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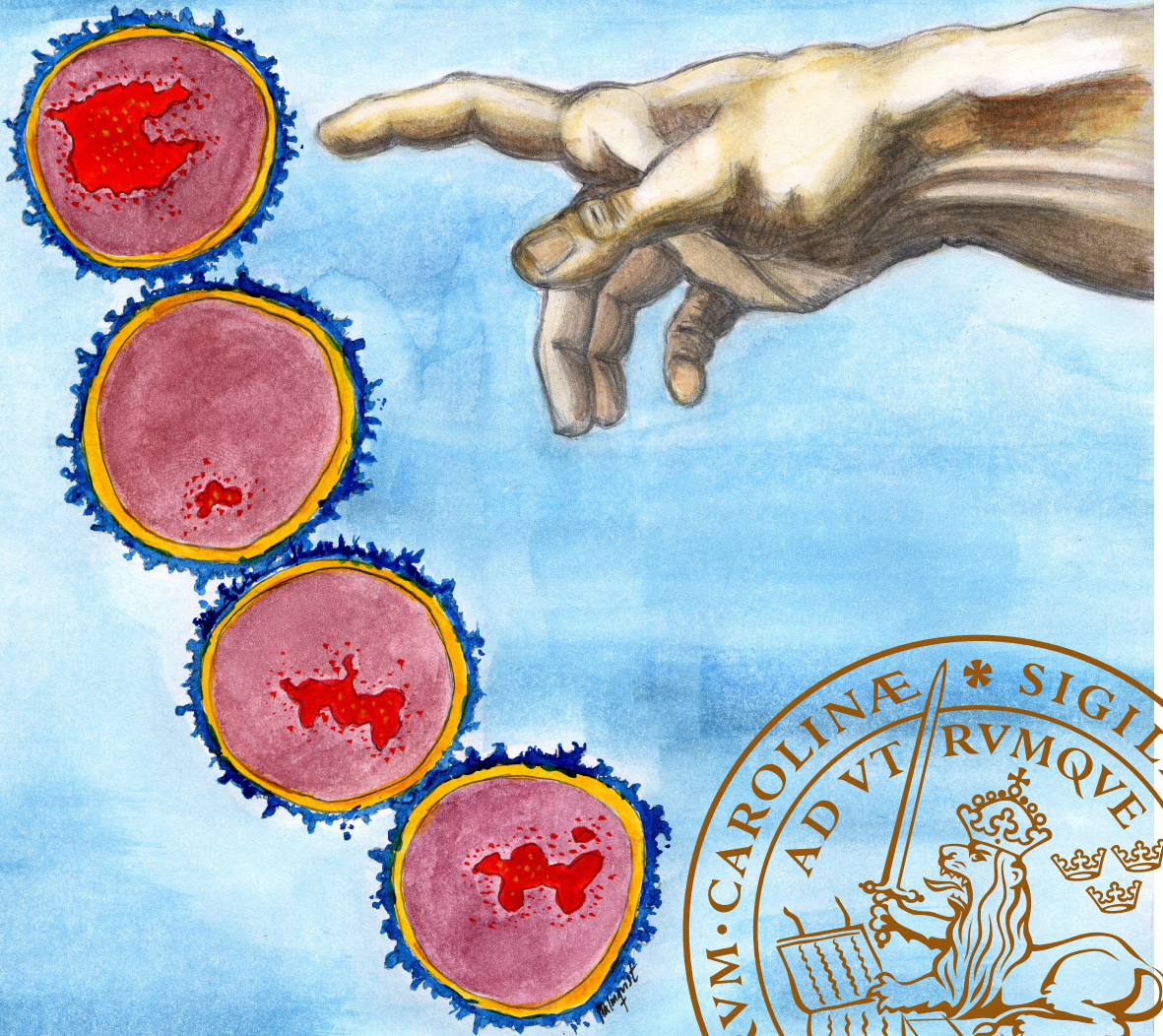
PO Box 117
221 00 Lund
+46 46-222 00 00

Interactions between β -haemolytic streptococci and the human host

Heart, skin and beyond

ANNA BLÄCKBERG

DEPARTMENT OF CLINICAL SCIENCES, LUND | FACULTY OF MEDICINE | LUND UNIVERSITY



Interactions between β -haemolytic streptococci and the human host

Interactions between β -haemolytic streptococci and the human host

Heart, skin and beyond

Anna Bläckberg



LUND
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DOCTORAL DISSERTATION

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Faculty opponent

Prof. Steinar Skrede

Haukeland University Hospital, University of Bergen, Norway

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Interactions between β -haemolytic streptococci and the human host

Heart, skin and beyond

Anna Bläckberg



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Main supervisor: Magnus Rasmussen

Co-supervisor: Rolf Lood

Anna Bläckberg
Department of Clinical Sciences, Lund
Division of Infection Medicine
Faculty of Medicine
Lund University
Biomedical Centre, B14
221 84 Lund, Sweden
email: anna.blackberg@med.lu.se

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To my family and friends for their love and support

Now is the time to understand more, so that we may fear less

Marie Curie

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Abbreviations

ADN-B	anti-deoxyribonuclease-B
ASO	anti-streptolysin O
AST	antibiotic susceptibility testing
BHS	β-haemolytic streptococci
C4BP	C4-binding protein
CDC	Centers for Disease Control and Prevention
CFU	colony-forming unit
CLSI	Clinical and Laboratory Standards Institute
DNA	deoxyribonucleic acid
ELISA	enzyme-linked immunosorbent assay
ESC	European Society of Cardiology
EUCAST	European Committee on Antimicrobial Susceptibility Testing
FHL1	Factor H-like protein 1
GAS	group A streptococci
GBS	group B streptococci
GCS	group C streptococci
GGs	group G streptococci
GRAB	protein G-like α ₂ -macroglobulin-binding
HBP	heparin-binding protein
IE	infective endocarditis
IVlg	intravenous immunoglobulin
LTA	lipoteichoic acid
MAC	membrane-attack-complex
MALDI-TOF MS	matrix-assisted laser desorption/ionization-time-of-flight mass spectrometry
MBL	mannose-binding lectin
MASP	MBL-associated serine protease
MIC	minimum inhibitory concentration
MLST	multilocus sequence typing
NBHS	non-β-haemolytic streptococci
NSTI	necrotizing soft tissue infection
PBPs	penicillin-binding proteins
PCR	polymerase chain reaction
rRNA	ribosomal ribonucleic acid
<i>S. aureus</i>	<i>Staphylococcus aureus</i>
<i>S. agalactiae</i>	<i>Streptococcus agalactiae</i>
<i>S. dysgalactiae</i>	<i>Streptococcus dysgalactiae</i>
<i>S. pyogenes</i>	<i>Streptococcus pyogenes</i>
SIRS	systemic inflammatory response syndrome
SLO	streptolysin O
SLS	streptolysin S
Spes	streptococcal pyrogenic exotoxins
SRIE	Swedish Registry of Infective Endocarditis
STSS	streptococcal toxic shock syndrome
TTP	time to positivity
WGS	whole genome sequencing
WHO	World Health Organization

Abstract

Group C and G streptococci (GCS/GGS) and group A streptococci (GAS) belong to β -haemolytic streptococci (BHS). GCS/GGS can further be species determined to *S. dysgalactiae*, whereas *S. pyogenes* are GAS. *S. dysgalactiae* and *S. pyogenes* cause similar diseases, (e.g., skin and soft tissue infections, erysipelas, bacteraemia, and infective endocarditis (IE)). Erysipelas often presents as a sharply demarcated oedematous erythema and often reoccurs in the same host. The diagnosis relies on the clinical presentation since cultures from blood and/or wounds often are negative. IE due to BHS is rare but is a challenging infection to treat. Combination therapy using a β -lactam and an aminoglycoside has been employed to treat the condition, but the evidence for synergy between the two antibiotics is weak.

This thesis began investigating erysipelas and predominantly found GCS/GGS, but also GAS as important pathogens to erysipelas, (Paper I). To further investigate the disease panorama of GCS/GGS, the second study comprised cases of IE due to *S. dysgalactiae* from a nationwide registry. IE with *S. dysgalactiae* was found to have an acute onset of symptoms with substantial mortality and embolic event rate, (Paper II). Synergy between penicillin G and gentamicin was observed in some blood isolates of *S. dysgalactiae*. However, in most cases, penicillin G alone showed bactericidal action so strong, that any further killing action of gentamicin was difficult to detect.

Recurrent infections with *S. dysgalactiae*, involving erysipelas but also bacteraemia, are common. The cell surface attached M protein is an important virulence determinant for the bacteria. There are different M proteins which render an antigenic diversity and facilitate the bacteria's evasion of the host defence system. In a prospectively based study, type-specific antibodies were developed in convalescent serum from patients with prior bacteraemia with *S. dysgalactiae*, (Paper III). However, further analysis with bactericidal and phagocytosis assays could not establish that these evolved antibodies opsonised the bacteria or enhanced the killing of the bacteria.

The quest for prognostic factors in bacteraemia with BHS is challenging. Time to positivity (TTP) from blood cultures may reflect bacterial concentration in blood and was identified as an independent prognostic factor for 30-day mortality in invasive infections due to both *S. pyogenes* and *S. dysgalactiae* respectively, (Paper IV and V).

All things considered, this thesis highlights the clinical and microbiological aspects of infections caused by BHS and their interactions with the human host. Recurrent infections due to the bacteria are common, and a lack of development of opsonising antibodies may partially explain the presence of recurrent bacteraemia with *S. dysgalactiae*.

Preface

I began my research career trying to identify the aetiology of erysipelas. I, therefore, became acquainted with β -haemolytic streptococci, or more precisely *Streptococcus dysgalactiae* and *Streptococcus pyogenes*. The two pathogens share many virulence determinants and cause similar diseases. During my clinical practice, it became evident that despite infections with *S. dysgalactiae* and *S. pyogenes* being relatively common, knowledge of diagnostic procedures, prognostic factors, and treatment options is limited. My curiosity led me to further investigations of the clinical and microbiological aspects of the two bacteria. My thesis comprises studies of translational research where I observed a dilemma at the clinic and brought the bacteria back to the laboratory. Findings from the laboratory were correlated with clinical characteristics of diseases caused by *S. dysgalactiae* and *S. pyogenes*.

This thesis aimed to investigate clinical features, patterns of resistance, antibody responses, and prognostic factors in infections caused by *S. dysgalactiae* and *S. pyogenes*. The thesis is structured into seven different parts. The first section encompasses important historical aspects in the fields of bacteriology and immunology. The next section comprises aspects of microbiology, species determination and methods to find the aetiology of different diseases. The third section describes antibiotic susceptibility testing and antibiotic synergy. The fourth section covers a presentation of diseases and important molecules of *S. dysgalactiae* and *S. pyogenes*. The fifth section describes important aspects of immunology, as well as a description of different parts of the interactions between the human host and *S. dysgalactiae* and *S. pyogenes*. A summary of the present investigations is discussed in the sixth section. The final sections summarize the major findings of this thesis put into a broader perspective and elaborate on future perspectives related to the present research.

Introduction

The following section provides a historical background of some of the aspects of bacteriology and immunology that are important for this thesis. Methods from the laboratory, e.g., Lancefield grouping, are still relevant today and essential for the grouping of β -haemolytic streptococci (BHS). Group A streptococci (GAS) and group C and G streptococci (GCS/GGS) belong to BHS. In recent years the molecular technique has improved making further species determination within these groups possible. *Streptococcus pyogenes* carries group A antigen, whereas *Streptococcus dysgalactiae* usually expresses group C or G antigen. This thesis investigates the clinical and microbiological aspects of GAS, *S. pyogenes* and GCS/GGS, *S. dysgalactiae*. Since species and group determination was not always performed or has been available, the bacteria are either referred to as their Lancefield group or species throughout this thesis.

Brief history

“Many were attacked by the erysipelas all over the body when the exciting cause was a trivial accident...flesh, sinews, and bones fell away in large quantities...there were many deaths”

Descamps V, Lancet, 1994¹

Observations of the progression of different infections have been documented throughout the years. Erysipelas, Greek for “red skin” was described during the early age of *Hippocrates* in the 5th century BC. “Erysipelas” is quite an adequate description since it generally presents an acute onset of erythema with enlargement and sometimes blisters. However, microorganisms causing this disease were first identified during the 19th century.

Developed in 1881, *Robert Koch's* postulates assessed different criteria to determine whether an organism is causing a specific disease. Moreover, in those criteria, *Koch* described that the microorganism should be cultured from the putatively infected person. Injection of the cultured organism into a healthy animal or human should then generate the same disease. Thereafter, the same pathogen should be possible to identify

again². Additionally, *Koch* developed a solid culture medium method for culturing bacteria resulting in the isolation of pure cultures of bacteria and sub-culturing them on different broth media. Furthermore, the molecular *Koch's* postulates comprise genetic analysis of the bacterial pathogenesis³.

Theodor Billroth observed rounded microorganisms, organized in chains when investigating cultures obtained from wound and skin infections in the early 1870s⁴. To describe his findings, he applied the term *Streptococcus*. *Streptococcus* comes from the Greek *streptos* denoting “chain” and *kokkos* meaning “grain, berry”. In 1919, *James Brown* presented a method for grouping streptococci based on their haemolysis on blood agar⁵. α -haemolysis, sometimes called green haemolysis, refers to partial haemolysis and is caused by a hydrogen peroxidase. β -haemolysis is defined as the complete lysis of red blood cells and degradation of haemoglobin in the media adjacent to the colonies. The mechanism is suggested to be a result of the activity by streptolysin S. γ -haemolysis refers to non-haemolytic streptococci and the media around these colonies remain unchanged, (Fig. 1).



Figure 1. Different types of haemolysis are displayed on a blood agar plate. From left to right: α -, β and γ -haemolysis. Photo 1 by Anna Bläckberg.

“Hemolytic streptococci can be differentiated serologically by means of the precipitin reaction in distinct and sharply defined groups which are not disclosed by the agglutination reaction. The test is relatively simple and gives results which are strikingly uniform and consistent.”

Lancefield R, J Exp Med, 1933⁶

Rebecca Lancefield was a great pioneer of her time and is sometimes referred to as the “PI in the Scotland Yard of Streptococcal Mysteries”⁷. In 1933, *Lancefield* established a further classification of the streptococci based on the presence of the carbohydrate

antigens on the bacterial surface⁶. Lancefield grouping comprises 20 groups, where BHS often carry either group A, B, C or G antigen. Moreover, in 1937, *James Sherman* classified the streptococci into either the pyogenic division, viridans division, lactic division, or enterococci⁸. BHS belong to the pyogenic division.

We are constantly exposed to different microorganisms and some of them can cause severe infections. Long-lasting immunity, against different microorganisms responsible for epidemics, was thought to be encountered in persons surviving the epidemics. In the late 18th century, *Edward Jenner* described that prior inoculation with cowpox virus seemed to confer protection against smallpox, a mechanism called “vaccination”⁹. The term vaccine is derived from the Latin word *vacca* for cow. Several years after, in the 1890s, *Emil von Behring* and *Shibasaburo Kitaso* described an “antitoxic activity” when they analysed serum from animals immune to diphtheria or tetanus resulting in protection against the toxins of diphtheria or tetanus¹⁰. *Paul Ehrlich* further developed this theory and introduced the development of antiserum as a treatment for diphtheria and tetanus. This mechanism of “antitoxic activity” is a way of protective immunity in which antibodies bind to and neutralizes toxins. The term antibody was first mentioned by *Paul Ehrlich*^{11, 12}, who also described the “lock and key mechanism” of antibody-antigen interaction, which was later confirmed by *Linus Pauling* in 1940¹³. In 1923, *Michael Heidelberger* and *Oswald Avery* demonstrated that these antibodies are proteins¹⁴. In 1948, the Swedish immunologist *Astrid Fagraeus* displayed the function of plasma cells and their generation of antibodies¹⁵.

“The specific serologic types of group A streptococci are based, not on carbohydrates, but on protein components, designated M antigens, which determine the production of protective antibodies specific for each type”

Lancefield R, J Immunol, 1962¹⁶

Concurrently, *Rebecca Lancefield* observed the persistence of type-specific antibodies against GAS in human sera¹⁷. Since then, knowledge of the molecular structure of the antibodies has increased¹⁸⁻²⁰. Focus on the development of protective antibodies and effective vaccines to prevent *S. pyogenes* infections have been in the spotlight for several decades, but still, there is no licensed vaccine. Clinical trials to develop a GAS vaccine dates back to 1923²¹. Approaches to vaccine using heat-killed streptococci or cell walls of GAS were initially undertaken²². The M protein is an important virulence determinant for both *S. pyogenes* and *S. dysgalactiae*, and recent studies have tried to develop GAS vaccines containing type-specific M protein epitopes^{23, 24}. In summary, the evidence of protective immunity following streptococcal bacteraemia is still inconclusive, and different ways to prevent infections due to BHS are investigated.

Disease and aetiology

Cultures from blood, throat, wounds, and so forth may be obtained to find the bacteria responsible for causing a disease in a patient. The procedure to find the causative pathogen is not always successful. There are several different diagnostic procedures to process cultures and determine species as described below.

Blood culturing

Bloodstream infections represent a major problem causing high mortality and morbidity throughout the world. Blood culture remains the gold standard to detect bacteraemia and makes it possible to discriminate bacterial species as well as antibiotic susceptibility of the isolated organism. The standard procedure of blood culturing includes four bottles (two sets), of which each set consists of paired aerobic and anaerobic culture bottles^{25, 26}. Adequate volume sampling is important since the density of bacteria and fungi can be very low in patients with bacteraemia or fungemia²⁷⁻²⁹. Each culture bottle should contain at least 10 ml of blood, corresponding to a total of 40 ml of blood for bacterial culturing. However, filling the bottle > 10 ml may also result in falsely flagged positively by the blood culture system³⁰. The blood culture bottles are incubated at 35–37°C for a maximum duration of 5–7 days^{31, 32}. Different laboratories utilize different automated blood culture system³³. The automated blood culture system BACTEC FX from Becton Dickinson has been used continuously since 2014 in Region Skåne, Sweden. The system uses fluorescent CO₂ sensors that detect growth by the metabolic activity of bacteria growing in blood cultures.

Time to positivity from blood cultures

“Making use of the hidden information”

Lamy B, CMI, 2018³⁴

Time to positivity from blood cultures (TTP) is defined as the time interval between the start of incubation of the blood culture bottle and the detection of growth, using an automated monitoring system (as previously mentioned, in Region Skåne the BACTEC FX blood culture system is utilized). A short TTP may correlate with a higher bacterial concentration which in turn may be associated with severe infection and elicit a stronger immune response by the host. Short TTP has been implied to be an important prognostic factor for outcomes, such as 30-day mortality as well as distinguishing infective endocarditis in bacteraemia with several major pathogens³⁵⁻³⁷. TTP is also an important tool and implemented in clinical practice when diagnosing catheter-related bloodstream infections³⁸. However, many circumstances may affect TTP, such as inadequate blood volume, logistic transportation of blood culture bottles, polymicrobial growth, and so forth.

The Gram-stain

“The experiments resulted from the accidental observation that aniline-gentian violet preparations of tissues after treatment with iodine-potassium iodide, are completely and rapidly decolorized in alcohol”

Gram C, Fortschr Med, 1884³⁹

In 1884, *Hans Christian Gram* described a method to differentiate microorganisms based on their cell wall composition and reactions to different series of staining and decolourization steps³⁹. The Gram stain is still utilized today, with some steps of modification during the years. The procedure includes a primary stain of crystal violet, followed by the addition of iodine, decolourization with ethanol or acetone and in the end counterstaining with e.g., safranin⁴⁰. Microorganisms with cell walls containing thick layers of peptidoglycan retain the primary dye, stain purple, and are called Gram-positive bacteria. Gram-negative bacteria have thinner layers of peptidoglycan and retain only the counter stain resulting in a pink staining⁴¹. The microscopic view of BHS reveals a pattern of growth and shape as chains. The bacteria stain purple due to their thick cell wall and fall into the category of Gram-positive bacteria, (Fig. 2).

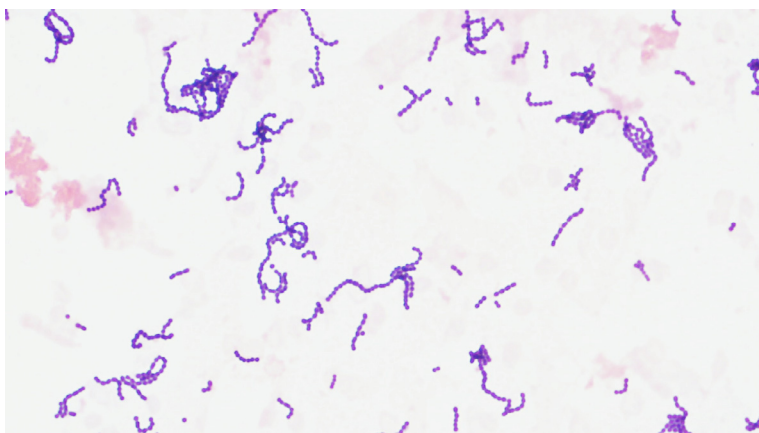


Figure 2. The microscopic view of *S. pyogenes*. BHS grow in chains and are Gram-positive bacteria. Photo 2 courtesy of Torgny Sunnerhagen.

Lancefield grouping

The serologic method for classifying BHS, based on the presence of their cell wall polysaccharide refers to the Lancefield grouping⁶. As previously mentioned, *S. pyogenes* expresses group A antigen, whereas *S. dysgalactiae* most often carries group C or G antigen. This method of grouping has during the years been improved and new commercial latex agglutination test kits are used which allows easy and rapid detection of BHS⁴².

Rapid strep test for early detection of β -haemolytic streptococci

Conventional cultures from blood and wounds require 24–48 hours for isolation and subsequent detection of viable organisms. Rapid antigen tests have been implemented to enable early detection of GAS from swabs^{43,44}. In Region Skåne, Sweden, the OSOM Strep A test is used which can detect viable or nonviable organisms directly from throat swabs within 5 minutes. The test relies on a colour immunochromatographic assay. There are several rapid diagnostic tests for detection of GAS from throat swabs on the market and implemented in clinical practice⁴⁵. The quick test enables decision-making and may assign treatment plans for patients with strep throat.

MALDI-TOF MS

In 1975, *Catherine Fenselau* and *John Anhalt* suggested a method based on mass spectrometry to improve species identification of bacteria⁴⁶. A decade later, the technique matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS) was introduced and developed for rapid microbiological species identification of bacterial isolates⁴⁷⁻⁴⁹. A given species has a unique set of protein content and the procedure involves colonies from agar plates that are smeared onto a metal plate to which a special solution of a matrix is later added, and the plate is left to dry. The plate is later irradiated by using a UV laser beam resulting in ionization and sublimation of the sample. The generated ions are separated based on their mass-to-charge ratio (m/z) during time-of-flight in the mass spectrometer. Ions with the smallest mass and highest charge will travel faster to the detector followed by ions with larger masses and lower charges. This reflects the protein profile in the sample. Altogether, this results in a generation of a “mass spectrum” which is compared and matched against certain protein profiles in a database to identify a certain species level⁵⁰. It took 30 years after the MALDI-TOF MS was developed until it was utilized in routine clinical practice and commercially introduced^{51, 52}. MALDI-TOF MS has a relatively low cost, it is fast, and has enabled species determination of bacteria that were previously not possible to identify⁵³⁻⁵⁵. MALDI-TOF MS has improved species determination within BHS, separating *S. pyogenes* from *S. dysgalactiae*⁵⁶, but can formally not separate subspecies within *S. dysgalactiae*⁵⁷. Moreover, MALDI-TOF MS may have difficulties in separating *S. dysgalactiae* from *S. canis*. However, by a refinement of certain reference spectra of MALDI-TOF MS, the identification of *S. dysgalactiae* and *S. canis* can be improved⁵⁸.

Sequencing

Sequencing is used to determine the structure in a certain nucleotide sequence of a DNA or RNA molecule. *Frederick Sanger* developed the chain terminator sequencing “Sanger sequencing” in 1977, which was the first reliable method for DNA sequencing⁵⁹. The method involves a double-stranded DNA that is denatured into two single-stranded DNA (ssDNA), and a primer that binds to the DNA corresponding to the beginning of the sequencing. It also involves polymerase solutions (four deoxynucleotide triphosphate (dNTP), one type of di-deoxynucleotide triphosphate (ddNTP), and a DNA polymerase⁵⁹).

Sequencing 16S ribosomal ribonucleic acid (rRNA) is important to determine species within BHS. 16S rRNA is present in all bacteria and encodes the 30S which is a small subunit in the prokaryotic ribosome. The method of this sequencing is therefore very valuable to distinguish certain species of bacteria even after antibiotic treatment has been administered when the bacterial isolate can no longer be cultured⁶⁰. 16S rRNA can be useful in distinguishing different species within BHS, but not to the level of subspecies. In those cases, multilocus sequence typing (MLST) may be applied. MLST is a DNA sequence-based method and involves sequencing of multiple loci, often house-keeping genes within the bacterial genome⁶¹. A sequence type or lineage is assigned by comparing the results to other already known isolate profiles in a database. This enables species identification in species and subspecies that are closely related. MLST is used for further characterization of *S. pyogenes*⁶² and is also available for delineation of *S. dysgalactiae*⁶³. In addition, whole genome sequencing (WGS), can discriminate closely related species and subspecies. The method analyses the entire DNA sequence of an organism and provides a genomic fingerprint which can enable a better understanding of clonal groups in bacterial infections^{64, 65}.

emm typing

“As a general rule, a single M antigen is specific for the type concerned”

Lancefield R, J Immunol, 1962¹⁶

Rebecca Lancefield developed a serotyping system based on the antigenic variation of the M protein to distinguish different *emm* types of GAS¹⁶. *Frederick Griffith* developed another system, called the T protein agglutination system with the purpose of typing⁶⁶. The M protein system is more utilized in the scientific world. Both *S. pyogenes* and *S. dysgalactiae* express the M protein, and this is an important virulence factor for the bacteria. *emm* typing involves sequencing of the *emm* gene that encodes the M protein. The method involves sequencing of the 5'- end of the *emm* gene which encodes the hypervariable N-terminus of the M protein⁶⁷. There are currently more than 90 different *emm* types among *S. dysgalactiae*⁶⁸ and > 250 different *emm* types among *S. pyogenes*^{68, 69}. Centers for Disease Control and Prevention (CDC) has set up a database for *emm* typing⁷⁰.

ELISA

Sometimes there is a need to detect levels of antibodies following an infection. Enzyme immunoassay (EIA) and enzyme-linked immunosorbent assay (ELISA) incorporate an immunoassay technique which is routinely utilized in the laboratory and integrated into diagnostic methods globally⁷¹. *Anton Schurs* and *Bauke van Weemen*, from the Netherlands, developed the EIA technique in the 1970s⁷², and almost concurrently the ELISA method was developed and conceptualized in Sweden by *Peter Perlman* and *Eva Engvall*⁷³. The ELISA methodology is used to detect any presence of antibodies, antigens and/or different proteins in biological samples. The basic principle for ELISA is the immobilization of an antigen to a solid surface. A multi-well 96-plate is often used for that purpose. The antigen is later complexed with an antibody, which in turn is conjugated with a molecule that enables detection by another enzyme or a fluorophore. There are four different assays of ELISA. The indirect ELISA carries a two-step process for detection, in which a primary antibody binds to the antigen, followed by a secondary antibody that binds to the primary antibody for detection. In experimental settings, the primary antibody can be substituted with serum. Altogether, the ELISA methodology is easy to perform and has high sensitivity and specificity to detect antigens in biological samples⁷⁴⁻⁷⁶. Serologic testing is sometimes used to diagnose different infections due to BHS. The anti-streptolysin O (ASO) test, and the anti-deoxyribonuclease-B (ADN-B) test are specific for both *S. dysgalactiae* and *S. pyogenes*. Increased titres of both ASO and ADN-B have been observed in patients with erysipelas⁷⁷, but in most of the cases, no serologic evidence for a BHS aetiology of the infection can be found⁷⁸.

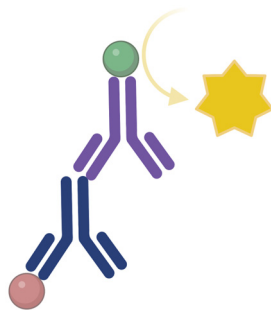


Figure 3. Schematic view of indirect ELISA.

The antigen (pink) is immobilized into a microtitre plate. A primary antibody (blue) is subsequently added and binds to the antigen. This is followed by a secondary antibody (purple) that binds to the primary antibody. The secondary antibody is typically conjugated to an enzyme, such as horseradish peroxidase (HRP) (green). A substrate is added which reacts with the enzyme conjugate, a colour is developed, and a measurable by-product is produced. The colour is proportional to the amount of bound antigen. Figure 3 was created with Biorender.

Antibiotics

β -lactam antibiotics

β -lactam antibiotics were early discovered and remain the most utilized antibiotic in clinical practice throughout the world. β -lactam antibiotics have a β -lactam ring and exert their bactericidal activity by inhibiting the cell wall of the bacteria^{79, 80}. The targets for the β -lactam antibiotics are the penicillin-binding proteins (PBPs). PBPs are enzymes that catalyse several reactions involved in the crosslinking of the peptidoglycan by the bacteria⁸¹. When β -lactam antibiotics bind to the PBPs, the last step in the peptidoglycan synthesis is interrupted resulting in cell lysis and eventually cell death^{79, 82}.

Penicillin

“Streptococcus pyogenes is also very sensitive. There were small differences in the titre with different strains, but it may be said generally that it is slightly more sensitive than Staphylococcus”

Fleming A, Br J Exp Pathol, 1929⁸³

Penicillin G, also known as benzylpenicillin, was the first discovered and marketed group of β -lactam antibiotics⁸⁴. In 1928, *Alexander Fleming* discovered by chance that the growth of *Staphylococcus aureus* on an agar plate could be inhibited by a mould, belonging to the *Penicillium* genus. He determined that this mould had an antibacterial effect on Gram-positive bacteria and named the agent *penicillin*⁸³. Penicillin G is employed for the treatment of several streptococcal infections, e.g., tonsillitis, pneumonia, infective endocarditis, meningitis, and skin and soft tissue infections. Since its bactericidal activity correlates with time over the minimum inhibitory concentration (MIC), it is important to administer the antibiotic equally during the day. In contrast to other pathogens, (e.g. *S. aureus*, *Escherichia coli*) that have evolved resistance mechanisms towards penicillin, such as producing β -lactamases that hydrolyse the β -lactam ring and deactivate penicillin^{85, 86}, *S. dysgalactiae* and *S. pyogenes* have remained uniformly sensitive to penicillin^{87, 88}. Quite recently though, four isolates of *S.*

dysgalactiae causing human infections exhibited decreased susceptibility to penicillin, with the detection of identical mutations in the PBPs⁸⁹. Reduced penicillin susceptibility, with certain amino acid substitutions in the PBPs, has been noted in isolates of *S. pyogenes*^{90, 91}, but these mutations have not shown any trending increase.

Aminoglycosides

Aminoglycosides are members of the protein synthesis inhibitors. The first member of the class to be discovered was streptomycin in 1943. Since then, several members have been discovered, and gentamicin is commonly used in clinical practice in Region Skåne, Sweden. Gentamicin binds to the 30S ribosomal subunit. This binding to the 16S rRNA leads to a misreading between transfer RNA and messenger RNA of the ribosome. This results in a defect protein synthesis⁹²⁻⁹⁵. Accumulation of misreading during protein synthesis cause changes in the cytoplasmic membrane which permits further uptake of aminoglycosides and eventually cell lysis^{96, 97}. Aminoglycosides are concentration-dependent killers, thereby requiring high concentration to eradicate the microorganism. The bactericidal activity of aminoglycosides is dependent on both the peak concentration and the time the concentration is over the MIC, of the bacterium. This results in the area under the curve of the antibiotic concentration over time being a good measure of the expected effect. Aminoglycosides have several side effects and can be highly ototoxic and nephrotoxic, for which the risk increases with several doses⁹⁸⁻¹⁰⁰. The addition of an aminoglycoside to a β -lactam antibiotic is often used for treating infective endocarditis due to Gram-positive bacteria, based on presumptive synergy^{101, 102}.

Clindamycin

Clindamycin belongs to the group of lincosamides, which is a class of antibiotics that once inside the bacterial cell, binds to the 50S ribosomal subunit of the ribosome of the bacteria. The binding results in blocking of the peptide bond formation on the ribosome and thereby interfering with the synthesis of proteins^{103, 104}. Clindamycin is often added together with penicillin in the treatment of necrotizing soft tissue infections, partially as it has been shown to inhibit the production of exotoxins *in vitro*^{105, 106}. Increased resistance to clindamycin has been observed for *S. pyogenes*¹⁰⁷, but in particular for *S. dysgalactiae*¹⁰⁸. This is important information when a streptococcal infection is solitarily treated with clindamycin.

Antibiotic susceptibility testing

Antibiotic susceptibility testing (AST) of bacteria is performed *ex vivo* in clinical practice. Bacteria can be inherently resistant to antibiotics or evolve resistance mechanisms to certain antibiotics during and after treatment. Therefore, it is of great importance to know the patterns of resistance for the bacteria when treating the patients. Susceptibility testing can be performed in liquid media (broth) and/or on solid media (agar). By these two methods, one can determine the inhibition of growth and the bactericidal effect of the antibiotic on the bacteria. The European Committee on Antimicrobial Susceptibility Testing (EUCAST) harmonizes clinical breakpoints for existing antimicrobial agents and incorporates guidelines and protocols for performing AST¹⁰⁹. Of note, in addition to EUCAST, the Clinical and Laboratory Standards Institute (CLSI) is the American representative organization for providing AST¹¹⁰. The two reference methods are similar with some differences.

Broth dilution

Broth dilution test has been utilized throughout the years to provide AST for different agents and comprises two-fold dilutions of antibiotics (e.g., 16, 8, 4, 2 µg/mL) in a liquid growth medium containing tubes. These tubes are later inoculated with a bacteria solution of a standardized concentration and incubated overnight under appropriate conditions¹¹¹. The method of broth dilution is recommended by EUCAST and corresponds with the recommendations from the International Standards Organisations¹⁰⁹. Bacterial growth is distinguished as evidenced by turbidity in the medium. The principal advantage of this method is the ability to generate concrete results, e.g., MIC which is the lowest antibiotic concentration that inhibits bacterial growth. Fig. 4 visualizes the method of broth dilution.

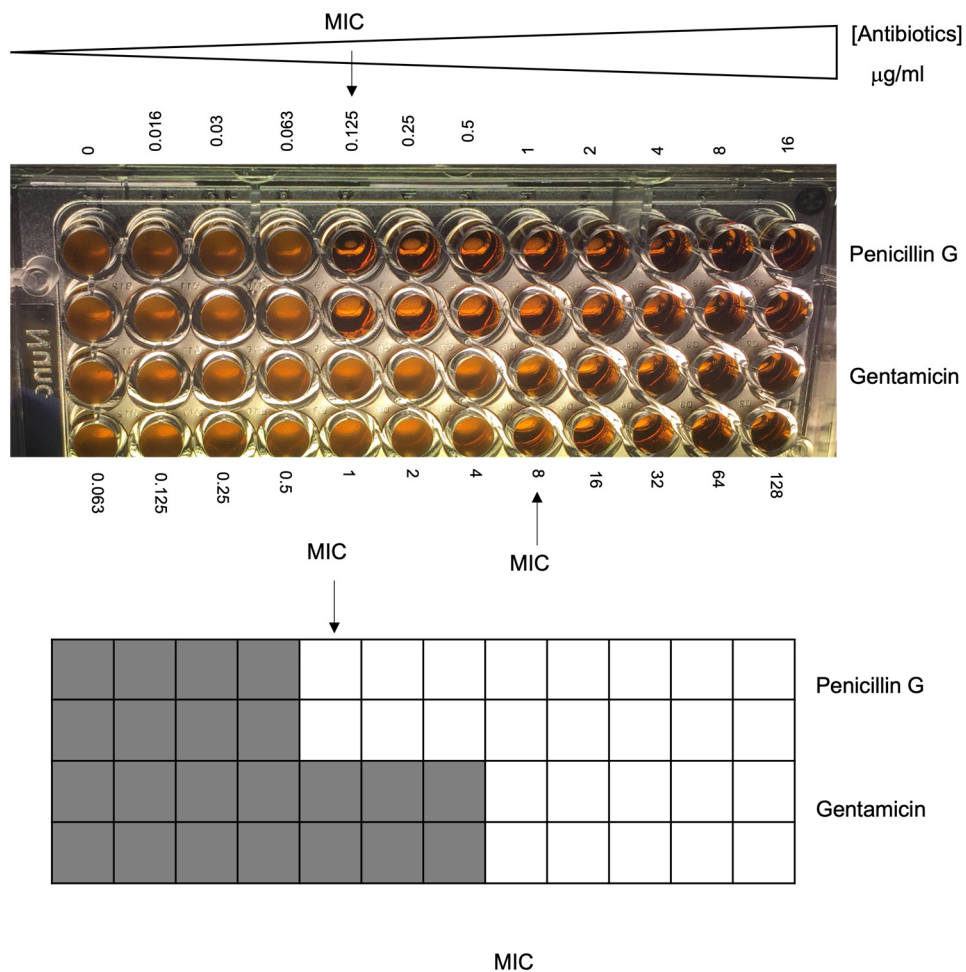


Figure 4. Broth dilution method.

The broth dilution method comprises a liquid growth medium with different antibiotic concentrations. The first evident clarity of the wells indicates the MIC for the two different antibiotics respectively. Photo 4 by Anna Bläckberg.

The antimicrobial gradient method

The antimicrobial gradient diffusion method utilizes a strip, with a concentration gradient of the antimicrobial compound in question to determine the antimicrobial susceptibility of the bacterial isolate. This antibiotic gradient may be, e.g., the Etest (bioMérieux), and the MIC test strip (Liofilchem). An agar plate is streaked with a standardized inoculum of bacteria, the gradient is applied, and the plate is later incubated overnight at appropriate conditions. The MIC can be imprinted on the MIC reading scale in $\mu\text{g/mL}$ and is determined by the intersection of the lower ellipse, which is the area of inhibition, (Fig. 5).

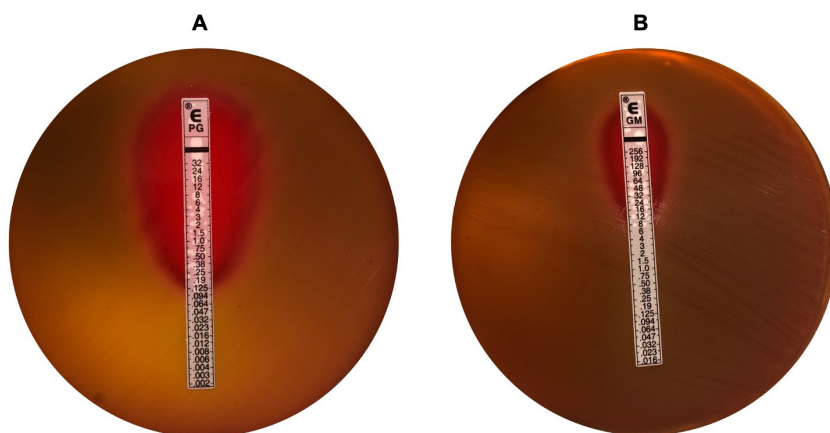


Figure 5. The Etest methodology of *S. dysgalactiae* streaked on agar plates.

The agar plates are inoculated with the same strain of *S. dysgalactiae*. The MIC is measured as the inhibition zone that intersects the graded antibiotic strip respectively. The Etest strip carrying the penicillin gradient results in a large ellipse (A), whereas the Etest strip with gentamicin, presents a smaller ellipse (B). Photo 5 by Anna Bläckberg.

The disk diffusion method

In contrast to the antimicrobial gradient method which gives an exact MIC value, the disc diffusion method gives a qualitative measurement of antibiotic susceptibility, stated as “susceptible”, “increased exposure” or “resistant”, in some species for some antibiotics, according to the EUCAST breakpoints^{109, 112}. The disc diffusion method comprises a standard bacterial inoculum on an agar plate with an antibiotic disk in the centre. The plate is incubated overnight at appropriate conditions and later a zone may be visualized around the antibiotic disk, indicating inhibition of bacterial growth.

Antibiotic synergy

“When two drugs act simultaneously upon a uniform microbial population the result may be synergy, addition, indifference or antagonism”

Simmons NA, J Antimicrob Chemother, 1975¹¹³

Combinations of antimicrobial agents have been and are widely used in different settings, such as to obtain broad-spectrum coverage for the initial therapy and for polymicrobial infections, to reduce the development of resistance mechanisms, and lastly acquire antimicrobial synergistic activity. Antibiotic synergy is suggested to occur when the effect of a combination of antimicrobial agents is bigger in bactericidal or bacteriostatic activity compared to each antimicrobial substance alone¹¹³. The general agreement is that it can be difficult to distinguish antibiotic synergy *in vitro* and the pursuit of synergy must be evaluated in each case. IE is a condition with fatal outcomes from which one might benefit from a strong bactericidal effect. Combination therapy has been used throughout the years to treat IE, e.g., due to enterococci and non- β -haemolytic streptococci (NBHS)^{101, 102, 114-116}. Antibiotic synergy can be measured in several different ways as described below.

Time-kill assays

To perform time-kill assays the antibiotic susceptibility for the two different antimicrobial substances for the bacteria is performed, using broth dilution method as advocated by the EUCAST. The bacterial inoculum is added in nutrient broth in which the obtained concentrations of the different antibiotics are applied, solitary but also in combination, and incubated under appropriate conditions. Bacterial killing is measured by subculturing the bacteria at different time points, e.g., 6 and 24 hours after incubation. Synergy is defined and measured at a certain level of difference in cfu/mL between the combination of the two antibiotics and the most effective single antibiotic^{117, 118}.

Etest synergy methodology

Antibiotic synergy can be measured on a solid medium in different ways, a non-cross synergy test and a cross or 90° angle test. The two tests employ two different antibiotic strips of which the MIC for each antibiotic alone is already known. In a non-cross synergy test, one antibiotic is infused into the agar and another strip is overlaid. The resulting ellipse or inhibition zone is then analysed. The cross or 90° angle test is placed simultaneously but in a form of a cross with the two previously determined MIC-valued forming the 90° angle¹¹⁹, (Fig. 6).

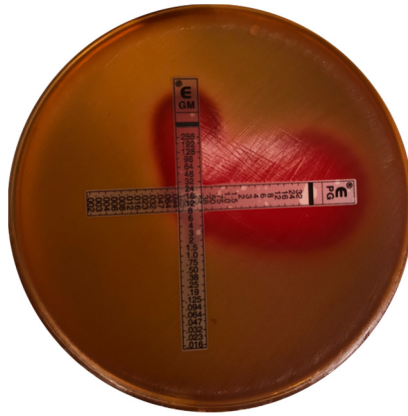


Figure 6. Etest synergy methodology with cross or 90° angle test.

The agar plate is inoculated with a bacterial strain of *S. dysgalactiae*. The Etest strips of penicillin and gentamicin are overlayed on the agar plate in a cross-approach. MIC is measured as the inhibition zone that intersects the graded antibiotic strip for each Etest strip respectively. Photo 6 by Anna Bläckberg.

The obtained MIC values in the combined approach are calculated against each antibiotic alone and synergy can be determined as described by the equation below¹¹⁹.

$$FIC^{Index} = FIC^A \left[\frac{MIC^{A+B} \text{ combination}}{MIC^A \text{ alone}} \right] + FIC^B \left[\frac{MIC^{B+A} \text{ combination}}{MIC^B \text{ alone}} \right]$$

The fractional inhibitory value (FIC) of each antibiotic can be calculated by distinguishing the MIC values of each inhibitory zone for the antimicrobial alone and in combination. The fractional inhibitory index (FIC^{Index}) is calculated by the sums of FIC^A and FIC^B . A value < 0.5 suggests the presence of synergy.

Synergistic antibiotic activities against β -haemolytic streptococci

Combination therapy may be imperative when strong bactericidal action is desired. As previously mentioned, combination therapy is recommended in IE due to some bacteria^{101, 102, 114-116}. IE due to BHS is rare but has an acute onset of presentation¹²⁰⁻¹²². Combination therapy with a β -lactam and an aminoglycoside for treating IE due to BHS is suggested in the current European and American guidelines^{123, 124}. This is partly based on previous publications implying synergy *in vitro* between penicillin G and an aminoglycoside on isolates of GGS^{125, 126}. The Swedish guidelines recommend monotherapy with penicillin G for IE due to BHS¹²⁷. This is partly based on a lack of any certain beneficial effect with combination therapy in IE due to group B streptococci (GBS)¹²⁸. Aminoglycosides are also notorious for their adverse effects including nephrotoxicity and ototoxicity^{129, 130}. As previously mentioned, clindamycin is often added in the treatment of necrotizing soft tissue infections due to BHS, partly due to

the observation of greater efficacy of clindamycin in mouse models with necrotizing soft tissue infections¹³¹. However, there are also reports that a combination of penicillin and clindamycin did not show any evident bactericidal advantage over each agent alone in the killing of *S. pyogenes* performed in time-kill assays¹³². In addition, older studies have reported antagonism of penicillin together with protein synthesis inhibitors both *in vitro*¹³³ and *in vivo*¹³⁴. Of note, results from studies *in vitro* cannot be directly implemented *in vivo*. Therefore, synergy may be observed *in vitro*, but may not be clinically effective *in vivo*. Combination therapy may be justified in patients with severe infections, but the risk of unwanted side effects should be considered and the need for synergistic bactericidal action can be evaluated in each case.

Antimicrobial resistance has emerged as one of the most critical public health problems worldwide, according to the World Health Organization (WHO)¹³⁵. Bacteria have evolved versatile resistance mechanisms to antibiotics, such as enzymatic inactivation, modifications of drug targets, reduced permeability of the outer membrane, and active drug efflux¹³⁶. *S. pyogenes* and *S. dysgalactiae* have remained sensitive to penicillin. On the other hand, they have evolved resistance mechanisms to other classes of antibiotics, in particular to erythromycin and clindamycin¹³⁷⁻¹³⁹.

Streptococcus dysgalactiae and *Streptococcus pyogenes*

S. dysgalactiae and *S. pyogenes* are the two main bacteria of this thesis. This chapter begins by describing the historical aspects of species determination, as well as microbiological characteristics, and diseases of *S. dysgalactiae* and *S. pyogenes*. This is followed by a brief description of the important molecules that these bacteria possess to interact with the host defence system.

Classification

S. dysgalactiae and *S. pyogenes* are members of the genus *Streptococcus* and belong to the phylum *Firmicutes*. *Friedrich Rosenbach* was the first to use the term *S. pyogenes* when isolating the organism from a wound and skin infection in 1884¹⁴⁰. *S. pyogenes* expresses group A antigen (GAS) and almost all strains are β -haemolytic. The “*pyo*” denoting “pus” and “*genes*” meaning “to produce” in Greek.

Another β -haemolytic pathogen is *S. dysgalactiae* which usually carries group C or G antigen (GCS/GGS). *S. dysgalactiae* was initially recognized as causing bovine mastitis. The *dys* denoting “ill, hard” and *galactia* pertaining to “milk secretion”, and *dysgalactiae* meaning “loss or impairment of milk”. *S. dysgalactiae* was first mentioned by *Diernhofer*¹⁴¹. Furthermore, *Frost* and *Engelbrecht* described *Streptococcus equisimilis* for human BHS carrying group C antigen in 1936¹⁴². Alongside the recognition of the Lancefield grouping, this was the preferred nomenclature used. Years later, results from DNA-DNA hybridization data indicated similarities between *S. dysgalactiae*, *S. equisimilis* and large colony forming group C, G, or L streptococci. The species were transferred into a single species, *S. dysgalactiae*. This was revived and recognized in the approved lists of bacterial names in 1983^{143, 144}. Thirteen years later, *Vandamme et al* proposed a new classification and proposed that *S. dysgalactiae* should be classified into distinct subspecies as they show different phenotypic characteristics¹⁴⁵. Furthermore, *S. dysgalactiae* was divided into two subspecies; *S. dysgalactiae* subsp. *equisimilis* (refers to human origin, presents β -haemolysis, or α -haemolysis, and reacts with group A, C, G or L antigen) and *S. dysgalactiae* subsp. *dysgalactiae* (proposed for animal origin, can

present β -haemolysis, α -haemolysis, or γ -haemolysis and expresses group C antigen¹⁴⁶). The division of *S. dysgalactiae* is complex and results from whole genome sequencing data have shown both host-restricted and lineage-specific restricted distribution of the subspecies of *S. dysgalactiae*¹⁴⁷. Transmission of *S. dysgalactiae* is usually person to person, and sites of colonization or focal infections may serve as reservoirs for transmission¹⁴⁸. *S. pyogenes* is transmitted directly by air droplets, and indirectly through contaminated surfaces^{61, 149}. Similar to *S. pyogenes*, outbreaks of community- or hospital-acquired infection have been observed with *S. dysgalactiae*¹⁵⁰. Human infections due to *S. dysgalactiae* subsp. *dysgalactiae* are rare and have predominantly been reported in patients after animal contact^{148, 151-155}. In addition to *S. dysgalactiae*, *Streptococcus equi* and *Streptococcus canis* carry group C and G antigens respectively^{156, 157}. These species are often of animal origin but can cause diseases in humans¹⁵⁸. Table 1 summarizes some of the different groups, species, subspecies, and types within BHS^{68, 145, 146, 159-161}.

Table 1. β -haemolytic streptococci

Group	Species	Subspecies	Haemolysis	Types
A				
	<i>S. pyogenes</i>		β	> 250 <i>emm</i> types ^{68, 69}
	<i>S. dysgalactiae</i>			
		<i>S. dysgalactiae</i> subsp. <i>equisimilis</i>		*
B				
	<i>S. agalactiae</i>		β	10 serotypes ¹⁶²
C				
	<i>S. dysgalactiae</i>		α , β , γ	
		<i>S. dysgalactiae</i> subsp. <i>equisimilis</i>		*
		<i>S. dysgalactiae</i> subsp. <i>dysgalactiae</i>		
	<i>S. equi</i>		β	
		<i>S. equi</i> subsp. <i>equi</i>		
		<i>S. equi</i> subsp. <i>zooepidemicus</i>		
		<i>S. equi</i> subsp. <i>ruminantium</i>		
G				
	<i>S. dysgalactiae</i>		β	
		<i>S. dysgalactiae</i> subsp. <i>equisimilis</i>		*
	<i>S. canis</i>		β	
L				
	<i>S. dysgalactiae</i>		β	
		<i>S. dysgalactiae</i> subsp. <i>equisimilis</i>		*

The number of *emm* types is constantly shifting, and the exact number of *emm* types are therefore fluctuating. * *S. dysgalactiae* expresses > 90 *emm* types and they can be found in group A, C, G or L streptococci. The current *emm* types can be found in the StrepLab database, at CDC^{68, 70}.

Microbiological aspects

S. pyogenes and *S. dysgalactiae* are facultative anaerobic cocci and display white-greyish colour with a diameter of ≥ 0.5 mm, surrounded by a β -haemolysis on a blood agar plate, 24 hours after incubation in 5% CO₂ at 35–37°C, (Fig. 7).



Figure 7. *S. dysgalactiae* streaked on a blood agar plate.

White-greyish colonies are displayed surrounded by β -haemolysis after 24 hours of incubation in 5% CO₂ at 35–37°C. Photo 7 by Anna Bläckberg.

The PYR test refers to the presence of the enzyme pyrrolidonyl arylamidase. Since *S. pyogenes* is positive and *S. dysgalactiae* negative, the bacteria can be separated by this test¹⁶¹. Another way to differentiate the pathogens is that *S. pyogenes* is susceptible to bacitracin whereas *S. dysgalactiae* is resistant. However, bacitracin-resistant strains of *S. pyogenes* have been reported throughout Europe^{163, 164}. Of note, streptococci are difficult to classify purely based on the presence of the carbohydrate antigen, as many of the streptococci within NBHS (e.g., *Streptococcus anginosus*) may carry A, C, G or F antigen, and may also display α -, β - or γ -haemolysis on a blood agar plate¹⁶⁵. Other bacteria such as enterococci are also able to display β -haemolysis and are also PYR positive. Enterococci can be relatively easily distinguished from *S. pyogenes* due to their differences in colony morphology and results from a combination of phenotypic testing.

Emergence of invasive diseases

The burden of invasive diseases due to both *S. pyogenes* and *S. dysgalactiae* varies in time and by geographical distribution. The estimated annual incidence of invasive diseases due to *S. pyogenes* ranges from 2.2–3.8 per 100 000 inhabitants¹⁶⁶⁻¹⁶⁸, with a case-fatality

rate of 14–16%¹⁶⁷⁻¹⁷⁰ in high-income countries. *S. pyogenes* still causes invasive diseases with millions of deaths yearly in less developed countries¹⁷¹, and since 1980 an increase in infections with *S. pyogenes* has also been reported in developed countries¹⁷². A plausible explanation for the increase of invasive diseases may be due to fluctuations of different *emm* types both temporally and geographically. *emm1*, expressed by *S. pyogenes*, is the most common *emm* type but has also been associated with causing invasive diseases with high mortality^{168, 173}. *emm3* has also been associated with invasive infections with high mortality rates¹⁶⁸. These two *emm* types have also been found in patients with necrotizing soft tissue infections¹⁷⁴.

S. dysgalactiae has historically been perceived as non-pathogenic flora of the upper respiratory tract, the gastrointestinal tract and the female genital tract, and was not recognized as an important human pathogen until 1980s¹⁷⁵. However, in recent years the burden of invasive *S. dysgalactiae* diseases approximates that of invasive *S. pyogenes* diseases. The annual incidence of invasive diseases due to *S. dysgalactiae* reaches from 2.2–6.3 per 100 000 inhabitants¹⁷⁶⁻¹⁸⁰, with a case-fatality rate ranging from 2–18%^{179, 181-183}. Prolonged survival in adults and improved species determination with MALDI-TOF MS may be an explanation for the high detected rate of invasive *S. dysgalactiae* diseases. The distribution of *emm* types among isolates of *S. dysgalactiae* shows greater diversity than among *S. pyogenes*. In a study from the United States, the most predominant *emm* types were stG6 and stG2078¹⁸⁴, which was dissimilar from a study from Finland in which stG480 and stG485 were the most common¹⁸³. A study from Japan linked certain *emm* types, (stG2078 and stG10), to invasive diseases¹⁸⁵, whereas other studies have not established such a distinct correlation^{186, 187}.

Streptococcal infections at a glance

Both *S. pyogenes* and *S. dysgalactiae* give rise to a wide range of infections, from mild tonsillitis to sepsis and severe soft tissue infection. The species cause similar infections with some exceptions. *S. dysgalactiae* often affects elderly people with comorbidities, while *S. pyogenes* affects a younger age group. Table 2 summarizes different diseases the pathogens cause, and some will be separately discussed below.

Table 2. Streptococcal infections at a glance

Disease	<i>S. dysgalactiae</i>	<i>S. pyogenes</i>
Erysipelas	✓	✓
Sepsis	✓	✓
Necrotizing soft tissue infection	rare	✓
Infective endocarditis	✓	rare
Recurrent bacteraemia	✓	✓
Tonsillitis	✓	✓
Pneumonia	✓	✓
Postpartum endometritis	✓	✓

Table 2 summarizes a brief selection of important diseases caused by BHS. Recurrent bacteraemia will be described in the following chapter and will therefore not be separately discussed in this section.

Erysipelas

Erysipelas is a common skin infection with low mortality but substantial morbidity¹⁸⁸. The reported incidence of erysipelas in European countries estimates up to 200 per 100 000 inhabitants per year¹⁸⁹. Erysipelas has an acute onset of presentation. It affects the upper dermis layer of the skin presenting oedematous erythema with a sharp demarcation from the surrounding tissue and involves the superficially located lymphatic vessels. Development of blisters may also appear, (Fig. 8).

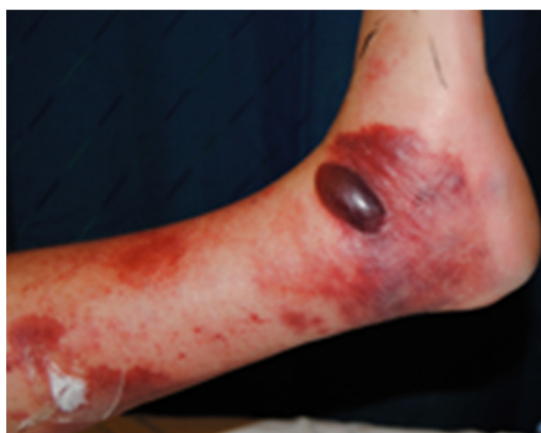


Figure 8. Erysipelas of the lower limb.

Clinical manifestation of erysipelas with the presence of blisters. Photo 8 by Anna Bläckberg.

The skin infection is often accompanied by a systemic involvement comprising fever, chills and malaise¹⁹⁰. The terms erysipelas and cellulitis are often used for the same disease. Erysipelas is usually assigned for skin infections to the superficial skin layers, (e.g., epidermis, dermis, and upper subcutis), whereas cellulitis is considered to have a

deeper involvement, (extending from the dermis into the subcutaneous tissue), and seldom presents a demarcation line¹⁹¹. The definitions and clinical presentations are overlapping, and some clinicians use the term cellulitis also for more cutaneous infections and erysipelas only for facial cutaneous infections. To distinguish erysipelas from cellulitis or even necrotizing soft tissue infections may be challenging¹⁹². Cellulitis is more often associated with a more diverse variety of organisms, e.g., *S. aureus* is often recognized¹⁹³. BHS have been more associated with erysipelas and cellulitis¹⁹⁴⁻¹⁹⁷, and predominantly GCS/GGS have been implicated as important pathogens^{195, 198}. But again, the diagnosis of skin and soft tissue infection relies on the clinical presentation, and different terms are used for the same diseases making the comparison between studies difficult. Bacteraemia is rare in erysipelas, (81-87% of blood cultures are negative), and obtaining blood cultures may not be compulsory in uncomplicated erysipelas¹⁹². Erysipelas has thus remained a clinical diagnosis during the years. When and if skin lesions are present, the causative agent is rarely found from obtained cultures¹⁹². Other methods such as cultures from needle aspirates, and or punch biopsies have identified BHS in a minority of cases^{78, 194, 199}. Other analyses may be serologic testing^{77, 78, 194} and or direct immunofluorescence²⁰⁰. These methods are often time-consuming, difficult to analyse and therefore hard to implement in clinical routine practice. As the condition is an infection of the skin, punch biopsies from infected sites may generate the bacteria by the PCR technique. However, in most of the cases, no bacterial agent to cellulitis could be detected using this movement and molecular technique²⁰¹. Furthermore, if pathogenic bacteria are found, it may also be difficult to assess their clinical relevance.

Recurrence of erysipelas is common, often affects the same anatomic site and is present in up to 40% of cases^{202, 203}. Two- and five-year recurrence rates have been estimated in up to 17%, and 57% of patients with a previous history of cellulitis²⁰⁴⁻²⁰⁶. The major risk factor for recurrent erysipelas is lymphedema^{202, 207}. Other risk factors may be previous radiation, venous insufficiency, and the presence of disruption of cutaneous barriers resulting in entrance sites for the bacteria^{202, 208, 209}. Colonization of BHS has been recognized in up to 44% of patients presenting with erysipelas, in which *S. dysgalactiae* has been the most predominant bacteria²¹⁰. Some patients still carry the organism after concluded antibiotic treatment. Another plausible explanation for recurrence may be the ability of both GAS and GCS/GGS for intracellular uptake into and persistence in epithelial and endothelial cells creating a streptococcal reservoir^{211, 212}. These intracellular reservoirs are likely not eliminated by penicillin since penicillin does not inevitably reach sufficient bactericidal intracellular concentration²⁰⁹. Treatment with antibiotics is often directed towards the streptococci with penicillin. However, when the depth of the infection in the tissue is unknown, antibiotic agents

may be administered to establish a broader coverage including *S. aureus*²¹³. Preventing the recurrence of erysipelas is challenging. Prophylactic administration of low-dose penicillin has been proven to prevent recurrences at a low cost, but these results have been based on smaller studies^{214, 215}. Other studies have not observed any significant reduction of recurrent erysipelas with the administration of prophylactic antibiotics²¹⁶. Occasionally some patients experience relapses of erysipelas although they are on long-term prophylaxis with penicillin^{217, 218}. In addition, erysipelas often reoccurs within a short time after prophylaxis has been discontinued^{203, 219}. Other interventions to prevent recurrent erysipelas/cellulitis have been compression therapy. In a randomized controlled trial study, compression therapy has proven to reduce the recurrence of cellulitis in the lower limbs with chronic oedema²²⁰.



Figure 9. Erysipelas of the lower limb.

Typical edematous erythema and swelling of the lower limb. The right leg is shown for comparison. Photo 9 by Anna Bläckberg.

Sepsis

Sepsis is a major health problem causing high morbidity and mortality with increasing incidence globally²²¹⁻²²³. Gram-negative bacteria have traditionally been perceived as the most common causative pathogens in sepsis, but more recently, Gram-positive bacteria

have been associated with sepsis^{224, 225}. As previously mentioned, *S. dysgalactiae* and *S. pyogenes* cause sepsis with high mortality and morbidity throughout the world^{171, 178}.

The term *sepsis* has been used since ancient Greek and comes from the Greek word “σηπῶ”, denoted “I rot”^{226, 227}. The definition of sepsis has been re-evaluated for several years based on pathophysiology, diagnosis, and degrees of severity. In 1992, *Roger C Bone* and the American College of Chest Physicians and the Society of Critical Care Medicine (ACCP/SSCM) Consensus Conference Committee, published their definition of sepsis (**Sepsis-1**)²²⁸. Sepsis was defined as the systemic inflammatory response syndrome (SIRS) in the presence of infection. The SIRS criteria comprised tachycardia, tachypnoea, hypothermia or hyperthermia, and leucocytosis. Furthermore, sepsis was divided based on the degree of severity. Severe sepsis was defined as organ dysfunction accompanied by hypoperfusion or hypotension and septic shock was defined as organ dysfunction with persisting hypotension despite adequate fluid resuscitation. Since then, the criteria for sepsis have been evaluated. Although the SIRS criteria may occur outside the presence of infection, the evidence of changing the pre-existing criteria was low and the definition of sepsis was only slightly altered in 2001, then comprising an expansion of signs and criteria for diagnosing sepsis, (**Sepsis-2**)²²⁹. The **Sepsis-3** criteria were proposed and published in 2016 where *Singer et al* stated that “Sepsis is a life-threatening organ dysfunction caused by a dysregulated host response to infection”²³⁰. As it was considered that sepsis was always to be perceived as severe, the term severe sepsis was removed and the scoring system, Sequential Organ Failure Assessment (SOFA) was applied to assess the definition of organ dysfunction^{231, 232}. **Sepsis-3** is still in use today and the use of the SOFA score has been validated to be an important predictor for mortality as well as for staging of severity of sepsis^{233, 234}.

Streptococcal toxic shock syndrome (STSS) was first described by *Cone et al*²³⁵. and in 1993 the case definition was established²³⁶. STSS involves parameters that largely mirror those that define septic shock but coupled with evidence of GAS pathology^{230, 236}. STSS indicates multiorgan failure and is often present in severe soft tissue infection due to GAS. STSS has also been frequently encountered in severe infections due to GCS/GGS²³⁷⁻²⁴³. Bacteraemia, necrotizing soft tissue infection, and puerperal sepsis due to BHS are often associated with STSS¹⁷³.

Necrotizing soft tissue infection (NSTI)

“The infection essentially produces a gangrene of the subcutaneous tissue, subsequently it causes death of a part of the overlying skin”

Meleney FL, Archives of Surgery, 1924²⁴⁴

Necrotizing soft tissue infection (NSTI) is a progressive deep infection of the subcutaneous tissue associated with serious and life-threatening conditions with a high mortality rate. The symptoms were initially described by *Hippocrates* in the 5th century BC and the condition has thereafter been described in a multiplicity of terminology. For example, in 1871, the surgeon *Joseph Jones* described it as “hospital gangrene”²⁴⁵, and in 1918, *Pfanner* described a patient with β -haemolytic streptococcal infection to the skin as a necrotizing erysipelas²⁴⁶. In 1924, *Meleney* published a series of patients with rapidly developed gangrene and described the infection as “haemolytic streptococcus gangrene”, as he considered the causative organism to be what he called haemolytic streptococci²⁴⁴. The condition often involves extensive necrosis of subcutaneous tissue and local ischemia and can be caused by other bacteria that are not haemolytic. *Wilson* considered this and was the first to use the term “necrotizing fasciitis” in 1952²⁴⁷. This term has later been used in succeeding publications²⁴⁸⁻²⁵⁰, and the term NSTI is now used to encompass infections that also involve other soft tissues than fascia. *S. pyogenes* is associated with NSTI, but *S. aureus* and other Gram-negative bacteria are also linked to the condition^{248, 251}. NSTI due to GCS/GGS has also been described^{174, 178, 252}. NSTI is often accompanied by STSS and severe pain. Penicillin is the primary treatment for NSTI due to BHS. Further addition of clindamycin has proven to be valuable, and this combination therapy has shown lower mortality rates compared to penicillin monotherapy^{253, 254}. It has been suggested that intravenous immunoglobulins (IVIg) may inhibit pro-inflammatory responses of superantigens from *S. pyogenes* and aid the opsonisation of the bacteria^{255, 256}. Administration of IVIg has proven to be effective in the treatment of patients with NSTI and STSS^{254, 257-260}. In a Cochrane review from 2013, *Alejandria et al* showed that administration of IVIg significantly reduced the mortality rate in patients with bacterial sepsis²⁶¹. However, the studies included were small and the overall results were not robust enough to implement IVIg in the recommended treatment of NSTI in the current guidelines from the Infectious Diseases Society America¹⁹².

Infective endocarditis (IE)

Infective endocarditis (IE) is an infection of the heart valves, the endocardium or intracardiac devices. In 1885, *Sir William Osler* described the condition as a “mycosis” that could be spread throughout the body²⁶². In developed countries, the incidence is 2.6–7.0 cases per 100 000 inhabitants per year with a mortality rate of 10–30%^{124, 263}. Diagnosing IE may be difficult and is summarized by clinical findings, symptoms, microbiological findings, and results from imaging. In 1994, *David Durack* proposed criteria to adequately diagnose IE, the so-called “Duke Criteria”²⁶⁴. These criteria have during the past years been evaluated and resulted in the utilization of the modified

Duke criteria which are commonly used today²⁶⁵, with the addition of the European Society of Cardiology (ESC) 2015 modified diagnostic criteria¹²³. Table 3 summarizes these criteria. *S. aureus* is an important pathogen in causing IE, followed by streptococci from the oral flora^{266, 267}. IE due to BHS is rare, constituting up to 3% of all cases of IE in some countries²⁶⁸. Although endocardial infections with BHS is rare, these cases constitute challenges in clinical practice and have an acute presentation with a substantial embolization rate^{122, 269, 270}. A study from Norway investigated sixteen cases of IE due to *S. pyogenes* and *S. dysgalactiae* that occurred in the period 1999–2013. The embolic event and 30-day mortality rate was 50% and 25% respectively¹²⁰. Most of the patients were treated with a combination of a β -lactam and an aminoglycoside. Recently, a study described cases of IE due to BHS based on results from data from the International Collaboration on Endocarditis Cohort Study²⁶⁸. This is a large multinational database comprising patients with IE recruited from 64 sites and 28 countries using a standardized case report form. BHS with GCS/GGS were more common than IE due to GAS. In contrast to the other bacteria within BHS, only patients with GCS/GGS aetiology had any form of relapse²⁶⁸. Altogether, BHS with IE is characterized by an acute presentation, often requiring surgery, and has a high rate of embolization. Findings of BHS in blood cultures do not, according to the modified Duke criteria, count as a major criterion. Current European and American guidelines consider some streptococcal species and their prevalence of IE, but not all streptococci are encountered in that calculation^{123, 124}. Therefore, it may be difficult to know when further investigations of looking for a cardiac focus of infection should be performed in patients with BHS bacteraemia. In a study by *Sunnerhagen et al*, a score for directions on when to perform echocardiography was proposed in patients with bacteraemia with NBHS²⁷¹. In addition, a retrospectively based study from Denmark proposed a flowchart for when to perform echocardiography in patients with IE due to *S. dysgalactiae* and *S. pyogenes* respectively, based on numbers of positive blood cultures, risk factors and the prevalence of IE²⁷². Patients with *S. pyogenes* bacteraemia were regarded as having a low risk of having IE and echocardiography was not recommended unless they had ≥ 3 positive blood cultures and risk factor/-s associated with IE. Bacteraemia with *S. dysgalactiae* was considered as a moderate risk for having IE and echocardiography was suggested with the presence of ≥ 3 positive blood cultures and/or risk factor/-s associated with IE. This stratification of low risk (< 3%) and moderate risk (3-10%) for the diagnosis of IE was based on cases of streptococcal bloodstream infections from Danish nationwide registries²⁷².

Table 3A. Definition of IE according to the modified Duke Criteria²⁶⁵

Definitive 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

Table 3B. Terms used in the modified Duke criteria²⁶⁵, with addition of the ESC 2015 modified diagnostic criteria¹²³

Major criteria	
Blood culture positive for IE	Typical organisms consistent with IE from 2 separate blood cultures or Microorganisms consistent with IE from persistently positive blood cultures or Single positive blood culture for <i>Coxiella burnetii</i> or IgG titre > 1: 800
Evidence of endocardial involvement	
Echocardiogram positive for IE	Oscillating intracardiac mass on valve or supporting structures, in the path of regurgitant jets, or on implanted material in the absence of an alternative anatomical explanation or Abscess or New partial dehiscence of prosthetic valve
New valvular regurgitation	
Identification of paravalvular lesion by cardiac CT ¹²³	
Abnormal activity around the site of prosthetic valve implantation detected by ¹⁸ F-FDG PET/CT (only if the prosthesis was implanted for > 3 months), or radiolabelled leucocytes SPECT/CT ¹²³	
Minor criteria	
Predisposition	Predisposing heart conditions or intravenous drug use
Fever, temperature > 38° C	
Vascular phenomena	Major arterial emboli, or septic pulmonary infarcts, or mycotic aneurysm, or intracranial hemorrhage, or conjunctival hemorrhages, or/and Janeway's lesions
Immunological phenomena	Glomerulonephritis, Osler's nodes, Roth's spots, and rheumatoid factor
Microbiological evidence	Positive blood culture, but does not meet the criteria for a major criterion or Serological evidence of active infection with organism consistent with IE
Identification of recent embolic events or infectious aneurysms by imaging only (silent events) ¹²³	

Table 3B presents the modified Duke Criteria²⁶⁵, with the addition of the proposed ESC 2015 modified diagnostic criteria¹²³. Abbreviations used are; computed tomography (CT), fluorodeoxyglucose (FDG), positron emission tomography (PET), and single photon emission computerized tomography (SPECT).

Tonsillitis

Tonsillitis encounters an acute inflammation of the pharynx and tonsils. *S. pyogenes* has been known to cause this disease²⁷³, but other BHS have also been encountered²⁷⁴. In a study from Sweden, the asymptomatic carrier rate of GAS was 6.4% in persons < 10 years of age, 9.4% in persons 10–15 years of age, and 0.2% in persons ≥16 years of age²⁷⁵. In the present study, the carrier rate of GCS/GGS was 0.04%, 3.1%, and 2.7% respectively for the same age categories²⁷⁵. Additionally, the prevalence of GAS in symptomatic patients was 25% in patients 10–15 years of age and 14.1% in patients ≥16 years of age²⁷⁵. Concurrently, the prevalence of GCS/GGS was 6.3% and 4.5% respectively in the same age categories²⁷⁵.

Pneumonia

Community-acquired pneumonia (CAP) due to *S. pyogenes* was often reported during the prebiotic era in the 20th century^{276, 277}. Today it is a rare entity, sporadically detected following viral infection, e.g., influenza²⁷⁸⁻²⁸⁰. A study from Spain observed an annual incidence of *S. pyogenes* pneumonia of 0.29–2.29 per 100 000 inhabitants with a case-fatality rate of 20%. The most prevalent type was *emm1*²⁸¹. Pneumonia due to *S. dysgalactiae* seems to be a rare presentation^{282, 283}.

Postpartum endometritis

Postpartum endometritis is an infection of the uterine lining and is one of the major causes of maternal infection following delivery. Since the 17th century puerperal sepsis following abortion or vaginal delivery has been described with mortality rates of up to 40%²⁸⁴⁻²⁸⁶. *S. pyogenes* has been known to cause postpartum endometritis, but endometritis with *S. dysgalactiae* does also occur^{287, 288, 289}. Cultures from blood and cervix are usually obtained. Surveillance with *emm* typing may be of importance when nosocomial outbreaks occur²⁹⁰.

Bacterial molecules to fight the human host defence

S. dysgalactiae and *S. pyogenes* possess similar molecules to fight the human host defence. The pathogens express fibronectin-binding proteins which mediate adherence to the human epithelial cells and facilitate internalization into host cells²⁹¹⁻²⁹⁴. The presence of hyaluronic acid capsule has been shown to confer phagocytosis by both masking

adhesins on the bacterial cell wall and resisting complement-mediated opsonophagocytic killing^{295, 296}. Horizontal genetic transfer between *S. dysgalactiae* and *S. pyogenes* may occur to acquire genetic material²⁹⁷. Table 4 summarizes a brief selection of molecules that either *S. dysgalactiae* and/or *S. pyogenes* possess and that interact with the human host. Several of these molecules will be specified in separate sections below.

Table 4. Important molecules of *S. dysgalactiae* and *S. pyogenes* that interact with the host defence

Molecule	<i>S. dysgalactiae</i>	<i>S. pyogenes</i>	Function
Fibronectin-binding proteins	✓	✓	Adhesion. Invasion ²⁹¹⁻²⁹⁴
Capsule	✓	✓	Resist phagocytosis ^{295, 296}
M protein	✓	✓	Evascape from the host defence system ²⁹⁸⁻³⁰³
Protein G	✓	-	Antibody binding ^{304, 305}
IdeS	-	✓	Antibody degradation ^{306, 307}
Streptolysin S	✓	✓	β-haemolysis. Tissue damage ³⁰⁸⁻³¹⁰
Streptolysin O	✓	✓	Tissue damage ^{311, 312}
Streptokinase	✓	✓	Bacteria spread in the tissue ^{148, 313, 314}
Superantigens	speA, speC, speG, speK, speL, speM, ssa, smeZ	speA, speC, speG, speH, speI, speJ, speK, speL, speM, ssa, smeZ	Proinflammatory response. Non-specific activation of T-cells ^{147, 315-317}
SIC	sicG	✓	Inhibition of complement ³¹⁸⁻³²¹
EndoS	EndoSd	✓	Antibody deglycosylation, loss of function ^{322, 323}
C5a peptidase	✓	✓	Inhibition of complement ^{147, 324, 325}
SpeB	-	✓	Tissue necrosis ³²⁶

The molecules may also have other functions in fighting the immune defence. Some of these molecules are present in *S. dysgalactiae* subsp. *equisimilis* but not in *S. dysgalactiae* subsp. *dysgalactiae*. Abbreviations used are; Immunoglobulin G-degrading enzyme of *S. pyogenes* (IdeS), streptococcal pyrogenic exotoxin (Spe), streptococcal inhibitor of complement (SIC), sic-like gene (sicG), streptococcal cysteine protease (SpeB).

M protein

The M protein is a cell surface attached protein and is a major virulence determinant for both *S. dysgalactiae* and *S. pyogenes*. The M protein is encoded by the *emm* gene and is the basis of the Lancefield serotyping. As previously mentioned, there are more than 90 different *emm* types among *S. dysgalactiae*^{68, 327} and > 250 different *emm* types among *S. pyogenes*^{68, 69}. The M protein is an α-helical coiled-coil protein that extends up to 0.6 μm from the cell wall^{299, 328}. It consists of various domains. The N-terminal part, on which molecular typing is based, consists of a hypervariable domain. This region is a target for type-specific protective antibodies, but intriguingly has also been shown in experimental studies sensitive to proteolysis and thereby weakly immunogenic³²⁹. This

Apart from these classical M proteins, there are *M-like proteins*, a term that was first presented by *Bessen* and *Fischetti* in 1992³³⁴. The fact that GAS were able to express more than one M protein was initially described in the 1960s by *Wiley* and *Wilson*³³⁵. The M-like proteins are encoded by the *enn* or *mrp* genes in contrast to the M protein, which is encoded by the *emm* gene, as previously mentioned. These *emm*-like genes as well as the *emm* gene are found within the chromosomal locus, regulated by *mga* (multiple gene activator) regulon²⁹⁸. This regulon coordinates the expression of the M protein, C5 peptidase and many other molecules³³⁶.

M protein and interaction with host ligands

The M protein has a variety of different immunomodulatory assets. Figure 11 summarizes different interactions between the M protein and the human host. Some of these interactions are described in separate sections below.

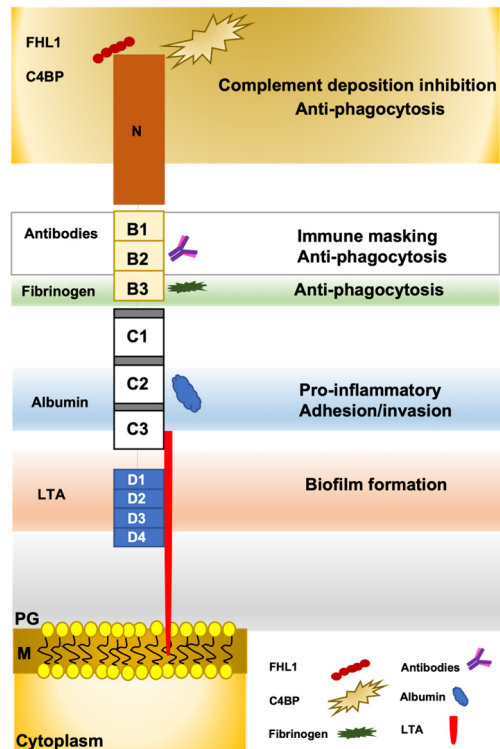


Figure 11. Interactions between the M protein and different host ligands.

Schematic outlined how the M protein interplays with the immune defence, inspired by *Frost et al*²⁹⁸, and by *Bisno et al*³³⁷. Some of these proteins have also other immunomodulatory assets. Abbreviations used are; Factor H-like 1 protein (FHL1), C4-binding protein (C4BP), lipoteichoic acid (LTA), peptidoglycan (PG), and membrane (M). Figure 11 by Anna Bläckberg.

Inhibition of the activation of the complement system

Many M proteins can bind to C4-binding protein (C4BP). This protein inhibits the classical pathway complement activation³⁰². Moreover, different M proteins can bind to another complement inhibitory protein, factor H-like protein 1 (FHL1)³⁰¹. Bound FHL1 and/or bound C4B to the hypervariable region of the M protein results in the protection of the bacteria against complement activation. This inhibition of complement activation results in partial resistance to phagocytosis³⁰⁰.

Interaction with fibrinogen and albumin

The M protein may bind to fibrinogen. This binding may in turn block the deposition of complement and indirectly inhibit complement activation^{338, 339}. The C-domain of the M protein may bind to human serum albumin³⁴⁰. This binding may have a role for the bacteria to avoid antibody opsonisation and resist phagocytosis³⁴¹.

Biofilm formation

The different M proteins possess the ability to form a complex with lipoteichoic acid (LTA) resulting in enhancement of the hydrophobic surface of the streptococci which may contribute to adhesion and biofilm formation³⁴². The ability to form biofilm enables the bacteria to survive and proliferate in hostile environments.

Immunoglobulin binding

The M protein can interact with the Fc region of the IgG and thereby block their function and partially resist phagocytosis³⁴³. The ability of the bacteria to bind and coat different host molecules may result in a “masking” of antigenic epitopes which enables the bacteria to avoid opsonisation and thereby evading the host immune system. On the other hand, it is believed that type-specific antibodies may be directed towards the hypervariable domain and confer protection after infection with *S. pyogenes*. This may result in protective immunity against the certain *emm* type³⁴¹. Studies from mouse models have discriminated development of antibodies towards the C-terminal of the M protein following invasive *S. pyogenes* infection, but these antibodies did not seem to opsonise and promote phagocytosis of the bacteria³³⁰, but other studies indicate that antibodies towards the C-terminal of the M protein may be protective³⁴⁴. Moreover, the orientation of the IgGs at the bacterial surfaces may be dissimilar from different sites, e.g., in saliva, the IgGs have mostly been found to bind to *S. pyogenes* via Fc³⁴⁵. In blood, the IgGs were preferentially bound to the bacteria via Fab facilitating phagocytosis of the bacteria³⁴⁵.

Protein G

Protein G is a cell wall protein that is expressed by *S. dysgalactiae* and has unique protein-binding properties³⁰⁴. Protein G can bind to immunoglobulin G, as well as to serum albumin. The protein has an affinity for all human IgG subclasses, whereas there is no binding to IgM, IgA or IgD. The main function of protein G is binding to the Fc region of the IgG, but interaction with the Fab fragments of the IgG has also been established. The latter binding may therefore confer protection against opsonisation^{304, 305, 346}. Protein G has also an affinity to α_2 -macroglobulin (proteinase inhibitor of human plasma)^{347, 348}. Altogether, protein G is important for the bacteria in both evading the immune system by its binding to immunoglobulins and conferring protection against proteolytic degradation. *S. pyogenes* has a protein G-like α_2 -macroglobulin-binding (GRAB) protein that protects the bacteria from proteolysis but lacks the ability of binding to immunoglobulins³⁴⁹.

IdeS

IdeS is an IgG-degrading enzyme expressed by *S. pyogenes*³⁰⁶. IdeS cleaves the IgG molecule just beneath the hinge region which generates one F(ab')₂ fragment and two monomeric Fc fragments³⁰⁶. This degradation of IgG results in an accumulation of Fc and F(ab')₂ fragments that retain the ability to bind to surface antigens of the bacteria but have difficulties in activating the complement system and facilitating phagocytosis by immune cells³⁰⁷. Due to this highly unique enzymatic reaction, the use of IdeS as a therapeutic agent has been discussed as a future treatment for clinical conditions involving pathogenic immunoglobulins.

Toxins

Streptolysin S (SLS) is responsible for causing the β -haemolysis³⁰⁸. This is an oxygen-stable oligopeptide, whereas the other cytotoxin, streptolysin O (SLO) is a cholesterol-binding bacterial exotoxin. SLO binds to the cholesterol in eukaryotic cell membranes eventually causing cell lysis and tissue damage^{311, 312}. The exact mechanism of SLS is not fully understood, but it has been suggested that the peptide contributes to soft tissue damage, and tissue necrosis, and indirectly mediates cell death of macrophages^{309, 310}.

Streptokinase

S. dysgalactiae and *S. pyogenes* express streptokinase. This enzyme can bind to and activate the circulatory plasminogen into plasmin³⁵⁰. Plasmin is a central proteinase in the fibrinolytic system and contributes to tissue damage and bacterial spread across barrier^{148, 313, 314}.

Superantigens

Streptococcal pyrogenic exotoxins (Spe) are superantigens. They bind to the T-cell receptor and MHC class II which results in co-stimulation of a large number of T-cells³⁵¹. This stimulation mediates the massive release of proinflammatory cytokines and contributes to the pathogenesis of invasive diseases. Production of superantigens has been associated with multi-organ failure and STSS³⁵². *S. dysgalactiae* also expresses superantigens, such as SpeA³⁵³ and SpeG. However, the exact role superantigens play in the pathogenesis of severe infections with *S. dysgalactiae* remains unclear^{182, 354}.

SIC and *sicG*

Streptococcal inhibitor of complement (SIC) is an extracellular protein that is expressed by some types of *S. pyogenes*, mostly *emm1*, and is known to inhibit the membrane attack complex of complement^{318, 319}. Like *S. pyogenes*, some types of *S. dysgalactiae* possess a streptococcal inhibitor of complement-mediated cell lysis-like gene, denoted *sicG*. The presence of this gene has been associated with severe soft tissue infections^{321, 355}.

EndoS

EndoS is expressed by *S. pyogenes* and some types of *S. dysgalactiae* express EndoS^{322, 323}. This secreted endoglycosidase is important in its ability to hydrolyse the N-linked glycan on IgG. This hydrolyzation results in the loss of IgG glycan and affects the binding between the Fc region of IgG and the Fc receptor on phagocytes³⁵⁶. This reduces complement activation through the classical pathway and enables escape from the mediated antibody response.

Aspects of the immune defence

We are constantly surrounded by organisms and some of them can cause invasive diseases. The human host possesses many defence mechanisms to fight these organisms. The innate immune system encompasses the first barrier of defence. It relies on epithelial surfaces, secreted proteins, and cell-associated receptors to detect infection and separate pathogens from host tissue. The adaptive system is on the other hand based upon antigen-binding properties of immunoglobulins and lymphocytes resulting in the identification and efficient eradication of the invading microbe. In addition, the adaptive system acquires an immunologic memory. It remembers the initial antigens that elicited a primary immune response, and this leads to an enhanced immune response when encountering the same pathogens again. As previously mentioned, infections with BHS tend to reoccur, this is true for erysipelas but also for bacteraemia with *S. dysgalactiae*. This chapter begins by describing different important sections of the immune defence to fight bacterial infections. This is followed by a detailed description of the humoral response and development of antibodies following invasive infections with BHS. Lastly, the interplay between these developed antibodies and BHS is further discussed.

The first line defence

Anatomical and chemical barriers are the first line of defence that a microorganism meets when invading the human defence. The anatomical barrier consists of epithelia lining the external and internal parts of the body. This could be the skin but also the respiratory and gastrointestinal tract. The chemical barrier consists of mucous, cilia, and antimicrobial peptides (AMPs). AMPs are expressed in different parts of the body and can disrupt bacterial cell walls and bacterial cell membranes. AMPs are also expressed by cells and are crucial constituents of the innate system, and involved in the chemotactic and immunomodulating actions during an infection³⁵⁷.

The innate system

Once the invading microbes succeed to overcome the anatomical and chemical barrier of the immune system, the host defence has indirect strategies to fight them. Some pathogens express pathogen-associated-molecular-patterns (PAMPs). These PAMPs can be recognized by pattern-recognition receptors (PRRs) that the host's immune cells possess^{358, 359}. PRRs include Toll-like receptors (TLRs)^{360, 361}, NOD-like receptors (NLRs)^{362, 363}, retinoic acid-inducible gene I (RIG-I)-like receptors (RLRs)^{364, 365}, and C-type lectin receptors (CLRs)^{366, 367}. This recognition of PAMPs by PRRs induces an efficient immune response which ultimately may result in the eradication of the invading microbe. The host's immune cells carrying such receptors are often called phagocytes and include monocytes, macrophages, neutrophils, and dendritic cells. The microbes are internalized into a membrane-enclosed endocytic vesicle, a phagosome and are eliminated and killed within the phagocyte. This is a complex process called phagocytosis and was first discovered and described by *Élie Metchnikoff*, in the 20th century³⁶⁸. This microbial recognition and killing activity constitute a central part of the innate immune system^{369, 370}. Further enhancement of phagocytosis occurs when certain proteins bind to the pathogen surface which promotes early identification of the invading microbe by the host's cells. Opsonisation refers to the coating of a pathogen with soluble host proteins called opsonins. Phagocytic cells carrying specific receptors recognize these opsonins and can thereby easier eradicate opsonised microbes³⁷¹. Opsonins may be immunoglobulins and/or complement proteins³⁶⁸.

The complement system

The complement system is a major counterpart of the immune system but also acts as a bridge to the adaptive immune system. This collection of soluble proteins was first described by *Buchner et al*³⁷². The complement system is composed of more than 30 plasma proteins that are mostly synthesized in the liver and circulate in the plasma or on cell surfaces as inactive precursors³⁷³. Complement activation comprises a cascade of proteolysis. Within complement activation, there are three different pathways. Each pathway results in the formation of C3 convertase which is the centre protein of the complement cascade. Further cleavage of C3 convertase results in C3a and C3b³⁷⁴. C3a acts as an anaphylatoxin and can recruit inflammatory cells. C3b acts as an opsonin and enables the eradication of the pathogen by phagocytes expressing receptors for C3b. C5 convertase can be cleaved into C5a and C5b. C5a acts as a chemoattractant and recruits

immune cells while C5b triggers the membrane-attack-complex (MAC) in which pores are inserted in the bacterial cell membranes leading to cell lysis³⁷⁵.

The classical pathway is an antibody-triggered pathway that was the first to be discovered and described in the 1940s by *Louis Pillemer*³⁷⁴. The classical pathway is initiated when C1q, (the first complement protein of the cascade), interacts with the surface of the pathogen or forms an antigen-antibody complex with either IgM or IgG. Further cleaving results in the formation of C3 convertase. In 1954, *Louis Pillemer* proposed the alternative pathway, which became highly controversial and was first rejected by the research field. A decade later, the alternative pathway was accepted and established as a part of the scientific body of knowledge³⁷⁶⁻³⁷⁸. The pathway can be spontaneously activated which results in the formation of a unique C3 convertase³⁷⁹. In the late 20th century, the third pathway was discovered. This is called the lectin-pathway which is initiated by mannose-binding lectin (MBL) that recognizes and binds to the surface of the pathogen^{380, 381}. When MBL binds to carbohydrate ligands on the pathogen surface, MBL-associated serine protease 1 (MASP-1) is induced which in turn cleaves and activates MASP-2. MASP-2 can cleave C4 allowing a conformational change eventually resulting in a generation of C3 convertase^{382, 383}.

The adaptive immune system

The keystones of the adaptive immune system are adaption, specificity, and memory. The term immunity refers to the immunomodulatory development and actions of the adaptive immune system which results in the protection against the recurrence of an infection you have already encountered. The adaptive system is made up of T-and B-lymphocytes, and antibodies. There are two different ways in which the adaptive immune system carries out the immune response, cell-mediated immune response (cellular immunity) and antibody response (humoral immunity). T-lymphocytes are classified into cytotoxic T-cells and helper T-cells. The helper T-cells mediate adaptive immunity to invading microbes and regulate both B-lymphocytes and cytotoxic cells³⁸⁴⁻³⁸⁷. Humoral immunity is established by B-lymphocytes that produce antibodies. These antibodies may neutralize the pathogen and block the actions of the toxins the pathogens express. The antibodies may also opsonise the pathogen either directly or through activation of the complement system. Phagocytes carrying receptors for the Fc region of IgG molecules (FcγRs) or for the C3b fragments recognize and ingest targets coated with IgG and/or C3b fragments. This facilitation of phagocytosis is as earlier mentioned called opsonisation. The B-cell receptor (BCR) can bind to the antigen directly but also deliver the antigen to intracellular sites in which it is degraded,

producing antigenic peptides bound to MHC class II that are returned to the surface of the B-lymphocyte^{388, 389}. If this MHC II complex is recognized by antigen-specific helper-T-cells, these cells stimulate the B-lymphocyte leading to proliferation and differentiation into antibody-secreting plasma cells and memory B-cells^{390, 391}.

Immunoglobulins

Immunoglobulins or antibodies are arranged according to different classes structured on and adapted to the function of the compartment of the body. The different classes, also referred to as isotypes, are IgA, IgD, IgE, IgG and IgM.

The Ig molecule is composed of two identical heavy chains and two identical light chains that are connected by disulphide bonds. The light chain is composed of a variable domain, V_L , and a constant domain, C_L . The heavy chain consists of one variable domain, V_H and three constant domains, C_{H1-3} (Fig. 12). Each domain consists of approximately 110 amino acids, folded as a cylinder composed of two extended layers of β -sheets. The light chains are of two types, lambda (λ) or kappa (κ). The heavy chain is made up of five different types α , δ , ϵ , γ and μ respectively which defines the classes of the immunoglobulins³⁹².

The Ig molecule can be regarded in three different parts based on the function of the Ig molecule. There are two identical antigen-binding parts, (Fab) and a constant region (Fc) that are linked by a flexible hinge region. Within the domains of V_H and V_L , there are hypervariable regions, and this is the binding site for the antigen³⁹³. Since it is the combination of both the heavy chain and the light chain that makes up the antigen binding site, the immune system has a combinatorial diversity generating different antibodies based on the combination of the heavy-and light-V region. Digestion of the Ig molecule with the enzyme, papain, results in the separation of both Fab from Fc generating two Fab fragments and one Fc fragment. Another protease pepsin digests the Ig molecule leaving the hinge region intact generating a $F(ab')_2$ fragment. Since the Fab region of the antibodies is important in protective immunity and antigen recognition, pathogens have evolved mechanisms to overcome this antigen detection. For example, *Finnegoldia magna* (formerly known as *Peptostreptococcus magnus*) possesses a cell wall molecule called protein L that has an affinity for the light chain (λ) of the IgG molecule, thereby binding to the Fab fragment of the antibody³⁹⁴⁻³⁹⁶, (Fig. 12).

Immunoglobulin G

IgG is the most abundant antibody in the blood, with a concentration of 7–16 mg/ml. The total molecular weight is 150 kDa, 50 kDa for each heavy chain and 25 kDa for each light chain. IgG is found in four different subclasses, (IgG1, IgG2, IgG3, and IgG4) dependent on the structure of the heavy chain, γ . IgG may bind to the antigen through the Fab region and coat the surface of an invading pathogen. As previously mentioned, the Fc region of certain isotypes can bind to Fc γ Rs that are expressed on the surface of phagocytic cells. This recognition facilitates phagocytosis of the pathogen. IgG is also able to activate the complement system. The Fc region binds to C1q and activates the classical pathway which eventually results in the formation of C3 convertase. The Fc region of the Ig molecule facilitates transportation, which is why IgG can be transported across the placenta providing an immune defence to the foetus and the new-born.

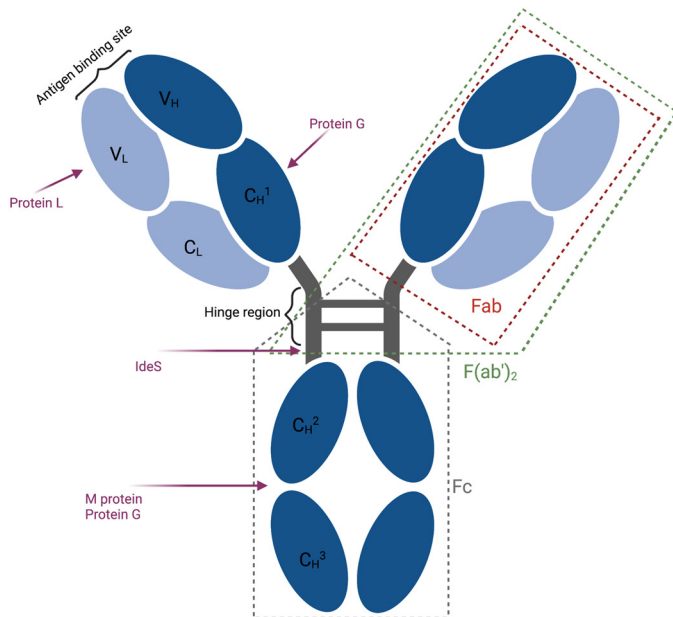


Figure 12. Immunoglobulin G.

To the left molecules interacting with the immunoglobulin, the arrows indicate the location sites. Figure 12 was created with Biorender.

Antibody responses following infection with BHS

Both *S. dysgalactiae* and *S. pyogenes* possess many molecules that interact with the human host defence system. *S. dysgalactiae* expresses protein G which mainly has an affinity for the Fc region of the antibody, preventing the pathogen from being eliminated by the host's cells^{314, 346}. *S. pyogenes* possesses IdeS. This is an IgG-cleaving enzyme that cleaves the Ig molecule in a way like the previously mentioned enzyme pepsin. The cleaving is beneath the hinge region and generates two separate chains of the Fc region and a F(ab')₂ fragment (Fig. 12). This splitting of the IgG molecule prevents the normal function of the IgG^{307, 397}. Furthermore, the binding of the M protein to the IgG enables the bacteria to avoid phagocytosis^{398, 399}, (Fig. 12).

"Persistence of type-specific antibodies in man following infection with group A streptococci"

Lancefield R, J Exp Med, 1959¹⁷

Recurrent bacteraemia with *S. pyogenes* is rare and there have been many plausible explanations for this phenomenon. In 1927, *Todd* performed an *in vitro* assay that involved inoculation of *S. pyogenes* into donor whole blood to investigate immunity⁴⁰⁰. This bactericidal assay was further established by *Lancefield* and is sometimes referred to as the "*Lancefield* whole blood killing assay". Years later the bactericidal test is still used but has been improved with the addition of several modifications to assess opsonisation and killing of different types of GAS^{401, 402}. Initially the method was used to identify serotypes of GAS with type-specific antisera prepared in rabbits⁴⁰³, and in 1959 *Lancefield* described the persistence of type-specific antibodies in some individuals 4–32 years after a GAS infection¹⁷. These antibodies were thought to be directed towards the hypervariable region of the *emm* type and confer immunity against that certain M type. The development of type-specific antibodies towards the M protein has been described in several other studies^{404, 405}. In addition, a lack of opsonising antibodies was present in a patient with recurrent bacteraemia with *S. pyogenes*⁴⁰⁶. Since the *Lancefield* study of 1959¹⁷, there is currently one study that has confirmed the persistence of opsonic antibodies following GAS infection⁴⁰⁷. *Bencivenga et al* reported the persistence of opsonic antibodies in one out of two patients with previously confirmed rheumatic fever⁴⁰⁷. Type-specific immunity following infection with GAS seems to rely on the development of antibodies towards the hypervariable terminal part of the M protein⁴⁰⁸, and cross-immunity between different *emm* types does not seem to be present⁴⁰⁹. However, the presence of protective antibodies towards the conserved epitopes of the M protein has been observed in repeated infections with GAS³⁴⁴. Similar findings from studies regarding immunity following GCS/GGS do not seem to be

present. In a murine model, in which cellulitis was induced by a strain of GGS, there was no evidence of acquired protective immunity by opsonic antibodies⁴¹⁰. Recurrent bacteraemia with *S. dysgalactiae* occurs in up to 10% of cases^{183, 327, 411, 412}. The second episode often occurs within 6 months from the first episode, and the same *emm* type from the first episode is often encountered⁴¹². In addition, recurrent bacteraemia with *S. dysgalactiae* seems to come from the same clonal origin as the first episode⁶⁵. This may indicate a carriage between the episodes of bacteraemia. Perianal colonization with *S. dysgalactiae* may confer such carriage and could be a potential risk factor for recurrent infection²¹⁰. The interplay between BHS and the human host seems to be essential when infections with BHS reoccur.

Present investigations

Understanding the pathophysiology of infection is important to find the organism responsible for the disease. This knowledge is also of importance when it comes to diagnostic modalities and administering adequate therapy for infections. In this section, papers I–V will be briefly and separately discussed. For complete papers, see the articles in appendices I–V.

The overall aims of this thesis were to further investigate:

- Microbiological findings and clinical features of erysipelas (Paper I)
- Clinical characteristics, and presence of antibiotic synergy in IE due to *S. dysgalactiae* (Paper II)
- Antibody responses following invasive infections with *S. dysgalactiae* (Paper III)
- Prognostic factors in invasive infections with *S. pyogenes* and *S. dysgalactiae* (Paper IV and V)

Paper I

Erysipelas, a large retrospective study of aetiology and clinical presentation

Erysipelas is a common but also ambiguous infection of the skin. Ambiguous may refer to the difficulties in the terminology, as well as the lack of conclusive and consistent findings from microbiological cultures. Erysipelas has therefore remained a clinical diagnosis and typically presents as an acute progressively oedematous erythema that is sharply demarcated from adjacent unaffected skin. However, all that is red is not erysipelas/cellulitis. Deep vein thrombosis, stasis dermatitis, and other infections can also mimic the symptoms of erysipelas. Since it is an infection of the skin, one may hypothesize that streptococci can be found from punch biopsies from the affected skin. Punch biopsies were performed in eight clinically diagnosed patients with erysipelas. In seven punch biopsies, no organisms were discovered, but in one patient the organism *Vibrio vulnificus* was found by the PCR technique. This patient had acquired a *V. vulnificus* wound infection, vibriosis, or in Swedish called “badsårsfeber”. Identifying

streptococcal DNA from punch biopsies from affected skin by the PCR-approach did not seem to be successful. These findings were later in accordance with other studies. *Crisp et al* performed skin biopsy specimens from paired infected and non-infected sites of 49 subjects and were not able to identify *S. pyogenes* or *S. dysgalactiae* with the PCR technique used²⁰¹.

Alongside undertaking punch biopsies, medical records of patients with erysipelas were studied trying to identify the aetiology of the disease. These episodes of erysipelas occurred in Region Skåne, Sweden during a seventh-year study period. Patients with a diagnosis of cellulitis were not included since this diagnosis was considered to refer to a deeper infection of the skin. Demographics and microbiological findings were extracted from medical records and analysed.

In accordance with other studies of erysipelas, the presence of an underlying disease or predisposing factor for erysipelas was common whereas only 128 patients (13%) had neither an underlying disease nor predisposing factor. The most common location for erysipelas was the leg (68%) followed by the arm (12%) and the face (9%). Wound cultures were obtained in 343 episodes (30%). GAS were identified in 53 cultures (23%), and in 64 episodes (26%) GCS/GGS were identified. Additionally, *S. aureus* was identified in 153 wound cultures (63%). In 555 episodes (49%) blood cultures were obtained, of which only 50 (9%) were positive. The low rate of positive blood cultures in erysipelas was not unexpected, and blood culturing is not mandatory in patients with uncomplicated erysipelas according to the guidelines¹⁹². GCS/GGS were the most common pathogens from blood cultures of which all were species determined to *S. dysgalactiae* subsp. *equisimilis*, followed by *S. pyogenes*, *S. agalactiae*, and *S. aureus*. Other studies have suggested GGS as important pathogens in erysipelas¹⁹⁵. This has also been demonstrated in more recent studies, suggesting a predominance of GCS/GGS causing erysipelas/cellulitis¹⁹⁸. Paper I indicated BHS aetiology in only 13% of the cases which is lower compared to other studies. During the same period, a Norwegian study prospectively included patients with erysipelas. BHS were confirmed in 72%, (146/203), by combining results from serologic testing, blood cultures and/or cultures from tissue¹⁹⁸. This rate increased to 85% when cases of “probable BHS caused erysipelas” were considered, (confirmed by penicillin response, or findings of BHS in superficial cultures)¹⁹⁸. The exact role of *S. aureus* in erysipelas/cellulitis seems to be undetermined based on the results from Paper I. However, the patients with *S. aureus* bacteraemia had another infection than erysipelas. Moreover, the six patients who had co-cultures with BHS and *S. aureus* had bacteraemia with BHS. In addition, since all patients with a diagnosis of erysipelas were included, these patients might have been excluded if patients with a primary diagnosis of erysipelas would have been included.

In conclusion, the results from this thesis indicate that GCS/GGS are important pathogens to erysipelas. Erysipelas tends to reoccur, and this is more probable when the causative agents are GCS/GGS compared to GAS. The difficulties in finding the aetiology of erysipelas highlight the importance to improve diagnostic procedures to better assess antibiotic therapy.

Paper II

Infective endocarditis due to Streptococcus dysgalactiae: clinical presentation and microbiological features

Infective endocarditis (IE) due to BHS is rare but has been described and recognized in earlier publications. In 2016, *El Rafei et al* described IE due to BHS as an acute onset of presentation with a high rate of embolization. The study encompassed 49 cases of IE, but only eight cases were due to GCS/GGS, and further species determination was not performed¹²². At the beginning of Paper II, only a few case reports of IE due to *S. dysgalactiae* had been published. However, during data collection, another research group from Norway published nine cases of IE due to *S. dysgalactiae*. The results were in accordance with previous findings and showed that IE with *S. dysgalactiae* has a high embolic event and mortality rate¹²⁰. Most of the patients received a combination therapy of a β -lactam and an aminoglycoside in the two described studies. This is in accordance with the European and American guidelines that suggest combination therapy of a β -lactam and an aminoglycoside for the first 2 weeks of a 4–6 weeks course of antibiotic therapy in IE due to BHS^{123, 124}. The rationale behind this has been based on *in vitro* studies showing better bactericidal action on isolates of GGS with the addition of gentamicin to penicillin G compared to penicillin G monotherapy¹²⁵. In addition, a more favourable outcome with combination therapy compared to monotherapy has also been suggested in IE due to BHS⁴¹³. However, this has not been confirmed in other more recent studies. *El Rafei et al* did not detect any favourable outcome in patients treated with adjunctive aminoglycosides. In contrast, this group seemed to have a higher rate of acute kidney injury (41%) compared to patients with monotherapy (22%). In addition, *Ruppen et al* did detect an initially faster killing in time-kill assays with the addition of gentamicin to penicillin G on blood isolates of GBS, but this was only observed within the first few hours⁴¹⁴. Moreover, *Sendi et al* did not detect any better outcome in patients with IE due to GBS who received adjunctive aminoglycosides compared to monotherapy¹²⁸. Swedish guidelines recommend monotherapy with penicillin G, due to a lack of evident benefits of combination therapy, thus avoiding potential toxic actions of aminoglycosides.

Clinical characteristics of cases of IE due to *S. dysgalactiae* were compared with cases of IE due to other major pathogens. Since IE due to *S. dysgalactiae* is rare, a nationwide study was conducted. Cases of IE occurring in Sweden are continuously reported to the Swedish Registry of Infective Endocarditis (SRIE). SRIE is managed by the Swedish Society of Infectious Diseases and since its inception, departments of infectious diseases throughout Sweden have participated in reporting cases treated at their respective clinics. In addition to the parameters from the SRIE, medical records of included patients with IE due to *S. dysgalactiae*, were collected from each clinic after obtaining written consent. In addition, blood isolates of *S. dysgalactiae* were collected from the different microbiological departments to establish species determination by MALDI-TOF MS. Antibiotic susceptibility testing and the presence of any possible synergistic effect between penicillin G and gentamicin were also performed on collected isolates of *S. dysgalactiae*.

Patients with IE due to *S. dysgalactiae* had a similar presentation as IE due to *S. aureus*. The condition has an acute onset of presentation, often requiring surgery and has a high rate of embolic events. Patients with IE due to *S. dysgalactiae* were older compared to patients with IE due to the other major pathogens. stC74a and stG62647 were the most common *emm* types in the study cohort, but the distribution was diverse and no correlation between hospital mortality and certain *emm* types was distinguished. However, the study was likely underpowered to detect such differences. The antibiotic synergy between penicillin G and gentamicin was not detected with the Etest methodology. In four out of nine isolates of *S. dysgalactiae*, antibiotic synergy was detected utilizing time-kill methods, but in most cases, further additive killing with gentamicin could not be discriminated, since the action of penicillin G alone was bactericidal enough to kill the bacteria within 24 hours. There was no statistical difference in outcome with patients receiving combination therapy compared to monotherapy, however, the study was likely underpowered to detect such difference. This study involves 50 cases of IE due to *S. dysgalactiae* which is considerably larger compared to other published studies, but of course limited in its retrospective design.

In conclusion, the results from Paper II indicate that IE due to *S. dysgalactiae* occurs in elderly people and has an acute onset of presentation. Synergy between penicillin G and gentamicin was demonstrated *in vitro* against some blood isolates of *S. dysgalactiae*. However, in most of the cases, penicillin G alone displayed bactericidal action so strong, that further additive killing with gentamicin could not be detected by the time-kill method used. The results from this study together with other published studies reporting a lack of favourable outcomes with combination therapy do not provide any robust evidence of adjunctive gentamicin in IE due to BHS. In severe infections, one might tend to either change or add an antibiotic. The need for potential strong

bactericidal action with combination therapy may be evaluated in everyone, nevertheless, administering a potential toxic additive antibiotic should be carefully considered and reassessed in clinical practice. The current Swedish guidelines already suggest monotherapy in IE due to BHS. It may now be the time to consider abandoning the recommendation of adjunctive aminoglycosides in IE due to BHS in the current international guidelines as well.

Paper III

*Lack of opsonic antibody responses to invasive infections with *Streptococcus dysgalactiae**

Infections with *S. dysgalactiae* tend to reoccur. Patients may have 3–4 episodes of erysipelas per year. The tendency to reoccur is not only true for erysipelas, but also for bacteraemia with *S. dysgalactiae*. Recurrent bacteraemia with *S. pyogenes* seems to be a very rare presentation. The persistence of protective opsonic type-specific antibodies following GAS infection¹⁷, as well as the lack of development of these antibodies in recurrent bacteraemia with *S. pyogenes*⁴⁰⁶ suggest that the activity of opsonic type-specific antibodies is important in preventing infections with *S. pyogenes*. However, knowledge of immunity following infections with GCS/GGS is limited. Most of the reoccurrence of bacteraemia with *S. dysgalactiae* strikes within 6 months from the first episode, and the same *emm* type is often encountered in the second episode⁴¹².

The purpose of this study was to further investigate any presence and function of type-specific antibodies following bacteraemia with *S. dysgalactiae*. Furthermore, this study aimed to determine if these antibodies could confer type-specific protective immunity. To answer all these questions, patients with *S. dysgalactiae* bacteraemia in Region Skåne were prospectively enrolled. Acute sera were collected within 5 days from hospital admission and convalescent sera were obtained 4–6 weeks after onset of hospitalization. Medical records of included patients were reviewed, and sera were analysed for the development of type-specific antibodies. Blood isolates of *S. dysgalactiae* were *emm* typed. Fragments of the M protein, corresponding to the two predominantly *emm* types were synthesised to establish recombinant M proteins. ELISA was performed to detect levels of IgG in the paired acute and convalescent sera. Furthermore, bactericidal assay, as originally described by *Lancefield*^{17, 403}, was applied to detect any enhancement of further opsonisation and killing of the bacteria with the presence of convalescent compared to paired acute sera. In addition, a phagocytosis assay, “the PAN-method”, developed by *de Neergaard et al*¹⁵, was undertaken to investigate any further phagocytosis of the bacteria with the presence of convalescent compared to paired acute sera.

Recurrent bacteraemia with *S. dysgalactiae* was recognized in one patient during the study period. There might have been more patients with recurrent bacteraemia, but the study period was limited. Overall, there were increased levels of IgG in the convalescent sera compared to the paired acute sera towards the bacteria. In addition, increased levels of IgG in convalescent sera compared to paired acute sera were observed towards fragments of the recombinant M protein. Additionally, in one patient with recurrent bacteraemia, type-specific antibodies had been developed in the convalescent sera. However, there was no robust evidence that these antibodies enhanced the killing of the bacteria in the bactericidal assay, nor did these evolved antibodies improve any significant phagocytosis of the bacteria in the phagocytosis assay.

The fact that there was a development of type-specific antibodies but that these failed to opsonise the bacteria may partially explain the tendency of reoccurrence. But as in the study of Lancefield of 1959¹⁷, other immunological aspects that may affect the results were not considered. Some of the included patients had findings of *S. dysgalactiae* perianal or from a wound, and this turned out to be the same *emm* type as in blood, suggesting a carriage or colonisation. A Swedish study by Trelle *et al* detected carriage of BHS in patients with erysipelas, (11/25 patients), in which some patients, (3/9 patients), still were colonized with BHS after concluded antibiotic treatment²¹⁰. Skin and soft tissue infections caused by BHS, e.g., pyoderma reoccur, although, type-specific antibodies to M protein have been detected in patients with GAS pyoderma^{409, 416}. In a study from Australia, the development of type-specific antibodies was observed in mice with induced skin infections with different types of GAS⁴⁰⁹. But the profound immunity was short-lived as there was no presence of signs of development of B-memory cells. Nevertheless, reinfection with the same type within three weeks generated B-cells and long-term immunity was accomplished.

In conclusion, this thesis detected the development of type-specific antibodies following invasive disease with *S. dysgalactiae*. These antibodies failed to opsonise and did not clearly improve the phagocytosis of the bacteria. This lack of opsonising antibodies may partially explain why bacteraemia with *S. dysgalactiae* tends to reoccur. In addition, the lack of antibodies towards the conserved region of the M protein provides a plausible explanation for why cross-immunity against different *emm* types following bacteraemia with *S. dysgalactiae* does not seem to be present. Overall, the results from this study underline the complexity of the antibody responses to BHS.

Paper IV and Paper V

Time to blood culture positivity: an independent predictor of mortality in Streptococcus pyogenes bacteremia

Time to positivity of blood cultures in bloodstream infections with Streptococcus dysgalactiae and correlation to outcome

Finding novel prognostic biomarkers in sepsis is challenging. Heparin-binding protein (HBP) was found to be a valuable tool for detecting infection-induced organ dysfunction in patients with sepsis, (with no regard to aetiology)⁴¹⁷. Other prognostic biomarkers may be C-reactive protein, procalcitonin and so forth, but these biomarkers, including HBP, (although HBP was able to discriminate infection-related organ dysfunction), may also be elevated in non-infectious diseases. TTP is a valuable prognostic factor in bacteraemia with different bacteria^{35, 36}. TTP is the time interval from the insertion of the blood culture bottle into the incubator and the detection of growth, utilizing an automated system. Short TTP may indirectly reflect a higher bacterial concentration and be associated with a severe infection that triggers a heavier immune response by the host. The use of the differential TTP as a part of diagnosing intravascular catheter-related caused bacteraemia is supported by the current guidelines from the Infectious Disease Society of America⁴¹⁸. A time of 120 minutes of differential TTP has been suggested to have a high sensitivity and specificity for diagnosing catheter-related bacteraemia³⁸.

Short TTP has also been suggested to be a tool for determining the risk of IE^{419, 420}, as well as a prognostic factor for 30-day mortality in patients with bacteraemia with major pathogens³⁵. Some researchers suggest that TTP may give a clue into which selected microorganisms are involved⁴²¹, however, this remains to be determined. These studies are of the retrospective design, and knowledge of TTP and correlation with BHS is limited.

Paper IV and V investigated if there was any correlation between levels of TTP and outcome in patients with BHS bacteraemia. Episodes of bacteraemia with *S. pyogenes* and *S. dysgalactiae* occurring in Region Skåne, Sweden were identified and retrospectively studied. Medical records of included patients were reviewed and information on TTP for each episode was obtained from the Department of Clinical Microbiology, Skåne, Sweden. Since IE due to BHS is a rare condition, the study did not have enough power to correlate levels of TTP with the diagnosis. The primary

outcome was set as 30-day mortality, and secondary outcomes as the development of sepsis with or without septic shock within 48 hours from blood culturing.

Short TTP, in patients with *S. pyogenes* bacteraemia, was correlated with 30-day mortality, even after adjustments for covariables such as age, focus of infection, and comorbidities. Focus of infection to the lungs was associated with a fatal outcome. This is in line with other publications, suggesting that pneumonia due to *S. pyogenes* has a severe progression with high mortality rate²⁸¹. Furthermore, any correlation between *emm* types and outcomes was not observed in this study. This may be a bit surprising as several other studies have identified an association of *emm1* and *emm3* to more severe infections¹⁶⁸. The study did perhaps not have enough power to detect such difference, or the lack of such correlation may reflect that different *emm* types do not have to be associated with certain diseases and/or outcomes.

As for *S. pyogenes* bacteraemia, short TTP was correlated with 30-day mortality in patients with *S. dysgalactiae* bacteraemia. The rate of unknown focus was substantially high, and this is in line with another study, that reported a high presence of bacteraemia with unknown origin in patients with *S. dysgalactiae* bacteraemia in Switzerland⁴²².

A recent study from *Hamilton et al*, investigated TTP and its correlation to outcome in bloodstream infections with different organisms³³. Data were from a multicentre randomized controlled trial. In contrast to other described studies, they did not discriminate any association between mortality and TTP in bacteraemia with different organisms. However, candida and BHS seemed to be an exemption from this conclusion, in which longer TTP was associated with a worse outcome for fungemia while shorter TTP was associated with a fatal outcome for BHS bacteraemia³³.

In conclusion, both Paper IV and Paper V indicate that TTP may be a valuable additive prognostic tool and marker for point of care in patients with bacteraemia with BHS. Thus, there are several factors that may influence TTP and the actual value of TTP must be carefully interpreted.

Concluding remarks

- Erysipelas due to GCS/GGS affects older patients with a higher rate of reoccurrence, compared to erysipelas caused by GAS.
- IE due to *S. dysgalactiae* has an acute onset of symptoms with a high rate of embolization. Antibiotic synergy between penicillin G and gentamicin is observed in isolates of *S. dysgalactiae*. However, in most cases penicillin G alone has a bactericidal action so strong that any additional killing with gentamicin is difficult to detect. There is no robust evidence to recommend combination therapy in IE due to *S. dysgalactiae*.
- Type-specific antibodies are developed following invasive infections with *S. dysgalactiae*. These antibodies do not seem to be efficient at opsonising the bacteria. The lack of development of opsonic antibodies may partially explain why bacteraemia with *S. dysgalactiae* reoccurs in the same host.
- TTP is independently associated with 30-day mortality rates in patients with bacteraemia with *S. pyogenes* and *S. dysgalactiae* respectively. TTP may be a useful additional tool in determining the prognosis in patients with invasive diseases due to BHS.

Future perspectives

Each episode of erysipelas can damage the lymphatic vessels and may eventually result in irreversible lymphedema. This in turn is a major risk factor for recurrence of the disease. Optimal management of infections due to BHS is therefore needed to prevent reoccurrence. In addition to adequate antibiotic treatment, compression therapy is important to avoid this vicious circle of erysipelas. Corticosteroid therapy has been suggested for a more favourable outcome in patients with erysipelas, however, the studies have been small and to fully establish any effective treatment a double-blind study should be performed. Future aspects may therefore focus on developing strategies to prevent the reoccurrence of infections with BHS and to find novel strategies capable of treating but also eliminating the streptococci. In patients with recurrent erysipelas, it may be favourable to obtain perianal cultures and cultures from the throat after concluded antibiotic treatment, to look for any host-specific colonisation. If such persistence occurs, a combination of antibiotic treatments may be administered to eradicate the bacteria. Since the same *emm* type often is encountered in patients with recurrent *S. dysgalactiae* bacteraemia, this eradication therapy would potentially not only decrease the reoccurrence rate of erysipelas but also of bacteraemia.

Today invasive diseases due to *S. dysgalactiae* almost equal the burden of invasive diseases due to *S. pyogenes*. *emm* typing is mandatory for invasive infections with *S. pyogenes* in Region Skåne. This has in turn increased knowledge of outbreaks related to the emergence of clonal outbreaks of new or common *emm* types. This epidemiological surveillance system is not established for invasive diseases caused by *S. dysgalactiae*. Several studies have not shown any significant correlation between specific *emm* types and outcomes in bacteraemia with *S. dysgalactiae*, but since we do not routinely perform *emm* typing any possible changes in the epidemiology are difficult to determine. In addition to *emm* typing, the use of WGS may be useful to detect clonal bacterial infections connected to an epidemiological pattern. Genomic analysis of BHS can contribute to identifying important virulence factors or resistance genes and to further understanding of the phylogenetic relationship within BHS. All things considered, the quest to find novel strategies and approaches to tackle infections due to BHS is challenging, but also within reach. The results of this thesis highlight different clinical and microbiological aspects of infections due to BHS and may in turn provide better future diagnostic and prognostic modalities for these infections.

Sammanfattning

Bakterier är en grupp encelliga organismer där vissa har förmåga att attackera det mänskliga immunförsvaret och orsaka sjukdom. Erysipelas, eller i dagligt tal rosfeber är en vanligt förekommande infektion som drabbar människor i olika åldrar. Sjukdomsbilden innebär en rodnad, värmeökning och svullnad i huden. Rodnaden är kraftigt avgränsad och ibland kan det även uppstå blåsor i det drabbade hudområdet. Ofta föreligger det sår i huden som skulle kunna vara ingångsport för bakterier, men i andra fall kan detta saknas. Odlingar från blod och/eller eventuella sår tas innan administrering av antibiotika, men i majoriteten av fall påvisas inte några bakterier. Därför är det svårt att fastställa vilka bakterier som ligger bakom erysipelas. I delarbete I kunde vi konstatera grupp A streptokocker (GAS) och framför allt grupp C och G streptokocker (GCS/GGS) som viktiga bakterier som kan orsaka erysipelas. Dessa bakterier tillhör gruppen beta-hemolytiska streptokocker (BHS). Streptokocker ur gruppen BHS kan delas in i grupp A, C och G utifrån hur de reagerar med särskilda sockerarter. Denna indelning kallad Lancefield- gruppering etablerades på 1950-talet av *Rebecca Lancefield*. Därefter har man med hjälp av tekniken masspektrometri kunnat ytterligare förfina kartläggningen av olika bakterier och artidentifiera bakterier som tillhör grupperna GAS, GCS eller GGS. Tekniken kallas matrix-assisted time of flight mass spectrometry (MALDI-TOF MS). Bakteriearten *Streptococcus pyogenes* uttrycker grupp A antigen och *Streptococcus dysgalactiae* bär oftast grupp C eller G antigen.

Både *S. dysgalactiae* och *S. pyogenes* kan orsaka ett flertal olika infektioner, allt från halsfluss till svåra hud- och mjukdelsinfektioner, sepsis, och hjärklaffsinfektion. Historiskt sett har *S. pyogenes* varit mer känd för allmänheten och kopplad till sjukdomsfall av barnsängsfeber och lunginflammation. Under senare år har dock svåra infektioner orsakat av *S. dysgalactiae* blivit allt vanligare och utgör nu en liknande sjukdomsburda jämfört med infektioner orsakade av *S. pyogenes*. Infektiös endokardit eller hjärklaffsinfektion är en allvarlig infektion som framför allt kan orsakas av *S. dysgalactiae*, men även av *S. pyogenes*. Infektionen är som namnet avslöjar, en infektion på hjärtats klaffar och innebär att små ”bakterieklumpar”, även kallade vegetationer, får fäste på antingen en klaff, inopererad pacemaker eller på hjärtats insida, endokardiet. Infektiös endokardit med BHS är en ovanlig sjukdom och har ett akut och aggressivt förlopp. Tidig antibiotikabehandling är mycket viktigt för överlevnad och

tillfrisknande. Kombinationsbehandling med två olika sorters antibiotika används ofta vid svåra infektioner för att åstadkomma en bättre avdödning av bakterierna jämfört med om ett preparat skulle användas ensamt. Detta kallas för antibiotikasynergi. Internationella terapiriktlinjer rekommenderar en kombination av två olika antibiotikapreparat, penicillin och aminoglykosider, för behandling av infektiös endokardit orsakat av BHS. Bakomliggande evidens för detta är delvis baserat på äldre studier som har visat synergi *in vitro*, det vill säga i laboratoriet. Aminoglykosider kan dock ge svåra biverkningar. I Sverige rekommenderar man behandling med enbart penicillin vid infektiös endokardit orsakat av BHS, baserat på avsaknad av tillräcklig vetenskaplig evidens för kombinationsbehandling. I delarbete II undersökte vi infektiös endokardit orsakat av *S. dysgalactiae* och ställde våra resultat i jämförelse med infektiös endokardit orsakat av andra mer vanliga organismer. Vi kunde fastställa att infektiös endokardit med *S. dysgalactiae* framför allt drabbar äldre patienter, och har ett akut och aggressivt förlopp med en relativt hög dödlighet. Vidare undersökte vi om det föreligger en synergi mellan penicillin G och gentamicin (aminoglykosid) på bakterieisolat av *S. dysgalactiae* i provrör. I flera fall kunde vi urskilja en bättre avdödning när gentamicin var närvarande, men i majoriteten av fallen hade penicillin G ensamt en så potent effekt att ytterligare avdödning med gentamicin inte kunde detekteras med metoden. Sammantaget fann vi inga starka belägg för att det var bättre med kombinationsbehandling vid infektiös endokardit orsakat av BHS.

Kunskap om interaktionen mellan bakterier och det mänskliga immunförsvaret är viktig för att förstå hur vissa organismer kan orsaka sjukdom. *S. dysgalactiae* kan ofta upptäckas upprepande gånger i blodet. Återkommande fynd av bakterien i blodet, kallas återkommande bakteriemi. Under 1950-talet upptäckte *Rebecca Lancefield* antikroppar mot GAS i blod från människor som tidigare haft infektion med bakterien. Man trodde att dessa antikroppar var typspecifika och kunde skydda mot framtida infektioner med GAS. Typspecifika antikroppar skulle nämligen kunna fästa till ytstrukturer på bakterien, så kallad opsonisering, och därmed underlätta för immunförsvarets celler att avdöda bakterien. Detta skulle delvis kunna förklara varför man nästan aldrig får återkommande bakteriemi med *S. pyogenes*. Liknande data har inte studerats när det gäller bakteriemi med *S. dysgalactiae*. I delarbete III inkluderade vi därför patienter med *S. dysgalactiae* i blodet och erhöll initiala blodprov (akut serum) vid inläggning med förnyad blodprovstagnation 4–6 veckor efter att patienten blivit utskriven (konvalescentserum). Blodproven analyserade med hjälp av olika mikrobiologiska samt immunologiska metoder för att utforska om det förelåg en utveckling av typspecifika antikroppar mot *S. dysgalactiae*. Vi konstaterade en utveckling av typspecifika antikroppar i konvalescentserum hos patienter med *S. dysgalactiae* bakteriemi. Dessa typspecifika antikroppar bidrog dock inte till någon

bättre avdödning av bakterien i avdödningsexperiment. Avsaknad av opsoniserande antikroppar skulle delvis kunna förklara hur *S. dysgalactiae* kan orsaka återkommande infektioner i samma värd.

När patienter kommer in till sjukhuset med misstänkt bakteriell infektion tas flera olika blodprov vilket ofta inkluderar blododlingar. Bakteriemi med *S. dysgalactiae* eller *S. pyogenes* är vanligt förekommande och kan vara mycket farligt. Det är dock svårt att förutsäga vilka patienter med fynd av BHS i blodet som kommer att utveckla en sjukdom med dödlig utgång. Tid från att blododlingsflaskan ställs i inkubatorn till detektion av bakterietillväxt, med hjälp av ett automatiserat blododlingssystem, så kallad tid till positivitet (TTP) har visat sig vara ett viktigt prognostiskt verktyg för att avgöra hur stor risk för allvarlig sjukdom bakterierna i blodet utgör. Kort TTP kan innebära en större bakteriekoncentration vilket kan avspegla en svårare infektion hos patienten. Det finns flertal faktorer som kan påverka TTP och informationen bör tolkas med försiktighet. I delarbete IV och V identifierade vi episoder av bakteriemi med *S. pyogenes* och *S. dysgalactiae* under en fyra-årsperiod. Medicinska journaler granskades och information om TTP inhämtades och ställdes i relation med det primära utfallet, som var död inom 30 dagar från det att blododlingen togs. Vi kunde konstatera att vid fynd av *S. pyogenes* eller *S. dysgalactiae* var TTP en oberoende prognostisk faktor för att dö inom 30 dagar. TTP skulle därför kunna vara ett användbart verktyg i den kliniska vardagen för att avgöra vilka patienter som kommer att utveckla en svår sjukdom.

Sammanfattningsvis belyser min doktorsavhandling mikrobiologiska, immunologiska och kliniska perspektiv kring sjukdomar orsakade av *S. dysgalactiae* och *S. pyogenes*. Min förhoppning är att resultaten som presenteras i avhandlingen leder till ökad kunskap och förståelse kring infektioner orsakade av BHS och att detta kan möjliggöra en mer effektiviserad handläggning och optimerad behandling av dessa sjukdomar i framtiden.

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Interactions between β -haemolytic streptococci and the human host

*Nothing in life is to be feared,
it is only to be understood.
Now is the time to understand more,
so that we may fear less.*

Marie Curie

