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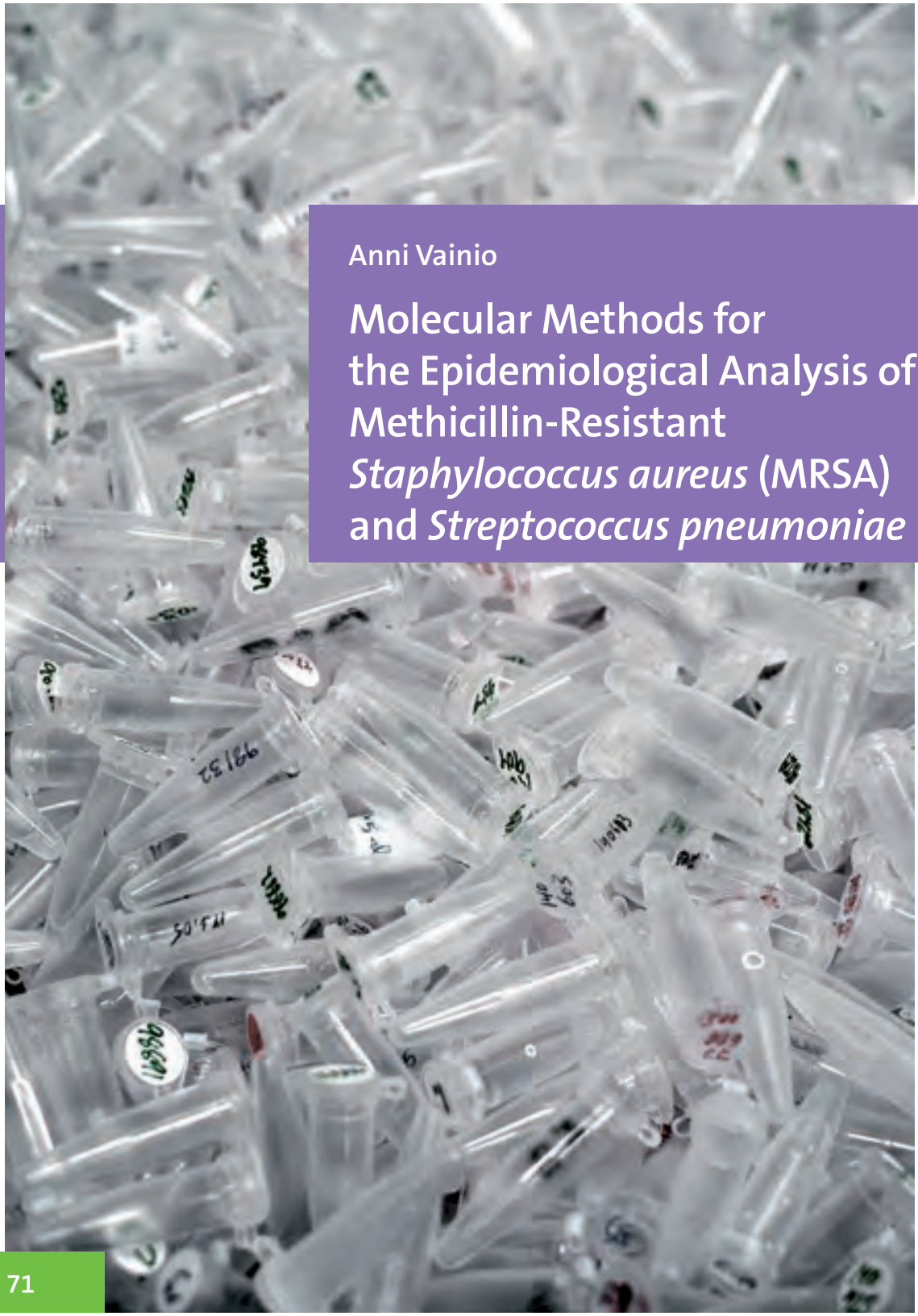

RESEARCH

Anni Vainio

Molecular Methods for the Epidemiological Analysis of Methicillin-Resistant *Staphylococcus aureus* (MRSA) and *Streptococcus pneumoniae*

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Molecular Methods for the Epidemiological Analysis of Methicillin-Resistant *Staphylococcus aureus* (MRSA) and *Streptococcus pneumoniae*

ACADEMIC DISSERTATION

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Abstract

Anni Vainio. Molecular Methods for the Epidemiological Analysis of Methicillin-Resistant *Staphylococcus aureus* (MRSA) and *Streptococcus pneumoniae*. National Institute for Health and Welfare (THL), Research 71, 164 pages. Tampere, Finland 2012. ISBN 978-952-245-554-3 (printed); ISBN 978-952-245-555-0 (pdf).

Methicillin-resistant *Staphylococcus aureus* (MRSA) and *Streptococcus pneumoniae* are major health problems worldwide, both found in symptomless carriage but also causing even life-threatening infections. The aim of this thesis was to characterise MRSA and *S. pneumoniae* in detail by using several molecular typing methods for various epidemiological purposes: clonality analysis, epidemiological surveillance, outbreak investigation, and virulence factor analysis.

The characteristics of MRSA isolates from the strain collection of the Finnish National Infectious Disease Register (NIDR) and pneumococcal isolates collected from military recruits and children with acute otitis media (AOM) were analysed using various typing techniques. Antimicrobial susceptibility testing, pulsed-field gel electrophoresis (PFGE), multilocus sequence typing (MLST), *spa* typing, staphylococcal cassette chromosome *mec* (SCC*mec*) typing, and the detection of Panton-Valentine leukocidin (PVL) genes were performed for MRSA isolates. Pneumococcal isolates were analysed using antimicrobial susceptibility testing, serotyping, MLST, and by detecting pilus islet 1 (PI-1) and 2 (PI-2) genes. The epidemiological background data for the pneumococcal pneumonia outbreak investigation were retrieved by interviewing all the military recruits involved in the outbreak. In addition, the medical records of five hospitalised recruits were reviewed.

Forty-four previously recognised epidemic MRSA strains (EMRSA) in Finland were grouped into 26 PFGE clusters, 20 MLST sequence types (ST) belonging to 12 clonal complexes (CC), and 27 *spa* types divided into four *spa* clonal complexes (*spa* CC). The CC8, *spa* CC 051/008, and SCC*mec* type IV were the most prevalent among these strains. In addition, 52% of the strains were multiresistant to antimicrobials. FIN-4 and FIN-16 isolates mainly associated with *spa* type t172 (2006: 81% and 1997–2006: 92%) and t067 (2006: 93% and 1997–2006: 80%), respectively. The 124 MRSA blood isolates from 1997–2006 were divided into 19 PFGE types and 38 *spa* types. The most prevalent *spa* types were t067 (32%), t041 (13%) and t172 (10%).

Typeability, discriminatory power, concordance, Wallace coefficient, and the consumption of time and money was compared between MRSA typing methods. The overall typeability of the methods was high, above 89%. PFGE clusters, MLST and *spa* typing showed a high discriminatory power. The concordance was highest

between MLST and PFGE clusters. *spa* typing demonstrated a high probability to predict PFGE, MLST ST and MLST CC, as well as SCC*mec* types, depending on the study material. Compared to PFGE, *spa* typing was found to be more expensive but approximately four times faster to perform.

S. pneumoniae serotype 7F and genotype ST2331 associated with an outbreak of pneumonia and nasopharyngeal carriage among 43 military recruits in Finland in 2006. Of these recruits, five (12%) were hospitalised due to pneumonia and two of them were found to have positive blood cultures for *S. pneumoniae*. Forty-two per cent of the military recruits carried *S. pneumoniae* in their nasopharynx. Half of these isolates had the same serotype 7F, ST2331.

Among the 75 pneumococcal isolates from middle ear fluid (MEF) and/or nasopharyngeal aspirate (NPA) samples of children with acute otitis media (AOM), 14 different serotypes were detected. PI-1 genes were present among 20% of the isolates. PI-2 genes were not found. The 52 isolates from 33 children genotyped by MLST belonged to 30 different STs and 18 known CCs. PI-1 was associated with genotype CC490 and serotypes 6A, 6B and 9V.

Several international community- and hospital-associated MRSA clones were recognised in Finland. The genetic diversity among MRSA FIN-4 isolates and among FIN-16 isolates was low. Overall, MRSA blood isolates from 1997 to 2006 were genetically diverse. *spa* typing was found to be a highly discriminatory, rapid and accurate typing method and it also qualifies as the primary typing method in countries with a long history of PFGE-based MRSA strain nomenclature. However, additional typing by another method, e.g. PFGE, is needed in certain situations to be able to provide adequate discrimination for epidemiological surveillance and outbreak investigation.

An outbreak of pneumonia was associated with one pneumococcal strain among military recruits, previously healthy young men living in a crowded setting. The pneumococcal carriage rate after the outbreak was found to be exceptionally high.

PI-1 genes were detected at a rather low prevalence among pneumococcal isolates from children with AOM. However, the study demonstrated that PI-1 has existed among pneumococcal isolates prior to pneumococcal conjugate vaccine and the increased antimicrobial resistance era. Moreover, PI-1 was found to associate with the serotype rather than the genotype.

Keywords: clonality; methicillin-resistant *Staphylococcus aureus*; molecular typing; outbreak; pilus; *Streptococcus pneumoniae*; surveillance

Tiivistelmä

Anni Vainio. Molecular Methods for the Epidemiological Analysis of Methicillin-Resistant *Staphylococcus aureus* (MRSA) and *Streptococcus pneumoniae*. [Metisilliini-resistentti *Staphylococcus aureus* (MRSA) ja *Streptococcus pneumoniae*: molekyylimenetelmien käyttö epidemiologisissa analyyseissa]. Terveyden ja hyvinvoinnin laitos (THL), Tutkimus 71, 164 sivua. Tampere 2012. ISBN 978-952-245-554-3 (painettu); ISBN 978-952-245-555-0 (pdf).

Metisilliini-resistentti *Staphylococcus aureus* (MRSA) ja *Streptococcus pneumoniae* aiheuttavat merkittäviä terveysongelmia maailmanlaajuisesti. Molemmat voivat löytyä oireettomilta kantajilta, mutta ne voivat aiheuttaa myös hengenvaarallisia infektioita. Väitöskirjan tavoitteena oli karakterisoida MRSA- ja *S. pneumoniae*-bakteereita tarkemmin käyttämällä useita tyypitysmenetelmiä erilaisiin epidemiologisiin tutkimuksiin: klonaalisiin analyyseihin, epidemiologiseen seurantaan, epidemia-selvityksiin ja virulenssitekijöiden analysointiin.

Tartuntatautirekisterin kantakokoelman MRSA-kantojen ja varusmiehiltä sekä akuuttia välikorvantulehdusta sairastavilta lapsilta kerättyjen pneumokokkikantojen ominaisuuksia analysoitiin käyttämällä erilaisia tyypitysmenetelmiä. MRSA-kannoille käytettiin antibioottiherkkyystestausta, pulssikenttägeelielektroforeesia (PFGE), MLST-menetelmää, *spa*- ja SCC*mec*-tyypitystä sekä Panton-Valentin-leukosidiini- (PVL) geenien havaitsemista. Pneumokokkikantoja analysoitiin antibioottiherkkyystestauksella, serotyypityksellä, MLST-menetelmällä ja määrittämällä pilus-1 (PI-1) ja -2 (PI-2) geenien läsnäoloa. Pneumokokin aiheuttamassa keuhkokuume-epidemiassa taustatieto hankittiin haastattelemalla kaikki epidemiaan liittyvät varusmiehet. Lisäksi tarkasteltiin viiden sairaalahoitoon joutuneen varusmiehen sairaskertomuksia.

Neljäkymmentäneljä jo aikaisemmin tunnistettua suomalaista epideemistä MRSA-kantaa (EMRSA) jakaantui 26 PFGE ryhmäksi ja 20 MLST sekvenssityypiksi (ST), jotka kuuluivat 12 klonaaliseen kompleksiin (CC). Lisäksi näiden kantojen joukosta löydettiin 27 *spa*-tyyppiä, jotka jakaantuivat neljään *spa*-klonaaliseen kompleksiin (*spa* CC). EMRSA-kantojen joukossa yleisimpiä olivat CC8, *spa* CC 051/008 ja SCC*mec*-tyyppi IV. Lisäksi 52 % kannoista oli resistenttejä useille antibiooteille. FIN-4-kannat yhdistettiin pääasiassa *spa*-tyyppiin t172 (2006: 81 % ja 1997-2006: 92 %) ja FIN-16-kannat *spa*-tyyppiin t067 (2006: 93 % ja 1997-2006: 80 %). Vuosilta 1997-2006 verestä eristetyistä MRSA-kannoista 124 jakaantui 19 PFGE-tyypiksi ja 38 *spa*-tyypiksi. Yleisimmät *spa*-tyypit olivat t067 (32 %), t041 (13 %) ja t172 (10 %).

Tyypityksen onnistumista, vastaavuutta, Wallace-kerrointa, sekä tyypityksiin kuluvaan aikaa ja kustannuksia vertailtiin eri MRSA-tyypitysmenetelmien kesken. Kaiken kaikkiaan yli 89 % kannoista saatiin tyypitettyä. Kaikkein erottelevimpia menetelmiä olivat PFGE-menetelmä (ryhmätasolla), MLST ja *spa*-tyypitys. Vastaavuus oli korkeinta

MLST- ja PFGE-menetelmien (ryhmätasolla) välillä. *spa*-tyypitys ennusti PFGE-, MLST ST-, MLST CC- ja SCC*mec*-menetelmien tyypitystulosta suurella todennäköisyydellä, mutta tutkimusmateriaalista riippuen. *spa*-tyypitys oli kalliimpaa mutta nopeampi suorittaa kuin PFGE.

Pneumokokin serotyppi 7F ja genotyyppi ST2331 yhdistettiin keuhkokuume-epidemiaan ja nenänielun kantajuuteen 43 varusmiehellä Suomessa vuonna 2006. Viisi (12 %) varusmiehistä joutui sairaalahoitoon keuhkokuumeen vuoksi ja kahdella heistä kasvoi pneumokokki veriviljelynäytteestä. Varusmiehistä 42 % kantoi pneumokokkia nenänielussaan. Puolet varusmiesten nenänieluista löydetyistä pneumokokeista olivat samaa serotyppiä 7F, ST2331.

Akuuttia välikorvantulehdusta sairastavien lasten välikorvan eritteestä ja/tai nenä-nielun aspiraatiosta eristetyistä 75 pneumokokkikannasta löytyi 14 erilaista sero-tyyppiä. Kannoista 20 % kantoi PI-1-geenejä. PI-2-geenejä ei löydetty ollenkaan. Viisikymmentäkaksi kantaa, jotka eristettiin 33 lapselta ja genotyypitettiin MLST-menetelmällä, kuuluivat 30 eri sekvenssityyppiin ja 18 tunnettuun klonaaliseen kompleksiin. PI-1 yhdistettiin genotyyppiin CC490 ja serotyyppeihin 6A, 6B ja 9V.

Useita kansainvälisiä sairaalaperäisiä ja avohoitoon liittyviä MRSA-klooneja on tunnistettu Suomessa. Geneettinen monimuotoisuus oli FIN-4 ja FIN-16 MRSA-kantojen joukossa alhainen. Yleisesti ottaen verestä eristetyt MRSA-kannat vuosilta 1997-2006 olivat geneettisesti erilaisia. *spa*-tyypitys oli hyvin erottelukykyinen, nopea ja tarkka tyypitysmenetelmä ja se täyttää vaatimukset ensisijaisena tyypitysmenetelmänä myös maissa joilla on pitkä historia MRSA-kantojen nimeämisestä PFGE-tyypityksellä. Lisätyypitysmenetelmiä, esimerkiksi PFGE-menetelmää, kuitenkin tarvitaan tietyissä tilanteissa, jotta saavutetaan riittävä erottelukyky epidemiologista seurantaa ja epidemiaselvitystä varten.

Ahtaissa oloissa elävien, aiemmin terveiden varusmiesten keuhkokuume-epidemiaan liittyi yksi pneumokokkikanta. Epidemian jälkeen varusmiesten pneumokokki-kantajuuden taso oli poikkeuksellisen korkea.

Melko pieneltä osalta pneumokokkikannoista, jotka oli eristetty akuuttia välikorvantulehdusta sairastavilta lapsilta, löydettiin PI-1-geenejä. Tutkimus kuitenkin osoitti, että PI-1 on ollut olemassa jo ennen pneumokokin konjugaattirokotteen käyttöä ja lisääntynyttä antibioottiresistenttiyttä. Lisäksi PI-1 liittyi ennemmin serotyyppiin kuin genotyyppiin.

Avainsanat: epidemia; klonaalisuus; metisilliini-resistentti *Staphylococcus aureus*; molekyylityypitys; pilus; seuranta; *Streptococcus pneumoniae*.

Contents

List of original papers

This thesis is based on the following original publications, which are referred to throughout the text by the Roman numerals given below (I-IV).

I Vainio A, Kardén-Lilja M, Ibrahem S, Kerttula AM, Salmenlinna S, Virolainen A, Vuopio-Varkila J. Clonality of epidemic methicillin-resistant *Staphylococcus aureus* strains in Finland as defined by several molecular methods. Eur J Clin Microbiol Infect Dis. 2008 July;27(7):545-55.

II Vainio A, Koskela S, Virolainen A, Vuopio J, Salmenlinna S. Adapting *spa* typing for national laboratory-based surveillance of methicillin-resistant *Staphylococcus aureus*. Eur J Clin Microbiol Infect Dis. 2011 Jun;30(6):789-97.

III Vainio A, Lyytikäinen O, Skyttä R, Kaijalainen T, Teirilä L, Rantala M, Lehtinen P, Ruuska P, Virolainen A. An outbreak of pneumonia associated with *S. pneumoniae* at a military training facility in Finland in 2006. APMIS. 2009 Jul;117(7):488-91.

IV Vainio A, Kaijalainen T, Hakanen AJ, Virolainen A. Prevalence of pilus-encoding islets and clonality of pneumococcal isolates from children with acute otitis media. Eur J Clin Microbiol Infect Dis. 2011 Apr;30(4):515-9.

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Abbreviations

AOM	acute otitis media
AR	adjust Rand
agr	accessory gene regulation
bp	base pair
BURP	based upon repeat pattern
CA-MRSA	community-acquired methicillin-resistant *Staphylococcus aureus*
CBP	choline-binding protein
CC	clonal complex
ccr	cassette chromosome recombinase
CI	confidence interval
CLSI	Clinical and Laboratory Standards Institute
CPS	capsular polysaccharide
DI	discriminatory index
eBURST	based upon related sequences
EARS-net	European Antimicrobial Resistance Surveillance Network
EMRSA	epidemic methicillin-resistant *Staphylococcus aureus*
EUCAST	European Committee on Antimicrobial Susceptibility Testing
HA-MRSA	hospital-acquired methicillin-resistant *Staphylococcus aureus*
ICU	intensive care unit
IPD	invasive pneumococcal disease
J	joining region of SCC*mec*
LytA	N-acetylmuromyl-L-alanine amidase (hydrolytic enzyme)
LytC	*β-N*-acetylmuramidase
MSCRAMM	microbial surface components recognising adhesive matrix molecule
mecA	gene coding for penicillin-binding protein 2a (PBP2a)
MEF	middle ear fluid
MIC	minimum inhibitory concentration
MLST	multilocus sequence typing
MLVA	multilocus variable number tandem repeat analysis
MSSA	methicillin-sensitive *Staphylococcus aureus*
MRSA	methicillin-resistant *Staphylococcus aureus*
NIDR	National Infectious Disease Register
NPA	nasopharyngeal aspirate

ORF	open reading frame
PavA	pneumococcal adherence and virulence factor A
Pht	pneumococcal histidine triad protein
PBP	penicillin-binding protein
PCR	polymerase chain reaction
PCV	pneumococcal conjugate vaccine
PFGE	pulsed-field gel electrophoresis
PI-1	pilus islet 1
PI-2	pilus islet 2
PNC	*Streptococcus pneumoniae*
PpmA	putative proteinase maturation protein A
PrtA	serine protease of *S. pneumoniae*
PsaA	pneumococcal surface antigen A
PspA	pneumococcal surface protein A
PspC	pneumococcal surface protein C
PVL	Panton-Valentine leukocidin
RFLP	restriction fragment length polymorphism
Spa	staphylococcal protein A
SCC*mec*	staphylococcal cassette chromosome *mec*
SLV	single locus variant
SLST	single-locus sequence typing
SNP	single nucleotide polymorphism
ST	sequence type
THL	National Institute for Health and Welfare (Terveyden ja hyvinvoinnin laitos, THL)
TSS	toxic shock syndrome
TSST	toxic shock syndrome toxin
UK	United Kingdom
US	United States
VISA	vancomycin intermediate-resistant *S. aureus*
VNTR	variable number tandem repeat
VR	variable region
VRSA	vancomycin-resistant *S. aureus*
W	Wallace coefficient

1 INTRODUCTION

Staphylococcus aureus is able to cause a wide variety of different diseases, ranging from superficial skin inflammation to severe invasive infections such as bacteraemia (355). Methicillin-resistant *Staphylococcus aureus* (MRSA) has become a major problem worldwide. MRSA can cause both community-acquired and nosocomial infections. In Finland, an increase in the annual numbers of MRSA cases was observed from 121 in 1997 to 1772 in 2008. However, the MRSA situation improved in 2009, when 1267 cases were registered in the National Infectious Diseases Register (358). Accurate genotyping methods are required for national MRSA surveillance, outbreak investigations and international comparisons. Several different genotyping techniques for MRSA have been used in Finland. Macrodigestion of the whole bacterial chromosome, pulsed-field gel electrophoresis (PFGE), served as a primary typing method to distinguish different MRSA strains for several years (353). However, *spa* typing, which analyses the polymorphic X-region of protein A of *S. aureus*, replaced PFGE, and is now used as the first-line typing tool in Finland (329, 351). Multilocus sequence typing (MLST) of seven housekeeping genes and analysis of a methicillin resistance genetic element, staphylococcal cassette chromosome *mec* (SCC*mec*), are used as additional typing methods. In addition, MLST and SCC*mec* typing are valuable for understanding the evolutionary relationship between MRSA clones (96, 98, 267, 269).

Streptococcus pneumoniae causes severe invasive infections such as meningitis, bacteraemia and pneumonia, and non-invasive infections such as sinusitis and acute otitis media worldwide (304). Pneumococci are also normal inhabitants of nasopharyngeal mucous membranes in healthy children and adults (263). Although pneumococcal conjugate vaccine (PCV) is increasingly being used, pneumococcal infections caused by non-vaccine serotypes and the antimicrobial resistance of *S. pneumoniae* strains have become a major public health issue (45, 89, 150). In Finland, over 800 new bacteraemic *S. pneumoniae* infections are registered annually (358). Careful monitoring of the serotype distribution, antimicrobial susceptibility pattern and clonality of the strains is needed for active national surveillance of the disease burden and the efficacy of the recently launched national immunisation of all children with PCV. Molecular typing methods, such as MLST, provide excellent tools for genotyping of the strains and for better understanding of pneumococcal transmission in outbreaks (99). In addition, analysis of pneumococcal virulence protein genes is of interest for understanding the pathogenesis of pneumococcal diseases.

The purpose of this thesis was to characterise *Streptococcus pneumoniae* and methicillin-resistant *Staphylococcus aureus* in detail by using several molecular typing methods for various epidemiological purposes. Firstly, the aim was to examine the characteristics and clonality of EMRSA, to evaluate and compare typing methods for national laboratory-based surveillance of MRSA. Secondly, the purpose was to study the outbreak and carriage of *S. pneumoniae* in a crowded community setting, and to study virulence factors of *S. pneumoniae* isolated from children with AOM infection.

2 REVIEW OF THE LITERATURE

2.1 *Staphylococcus aureus*

2.1.1 Characteristics of *S. aureus*

Staphylococcus aureus is a Gram-positive, facultative anaerobic, catalase- and coagulase-positive, coccus-shaped bacterium. *S. aureus* is a human pathogen and can also be found among domestic animals such as pigs, dogs and horses. The typical 24-h *S. aureus* colonies are large, cream-yellow to orange pigmented, and β-haemolytic on blood agar (16). The outermost layers of *S. aureus* are important for protection against many different harmful molecules in their environment. The cell wall of *S. aureus* consists of peptidoglycan and teichoic acids, which are connected to either pepticoglycan or cytoplasmic membrane (396). In addition, many staphylococcal surface proteins are anchored to the cell wall. More than 90% of all clinical isolates of *S. aureus* strains are covered by a polysaccharide capsule, and a total of 11 putative capsule serotypes have been described so far, of which the most prevalent serotypes are 5 and 8. Both of these serotypes are prevalent among isolates from clinical infections as well as from commensal sources (262). The *S. aureus* genome roughly consists of a single circular chromosome with insertion sequences, transposons and genomic islands (12). Prophages and pathogenicity islands, in particular, are considered to play important roles in the evolution and virulence of *S. aureus* (12). To date, several complete *S. aureus* genomes have been sequenced by various genome sequencing projects (170, 250). Genomes of *S. aureus* are approximately 2.9 megabase pairs (Mbp) in size with a relatively low G+C content (12).

2.1.2 Diseases and carriage

Staphylococcus aureus can cause a wide variety of diseases, ranging from mild skin infections to fatal forms of bacteraemia. The most common infection by *S. aureus* is superficial skin inflammation with a furuncle or boil. Other skin and subcutaneous infections caused by *S. aureus* include folliculitis, carbuncles, cellulitis, mastitis and impetigo. *S. aureus* can also cause chronic skin and soft tissue infections within human populations with some underlying disorders. More severe *S. aureus* infections include osteomyelitis, pneumonia, arthritis, scalded skin syndrome, endocarditis, myocarditis, pericarditis, and bacteraemia (64, 354). Community-acquired pneumonia caused by *S .aureus* is not common, but does occur. In hospital settings, *S. aureus* can cause pneumonia, with 15–20% mortality. *S. aureus* is reported to be the most common cause of nosocomial pneumonia and surgical site infections, and was the third most common cause of nosocomial blood stream

infections in intensive care units (ICUs) in the US from 2000 to 2004 (354). In addition, in 2005, there were an estimated 478 000 hospitalisations with a diagnosis of *S. aureus* infection and 14 million outpatient healthcare visits for suspected *S. aureus* skin and soft tissue infections in the US (50).

S. aureus is a leading cause of bacteraemia and is associated with high morbidity and mortality (65). Bacteraemia often originates from different infection sources such as pneumonia, osteomyelitis, deep tissue abscesses and septic pulmonary emboli (302). Bacteraemia can also originate from foreign bodies such as intravenous catheters and endoprostheses (354). In the Calgary Health Region in Canada, the annual incidence of *S. aureus* bacteraemia was 19.7 cases/100 000 population during 2000–2006, and in Olmsted County, Minnesota, 33.4 cases/100 000 population during 1998–2005 (92, 209). According to the European Antimicrobial Resistance Surveillance Network (EARS-Net) (former European Antimicrobial Resistance Surveillance System, EARSS), rates of *Staphylococcus aureus* bacteraemia in Europe in 2003 ranged from 14 cases/100 000 inhabitants in Spain to 32 cases/100 000 inhabitants in Ireland (361).

In Finland, the annual incidence of bloodstream infection caused by *S. aureus* rose by 55% during 1995–2001 (from 11 cases/100 000 population in 1995 to 17 in 2001) (220). According to the National Infectious Disease Register (NIDR), *S. aureus* has annually caused approximately 1200 bacteraemic infections in the past five years (Figure 1) (359). In addition, *S. aureus* was reported to be the second most common pathogen to cause bloodstream infection among the working-age population (from 15 to 64 years old) and the elderly (65 years old and older) during 1995–2009 (358).

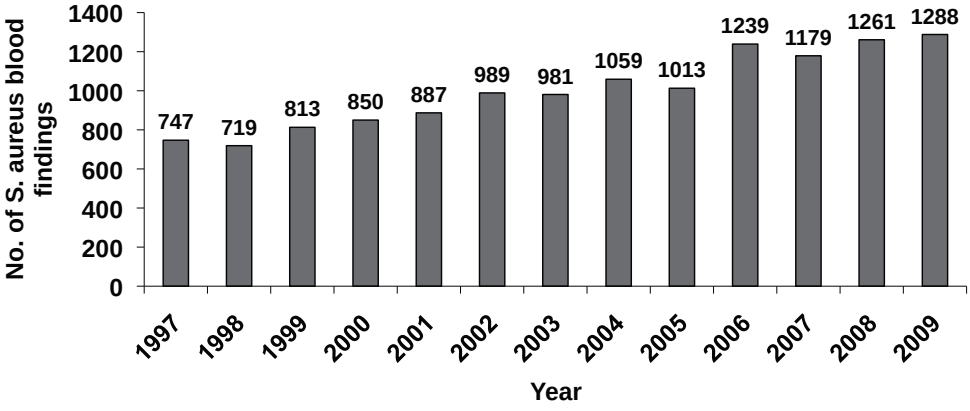

Figure 1. *S. aureus* blood culture findings in Finland during 1997–2009 (358).

Several diseases caused by *S. aureus* are toxin-mediated, including food poisoning, impetigo, toxic shock syndrome (TSS) and necrotising pneumonia. Staphylococcal enterotoxins ingested via contaminated food cause self-limiting staphylococcal food poisoning. The symptoms of staphylococcal food poisoning include nausea, vomiting, headache, and less commonly diarrhoea (354). TSS is caused by toxic shock syndrome toxin 1, which is a potent superantigen (321, 368). Menstrual TSS is typically associated with use of highly absorbent tampons among previously healthy women (327). Non-menstrual TSS may result from any primary staphylococcal infection, or from colonisation with a toxin-producing strain of *S. aureus* (207). The symptoms of TSS include high fever, hypotension, rash and the involvement of multiple organ systems (77, 207).

S. aureus colonises the skin and mucosal surfaces of humans. Although multiple sites in the body can be colonised by *S. aureus*, the anterior nares of the nose are the most consistent carriage site in humans (385). Other typical sites for *S. aureus* colonisation include the skin, perineum, pharynx, and less frequently the gastrointestinal tract, vagina and axillae (385). In longitudinal *S. aureus* carrier studies, three different carrier patterns have been historically assigned: persistent carriers, intermittent carriers and noncarriers (192). However, the criteria used to assign an individual to these carriage patterns vary and most of the studies have used a cross-sectional study design with a single nasal culture. In 2009, van Belkum and co-workers suggested that there are only two types of nasal *S. aureus* carriers: persistent and other (376). In studies performed in the United States, the prevalence of nasal colonisation with *S. aureus* has been estimated to be around 30%, and it was found to be highest among 6- to 7-year-old children (50, 202). In a recent study in Lebanon, the overall *S. aureus* nasal carriage rate between 2006 and 2007 was 38.4% (134). Nasal carriage of *S. aureus*, especially persistent nasal carriage, has been identified as a risk factor for the development of infections in various settings. It is especially a major risk factor in certain groups of patients, such as patients undergoing surgery and haemodialysis, and patients with intravascular devices and HIV infection (192).

2.1.3 Virulence factors and pathogenesis

In general, the ability of bacteria to cause disease in humans is mainly due to evasion of the host immune system. *S. aureus* can express an extensive number of different virulence factors, playing a role in the pathogenesis of infection. The form and severity of the disease are a result of the complicated interplay between the activities of *S. aureus* virulence factors of the infecting strain and host defence. A virulence factor may have several functions in pathogenesis, and multiple virulence factors may perform the same function. Staphylococcal pathogenesis is

multifactorial, involving three classes of factors that are directly or indirectly injurious: secreted proteins, cell-surface-bound proteins, and cell surface components. Secreted proteins, including superantigens (e.g. toxic shock syndrome toxin-1, enterotoxins A-D), cytotoxins [e.g. Panton-Valentine leukocidin (PVL), α-,β-,δ-,γ-haemolysin], and tissue-degrading enzymes (e.g. lipases, proteases), enable bacteria to invade and destroy the local cellular and structural elements of host tissue and organs (123, 257).

PVL toxin is encoded by two genes located on the prophage (81). It is lethal to neutrophils and causes tissue necrosis by forming pores in cell membranes, and it is associated with skin and soft tissue infections and severe necrotising pneumonia (204, 214). PVL production has also been associated with community-acquired methicillin-resistant *S. aureus* (CA-MRSA) strains (381). *S. aureus* has numerous surface proteins termed microbial surface components recognising adhesive matrix molecules (MSCRAMMs), including fibronectin-binding protein, fibrinogen-binding protein, collagen-binding protein, other adhesins, and antiopsonins (257). MSCRAMMs mediate adherence to host tissues, bacterial cells, the extracellular and to inert surfaces and appear to play a key role in the initiation of endovascular infections, bone and joint infections, and prosthetic-device infections (109, 123).

The best known surface protein of *S. aureus* is staphylococcal protein A (Spa). Spa was isolated first time from *S. aureus* after lysostaphin digestion in 1972 (335). It comprises five nearly identical Ig-binding domains, a polymorphic region X and C-terminal cell wall attachment sequence (133, 334, 369). The structure of *spa* gene is represented more thoroughly in the section 2.3.8. The X region of the *spa* gene contains a highly polymorphic sequence that is composed of repeats of 24 bp (112). Spa binds to the Fc region of IgG and blocks its normal function. This inhibits phagocytosis and can disguise the bacterium from the innate immune system by preventing opsonisation-dependent activation of the complement cascade (395). Protein A has also been reported to be capable of binding to platelets via the gC1qR/p33 receptor and the von Willebrand factor (142, 252). In addition, *spa* typing is a widely used genotyping method to compare *S. aureus* isolates, due to the highly variable X-region (111). Cell surface components, including the polysaccharide capsule and components of the cell wall peptidoglycan, have different activities in the pathogenesis of *S. aureus.* Mucoid capsules can block phagocytosis by masking complement factor C3b bound to the cell wall (72). However, the role of the capsules of *S. aureus* in pathogenesis is controversial.

The genes coding for staphylococcal virulence factors are controlled by a complex regulatory network. The surface protein genes are expressed shortly after the initiation of the exponential growth phase, when they are needed during the establishment of infection to aid in adherence to host tissues and to protect the

bacteria from host defences such as opsonisation-phagocytosis and complement-mediated killing. Many of the genes encoding secreted enzymes and cytotoxins are expressed primarily during the postexponential growth phase to aid in spreading, the acquisition of nutrients and killing of phagocytes (257). The accessory gene regulation (*agr*) locus is the main global regulatory system of *S. aureus* (395). In addition, three other two-component signalling modules are known to be involved in the regulation of staphylococcal virulence genes. Moreover, environmental factors such as pH, temperature, O_2 and CO_2 levels also affect the overall regulatory system by helping the bacterium to recognise and respond appropriately to its local environments (257, 395).

Although *S. aureus* has traditionally been regarded as an extracellular pathogen, multiple studies have shown that it can survive in a variety of eukaryotic cells, including endothelial cells, epithelial cells, fibroblasts, osteoblasts and keratinocytes. In addition, bacterial survival within human monocyte-derived macrophages and neutrophils has also been demonstrated (115). Intracellular survival of *S. aureus* can contribute to the persistent and /or recurrent nature of certain infections. *S. aureus* mutants known as 'small colony variants' (SCVs) are slow-growing colonies that are 10-fold smaller than normal and have certain characteristics such as decreased pigment formation, low coagulase activity, reduced haemolytic activity, decreased toxin production and resistance to aminoglycosides. Small colony variants have the ability to persist intracellularly and to cause persistent and recurrent infections (393).

2.1.4 Methicillin-resistant *Staphylococcus aureus* (MRSA)

In 1942, two years after the introduction of penicillin for medical use, the first penicillin-resistant *S. aureus* isolates were observed. Since 1960, approximately 80% of all *S. aureus* isolates have been penicillin-resistant. In addition, *S. aureus* developed methicillin resistance in 1961, only two years after its introduction. The resistance to methicillin and all other β–lactam antibiotics developed due to the acquisition of the *mecA* gene (81). This gene encodes a 78 kilodalton (kDa) additional penicillin-binding protein (PBP) 2a. PBPs are transpeptidases that catalyse the formation of cross-bridges in bacterial cell wall peptidoglycan. In methicillin-sensitive *S. aureus* (MSSA), β–lactam antibiotics bind to the native PBPs of the cell wall, disrupting the synthesis of the peptidoglycan layer and resulting in the death of the bacterium. Since PBP2a has a low affinity for all β–lactam antibiotics, synthesis of the peptidoglycan layer is not disrupted and MRSA can continue to grow normally (22). The *mecA* gene is regulated by the repressor MecI and the trans-membrane β–lactam-sensing signal-transducer MecR1. In the absence of β–lactam antibiotics, MecI represses the transcription of both *mecA* and *mec*R1-*mec*I. In the presence of the β–lactam antibiotics, MecR1 is autocatalytically cleaved and the metalloprotease domain of the MecR1 becomes active. This metalloprotease

cleaves MecI, allowing the transcription of *mecA* and subsequent production of PBP2a (22).

The *mecA* gene is located on a mobile genetic element ranging from 20 kilobase pairs (kbp) to more than 100 kbp in size and designated the staphylococcal cassette chromosome *mec* (SCC*mec*) (166). SCC*mec* is considered to have originated from other bacterial species and integrated into the chromosome of *S. aureus.* In addition, it is hypothesised that SCC*mec* is distributed through horizontal transmission between staphylococcal species (165, 183, 248, 295). SCC*mec* consists of the *mec* complex, cassette chromosome recombinase (*ccr*) complex, joining regions (J), and directly repeated 15-base-pair (bp) core sequences at the both ends. SCC*mec* is present at a specific site on the chromosome, the SCC*mec* attachment site (*att*B*scc*), at the 3' end of an open reading frame with an unknown function (*orf*X) (Figure 2) (165, 166).

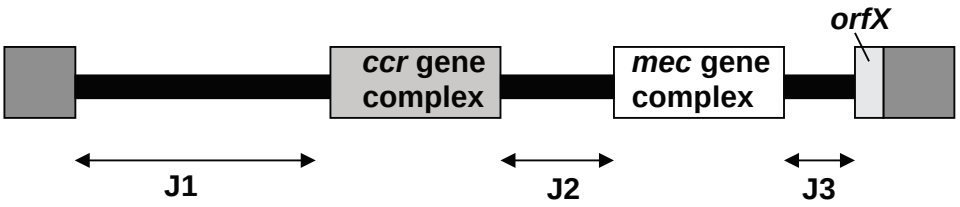

Figure 2. Simplified diagram of SCC*mec*. Grey regions indicate chromosome regions that are outside the SCC*mec* elements. J1-J3 indicates joining regions. The diagram is based on information from (155, 166).

Currently, ten main types of SCC*mec* (types I-X) and several variants have been designated based on differences in structure and size (24, 81, 151, 166, 167, 193, 212, 268, 403). SCC*mec* types I, IV, V, VI, and VII mainly cause β–lactam antibiotic resistance, while SCC*mec* types II and III cause multiresistance due to additional drug resistance genes integrated into SCC*mec* (81). The SCC*mec* type is classified by the *mec* complex (a region containing *mecA*, *mec*R, IS/*mec*I, IS431) and the *ccr* gene complex (a region containing recombinase genes such as *ccr*A, *ccr*B or *ccr*C). SCC*mec* can be further classified into subtypes based on different J regions (JI-J3), which border the *mec* and *ccr* complexes (81, 82, 166). *S. aureus* can also harbour additional resistance genes at other sites of the genome, such as transposons and plasmids (216). In general, the majority of community-acquired MRSA (CA-MRSA) isolates harbour SCC*mec* type IV, V or VII, while SCC*mec* types I, II and III are considered to mainly occur among hospital-acquired MRSA (HA-MRSA) isolates (81, 249).

Most MRSA strains have heterogeneous expression of methicillin resistance (300). The majority of cells are susceptible to low concentrations of methicillin, and a small proportion of cells can grow at high methicillin concentrations. Such highly resistant subclones may be partly associated with expression of the *mecA* gene, which apparently requires the genetic inactivation of the MecI repressor, and due to mutation events in other genes of the staphylococcal genome (22, 254, 305). Many of these chromosomal genes affecting methicillin resistance levels, independent of SCC*mec*, have been identified, and many of them are involved in peptidoglycan biosynthesis, such as *fem* (factors essential for methicillin resistance) and *aux* (auxillary) factors (22, 23, 78). Staphylococci are also able to acquire *mecA*-independent methicillin resistance, for instance due to overexpression of PBP2 and/or PBP4, and changes in their penicillin affinity. Low-level methicillin resistance of borderline resistant *S. aureus* (BORSA) strains results from the hyperproduction of penicillinase (52, 229). The challenge is to distinguish penicillinase hyper-producing strains from *mecA*-positive MRSA strains, without detecting the *mecA*-gene by PCR.

In 2003–2004, approximately 1.5% (4.1 million persons) of the US population were estimated to be colonised in the nose with MRSA, and in 2004, 64% of *S. aureus* infections in intensive-care units were caused by MRSA (50). In 2005, the incidence of invasive MRSA disease in 9 US communities was 31.8 cases/100 000 people (191). MRSA is also an increasing problem throughout Europe. In 2006, the proportion of MRSA isolates among blood and cerebrospinal fluid isolates was reported as 25% or higher in 12 out of 28 countries [mainly southern European countries, the United Kingdom (UK) and Ireland], while in northern parts of Europe the proportion was below 4% (90). In Finland, the annual number of MRSA cases has increased from 121 in 1997 to 1772 in 2008 (Figure 3) (359). However, the MRSA situation improved in 2009, when 1267 cases were registered in the National Infectious Diseases Register (NIDR) and the situation remained unchanged in 2010 (359, 360). In both years a quarter of these were diagnosed from samples taken from the nose or the nostrils (358, 360). The proportion of MRSA strains among *S. aureus* findings from blood rose above 3% in 2004 and has been around 2% in 2009-2010 (Figure 4) (358, 360).

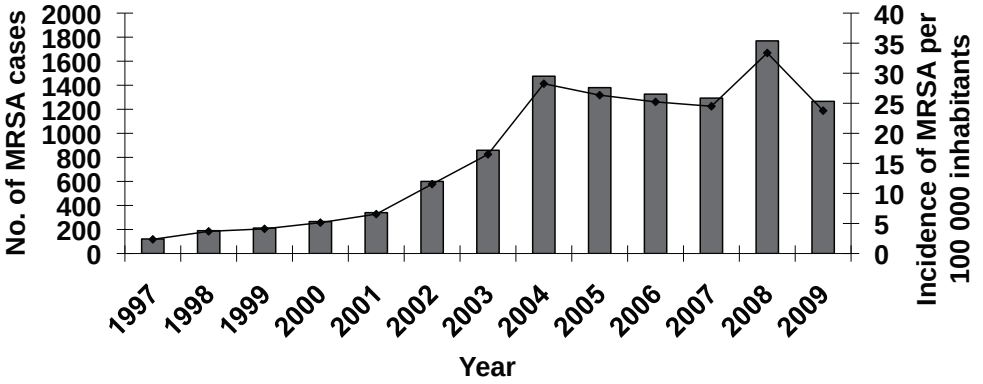

Figure 3. Annual number of MRSA cases and incidence/100 000 inhabitants in Finland during 1997–2009 (359).
MRSA cases: laboratory notifications included both asymptomatic carriers and cases with clinical disease.

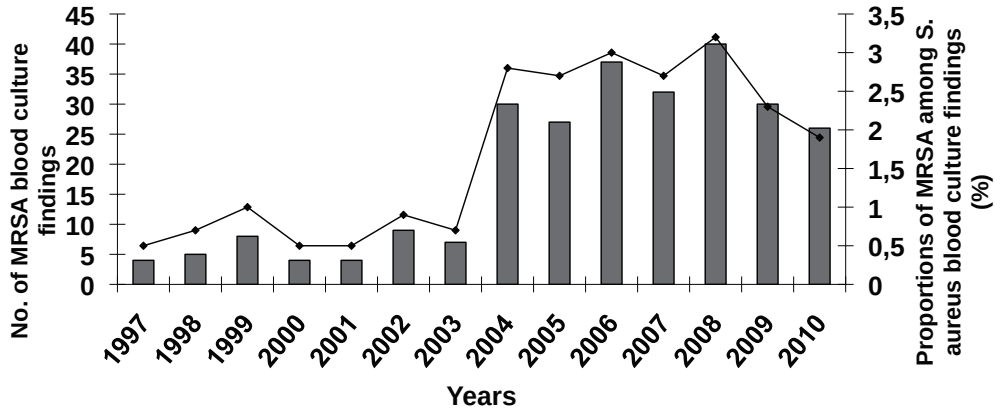

Figure 4. Annual number of MRSA blood culture findings and the proportion of MRSA among all the *S. aureus* blood culture findings during 1997–2010 (358, 360).

To date, there is no convincing evidence that MRSA is generally more virulent than MSSA. SCC*mec* does not contain any virulence genes, and thus is not directly associated with the possibly enhanced virulence of MRSA. However, some epidemiological studies, including a meta-analysis, have revealed increased morbidity and/or mortality from nosocomial MRSA (67, 233). In contrast, other studies have demonstrated no increase in mortality with nosocomial MRSA bacteremia or ventilator-associated pneumonia compared with MSSA infections (66, 401). However, according to a recent meta-analysis, colonisation by MRSA was associated with a 4-fold increase in the risk of infection compared with MSSA (306). Other causes than differences in virulence may explain the poorer outcomes with MRSA, such as the severity of illness, inadequate or ineffective therapy, and prolonged hospital stays (299, 306, 325). Although the question of whether MRSA is more virulent than MSSA remains unresolved, it is clear that MRSA infections are associated with greater costs and limited treatment options (66, 94, 291).

In 1997, a new threat emerged from Japan: vancomycin intermediate-resistant *S. aureus* (VISA)(156). More VISA cases were subsequently reported from other countries around the world. The mechanism of resistance in VISA strains is mediated by mutations and altered expression of certain genes, resulting in a thickened cell wall that prevents vancomycin from properly functioning (71). Vancomycin-resistant MRSA (VRSA) was first noticed in 2002, and around ten VRSA cases have since been reported in the US. In addition to the *mecA* gene, VRSA strains harbour a plasmid-borne transposon Tn1546 element with a *vanA* gene cluster following conjugation from a glycopeptide-resistant *Enterococcus* strain (275, 330). Although VISA and VRSA strains seem to be rare and limited, the potential for spread of such isolates should not be underestimated.

2.2 *Streptococcus pneumoniae*

2.2.1 Characteristics of *S. pneumoniae*

Streptococcus pneumoniae or pneumococcus is a Gram-positive, facultatively anaerobic, catalase-negative diplococcus bacterium that typically produces greenish haloes (alpha-haemolysis) on blood agar. Pneumococci may have a mucoid colonial appearance due to the production of varying amounts of capsular polysaccharide (304). In humans, pneumococcus can cause mild to more severe infections, and the asymptomatic nasopharyngeal carriage of pneumococci is common (304). The polysaccharide capsule forms the outermost layer of *S. pneumoniae*. Thus far, 91 structurally and serologically different capsular polysaccharide types have been recognised (272). The distribution of serotypes differs according to age and geographical area, and specific capsule types are associated with the capacity to cause severe disease (41). Multiple studies have shown that some serotypes and sequence types have a greater invasive potential than others (40, 303, 318). Under the thick polysaccharide capsule there is the cell wall, consisting of peptidoglycan and teichoic and lipoteichoic acids, which contain phosphoryl choline residues as structural components. Teichoic acids are linked to the peptidoglycan, whereas lipoteichoic acids are linked to the cell membrane (25, 83). In addition, at least three different sets of pneumococcal surface proteins are known to anchor to the cell wall: choline-binding proteins, peptidoglycan-attached proteins and lipid-attached proteins (25, 172).

In 2001, Tettelin et al. first described the whole genome of a pneumococcal serotype (357). Since then, several pneumococcal complete genomes have been sequenced, including invasive and non-invasive strains (152, 170, 250). The *S. pneumoniae* genome consist a single circular chromosome with a size ranging between 2.03 and 2.24 Mbp, depending on the strain. The average G+C content of the pneumococcal genome is 40%. TIGR4, which is a virulent pneumococcal isolate, has 2236 open reading frames (ORF), two-thirds of which have assigned roles for their predicted gene products. Approximately 20% of ORFs only exist in *S. pneumoniae*. Pneumococci have a large number of insertion sequences, which comprise up to 5% of the entire genome (87, 160, 357).

2.2.2 Diseases and carriage

Pneumococcus is an important human pathogen causing a wide variety of infections. It can cause mucosal infections such as sinusitis and acute otitis media (AOM) and severe invasive infections such as septicaemia, meningitis, pneumonia, arthritis, pericarditis and peritonitis (263).

Invasive pneumococcal disease (IPD) is most frequent in children less than 2 years of age, adults aged at least 65 years, and in immunocompromised individuals. Each year, 1 million children younger than 5 years of age die from pneumococcal diseases, mostly in African and Asian countries (261). Before the year 2000, *S. pneumoniae* infections annually caused approximately 60 000 cases of invasive diseases, including 3300 cases of meningitis in the US. The incidence of sterile-site infections varied from 21 to 33 cases per 100 000 population. However, since the introduction of the 7-valent pneumococcal conjugate vaccine (PCV7) in 2000, the incidence of IPD has declined, and in 2002 the incidence of invasive disease was 13 cases per 100 000 population in the US (48, 286). In developed countries, during 1995–2006, mortality from invasive pneumococcal diseases ranges from 5% to 30%, depending on age, genetic background, geographical location and the underlying medical condition, but mortality can be as high as 50% in Africa (35, 48, 168, 176, 190, 303). The risk factors for IPD are presented in Table 1. In addition, a recently published study has demonstrated that asthma increases the risk of invasive pneumococcal infections (189).

Table 1. Risk factors for pneumococcal pneumonia and invasive pneumococcal disease. Adapted and modified from (378).

Definite risk factors[a] (high risk)	**Probable risk factors[b] (moderate risk)**	**Possible risk factors[c] (low risk)**
<2 years or >65 years	Genetic polymorphisms of the host	Recent exposure to antibiotics
Asplenia or hyposplenia	Isolated populations	Defects in cellular immunity and neutrophil defects
Alcoholism	Poverty, crowding, low pneumococcal vaccine use	Diminished cough reflex, aspiration pneumonitis
Diabetes mellitus	Cigarette smoking	Proton-pump inhibitors and other gastric-acid inhibitors
Antecedent influenza	Chronic lung disease	Large organism burden in upper airways
Defects in humoral immunity (complement or immunoglobulin)	Severe liver disease	Child day care
HIV infection	Other antecedent viral infections	
Recent acquisition of a new virulent strain	Poor mucociliary function	

[a] Many clinical studies

[b] Some clinical and laboratory studies

[c] Few clinical studies

In Europe, most of the northern countries have extensive surveillance systems for IPD. In 2007, the reported incidence of IPDs was 21 cases/100 000 in Norway and 16/100 000 in Sweden (91). In Sweden, the reported IPD cases ranged from 1330 to 1790 cases per year during 2005–2009 (338). In Finland, during 1995–2002, the overall annual incidence of IPD was 10.6 per 100 000 persons (188). In addition, according to the International Circumpolar Surveillance System for invasive pneumococcal disease, the crude annualised incidence of IPD was 12.9 cases per 100 000 persons during 2000–2005 in Finland (39). *S. pneumoniae* is the fourth

most common blood culture finding in Finland (358). The annual number of new invasive (from blood or cerebrospinal fluid (CSF)) *S. pneumoniae* cases in Finland increased from 589 in 1997 to 926 in 2008 (Figure 5). However, the situation improved slightly in 2009 and 2010, when 854 and 836 cases were registered, respectively (359).

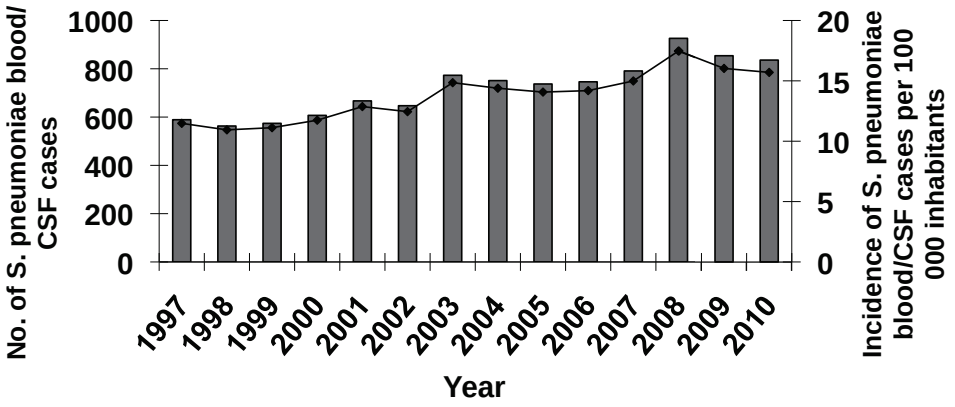

Figure 5. Number of *S. pneumoniae* blood/CSF cases and incidence/100 000 inhabitants in Finland during 1997–2010 (359).

S. pneumoniae is the most common cause of community-acquired pneumonia (6). Outbreaks of pneumococcal pneumonia can occur in crowded institutional settings, such as day care centres, nursing homes, military forces and men's shelters, and within urban neighbourhoods (70, 121, 301). Several risk factors have been recognised for pneumococcal pneumonia and invasive pneumococcal disease (Table 1) (190, 219, 378). In Finland, the total annual incidence of pneumococcal community-acquired pneumonia among children and the elderly (≥60 years) was estimated at 6.4/1000 and 8/1000 population, respectively (146, 177).

Acute otitis media (AOM) is one of the most common infectious diseases among young children in the developed countries, causing a considerable cost burden to the health care system (198, 253). According to a Finnish study, 42% of children have had one episode of AOM by one year of age and 71% by two years of age (5). It has been estimated that approximately 250 000 AOM episodes occur annually among children under 5 years of age in Finland (314). *S. pneumoniae* is still the major bacterial pathogen causing AOM infections, identified in 26–60% of AOM cases worldwide (132, 186, 298).

Antibiotic resistance in *S. pneumoniae* strains has become a major public health issue. According to data from the European Antimicrobial Resistance Surveillance Network (EARS-Net), 7% of the invasive *S. pneumoniae* isolates reported by 27 European countries were non-susceptible to penicillin in 2009. High levels (above 25%) of invasive penicillin-non-susceptible *S. pneumoniae* were mainly reported from southern and eastern Europe, while most of the northern countries reported low levels, below 5% (89). However, in Finland, erythromycin and penicillin non-susceptibility increased from 16% to 28% and from 8% to 16%, respectively, among pneumococcal isolates recovered from blood and cerebrospinal fluid samples between 2002 and 2006 (331).

The proportion of penicillin- and macrolide-resistant pneumococcal strains causing AOM has been estimated to be between 30% and 70% globally (210). However, after the introduction of the 7-valent pneumococcal conjugate vaccine (PCV7), a decline in the incidence of invasive pneumococcal infections and in the rates of antibiotic resistance was detected in the US (31, 74, 203, 387). In addition, PCV7 has been reported to reduce the number of pneumococcal AOM episodes in the US and also in Finland (46, 101, 108). Several studies have demonstrated a reduction in the carriage of 7-valent pneumococcal conjugated vaccine serotypes and replacement by non-vaccine serotypes (74, 150, 273). An increase in the incidence of pneumococcal disease caused by non-PCV7 serotypes has also been observed (9, 45, 150, 215).

S. pneumoniae can colonise the nasopharyngeal niche of healthy children and adults. Colonisation typically leads to asymptomatic carriage, although in some cases colonisation is followed by disease (33). Pneumococcal carriage starts during the first months of life and is highest among young children (33, 125, 349). The pneumococcal carriage rate of healthy children varies widely from 2% to 70% (28, 34, 60, 350). In Finland, the carriage of *S. pneumoniae* in healthy children younger than 2 years of age was reported to vary from 9 to 43%, and increased gradually with age (349). The proportion increased during respiratory infections without AOM to 22–45%, during AOM to 45–56%, and during pneumococcal AOM almost every children carried *S. pneumoniae* in the nasopharynx (97–100%) (349). The pneumococcal serotypes found in the middle ear and in the nasopharynx are usually the same (93, 389). Pneumococcal infection is usually caused by one serotype, which is acquired only shortly before the infection. However, during pneumococcal carriage, the sequential acquisition of more than one serotype is common (125, 263, 348). The common risk factors for pneumococcal carriage are ethnicity, crowding, environmental features and socioeconomic features. Environmental and socioeconomic risk factors include family size, income, smoking and recent antibiotic use (33). In addition, a recent study demonstrated that asthma can be a significant risk factor for pneumococcal carriage (178).

2.2.3 Virulence factors and pathogenesis

Pneumococcal infection is a complex interplay between virulence factors of the infecting strain and the host defence system. The pneumococcus produces a range of factors (colonising and virulence) that are involved in the disease process. The pathogenic route of *S. pneumoniae* infection is presented in Figure 6.

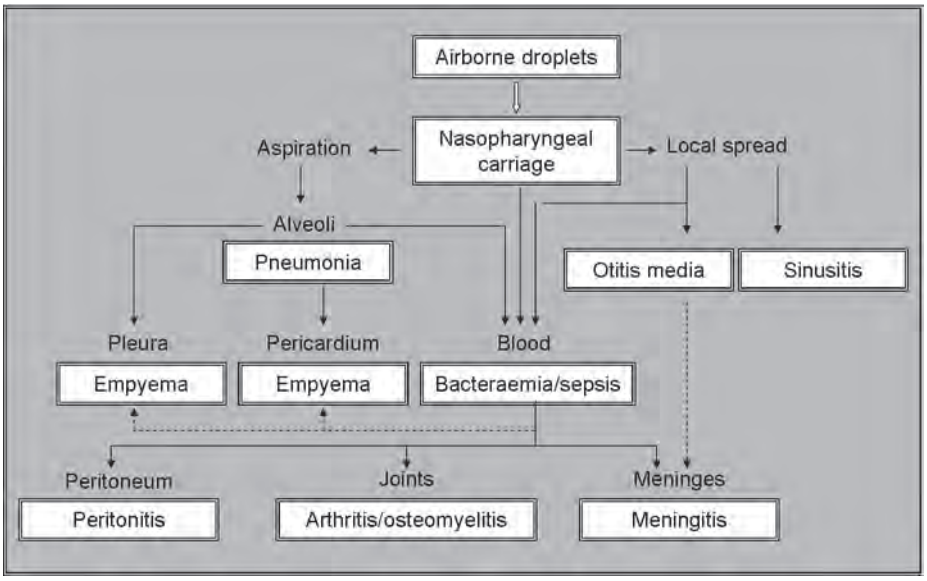

Figure 6. Pathogenic route of *S. pneumoniae* infection. Adapted from (33).

Pneumococcal disease usually occurs with preceding nasopharyngeal colonisation with a homologous strain (104, 125). Several pneumococcal adhesins are involved in the process of adhesion to human cells. Invasion of the lower respiratory tract is an important event in pneumococcal infection, which may lead to bloodstream invasion (118). To cause meningitis, blood-borne bacteria must localise in and cross the blood-brain barrier (199, 294). In addition to capsular polysaccharides, multiple virulence factors contribute to invasive disease. Some successful clones may have gathered a collection of genes providing a selective advantage in the invasion process (118).

By preventing phagocytosis, the polysaccharide capsule is the most important pneumococcal virulence factor (364). The capsule is also crucial in colonisation and dissemination from the respiratory tract. It can prevent mechanical removal by mucus, inhibit autolysis and reduce exposure to antibiotics (251, 378). Pneumococci are able to exchange their capsular type through horizontal recombination of

pneumococcal DNA surrounding the capsular loci by natural transformation (11, 57). Capsular switching most presumably occurs during nasopharyngeal carriage, when intermixing of clones expressing different types of capsular polysaccharides is possible (57, 279).

The pneumococcal exotoxin pneumolysin is a pore-forming cytotoxin that is expressed by almost all invasive pneumococcal strains. Pneumolysin is the most extensively studied pneumococcal protein virulence factor. It has been shown to have several roles in infection, including its ability to activate complement, lyse the host cells, induce the production of chemokines and cytokines, activate CD4+ T cells, impair the respiratory burst of phagocytic cells, and activate inflammation (158, 237, 378, 404).

The roles of several LPXTG-anchored surface proteins in virulence have been investigated, including hyaluronidase and neuraminidase. Hyaluronidase degrades hyaluronic acid in the mammalian connective tissue and extracellular matrix, and may aid bacterial spread and colonisation (171, 237). Neuraminidase may cause direct damage to the host cell or it may unmask potential binding sites for the organism by cleaving the N-acetylneuraminic acid from compounds such as mucin, glycolipids, glycoproteins, and oligosaccharides on cell surfaces and in body fluids, contributing to increased adhesion and invasion (43, 171, 362, 367).

Some of the pneumococcal surface proteins are directly attached to the lipids of the bacterial cytoplasmic membrane. Pneumococcal surface antigen A (PsaA) is a surface-exposed multi-functional lipoprotein. It mediates metal ion uptake by acting as a part of an ATP-binding cassette (ABC)-transporter. It is also an adhesin that plays major roles in pneumococcal colonisation and virulence (289). Two surface-exposed lipoprotein peptidyl prolyl isomerases, SlrA and PpmA, have also been demonstrated to have a role in virulence and colonization (69, 148). In addition, PpmA has been shown to be immunogenic in humans (370). PiaA and PiuA are lipoproteins that are also involved in virulence by acting as components of two separate iron-uptake ABC transporters (386).

Choline-binding proteins (CBPs) are attached to the pneumococcal cell surface via terminal choline residues of teichoic/lipoteichoic acids that are present on the surface of the bacteria. Several CBPs have been demonstrated to have a role in virulence, including cell wall hydrolytic enzymes such as *N*-acetylmuromyl-L-alanine amidase (LytA), *β*-*N*-acetylglucosamidase (LytB), *β*-*N*-acetylmuramidase (LytC), and phosphorylcholine esterase (CbpE/Pce), as well as pneumococcal surface protein A (PspA) and C (PspC) (29, 124, 171). PspA and PspC are widely studied multifunctional virulence factors of pneumococci. PspA has the ability to inhibit the alternative pathway of complement activation (293, 363). PspA also

binds to lactoferrin, which has an important role in innate immunity (137). The main virulence functions of PspC are its ability to act as an adhesin by binding to the polymeric immunoglobulin receptor and to limit opsonophagocytosis by binding the complement regulatory protein factor H and blocking C3b fixation (76, 138, 169, 402).

Several other surface proteins have been identified without any recognised anchor motifs, including pneumococcal adherence and virulence factor A (PavA), two glycolytic enzymes, glyceraldehyde-3-phosphate dehydrogenase and enolase, and the pneumococcal histidine triad protein (Pht) family (237). PavA is an adhesin that mediates attachment to endothelial cells by binding to the fibronectin, and it is essential for pneumococci to escape phagocytosis (256, 284). Glyceraldehyde-3-phosphate dehydrogenase and enolase are plasminogen-binding proteins (26, 27). The Pht family members have the ability to provide protection against infection when used as vaccines (1, 392). In addition, Pht proteins may have a role in complement evasion (230, 265).

Recently, adhesive pili have also been reported to exist on the surface of pneumococci, referred to as pilus islet 1 (PI-1) and 2 (PI-2) (13, 17). The *rlrA* pathogenicity islet, coding for PI-1 genes, was already identified in a genome-wide screen in 2003 (143). The pneumococcal *rlrA* pathogenicity islet includes three structural protein genes (*rrgA*, *rrgB*, and *rrgC*), three genes coding for sortase enzymes (*srtB*, *srtC*, and *srtD*) responsible for linking structural subunits, and the *rlrA* gene coding for a positive regulator of *rlrA* pathogenicity island genes. The gene cluster is flanked by insertion sequences characteristic of a mobile element (143) (Figure 7). The three genes coding for structural proteins (*rrgA*, *rrgB*, and *rrgC*) have homology to the LPXTG family of cell wall-anchored surface proteins (143).

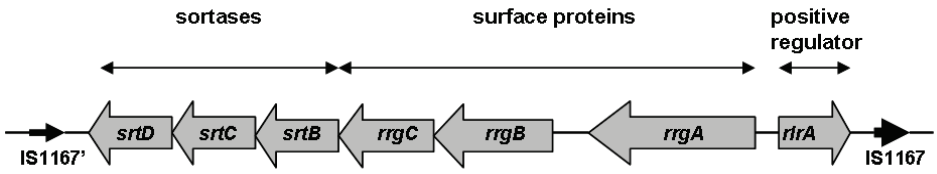

Figure 7. Schematic presentation of the *rlrA* pathogenicity islet. The locus includes three sortase genes (*srtB, srtC, srtD*), three surface protein genes (*rrgA, rrgB, rrgC*) and a gene for a positive regulator (*rlrA*). IS1167/IS1167' codes for insertion sequences of the *rlrA* pathogenicity islet. Adapted from (143).

Sequence comparison of 15 *rlrA* islets revealed the presence of 3 clade types, with an overall similarity of 88–92% (243). The molecular architecture of PI-1 has been identified. The native pili are flexible filaments that can be approximately 6 nm wide and over 1 µm long, consisting of a shaft composed of RrgB monomers, with RrgA at its distal and RrgC at its proximal end. It has been suggested that RrgC anchors the pilus to the peptidoglycan cell wall and RrgA has a role as an adhesin (153, 154).

PI-2 codes for a second functional pilus in pneumococcus between flanking sequences *pepT* and *hemH* (13). PI-2 consists of two structural protein genes (*pitA* and *pitB*), two sortase genes (*srtG1* and *srtG2*) and a gene coding for putative signal peptidase (*sipA*) (Figure 8) (13). Sequence alignment of nine PI-2s defined an overall similarity of approximately 99% (13).

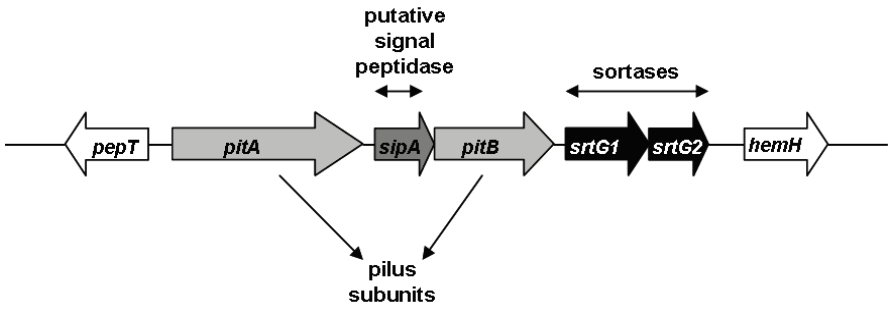

Figure 8. Schematic presentation of PI-2. The locus includes two sortase genes (*srtG1* and *srtG2*), two pilus subunit genes (*pitA* and *pitB*) and a gene for a putative signal peptidase (*sipA*). *hemH* and *pepT* are the flanking sequences of the locus. Adapted from (13).

Both these pilus islets seem to be present in some, but not all, clinical isolates. In addition, some strains are capable of expressing both PI-1 and PI-2 (13). Moreover, the isolates harbouring pilus islets are variable in terms of serotypes and genotypes. The prevalence of PI-1 varies from 21% to 30.6% among invasive and non-invasive isolates (2, 18, 242, 243). PI-1 has been reported to correlate with the pneumococcal genotype rather than the serotype (2, 242). In addition, a recent study demonstrated a correlation between PI-1 and antibiotic resistance (242). In murine models of pneumococcal infection, PI-1 has been shown to be involved in adherence to epithelial cells and virulence (17). The prevalence of PI-2 has been identified to be 21% among invasive isolates, 16% among both invasive and nasopharyngeal isolates, and 7% among MEF isolates (13, 242, 406). PI-2 has been shown to play a role in adherence to epithelial cells (13).

Pneumococcus requires genetic variability, adaptability and modulated expression of virulence factors to be able to survive and succeed in different host microenvironments. *S. pneumoniae* is one of the bacterial species having the ability to take up DNA from the external environment (natural genetic transformation)(11) and incorporate it into their genomes by homologous recombination (144). *S. pneumoniae* is competent (a state in which cells are able to take up free DNA) for only a short time period, and most probably regulates the expression of genes encoding competence proteins in response to certain cellular and/or environmental signals (174). Natural genetic transformation allows pneumococci to take up DNA from other *S. pneumoniae* and closely related streptococcal species. In addition, there is recent evidence that *S. pneumoniae* does not only rely on the accidental release of DNA for transformation but can trigger active release. The release involves a killing mechanism that could be used by competent cells to acquire DNA from non-competent (a state in which cells are not able to take up free DNA) pneumococci. This phenomenon is termed pneumococcal fratricide (145). In fratricide, the competent cells express a set of murein hydrolases (LytA, CbpD, and LytC) that attack the cell wall of non-competent target cells, resulting in cell lysis and the release of DNA, which can be taken up by competent cells (174). Through this behaviour, pneumococci can gain access to transforming DNA and nutrients, promote the release of virulence factors and kill competitors (145). Capsular switching of pneumococci is one of the known consequences of pneumococcal genetic transformation (57). It has recently been shown that killing is not limited to *S. pneumoniae*, but that related species such as ***Streptococcus mitis*** and ***Streptococcus oralis*** can also be attacked by competent pneumococci (173).

2.3 Bacterial typing

2.3.1 Reasons for bacterial typing

Bacterial typing methods are used as means to study the spread and population dynamics of bacteria in clinical and environmental settings. There are several reasons for typing bacteria for epidemiological purposes. Typing methods contribute essential information to epidemiological surveillance of infectious diseases. Surveillance is defined as an ongoing systematic process of data collection, analysis and interpretation of data, and dissemination of results, to be able follow disease trends and design ways to fight them (344, 375). Typing is an important tool for infectious disease outbreak investigations, where the aim is to define a local and temporal increase in the incidence of infection by a certain bacterial species. The typing of outbreak strains facilitates the development of outbreak control strategies, defining of the extent of epidemic spread of bacterial clones and the number of clones involved in transmission and infection, monitoring of the reservoirs of epidemic clones, and control evaluations of the efficacy of control measures, such as monitoring vaccine efficacy (344, 375, 380). By using typing methods, distinct epidemiological markers associated with pathogenic isolates can be detected (380). As an example, the presence of the gene coding for PVL in *S. aureus* strains is speculated to be linked to severity of necrotising pneumonia in young patients (119). Some molecular typing methods may be applied to study bacterial populations, evolution and the phylogenetic relationships between species (221, 375).

2.3.2 Criteria for evaluation, validation and comparison of typing methods

Several criteria should be considered when evaluating, validating and comparing typing methods. These can be divided into performance and convenience criteria (Table 2). The selection of the optimal and most applicable typing method depends on the purpose of the analysis (110, 332, 344, 374, 375). After the typing method has been selected, it is very important to carefully match this technology with the speed at which the molecular changes occur (374). The use of typing tools in epidemiological studies requires understanding of both the strengths and limitations of the chosen typing method as well as the epidemiological study design to be able to address the research question (110, 346). In addition, laboratory and epidemiological evidence validate each other, and typing results must therefore be interpreted in the context of the epidemiological evidence as well as the characteristics of the bacteria (110). To enable comprehensive epidemiological surveillance and international comparability, often one typing method alone is not sufficient, and a combination of various molecular typing techniques is used (86, 231, 388).

Table 2. Criteria for the evaluation, validation and comparison of typing methods.

Criteria	Explanation/purpose*
Performance criteria	
Stability	The molecular marker of the typing method should remain stable during the study period, and not vary to a degree that confuses the epidemiological picture (375, 380).
Typeability	The ability of a method to assign an unambiguous type to all isolates tested by it (332).
Reproducibility	The ability of a typing method to assign the same type to a strain tested on independent occasions, separated in time and/or place (366).
Discriminatory power	The ability to differentiate among epidemiologically unrelated isolates, ideally assigning each to a different type (162).
Wallace coefficient	A value indicating the probability that two strains classified as the same type by one method are also classified as the same by another method (371).
Concordance	A coefficient for quantifying the correspondence between typing methods (161, 297).
Test population	A well-defined and appropriate size (preferably >100) of the test population is a prerequisite for evaluating typeability, discriminatory power and concordance between typing methods (375).
Convenience criteria	
Flexibility	Typeability of a wide range of different species with minimal modifications of the method (375, 380).
Rapidity	The total time required to obtain to the final typing results from bacterial isolates (375).
Accessibility	The availability of reagents, equipment and the required skills in a given laboratory (375).
Ease of use	The technical simplicity, workload, suitability for analysing a large number of isolates, and ease of interpreting the results (375).
Cost	Consists of numerous factors, e.g. outlay for the equipment, reagents, personnel costs, software for interpreting the results and data storage (122, 375).
Computerised analysis and data storage	Possibility to computer-assistant analysis and interpretation of results. Potential to incorporate typing results into electronic databases enabling national and international comparison (e.g. MLST and *spa* typing) (100, 140, 222, 375).

* Reference describes an example or description of the criterion.

2.3.3 Characteristics of typing methods

Typing methods can be classified into phenotyping and genotyping methods. Phenotyping techniques detect the characteristics expressed by bacteria, while genotyping methods assess the variation in the chromosomal or extrachromosomal nucleic acid composition of bacterial isolates (375, 380). Phenotyping involves methods for detecting differences in biochemical reactions, morphology, and the environment. Biotyping is a widely used phenotyping method to assess biochemical characteristics varying within a given species, and is usually used in diagnostics and species identification and to separate the members of a particular species (332). Other commonly used phenotyping methods include serotyping, antimicrobial susceptibility testing, and bacteriophage and bacteriocin typing. Less frequently used phenotyping methods include multilocus enzyme electrophoresis (MLEE), mass spectrometry (MS) and SDS-PAGE of cellular and extracellular components (332, 375, 380).

Different genotyping methods are likely to reveal different degrees of genetic variability. Genetic diversity arises by various molecular processes, including the accumulation of spontaneous point mutations, diverse types of genetic rearrangements, and the loss and acquisition of chromosomal and extra-chromosomal DNA sequences (380). Genotyping methods define variation in the genomes of bacterial isolates with respect to the composition, overall structure, or precise nucleotide sequence. The increasing availability of bacterial genome sequences has had a significant impact on the evolution and improvement of genotyping methods (375).

Bacterial genotyping methods can be classified into three main categories: hybridisation-mediated, fragment-based and sequence-based methods (375). Hybridisation-mediated methods include ribotyping, which has been used for a long time, and novel DNA array-based methods such as DNA macro- and microarrays, cDNA microarrays and oligonucleotide microarrays (129, 375). Fragment-based methods can be separated into methods where fragments are generated by cleavage of DNA using restriction enzymes, including methods such as plasmid typing, restriction fragment length polymorphism (RFLP), restriction endonuclease analysis (REA), and pulsed-field gel electrophoresis (PFGE) (149, 225, 270). PCR fingerprinting (e.g. BOX, arbitrarily primed PCR), repetitive sequencing-based PCR (REP-PCR), and multilocus variable number tandem repeat analysis (MLVA) (217, 373, 375) are fragment-based methods where fragments are generated by amplification of DNA. A combination of both restriction enzyme digestion and DNA amplification includes methods such as PCR-RFLP and amplified fragment length polymorphism (AFLP) (375, 394). Sequence-based methods include single-

locus sequence typing (SLST), multilocus sequence typing (MLST), single nucleotide polymorphism (SNP) genotyping and genome sequencing, which is currently still too expensive and time demanding to be used in routine genotyping (213, 222, 375, 380).

The interpretation of typing data is an important phase in the typing process. Depending on the typing method, the interpretation of the experimental data leading to correct identification can be complicated and demanding. This may be due to technical factors relating to the typing method used or the fact that an epidemic strain can evolve during an ongoing outbreak (380). Some typing methods are more stable than others. For example, PFGE patterns of MRSA strains have been demonstrated to be relatively stable over periods of weeks to months (32). However, the use of PFGE in long-term laboratory-based surveillance can be misleading (351, 353). Interpretation rules should provide well-defined guidelines for unambiguous data interpretation, whether the strain is unique or a part of an outbreak (352, 353). Although computer-assisted analysis is nowadays common, most image-based methods generate complex band patterns, the interpretation of which remains subjective. Sequence-based typing methods allow unambiguous data interpretation, and therefore also better international comparison (100, 140, 222).

International databases are nowadays extremely important due to the worldwide spread of bacterial clones. Such databases rely on standardised typing methods and quality control ring trials for all participating laboratories. Significant efforts have been made to harmonise typing methods of multiple bacteria at the international level and to establish a standardised nomenclature (3, 218, 247, 276). There are several international databases, servers and networks to ease the comparison of typing data. These help in local infection control, as well as national and international surveillance of bacteria. Sequence-based methods, such as MLST of several different bacteria and *spa* typing of *S. aureus*, have useful international databases, MLST.net and SeqNet.org (SpaServer), respectively (238, 326). PulseNet for different foodborne pathogens and SalmGene for *Salmonella* species are molecular epidemiology databases with typing data and information concerning the clinical and/or epidemiological features associated with the isolates analysed. There are several different laboratory networks that base the strain comparison on PFGE methods, including PulseNet, SalmGene, HARMONY for *S. aureus*, and Listernet for *L. monocytogenes* (63, 79, 287, 313, 375).

The typing methods used in this thesis are discussed in more detail below.

2.3.4 Serotyping

Serotyping is a classic and important phenotyping method that has been developed since the early days of microbiology and successfully used for taxonomic grouping and epidemiological studies on a number of bacterial species. Serotyping is still important for typing *Salmonella*, *Legionella*, *Shigella* and *S. pneumoniae*. Traditionally, serotyping uses a series of antibodies to detect antigens on the surface of the bacterial cell. There are many ways in which serotyping can be performed and antibody-antigen reactions detected. Direct antibody-antigen agglutination is a frequently used method in which a bacterial cell suspension is mixed with panels of antibodies and the serotype is determined according to the agglutination profiles (332, 375). In addition, different types of molecular serotyping methods have been developed (37, 340).

Serotyping continues to be a valuable typing technique for *S. pneumoniae*, especially in monitoring vaccine efficacy and detecting the emergence of non-vaccine strains. The standard method for pneumococcal serotype determination has been the Quellung reaction, in which test antibodies bind to the corresponding capsular antigens and induce swelling of the capsule, which can be observed with light microscopy (186, 339). Several alternative methods to the capsular reaction test have been described for serotyping pneumococci, including a latex agglutination test, slide-agglutination method, a latex bead-based cytometric immunoassay, counterimmunoelectrophoresis (CIEP), enzyme-linked immunosorbent assay (ELISA), and recently a number of DNA techniques (37, 147, 224, 244, 271, 337, 384). PCR-based serotyping methods using primers that amplify serotype-specific genes coding for the capsule have been demonstrated, such as multiplex PCR-based serotyping, real-time PCR serotyping, PCR-based reverse line-blot hybridisation serotyping, and PCR restriction fragment length polymorphism (RFLP) (19, 37, 47, 194, 384). In addition, for *S. pneumoniae* serotyping, microarray analysis and few sequence-based assays have been described (21, 195, 196).

2.3.5 Antimicrobial susceptibility testing

Clinical microbiology laboratories commonly perform antibiotic susceptibility testing, providing important information for a clinician to choose the optimal antimicrobial treatment for a patient. Susceptibility testing results are also used in surveillance studies and by infection control personnel to detect and control the spread of antibiotic-resistant organisms (307, 308). In addition, antibiograms can be used as a typing method. Antimicrobial susceptibility testing can be performed by using either disk diffusion or broth microdilution methods with a variety of measurements systems. Susceptibility testing can measure the minimum inhibitory concentration (MIC) of an antimicrobial agent that prevents bacterial growth (in mg/L or µg/ml), or the test can provide qualitative results, as in the disk diffusion

method, categorising bacteria into three susceptibilities: susceptible (S), intermediate (I), and resistant (R) (365). To interpret an antibiogram, breakpoints for the separate susceptibility categories need to be determined. Many organisations such as the Clinical Laboratory Standards Institute (CLSI) and European Committee on Antimicrobial Susceptibility Testing (EUCAST) have worked to standardise and harmonise antimicrobial susceptibility testing protocols and breakpoints (56, 102).

Discrimination in antibiogram-based typing is dependent on the diversity, stability and relative prevalence of resistance mechanisms of the study isolates (375). In most epidemiological studies, antibiogram-based typing has relatively limited value due to its poor discriminatory power (352). In addition, antimicrobial resistance is often associated with mobile genetic elements (e.g. transposons and plasmids) that are rather instable and under selective pressure. Therefore, isolates that are not genetically and epidemiologically related may have the same susceptibility pattern. Resistance characteristics have been used to characterise certain global MRSA clones and to distinguish CA-MRSA and HA-MRSA, in addition to other typing methods (382, 390). However, the separation of CA-MRSA and HA-MRSA based on the antibiotic phenotype is becoming more problematic due to the migration of CA-MRSA into healthcare settings and the recent increasing antibiotic resistance of CA-MRSA strains (226).

2.3.6 Virulence gene detection and typing based on the polymerase chain reaction (PCR)

The polymerase chain reaction (PCR) was first described by Saiki *et al*. in 1985 and Mullis and Faloona in 1987 (245, 309). PCR is an *in vitro* reaction where a targeted nucleic acid sequence is amplified by using oligonucleotide primers, thermostable DNA polymerase, deoxynucleoside triphosphates (dNTPs), divalent cations (usually Mg^{2+}), a buffer to maintain the pH, monovalent cations (KCl), and template DNA (316). A large number of variations on the method have been described. In multiplex PCR, two or more primer sets are designed for the simultaneous amplification of different target DNA in a single reaction tube, which increases the efficiency of PCR and reduces the reagent costs (51, 255, 332). Real-time PCR amplifies and detects the target DNA sequence in real time and simultaneously by using specific dyes and fluorescent measurements (201, 391).

Conventional, real-time, and multiplex PCR methods have been used to detect virulence factors (pathotypes) from a variety of bacterial isolates and clinical specimens for different purposes. PCR-based virulence gene detection has been used, for instance, for species identification and to detect distinct virulence markers associated with pathogenic isolates within a species (8, 14, 214, 328).

PCR techniques are widely used in the identification of multiple *S. aureus* virulence factors, to determine the distribution of virulence factors among *S. aureus* strains, the relationship between the genetic background of *S. aureus*, and the association of virulence factors with certain forms and the severity of certain disease (380). Several PCR assays have been developed to detect PVL, a necrotising cytotoxin of *S. aureus* that has been associated with necrotic lesions of the skin and subcutaneous tissues and severe necrotising pneumonia (4, 204, 214, 227). PCR techniques have also been used to identify several other virulence factors of *S. aureus*, such as enterotoxins, exfoliative toxins, TSST-1 and different adhesin genes (211, 240, 405).

To date, several structural differences in SCC*mec* elements of *S. aureus* have been identified (167). The typing of SCC*mec* is used in epidemiological studies to discriminate MRSA strains or to define an MRSA clone in combination with the genotype (sequence type) (320, 346). In addition, SCC*mec* typing is used for evolutionary studies on MRSA (81). SCC*mec* types can be detected by identifying specific genes or gene alleles that are required for classification by PCR. Typically, first the type of *ccr* and class of *mec* are determined, and SCC*mec* types can then be subtyped by investigating differences in J regions. Multiplex PCR is usually used to assign *ccr* types, *mec* classes and J regions (320).

Pneumolysin is the most frequently applied virulence factor for pneumococcal identification and has been used to demonstrate pneumococcus directly from blood, cerebrospinal fluid, serum, pleural fluid, middle ear fluid, and a nasopharyngeal specimen (205, 315, 319, 379, 389). In addition, various other pneumococcal virulence factors have been demonstrated by using PCR, such as autolysin (LytA) and PsaA (235, 328). Pilus islets 1 (PI-1) and 2 (PI-2) have been shown to be involved in the virulence of pneumococcus, and the prevalence of PI-1- and PI-2-genes has been determined by PCR in multiple studies (2, 13, 18, 242, 243, 406).

2.3.7 Pulsed-field gel electrophoresis (PFGE)

Pulsed-field gel electrophoresis (PFGE) is an electrophoretic method used to separate large DNA molecules ranging from 10 kbp to 10 Mbp after rare-cutting restriction enzyme digestion of the total genomic DNA. PFGE can separate large DNA molecules due to an alternating electrical field at different angles in a flat agarose gel (149, 213, 323). Random genetic events including deletions, insertions and point mutations can alter the restriction sites and affect the banding pattern of the strain. PFGE was originally used for electrophoretic separation of the chromosomes of lower eukaryotes, and is now considered as a valuable typing tool for a wide variety of bacterial species such as *S. aureus*, *S. pneumoniae*, *C. difficile*, *Streptococcus* group A and B, and foodborne pathogens such as enterohaemorrhagic *Escherichia coli* (EHEC), *Salmonella*, *Shigella*, *Listeria* and *Campylobacter* (63,

288, 323, 353, 375). PFGE is still considered a successful typing method due to its high discriminatory power and reproducibility (110, 346, 352). However, it has some shortcomings, as it is a technically demanding, low-throughput and slow method, the interpretation of band profiles is subjective and interlaboratory comparison of strain profiles is difficult (346).

PFGE has been the 'gold standard' genotyping method for MRSA for over a decade, and it has been used widely for local outbreak investigation, long-term surveillance of MRSA infections at regional and national levels and for international comparisons (346). In Europe, harmonisation efforts have been made to standardise the PFGE typing protocol of MRSA and enable multicentre comparison of PFGE data (54, 247). Although PFGE has been valuable and the main typing method for MRSA for long time, weaknesses including the technical aspects, misleading interpretation in long-term epidemiological investigations, and the emergence of non-typeable isolates (e.g. livestock-associated ST398) have reduced the use of PFGE typing for MRSA (346, 351, 353).

PFGE is also a widely applied genotyping method for *S. pneumoniae*, and has been used to subtype, compare and characterise certain pneumococcal serotypes such as vaccine serotypes, vaccine-related serotypes, non-vaccine serotypes, and penicillin-resistant and penicillin-non-susceptible serotypes (86, 88, 116, 264). In addition, studies such as analysis of the genetic relatedness of pneumococcal isolates from paired blood and respiratory specimens, and comparison of pneumococcal nasopharyngeal isolates have used PFGE for characterising of the isolates (141, 399). Moreover, PFGE can be used to determine invasiveness and to differentiate pneumococcal clones (264).

2.3.8 Single-locus sequence typing (SLST)

Single-locus sequence typing (SLST) is a term for a variety of typing methods in which the sequencing of a single genetic locus provides useful typing results. To be able to provide sufficient discriminatory power, the target locus for SLST must be highly variable (375). SLST methods are rapid, easy to use and have high throughput, discriminatory power and reproducibility. The typing results of SLST are accurate, unambiguous and easily comparable (110, 332, 346).

One of the epidemiologically significant SLST schemes is *emm* typing of *S. pyogenes*, which is regarded as the 'gold standard' method for genotyping of streptococci. It is based on the sequencing of a 180 bp part of the hypervariable 5' terminus of the *emm* gene (103). At present, more than 100 *emm* sequence types and an even higher number of subtypes have been identified and stored in the database (49).

spa typing is a reliable tool for typing *S. aureus* and has become the most popular MRSA typing method (346). The method is based on sequencing of the polymorphic X region of the protein A gene (*spa*), present in nearly all *S. aureus* strains. The X region comprises of a variable number of typically 24 bp repeats flanked by well-conserved regions (Figure 9) (111, 112). The variable number of tandem repeats (VNTR) structure of the X region of the *spa* gene is explained by the slipped strand mispairing model. According to this model, illegitimate basepairing due to stretches and loops in short repeated unit motifs occurs during DNA replication, which leads the DNA polymerase to delete or insert repeat units (372). Many studies have evaluated the usefulness of *spa* typing for diverse epidemiological purposes and confirmed its ease of use, speed, high discriminatory power, reproducibility and typeability, and the portability of results (3, 135, 197, 329, 343, 351). In addition, due to the repeat structure, the *spa* gene characterises micro- and macrovariations, enabling the use of *spa* typing in both long- and short-term epidemiological studies, locally and globally (136, 197, 375). However, because *spa* typing is based on DNA sequences in hypermutable regions, it should be noted that mutants may arise even during an outbreak and therefore falsely suggest that the outbreak has multiple sources rather than one (375). Related *spa* types can be clustered (*spa* CC) according to the repeat structure by using the based upon repeat pattern (BURP) algorithm, which takes into account duplication and excision of the repeats (232). *spa* typing is suitable for computerised analysis, and at least two commercial software packages are currently available for *spa* typing (136). In addition, standardised nomenclature and Internet shared database (SpaServer) support and facilitate the use of the method (140, 326). The central *spa* server is organised by the SeqNet.org typing network, which currently includes 45 laboratories from 25 European countries (113).

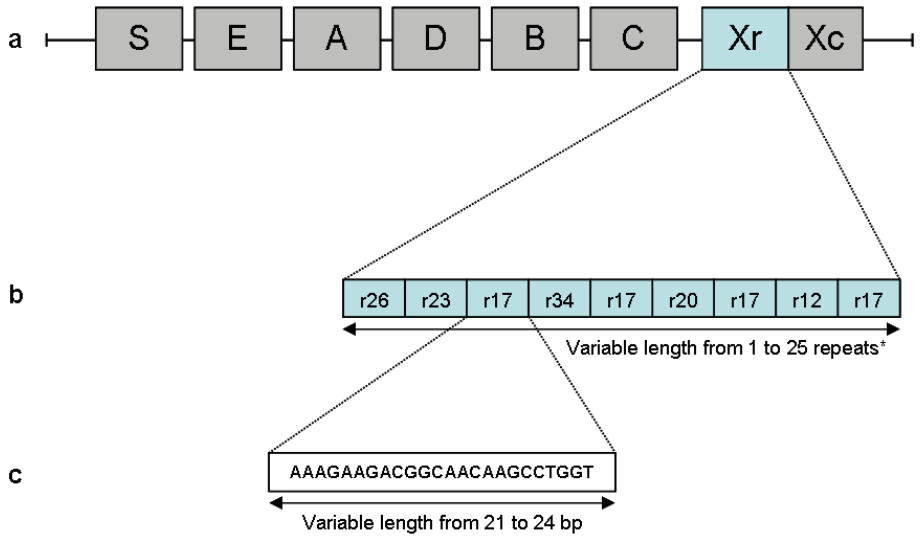

Figure 9. (a) Schematic diagram of the *spa* gene. S is the signal sequence; A to D are IgG-binding domains, E is a region homologous to A-D, X is the C-terminal part, divided into two regions, the VNTR region (Xr) and a constant region coding for cell wall attachment (Xc). (b) The repeat structure of the Xr region. The *spa* type presented is t067. (c) The DNA sequence of *spa* repeat 17. Adapted and modified from (136).
* SpaServer accessed July 2011.

2.3.9 Multilocus sequence typing (MLST)

Multilocus sequence typing (MLST) compares the nucleotide sequences of internal 400- to 500-bp regions of a series of housekeeping genes (usually five to ten), which are present in all isolates of a particular species (100, 222). For each gene fragment, the different sequences are assigned to distinct allele identification numbers and the combination of the numbers defined for all alleles generates the sequence type (ST). Isolates that have the same allelic profile can be considered as members of the same clone. The eBURST (based upon related sequences) program is used to cluster STs into clonal complexes (CC). The algorithm behind the program assigns the central genotype of CCs, which is the one with the highest number of single-locus variants (SLVs) (107). MLST has been applied to a large number of bacterial species (221, 238). It is useful tool for epidemiological analysis and surveillance of pathogens, and especially to study their population structure and evolution (221). MLST may be a more significant tool for population genetics and dynamics than for bacterial epidemiology, since its targets are the slowly evolving housekeeping genes. MLST has standard nomenclature and the data generated are fully portable both within and between laboratories. MLST data are available and can be shared globally via the

Internet (238, 285). However, MLST also has some drawbacks. It often fails to detect the variability of closely related strains and thus has only moderate discriminatory power. In addition, sequencing of several genes is time-consuming and costly (346).

MLST is particularly suitable for subtyping bacterial species with high a rate of genetic recombination, such as *S. pneumoniae* and *N. meningitidis* (99, 222). More than 5000 different allelic profiles are currently available for *S. pneumoniae* in the MLST database (238, 285). MLST has been used to identify antibiotic-resistant clones and virulent clones of pneumococcus causing invasive diseases (99, 117, 206, 331). MLST has been applied in research on *S. aureus* for ten years, and has been extremely useful for understanding the population structure of the species (96, 98). To date, more than 1700 MLST-based STs of *S. aureus* have been reported on the MLST website (238). MLST has been also used in several studies to understand the evolution of MRSA (95, 96, 98, 258, 269, 296). All major MRSA and MSSA clones are named according to their MLST and staphylococcal cassette chromosome (SCC*mec*) types (98). Some MRSA clones are associated with CA-MRSA or HA-MRSA infections (44, 98, 106, 381). In addition, several pandemic MRSA clones have also been described (95, 269).

3 AIMS OF THE STUDY

The general purpose of this thesis was to characterise two medically important bacterial pathogens, methicillin-resistant *Staphylococcus aureus* (MRSA) and *Streptococcus pneumoniae*, in detail by using several molecular typing methods for various epidemiological purposes: clonality analysis, epidemiological surveillance, outbreak investigation, and virulence factor analysis.

The specific objectives of this study were:

1. To evaluate the typeability, discriminatory power, and concordance of different genotyping methods of epidemic MRSA in determining the characteristics and clonality of strains for epidemiological surveillance and outbreak investigations of MRSA in Finland (I).

2. To search for a rapid typing scheme for national surveillance, epidemiological investigations and international comparisons of MRSA in Finland and to characterise Finnish MRSA isolates in more detail (II).

3. To study the cause and extent of a pneumococcal pneumonia outbreak among military recruits in Finland, and to define the pneumococcal carriage rate and molecular characteristics of the detected *S. pneumoniae* isolates (III).

4. To analyse the prevalence of pilus-encoding islets and the clonality of pneumococcal isolates associated with nasopharyngeal carriage and/or acute otitis media in children with AOM in Finland, in order to elucidate the association of pili with the disease potential of *S. pneumoniae* in AOM infections (IV).

4 MATERIALS AND METHODS

4.1 Bacterial isolates

The bacterial isolates used in the different studies are presented in Table 3. Since, 1995, all clinical microbiology laboratories across Finland have notified all MRSA findings in the National Infections Disease Register (NIDR) at the National Institute for Health and Welfare (THL). In this register, MRSA notifications concerning isolates from blood and cerebrospinal fluid from the same patient within a time interval of three months are merged into a single case. Before 2007, MRSA notifications from other sources were combined into a single case within a time interval of three years, and thereafter the time interval has been 50 years. Clinical laboratories send all MRSA isolates, corresponding to the notifications, to the Staphylococcal Reference Laboratory at THL for genotyping. MRSA isolates used for publication I and II were from the Strain Collection of NIDR. Pneumococcal isolates for publication III were isolated from blood and nasopharyngeal samples of military recruits of the Finnish Border Guard in the Kainuu Border Guard District involved in an outbreak investigation of pneumococcal pneumonia. *S. pneumoniae* isolates for publication IV were collected from MEF and NPA samples of children with AOM. Samples were obtained at the initial visit to the Department of Otolaryngology of Helsinki University Central Hospital (389).

Control strains used are described under the corresponding typing method.

Table 3. Isolates and methods used in the study

Publication	Bacteria	Source	Selection criteria for isolates	No. of isolates	Year/years	Typing methods used
I	MRSA	The strain collection of the Finnish NIDR	Representatives of the epidemic MRSA strain types	44	1991–2004	- Antimicrobial susceptibility testing - PFGE - MLST - *spa* typing - SCC*mec* typing - PVL-PCR
II	MRSA	The strain collection of the Finnish NIDR	1) All blood isolates 2) All sporadic isolates 3) FIN-4 isolates; the 1st isolate/every month (1997–2006); every 11th isolate/each month (2006) 4) FIN-16 isolates; the 1st isolate/every month (1997–2006); every 11th isolate/each month (2006)	1) 124 2) 49 3) 220 4) 196	1) 1997–2006 2) 2006 3) 1997–2006 4) 1997–2006	- PFGE - MLST - *spa* typing - SCC*mec* typing
III	PNC	Military recruits of the Finnish Border Guard	Outbreak investigation and defining the carriage rate; positive blood and nasopharyngeal cultures	20	2006	- Antimicrobial susceptibility testing - Serotyping - MLST - Pilus islet 1-PCR
IV	PNC	Finnish children with AOM	Isolates from the middle ear fluid (MEF) and/or nasopharyngeal aspirate (NPA) of children with AOM	75	1990–1992	- Antimicrobial susceptibility testing - Serotyping - MLST - Pilus islet 1- and 2-PCR

* PNC, *Streptococcus pneumoniae*

4.2 Epidemiological background data

For publication III, the epidemiological background data were retrieved by interviewing all the military recruits involved in a pneumococcal pneumoniae outbreak investigation. The epidemiological background data included the preceding symptoms, medical history, medication and smoking habits of the military recruits and were collected by a local infection control nurse with informed consent. In addition, the medical records of five hospitalised recruits were reviewed.

A confirmed pneumococcal pneumonia case was defined as having a positive blood culture for *S. pneumoniae*, in addition to the signs and symptoms. A non-bacteraemic pneumonia case was defined as having respiratory symptoms with fever and a lobar infiltrate in a chest radiograph.

4.3 Bacterial identification

All *S. aureus* isolates suspected of being MRSA and received from clinical laboratories were confirmed at the Staphylococcal Reference Laboratory at THL by detecting *mecA* and *nuc* genes using PCR until 2005. Thereafter, clinical microbiology laboratories have identified the isolates as *Staphylococcus aureus* by standard procedures (16) and confirmed methicillin resistance either by detecting the *mecA* gene using molecular methods or by testing oxacillin MIC by Etest (BioMérieux, France). According to the Finnish Study Group for Antimicrobial Resistance (FiRe), if the oxacillin MIC is above 64 mg/l, detection of the *mecA* gene is not required.

Identification of the pneumococcal isolates was performed in clinical laboratories (two isolates from blood for publication III) and in THL (nasopharyngeal swab specimens for publication III, isolates from MEF and NPA for publication IV) by using conventional methods (304), optochin susceptibility and bile solubility testing.

4.4 Antimicrobial susceptibility testing

The antimicrobial susceptibility of MRSA isolates was tested according to the guidelines of the Clinical and Laboratory Standards Institute (CLSI) (former National Committee for Clinical Laboratory standards, NCCLS) (publication I). The antimicrobial agents oxacillin, ampicillin, penicillin, cephalexin, cefuroxime, gentamicin, tobramycin, erythromycin, clindamycin, chloramphenicol, ciprofloxacin, rifampicin, fusidic acid, mupirocin and vancomycin were tested by the disk diffusion method. Oxacillin and vancomycin MICs were determined by the E-test

(AB Biodisk, Solna, Sweden) according to the recommendations of the manufacturer. If the isolates were resistant to three or more antimicrobial agents groups, they were considered multiresistant.

MIC testing of pneumococcal isolates was performed by the agar plate dilution technique. The antimicrobial agents tested for publication III were erythromycin, levofloxacin, clindamycin, ceftriaxone, tetracycline, penicillin and moxifloxacin, and for publication IV they were erythromycin, levofloxacin, clindamycin, ceftriaxone, tetracycline and penicillin (280). Breakpoints were defined according to the guidelines of CLSI.

4.5 Detection of serum antibodies and urinary antigens

Serum antibodies to *Mycoplasma pneumoniae, Chlamydia pneumoniae, Francisella tularensis* and common respiratory viruses (adenovirus, parainfluenza virus, influenza A and B virus, enterovirus, respiratory synctial virus and Puumala virus) were analysed in the Central Hospital of Kainuu by enzyme immunoassay (EIA) and complement fixation methods from acute-phase sera of recruits. *Legionella pneumophila* antigens from urine were detected by the BinaxNOW® Legionella Urinary Antigen test (Binax Inc., Scarborough, ME, USA).

4.6 Serotyping

Pneumococcal isolates were serotyped by counterimmunoelectrophoresis or by latex agglutination (the neutral serogroups/types 7 and 14). The capsular swelling (Quellung) test was used for confirmation when needed. All Omni, pool, group/type and factor antisera were acquired from Statens Seruminstitut, Copenhagen, Denmark.

4.7 Isolation of DNA and primers

Chromosomal DNA extraction of MRSA isolates was performed by using the guanidium thiocyanate-EDTA-sarkosyl-reagent (GES reagent) or the rapid lysis buffer method (HainLifescience GmbH, Nehren, Germany) (281) (publications I and II). The genomic DNA of pneumococcal isolates was isolated with the DNeasy tissue kit (Qiagen GmbH, Hilden, Germany) or lysis method (239) (publications III and IV).

The primers used in the publications are presented in Tables 4A and 4B.

Table 4A. Primers used in MRSA studies (publications I and II).

Target	Method	Reference
Protein A (*spa*)	*spa* typing	(3)
Carbamate kinase (*arcC*)	MLST	(68, 96)
Shikimate dehydrogenase (*aroE*)	MLST	(96)
Glycerol kinase (*glpF*)	MLST	(96)
Guanylate kinase (*gmk*)	MLST	(96)
Phosphate acetyltransferase (*pta*)	MLST	(96)
Triosephosphate isomerase (*tpi*)	MLST	(96)
Acetyl coenzyme A acetyltransferase (*yqiL*)	MLST	(96)
mec complex locus A	SCC*mec* typing	(267)
mec complex locus B	SCC*mec* typing	(267)
mec complex locus C	SCC*mec* typing	(267)
mec complex locus D	SCC*mec* typing	(267)
mec complex locus E	SCC*mec* typing	(267)
mec complex locus F	SCC*mec* typing	(267)
mec complex locus G	SCC*mec* typing	(267)
mecA	MRSA identification, SCC*mec* typing	(157, 193, 246, 267)
ccrA1-ccrB	SCC*mec* typing	(164, 193)
ccrA2-ccrB	SCC*mec* typing	(164, 193)
ccrA3-ccrB	SCC*mec* typing	(164, 193)
ccrA4-ccrB4	SCC*mec* typing	(193)
ccrC	SCC*mec* typing	(193)
mecA-mecI (*mec* class A)	SCC*mec* typing	(193, 266)
mecA-IS1272 (*mec* class B)	SCC*mec* typing	(183, 193, 266)
mecA-IS431 (*mec* class C)	SCC*mec* typing	(183, 193)
mecR1 (PB domain)	SCC*mec* typing	(183, 266)
mecR1 (MS domain)	SCC*mec* typing	(183, 266)
SCC*mec* subtype IVa	SCC*mec* typing	(266)
SCC*mec* subtype IVb	SCC*mec* typing	(266)
PVL genes *lukS*-PV and *lukF*-PV	PVL-PCR	(214)
Thermostable nuclease (*nuc*)	MRSA identification	(36)

Table 4B. Primers used in pneumococcal studies (publications III and IV).

Target	Method	Reference
Shikimate dehydrogenase (*aroE*)	MLST	(97)
Glucose-6-phosphate dehydrogenase (*gdh*)	MLST	(99)
Glucose kinase (*gki*)	MLST	(99)
Transketolase *(recP*)	MLST	(97)
Signal peptidase I (*spi*)	MLST	(282)
Xanthine phosphoribosyltransferase (*xpt*)	MLST	(97)
D-alanine-D-alanine ligase (*ddl*)	MLST	(30), B. Pichon, personal communication
rlrA	Pilus islet 1-PCR	(2)
rrgC	Pilus islet 1-PCR	(336)
pitA-sipA	Pilus islet 2-PCR	(13)

4.8 SCC*mec* typing by PCR

For publication I, SCC*mec* typing of MRSA isolates was initially performed by multiplex PCR, as previously described (267). Non-typeable strains were further analysed for *ccr* (*ccrAB* and *ccrC*) and the *mec* gene complex by PCR, as described by Okuma and co-workers (266). For publication II, the *ccr* types (*ccrAB1* to *ccrAB4*, or *ccrC*) and *mec* class (A, B, or C) within SCC*mec* were detected by multiplex PCR (M-PCR 1 and M-PCR 2), as previously described (193).

4.9 Virulence gene detection by PCR

4.9.1 PVL

The presence of PVL genes, *lukS*-PV and *lukF*-PV, of *S. aureus* isolates was detected by PCR (214). PVL encoding genes were amplified either alone or with *nuc* and *mecA* genes as a multiplex PCR (36, 246). Three strains were used as controls: CCUG 46923 (*nuc*+, *pv*l+), FIN-11 (ST80) (*nuc*+, *pvl*+, *mecA*+) and FIN-3 (ST5) (*nuc*+, *mecA*+).

4.9.2 Pilus islet 1- and 2- PCR

The presence of pneumococcal PI-1 genes (*rlrA* and *rrgC*) and PI-2 genes (*pitA-sipA*) was detected by PCR. The following strains were used as controls: IH152966-1 [from NIDR, penR, serotype 19F, ST236, *rlrA*+, *rrgC*+, (*pitA-sipA*)+] and IHU60124 [AOM strain, serotype 6B, ST1752, *rlrA*-, *rrgC*-, (*pitA-sipA*)-].

4.10 Pulsed-field gel electrophoresis (PFGE)

Pulsed-field gel electrophoresis (PFGE) was performed according to the harmonised protocol developed by consensus in ten European laboratories in 2003 (247). The strain NCTC 8325 was used as a reference standard. PFGE patterns were analysed with BioNumerics software (version 4.6 and 5.1, Applied Maths, Kortrijk, Belgium). The Dice coefficient was used to analyse the similarity of the banding patterns, and cluster analysis was performed by the unweighted pair group method using arithmetic averages (UPGMA). In general, the criterion of a difference of ≤ 6 bands or a similarity cut-off of 80% was used to determine a PFGE type (345, 353). If a seven or more band difference occurred, the PFGE types were interpreted as different. In publication I, PFGE types having a 1–6 band difference in their PFGE band profile were considered to belong to the same PFGE cluster and were assigned the same FIN number, including strains with a 1–2 band difference. Strains with a 3–6 band difference were named by letters after the FIN number. In publication II, PFGE types having a 1–6 band difference in their PFGE band profile were considered to belong to the same PFGE type, but were interpreted as different if a seven or more band difference occurred. PFGE subtypes were not defined. PFGE profiles differing by more than six bands, compared to any other profile in the local PFGE database, were interpreted as sporadic types. The interpretation criterion for a 'PFGE cluster' in publication I corresponds with the criterion for a 'PFGE type' in publication II.

4.11 Sequencing methods

4.11.1 *spa* typing

The polymorphic region of protein A gene (*spa*) was amplified by PCR and sequenced as described earlier (3, 343). Ridom StaphType software (Ridom GmbH, Würzburg, Germany) was used to analyse the sequences (140). Clustering of related *spa* types into clonal complexes (*spa* CC) was carried out by using the repeat pattern (BURP) algorithm of the Ridom StaphType software package (232). *spa* types were clustered if the calculated cost between members of a group was less than or equal to six (publication I) or four (publication II). *spa* types shorter than five repeats were excluded (publication II). A bioneighbour-joining tree of *spa* types was built by using the program Splits Tree4 (version 4.10). Cost values from the Ridom StaphType program were used to analyse the distances between *spa* types.

4.11.2 Multilocus sequence typing (MLST)

Multilocus sequence typing (MLST) was performed for MRSA and pneumococcal isolates as described previously (30, 96, 97, 99, 331). Seven housekeeping genes of MRSA isolates [carbamate kinase (*arcC*), shikimate dehydrogenase (*aroE*), glycerol

kinase (*glpF*), guanylate kinase (*gmk*), phosphate acetyltransferase (*pta*), triosephosphate isomerase (*tpi*) and acetyl coenzyme A acetyltransferase (*yqiL*)] and pneumococcal isolates [shikimate dehydrogenase (*aroE*), glucose-6-phosphate dehydrogenase (*gdh*), glucose kinase (*gki*), transketolase *(recP*), signal peptidase I (*spi*), xanthine phosphoribosyltransferase (*xpt*) and D-alanine-D-alanine ligase (*ddl*)] were amplified by PCR. The PCR products were purified using the QiaQuick PCR purification kit (Qiagen), the GeneClean Turbo kit (Q-Bio-Gene; MB Biomedicals, OH), or the Edge Bio QuickStep™ 2 PCR Purification kit (Edge Bio Systems, Gaithersburg, USA). The sequences were determined with an ABI Prism 310 Genetic Analyzer by using BigDye fluorescent terminator chemistry (Applied Biosystems, UK) (publication I) or by using full service sequencing offered by the Molecular Medicine Sequencing Laboratory (Biomedicum, Helsinki) (publications II–IV). The forward and reverse sequencing primers for each gene were the same as those for initial PCR. To analyse and trim the sequences, the Vector NTI Advance 10 software suite (Invitrogen Corporation, Carlsbad, CA) was used. The trimmed sequences were compared against the MLST database and the sequence types (STs) were assigned. The clonal complex (CC) for each ST was determined against the entire *Staphylococcus aureus* or *Streptococcus pneumoniae* database by eBURST V3 analysis using default stringent parameters. New allele sequence traces and STs were submitted to the MLST database.

4.12 Cost and time analysis

In publication II, the mean hands-on time and total time used for processing one set of isolates for PFGE (n = 12) and *spa* typing (n = 24) was estimated by stopwatch analysis. Each step of the process was timed at least three times by three different persons. The hands-on time included the time used for analysing the results. The material costs of both methods were calculated based on material usage per isolate. The Molecular Medicine Sequencing Laboratory (Biomedicum, Helsinki) provided the sequencing service for *spa* typing.

4.13 Comparisons of typing methods and statistical analysis

The discriminatory power of typing methods (discriminatory index, DI) and confidence intervals for DIs was calculated by using Ridom StaphType software (publications I and II) (131, 162). DI indicates the ability of two unrelated strains to fall into different typing groups and it depends on the total number of strains in the strain collection, the total number of strain types tested by the test method and the relative frequencies of these types (162). In publication I, DI calculation of SCC*mec* typing only included the SCC*mec* types, not the subtypes.

To assess the quantitative correspondence between typing methods, three different coefficients were used: Rand, Adjusted Rand (AR) and Wallace (W) (161, 297, 371). Ridom StaphType was used to analyse the Rand coefficient. AR and W coefficients were calculated via the Internet (www.comparingpartitions.info). The Rand and AR coefficients explore the concordance between typing methods, while the W coefficient value demonstrates the probability that two strains categorised as the same type by one method are also categorised as the same by another method. A high value for the W coefficient indicates that the result obtained by a given method could have been predicted from the result of the other method.

In publication III, statistical data were analysed by Intercooled Stata 9.1 for Windows (StataCorp, College Station Texas, the USA). The risk ratios (RR) were calculated using univariate analysis and 95% confidence intervals (CI) for exposures of interest. The chi-squared test was used to compare categorical data. In publication IV, statistical analysis was performed with the free GraphPad software (http://www.graphpad.com). For categorical data, proportions were compared using Fisher's exact test. In publications III and IV, *P* values <0.05 were considered statistically significant.

4.14 Ethical considerations

THL conducts infectious disease surveillance and research by legislation and has the rights to use data from the NIDR. No ethics committee approval was therefore required for publications I and II. For publication III, written informed consent was obtained from the all recruits involved in a point-prevalence survey. The experiments described in publication IV were carried out by using previously isolated strains of pneumococci. Written informed consent was obtained from the parents of all children before enrolment and the study protocol was approved by the ethics committees of THL (formerly Kansanterveyslaitos, KTL) and the University of Helsinki.

5 RESULTS

5.1 Molecular characteristics of MRSA strains (I, II)

5.1.1 Molecular characteristics of epidemic MRSA strains in Finland (I, II)

The molecular characteristics of epidemic MRSA strains in Finland were investigated in detail by using several typing methods in publication I. During a thirteen-year period (from 1991 to 2004), 44 Finnish epidemic MRSA (EMRSA) strains were detected by PFGE. When these strains were reanalysed using PFGE with the looser PFGE interpretation criteria, these 44 EMRSA strains could be grouped into 26 PFGE clusters. Amongst the 44 EMRSA strains, 20 MLST STs with 12 CC, and 27 *spa* types with four *spa* CC and 7 singletons (*spa* types), were detected (Table 5 and Figure 10). In addition, most of the EMRSA strains possessed SCC*mec* type IV (43%), were multiresistant (52%) and PVL negative (95.5%).

Table 5. Characteristics of EMRSA isolates from 1991–2004.

MLST CC	**MLST ST**	***spa* CC**	***spa***	**PFGE cluster**	**SCC *mec***	**Resistance to multiple antibiotics[a]**	**PVL**	**Year of isolation**
1	1	singleton	t127	FIN-19	IV	no	-/+ [c]	2001
5	5	002	t088	FIN-26	V	no	-	2004
5	5	002	t002	FIN-3	II	yes	-	1992
5	5	002	t002	FIN-3	II	yes	-	1999
5	5	002	t001	FIN-8	IVA	yes	-	1994
5	125	002	t067	FIN-16	IA	yes	-	2001
8	8	051/008	t008	FIN-15	IV	no	-	2000
8	8	051/008	t008	FIN-18	IV	yes	-	2000
8	8	051/008	t008	FIN-25	IV	no	+	2004
8	8	051/008	t596	FIN-7	IV	no	-	1997
8	8	051/008	t008	FIN-7	IV	no	-	1993
8	239	234/037	t037	FIN-13	III	yes	-	1998
8	239	234/037	t030	FIN-2	III	yes	-	1994
8	239	234/037	t037	FIN-2	III	yes	-	2000
8	239	234/037	t037	FIN-2	III	yes	-	1998
8	239	234/037	t234	FIN-2	IIIA	yes	-	1992
8	239	234/037	t573	FIN-2	IIIA	yes	-	1993
8	239	234/037	t030	FIN-9	IIIA	yes	-	1994
8	241	234/037	t037	FIN-2	IIIB	yes	-	1998

Table 5. Continues.

MLST CC	MLST ST	*spa* CC	*spa*	PFGE cluster	SCC *mec*	Resistance to multiple antibiotics[a]	PVL	Year of isolation
8	247	051/008	t051	FIN-1	IVA	yes	-	1992
8	247	051/008	t051	FIN-1	IVA	yes	-	1993
8	247	051/008	t051	FIN-1	IVA	yes	-	1993
8	247	051/008	t051	FIN-1	IVA	yes	-	1991
8	247	051/008	t051	FIN-1	IA	yes	-	1992
8	572	051/008	t562	FIN-1	IA	yes	-	1994
9	27	singleton	t100	FIN-22	V	no	-	2003
12	12	singleton	t561	FIN-14	IV	no	-	1998
22	22	singleton	t022	FIN-12	IV	no	-	1997
30	30	234/037	t018	FIN-5	IV	no	-/+ [c]	1993
30	36	234/037	t018	FIN-5	II	yes	-	1995
45	45	065	t015	FIN-10	IV	no	-	1996
45	45	065	t330	FIN-10	IV	no	-	1997
45	45	065	t004	FIN-10	IV	no	-	1998
45	45	065	t563	FIN-10	IV	no	-	1996
45	45	065	t560	FIN-10	V	no[b]	-	2000
45	46	065	t065	FIN-17	V	no	-	2000
59	375	singleton	t172	FIN-4	IV	no	-	1997
59	375	singleton	t172	FIN-4	IV	no	-	1993
59	375	singleton	t172	FIN-4	IV	no	-	1994
72	72	singleton	t148	FIN-20	IVA	no	-	2001
80	80	singleton	t044	FIN-11	IV	no[b]	+	1997
228	111	002	t041	FIN-24	IV	yes	-	2004
228	228	002	t041	FIN-21	I	yes	-	2002
228	228	002	t001	FIN-23	IV	yes	-	2004

[a] Multiresistant: resistant to ≥ 3 antibiotics; Non-multiresistant: resistant to 1–2 antibiotics

[b] Some isolates can also be multiresistant

[c] Both PVL-positive and -negative isolates have been identified among the groups FIN-19 and FIN-5

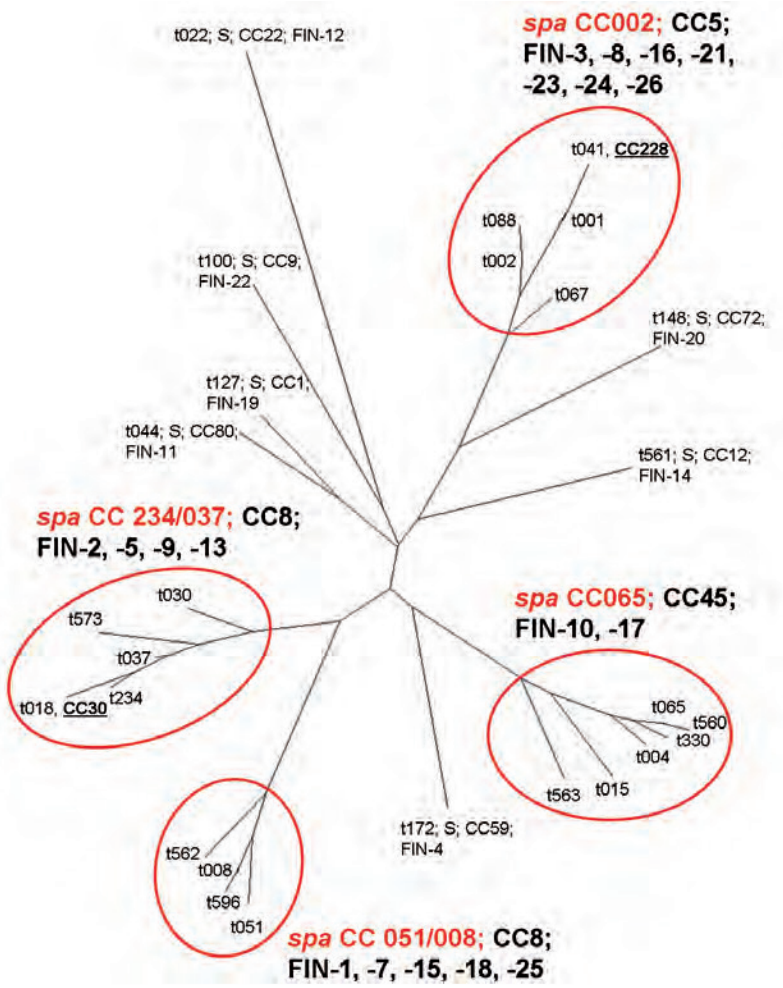

Figure 10. A bioneighbour-joining tree of 27 *spa* types of Finnish EMRSA isolates (N = 44). *spa* CCs are shown by red circles. S indicates a singleton *spa* type. MLST CCs and PFGE groups are mentioned after the *spa* CC or S. In two *spa* CCs, an additional MLST CC is found, besides the most common MLST CC. These MLST CCs are bolded and underlined after the *spa* type.

Nine *spa* types were found among 220 FIN-4 MRSA isolates between the years 1997 and 2006 (Table 6). One of the isolates was non-typeable. The majority (87%, 191/220) of the isolates were of *spa* type t172. The *spa* types belonged to one *spa* cluster, *spa* CC 172, and one of the isolates was a singleton. All of the three different STs belonged to one clonal complex (CC), CC59. Among eight *spa* types other than t172, seven possessed SCC*mec* type IV and one isolate was non-typeable. The non-typeable *spa* type also had a non-typeable SCC*mec* type.

Among the 196 FIN-16 isolates, 12 *spa* types were found between the years 1997 and 2006 (Table 6). All isolates belonged to one *spa* cluster, *spa* CC 002. *spa* type t067 (88%) was the most prevalent. All of the three different STs belonged to CC5. Among 11 *spa* types other than t067, eight possessed SCC*mec* type IV and the remaining three carried SCC*mec* type I, V, and a non-typeable SCC*mec* type. The both non-typeable *spa* types had also non-typeable SCC*mec* type.

Table 6. *spa* typing, MLST, and SCC*mec* results of MRSA FIN-4 and FIN-16 isolates in Finland between 1997 and 2006.

PFGE type	*spa* type[a] (No. of isolates)	*spa* CC[b]	MLST ST[c]	MLST CC[c]	SCC*mec*[a,c]
FIN-4	NT (1)	-	375	59	NT
	t172 (191)	172	375[d]	59[d]	IV[d]
	t216 (1)	172	87	59	IV
	t458 (1)	excluded	375	59	IV
	t529 (3)	excluded	375	59	IV
	t976 (1)	172	59	59	NT
	t2380 (4)	172	375	59	IV
	t3424 (2)	excluded	375	59	IV
	t3627 (15)	172	375	59	IV
	t3718 (1)	singleton	375	59	IV
FIN-16	NT (2)	-	5, 125	5	NT
	t002 (8)	002	5	5	IV
	t067 (172)	002	125	5	IV
	t214 (3)	002	5	5	IV
	t242 (1)	002	5	5	V
	t442 (3)	002	146	5	IV
	t535 (1)	excluded	5	5	NT
	t548 (1)	002	146	5	IV
	t777 (1)	excluded	125	5	IV
	t952 (1)	002	5	5	I
	t1094 (1)	002	5	5	IV
	t1991 (1)	excluded	125	5	IV
	t3109 (1)	002	125	5	IV

[a] NT, non-typeable
[b] *spa* types were clustered if the calculated cost (using Ridom StaphType software) between members of a group was ≤ 4; *spa* types shorter than five repeats were excluded.
[c] Eight FIN-4 and 11 FIN-16 representative MRSA strains were selected for MLST and SCC*mec* typing, based on different *spa* typing results compared to the most common *spa* type found among the PFGE types. In addition, five FIN-16 strains with *spa* type t067 were selected for MLST.
[d] MLST and SCC*mec* typing results are taken from the result of the representative FIN-4 EMRSA strain (index case).

5.1.2 Molecular characteristics of MRSA blood isolates in Finland (II)

The molecular characteristics of 124 Finnish MRSA blood isolates were studied in detail during 1997–2006 in publication II. Among 124 MRSA blood isolates, 19 different previously identified EMRSA strain types were detected by using PFGE (Table 7). In addition, six sporadic PFGE types were identified. *spa* typing was able to identify 38

different *spa* types, which divided into eight *spa* CC, including one without a founder, and 10 singleton *spa* types (12 isolates). The most prevalent PFGE types were FIN-16 (34%), FIN-21 (15%) and FIN-4 (10%). Concordant with the PFGE types, the most prevalent *spa* types were t067 (32%), t041 (13%) and t172 (10%).

Table 7. Characteristics of MRSA blood isolates in Finland between 1997 and 2006.

PFGE type	*spa* type (No. of isolates)	*spa* CC	No. of isolates (%) (N=124)	Isolation year/years
FIN-1	t562	singleton	1 (1%)	2000
FIN-2	t037	012	4 (3%)	1998–1999, 2004
FIN-3	t002	002	2 (2%)	2005, 2006
FIN-4	t172 (12)	no founder	13 (10%)	1999, 2001–2004, 2006
	t976 (1)	no founder		2005
FIN-5	t012	012	3 (2%)	2006
	t018	012		1997
	t2163	singleton		2003
FIN-7	t008 (3)	008	8 (6%)	2001, 2005, 2006
	t024	008		2005
	t068 (2)	008		2003, 2004
	t121	008		2006
	t596	008		2006
FIN-9	t030	012	1 (1%)	2005
FIN-10	t015 (3)	015	8 (6%)	2005, 2006
	t550	singleton		2005
	t596	008		2002
	t630	015		2006
	t1644	excluded		2004
	t3020	excluded		2004
FIN-11	t044 (2)	376	3 (2%)	1999, 2005
	t376	376		2006
FIN-12	t032	singleton	2 (2%)	2000, 2006
FIN-13	t037	012	2 (2%)	1998
FIN-14	t160	singleton	1 (1%)	1999
FIN-15	t008 (2)	008	3 (2%)	2001, 2004
	t689	singleton		2004
FIN-16	t067 (39)	067	42 (34%)	2002–2006
	t442	067		2006
	t1084	067		2005
	t3148	067		2005
FIN-19	t127 (2)	singleton	3 (2%)	2002, 2004
	t267	376		2006
FIN-20	t148	singleton	1 (1%)	2005
FIN-21	t041 (14)	2521/041	18 (15%)	2004–2006
	t1628 (2)	2521/041		2006
	t2521	2521/041		2004
	t3181	2521/041		2004
FIN-24	t041	2521/041	1 (1%)	2005
FIN-25	t008	008	1 (1%)	2006
sporadic	t037 (2)	012	7 (6%)	2002, 2004
	t041	2521/041		2004
	t067	067		2002
	t355	singleton		2006
	t583	015		2002
	t2099	singleton		2006

5.1.3 Clonality of MRSA (I, II)

Finnish EMRSA isolates were compared with international MRSA clones based on the literature and the MLST database. At the time of analysis the 44 Finnish EMRSA strains were divided into 12 known MLST CCs by eBURSTv3. However, the same data can now be divided into 10 known clonal complexes (Figure 11). Various international MRSA clones have been recognised in Finland and are presented in Table 8.

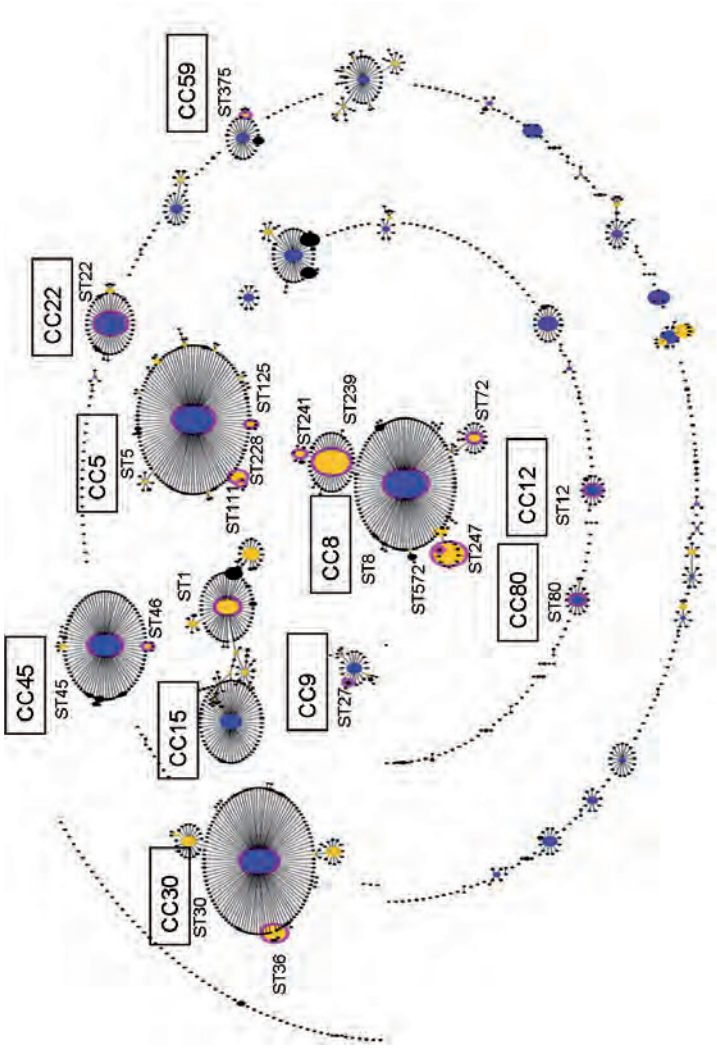

Figure 11. eBURST diagram displaying a 'population snapshot' of the *S. aureus* isolates from the MLST database (December 2010). The diagram was manually edited, and only STs (with magenta halo) and CCs detected among Finnish EMRSA strains were marked. Blue circle = founder, yellow circle = subgroup founder, black circle = all the other STs

Table 8. Finnish EMRSA strains with corresponding international clones.

MLST CC	**MLST ST**	***spa* type**	**SCC *mec***	**Resistance to multiple antibiotics**	**PVL**	**PFGE cluster**	**Related international MRSA clones based on the literature**
1/5[a]	1	t127	IV	no	-/+ [b]	FIN-19	USA400[c] (81, 356)
5	5	t002	II	yes	-	FIN-3	New York/Japan Clone[d] (USA100) (81, 356, 398)
5	5	t001	IVA	yes	-	FIN-8	Paediatric clone[d] (USA800) (81, 356, 398)
5	125	t067	IA	yes	-	FIN-16	Spanish epidemic clone[d] (10, 274, 388)
8	247	t051	IA	yes	-	FIN-1	Iberian clone[d] (81, 356, 398)
8	239	t234, t573 t030	IIIA	yes yes	- -	FIN-2 FIN-9	Brazilian clone[d] (81, 356, 398)
8	239	t037, t030 t037	III	yes yes	- -	FIN-2 FIN-13	Hungarian clone[d], Russian clone[d] (81, 356, 398)
8	8	t008 t008 t008	IV	no no yes	- - -	FIN-7 FIN-15 FIN-18	EMRSA-2/-6 (USA500)[d] (81, 356)
8	8	t008	IV	no	+	FIN-25	USA300[c] (81, 85, 356)
22	22	t022	IV	no	-	FIN-12	EMRSA-15 clone[d] (81, 175, 398)
30	30	t018	IV	no	-/+ [b]	FIN-5	Southwest Pacific clone[c] (USA1100) (81, 356)
30	36	t018	II	yes	-	FIN-5	EMRSA-16 clone[d], (USA200) (81, 175, 398)
45	45	t015, t563, t330, t004	IV	no	-	FIN-10	Berlin clone[d] (USA600) (81, 95)
80	80	t044	IV	no	+	FIN-11	European and Middle Eastern clone[c] (81, 381)
228/5[a]	228	t041	I	yes	-	FIN-21	Italian Clone/South German Epidemic Strain[d] (81, 241, 324)

[a] The latter CC is according to the MLST database (December 2010)

[b] PVL-positive and -negative isolates have been identified among the FIN-19 and FIN-5 clusters.

[c] Proposed CA-MRSA clone

[d] Proposed HA-MRSA clone

5.2 Molecular typing of MRSA (I, II)

5.2.1 Typeability, discriminatory power, concordance and Wallace coefficient of typing methods (I, II)

A combined analysis of typeability, discriminatory power, concordance and Wallace of MRSA typing methods was performed based on data from publications I and II. The overall typeability of MRSA typing methods was high, >89% (Table 9). The discriminatory power varied between genotyping methods (Table 10). In general, PFGE, *spa* typing and MLST had a high DI, while SCC*mec* typing had the weakest DI. Minor variations in DIs of *spa* typing and PFGE methods were detected between publications I and II.

Table 9. Typeability of MRSA genotyping methods.

Method	Publication	No. of isolates	No. of non-typeable isolates	Typeability
PFGE	I	44	0	100%
PFGE	II	589	0	100%
spa typing	I	44	0	100%
spa typing	II	589	3	99.5%
MLST	I	44	0	100%
MLST	II	24	0	100%
SCC*mec*	I	44	0	100%
SCC*mec*	II	19	2	89.5%

Table 10. Discriminatory power of MRSA genotyping methods.

Method	Publication	No. of isolates	No. of different types/groups	DI	95% CI
PFGE	I	44	44	1	[1.0-1.0]
PFGE cluster[a]	I	44	26	0.952	[0.922-0.983]
PFGE type[a]	II	124	26	0.847	[0.798-0.895]
MLST	I	44	20	0.936	[0.906-0.965]
MLST CC	I	44	12	0.785	[0.679-0.892]
spa	I	44	27	0.968	[0.948-0.988]
spa	II	124	38	0.866	[0.819-0.914]
spa CC	I	44	11	0.846	[0.798-0.893]
spa CC	II	124	17	0.811	[0.761-0.861]
SCC*mec*	I	44	5	0.637	[0.504-0.771]

[a] The interpretation criterion of 'PFGE cluster' in publication I corresponds to the criterion of 'PFGE type' in publication II

Generally, adjusted Rand showed much lower concordance values between typing methods than Ridom, which uses a coefficient that is formally equivalent to the Rand coefficient. According to AR, concordance was highest between MLST and PFGE clusters (AR = 0.641) in publication I (Table 11). The next highest was between *spa* CC and MLST CC (AR = 0.584), and *spa* CC and MLST (AR = 0.548). SCC*mec* typing showed the lowest concordance with all other typing methods (AR = 0.017–0.141). In publication II, the concordances between typing methods were higher than in publication I, as calculated by AR (Table 11). Concordance among MRSA blood isolates was highest between *spa* CC and the PFGE type (AR = 0.845).

Table 11. Concordances between MRSA typing methods as calculated by adjusted Rand (AR) and Ridom software.

Typing methods[a]	**Concordances of 44 EMRSA**		**Concordances of 124 blood MRSA**	
	AR	Ridom	AR	Ridom
MLST-PFGE cluster	0.641	0.961	-	-
spa CC-MLST CC	0.584	0.874	-	-
spa CC-MLST	0.548	0.910	-	-
spa-MLST	0.507	0.955	-	-
spa-PFGE cluster/type[b]	0.432	0.958	0.812	0.954
spa CC-PFGE cluster/type[b]	0.430	0.893	0.845	0.963
MLST CC-MLST	0.403	0.850	-	-
MLST CC-PFGE cluster	0.309	0.833	-	-
spa CC-*spa*	0.304	0.877	0.787	0.949
spa-MLST CC	0.205	0.815	-	-
SCC*mec*-*spa* CC	0.141	0.652	-	-
SCC*mec*-MLST	0.138	0.672	-	-
SCC*mec*-PFGE cluster	0.094	0.659	-	-
SCC*mec*-*spa*	0.075	0.656	-	-
SCC*mec*-MLST CC	0.017	0.586	-	-

[a] *spa* types were clustered if the calculated cost (using Ridom StaphType software) between members of a group was ≤ 6 (publication I) or ≤ 4 (publication II) and *spa* types shorter than five repeats were excluded.

[b] The interpretation criterion of 'PFGE cluster' in publication I corresponds with the criterion of 'PFGE type' in publication II

In publication I, PFGE clusters completely predicted MLST CC (W = 1.00) and *spa* CC (W = 1.00) calculated by the Wallace coefficient. In addition to *spa* CC (W = 1.00), *spa* types highly predictied MLST CC (W = 0.9667), MLST ST (W = 0.8) and SCC*mec* (W = 0.8). MLST ST fully predict *spa* CC (W = 1.00) and

MLST CC (W = 1.00). SCC*mec* poorly predicted all typing methods. Overall, PFGE clusters and *spa* types were poorly predicted by other typing methods in publication I. In publication II, the *sp*a type highly predicted the PFGE result (W = 0.9009) (Table 12). In addition, PFGE had high probability of predicting *spa* CC (W = 0.9870).

Table 12. Wallace coefficients for typing methods among 124 MRSA blood isolates.

Typing Method	***spa***	***spa* CC**	**PFGE**
spa		1.000	0.9009
***spa* CC**	0.6975		0.7810
PFGE	0.7853	0.9870	

5.2.2 Short- and long-term correspondence between PFGE and *spa* typing methods (II)

The short- and long-term correspondence between PFGE and *spa* typing was studied in detail among FIN-4, FIN-16 and MRSA blood isolates in publication II. The short-term correspondence was high among FIN-4 and FIN-16 isolates, the majority of which in 2006 had only one *spa* type: t172 (81%) and t067 (93%), respectively. In addition, most of the FIN-4 and FIN-16 isolates belonged to one *spa* CC, namely *spa* CC 172 (94%) and *spa* CC 002 (98%), respectively. However, the correspondence between PFGE and *spa* typing among sporadic isolates (determined by PFGE) from 2006 was rather low. PFGE and *spa* typing also corresponded well in the long term (between 1997 and 2006) among FIN-4 and FIN-16 isolates. Among FIN-4 isolates, 92% had *spa* type t172 and 99% belonged to *spa* CC 172. Of the FIN-16 isolates, 80% had *spa* type t067 and 96% belonged to *spa* CC 002. The correspondence between PFGE and *spa* typing of the MRSA blood isolates varied between 1997 and 2006 (Table 9). FIN-4, -5, -7, -10, -11, -15, -16, -19 and -21 showed variation in *spa* types. However, within five of these PFGE types (FIN-4, -7, -11, -16 and -21), the *spa* types belonged to same *spa* CC. The correspondence between PFGE and *spa* typing was high among the two most prevalent MRSA blood isolates, FIN-16 and FIN-21. Of the FIN-16 isolates, 93% had *spa* type t067, and of the FIN-21 isolates, 86% had *spa* type t041. FIN-10 was the most variable PFGE type; of the eight FIN-10 isolates, six different *spa* types were detected.

5.2.3 Cost and time analysis (II)

Cost and time consumption were compared between PFGE and *spa* typing methods. The total time required to type 12 isolates using PFGE was 40 h, and the time for 24 isolates by *spa* typing was 9 h 15 min. For both methods, the hands-on time needed was 6 h 30 min. The material cost for PFGE was approximately €5/isolate and for *spa* typing approximately €9/isolate.

5.2.4 Nomenclature of MRSA in Finland (I, II)

The nomenclature changes for MRSA strains in Finland were based on changes in typing methods described in publications I and II. In March 2005, new MRSA nomenclature was gradually introduced (Figure 12). Based on the new PFGE interpretation criteria of EMRSA strains, the 26 PFGE clusters were assigned FIN names with a numeric code (e.g. FIN-16). The numeric code was given in chronological order starting from the strain isolated first. Within each cluster of PFGE types, those with a 3–6 band difference were indicated by letters after the FIN number (e.g. FIN-1b). The new complete MRSA name consisted of the FIN name (based on the PFGE type) combined with the MLST sequence type and SCC*mec* type [e.g. FIN-4 (375;IV)]. At the beginning of 2009, the nomenclature of MRSA strains changed again due to the replacement of PFGE with *spa* typing as the first-line typing method. The new MRSA strain name is based on the *spa* type (e.g. t172).

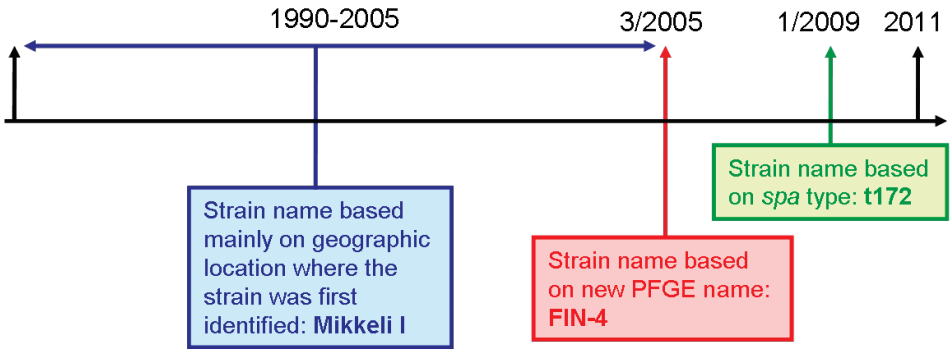

Figure 12. Changes in the nomenclature of EMRSA strains in Finland since the early 1990s in relation to this thesis research.

5.2.5 The current scheme for molecular typing of MRSA in Finland (II)

In Finland, clinical laboratories send MRSA isolates corresponding to the NIDR-reported MRSA cases to the Staphylococcal Reference Laboratory at THL for genotyping. The current typing scheme for MRSA was introduced at the beginning of 2009 and includes two typing categories: primary typing and additional typing. The MRSA typing scheme is presented in Figure 13. Primary typing is performed for all MRSA strains. Additional typing with different typing methods is carried out in specific instances. PFGE is performed if a new *spa* type is encountered, in Finland or according to SpaServer, in situations where the *spa* type is known to be associated with multiple PFGE types, or on occasions when the isolate is not typeable with *spa* typing. In addition, if a new *spa* type is encountered from at least five persons, additional typing such as MLST, SCC*mec* and PVL-PCR is performed. Moreover,

the presence of the PVL-encoding gene is defined from strains isolated from deep wounds, abscesses or puncture specimens.

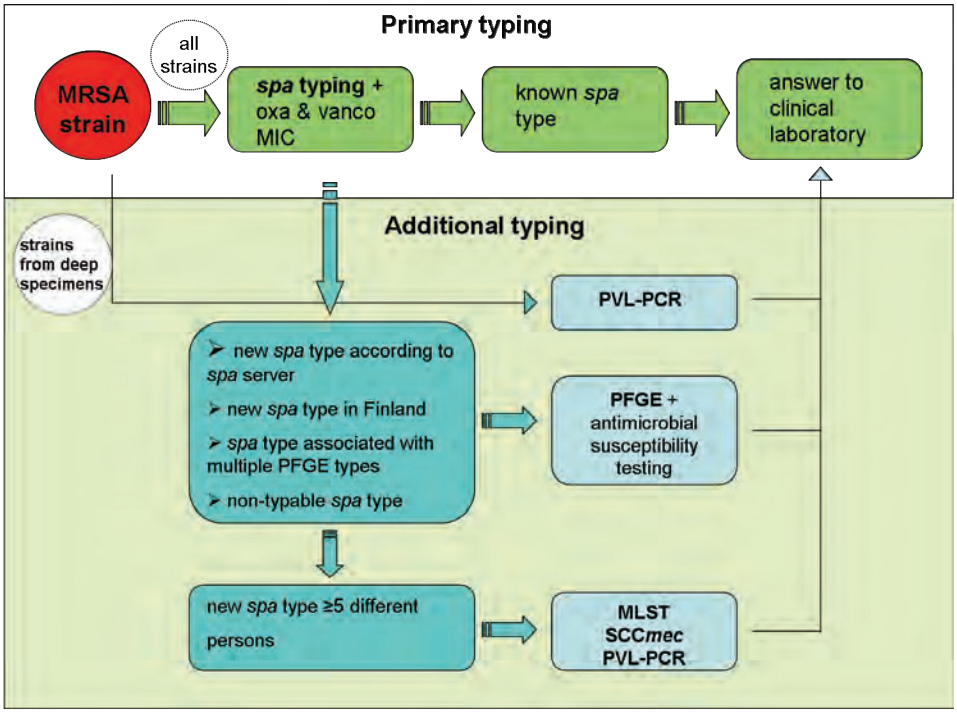

Figure 13. MRSA typing scheme in Finland from the beginning of 2009.

5.3 Molecular characteristics of *Streptococcus pneumoniae* strains and pneumococcal pneumonia outbreak investigation (III, IV)

5.3.1 Molecular characteristics of *S. pneumoniae* in community-acquired pneumonia and nasopharyngeal carriage (III)

The molecular characteristics of *S. pneumoniae* isolates detected from the blood and/or nasopharynx of military recruits were examined in detail in publication III. Of the 43 military recruits sharing the same housing during their military service, two had confirmed pneumococcal pneumonia defined as positive blood culture for *S. pneumoniae*. Both of the isolates were of serotype 7F, ST2331, CC2331, susceptible to penicillin and other commonly used antimicrobial agents. Neither of the isolates carried pilus islet 1 (PI-1).

Of the 43 military recruits, 18 (42%) carried *S. pneumoniae* in their nasopharynx. Nine (50%) of these isolates had the same serotype and genotype as the blood

isolates from the hospital recruits: 7F and ST2331. Other serotype-genotype combinations detected were: 9N-ST525 (n = 5, 28%), 23F-ST36 (n = 3; 17%) and 16-ST30 (n=1, 6%). All the nasopharyngeal isolates were susceptible to penicillin and other commonly used antimicrobial agents, and did not carry PI-1 in their genome.

5.3.2 Molecular characteristics of *S. pneumoniae* in acute otitis media and nasopharyngeal carriage (IV)

The molecular features of pneumococcal isolates found from MEF and/or NPA samples were defined by using various typing methods in publication IV. Among the 106 children with clinically diagnosed AOM, 56 (53%) children were found to be culture-positive for *S. pneumoniae*. These children were divided into three groups on the basis of the site of pneumococcal isolation: (1) MEF+/NPA+ (n = 19); (2) MEF+/NPA- (n = 3); and (3) MEF-/NPA+ (n = 34). All 75 pneumococcal isolates were susceptible to penicillin (MIC $\leq$ 0.06 µg/mL), one was non-susceptible to erythromycin (MIC $\leq$ 0.5 µg/mL) and two to tetracycline (MIC $\leq$ 4 µg/mL). Among these 75 isolates, 14 different serotypes were detected. The most prevalent serotypes were 19F (27%) and 23F (25%).

Of the 75 isolates, 15 (20%) were positive for PI-1 genes (*rrgC* and *rlrA*). PI-2 genes were not found in any of the isolates. PI-1 genes were detected among serotypes 6B (6/7 isolates), 6A (4/5 isolates), 9V (2/2) isolates, 23F (2/19 isolates), and 38 (1/1 isolate). Among MEF+/NPA+, 20% (8/41) of the pneumococcal isolates from four children contained PI-1 genes, and among MEF-/NPA+ the corresponding proportion was 21% (7/34). None of the three children with MEF+/NPA- had pneumococcal isolates with PI-1 genes.

Of the 75 isolates from 56 children, 52 isolates from 33 children were genotyped by MLST. To select the isolates for MLST, several criteria were used: 1) all isolates from children with MEF- and NPA-positive samples; 2) all PI-1-positive isolates; 3) one isolate for each serotype; 4) positive and negative PI-1 gene results of serotypes 6A, 6B, and 23F. Many of the isolates shared several of these criteria. The 52 isolates divided into 30 different STs and into 18 known CCs (Table 13). In addition, three STs had no predicted founder and two were singletons. The most prevalent CCs were CC439 (11/52; 21%) and CC490 (6/52; 12%). Nine of the 30 STs were related to known international pneumococcal clones (Table 13). Among MEF+/NPA+ children, the serotype and sequence type were the same in both samples.

PI-1 genes were found among six CCs: CC138, CC156, CC393, CC439, CC490 and CC522 (Figure 14). PI-1 genes were most prevalent in CC490 (6/6). PI-1 was

associated with genotype CC490 ($p = 0.002$) and with serotypes 6A ($P < 0.005$), 6B ($P = 0.0002$), and 9V ($P < 0.05$). PI-1 was not associated with the pneumococcal conjugate vaccine 7 (PCV7) or 10 (PCV10) serotypes compared to non-PCV7/PCV10 serotypes (PCV7 and PCV10, 10/56 vs. 5/19; $P = 0.51$). In addition, PI-1 was not associated with the PCV13 serotypes (14/56 vs. 1/19; $P = 0.10$).

Table 13. Molecular characteristics of the 52 pneumococcal isolates from 33 children.

CC[a]	ST[b]	Related international (PMEN) clone	PI-1 genes	Serotype carried by the child	Site of isolation	Age (mo)
15	15	SLV of England^{14}-ST9	-	19F	MEF+/NPA+[c]	24
43	43	none	-	19F	MEF+/NPA+	41
	526	none	-	19F	MEF+/NPA+	19
62	500	none	-	11A	MEF+/NPA-	41
66	2216	none	-	15A	MEF-/NPA+	10
72	72	none	-	24	MEF+/NPA+	10
100	100	none	-	6A	MEF-/NPA+	13
113	4126	SLV of Netherlands^{18C}-ST113	-	18C	MEF-/NPA+	13
124	124	Netherlands^{14}-ST124	-	14	MEF+/NPA+	72
	124	Netherlands^{14}-ST124	-	14	MEF+/NPA+	28
138	138	none	+	6B	MEF-/NPA+	50
	4594	none	+	6B	MEF-/NPA+	9
156	162	SLV of Spain ^{9V}-ST156	+	9V	MEF+/NPA+	52
180	180	Netherlands^{3}-ST180	-	3	MEF-/NPA+	23
199	199	Netherlands^{15B}-ST199	-	19F	MEF+/NPA+	11
393	310	None	+	38	MEF-/NPA+[d]	42
439	33	SLV of Tennesssee^{23F}-ST37	-	23F	MEF+/NPA+	9
	36	none	-	23F	MEF+/NPA-	39
	37	Tennesssee^{23F}-ST37	-	23F	MEF+/NPA+	50
	37	Tennesssee^{23F}-ST37	-	23F	MEF+/NPA+	9
	355	SLV of Tennesssee^{23F}-ST37	-	23F	MEF+/NPA+	20
	515	none	+	23F	MEF+/NPA+	33
460	460	none	-	6A	MEF-/NPA+	21
490	208	none	+	6B	MEF+/NPA+	41
	488	none	+	6A	MEF-/NPA+	32
	490	none	+	6A	MEF-/NPA+	32
	490	none	+	6A	MEF-/NPA+	50
	518	none	+	6A	MEF-/NPA+	36
522	522	none	+	6B	MEF+/NPA+	13
1523	1340	none	-	19F	MEF+/NPA+	53
NPF	482	none	-	19A	MEF+/NPA+	12
NPF	1752	none	-	6B	MEF+/NPA-	11
S	1069	none	-	19A	MEF+/NPA+	50

[a] CC named after the ST with the highest number of single-locus variants in the MLST database (November 2009). S, singleton; NPF, no predicted founder.
[b] New STs are underlined.
[c] non-susceptible to erythromycin
[d] non-susceptible to tetracycline

PMEN, Pneumococcal Molecular Epidemiology Network; SLV, single-locus variant

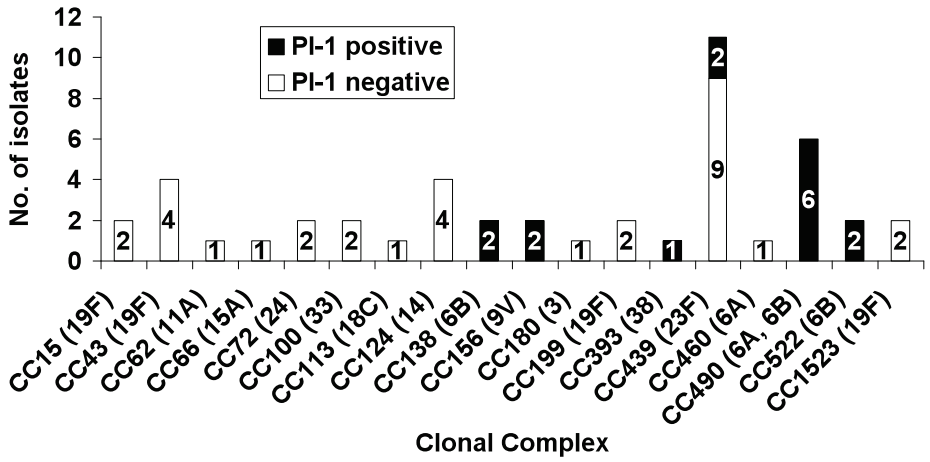

Figure 14. Clonal complex distribution of 47 study isolates and the prevalence of PI-1 among the CCs. Corresponding serotypes are presented in parentheses. In addition to known CCs, three isolates with no predicted founder and two singletons were detected.

5.3.3 Pneumococcal pneumonia outbreak investigation (III)

An outbreak investigation of pneumococcal pneumonia was conducted among military recruits participating in a one-week hard encampment. Five (12%) of the 43 military recruits were hospitalised with pneumonia. Two (5%) of 43 recruits were diagnosed as confirmed pneumonia cases and three (7%) as non–bacteraemic pneumonia cases. Pneumococcal isolates were cultured from the blood of confirmed pneumonia cases. Three of the five hospitalised recruits also had radiographically confirmed sinusitis (both confirmed pneumococcal pneumonia cases and one non-bacteraemic pneumonia case). Eighteen (42%) of the 43 recruits carried *S. pneumoniae* in their nasopharynx. *Staphylococcus aureus* was found from six nasopharyngeal cultures of the 43 recruits and β-haemolytic streptococci (groups G and C) from two nasopharyngeal cultures. None of the hospitalised recruits carried *S. pneumoniae* or *S. aureus* at the time the nasopharyngeal swab was taken for the point-prevalence study. Three of the 43 recruits were nasopharyngeally co-colonised by *S. aureus* and *S. pneumoniae*. In addition, group G streptococci were detected in one these co-colonised recruits. A group C streptococcus was isolated in the nasopharynx of one of the non-bacteraemic pneumonia cases.

Acute-phase sera of four of the five hospitalised recruits tested negative for viral and *M. pneumoniae* antibodies, and urine samples were negative for *L. pneumophila*.

All recruits were previously healthy young men with a median age of 20 years (range 19 to 21) without any continuous medications or underlying chronic diseases.
In order to study the preceding symptoms of the 43 recruits, they were divided into three different groups: hospitalised recruits (n = 5), pneumococcal carriers (n = 18) and non-pneumococcal carriers (n = 20). Symptoms that were asked about were fever, rhinitis, sore throat and cough. Fever (5/5, 100%) and cough (4/5, 80%) were the predominant preceding symptoms among the hospitalised recruits, and rhinitis was a common preceding symptom in all three groups (Table 14). Of the 43 recruits, 35 (81%) had some respiratory symptoms and 8 (19%) did not have any respiratory symptoms. Three of the 18 pneumococcal carriers had no preceding symptoms. The onset of respiratory symptoms, the dates of encampment and hospital stays are represented in an epicurve (Figure 15).

Table 14. The preceding respiratory symptoms of 43 military recruits.

Respiratory symptoms	Hospitalised recruits	Pneumococcal carriers	Non pneumococcal carriers	Total
Fever	5/5 (100%)	7/18 (39%)	5/20 (25 %)	17/43 (40 %)
Rhinitis	3/5 (60 %)	13/18 (72 %)	13/20 (65 %)	29/43 (67 %)
Sore throat	2/5 (40 %)	5/18 (28 %)	8/20 (40 %)	15/43 (35 %)
Cough	4/5 (80 %)	8/18 (44 %)	4/20 (20 %)	16/43 (37 %)
None	0/5 (0 %)	3/18 (17 %)	5/20 (25 %)	8/43 (19 %)

Data on smoking habits was obtained from 42 of the 43 recruits. Twenty-three (55%) of the 42 recruits were active smokers, and 12 (52%) of the 23 smoking recruits were pneumococcal carriers. Cigarette smoking was not seen to be a significant risk factor for overall pneumococcal carriage, nor for confirmed or non-bacteraemic pneumonia.

All the hospitalised recruits were treated with antimicrobial agents. In addition, two of the non-hospitalised recruits were treated for their symptoms as out-patients with antimicrobial agents before the encampment and four during or after the encampment, but none due to pneumonia. The usage of antimicrobial agents during the previous month was not associated with pneumococcal carriage (RR, 0.4; 95% CI, 0.11–1.50; P = 0.11).

All the recruits with confirmed and non-bacteraemic pneumonia fully recovered and no more pneumonia cases were detected.

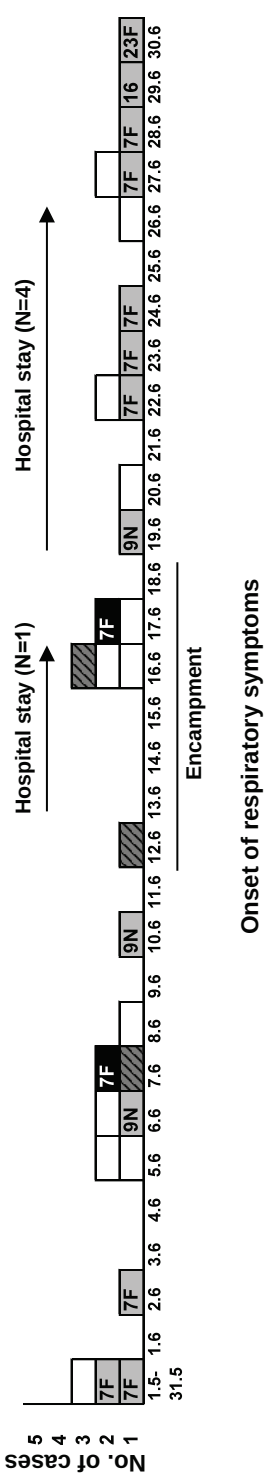

Figure 15. Epicurve of the onset of respiratory symptom in 30 military recruits between 1.5–30.6.2006. Each box indicates one case. Serotypes of pneumococcal isolates cultured from the nasopharynx and blood are marked in the box. Black box, confirmed pneumococcal pneumonia cases; dark grey striped box, non-bacteraemic pneumonia cases; grey box, pneumococcal carriers with respiratory symptoms; white box, non-carriers with respiratory symptoms. In addition to cases marked in the epicurve, 8/43 recruits had no respiratory symptoms (3 pneumococcal carriers, 5 non-pneumococcal carriers) and 5/43 recruits had respiratory symptoms but the onset day was lacking (2 pneumococcal carriers, 3 non-pneumococcal carriers). The blood samples of the hospitalised recruits were taken on 19.6. and the nasopharyngeal swab specimens from all the recruits were taken on 29.6.2006 and 5.7.2006.

6 DISCUSSION

6.1 *Staphylococcus aureus*

6.1.1 Molecular characteristics and clonality of MRSA

Most of the EMRSA strains, isolated during the years 1991–2004, belonged to MLST CC8, which can be divided into five STs, two *spa* CCs and eight PFGE clusters. MLST CC8 included several internationally distributed clones such as Iberian (ST247-I), Brazilian (ST239-IIIA), Hungarian (ST239-III) and USA300 (ST8-IV) clones (356, 398). The PVL-positive USA300 clones have been associated with CA-MRSA infections in several European countries and in USA (84, 98, 139). MLST CC5 included two different STs: ST5 and ST125. These STs are related to New York/Japan (ST5-II) and paediatric clones (ST5-IVA), which are well-known HA-MRSA-associated pandemic clones (81, 398). Two major HA-MRSA clones, UK EMRSA-15 and -16, were also identified in Finland. These clones were first identified in the UK at the beginning of the 1990s and are now distributed worldwide (175). Thus, many international clones have been identified in Finland. In addition, according to the *S. aureus* MLST database, most of the Finnish MRSA strains belong to the major clonal complexes (238). However, some of the Finnish EMRSA strains were isolated from local outbreaks with sporadic PFGE profiles and rare MLST results. FIN-22 (ST27-V), which caused an outbreak in a long-term care facility in northern Finland in 2003, is one example (185).

More than half of the Finnish EMRSA strains were multiresistant. In particular, SCC*mec* types II and III cause additional non-β-lactam antimicrobial resistance to multiple classes of antimicrobials, due to the additional drug resistance genes integrated into SCC*mec*, i.e. integrated plasmids and two transposons (81). Moreover, *S. aureus* can also harbour resistance genes on other sites of the genome, as well as on plasmids (216). In this study, the antimicrobial susceptibility profiles mainly corresponded with the assumed resistance characteristics of the SCC*mec* complex of the strain. However, disconcordance between the antibiogram and SCC*mec* type was noted within three PFGE clusters, which could result from additional resistance genes found elsewhere in the genome. Unfortunately, the antibiograms were not included in publication II due to technical reasons.

Only two of the EMRSA strains, FIN-11 (ST80-IV) and FIN-25 (ST8-IV), were positive for PVL encoding genes. ST80 is a widely disseminated PVL-positive ST in European countries, and ST8 in the United States (USA300) (85, 381). Both of these strain types have been associated with CA-MRSA worldwide. The PVL-positive

FIN-11 (ST80-IV) strain has also been verified to have community acquisition in Finland (181, 182, 184, 311). In addition, the presence of PVL encoding genes varied in some of the strains, such as in FIN-19 (ST1-IV) and FIN-5 (ST30-IV). These strains are related to two PVL-positive CA-MRSA clones, USA400 (ST1-IV) and Southwest Pacific (USA1100) (ST30-IV), which are disseminated worldwide (81). In accordance with this study, PVL-positive and -negative strains have been identified among the USA400 strains (266). SCC*mec* types I, II, III and rarely IV are typically considered as markers for HA-MRSA (81). In contrast to HA-MRSA, the majority of CA-MRSA isolates tend not to be multiresistant, and SCC*mec* types IV, V and VII, as well as PVL, are typically present (53, 81). However, the distribution of these markers among CA-MRSA and HA-MRSA is not complete. The presence of PVL among CA-MRSA is more variable, and in Denmark, for example, the prevalence of PVL ranged from 17% to 100% (208). In addition, PVL has also been detected in the hospital environment (200). During recent years, the distinction between HA-MRSA and CA-MRSA has started to disappear. CA-MRSA strains such as USA300 can also cause infections in hospitals, and clones traditionally linked to hospitals, such as EMRSA-15, can cause infections in the community (81, 200, 260).

EMRSA strains FIN-4 and FIN-16 were investigated in more detail in publication II. The study revealed that FIN-4 isolates as well as FIN-16 isolates were genetically closely related analysed by *spa* typing. In addition, both FIN-types belonged to only one MLST CC, CC59 (FIN-4) and CC5 (FIN-16). Moreover, SCC*mec* IV was predominant among FIN-4 isolates, whereas variation in SCC*mec* types was noted among FIN-16 isolates. One small cluster was recognised among the FIN-4 isolates with *spa* type t3627 (closely related to t172) in 2006. The strains were isolated from the same healthcare centre during June and July, and were also found in 2002 and 2003 from the same healthcare district. FIN-4 isolates comprised 10% of all MRSA isolates between 2004 and 2006 in Finland. In addition, FIN-4 isolates are known to have community acquisition and are found throughout Finland (181). According to the Ridom SpaServer, *spa* type t172 has been identified in several European countries, but to the best of our knowledge, it does not belong to the most prevalent type of strains in clinical settings outside the Nordic countries (326). Recently, MRSA isolates with *spa* type t172 were detected as the most commonly found MRSA in municipal wastewater in Sweden. These isolates probably originated from human carriage, because the genotypes of the isolates corresponded to those in clinical settings (42). Outbreaks caused by FIN-16 seem to be responsible for the increase in reported MRSA cases observed from 1997 to 2004 in Finland (184). In addition, between 2004 and 2006, FIN-16 comprised nearly one-third of all MRSA isolates in Finland (310). FIN-16 is related to the highly prevalent Spanish clone ST5-IV with *spa* type t067 (388). Several Spanish studies have reported that this clone is associated to HA-MRSA infections in Spain (10, 274, 388). *spa* type t067

has also been sporadically identified from Sweden and countries of Central Europe, such as France and Germany (130, 231, 241, 277). A German study reported one occasion where the Spanish clone was found from a patient who was repatriated from the Canary Islands (241). In addition, according to Ridom SpaServer, *spa* type t067 isolates have been identified in several European countries and in the US (326). According to recent MRSA data from Finland, t067 and t172 were the two most prevalent *spa* types in 2009 and 2010 (358, 360). *spa* types t067 and t172 respectively comprised 28% and 14% of cultured and confirmed MRSA cases in 2010. In 2010, *spa* type t172 was found from 16 hospital districts and *spa* type t067 from 9 hospital districts, most commonly in the Pirkanmaa hospital district (360).

In Finland, 124 genetically diverse EMRSA blood isolates were detected between 1997 and 2006. The most common *spa* types were t067 and t041. FIN-16 (t067) and FIN-21 (t041), internationally distributed and multi-drug-resistant EMRSA strains, were related to the increase in reported MRSA cases detected from 1997 to 2004 (184). According to a recent study of European invasive *S. aureus* covering the years 2006 and 2007, *spa* type t041 was also found to be frequent among invasive MRSA isolates (130). The study additionally demonstrated that *spa* type t041 was the most or the second most common invasive MRSA finding in Italy, Slovenia, Croatia, Austria, and Hungary. On the other hand, *spa* type t067 was the sixth most frequent finding among the study isolates and was the predominant finding in Spain (130). In 2010, fifteen MRSA isolates were cultured from blood in Finland. Six of these had *spa* type t067, and the rest represented eight different *spa* types (360). However, none of the *spa* types were t041 (unpublished data).

6.2 *Streptococcus pneumoniae*

6.2.1 Molecular characteristics of *S. pneumoniae*

Pneumococcal serotype 7F was the most prevalent pneumococcal serotype detected among military recruits. According to Finnish surveillance data on invasive pneumococci, serotypes 7F, 9N, and 23F have been among the 10 most prevalent serotypes causing invasive diseases for several years. To date, three invasive isolates of ST2331 with 7F/7 have been reported to the international MLST database (238). In addition, four isolates with ST2331 were found among adult patients with community-acquired pneumonia in Japan (163). Although serotype 7F has been associated with decreased risk of death due to IPD and is rarely found among healthy carriers, it has a high invasive disease potential (336, 383). PI-1 genes have been shown to be absent from serotype 7F, but the prevalence of PI-2 has been shown to be 89% among serotype 7F (2, 406). Unfortunately, PI-2 genes were not studied from the isolates of our material.

The pneumococcal serotypes isolated from children with clinically diagnosed AOM were the common ones known to be associated with AOM infections among children (101). The 10-valent pneumococcal conjugate vaccine (PCV10) would have covered 75% of the serotypes detected. This is of special interest, because in Finland the large-scale use of PCV10 began in September 2010, and consequences of the vaccination will be seen in the near future. Several studies have showed an increasing trend of *S. pneumoniae* infections caused by non-PCV7 serotypes after introducing the PCV7 (45, 150, 333).

The MLST STs of the MEF and NPA isolates showed great heterogeneity. Nine of the 30 MLST STs were related to international clones [Pneumococcal Molecular Epidemiology Network (PMEN clones)] (283). Two of these, ST156/CC156 and ST199, have also been observed among isolates from MEF of children with AOM in previous studies (242, 397).

In the early 1990s, the overall antimicrobial resistance of *S. pneumoniae* was very low in Finland (223). The study isolates from MEF and NPA were collected in 1990–1992, and all the isolates were susceptible to penicillin and almost all to other commonly used antimicrobials. However, the antimicrobial resistance of *S. pneumoniae* has also increased in Finland, and between 2002 and 2006 the penicillin non-susceptibility of invasive pneumococcal isolates increased from 8% to 16% and erythromycin non-susceptibility from 16% to 28% (331).

So far, the prevalence of pneumococcal PI-1 genes has mainly been studied among invasive isolates. According to previous studies, the prevalence of PI-1 among pneumococcal isolates cultured from invasive and community-acquired pneumonia diseases has been around 30% (2, 163, 243). The frequency of PI-1 among the MEF isolates has been shown to be the same 30% (242). In the US, the prevalence of PI-1 among nasopharyngeal isolates was shown to decrease from 24% to 15% after the introduction of PCV7. However, in 2007 it increased to pre-PCV7 level (292). In 2008, Bagnoli et al. described a second pilus type, referred as PI-2, in *S. pneumoniae* (13). The prevalence of PI-2 has been shown to be 16% for both invasive and nasopharyngeal isolates, and 7% for the MEF isolates (13, 242). In the US, the prevalence of PI-2 was shown to increase among invasive isolates from 3.6% in 1999 to 21% in 2006, after introducing the PCV7 (in 2000) (406). This study demonstrated that only 20% of the MEF and/or NPA isolates were positive for PI-1 and none for PI-2.

Previous reports have demonstrated a positive correlation between PI-1 and antimicrobial resistance (2, 242, 243). Thus, the lower prevalence of PI-1 in our material, compared to other studies could be due to low antimicrobial resistance in

the period in which the isolates were collected. PI-1 has been found to be associated with the PCV7 vaccine serotypes 4, 6B, 9V, 14, and 19F, whereas PI-2 was mainly found from non-PCV7 serotypes (1, 7F 11A, 19A), except for the serotype 19F (2, 18, 406). However, the presence of PI-1 was not associated with the PCV7 serotypes compared to the non-PCV7 serotypes in this study.

Several investigations have demonstrated that the presence of PI-1 and PI-2 appears to be a clonal property rather than serotype-associated (2, 13, 18, 242, 243, 292, 406). In contrast to these reports, in this study PI-1 associated more commonly with certain serotypes than genotypes. On the other hand, no STs or CCs that have been reported as PI-2 positive were found, which may explain the absence of PI-2. The lack of PI-2 could be also due to the small number of isolates. The low antimicrobial resistance of the isolates hardly explains the missing of PI-2, because Zähner at al. have reported that antimicrobial resistance is not a consistent property of PI-2-positive isolates (406).

PI-1 is not considered as a recently acquired feature of *S. pneumoniae*, but has existed among pneumococcal isolates prior to pneumococcal conjugate vaccine and the increase in antimicrobial resistance (292). This was also demonstrated in our study. Recently, Zähner et al. reported that PI-2 was also present among invasive pneumococcal isolates prior to pneumococcal conjugate vaccine, although clearly less than in 2006 (406). However, the recent increasing prevalence of PI-1 and PI-2 among pneumococcal isolates could be due to an intrinsic selective advantage of the pili, or to their association with certain genotypes carrying some other advantageous feature, such as antimicrobial resistance (292).

6.2.2 Pneumococcal pneumonia outbreak investigation

Streptococcus pneumoniae is a common cause of community-acquired bacterial pneumonia, and it can cause outbreaks particularly in crowded settings such as in nursing homes (121). *S. pneumoniae* is reported to be the leading cause of pneumonia hospitalisation among military forces in the US (7, 70, 126, 127, 317).

According to previous reports from Finland, the pneumococcal carriage among healthy military recruits has been reported to be low, ranging from 1% to 7% (178, 180, 400). However, the pneumococcal carriage rate among healthy men has been reported to be up to 10 times higher during military service than at the time of starting service (180). In this study, the pneumococcal carriage among recruits during the outbreak was remarkably high (42%). In a recent study from Israel, the pneumococcal carriage rate among healthy recruits during the pneumococcal

outbreak was also reported to be very high (44%). In that study setting, the carriage rate dropped to <1% after the vaccination and antimicrobial intervention (15).

There are several risk factors for military recruits to acquire respiratory infections, including pneumococcal diseases: physical and psychological stress, which may lead to immune depression, lack of sleep, crowded living conditions, and the mixing of people from diverse geographical locations (58, 59). This study demonstrated that the military recruits had to tolerate hard physical stress with temporarily poor nutrition along with dehydration in the forest encampment, which may have lowered their immune defence system and increased the risk of respiratory infections (120). The crowded living conditions may also have facilitated the transmission of pneumococci among the recruits and increased the nasal carriage rate (159). Although the rather high CRP values along with the white blood cell counts at the admission of recruits with hospitalised pneumonia clearly indicated the bacterial aetiology of the disease, the preceding viral respiratory infection may have increased the susceptibility to pneumococcal infection (187, 228, 378). However, no clear microbiological evidence of viral co-infection in the hospitalised recruits was detected, although the recruits complained of preceding respiratory symptoms. Unfortunately, the possible viral aetiology could not be confirmed because of the lack of convalescent sera results and since no NPA samples were taken at the time of preceding respiratory symptoms. Some of the recruits had respiratory symptoms before the encampment, although most of the recruits had their onset of respiratory symptom during or after the encampment. Recruits with respiratory symptoms may have transmitted pneumococci and/or possible viruses to other recruits during the encampment under strenuous conditions.

In general, cigarette smoking (active and passive) is one of the well-known risk factors predisposing subjects to nasopharyngeal colonisation by respiratory pathogens and for certain respiratory tract infections (128, 347). It has also been proposed that cigarette smoking is the strongest independent risk factor for invasive pneumococcal disease among immunocompetent, nonelderly adults (259). In addition, it has been demonstrated that the carriage rates of *S. pneumoniae* are significantly higher among cigarette smoking recruits than non-smoking recruits (179). However, in this study cigarette smoking was not a significant risk factor for overall pneumococcal carriage, nor for confirmed or non-bacteraemic pneumonia. This could be due to the limited sample size. Although recent exposure to the antibiotics seems to be a risk factor for pneumococcal colonisation and invasive disease due to antibiotic-induced changes in the nasopharyngeal flora (114, 378), the use of antibiotics during the previous month was not associated with pneumococcal carriage in this study.

Community-acquired pneumococcal pneumonia outbreaks can be halted using antibiotic prophylaxis, pneumococcal vaccination, a combination of these two measures or without any intervention (15, 70, 75, 234, 317). No intervention for recruits was used to control this outbreak, because no additional pneumonia cases were detected.

A key question of the study is why pneumococci with the same serotype and genotype caused life-threatening invasive disease in only a minority of the previously healthy military recruits, while others remained asymptomatic carriers for the same strain. This might be related to human genetic susceptibility. Genetic variation in immune response genes is associated with susceptibility to and the severity of infectious diseases (38, 62). Increasing numbers of studies have focused on finding genes related to the susceptibility to pneumococcal diseases. The genes encoding molecules involved in the immune response, such as Toll-like receptors, mannose-binding lectin and cytokines, are of special interest (38). Very rare mutations and common polymorphisms have previously been described in association with invasive pneumococcal disease (38, 278).

6.3 Bacterial typing

6.3.1 Molecular typing in national epidemiology and laboratory-based surveillance of MRSA in Finland

Along with the other Nordic countries, Finland has been a low-incidence MRSA country for long time. However, within last ten years, both the incidence and the diversity of circulating MRSA strains have increased (184, 359). At the same time, MRSA typing has become more challenging. An accurate and straightforward typing method with high discriminatory power is needed to enable comprehensive nationwide laboratory-based MRSA surveillance, to detect local outbreaks and to perform international comparisons. In addition, the typing method should be rapid, relatively inexpensive and easy to perform. In Finland, the nomenclature of EMRSA strains has been influenced by the typing methods used at a given time. In the 1990s, the typing of MRSA was based on phage typing, antibiograms, ribotyping and PFGE. However, by the turn of the century, PFGE was used as the primary typing method for EMRSA identification. From the early 1990s to 2005, EMRSAs were named according to the geographical location where they were first identified. However, the naming was changed in spring 2005 to FIN names with a numeric code based on PFGE results. This change was made to avoid stigmatising geographical locations. In this study, *spa* typing was validated as the first-line MRSA typing tool from the beginning of 2009. Since then, the nomenclature has been based on *spa* typing results. *spa* typing has fulfilled the requirements for nationwide MRSA surveillance. In addition, it is useful in both outbreak investigations and international comparisons.

However, additional typing methods such as PFGE, MLST and SCC*mec* typing are still needed for certain circumstances. Other previous studies have come to a same conclusion (231, 277, 388).

6.3.2 PFGE versus *spa* typing

Since the mid-1990s, PFGE has been the gold-standard method for distinguishing different MRSA strains in order to monitor their spread (247, 343). However, PFGE is a technically demanding and a time-consuming method. In addition, its interpretation leaves room for subjectivity, and the comparison of results between laboratories remains difficult, despite the standardised protocols and interpretation criteria (247, 353). Moreover, PFGE was originally developed for outbreak investigations and its high discriminatory power can mislead the interpretation in long-term epidemiological investigations (32, 351, 353).

In recent years, sequence-based *spa* typing has been shown to be reliable tool for typing MRSA isolates. Compared to PFGE, *spa* typing is more rapid, easier to use, and data interpretation is unambiguous. In addition, the data are easily exportable and used for international comparisons (3, 136, 329). Although *spa* typing has recently become a prime alternative to PFGE for typing *S. aureus* strains, in some instances the discriminatory power of *spa* typing is not as high that of PFGE, and it is not sufficient to discriminate outbreak strains (231, 277, 388). In addition, *spa* typing is based on only a single locus in the genome, and clustering of *spa* typing data can be complicated (322). The BURP algorithm is used to group *spa* types, and previous studies have shown that the concordance between *spa* groups (*spa* CC) and alternative methods is high (135, 343). However, 'group violations' associated with certain BURP groups and clonal lineages have been demonstrated (135, 342). These differences are possibly caused by recombination events in the *spa* locus, and the results from the BURP grouping method must therefore be interpreted with caution (135, 295).

spa typing was more rapid to perform, but more expensive compared to PFGE. Other studies have shown similar results (55, 122, 322, 341). However, the time required for PFGE and *spa* typing depends, though not directly, on the number of isolates that are processed at a time. In this study, due to practical reasons, the number of isolates processed at a time was 24 in *spa* typing, but only 12 in PFGE. In addition, the measurements demonstrated that the time required for the interpretation of PFGE patterns varied considerably.

The typeability of PFGE among MRSA isolates was 100%, and only three isolates were non-typeable by *spa* typing (99.5%). The typeability of PFGE among human MRSA isolates has also been virtually 100% in other studies (80, 105, 231). The

typeability of *spa* typing has additionally been shown to be mainly 100%, although Faria et al. demonstrated a lower typeability (98.3%) (80, 105, 231). Non-typeability by *spa* typing might be a consequence of mutations in the flanking conserved regions of the X region, which is used for primer design, leading to amplification problems (197). Although the typeability of PFGE has been 100% among human MRSA strains, certain livestock-associated MRSA strains (ST398) are not typeable by this method (20). However, other restriction enzymes than *Sma*I can be used to differentiate livestock-associated MRSA strains by PFGE (290). Strain identification is crucial before bacterial typing to ensure accurate typing results. Bacterial identification must especially be verified in cases of non-typeable typing results.

PFGE and *spa* typing both had good discriminatory power (DI values). PFGE had a higher discriminatory power than *spa* typing in publication I. However, this result was biased, because the data were already previously recognised and selected for this study. When the blood isolates of MRSA strains were typed, *spa* typing had a higher DI than PFGE, although the discriminatory power of *spa* typing, evaluated in other studies, was found to be similar or somewhat lower compared to PFGE (135, 197, 277, 343). However, PFGE results were not interpreted at the subtype level, which affected the DI of this method. Although the two methods measure different markers of genetic variation of *S. aureus*, they both have a good ability to discriminate isolates. *spa* typing targets the polymorphic repeat region of the *spa* gene, whereas PFGE targets the whole genome of *S. aureus*. Epidemiological interpretations of related PFGE patterns depend on the temporal scale of the study (32). In addition, the mutation rate of the given bacterial clone affects the pattern stability of PFGE (32, 351). Although the *spa* region has been shown to be rather stable, the relationship between variability in the *spa* region and the overall evolution of the *S. aureus* genome is not yet fully understood, and it may vary between *S. aureus* clones (111, 197, 329).

Besides a high discriminatory power, *spa* typing has been shown to be in good concordance with PFGE at the type level or between clusters (105, 135, 197, 343). This was also the case this study, in which the results were strongly correlated between PFGE (at the cluster level) and *spa* typing, and between PFGE (at the cluster level) and *spa* CC analysed by Ridom software. However, the concordance between these methods was lower when analysed by AR, especially among the 44 EMRSA strains studied. Faria et al. reported that the concordance measure, Rand's coefficient, used in Ridom StaphType software leads to overestimation of the agreement between two typing methods and should be avoided (105, 297). The AR coefficient corrects Rand's coefficient and is more suitable for concordance analysis. This work, as well as previous studies, has shown low AR values for typing methods of staphylococci (105, 236). A clear difference was noted in concordances calculated with AR between publication I and II. This could be explained by the difference

between the two data sets used. In publication 44, representatives of all Finnish EMRSA strain types were used. These strains were already known to be different based on old strict PFGE interpretation criteria, whereas publication II covered heterogeneous MRSA blood isolates (n = 124) between 1997–2006.

The short- and long-term correspondence between PFGE and *spa* typing was high among FIN-4 and FIN-16 isolates. However, the long-term correspondence between PFGE and *spa* typing varied among the MRSA blood isolates. Among the sporadic isolates, the short-term correspondence between the two methods was rather low. Although many FIN-types showed variation in *spa* types among MRSA blood isolates, the correspondence between PFGE and *spa* CC was high. In addition, FIN-16 and FIN-21, which were the most prevalent *spa* types among MRSA blood isolates, showed high correspondence between PFGE and *spa* typing.

PFGE types and clusters could convincingly predict *spa* CC calculated by the Wallace coefficient. In addition, *spa* types were highly predictive of the PFGE type in publication II. However, in publication I the *spa* types had only a 57% probability of predicting PFGE clusters. Similarly, Faria et al. reported that the predictive power between PFGE (at type and subtype level) and *spa* type was low among MRSA isolates (105). They also found that the PFGE type and PFGE subtype highly predicted the *spa* CC.

Overall, in publications I and II the interpretation of the PFGE band profiles was challenging due to the long time frame of the studies. For publication II, PFGE typing was performed as part of earlier national MRSA surveillance and PFGE profiles were used again for methodology comparisons. The genetic changes in the bacterial genome within the bacterial population during long time frame and the high discriminatory power of PFGE may have misled the interpretation.

The use of a different PFGE nomenclature system between publication I and II may cause confusion. For clarification, in publication I, PFGE profiles with a 3–6 band difference were indicated by letters after the FIN number, whereas in publication II subtypes were not assigned. Therefore, the ‘PFGE type’ in publication II corresponds with the ‘PFGE cluster’ in publication I. However, the interpretation criteria used for analysing the PFGE profiles were the same in both studies.

Recently, MLVA was validated for *S. aureus*. MLVA has been shown to be an efficient, relatively inexpensive and highly discriminatory genotyping method for *S. aureus* strains (322). In addition, it is reported that there is congruence between MLVA, *spa* typing and PFGE (322). Moreover, MLVA would be better method for livestock-associated MRSA, since ST389 strains, also detected in Finland, are non-typeable by PFGE (Sma I digestion) (20, 73, 312, 377). A recent study revealed that

MLVA could be used to discriminate LA-MRSA ST398, although PFGE was more discriminatory than MLVA. MLVA would also be a useful tool for MRSA typing in Finland.

6.3.3 Characteristics of MLST and SCC*mec* typing methods

MLST showed a high typeability and discriminatory power (94%), whereas SCC*mec* typing clearly had a lower typeability and discriminatory power (64%) compared to all the other typing methods used. The discriminatory power of MLST STs among *S. aureus* isolates has also been observed to be high (over 90%) in several other studies (61, 105, 343). The high discriminatory power of MLST is somewhat surprising, because MLST is generally considered to have only moderate discriminatory power due to the low mutation rate of the seven housekeeping genes (332, 346). Because of this, MLST is considered to be especially useful for long-term epidemiological studies and in investigating the population structure and evolution of pathogens (96, 221). One explanation for the high discriminatory power of MLST in the reference studies for methicillin-sensitive and -resistant *S. aureus* is the diversity of strain collections used. The strains selected for our study and the three other studies were highly diverse (61, 105, 343).

In general, SCC*mec* typing is a low discriminatory technique due to the restricted number of variants generated by the method. The low discriminatory power of SCC*mec* typing detected in our study was a consequence of only five different SCC*mec* types being used, because the SCC*mec* subtypes were not included in the DI calculations. Faria et al. detected a higher discriminatory power for SCC*mec* typing among MRSA isolates, but they also included the subtypes in the DI calculations (105). The concordance between SCC*mec* typing and other methods was low. SCC*mec* typing is based on analysing a mobile genetic element, in contrast to PFGE, MLST and *spa* typing methods, which analyse more stable parts of the genome. In addition, SCC*mec* typing is used in epidemiological studies to analyse MRSA strain transmission and evolution (346). Variation in SCC*mec* types within the same PFGE cluster, MLST CC and *spa* CC was observed in publication I and among FIN-16 PFGE types in publication II. This could be a consequence of different ancestors or the susceptibility of staphylococcal isolates to receiving, recombining and/or replacing different SCC*mec* elements (403).

7 CONCLUSIONS

In this thesis, methicillin-resistant *Staphylococcus aureus* and *Streptococcus pneumoniae* were investigated in detail by using several molecular typing methods for various epidemiological purposes: to study the characteristics and clonality of EMRSA, to evaluate and compare typing methods for national laboratory-based surveillance of MRSA, to study the outbreak and carriage of *S. pneumoniae* in a crowded community setting, and to elucidate the association of pili with the disease potential of *S. pneumoniae* in children with AOM infection.

Among Finnish EMRSA strains during 1991–2004, CC8 was the most prevalent MLST CC, and most of the strains belonged to *spa* CC 051/008. SCC*mec* type IV was predominant and over half of the strains were multiresistant to antimicrobials. Only two of the EMRSA strains were positive for PVL encoding genes. Among two EMRSA strains, PVL-positive and -negative isolates were detected. Several international CA-MRSA and HA-MRSA clones were recognised in Finland. MRSA FIN-4 isolates as well as FIN-16 isolates were genetically closely related analysed by *spa* typing. In addition, both FIN-types belonged to only one MLST CC. Overall, MRSA blood isolates from 1997 to 2006 were genetically diverse. However, *spa* type t067 was the predominant *spa* type.

The discriminatory power of *spa* typing, PFGE and MLST was high. The overall concordance values of the typing methods differed when assessed by two different calculation methods. The adjusted Rand coefficient showed clearly lower correlations for all comparisons. However, both methods agreed that the concordance was the highest between PFGE clusters and MLST. *spa* typing had a high probability to predict PFGE clusters, MLST ST and CC, and SCC*mec* types, depending on the study material. *spa* typing recognised internationally successful strains to also be common in Finland, and was found to be more expensive but approximately four times faster to perform than PFGE.

An inexpensive, rapid, discriminatory and accurate typing method is required for national surveillance and outbreak identification, and to perform international comparisons. According to these results, *spa* typing was found to qualify as the primary typing method for laboratory-based MRSA surveillance in countries with a long history of PFGE-based strain nomenclature, and was implemented in Finland as a first-line typing tool from the beginning of 2009. Simultaneously, the nomenclature of MRSA was changed to be based on *spa* typing results. However, additional typing methods are still needed in certain situations to provide adequate discrimination or to characterise isolates with a newly recognised *spa* type in

Finland. In 2009, additional typing by PFGE was needed for approximately one fifth of the isolates in Finland.

Streptococcus pneumoniae serotype 7F and genotype ST2331 was associated with an outbreak of pneumonia and nasopharyngeal carriage among military recruits. Five of the recruits were hospitalised with pneumonia and two of them had positive blood cultures for *S. pneumoniae*. An exceptionally high pneumococcal carriage rate was detected among the recruits. The outbreak strain covered 55% of all the pneumococcal findings. Although the viral aetiology of the recruits remained unclear, a possible viral respiratory infection along with the hard physical stress and crowded living conditions may have exposed the recruits to this outbreak. However, cigarette smoking was not found to be a significant risk factor for overall pneumococcal carriage, nor for confirmed or non-bacteraemic pneumonia. Moreover, previous antimicrobial use was not seen to be associated with pneumococcal carriage.

All the pneumococcal isolates cultured from MEF or NPA of the children with AOM were susceptible to penicillin. The pneumococcal serotypes detected among these isolates were the common ones known to cause AOM infection among children. High heterogeneity was observed among these strains analysed by MLST. The pneumococcal AOM isolates detected in the time prior to pneumococcal conjugate vaccine and the increase in antimicrobial resistance were found to carry PI-1 genes at a rather low prevalence, and PI-2 genes were not found at all. PI-1 was found to associate with the serotype rather than the genotype. This low prevalence of PI-1 compared to other studies could be due to low antimicrobial resistance at the time when the isolates were collected. Based on this thesis, the association of pili with the disease potential of *S. pneumoniae* in AOM infections remains unsolved.

Comparing *Staphylococcus aureus* and *Streptococcus pneumoniae* in general, several similarities and differences can be observed between these bacteria. Both are Gram-positive cocci-shaped bacteria and can cause mild to severe infections in humans. In addition, both can colonise the same ecological niche, the nasopharynx. However, the most common diseases caused by these bacteria are different. *S. aureus* commonly infects the skin and subcutaneous tissue, whereas *S. pneumoniae* is a common cause of mucosal infections such as sinusitis and AOM. From the various invasive infections they can cause, pneumonia and bacteraemia are common to both. In 2010, *S. aureus* was the second and *S. pneumoniae* the fourth most common blood culture finding in Finland. The antimicrobial resistance of these bacteria has become a major public health issue. Currently, polysaccharide and conjugate vaccines are available to prevent infections with several *S. pneumoniae* serotypes, but there are no licensed prophylactic vaccines for human use for the prevention of *S. aureus* disease. Both *S. aureus* and *S. pneumoniae* have extensive

numbers of different virulence factors, which play an important role in the pathogenesis of infections. The typing of these two important pathogens using various typing techniques can aid in the detection of outbreaks and in conducting surveillance. The purpose of the analysis should be considered carefully when choosing typing methods for these bacteria. In addition to the first-line typing methods, *spa* typing for *S. aureus* and serotyping for *S. pneumoniae*, techniques such as MLST and PFGE can be used for both bacteria. Moreover, determination of the presence of virulence factors encoding genes such as PVL encoding genes in *S. aureus* and pilus protein encoding genes in *S. pneumoniae*, can be used to investigate their association with the pathogenesis of the disease. *S. pneumoniae* is a diverse bacterium due to its ability to naturally take up DNA (natural genetic transformation). Thus, serotyping of pneumococcal isolates can be challenging due to capsular switching. Natural genetic transformation must also be taken into account when using other typing methods such as MLST and PFGE, because the speed at which molecular changes occur is potentially faster in *S. pneumoniae* than in *S. aureus*.

8 FUTURE CONSIDERATIONS

These MRSA studies add to our understanding of the molecular epidemiology of MRSA strains in Finland and the importance of an appropriate genotyping method to be able to perform high-level laboratory-based surveillance of MRSA. A thorough knowledge of the characteristics of MRSA strains in Finland enables international comparisons. Although the number of annual MRSA cases has stabilised during the last couple of years, an increase in MRSA cases is possible at any time. In particular, the increasing number of elderly people in Finland will place a greater burden on the health care system, including a potential for more MRSA cases. Besides active MRSA control measures, rapid and adequate laboratory-based MRSA surveillance is still needed. However, if the number of new MRSA cases notably increases, the typing of MRSA strains has to be limited and typing should be directed to outbreak investigations. The near future will provide new typing techniques for MRSA due to the rapid development of new typing techniques. For example, MLVA will presumably offer assistance for MRSA typing in Finland in the near future. In addition, for research use, the DNA-based microarray technique and single nucleotide polymorphism (SNP) genotyping could offer a powerful tool for detecting virulence and resistance genes in *S. aureus*.

Most of the MRSA strains detected in Finland are related to international clones, and the increasing amount of immigration and travelling will bring more foreign clones to Finland. Thus, travel- and immigration-related MRSA infections would warrant more detailed research. For example, Spain is a popular destination among Finnish travellers and over 400 000 people travel to Spain each year. Interestingly, *spa* type t067 is the most prevalent *spa* type in Finland and in Spanish hospitals. The connection between the *spa* type t067 MRSA strain in Spain and Finland would be interesting to study further. This type of investigation could be performed by adding a travel information field to the national infectious disease register form or by undertaking a structured questionnaire for patients with *spa* type t067. In addition, further studies on the virulence genes of this clone compared to other clones would shed more light on why this clone has such a powerful ability to spread.

Animals may serve as a reservoir for MRSA and may transmit it to humans. In particular, CC398, has been reported to be common in animals such as pigs and horses, has been detected in humans, also in Finland. Thus, the transmission and spread of livestock MRSA, especially CC398, among animals and to humans needs further investigation.

Our study confirmed that outbreaks of invasive pneumococcal disease can occur in crowded environments such as in military encampments among previously healthy young men. Why pneumococci with the same sero- and genotype can cause severe invasive disease in some previously healthy young men while others only remain as carriers of the same strain is the main question raised by this study. Thus, further research could be targeted at human genetic susceptibility to pneumococcal disease. Future approaches will utilise genome-wide association studies to identify previously unsuspected genetic relations with pneumococcal diseases.

Since the low prevalence of PI-1 among pneumococcal isolates from children with AOM could be due to low antimicrobial resistance at the time when the isolates were collected, it would be interesting to further examine the changes and pilus prevalence of AOM isolates in Finland nowadays, following the increase in pneumococcal antimicrobial resistance and the large-scale pneumococcal conjugate vaccination. Because the role of pili in the pneumococcal AOM infections remained unclear, the significance of the pilus proteins as a virulence factor in AOM infections and their association with the spread of antimicrobial resistance should be studied in more detail.

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