lunginflammation och oförklarad blodförgiftning hade den högsta dödligheten. Återfall inträffar i knappt 10% av fallen. Det är vanligt med resttillstånd, t ex hade 60%, av patienter med ledinfektion någon form av problem en månad efter avslutad antibiotikabehandling.

I delstudie 3 visade vi att individer med allvarlig stafylokockinfektion har mycket varierande nivåer av antikroppar. En orsak är åldern, patienter över 65 år har lägre halter. De som dog eller hade ett komplicerat sjukdomsförlopp hade lägre nivåer.

Detta skulle kunna betyda att antikroppar trots allt har betydelse för försvar mot stafylokockinfektion.

I delstudie 4 undersökte vi friska människor och förutom blodprov för antikroppar tog vi också en näsodling för stafylokocker. Det är vanligt att i övrigt friska personer har stafylokocker i näsöppningen. Vi visade att även hos friska varierade antikroppsnivåerna mycket, men generellt var nivåerna lägre än hos patienter. Personer som var näsbärare av bakterien hade högre nivåer av alla testade antikroppar än icke-näsbärare.

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