Aspects of infective endocarditis. Molecules, microbiology, management, and more.

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2020

Document Version:
Publisher's PDF, also known as Version of record

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DEPARTMENT OF CLINICAL SCIENCES | LUND UNIVERSITY

Printed by Media-Tryck, Lund 2020 NORDIC SWAN ECOLABEL 3041 0903

LUND UNIVERSITY

FACULTY OF MEDICINE

Division of Infection Medicine
Department of Clinical Sciences

Lund University, Faculty of Medicine
Doctoral Dissertation Series 2020:97
ISBN 978-91-7619-959-6
ISSN 1652-8220

Aspects of infective endocarditis

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Molecules, microbiology, management, and more

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LUND
UNIVERSITY

DOCTORAL DISSERTATION

by due permission of the Faculty of Medicine, Lund University, Sweden.
To be defended at Belfragesalen (BMC D15) on the 16th of October 2020 at 09.00.

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Rennes, France

Organization LUND UNIVERSITY Department of Clinical Sciences Division of Infection Medicine	**Document name** Doctoral dissertation **Date of issue** 16th of October
Author(s) Torgny Sunnerhagen	Sponsoring organization

Title and subtitle **Aspects of infective endocarditis – molecules, microbes, management, and more**

Abstract

Endocarditis, or heart valve infection, can be caused by a number of pathogens, many of which are Gram-positive bacteria. The diagnosis is based on imaging techniques such as echocardiography and on blood culture. The implementation of fast and accurate species identification methods, such as the matrix-assisted laser desorption/ionisation-time of flight mass spectrometry (MALDI-TOF MS) in routine use for bacteria found in blood culture, has meant that bacteria previously thought to be rare have become increasingly recognised in the clinic. Some of these newly recognised bacteria are the aerococci, a genus of bacteria consisting of eight identified species, first identified in 1956. Other areas where MALDI-TOF MS and other new bacteriological methods have been helpful are the differentiation between the groups of NBHS (non-beta-haemolytic streptococci), also known as alpha streptococci, and in the identification of other Gram-positive cocci such as *Abiotrophia*, *Gemella*, and *Granulicatella*.

This thesis consists of six different studies on endocarditis and endocarditis-causing Gram-positive bacteria. The first of these covers *Aerococcus urinae*. Using mass spectrometry, two distinct LPATG-anchored proteins named Asp 1 and Asp 2 were identified on the surface of the bacterium. The presence of these proteins was also confirmed using antibodies generated against recombinantly expressed Asp 1 and Asp 2. After sequencing 25 *A. urinae* genomes, six different variants of *asp* genes, named *asp*1-6, were found. All sequenced isolates contained one or two of these *asp*-genes located in the same region of the chromosome designated Locus Encoding Aerococcal Surface Protein (LASP).

The possible synergy between benzylpenicillin and gentamicin against bacteria has long been an argument used in guidelines recommending combination therapy in infective endocarditis (IE). Two of the studies in this thesis look at this, one of which also describes the characteristics of IE caused by aerococci. Bactericidal synergy was shown against 14 of 24 streptococcal isolates and against 7 of 15 tested aerococcal isolates. The characterisation of aerococcal IE (based on data from the Swedish Endocarditis Registry) showed, amongst other things, that the mean age was significantly higher than in IE caused by NBHS or *Staphylococcus aureus*.

By using a cohort of Swedish patients with NBHS-bacteraemia with or without IE, the HANDOC score was constructed: one point given for heart murmur or heart valve disease (H); one point given for an aetiology of *Streptococcus bovis*-group, *Streptococcus sanguinis*-group, or *Streptococcus mutans*-group, and one point subtracted for *Streptococcus anginosus*-group bacteraemia (A); one point added if the number of positive blood cultures was two or more (N); one point added for a duration of symptoms of seven days or more (D); one point if only one species was present in the blood culture (O); and one point added for a community-acquired infection (C). Using a cut-off of two points, the sensitivity was 100% for detecting IE and the specificity was 76%. The HANDOC score was then validated in a second cohort of Danish patients with NBHS in blood culture. The HANDOC score and the previously published DENOVA score (originally developed to distinguish IE from non-IE in enterococcal bacteraemia) were then applied in cases of bacteraemia with *Aerococcus*, *Abiotrophia*, *Gemella*, and *Granulicatella*. The sensitivities of HANDOC and DENOVA were 97% and 93%, respectively, with specificities of 85% and 90%. Thus, HANDOC can possibly be used to decide whether or not to perform IE diagnostics in cases of NBHS bacteremia, and both HANDOC and DENOVA can possibly be used for the decision to perform IE diagnostics in cases of bacteremia with *Aerococcus*, *Abiotrophia*, *Gemella*, or *Granulicatella*.

Key words Aerococcus, Streptococcus, Gemella, Granulicatella, Abiotrophia, bacteraemia, bacteremia, endocarditis, antibiotic synergy

Classification system and/or index terms (if any)

Supplementary bibliographical information		**Language** English
ISSN and key title 1652-8220		**ISBN** 978-91-7619-959-6
Recipient's notes	**Number of pages** 78	Price
	Security classification	

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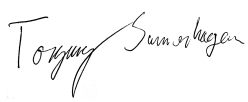
Signature  Date 2020-09-10

Aspects of infective endocarditis

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Cover image: *Aerococcus urinae* colonies on blood agar. Photo by Torgny Sunnerhagen and Anupam Das.

Faculty of Medicine
Department of Clinical Sciences, Lund

ISBN 978-91-7619-959-6
ISSN 1652-8220

Printed in Sweden by Media-Tryck, Lund University
Lund 2020

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Original articles

1. Senneby, E., **Sunnerhagen, T.**, Hallström, B., Lood, R., Malmström, J., Karlsson, C., Rasmussen, M. (2019). Identification of two abundant *Aerococcus urinae* cell wall-anchored proteins. *International Journal of Medical Microbiology,*

2. **Sunnerhagen, T.**, Nilson, B., Olaison, L., Rasmussen, M. (2016). Clinical and microbiological features of infective endocarditis caused by aerococci. *Infection*, *44*(2), 167–173.

3. **Sunnerhagen, T.**, Nilson, B., & Rasmussen, M. (2015). Antibiotic synergy against viridans streptococci isolated in infective endocarditis. *International journal of antimicrobial agents*, 45(5), 550–551.

4. **Sunnerhagen, T.**, Törnell, A., Vikbrant, M., Nilson, B., Rasmussen, M. (2017). HANDOC: a handy score to determine the need for echocardiography in non-β-hemolytic streptococcal bacteremia. *Clinical Infectious Diseases*, 66(5), 693–698.

5. **Sunnerhagen, T.**, Højgaard Andersen, M., Bruun, NE., Bundgaard, H., Karmark Iversen, K., Rasmussen, M. (2019) External validation of the HANDOC score–high sensitivity to identify patients with non-beta-haemolytic streptococcal endocarditis. *Infectious Diseases,* 52 (1), 54–57.

6. Berge, A., Kronberg, K., **Sunnerhagen, T.,** Nilson, B. K. B., Giske, C. G., Rasmussen, M. (2019). Risk for endocarditis in bacteraemia with *Streptococcus*-like bacteria: a retrospective population-based cohort study. *Open forum infectious diseases,*6 (10).

Articles not included in the thesis

1. **Sunnerhagen, T.**, Hammarlund, P., Rasmussen, M. (2015). A case of suspected infective endocarditis with Lactococcus garvieae: lack of *in vitro* synergy between ampicillin and gentamicin. *JMM Case Reports*, *2*(1).

2. Oskooi, M., **Sunnerhagen, T.**, Senneby, E., Rasmussen, M. (2018). A prospective observational treatment study of aerococcal urinary tract infection. *Journal of Infection*, *76*(4), 354–360.

3. Nygren, D., Älverbrandt, M., **Sunnerhagen, T.**, Fagman, E., Ostenfeld, E., Rasmussen, M. (2018). Aortitis caused by *Abiotrophia defectiva*: Description of two cases. *Infectious Disease Reports, 10*(3).

Abbreviations

CT – Computed tomography

FICI – Fractional inhibitory concentration index

IE – Infective endocarditis

LASP – Locus encoding aerococcal surface protein

MALDI-TOF MS –Matrix-assisted laser desorption/ionization-time of flight mass spectrometry

MBC – Minimal bactericidal concentration

MH-F – Müller-Hinton medium, fortified

MIC – Minimal inhibitory concentration

MLST – Multi-locus sequence typing

MRI – Magnetic resonance imaging

NBHS – Non-beta-haemolytic *Streptococcus*

TEE – Trans-esophageal echocardiography

TTE - Trans-thoracic echocardiography

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Endocarditis, or heart valve infection, can be caused by a number of pathogens, many of which are Gram-positive bacteria. The diagnosis is based on imaging techniques such as echocardiography and on blood culture. The implementation of fast and accurate species identification methods, such as the matrix-assisted laser desorption/ionisation-time of flight mass spectrometry (MALDI-TOF MS) in routine use for bacteria found in blood culture, has meant that bacteria previously thought to be rare have become increasingly recognised in the clinic. Some of these newly recognised bacteria are the aerococci, a genus of bacteria consisting of eight identified species, first identified in 1956. Other areas where MALDI-TOF MS and other new bacteriological methods have been helpful are the differentiation between the groups of NBHS (non-beta-haemolytic streptococci), also known as alpha streptococci, and in the identification of other Gram-positive cocci such as *Abiotrophia*, *Gemella*, and *Granulicatella*.

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Introduction

The work included in this thesis is diverse but the general theme of endocarditis and endocarditis-causing organisms is a connecting thread. Ever since the beginnings of bacteriology in the 19th century, there has been an aim to differentiate and classify bacteria. Even though the famous postulates by Robert Koch have been modified and adapted, the underlying line of thought that different pathogens cause different diseases still remains relevant[1]. In recent decades, there has been great improvements in the technology used to identify bacteria, leading to a number of species being identified in clinical samples that were previously thought to be very rare. Despite this, the finding of newly discovered or re-classified bacterial species or subspecies has not always led to an increased understanding about the diseases they cause or the way they do so. In this thesis, the overall aims are thus to describe the characteristics of several similar bacteria and the diseases that they cause. They all share the characteristics of being bacteria that were rarely identified in clinical practice until recently (making them emerging pathogens) or bacteria where detailed classification was not possible or considered necessary until recently.

Bacteriology and bacteriological methods

In clinical bacteriology, a major goal is to detect and identify the pathogenic bacteria causing an infection and to determine what antibiotic treatment is likely to be effective against them. This chapter will describe methods that are used to this end in clinical bacteriology (especially as pertaining to the Gram-positive bacteria studied in this thesis) as well as some background on methods used in experimental bacteriology.

Species identification

The Gram stain

The Gram stain was originally described in 1884 by the bacteriologist Hans Christian Gram. It involves staining the bacteria with crystal violet, adding iodine which binds to crystal violet and traps it in the cell. Ethanol or acetone is then added to decolorize. A counterstain (usually safranin) is then added. Gram-positive bacteria are those that retain the original crystal violet stain whereas Gram-negative bacteria lose the crystal violet during decolorization and only retain the counter stain.[2]. Just as Gram-negative bacteria are diverse, Gram-positive bacteria constitute a diverse group of bacteria that still have several characteristics in common. Morphologically, Gram-positive bacteria share the basic components of the cytosol, the cell membrane, and an outer cell wall. This is a main morphological difference from Gram-negative bacteria which have two membrane layers and a much thinner wall in between[3,4]. The thicker cell wall of Gram-positive bacteria contains peptidoglycan, and is the reason for Gram-positive bacteria retaining the Gram stain and Gram-negative bacteria not doing so[5].

Blood culture procedures

Culturing bacteria from blood is the most common way of detecting bacteraemia, and can combine bacterial detection with species (and subspecies) determination and antibiotic sensitivity testing. For intravascular and cardiac infections, as well as infections originating at other sites where bacteraemia is suspected, taking at least two sets of blood cultures is recommended. A set generally consists of one aerobic and one anaerobic culture bottle[6,7]. As approximately 10 ml of blood is recommended to be used per bottle, this equals 40 ml of blood for bacterial culturing[6]. The volume of blood taken is important, and many studies have shown the relationship between the volume of blood taken and the likelihood of finding the pathogen in question[8–10]. The blood culture bottles are then incubated at 35-37ºC for 5 days. In the systems used at Skåne University Hospital (BacT/Alert from bioMérieux for the years 2009-2014 and BACTEC FX from Becton Dickinson from December 2014 and on), CO_2 levels in the bottles are detected by a sensor and the system gives an alert when it detects increased levels of CO_2 indicating growth of bacteria. The procedure then involves direct Gram staining of the blood culture broth as well as inoculation into blood agar, chocolate agar, and agar plates optimized for anaerobic growth. Even though species identification is sometimes possible directly from the blood culture broth, the amplification step on agar is often necessary for species identification and for antibiotic sensitivity testing[6].

MALDI-TOF MS

MALDI-TOF MS stands for Matrix-Assisted Laser Desorption Ionisation - Time Of Flight Mass Spectrometry. MALDI was first used in the 1980s to identify small molecules, with Tanaka et al. describing protocols for ionising larger proteins in 1987 [11–13]. The principle behind MALDI-TOF MS is that the analyte (a sample from a bacterial colony in the clinical use of MALDI-TOF MS) is placed on a metal plate together with a matrix consisting of a saturated solution of a low-mass organic compound, often an acid. The sample is then irradiated with a laser beam. This causes the ionization of the analyte and a sublimation to gas phase. The ionized molecules are then analysed using the mass spectrometer, with the time of flight being used to sort the molecules by weight. This procedure is repeated many times per sample, with the laser targeting different parts of the spot formed by the analyte and matrix. This means that an average of the sample can be calculated and matched against a database of protein profiles for different species[14].

The introduction of MALDI-TOF into routine use in clinical bacteriology represented a big shift in species identification. Before its introduction, the main way of identifying bacteria in culture (whether blood culture as described above, or bacteria cultured from urine, sputum or other sources) was based on Gram stain

followed by phenotypical characterisation consisting mainly of colony morphology and the ability to tolerate different environments such as high salt concentration, the ability to utilise different carbon sources, and enzymatic activities. This process yielded quite good exactness in many cases, but had difficulties in identifying some bacterial species, and was time consuming to perform. The use of MALDI-TOF MS has shortened the time it takes to get species determination, enabled the possibility to routinely identify species that were not possible to identify before, and decreased the granularity of bacterial species identification in clinical microbiology[14–18].

Sequencing

Sequencing is the use of various methods to determine the nucleotide sequence of a DNA or RNA molecule such as a chromosome, plasmid, or ribosome. Though there were methods used to determine RNA sequences in the early 1970s and a whole gene of a bacteriophage was determined in 1972[19], the first reliable method for DNA sequencing was the so-called chain termination method (or Sanger sequencing) described in 1977. In this method, the sequence is obtained by using a single-stranded DNA fragment, a primer binding to the DNA at the site where sequencing should begin, and four separate solutions containing all four dNTP (deoxynucleotide triphosphate) as well as one of four di-deoxynucleotide triphosphates (ddTTP, ddATP, ddGTP, or ddCTP), and a DNA polymerase. When the polymerase adds nucleotides to the single-stranded DNA, it will stop when it adds a ddNTP instead of dNTP and the DNA chain will end, giving fragments of various lengths. The ddNTP can be labelled in various ways to enable determination of what nucleotide is at the end of the sequence. This means that the nucleotide sequence of the DNA molecule can be determined in an accurate way[20].

16S rRNA sequencing is a very important application of sequencing in clinical bacteriology. The 16S rRNA is the RNA of a component of the small subunit of the prokaryotic ribosome and is present in all bacteria. Due to this and the fact that the 16S rRNA is mostly species specific, sequencing of it both makes it possible to detect bacteria in clinical samples even after antibiotics have made culturing difficult and to determine the species of the bacteria[21–23]. When the goal is to separate more closely related species, 16S RNA sequencing sometimes has problems identifying which species it is. In those cases, multilocus sequence typing (MLST) is an alternative. MLST involves sequencing multiple loci, usually housekeeping genes, which can enable an accurate species identification even between closely related species and in cases of horizontal transfer of genes. The data from the typing is then compared to a database of know sequences from different species and subspecies to generate the probability that the sample belongs to a given species[24,25].

Sequencing the whole genome of an organism is sometimes done to provide even more detail than is provided by MLST. The introduction of so-called next generation sequencing techniques has changed both research and clinical bacteriology in regard to this. Though the specific protocols differ between methods, the main benefit compared to chain termination sequencing is the much higher throughput compared to chain termination sequencing (in some cases at the cost of more errors), and many methods (such as Illumina sequencing) use a synthetisation technique instead of hybridisation to achieve this[26–28].

Antibiotic sensitivity testing

Resistance to antibiotics is an increasing problem in treating infectious diseases, and has been declared by the World Health Organization to be one of the top three most important health issues[29]. Due to the fact that bacteria (both on the species level and individual isolates) have differing levels of susceptibility to different antibiotics, due to either intrinsic antibiotic tolerance or to acquired resistance, testing of antibiotic sensitivity is a central part of clinical bacteriology[30]. While antibiotic resistance is not a general problem when treating the bacteria discussed in this thesis such as, *Aerococcus*, *Gemella*, *Granulicatella*, *Abiotrophia* and the non-beta-haemolytic members of *Streptococcus*, antibiotic susceptibility still varies. In this chapter, aspects of antibiotic sensitivity testing and antibiotic synergy as pertaining to substances relevant to the studies in this thesis will be covered.

General principles and guidelines

The testing of antibiotic susceptibility of a microbial sample is done *ex vivo* in clinical practice and shows how well the antibiotic substances can hinder bacterial proliferation. The clinician who receives the results from the susceptibility testing is generally concerned whether or not the patient with the infecting organism will be cured when given a certain antibiotic. These are two similar but not identical aspects that form one of the important foundations for designing, performing and interpreting antibiotic susceptibility tests.

Susceptibility testing can be done in liquid media (broth) or on solid media (agar), and can look at the inhibition of growth (inhibitory effect) or on the killing of bacteria (bactericidal effect). Broth dilution was the original way of determining antibiotic susceptibility and is still in use[31] The method involves two-fold dilutions of antibiotic (e.g., 4, 2, 1, 0.5, 0.25 µg/mL) in a broth capable of sustaining bacterial growth. A standardised bacterial inoculum is then added to the tubes. After incubation at appropriate conditions, bacterial growth can be seen as

turbidity in the medium. The lowest antibiotic concentration that inhibits growth is the minimal inhibitory concentration (MIC). The European committee on antibiotic susceptibility testing (EUCAST) has guidelines recommending how to perform susceptibility testing, both when a specific protocol exists for the bacteria (such as NBHS and *Aerococcus*) and when there is no genus- or species-specific protocol (such as for *Granulicatella*, *Gemella* or *Abiotrophia*). For fastidious organisms, such as *Aerococcus* or *Streptococcus,* MH-F agar or broth is recommended for susceptibility testing. This is Mueller-Hinton medium supplemented with 5% defibrinated horse blood and 20 mg/ml β-NAD (β-nicotinamide adenine dinucleotide). Incubations are to be performed in an atmosphere with 5% CO_2 and at 37ºC[32].

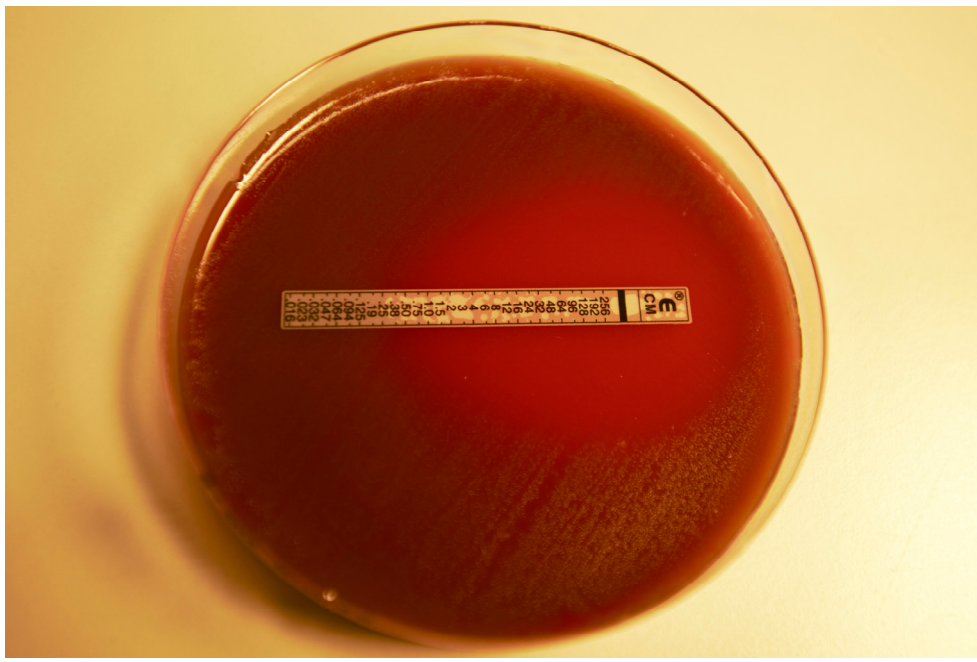

Figure 1. Etest inhibiting growth at higher antibiotic contentrations but allowing growth at lower concentrations.

The main ways of testing on agar are the antimicrobial gradient method and the disk diffusion test. The antimicrobial gradient method employs a plastic or paper strip imbued with an antibiotic with a concentration gradient along its length. When this strip is placed on an agar plate with a standardized inoculum of bacteria and incubated at appropriate conditions, the MIC is detectable as the intersection of the inhibition zone and the growth zone at the strip. Testing antibiotic susceptibility using the disc diffusion method is similar. In this method, several discs infused with a specific antibiotic each at a standardised concentration is placed on an agar plate with a bacterial inoculum. After incubation, the zones of

inhibition are measured to the nearest millimetre. This gives a qualitative measurement of antibiotic susceptibility (susceptible, intermediate, or resistant) rather than an exact MIC value[33,34].

Penicillin and aminoglycosides

The antibiotic properties of penicillin was originally discovered by Alexander Fleming who noted that the growth of *Staphylococcus* was inhibited around colonies of *Penicillum* mould on an agar plate[35]. The mode of action involves the inhibition of the D-alanine carboxypeptidase mediated through the beta-lactam ring, thus inhibiting cell wall synthesis[36,37]. One of the commonly used penicillin molecules is benzylpenicillin (also known as penicillin G), which is mostly effective against Gram-positive bacteria. As it is inactivated by gastric acid, it is administered intravascularly. Due to its high plasma concentration, it is used for infections such as endocarditis. However, since it is susceptible to beta-lactamases produced by some bacteria, resistance testing is important. Pharmacologically, the bactericidal effect is correlated with the time the antibiotic concentration is above the MIC value. Due to this, the dosing schedules often aim to spread the doses evenly during the day rather than maximising the peak concentration[38].

Another class of antibiotics are the aminoglycosides, of which gentamicin is one, originally derived from actinomycetes[39]. The antibacterial action of aminoglycosides comes from the binding to the16S rRNA of the ribosome[40]. This causes mistranslation and thus error-prone protein synthesis with polypeptides formed using the wrong amino acids. These are then released and can cause damage to the cell membrane and elsewhere[41]. The aminoglycosides have a poor bioavailability when taken orally and are thus given intravenously. Bactericidal effect is an effect of the area under the curve (AUC) of the concentration. Due to this and the fact that detrimental side effects such as nephrotoxicity are more frequent when aminoglycosides are given as multiple doses, the usual regimen is a single-dose-daily schedule[39,42].

Antibiotic synergy

The concept of antibiotic synergy is not a new one and refers to cases when the effect of a combination of antibiotics is a large increase in bactericidal or bacteriostatic activity compared to each antibiotic substance in isolation [43,44]. Testing of bactericidal synergy can be done using so-called time-kill assays where a bacterial inoculum is placed in nutrient broth with different concentrations of the tested antibiotics in combination and alone. The killing of bacteria is then measured by subculturing the bacteria at different time points. Another method using nutrient broths is the checkerboard assay. In this method, tubes of liquid media (or wells in a microtitre plate) are organised in a square with the

concentration of one antibiotic decreasing from left to right and the concentration of the other antibiotic decreasing from top to bottom. The MIC values of each antibiotic alone and in combination can be elucidated from the inhibitory zone and the FIC value (fractional inhibitory concentration) of each antibiotic can be calculated. The formula for the FIC value for antibiotic A is $FIC_A=MIC_{A+B}/MIC_A$, for antibiotic B the formula is thus $FIC_B=MIC_{B+A}/MIC_B$. The sums of FIC_A and FIC_B are added to give the FICI (fractional inhibitory index) which is an indication of the degree of inhibitory synergy. FICI is also possible to measure on solid medium such as agar plates. Antibiotic gradient strips can be used in this method and placed on top of each other in various combination, or with one antibiotic being infused in the agar and the other placed as an antibiotic gradient strip or an antibiotic diffusion disk[44–47].

Synergy between aminoglycosides, such as gentamicin, and penicillin, such as benzylpenicillin, has been shown against streptococci and enterococci[48–53]. The proposed mechanism is a weakening of the cell wall by penicillin, enabling more aminoglycoside to enter the bacterial cell[54]. There has been criticism of the methods mentioned above, pointing out problems such as the theoretical difference between inhibition of growth and killing, and the fact that two antibiotics with different pharmacokinetic and pharmacodynamic properties might not be present at relevant conditions for long *in vivo* even if they show synergy *in vitro*[55].

Classification of Gram-positive bacteria

The cell wall of Gram-positive bacteria is made up of peptidoglycan with lipoteichoic acid interspersed in the inner part of the cell wall (and linking it to the membrane) and wall teichoic acid in the outer part of the cell wall[56–58]. Many Gram-positive bacteria have proteins that are anchored to the cell wall, and a common factor of these proteins in species related to *Staphylococcus* and *Streptococcus* is the LPXTG sequence in the C-terminal end[59], a signal peptide, a hydrophobic portion, and a charged tail[60,61]. These, collectively, are the cell wall sorting signal, which is highly conserved[62]. The sorting signal is recognized by sortase, a membrane-associated enzyme that cleaves the peptide bond between the threonine and glycine residues and covalently binds the threonine to the peptidoglycan[60,63]. Other variants of cell surface proteins in Gram-positive bacteria are transmembrane proteins, lipoproteins covalently attached to membrane lipids, and cell wall proteins attached to cell wall domains through other methods than LPXTG-domains[64]. Regardless, many proteins considered virulence factors are LPXTG proteins, such as protein M and GRAB in *Streptococcus pyogenes* and protein A in *Staphylococcus aureus*[65–69].

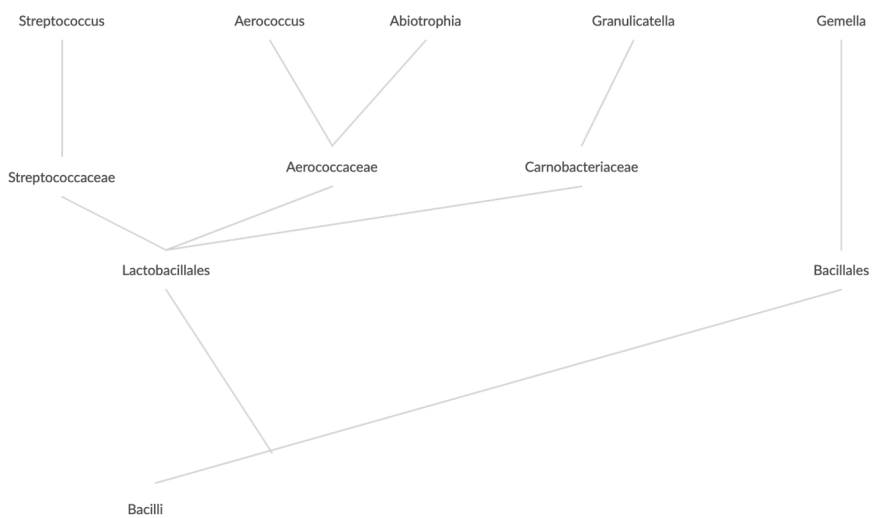

Figure 2. Cladogram of the bacterial genuses discussed in the thesis

Aerococcus - a little known genus

Aerococcus is a genus of Gram-positive bacteria in the Lactobacillales group. They were originally isolated from dust in 1953, with the type species being *Aerococcus viridans*. *Aerococcus* in clusters are morphologically similar to staphylococci but unlike them, aerococci are catalase-negative, and the colonies produced on blood agar are similar to those of streptococci[70]. In 1989, a new species called *Aerococcus urinae* (initially described as an *Aerococcus*-like organism) was described and was found to cause urinary tract infections and sepsis[71–74]. Since then, further species such as *A. sanguinicola*[75], *A. christensenii*[76], *A. urinaehominis*[77], *A. urinaeequi*[78], *A. suis*[79], and *A. vaginalis* have been found[80].

A. urinae was thought to be a very rare cause of human infection and was rarely identified. One cause of this might have been the fact that they are morphologically similar to staphylococci when grown in liquid medium and similar to alpha-haemolytic streptococci when grown on blood agar[81]. Furthermore, the Vitek system frequently misreports *A. urinae* as *A. viridans*, while the API system and BBL-Crystal-GP provided a more certain identification. Both Vitek and API have problems with identifying *A. sanguinicola*[82,8384]. Sequencing the 16S rRNA gene or intergenic spacer regions is a precise method for identifying the different *Aerococcus* species and separating them from each other[85], but MALDI-TOF MS has been shown to be a very accurate method that is quicker and more easily used in a clinical laboratory[86,87].

Since *A. viridans* was first discovered, it has been shown that different subspecies can cause infections in lobsters (gaffkaemia), turtles, pigs, and cows, as well as reports of human infections[88–95], though the validity of species determination in case reports of human infections has been put into question[96]. Nevertheless, some case reports show infections with *A. viridans* where the species identification is certain[97,98].

The virulence factors of aerococci are mostly unstudied, but some data exist. It has been shown that *A. urinae* and *A. sanguinicola* can produce biofilm and cause antibody- and fibrinogen-dependent platelet aggregation [99,100]. Genetic homologs for capsular polysaccharides have been identified in *A. urinae* and *A. sanguinicola*[101], and a capsule has been identified in strains of *A. viridans* that have infected lobsters[89].

A. urinae has been shown to colonize the urinary tract of people with a urinary catheter, and the genital area and urinary tract might be the natural habitat of the species[102]. There have been many reports of aerococci causing urinary tract infection in adults[71,82,83,103–106], but cases of malodorous urine in otherwise symptomless children have also been identified[107,108]. Cases of more severe infection such as endocarditis[100,103,109–113], spondylitis[114,115], arthritis and other types of invasive infection have also been described[100,113,115,116]. The median age of

patients with infections by *A. urinae* and *A. sanguinicola* is quite high, with a median age of over 80 being reported in most studies[100,106,113,115].

Though initial studies suggested that *A. urinae* was resistant to trimethoprim, this might be due to the medium used when testing[82,117–119]. Urinary tract infections due to aerococci seem to recover clinically and microbiologically at slightly lower frequencies than infection due to other pathogens such as *Escherichia coli*, something that might be due to the patients with aerococcal urinary tract infections being older. Another possibility is that the aerococci might tolerate the antibiotics better despite *in vitro* testing showing them as sensitive, possibly due to biofilm formation[102,106]. Strains of both *A. urinae* and *A. sanguinicola* are generally very sensitive to penicillin, and have low MIC for cephalosporins and carbapenems[84,119–123]. Bactericidal synergy between penicillin and gentamicin against *A. urinae* has been shown in case reports, though later more robust investigations have shown that synergy is not universal[111,112].

Abiotrophia and *Granulicatella* - nutritionally variant

Abiotrophia and *Granulicatella* were originally identified as so-called nutritionally variant streptococci and were considered part of the alpha-haemolytic streptococci until they were moved to the genus *Abiotrophia* in 1995, and then split into *Abiotrophia* and *Granulicatella* in 2000[124–126]. They lack Lancefield antigens but are serotypeable, with different species generally reacting to specific types of serum[127]. Invasive infection in humans has been described with *A. defectiva*, *G. elegans*, *G. adiacens*, and *G. para-adiacens*[128–130]. Species identification has traditionally been regarded as difficult[131–135] and correctly identifying the species in clinical samples has been shown to be more effective when methods such as 16S rRNA sequencing or, more recently, MALDI-TOF MS have been used in addition to or instead of traditional phenotypical classification[129,136–138].

Both *Granulicatella* and *Abiotrophia* are part of the oral flora and have been found to cause diverse invasive infections [131–135]. *Abiotrophia* has been reported in infections ranging from endocarditis, spondylitis, and joint infections to central nervous system infections and keratitis[131,139–144]. *Granulicatella* is also known to cause invasive infections with a predilection for endocarditis, but is also a cause of meningitis, brain abscesses, implant associated infections, and osteomyelitis with many infections appearing after dental treatment[145–150]. The ability to cause endocarditis as well as osteomyelitis and implant associated infections might be connected to biofilm formation or to the ability of *G. adiacens* to bind fibrinogen [151–155].

Biofilm formation also affects the antimicrobial susceptibility, with sensitivities for both beta-lactam antibiotics and others decreasing greatly in the biofilm. In

planktonic phase, *G. elegans* is generally susceptible to penicillin, with *G. adiacens* and *A. defective* being less sensitive. Ceftriaxone has shown good effect against *A. defectiva* and *G. elegans* but not *G. adiacens*, with all three species being sensitive to vancomycin. They are also often sensitive to amoxicillin and ampicillin[152,156–158].

Table 1. List of non-beta-haemolytic *Streptococcus* (NBHS) species and their commonly accepted group classifications

Group	Species
Mitis	*S. mitis*
	S. pneumoniae
	S. pseudopneumoniae
	S. oralis
	S. peroris
	S. infantis
	S. australis
	S. parasanguinis
Sanguinis	*S. sanguinis*
	S. gordonii
	S. cristatus
Anginosus	*S. anginosus*
	S. constellatus
	S. intermedius
Salivarius	*S. salivarius*
	S. thermophilus
	S. vestibularis
Mutans	*S. mutans*
	S. sobrinus
	S. ratti
	S. macacae
Bovis	*S. gallolyticus*
	S. infantarius
	S. equinus

Streptococcus - not only beta-haemolysis

Bacteria of the genus *Streptococcus* have long been known to cause infections. Though they are Gram-positive and in the Lactobacillales family, there have been numerous ways of classifying them such as the Lancefield system of carbohydrate antigens[159], dividing them into α-haemolytic, β-haemolytic, and γ-haemolytic streptococci, as well as taxonomy based on the genetic similarity of the species[160,161]. The streptococci that are relevant to this thesis are mainly those that are α-haemolytic or γ-haemolytic, that is: non-β-haemolytic streptococci (NBHS). The phenotypic methods based on haemolysis and Lancefield antigens are still in common practice but have problems that are apparent upon a closer look. Species such as *S. pyogenes* are easy to classify as they uniformly express Lancefield A antigen and are β-haemolytic, the haemolysis being mediated through secreted streptolysin S and streptolysin O[159,162,163].

The NBHS are generally harder to classify using haemolysis and Lancefield antigens grouping systems, and there is not always consensus on what bacteria to include in different groups[160]. The bacteria in the *S. anginosus* group (also known as the *S. milleri* group) are an illustrative example of this. Some isolates considered to be *S. anginosus* have shown β-haemolysis, some α-haemolysis, and some show no haemolysis at all. These isolates can also have group A, C, G, or F Lancefield antigens, with some isolates having none at all[164,165]. Species that are considered to be in the *S. anginosus* group are *S. anginosus*, *S. constellatus*, and *S. intermedius*[166].

The question of which species to include in the *S. mitis* group has practical considerations as the spectrum of diseases they cause differ.[167–171]. Bacteria commonly included in the *S. mitis* group are *S. mitis, S. pneumoniae, S. pseudopneumoniae, S. oralis, S. peroris, S. infantis, S. australis, S. parasanguinis*[172]. *S. massiliensis* is sometimes placed in the *S. mitis* group, and *S. sanguinis*, *S. gordonii*, and *S. cristatus* are sometimes considered to be in the *S. mitis* group but are lately often placed in the separate *S. sanguinis* group (or *S. gordonii* group)[171–173]. MALDI-TOF MS has been useful in differentiating the different species in the group[174,175].

Both the *S. salivarius* group (consisting of *S. salivarius*, *S. vestibularis*, and *S. thermophilus*) and the *S. mutans* group (consisting of *S. mutans*, *S. sobrinus*, and *S. rattus*) are NHBS that are part of the normal flora and represent bacteria where the species determination by MALDI-TOF has improved during the recent years[176–178].

The S. bovis group share the D group antigen, and species that are generally considered to be in the *S. bovis* group are *S. gallolyticus*, *S. infantarius*, *S. bovis*, *S. pasteurianus*, *S. equinus*, *S. macedonicus*, and *S. lutetiensis*[179–181].

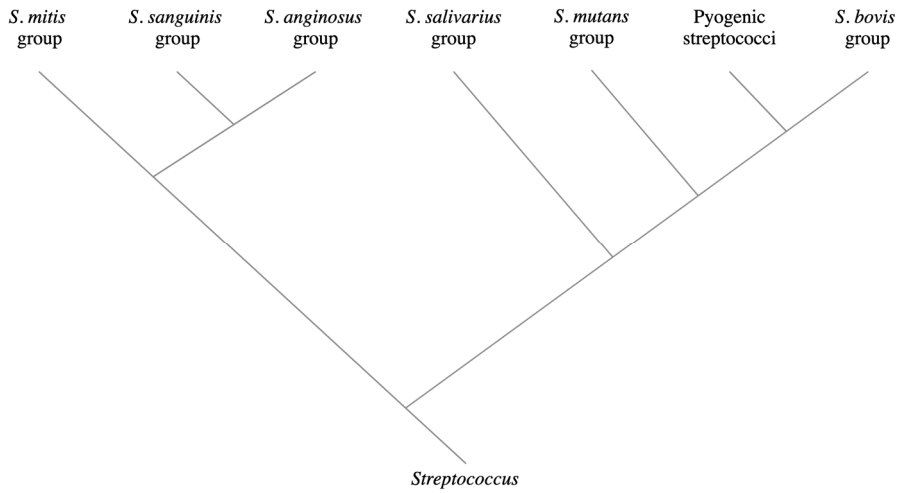

Figure 3. Relationship between the *Streptococcus* groups described in the thesis[171,173,176]

S. anginosus group bacteria are commonly found in the oral cavity as part of the normal flora, but also as parts of the gastrointestinal and genitourinary flora[182,183]. Haemolysis is mediated by streptolysin S (in strains with β-haemolysis) which has a high level of similarity to streptolysin S from *S. pyogenes*[184] whereas intermedilysin from *S. intermedius* is similar to streptolysin O[185]. Common infections caused by *S. anginosus*-group bacteria are soft tissue infections and liver abscesses[186], but cases of IE also occur[167,187].

The *S. mitis* group of streptococci have been have also been regarded as NBHS with a propensity to cause IE, but with the division of the former group into the *S. mitis* group and the *S. sanguinis* group (where *S.* sanguinis is known to produce biofilm) it is clear that the propensity to cause endocarditis is higher in the *S. sanguinis* group [167–171,188,189]. *S. pneumoniae* is a common cause of pneumonia and is also known for causing meningitis. The closely related *S. mitis* is a part of the oral flora and a rarer cause of invasive disease and lacks some of the virulence factors present in *S. pneumoniae*[190,191]. The capsule that is present in *S. pneumoniae* is one of these differences, though there have been findings that indicate that *S. mitis* in some cases may express a capsule and that the type of capsule might be of importance in infections[192–194].

Both the *S. salivarius* group and the *S. mutans* group are part of the oral flora[176]. While *S. salivarius* (together with *S. sanguinis*, *S. mitis*) is associated with plaque formation[195], *S. mutans* (and to some degree *S. salivarius*) is associated with caries and is a producer of biofilm[176,196,197]. Both *S. salivarius* group and *S. mutans* group streptococci have been reported to cause IE, with *S. mutans* seeming to be more

likely to do so[167,170,188,198]. Other types of invasive infections that have been noted are meningitis and joint infections[199–202].

The *S. bovis* group of streptococci is somewhat of an outlier compared to other NBHS in that it is found in the gastrointestinal tract in addition to the oral cavity[203]. An association between *S. bovis* group bacteria and gastrointestinal malignancies has been known for a long time[204], though the extent of the association and what types of malignancies are associated has been debated[203,205–207]. Similar to other types of NBHS the *S. bovis* group is an important cause of IE, both with and without valve prostheses[170,208–210].

The NBHS have many similarities, but also important differences such as penicillin non-susceptibility being more common in the *S. mitis* and *S. salivarius* groups whereas the *S. anginosus*, *S. sanguinis*, and *S. bovis* group have been found to be very susceptible. The NBHS are also generally susceptible to clindamycin and vancomycin[211–213].

Gemella - the odd one out

Gemella was originally identified as a separate genus in 1961 when *Gemella haemolysans* was separated from *Neisseria* where it had originally been placed. The separation was based on *Gemella* being oxidase-negative, and they were defined as Gram-negative, facultatively aerobic and oxidase-negative cocci growing in pairs[214,215]. It was later found to be able to retain Gram stain and to have an architecture resembling Gram-positive bacteria, though with a somewhat thin cell wall[216,217]. *Gemella* has had many different places in the tree of taxonomy, with the type species *G. haemolysans* originally being considered to be one of the streptococcaceae[216], and what is now classified as *G. morbillorum* was previously classified as *S. morbillorum*[218]. It is now placed amongst the bacilli, though it nevertheless has several similarities to the bacteria in the lactobacillales genus[219].

At the time of writing, seven species of *Gemella* have been identified in humans: *G. haemolysans*, *G. bergeriae*[220], *G. morbillorum*[218,221], *G. taiwanensis*[222], *G. asaccharolytica*[223], *G. parahaemolysans*[222], and *G. sanguinis*[224].

Though they are a part of the normal oral flora[134,225–228], both *G. haemolysans*, *G. bergeriae*, *G. sanuinis*, *G.* taiwanensis, and *G. morbillorum* have been reported to cause endocarditis[229–237]. *Gemella* is also known to cause abdominal abscesses, ocular infections, spondylodiscitis, joint infections, and spinal and cerebral infections[238–245].

Gemella are generally sensitive to beta-lactam antibiotics and aminoglycosides but resistance to macrolides and lincosamides has been found[246,247].

Some aspects of infective endocarditis

Infective endocarditis (IE) is a disease that has been recognised for quite some time, with a theory proposed by Sir William Osler in his 1885 Gulstonian Lectures that susceptible patients acquired a "mycotic" growth with spread of the microorganisms to other parts of the body[248]. It is one of the many forms of invasive bacterial infection, with a mortality close to 100% before the introduction of antibiotic treatment, and with a mortality of 15-20% even now[249,250]. The key feature of it is an infection of the heart valves (either native or prosthetic), the endocardium, or an indwelling cardiac device[251]. Even though blood is traditionally seen as a sterile site, the cardiac endothelium is regularly exposed to bacteria during activities such as tooth-brushing[252]. Normally, it is able to withstand the bacteria encountered during these episodes. If endothelial injury is present, the release of cytokines and tissue factors may cause a thrombus to form, allowing bacterial adherence and thus starting the endocarditis[253]. As the bacterial growth continues, additional damage to the endothelium occurs, starting new cycles of thrombus formation and the formation of a vegetation. There is also speculation that some bacteria also form a biofilm (a structured matrix of polysaccharides, DNA, and proteins), which helps with adherence and can increase the tolerance to antibiotics. In cases of endocarditis on prosthetic valves, the presence of biofilm is less controversial[254,255].

Epidemiology and microbiology

While endocarditis remains potentially deadly, the incidence is relatively low at 3-10 cases per 100 000 persons per year[256–259]. A difference in the epidemiology of endocarditis exists between high- and low-income countries, with rheumatic heart disease being a much more common risk factor in some low- and middle-income countries[260,261]. The patients are often younger and the endocarditis caused by streptococci from the oral flora, which contrasts to patients in high-income countries where instead *Staphylococcus aureus* is the most common cause of IE[169,262–265].

Cardiac prostheses such as prosthetic valves are a major risk factor for IE, and prosthetic valve endocarditis is an increasing problem in high-income countries.

Other foreign bodies such as pacemakers and implantable cardioverter-defibrillators (ICD) are also an increasingly common risk factor for IE in high-income countries [256,266,267]. The microbial spectrum is somewhat different in high-income countries as compared to low-income ones, with most studies describing *S. aureus* as being the most common cause of IE and with streptococci as the second most common group[169,250,258,268–270]. The streptococci most commonly associated with IE are the so-called viridans group streptococci (including *Streptococcus mitis, Streptococcus mutans, Streptococcus sanguinis, Streptococcus anginosus*, and *Streptococcus salivarius*) and the group D streptococci (including amongst others *Streptococcus bovis*)[251,271], but other streptococci such as *S. dysgalactiae* are also found[272]. The third main group of bacteria causing IE is the enterococci with most cases being caused by *E. faecalis*[251]. More uncommon causes causing IE are the Gram-negative bacteria in the HACEK group (consisting of the *Haemophilus*, *Aggregibacter*, *Cardiobacterium*, *Eikenella*, and *Kingella* genera)[273], and Gram-positive bacteria such as *Abiotrophia*, *Aerococcus*, *Corynebacterium*, *Gemella*, and *Granulicatella* [112,138,274–278].

Diagnosing endocarditis

The diagnosis of IE is based on a combination of clinical findings, microbiological data, and imaging results. The modified Duke criteria include these three domains and are the most commonly used definition of endocarditis[279,280]. Microbial diagnosis is most commonly made through blood cultures, but can also be made by culturing valve biopsies, serology, or PCR. When using blood cultures for microbial diagnostics in IE, taking more cultures increases the sensitivity[8,281–283]. Blood cultures are the main method for more common causes of IE such as coagulase-negative *Staphylococcus*, *S. aureus*, NBHS, and other streptococci, *Enterococcus*, and the HACEK bacteria. Microbiological diagnosis of blood culture negative IE is more challenging, but methods such as serology and PCR on heart valves can identify bacteria such as *Coxiella burnetii*, *Bartonella* spp., and *Tropheryma whipplei*. In some cases, specialised growth medium and specific incubation condition can be used, such as in IE caused by mycobacteria or fungi[282].

Table 2.
The modified Duke criteria for IE[279,280].

Major criteria			
	Blood culture positive for IE		Typical microorganisms consistent for IE from 2 separate blood cultures
		or	Microorganisms consistent with IE from persistently positive blood cultures
		or	Single positive blood culture for *Coxiella burnetii* or antiphase I IgG antibody titer >1:800
	Evidence of endocardial involvement		
	Echocardiogrram positive for IE		Oscillating intracardiac mass on valve or on supporting structures, in the path of regurgitant jets, or on implanted material in the abscence of an alternative anatomical explanation
		or	Abscess
		or	New partial dehiscence of prosthetic valve
Minor criteria			
	Predisposition		Predisposing heart condition
		or	Intravenous drug use
	Fever, temperature >38ºC		
	Vascular phenomerna		
	Immunologic phenomena		
	Microbiological evidence		Positive blood culture that does not meet the criteria for a major criterion
		or	Serological evidence of active infection with organism consistent with IE

Echocardiography is the main method used to localise vegetations on the heart valves as well as to identify valvular damage and abscesses. TTE (transthoracic echocardiography) can be used to find microbial vegetations, but the sensitivity and specificity of TEE (transoesophageal echocardiography) makes it the preferred modality in IE investigations[284,285]. When the suspicion of IE is high, a TEE with normal findings can be repeated after 7-10 days. Further investigations can add information but only in a minority of cases[286,287]. Echocardiography is also the main method of identifying infections of cardiac devices[288,289].

The question is when to raise the suspicion of IE? Some factors are known to increase the risk that a bacteraemia is associated with IE. Much of the research that has been done concerns *S. aureus* bacteraemia, but also *Enterococcus* and streptococcal species. The time it takes until a blood culture is reported positive by the automated detection systems ("time to positivity") has been shown to be inversely related to the risk of IE in case of *S. aureus* bacteraemia, that is, a shorter

time until the culture turns positive means a higher risk of IE[290–292]. Other factors known to be associated with an increased risk that a bacteraemia is caused by IE are the presence of a cardiac implantable electronic device, secondary foci (such as spondylodiscitis), embolization, or if the patient is receiving haemodialysis[293–295]. The implementation of these results, together with other data, into formalised score systems were later published as the PREDICT and VIRSTA scores in an attempt to guide whether or not to suspect IE in case of *S. aureus* bacteraemia[296,297]. For *Enterococcus* bacteraemia, known risk factors for IE are male sex, community acquisition, the presence of a valvular prothesis, unknown focus of infection, monobacterial infection, and multiple positive blood cultures. Some of these factors were included in the NOVA score which attempted to identify patients with bacteraemia but with a low risk of IE where IE investigations were unnecessary. As can be seen, there are many factors that are common between bacteria that increase the likelihood that bacteraemia is associated with IE, and some that seem to be more important when the bacteraemia is caused by certain bacteria [298–303]. The NOVA score was later refined and the DENOVA score published, which increased the specificity for detecting IE while retaining a high sensitivity[304].

Other imaging modalities may also be used in process of diagnosing IE. Multislice computed tomography (CT) was originally used to diagnose complications of IE, such as ischemic lesions caused by embolism[305]. Cardiac tomography can also be gated by electrocardiogram to investigate lesion in cases of suspected prosthetic valve IE or to explore the anatomy preoperatively[306,307]. CT can also be used in cardiac imaging to find valve abnormalities and vegetations, and may be superior to TEE in patients with calcified valves or in the assessment of the extent of perivalvular abscesses or pseudoaneurysms[308–310]. MRI is also a modality that can be used to diagnose asymptomatic embolization, which can affect both diagnosis and choice of management[311,312]. Both CT and MRI (magnetic resonance imaging) imaging of the brain can be used to detect embolization both to confirm a suspicion of IE (as it confirms one of the Duke criteria) and to aid in the prognostication. Nuclear imaging is another modality that is used in the management and diagnosis of IE, and the finding of increased uptake around the valves using 18F-fluorodeoxyglucose positron emission tomography-CT has been proposed as a major criterion for the diagnosis of IE, though not without criticism[308,313–315].

Treatment and other clinical aspects

Treatment of endocarditis is based on antibiotic therapy with surgery performed when needed to replace damaged valves and as source control[316,317]. Treatment has traditionally been performed as long regimes of intravenous antibiotics. For bacteria such as *Enterococcus* and *Streptococcus*, therapies combining beta-lactam

antibiotics and aminoglycosides have been common with the possibility of shortening the necessary treatment time being given as the reason[313,318]. Both European and American guidelines have also recommended a combination therapy for IE caused by *Abiotrophia* and *Granulicatella* [313,318]. This is something that has been put into question by recent studies, and combinations of different beta-lactams or beta-lactams combined with daptomycin have been tried[319,320]. Recent studies indicate that oral treatment might be a safe and effective option for IE in stable patients without complicating factors[321,322]. In cases of prosthetic valve endocarditis, the antibiotic treatment is often longer than in cases of native valve endocarditis, though the antibiotics of choice remain the same except when the causative organism is *S. aureus*, in which case the addition of rifampicin and gentamicin is recommended[255].

Complications that might occur depend on the location of the infection, underlying conditions, and on the infecting pathogen. Common serious complications include valve damage with consequent heart failure, embolization to end arteries with ischemia and stroke, and metastatic infections such as spondylodiscitis. These complications are the reason for much of the mortality and morbidity of endocarditis[255,295,323]. The question of when to perform surgery is one of the major ones in the management of IE. In cases such as patients with valve damage and heart failure, high risk of embolization, or infections with highly resistant microorganisms, the consensus is that surgery is to be performed. Results from different studies diverge on the importance of early surgery, possibly due to different patient characteristics in different studies. A recent meta-analysis does show that early surgery decreases mortality, and other studies emphasise the importance of surgery in cases of heart failure. There is also some evidence that the type of infectious agent might affect surgical outcome. As the decision on if and when to perform surgery, what kind of imaging to use and when, and what kind of antibiotics to use and for how long are difficult, a close collaboration between different specialities is needed to handle this complicated infection properly[313,324–332].

Present investigations

Paper I

Identification of two abundant *Aerococcus urinae* cell wall-anchored proteins

When work started on this paper, very little was known about the virulence mechanisms of *A. urinae*. The aim was to explore the surface proteome in the hope of finding possible mechanisms through which the bacteria cause infection. Surface proteins are an interesting subject to study as they are often important for virulence, such as being used for adhesion to surfaces, attachment to cells, or due to being a part of a capsule. They are also often important in the immune response and potentially of interest as a vaccine target. The initial study was done through mass spectrometry, and two surface proteins named Aerococcal surface protein (Asp) 1 and 2 were identified and found to quantitatively dominate the bacterial surface. The presence of these protein was also shown using ELISA with serum from rabbits immunized with recombinantly expressed Asp 1 and 2. These proteins had a signal sequence in the amino-terminal end as well as a wall-sorting sequence in the carboxy-terminal end with an LPATG-motif, a lipophilic domain and a positively charged tail. In total, twenty-five *A. urinae* genomes were sequenced using an Ilumina HiSeq system. Six different variants of the genes (*asp*1-6) were found. All studied isolates had one or two *asp*-genes located in the conserved locus denoted Locus encoding Aerococcal Surface Proteins (LASP). The original plan was to study their function by knocking out the *asp* 1 and *asp* 2 genes. Sadly, despite a great deal of effort, we were unsuccessful in knocking out the *asp* 1 and *asp* 2 genes and we had to settle for a more descriptive study. Cell wall-anchored proteins with LPXTG-domains are a common virulence factor in Gram-positive bacteria. Thus, despite our goals being to characterise the function of the Asp proteins even further, the finding and characterisation of these proteins and genes are an important step in the mapping of aerococcal molecular virulence mechanisms.

Paper II

Clinical and microbiological features of infective endocarditis caused by aerococci

Antibiotic treatments for IE combining beta-lactam antibiotics with aminoglycosides have been common for decades, despite limited evidence for the usefulness of such combinations. The reasoning used is a supposed bactericidal synergy between beta-lactam antibiotics and aminoglycosides against Gram-positive bacteria, something which has been shown *in vitro* for certain bacteria such as *Entercococcus* but has not been demonstrated for many other species. The lack of evidence is more pronounced when it comes to emerging pathogens such as *Aerococcus* where the only study published before this was a description of two cases of IE with killing kinetics described[111]. This lack of knowledge combined with the lack of more systematic descriptions of endocarditis caused by aerococci formed the background behind this study. The fact that previous studies describing aerococcal IE were mostly case reports increased the importance of a more organised study such as this, as case reports are more likely to be written in cases of a dramatic course of disease or in cases with unusual types or localisations of infection thus skewing the published data. The Swedish Registry of Infective Endocarditis (SRIE) was used to find cases of aerococcal IE between 2002 and 2014. Fourteen cases of IE caused by *Aerococcus urinae* and two cases of IE caused by *A. sanguinicola* were identified and the species identification was confirmed using MALDI-TOF MS. The medical data was then studied and compared to cases of IE reported to the SRIE caused by other bacteria. Antibiotic sensitivity to various antibiotics was tested using Etests and synergy between benzylpenicillin and gentamicin was tested using broth microdilution with different concentrations of benzylpenicillin and measuring at 0, 6, and 34 hours.

The data showed that patients with aerococcal IE on average were older than patients with IE caused by non-beta-haemolytic streptococci or *Staphylococcus aureus*. 75% of the patients with aerococcal IE were men. An interesting aspect of the study is that all patients had received a combination of penicillin and an aminoglycoside. *In vitro* bactericidal antibiotic synergy was, however, only shown for 7 of the 16 isolates, which makes this the first study to systematically describe antibiotic synergy against aerococci. The reasoning behind the decision to treat the patients with a combination of a penicillin and an aminoglycoside is not known with any certainty, but it is possible to speculate. Perhaps the decision was based on the presumed synergy between penicillin and aminoglycosides against enterococci and streptococci? Regardless, it shows the importance of studying pathogenic bacteria systematically instead of relying on case reports, and instead of assuming that they behave identically to their relatives. This study provides valuable clinical information about aerococcal IE as well as an indication that

meaningful antibiotic synergy might be possible but also definitely not certain in a given isolate.

Paper III

Antibiotic synergy against viridans streptococci isolated in infective endocarditis

Guidelines for IE caused by viridans (or non-beta-haemolytic) streptococci have often recommended combining beta-lactam antibiotics with aminoglycosides. The basis for this has been clinical practice as well as a limited amount of animal studies where antibiotic synergy has been shown, together with old clinical studies where the controls were not fully comparable to the patients receiving combination treatment. Since aminoglycosides have well known side effects such as ototoxicity, we wanted to determine if it was possible to show bactericidal synergy between benzylpenicillin and gentamicin *in vitro* as well as see if bactericidal synergy was associated with bacteriostatic synergy. The idea was to see if it was possible to determine if this synergy was possible to detect using methods that would be easy to implement in routine use at clinical microbiology laboratories. This would allow the use of gentamicin to be restricted to cases with proven synergy and spare other patients a potentially toxic treatment.

24 of viridans group streptococci were collected from patients that had been treated for IE at Skåne University Hospital and the species determination confirmed by MALDI-TOF MS. Antibiotic sensitivity was then tested using Etests and broth microdilution. Fractional inhibitory concentration (FIC) for benzylpenicillin and gentamicin was then tested using Etests. Bactericidal synergy was tested using broth microdilution with two different concentrations of benzylpenicillin combined with gentamicin, measured at 0, 6, and 24 hours.

Bactericidal synergy was shown in 14 of 28 isolates. Bacteriostatic synergy as determined by FIC was shown in 2 out of 24 isolates, none of which sowed bactericidal synergy. This means that antibiotic synergy is present in some isolates at least *in vitro* but the clinical applicability is uncertain. The question if *in vitro* synergy can be assumed to correlate to an increased synergy *in vivo* remains, as does the question if lack of synergy *in vitro* (as defined in this study) means that there is no appreciable effect *in vivo* if gentamicin is added to benzylpenicillin in treatment. The difference between FIC values and bactericidal synergy might be due to differences between solid and liquid media. Regardless of the mechanistic reason for the difference, it means that there is no easy way of testing antibiotic synergy in clinical practice. In the 2015 European guidelines for IE, a combination of a beta-lactam antibiotic and an aminoglycoside is one of the recommended antibiotic treatments for streptococcal IE in cases where the renal function is good.

In one of the studies cited in the guidelines, relapse was noted in a case of endocarditis with a bacterium where no *in vitro* synergy was seen. This, together with the clinical studies cited presenting patients with complications from the ototoxic and nephrotoxic effects of aminoglycosides show the need of ascertaining whether or not there truly is synergy if a combination treatment is to be used[313,333–335]. Data from the POET (Partial Oral Treatment of Endocarditis) study show that short intravenous antibiotic therapy followed by oral antibiotic treatment is a promising alternative to short-term combination therapy with beta-lactams and aminoglycosides if no good way to determine actual synergy can be found[321].

Paper IV

> HANDOC: a handy score to determine the need for echocardiography in non-β-haemolytic streptococcal bacteraemia

As can be inferred from the Duke criteria, a combination of echocardiography and microbiological testing is used to diagnose endocarditis. A question thus arises when a clinician is confronted with a blood culture showing growth of a bacterium that can cause endocarditis: when should you perform further investigations for IE and when should you look elsewhere for the source of the bacteraemia? Scoring systems that try to identify patients with low risk for IE (and thus without need for further IE investigations) have been developed for IE caused by *Staphylococcus aureus* and enterococci with varying degrees of success. Despite non-beta-haemolytic streptococci (NBHS) being a quite common cause of IE, they also cause other infections where different investigations are needed. To rectify this, we decided to perform a retrospective study of patients with bacteraemia caused by these streptococci. Patients in Skåne showing growth of NBHS in blood culture between 2012 and 2014 were identified. Patients under 18 years of age were excluded, as were patients with neutropenia or where the medical records were unavailable. They were then divided into two groups with the patients with blood cultures from January 2012 to June 2013 used to generate the score. Species identification was confirmed using MALDI-TOF MS. The first cohort consisted of 339 patients of whom 26 had IE as defined by the modified Duke criteria. Since IE can have many symptoms and the diagnostic process can have ambiguous results, patients were put into three groups. One group was those where IE was confirmed according to the Duke criteria, one group included patients where IE could be excluded (i.e., by TEE without signs of IE), and one group consisted of patients that did not meet the Duke criteria for IE but where IE could not strictly be excluded (i.e., patients where TTE and TEE was not performed but received a long antibiotic treatment that might be curative for IE).

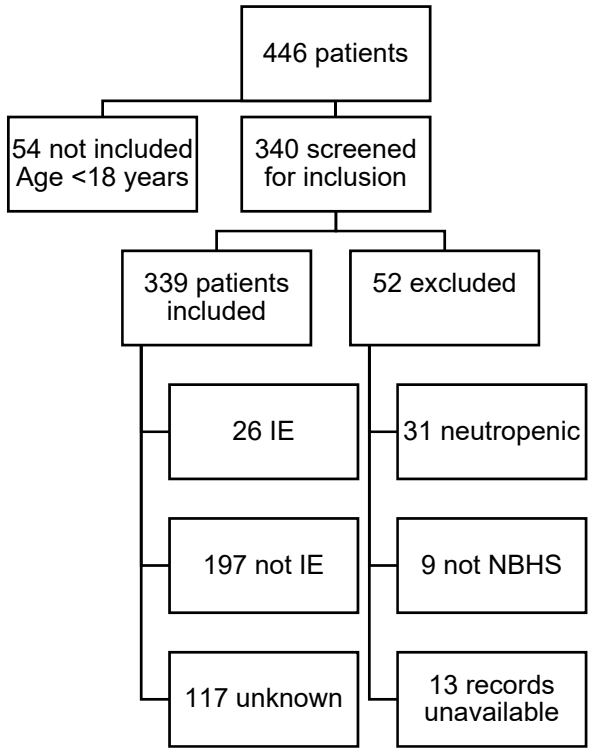

Figure 4. Flowchart describing the inclusion process for the study.

Several factors differed between those that had IE and those that did not. Among these factors, the presence of heart murmur or heart valve disease (H), aetiology with the groups of *Streptococcus mutans*, *Streptococcus bovis*, *Streptococcus sanguinis*, or *Streptococcus anginosus* (A), number of positive blood cultures ≥2, duration of symptoms of 7 days or more, only one species found in blood culture, and community acquisition of the infection were used to form the HANDOC score. One point was given for each of the components in the score except aetiology of *S. anginosus* where one point was subtracted instead. Using a cut-off between 2 and 3 points, the sensitivity was 100% and the specificity was 73% for identifying patients with IE.

To validate the HANDOC score, the second half of the population was used, which comprised of patients whose blood cultures were received between July 2013 and December 2014. 522 blood cultures were received during that period, and the same inclusion and exclusion criteria as in the main cohort were used. When comparing patients with and without IE in this cohort, the HANDOC score had a sensitivity of 100% and a specificity of 76%. This means that it might be possible to use HANDOC in clinical practice to identify patients with a very low

risk of IE and thus direct the use of echocardiography resources to cases where the risk is higher. A criticism that was published was regarding the group where the IE status was classified as unknown. Similar studies have generally classified all cases which did not fulfill the Duke criteria as non-IE. While it remains possible that cases of IE were missed and placed in the "unknown" group, this reflects the clinical difficulties in establishing a certain IE diagnosis. The few patients in the "unknown" group who received a clinical diagnosis of IE all had HANDOC scores of 3 or more, which might indicate that HANDOC would not miss cases of infection where the IE status is uncertain[336,337]. Another possible problem is the overlap between the Duke criteria and the HANDOC score, such as two positive blood cultures being a criterion for IE in the Duke criteria as well as one of the parts of the HANDOC score. While this causes a possible circle reasoning, only one of the IE cases in the study would have been classified otherwise if they had had only one positive blood culture. That patient had 6 out of 6 possible points in HANDOC and indicates that the success of HANDOC is not only due to the criteria being similar to the Duke criteria. Similarities between the scoring systems are difficult to avoid as they concern the same clinical condition. The division of the NBHS into groups is one of the strengths of the study and shows the usefulness of improved microbiological capabilities. Separating the *S. mitis* group from the *S. sanguinis* group can be seen as somewhat controversial as they have traditionally been placed together. However, new genetic data as well as their different predilections for causing IE show that separating them into different groups makes sense both from a scientific and a practical point of view. Looking at single streptococcal species might have revealed even more differences in the risk for IE in bacteremia, but would have necessitated a larger cohort and even more certain species determination. All in all, HANDOC represents a scientific study of a potential implementation of a systematic use of microbiological and clinical data into clinical use. The usefulness might be greatest in hospitals where there is no in-house capability for TEE at all and where the patients thus must be sent to other hospitals when TEE is indicated.

Paper V

> External validation of the HANDOC score–high sensitivity to identify patients with non-beta-haemolytic streptococcal endocarditis

The investigations presented in Paper IV showed that the HANDOC score had a very high sensitivity and a good specificity. A drawback with regards to generalizing the results is that both the group of patients used to generate the HANDOC score and the group used to validate it come from the same geographical area. This means that one can expect similar patient characteristics in

both groups, but also that patient selection, routines for when to take blood cultures, availability of TEE, and possibly also the physicians that documented their findings in the medical records were the same in both groups, which possibly limits the generalisability. A study was thus performed where the patient data was collected at Herlev-Gentofte Hospital and North Zealand Hospital in Denmark. Patients with NBHS in blood culture between March and September 2016 were included as part of an ongoing study where data on all patients with Gram-positive cocci in blood cultures was collected. TTE and TEE were recommended according to local guidelines when blood culture showed growth of Gram-positive bacteria typically found in IE. I thus went through the medical records of the patients with NBHS and classified the patient data and microbiological findings in the same way as in Paper IV. The HANDOC score was applied without modifications. In total 68 patients were included, 16 of which had confirmed IE. When the HANDOC score was used to separate patients with IE from those without, in this setting, the sensitivity was 100% and the specificity 59%. This shows an excellent sensitivity and an acceptable specificity. The lower specificity and positive predictive value in this study as compared to Paper IV might plausibly be due to blood cultures being taken from a different proportion of patients as well as there being a lower amount of polymicrobial findings in culture, indicating either a better technique for blood drawing or different laboratory conditions. Another limitation is the small size of the study population, limiting the power in regards to evaluating HANDOC in cases of less common NBHS variants. Just as in Paper IV, the small size of the study population hinders any analysis of single NBHS species. Despite these limitations, I consider this study an indication that HANDOC might be of use in other regions as well.

Paper VI

> Risk for endocarditis in bacteraemia with *Streptococcus*-like bacteria, a retrospective population-based cohort study

Gram-positive bacteria are a known and common cause of IE. The amount of information available about IE caused by different species is uneven, with the risk of IE by bacteria such as *Staphylococcus aureus* and *Enterococcus* being quite well studied. The risk of IE due to streptococci was explored in Papers IV and V. As described in Paper II, aerococci are also a possible cause of IE. The aim of this paper was to study the risk of IE in bacteraemia with so-called *Streptococcus*-like bacteria such as *Aerococcus*, *Abiotrophia*, and *Granulicatella* in the Lactobacillales order as well as *Gemella* in the Bacillus order. Blood cultures positive for *Aerococcus*, *Gemella*, *Granulicatella*, or *Abiotrophia* between January 2012 and December 2017 were identified in the databases of the Clinical

Microbiology Laboratory in Region Skåne and from Karolinska University Hospital as part of a collaborative study. Patients under the age of 18 were excluded, as were those whose medical records were unavailable. Information from the medical records was collected and the data was categorised according to the NOVA[301], DENOVA[304], and HANDOC[188] scores. These scoring systems were originally designed for separating patients with and without IE in bacteraemia with enterococci (NOVA and DENOVA) or streptococci (HANDOC). The aetiology (A) variable in HANDOC originally refers to which streptococcal group is found in blood culture. To be able to apply the score to other bacteria, the proportion of IE in bacteraemia with *Abiotrophia*, *Aerococcus*, *Gemella,* and *Granulicatella* was compared to the proportions in the HANDOC study. *Abiotrophia defectiva* was found to be significantly more likely to cause IE while *Aerococcus sanguinicola* was less likely to do so. Thus one point was given for *A. defective* for A in the HANDOC score, one was subtracted for *A. sanguinicola* and the remaining species were given zero points. In addition to testing the risk cores, outcome and risk factors for IE were also studied.

The risk for IE in bacteraemia with *Abiotrophia* was found to be 21% in this study, which is higher than many other species that are known for causing IE, such as *S. aureus*, *E. faecalis* and NBHS. The NOVA score had a sensitivity of 100% but the specificity was only 15,5%. Both the HANDOC score and the DENOVA score had a high sensitivity (97% and 100%, respectively, and also a high specificity (85% and 61%, respectively). This study is the first systematic study of the risk of IE in bacteraemia cases with *Abiotrophia*, *Granulicatella* and *Gemella*, and the second published study on risk of IE in bacteraemia cases with *Aerococcus*. The results show that the risk of endocarditis is highly species-dependant. The most obvious example is the very high risk of IE in bacteraemia with *Abiotrophia*, but also the difference in risk of IE between *Aerococcus urinae* and *Aerococcus sanguinicola*. To conclude, this study showed that it should be possible to use both HANDOC and DENOVA to inform the need for IE investigations in bacteraemia with *Streptococcus*-like bacteria (SLB), and also that the characteristics of IE caused by SLB is similar to IE caused by enterococci and streptococci. The study also shows the utility of exact species determination in clinical decision making.

Conclusions and future perspectives

The general awareness of the bacteria studied in this thesis was low when I started the work presented herein, especially regarding the *Aerococcus* genus and aerococcal infections. With MALDI-TOF MS having become the primary method of species determination in clinical bacteriology, the number of species and subspecies that are possible to identify and report to the clinician has increased tremendously. Despite this, there was of a lack of scientific data about what these emerging pathogens represented clinically. During the course of this work, *Aerococcus* has gone from a pathogen that was mostly known by a small group of bacteriologists to a clinically known and relevant genus, and the groups of NBHS have been recognised to have different predilections to cause IE and other disease. The work on determining the clinical differences in bacteraemia caused by other Gram-positive cocci such as *Gemella*, *Granulicatella*, and *Abiotrophia* has also increased during this time. While the classification and identification of these bacteria has been more or less known, the works in this thesis (along with many other scientific investigations) have shown the clinical utility of the improved species identification available. The works presented here have studied the case of species identification in endocarditis diagnosis in particular, but further studies might show the utility in other cases.

The results from Papers IV-VI have implications beyond the need for accurate species determination in clinical bacteriology. Though some studies have recommended echocardiography in all cases of blood stream infection with *Streptococcus* and similar species[338], the data from these studies suggest that echocardiography could be omitted in certain cases based on bacterial species but also clinical findings. The clinical findings in question, such as the finding of a heart murmur or the presence or lack of a focal infection, are parameters that have been known to clinicians and described scientifically before but have not been aggregated and formalised until recently. The implementation and optimisation of such management scores and algorithms warrant further study.

In addition, the further characterisation of new bacteria such as *Aerococcus urinae,* both molecularly and clinically, has provided clinically important data and venues for further studies. The LASP genes encoding the Asp proteins are arranged in a way that has a striking similarity to the M proteins of *Streptococcus pyogenes*. It is possible that the Asp proteins have a similar function in aerococci, or that they

function as adhesion factors to urothelium or other surfaces. Further studied are needed to determine this.

In a perhaps more visionary perspective, this thesis shows the interconnectedness of microbiology in the laboratory and the practice of treating infectious diseases clinically. Many of the projects in this thesis have utilized data that is already possible to obtain from clinical microbiology laboratories. This, in my opinion, stresses the need for utilization of the information that is available. There is a trove of information available that might improve patient outcomes if only we knew how to utilise it. If identification—not only on the species level, but also on the subspecies level—becomes generally available, the prospect of further tailoring diagnostic procedures and treatments becomes possible. Examples of this could be choosing treatment length, dosing regimens and antibiotic combinations based on bacterial species, antibiotic synergy patterns, as well as the existence of virulence factors or the ability of the bacterial isolate to produce biofilm. The continuing research into these areas have great potential and is necessary in the face of threats such as antibiotic resistance.

Populärvetenskaplig sammanfattning

Bakterier är en sorts mikroorganismer där en del orsakar sjukdom hos människor och djur, och en del lever på och kring oss utan att orsaka skada. En del bakterier lever i munnen, andra på huden, i tarmen eller kring urinvägarna. I denna avhandling berörs bakterierna i släktena *Aerococcus* och *Streptococcus*, samt de besläktade bakterierna *Abiotrophia*, *Gemella* and *Granilucatella*. Alla dessa bakterier finns normalt sätt på och i oss men kan i vissa situationer orsaka sjukdom.

Bakterier är encelliga varelser som är betydligt mindre än kroppens egna celler. Gemensamma drag är att de har sitt DNA i en enda kromosom och oftast har en cellvägg runt sitt cellmembran. Ett sätt att dela upp bakterier är i så kallat Gram-positiva och Gram-negativa bakterier. Uppdelningen baseras på hur bakterierna färgas av ett speciellt färgämne och beror på hur deras cellväggar och cellmembran är ordnade. Gram-negativa bakterier har en tunn cellvägg av peptidoglykan kring sitt cellmembran med ett andra membran utanför, medan Gram-positiva bakterier enbart har cellmembranet samt en tjock cellvägg av peptidoglykan. Bakterierna *Aerococcus*, *Abiotrophia*, *Gemella*, *Granilucatella* och *Streptococcus* är alla Gram-positiva bakterier. Gram-färgning ger dock bara en väldigt grov uppdelning av bakterier och man har även använt sig av saker så som vilken form bakterier har, under vilka betingelser de växer och vilka ämnen de kan bryta ner för att klassificera och identifiera dem. Även dessa sätt har dock begränsningar, och man hade därför svårt att identifiera aerokocker, och tolkade *Abiotrophia* och *Granilucatella* som en sorts streptokocker. Med så kallad MALDI-TOF-teknik där man analyserar proteinmönstret i bakterien har detta blivit mycket lättare och man ser nu att framför allt aerokocker är klart vanligare än man hade trott. Eftersom man tidigare har haft svårt att hitta och identifiera aerokocker i prov från människor har man heller inte studerat dem särskilt mycket. Det gör att man bland annat inte har vetat vilka proteiner som sitter i deras cellvägg. Arbetet med att studera dessa är ett av projekten som beskrivs i avhandlingen. Här identifierades två proteiner hos bakterien *Aerococcus urinae* och vi kunde konstatera att de satt i bakteriens cellvägg och var mycket lika varandra.

Dessa bakterier kan orsaka olika sjukdomar där en av de mest allvarliga är hjärtklaffsinfektion, endokardit. När detta sker bildar växer bakterierna på hjärtats klaffar i klumpar, så kallade vegetationer, samt på hjärtats insida, endokardiet.

Innan antibiotikabehandling fanns var detta en sjukdom som i praktiken alltid ledde till döden. Detta förändrades när penicillin och andra antibiotika kom, men dödligheten är fortfarande hög. Traditionellt har man valt att behandla endokardit orsakad av dessa bakterier med långa antibiotikakurer med en kombination av betalaktamantibiotika (där penicillin ingår) och aminoglykosider (som är en annan sorts antibiotika). Anledningen har varit att man har tänkt att kombinationen av dessa antibiotika har en så kallad synergistisk bakteriedödande effekt, där de olika typerna av antibiotika ihop har en klart kraftigare effekt en de har var och en för sig. För aerokockendokardit har det funnits väldigt lite systematisk beskrivning av förlopp och inga studier kring om kombinationsbehandling har effekt. I en av studierna gjordes därför en systematisk genomgång av data från svenska endokarditregistret där fall av aerokockendokardit jämfördes med endokardit orsakad av andra bakteriearter. Dessutom jämfördes kombinationsbehandling (i provrör) med penicillin och aminoglykosid mot vardera antibiotikum för sig. Man såg att det var en överrepresentation av äldre män bland patienterna med aerokockendokardit och att synergi sågs hos vissa men långt ifrån alla isolat. En annan studie i avhandlingen undersökte på liknande sätt antibiotikasynergi mot streptokockisolat från patienter med endokardit. Även i denna studie såg man antibiotikasynergi hos vissa men inte alla isolat.

Ett praktiskt problem för sjukvården är att bakterier från släkterna *Aerococcus*, *Abiotrophia*, *Gemella, Granulicatella* samt *Streptococcus* inte bara orsakar endokardit utan även andra sorters infektioner. När man hittar dem i blodet är det således inte helt enkelt att veta vilken sorts infektion som ligger bakom. Ett sätt att ta reda på detta är genom att undersöka hjärtat med ultraljud där sensorn placeras i matstrupen i hjärthöjd, så kallad transesofakal ultraljudsundersökning (TEE). Detta är dock en undersökning där kompetensen för att genomföra den är begränsad till vissa sjukhus och med begränsad kapacitet, samt som dessutom är en inträngande och obehaglig undersökning för patienten. Tre av studierna i avhandlingen berör därför endokardit och när risken för detta är så hög att utredning behövs. En population med patienter som hade streptokocker i blodet studerades därför och riskklassificeringssystemet HANDOC togs fram. Genom att bedöma om patienten hade hjärtklaffsjudkom eller blåsljud på hjärtat (H, "heart murmurs or heart valve disease"), vilken streptokockart som låg bakom infektionen (A, "aetiology"), antal fynd i blododling (N, "number of positive cultures"), symptomduration (D, "duration"), om det var en eller flera bakteriearter i odlingarna (O, "only one species") samt om infektionen var samhällsförvärvad (C, "community acquired") kunde man skilja vilka patienter som hade endokardit från de som inte hade det med hundraprocentig sensitivitet och god specificitet. Med sensitivitet menas andelen av de som faktiskt hade endokardit som metoden korrekt identifierade som personer med endokardit, och med specificitet menas andelen av de som inte hade endokardit som metoden korrekt identifierade som personer utan endokardit. Detta innebär att HANDOC skulle kunna användas för att på ett säkert sätt sortera ut patienter som har så låg risk för endokardit att

endokarditutredning inte behöver göras. Detta poängsystem prövades och kvaliteten bekräftades sedan externt i en studie med data från en grupp danska patienter. Även i detta fall såg man en hundraprocentig sensitivitet och en hög specificitet.

I avhandlingens sista arbete testades HANDOC samt riskstratifieringssystemet DENOVA (framtaget för att bedöma risk för endokardi vid bakteriemi med *Enterococcus*) på patienter med *Abiotrophia*, *Aerococcus*; *Gemella* och *Granulicatella* i blodet. Patientdata samlades in från journaler i Skåne och Stockholm och patienter med och utan endokardit testades i poängsystemen. DENOVA hade en hundraprocentig sensitivitet och HANDOC en sensitivitet på nästan hundra. Båda hade en acceptabel specificitet, där specificiteten för HANDOC var högre.

Sammanfattningsvis har jag i denna avhandling studerat ytproteiner hos de allt mer uppmärksammade och endokarditorsakande bakterierna *Aerococcus*, testat antibiotikasynergi mot *Aerococcus* och *Streptococcus*, tagit fram samt validerat riskbedömningssystem för endokardit med *Streptococcus* och slutltligen utvärderat riskbedömningssystem för endokardit med *Abiotrophia*, *Aerococcus*, *Gemella* och *Granulicatella*. Min förhoppning är att resultaten från dessa studier kommer att öka förståelsen för dessa bakterier och att de på sikt kommer att leda till effektivare handläggning av dem i sjukvården, både vid patientmöten och inom klinisk mikrobiologi.

Acknowledgements

There are many people without which this thesis would not have been possible. If you are holding this book, the likelihood is high that you have contributed in some way.

To my supervisors

I would first like to extend my deeply felt thanks to my supervisor **Magnus Rasmussen**. Beginning with my master thesis many years ago, you have continuously supported me. Guiding me through my PhD with encouragement and scientific discussions as well as guiding me through life as a PhD student, you have always been there when I needed. The invaluable lesson on the importance of the coffee break has also stuck with me. You have been a supporting supervisor, a great scientist, and an inspiration.

I would also like to thank my co-supervisor **Rolf Lood**. Since my first day as a PhD student you have been there to guide me and help me when needed. When I was working for months on end on projects that seemed to go nowhere, you taught me the delicate balance between not giving up when facing problems and knowing when to pursue a different path.

To the MOL-group

Thanks to everyone in the MOL-group, both past and present! A special thank you to **Oonagh Shannon** for taking me on as a bachelor student and encouraging my research interests. Thank you **Gisela** for making sure that I got through my time at BMC in one piece and with access to everything from blood agar plates to the proper delivery address despite my sometimes messy desk. Thank you as well to **Lisa, Frida**, **Sinead**, **Lisbeth**, **Hossein**, **Karin**, **Erik**, **Olivia**, **Yashuan**, **Anna**, **Karl**, and **Eleni** for all the help and stimulating discussions.

Thank you, **Anita Berglund**, for being the one that holds everything together at BMC. Brightening my day even when I had misplaced grant money will be an enduring memory for me. A big thank you to **Lars Björck** for setting up the fantastic environment of BMC B14 as well as for introducing me to the amazing world of research in infectious diseases.

The work-life balance in research can be difficult, and would be even more so if not for all the great colleagues who have provided support and stimulating fika discussions. A sincere thank you to everyone who has contributed to that!

To my co-authors without whom I would not be where I am: thank you!

To my family

Lastly, I would like to thank my family and loved ones. Mere words are maybe not enough to convey all that you have done for me in both ups and downs. This thesis would certainly not exist without your support and not without you believing in me.

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