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RAPID DIFFERENTIATION OF PNEUMOCOCCI AND VIRIDANS GROUP STREPTOCOCCI BY MALDI-TOF MASS SPECTROMETRY AND A RAPID NUCLEIC ACID AMPLIFICATION TEST IN A CLINICAL MICROBIOLOGY LABORATORY

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ACADEMIC DISSERTATION

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ABSTRACT

Streptococcus pneumoniae is one of the most significant human bacterial pathogens. It is a major causative agent of community-acquired pneumonia. It is also the most common cause of bacterial otitis media and among most common bacteria causing meningitis in children. The close relatives of *S. pneumoniae*, the Viridans group streptococci form a central part of the human oral microbiome, but they are also a leading cause of endocarditis. The fast and reliable identification of these bacteria from clinical specimens would therefore be of prime importance for a clinical microbiology laboratory, allowing for the laboratory to provide helpful information to clinicians for identifying the infection focus and targeting antibiotic treatment.

The close relationship between *S. pneumoniae* and Viridans group streptococci, especially their subgroup Mitis group streptococci, complicates the reliable species level identification of these bacteria. The identification of streptococci by the traditional biochemical methods is both unreliable and time-consuming: the identification takes 1-2 days after bacterial growth has been detected on a plate or in a blood culture bottle. Even sequencing of the 16S ribosomal RNA gene commonly used as a reference method for bacterial identification cannot reliably differentiate between pneumococci and other Mitis group streptococci or identify other Mitis group streptococci to the species level. For the identification of pneumococci, detection of pneumococcal-specific genes is considered a "golden standard". For the Viridans group streptococci, multi-locus sequence typing (MLST), based on the analysis of the sequences of several genes, is considered to be the most reliable method of identification. However, the MLST is too laborious and time-consuming for the identification of streptococci in a routine clinical microbiology laboratory.

Within the last decade, MALDI-TOF mass spectrometry has enabled the fast and reliable identification of most bacterial species. However, exceptionally closely related bacterial groups pose a challenge even to the MALDI-TOF technology. This holds true even for pneumococci and Mitis group streptococci. In this study, the earlier database and algorithm versions of the commonly used MALDI-TOF systems were shown to be unable to reliably differentiate between pneumococci and Mitis group streptococci. Therefore, rapid molecular detection tests, such as GenomEra *S. pneumoniae*™ evaluated in this study, are still needed for rapid detection of pneumococci. However, it seems that the addition of more pneumococcal and Mitis group strains in the databases and new interpretation algorithms allow for reliable differentiation between pneumococci and the Mitis group streptococci. Altogether, the combination of rapid gene detection tests and

MALDI-TOF technology with enhanced databases and identification algorithms enables fast, reliable and cost-effective differentiation between pneumococci and Mitis group streptococci and the reliable group level identification of other Mitis group streptococci. This is a sufficient level of identification in a clinical microbiology laboratory, enabling the clinician to evaluate the clinical significance of the finding and target further investigations and antibiotic treatment more specifically.

TIIVISTELMÄ

Pneumokokki (*Streptococcus pneumoniae*) on merkittävimpiä ihmisen bakteeripatogeenejä: se on merkittävä avohoitosyntyisen keuhkokuumeen ja lasten välikorvatulehdusten aiheuttaja ja aiheuttaa myös suuren osan lasten aivokalvontulehduksista. Sen lähisukulaiset, viridans-ryhmän streptokokit, taas muodostavat merkittävän osan ihmisen suun normaalifloorasta, mutta ovat myös keskeisiä sydänkalvontulehduksen aiheuttajia. Pneumokokkien ja viridans-ryhmän streptokokkien nopea ja luotettava tunnistus kliinisen mikrobiologian laboratoriossa olisi kuitenkin ensiarvoisen tärkeää, koska tunnistustulos voi auttaa lääkäriä tunnistamaan infektiofokuksen ja suuntaamaan antibioottihoidon oikein.

Pneumokokin ja viridans-ryhmän, erityisesti sen alaryhmän, mitis-ryhmän streptokokkien lähisukulaisuus vaikeuttaa näiden bakteerien luotettavaa erottamista toisistaan ja tunnistamista lajitasolle. Perinteisillä biokemiallisilla tunnistusmenetelmillä pneumokokkien ja viridans-ryhmän streptokokkien tunnistus vie yhdestä kahteen vuorokautta siitä, kun maljalla tai veriviljelypullossa on havaittu bakteerikasvua. Edes bakteerien tunnistuksessa yleisesti käytetty 16S rRNA-geenin sekvensointi ei kykene luotettavasti erottelemaan pneumokokkeja mitis-ryhmän streptokokeista eikä viridans-streptokokkeja lajitasolle. Pneumokokkien tunnistuksessa referenssimenetelmänä käytetäänkin pneumokokeille tyypillisten geenien monistusta. Viridans-ryhmän streptokokkien kohdalla tällaisena "kultaisena standardina" käytetään usean geenin nukleiinihappojärjestyksen analysointiin perustuvaa tyypitystä (MLST). Useiden geenien sekvenssianalyysiin perustuva tunnistus on kuitenkin liian kallista ja aikaa vievää kliinisen mikrobiologian laboratorion tarpeisiin.

Viimeisen vuosikymmenen aikana MALDI-TOF-massaspektrometria on mahdollistanut useimpien bakteerien nopean ja luotettavan tunnistamisen. Toisilleen poikkeuksellisen läheistä sukua olevat bakteerit ovat kuitenkin haasteellisia tunnistettavia myös MALDI-TOF:lla. Tämä pätee myös pneumokokkeihin ja Mitis-ryhmän streptokokkeihin. Tässä työssä osoitettiin, että varhaisempia tietokantaversioita ja algoritmejä käytettäessä tavallisimmin käytetyt MALDI-TOF-laitteistot eivät kyenneet luotettavasti erottamaan pneumokokkeja Mitis-ryhmän streptokokeista. Tämän vuoksi geenimonistustestit kuten tässä työssä arvioitu GenomEra *S. pneumoniae*™ ovat tarpeen pneumokokkien nopeassa osoituksessa. Tämän tutkimuksen kolmas osatyö osoitti kuitenkin, että uusien pneumokokki- ja Mitis-ryhmän streptokokkikantojen lisääminen ja uusien tunnistusalgoritmien kehittäminen mahdollistavat pneumokokkien ja Mitis-ryhmän streptokokkien luotettavan erottamisen toisistaan myös MALDI-TOF:lla. MALDI-TOF:n ja nopean nukleiinihappotestin käyttö mahdollistavat pneumokokkien ja

Viridans-streptokokkien nopean, luotettavan ja kustannustehokkaan erottamisen sekä Viridans-streptokokkien luotettavan tunnistuksen ryhmätasolle. Tämä tunnistustaso on riittävä kliinisen mikrobiologian laboratorion tarpeisiin, sillä hoitava lääkäri pystyy sen perusteella arvioimaan löydöksen kliinistä merkitystä ja kohdistamaan mahdollisia jatkotutkimuksia ja antibioottihoitoa tarkemmin.

ACKNOWLEDGEMENTS

CONTENTS

LIST OF ORIGINAL PUBLICATIONS

This thesis is based on the following publications:

I Kärpänoja P*, Harju I*, Rantakokko-Jalava K, Haanperä M, Sarkkinen H. 2014. Evaluation of two matrix-assisted laser desorption ionization-time of flight mass spectrometry systems for identification of viridans group streptococci. Eur J Clin Microbiol Infect Dis. 33:779-788

II Harju I, Lange C, Kostrzewa M, Maier T, Rantakokko-Jalava K, Haanperä M. 2017. Improved differentiation of *Streptococcus pneumoniae* and other mitis group streptococci by MALDI Biotyper using an improved MALDI Biotyper database content and a novel result interpretation algorithm. J Clin Microbiol. 55:914-922

 *) Pauliina Kärpänoja and Inka Harju have contributed equally to this study.

III Hirvonen JJ, Seiskari T, Harju I, Rantakokko-Jalava K, Vuento R, Aittoniemi J. 2015. Use of an automated PCR assay, the GenomEra S. *pneumoniae*, for rapid detection of *Streptococcus pneumoniae* in blood cultures. Infect Dis (Lond). 47:796-800

The publications are referred to in the text by their roman numerals.

ABBREVIATIONS

16S rRNA	16 small subunit ribosomal ribonucleic acid
bp	base pairs
CAP	community-acquired pneumonia
cps	capsular polysaccharide
Da	Dalton
DB	database
Ddl	D-alanine-D-alanine ligase
ESI-MS	Electron spray ionization – mass spectrometry
GPC	gram-positive cocci
GroEL	chaperonin-60
GroES	chaperonin-10
IL	interleukin
IVD	in vitro diagnostics
LPA	line probe assay
LytA	pneumococcal autolysin
MALDI-TOF	matrix-assisted laser-desorption-ionization time-of-flight
MGS	Mitis group streptococci
MLST	multilocus sequence typing
NAAT	nucleic acid amplification test
NASBA	nucleic acid sequence based amplification
PavA	pneumococcal adhesion and virulence protein A
Ply	pneumolysin
PCR	polymerase chain reaction
PsaA	pneumococcal surface antigen A
Psp	pneumococcal surface protein
RecA	subunit of bacterial recombinase
RNA	ribonucleic acid
RnpB	RNA subunit of endoribonuclease B
RpoB	β-subunit of RNA polymerase
rpm	rounds per minute
RUO	research use only
SodA	manganese-dependent superoxide dismutase
TNF-α	tumour-necrotizing factor alpha
Tuf	elongation factor Tu
VGS	viridans group streptococci
VSB	viridans streptococcal bacteraemia
VSSS	viridans streptococcal shock syndrome

1 INTRODUCTION

The human microbiome has been the object of intense scientific interest in recent decades. The study of human microbiome was long held back by the limitations of bacterial culture methods. These limitations include the inability of bacterial culture to detect small amounts of bacteria or the many bacteria which cannot be grown in laboratory conditions due to their specific growth requirements (Amann *et al.* 1995, Riesenfeld *et al.* 2004). The development of molecular methods suitable for simultaneous detection of hundreds or thousands of microbial species, such as shotgun sequencing, has therefore been instrumental for the study of the human microbiome (Kim *et al.* 2015). Starting from 2000s, several large-scale projects were started in order to study human microbiome. Among the largest of these international research consortia are Metagenomics of the Human Intestinal Tract and Human Microbiome Project. According to the results gained in the Metagenomics of the Human Intestinal Tract, it is estimated that there are over 1000 bacterial species present in the human gut (Qin *et al.* 2010). In the Human Microbiome Project the microbial diversity in a variety of body sites was studied (Human Microbe Project Consortium 2012, Lloyd-Price *et al.* 2017). In this and in a previous study by Costello *et al.* (2009) the human mouth was found to house an exceedingly high variety of bacterial species, with the members of bacterial genus *Streptococcus* dominant in most habitats within the human mouth.

From human perspective, the genus *Streptococcus* is one of the most important bacterial genera, because it includes some of the most important bacterial pathogens as well as central members of the healthy human microbiome. The best-known members of the genus *Streptococcus* are pathogenic organisms such as pyogenic streptococci that cause tonsillitis, erysipelas, wound infections and bacteraemia and are traditionally typed according to the Lancefield antigens they present, such as A, C or G. Another important member of the genus is *Streptococcus agalactiae*, presenting Lancefield antigen B and most typically causing bacteraemia in the new-born babies. All these streptococci present a clear zone around the colonies when growing on blood agar plates. This clearing is caused by cytotoxins (streptolysins) secreted by these bacteria lysing red blood cells in the agar. This phenomenon is called beta-haemolysis and these pathogenic streptococci are duly called beta-haemolytic streptococci.

However, there is another pathogen within the genus *Streptococcus* that is also of prime importance as a human pathogen: *Streptococcus pneumoniae*. Unlike pyogenic streptococci, *S. pneumoniae* does not produce the typical clear zone around its colonies on blood agar but rather a faintly greenish colouring diffused into the agar surrounding the colony. This phenomenon is

called alpha-haemolysis and is caused by the streptococci producing hydrogen peroxide, which oxidizes the haemoglobin in the blood agar to methaemoglobin. Despite its lack of beta-haemolysis, *S. pneumoniae* can cause infections using other virulence factors, such as pneumococcal autolysin (LytA) and the pneumolysin (Ply) (Alonso de Velasco *et al.* 1995, Jedrzejas 2001). *S. pneumoniae* is an important causative agent of community-acquired pneumonia and bacterial otitis media. It also causes a significant proportion of cases of meningitis (Krzyściak *et al.* 2013, Feldman *et al.* 2014).

Despite its virulence, *S. pneumoniae* is more closely related to alpha-haemolytic Viridans group streptococci (VGS) than to the beta-haemolytic streptococci. VGS are an integral part of the healthy human microbiome in the respiratory, gastrointestinal and urogenital tracts. Although VGS are mostly commensal, they can also cause infections, most notably endocarditis, predominantly in patients with predisposing factors (Doern and Burnham 2010, Krzyściak *et al.* 2013). The VGS are divided into five groups: *Streptococcus anginosus, Streptococcus bovis, Streptococcus mitis, Streptococcus mutans and Streptococcus salivarius* (Kawamura *et al.* 1995, Spellerberg *et al.* 2011). Of all the VGS groups, the taxonomy of the *S. mitis* group has perhaps been most challenging: in addition to twelve closely related commensal species of the upper respiratory tract it also contains the important pathogen *S. pneumoniae* that is phylogenetically close to these commensal species.

In clinical microbiology laboratories, in which pathogenic microbes are isolated and identified from patient samples, VGS have traditionally been differentiated from pneumococci based on their optochin resistance and lack of bile solubility, as well as by using commercial biochemical test panels. However, Ikryannikova *et al.* (2011) has reported misidentification of optochin-susceptible *S. mitis* strains as *S. pneumoniae*. Commercial biochemical test panels have also been shown to be limited in their ability to identify streptococci reliably to the species level (Bascomb and Manafi 1998, Summanen *et al.*, 2009, Chatzigeorgieau *et al.*, 2011, Teles *et al.* 2011). Biochemical identification methods are also time-consuming, generally taking 1-2 days to complete.

The advent of molecular methods has enabled more reliable identification of many clinically relevant bacteria. However, the sequencing of 16S rRNA gene, which is widely used as a reference identification method in microbiology, has been proven to be of limited use in the species identification of VGS species, because of the tight interrelatedness of these species (Haanperä *et al.* 2007). Because of limitation of the 16S rRNA sequencing, multi-locus sequence typing (MLST) approach targeting several streptococcal housekeeping genes (Kawamura *et al.* 1999, Whatmore *et al.* 2000, Hoshino *et al.* 2005, Chi *et al.* 2007, Kilian *et al.* 2008, Do *et al.* 2009) has been established as a golden standard for streptococcal identification and

phylogenetic studies. Genes conferring to virulence in pneumococci have also been targeted for development of various nucleic acid amplification assays (NAATs) in order to identify pneumococci isolated from cultured clinical samples (Rudolph *et al.* 1993, Domiguez *et al.* 2001).

Because NAATs still remain relatively expensive, there has been wide interest in assessing the potential of matrix-assisted laser desorption/ionization-time of flight (MALDI-TOF) mass spectrometry, which in recent years has established itself as fast and cost-effective identification method in clinical microbiology laboratories (Seng *et al.* 2009, Bizzini *et al.* 2010, Neville *et al.* 2011). Although the instrument cost of MALDI-TOF mass spectrometry is high, the reagent costs are very low compared to molecular methods, and only minimal hands-on time is required. However, the greatest benefit of MALDI-TOF technology for a clinical microbiology laboratory is that it is possible to acquire bacterial identification within minutes compared to one or two days typically required by traditional biochemical methods. Such rapid diagnostic methods are especially important in a clinical laboratory setting, where laboratory diagnosis can have direct impact on the patient receiving effective treatment.

Identification of *Streptococcus pneumoniae* and VGS by MALDI-TOF MS has been widely studied (van Veen *et al.* 2010, de Bel *et al.* 2010, Neville *et al.* 2011, Scholz *et al.* 2012, Martiny *et al.* 2012, Werno *et al.* 2012, Davies *et al.* 2012, Lopez Roa *et al.* 2013, Ikryannikova *et al.* 2013, Dubois *et al.* 2013, Branda *et al.* 2013, Woods *et al.* 2014, Angeletti *et al.* 2015, Isaksson *et al.* 2015). However, the differentiation of *S. pneumoniae* and other Mitis group streptococci, especially *S. mitis* and *S. pseudopneumoniae* has proven challenging. Researchers have suggested potential solutions to this problem; such as expanding the databases of these MALDI-TOF systems to include recently described Mitis group species such as *Streptococcus tigurinus* (Isaksson *et al.* 2015) and improving the algorithms used to calculate closest matches (Rychert *et al.* 2013, supplementary material).

In this study, we aimed to assess rapid commercial methods for their potential for fast, reliable and cost-effective identification of *S. pneumoniae* and VGS in a routine clinical microbiology laboratory. The performance of two MALDI-TOF systems, MALDI Biotyper and VITEK MS, for identification of VGS were compared. We also evaluated the effect of using a novel algorithm utilizing "list scores" combined with the addition of new streptococcal strains to the database to the ability of MALDI Biotyper to differentiate between pneumococci and Mitis group streptococci. Furthermore, the potential of a commercial NAAT, GenomEra Pneumococcus™, for rapid identification of *S. pneumoniae* directly from positive blood culture bottles was investigated.

2   REVIEW OF THE LITERATURE

2.1 THE GENUS *STREPTOCOCCUS*

The genus *Streptococcus* comprises catalase-negative gram-positive cocci, typically occurring in chains or pairs. It is one of the clinically most important bacterial genera. It contains important pathogens such as *Streptococcus pyogenes* and *Streptococcus pneumoniae* as well as numerous members of healthy human microbiota. Streptococci have traditionally been divided into pyogenic and viridans streptococci based on the type of haemolysis they produce on blood agar plates. So-called pyogenic streptococci such as *S. pyogenes*, *Streptococcus dysgalactiae*, *Streptococcus agalactiae* and *Streptococcus equi* can cause infections in humans and/or animals and produce a clearing of the agar around their colonies, called beta-haemolysis (Spellerberg and Brandt 2011).

Conversely, the viridans group streptococci contain mostly species belonging to the healthy human microbiota, especially in the mouth and upper respiratory tract. The name viridans group streptococci refers to the greenish coloration, called alpha-haemolysis, which these bacteria often develop around their colonies. However, this alpha-haemolysis is not typical for all species within this group. Many VGS species form also non-haemolytic or beta-haemolytic colonies. Members of the *Streptococcus anginosus* group may present the Lancefield antigens A, C and G typical for the pyogenic streptococci and members of the *Streptococcus bovis* group may exhibit the Lancefield antigen D also present in enterococci (Doern and Burnham 2010, Spellerberg and Brandt 2011). In addition to commensal and only opportunistically pathogenic species, the viridans group also includes the important pathogen *S. pneumoniae*.

2.2 TAXONOMY AND CLASSIFICATION

The taxonomy of streptococci has been in flux since the start of modern bacteriology, taxonomical changes continuously spurred by technical developments in streptococcal typing and identification. In 1937, Sherman suggested dividing the organisms then classified as streptococci into four groups: pyogenic streptococci, viridans streptococci, lactic streptococci and enterococci. He excluded pneumococci on the basis of their bile-solubility, the members of the genus *Leuconostoc* because of their lactic-acid production and obligate anaerobic gram-positive cocci, then grouped as "anaerobic streptococci" but now considered to belong to several different genera such as *Peptostreptococcus*, *Finegoldia* and *Micromonas*. Sherman's groupings into pyogenic and viridans streptococci are still relevant today, but strains showing

phenotypical characteristics similar to his enterococcus group are nowadays classified into the genus *Enterococcus* with the advent of 16S rRNA sequencing (Schleifer *et al.* 1984, Ludwig *et al.* 1985). Similarly, the two species of his "lactic streptococci" group were moved into the new genus *Lactococcus* in the 1980s, also based on the comparison of 16S rRNA sequences by Schleifer *et al.* (1986). Sherman's classification was based on whether the strains presented a clearing around the colonies known as beta-haemolysis (first described by Schottmüller in 1903 and Brown in 1919 in publications that are no longer readily available, Facklam 2002) and on specific antigens presented by beta-haemolytic streptococci reported by Lancefield in 1933, as well as salt and thermal tolerance along with other biochemical properties. Detection of haemolysis and Lancefield antigen typing remain a cornerstone for identification of beta-haemolytic even today, but reliable identification of VGS and pneumococci has remained challenging ever since Sherman's days.

The taxonomical classification within VGS has seen several upheavals since the 1990s, mainly due to the advent of modern molecular typing methods (Facklam 2002, Arbique *et al.*, 2004, Doern *et al.* 2010, Spellerberg and Brandt 2011). Currently, the VGS are divided into five groups: *Streptococcus anginosus*, *Streptococcus bovis*, *Streptococcus mitis*, *Streptococcus mutans* and *Streptococcus salivarius* on basis of comparison of 16S rRNA gene sequences (figure 1) (Kawamura *et al.* 1995, Whiley *et al.* 1998, Spellerberg *et al.* 2011). Other researchers basing their groupings more on phenotypic characters have, however, distinguished *Streptococcus sanguinis*, *Streptococcus parasanguinis* and *Streptococcus gordonii* from other mitis group streptococci, to which they are related based on their 16S rRNA sequences, into a separate *Streptococcus sanguinis* group (Facklam 2002, Doern and Burnham 2010).
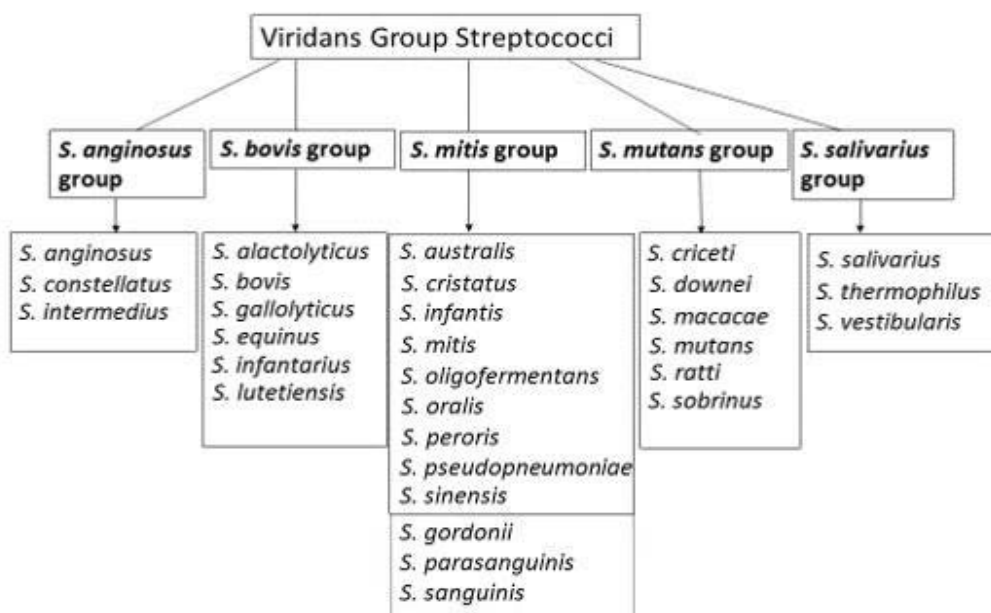
Figure 1. Subgroups of Viridans group streptococci

The first description of the *Streptococcus mitis* was presented by Andrewes and Horder in 1906 based on the phenotypical characteristics that could be studied with the methodology available at the time. They classified into *Streptococcus mitis* group commensal streptococci that formed short chains and grew well at 20°C and were able to ferment both lactose and sucrose and which, in contrast to the other VGS species he named *Streptococcus salivarius*, were usually unable to curdle milk or ferment inulin or raffinose. Although none of the strains studied by Andrewes and Horner have been preserved, Kilian *et al.* (1989 (1)) have speculated that the strains they designated as *S. mitis* were likely to comprise several of the currently recognized streptococcal species.

In 1943 Sherman *et al.* criticized the existing species descriptions within VGS for their vagueness: "As compared with the other major groups of the genus, the taxonomy of the viridans streptococci is in a highly unsatisfactory condition; but the common impression that the viridans streptococci compose a hopelessly heterogeneous conglomeration is largely due to the general failure to determine the basic nature of the organisms dealt with." On basis of their comparison of biochemical and morphological properties between VGS, Sherman concluded that although *S. mitis* group displayed relatively heterogeneous phenotypical properties, its members could still be reliably distinguished from *S. salivarius* and other recognised VGS. Although he was unable to ascertain it due to the lack of knowledge on the nature and mode of

transfer of genetic information at the time, Sherman hypothesised that *S. mitis* group was likely comprised of several species.

Despite Watson and Crick deciphering the structure of deoxyribonucleic acid (DNA) in 1953, the use of genetics in bacterial phylogenetic studies really took off only in the 1980s after the development of the polymerase chain reaction (PCR) that enabled the amplification of DNA so that it could be feasibly studied (Saiki *et al.* 1985, Mullis *et al.* 1986, Saiki *et al.* 1988). However, Coykendall *et al.* (1978) and Gilmour *et al.* (1987) studied the phylogenetic relationships among the *S. mitis* group using DNA-DNA hybridization and multilocus enzyme electrophoresis even before the advent of PCR-based methods.

The *S. mitis* group currently includes the important pathogen *S. pneumoniae and* twelve other validly described species: *S. australis, S. cristatus* (formerly *S. crista), S. gordonii, S. infantis, S. mitis, S. oligofermentans, S. oralis, S. parasanguinis* (formerly *S. parasanguis*), *S. peroris, S. pseudopneumoniae* (Arbique *et al.* 2004)*, S. sanguinis* (formerly *S. sanguis*) and *S. sinensis* (Spellerberg and Brandt 2011). Furthermore, recently described species *S. dentisani* (Camelo-Castillo *et al.* 2014) and *S. tigurinus* (Zbinden *et al.* 2012) seem to belong to the mitis group as well. Recently, Jensen *et al.* (2016) have suggested re-evaluation of the taxonomy of the *S. mitis* group, based on whole-genome analyses. They have proposed reclassification of *Streptococcus dentisani* as *Streptococcus oralis* subsp. *dentisani* comb. nov., *Streptococcus tigurinus* as *Streptococcus oralis* subsp. *tigurinus* comb. nov. and *Streptococcus oligofermentans* as a later synonym of *Streptococcus cristatus*.

2.3   CLINICAL SIGNIFICANCE

2.3.1 INFECTIONS CAUSED BY STREPTOCOCCUS PNEUMONIAE

Streptococcus pneumoniae is one of the most important bacterial pathogens. Asymptomatic pneumococcal carriage in the nasopharynx is very common, especially in children. In a recent study by Hamaluba *et al.* (2015), 47 % of children, 9 % of parents of small children and 2.2 % of older adults were found to be carriers. The elderly individuals also seem to be more susceptible to pneumococcal colonization, at least in the rat model (Theravanjan *et al.* 2016). However, it is also among the leading causative organisms of such important invasive infections as community-acquired pneumonia (CAP), pneumonia in young children (especially important in developing countries), meningitis in young children and otitis media (Feldman *et al.* 2014). These invasive pneumococcal infections can also lead to bacteraemia, in which the pathogenic bacteria spread through the blood stream. According to the infectious diseases statistics compiled by the

National Institute of Health and Welfare there were in the year 2018 over 800 diagnosed cases of invasive pneumococcal infections in which *S. pneumoniae* had been isolated from blood or cerebrospinal fluid (www.thl.fi, referred in 28.3.2019). Of the non-invasive pneumococcal infections, the most significant is by far otitis media, which is very common in young children and causes a significant burden to health-care systems both in developing and developed countries.

Pneumonia is one of the most common infectious causes of death worldwide (Lozano *et al.* 2012). In the latter half of the 20th century, mortality rate in bacteremic CAP has been estimated to have been on average 12 % (Ludwig *et al.* 2012). The incidence of pneumococcal pneumonia is probably underestimated due to diagnostic difficulties: the pathogen is detected only in bacteremic cases, unless special tests such as urine pneumococcal antigen tests are performed (Said *et al.* 2013). However, the use of these antigen tests is limited to adult patients, since high rates of nasal pneumococcal carriage in children can result in false positive reactions (del Mar García-Suarez *et al.* 2007).

Pneumococci often causes secondary bacterial respiratory infections in patients with influenza (Chertow *et al.* 2013). This is illustrated by Cilloniz *et al.* (2012), Martin-Loeches *et al.* (2011), Rice *et al.* 2012 and Muscedete *et al.* (2013) who have reported increased rates of community-acquired pneumonia, often caused by pneumococci, in patients infected with H1N1 influenza in 2009.

In the Global Burden of Disease Study 2013 Vos *et al.* (2015) estimated the global prevalence of pneumococcal meningitis in 2013 to have been between 5 million and 11 million and to have caused between 500 000 and 900 000 years lived with disability, mainly because of hearing loss and intellectual disability. *S. pneumoniae* was also estimated to be the most common causative agent of meningitis worldwide in 2013. In the same study, pneumococcal meningitis was placed fifth among the identifiable causes of intellectual disability worldwide after neonatal encephalopathy due to birth asphyxia and trauma, unbalanced chromosomal rearrangements, preterm births complications and Down syndrome. It was also estimated to be the most common infectious cause of intellectual disability. Vos and collaborators also estimated otitis media to be the most common and pneumococcal meningitis the second most common infective cause of hearing impairment worldwide. Vos *et al.* (2015) also estimated that pneumococcal meningitis had caused 35 000 years lived with disability for visual impairment.

Otitis media is one of the most common childhood infections. Otitis media is often preceded by viral upper respiratory infections that facilitate the invasion and infection of the middle ear caused by bacterial pathogens as *Streptococcus pneumoniae*, *Haemophilus influenzae* and *Moraxella catarrhalis* that are common residents of the nasopharynx in young children

(Ruuskanen *et al.* 2003). In developed countries otitis media is usually effectively treated with antibiotics, therefore serious sequelae are rare in these countries. However, in developing countries, adverse outcomes of untreated otitis media are common, often resulting in some degree of hearing loss. In the Global Burden of Disease Study 2013 Vos *et al.* (2015) estimated the global prevalence of otitis media in 2013 to have been about 85 million and to have caused between 1 million and 2.7 million years lived with disability, mainly because of hearing impairment. A significant portion of these infections can be assumed to be caused by pneumococci.

2.3.2 INFECTIONS CAUSED BY VIRIDANS GROUP STREPTOCOCCI

Viridans group streptococci form an integral part of the human microbiome. Members of *S. mitis*, *S. mutans* and *S. salivarius* groups typically colonise the oral cavity, whereas members of *S. bovis* group inhabit the intestine and members of *S. anginosus* group can be found both in the oropharynx and in the intestine. *S. mutans* is well-known for its role in the pathogenesis of dental caries (Krzysciak *et al.* 2014), but other members of the VGS are a part of the normal human oral and intestinal bacterial flora. They can, however, cause serious infections in immunocompromised hosts or in patients with underlying heart disease.

According to Pant *et al.* (2015) *Streptococcus* species were the second most common causative organisms of infective endocarditis in the US 2000-2011 after *Staphylococcus* species. Gupta *et al.* (2016) reported that this was also the case for children in the US 2010-2011. In the same article, Gupta *et al.* also reported that VGS were still the most common causative organisms in children with underlying heart disease.

The frequency of streptococcal endocarditis has decreased in the past 50 years in developed countries, whereas the incidence of infective endocarditis caused by *Staphylococcus* and *Enterococcus* species has increased, which might be connected with the increased use of intravascular catheters and prosthetic vascular grafts in the case of coagulase-negative staphylococci, increased intravenous drug abuse in the case of *Staphylococcus aureus* and increased median patient age and increased prevalence for diabetes for *Enterococcus* species (Slipczuk *et al.* 2013). In a review by Elder *et al.* (2015) it was noted that in paediatric patients the proportion of VGS as a cause of infective endocarditis had steadily decreased since the 1950s. They attributed this relative reduction to the improved surgical treatment options for congenital heart diseases and the decreased incidence of rheumatic fever and rheumatic heart disease resulting from it.

The role of VGS in causing invasive infections in patients treated for cancer with chemotherapy was first highlighted by Pizzo *et al.* in 1978 for adults and Hoecker *et al.* in the same year. The damage to mucous membranes and granulocytopenia induced by chemotherapy allowed VGS to cause invasive

infection when entering the blood stream from damaged mucosal membranes. Transient VGS bacteraemia (VSB) is common in connection with dental treatment or periodontal disease, but immunocompetent hosts can quickly clear it. VSB has also been found to be an important problem in stem cell transplant recipients (Henslee *et al.* 1984, Bilgrami *et al.* 1998). VGS bacteraemia has been studied in paediatric patients with acute myeloid leukaemia in recent years (Brunet *et al.* 2006, Johannsen *et al.* 2013, Lewis *et al.* 2014). Nielsen *et al.* (2016) found also the children suffering from acute lymphoblastic leukaemia to be at high risk of VSB.

Han *et al.* (2006) has investigated the species variety and antibiotic susceptibility of the VGS isolated from blood cultures of cancer patients and has found out that *S. mitis* was the most common species. In this study, *S. mitis* also displayed more resistance to penicillin and fluoroquinolones than strains belonging to other VGS species. *S. mitis* was also shown to cause a disproportionately high percentage of bacteraemia accompanied by viridans streptococcal shock syndrome (VSSS) both in Han's study and in a study published by Shelburne *et al.* in 2014. *S. mitis* was also the most common causative organism in all the eight VSSS cases in paediatric cancer patients reported by Nielsen *et al.* (2016). In VSSS the patient develops hypotension and/or acute respiratory distress syndrome. The mortality rate of VSSS has been reported to range from 0 % to 37 % (Gassas *et al.* 2004, Nielsen *et al.* 2016).

2.4 VIRULENCE FACTORS

2.4.1 VIRULENCE FACTORS OF *STREPTOCOCCUS PNEUMONIAE*

Streptococcus pneumoniae colonises the mucous membranes of the oropharynx of more than 60 % of healthy people (Mitchell *et al.* 2003) but can cause disease when it gains access to the middle ear, the lower respiratory tract or the brain. For successful oropharyngeal colonisation *S. pneumoniae* needs the ability to adhere to the mucosal surfaces. In addition, in order to cause disease *S. pneumoniae* needs several virulence factors that allow it to attach to the host cells, invade the host tissues and avoid the host immune system. (Alonso de Velasco *et al.* 1995, Jedrzejas *et al.* 2001, Mitchell *et al.* 2003). The virulence factors of *S. pneumoniae* and Viridans group streptococci are also central to this study because the genes encoding for pneumococcal virulence factors have been the principal targets of most *S. pneumoniae* nucleic acid amplification assays. It is therefore important to be aware to which extent these virulence factors are shared by *S. pneumoniae* and VGS in order to ascertain the specificity of these nucleic acid amplification tests.

The polysaccharide capsule is considered to be one of the main virulence factors of *S. pneumoniae*. The polysaccharide capsule is believed to impair the phagocytosis by neutrophils (Jonsson *et al.* 1985, Hyams *et al.* 2010) by reducing complement deposition, inhibiting CRP binding and covering

antigens on the pneumococcal cell surface (Skov-Sorensen *et al.* 2016). Magee *et al.* (2001) and Nelson *et al.* (2007) also found out that the *S. pneumoniae* strains that lacked the polysaccharide capsule were less efficient in colonising the mucosal surfaces. The extreme diversity of the capsular polysaccharide helps the pneumococci to evade the human immune system by cycling the capsular antigens that the human antibody-response is largely targeted to. The differences in the capsule polysaccharide are also the basis of pneumococcal serotypes that are important for pneumococcal epidemiology and vaccine development. Before the advent of antibiotic treatment, the serotyping of pneumococci was also crucial for anti-serum treatment of pneumococcal infections (Geno *et al.* 2015). Currently, there are 98 recognised pneumococcal capsular serotypes (Skov-Sorensen *et al.* 2016, Geno *et al.* 2017). Infection with different pneumococcal serotypes together with the different host factors are also known to generate variant cytokine profiles, although common themes in these profiles can also be found in all pneumococcal lung infections in a mice model (Jonczyk *et al.* 2016)

In addition to the polysaccharide capsule, the pneumococcal cell wall is also important for pathogenesis. In addition of helping with the attachment to lung cells (Cundell *et al.* 1995), the cell wall of *S. pneumoniae* also plays a role in the development of the inflammation in invasive pneumococcal infections (Tuomanen *et al.* 1985). The fact that the phosphorylcholine contained in the pneumococcal cell wall binds to the receptor of the platelet-activating factor might also partly explain why pneumococcal pneumonia so often develops after viral infection, since the platelet-activating factor is activated during viral infections (Ishizuka *et al.* 2003, Mitchell 2003).

Pneumococcal surface proteins also play an important role both in pneumococcal colonisation pathogenesis. The pneumococcal surface proteins are divided into three groups based on their linkage to the cell surface: LPXTG-anchored proteins, lipoproteins and choline-binding proteins (Mitchell *et al.* 2003). LPXTG-anchored proteins derive their name from how they are anchored to the cell wall using an LPXTG-type amino-acid motif. Neuraminidase and hyaluronidase are among the most important of these proteins. Neuraminidase seems to play a role in the nasopharyngeal colonisation by *S. pneumoniae* (Tong *et al.* 2000). Hyaluronidase can break down the hyaluronic-acid in connective tissue and extracellular matrix and has been found to augment pneumolysin-mediated injury to human ciliated epithelium (Feldman *et al.* 2007).

The pneumococcal lipoproteins considered to be most important for adhesion and pathogenesis are pneumococcal surface antigen (PsaA) and pneumococcal adhesion and virulence protein A (PavA). PsaA is part of a manganese-transport chain (Lawrence *et al.* 1998) and has been found to have the potential to assist in the invasion of the blood stream by *S. pneumoniae* (Hu *et al.* 2013). Mutants lacking the *psa* and Mn^{2+} transport, have been found

to be unable to cause infection (Marra *et al.* 2002). PavA is a protein present on the pneumococcal cell surface and is known to associate with fibronectin (Mitchell 2003). Kadioglu *et al.* (2010) conclude that PavA is involved in successful pneumococcal colonization of mucosal surfaces and in translocation of pneumococci across host barriers, making it important both for establishing nasopharyngeal colonisation and in invasive infections.

The most important pneumococcal virulence factors among the choline-binding proteins are autolysin (LytA) and pneumococcal surface proteins A and C (PspA and PspC). The pneumococcal autolysin is involved in releasing pneumolysin out of the cell (Mitchell 2003). PspA is expressed by all clinically important pneumococcal serotypes and is highly variable among them (Crain *et al.* 1990). Both PspA and PspC have been found to interfere with the functioning of the human complement system (Mitchell *et al.* 2003). Mutant strains lacking PspA and PspC have been found to have reduced ability to adhere to the nasopharyngeal surfaces and to translocate to the lungs and bloodstream (Ogunniyi *et al.* 2007). Quin *et al.* (2007) reported also that the PspA and PspC deficient mutant were cleared from the bloodstream more easily than non-mutated strains. Keller *et al.* (2016) found that a triple deletion of the common virulence factors PspA, PspC, and Ply completely impaired virulence in the chinchilla otitis media model.

Pneumolysin is a cytoplasmic pore-forming toxin released from pneumococcal cells by autolysin during pneumococcal autolysis and growth (Mitchell and Mitchell 2010). Pneumolysin has been found to contribute to the pathogenesis of pneumococcal infections in several animal models (Mitchell *et al.* 2003, Hirst *et al.* 2004 and Zafar *et al.* 2017). Zafar *et al.* (2017) found that pneumolysin increased inflammation and pneumococcal shedding in mouse pups and also improved the environmental survival of *S. pneumoniae*, therefore allowing for more efficient transmission from one mouse pup to another.

Pneumolysin can induce inflammation by boosting the production of inflammatory cytokines such as tumour-necrotizing factor alpha (TNF-α) and interleukins IL-1β (Houldsworth *et al.* 1994), IL-8 (Cockeran *et al.* 2002, Lüttge *et al.* 2012) and IL-17 (Basset *et al.* 2007). Pneumolysin is also known to be able to activate the complement system (Mitchell *et al.* 2003). These effects may be behind its association with increased infiltration of neutrophils into the lungs outlined in Hirst *et* al. (2004). Pneumolysin plays a role in the evasion of the immune system by pneumococci (Quin *et al.* 2007), which might result partly from its cytotoxic effects on leukocytes. Gilley *et al.* (2016) noticed that pneumococci invading the myocardium kill infiltrated macrophages by pneumolysin-mediated necroptosis. Pneumolysin is also toxic to both pulmonary endothelial (Rubins *et al.* 1992) and epithelial (Rubins *et al.* 1993) cells. As a pore-forming toxin, pneumolysin can perforate lipid membranes of epithelial and endothelial cells. This allows it to impair

their barrier function within the blood vessels (Mitchell and Mitchell, 2010). Braun *et al.* (2007) found that pneumolysin could cause the death of nerve cells by impairing the function of their mitochondrial membranes.

2.4.2 VIRULENCE FACTORS OF VIRIDANS GROUP STREPTOCOCCI

Of all the VGS species, the virulence factors of *S. mitis* and *S. mutans* have been most widely studied, since these species are also considered to display most pathogenic potential. This far, the complete genomes of two *S. mitis* strains have been sequenced. The first of them was a commensal strain *S. mitis* B6 (Denapaite *et al.* 2010) and the second a clinical *S. mitis* strain SVGS_061, isolated from the blood culture of a patient who had developed a VSSS (Petrosyan *et al.* 2016).

The analysis of the genomes of *S. pneumoniae* and *S. mitis* has revealed that these close relatives also share most of the virulence factors known to contribute to the pathogenicity of *S. pneumoniae* (Johnston *et* al. 2010, Mitchell 2011). In their analysis of the genome of *S. mitis* B6, Denapaite *et al.* (2010) found that *S. mitis* B6 shared with *S. pneumoniae* especially most virulence factors involved in colonization and adherence, suggesting these genes would be needed also for a commensal lifestyle typical for *S. mitis*. Most of the regulatory proteins involved in virulence in *S. pneumoniae* were also found in the *S. mitis* B6 genome. Denapaite *et al.* (2010) concluded that the only essential pneumococcal virulence genes that were absent in *S. mitis* B6 were pneumolysin *ply*, the choline-binding proteins *pspA, pspC, pcpA*, and the hyaluronidase *hlyA* in addition to the genes involved in the synthesis of the polysaccharide capsule.

Mitchell (2011) proposes that the absence of the polysaccharide capsule might be the essential factor impairing the pathogenicity of *S. mitis*, since the lack of the capsule would render *S. mitis* vulnerable to attacks by neutrophils. However, many other *S. mitis* strains have been found to contain capsule loci (Kilian *et al.* 2008, Johnston *et al.* 2010, Petrosyan *et al.* 2016), as well as genes encoding pneumolysin (Whatmore *et al.* 2000, Neeleman *et al.* 2004, Johnston *et al.* 2010, Kilian *et al.* 2014, Morales *et al.* 2015) and hyaluronidase (Johnston *et al.* 2010).

Several choline-binding proteins were also detected in many *S. mitis* strains by Kilian *et al.* (2014), although the genes for certain choline-binding proteins known to be involved in attachment to host cells in *S. pneumoniae*, such as PspA, were not found, in line with previous studies (Hakenbeck *et al.* 2009, Denapaite *et al.* 2010, Johnston *et al.* 2010). Recently, Skov Sørensen *et al.* (2016) explored the expression of polysaccharide capsule genes in several VGS species, finding that the genes involved in capsule polysaccharide synthesis were expressed in all the VGS strains they studied. In addition to the lack of some crucial pneumococcal virulence factor genes in *S. mitis*, part of the explanation for the lesser pathogenic potential of *S. mitis* might be the fact

that many regions of the *S. mitis* genome are inverted in respect to the replication of origin compared to *S. pneumoniae*, presumably affecting the temporal expression patterns of these genes (Denapaite *et al.* 2010, Mitchell 2011).

In addition to the specific virulence factors, *S. mitis* has a high competence for genetic transformation (Denapaite *et al.* 2010, Mitchell *et al.* 2010), which presumably helps to generate the antigenic variation necessary for the evasion of the activity of the strain-specific secretory immunoglobulin A against *S. mitis* found in the human saliva (Kirchherr *et al.* 2005, Kirchherr *et al.* 2007, Johnsborg *et al.* 2008). In addition of the genes involved in competence and transformation, Denapaite *et al.* (2010) found in the *S. mitis* B6 genome genes for bacteriocins facilitating access to foreign DNA by killing and lysing other bacteria. On the other hand, Killian *et al.* (2008) found out that many *S. mitis* strains lacked some of the 22 genes that are considered essential for competence for genetic transformation in pneumococci. In contrast, all those 22 genes were found in all the *S. pneumoniae* and *S. pseudopneumoniae* strains they studied.

Apart from *S. mitis*, the most widely studied member of the *S. mitis* group is *S. pseudopneumoniae*. Shahinas *et al.* published in 2011 the first whole-genome sequence of *S. pseudopneumoniae*. In 2013 Shahinas *et al.* published a wider comparative analysis of the *S. pseudopneumoniae*. In this 2013 study they found that *S. pseudopneumoniae* shared many virulence genes with *S. pneumoniae*, including the genes encoding for pneumolysin (Ply) and autolysin (LytA). However, similarly to the *S. mitis* strains studied by Denapaite *et al.* (2010), Johnson *et al.* (2010) and Kilian *et al.* (2014), *S. pseudopneumoniae* was lacking the genes for choline-binding proteins PspA, PspC and PcpA, which in *S. pneumoniae* are important cell-surface proteins crucial for the attachment to the host cells. On the other hand, *S. pseudopneumoniae* was found to have a wide array of other choline-binding genes, which are believed to be involved in the murine synthesis and cell separation (Shahinas *et al.* 2013). The variety of choline-binding proteins in *S. pneumoniae* and in other MGS have been studied by Hakenbeck *et al.* (2009), who found them to be highly polymorphic and to have undergone several duplications and recombination events in the course of the evolution of these species.

In addition to the above-mentioned virulence factors, Bek-Thomsen *et al.* (2012) have recently studied the evolution of zinc metalloproteases in VGS and *S. pneumoniae*. Morales *et al.* (2015) have investigated the gene for pneumococcal autolysin (*lytA*) in *S. pneumoniae* and other Mitis group streptococci and come to the conclusion that the *lytA* and *plyA* (pneumolysin) genes tend to occur together in the same genomic islands and have recognised eight different types of these *lytA-plyA* genomic islands in *S. pneumoniae* and in several other Mitis group species. The close association of *lytA* and *plyA* is

not surprising considering the autolysin is essential for the release of pneumolysin from the pneumococcal cells (Mitchell *et al.* 2003) during autolysis, although newer research has shown, that pneumolysin can also be released during the growth, without the involvement of autolysin (Mitchell and Mitchell 2010).

Of the other virulence factors, the role of hydrogen peroxide and NADH oxidase in both *S. pneumoniae* and in other *S. mitis* group streptococci has been most widely studied. Auzat *et al.* (1999) found that the competence for genetic exchange or virulence could be modulated by oxygen via the oxygen-sensing NADH oxidase. Recently, Ge *et al.* (2016) discovered that *S. sanguinis* mutants lacking the NADH oxidase gene (*nox*) had higher intracellular hydrogen peroxide (H_2O_2) and were more susceptible to environmental stress and reduced survival in serum, resulting in impaired competitivity and an impaired ability to cause endocarditis in rabbits.

Duane *et al.* (1993) found that all the *S. pneumoniae* strains they studied produced hydrogen peroxide which was toxic to the rat alveolar epithelial cells. Okahashi *et al.* (2016) also found that hydrogen peroxide (H_2O_2) produced by *S. oralis* was able to kill human monocytes and epithelial cells. However, in a study by Zahlten *et al.* (2015) it was discovered that the oxidative stress induced by pneumococcal infection in mouse and human lung tissue was independent of H_2O_2 and pneumolysin produced by *S. pneumoniae* and instead depended on the pneumococcal autolysin LytA.

Of the other VGS species, the virulence factors of *S. mutans* have attracted most scientific interest. In contrast to the *S. mitis* group streptococci, *S. mutans* doesn't seem to share many virulence factors with *S. pneumoniae*. (Krzysciak *et al.* 2014). The most important pathogenicity determinant it shares with *S. pneumoniae* are the genes for fibrinogen-binding PavA-like (pneumococcal adhesion and virulence A) protein and for SloC belonging to the lipoprotein receptor antigen family (Mitchell 2003). The operon in which *sloC* is contained within *S. mutans* is similar to *S. pneumoniae* operons *psaBCAD* and *adcCBA* involved in the transport of divalent cations, such as iron and manganese (Dintilhac *et al.* 1997, O'Rourke *et al.* 2010). The Psa operon has been shown to be important for pneumococcal virulence (Marra *et al.* 2002). Kitten *et al.* (2000) showed that the *S. mutans* strains deficient in *sloC* could still cause dental caries but their ability to cause endocarditis was reduced in a rat model. In addition to these virulence factors shared with *S. pneumoniae*, *S. mutans* also have other pathogenicity determinants important *for* attachment and biofilm formation, such as glucosyltransferases and glucan-binding proteins (Krzysciak *et al.* 2014).

2.5 PHYLOGENY OF *STREPTOCOCCUS PNEUMONIAE* AND VIRIDANS GROUP STREPTOCOCCI

The parallel evolution of *S. pneumoniae* and other species in the viridans group has been puzzling the scientists for decades, since early observers already recognised that the species distinctions within these species were far from clear-cut (Sherman *et al.* 1943) and yet the pathogenic potential of *S. pneumoniae* and other members of the *S. mitis* group differs immensely. Thanks to the development of more efficient methods for nucleic acid amplification and sequencing within the last 25 years, researchers have been able to gain significant new insights into the phylogeny of this intriguing bacterial group which contains both one of the most important bacterial pathogens as well as integral members of healthy oral microbiota.

In a ground-breaking study published in 1995, Kawamura *et al.* constructed the first comprehensive phylogenetic tree based on 16S rRNA including all the VGS species then recognised. The results of their study supported the division of VGS into five groups: *anginosus*, *bovis*, *mitis*, *mutans* and *salivarius*. They also found that within the *S. mitis* group, *S. sanguis* and *S. parasanguis* (later re-named as *S. sanguinis* and *S. parasanguinis*) were more closely related to each other than to other streptococci within the *S. mitis* group and that *S. pneumoniae*, *S. mitis*, and to a lesser degree, *S. oralis* were closely related, exhibiting sequence homology of over 99% to each other. However, the level of DNA-DNA hybridization between these three species was found to be significantly lower, under 60 %, well below the traditional species separation threshold value of 70 % (Wayne *et al.* 1987). This striking difference between the conservation of the 16S rRNA gene and more diverse total DNA within these three species demanded an explanation.

Because the highly conserved 16S rRNA sequences did not provide for an optimal tool for the species level identification of the species within the *S. mitis* group, different housekeeping genes were selected by researchers to be used in combination in a multi-locus sequence typing approach for identification of these species (Kawamura *et al.* 1999, Whatmore *et al.* 2000, Hoshino *et al.* 2005, Kilian *et al.* 2008, Do *et al.* 2009, Rasmussen *et al.* 2016). The publishing of the whole genome sequences of several *S. pneumoniae* (Hoskins *et al.* 2001, Tettelin *et al.* 2001, Lanie *et al.* 2007, Donati *et al.* 2010) strains as well as *S. pseudopneumoniae* strain IS 7493 (Shahinas *et al.* 2011, 2013) and *S. mitis* strains B6 (Denapaite *et al.* 2007) and SVGS_061 (Petrosyan *et al.* 2016) also provided more insights into the evolutionary history of the *S. mitis* group.

Comparing the partial sequences of the housekeeping genes *ddl*, *gdh*, *rpoB* and *sodA* of both pneumococcal and non-pneumococcal members of the *S. mitis* strain, Kilian *et al.* (2008) constructed a phylogenetic tree of the *S. mitis*

group. In this tree, *S. infantis* and *S. oralis* strains were both clustered separately from the *S. mitis*, *S. pseudopneumoniae* and *S. pneumoniae* strains, which were clustered significantly closer to each other than to the other members of the group. However, the strains within these three species were also found to display remarkable polymorphism in these genes: nearly all *S. pseudopneumoniae* and *S. mitis* strains had their own unique alleles of these genes, whereas shared alleles were found almost exclusively among the pneumococcal strains. *S. pneumoniae* strains were thus grouped much more closely together in this tree than the more diverse *S. mitis* and *S. pseudopneumoniae* strains. When performing DNA-DNA hybridization between different *S. mitis* strains the researchers discovered that among the *S. mitis* strains, most isolates had hybridization values well below 70 %, thus constituting separate species following the traditional taxonomical criteria (Wayne *et al.* 1987). Designating most *S. mitis* strains as separate species would naturally be highly impractical, and the strains have therefore remained within the *S. mitis* species. Kilian *et al.* (2008) hypothesized that the genetic differences between individual lineages of *S. mitis* might result from their sexual separation resulting from mostly vertical transfer from parents to offspring. However, both adults and infants are found to be colonized with numerous, highly variant, *S. mitis* clones at different sites on the oropharyngeal surfaces (Hohwy *et al.* 2001, Kirchherr *et al.* 2005) and few of these clones seem to persist for more than a few weeks (Fitzsimmons *et al.* 1996, Hohwy *et al.* 2001, Kirchherr *et al.* 2005). In addition to that, the infants and their mothers seem to only infrequently share the same clones (Kirchherr *et al.* 2005). These findings seem to suggest that the sexual isolation hypothesis is inadequate in trying to explain the diversity within this species.

The homological recombination plays an important role in creating the genetic landscape within the VGS. Extensive genetic transfer has been reported to occur both within species and between species in *S. anginosus* and *S. mitis* groups, but in the study conducted by Hoshino *et al.* (2005), recombination was only found between the strains belonging to the same phylogenetic cluster (for example, between *S. gordonii* and *S. sanguinis* and between *S. anginosus* and *S. intermedius*). This finding is not surprising in the light of the fact that efficient recombination requires a certain degree of sequence similarity between the strains involved (Lawrence 2002). Kilian *et al.* (2008) also found several strains grouped as *S. oralis* on the basis of the housekeeping genes and DNA-DNA hybridization to contain copies of the 16S rRNA gene typical for both *S. mitis* and *S. oralis*, suggesting gene transfer from *S. mitis* to *S. oralis*. As described in more detail in the section on the virulence genes, both *S. pneumoniae* and other MGS species are competent for transformation (Håvarstein *et al.* 1996, Pestova *et al.* 1996, Morrison *et al.* 1997, Kilian *et al.* 2008, Whatmore *et al.* 1999, Denapaite *et al.* 2010).

Combining the analysis of both 16S rRNA gene sequences, MLST using housekeeping genes and DNA-DNA hybridization, Kilian *et al.* (2008) came to the conclusion that commensal *S. mitis* lineages had developed from pathogenic ancestors resembling current *S. pneumoniae* strains through loss of virulence genes among other parts of the genome after a previous evolutionary split between the *mitis-pneumoniae-pseudopneumoniae* and other species in the *S. mitis* group such as *S. oralis* and *S. infantis*. They also proposed that the split between pathogenic *S. pneumoniae* and commensal *S. mitis* occurred concurrently with the development of the immunoglobulin A (IgA) subclass in the common ancestor of humans, chimpanzees and gorillas, about 6 to 7 million years ago (Kawamura *et al.* 1992). This is supported by the fact that *S. mitis* has not been isolated from other primates or other mammal groups and that *S.* pneumoniae has been found to cause infections in chimpanzees (Chi *et al.* 2007 (2), Denapaite and Hakenbeck 2011). In another study, Kilian *et al.* (2014) suggest that continuous unidirectional gene transfer from *S. mitis* lineages to *S. pneumoniae* has been helping *S. pneumoniae* to evade the human immune system by ensuring the structural variety of the capsular polysaccharides by importing fragments of the *cps* operon from *S. mitis* and other VGS members of oropharyngeal microbiota. This theory has recently been corroborated by findings of Skov Sørensen *et al.* (2016) when they studied the expression of capsular polysaccharide across different VGS species.

S. *pseudopneumoniae* was first described by Arbique *et al.* in 2004 comprising some of the strains that historically would have been described as "atypical pneumococci". *S. pseudopneumoniae* has in several phylogenetic studies been found to inhabit an intermediary position between *S. pneumoniae* and *S. mitis* (Kilian *et al.* 2008, 2014, Rolo *et al.* 2013) In their analysis of the genome sequence of *S. pseudopneumoniae* strain IS7493, Shahinas *et al.* (2013) found that the genome of *S. pseudopneumoniae* IS7493 resembled that of *S. pneumoniae* R6 but that there was evidence of several genetic recombinations having taken place in IS7493. Rolo *et al.* (2013) also discovered signs of extensive recombination with both *S. pneumoniae* and *S. mitis* in their analysis of the genetics of several *S. pseudopneumoniae* strains. Do *et al.* (2009) found the population structure of *S. oralis* to resemble that of *S. mitis* with highly variant individual lineages and evidence of significant inter- and intraspecies recombination.

A genetic landscape like that of the MGS has been revealed for other oral VGS, such as members of *S. salivarius* group (Delorme *et al.* 2007, 2015) and *S. mutans* (Nakano *et al.* 2007). These oral streptococci also seem to be genetically diverse and to show wide-spread genetic recombination. Interestingly, *S. thermophilus*, a member of *S. salivarius* group inhabiting the mammary mucosa of cows and used commercially in dairy industry for fermenting yoghurt, seems to have recently evolved from human oral species

S. vestibularis (Pombert *et al.* 2009) and displays far less genetic variation than the members of this group inhabiting the human oral mucosa. When looking at *S. mutans*, when compared to MGS, individual seem to carry far fewer clones that are frequently transmitted to infants from parents and other family members (Momeni *et al.* 2016).

2.6 IDENTIFICATION METHODS

2.6.1 BIOCHEMICAL IDENTIFICATION METHODS

The identification of *S. pneumoniae* and VGS in clinical microbiology laboratory has been based on colony and microscopic morphology combined with various biochemical tests. Both *S. pneumoniae* and many VGS form alpha-haemolytic colonies on sheep blood agar, whereas many VGS display no haemolysis and members of the *S. anginosus* group of VGS often grow as very small beta-haemolytic colonies. Many *S. pneumoniae* strains require incubation in 5 % CO_2, and the growth of many VGS isolates is also enhanced in the presence of elevated level of CO_2. (Spellerberg and Brandt 2011). VGS typically grow as chain of cocci, but *S. pneumoniae* is often also growing as pairs of elongated cocci. Some species may also grow as short rods in certain conditions, such as *S. mutans* grown in acidic conditions (Spellerberg and Brandt 2011) and members of *S. anginosus* group incubated in 5 % CO_2 (author's unpublished experience).

For differentiating between *S. pneumoniae* and closely related VGS, testing optochin sensitivity and bile solubility have been considered essential (Spellerberg and Brandt 2011). *S. pneumoniae* strains are typically optochin sensitive and bile-soluble, in contrast to other VGS, which are typically optochin resistant and insoluble to bile. Wessels and co-workers (2012) reported that optochin testing in a CO2-atmosphere and tube bile solubility testing give consistent results for *S. pneumoniae*. However, in another study (Ikryannikova *et al.* 2011) 21 optochin susceptible isolates were identified as *S. mitis* by MLST. Commercial biochemical test panels have limitations in identifying streptococci to species level (Bascomb and Manafi 1998, Summanen *et al.* 2009, Chatzigeorgieau *et al.* 2011, Teles *et al.* 2011).

2.6.2 PNEUMOCOCCAL SEROTYPING

Over 90 different capsular serotypes of *S. pneumoniae* have been identified this far (Habib *et al.* 2017). The detection of pneumococcal serotypes is important for monitoring the epidemiology of pneumococcal infections. During the 2000s, it has also become central to monitoring serotype replacement in response to pneumococcal vaccinations (Weinberger *et al.* 2011). In Finland, clinical microbiology laboratories are obliged to submit all pneumococcal strains isolated from blood and cerebrospinal fluid to the National Institute of Health and Welfare for serotyping (https://thl.fi/fi/web/infektiotaudit/laboratoriotoiminta/laboratoriotutkimu

kset, referred 29.3.2019). Quellung reaction, first described by Fred Neufeld in 1902, is considered a golden standard of pneumococcal serotyping. Nowadays, multiplex-PCR is also applied to pneumococcal serotyping. The use of MALDI-TOF mass spectrometry for serotyping has also been investigated, but this far it has not been shown to be a reliable tool for serotyping, because different serotypes have not been shown to reproducibly display typical peaks (Ercibengoa *et al.* 2019).

2.6.3 MALDI-TOF MASS SPECTROMETRY

In mass spectrometry, chemicals are ionized by various methods, such as directing laser beams on the sample or spraying it with electrons. The mass-charge ratio of the various components of the sample is then plotted as a mass spectrum, showing the relative proportions of these components in the sample in addition to the masses of each component. Mass spectrometry has many applications in physics, chemistry and life sciences and can be applied to such varied uses as enrichment of uranium for nuclear technology applications and analysing protein composition of biological samples. Different methods have been developed for ionization and for separation of the ionized sample components according to their mass-charge ratios. Different mass spectrometry methods can also be combined in tandem mass spectrometry. Mass spectrometry can also be used together with such chemical separation methods as gas or liquid chromatography.

The first modern mass spectrometers were used for discovery and identification of the different isotopes of chemical elements by Francis William Aston in 1919. During the World War II, a type of sector mass spectrometer called calutron was used by Ernest O. Lawrence to separate the isotopes of Uranium, enabling the development of nuclear weapons in Manhattan project (Parkins 2005). However, these types of mass spectrometers were not suitable for analysis of the chemical composition of biological materials. The study of large biological chemical compounds, such as proteins, would have required softer ionization methods in order to maintain sufficient integrity of the molecules.

The analysis of biological macromolecules by mass spectrometry only became possible by development of Matrix-assisted laser desorption/ionization mass spectrometry (MALDI) during the 1980s by Karas *et al.* (1985, 1987) and Koichi Tanaka (Tanaka *et al.* 1988). MALDI-TOF mass spectrometry utilizes ionization technique in which pulsed laser beams are used to softly ionize large molecules such as proteins or peptides, allowing them to maintain their integrity during ionization. Before ionization, the sample is injected on a target plate and overlaid with a matrix solution containing organic acid such as α-cyano-4-hydroxycinnamic acid (HCCA) or 2,5-dihydroxybenzoic acid and a counter acid such as trifluoroacetic acid (TFA) dissolved in a mixture of an organic solvent such as acetonitrile and

deionised water. The solvents are then left to evaporate, leaving the sample covered in recrystallized matrix. The matrix is used to protect the sample molecules during the ionization.

After being ionized by the pulsed laser, the ionized molecules are then launched on a flight path and their flight time measured by a detector in a process called time-of-flight (TOF). The measured flight time of the ionized molecules is then used to calculate their mass-charge ratio and create a mass spectrum showing the relative abundance of molecules of various masses contained in the sample. Time-of-flight mass spectrometry is useful in analysis of biological molecules because it allows for detection of molecules over a wide mass range. In case of microbial identification, the mass range measured is typically between 2 000 and 20 000 Da.

Within the last ten years the matrix-assisted laser desorption/ionization-time of flight mass spectrometry (MALDI-TOF MS) has established itself as a fast and cost-effective identification method for a wide range of microbes, both bacteria and fungi. (Seng *et al.* 2009, Bizzini *et al.* 2010, Neville *et al.* 2011, Martin *et al.* 2017). Although Anhalt and Fenselau had investigated the use of mass spectrometry for bacterial identification already in 1975, mass spectrometry for microbial identification wasn't widely studied before the late 1990s and early 2000s when new MALDI-TOF technology enabled more gentle treatment of microbial cells (Claydon *et al.* 1996, Krishnamurty *et al.* 1996, Fenselau and Demirev 2001). Earlier mass spectrometry methods had disintegrated the microbial proteins into peptides, thus yielding them unsuitable for identification purposes. With the MALDI-TOF technology, the bacterial proteins are better conserved, allowing for identication based on comparison of signature of protein spectra typical for different species (Fenselau and Demirev 2001). The mass range detected for microbiological applications is typically between 2 000 and 20 000 Da. An example of MALDI-TOF mass spectrum for a bacterial strain is shown in figure 2.
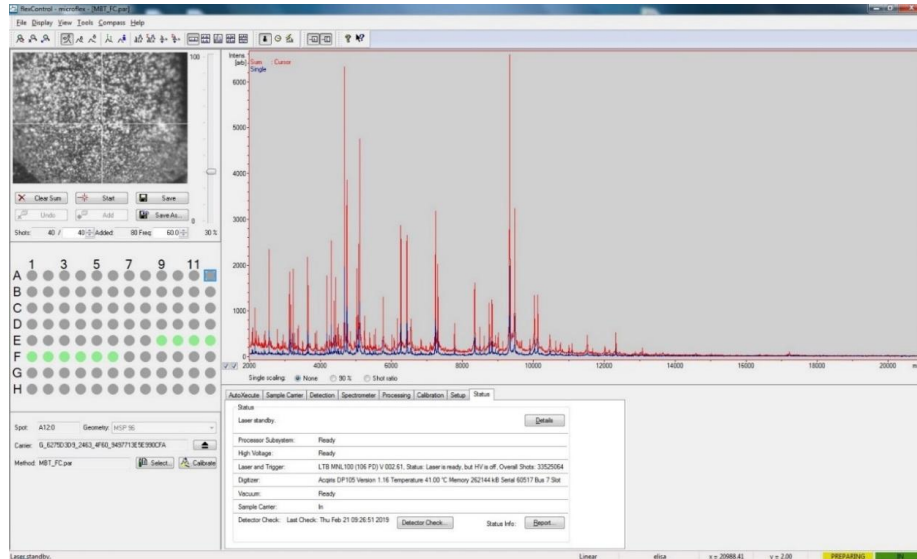
Figure 2. An example of a MALDI-TOF mass spectrum of a bacterial strain

For microbial identification, the protein spectra need to be compared to a database composed of protein spectra of well-characterised reference strains by a dedicated software utilising an algorithm which calculates the similarity of the sample spectra to the reference spectra of the database. This similarity is then expressed in a numerical form, as percentage on the VITEK MS platform (BioMérieux) and as a score value ranging from zero to three in the MALDI Biotyper system (Bruker Daltonics). The standard log(score) algorithm employed by MALDI Biotyper gives the same relative weight to similarity across the whole mass range of 2 000-20 000 Da. In contrast, the algorithm employed in VITEK MS weighs specific peaks in the mass areas showing particularly high variability when calculating the identification results (Rychert *et al.* 2013).

Within the last decade MALDI-TOF mass spectrometry has been widely studied and adopted in clinical microbiology laboratories for identification of pathogenic bacteria (Seng *et al.* 2009, Bizzini *et al.* 2010, Neville *et al.* 2011). In contrast to nucleic acid amplification tests (NAATs), identification by MALDI-TOF mass spectrometry is usually performed on microbial biomass extracted from a culture medium, such as culture plates or blood culture bottles. The main benefits of MALDI-TOF mass spectrometry compared to traditional biochemical methods include its quick time to identification (minutes compared to hours to days when using biochemical methods), low reagent costs and specificity (Martin *et al.* 2017, Ródriguez-Sánchez *et al.* 2019). MALDI-TOF mass spectrometry also has lower reagents costs than NAATs. Neville *et al.* (2011) estimated the cost of MALDI-TOF identification to be only 0.45 Australian dollars (roughly 0.70 €) per isolate, which is

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significantly less than typical costs of NAATs, which typically cost 10-100 hundred times more per isolate.

Another benefit of MALDI-TOF mass spectrometry in comparison to NAATs is that it can be used to identify any bacteria or fungi included in the database of the instrument, unlike NAATs that target a specific gene or a set of genes for detection of a limited number of organisms at time. Since the databases of the MALDI-TOF instruments today include spectra from thousands of bacterial species, MALDI-TOF mass spectrometry can be used to reliably identify almost any bacterium isolated in a clinical microbiology laboratory, greatly decreasing the need for several separate identification tests for different group of bacteria (Martin *et al.* 2017).

On the other hand, the technical limitations of MALDI-TOF for use in microbiology include the fact that enrichment by culture methods is typically required in order to generate enough bacterial biomass for identification and its difficulty in identifying several bacteria from mixed cultures. MALDI-TOF mass spectrometry also cannot reliably distinguish between certain very closely related bacterial species such as *Escherichia coli* and *Shigella* sp. or the different species of Mitis group streptococci. It is interesting to note, that the species that are difficult to differentiate by MALDI-TOF are typically also difficult to reliably identify to species level using 16S rRNA sequencing, which is generally considered a golden standard in bacterial identification.

MALDI-TOF mass spectrometry has also been successfully applied to identification of other clinically relevant microbial groups, such as mycobacteria, yeasts and filamentous fungi (Martin *et al.* 2017). The extraction of bacterial biomass for MALDI-TOF identification from certain bacterial groups containing mycolic acid in their cell walls, such as mycobacteria and aerobic actinobacteria like *Nocardia* and *Streptomyces*, has proved challenging (Martin *et al.* 2017, Rodríguez-Sánchez *et al.* 2019). Another drawback is the inability of MALDI-TOF to distinguish between different species of the *Mycobacterium tuberculosis* complex. However, the development of modified glass bead extraction methods and introduction of identification modules specifically designed for identification of mycobacteria have allowed for reliable identification of atypical mycobacteria (Rodríguez-Sánchez *et al.* 2016, Alcaide *et al.* 2018). When it comes to the MALDI-TOF identification of clinically relevant fungi, yeast species can be identified utilising the standard sample processing protocols (Chao *et al.* 2014). In contrast, MALDI-TOF identification of filamentous fungi such as moulds and dermatophytes often requires prior incubation in an enrichment broth in order to produce more homogenic mycelia (Cassagne *et al.* 2016).

For diagnosis of bacteraemia, enrichment of blood specimens in an automated blood culture system is still necessary, but the time to identification of microbes growing in positive blood culture has been significantly reduced in recent years by adoption of rapid identification methods such as automated

real-time PCR and isothermal amplification assays and MALDI-TOF mass spectrometry. Whereas identification of microbes growing in a blood culture bottle using traditional biochemical methods would typically take 1 to 2 days in the case of aerobic bacteria and 4 to 7 days for anaerobic bacteria (Tabak *et al.* 2018), performing NAAT or MALDI-TOF identification directly from a positive blood culture can reduce this time to 30 to 120 minutes (Ferreira *et al.* 2011, Kok *et al.* 2011, Dixon *et al.* 2015). This has been showed in studied by Martiny *et al.* (2013) and Clerc *et al.* (2013) to allow for earlier initiation of targeted antibiotic treatment. In a study carried out by Clerc *et al.* (2013) early MALDI-TOF identification directly from positive blood culture bottle led to the adjustment of antibiotic treatment in 35 % of the bacteraemia cases. In the same study it was also noticed, that these changes in antibiotic treatment typically led to a switch to a narrower-spectrum antibiotic, significantly reducing the use of carbapenems and other broad-spectrum antibiotics. Limiting the unnecessary use of broad-spectrum antibiotics is in turn important for combating the spread of antibiotic resistance, since exposure to these antibiotics selects for strains harbouring genes coding for resistance to them. Dixon *et al.* (2015) also found that MALDI-TOF identification of bacteria isolated from blood-stream infection resulted in a reduction of subsequent length of stay and treatment costs. A rapid identification of potential contaminants is also important, in many cases preventing the starting of unnecessary antibiotic treatment (Martiny *et al.* 2013). Thus, it is important to develop these techniques so that all significant pathogens could be identified directly from positive blood culture bottles using these techniques.

Identification of *Streptococcus pneumoniae* and VGS by MALDI-TOF MS has been widely studied (van Veen *et al.* 2010, de Bel *et al.* 2010, Neville *et al.* 2011, Scholz *et al.* 2012, Martiny *et al.* 2012, Werno *et al.* 2012, Davies *et al.* 2012, Lopez Roa *et al.* 2013, Ikryannikova *et al.* 2013, Dubois *et al.* 2013, Branda *et al.* 2013, Woods *et al.* 2014, Angeletti *et al.* 2015, Isaksson *et al.* 2015, Van Prehn *et al.* 2016). What has emerged from these studies has been that whereas other members of VGS can be more reliably distinguished from *S. pneumoniae* by MALDI-TOF, reliable differentiation between *S. pneumoniae* and other members of *S. mitis* group has proven more challenging because of the close relationship of these species. Within VGS, reliable species level identification has proved challenging, since the protein spectra of the species included in the same VGS subgroup (*S. anginosus*, *S. bovis*, *S. mitis*, *S. mutans*, *S. salivarius* or *S. sanguinis* groups) typically resemble each other, reflecting their close genetic relationship.

2.6.4 MOLECULAR DIAGNOSTCS

Within last couple of decades, diagnostic assays based on the detection of nucleic acid sequence specific for certain pathogenic microbes have been widely adopted in clinical microbiology laboratories. Advances in diagnostic techniques, such as real-time PCR and isothermal amplification, have reduced the hands-on-time required, as well time-to-results and cost of these assays, allowing for their use also in large-scale routine diagnostic investigations. In addition to the development of more integrated and automated nucleic acid amplification platforms, their miniaturization has been instrumental in the spreading of nucleic acid amplification techniques (NAATs). Compared to immunochromatographic antigen detection tests, the nucleic acid amplification tests have the benefit of greater sensitivity, reducing the need for further confirmatory tests. Although NAATs target specific genes, development of multiplex nucleic acid amplification tests has allowed for detection of several target genes simultaneously in the same assay. This, along with automatization and miniaturization of the nucleic acid amplification platforms has greatly enhanced their appeal to clinical microbiology laboratories, enabling the detection of several key pathogens from a single sample. Such multiplex NAAT panels are typically tailored to include key bacterial pathogens typically isolated from a certain specimen type, such as stool or cerebrospinal fluid.

The 16S rRNA gene, which is widely used in molecular identification of bacteria, is highly conserved among the species within a certain group of VGS (such as *S. mutans* group or *S. mitis* group) (Kawamura *et al.* 1995, 1999, Hoshino *et al.* 2005) and might even be present in several variant copies in some strains (Kilian *et al.* 2008), yielding it suboptimal as an identification tool for this group of bacteria to the species level. However, Haanperä *et al.* (2007) showed that pyrosequencing of the most variable part of the 16S rRNA gene, located in its 5' end, could be used for identification of VGS to the subgroup level. Pyrosequencing, which is a rapid and low-cost sequence generation method, has also been applied for identification of streptococci amplifying the RNase P RNAse gene, *rnpB* (Innings *et al.* 2005). Therefore, several other genes have been targeted in identification and phylogenetic studies, often using a multi-locus sequence typing (MLST) approach (Kawamura *et al.* 1999, Whatmore *et al.* 2000, Hoshino *et al.* 2005, Chi *et al.* 2007, Nakano *et al.* 2007, Kilian *et al.* 2008, Do *et al.* 2009, Thompson *et* al. 2013, Delorme *et al.* 2015, Rasmussen *et al.* 2016). Some of the most commonly used targets include the housekeeping genes for D-alanine-D-alanine ligase (*ddl*) (Garnier *et al.* 1997, Kawamura *et al.* 1999, Kilian *et al.* 2008), heat shock proteins chaperonin-10, GroES and chaperonin-60, GroEL (*groESL*) (Teng *et al.* 2002), RNA subunit of endoribonuclease B (*rnpB*) (Täpp *et al.* 2003, Innings 2005, Isaksson *et al.* 2015), the β-subunit of RNA polymerase (*rpoB*) (Drancourt *et al.* 2004), manganese-dependent

superoxide dismutase (*sodA*) (Poyart *et al.* 1998, Poyart *et al.* 2002), elongation factor Tu (*tuf*) (Picard *et al.* 2004) and a conserved subunit of bacterial recombinase, *recA* (Zbinden *et al.* 2011, Sistek *et al.* 2012). Of these, the *recA* gene is a housekeeping gene which has been selected for a candidate gene for species differentiation between *S. pneumoniae* and MGS as well as within SMG because of its high interspecies variation within MGS. A 756bp fragment of *recA* amplified in a real-time PCR assay developed by Sistek *et al.* 2012 was shown to allow good differentiation between *S. pneumoniae* and *S. pseudopneumoniae*. However, MLST is too expensive and time-consuming to use as a routine identification tool in a clinical microbiology laboratory and therefore remain more of a research tool.

Shahinas *et al.* (2013), Kilian *et al.* (2014) and Rasmussen *et al.* (2016) have explored the use of whole genome sequencing as a tool for investigating the phylogenetic relationships among clinical strains of the MGS. These analyses have provided more insights into the genomic organisation for the MGS, corroborating the previous evidence for wide-spread genetic recombination and providing new information especially on the presence and organisation of virulence genes in members of SMG, as discussed previously. Recently, Zheng *et al.* (2016) have published StreptoBase, an online database and collection of information for the identification of SMG (http://streptococcus.um.edu.my).

The strategies based on MLST and whole genome sequencing discussed above are at least currently too expensive and cumbersome for differentiation of *Streptococcus pneumoniae* and Viridans group streptococci in a routine clinical microbiology laboratory. In contrast, several nucleic acid amplification tests based on PCR and intended for detection of *S. pneumoniae* directly from patient samples have therefore been developed.

In an early pneumococcal PCR study, Rudolph *et al.* (1993) amplified pneumolysin and autolysin genes from blood samples, with a sensitivity of 37.5 % for whole blood samples. Zhang *et al.* (1995) developed a PCR test to detect a part of the pneumococcal PBP 2B gene from the whole blood of patients with bacteremic bacterial pneumonia, obtaining a sensitivity of 80 %. Dagan *et al.* (1998), Toikka *et al.* (1999), Lorente *et al.* (2000) and Dominguez *et al.* (2001) used traditional PCR and agarose gel electrophoresis to amplify pneumococcal pneumolysin gene directly from blood samples of patients with suspected community-acquired pneumonia, but all of these reported low sensitivity ranging from 26.6 % (Dominguez *et al.* 2001) to 55 % (Lorente *et al.* 2000). Salo *et al.* (1995) and Menendez *et al.* (1999) had reported improved sensitivity of PCR test detecting fragment of pneumolysin genes when using nested PCR from serum samples from patients with community-acquired pneumonia compared to blood cultures, but considered the multiple steps required to process the blood samples and extract the DNA a significant drawback of their method. In general, the PCR assays they studied tended to require fractioning the blood samples and only show high sensitivity when

analysing samples from patients for whom bacterial culture from blood was also positive.

In 2004, Sheppard *et al.* developed a real-time PCR assay for detection of *S. pneumoniae* targeting the pneumococcal autolysin-gene, but in line with the results of previous studies, reported a sensitivity of only 42.9 % when analysing EDTA blood samples from patients with blood-culture-confirmed pneumococcal infections. In 2009, Smith *et al.* compared the performance of a PCR protocol targeting both pneumolysin and autolysin genes to Binax NOW S. pneumoniae urinary antigen test in diagnosing pneumococcal infections in adults with community-acquired pneumonia and found the dual-PCR assay to be significantly less sensitive than the urine antigen detection test. Abdeldaim *et al.* (2010) evaluated the performance of three real-time PCR assays, targeting genes for autolysin and pneumolysin as well as *S. pneumoniae* 9802 gene fragment (Spn9802). They reported that the assay targeting the ply gene was too unspecific for clinical use, whereas autolysin and Spn9802 assays showed higher specifities (100 % and 98 %, respectively), although also lower sensitivities than pneumolysin assay.

More recently, Clancy *et al.* (2015) have also designed an isothermal nucleic acid amplification assay utilizing rapid recombinase polymerase amplification technique (RPA), targeting the pneumococcal gene encoding leader peptidase A. They had only analysed a small number of clinical blood samples but reported that the assay was positive for 8 of the 11 blood culture-positive patient blood samples. As they allow for fast nucleic acid amplification, several other assays based on isothermal amplification methods have also been designed, especially for diagnosis of pneumococcal meningitis. These assays are often incorporated into multiplex panels simultaneously targeting several bacterial pathogens which often cause meningitis. Examples of such multiplex assays include an assay designed by Clancy *et al.* (2016) utilizing nucleic acid sequence-based amplification (NASBA) and one designed by Soysal *et al.* (2017) based on line probe assay (LPA) technology.

The ABACUS GenomEra *S. pneumoniae* test (Abacus Diagnostica, Turku, Finland) is an identification test based on automated nucleic acid amplification reaction using primers specific for *Streptococcus pneumoniae*. The target gene of these primers has not been disclosed by the manufacturer. The Genomera CDX instrument automatically identifies the inserted test chips by reading barcodes on the chips. The chips are then sealed thermally in order to minimize the risk of cross-contamination. The nucleic acid amplification reaction is performed by automatically transporting the test chips between blocks heated to different temperatures for denaturing, annealing/extension and measurement. The speed of the amplification is further enhanced by using heat-conducting metallic background for the test chips and by inclusion of extreme-temperature blocks in the thermocycling. The amplification products are detected by homogenous fluorescence measurement using enhanced

competitive hybridization technique (Hagren *et al.* 2008). In enhanced competitive hybridization, two partially complementary oligonucleotide probes bind to the target DNA more tightly than to each other, allowing for more sensitive detection of the target DNA. Furthermore, the use of heat-resistant, time-resolved fluorescent lanthanide label on the label probe effectively reduces sample autofluorescence (Hirvonen 2017). The whole assay can be completed in 50 minutes and up to four samples can be analysed simultaneously. In addition to GenomEra S. pneumoniae assay, there are currently commercially available GenomEra assays for the detection of MRSA/SA, *Clostridium difficile*, *Streptococcus agalactiae* and norovirus.

3 AIMS OF THE STUDY

The general aim of this work was to investigate whether MALDI-TOF mass spectrometry and nucleic acid amplification methods could be used to enable the rapid and reliable identification of *Streptococcus pneumoniae* and differentiation between *S. pneumoniae* and mitis group streptococci in a clinical microbiology laboratory.

The specific aims were:

I To compare the performance of two MALDI-TOF systems, MALDI Biotyper and VITEK MS for identification of *Streptococcus pneumoniae* and viridans group streptococci.

II To investigate whether the performance of MALDI Biotyper system for differentiation between *S. pneumoniae* and mitis group streptococci could be enhanced by the combined addition of more strains into the MALDI Biotyper database and the incorporation of novel result interpretation algorithm.

III To investigate the performance of ABACUS GenomEra *S. pneumoniae* nucleic acid amplification test for direct detection of *Streptococcus pneumoniae* directly from positive blood culture bottles.

4 MATERIALS AND METHODS

4.1 BACTERIAL STRAINS AND CULTURE METHODS

4.1.1 BACTERIAL STRAINS

Both reference streptococcal strains and *Streptococcus pneumoniae* and Viridans group streptococcal strains isolated from patients with invasive streptococcal infections at the Clinical Microbiology Department of Turku University Hospital were utilised in this study. The strains and sample materials used in this study are listed in original publications (I-III) and summarized in the figure 3.
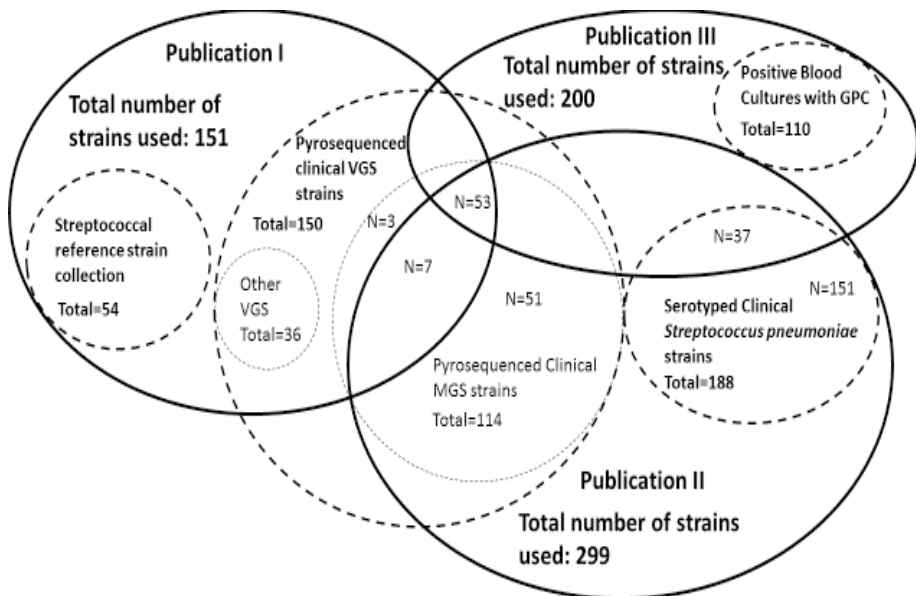
Figure 3. A schematic diagram of the strains and specimens used in the different publications included in this study. N = Number of strains or specimens, GPC = gram-positive cocci, VGS = Viridans group streptococci, MGS = Mitis group streptococci.

For the first part of this study, the performance of MALDI Biotyper (Bruker Daltonics, Bremen, Germany) and VITEK MS (bioMérieux, Marcy-l'Etoile, France) MALDI-TOF mass spectrometry systems for identification of Viridans group streptococci was compared. This comparison was performed by identifying a collection of 54 streptococcal reference strains and 97 clinical Viridans group streptococcal strains isolated from positive blood cultures on both MALDI-TOF systems. The 16S rRNA gene of these clinical Viridans group streptococcal strains had previously been partially sequenced using pyrosequencing as described in Haanperä *et al.* 2007.

In the second part of this study, comparing the performance of different MALDI Biotyper database versions and identification algorithms in differentiation of *S. pneumoniae* and Mitis group streptococci, 111 MGS strains isolated from invasive infections (60 of which had also been included in the earlier parts of this study) were analysed (II, figure 3). The *S. mitis/S. sanguinis* group strains had been preliminarily identified as belonging to *S. mitis* or *S. sanguinis* groups by biochemical identification by VITEK2 instrument (BioMérieux, Marcy-l'Etoile, France) and confirmed as such by partial sequencing of the 16S rRNA gene. A collection of 188 serotyped *S. pneumoniae* strains isolated from blood culture specimens, 37 of which had also been included in the second part of this study, was also included.

In order to evaluate the ability of the automated PCR assay GenomEra S. pneumoniae (Abacus Diagnostica, Turku, Finland) to differentiate between S. pneumoniae and closely related other Mitis group streptococci, 53 clinical MGS strains (44 *S. mitis* group streptococcal strains and 9 S. sanguinis group strains) that had been included in the first part of this study, were also included in the third part of this study (III, figure 3). A collection of 37 serotyped *S. pneumoniae* strains isolated from positive blood cultures at the Clinical Microbiology Laboratory of Turku University Hospital and a collection of positive blood culture specimens isolated at FIMLAB laboratories was also included in the third part of this study.

4.1.2 CULTURE CONDITIONS

For MALDI-TOF identification (I-III), streptococcal strains were stored in a mixture of skimmed milk and glycerol at -70°C and subsequently cultured on BD BBL™ Trypticase™ Soy Agar with 5 % Sheep Blood (Becton Dickinson, Franklin Lakes, New Jersey, USA). After incubation at 35°C with 5 % CO_2 for 17-24 h, the strains were re-inoculated from these initial culture plates on BD BBL™ Trypticase™ Soy Agar with 5 % Sheep Blood (Becton Dickinson, Franklin Lakes, New Jersey, USA). The resulting pure cultures were incubated at 35°C with 5 % CO_2 for 17-24 h before analysis with MALDI-TOF mass spectrometry.

For evaluation of identification of *S. pneumoniae* with automated PCR assay GenomEra *S. pneumoniae* (Abacus Diagnostica, Turku, Finland) (III), the streptococcal strains isolated at the Clinical Microbiology Department of Turku University Hospital were stored and incubated at Clinical Microbiology Laboratory of Turku University Hospital as described above. The strains were then re-inoculated from these initial culture plates on sheep blood agar (LabM Ltd, Heywood, Lancashire, UK) and incubated at 37°C with 5 % CO_2 for 18 h at FIMLAB Laboratories.

Positive blood culture specimens yielding gram-positive diplococci or gram-positive cocci growing in chain formations were also collected at FIMLAB Laboratories for evaluation of identification of *S. pneumoniae* with

automated PCR assay GenomEra *S. pneumoniae* (Abacus Diagnostica, Turku, Finland) (III). These blood culture specimens had been incubated on BacT/Alert automated blood culturing system (bioMérieux, Marcy l'Etoile, France). For comparative identification methods (Vitek MS, bioMérieux and StrepID 32, BioMérieux), these positive blood culture specimens were also subcultured on blood and chocolate agars (LabM Ltd) and fastidious anaerobe agar (LabM Ltd).

4.2 METHODS USED IN THIS STUDY

The methods used in this study are summed in table 1.

Table 1. Summary of the methods and instruments used

Publication	I	II	III
MALDI-TOF identification	MALDI Biotyper, VITEK MS	MALDI Biotyper	MALDI Biotyper
ABACUS GenomEra *S. pneumoniae* test	No	No	Yes
VITEK 2 identification	Yes	Yes	Yes
Optochin sensitivity testing	Yes	Yes	Yes
Bile solubility testing (pneumococcal strains)	No	Yes	Yes
Pneumococcal serotyping	No	Yes	Yes
Pyrosequencing	Yes	Yes	Yes
recA sequencing	No	Yes	No

4.2.1 BIOCHEMICAL IDENTIFICATION METHODS

The *S. pneumoniae* strains used in this study had been identified as pneumococci by optochin sensitivity testing using optochin discs (Optochin 10 μg, DIATABS™, ROSCO Diagnostica A/S, Taastrup, Denmark). Optochin sensitivity testing was also performed for all VGS strains included in this study. All *S. pneumoniae* strains had also been serotyped and confirmed as *S. pneumoniae* by optochin sensitivity and bile solubility testing at the Finnish National Institute for Health and Welfare. All clinical streptococcal strains used in this study had also been presumptively identified by an automated biochemical identification system (VITEK 2, BioMérieux). For the second part of this study, the strains isolated from positive blood culture bottles were also identified by a biochemical test panel, StrepID 32 (BioMérieux).

4.2.2 PNEUMOCOCCAL SEROTYPING

All *S. pneumoniae* strains used in this study were confirmed as pneumococci by the Finnish National Institute for Health and Welfare with a multiplex PCR specific for *S. pneumoniae* as described by Siira *et al.* (2012). Pneumococcal serotyping based on Quellung reaction had also been performed at the Finnish National Institute for Health and Welfare for all pneumococcal strains included in this study.

4.2.3 IDENTIFICATION USING MALDI-TOF MASS SPECTROMETRY

For MALDI-TOF identification using MALDI Biotyper (Bruker Daltonics, Bremen, Germany) (I-III), the isolates were initially inoculated directly from colonies to the steel target plates as duplicates. On top of each of these spots, 1 μl of matrix solution (α-cyano-4-hydroxycinnamic acid in 50 % acetonitrile/2.5 % trifluoroacetic acid, Bruker Daltonics, Bremen, Germany) was added. Bacterial Test Standard calibrant (Bruker Daltonics) was used as a quality control on each plate. Bacterial Test Standard consists of a freeze-dried reference strain *Escherichia coli* ATCC 25922 to which RNAse A and bovine serum albumin was utilized have been added to provide coverage of higher molecular weight range (10 000-20 000 Da) of the instrument's measurement area of 2 000-20 000 Da. The isolates yielding an identification score between 1.7 and 1.999 were designated as "good identification to the genus level" by the MALDI Biotyper software. The isolates yielding a score value <1.7 were designated as "no reliable identification". All strains yielding identification results with a score value under 2.0 were subsequently reanalysed using the standard extraction protocol recommended by Bruker Daltonics.

In the extraction protocol recommended by Bruker Daltonics, bacterial biomass was collected with 1 μl plastic loop. It was then suspended in 300 μl chromatography-grade deionized water, to which 900 μl of absolute ethanol was added. Repeated centrifugation (2 min, 13 000 rpm) was then performed, with disposal of supernatant after each round. The resulting pellet was suspended in 10-30 μl of 80 % formic acid, the volume of formic acid added depending on the size of the pellet. After two minutes' incubation at room temperature, a volume of acetonitrile equal to the volume of formic acid was added. Finally, a centrifugation step (2 min, 13 000 rpm) was performed. We then added 1 μl of bacterial extract on two consecutive spots on the steel target plate. When the spots had dried, they were overlaid with 1 μl of matrix solution.

For MALDI-TOF identification using the VITEK MS instrument (BioMérieux, Marcy l'Etoile, France) (I and III), bacterial colonies were inoculated as duplicates on disposable target plates (BioMérieux, Marcy l'Etoile, France) using a 1 μl plastic loop. On one of these spots, 25 % formic acid was added as recommended by the manufacturer for identification of yeasts. When spots were dry, 1 μl of the matrix solution (α-cyano-4-

hydroxycinnamic acid (VITEK MS CHCA, BioMérieux) was added on each of them. *Escherichia coli* ATCC 8739 was used as a calibrant and internal control following manufacturer's instructions.

If the analysis yielded a percent probability match of under 60 (the best possible probability match being 99.9), the isolate was considered non-identified and the same sample preparation protocol was repeated.

For this study, the MALDI-TOF identifications were performed using both the Microflex LT instrument (Bruker Daltonics, Bremen, Germany), (figure 4) at the Clinical Microbiology Department of Turku University Hospital (I-III) and VITEK MS instrument (BioMérieux, Marcy l'Etoile, France, figure 5) at the Department of Clinical Microbiology of Päijät-Häme Social and Health Care Group in Lahti (I) and at FIMLAB laboratories in Tampere (III).

Figure 4. Microflex LT instrument, Bruker Daltonics

Figure 5. VITEK MS instrument, BioMérieux (source: BioMérieux)

The database versions and algorithm utilised in various publications contained in this study are outlined in Table 2.

Table 2. MALDI-TOF databases and algorithms used in this study, including the number of *S. pneumoniae, S. mitis* and *S. oralis* reference spectra in different database versions of MALDI Biotyper

Publication	I	II	III
VITEK MS IVD database version	VITEK MS IVD database v2/ VITEK MS SARAMIS		VITEK MS IVD database v2
MALDI Biotyper database versions (strains)	3.3.1.0 (4613 strains)	3.3.1.0 (4613 strains) 4.0.0.1 (5627 strains)	3.3.1.0 (4613 strains)
The number of *S. pneumoniae* reference spectra in the MALDI Biotyper database version tested	8	8 (3.3.1.0) 30 (4.0.0.1)	8
The number of *S. mitis* reference spectra in the MALDI Biotyper database version tested	1	1 (3.3.1.0)) 39 (4.0.0.1)	1
The number of *S. oralis* reference spectra in the MALDI Biotyper database version tested	4	4 (3.3.1.0) 38 (4.0.0.1)	4
Algorithm used for MALDI-TOF identification with MALDI Biotyper	log(score)	log(score), list(score) (used only in conjunction with 4.0.0.1)	log(score)

For comparison of two MALDI-TOF identification systems in the first part of this study, the identifications were performed using both Flex Control 3.0 software and MALDI Biotyper DB Update 3.3.1.0 containing 4 613 reference entries on Microflex LT (Bruker Daltonics, Bremen, Germany) and VITEK MS IVD v2 database on VITEK MS instrument (bioMérieux, Marcy l'Etoile, France). For the first part of this study, the streptococcal type strains not adequately identified when using VITEK MS IVD v2 database were also subsequently identified with VITEK SARAMIS (RUO) database. MALDI Biotyper DB Update 3.3.1.0 contained reference spectra for 53 of the 54 species included in the reference strain collection tested. In contrast, only 38 of the 54 streptococcal species in the reference strain set were included in the VITEK MS IVD v2 database.

For the second part of this study, the clinical pneumococcal and *Streptococcus mitis* group strains were identified by MALDI-TOF using both the Microflex LT instrument (Bruker Daltonics, Bremen, Germany) at the Clinical Microbiology Department of Turku University Hospital. Spectra were analysed using MALDI Biotyper software version 3.0 (RUO) for both database versions 3.3.1.0 containing 4 613 reference entries and 4.0.0.1 containing 5 627 reference. Of these database versions, MALDI Biotyper database version 3.3.1 (4 613 entries) contained 1 *S. mitis*, 4 *S. oralis* and 8 *S. pneumoniae* strains. The updated database version 4.0.0.1 (5 627 entries) contained 39 *S. mitis*, 38 *S. oralis*, and 30 *S. pneumoniae* isolates (table 2).

For result interpretation, the standard MALDI Biotyper data interpretation rules [using log(scores) for the best and second-best matches of the ranking list] were applied to both database versions. Using MALDI Biotyper database version 4.0.0.1, the first 10 positions of the ranking lists were additionally used to calculate a new supplementary list(score) as described in the third part of this study in more detail. At the first step, each log(score) of the first 10 positions of a ranking list was multiplied by a factor of 10 (1st position), a factor of 9 (2nd position), . . ., a factor of 2 (9th position), and a factor of 1 for the 10th position to calculate weighted log(scores). In the second step, each weighted log(score) was summarized for each single species appearing in the ranking list. List(scores) were then compared between all species appearing in the ranking list. Such list(scores) can be applied to differentiation of closely related microorganisms. It naturally requires a database content of more than 10 strains per species in order to be useful.

For the third part of this study, MALDI-TOF identification of the isolates belonging to the streptococcal collection had been performed using Flex Control 3.0 software and MALDI Biotyper DB Update 3.3.1.0 containing 4 613 reference entries on Microflex LT (Bruker Daltonics, Bremen, Germany at the Clinical Microbiology Department of Turku University Hospital. Conversely, MALDI-TOF identification of the strains isolated from the positive blood culture bottles was performed at FIMLAB laboratories in Tampere using a VITEK MS IVD v2 database on VITEK MS instrument (bioMérieux).

4.2.4 ABACUS GENOMERA S. PNEUMONIAE ASSAY

For the third part of this study, the pneumococcal nucleic acid amplification assays were run on GenomEra CDX (Abacus Diagnostica), which is a compact fully automated thermal cycler (figure 6), at FIMLAB Laboratories at Tampere, Finland.

For the pneumococcal and *S. mitis*/*S. sanguinis* group streptococcal culture collection strains, bacterial biomass collected from a single bacterial colony from a pure culture plate was suspended in 1 ml of GenomEra Sample Buffer. For the positive blood culture bottles, 25 µl of blood was diluted in 1 ml of GenomEra Sample Buffer. 35 µl of these sample suspensions was then

transferred to a GenomEra *S. pneumoniae* test chip, which contained all the PCR reagents and an internal amplification control.

Figure 6. ABACUS GenomEra CDX instrument

The software used for the interpretation of the nucleic acid amplification results from the GenomEra CDX instrument calculates numerical signal values for the target sequence, ranging from a -15 (negative) to +100 (strong positive). The results that fall in the area between -5 and +5 are considered inconclusive.

4.2.5 PYROSEQUENCING

All the Viridans group streptococcal strains used in this study were identified by pyrosequencing of the 16S rRNA gene at the Finnish National Institute for Health and Welfare as described by Haanperä *et al.* (2007). Sequence data from previously sequenced isolates were re-analysed for the third part of this study in order to evaluate whether any of these isolates belonged to the newly described species *Streptococcus dentisani* (Camelo-Castillo *et al.* 2014) or *Streptococcus tigurinus* (Zbinden *et al.* 2012).

4.2.6 *recA* GENE SEQUENCING

In the second part of this study, in addition to pyrosequencing of the 16S rRNA gene, *recA* sequencing according to Sistek *et al.* (2012) was performed for 24 SMG strains. The sequencing of the recA gene was performed in order to confirm whether these isolates belonged to the species *Streptococcus pseudopneumoniae*. These strains were selected for the *recA* gene sequencing because they did not fall clearly into any specific SMG species based on the results of pyrosequencing of the 16S rRNA gene. In contrast, their 16S rRNA sequences differed by the same number of nucleotides (1 to 3 nucleotides) from those of the type strains of *S. pneumoniae*, *S. pseudopneumoniae*, *S. mitis*, and *S. oralis*

5 RESULTS

5.1 ASSESSMENT OF THE PERFORMANCE OF MALDI BIOTYPER AND VITEK MS FOR IDENTIFICATION OF VIRIDANS GROUP STREPTOCOCCI (I)

The results of the MALDI-TOF identifications performed in this study are shown in Table 3.

For comparison of the MALDI Biotyper (Bruker Daltonics) and Vitek MS (BioMérieux) MALDI-TOF systems (I), MALDI Biotyper system using database version 3.3.1.0 containing 4613 strains gave species- or genus-level identification with score value ≥1.7 for 97 % (149/151) of the streptococcal strains studied. 46 % of the strains (68/151) were identified using only direct spotting and 54 % (81/151) using formic acid extraction by MALDI Biotyper. VITEK MS identified 89 % (135/151) of the streptococcal strains with an acceptable confidence level (≥ 60 %). For 86 % of these strains (116/135), the identification was reached by direct colony method, whereas 14 % (19/135) required on-target extraction with formic acid extraction for identification.

For the clinical VGS strains tested in the first part of this study, 95 of 97 strains had been identified at least to the group level (*S. anginosus/S. bovis/S. mitis/S. salivarius/S. sanguinis* groups) by partial sequencing of the 16S rRNA gene. The remaining two strains were ambiguously identified by pyrosequencing as *S. oralis/S. gallolyticus*. These strains were identified by both evaluated MALDI-TOF methods as *S. parasanguinis*.

For the streptococcal reference collection strains, MALDI Biotyper correctly identified 94 % of them (51/54) to the species level. One reference isolated remained unidentified and two type strains were misidentified. *Streptococcus infantarius* ssp. *infantarius* CCUG43820 was misidentified by MALDI Biotyper as *Streptococcus equinus* and *Streptococcus mitis* DSM12643 as *Streptococcus pneumoniae*. VITEK MS system with the database version VITEK MS IVD v2 consistently identified 69 % (37/54) of the streptococcal type strains. VITEK MS could provide no identification for 9 of the 54 reference strains tested. Further eight reference strains were misidentified by VITEK MS system when the database version VITEK MS IVD v2 was used. For two of the non-identified strains and three of the misidentified strains correct identification could subsequently be obtained by utilising the VITEK MS SARAMIS (RUO) database.

Table 3. MALDI-TOF identification results of streptococcal strains used in this study acquired by different MALDI-TOF instruments, database versions and algorithms (DB = database, v. = version, N = number of strains, VGS = Viridans group streptococci, MGS = Mitis group streptococci)

	MALDI-TOF system and database version	VITEK MS IVD DB v. 2		MALDI Biotyper DB v. 3.3.1.0 (4613 strains)		MALDI Biotyper DB v. 4.0.0.1 (5627 strains) with log(score) algorithm		MALDI Biotyper DB v. 4.0.0.1 (5627 strains) with list(score) algorithm	
	Publication	n	%	n	%	n	%	n	%
Streptococcal strains yielding an acceptable identification [a]	I	135/151	89	149/151	97				
	II			299/299	100	299/299	100	299/299	100
Streptococcal reference strains correctly identified to the group or species level	I	37/54	69	51/54	94				
Clinical VGS strains identified correctly to the species group level[b]	I	88/97	93	85/97	89				
Clinical VGS strains correctly identified to the species level[c]	I	35/36	97	27/36	75				
Clinical Viridans group non-pneumococcal strains misidentified as *S. pneumoniae*[d]	I	0/97	0	36/97	37				
S. pneumoniae strains correctly identified to the species level	II			188/188	100	188/188	100	188/188	100
Clinical MGS strains correctly identified to the species group level[e]	I	63/63	0	27/63	43				
	II			35/101	35	100/101	99	101/101	100

a) ≥ 1.7 for MALDI Biotyper, ≥ 60 % for VITEK MS
b) 95 of the 97 clinical VGS strains had been identified at least to the species group level by partial 16S rRNA sequencing
c) 36 of the 97 clinical VGS strains unequivocally identified to the species level by partial 16S rRNA sequencing
d) All the strains misidentified as Streptococcus pneumoniae were identified as non-pneumococcal S. mitis/S. sanguinis group species by partial 16S rRNA sequencing
e) All misidentified strains were misidentified as Streptococcus pneumoniae. The 10 strains belonging to the S. sanguinis subgroup of MGS were excluded from this analysis, since they were all correctly identified to the species level even when using the MALDI Biotyper database version 3.3.1.0

MALDI Biotyper identified 85 of the 97 clinical Viridans group strains used in the first part of this study (89 %) and VITEK MS 88 (93 %) to the group level consistently with the partial 16S rRNA gene sequencing. Of the 36 strains for which partial 16S rRNA sequencing had unequivocally suggested a species level identification, MALDI Biotyper identified 27 (75 %) as the same species as pyrosequencing. VITEK MS identified 35 of these 36 strains (97 %) to the same species as partial 16S rRNA sequencing. One of the 36 strains could not be identified by VITEK MS.

MALDI Biotyper misidentified 36 of the 97 clinical Viridans group streptococcal strains included in the first part of this study as *S. pneumoniae* (table 3). In contrast, VITEK MS misidentified none of these strains as *S. pneumoniae*. All the clinical Viridans group streptococcal strains misidentified by MALDI Biotyper had been identified by partial 16S rRNA sequencing as members of either *S. mitis* or *S. sanguinis* groups. (There were altogether 63 strains identified by pyrosequencing as belonging to these groups included in the first part of this study, figure 3.) Optochin testing was performed for all the strains identified as *S. pneumoniae* by MALDI Biotyper. All but one of them proved to be optochin-resistant, which is consistent with the pyrosequencing results. When re-examining the pyrosequencing data (Haanperä *et al.* 2007), all these strains were also found to contain adenine at the position 203 in 16S rRNA gene, which had been reported not to be the case for *S. pneumoniae* by Scholz *et al.* in 2012.

5.2 THE PERFORMANCE OF MALDI BIOTYPER AND ABACUS GenomEra™ *S. PNEUMONIAE* TEST FOR DIFFERENTIATION OF CLINICAL PNEUMOCOCCAL AND MITIS GROUP STREPTOCOCCAL STRAINS (I, II, III)

In the first part of this study, a collection of 63 clinical streptococcal strains identified as non-pneumococcal members of *S. mitis* species group (including members of *S. sanguinis* subgroup) by partial 16S rRNA sequencing were identified with both MALDI Biotyper with database version 3.3.1.0 (4613 strains) (Bruker Daltonics) and VITEK MS with database version IVD vs. 2 (BioMérieux) MALDI-TOF systems in (I). MALDI Biotyper misidentified 36 of these strains as *S. pneumoniae*, whereas VITEK MS misidentified none of these strains as *S. pneumoniae* (table 3). No clinical pneumococcal strains were included in this part of the study.

In the second part of this study (II), the effect of adopting an updated database version (4.0.0.1, 5627 strains) containing an increased number of reference entries for *S. pneumoniae*, *S. mitis* and *S. oralis* (table 2) combined with a novel identification algorithm (list(score)) for the ability of MALDI Biotyper to differentiate between *S. pneumoniae* and other members *S. mitis/S. sanguinis* group streptococci was studied. For this goal, a wider selection of 111 clinical Mitis/Sanguinis group streptococcal strains and 188 pneumococcal strains were identified using MALDI Biotyper system.

The results of assessment of the performance of MALDI Biotyper for differentiation of pneumococci and mitis group streptococci using an enhanced database and a novel result interpretation algorithm (II) are summarized in Table 3. All *S. pneumoniae* strains were correctly identified as *S. pneumoniae* with all MALDI Biotyper database versions and both the standard log(score) algorithm and the novel list(score) algorithm. For the *S. mitis* and *S. sanguinis* group strains, only 35/101 strains (35 %, 10 of which were members of *S. sanguinis* subgroup) were correctly identified to the group level with database version 3.3.1 containing 4613 bacterial strains and standard log(score) algorithm, whereas 66/101 (65 %) strains were misidentified as *S. pneumoniae*, consistent with the results of the first part of this study, in which 27 of the 63 *S. mitis* and *S. sanguinis* group strains tested (75 %) were misidentified using the same database version and algorithm (table 3). Using the newer database version 4.0.0.1 (5627 strains) with the standard log(score) algorithm, only one of the 101 Mitis group strains tested was misidentified, whereas even that strain was correctly identified when combining the newer database version 4.0.0.1 (5627 strains) with the novel list(score) algorithm.

For the evaluation of the ability of the ABACUS GenomEra™ *S. pneumoniae* nucleic acid amplification test to reliably differentiate between *S. pneumoniae* and other members of *S. mitis*/*S. sanguinis* species group streptococci (III), 37 clinical pneumococcal strains and 53 members of the *S. mitis*/*S. sanguinis* species group also isolated from clinical infections were included in the third part of this study (figure 3). All 37 *S. pneumoniae* strains included were positive for *S. pneumoniae* target in ABACUS GenomEra™ *S. pneumoniae* assay, whereas all 44 *S. mitis* group streptococcal strains and 9 *S. sanguinis* group strains gave negative results. The turnaround time to the results was 55 minutes.

5.3 THE PERFOMANCE OF ABACUS GenomEra™ S. PNEUMONIAE TEST FOR DETECTION OF PNEUMOCOCCI DIRECTLY FROM POSITIVE BLOOD CULTURE BOTTLES (III)

For the evaluation of the performance of ABACUS GenomEra™ Pneumococcus test, detection of pneumococci directly from positive blood culture bottles, 46 of the 110 positive blood culture specimens (41.8 %) the bacteria in the specimens were identified as *S. pneumoniae* by VITEK MS (BioMérieux, Marcy-l'Etoile, France), Strep ID 32 (BioMérieux) and optochin susceptibility testing. Other streptococcal species, enterococci, anaerobic gram-positive cocci along with *Acinetobacter lwoffii*, *Corynebacterium amycolatum* and *Veillonella parvula* were recovered from the remaining positive blood culture bottles (64 specimens). All 46 positive blood culture specimens containing *S. pneumoniae* gave strongly positive (+100) signals for *S. pneumoniae* target in ABACUS GenomEra™ *S. pneumoniae* assay, whereas all 64 positive specimens containing other bacterial species remained negative (signal level -15). All positive blood culture specimens containing *S. pneumoniae* yielded positive results within 55 minutes.

6 DISCUSSION

Rapid identification of *Streptococcus pneumoniae* using NAATs or MALDI-TOF mass spectrometry has proven challenging because of its close genetic relationship to streptococci belonging to *Streptococcus mitis/Streptococcus sanguinis* group, which form an important part of normal oral microbiota (Haanperä *et al.* 2007). It has also been reported in several studies that differentiation between *S. pneumoniae* and *S. mitis* group streptococci poses a challenge in direct identification of bacteria from positive blood culture bottles using MALDI-TOF mass spectrometry (La Scola *et al.* 2009, Prod'hom *et al.* 2010, Kok *et al.* 2011, Farina *et al.* 2015). Direct pneumococcal antigen testing has been proposed as a confirmatory test for rapid identification of *S. pneumoniae*, but false positive results caused by *S. mitis* group streptococci cross-reacting with the pneumococcal C polysaccharide antigen are common (Petti *et al.* 2005).

In the early studies on the ability of the MALDI-TOF mass spectrometry to identify streptococci, identification of VGS remained unreliable, probably largely due to the limited number of VGS strains contained in the earlier versions of the databases of MALDI-TOF instruments (van Veen *et al.* 2010, de Bel *et al.* 2010, Neville *et al.* 2011, Scholz *et al.* 2012, Martiny *et al.* 2012, , Davies *et al.* 2012, Lopez Roa *et al.* 2013, Ikryannikova *et al.* 2013, Dubois *et al.* 2013, Branda *et al.* 2013, Woods *et al.* 2014).

Based on the results of the comparison between MALDI Biotyper with database version 3.3.1.0 and VITEK MS with database version IVD vs. 2, VGS species could be reliably identified to the group level (to *S. anginosus* group, *S. bovis* group, *S. mitis* group, *S. mutans* group and *S. salivarius* groups) by MALDI-TOF mass spectrometry. In contrast, reliable identification of VGS to the species level within a specific group remained elusive (I). When using the earlier database version 3.3.1.0 containing 4 613 reference entries, in which only a few reference spectra for both *S. pneumoniae* and *S. mitis* group streptococci were included, MALDI Biotyper (Bruker Daltonics) could not sufficiently differentiate between these closely related species. Compared to MALDI Biotyper with the earlier database version (containing 4613 database entries), Vitek MS system with VITEK MS IVD database v2 (BioMérieux) was better able to reliably distinguish between *S. pneumoniae* and *S. mitis* group streptococci. This has also been found to be the case in the study by Angeletti *et al.* (2015) carried out using the same database versions. In their study in 2015 Isaksson *et al.* also found that among the VGS, MALDI-TOF had most difficulties with the identification of the MGS. On the other hand, for the VGS as a whole, VITEK MS performed worse than MALDI Biotyper for species or group level identification. This probably reflected the fact that at the time of the study, compared to the MALDI Biotyper database, the VITEK MS database

was weighed more towards the clinically most important bacterial species and therefore contained more reference spectra for *S. pneumoniae*. Another factor might have been the difference between the algorithm utilised by the two MALDI-TOF mass spectrometry platforms. Whereas the standard log(score) algorithm employed by MALDI Biotyper compares the whole sample spectrum to the reference spectra in a similar manner, the algorithm employed in VITEK MS weighs specific peaks in the mass areas showing particularly high variability when calculating the identification results (Rychert *et al.* 2013).

Based on the results of the comparison between MALDI Biotyper with database version 3.3.1.0 and VITEK MS with database version IVD vs. 2, we hypothesized, that a combination of MALDI-TOF and NAAT identification techniques might still be necessary in a clinical microbiology laboratory, since obtaining good-quality mass spectra directly from blood culture bottles can sometimes be more challenging for streptococci and other gram-positive bacteria than for gram-negative bacteria (Kok *et al.* 2011). We therefore also wanted to test a rapid automated NAAT ABACUS GenomEra *S. pneumoniae* assay for confirmation of *S. pneumoniae* in conjunction with MALDI-TOF mass spectrometry. The results proved that ABACUS GenomEra can reliably distinguish between *S. pneumoniae* and *S. mitis* group streptococci directly from positive blood culture bottles. Since *S. pneumoniae* is one of the most important pathogens causing bacteraemia, the ability to identify it quickly and correctly also from positive blood culture bottles is of prime importance.

Because it was noticed that MALDI Biotyper misidentified most of the Viridans group streptococci belonging to *S. mitis* and *S. sanguinis* groups as *S. pneumoniae* when using the MALDI Biotyper database version 3.3.1 containing 4613 bacterial strains, we decided to concentrate on the differentiation between *S. pneumoniae* and *S. mitis* and *S. sanguinis* group streptococci. We hypothesized that adding more *S. pneumoniae* and *S. mitis* and *S. sanguinis* group streptococcal spectra to the MALDI Biotyper database and/or the application of a novel list(score) algorithm tailored to the differentiation between these bacteria might enhance the identification of these closely related species.

The addition of 22 new *S. pneumoniae* strains, 38 new *S. mitis* strains and 34 new *S. oralis* strains into the MALDI Biotyper database version 4.0.0.1 (DB_5627) resulted in significantly improved differentiation between S. pneumoniae and S. mitis and S. sanguinis group, with only one isolate of 101 misidentified as S. pneumoniae when using the DB_5627 with the standard log(score) algorithm. When using the DB_5627 with the novel list(score) algorithm, all the S. mitis/S. sanguinis group strains tested were identified correctly to the group level. This was also the case when using the newer MALDI Biotyper database version DB_5989 with the standard log(score) algorithm.

It is interesting to note that Slotved *et al.* (2017) received markedly worse identification results in their study in which they had identified 47 *S. pneumoniae* and 37 other Mitis group streptococcal strains using MALDI Biotyper with a newer database version containing 6903 entries. In their study, all pneumococcal isolates with a serotypeable capsule were correctly identified, while one non-capsular isolate was incorrectly identified as *S. pseudopneumoniae*, whereas four of the nine *S. pseudopneumoniae* strains were incorrectly identified as *S. pneumoniae*. Of the 20 *S. mitis* strains they tested, three were misidentified as *S. pneumoniae*. This difference between our and their results may be partially be explained by the fact that unlike us, they did not perform formic acid extractions in addition to direct colony spotting. However, the difference is probably also largely due to the fact that their set of MGS strains contained nine strains of *S. pseudopneumoniae*, whereas none of the clinical strains in our study was identified as *S. pseudopneumoniae* by partial 16S rRNA sequencing or *recA* gene sequencing. There were also no non-serotypeable *S. pneumoniae* strains among our clinical isolates, whereas in their study 25 of the 47 *S. pneumoniae* strains tested were non-capsular and therefore non-serotypeable. The lack of *S. pseudopneumoniae* and non-capsular *S. pneumoniae* strains among our clinical strains, which were isolated from invasive infections supports the notion that *S. pseudopneumoniae* and non-capsular pneumococci are typically not involved in invasive infections (Park et al. 2014, Keller *et al.* 2016 (2), Bradshaw *et al.* 2018). In another study by van Prehn *et al.* (2016) it was also found that MALDI Biotyper (with database version containing 5627) misidentified three of the 13 *S. pseudopneumoniae* tested as *S. pseudopneumoniae*, in contrast to VITEK MS, which identified correctly all the *S. pseudopneumoniae* strains tested in their study. Although the difficulties with reliable identification of *S. pseudopneumoniae* are not probably highly relevant for diagnosis of invasive infections, they are probably more relevant when detecting pneumococcal carriage.

Several authors have also suggested detection of specific peaks in order to help with the differentiation of *S. pneumoniae* and MGS and aiding in species-level identification of non-pneumococcal MGS (Werno *et al.* 2012, Ikryannikova *et al.* 2013, Chen *et al.* 2015, Marín *et al.* 2018). Although useful for research laboratories, adoption of such closer analysis of the sample spectra in a clinical microbiology laboratory would require a more automated data analysis than is currently available. The results of the studies detecting specific peaks also seem generally less reproducible than identification results based on the analysis of the whole protein spectra, probably because the exact mass of a single peak is more sensitive to intraspecies variations and differences in growth conditions.

7 CONCLUSIONS AND FUTURE PERSPECTIVES

Our study has shown that VGS streptococci can reliably be identified to group level (to *S. anginosus* group, *S. bovis* group, *S. mitis* group, *S. mutans* group or *S. salivarius* group) by MALDI-TOF mass spectrometry. *S. pneumoniae* and *S. mitis* group streptococci can also be reliably distinguished from each other by MALDI-TOF mass spectrometry when using a database containing reference spectra of several species of both *S. pneumoniae* and *S. mitis* group streptococci, preferably combined with an identification algorithm weighing first few reference matches, such as list-score algorithm tested in the third part of this study for MALDI Biotyper, or weighing specific peaks when calculating the identification results, such as the algorithm employed in VITEK MS (Rychert *et al.* 2013).

Based on the results of our study, we would suggest that including more *S. pneumoniae* and *S. mitis* group streptococci in the databases of MALDI-TOF mass spectrometry instruments and developing specific algorithms has significantly improved the ability of MALDI Biotyper system to distinguish between these closely related streptococci, allowing for reliable differentiation of *S. pneumoniae* and *S. mitis* group streptococci colonies from culture plates. It would also be interesting to know whether the application of the list(score) algorithm tested in this study would aid in more reliably identifying even *S. pseudopneumoniae* strains, which were unfortunately not included in our study sample.

In addition to MALDI-TOF mass spectrometry, adoption of rapid automated NAATs such as ABACUS Genomera *S. pneumoniae* evaluated in this study also have the potential to significantly accelerate the identification of *S. pneumoniae*. Combining MALDI-TOF mass spectrometry and such rapid targeted NAATs for identification of streptococci directly from positive blood culture bottles could enable their identification within an hour of the blood culture system has signalled for growth, whereas identification of *S. pneumoniae* and VGS using traditional biochemical identification methods typically takes two days after growth has been detected in the blood culture bottle. In the future, it would be interesting to evaluate the effect that the adaption of a rapid identification strategy combining MALDI-TOF mass spectrometry with NAATs performed directly from positive blood culture bottles has had for the turn-around time for the identification of streptococci in a clinical microbiology laboratory. In near future, it may be possible to accelerate the identification process even further by utilising more sensitive NAATs that can identify bacteria directly from patient's blood without need for enrichment by blood culture. Combination of MALDI-TOF or electron-

spray ionization mass spectrometry (ESI-MS) with other technologies such as flow cytometry or chromatography also hold potential for faster and more accurate infection diagnostics.

Accurate species-level identification among non-pneumococcal *S. mitis* group or other VGS strains will likely remain an elusive goal even with the development of even more sophisticated molecular identification methods in the future. This is a result of their nature as a very variable species group or species spectrum for which human-imposed species definitions, which don't consider the extent of horizontal gene transfer and genome rearrangements likely happening within even the same strain over time, are ill-fitting. This is to a lesser degree true also for other Viridans group streptococci, for which reliable group level identification can be obtained by MALDI-TOF mass spectrometry, but for many of which more detailed species-level identification has also proven challenging, even when using such laborious and time-consuming methods as MLST.

Fortunately, for Viridans group streptococci, group-level identification seems to be enough for the purpose of evaluating the clinical significance of the isolated organism, since members of the same species group tend to reside in similar sites in the human body and resemble each other in their pathogenic potential. After 75 years and countless technological innovations in the field of microbiology, these words of Sherman *et al.* on Viridans group streptococci in the seminal paper of 1943 still ring true: "It is idle to try to define a "species" of bacteria, or even say whether or not such an entity can be defined… However, the classification of bacteria is of value for practical purposes, the "species" being units which can be identified and differentiated from other closely related groups with some degree of certainty and ease… Although tedious and detailed studies are essential in order to define more definitely the limits of a species, the main value of such work as this is to arrive at some simple tests which can be applied in the practical problem of quickly identifying the organism."

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