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On Invasive staphylococcal infections. Penicillin susceptibility, treatment and outcome.

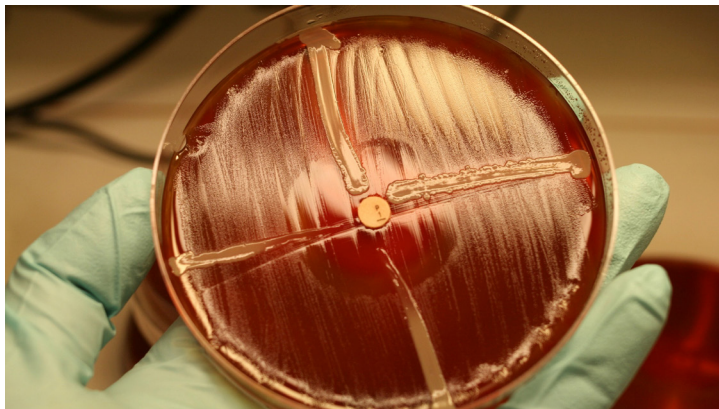Hagstrand Aldman, Malin

2022

On invasive staphylococcal infections

Penicillin susceptibility, treatment and outcome

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Organization LUND UNIVERSITY	Document name DOCTORAL DISSERTATION
	Date of issue 29th of April 2022
Author(s) Malin Hagstrand Aldman	Sponsoring organization

Title and subtitle On invasive staphylococcal infections - Penicillin susceptibility, treatment and outcome

Abstract

Staphylococci causes a wide range of different infections such as skin infections, endocarditis and prosthetic joint infections. *Staphylococcus aureus* is one of our most common causes of blood stream infections, with approximately 600 cases each year in Skåne. *Staphylococcus lugdunensis* is less common but causes infections resembling those caused by *S. aureus*. In Sweden, cloxacillin is the standard treatment for staphylococcal infections. However, cloxacillins have many disadvantages compared to penicillin G, such as high protein binding, liver toxicity and vessel irritation.

Our aim was

- To evaluate methods for penicillin susceptibility testing in *S. aureus* and *S. lugdunensis* and to describe the frequency of susceptible isolates.
- To describe the clinical presentation of infectious endocarditis and prosthetic joint infections caused by *S. lugdunensis*.
- To study the outcome of bacteraemia caused by penicillin susceptible *S. aureus* when treated with penicillin G compared to cloxacillin.

Methods

In the first two studies (Paper I and II) we used several methods for penicillin susceptibility testing *S. aureus* and *S. lugdunensis*. This included methods advocated by EUCAST or CLSI including disk diffusion, zone edge appearance, E-test, clover-leaf test and PCR, to evaluate the accuracy of the methods and also to describe the prevalence of penicillin susceptible isolates in *S. aureus* and *S. lugdunensis*.
Paper III was a retrospective study on *S. lugdunensis* endocarditis, using data from the Swedish National Registry of Infectious Endocarditis to describe the clinical manifestations. In paper IV we investigated isolates from prosthetic joint infections caused by *S. lugdunensis* and their ability to form biofilm. We investigated the correlation of ability to form biofilm and clinical outcome.
Paper V was a retrospective study comparing the outcome of penicillin-susceptible *S. aureus* bacteraemia when treated with penicillin G or cloxacillin. A ranking scale was constructed to give a more patient orientated outcome scale.

Results

29% of blood cultures with *S. aureus* and 67% of invasive isolates with *S. lugdunensis* are susceptible to penicillin G in Skåne. There are reliable methods for penicillin G susceptibility testing in both species.
S. lugdunensis seems to cause an aggressive form of endocarditis but embolization frequency and need of surgery was not as frequent as previously described. The ability to form biofilm in *S. lugdunensis* isolates was correlated to relapsing infections.
The overall outcome in PSSA bacteraemia is worse when treated with cloxacillin compared to penicillin G with an adjusted OR 2.43, *p*=0.005 for having any treatment complication.

Key words: *Staphylococcus aureus*, *Staphylococcus lugdunensis*, bacteraemia, penicillin G, cloxacillin, antimicrobial therapy, infectious endocarditis, prosthetic joint infections,
Classification system and/or index terms (if any)

Supplementary bibliographical information	**Language** English
ISSN and key title 1652-8220	**ISBN** 978-91-8021-220-5

Recipient's notes	**Number of pages** 85	Price
	Security classification	

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On invasive staphylococcal infections

Penicillin susceptibility, treatment and outcome

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Knowledge is proud of knowing so much;
Wisdom is humble that it knows no more

William Cowper

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Preface

I have always been a curious and stubborn kind of person. This may occasionally have caused me some trouble, but it has also led me into good things, like research. The start of this thesis was pure curiosity on something unknown to me, that *S. aureus* had previously been susceptible to penicillin. Infections caused by *S. aureus* are a common and serious and repeatedly met as a specialist in infectious diseases. It seemed thrilling to me to find out if there still was any penicillin susceptible isolates in Skåne. Since then, I have just continued with new questions along the way. This thesis contains the studies evolved from those questions and a subjective non-systematic review of the scientific literature.

Thesis at a glance

Paper	Study question	Methods	Results	Conclusions
I	Prevalence and clinical presentation of penicillin susceptible *S. aureus* in Skåne.	Penicillin susceptibility tests in retrospective and prospective collected *S. aureus* isolates. Assesment of medical records.	29 % of SAB in Skåne 2014/15 were penicillin susceptible.	A high frequency of *S. aureus* isolates are penicillin susceptible, and it is a quick test to do in clinical routine.
II	Prevalence of penicillin susceptibility in invasive infections caused by *S. lugdunensis*. Evaluation of penicillin susceptibility tests.	Retrospective collected isolates. Susceptibility tests according to EUCAST and CLSI with additional, nitrocefin tests, *blaZ* PCR, appearance of zone edge. Assessment of medical records.	67% of invasive isolates were penicillin susceptible. The method according to EUCAST was accurate but the CLSI method resulted in one major error. The zone edge appearance can improve the CLSI method.	A high frequency of *S. lugdunensis* was penicillin susceptible. The penicillin susceptibility test according to EUCAST is preferred.
III	The clinical presentation of infectious endocarditis caused by *S. lugdunensis*.	Retrospetive analysis from Swedish National Registry of Infective Endocarditis. Data compared with infectious endocarditis caused by *S. aureus* and other CoNS from the same registry.	In infectious endocarditis caused by *S. lugdunensis*, 70% affected native valves and the embolizing frequency was 7%. The 30-day mortality rate was 20%, higher than *S. aureus* (9%) and other CoNS (7%).	The study indicates that *S. lugdunensis* is an aggressive pathogen in infective endocarditis, but the embolization frequency was lower than previously reported.
IV	Clinical presentation of prosthetic joint infections caused by *S. lugdunensis* and the correlation between biofilm formation in isolates and the clinical outcome.	Retrospective cohort in Skåne between 2015-2019 with *S. lugdunensis* from sterile sites. Biofilm formation conducted in 96-wells plates and quantified with absorbance.	Prosthetic joint infection (PJI) caused by *S. lugdunensis* is often an acute infection. Biofilm formation was more robust in isolates that caused *late acute hematogenic* PJI and in relapsing infection.	*S. lugdunensis* is an aggressive cause of PJI and biofilm formation is stronger in isolates causing relapsing infections.
V	Outcome in *S. aureus* bacteraemia (SAB) when treated with cloxacillin compared to penicillin G	Retrospective analysis of 684 patients with penicillin susceptible SAB, between 2018-2020. Outcome analysed with logistic regression and propensity score weighted analysis.	The OR for cloxacillin treatment to have any complication compared to penicillin G was 2.4, *p*=0.005	Penicillin G treatment in *S. aureus* bacteraemia can and should be encouraged.

List of paper included

Papers included in the thesis, which will be referred to in the text by their Roman numbers I-V.

I. **Hagstrand Aldman M**, Skovby A & Påhlman LI., Penicillin-susceptible *Staphylococcus aureus*: susceptibility testing, resistance rates and outcome of infection. *Infectious Diseases.* 2017 Vol 49, (6) 454-460

II. **Hagstrand Aldman M,** and Påhlman LI., Evaluation of penicillin G susceptibility testing methods for *Staphylococcus lugdunensis*. *The Journal of Antimicrobial Chemotherap*y. 2020 May 1;75(5):1206-1211

III. **Hagstrand Aldman M**, Rasmussen M, Olaison L, Påhlman LI., Endocarditis due to *Staphylococcus lugdunensis*—A retrospective national registry-based study. *Eur. J. Clin. Microbiol. Infect*. *Dis*. 2021 May;40(5):1103-1106

IV **Hagstrand Aldman M**, Thompson O, Påhlman LI., Biofilm formation correlates with clinical failure in prosthetic joint infections caused by *Staphylococcus lugdunensis.* Submitted 2022

V. **Hagstrand Aldman M**, Kavyani R, Kahn F, Påhlman LI., Treatment outcome with penicillin G or cloxacillin in penicillin susceptible *Staphylococcus aureus* bacteraemia -A retrospective cohort study. *International Journal of Antimicrobial Agents* 2022 Mars 11, Manuscript Accepted

Abbreviations

blaZ	gene coding penicillinase
CCI	Charlson Comorbidity Index
CI	Confidence interval
CLSI	The Clinical and Laboratory Standards Institute
CoNS	Coagulase negative staphylococci
EUCAST	European Committee on Antimicrobial Susceptibility Testing
fT>MIC	time that the free, unbound antibiotic concentration is above the MIC-value
ICD	Intra cardiac device
IDSA	infectious Diseases Society of America
IE	Infective endocarditis
MIC	Minimal Inhibitory Concentration
MRSA	Methicillin Resistant *S. aureus*
MSSA	Methicillin Susceptible *S. aureus*
PBS	Pitt bacteraemia score
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PJI	Prosthetic joint infection
PSSA	Penicillin susceptible *S. aureus*
RAF	Referensgruppen för antibiotikafrågor
SAB	*Staphylococcus aureus* Bacteraemia
TSBG	Tryptic Soy Broth Glucose

Background

Genus Staphylococcus

Bacteria were first described in 1676 by the Dutch scientist Leeuwenhoek.[1] He called them "small animals" and it was not until the year 1828, that the German microscopist Ehrenberg, used the name bacteria for the first time.[2] The name is a Latinisation of the Ancient Greek word bakterion, meaning rod, staff. Later that century, Koch, a German scientist, proposed the rules for showing causality between a specific bacterium and the corresponding disease, called the Koch´s postulates, which was the beginning of modern microbiology.[3]

In 1882 Alexander Ogston, a Scottish surgeon, isolated and named the group of bacteria that he called staphylococci.[4-6] He isolated the bacteria from abscesses, grew them in vitro, and fulfilled Koch´s postulate, as the bacteria later caused infection when inoculated in mice. At that time, his result evoked scepticism and it took some time to get the work published.[5, 6]

Staphylococci are facultative anaerobic, gram-positive cocci and in a microscope they are looking like a bunch of grapes (Greek, staphylé, bunch of grapes), small clusters or chains. The genus staphylococcus consists of more than 50 different species and subspecies[7] and among these there are one of our most dreaded pathogens but also several common inhabitant of the skin. In this thesis, I am just going to concentrate on two species that possess several similarities from a clinical aspect, namely *Staphylococcus aureus* and *Staphylococcus lugdunensis.* So alike and so different.

First, we roughly divide the genus staphylococcus into two categories: coagulase-positive and coagulase-negative. The enzyme coagulase is a virulence factor that clots blood and is a potential way to evade the human immune system. The species *S. aureus* harbours this enzyme, but not the other staphylococci, which are therefore named, coagulase-negative staphylococci (CoNS).

CoNS, assessed as harmless skin commensals, can on special occasions, most often on indwelling foreign material, cause severe infections. This is true with at least two exceptions, *S. lugdunensis* and *Staphylococcus saprophyticus*. *S. saprophyticus* is a well-known pathogen causing urinary tract infections, but not any other infections in humans and will not be further investigated in this thesis.

Staphylococcus aureus

S. aureus is sometimes referred to as an opportunistic bacterium, though it can be a skin commensal and just occasionally causes infection in its carriers. The incidence of *S. aureus* skin or nasal carriage differs between countries[8] and in Sweden it has been reported between 21-29%.[9, 10]

It is also one of our most common pathogens in human bacteraemia.[11, 12] The incidence of *S. aureus* bacteraemia (SAB) in humans has been reported to 10-30 per 100,000 person-year in the industrialized world,[13] and so also in Sweden.[14] In Denmark a longitudinal study could show an increasing incidence over 33 years, from 3 to 20 per 100,000 person-years,[15] Swedish data, between 2000-2008, also shows an increasing incidence from 15 to 30 per 100,000 person years.[16]

In the pre-antibiotic era, the fatality rate was as high as 82% for bacteraemia caused by *S. aureus*,[17] but after the introduction of antibiotics, the mortality rate decreased to approximately 20%.[18] [19] This has been unchanged for years until recently, when a study showed a decreased mortality rate, despite the fact that the more recent cohort was older and had higher comorbidities (in the previous cohort 18% vs. the newer cohort 13%).[20]

Clinical manifestations

As mentioned before, *S. aureus* can cause a wide variety of infections. Rather harmless skin infections are by far the most common clinical manifestations but bacteraemia with *S. aureus* is a common infection in the hospital ward and not seldom somewhat complicated to treat. These infections can be community acquired or nosocomial infections most often due to central or peripheral venous catheters or as ventilator associated pneumoniae.[21, 22]

The prevalence of diagnoses differs between different cohorts studied, but the most common primary foci reported are skin and soft tissue infections (SSTI) (19-27%) [14, 22], catheter-related infections (14-25%),[14, 22, 23] infective endocarditis (8-22%),[24-26] osteoarticular infections (15-21%)[14, 27] and prosthetic joint infection (2-10%).[28, 29] Surprisingly often, unknown focus is reported, 19-63%,[14, 22, 23] and if this is a result of unthorough examination or because it sometimes is hard to reveal the focus is not known. Although it has been shown that mortality rate decreases when an infectious specialist examine the patients,[30-32] unthorough examination can be part of the explanation.

Which type of infection the patient gets, can of course be a matter of chance, but most probably a wide variety of different factors are involved. Several risk factors in the specific patient along with virulence factors that the specific isolate harbours are probably a more reasonable answer.

Risk factors of getting SAB

To be a nasal carrier of *S. aureus* has been shown to be a risk factor for clinical infections.[33, 34] Higher frequency of carriage has been reported in males,[33, 35] persons with atopic dermatitis,[36, 37] and in patients with haemodialysis.[38, 39] This might explain that males consistently have a higher incidence of SAB than female.[22, 40-43] The carriage of *S. aureus* might partly even explain the risk factor for acquiring SAB in patients in haemodialysis. Haemodialysis is a well described risk factor by several previous studies, and the incidence reported is approx. 22-46 per 1000 person years compared with the incidence in the control group reported to 0.5 per 1000 person years.[44, 45]

Risk factors for adverse outcome

Age is a strong risk factor for death in SAB[40] and the 30-day mortality rate increases from 37% at the age of 65 years to 57% in patients aged ≥ 85 years.[46] Several reports state that the mortality rate is higher in women[22, 47-49] while others do not.[50] This is not fully understood and more studies on this subject are needed. There has also been a study addressing the question if outcome could be correlated to penicillin resistance or methicillin resistance of the isolate in *S. aureus* infections.[51] This study could not show any statistical significance in mortality rate between those with infections caused by penicillin resistant and penicillin susceptible isolates. On the other hand, a Danish study showed significant higher mortality in patients with infections caused by penicillin resistant *S. aureus* than those patients with infections from penicillin susceptible isolates.[52] A German study later confirmed this result showing a higher OR for mortality in infections caused by strains harbouring the *blaZ* gene compared to infections caused by strains without the *blaZ* gene.[25]

Not only a high mortality has been reported, but also, a high frequency of relapse, 11-24% in SAB.[14, 53-55] Some previously described risk factors for adverse outcome are alcoholism, immunosuppressive treatment, endocarditis and pneumonia,[40] unknown focus of infection,[22, 23] renal failure, dialysis and other severe comorbidity such as diabetes and paraplegia.[23] None of these seem to be controversial from a medical perspective. On the contrary, a Danish study showed a 90-day case fatality rate at 18% for SAB in patients with end-stage kidney disease, lower than 34%, showed in the control group.[45] Even though the impact of antibodies against *S. aureus* is not revealed, carriers have a lower mortality than non-carriers.[56]

Uncomplicated/Complicated SAB

SAB is often divided in two clinical categories according to the severity of the disease and these correspond to different treatment approaches. It is *uncomplicated* and *complicated* SAB. Several attempts have been done to define *uncomplicated* SAB, but it seems as if these criteria often must be adjusted to the local setting.[57-60] IDSA have a definition on *uncomplicated* SAB based on whether it fulfils these criteria:[61]

- o Negative follow up blood culture, 48-96 h after the index blood culture
- o Defervescence by 72 h after appropriate antibiotic therapy
- o Exclusion of endocarditis
- o Absence of major prosthetic/implant devices
- o Absence of metastatic infection, such as septic embolization

Since 2018, Region Skåne have a local recommendation, based on the IDSA definition, for the treatment of *uncomplicated* SAB.[62] The IDSA definition is modified as follows: negative follow up culture 48-72 h after treatment initiation and major prosthetic/implant devices are defined as any intracardial device or prosthetic valves. Any prosthetic joints are not taken in consideration as a major prosthetic/implant device.[62] The treatment recommendation for *uncomplicated* SAB is 10-14 days of antibiotics intravenously (*iv.*). The evidence for this regime is not rock solid, although there are several researchers that have shown a higher risk of relapse with shorter treatment duration and none that have proved the opposite yet.[63, 64]

There have been a few observational reports with shorter duration in uncomplicated SAB, but the numbers are small, and a lack of control group makes these data hard to relay on.[21, 32] Recently a retrospective study from Denmark compared short-course treatment (6-10 days) vs. long-course treatment (11-16 days), were no significant difference in 90 day mortality rate could be shown.[65] Some major concerns about the study have been raised by Tong et al. such as, patients included in the long-course being sicker, having higher C-reactive protein, having undergone echocardiograms to a larger extent and in the short-course having more intravenous device-related infections. Further on, the mortality rate was unexpectedly high (17-23%) for being rated "low risk" SAB.[66] Another study from Abbas et al. in 2019 [67] tried to address the same question, a paper with lots of complicated statistic work but the most interesting question is not answered: Does short treatment, less than 14 days of antibiotics, in uncomplicated SAB, have the same outcome as long treatment, namely ≥14 days? The study included 149 patients with uncomplicated SAB, 46 of which were having a short treatment, ≤ 14 days. The result showed a significantly lower 90-day mortality rate in treatment duration ≥ 14 days for

complicated SAB, but no difference in 90-day mortality rate between the two treatment durations in uncomplicated SAB. The study was underpowered to show any small differences in uncomplicated SAB between the treatment groups which is a major limitation. Mortality rate in the whole group of uncomplicated SAB was 27%, which can be considered high as selected as "low risk" patient.

The group referred to as complicated SAB are those diagnoses of deep-seated infections like, infective endocarditis, spondylodiscitis, and bone and joint infections. All these diagnoses have different recommendations concerning antibiotic duration. For example, the recommendation for infective endocarditis caused by *S. aureus* is 2-4 weeks of *iv.* antibiotics depending on which valve involved.[68] Furthermore, in spondylodiscitis, treatment duration is 1-2 weeks of *iv.* antibiotic followed by oral treatment till a total time of 3 months.[69]

Choice of treatment in SAB

Which is the most favourable antibiotic choice in SAB, is a question yet to be answered, but which has been extensively analysed. A high frequency of relapse has been described in treatment with vancomycin compared to other anti-staphylococcal beta-lactams.[70, 71] These results, reported repeatedly, are well accepted and in Sweden it is not encouraged to use vancomycin is MSSA infections.

Numerus studies have also been made to reveal any outcome differences between various beta-lactams. In a Danish study, conducted by Nissen et al.,[72] penicillin G and dicloxacillin were compared with cefuroxime. The main outcome showed a mortality rate significantly higher in the cefuroxime treatment group (39% vs 20%). Albeit being statistically correct, the cefuroxime dose, in this study, was half of the recommended dose and this may have had an impact on the final result. In another Danish publication from 2013, Rasmussen et al. showed a significantly higher 90-day mortality rate, in a propensity-score matched cohort, for cefuroxime treatment compared to dicloxacillin. Neither antibiotic doses nor interval between doses were presented. Since this publication has patients from the same time period and geographical area as Nissen et al., it can be assumed that they probably have the same patient cohort and one can wonder if cefuroxime was underdosed here as well. The mortality rate is also surprisingly high in both treatment groups, 25% and 38% respectively.[73]

Forsblom et al. compared the first week of treatment with cloxacillin to cephalosporins, including cefuroxime and ceftriaxone, as a predictor of 28- and 90-day mortality rate. No differences were shown.[74] A retrospectively conducted study from Israel, tried to elucidate the differences in 30- and 90-day mortality rates between cloxacillin/cefazolin and other beta-lactam treatments of SAB. They could not show any differences between the groups.[75] The study had several limitations as there was no analysis between the different groups concerning diagnosis, length of

antibiotics, previous comorbidities and other outcomes. The overall mortality rate was also remarkably high, between 32-42%. Paul et al. published a study in 2011 with the question if all beta-lactams were similarly effective in the treatment of *S. aureus*. Their summarised answer was that cloxacillin was superior compared to cephalosporines like cefuroxime, ceftriaxone and cefotaxime, but just showed for the empirical treatment. The analysis for definitive treatment could not show any differences between the groups of antibiotics.[75]

Surprisingly few studies have been made comparing penicillin G and isoxazolyl penicillins, despite the fact that penicillin G previously has been the treatment of choice, according to many guidelines in susceptible isolates.[76-79] Over the years guidelines have changed their recommendation due to the insecurity in the laboratory techniques testing penicillin susceptibility,[80] even though it is shown that susceptibility tests are reliable.[81-83]

The reappearance of penicillin-susceptible *S. aureus* has lately been elucidated.[84-88] A few publications have examined penicillin G as a treatment option in comparison with other anti-staphylococcal treatments. Henderson et al. published a large retrospective cohort study in 2019, showing that penicillin G treatment was superior to flucloxacillin treatment. The major limitations to this study were that neither information about the duration of therapy, combination treatments nor doses or intervals of antibiotics were registered, which may influence the outcome.[89] A small Japanese study compared penicillin G and ampicillin with cefazolin, without any outcome differences between the groups, although as a total 44 patients were examined.[90] Shah et al. showed a comparable outcome between nafcillin, cefazoline and penicillin G treatment in SAB, but the frequency of adverse events was significantly higher in the nafcillin group.[91]

Staphylococcus lugdunensis

This is a pathogen quite recently described, i.e. in 1988,[92, 93] and the name derives from Lugdunum, the Latin name for the French city Lyon where *S. lugdunensis* was first discovered. Like other CoNS, *S. lugdunensis*, is a skin commensal, found in 22-67% of healthy control persons, mainly found in the groin and on lower extremities.[10, 94]

Unlike other CoNS, it is known to be more virulent when causing infections. In this way resembling those infections caused by *S. aureus*, such as infective endocarditis, skin and soft tissue infections, arthritis, and prosthetic joint infections[95-98] and is rarely a contaminator when cultured from sterile loci.[99, 100] The latter, probably due to its normal habitat, the groin, not in the direct vicinity of where blood cultures normally are obtained.

Although the incidence of *S. lugdunensis* infection is much lower than *S. aureus,* the number of registered isolates have varied between 2008-2019, in Skåne. Blood cultures yielded 10-15 isolates yearly with a peak of 25 isolates in 2017 (unpublished data) to be compared with approx. 600 *S. aureus* single-patient isolates in blood cultures yearly in Skåne. In cultures from skin and soft tissue, during the same time period, *S. lugdunensis* was found in 100-700 single-patient isolates every year. In Denmark the total incidence of all sorts of infections with *S. lugdunensis*, has been reported to 53 per 100,000 per year.[101] A reported risk factor of bacteraemia caused by *S. lugdunensis* is haemodialysis.[102]

In contrast to other CoNS, *S. lugdunensis* has a well-preserved susceptibility to most antibiotic agents, including penicillin G.[97] Although the susceptibility ratio differs in different parts of the world, probably according to antibiotic consumption, *S. lugdunensis* is still far more susceptible than other CoNS species.[95, 103, 104] In Scandinavia where the antibiotic consumption is relatively low, the susceptibility rate is still high to most antibiotics.[105] One reason for this preserved susceptibility might be due to the low degree of genetic diversity between the isolates.[97, 106, 107] But one case report has elucidated the risk of emergence of resistance during antibiotic treatment.[108]

Clinical manifestations

As mentions above, *S. lugdunensis* causes a variety of different infections, and endocarditis was the first clinical manifestation described.[93] After this first report several has followed, most of them with one, or a few, cases together with a short review of the, at time, spare literature. All these cases have horrifying examples of a rapid aggressive infection, not seldom ending with valve destruction and death.[109-114] This has led to the fact that these infectious endocarditis are considered particularly severe and in need of acute surgery.[68, 96] The most common clinical manifestation is skin and soft tissue infection[100, 101] and other clinical manifestations registered are bone and joint infections, both in native joints and prosthetic joints.[115] Recently several observations have elucidated *S. lugdunensis* as an emerging pathogen in prosthetic joint infections.[116-121] In these prosthetic joint infections high rates of relapse have been demonstrated (13-21%).[120, 122] Mortality rate in *S. lugdunensis* bacteraemia has been reported to 13-24%.[102, 111, 113]

Endocarditis

The overall incidence of infective endocarditis (IE) ranges from 3-7 per 100,000 person-year.[80]

Infective endocarditis is a criteria-based diagnosis, based on a publication from Duke University Medical Centre, and revised by Li et al.[123, 124] Modified and simplified Duke criteria shown in Figure 1. The major and minor criteria reflect the weight they individually contribute to the diagnosis. One of the major criteria: "typical microorganism from two separate blood cultures" is often fulfilled for *S. aureus*, but not for *S. lugdunensis*, even though infective endocarditis are similarly frequent in bacteraemia with both species.[25, 26, 104, 114, 125] Although some researchers support the idea that *S. lugdunensis* should be considered as a typical pathogen in the same way as *S. aureus* in this matter.[114]

Several studies have been made trying to aid the clinicians in how to exclude endocarditis in SAB without conducting a transoesophageal echocardiogram (TEE).[42, 126-128] Healthcare-associated SAB has been reported to have a lower frequency of IE, suggesting that echocardiography are not required for these cases [42]. In Skåne it is recommended to do a transthoracic echocardiogram (TTE) in all patients with SAB.[62]

In patients with SAB approximately 4-16 % are diagnosed with endocarditis and this is one of the most frequent occurring deep seated infection.[29, 52, 125, 129] Septic embolization in infective endocarditis caused by *S. aureus* have been reported in a frequency of 46%.[112] The mortality rate in IE has been reported higher than in general SAB 46-71% vs 6-24 % .[52, 129-131]

Definite infective endocarditis:
2 major criteria or 1 major criterion and 3 minor criteria or 5 minor criteria
Possible infective endocarditis:
1 major criterion and 1 minor criterion or 3 minor criteria

Major criteria

o Blood culture positive for IE,
  o two separate blood cultures with microorganism consistent with IE:
    o *S. aureus*
  o Microorganism consistent with IE from persistently positive blood cultures,
    o ≥2 positive blood samples drawn > 12h apart;
    o 3 or majority ≥ 4 separate blood cultures
  o Single positive blood culture for *C. burnetti* or IgG titre >1:800
o Evidence of endocardial involvement
o Echocardiogram positive for infective endocarditis
o New valvular regurgitation

• Minor criteria
  o Predisposition, predisposing heart condition or injection drug use
  o Fever, > 38.5° C
  o Vascular phenomena: major arterial emboli, septic pulmonary infarcts, mycotic aneurysm, intracranial haemorrhages, conjunctival haemorrhages, and Janeway´s lesions
  o Immunologic phenomena: glomerulonephritis, Osler´s noduli, Roth´s spots and rheumatic fever
  o Microbiological evidence: positive blood culture but does not meet a major criterion noted above

Figure 1. Showing the modified Duke´s clinical criteria for the diagnosis of infective endocarditis

The incidence of IE in *S. lugdunensis* bacteraemia is noted between 6-46% from different reports.[104, 113, 114, 132] Infective endocarditis caused by *S. lugdunensis* has previously exclusively been described by case reports, small case series or reviews, and no population-based study has been published, which makes the incidence numbers hard to interpret. The literature bear witness of an aggressive form of IE with high rate of valve destruction and prompt need for surgery. The mortality rate in IE has been reported to 13-39 %.[110-112] Need of surgical intervention and occurrence of septic embolization has also been reported surprisingly high 61-75% and 17-33% respectively.[110, 111 109, 133, 134] This can of course be a result of bias by publication but is an indication that this is a pathogen of significance.

Virulence factors involved in endocarditis are, the ability to attach to and invade endothelial surface and to form biofilm at the place. Both *S. aureus* and *S. lugdunensis* possess abilities to do this. The exact mechanism of this is not revealed but *S. aureus* binds to damaged heart valves via two cell-wall anchored proteins, clumping factor A and the von Willebrand factor binding protein. The virulence factors involved in this process in *S. lugdunensis* have been suggested to involve von Willebrand factor binding protein and the binding of fibrinogen.[135-138]

Prosthetic joint infections

The incidence of prosthetic joint infections (PJI) in knee and hip protheses is 1-3 % of those going through arthroplasty.[139, 140] With a growing older population, prosthetic joints are expecting to increase and so also the absolute number of infections.[141] Staphylococci are the most frequent bacteria causing PJI including both *S. aureus* and CoNS.[142] Men have a higher risk of getting surgical site infections following joint arthroplasty, but the reason for this is not fully understood.[143] They are also at higher risk for infections with staphylococci in general which in part can be the explanation for this.[22, 40, 41, 43] Other risk factors showed for PJI are prior surgery on the index joint, obesity and rheumatoid arthritis.[43]

Prosthetic joint infection occurs when bacteria have established growth on arthroplasty in the joint and cause an infection. Even though this seems crystal clear, it has previously not been internationally defined and therefore it is sometimes tricky to compare different studies. The definition of PJI by IDSA was published in 2013 and together with EBJIS (European bone and joint infection society) definition, published in 2021, will make it easier to compare studies.[144-146] The definition by EBJIS define the probability of true PJI, definitions shown in Table 1, modified to those results available in Skåne. Unfortunately, EBJIS definitions neither address the timespan correlated to surgery, nor the duration of infection. On the contrary, the IDSA definitions reflects the different treatment options possible according to

when the infection appears and for how long the suspected infection has existed.[145] Acute PJI, defined as symptoms less than 3 weeks in an earlier well-fixed prosthesis or no more than one month postoperative, is recommended debridement, antibiotic and implant-retention strategy (DAIR).[145] If symptoms duration last more than 3 weeks, it is regarded as a chronic infection and the surgical intervention and antibiotic strategy recommended are thereby different. In these cases, surgical options are either, one stage replacement, 2-stage replacement or even amputation. Patients not fit for surgery, due to comorbidities or age, are recommended suppressive antimicrobial treatment according to the susceptibility test.[145] It is not a far-fetched thought that the infections with acute onset could be caused by more virulent strains than the non-acute onset chronic infection.

Over the last years, several reports have described *S. lugdunensis* as an emerging pathogen in bone and joint infections, including PJI.[119, 120, 122, 147] In a large database study of hip and knee periprosthetic joint infection, 4% of the infections were caused by *S. lugdunensis* and by their definitions 75% where late infections.[148]

Table 1. EBJIS definitions with the modification just showing analyses that are clincallly availible in Skåne.

	Infection Unlikely All findings negative	Infection Likely Two positive findings	Infection Confirmed Any positive finding
Clinical and blood workup			
Clinical features	Clear alternative reason for implant dysfunction	a) Radiological signs within first 5 years after implantation b) Previous wound healing problems c) History of recent fever or bacteraemia d) Purulence around the prosthesis	Sinus tract with evidence of communication to the joint or visualisation of the prothesis
C-reactive protein		>10mg/L	
Synovial fluid cytological analysis			
Leucocytes in synovial fluid (cells/mikroL)	<1500	>1500	>3000
Microbiology			
Aspiration		Positive culture	
Intraoperative (fluid or tissue)	All cultures negative	Single positive culture	>Two positive cultures

Virulence factors in *S. aureus* and *S. lugdunensis*

All bacteria possess virulence factors to be able to survive in their normal habitat. This can be abilities to thrive under different conditions and to evade attacks from the immune system or from competing bacteria and fungi, and to get hold of different vital nutrients. The virulence factors can be harmful to us as a host/infected patient or harmful to competing bacteria. Some virulence factors are similar in these two species, and some are not. Phylogenetically these species are closely related to each other,[135] but there are also major differences between the species. While the genome in *S. aureus* is open for acquisition of foreign DNA, the genome in *S. lugdunensis* is closed and show a low genetic diversity.[106, 135] This may in part explain that the repertoire of virulence factors in *S. lugdunensis* is less than in *S. aureus*.[135] This may also be an explanation to why, although *S. lugdunensis* is a more prevalent skin inhabitant, the infections caused by *S. aureus* are dominating the scene.

It is believed that acquired virulence factors comes with a cost for the bacteria harbouring them. This can lead to that genes of no use, are excreted and lost.[149] On the contrary a Danish study of infective endocarditis showed that patients infected with penicillin G susceptible *S. aureus* strains had lower mortality rate compared with those infected with penicillin resistant strains.[52] This was also confirmed by a German study.[25]

Coagulase, a molecule excreted by *S. aureus*, converts fibrinogen to fibrin via activation of prothrombin. *S. aureus* also harbours a cell wall linked protein, clumping factor A that converts fibrinogen to fibrin directly. *S. lugdunensis* also possess a cell wall anchored clumping factor, distinctive from *S. aureus* but with the similar activity. This clumping factor is not present in all isolates but when it is, if tested for coagulase in a clinical laboratory, isolate can be mistaken for a *S. aureus* strain. The clumping factor is a virulence factor shown to be important in the mechanism of bacteraemia and endocarditis.[135]

Panton-Valentine Leucocidin (PVL), is a virulence factor in *S. aureus*, found in 1932 by Panton and Valentine.[150, 151] This is a two-unit poor forming toxin, inducing apoptosis in human phagocyting leukocytes *in vitro*.[152, 153] The clinical significance has been elucidated especially in young children with necrotizing pneumonia.[154] It has also been shown that skin infections caused by PVL positive isolates correlate to the presence of major abscesses compared to PVL negative isolates, but not associated with the cure rate of infection.[155]

Lugdunin, a virulence factor, shown by Zipperer et al., is an antibiotic peptide produced by *S. lugdunensis*. It has been shown that this peptide, has a bactericidal activity against other pathogens such as enterococci and *S. aureus*.[156] This study also showed that persons that are carries of *S. lugdunensis* had a 6-fold lower risk of

being *S. aureus* carriers. The lugdunin stimulates the immune response in the keratinocytes, which protects against colonisation of *S. aureus*. [157] This virulence factor is a good example of a peptide made to outcompete other species in the same habitat and a potential compound that could be developed to a new antibiotic drug for humans. For this reason, it have been speculated that deliberate colonisation of *S. lugdunensis* could be a prevention for *S. aureus* colonization.[156] Since *S. lugdunensis* can be a potential aggressive pathogen, this seems doubtful.

Biofilm formation

Biofilm formation is a process that starts when planktonic bacteria have adhered to a surface and then form an environment to thrive in. Natural biofilm formation is present on our own teeth, twice a day exposed to mechanical influence of our toothbrushes. When speaking about biofilm in medicine we most often mean biofilm formation in relation to foreign material.

The initial reversible step in biofilm formation is the adhesion to the surface via hydrogen bonds, van deer Waals forces and electrostatic bonds. The sessile bacteria then produce a species specific extracellular matrix containing polysaccharides, proteins, DNA and cell debris.[95, 158] This extracellular matrix forms a complex microbial community with the encapsulated bacteria. The matrix also contributes to the antimicrobial resistance by its structural barrier and by binding directly to the antibiotic substances per se.[159] Furthermore, bacteria living in the biofilm community have a different gene expression than those living in planktonic form. This leads to a declining bacterial cell growth and subsequently lower the susceptibility to cell-wall active antibiotics such as the beta-lactams. [159]

The altered gene expression can also enhance the facilitation of fibrin attachment and then contribute to the formation of capsules, with encased bacteria, and also evade the immune system by intricate immune modelling systems.[160]

The ability to form biofilm differs between species and between isolates of the same species.[158] Biofilm formation is a known virulence factor in staphylococci and is considered to play a significant role in some infections like PJI and endocarditis.[161] Speaking against the clinical importance of the latter, is that the recommended antibiotics used in endocarditis in native valves are not biofilm active.[68] Although this can be due to the presence of human endothelial cells that regenerate in contrast to the prosthetic hardware infected in PJI.

The clinical significance of biofilm formation in PJI has been elucidated by a study from Gothenburg, were a more robust biofilm formation in *S. aureus* and *S. epidermidis* was correlated to worse outcome.[162] Morgenstern et al. also showed a trend towards decreasing cure rate with increasing ability to form biofilm, in orthopaedic devise infections by *S. epidermidis*.[163] Furthermore, it is shown that

there is an association of biofilm formation capacity in *E. faecalis* and catheter related infections.[164]

S. lugdunensis have been described to possess the ability to form biofilm in tested strains. [165] Genes controlling the process and factors affecting the biofilm formation in *S. lugdunensis*, are partly revealed.[118, 165-169] Biofilm formed by *S. lugdunensis* is to a larger extent protein based compared to biofilm formed by *S. aureus* which is dominated by polysaccharides.[95]

Frank et al. showed that the biofilm formation increased by the presence of nafcillin in 93% of the tested *S. lugdunensis* isolates.[170] They could also show that those *S. lugdunensis* isolates susceptible to vancomycin had an impaired minimal bactericidal concentration (MBC), indicating a tolerance and reduced killing capacity for vancomycin.[95] In contrast to previous studies, Ausbacher et al. could show removal of mature biofilm formed by *S aureus*, when penicillin G was added in their test milieu.[171]

Penicillin resistance

One can ascertain that antimicrobial excretion and resistance in bacteria are virulence factors, not only in the interaction with humans, but to outmanoeuvre other competing bacteria and fungi in their normal habitat. In the very beginning when penicillin was discovered, almost all *S. aureus* strains were penicillin susceptible.[172] Just a few years after the introduction of penicillin G, a number of reports concerning penicillin resistance were published[173, 174] and also Fleming, warned of the development of resistance when treating bacteria with penicillin in his speech as a Nobel laureate.[175]

Staphylococci can be resistant to penicillin in two different ways. Either they produce penicillinase, coded by the *blaZ* gene, that degrades the penicillin, or they have an altered penicillin binding protein (PBP), which makes the binding to the majority of beta-lactams insufficient. The strains with altered PBP are referred to as Methicillin resistant *S. aureus*, MRSA.

BlaZ gene-penicillinase
The most common way of resistance to penicillin is due to the enzyme penicillinase that hydrolyse the beta-lactam ring, thus making penicillin inactivated and useless. Penicillinase is coded by the *blaZ* gene, located either on a plasmid or on the chromosome.[172, 176] In the presence of penicillin, a sensor protein BlaR1 trigger a signalling cascade system resulting in cleavage of the repressor BlaI and subsequently the penicillinase production is induced.[177] The production is induced in 30-60 minutes[178, 179] and the penicillinase is excreted extracellular, also able to help surrounding bacteria. This in contrast to Gram-negative bacteria that have their beta-lactamases mostly in the periplasmatic space.[172]

There are four different types of *blaZ* genes, from A-D, and they possess different degrees of activity in forming penicillinase.[180, 181] Both *S. aureus* and CoNS can harbour the *blaZ* gene and they share the same ancestor gene.[176] The genetic differences between the *blaZ* genes carried on plasmids and on the chromosome, from different staphylococci, indicate that the evolution has been separated long ago. There are also indications that the exchange of the *blaZ* gene between species and strains is an extremely rare event.[176]

Altered PBP

The other way of possessing beta-lactam resistant is to have an altered penicillin binding protein (PBP). This alternation is coded by the *mecA* gene located on the bacterial chromosome within the SSC*mec* (Staphylococcal cassette chromosome *mec*).[182] This *SSCmec* is a mobile element that can be transferred to other *S. aureus* strains. Those strains that possesses this gene are called MRSA. The altered PBP, called PBP2a, has a reduced affinity for almost all beta-lactams, except for ceftobiprole and ceftaroline.[7] The specific PBP2a expression is induced by the presence of beta-lactams indicating that the production bears a cost for the bacterium.[183] It has been shown that penicillin G has a higher affinity for PBP2a than isoxazolyl penicillins, and this has even led to the somewhat wild suggestion that these strains could be treated with penicillin G along with a beta-lactamase inhibitor, if the strains are penicillinase producers.[184] Although the binding of penicillin G to the PBP2a enzyme is very slow and in therapeutic concentrations of antibiotics *in vivo*, the amount of bound penicillin G to PBP2a is negligible compared to the bacterial generation time, which makes it a doubtful antibiotic for clinical situations.[183]

Antibiotics

The first antibiotic, treating syphilis, was discovered in 1909 by Paul Ehrlich, a German scientist, accompanied by Hata, a Japanese microbiologist. This medicine, Salvarsan, was the standard of care for syphilis, until penicillin was available in the mid-1940s.[185]

In 1928, Fleming discovered the potential effect of the mould called *Penicillin notatum* on bacterial growth. He published the results in 1929, but there was hardly any response from the research community.[186] The first beta-lactam antibiotic was found, and this was one of the greatest steps for mankind. When Florey, Chain and co-workers, published the effect of penicillin in *S. aureus* infections in mice, there was increased interest and in 1943 a large-scale production of penicillin, using *Penicillium chrysogenum,* for clinical use was developed.[186] This would be a gamechanger in the treatment of infectious diseases.

Antibiotics acts in different ways but they all must be able to exceed the minimal inhibitory concentration (MIC) of the targeted bacteria in the blood stream or at the site of infection. The MIC-value is determined by the minimal concentration of antibiotic needed to kill all visible bacteria in the growth medium. In these investigations the bacterial concertation is standardized, and the incubation temperature as well as the incubation time.[187] To be able to measure the effect of different antibiotics, killing assays have been done and has revealed that different groups of antibiotics have different dosing strategies for best antibiotic effect.[188]

In beta-lactams the best corresponding effect is the time period when the antibiotic concentration is above the MIC-value of the targeted bacteria. It is recommended that the *in vivo* free antibiotic concentration for penicillins shall be above the MIC-value more than 50% of the time.[188]

Beta-lactams

Penicillin was the first beta-lactam used, even though its way of action was not discovered until 1965, by Wise and Park.[189] All beta-lactams have a lactam-ring, which is essential for the interaction with the bacterial PBP. The PBP is an enzyme that facilitate the process of crosslinking the peptidoglycan production in the cell wall. These cross-linkings anchor the cell-wall layers to each other and are crucial for cell-wall stability.[190]

Different bacterial species possess variable numbers of variants of the PBP enzyme.[190] Therefore, different beta-lactam antibiotics have dissimilar affinity to the PBPs in different species.[190] *S. aureus* possess genes for several different PBPs and they have slightly different targets in the peptidoglycan biosynthesis. They can all be expressed simultaneously or induced by the presence of antibiotics, e.g. the PBP2a.[190]

The penicillin molecule possess similar structure with the natural substrate of the PBP, the D-Ala-D-Ala dipeptide, see Figure 2. When penicillin and PBP are forming a covalent complex, it leads to an inactivating of the PBP enzyme.[183] This inhibition is strong and hydrolyses slow. The regeneration of PBP is also slow and this together is leading to cell wall instability with cell lysis and death as a consequence.[7, 191] As such, penicillin is bactericidal only to actively growing and dividing susceptible bacterial strains.[192]

Nowadays there are several other groups of natural and synthetic beta-lactams such as cephalosporines, monobactams and carbapenems, as such, or in combination with different beta-lactamase inhibitors.
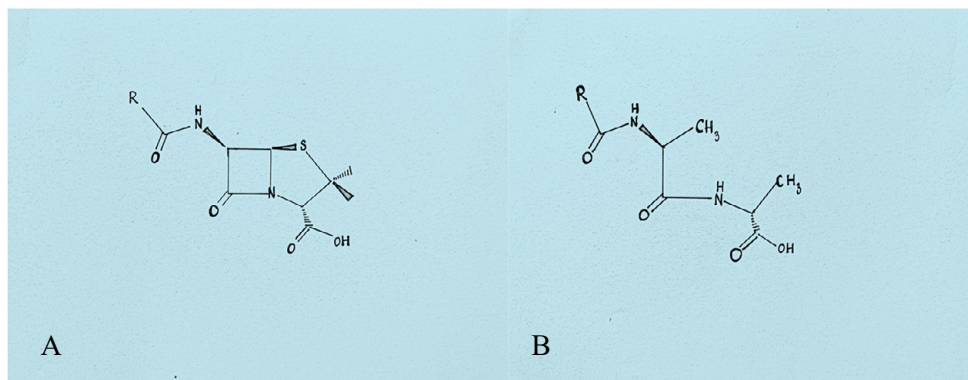
Figure 2. Panel A. Shows penicillin G molecule structure and B the normal substrate for the transpeptidase PBP, D-Ala-D-Ala dipeptid. R is the side chain.

Penicillins

All penicillins consists of three chemical components: a thiazolidine ring, a beta-lactam ring and side chain. The side chain determine the penicillin´s pharmacokinetic properties and spectrum of activity.[191] The beta-lactam ring is the unstable part that makes the binding to the PBP and is also the target for penicillinases. When penicillin is hydrolysed by the penicillinase, opening the beta-lactam ring formation, it will result in the biologically inactive penicilloic acid.[193] Changes in the acyl side chain can make a steric protection against the penicillinase.[191, 194]

Due to the increasing resistance to penicillin G among *S. aureus* strains, penicillinase-stable penicillins were developed. One of the first registered was methicillin accompanied by nafcillin.[194] Methicillin does not belong to the later developed acid stable isoxazolyl group and nor does nafcillin, but both were used as anti-staphylococcal antibiotics exclusively and nafcillin still is.

Penicillin G

Among those few early antibiotics, penicillin G was a major clinical success, which is still extensively used today. In penicillin G the side chain includes a benzyl ring giving it the commonly used name benzylpenicillin, see Figure 3. In Sweden, we quantify the penicillin G in grams, but International Units (IU) are used in many other countries and one IU correspond to 0.6 grams.[195]

Penicillin G does not resist the gastric digestion, making it unable to administrate orally and has to be administered intravenously or intramuscular.[196] On the contrary phenoxymethylpenicillin (penicillin V) is acid stable and often given as the oral choice to penicillin G. The penicillin V is also more resistant to the penicillinase produced by *S. aureus* than penicillin G.[195] As most penicillins, penicillin G is excreted by the kidneys and is to be reduced with renal impairment.[197]

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Penicillin G is known as a non-toxic antibiotic even at high doses, but the most common side effect is rashes, noted in 2% of the users.[197] Even though a rather rare event it is also known to lower the threshold for seizures, at high doses.[196, 198] Anaphylaxis is a rare but serious adverse event, reported in less than 1 in 1000 treated patients.[197]

Isoxazolyl penicillins

Today there are several different penicillinase-stable penicillins, belonging to the acid stable isoxazolyl penicillins, in clinical use, i.e., oxacillin, flucloxacillin, dicloxacillin and cloxacillin. The use of the different isoxazolyl penicillins differ according to geographical location. Cloxacillin is the most common *iv.* isoxazolyl penicillin used in Sweden today and also used in parts of Europe, while Australia uses flucloxacillin *iv.* In the USA, nafcillin and oxacillin are other common options, and in Japan, isoxazolyl penicillins or nafcillin are not available at all.[90] These differences may be due to different judgments of safety data, and the pharmaceutical companies' decisions of where to launch their products.[195]

One major difference between isoxazolyl penicillin and the penicillin G molecule, is the hydrophobic side chain which makes it resistant to penicillinase and acid stable.[195] This side chain also binds the antibiotic molecule to plasma proteins to a higher extent than what the penicillin G molecule does.[193] The cloxacillin molecule shown in Figure 3. When penicillin G have a protein binding of approximately 30-50%, isoxazolyl penicillins binds up till 93-97%, leaving only a few percent active antibiotic in the blood stream.[199] Although there is a recently published study which concluded that the unbound, active substance of flucloxacillin in patients was higher than expected from a theoretical point of view and compared to previous studies on healthy controls.[200] The different isoxazolyl penicillin possess basically the same pharmacokinetic properties with just a slight differences between them.[199]

Cloxacillin is excreted in the bile to a larger extent than penicillin G and renal excretion is less prominent. Therefore, some researchers advocated not to adjust for renal impairment.[201] Although, there are reports of severe side effects, such as delirium and coma, when no adjustments due to the renal impairment were done.[198, 202]

Common side effects registered (>1/100, <1/10) are, rashes, thrombophlebitis, and diarrhoea.[201] It is also known to cause, renal impairment, bone marrow depression[201] and liver toxicity.[203] One study in children reports of discontinuing of cloxacillin, due to side effects such as leukopenia and hepatitis in 53% of the cases.[204]
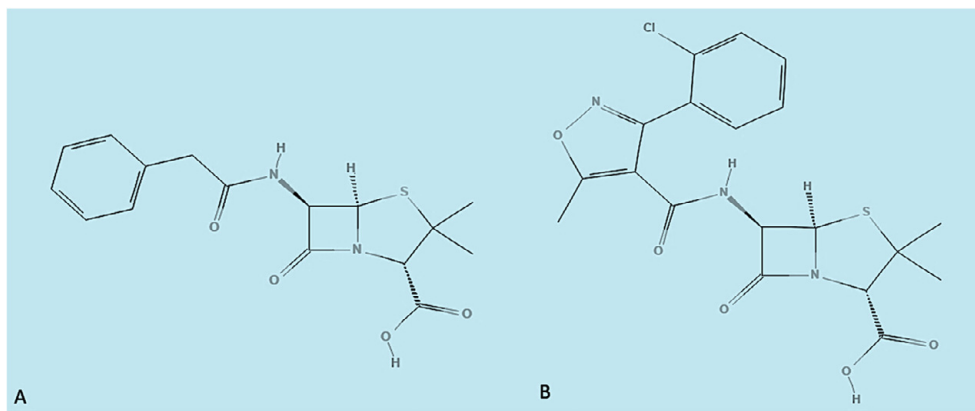
Figure 3. molecular structure of A, penicillin G and B, cloxacillin.
National Center for Biotechnology Information (2022). PubChem Compound Summary for CID 5904, Penicillin g.
Retrieved March 13, 2022 from https://pubchem.ncbi.nlm.nih.gov/compound/Penicillin-g.PubChem Compound
Summary for CID 6098, Cloxacillin. https://pubchem.ncbi.nlm.nih.gov/compound/Cloxacillin.

Differences between penicillin G and cloxacillin

When comparing penicillin G with cloxacillin there are some practical and theoretical differences. It is well established that when cloxacillin is given intravenously, the risk of thrombophlebitis is substantial.[205, 206] This is an additional drawback when treating old patients with small fragile blood vessels as it is and, in these situations, a central venous catheter is often preferable. This problem is not at all as common when treating patients with penicillin G, even though stated as a common side effect in FASS.[197] Penicillin G is given as a short injection and cloxacillin as an infusion, leading to more work for the nurses and in Skåne, cloxacillin is recommended to be dosed four times daily compared to three times with penicillin G.

Pharmacokinetic and pharmacodynamic differences

Pharmacokinetics describes the way the human body interact with the drug, in our case the antibiotics. Included in this is the absorption, distribution, metabolism and elimination of the drug. Pharmacodynamics describes how the drug interacts with the body, or in our case mainly with the bacteria.

Since the best effect of beta-lactam antibiotic is based on the MIC-value in the specific species, the free concentration of the antibiotic in blood and the time this free concentration is above the MIC-value of the targeted pathogen, we can compare these properties for cloxacillin and penicillin G in *S. aureus*. In *S. aureus* the MIC-value for cloxacillin is higher than the MIC-value for penicillin in susceptible strains, indicating that lower concentration of penicillin G is needed to achieve bacterial inhibition. Figure 4 A shows the histogram for cloxacillin and 4 B the histogram for penicillin G.

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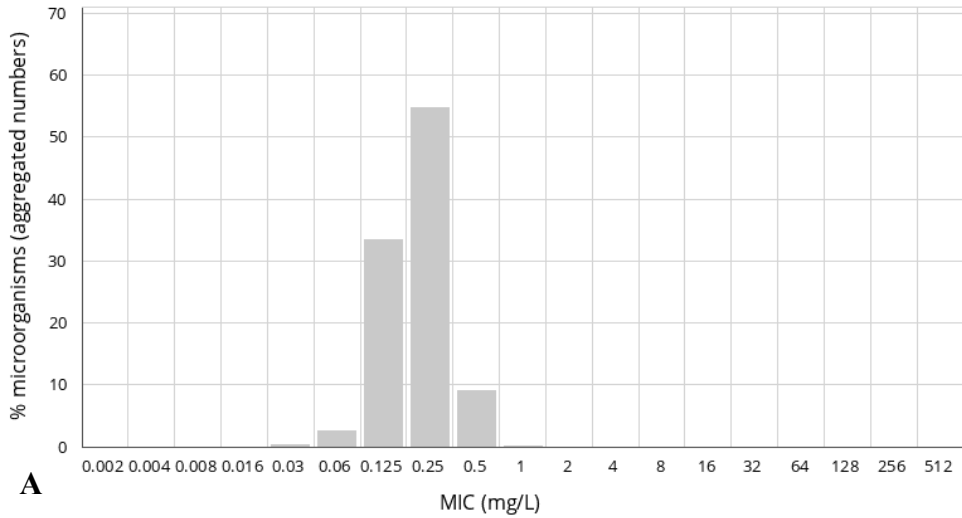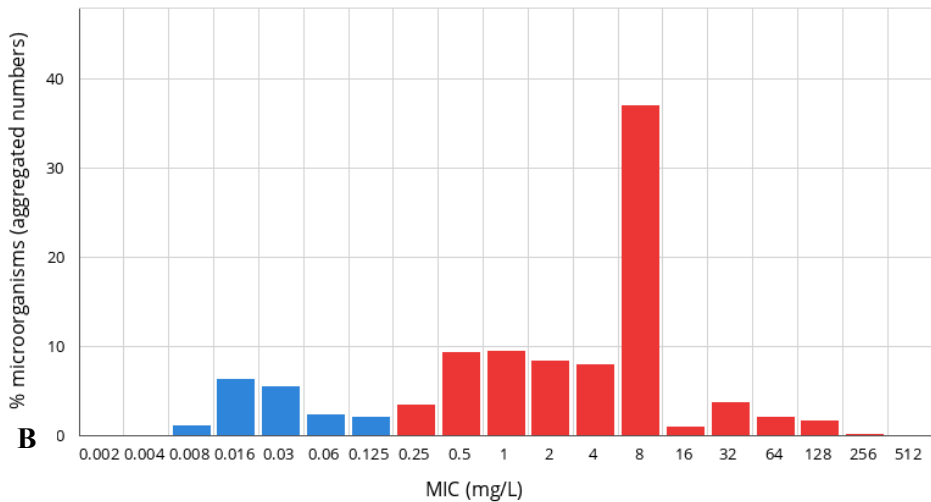
Figure 4. Histogram in S. aureus for cloxacillin (A) and penicillin G (B) , respectively. The blue represent susceptible strains and the red represent resistant strains. Grey represent the distribution of wild-type strains for *S. aureus*. Published with courtesy of EUCAST, European Committee on Antimicrobial Susceptibility Testing. Data provided by EUCAST MIC distribution website, last accessed 15 Feb 2022. http://www.eucast.org

The maximum concentration of cloxacillin in blood, when given 2g *iv.* is 100mg/L and the half-life is 30 min.[201, 207] The protein binding is as high as 92-96% and therefore only a small amount of the antibiotic is active. The maximum concentration of penicillin G in blood when given 3g *iv.* is 300mg/L and the half-

life 30-60 min. The protein binding is 30-50%.[197, 199, 207] The high protein binding in cloxacillin, giving a small amount of active antibiotic, and the short half-life, makes the theoretical time above the MIC-value much shorter for cloxacillin than for penicillin G. Although, this is a very simplified model since the volume of distribution and the antibiotic protein binding equilibrium is not considered. This can although give a hint of some of the different properties between the two compounds, summarised in Table 2.

Table 2. Summerises the time free antibiotic concentration is above the MIC-value (fT>MIC) for *S. aureus* in a crude model. The MIC values in the table represent the highest MIC and the mode MIC for *S. aureus* for the different antibiotics.

	C_{max} mg/L	$T_{1/2}$ min	Protein binding	fT>MIC, MIC 0.5	fT>MIC MIC 0.25
Cloxacillin 2gx3	100	30	94%	25%	31%
Cloxacillin 2gx4	100	30	94%	33%	42%
				fT>MIC MIC 0.125	fT>MIC MIC 0.064
Penicillin G 1gx3	32 after 1h	45	65%	58%	69%
Penicillin G 3gx3	300 (50 after 1h)	45	65%	78%	88%

Antibiotic treatment of staphylococci infections

To summarize; This is a complex assemblage of different types of infections. The recommended antibiotic duration, antibiotic administration and sometimes the need of surgery differ between the diagnoses. The best choice of antibiotics has still not been convincingly showed, but penicillin G seems to have many advantages compared with cloxacillin. Despite this, cloxacillin is the most commonly used anti staphylococcal antibiotic in Sweden today.

Aims

The overall aim was to elucidate some clinical aspects of different types of staphylococcal infections, prevalence of penicillin-susceptible staphylococci and the effect of penicillin treatment.

The specific aims in the different papers were:

- To determine the frequency of penicillin-susceptible *S. aureus* isolates from blood and wound cultures in Skåne, Sweden, and to evaluate, methods for penicillin testing in *S. aureus*. We also wanted to investigate if penicillin-susceptible isolates were associated with higher mortality.

- To retrospectively assess penicillin susceptibility rates among *S. lugdunensis* isolates and to evaluate different methods for penicillin susceptibility testing according to CLSI and EUCAST guidelines. A secondary aim was to describe the clinical presentation of *S. lugdunensis* infections from isolates included in the study and to review the antibiotic treatment given.

- To describe the clinical presentation of infective endocarditis caused by *S. lugdunensis*, and to compare with the clinical presentation of infective endocarditis caused by other CoNS and *S. aureus* registered in the National Swedish Registry of Infective Endocarditis. A secondary aim was to present the antibiotic treatment in endocarditis caused by *S. lugdunensis*.

- To describe the clinical presentation of prosthetic joint infections caused by *S. lugdunensis* and to correlate the ability to form biofilm with the outcome.

- To compare 90-days mortality in *S. aureus* bacteraemia in patients treated with penicillin G or cloxacillin. A secondary aim was to investigate the difference between the two antibiotics with a combined patient orientating outcome.

Methods

Microbiological methods

Susceptibility testing

Some of the methods to determine the susceptibility of antibiotics in a particular bacterial strain used in this thesis are listed below. In the very beginning of bacteriology every laboratory had its own way of determining this, but later on, the methods became more standardized and today most of the laboratories follow standard methods described by the European EUCAST or the American CLSI. These institutions also provide standard templates to be able to interpret the result of each strain tested into susceptible or resistant.[208, 209] Below I explain the methods and their implications. The pros and cons of every method are discussed in the discussion part of the thesis.

Disk diffusion method

In this method a standardised bacterial suspension at 0.5 McFarland is conducted. The 0.5 McFarland standard correspond to the turbidity of bacterial suspension of approximately 1-2 $\times 10^8$ colony forming units (CFU).[210] It can be measured at optical density (OD) at 600 nm with absorbance result between 0.08-0.1. This standard can also be constructed with a mixture of barium chloride and sulfuric acid, for visual comparison.[210]

The bacterial suspension is spread on an agar plate where the antibiotic disk is placed in the centre. The agar plate is then incubated at the specified temperature and during the specified time period advocated by the EUCAST or CLSI, depending on protocol followed. The antibiotic is diffusing in the agar layer and inhibit bacterial growth where the antibiotic concentration is high enough for killing the tested bacterial strain. This makes a zone around the antibiotic paper disk with inhibition of bacterial growth.

The diameter with no bacterial growth is then measured in mm and compared with a reference standard from the European EUCAST, or the American CLSI respectively.[187, 208, 211] The interpretation is "susceptible" or "resistant" as standard.

In some species and for some antibiotics, there can also be the interpretation I "increased exposure" which means that the patient have to have a higher dose and/or more frequent doses of antibiotic to get the concentration needed to cure the addressed bacteria. The method is standardised both regarding to the agar content, antibiotic content in the disk and the quantity of agar in each plate. All this strictly regulated methods are to be able to get reliable and repeatable results.[211]

When it comes to penicillin G susceptibility test in *S. aureus*, not only the zone diameter is of importance, but also the appearance of the zone edge. When testing a penicillin susceptible strain, the zone edge appearance is fuzzy and when testing a penicillinase producing strain the zone edge is sharp. The fuzzy edge contains colonies that are partly lysed by the penicillin and therefore giving this appearance.[195] For the isolates producing penicillinase, they all help each other by excreting penicillinase in the zone edge, making a sharp edge or a heaped edge,[195, 212, 213] shown in Figure 5. This is the advocated method to determine penicillin susceptibility in *S. aureus* according to EUCAST.[209] The CLSI first advocates the chromogenic nitrocefin test and then zone edge appearance before reporting as susceptible in *S. aureus*. When it comes to penicillin susceptibility test in *S. lugdunensis,* CLSI state that just diameter is taken in to account and further penicillinase tests are not necessary since isolates producing beta-lactamase will be resistant in their tests.[208]
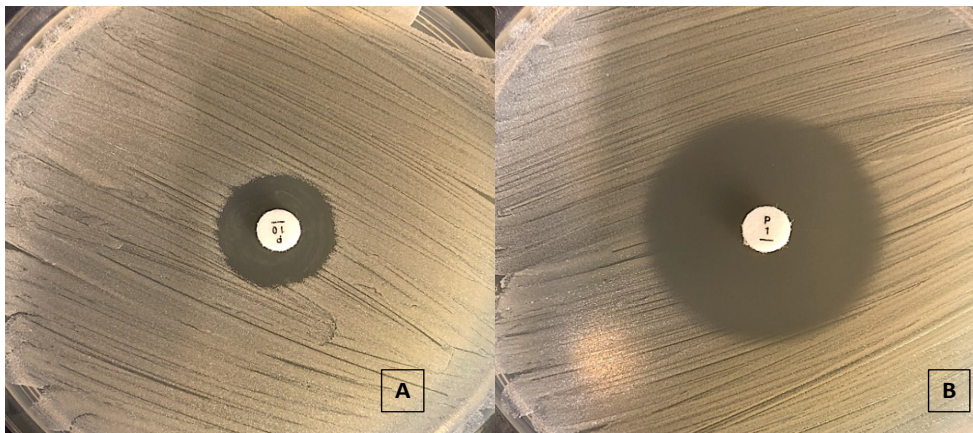
Figure 5. Disk diffusion test with penicillin G. Picture A, shows a sharp zone edge appearance (resistant) and B, shows a fuzzy zone edge appearance (susceptible).

MIC determination with E-test

The golden standard to evaluate the minimal inhibitory concentration (MIC) is with broth microdilution in tubes with different antibiotic concentration. This method is both time and material consuming and often replaced by the easier E-test, which is

a paper strip impregnated with antibiotic in a gradient. The paper strip is then placed on an agar plate where the suspension of testing bacteria has been spread. This results in an epsilon (E) shaped recess in bacterial growth, therefore called E-test. The method is easy to use and not as time consuming as the broth microdilution method. The epsilon test for penicillin G was used in paper II.

Clover-leaf test

This is a test to determine if a strain is excreting beta-lactamase to its surrounding. An agar plate is covered with the indicator strain, susceptible to the tested antibiotic, penicillin G.[214] The tested strain is applied as a line from the centre to the periphery of the agar plate. Optimally a negative and positive control strain is applied in 90 degrees to the tested strain to form a cross. In the middle an antibiotic paper disk, penicillin 1U, is placed. If the tested strain produce beta-lactamase, the indictor strain will be able to thrive along the tested strain, where the beta-lactamase is excreted, forming a rounded shape towards the antibiotic disk, see Figure 6. We used a penicillin susceptible *S. pneumoniae* strain as indicator and conducted the test on blood agar plates. If a penicillinase producing strain is applied in all four lines it will result in a clover leaf shape, explaining the name of the method. This method was used in paper I.
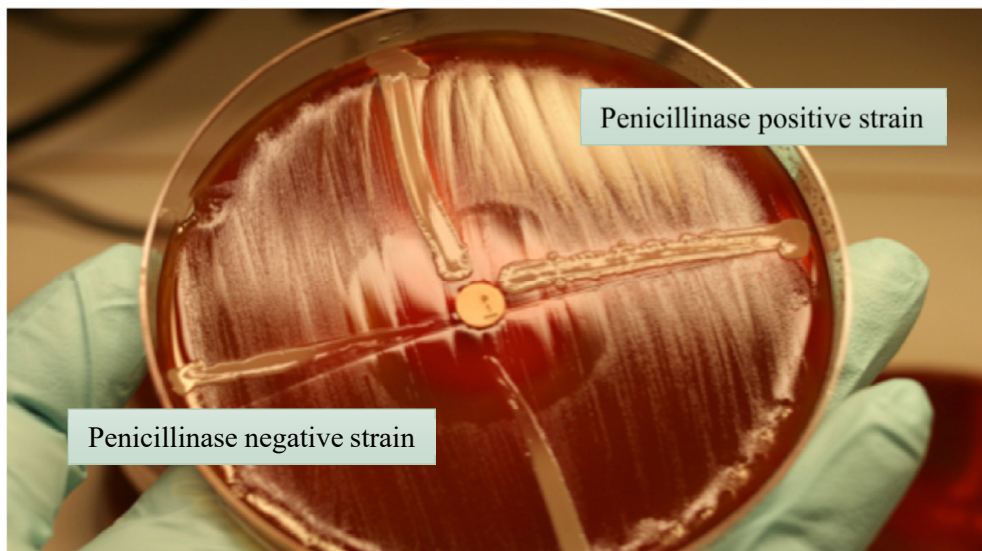
Figure 6. Clover-leaf test were two strains are penicillinase producing (in the upper region and right of the plate) and two non-penicillinase producing strains (in the lower and to the left.) A growth of indicator strain along the penicillinase producing strain is seen.

PCR-method

The polymerase chain reaction (PCR) is a method where specific genes or parts of the DNA are amplified. Which part of the DNA that is amplified is determined by the *primer*, a short sequence matching the DNA where the determined gene is located. Except for the primers, one in each direction, the PCR process needs, heat stable DNA polymerase that can multiply the DNA sequence and base pairs as building blocks. This gene fragment is then multiplied in a cyclic process where every cycle result in several new completed gene specific DNA sequences.

In my research, I used this method to multiply the *blaZ* gene. This gene encodes for the enzyme penicillinase, which degrades penicillin, in staphylococcal species, such as, *S. aureus* and *S. lugdunensis*[176]. Those strains that do not harbour this gene are unable to produce penicillinase. Furthermore, I have also amplified the *nuc* gene, encoding the thermostable nuclease, species specific for *S. aureus* .This nuclease is an enzyme excreted extracellular by *S. aureus* which degrades DNA and RNA. [215] In paper I the PCR of the *nuc* gene worked as a proof that the PCR method was functioning and that the *blaZ* negative samples contained bacterial DNA from *S. aureus* and that these isolates were true *blaZ* negative.

In paper II when penicillin susceptibility testing was conducted in *S. lugdunensis,* these strains do not possess the *nuc* gene and the positive control proving that the samples contained bacterial DNA was made by amplifying the 16S gene. The 16S gene is coding for one part of the procaryote ribosome. PCR of this gene is often used to determine which bacterial species it is, since every species have a unique genetic code in this 16S gene region.

Biofilm formation

To conduct the study on biofilm formation in *S. lugdunensis* we followed a protocol recommended for staphylococci presented by Stepanovic et al.[216] In general, bacteria grown overnight were suspended in growth medium, Tryptic soy broth with glucose (TSBG), in a 96-well plate and incubated for 24 h. The protocol by Stepanovic was followed with the modifications of washing the initially formed biofilm with PBS (Phosphate buffered saline) with micropipette and fixation the biofilm in 60° C air. After the biofilm was dyed with crystal violet, the superfluous dye was washed with PBS 6 times. The resolved biofilm was measures as the optical density (OD), at 550 nm. The OD of crystal violet detected by the spectrophotometry is assumed to correlate with the amount of biofilm produced by each isolate in each well.

This is a strict in vitro method and the influence of the immune system in the process is not tested. Negative controls in the investigations are the plain growth medium without adding the bacterial cells. All isolates were tested in triplicates.

Epidemiological and Statistical methods

Study settings

Retrospective studies

All studies were conducted as retrospective studies on patients with different staphylococcal infections. Patient included in the studies were collected due to the presence of either *S. aureus* or *S. lugdunensis* isolates from a clinical cultured sample. The clinical data were collected from medical records in paper I, II, IV and V and from the National Swedish Registry of Infective Endocarditis in paper III. In Paper I we collected bacteriological strains of *S. aureus* prospectively, but patient characteristics were collected retrospectively.

Statistical methods

All statistical analyses were performed using SPSS software 25 and 27 (SPSS, Armonk, NY, USA). Variance analyses were tested with chi-squared test for categorical variables, and Mann-Whitney U for continuous variables, when appropriate. All tests were two tailed and p-values < 0.05 were considered significant.

In Paper II we used methods to determine sensitivity, specificity, positive predictive value and negative predictive value. These can be demonstrated in a two-by two table, see Table 3. Here exemplified by figures from Paper II, the penicillin susceptibility test in *S. lugdunensis* according to CLSI.

Table 3. Example from paper II, outcome of susceptibility test according to CLSI with *S. lugdunensis* strains. Penicillin G disk containing 10 U.

	blaZ positive	*blaZ* negative	Sum
Phenotype R	36 (True resistant)	0 (False resistant)	36
Phenotype S	1 (False susceptible)	75 (True susceptible)	76
Sum	37	75	112

Sensitivity = 36/(1+36) Probability that a true resistant isolate also becomes resistant in the phenotypically test. If this is low, isolates can be interpreted as susceptible but are resistant.

Specificity = 75/(0+75) Probability that true susceptible isolate is phenotypically susceptible in the test. If the specificity is low, some of the susceptible strains will be interpreted as resistant.

Positive predictive value- 36/ (36+0) Probability that those with a resistant test are true resistant.

Negative predictive value- 75/ (1+75) Probability that those with a susceptible test result is true susceptible.

Survival analysis

In Paper III, we conducted a time-to-event (death) analysis in endocarditis caused by three different bacterial species. This time-to-event was calculated during the first 30 days after the first day of hospital admission in a Kaplan-Meier analysis. This is a regression analysis where no adjustments are made of other contributing variables, and it is assumed that the risk to event is the same over time.

Logistic regression

Logistic regression was used in Paper IV and V and is a statistical method that try to overcome some drawbacks of a retrospective design of a study and thereby tries to mimic a randomized trial.

First, logistic regression is a statistical method trying to distinguish how much each variable impacts the binary outcome as such. In this statistical method you can adjust for different variables that can be confounding factors and influence the outcome or factors that are imbalanced between the compared groups. The logistic regression yields an odds ratio that indicates on how much the single factor is contributing to the outcome. The logistic regression can be done as a univariate regression just looking at one variable or multivariate regression where several factors can be adjusted for. As a rule of thumb, you can adjust for one variable for every 10 patients in the outcome. In Paper V we compared the treatment outcome between penicillin G and cloxacillin. The adjustment were made with factors known to impact the outcome such as, age, co-morbidity index,[217, 218] Pitt bacteraemia score[219] and factors that differed between the treatment groups as complicated SAB, unknown SAB, treatment duration and inadequate treatment duration.

Propensity score analysis

In Paper V we calculated a propensity score for outcome analysis. Propensity score is another method trying to mimic a randomized trial not shown better than logistic regression but another way of calculate.[220] Propensity score is a probability to get the study treatment. When calculated, variables that are clinical considered to influence the choice of treatment are included. In our study these variables were age,

gender, co-morbidity index, Pitt bacteraemia score, *iv*. drug use and presence of prosthetic heart valves or pacemaker/ICD.

The propensity score can then be used in the final outcome analysis in different ways; to match patients in both groups, stratify patients, adjust as a variable in a logistic regression or make an inverse probability treatment weighting (IPTW).[221] The latter was conducted in paper V. This weight to every patient, reflects how many times the case shall be counted as a participant in the final analysis, giving a larger "pseudo population". Every case is presented according to their weight in the invers propensity score as followed, treatment group 1/p and non-treatment group 1/(1-p) where p is the propensity score.

Results

Paper I

Setting

In paper I, clinical isolates of *S. aureus* were identified with the database at the Department of Medical Microbiology in Skåne. We prospectively collected 141 isolates from skin infections, 140 isolates from bacteraemia during 2014/2015 and retrospectively 100 isolates from bacteraemia during 2008/2009. We conducted penicillin susceptibility tests with disk diffusion, noted zone edge appearance, did clover-leaf assay, and in all isolates performed PCR for the *bla*Z gene. Medical records from those patients with isolates from *S. aureus* bacteraemia, were reviewed. Mortality rate at 30 days and 90 days was noted.

Result

This study showed that 29% (*n*=41) of the *S. aureus* isolates from bacteraemia 2014-2015 was penicillin susceptible and 57% (*n*=57) of the *S. aureus* isolates from bacteraemia in 2008-2009 was penicillin susceptible. The isolates from skin infections were penicillin susceptible to a rate of 21%. The method including both zone diameter and zone edge appearance matched the result from the *bla*Z PCR. In eight isolates the zone diameter were ≥26mm but they all had sharp zone edge and negative cloverleaf tests, therefore interpreted as penicillin resistant strains, histogram shown in Figure 7. We could not find any difference in mortality rate between patients infected with penicillin susceptible strains compared with those infected with resistant strains. The overall 30-day mortality rate was 21%.
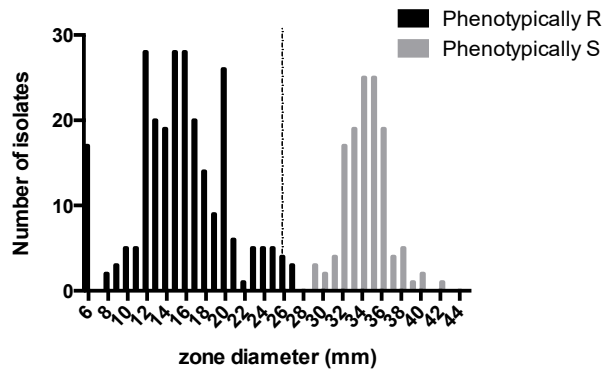
Figure 7. Histogram of *S. aureus* strains tested with penicillin G 1 U in disk difussion test. Grey bars represent strains phenotypically susceptible to penicillin G and black represents phenotypically resistant strains.

Paper II

Setting

Patients registered with a *S. lugdunensis* isolate from sterile locations, between 2015-2017, were identified from the database in Department of Medical Microbiology in Skåne. Isolates were retrospectively collected from stored stocks and cultured. These isolates were then tested for penicillin G susceptibility with the different methods according CLSI and EUCAST recommendations.[208, 209] We added the judgement of zone edge appearance, MIC-value by E-test, nitrocefin test and PCR of the *blaZ* gene. The *blaZ* PCR was used as reference method. Medical records from the patients were reviewed and information of comorbidity, age, sex, and outcome were registered.

Result

112 strains of *S. lugdunensis* were included in the study and 67% (*n*=75) were *blaZ* negative. The method according to EUCAST was accurate but the CLSI method yielded one major error. If the zone edge appearance was added to the CLSI-method, it resulted in 100% accuracy. The correlation between isolates tested with penicillin 1U, 10 U and MIC-value by E-test is shown in Figure 8.
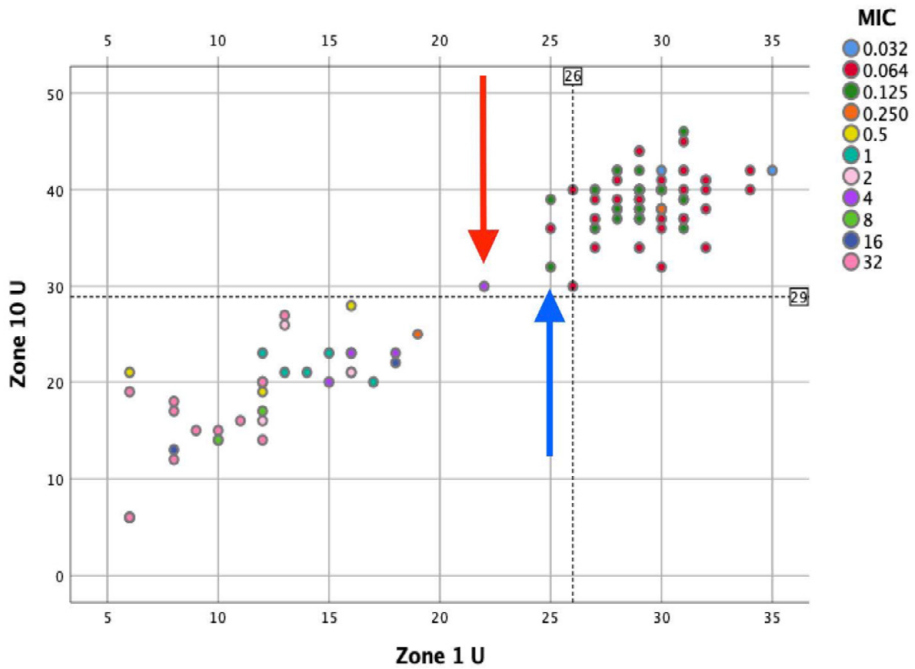
Figure 8. All isolates shown as an individual dot with the results from penicillin 10U, 1 U in the diagram. The MIC-value is marked with colour according to the key. Red arrow shows the isolate that falsely is interpreted as susceptible in the CLSI method, blue arrow indicates those isolates falsely interpreted as resistant according to EUCAST method.

The sensitivity, specificity, PPV, and NPV according to every method used are shown in Table 4, note here corrected numbers according to the errata. All but one isolate were susceptible to isoxazolyl penicillin. The most common type of infection, in 55%, was foreign body infection. Infections were more common in men, 69% (*n*=77). The 30-day mortality rate was 12%.

Table 4. Sensitivity, specificity and predictive values of phenotypic tests for the detection of penicillin resistance in *S. lugdunensis*. PCR of the *bla*Z gene was used as reference method. Updated table with corrected numbers.

Penicillin G	Sensitivity (%)	Specificity (%)	PPV	NPV
1U disk	100	96	92.5	100
10U disk	97.3	100	100	98.7
10U disk+ zone edge	100	100	100	100
Nitrocefin	91.9	98.7	97.1	96.1
Etest	100	98.7	97.4	100

PPV=Positive predictive value, NPV=Negative predictive value

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Paper III

Setting

The National Swedish Registry of Infective Endocarditis was used to retrospectively identify patients with endocarditis caused by *S. lugdunensis*, *S. aureus* and CoNS between 2008-2018. The registry has changed over time and during the first years the registry only had tick boxes to confirm different diagnoses and none to negate information. We did not impute information but missing information about comorbidities were interpreted as a negation of the condition. All antibiotic susceptibility data were received by personal contact with the local microbial laboratory. The comparison between the three different groups of infective endocarditis were analysed with Chi-squared test or Mann-Whitney U when appropriate. For survival analysis a Kaplan-Meier curve and log-rank test were conducted.

Result

In total, we found 30 cases of endocarditis caused by *S. lugdunensis*, 262 cases caused by other CoNS and 1892 cases caused by *S. aureus*. In endocarditis caused by *S. lugdunensis* the proportion of native valve engage was high (70%), and the most common localisation was the aortic valve (60%). In the cases infected with *S. lugdunensis*, 90% fulfilled the Duke´s criteria for definitive endocarditis. The embolization frequency with *S. lugdunensis* was low (7%, *n*=2) both compared to *S. aureus* (48%, *p*<0.001) and CoNS (24%, *p*=0.033). All *S. lugdunensis* isolates were susceptible to isoxazolyl penicillin, except two isolates with missing data and the most common antibiotic treatment was isoxazolyl penicillin (60%). The in-hospital mortality was comparable between the groups, but death occurred earlier, in median after 9 days, in the *S. lugdunensis* group, data shown in Table 5. This makes the all-cause mortality rate at 30 days higher in the *S. lugdunensis* group (20%, *n*=6) compared with other CoNS (7%, *n*=17) and *S. aureus* (9%, *n*=166) *p*=0.016, illustrated in Figure 9.

Table 5. Basic data and outcome data of included patients.

Bacteria (n)	*S. lugdunensis* *n*=30	CoNS *n*=262	*P*-value CoNS vs *S. lugdunensis*	*S. aureus* *n*=1892	*P*-value *S. aureus* vs *S. lugdunensis*
Background data					
Age (years); median (IQR)	73 (65-84)	72 (61-80)		66 (45-79)	*p*= 0.01
Gender-female	11 (37%)	86 (33%)		725 (38%)	
Diabetes	9 (30%)	58 (22%)		349 (18%)	
Cancer last 5 years	6 (20%)	46 (18%)		173 (9%)	*p*=0.042
IV drug users	0 (0%)	9 (3%)		448 (24%)	*p*=0.002
Prosthetic valve	8 (27%)	115 (44%)		255 (14%)	*p*=0.037
Pacemaker/ICD	1 (3%)	74 (28%)	*p*=0.031	324 (17%)	*p*=0.046
Native valve disease	5 (17%)	55 (21%)		222 (12%)	
Treatment delay, days median (IQR)	9 (4-15)	10 (3-26)		5 (2-9)	*p*<0.001
Duke´s criteria					
Definite	27 (90%)	194 (74%)		1544 (82%)	
Possible	3 (10%)	67 (26%)		338 (18%)	
Localization					
Aortic	18 (60%)	121(46%)		577 (31%)	*p*=0.001
Mitral	10 (33%)	76 (29%)		596 (32%)	
Tricuspid	1 (3%)	22 (8%)		441 (23%)	*p*=0.01
Type of infection					
Prosthetic IE	6(20%)	110(42%)	*p*=0.02	245 (13%)	
Pacemaker/ ICD IE	1(3%)	48 (18%)	*p*=0.01	179 (9%)	
Native valve IE	21 (70%)	90 (35%)	*p*=0.0001	1103 (58%)	
Community acquired	25 (83%)	179 (68%)		1543 (82%)	
Outcome					
Antibiotic treatment, median days (IQR)	31 (18-37)	35 (28-42)	*p*=0.046	30 (28-40)	
Embolization	2 (7%)	62 (24%)	*p*=0.033	907 (48%)	*p*<0.001
Surgical intervention	7 (23%)	111(42%)	*p*=0.044	455 (24%)	
Day of surgery, median (IQR)	5 (1-9)	12 (5-20)		12 (7-23)	
Mortality at 30 days In hospital mortality	6 (20%) * 7 (23%)	17(7%) 49 (19%)		166 (9%) 268 (14%)	
Day of death in hospital, median (IQR)	9 (8-23)	36 (28-47)	*p*=0.007	25 (14-39)	*p*=0.016

*p=0.016
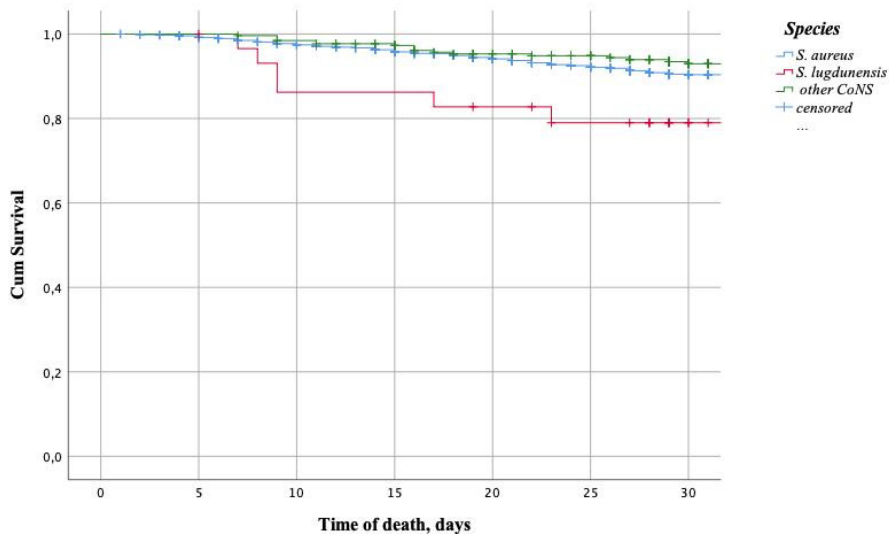
Figure 9. Kaplan-Meier curve on mortality at 30 days in infective endocarditis.

Paper IV

Setting

Patients with *S. lugdunensis* cultured in sterile loci, between 2015-2019, were identified from the database at Department of Medical Microbiology at Skåne University Hospital. Isolates were collected and tested for biofilm formation according to previously described method.[216] Medical records were reviewed and patients with prosthetic joint infections (PJI) were selected for a deeper analysis. To compare groups and for outcome analyses, Mann-Witney U test, Chi-squared test and logistic regression were conducted. The type of infections were classified according to when and how the PJI appeared. The *acute postoperative* infection was defined as infection occurring within one month postoperative. *Late acute hematogenic* infection was defined as symptom duration less than 3 weeks in a previously well-functioning prosthetic joint and lastly *chronic infection* was defined as symptom duration longer than 3 weeks starting more than one month after implantation.

Result

The aim was to describe prosthetic joint infections caused by *S. lugdunensis* and to correlate the isolates biofilm formation to outcome. We retrospectively collected 141 isolates of *S. lugdunensis* and 36 of these were from prosthetic joint infections (PJI). The most common form of PJI was postoperative infection (*n*=20, 57%). Surgical treatment was done in 97% of the patients (*n*=33). The most common antibiotic treatment was vancomycin (*n*=15, 42%). The overall cure rate in PJI was 81% (*n*=29). All of the 141 isolates, but two, formed biofilm. The biofilm formation capacity in PJI was correlated to type of infection, were isolates from *late acute hematogenic* infections produced more robust biofilm than those in *acute postoperative* infection, shown in Figure 10 A. There was also significantly more robust biofilm formation in isolates causing relapsing infections in PJI, shown Figure 10 B. A correlation between robust biofilm formation and relapsing infection, in PJI, was shown with the OR 3.7 (95% CI 1.21-11.57, *p*=0.02).
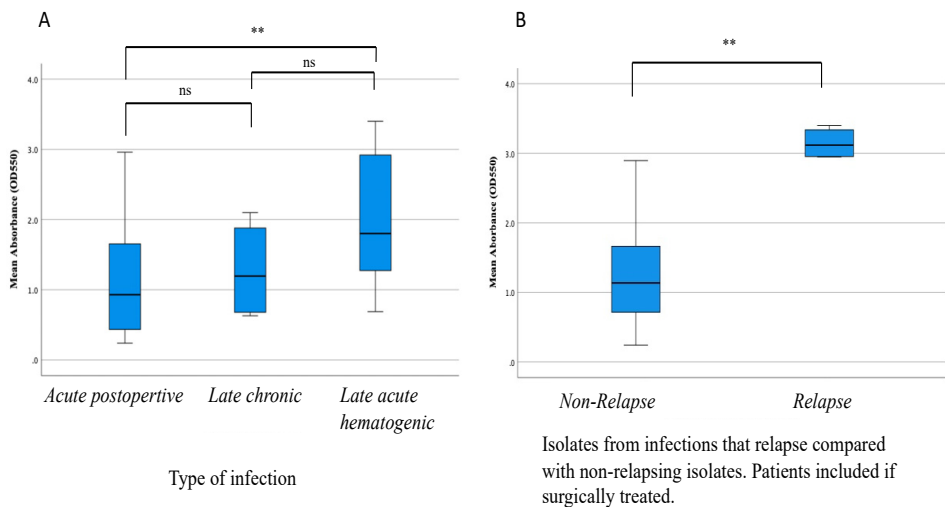

Figure 10. Panel A, type of infection correlated to biofilm forming capacity. Panel B, biofilm formation in relapsing isolates compared to non relapsing isolates in those patients that underwent surgical treatment

Paper V

Setting

All patients with blood steam infections caused by penicillin susceptible *S. aureus* between 2018-2020 were registered. An outcome scale, ranking from best to worst, was conducted for the purpose. The best outcome was to have survived without any complications within 90 days after finished antibiotic treatment, followed by adverse events registered during the treatment but without any changes in treatment regime, followed by changes or addition of antibiotics due to adverse events or treatment failure, relapsing infection within 90 days after treatment completion, and last death within 90 days. Clinical data was noted, and the worst outcome rank was registered for every patient. A comparison with patients treated with penicillin G and cloxacillin was made with logistic regression and propensity score analyses with inverse probability treatment weighting (IPTW). Follow up time was at least 6 months.

Result

We included 384 patients with blood stream infections caused by penicillin susceptible *S. aureus*. Cloxacillin treatment was given to 316 patients and penicillin G to 68 patients. The background data differed between the groups with a higher frequency of complicated SAB in the penicillin G group. The 90-day mortality rate was comparable between the groups (19%, *n*=61, 13%, *n*=9, *p*=0.24). The cases were ranked according to the predefined ranking system, outcome presented in Table 6. The overall outcome, having any complication was more common in the cloxacillin group (45%, *n*=142 vs 29%, *n*=20, *p*=0.02). Cloxacillin had the OR 2.43 of having any complication in the adjusted logistic regression analysis (95% CI 1.3-4.53, *p*=0.005). Results shown in Table 7. The propensity score weighted analysis confirmed the result.

Table 6. Outcome according to the ranking scale.

Outcome	Penicillin G *n*=68 (%)	Cloxacillin *n*=316 (%)	*P*-value
1. Alive at 90 days, no complications	48 (71)	174 (55)	0.02
2. Adverse events	5 (7)	29 (9)	0.23
3. Change or addition of antibiotic agent	4 (6)	33 (10)	0.25
4. Relapse within 90 days	2 (3)	19 (6)	0.31
5. Death within 90 days	9 (13)	61 (19)	0.24

Table 7 . Logistic regression comparing the outcome of any treatment complication (rank 2-5) with alive at 90 days without any complications (rank 1) between cloxacillin and penicillin G.

Variable	Unadjusted			Adjusted		
	OR	95% CI	*P*-value	OR	95% CI	*P*-value
Background						
Gender (female=1)	0.87	0.58-1.32	0.52			
Age, years[a]	1.02	1.01-1.04	0.001	1.02	1.01-1.04	0.004
CCI[b]	1.10	1.00-1.20	0.022	1.09	0.99-1.21	0.087
Iv-drug user	0.53	0.17-1.74	0.30			
Infection						
PBS	1.07	0.94-1.23	0.315	0.98	0.85-1.14	0.83
Uncomplicated SAB	0.53	0.33-0.84	0.007			
Complicated SAB	1.82	1.20-2.74	0.005	1.51	0.87-2.63	0.14
Endocarditis	2.24	1.15-4.37	0.018			
Device-related	0.89	0.55-1.44	0.63			
Spondylodiscitis	0.74	0.34-1.58	0.43			
Unknown SAB	0.88	0.52-1.51	0.648	1.13	0.59-2.18	0.711
Treatment						
Penicillin G	reference					
Cloxacillin	1.96	1.11-3.45	0.020	2.43	1.30-4.53	0.005
Treatment duration *iv*, days	1.06	1.04-1.09	<0.001	1.07	1.04-1.10	<0.001
Inadequate treatment duration	1.24	0.67-2.29	0.494	2.23	1.11-4.44	0.023

a) For every year increase. b) For each point increase. OR-odds ratio, CI-confidence interval, CCI-Charlson comorbidity index, PBS-Pitt bacteriemia score.

Discussion

Methodological considerations

Many microbiological methods are part of subjective skills and judgement. It is of course more reliable if the methods used are repeated by the same person for a thousand times, than by a thousand persons to do the same method only once. Even though you have some experience, a new method takes time to optimize and to minimizing the possibility of receiving results, not possible to repeat by others. All the microbiological methods used in this thesis have been done repeatedly to minimize the risk of subjective impact on the outcome.

Disk diffusion method

This is one of the most common methods used, in routine in clinical microbial laboratories, to determine the clinical bacterial isolates susceptibility to several antibiotics at the same time.

This said, there are several steps that must be addressed. There are both risks when conducting the method and when interpreting the result. First there is always a risk of variation in every man-made step, when making a solution of 0.5 McFarland, when applying the bacteria on the agar plate or in the determination of the zone diameter with a calliper. All of these steps in this method are made every day in the clinical laboratory and their accuracy is vital to our ability to choose the right antibiotic treatment.

When it comes to the penicillin G susceptibility test in staphylococci, the test with the highest risk of uncertainty, in my investigations, is the assessment of the zone edge appearance, where subjective interpretations are made. For this test a certain experience is important so the experiment can be repeated over and over again, with the same result, for the same isolate. As noted before, the zone edge can be sharp or "heaped-up" not to be mixed up with the fuzzy zone appearance. [195] Several studies have addressed this issue and ended up in different conclusions if this zone edge judgment is reliable enough. [81, 82, 222-224] Although, some of the differences in interpretation can be explained by different methods used. Both EUCAST and CLSI advocate considering the zone edge appearance, in the judgment, when testing *S.*

aureus.[208, 209] The CLSI recommends a 10U penicillin disk in their method and some studies have found this guideline inferior to the one advocated by EUCAST.[82, 224]

We tried to minimize the test uncertainty, having no more than two persons interpreting the zone edge in both Paper I and II. When interpreting the zone edge appearance and zone diameter, the *blaZ* PCR result was not known, minimizing the risk of interpretation bias.

This points out that experience of the method is important when taken into clinical practice in a reference laboratory. In Skåne, *S. aureus* is only answered penicillin susceptible after an additional negative *blaZ* PCR is conducted.

Clover-leaf test

This method is somewhat time consuming but an additional way to determine the isolate for beta-lactamase production. Interpretation can be hard if the indicator strain grows weakly on the agar plate and can then make a false negative result. The consequences of a misinterpretation like that can be devastating and might end up with a patient getting insufficient antibiotic treatment. Even this method has been under debate concerning its accuracy[222, 224] but is used in e.g. Denmark and Finland (personal communication).

In in paper I, we used a penicillin susceptible *Streptococcus pneumoniae* strain as indicator strain. This makes it easier to separate the strains and interpret the result, but harder to keep the *S. pneumococcus* strain alive. All isolates were tested with a positive strain for comparison to be able to minimize the misinterpretations.

MIC determination with E-test

It has been a vivid debate about the E-tests accuracy and especially when antibiotic combination has been tested.[225] We tested strains of *S. lugdunensis* for penicillin G with an E-test as a comparative test for susceptibility. Since this is the method used in the local clinical Medical Microbiology Laboratory on demand, it was relevant to do the test. This is one of the tests advocated by CLSI, is susceptibility tests for penicillin in *S. lugdunensis*.[208]

PCR-method

Polymerase chain reaction is a method to amplify small parts or genes of DNA. Just a very small amount of DNA is enough to give a positive result. This is therefore a good method to detect fragments of DNA even in non-living organisms. These are the positive and negative aspects of the PCR method as it is a strict genotypical method and not phenotypical and cannot distinguish between living or dead

bacteria. Further on, the PCR result does not indicate whether the gene is able to be transcribed or whether the protein is expressed. In addition, genes cannot be detected if there is a mutation in the targeted DNA region where the primer attaches. The method is widely used and popular in detecting different resistance genes. Since this method is rapid and can be conducted in a few hours compared with normal susceptibility test, it is sufficient to use in detecting widely spread strains with a conserved gene, such as the *mecA* gene in MRSA.

In our experiments when testing penicillin susceptible strains, it is problematic that the PCR result becomes negative. Therefore, reliable methods are of great importance and must be accurate; a negative result must be a true absence of the *blaZ* gene. If an isolate would have an altered *blaZ* gene not yielding a positive PCR result and therefore be interpreted as a susceptible strain could be devastating. Since we combined the PCR result with several different phenotypical tests, we could conclude that no *blaZ* negative strains did produce any active penicillinase.

To be certain that the PCR result yielded is a true result, a positive and negative control was added to the tests. The positive control shows that the enzyme, primer and method as a hole are all functioning, and that a negative result is a lack of the gene of interest, rather than a failed PCR method. The negative control shows that the batch with water, enzymes and primers are not contaminated with DNA fragments, and even with a functioning method the result will be negative. When analysing the result, you put the PCR product on an agarose gel. This step is crucial and need both concentration and thoroughness.

Biofilm formation

In paper IV we conduct biofilm formation trials. This is not a standardised method and is user dependent. Even though following a protocol the washing steps are very delicate concerning the way the pipette is used. To diminish this, the same person performed all steps and we tried to conduct the procedure the same way every time. There is also a step when washing the biofilm after dyeing that can be affected of subjective influence. To overcome this, we decided to always wash six times with the PBS to be able to compare the results between different 96-wells plates made. The isolates were also tested in triplicates to establish a mean value of absorbance and diminish the impact of a single value. Furthermore, the biofilm formation experiments were conducted blinded to the clinical outcome results.

Biofilm formation experiments can be conducted in several other ways and there are more sophisticated methods where the *in vitro* method is more alike the *in vivo* milieu. The method chosen for biofilm formation was a rather simple but straight forward method easy to conduct when analysing many strains, as done in Paper IV. It is important to bear in mind that this is an *in vitro* method and we do not know if

the result would be the same *in vivo*, where the biofilm is attached to metallic devices and with the immune system present.

Retrospective studies

The best accomplished type of study is the prospective randomized blinded trial. This format diminishes all known and unknown potential biasing and unknown confounding factors. These trials are often expensive, need thorough planning and must often be conducted over long time. Much easier is the retrospective design where all facts are available as soon as the hypothesis is designed. One major drawback to this design, though, is the lack of randomization and therefore the risk of unbalanced study populations. Another limitation is that is not possible to adjust for any unknown confounder. Even so, this is a quick, cheap and easy way to conduct studies.

Retrospectively conducted studies, through medical records, are a good way of easily follow a large number of patients with a certain disease. There are obviously several pitfalls when assessing medical records and drawing any advanced conclusions is sometimes risky. You can only answer questions where the facts are already registered in the medical records. The primary task of medical records is to be a tool for the clinical everyday work. It is important to bear in mind that this is not a research tool, it is not as objective as one could wish for, and valuable information can be missing. The data presented in the medical record is also interpretations of signs and symptoms judged by the clinicians. The registration can also be biased to what the clinician finds interesting in the actual case, what is worth noting in the medical records. If a very common side effect appears, this may not be noted in the medical records due to lack of excitement.

You are also directed to the facts that is noted in the records; all missing data are lost. Other challenges to retrospective studies are deciding in advance what to register, knowing what questions one wants to be answered and where in the medical records they are listed.

On the other hand, one major advantage in retrospective studies is that, when investigating rare events (or rare bacterial infections), all data is there, and you do not have to wait until the next event will happen. This was the case in paper II, III and IV. None of these studies would have been finished by this time if conducted prospectively.

Another drawback with prospective studies is that the inclusion and exclusion made to make a uniform population, can result in that several participants are excluded. In this aspect the retrospective studies are more real-life data, truer to what the treatment groups looks like in the real life clinical everyday work.

At first, we thought of doing a prospective study on penicillin G vs. cloxacillin treatment in SAB but this was rejected due to that it would be time consuming and become a very expensive study. There was also indications that a large prospective study comparing flucloxacillin with penicillin G in SAB already had been planned in Australia.[226]

To diminish the biased interpretation in the retrospective studies, we tried to specify all variables that should be registered and define signs and symptoms before starting the registration. This was most important in paper III and V where all data is from a registry or medical records. In paper III, there was only one person interpreting all data and in paper V there were two persons registering the basic data. No statistical analyses were made during the registration, reducing the risk of being biased by already registered data.

Statistical considerations

In a Kaplan-Meier curve analysis, it is assumed that the risk of outcome is consistent over time. This can be argued when the follow-up time is long and the differences in other risk factors are high, such as age or comorbidity. In our cohort the time span was short (30 days), diminishing the influence one of these factors. Although this is a crude method since it does not adjust for other confounding factors that might influence the outcome.

There are several statistical ways to try to overcome the unbalanced variables in retrospective studies and to try to mimic a randomized controlled trial. One way is the logistic regression where adjustment is done according to variables that impacts the outcome. The choice of variables to adjust for is a delicate step in this analysis. Variables eligible are those that are clinically conceivable to impact the outcome. Adjustment can also be done in variables that are imbalanced between the groups.

Propensity score is a probability of getting a certain choice of treatment, calculated from variables that might have influenced this choice. This is a subjective step in the analysis and must be handled with prudence. One can be tempted to try as many combinations as possible to get a propensity score that in the final outcome analysis confirm your hypothesis. Therefore, it is important to try to identify and include variables only clinically relevant when conducting the propensity score. Since the penicillin G study population was small, we did not conduct a propensity score matched analysis. This type of analyses select matched pairs and the risk of getting too small a population in final analysis was imminent.

We used the propensity score in an inverse probability treatment weighted analysis (IPTW), and even though extensively used, it can inflate factors not included in the propensity score analysis. When the pseudo population is conducted, basic variables must be checked if balanced between the treatment groups. If imbalance is noted,

this must be compensated in the final analysis. In Paper V, we used IPTW analysis to confirm our result from the logistic regression, that patients treated with cloxacillin had a worse outcome than those treated with penicillin G. Our population was after the IPTW analysis balanced according to these factors included in the propensity score, but still imbalanced in several other variables important to the outcome. These variable, complicated SAB, unknown SAB, inadequate antibiotic duration, and treatment length were adjusted for in the final outcome analysis. If the study population would have been larger, a matched analysis would have been conducted instead.

Findings and implications

In paper I we examined how many of the *S. aureus* isolates in blood cultures, that were still penicillin susceptible. To our surprise, a high frequency of *S. aureus* isolates was penicillin susceptible (29%). There are several practical and theoretical arguments that penicillin G would be more favourable than cloxacillin in SAB. The ability to give a longer time with free antibiotic concentration above the MIC-value, when treating *S. aureus*, is one major argument. The result in paper V indicated that the overall outcome was better in the penicillin G group, despite small number of participants in this group.

There have also been concerns about the penicillin susceptibility method in *S. aureus*. [80] Since the frequency of penicillin susceptibility in *S. aureus* isolates diminished over the years and the lack of evidence that penicillin G would be a better treatment option, the indication of still conducting this penicillin susceptibility test in *S. aureus* subsided.

In 2021, a brief report described a possible misinterpretation of penicillin susceptibility test in a *S. aureus* isolate from repeated blood cultures.[227] It would be devastating if this was the case, but when inspecting the picture of a "single colony" culture, it looks contaminated, and when cultivated to five different plates with a single colony in each plate, it is clear that there is more than two different strains present. In this case report, strains are also interpreted as susceptible despite that the zone diameter is less than 26 mm, generally regarded as resistant according to EUCAST.[228]

This phenomenon has not been reported during the 3 years we have had this test in clinical routine. Neither have any of the cases assessed in paper V had any relapses or treatment failure with strains later recognised as penicillin resistant.

The early literature, treatment experiences from other countries and the theoretical arguments were convincing that penicillin G treatment of *S. aureus* would be as good as, and not worse, than the existing cloxacillin treatment in penicillin

susceptible strains.[72, 79, 229] Today Skåne is the only region in Sweden that systematically test all *S. aureus* strains from blood cultures, for penicillin susceptibility. In 2019 we started treating *S. aureus* bacteraemia with penicillin G on a more regular basis in Skåne.

It was important to investigate the outcome of those that received penicillin G instead of cloxacillin in PSSA bacteraemia. A few studies recently published indicated that penicillin G would be a safe alternative to cloxacillin treatment in SAB.[72, 89-91] In paper V we could conclude, in line with these studies, that penicillin G was a good alternative to cloxacillin and in respect of adverse events, penicillin G seemed to be a better alternative than cloxacillin. This is the first study that has compared cloxacillin treatment with penicillin G head-to-head. Also, the first study where both diagnoses and antibiotic length are included in the treatment evaluation along with the antibiotic choice.

We did conduct a totally new outcome ranking scale for this study. There has been other advocated ranking scales to evaluate a more complex outcome than death, such as the DOOR scale (desirability of outcome ranking) in infections like SAB.[230] Our major argument not to use this was that the rank was a sum of how many different adverse effects every patient had. As an example, a patient that had an adverse event of antibiotic treatment would be in the same rank as those who had a relapsing infection. In our opinion this was a rather crude method. Our ranking system has not been evaluated before, and this is a limitation to the study. Nevertheless, this was an attempt to make a ranking system from a patient perspective. Even though a retrospective investigation with a small number of patients treated with penicillin G, it is an important study with convincing outcome.

The conserved antibiotic susceptibility in *S. lugdunensis* had been described before and is well known.[105, 111] Today, there is no penicillin susceptibility test for *S. lugdunensis* or other CoNS at the Department of Medical Microbiology in Skåne. It is crucial that the susceptibility tests in clinical practice is accurate and with a high sensitivity. If the sensitivity is low, several strains that are resistant will be interpreted as susceptible. In paper II we evaluated different susceptibility tests for penicillin G in *S. lugdunensis*. This resulted in one major error for the methods advocated by CLSI.[208] Naturally this was a bit distressing, that a method used as a recommendation in a standard laboratory could yield a false susceptible strain. We tested the strain repeatedly which resulted in the same conclusion. This experiment has now been repeated by Teh et al. showing the same result in two strains tested with penicillin G 10 U and interpreted as false susceptible.[231] This group also interpreted the zone edge appearance with a 100% conformity with the presence of the *blaZ* gene, in line with our conclusions. In our hands the judgement of zone edge appearance could be of additional value in the method advocated by CLSI. In contrast to our findings McHardy et al. did not find the zone edge appearance to be a reliable method when testing penicillin susceptibility in *S. lugdunensis*.[223]

As an overall implication of paper II this result may guide the clinical laboratory to start adding penicillin G as a routine susceptibility test in *S. lugdunensis*.

In paper III we examined infectious endocarditis caused by *S. lugdunensis* in Sweden during 2008-2018. Endocarditis caused by *S. lugdunensis* has been considered as severe with prompt need of surgery, massive embolization and high mortality. These "facts" are exclusively based on previous publications.[109, 110, 112, 113] It is a rather newly discovered CoNS and when causing severe IE, this is of course a pressing need for publications. No previous published studies have been done with a total cohort but with one or a few sensational cases and a short literature review. Our study is the first cohort study, even though the national registration is far from complete. We could conclude that the mortality rate was high, but the embolization frequency was much lower than in previously published studies.[110-112] We could also note that the use of penicillin G was surprisingly low. This concludes that the knowledge of the conserved susceptibility in *S. lugdunensis* have to be elucidated to our colleagues. This is a retrospective register study with all its drawbacks. Still, it can be of value to know that it is an aggressive disease with high mortality rate and need of surgery.

In paper IV we presented prosthetic joint infections caused by *S. lugdunensis*. Since patients included in previous studies were few, it is hard to compare our results with previous studies. The relapse rate has been reported between 13-21% and in our cohort 11% relapsed in those surgically treated patients.[120, 122] Biofilm formation was more robust in those isolates causing an *acute hematogenic* infection indicating that this is an important virulence factor to establish PJI. Furthermore, we could show a correlation between isolates ability to form biofilm and risk of relapsing infection. This was in analogy with what others have showed before in prosthetic joint infections caused by other species.[162] Paper IV indicates that the ability to form biofilm is a virulence factor of significance.

Also, in paper IV the most common antibiotic treatment was vancomycin despite that all isolates were isoxazolyl penicillin susceptible. This indicates that the conserved antibiotic susceptibility in this species is largely unknown. This may in this study partly be explained by the large number of polymicrobial cultures.

Both paper III and IV have the largest cohort described, consisting of 30 and 36 cases, respectively. Since these are the largest cohort just containing small numbers, one must be humble when interpreting the results.

Conclusions

- Susceptibility test for penicillin in *S. aureus* is reliable and easy to conduct if following the EUCAST recommendations.

- The frequency of penicillin susceptible *S. aureus* in bacteraemia in Skåne was 29%, higher than expected.

- Treatment outcome of bacteraemia with penicillin susceptible *S. aureus*, was overall better when treated with penicillin G compared to cloxacillin.

- Penicillin susceptibility test for *S. lugdunensis* was accurate according to EUCAST but the method according to CLSI had one major error.

- Endocarditis due to *S. lugdunensis* is an aggressive infection with high mortality at 30 days, but embolization seems to be a rarer event than previously described.

- *S. lugdunensis* is able to cause prosthetic joint infections and have a good ability to form biofilm. The ability to form a robust biofilm is associated with relapsing infections implicating that biofilm formation is a virulence factor of significance.

Populärvetenskaplig sammanfattning

Bakteriesläktet stafylokocker innehåller ett 50-tal olika arter. De flesta av dessa bär vi eller våra husdjur på, på huden utan att de orsakar någon skada. Det finns dock några undantag och den vanligaste sjukdomsalstraren kallas för den gula stafylokocken, *Staphylococcus aureus*. Det är en av dem vanligaste bakterierna som orsakar infektioner hos oss människor. Vi kan få allt från små hudinfektioner till svåra hjärtklaffinfektioner eller ledprotesinfektioner av denna bakterie. En släkting till *S. aureus* är *Staphylococcus lugdunensis*, den är mycket mer ovanlig men kan orsaka lika aggressiva infektioner som *S. aureus*.

När penicillinet upptäcktes på sent 20-tal så var alla *S. aureus* känsliga för penicillin. Bara några år efter att penicillinet börjat användas som behandling, i mitten på 40-talet, upptäckte man de första resistenta stammarna. Snabbt spreds de resistenta klonerna och på sextiotalet övergav många behandling med penicillin vid *S. aureus* infektioner, till förmån för det nyare antibiotikumet, cloxacillin.

I min första artikel undersökte jag andelen *S. aureus* som är känsliga för penicillin G från kliniska isolat från blod och sår odlingar under 2014/2015, i Skåne. Jag jämförde också gamla sparade isolat, från 2009, för att se hur penicillinkänsligheten såg ut tidigare. Vi kunde visa att hela 29% av *S. aureus* isolaten från 2014/2015, i odlingar från blodet, var känsliga för penicillin. Odlingarna från 2009 visade att 57% av *S. aureus* isolaten var känsliga för penicillin.

I min andra artikel kontrollerade vi hur resistensbestämningen av penicillin fungerade på *S. lugdunensis* och hur många av de kliniska isolaten i Skåne, som var penicillinkänsliga. Av de undersökta bakterieisolaten var 67% känsliga för penicillin. Vi kunde visa att den europeiska metoden från organisationen EUCAST, var bra och tillförlitlig men att den amerikanska metoden från CLSI gav ett förödande fel. Detta fel skulle medföra att ett av isolaten som var penicillin resistent skulle svarats som känslig i USA eller där denna metod används. Det skulle riskera att patienten inte får adekvat behandling för sin allvarliga infektion.

I min tredje artikel undersökte jag hjärtklaffinfektioner som orsakats av *S. lugdunensis*. Data kom från det Svenska Registret för infektiös endokardit (klaffinfektioner) under perioden 2008–2018. Vi hittade 30 patienter med hjärtklaffinfektion orsakad av *S. lugdunensis* under den angivna tidsperioden. Vi jämförde kliniska data från dessa infektioner med 1892 fall av hjärtklaffinfektioner med *S. aureus* och 262 fall orsakade av andra stafylokocker, från samma tidsperiod

och register. Våra slutsatser visade att *S. lugdunensis* gav en aggressiv infektion och att fler hade dött vid 30 dagar av *S. lugdunensis*-klaffinfektion än de som fick klaffinfektion av de jämförande bakterierna.

I fjärde artikeln har vi undersökt *S. lugdunensis* inblandning i ledprotesinfektioner. Vi har använt bakterieisolat som sparats från protesinfektioner under 5 år (2015–2019) och undersökte deras förmåga att bilda biofilm. Biofilm är ett slags gelémassa som bakterierna bildar när de sätter sig på kroppsfrämmande material. I denna gelémassa kan de hjälpa varandra att överleva och skydda sig mot både immunförsvaret och antibiotika. Vi undersökte sambandet mellan förmågan att bilda denna biofilm och resultatet av protesinfektionen efter behandlingen. Vi kunde visa att de patienter med isolat som kunde bilda stark biofilm hade en större risk att få en återkommande infektion, även om de behandlades rätt.

I det sista delarbetet så har vi jämfört utfallet hos de patienter som fått behandling med penicillin G med de som fått cloxacillin för sin *S. aureus* infektion. Vi kunde visa att de som fått penicillin klarade sig bättre och hade färre biverkningar än de som blev behandlade med cloxacillin.

Sammanfattningsvis; Vi har kunnat visa att det finns tillförlitliga tester för att undersöka penicillinkänslighet hos arterna *S. aureus* och *S. lugdunensis*. Behandling av *S. aureus*-infektioner med penicillin G är att föredra framför cloxacillin när isolaten är känsliga. *S. lugdunensis* endokardit har en hög mortalitet tidigt i förloppet men att embolier från infektionen är ovanligare än vad man tidigare trott för denna art. Vi har också visat att om *S. lugdunensis* isolat från protesinfektioner bildar mycket biofilm finns en ökad risk för återkommande infektioner hos patienterna.

Acknowledgements

Det finns många att tacka när man nu får chansen:

Först och främst, TACK Lisa Påhlman som gjort detta möjlig! Utan dig hade det inte blivit något alls. Du har fått stå ut med alla tvära kast i mitt liv och stått där som en fast och trygg punkt. Jag är så glad över att du har orkat vägleda mig, ingen hade passat mig bättre! Du har betytt mest av allt och alla i denna process.

Mina bihandledare:
Fredrik K, för att du alltid finns där både för lite småsnack och viktiga statistiska samtal, även när du har varit vansinnigt upptagen de sista åren. Ta hand om dig! Bengt, jag önskar att vi hade hunnit med mer, men tiden gick så fort, tack för alla spännande samtal om forskning, livet och allt däremellan.

Anita Berglund, för att du alltid bidrar med en så smittande glädje och såklart för allt det där praktiska, vad skulle B14 göra utan dig!

Gisela Hovold, Tack för allt laborativt stöd, du har ställt upp varje gång jag förvirrat irrat runt på labbet igen. Och alltid gjort det med ett leende!

Adrian Velicu, Tack för att du så förtjänstfullt och noggrant språkgranskat avhandlingen.

Alla i MOL-gruppen, Oonagh, Magnus R, Karin H, Magnus P, Frida, Linnea P, Cecilia, Anna B, Hanna B och alla andra, för fina fikastunder, viktiga samtal över labb-bänkarna och ibland även lite forskningssnack.

Lisa M o Olof T, alltid där för stöd och pepp både i klinik, forskning och livet. TACK!

Tack Alla mina fantastiska arbetskamrater, läkare, sjuksköterskor och undersköterskor på infektionskliniken i Lund. Ni förgyller mina dagar och nätter på jobbet!

Malin I, Tack för att du är en så förstående och uppmuntrande chef och en generös och positiv svägerska och vän, var vore vi utan dig (och Andreas)?

Mina vänner, Anna, Ida, ni som har varit med mig sedan tidernas begynnelse och fortfarande står ut. Mina rötter när det stormar!

Sanna och Stefan, för allt roligt vi gjort och gör, och för att ni alltid finns där med så mycket glädje och skratt! -Sanna för enastående mentorskapet, vi fortsätter väl som vanligt?

Malin, Karin och Viktoria, för alla resor, glädje och roliga minnen! Vänner i vått o torrt! Snart är vi väl på väg igen!

Alla fina vänner som delar vardag och fest med mig och min familj!

Mina nära;

Svärmor Margaret, för att du alltid ställer upp med outtröttlig kärlek.

Pappa Jan, Pelle, Nina, Petra, för att ni finns där för mig! Jag behöver er mer än ni kan tro! ♡

Min älskade familj; Martin, Signe, Hannes och Märta, för att ni är min kärleksfulla o galna familj! Tack för att ni ger mig perspektiv på livet.
Extra Tack till Signe för omslagsbilden, Märta för korrekturläsningen och Hannes för all uppmuntran och stöd längs vägen!
Martin, finaste du, för kärleken och allt det andra som betyder mest! ♥

Till er som inte fick vara med tills nu; Mamma, Nina och Nilla- Ni fattas mig!

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