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Do Polymorphisms in Innate Immunity Genes Predispose for Staphylococcus aureus Bloodstream Infection and Affect Severity and Outcome?

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Do Polymorphisms in Innate Immunity Genes Predispose for *Staphylococcus aureus* Bloodstream Infection and Affect Severity and Outcome?

Abstract

Single nucleotide polymorphisms (SNPs) in genes encoding several of the proteins involved in the human immune system may have an effect on the host's susceptibility for invasive *Staphylococcus aureus* (*S. aureus*) infections. In this study a comparison was made between two SNPs in each of the genes encoding the Toll-like receptor 2 (TLR2) and Toll interacting protein (TOLLIP), and these SNPs' correlation with the predisposition for, severity and outcome of sepsis by *S. aureus* infection. Blood samples from patients with *S. aureus*-caused sepsis at Ahus were collected in the period from the 1st of March 2011 through the 28th of February 2013. Control samples were collected from healthy carriers (elective, orthopaedic patients) of *S. aureus* during two periods; the first batch was collected from the 1st of March 2011 to 12th of June 2014 and the second from the 9th of September 2013 to 12th of June 2014 at Ahus. All samples were analyzed using PCR.

For TOLLIP rs5743942, TLR2 rs5743704 and TLR2 rs5743708, no statistically significant difference was observed in the distribution of SNPs (P> 0.05) between the sepsis group and the control group. For TOLLIP rs5743867 there was a significant difference (P=0.046) with Chi-square test, but neither the crude nor adjusted multinomial logistic regression model could detect a significant difference. SNP variant had no effect on the severity or mortality following sepsis.

The susceptibility, severity and mortality of *S. aureus* bloodstream infections cannot be attributed merely to these point mutations in the genes encoding the TLR2 and TOLLIP proteins. The underlying mechanisms of sepsis are probably more complex, and may involve specific characteristics of both the pathogen and the host that might have a certain "lock-and-key" aspect to it.

Sammendrag

Enkel nukleotid polymorfismer (SNPs) i gener som koder for flere av proteinene involvert I det humane immunsystem kan ha en effekt på vertens risiko for invasive *Staphylococcus aureus* infeksjoner. I denne studien ble to SNP'er i hver av genene for Toll-liknende reseptor 2 og Toll-interagerende protein korrelert med risikoen for-, alvorlighetsgraden- og utfallet av sepsis, forårsaket av *S. Aureus*. Blodprøver fra pasienter med *S. aureus* positiv blodkultur på Ahus ble samlet inn i perioden fra 1. Mars 2011 til 28. Februar 2013. Kontrollprøver ble samlet inn fra friske bærere (elektive, ortopediske pasienter) av *S. aureus* i to perioder. Første gruppe ble samlet inn fra 1. Mars

2011 til 12. Juni 2014, andre gruppe fra 9. September 2013 til 12. Juni 2014 på Ahus. Alle prøvene ble analysert ved hjelp av PCR.

For TOLLIP rs5743942, TLR2 rs5743704 og TLR2 rs5743708 var det ingen statistisk signifikant forskjell i distribusjon av alleler (P>0.05) mellom sepsisgruppen og kontrollgruppen. For TOLLIP rs5743867 var det signifikant forskell i Kji-kvadrat test (P=0.046) men ingen signifikant forskjell ved justert og ujustert multinomial logistisk regressjonsmodell. SNP variant hadde ingen effekt på alvorlighetsgraden og mortaliteten av sepsis.

Mottakelighet, alvorlighetsgrad og mortalitet forårsaket av *S. Aureus* infeksjon i sirkulasjonssystemet kan ikke tilskrives kun punktmutasjoner i disse polymorfismene i genene for TLR2 og TOLLIP. Mekanismene involvert i utviklingen av sepsis er antakelig mer komplekse, og involverer muligens spesifikke karakteristikker i både vert og bakterie med et visst lås-og-nøkkel aspekt ved seg.

Contents

Preface

This study is part of a larger project at Akershus University Hospital (AHUS), with the goal of characterizing molecular factors of *S. aureus* causing bloodstream infections (BSIs). Based on earlier work by Aamot *et al*. and Blomfeldt *et al*. it was evident that more clinical data was necessary for the study, and so a prospective study was organized.

This project was named "Project Invasive" and it was begun in March 2011. It includes all patients with *S. aureus* positive blood cultures at AHUS in a three year period from March 1st 2011 to February 28th 2013. The study is two-part, focusing on both bacterial characteristics and host characteristics of infection. The bacterial part is led by Anita Blomfeldt in her PhD project "Invasive *Staphylococcus aureus* infections – microbial genetics, epidemiology and clinical features" while the part regarding the host factors are headed by PhD Hege Vangstein Aamot.

This thesis is a sub-part of the host characteristics part led by H. V. Aamot, of the aforementioned prospective study.

The project was financed by AHUS strategical study grants, and all project work was done at the hospital labs under the direction of the Research and Development unit of the department for Microbiology and Infection Control.

This project was approved by the Regional Committees for Medical and Health Research Ethics (no. 2009/2149).

I would like to express my sincere gratitude to PhD Hege Vangstein Aamot, my primary supervisor for this project, and the Research and Development unit of the department for Microbiology and Infection Control at Akershus University Hospital for their help and advice in this process.

I would also like to thank my family and friends for their continued support and understanding.

Oslo, April 2015.

Abbreviations

MAF – Minor allele frequency

SNP – Single nucleotide polymorphism

PRR – Pattern recognition receptor

PAMP – Pathogen-associated molecular pattern

SNP – Single Nucleotide Polymorphism

BSI – Blood stream infection

MRSA – Methicillin resistant Staphylococcus aureus

TLR2 – Toll-like receptor 2

TOLLIP – Toll-interacting protein

PCR – Polymerase chain reaction

Introduction

Staphylococcus aureus

Staphylococcus aureus (*S. aureus*) was discovered in 1880 by a surgeon named Sir Alexander Ogston who routinely examined microscope slides from postoperative wound infections (Ogston, 1882). Because of the grape-like clusters of bacteria seen when viewed in the microscope he called them *Staphylococcus*, meaning "a bunch of grapes" in Greek. It was not until 1884 that the bacteria got the name *aureus*, when another scientist observed the yellow color the bacteria had when cultured (Rosenbach, 1884). After its discovery, *S. aureus* was identified as the cause of a large variety of human diseases ranging from minor skin infections to invasive, often deadly infections like sepsis, osteomyelitis, and endocarditis (Lowy, 1998; Moreillon, Que, & Glauser, 2005).

Bacteria are often divided into those that are gram positive, and those that are gram negative. This distinction is made based on the characteristics of their cell walls and how it affects staining. Gram positive bacteria like *S. aureus* have a cell wall composed of a thick peptidoglycan layer (Tortora, *et al*., 2010). Gram negative bacteria, in contrast, have a cell wall containing a thin layer of peptidoglycan and lipopolysaccharides (LPS), which are absent in gram positives (Tortora, *et al*., 2010). Peptidoglycan is a two-part structure containing disaccharides and polypeptides. The disaccharide part provides the backbone of the structure, while the peptide part provides the means to link the saccharides together in layers (Tortora, *et al*., 2010). Gram positive bacteria contain several layers of peptidoglycan and when stained the crystal structures that form are trapped in the peptidoglycan layer, making the bacteria appear blue or purple in the microscope (Tortora, *et al*., 2010).

The plasma membrane is located underneath the cell wall, it contains primarily phospholipids and proteins, and in prokaryotes it is less rigid than in eukaryotes, as most bacteria lack sterols in their membranes (Tortora, *et al*., 2010). Within the space enveloped by the plasma membrane is the cells cytoplasm, a medium containing enzymes, ions, lipids, carbohydrates, ribosomes and other substances (Tortora, *et al*., 2010). One of the large structures in the cytoplasm is the nucleoid, which contains the bacterial DNA required for the operation of the cell. In addition to the genetic information carried in the bacterial chromosome, genetic information can also be contained in what is called plasmids, which are circular double stranded DNA molecules (Tortora, *et al*., 2010). These can enter and exit the cell without harming it, and may carry genes that enable resistance to antibiotics, or genes for the production of toxins (Tortora, *et al*., 2010). Plasmids can also be transferred between bacteria, which may spread resistance to antibiotics (Tortora, *et al*., 2010).

The number of community acquired *S. aureus* infections has steadily increased during the past 25 years, and the bacteria is ranked as the second most frequent cause of nosocomial (hospital acquired) bloodstream infections (BSIs) (Pittet, *et al*., 1994; Steinberg, *et al*., 1996; Lowy, 1998; Richards, *et al*., 2000; Wisplinghoff, *et al*., 2004; Wertheim, *et al*., 2004).

A bit under one third of the population is what is called healthy carriers of *S. aureus*, meaning that they are colonized by the bacteria, but not necessarily develop infections (van Belkum, *et al*., 2009). The most frequent site of colonization is the front of the nose (Aly & Levit, 1987; Hoefnagels-Schuermans, *et al*., 1999).

Most of the nosocomial infections are endogenous, meaning that the genotypes of the carried and infecting strains are the same (Yu, *et al*., 1986; Luzar, *et al*., 1990; Von Eiff, *et al*., 2001). These infections usually occur when the bacteria winds up at vulnerable locations in the patient. *S. aureus* can spread from the infected locations to the blood, causing sepsis, which is a common secondary infection following *S. aureus* infections. Sepsis is difficult to treat, with high morbidity and a high mortality rate. The difficulty of treatment leads to prolonged hospital-stays, which is expensive. Frequent hospital visits, comorbidities, patient age and use of invasive procedures and implants may increase the number of BSIs, and there is a lack of effective vaccines. Efforts should perhaps instead be made to improve treatments that reduce the severity and length of infections.

S. aureus has shown a great ability to adapt and develop mechanisms to counter the effects of a large number of antibiotics and modern treatments, with some species virtually resistant to a broad range of antibiotics, like methicillin resistant *S. aureus* (MRSA) (van Belkum, *et al*., 2009). Being colonized with a MRSA causes no immediate threat to the carrier, but if the MRSA causes an infection in the host there would be fewer options for treatment. If the infection was of the more severe kind, then the MRSA could pose a very real problem.

Although some characteristics of the more infectious *S. aureus* strains have been identified, there is still uncertainty about which factors play a major role in the pathogenicity of the bacteria (van Belkum, *et al*., 2009). *S. aureus* has several ways of interacting with the host, and the relationship between the mechanisms of infection by the bacteria, and the hosts resistance and susceptibility to infection needs to be explored in order to effectively treat or defend against colonization (van Belkum, *et al*., 2009).

S. aureus is a common bacteria found in 20-30% of the population (van Belkum, *et al*., 2009; Tortora, *et al*., 2010; Kong & Jabra-Rizk, 2015). Most of the strains that cause disease are coagulase positive, meaning that they produce an enzyme that is able to clot fibrin in the blood, which contributes to

bacterium's pathogenicity (Tortora, *et al*., 2010). *S. aureus* has several ways of evading the host immune response to the infection. Many strains produce a protein that can block chemotaxis, which hinders the recruitment of neutrophils (a type of white blood cells) to the site of infection; it can often also produce toxins that directly kill the neutrophils at the site (Tortora, *et al*., 2010). The bacterial cell wall is lysozyme (an enzyme that breaks down bacterial cell walls) resistant, so that if the bacterium does get phagocytized, it can resist the detrimental effects of the phagocytes lysozyme (Tortora, *et al*., 2010).

Virulence is a term used to describe the pathogenicity of a microbe. The ability to bypass barriers such as skin and mucosa, and to which degree it has the ability to cause disease, are all contributing to a species' virulence (Tortora, *et al*., 2010). In the case of *S. aureus*, there are several virulence factors involved. Among these are enzymes like lipase and beta-lactamase which help the bacterium break down lipids and defend against antibiotics (beta-lactams), respectively (Tortora, *et al*., 2010). Other virulence factors include toxins like the Panton-Valentine leukocidine (PVL) bicomponent, which can cause necrotizing pneumonia, a severe disease in which cells of the respiratory system undergo autolysis (Tortora, *et al*., 2010).

Bacteremia and sepsis

When microbes are present in the bloodstream the condition is called a bacteremia (Tortora, *et al*., 2010). It is often a result of invasive medical procedures like the use of catheters and intravenous feeding tubes (Tortora, *et al*., 2010). Although bacteremia are relatively common, and rarely cause severe disease in healthy individuals, the condition may worsen in certain conditions like in the case of immunosuppressed individuals (Tortora, *et al*., 2010). In these cases the infection is termed a septicemia, which is a condition in which bacteria or their toxins persists in the circulatory system. The term sepsis is also used for this condition but is, according to Tortora, defined as "*a systemic inflammatory response syndrome (SIRS) caused by a focus of infection that releases mediators of inflammation into the bloodstream*" (Tortora, *et al*., 2010). The condition is termed a SIRS if at least two defined conditions are met: fever, increased heart or respiratory rate and increased white blood cells (Tortora, *et al*., 2010). Dellinger and co-workers define sepsis as "*the presence (probable or documented) of infection together with systemic manifestations of infection*" (Dellinger, *et al*., 2013). If the body is unable to effectively clear the SIRS the disease can progress into severe sepsis, which is a drop in blood pressure resulting in dysfunction of at least one organ (Dellinger, *et al*., 2013). The final, and often fatal, stage of progression is called septic shock, which is when blood pressure cannot be controlled by the addition of fluid to the bloodstream (Dellinger, *et al*., 2013).

Sepsis can be caused by both gram negative- and gram positive bacteria. Gram negative bacteria most often give rise to septic shock, due to their endotoxic nature which can drastically reduce blood pressure (Tortora, *et al*., 2010). Gram positive bacteria, however, are the most common cause of sepsis (Tortora, *et al*., 2010). The treatment of sepsis poses a medical challenge, because many of the symptoms exhibited by the disease is common in many diseases, and does not present themselves specifically in sepsis (Tortora, *et al*., 2010). Furthermore, once the condition is finally identified as sepsis, the administration of antibiotics may worsen the prognosis, especially in the case of gram negative bacteria, as the lysis of these microbes cause the release of endotoxins (present in the lipopolysaccharide layer of the bacterium), which can cause a drop in blood pressure (Tortora, *et al*., 2010).

A nationwide surveillance study on nosocomial BSIs in U.S. hospitals reported *S. aureus* as the second most frequent cause of BSI, with 20% of cases attributed (Wisplinghoff, *et al*., 2004). BSIs are generally serious infections with a high mortality rate. In a study done in a surgical intensive care unit between 1988 and 1990, an estimated 35% of all deaths were attributed to BSIs (Pittet, *et al*., 1994).

Immune system

The human immune system is comprised of the innate immune system and the adaptive immune system. The adaptive immune system is commonly referred to as the "second line of defense" and involves the activation of B and T cells, and antibodies (Bogen & Munthe, 2007). The innate immune system, the "first line of defense", involves the complement cascade, recruitment of leukocytes to sites of infection, and also all the physical and chemical barriers (e.g. skin, mucosa, and pH of the stomach) microbes have to pass through (Bogen & Munthe, 2007).

When a pathogen first passes through a mechanical barrier (e.g. skin), it is recognized by what is called resident leukocytes, often macrophages, which are monocytes that have further differentiated (Bogen & Munthe, 2007). These macrophages display on their surface pattern recognition receptors (PRRs) which binds to conserved areas of microbes, for instance portions of the LPS (lipopolysaccharide) layer of gram negative bacteria, or lipoteichoic acid in the cell wall of gram positive bacteria (Arancibia, *et al*., 2007). When the macrophages have recognized the bacteria, their gene expression is altered so that they start producing and releasing cytokines, which are signals of the immune system (Sand, *et al*., 2007). These signals have different effects on the immune system, and one of them is the recruitment of more cells to the site of infection through a cytokine mediated process called chemotaxis (Sand, *et al*., 2007; Bogen & Munthe, 2007).

Among the cells that are recruited are other phagocytes. These are cells that ingest pathogens and then break them down, mainly by enzymatic activity (by lysozyme) which breaks down the bacterial

cell wall (Tortora, *et al*., 2010). Dendritic cells are another type of cell recruited to the site of infection. These cells have the ability (along with macrophages) to present peptides produced by the destruction of pathogens to lymphocytes located in the lymph nodes (Bogen & Munthe, 2007). This mechanism involves the activation of the adaptive immune system (Bogen & Munthe, 2007).

Another important part of the innate immune system is the complement cascade. Complement is proteins circulating in the blood as inactive precursor proteins, which are cleaved by proteases at the onset of an infection, turning them into active proteins (Bogen & Munthe, 2007). As the name implies, the complement system complements other parts of the immune system, for instance by binding to microbes, targeting them for phagocytes (Bogen & Munthe, 2007). This is a process known as opsonization; hence some proteins of the complement cascade are also called opsonins (Sand, *et al*., 2007). In addition, when complement proteins binds to bacteria, they ultimately attack the bacterial cell wall through a complex biochemical cascade involving over 30 different complement proteins, which help to kill the bacteria (Bogen & Munthe, 2007).

Single nucleotide polymorphisms

Single nucleotide polymorphisms (SNPs) pronounced "snips" constitute the major genetic variation in the human genome (Alberts, *et al*., 2004). SNPs are point mutations in the genome that vary in nucleotide sequence between individuals. To be classified as an SNP, there must be more than 1% variation in the population at a specific genomic location (Nature Education, 2015). One individual may have an A-T pair at one location where another individual has a G-C pair (Alberts, *et al*., 2004). More than 3 million SNPs have been located in the human genome (Alberts, *et al*., 2004). Variations in the DNA sequences of humans can affect how humans develop diseases and respond to pathogens and other agents. Analysis of single nucleotide polymorphisms (SNP) may be used to compare regions of the genome between cohorts, in this case patients with and without *S. aureus* BSIs.

SNPs are referred to by their frequency in a population, which can vary between ethnic groups. This frequency is called the minor allele frequency (MAF), and it is defined by NCBI to be the second least common allele observed in a population (NCBI, 2015). NCBI operates with the term global minor allele frequency (GMAF) which is the second least common allele observed in a worldwide population of about 1000 individuals.

Toll-like receptor 2

Toll like receptors (TLRs) are a type of pattern recognition receptors (PRRs) which recognize structurally conserved regions of microbes, denoted pathogen-associated molecular patterns (PAMPs) (Chang, 2010). There are currently 11 known types of TLRs in the human genome and they recognize different types of ligands (Chang, 2010).

TLR2 is a membrane protein expressed on the surface of many white blood cells, including granulocytes (Kurt-Jones, *et al*., 2002; Chang, 2010). The protein binds to PAMPs of gram positive bacteria and activates host immune responses to infection (Takeuchi, *et al*., 1999). When TLR2 is bound to its ligand a series of downstream events including several adapter proteins leads to the release of NF-ƙB (which is a transcription factor) into the cell nucleus where it can alter the gene expression (Figure 1), resulting in the production of inflammatory cytokines (O'Neill, *et al*., 2009).

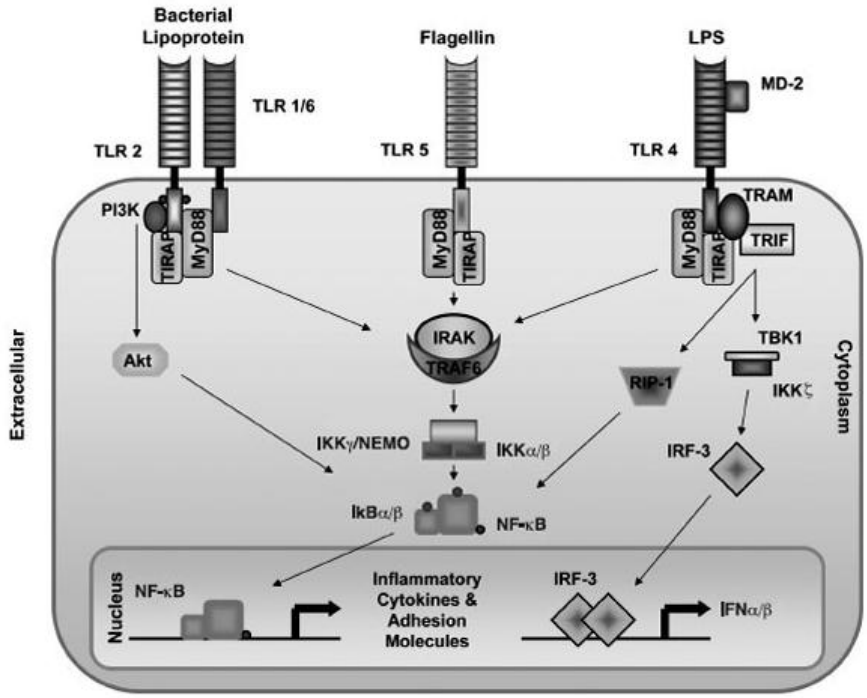

Figure 1. TLR signaling activation by bacterial ligands. Binding of a bacterial ligand to TLR2 creates a downstream signaling event that ultimately alters the gene expression through NF-ƙB, promoting the production and release of cytokines and adhesion molecules.
(http://www.getdomainvids.com/keyword/tlr2%20pathway/)

Toll like receptor 2 is encoded by the Toll-like receptor 2 gene which is conserved in drosophila and humans (Rock, *et al*., 1998). It is located on chromosome 4 and has a length of 2600 base pairs. The TLR2 protein contains 784 amino acids (NCBI, 2015). TLR2 has several SNPs, rs5743704 is a base change from C to A with GMAF A = 0.0096. This change causes what is known as a missense mutation (a type of non-synonymous base substitution), which means that it causes an amino acid change in the protein (Watson, *et al*., 2008). In the case of rs5743704, the amino acid substitution is a change from proline to histidine (Pro631His). Pro631His was associated with increased susceptibility to complicated skin and skin structure infections (cSSSIs) in an article by Stappers and co-workers (Stappers, *et al*., 2014). It has also been associated with reduced activation NF-ĸB (Ben-Ali, *et al*., 2011). Additionally, it has been associated with a higher risk of recurrent vulvovaginal candidiasis in women, and possibly a reduced production of IL-17 and IFNγ (Rosentul, *et al*., 2014), which are cytokines of the immune system (Bogen & Munthe, 2007).

Rs5743708 is a change from G to A with the GMAF being A = 0.0068. This is also a missense mutation causing an arginine to glutamine amino acid substitution (Arg753Gln). Rs5743708 is one of the most well-studied SNPs in the TLR2 gene and many articles is based on the results found in a study by Lorenz and co-workers, where they suggest that this specific SNP is correlated with increased susceptibility to gram positive septic shock (Lorenz, *et al*., 2000). It has also been shown to influence the risk of developing tuberculosis (Ogus, *et al*., 2004) and acute reactive arthritis after infection by *Salmonella enteritidis,* described by Tsui and co-workers (Tsui, et al., 2008). Woehrle and co-workers linked Arg753Gln to altered cytokine release after infection with *Candida* but not with gram positive sepsis (Woehrle, et al., 2008).

Toll interacting protein

Toll interacting protein, or TOLLIP, is an adapter protein that has been shown to interact with several proteins of the immune system, including TLR2 (Zhang & Ghosh, 2002). The TOLLIP gene is located on chromosome 11, consists of 3665 base pairs and translates to a 274 amino acid length protein (NCBI, 2015).

TOLLIP acts as an inhibitory protein by controlling the magnitude of response to the binding of a ligand to a TLR. This mechanism occurs downstream of the MyD88 and TIRAP (which are adapter proteins of the TLR signaling complex) of the TLR by inhibiting Interleukin-1 receptor associated kinase 1 (IRAK1) (Schimming, *et al*., 2007) shown in Figure 2.

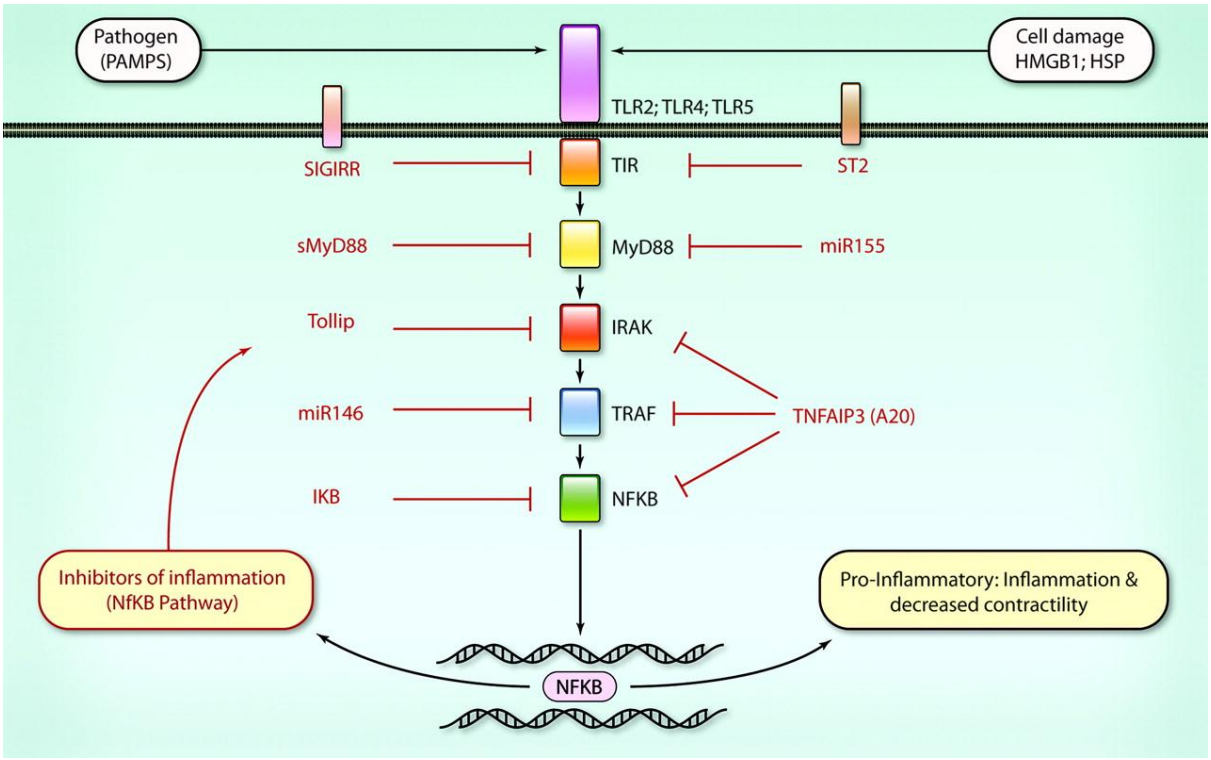

Figure 2. Overview of the different adapter proteins involved in the TLR adapter complex. Tollip inhibits IRAK, altering NF-ƙB activation.
(http://circres.ahajournals.org/content/110/1/126/F2.expansion.html)

SNPs in the TOLLIP gene are not very well studied, however a few SNPs has been described. Rs5743942 has a GMAF of G=0.3890 and is a polymorphism in an intron. Rs5743867 has a GMAF of G=0.2117 and is also a polymorphism in an intron. A variant of rs5743867 has recently been associated with decreased risk of sepsis in the Chinese Han population (Song, *et al*., 2011). Rs5743942 was associated with increased susceptibility to sepsis (Song, et al., 2011) and tuberculosis (Shah, *et al*., 2012).

Polymerase chain reaction

Polymerase chain reaction, or PCR, is a technique in which one selects a small part of a genome to amplify (Brown, 2007). Several components are needed for the process including primers, template, an instrument that is able to change heat in timed steps, nucleotides and polymerase (Sjøberg, 2006; Brown, 2007). PCR is based on DNA's ability to separate (or denature) at certain temperatures, and then anneal (renature) when the temperature falls again (Brown, 2007). Primers (forward and reverse), are small sequences of DNA that is complementary to each strand of the genomic DNA,

flanking the site of amplification (Brown, 2007). These are selected, primarily, based on specificity and melting temperature, meaning that they should be complementary only to one specific part of the genome and have a melting temperature that is different from the melting temperature of the amplified DNA section (Sjøberg, 2006). First, DNA is separated by increasing the heat of the block in the PCR instrument to about 95˚C, the temperature is then reduced to between 40˚C to 70˚C, where the primers attach to the complementary sequences (Sjøberg, 2006).This specific temperature, which is derived from the length and types of bonds in the primer sequence, is also selected with the temperature optimum of the polymerase in mind (72˚C for Taq DNA polymerase (Sjøberg, 2006)). A commonly used polymerase is the heat stable Taqman polymerase, isolated from the bacterium *Thermus aquaticus* (Sjøberg, 2006; Brown, 2007). The polymerase starts to synthesize the new strand from the primer with the added nucleotides, and in the first cycle it then continues indefinitely (Sjøberg, 2006). In the next cycle however, the primers bind to the newly synthesized strands, and these copies has defined ends, in other words, they end where the complementary strand begins (Sjøberg, 2006). This is why it is called a chain reaction, as it causes the number of DNA copies to increase in an exponential fashion (Brown, 2007). The principle of the PCR reaction can be seen in Figure 3.

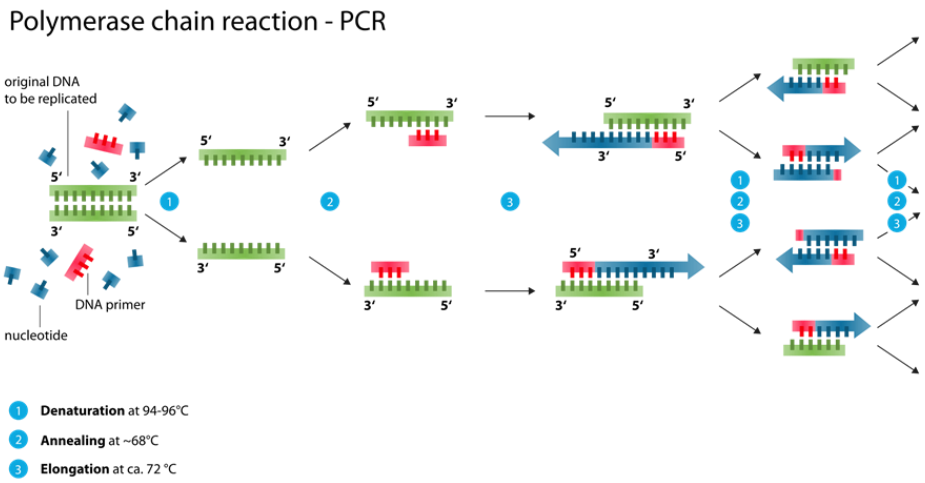

Figure 3. Principle of the PCR reaction. Primers attach to each strand of the melted template DNA (green). Polymerase synthesizes new strands starting at the primer 3' end. In the next cycle, new primers attach to the products from the first cycle. Subsequent reactions synthesize new strands of target DNA in an exponential fashion.

(http://en.wikipedia.org/wiki/Polymerase_chain_reaction)

Probes

PCR can be used for the detection of a certain base or variation, in our case a SNP (Sjøberg, 2006). This is facilitated by the use of a probe. A probe is a small sequence of DNA complementary to the allele of interest (Sjøberg, 2006). There are several types of probes. Only Taqman probes will be described further. The Taqman probes are marked with a fluorophore, which is a chemical compound that is able to absorb light of a specific wavelength and emit light at another wavelength (Sjøberg, 2006). The probe is designed in such a way that the fluorophore only emits light in the absence of what is called a quencher (Sjøberg, 2006). The quencher and the fluorophore are originally attached to the probe in close proximity to each other. However, when the polymerase of the PCR reaction synthesizes the new strand, the probe is cleaved by the 5' -> 3' exonuclease activity of the polymerase, separating the fluorophore from the quencher (Sjøberg, 2006), shown in Figure 4. This allows the fluorophore to emit light or a signal (Sjøberg, 2006). After the PCR is completed, the amount of signal in the end product can be measured. If there is no signal the relevant allele is not present and vice versa (Sjøberg, 2006).

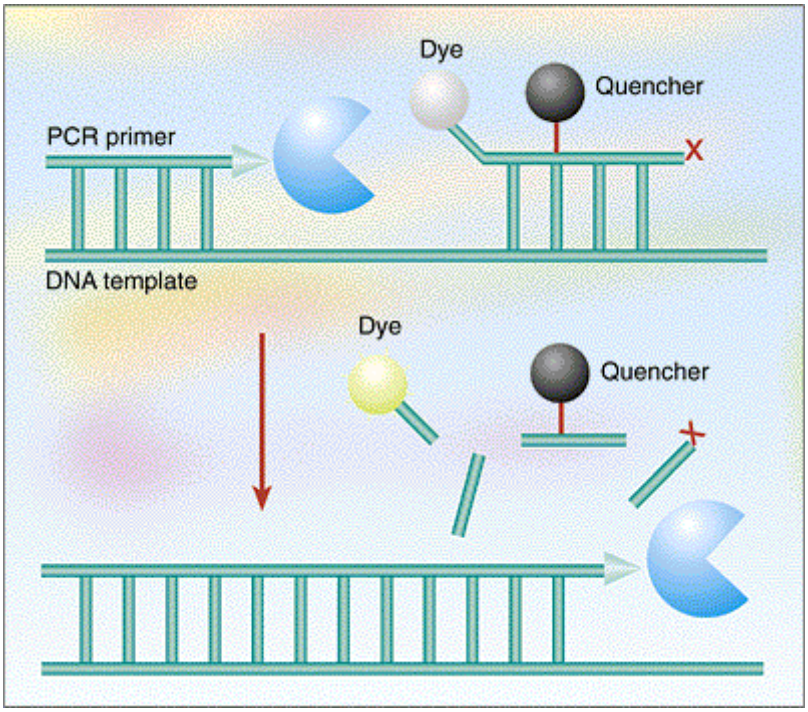

Figure 4. Principle of a Taqman PCR probe. The probe binds to the complementary sequence of the amplified PCR product. The fluorophore (dye) is separated from the quencher as the polymerase synthesizes the new strand, enabling it to emit signal.

(http://dyes.gene-quantification.info/)

Overview

It is important to establish an understanding of the many mechanisms of infection displayed by *S. aureus*, be it characteristics of the bacteria and its virulence factors, or host defenses against infection. Any *S. aureus* can potentially give rise to severe infections, and with better understanding of the underlying mechanisms, one can more easily employ strategies to reduce the incidence of infection by *S. aureus*, and counter the spread of antibiotic resistant strains. To get a better understanding of the mechanisms involved, one can compare traits in people that get infections versus people that do not get infections.

Aim

The aim of this study was to examine the possible association between SNPs in the genes encoding TLR2 and TOLLIP and predisposition, severity, and outcome of *S. aureus* blood stream infection. Specifically, two SNPs in TLR2; rs5743704 and rs5743708, and two SNPs in TOLLIP; rs5743867 and rs5743942 were investigated.

Materials and methods

Patient groups

All patients >18 years diagnosed with *S. aureus* infection in at least one positive blood culture from the 1st of March 2011 through the 28th of February 2013 at Akershus University Hospital (Ahus), Lørenskog, Norway were included in the study. Ahus is a 650 bed public secondary hospital with a catchment area of nearly 500.000 inhabitants or close to 10% of the Norwegian population. EDTA blood from these patients was gathered for analysis. Clinical information including sex, age, immune status, 30-days all-cause mortality, and severity of infection categorized as sepsis, severe sepsis or toxic shock, according to the criteria specified by Dellinger and co-workers (Dellinger, *et al*., 2013) were collected.

The control group consisted of patients with elective orthopedic surgery (knees, hips and lumbar surgery). Patients were included during two periods; the first batch was collected from the 1st of March 2011 to 12th of June 2014 and the second from the 9th of September 2013 to 12th of June 2014 at Ahus. The control patients were included based on certain criteria, namely that they 1) were of Caucasian ethnicity, 2) were patients healthy enough to undergo orthopedic surgery, 3) did not have ongoing infections and 4) they did not have postoperative infections within 1 year after surgery.

Extraction

The EDTA blood samples were centrifuged for 10 minutes at 3000g. DNA was extracted from white blood cells on a QIAGEN symphony instrument (QIAsymphony SP, cat. no. 9001297) using the buffy coat portion of the EDTA blood. A commercial QIAsymphony DNA Kit (QIAsymphony DSP DNA Mini Kit, cat. no.937236), available from the manufacturer of the instrument, was used for the extraction. DNA concentrations were measured after extraction in a few samples on a NanoDrop 1000 instrument (Thermo Scientific). All measured samples had a DNA concentration in the range 30ng/µL – 70ng/µL. Ideal concentrations for PCR were 10ng/µL, but instead of diluting all the samples to this concentration individually, the samples with purified DNA was diluted with a factor, resulting in concentrations between 1-20 ng/µL. This was done because the categorical nature of the results did not warrant exact PCR CT-values, and for efficiency. Samples without results due to low DNA concentrations were re-run on the PCR, eliminating false negatives, ensuring that all samples got a result.

PCR

A lot of time and effort went into optimizing the PCR process; at first the HT7900 was unable to cluster the samples, even after several attempts with varied concentrations of ingredients. A decision was made to try another assay/master mix manufacturer and PCR system from Matrix. This new

system gave results but offered considerable increased workload as the Matrix system did not support 384-well plates, and it was unknown whether Life Technologies' master mix would work on the Matrix PCR system. Ultimately, after a lot of trial and error, it was found that the problem was in the HT7900 instrument clustering algorithm, and not in the concentrations specified by the protocol. The results from the HT7900 were therefore imported into the software "Taqman Genotyper Software", available from Life Technologies which had an updated algorithm able to cluster the results correctly.

The included SNPs are presented in Table 1. The PCR reaction mix was prepared according to the protocol provided from Applied Biosystems, shown in Table 2, with a small modification of added water to adjust the final volume to 5µL per well. TaqMan genotyping master mix, TaqMan genotyping assay mix and nuclease-free water were added to the reaction mix. All reagents used were obtained from Applied Biosystems.

Table 1. Overview of included SNPs

	SNP Rs#	Amino acid substitution	Commercial Assay ID	Type of polymorphism	Frequency (GMAF*)	PubMed Articles
TLR2	5743704	Pro631His	C_25607736_10	A/C (missense)	GMAF: A=0,0096	PMCID: PMC4172055
TLR2	5743708	Arg753Gln	C_27860663_10	A/G (missense)	GMAF: A=0,0068	PMID: 18975326
TOLLIP	5743942	-	C_1582183_10	C/T (intron variant)	GMAF: G=0,3890	PMCID: PMC3428135
TOLLIP	5743867	-	C_1582169_10	C/T (intron variant)	GMAF: G=0,2117	PMCID: PMC3222042

*GMAF is the second least frequent allele observed in the NCBI global population of 1000, acquired from NCBI (NCBI, 2015).

Table 2. PCR Reaction mix

Component	5µL Reaction (384-well plate)
TaqMan Genotyping Master Mix (2X)	2,50µL
TaqMan Genotyping Assay Mix (20X)	0,25µL
Nuclease-free water	0,25µL
DNA	2µL
Total	**5µL**

The reaction mix was then added to a MicroAmp™ Optical 384-Well Reaction Plate, in addition to 2µL of 1-20ng/µL DNA. The plate was then covered with a MicroAmp Optical Adhesive Film before it was placed in a HT7900 Real-time PCR instrument (Life Technologies, catalog number 4329001). The HT7900 thermal cycling conditions were programmed according to the protocol provided, as shown in Table 3.

Table 3. PCR Program for HT7900

Step	Temp (˚C)	Duration	Cycles
AmpliTaq Gold, UP Enzyme Activation	95	10 min	HOLD
Denature	95	15 sec	40
Anneal/Extend	60	1 min	

A pre-PCR plate-read was run before starting the PCR reaction, and an endpoint run was done after the reaction had finished for removal of background signals. The PCR results were imported into the software "Taqman Genotyper software" for cluster analysis.

Samples were analyzed in parallels, and 10% of all samples were re-run as technical controls to verify the results.

Statistics

Statistical tests were done in the SPSS Statistics software, version 21, available from IBM. The sepsis and control group was compared with regards to the distributions of alleles in the two groups; the data was analyzed with Chi-Square test. In addition, multinomial logistic regression was done for one SNP, TOLLIP rs5743867. The α-level, or p-value, was set to 5%.

Statistical Power

The statistical power of a test is a measure of a tests ability to detect an effect if there actually is one, or in other words, the probability of correctly accepting the alternative hypothesis when the alternative hypothesis is true (Moore & McCabe, 2006).

Statistical power was calculated to establish the number of required samples. To detect a difference of 10% between patients (SNP frequency 20%) and controls (SNP frequency 10%) with a power of 80% and p=0.05, two-sided test, a total of 165 patients and 297 controls was needed.

In this study a total of 264 BSIs and 297 controls were collected, well above the sample size required to detect a difference.

Results

In the study period, there were 365 patients with *S. aureus* positive blood culture. Of these 29 was polymicrobial, 7 was from non-residents, 5 was deemed contaminated (no symptoms and left untreated) and 12 was from patients under 18. This summed up to a total of 312 patients with *S. aureus* positive blood cultures, of which 9 was unavailable for inclusion. Out of the remaining 303 cultures, 264 had extra blood for extraction. The control group consisted of 297 patients. Clinical characteristics of the patients are displayed in Table 4.

There were no statistically significant difference in SNP allele distribution between the control group and the sepsis group for TOLLIP rs5743942, TLR2 rs5743704 or TLR2 rs5743708 ($P>0.05$ for all comparisons), as shown in Tables 6 and 7. No statistically significant difference was observed in the distribution of alleles between the sepsis and severe sepsis categories, or in the 30-days all-cause mortality; alive vs. dead categories. The difference in the case of TOLLIP rs5743867 was statistically significant in the Chi-square test, however in the age- and sex-adjusted multinomial logistic regression model, shown in Table 5, it was not.

Comparing the sepsis group versus the control group in table 4 shows that the distribution of male and female patients, along with age and the age range for these patients vary in the two groups. The median age is higher in the sepsis group along with the upper age range. Additionally there are more men and fewer women in the sepsis group.

Table 4. Number of patients by category, age and range, and mortality.

	Controls	Sepsis total	Sepsis immuno-competent*	Sepsis	Severe sepsis and toxic shock	Sepsis immuno-competent*	Severe sepsis and septic shock immuno-competent*
Patients (N)	297	264	209	212	52	169	40
Median age (range)	66 [18-88]	71 [18-95]	70 [21-95]	-	-	-	-
Male (N)	136	166	130	137	29	107	23
Female (N)	161	98	79	75	23	62	17
30-days all-cause mortality	-	44	32	21	23	16	16

*Immunosuppressed patients are excluded

Table 5. Crude and adjusted multinomial logistic regression model for TOLLIP rs56743867

TOLLIP rs5743867	N	OR	95%CI	p-value	OR adjusted	95% CI adjusted	p-value adjusted
G/G*	6						
A/A	406	7.69	(0.89 to 66.41)	0.064	6.90	(0.75 to 63.27)	0.088
A/G	94	5.68	(0.64 to 50.51)	0.119	5.03	(0.53 to 47.57)	0.158

* Reference category

OR; odds ratio, CI; confidence interval

Table 6. Comparison of SNPs in control group and sepsis group, severity and mortality rates in all patients.

				Severity			30-days all-cause mortality		
	Controls	Sepsis total		Sepsis	Severe sepsis and toxic shock		Alive	Dead	
TLR2 rs5743704									
A/C	19 (6.4%)	14 (5.3%)	**P=0.582**	12 (5.7%)	2 (3.8%)	**P=0.601**	10 (4.5%)	4 (9.1%)	**P=0.219**
C/C	278 (93.6%)	250 (94.7)		200 (94.3%)	50 (96.2%)		210 (95.5%)	40 (90.9%)	
TLR2 rs5743708									
A/G	20 (6.7%)	22 (8.3%)	**P=0.472**	17 (8.0%)	5 (9.6%)	**P=0.709**	20 (9.1%)	2 (4.5%)	**P=0.319**
G/G	277 (93.3%)	242 (91.7%)		195 (92.0%)	47 (90.4%)		200 (90.9%)	42 (95.5%)	
TOLLIP rs5743867									
A/A	246 (82.8%)	210 (79.5%)	**P=0.166**	166 (78.3%)	44 (84.6%)	**P=0.409**	174 (79.1)	36 (81.8%)	**P=0.261**
A/G	50 (16.8%))	49 (18.6%)		41 (19.3%)	8 (15.4%)		43 (19.5%)	6 (6%)	
G/G	1 (0.3%)	5 (1.9%)		5 (2.4%)	0 (0%)		3 (1.4%)	2 (4.5%)	
TOLLIP rs5743942									
A/A	86 (29%)	77 (29.2%)	**P=0.850**	61 (28.8%)	16 (30.8%)	**P=0.951**	58 (26.4%)	19 (43.2%)	**P=0.073**
A/G	155 (52.2%)	142 (53.8%)		115 (54.2%)	27 (51.9%)		124 (53.4%)	18 (40.9%)	
G/G	56 (18.9%)	45 (17%)		36 (17%)	9 (17.3%)		38 (17.3%)	7 (15.9%)	

Table 7. Comparison of SNPs in control group and sepsis group, severity and mortality rates in all immune-competent patients.

				Severity			30-days all-cause mortality		
	Controls	**Sepsis total**		**Sepsis**	**Severe sepsis and toxic shock**		**Alive**	**Dead**	
TLR2 rs5743704									
A/C	19 (6.4%)	8 (3.8%)	**P=0.205**	7 (4.1%)	1 (2.5%)	**P=0.629**	7 (4.0%)	1 (3.1%)	**P=0.822**
C/C	278 (93.6%)	201 (96.2%)		169 (95.9%)	39 (97.5%)		170 (96.0%)	31 (96.9%)	
TLR2 rs5743708									
A/G	20 (6.7%)	17 (8.1%)	**P=0.551**	14 (8.3%)	3 (7.5%)	**P=0.870**	16 (9.0%)	1 (3.1%)	**P=0.260**
G/G	277 (93.3%)	192 (91.9%)		155 (91.7%)	37 (92.5%)		161 (91.0%)	31 (96.9%)	
TOLLIP rs5743867									
A/A	246 (82.8%)	160 (76.6%)	**P=0.046**	126 (74.6%)	34 (85.0%)	**P=0.286**	134 (75.7%))	26 (81.2%)	**P=0.152**
A/G	50 (16.8%))	44 (21.1%)		38 (22.5%)	6 (15.0%)		40 (22.6%)	4 (12.5%)	
G/G	1 (0.3%)	5 (2.4%)		5 (3.0%)	0 (0.0%)		3 (1.7%)	2 (6.3%)	
TOLLIP rs5743942									
A/A	86 (29%)	65 (31.1%)	**P=0.721**	53 (31.4%)	12 (30.0%)	**P=0.776**	52 (29.4%)	13 (40.6%)	**P=0.320**
A/G	155 (52.2%)	110 (52.6%)		90 (53.3%)	20 (50.0%)		97 (54.8%)	13 (40.6%)	
G/G	56 (18.9%)	34 (16.3%)		26 (15.4%)	8 (20%)		28 (15.8%)	6 (18.8%)	

Discussion

S. aureus is common in the human bacterial flora, present in a large number of people. It can be present without causing any symptoms, but it can also give rise to a battery of infections, ranging from minor infections with generally favorable outcomes, to more serious conditions like sepsis. *S. aureus* poses a medical challenge in terms of treatment and prevention, and it is one of the leading causes of hospital infections. It is of clinical interest to identify the factors involved in the development of sepsis, and to figure out why some people get this serious infection, while others do not. We believe that this is due to a "lock and key" interaction between bacterial traits and host factors. The bacterial aspect of this interaction is being investigated in Anita Blomfeldt's PhD project. As for the host factors, we selected two SNPs in TLR2 because of its importance in the recognition of *S. aureus* and two SNPs in TOLLIP as it is a negative regulator of TLR2. These SNPs have both previously been reported to be associated with several infections and sepsis/bacteremia.

In this study, TaqMan® SNP Genotyping Assays with PCR amplification and plate read analysis on the 7900HT Fast Real-Time PCR systemfrom Applied Biosystems was chosen to detect SNPs because of its ease of use and cost-efficiency. Primers and probes were designed by the producer of the commercial kits, which were all validated. Additionally, 384-well plates were available on the 7900HT instrument and the results gathered could be processed by computer software, further reducing the amount of time required to produce results.

Instead of using commercial PCR kits, all samples could have been sequenced. This was not opted for because of the increased workload required with such a method. Primers would have had to been designed, more steps are involved in a single run, and the results are harder and more time-consuming to analyze. The whole sequencing process in general is more complex and intensive, which in turn increases the risk of errors.

All PCR reactions were run in parallel to ensure correct results. In addition around 10% of all samples (30 samples from the control group and 30 samples from the sepsis group) were re-run on the PCR as technical controls. The results of these control-runs matched the results from the original runs, indicating that the results obtained in the study are indeed correct, and that they are reproducible.

The aim of this study was to examine polymorphisms of TLR2 and TOLLIP, and their effect on the predisposition, severity and outcome of *S. aureus* blood stream infections.

According to Song and co-workers (2011), people with the minor allele of TOLLIP rs5743867 has a reduced risk of developing sepsis. In our study there was a significant difference in distribution of alleles between the two groups when performing Chi-square test. However, in the multinomial

logistic regression analysis there was no difference, both for the crude and age- and sex-adjusted analysis. According to our study there are no statistical significant difference in the distribution of alleles between the control group and the sepsis group, as one might suspect given the results by Song. It is important to note however that in Songs study, all patients were from the Han Chinese ethnic group, and this may indicate that that there are other co-factors involved in the development of sepsis in the Han Chinese in addition to the variant of TOLLIP rs5743867.

Song (2011) also shows TOLLIP rs5743942 to increase the risk of developing sepsis, but those results were not significant after Bonferroni correction. Shah (2012) reports rs5743942 to be correlated with increased susceptibility to tuberculosis (before Bonferroni correction). TOLLIP rs5743942 was included in this study based on the results found by Song and because if a certain variant of the SNP is associated with tuberculosis, the same SNP might also be associated with susceptibility to *S. aureus* infection. However, there is no evidence supporting that hypothesis in this study. There is no statistical difference in the distribution of alleles between the sepsis group and the control group for TOLLIP rs5743942.

TLR2 rs5743704 causes a Pro631His amino acid substitution. It was associated with reduced activation of NF-ĸB by Ben-Ali (2011). NF-ĸB is a transcription factor that promotes the release of cytokines – important immune system signals - and one could speculate that the reduction in these signals would lead to increased susceptibility to sepsis. In this study then, one would expect a higher frequency of the Pro631His amino acid substitution in the sepsis group compared to the control group, if the change indeed does lower the activation of NF-ĸB, and if the reduction alone is enough to increase susceptibility to sepsis. Pro631His was also shown by Rosentul (2014) to be correlated with increased susceptibility to idiopathic recurrent vulvovaginal candidiasis. In our study however, there was no statistical difference in allele distribution between the two groups, indicating that Pro631His cannot be the only variable to take into account when assessing increased susceptibility to sepsis.

Finally, TLR2 rs5743708, which causes an Arg753Gln amino acid substitution, was described by Lorenz (2000), she suggested that this amino acid change predisposed individuals to life-threatening bacterial infections. In their study, 22 out of 91 patients had gram positive septic shock. Out of these 22 gram positive sepsis patients, 2 exhibited the Arg753Gln amino acid substitution. Their results were not statistically significant, and they reason that because Arg753Gln has a low frequency (3% in the populations they tested), it might still be a factor that increases the susceptibility to sepsis, even though they can't prove it statistically. While this might be true, it could also just be pure coincidence.

Additionally, rs5743708 was reported by Tsui (2008) to influence the risk of developing acute reactive arthritis, and Ogus (2004) to influence the susceptibility to tuberculosis. The results in our study, at any rate, does not support the notion of TLR2 rs5743708 having any effect on the predisposition, severity or outcome of an *S. aureus* sepsis, with no statistical difference observed between the distribution of alleles in the sepsis group and the control group. This observation also agrees with the results proposed by Woehrle and co-workers (2008), where they found no evidence of altered cytokine pattern following gram positive sepsis. Their results did however show a different cytokine pattern in the case of *Candida* sepsis.

In addition to the SNPs not having statistically different distributions in the control group and the sepsis group, none of the four SNPs had any effect on the severity of sepsis or the 30-days all-cause mortality rates of the patients either ($P>0.05$ for comparison of the distribution of alleles between sepsis vs. toxic shock and alive vs. dead for all SNPs).

Several of the patients in the sepsis group were immune-suppressed, which may have increased the number of people in the severe sepsis category without it being attributable to SNP variant, but instead other factors related to immune-compromised patients. Statistics were therefore calculated separately with and without immune-suppressed individuals included. There was however no statistically significant difference observed in allele distribution both when including immune-suppressed patients, and when excluding them. Both comparisons had P-values above 0.05 in all tests.

The statistical power calculation performed before the start of the study was made to ensure that an adequate number of controls were included in the study in order to correctly detect a difference in the distribution of alleles between the sepsis group and the control group. In this study, 264 patients were included in the sepsis group and 297 patients were included in the control group. This number of patients was above the necessary sample size needed for the study to have adequate power.

Limitations of the study include the fact that there are only patients from one hospital included, and as such the results may not be generalized to other hospitals. There is no consensus definition of sepsis and severe sepsis/toxic shock, and different articles may be using other definitions, this makes it difficult to compare results between studies. Additionally, all participants in our study are of Caucasian ethnicity, which makes it hard to draw any conclusions for other ethnicities.

It seems likely that whatever mechanisms are involved in the pathogenesis of *S. aureus,* it is not restricted to SNP variants in the genes for TLR2 and TOLLIP. SNPs vary in frequency between different

ethnic groups, which may be one of the reasons that there was no observable difference in this study, as it includes mainly Caucasians.

Conclusions

The results obtained in this study do not support the hypothesis that SNP in TLR2 and TOLLIP predispose for *S. aureus* BSI or affect severity and outcome. The interaction between host and bacterium when it comes to the severity and progression of sepsis is apparently more complex than specific genetic changes in these two proteins of the immune system. The interaction might depend on certain characteristics of the bacterium and the host to be present, which, when combined have a detrimental effect on the progression of sepsis in the human host.

Additional studies should focus on the characteristics of the bacteria that cause sepsis, and those findings should further be correlated with potential host factors that might influence the susceptibility to sepsis.

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Attachement

Statistical Power calculation

sampsi 0.2 0.1, p(0.8) r(1.8)

Estimated sample size for two-sample comparison of proportions

Test Ho: p1 = p2, where p1 is the proportion in population 1

and p2 is the proportion in population 2

Assumptions:

alpha = 0.0500 (two-sided)

power = 0.8000

p1 = 0.2000

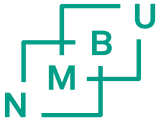
p2 = 0.1000

n2/n1 = 1.80

Estimated required sample sizes:

n1 = 165

n2 = 297



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