RESEARCH

Salha Ibrahem

Methicillin Resistance in Staphylococci:

Horizontal Transfer of Mobile Genetic Element (SCC*mec*) between Staphylococcal Species

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ACADEMIC DISSERTATION

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Cover photo: *Penicillium notatum* (or *P. chrysogenum),* a species of fungus used to produce the antibiotic penicillin. It is the first antibiotic discovered, and is used for treatment of bacterial (*staphylococcus*) infections. It was also the trigger for developing bacterial resistance mechanism. This photo is provided by and copyrighted to Dennis Kunkel Microscopy, Inc. (www.denniskunkel.com).

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بسم الله الرحمن الرحيم

In the name of God, the Most Gracious, Most Merciful

To my parents

ABSTRACT

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Regardless of the existence of antibiotics, infectious diseases are the leading causes of death in the world. Staphylococci cause many infections of varying severity, although they can also exist peacefully in many parts of the human body. Most often *Staphylococcus aureus* colonises the nose, and that colonisation is considered to be a risk factor for spread of this bacterium.

S. aureus is considered to be the most important *Staphylococcus* species. It poses a challenge to the field of medicine, and one of the most problematic aspects is the drastic increase of the methicillin-resistant *S. aureus* (MRSA) strains in hospitals and community world-wide, including Finland. In addition, most of the clinical coagulase-negative *staphylococcus* (CNS) isolates express resistance to methicillin.

Methicillin-resistance in *S. aureus* is caused by the *mecA* gene that encodes an extra penicillin-binding protein (PBP) 2a. The *mecA* gene is found in a mobile genomic island called staphylococcal chromosome cassette *mec* (SCC*mec*). The SCC*mec* consists of the *mec* gene and cassette chromosome recombinase (*ccr*) gene complexes. The areas of the SCC*mec* element outside the *ccr* and *mec* complex are known as the junkyard J regions. So far, eight types of SCC*mec* (SCC*mec* I- SCC*mec* VIII) and a number of variants have been described. The SCC*mec* island is an acquired element in *S. aureus***.** Lately, it appears that CNS might be the storage place of the SCC*mec* that aid the *S. aureus* by providing it with the resistant elements. The SCC*mec* is known to exist only in the staphylococci.

The aim of the present study was to investigate the horizontal transfer of SCC*mec* between the *S. aureus* and CNS. One specific aim was to study whether or not some methicillin-sensitive *S. aureus* (MSSA) strains are more inclined to receive the SCC*mec* than others. This was done by comparing the genetic background of clinical MSSA isolates in the health care facilities of the Helsinki and Uusimaa Hospital District in 2001 to the representatives of the epidemic MRSA (EMRSA) genotypes, which have been encountered in Finland during 1992-2004. Majority of the clinical MSSA strains were related to the EMRSA strains. This finding suggests that horizontal transfer of SCC*mec* from unknown donor(s) to several MSSA background genotypes has occurred in Finland.

The molecular characteristics of representative clinical methicillin-resistant *S. epidermidis* (MRSE) isolates recovered in Finnish hospitals between 1990 and

1998 were also studied, examining their genetic relation to each other and to the internationally recognised MRSE clones as well, so as to ascertain the common traits between the SCC*mec* elements in MRSE and MRSA. The clinical MRSE strains were genetically related to each other; eleven PFGE types were associated with sequence type ST2 that has been identified world-wide. A single MRSE strain may possess two SCC*mec* types III and IV, which were recognised among the MRSA strains.

Moreover, six months after the onset of an outbreak of MRSA possessing a SCC*mec* type V in a long-term care facility in Northern Finland (LTCF) in 2003, the SCC*mec* element of nasally carried methicillin-resistant staphylococci was studied. Among the residents of a LTCF, nasal carriage of MR-CNS was common with extreme diversity of SCC*mec* types. MRSE was the most prevalent CNS species. Horizontal transfer of SCC*mec* elements is speculated to be based on the sharing of SCC*mec* type V between MRSA and MRSE in the same person.

Additionally, the SCC*mec* element of the clinical human *S. sciuri* isolates was studied. Some of the SCC*mec* regions were present in *S. sciuri* and the *pls* gene was common in it. This finding supports the hypothesis of genetic exchange happening between staphylococcal species.

Evaluation of the epidemiology of methicillin-resistant staphylococcal colonisation is necessary in order to understand the apparent emergence of these strains and to develop appropriate control strategies. SCC*mec* typing is essential for understanding the emergence of MRSA strains from CNS, considering that the MR-CNS may represent the gene pool for the continuous creation of new SCC*mec* types from which MRSA might originate.

Keywords: antibiotic; methicillin-resistant *S. aureus;* methicillin-resistant coagulase-negative *staphylococcus*; horizontal gene transfer; staphylococcal chromosome cassette *mec* (SCC*mec*); molecular typing.

ملخص الأطروحة

البحث مقدم من د. صالحه عبدالله إبراهيم قدورة بعنوان مقاومة الميثسلين في البكتيريا العنقودية المكورة والانتقال الأفقي لعناصر الجزيرة الجينومية المقاومة بين أنواع العنقوديات. بإشراف كلية الطب، جامعة هلسنكي و المؤسسه الوطنيه للصحة والرعايه الاجتماعيه، البحث35 /2010.
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بالرغم من وجود المضادات الحيوية فإن الأمراض المعدية تعتبر مسبب رئيسي للوفيات في العالم. البكتيريا العنقودية المكورة تسبب التهابات عديدة ومتفاوتة الشدة، مع أنها يمكن أن تتواجد في أجزاء كثيرة من جسم الإنسان دونما ضرر، وغالبا ما يكون الغشاء الأنفي هو موطن البكتيريا العنقودية الذهبية *S. aureus*، وهذا الاستيطان يعتبر عامل خطر لانتشار هذه البكتيريا.

تعتبر البكتيريا العنقودية الذهبية *S. aureus* من أهم سلالات البكتيريا العنقودية المكورة staphylococci حيث تشكل تحديا حقيقيا للميدان الطبي، إن أحد أكثر الجوانب إشكالية في هذا الشأن هي الزيادة المطردة للبكتيريا *S. aureus* المقاومة للميثسلين في المستشفيات و المجتمع في العالم دون استثناء بما في ذلك فنلندا. وعلاوة على ذلك فإن معظم العنقوديات الإكلينيكية سلبية التخثر المعزولة (CNS) أظهرت مقاومة للميثسلين.

مقاومة الميثسلين في البكتيريا العنقودية الذهبية ناتج عن وجود جين *mecA* الذي يقوم بتشفير البروتين PBP/2a. وهذا الجين يقع على جزيرة جينومية على الكروموسوم وتسمى جزيرة SCC*mec*، وهذه الجزيرة تتكون من جينين مركبين وهما *ccr* و *mec* وثلاثة مناطق تسمى junkyard J regions تحيط بهما. حتى الأن تم وصف ثمانية أنواع من الجزر الجينومية SCC*mec* I-VIII و عدة فروع منها. إن جزيرة SCC*mec* غير موجودة اصلا في *S.aureus* بل تكتسبها من مصادر خارجية .حديثا بدا أن البكتيريا العنقوديه سلبية التخثر CNS قد تكون مكان تخزين لـ SCC*mec* وهي قد تكون أحد مصادرتزويد *S.aureus* بجينات المقاومة للمضاد الحيوي. من المعروف حتى الأن أن SCC*mec* موجودة حصريا في البكتيريا staphyloccci.

الهدف من هذه الآطروحة هو دراسة ظاهرة الانتقال الأفقي للعناصر الجينية الـ SCC*mec* بين *S. aureus* و CNS. وكان أحد الأهداف المحددة دراسة ما إذا كان هناك بعض البكتيريا الغير مقاومة للميثسلين MSSA تميل لاكتساب جين المقاومة دون غيرها. و قد تم دراسة ذلك من خلال مقارنة التركيبة الجينية لعينات إكلينيكية من البكتيريا MSSA أخذت من المستشفيات في مدينة هلسنكي وضواحيها في سنة 2001 مع التركيبة الجينية الوبائية للبكتيريا المقاومة EMRSA والتي ظهرت في فنلندا خلال الفترة 1992-2004. وأظهرت هذه الدراسة إن غالبية البكتيريا MSSA كانت ذات صلة جينية وثيقة بالبكتيريا المقاومة EMRSA. تشير هذه النتائج إلى أن الانتقال الأفقي للجزيرة SCC*mec* إلي البكتيريا MSSA من مصدر مجهول قد حدث فعلا في العينات الفنلندية.

كما أنه تم دراسة الخصائص الجزيئية للعينات الإكلينيكية المختارة للبكتيريا العنقوديه سلبية التخثر *S. epidermidis* المقاومة للميثسلين (MRSE) التي أخذت من مستشفيات في فنلندا، في الفترة الزمنية من 1990 إلى 1998 كما تم دراسة علاقتها الوراثية الجينية بين بعضها البعض

وبين عينات عالمية أخرى تم دراستها سابقا، و كذلك للتأكد من وجود صفات مشتركة بين عناصر الـجزيرة SCC*mec* في MRSE و MRSA. إن العينات الإكلينيكية من بكتيريا MRSE كانت متشابهة وذات صلة جينية ، ومما يحسن ذكره أن أحد عشر نوعا من الـ PFGE ارتبطت بتسلسل من نوع ST2 تم التعرف عليها عالميا. وأظهرت هذه الدراسة أن بكتيريا منفردة من MRSE قد يوجد بها نوعين من SCC*mec* III-IV، والتي تم التعرف عليها سابقا في الـبكتيريا MRSA.

بعد ستة أشهر من بدء تفشي بكتيريا *S. aureus* المقاومة للميثسلين (MRSA) التي تحمل نوع الجين SCC*mec*-V في مؤسسة رعاية كبار السن في شمال فنلندا (LTCF) في سنة 2003 قمنا بدراسة تفصيلية على التركيبة الجينية لـ SCC*mec* في البكتيريا العنقودية المقاومة للميثسلين الموجودة في انف المقيم في المؤسسة، فوجدنا أن MR-CNS البكتيريا المقاومة سائدة بين أغشية أنوف المقيمين في المؤسسة وأن البكتيريا الأكثر شيوعا هي MRSE. كما أنه وجدنا أن كلتا البكتيريا MRSE و MRSA تواجدتا معا في أنف نفس المريض وكلا نوعا البكتيريا يحملان نوع SCC*mec*-V وهذا يعطينا إشارة على احتمال الأنتقال الأفقي للنوع V بينهما.

وقمنا أيضا بدراسة التركيبة الجينية لـ SCC*mec* لبكتيريا *S. sciuri* المأخوذة من عينات إكلينيكية من الإنسان، وظهر أن هناك بعض مناطق أو اجزاء من SCC*mec* موجودة في الـ *S sciuri* و الجين *pls* كان شائعا فيها. هذه النتيجة تعزز الفرضية بوجود تبادل جيني يحدث بين سلالات البكتيريا العنقودية محل البحث.

إن التقويم الوبائي لبكتيريا العنقودية المقاومة للميثسلين المستوطنة ضروري لأجل فهم واضح لنشوء هذه السلالات ووضع استراتيجيات المكافحة المناسبة لها. إن تصنيف الـSCC*mec* ضروري وأساسي لفهم نشوء سلالات البكتيريا العنقودية المقاومة للميثسلين من CNS. مع الأخذ بالاعتبار احتمالية أن الـ MR-CNS ممكن أن تمثل المنبع الجيني لاستمرار تطور أنواع جديدة من الـ SCC*mec* الذي منه يمكن نشوء بكتيريا عنقودية مقاومة للميثسلين.

الكلمات الرئيسية: المضادات الحيوية; مقاومة الميثسلين; البكتيريا العنقودية الذهبية; البكتيريا العنقودية سلبية التخثر; الأنتقال جيني الأفقي; الجزيرة جينومية SCC*mec*; التحاليل الجزيئية.

TIIVISTELMÄ

Salha Ibrahem, Methicillin Resistance in Staphylococci: Horizontal Transfer of Mobile Genetic Element (SCC*mec*) between Staphylococcal Species [Stafylokokkien metisilliini-resistenssi: geneettisten elementtien (SCC*mec*) horisontaalinen siirtyminen]**.** Terveyden ja hyvinvoinnin laitos. Tutkimus 35/2010. 148 sivua. Helsinki, Finland 2010.
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Antibioottien olemassaolosta huolimatta tartuntataudit ovat yksi maailman yleisimpiä kuolinsyitä. Vaikka stafylokokit aiheuttavat monia vakavuudeltaan eriasteisia infektioita, voivat ne esiintyä myös harmittomina ihmisen elimistössä. *Staphylococcus aureus* löytyy yleisimmin nenän limakalvolta, mitä pidetään riskitekijänä kyseisen bakteerin leviämiselle.

S. aureusta pidetään tärkeimpänä stafylokokkilajina ja se asettaa haasteita lääketieteelle. Yksi sen ongelmallisimmista piirteistä on metisil-liiniresistenttien *S. aureus* (MRSA) –bakteerikantojen jyrkkä lisääntyminen sairaaloissa ja avohoidossa, niin Suomessa kuin maailmallakin. Lisäksi kliinisistä koagulaasinegatiivisista stafylokokki-kannoista (KNS) suurin osa on vastustuskykyisiä metisilliinille.

Metisilliiniresistenssi *S. aureus* -bakteerissa johtuu *mecA* –geenistä. Kyseinen geeni koodaa penisilliiniä sitovaa proteiinia (PBP) 2a, ja se löytyy liikkuvasta yksiköstä, jota kutsutaan stafylokokkaaliseksi kromosomikasetti *mec*:ksi (SCC*mec*). SCC*mec* koostuu *mec*-geenistä ja rekombinanteista kasettikromosomi geenikomplekseissta (*ccr*). Ne SCC*mec* elementtien alueet, jotka sijaitsevat *ccr* ja *mec* -kompleksien ulkopuolella, tunnetaan nimellä junkyard J –alueet. Tähän mennessä kahdeksan SCC*mec* (SCC*mec* I- SCC*mec* VIII) lajia, sekä useita eri variantteja on kuvattu, ja on saatu selville, että SCC*mec* -kasetti on hankittu elementti *S. aureuksessa*. Uusimpien tutkimustulosten valossa näyttää siltä, että KNS saattaisi olla vastustuskykyisiä SCC*mec* elementtejä *S. aureukselle* tarjoava reservuaari. . SCC*mec*:n tiedetään löytyvän vain stafylokokeissa.

Tämän tutkimuksen tavoitteena oli selvittää SCC*mec:*n horisontaalinen siirtyminen *S. aureuksen* ja KNS:n välillä. Erityisesti tavoitteena oli selvittää, ovatko jotkut metisilliinille herkät *S. aureus* (MSSA) –bakteerikannat taipuvaisempia vastaanottamaan SCC*mec*:n kuin muut. Tämä tehtiin vertaamalla Helsingin ja Uudenmaan sairaanhoitopiirin tervey-denhuollon laitoksista vuonna 2001 otettujen kliinisten MSSA -näytteiden geneettisiä piirteitä Suomessa vuosina 1992–2004 esiintyneisiin epideemisiin MRSA (EMRSA)-genotyyppeihin. Suurin osa kliinisistä MSSA -kannoista oli sukua EMRSA-kannoille. Tämä havainto viittaa siihen, että SCC*mec*:n horisontaalinen siirtyminen tuntemattomalta luovuttajalta (/luovuttajilta) useille MSSA genotyypeille on tapahtunut Suomessa.

Suomen sairaaloista vuosina 1990–1998 saatujen kliinisten metisilliiniresistenttien *S. epidermidis* (MRSE) -kantojen molekyylitason ominaisuuksia tarkasteltiin tutkimalla niiden geneettistä suhdetta sekä toisiinsa että kansainvälisesti tunnettuihin MRSE -klooneihin, jotta SCC*mec* elementtien yhteiset piirteet MRSE:ssa ja MRSA:ssa saataisiin selvitettyä. Kliiniset MRSE -kannat olivat geneettisesti toisilleen sukua; yksitoista PFGE -tyyppiä yhdistettiin maailmanlaajuisesti esiintyvään sekvenssi ST2:een. Yksi MRSE -kanta saattaa omata kaksi SCC*mec* -tyyppiä (III ja IV), jotka ovat tunnettuja MRSA-kantojen keskuudessa.

Kuusi kuukautta SCC*mec* tyyppi V:n omaavan MRSA tautiryppään puhkeamisen jälkeen pitkäaikaishoidon laitoksessa Pohjois-Suomessa (LTCF) vuonna 2003, tutkittiin nenässä esiintyvien metisilliiniresistenttien stafylokokkien SCC*mec* elementtien kantajuutta. LTFC:n asukkaiden keskuudessa nenässä kulkeutuva MR-KNS oli yleinen, joskin SCC*mec* tyypeissä oli valtavaa vaihtelua. MRSE oli yleisin KNS -laji. SCC*mec* elementtien horisontaalisen siirtymisen on arveltu perustuvan SCC*mec* tyyppi V:n siirtymisen MRSA:n ja MRSE:n välillä samassa henkilössä.

Tämän lisäksi tutkittiin myös ihmisen kliinisten *S. sciuri* – kantojen SCC*mec* -elementtiä. Useat SCC*mec* alueet olivat läsnä *S. sciurissa*, ja niissä *pls* -geeni oli yleinen. Tämä havainto tukee olettamusta geneettisen vaihdon tapahtumisesta stafylokokkilajien välillä.

Metisilliiniresistentin stafylokokkikolonisaation epidemiologian arviointi on tarpeen, jotta voidaan ymmärtää näiden kantojen ilmaantuminen ja kehittää asianmukaisia seurantastrategioita SCC*mec* geenikasetin tyypittäminen on olennaista KNS:stä kehittyvien MRSA -kantojen ymmärtämiselle ottaen huomioon sen, että MR-KNS saattaa edustaa geenipoolia, jossa syntyy jatkuvasti uusia SCC*mec* tyyppejä, joista MRSA saattaa mahdollisesti olla peräisin.

Asiasanat antibiootti; metisilliini-resistentti *S. aureus*; metisilliini-resistentti koagulaasi-negatiivinen staphylokokki; horisontaalinen geenin siirto; staphylokokkien kromosomi-kasetti *mec* (SCC*mec*); molekylaarinen tyypitys.

SAMMANDRAG

Salha Ibrahem, Methicillin Resistance in Staphylococci: Horizontal Transfer of Mobile Genetic Element (SCC*mec*) between Staphylococcal Species [Stafylokockernas meticillinresistens: horisontell överföring av ett mobilt genetiskt element (SCC*mec*) mellan stafylokockarter]. Institutet för Hälsa och Välfärd, Forskning 35/2010. 148 sidor. Helsingfors, Finland 2010.
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Infektionssjukdomar är den ledande dödsorsaken i världen idag fastän antibiotikan har uppfunnits. Stafylokocker orsakar flera slag av infektioner med varierande gravhet men bakterierna kan också förekomma fredligt på flera ställen av människokroppen. Oftast koloniserar *Staphylococcus aureus* näsans slemhinnor, något som anses vara en riskfaktor för spridning av bakterien.

S. aureus anses vara den viktigaste av arterna i *Staphylococcus*-släktet. Bakterien utgör en utmaning för läkarvetenskapen, speciellt som antalet meticillinresistenta *S. aureus*-stammar (MRSA) som påträffas på sjukhus och inom den öppna vården har ökat kraftigt i Finland och världen över. Också största delen av de koagulasnegativa stafylokockerna (KNS) från kliniska fynd är motståndskraftiga mot meticillin.

Meticillinresistensen hos *S. aureus* orsakas av *mecA*-genen som kodar för det extra penicillinbindande proteinet (PBP) 2a. *mecA*-genen finns i resistenskassetten staphylococcal chromosome cassette *mec* (SCC*mec*) som är ett mobilt genetiskt område i bakteriekromosomen. SCC*mec* består av två genkomplex: *mec*-komplexet där *mecA*-genen sitter och *ccr*-komplexet med rekombinasgenerna cassette chromosome recombinase (*ccr*). SCC*mec* består utöver *ccr*- och *mec*-komplexen av så kallade ”junkyard” eller J-regioner. För tillfället har åtta SCC*mec*-typer (SCC*mec* I-SCC*mec* VIII) och ett antal varianter av dessa beskrivits. SCC*mec* är ett förvärvat område i *S. aureus*. De senaste årens forskning tyder på att KNS fungerar som reservoar för gener som ger bakterierna motståndskraft mot antibiotika. SCC*mec* har påträffats endast hos stafylokocker.

Målet för det här forskningsprojektet var att undersöka den horisontella genöverföringen av SCC*mec* mellan *S. aureus* och KNS. Ett specifikt mål var att undersöka om vissa meticillinkänsliga *S. aureus*-stammar (MSSA) är mer benägna att ta upp SCC*mec* än andra. Metoden som användes gick ut på att jämföra genotyperna hos kliniska MSSA-isolat från Helsingfors och Nylands sjukvårdsdistrikt år 2001 med genotyperna hos de epidemiska MRSA-stammar (EMRSA) som påträffats i Finland under åren 1992-2004. Majoriteten av de kliniska MSSA-stammarna var besläktade med EMRSA-stammarna. Observationen tyder på att det i Finland förekommit horisontell genöverföring där SCC*mec* har flyttats över från en eller flera okända donatorer till flera MSSA-genotyper.

Även de molekylära karaktärsdragen hos en del av de kliniska meticillinresistenta *S. epidermidis*-bakterierna (MRSE) som påträffats på finländska sjukhus mellan år 1990 och 1998 undersöktes. Isolatens genetiska likhet kunde jämföras både inom gruppen och med internationellt kända genotyper av MRSE och gemensamma egenskaper hos SCC*mec*-kassetterna i MRSE och MRSA kunde påvisas. De kliniska MRSE-stammarna var genetiskt besläktade sinsemellan. Elva av PFGE-typerna kunde anknytas till sekvenstyp ST2, en genotyp som har påträffats runt om i världen. En av MRSE-stammarna bar sannolikt på två SCC*mec*-typer, typerna III och IV. Båda SCC*mec*-typerna fanns också bland MRSA-stammarna.

I en inrättning för långtidsvård i norra Finland bröt år 2003 en MRSA-epidemi ut som förorsakades av en MRSA-stam med en SCC*mec*-kassett av typen V. Sex månader efter utbrottet undersöktes SCC*mec*-kassetterna hos de meticillinresistenta stafylokocker som påträffades i prov från klienternas näshålor. En stor del av klienterna var MR-KNS-bärare och mångfalden av SCC*mec*-typer var mycket stor. MRSE var den mest utbredda av KNS-arterna. Horisontell överföring av SCC*mec* kan ha förekommit eftersom samma person bar på både MRSA- och MRSE-isolat med SCC*mec* av typen V.

Även SCC*mec*-kassetten hos kliniska *S. sciuri*-isolat undersöktes. Analysen visade att vissa delar av SCC*mec*-kassetten påträffades i *S. sciuri* och att *pls*-genen var allmän. Denna observation stöder hypotesen att stafylokockarterna utbyter genetiskt material sinsemellan.

För att förstå uppkomsten av meticillinresistenta stafylokocker och för att utveckla lämpliga kontrollåtgärder är det nödvändigt att undersöka stafylokockernas epidemiologi. Att granska SCC*mec* är ytterst viktigt för att förstå hur MRSA uppkommer från KNS, eftersom forskningen tyder på att MR-KNS-populationen fungerar som den genpool ur vilken ständigt nya SCC*mec*-typer bildas för att sedan möjligen överföras till *S. aureus*.

Nyckelord: antibiotika; meticillinresistent *S. aureus*; meticillinresistent koagulasnegativ stafylokock; horisontell genöverföring; stafylokockernas resistenskassett SCC*mec*; molekylär typning.

CONTENTS

LIST OF ORIGINAL PUBLICATIONS

This thesis is based on the following original publications, which are referred to in the text by their Roman numerals**.**

I. Ibrahem S., Salmenlinna S., Kerttula A-M., Virolainen-Julkunen A., Kuusela P., Vuopio-Varkila J. (2005). Comparison of genotypes of methicillin-resistant and methicillin-sensitive *Staphylococcus aureus* in Finland, *Eur J Clin Microbiol Infect Dis*. 24(5):325-328.

II. Ibrahem S., Salmenlinna S., Lyytikäinen O., Vaara M., Vuopio-Varkila J. (2008). Molecular characterization of methicillin-resistant *Staphylococcus epidermidis* strains from bacteraemic patients. *Clin Microbiol Infect*.14 (11):1020–1027.

III. Ibrahem, S., Salmenlinna, S., Virolainen, A., Kerttula, A.-M., Lyytikäinen, O., Jägerroos, H., Broas, M., Vuopio-Varkila, J. (2009). Carriage of methicillin-resistant staphylococci and their SCC*mec* types in a long-term-care facility. *J. Clin. Microbiol*. 47(1): 32–37.

IV. Juuti K., Ibrahem S., Virolainen-Julkunen A., Vuopio-Varkila J., Kuusela P.(2005). The *pls* gene found in methicillin-resistant *Staphylococcus aureus* strains is common in clinical isolates of *Staphylococcus sciuri*. *J Clin Microbiol*.43 (3):1415-9. Erratum in: *J.Clin Microbiol*. 2005 Jul; 43 (7):3589.

ABBREVIATIONS

ATCC	American type culture collection
CA-MRSA	Community-acquired methicillin-resistant *Staphylococcus aureus*
CC	Clonal complex
ccr	Cassette chromosome recombinase
CLSI	Clinical and Laboratory Standards Institute (formerly NCCLS)
CNS	Coagulase-negative *Staphylococcus*
DR	Direct repeats
EMRSA	Epidemic MRSA
HA-MRSA	Hospital-Acquired MRSA
HGT	Horizontal gene transfer
HUCH	Helsinki University Central Hospital
ICU	Intensive care unit
IR	Inverted repeates
IS	Insertion Sequence
kbp	kilo base pairs
KTL /THL	National Public Health Institute (Kansanterveyslaitos); name changed on 1.1.2009 to National Institute of Health and Welfare (Terveyden ja hyvinvoinnin laitos; THL)
LTCF	Long-term care facility
mecA	Gene coding for penicillin-binding protein2a (PBP2a)
MIC	Minimal inhibitory concentration
MLST	Multilocus sequence typing
MLVA	Multiple-locus variable-number tandem repeat typing
MPCR	Multiple PCR
MR-CNS	Methicillin-resistant-CNS
MRSA	Methicillin-resistant *Staphylococcus aureus*
MS	Membrane-spanning domain
MSSA	Methicillin-sensitive *Staphylococcus aureus*
NICU	Neonatal intensive care unit
ORF	Open reading frame
PB	Penicillin-binding domain
PBPs	Penicillin-binding proteins
PCR	Polymerase chain reaction
PFGE	Pulsed field gel electrophoresis

Pls	Plasmin-sensitive protein
PVL	Panton-Valentine leukocidin
SCC*mec*	Staphylococcal cassette chromosome *mec*
Spa	Staphylococcal protein A
ST	Sequence type
VISA	Vancomycin intermediate-resistant *S. aureus*
VNTR	Variable-number tandem repeat
VRSA	Vancomycin-resistant *S. aureus*

1 INTRODUCTION

Staphylococci are a diverse group of bacteria that can cause diseases ranging from minor skin infections to life-threatening bacteremia. They are also part of normal flora/microbiota in humans. Within the genus *Staphylococcus, S. aureus* is the main human pathogen and *S. epidermidis* the most important opportunistic pathogen. Overall, staphylococci are a major cause of both nosocomial and community-acquired infections[1].

β-lactams were the first group of antimicrobial agents used to treat staphylococcal infections. However, penicillin was soon discovered ineffective due to β-lactamases, that is, enzymes produced by staphylococci and capable of cleaving the β-lactam ring. The introduction of β-lactamase resistant β-lactams such as methicillin into medical practice in early 1960s was followed by the emergence of methicillin-resistant staphylococci. Nowadays, the majority of CNS isolated from hospitalised patients express resistance to methicillin. Among nosocomial isolates the incidence of methicillin resistant *S. aureus* (MRSA) is high in some countries; reaching even 70%[2]. In Finland, methicillin resistance among *S. aureus* is still relatively low but the annual number of MRSA cases has increased significantly in recent years, further challenging the prevention of transmission and infections caused by it. MRSA infections are treated with vancomycin, but the first high-level vancomycin-resistant isolates (VRSA) have already been described[3]. There are few effective antibiotics on the market for treating MRSA infections, and very few if any are expected to be developed in the near future[4].

Resistance to methicillin is determined by the *mecA* gene, which encodes an additional penicillin-binding protein (PBP2a) with low affinity for β-lactams. The *mecA* gene, which is not native to *S. aureus*,[5] is considered to have originated in CNS. In these, the *mecA* gene is carried in a chromosomal element called the SCC*mec*. The SCC*mec* varies in size (24-67 kbp), and eight main types of SCC*mec* (I-VIII) have been identified to date[6–14]. The current hypothesis is that SCC*mec* serves as a carrier *mecA* gene across staphylococcal species. In addition, it has been hypothesised that MRSA originated through the transfer of SCC*mec* into a limited number of MSSA lineages.

Structural typing of this chromosomal region is vital for understanding transmission routes and the genetic background of the strains. There are several reasons why research on staphylococci and the mobile element

SCC*mec* is justified and important. Firstly, our knowledge about commensal and clinical MSSA and methicillin-resistant coagulase-negative staphylococcal strains in Finland is sparse. Secondly, staphylococci are part of the normal human flora and possess great potential as pathogens. If antimicrobial resistance becomes common amongst these bacterial species, as has already happened with many CNS species, treatment of infections caused by the resistant strains becomes difficult and expensive. It may even increase the risk of mortality although this remains controversial. The carriage of multi-resistant staphylococci is often long-term which challenges preventive measures and requires continuous surveillance. Thirdly, detailed studies of staphylococci on the genetic level allow us to gain a deeper understanding of the mechanisms involved in the development and spread of resistance markers. Mapping bacterial factors linked to resistance and the capacity to spread may help in developing targeted diagnostics, and thus improve the treatment, surveillance and prevention of infections caused by this group of bacteria.

The aim of this study is to analyze the genetic characteristics and putative horizontal transfer of mobile genetic elements (*i.e.* SCC*mec*) in *S. aureus* and the coagulase-negative staphylococcal population in Finland.

2 REVIEW OF THE LITERATURE

2.1 Staphylococci

2.1.1 Genus *Staphylococcus*

Staphylococci were first detected and cultured by Koch (1878) and Pasteur in 1880[15], but Ogston and Rosenbach made the initial detailed studies: In 1881 Ogston named the clustered micrococci "staphylococci," from the Greek word “staphyle”, meaning bunch of grapes. In 1884, Rosenbach isolated two strains of staphylococci, which he named for the pigmented appearance of their colonies; *Staphylococcus aureus*, from the Latin aurum for gold, and *Staphylococcus albus* (now called *epidermidis*), from the Latin albus for white[16–18]. Nowadays, the genus *Staphylococcus* is classified as a member of the family *Micrococcaceae*, but the staphylococci are phylogenetically distantly related to any other genera in the family. A wide variety of genetic criteria indicates that the genus *Staphylococcus* forms a coherent and well-defined natural group that is very different from the genus *Micrococcus*. According to 16s RNA analysis, the genus *Staphylococcus* belongs to the broad *Bacillus-Lactobacillus-Streptococcus* cluster. The planococci, enterococci and bacilli are the closest relatives to staphylococci[19].

Forty species and 17 subspecies of the genus *Staphylococcus* have been identified[20–22]. Staphylococci are Gram-positive nonmotile, nonsporulating spherical bacteria (size range 0.5–1.2 µm) that occur in microscopic clusters resembling grapes. They are catalase-positive, oxidase-negative and facultative anaerobes (i.e. capable of both aerobic and anaerobic growth). Staphylococci are differentiated by their ability to produce coagulase in the coagulase-positive (*S. aureus*) and CNS [23–25]. In addition, *S. aureus* can be distinguished from the other staphylococcal species by the fermentation of mannitol and trehalose and by producing deoxyribonuclease (DNase). However, these tests cannot distinguish *S. aureus* from all other staphylococcal isolates. When grown on blood agar, cultures of *S. aureus* typically yield golden-yellow colonies that are usually beta-hemolytic.

In the clinical laboratory, the identification of staphylococcal species and subspecies has been facilitated by the availability of rapid and miniaturised kit identification systems, some of which may be processed and interpreted by automated instrumentation combined with the computer technology (e.g. the API ID32 Staph system, bioMerieux, Marcy l`Etoile,

France)[26–28]. Species and subspecies identification can give important information in assessing clinical significance and guiding etiological diagnosis and management of infections[21, 29–33].

2.1.2 Cell wall of staphylococci

The cell wall is the outermost component common to all bacteria (except for *Mycoplasma* species, which are bound by a cell membrane, not a cell wall). The bacterial cell wall is a single rigid net, and is cross-linked to itself in many places to provide structural support. Under the electron microscope it appears as a thick homogeneous structure (about 20 to 40 nm). The cell wall consists of the peptidoglycan (also called murein) with attached polysaccharides (i.e. capsule), proteins and teichoic acids[34–36] (Figure 1).

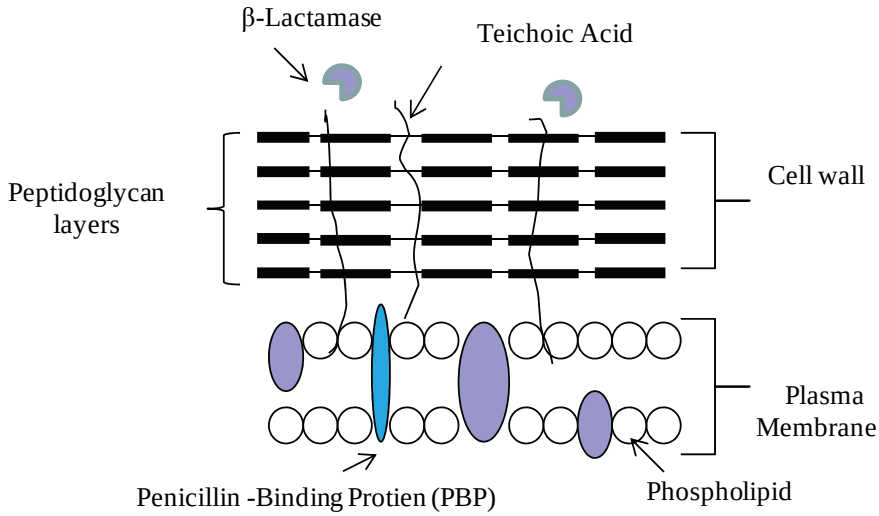


Figure 1. Schematic diagram of the basic structures of gram-positive bacteria cell wall (adapted from :http://www.cehs.siu.edu/fix/medmicro/genmicr.htm).

S. aureus peptidoglycan is a polymer that consists of long glycan chains. The glycan chain is composed of alternating N-acetylmuramic acid and N-acetylglucosamine molecules. Tetrapeptides consisting of both D- and L-amino acids join the adjacent glycan chains by the muramic acid residues. In CNS, the peptidoglycan differs from that of *S. aureus* mainly in the inter peptide bridges (some of the glycins are replaced by L-serine)[35]. The rigid peptidoglycan is important in defining the shape of the cell and

in preventing osmotic lysis. Peptidoglycan is a good target for antimicrobial agents because it is present in bacteria but not in human cells. Penicillin-binding proteins (PBPs) catalyze the cross-linking of peptidoglycan subunits during bacterial cell wall assembly. Teichoic acids are substituted polysaccharides containing ribitol or glycerol residues joined through diphosphoester linkages. These polysaccharides extend through the entire peptidoglycan layer and appear on the cell surface. The majority of *S. aureus* are enclosed in a polysaccharide capsule, eleven serotypes have been reported and 75% of human infections are due to serotypes 5 and 8[37, 38]. The importance of the staphylococcal cell wall in virulence, epidemiology and genomic evolution has been described in numerous studies[34, 37, 39–44].

2.1.3 Virulence factors of staphylococci

Bacteria are pathogens when they have the ability to cause disease. This ability is known as pathogenicity and virulence is a degree of the pathogenicity. Virulence factors are elements produced by a pathogen which contribute to pathogenicity. The surface proteins of *S. aureus* have been shown to be virulence factors in different infection models, although the mechanism of the action of many of them has not yet been revealed[45]. In the majority of diseases caused by *S. aureus*, the pathogenesis depends on the combined actions of several virulence factors. The virulence factors known to date include (i) surface structures, (ii) invasins, (iii) toxins, (iv) biochemical properties, and (v) determinants for resistance.

(i) The surface proteins (e.g. clumping factor, collagen binding protein, extracellular adherence protein, elastin-binding protein, fibronectin binding protein) promote attachment and colonisation of host tissues, or inhibit phagocytic engulfment (capsule and protein A) and promote immunological disguise (protein A, coagulase, and clotting factor). (ii) Invasins include several enzymes secreted by invasive bacteria that promote bacterial spread in tissues and play a role in pathogenesis. Among the most important enzymes are leukocidins (e.g. Panton-Valentine leukocidin (PVL), kinases and hyauronidase (iii) Membrane-damaging toxins disrupt eukaryotic cell membranes (hemolysins and leukotoxin) and superantigens contribute to the symptoms of septic shock (e.g. toxic shock syndrome toxin (TSST). (iv) Some biochemical properties increase staphylococcal survival in phagocytes (*i.e.,* catalase has the ability to convert hydrogen peroxide (H_2O_2) to water and oxygen and the carotenoid pigment has an antioxidant effect.). (v) The determinants for inherent and acquired

resistance to antimicrobial agents can also be considered virulence factors[46–48]. Examples of some of these virulence factors are given in the following: Plasmin-sensitive protein (Pls) is an example of the pathogenic potential of *S. aureus* reflected by surface proteins with adhesive and invasive functions. The *pls* gene has been shown to attenuate bacterial binding to immobilised fibronectin and immunoglobulin G [49], as well as the invasion of epithelial cells[50]. In addition, the *pls* gene appears to mediate adhesion to cellular lipids and glycolipids and to promote bacterial cell-cell interactions[51]. The *S. epidermidis aap* gene (accumulation-associated protein) is very similar to *pls* (36.1% identical and 62.8% similar) in amino acid sequence[52]. Josefsson and co-workers have reported that *pls* is a virulence factor for septic arthritis and sepsis in a mouse model[45]. Interestingly, the *pls* gene is located in the staphylococcal cassette chromosome *mec*, which contains the *mecA* gene that causes resistance to methicillin. The *pls* gene is associated with SCC*mec* type I MRSA[7]. However, it has also been detected in SCC*mec* type II, SCC*mec* type III[7, 53] and SCC*mec* type IV[54].

Another *S. aureus* surface protein, staphylococcal protein A (Spa) is one of the main surface proteins in *S. aureus*[55], and it is encoded by the *spa* gene. It has been suggested that Protein A is anti-phagocytic due to the way it binds the Fc fragment of IgG from several mammalian species[56]. Many regulators in a complex network are responsible for the expression of the *spa* gene[57–59]. The first identified regulatory locus was the *agr* gene, a repressor of *spa* transcription[59]. The effector molecule of the *agr*-dependent regulation is RNAIII, and in response to the accumulation of the auto-inducing peptide, AIP, it is synthesised at the end of the exponential phase of growth[57]. Sequencing the staphylococcal protein A, *spa* gene, can be used as a typing method for MRSA strains.

As an example of invasins/toxins, the PVL leukotoxin was first described by Panton and Valentine in 1932[60]. *pvl* genes encode a bicomponent pore-forming leukotoxin. Each leukotoxin is composed of class S protein (lukS-PV) and class F protein (lukF-PV). PLV causes lytic pores in the cell membranes of neutrophils, causing leukocyte destruction and the release of chemotactic factors that result in a massive inflammatory response[61]. So far, 11 leukotoxin proteins have been recognised[62]. *pvl*-positive *S. aureus* are usually associated with necrotising pyogenic cutaneous infections (abscesses, furuncles) and infrequently with cellulitis or tissue necrosis[63]. However, it has been noticed that many *S. aureus* isolates from humans suffering from septic arthritis, bacteraemia[64] and necrotising pneumonia[65–67] are *pvl*-positive.

Most patients developing necrotising pneumonia have no history of skin infection but commonly have a preceding 'flu-like' illness. Both MSSA and MRSA can possess *pvl* genes[68] Community-acquired MRSA (CA-MRSA) possessing *pvl* genes has been shown to cause severe acute hematogenous osteomyelitis in children[69], and the incidence of hospital-acquired MRSA(HA-MRSA) and/CA-MRSA possessing *pvl* genes is increasing[70].

Apart from the above mentioned virulence factors, biofilm formation is nowadays considered the most important virulence determinant of CNS[71–73]. Polysaccharide production by CNS is related to their ability to adhere to biomaterials[74–79]. Polysaccharide synthesis is controlled genetically by *ica*A and *ica*D, which encode an N-acetylglucosaminyl transferase enzyme that catalyses the synthesis of the capsular polysaccharide β-1,6-glucosamine glycan from *N*-acetylglucosamine[80]. In addition, it has been proposed that the genes *gehC* and *gehSE1* coding for lipases from *S. epidermidis* are involved in skin colonisation[73]. However, CNS is normally less virulent than *S. aureus* and express fewer virulence factors.

2.2 Clinical significance of staphylococci

2.2.1 Carriage

Colonisation means the presence of viable bacteria in or on a host but without any clear clinical expression. In general, the internal tissues, for example, blood, brain, heart, muscle etc., are normally free of microorganisms. Regardless, the surface tissues, skin and mucous membranes, become readily colonised by various microbial species due to being continuously in contact with environmental organisms. Staphylococci are widely distributed in nature and can be isolated from humans, animals and food products[28, 81–83]. *S. aureus* can colonise the skin and mucosal surfaces of humans[84-86]. The nose is the most common carriage site for *S. aureus* but other carriage sites like the pharynx and perineum, axillae, gastrointestinal tract and vagina, have been described[85-89]. In addition, *S. aureus, S. epidermidis* and *S. haemolyticus* share the same habits and may permanently or transiently colonise the anterior nares and further regions of skin and mucous membranes which may act as sources of bacteremia and other infections[90, 91]. These species can also survive on innate environments, such as medical devices and medical equipment in intensive care unit (ICU) surroundings, for weeks to months[83]. *S. hominis* is often

isolated from the axillae, head and extremities. *S. capitis* is often isolated from the head and arms. *S. simulans, S. xylosus, S. cohnii, S. saprophyticus* and *S. warneri* have occasionally been isolated from the skin[92].

Nasal carriage of *S. aureus* has often been studied using a cross-sectional (i.e. a group of people are examined on one occasion) design with a single nasal culture to classify an individual as a carrier or not. Longitudinal studies (i.e. chosen individuals are sampled frequently over a period) discriminate at least three *S. aureus* nasal carriage patterns, which can be described in healthy individuals: persistent carriage, intermittent carriage and non-carriage[84, 86, 93–95]. This approach has shown that about 20% of individuals are persistent *S. aureus* nasal carriers, approximately 30% are intermittent carriers, and about 50% are non-carriers[93, 96–98]. Children have higher persistent carriage rates than adults[84, 99, 100]. There seems to be a transition pattern from persistent carriage to intermittent or non-carriage states during adolescence[84, 86]. However, carriage of staphylococci has been identified as a risk factor for development of infections in various settings: in hospitalised patients, in patients after surgery, in patients receiving continuous ambulatory peritoneal dialysis and in patients receiving hemodialysis[91, 101].

2.2.2 Staphylococcal infections

An infection means successful multiplication of bacteria on or within a host, leading to an infectious disease when signs and symptoms result from damage or altered physiology associated with an infection[102]. *S. aureus* is a common cause of infection in both hospital and community settings[103]. More than 80% of all suppurative infections are caused by staphylococci. *S. aureus* causes diseases ranging from mild infection to very serious, even life-threatening, conditions. Most often it causes skin infections, including folliculitis, furuncles, impetigo and subcutaneous abscesses. It can also be responsible for scalded skin syndrome, soft-tissue infections like pyomyositis, septic bursitis, septic arthritis, bacteremia, toxic shock syndrome, endocarditis, osteomyelitis, pneumonia and food poisoning[104].

In the last two decades, CNS have also emerged as significant pathogens[105] especially in immunocompromiced patients, premature newborns and patients with implanted biomaterials. The most frequent CNS species associated with human infections is *S. epidermidis*, particularly in association with intravascular catheters. It is the predominant agent of

nosocomial bacteremia, prosthetic-valve endocarditis, surgical wound infections, central nervous system shunt infections, intravascular catheter-related infections, peritoneal dialysis-related infections, and infections of prosthetic joints. The second most frequently encountered CNS species is *S. haemolyticus,* and it is remarkable for its highly antibiotic-resistant phenotype. It has been implicated in native-valve endocarditis, septicemia, peritonitis, wound, bone and joint infections. Other CNS species are involved in a variety of infections; *S. saprophyticus* causes urinary tract infections, especially in women[106], and *S. sciuri* associated with human infections (e.g. boils, wound infections and endocarditis) have been reported[107–109]. It may also be the main source of the *mecA* gene[110]**.** *S. lugdunensis* has been implicated in arthritis, catheter infections and prosthetic joint infections[111, 112]. Osteomyelitis caused by *S. schleiferi* and *S. caprae* associated with bone and joint infections have been reported[113–116]. *S. hominis, S. warneri, S. capitis, S. simulans, S. xylosus* and *S. cohnii,* are significant opportunistic pathogens[102]. CNS strains are commonly multi-resistant.

2.2.3 Epidemiology of staphylococcal infections

The word epidemiology is derived from the Greek words “epi” (on or upon), “demos” (people or population), and “logos” (word or reason) [117]. Thus epidemiology is the study of the reasons, incidences and patterns of disease in different groups of people. The incidence of disease is the rate at which new cases occur in a population during a specified period. Epidemiological information is used for planning and evaluating strategies to prevent morbidity and to guide the management of patients who have fallen ill (http:// bmj.com). Every year in the United States, about 400,000 hospital patients are infected by *S. aureus*, around 100,000 of these die from complications due to their infections (CDC; http://www.cdc.gov/ncidod/dhqp/ar_mrsa.html). The European Prevalence of Infection in Intensive Care study (EPIC) conducted in 1992 found that 30% of all nosocomial infections were attributable to S. *aureus* and 19% to CNS[118].

In Finland, around 3–5% of hospital patients develop a hospital-acquired infection which leads to about 500 deaths each year. In the hospital infection surveillance programme SIRO, *S. aureus* was in blood culture positive hospital infections (11%) and surgical site infections (18%) the second most common microbe in 1999–2003. The number of *S. aureus*

findings from blood increased by nearly 70% from 1995 to 2004 (from 12 to 20 cases per 100,000 population) http://www.ktl.fi/hif/hif.pdf [119].

Most MRSA infections originated in hospitals and long– term care facilities[120, 121]. In the late 1970s and early 1980s, MRSA became endemic in hospitals worldwide. These infections are called hospital-associated MRSA (HA–MRSA) to differentiate them from infections caused by strains of *S. aureus* that develop in community settings. Community-acquired MRSA (CA-MRSA) infections were documented in the mid-1990s, occurring in individuals who have no previous risk factors for MRSA infections, such as exposure to hospital [72, 122, 123]. Since then, the rates of MRSA infections in hospitals and the community have increased[124–128]. The incidence of methicillin-resistance among nosocomial isolates of *S. aureus* is, however, very high in some countries reaching even 70%. Along with the other Nordic countries, Finland has been a low-incidence MRSA country for a long time. However, within the last ten years both the incidence rates and the diversity of circulating MRSA strains have increased. The yearly number of new MRSA cases was 89 in 1995 and had climbed as high as 1741 in 2008 (Figure 2) (http://www3.ktl.fi/stat/). In Finland, methicillin-resistance rates in CNS vary between 70% and 80%[129], and similar high rates of resistance are also reported from the United States, Canada and Latin America[130].

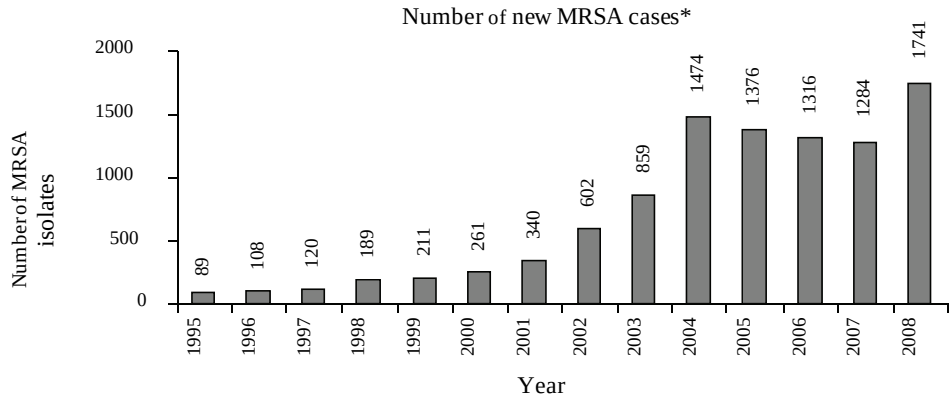


Figure 2. The number of new MRSA cases in Finland 1995 to 2008
*MRSA cases= laboratory notification including both asymptomatic carriers and cases with clinical disease.

2.3 Genomes of staphylococci

The *staphylococcus* genome consists of a single circular chromosome. The size of this circular chromosome varies from 2.4 to 3.07 million bp of DNA. The number of genes is about 2900 plus a collection of other genetic elements: plasmids, mobile elements, prophages and other variable elements. Kuroda and coworkers [131] completed the first whole genome sequence of two *S. aureus* strains in 2001**.** They used random shotgun sequencing to determine the genome sequences of MRSA strain N315 and VRSA strain Mu50. They found an elaborate combination of genes, many apparently acquired by horizontal gene transfer (HGT), indicating that these bacteria have a remarkable ability to acquire potentially useful genes from a variety of organisms. Eight genomic islands were identified on staphylococci. Seven of them are called pathogenicity islands that carry virulence genes[9, 123, 132–134]. The eighth one is the SCC*mec* which carries antibiotic resistance genes.

To date, 17 strains of *S. aureus* have been completely sequenced: Mu50, N315[131], MW2[132], NCTC8325[135, 136], MRSA 252, MSSA476[137], COL[133], USA300 (FPR3757)[138], USA300_TCH1516[139], RF122[140], Strain Newman[141], MU3[142], Strain 04–02981[143] , ED98[144], TW20 (0582)[145], HJ1 and HJ9[146]. The whole genome of two strains of *S epidermidis*; RP62A and ATCC12228[133], and one strain each of *S. haemolyticus* (JCSC1435)[147], *S .saprophyticus* ATCC1516[148], *S. carnosus* TM300[149] and *S. Lugdunsis* HKU09-01[150] have been completely sequenced to date (http://www.cbs.dtu.dk/services/GenomeAtlas/).

2.4 Antimicrobial agents

Antimicrobial agents, also known as antibiotics, are chemical compounds produced by microorganisms or are chemically synthesized. They are selectively toxic for microorganisms without causing the host any significant damage. The anti-microbial agents were ushered in with the accidental discovery of penicillin by Alexander Fleming in 1928; he noticed that growth of staphylococcal colonies was disrupted when growing near mould contaminating the agar plate. The year after, he published a paper describing this mould as *Penicillium notatum* and termed the active compound of it as penicillin[151], although it took over ten years before mass production of penicillin was achieved.

2.4.1 Modes of action and classification of antimicrobial agents

According to their mode of action antimicrobial agents can be divided into two major types: bactericidal agents which kill microorganisms directly, or bacteriostatic ones which inhibit growth of bacteria.

Antimicrobial agents obstruct the life cycle of bacteria in many ways (Table 1); by inhibiting cell wall synthesis, by inhibiting protein synthesis, or by inhibiting metabolic pathways. Antimicrobial agents are classified as being either narrow-spectrum (effective mainly against one or very few types of microorganism) or broad-spectrum (effective against several types of microorganism). The narrow-spectrum agents include penicillin G, glycopeptides, macrolides, nitrofurantoin, metronidazole, aztreonam, nalidixic acid, and phosphomycin. Broad-spectrum agents include the carbapenems, cephalosporins, β-lactam and β-lactam inhibitor combinations, and the fluoroquinolones[152]. The distinction between broad and narrow is not always clear, for example tetracycline and chloramphenicol are both usually classified as broad-spectrum agents, but the development of resistance to these drugs has imposed significant limitations on their spectrum of activity, thereby altering their precise classification (Table 1).

Table 1. Classification of antimicrobial agents based on the mechanism of action (adapted from[153–155])

Anti-microbial agent	Mechanism of action
β-lactams (penicillins) and semisynthetic β-lactams (methicillin, nafcillin, oxacillin), cephalosporins, imipenem, aztreonam, vancomycin, bacitracin	**Inhibition of cell wall synthesis** Inhibits steps in cell wall (peptidoglycan) synthesis and murein assembly
	Inhibition of protein synthesis
chloramphenicol, clindamycin, and erythromycin	Acts on 50S ribosomal subunit
aminoglycosides and tetracyclines	Acts on 30S ribosomal subunit
	Inhibition of nucleic acid synthesis
quinolones and ciprofloxacin	Inhibits DNA synthesis
rifampicin	Inhibits mRNA synthesis
clavulanic acid (often added to a β-lactam amoxicillin)	Inhibits bacterial β-lactamases
Growth factor analogs (sulfanilamide, sulfisoxazole acetyl, trimethoprim)	Inhibits folic acid metabolism (anti-folate)
polymyxin	Damages cytoplasmic membranes

2.4.2 Mechanisms of antibiotic resistance

One of the most significant events in clinical microbiology since the discovery of the antimicrobial agents has been the emergence of antimicrobial resistance in common pathogens, such as staphylococci, enterococci and gram-negative rods like *Klebsiella pneumoniae*, and *Pseudomonas* spp[156]. Bacterial resistance may result in treatment failure, which can have serious consequences, especially in critically ill patients.

Bacteria have evolved many strategies to resist the action of antibiotics. Bacteria may be intrinsically resistant to certain antibiotics, for example Gram-negative bacteria and penicillin, or to more than one class of antimicrobial agents, or they may acquire resistance by mutation[157] or via acquisition of resistance genes from other bacteria[155, 158]. Acquired resistance genes may allow bacteria to produce enzymes that destroy the antibacterial drug, to express efflux systems that prevent the drug from reaching its intracellular target, to modify the anti-microbial target site, or to produce an alternative metabolic pathway that bypasses the action of the drug[155].

2.4.3 β-lactam agents and resistance to β-lactams

The original group of β-lactam agents contains the penicillins. β-lactam agents are named after their characteristic ring structure (β-lactam ring) At present, β-lactams remain the most widely used antibiotics due to their comparatively high effectiveness, low cost, ease of delivery and minimal side effects. The targets of β-lactams in staphylococci are the four native PBPs[159, 160] which are enzymes involved in the synthesis of the cell wall. β-lactams can be inactivated by three primary resistance mechanisms[161].

1. By β-lactamases, enzymes produced by a large group of both gram-positive (e.g. staphylococci) and gram-negative bacteria, which inactivate β-lactams by hydrolysing the β-lactam ring.
2. By acquisition of low affinity PBP2a and by alteration of the native target of the antibiotic (e.g. staphylococci).
3. By prevention of access of the antibiotic to the target by way of altered permeability or forced efflux (e.g. *Pseudomonas).*

Genetically both mechanisms 1 and 2 are related to each other. They can also co-exist in the same cell and influence each other's expression[162–164].

2.5 Horizontal gene transfer (HGT)

Two types of gene transfer from one organism to another have been recognised; vertical and horizontal. Vertical gene transfer occurs between parents and offspring and HGT, also called lateral transfer, is a process in which a microbe can acquire genes from other microbes of the same species, or from different species, or even from different genus than that of the recipient. The significance of HGT for bacterial evolution was not recognised until the 1950s, when multidrug resistance patterns emerged on a worldwide scale[165]. Before the start of the genomics era, only a handful of horizontal gene transfer events were reported in the literature[166–169]. Currently, genomic sequencing is providing increasing evidence of extensive exchange of chromosomal genes. Horizontal gene transfer is now considered a major mechanism involved in bacterial evolution and responsible for the dissemination of numerous antimicrobial resistance determinants throughout different bacterial species. It is one of the reasons why antibiotic resistance genes spread so quickly[170].

2.5.1 Mechanisms of horizontal gene transfer

Theoretically, every organism is potentially capable of taking up DNA. Three mechanisms of gene transfer in bacteria have been recognised[171]:

(i) Conjugation (mating) has been well studied, especially in *E. coli*. Conjugation requires cell to cell contact, and is a process where plasmids or transposons transfer from donor to recipient cells[172]. Conjugation can occur between distantly related bacteria or even between different domains (between bacteria and plants, between bacteria and yeast or even between bacteria and mammalian cells)[173–175]. The exogenous genetic material can be integrated into the recipient genome through genetic recombination (Figure 3).

(ii) Transformation involves the uptake and incorporation of naked DNA, and it was first observed in *Streptococcus pneumoniae* in 1944 by Oswald Avery. The transformation of resistance genes in *Streptococcus spp.*, *Bacillus subtilis* and *Neisseria* species have served as good moudels for studying interspecies transformational events[176, 177].

(iii) Transduction whereby the host DNA is encapsulated into a bacteriophage which acts as the vector for its injection into a recipient cell[178]. Transduction is an important mechanism of genetic transfer among antimicrobial resistant bacteria. However, transfer of virulence factors and pathogenicity islands is even more common than transfer of resistance genes. One of the resistant pathogens most affected by transduction is MRSA[179].

2.5.2 Mobile genes

HGT between bacteria in the environment is one of the mechanisms for the generation of genetic diversity[180]. The mobile genetic elements play a primary role in the development and dissemination of antibiotic-resistance genes and allow bacterial populations to adapt rapidly to strong selection pressure [179, 181]. Elements involved in dissemination of resistance determinants among bacteria are numerous, and novel elements are still being discovered. The mobile genetic elements in bacteria consist of viruses, plasmids and transposable genetic elements (insertion sequences, IS, transposons and composite transposons). They are either self-transmissible or use mobile plasmids and viruses as vehicles for their dissemination[132–134, 137].

Plasmids are extrachromosomal genetic elements that are able to replicate independently of the host chromosome and are found in many bacteria and some yeasts, but they are not vital for growth. The copy number of plasmid in a cell remains constant from generation to generation[182]. Conjugative plasmids are considered self-transmissible. Plasmids frequently harbour antibiotic resistance and virulence genes. Many plasmids contain a large number of mobile genetic elements, such as IS elements, transposons, integrons and conjugative transposons[183, 184]. Most of the staphylococci carry one or more plasmids but there are some *S. aureus* strains that carry none[185].

Insertion sequences (ISs) are the simplest transposable elements and capable of inserting at multiple sites in a target molecule. The majority of ISs show short terminal inverted-repeat sequences (IR) of between 10 and 40 bp. Also on insertion, most ISs generate short directly repeated sequences (DRs) of the target DNA flanking the IS.

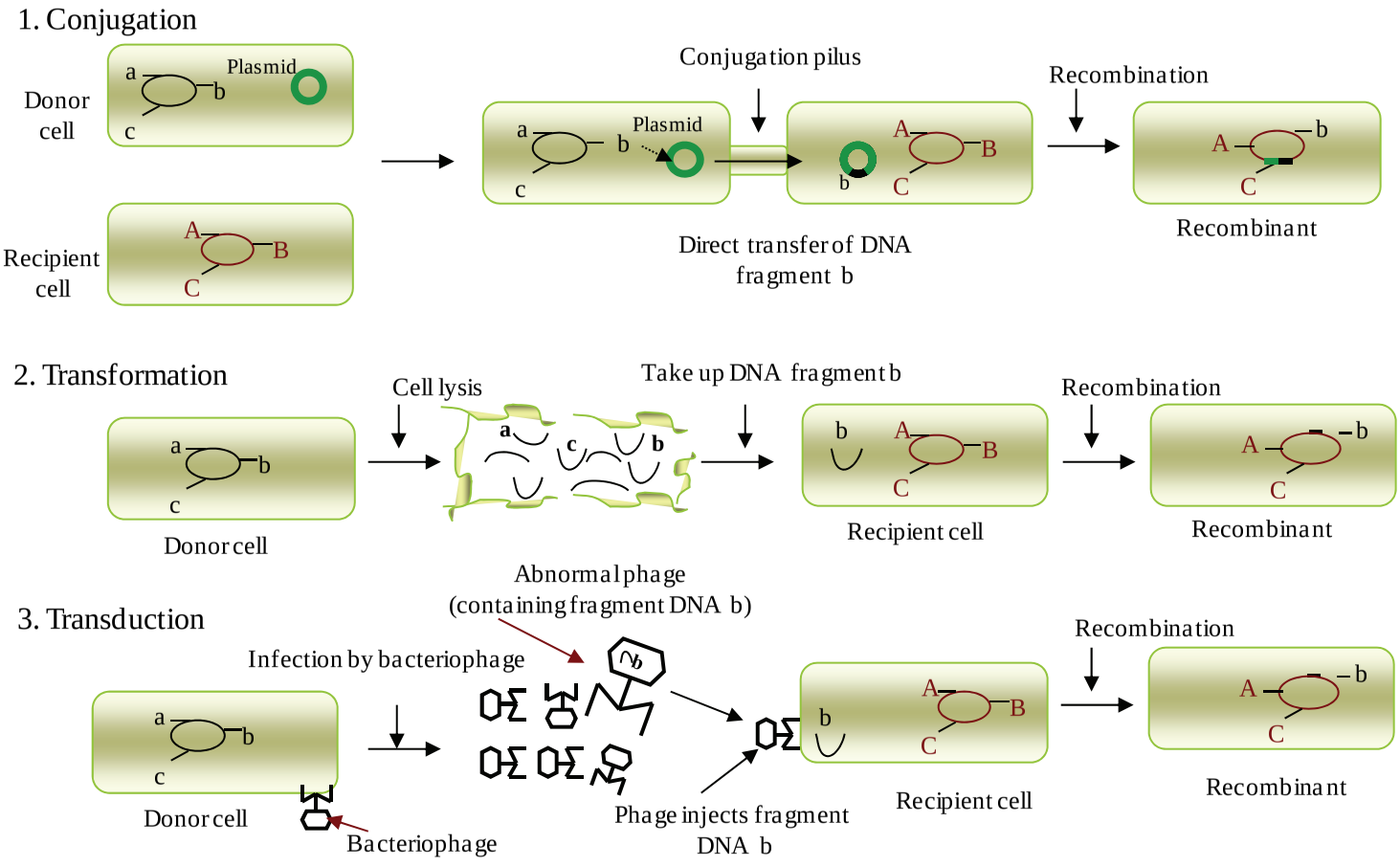


Figure 3. Diagram of different mechanisms for horizontal gene transfer. Adapted from (http://fig.cox.miami.edu/Faculty/Dana/bacfun.jpg**).**

The transposons (jumping genes) are mobile genetic elements, flanked by a pair of identical ISs, and can insert themselves within a structural gene. They may cause mutations or increase (or decrease) the amount of DNA in the genome. They are capable of jumping between different positions in the chromosome or between chromosomes and plasmids (e.g. Tn*5801*, is a site-specific transposon that encodes resistance to tetracycline in the MRSA strain Mu50). A lot of HGT has occurred during the evolution, therefore, it is even hard to clearly determine which organism is the donor and which one is the recipient in each case.

2.5.3 Evidence of horizontal gene transfer

The old general conception has held that HGT is rare, especially between distantly related organisms. The modern era of genomics has revealed increasingly numerous claims that HGT is frequent[186–189]. The first evidence that HGT might occur was the recognition that capsule specific genes could be transferred between pneumococci in infected mice[190]. HGT is most common among bacteria, especially those closely related to each other, occurring by all three mechanisms described above. Approximately 18% of the *E. coli* genome (about 750 genes) has been gained through HGT between two well-known and strongly related bacteria, *E. coli* and *Salmonella enterica*[187].

In the genus *Staphylococcus*, prophage-mediated conjugation is one of the most common mechanisms of genetic transfer. In this conjugation the presence of bacteriophages can increase the adhesiveness of the bacterial cell surface and therefore help in the conjugative transfer of genetic materials between two organisms [191]. Broad-host range conjugative plasmids with aminoglycoside resistance have been shown to transfer from streptococci to staphylococci[192]. The conjugative transposon harboring tetracycline resistance determinant (*tetM*) is transmissible between *S. aureus* and *Clostridia*[9]. Transposons (Tn*4001*) are found on the chromosome in staphylococci[193], and transposons closely related to it are found in enterococci[194] and streptococci[195].

In 1992, it was discovered for the first time that *Enterococcus faecalis* is able to transfer genes of vancomycin resistance to *S. aureus*. The transfer of the transposon Tn*1546* (vanA) conferring vancomycin resistance from *E. faecalis* to a clinical isolate of *S. aureus* was reported[196]. Both *E. faecalis* and *S. aureus* are common in hospital settings and face selective pressure to survive an attack by vancomycin. Conjugative mobilisation and

a self-transmissible plasmid transfer between staphylococcal species have been reported in many studies[197–203].

2.5.4 Significance of horizontal gene transfer

Horizontal gene transfer allows us to understand how asexual bacteria are able to exchange genetic material and create genetic diversity amongst their populations. Many studies propose that adaptation to the external environment and horizontal gene transfer among strains play important roles in the evolution of pathogens[40, 204, 205]. There is strong evidence that pathogenic bacteria can develop multi-drug resistance simply by acquiring antibiotic resistance genes from other bacteria[196, 206]. Recently, it has become increasingly evident that horizontal gene transfer is central to microbial activities that influence both our health and the environment.

2.6 Resistance mechanisms in bacteria in the 'pre-antibiotic' era

Bacteria have been present for billions of years, and they have developed exceptional defense mechanisms against each other. These mechanisms are used by bacteria against antibiotics.

The phenomenon of antibiotic resistance in some cases is innate to the microbe. Resistance mechanisms have been shown to be present before the introduction of antibiotics and they, obviously, arose by mutations of microbial genes or acquisition of genes from exogenous sources[165, 179, 207]. These resistance genes must have precursors in environmental microbes. The precursors of known antibiotic-resistance genes can be found in microbes living in very different environments and in various microbial groups.

Recently, shotgun sequencing of microbial genomes has discovered orthologues of known resistance genes in many pathogenic and nonpathogenic bacteria. These genes are closely related to the antibiotic-resistance genes. Although our understanding of the natural sources of resistance genes is increasing, their actual biochemical functions are still unknown. Most antibiotics are natural microbial products that are supposed to act as competitive or signalling agents in the microbial community. Several clinically important pathogens are ‘naturally’ resistant to antibiotics[208]. For example, *Pseudomonas aeruginosa* shows intrinsic resistance to many antibiotics. *Pseudomonads* are denizens of many hostile environments and can, proba-

bly, survive these conditions because of their ability to pump out various toxic organic compounds, including antibiotics[209].

2.7 Methicillin-resistance in staphylococci

In 1950, about ten years after the mass-production of penicillin, penicillinase-producing staphylococci became a therapeutic problem, especially in hospitals where numerous outbreaks were identified. Methicillin was introduced in 1959 to fight the growing problem of penicillin resistance. Immediately upon the introduction of methicillin into clinical practice, the first methicillin-resistant strains of staphylococci were identified, and the first cases of MRSA[210] and MRSE were published[211].

2.7.1 Evolution of MRSA

MRSA is born when a MSSA gains a genomic island known as SCC*mec*. Two hypotheses have been proposed to explain the evolutionary origin of MRSA strains. According to the clone hypothesis, MRSA isolates are descendants of a single original clone[212, 213]. Consistent with the second hypothesis, horizontal transfer of genes is a common evolutionary mechanism in *S. aureus.* The current whole genome sequencing of *S. aureus* strongly supports the latter proposal that MRSA strains have evolved a number of times by means of horizontal transfer of *mecA* into phylogenetically distinct MSSA precursor strains[214]. However, the mechanisms responsible for the possible horizontal transfer of *mecA* between staphylococcal species or between different gram-positive species are unknown. In addition, the frequency with which SCC*mec* is acquired is not completely known.

2.7.1.1 The origin of the *mecA* gene

The *mecA* gene is highly preserved among staphylococcal species[5, 215, 216]. The *mecA* gene is not native to *S. aureus* but acquired from an unknown donor by a HGT mechanism[5, 43]. It may have originated in the species *S. sciuri*, possibly an ancestral species and later transferred to other species (*S. aureus* and *S. epidermidis*)[109, 110]. To identify the source of the *mecA* gene, Cout and coworkers tested more than 200 isolates of different staphylococcal species that would react with a DNA probe internal to the *mecA* of *S. aureus*. A homologue of the gene *mecA* $_{aureus}$ was shown to be ubiquitous in isolates of the animal species *S. sciuri*. The *mecA* homologue identified in *S. sciuri* showed 79.5% DNA sequence similarity to the *mecA* gene of a

MRSA strain. However, the *mecA* gene of *S. sciuri* was not flanked with the regulatory genes and majority of the *S. sciuri* strains tested were susceptible to methicillin. Methicillin-resistant *S. sciuri* carry copies of both *mecA* $_{sciuri}$ and *mecA* $_{aureus}$ [217]. The *mecA* gene may thus be a native gene in the *S. sciuri,*[110] and the *mecA* of *S. sciuri* possibly is the originator of the *mecA* of *S. aureus.* Based on epidemiological and genetic evidence, it has been proposed that S. *sciuri pbpD* might represent the evolutionary precursor of the S. *aureus mecA* gene[218, 219]. The protein product of *pbpD* later became known as PBP4, one of the six PBPs detected in *S. sciur* [220]. The *S. sciuri* PBP4 share several biochemical properties with *S. aureus* PBP2A[221].

Recently, the whole genome of a methicillin-resistant *Macrococcus caseolyticus* strain JCSC5402 was completely sequenced[141], and it was found that *M. caseolyticus* harbours various antibiotic resistance determinants including those mediating resistance against β-lactams, aminoglycosides and macrolides. Additionally, it was revealed that one of the eight plasmids of *M. caseolyticus* harbours a *mecA* gene homologue, designated *mecA*$_m$, encoding a penicillin-binding protein similar to PBP2a of MRSA, with 72% amino acid sequence identity to *mecA* of *S. aureus*. The *mecA*$_m$ gene is flanked by β-lactam regulator genes, similar to *blaI-blaR1* (designated *mecI*$_m$-*mecR1*$_m$), and a β-lactamase gene similar to *blaZ* (*blaZm*). In 2010, Tsubakishita and co-workers revealed a new SCC*mec*-like element in *M. caseolyticus* strain JCSC7096[222]. This may be the ancestral form of the methicillin-resistance determinant of MRSA. However, the evolutionary origin of *mecA* and the formation of the *mec* element have remained largely a matter of speculation.

2.7.2 The genetic basis of methicillin resistance

As stated above, there are three mechanisms by which staphylococci become resistant to methicillin, one of them being the presence of an acquired penicillin-binding protein (PBP2a/PBP2`)[223]. Most clinical isolates of staphylococci are characterised by production of PBP2a[216, 224, 225]. Both susceptible and resistant strains of *S. aureus* produce four major PBPs: 1, 2, 3 and 4[216]. PBPs are membrane bound DD-peptidases that have evolved from serine proteases, and their biochemical activity is mechanistically similar to that of the serine proteases[159, 226]. These enzymes catalyze the transpeptidation reaction that cross-links the peptidoglycan of the bacterial cell wall. PBP2a is a unique transpeptidase with low affinity for β-lactams

Table 2. List and characteristics of strains from which the SCC*mec* has been sequenced. Strains with whole genome sequence are marked with *

Species	Strain	Country	Year of isolation	Size of genome (bp)	No of pro-phages	No of IS	No of trans-posons/plasmids	SCC*mec* type	GenBank accession number	Reference
S. aureus	N315*	Japan	1981	2,839,469	1	20	5/1	II	D86934	227
S. aureus	85/2082	New Zealand	1985					III	AB38513	7
S. aureus	NCTC10442	England	1961					I	AB33763	7
S. aureus	Mu50*	Japan	1996	2,903,636	2	23	3/1	II	BA000017	131
S. aureus	HDE288	Portugal	1996					VI	AF411935	232
S. aureus	MW2* (USA400)	USA	1998	2,820,462	2	6	0/1	IVa	BA000033	132
S. aureus	CA05	USA	1999					IV.1(IVa)	AB063172	11
S. aureus	8/6-3P	USA	1996					IV.4	AB063173	11
S. aureus	WIS	Australia	1995					V	AB121219	8
S. aureus	MRSA252* (EMRSA-16)	USA	1997	2,902,619	2	22	3/1	II	BX571856	137
S. aureus	AR13.1/330.2	Ireland	1999					II.E	AJ810120	233
S. aureus	AR43/3330.1	Ireland	1999					IV.E	AJ8101121	233
S. aureus	M03-68	Korea	2003					IVg	DQ106887	234
S. aureus	JCSC3063	Japan	2001					IIb	AB127982	235
S. aureus	JSGH17	Taiwan	1997-2002					V	AJ894415 AJ894416	236

S. aureus	COL*	UK	1960	2,813,862	1	6	0/1	I	CP000046	133
S epidermidis	RP62A*	USA	1979-1980	2,643,840	1	23	4/1	II	CP000029	133
S. aureus	USA300 * (FPR3757)	USA	2000	2,917,469	3	6	0/3	IV	NC-007793	138
S. aureus	ZH47	Switzerland	2003					SCC*mec*$_{ZH47}$	AM292304	237
S. aureus	JH1*	USA	2003	2,936,936			/1	II	NC009632	146
S. aureus	JH9 * (VISA)	USA	2003	2,937,129			/1	II	NC009487	146
S. aureus	PM1	Taiwan	2000-2006					VII (V_T)	AB462393	13
S. aureus	MU3* (VISA)	Japan	1997	2,880,168				II	AP009324	142
S. aureus	V14	India						III	AB425427	238
S. aureus	JCSC6826	Japan	2004					Pseudo-type II.5	AB435014	239
S. aureus	04-02981*	Germany	2004	2,821,452	2			II, *dru* deletion	CP001844	143
S. aureus	TW20(0582)*	UK	2003	3,075,806	3		5/2	III	FN433596	145
S. haemolyticus	JCSC1435*	Japan	2000	2,697,861	2	82	2/3	Novel type	AP006716	147
S. saprophyti-cus	TSU33	Japan	2003					*ccrA1*/*ccrB3* &class A	AB353724	240

that is found only in methicillin-resistant staphylococci and encoded by the *mecA* gene, which is located within a large chromosomal region initially designated as *mec* DNA. The first *mec*DNA region was sequenced by Ito *et al*[227] and the region was called a staphylococcal cassette chromosome *mec* (SCC*mec*)[228]. Since then, several SCC*mec* elements of MRSA strains have been sequenced, and more will be coming (Table 2).

Other genes independent of SCC*mec* have influenced methicillin resistance, such as the *bla* regulatory genes (*blaR1* and *blaI*) and factors essential for methicillin (*fem*) or auxiliary factors (*aux*). To date, over 20 *fem* or *aux* factors have been recognised[39, 162, 229, 230]. These factors are housekeeping genes and they are native in the genome of *S. aureus*. The majority of these genes are of unknown function but some of them encode the protein kinases and ABC transporters, possibly involved in signal transduction[231]or are membrane-associated proteins. None of these factors have been shown to influence PBP2a production.

2.7.2.1 Staphylococcal cassette chromosome *mec* (SCC*mec*)

SCC*mec* is one of the largest bacterial mobile elements known to date, its size ranging from 21 to 67 kbp, and it is exclusive to staphylococci [9, 241]. The SCC*mec* is considered to disseminate through horizontal transmission between staphylococcal species[227, 228]. The molecular structure of SCC*mec* consists of the *mec* gene complex, a pair of *ccr*-genes, and three junkyard regions (J1-J3). The J regions are located between and around the *mec* and *ccr* complexes, and contain different genes or pseudo genes. SCC*mec* elements are classified into types based on the characteristics of the *mec* and *ccr* complexes, and the variants of each SCC*mec* type are defined by differences in the J regions.

The SCC*mec* is integrated at a specific site in the staphylococcal chromosome (*attBcc*), which is located at the 3' end of the open reading frame X (*orfX*). The *orfX* encodes for a protein with an unknown function and is located near the origin of replication, between a gene encoding a protein involved in purine synthesis (*purA)* and the protein A encoding gene (*spa)*. The integrated SCC*mec* is bordered by a pair of 15 nucleotide direct repeats (DR-SCC and DR-B). The DR-SCC is found in the SCC*mec* directly next to *orfX*. DR-B is located at the other end of the SCC*mec* and is found outside the SCC*mec (attB)*. The SCC*mec* is also flanked by a pair of 27 bp inverted repeats (IRscc-L and IRscc-R) located at both ends of the

SCC*mec*. Most of the DRscc-R is located in, and partly cover, the IR next to *orfX* [8, 10, 131, 227].

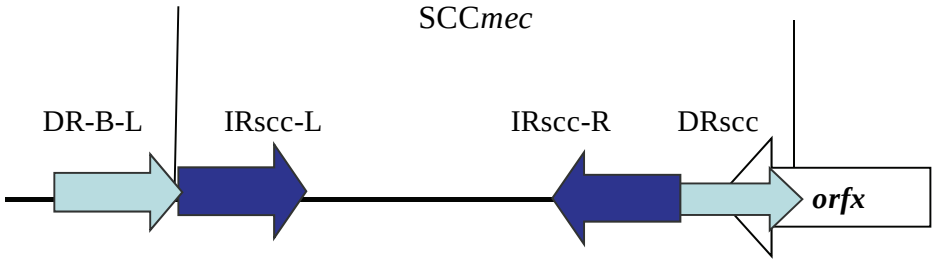


Figure 4. Schematic diagram of integration site sequence of SCC (*attB*SCC*)* as described by[131], *orfX* = Open Reading Frame, DR-B= Direct-repeats and IRscc= Inverted repeats.

2.7.2.1.1 The *ccr* gene complex

SCC*mec* carries specific cassette chromosome recombinase genes (*ccr*). The *Ccr* proteins belong to the invertase/ ressolvase family[7, 8, 10, 227] and carry characteristic catalytic motifs at the N-terminal domain. The *ccr* genes are responsible for the mobility of the element and its chromosomal integration and excision.

Three different *ccr* genes have been described: *ccrA*, *ccrB* and *ccrC*. The *ccr*A and *ccr*B are always found together and need each other to be functional[7]. They share about 80% identity. The *ccrC* gene is composed of only one recombinase gene and is able to induce mobility by itself[8]. To date, five types of *ccr* are known in staphylococci: type1 *(ccrA1* and *ccrB1*), type 2 *(ccrA2* and *ccrB2*), type 3 (*ccrA3* and *ccrB3*), type 4 (*ccrA4* and *ccrB4)* and type 5 (*ccrC*).

2.7.2.1.2 The *mec* gene complex

The *mec* gene complex is composed of an IS*431* (present in all *mec* classes) followed by the *mecA* gene and the regulatory genes *mecI* and *mecR1* or their truncated versions and insertion sequences, like IS*431* or IS*1272*. The *mecA* gene, which is 2,1 kb in length, is the structural gene that encodes for PBP2a, which has a low affinity for β-lactams[242, 243].

The regulatory genes *mecI* and *mecRI* are located upstream of *mecA* and transcribed in the opposite direction of the *mecA* gene[244]. The *mecR1-mecI* encodes the MecR1 single-transducer with a penicillin-binding domain (PB) and a memberan–spanning domain (MS) and MecI repressor. The *mecR1-mecI* is structurally and functionally closely related to the β-lactamase regulatory BlaR1 and BlaI[244, 245].

The BlaR1 and BlaI are able to control the expression of PBP2a when the *mecR1* and *mecI* genes are disabled[246–248]. The *mec* classes are defined by the arrangement of genes adjacent to *mecA*[233]. Six main classes of *mec* gene complex (A- E) and five subtype classes (A1, A.3, A.4 and B1, B2) have been described (Figure 5)[7, 8, 11, 12, 227, 228, 237, 249].

The class A *mec* consists of IS*431*, intact *mecA, mecR1 and mecI* [227]. The subtype of class A, class A1, has a 166 bp deletion in *mecR1* (MS). The class A.3 has an intact *mecR1* gene, but 119 bp of its *mecI* sequence is truncated by the insertion sequence IS*1182* at its 3`end. The class A.4 is similar to class A.3 but IS*1182* is inserted within the *mecI* and causes a 16 bp deletion of *mecI* directly upstream of the inserted IS*1182* [233].

The class B *mec* consists of IS*431*, *mecA* and *mecR1* partially deleted (PB totally and 64 bp of MS) by insertion of a partial copy of IS*1272*. The *mecI* gene is totally deleted by insertion of IS*1272*[228, 250]. The subtype class B1 consists of *mecA* and *mecR1* with partial deletion MS by insertion of the partial copy of IS*1272*. Class B2 is another variant of *mec* B with *mecR1* (MS) interrupted by Tn*400* [237].

The class C *mec* consists of IS*431*, *mecA*, *mecR1* (MS) and IS*431*; the *mecI* and *mecR1* (PB) genes are deleted and replaced by insertion of a copy of IS*431*[228]. The subtype class C1 is different to class C2 by the direction of IS*431* and the size of MS part the length of which in C1 is 968 and in C2 91 bp.

The class D *mec* consists of IS*431*, *mecA*, *mecR1 (*MS*). mecI* and *mecR1* (PB) are deleted without any insertion of an IS[228].

The class E consists of IS*431*, *mecA* and *mecR1 (*PB*). mecI* and *mecR1*,(MS) are deleted[251]. The genetic structures of all classes are shown in Figure 5.

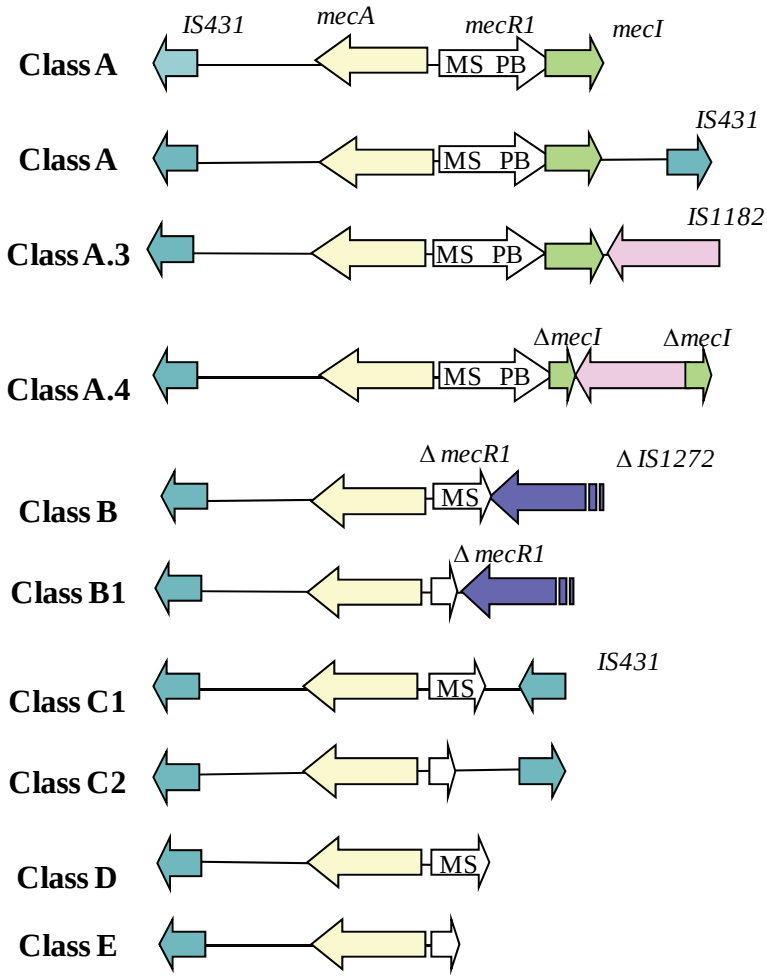


Figure 5. Schematic diagram of the different classes of *mec* gene complexes in staphylococci. Based on[9, 228, 233, 251]. MS: membrane-spanning domain and PB; Penicillin-binding domain.

2.7.2.1.3 The junkyard regions and additional resistance genes

The regions other than *ccr* and *mec* gene complexes in SCC*mec* are known as the junkyard J regions. The J regions can be divided into three regions based on their location in relation to the *ccr* and *mec* complex as follows: the J1 region is located between *ccr* and the chromosomal region flanking SCC*mec* (DR-B), the J2 region is located between *mec* and *ccr*, and the J3 region is located between *mec* and *orfX*. Certain J regions are commonly shared among certain types of SCC*mec* elements, but they are not always

specific to each SCC*mec* type. The difference in the J regions may be used to track the evolution and molecular epidemiology of SCC*mec*[9]. The size difference of the SCC*mec* types can be explained by the integration of various additional genetic elements in J regions of SCC*mec*. These additional elements include:

Genes encoding resistance; Tn*554* (encodes resistance to macrolides, lincosamides, streptogramin [MLS] and spectinomycin or cadmium resistance [*cadDX*]), integrated plasmid pUB*110* (encodes resistance to aminoglycosides), integrated plasmid pT*181* (encodes resistance to tetracycline), mercurial resistance operon (*mer*), copy of Tn*4001* (the resistance to aminoglycosides transposon) [7] and Tn*5405* carrying the genes *aphA-3*and *aadE,* which encode resistance to aminoglycosides[252].

Other genes not encoding resistance; *kdp* encoding for a potassium transport system, *pls* (plasmin-sensitive surface protein)[7] *hsd*R encoding an endonuclease, and the genes *hsd*S and *hsd*M encoding a DNA methyltransferase[253].

Five insertion sequences, IS*431,* IS*1272,* IS*1181,* IS*1182* and IS*256,* have been described in staphylococci[254, 255]. Briefly, IS*431* is a part of the *mec* gene complex which may be present as more than one copy in the same SCC*mec*. It has been found to be responsible for the deletion and rearrangement of the *mecA* regulatory genes, the mobility and accumulation of the resistance genes[7–9, 227, 256–258], and perhaps, to a degree, the expression of the *mecA*. IS*431* is regarded to be involved in the integration of exogenous material in the chromosome. Tn*4003* (trimethoprin resistance), *mer* operon, *cadDX* operon, *mupA* operon (mupirocin resistance gene) and other genes are flanked by copies of IS*431*[258, 259].

IS*1272* is a 1934 bp element consisting of two ORFs and 16 terminals IRs. A truncated copy of IS*1272* (Δ IS*1272*) has been found to truncate the *mecA* regulatory region[260]. IS*1272* is seldom detected in MSSA and some MRSA only carry a single partial copy of IS*1272*. On the contrary, IS*1272* is frequently found as multiple copies in *S. haemolyticus* while in *S epidermidis* it exists as truncated IS*1272*[250, 260, 261]. The *mecR1*- IS*1272* junction sequences of MRSA, *S. haemolyticus* and *S epidermidis* are identical. It has been suggested that IS*1272* is native to *S. haemolyticus*[7, 250, 260, 261].

IS*1181* is exclusive to *S. aureus* even though there are some isolates (as EMRSA-16) free from it[132, 137, 262, 263]. IS*1181* does not associate with any resistance genes.

IS*1182* is found as multiple copies in staphylococci[252, 259]. Transposon Tn*5405* is flanked by inverted copies of IS*1182*. IS*1182* has been found to be related to IS*1272* with 60% nucleotide similarity. IS*1182* inseration into the *mec* complex truncates the *mecI* gene[233].

IS*256* is in chromosome and on plasmids as multiple copies[264]. Transposon Tn*4001* is flanked by inverted copies of IS*256*[164, 193, 265]. It also plays a role in biofilm formation in *S. epidermidis*[266, 267].

2.7.2.2 Nomenclature and types of SCC*mec* elements

The SCC*mec* types are classified based on the combination of *ccr* type and *mec* class, and further classified into subtypes according to differences in their J region DNA. There are two systems for naming SCC*mec*. The first is by Roman numerals (I- VIII) given to SCC*mec* in the order of appearance. This nomenclature has been used widely (Ito, 2003). The second naming system is described by Arabic numerals for *ccr* types (1, 2, 3,…) and an upper case letter for *mec* class (A, B,…)[249]. To date, eight main SCC*mec* types have been recognised: types I (1B), II (2A), III (3A), IV (2B), V (5C2a), VI (4B), VII (5C2b) and VIII (4A)[6, 8, 9, 11–14]. These types are illustrated in Figure 6. In addition to the main types, a number of subtype SCC*mec* elements have been revealed[268].

Type I (34 kb) has *ccr* type 1, *mec* class B and the *pls* gene in the J1 region. It is found in a group of MRSA isolates, detected in the early 1960s[7]. It is also found in the pandemic Iberian clone[269]. The subtype IA (40 kb) differs from type I by the presence of an integrated plasmid pUB*110* flanked by two copies of IS*431* in the J3 region[232].

Type II or IIa (53kb) has *ccr* type 2, *mec* class A and *kdp* operon in the J1 region, Tn*554* in the J2 region, and integrated plasmid pUB*110* flanked by two copies of IS*431* in the J3 region. It has been identified in the pre-MRSA strains that were in 1982 in Japan[227]. The subtype IIb differs from type II by the presence of a copy of IS*256*, and absence of *kdp* operon and integrated plasmid pUB*110*. It has been found in MRSA strains isolated from healthy Japanese children[235].

The other five subtypes of the SCC*mec* II type (31-40 kb) have been found among the Irish MRSA strains isolated between 1971 and 2002[233]. In all these subtypes (IIA- IIE), the *kdp* operon in the J1 region is absent, and they share the J1 region of type IVb. Subtype IIA has class A.4 *mec*, type 2 *ccr*, Tn*554* in theJ2 region and integrated plasmid pUB*110* flanked by two copies of IS*431* in the J3 region. IIB differs from type II only by the absence of Tn*554*. IIC carries class A.3 *mec* and truncated Tn*554*. IID has a class A.4 *mec*, and IIE carry a class A.3 *mec*, and both lack the integrated plasmid pUB*110*. Recently, two subtypes (type II.5 and pseudo-type II.5) were identified[239].

Type II.5 has *ccrA2* and class A, and in its J1 region it does not carry the *kdp* operon. Pseudo-type II.5 has *ccrA2* and class B, and in its J1 region it does not carry Tn*6012 kdp* operon and the Tn*554*.

Type III (66,9 kb) was first identified in an MRSA strain isolated in 1985 in New Zealand. Type III carries a number of antimicrobial resistance genes in its J regions. It has class A *mec*, type 3 *ccr* and a copy of Tn*554* in the J2 region. The J3 region contains SCC*mercury* that consists of *ccrC*, *mer* operon and Tn*554*, and integrated plasmid pT*118*.

Type IIIA differs from type III by the presence of integrated plasmid pUB*110* with its flanking IS431 in the J2 region. Type IIIB lacks SCC*mercury* and integrated plasmid pT*118*[7, 232, 249].

The type IV to VIII SCC*mec* elements are characterised by their small size (21 to 31 kbp) and lack of resistance genes other than *mecA*. They are widely disseminated among CA-MRSA strains[6, 9, 11, 13, 236, 270, 271].

Type IV (IVa) harbours type-2 *ccr*, class B *mec* and the J1 region, which is specific for IVa[11]. Several subtypes of type IV have been reported[268]. Briefly, the differences between subtypes are in their J regions. In subtype IVb, the J1 region is specific for IVb[11], In subtype IVc, the J1 region is specific for IVc, and it carries Tn*4001* with its flanking IS256 in the J3 region[272, 273]. In subtype IVd, the J1 region is specific for IVd[235].

In subtype IVE, the J1 region is identical to IVc, and it has a unique left extremity sequence. In subtype IVF, the J1 region is identical to IVb, and it has a unique left extremity sequence[233]. Subtype IVg harbours *ccr2*, *mec* class B and J1 specific for IVg[234] and IVA carries integrated plasmid pUB*110* with its flanking IS*431* in the J3 region[232].

Type V harbours type-5 *ccr* (*ccr*C*)* and class C2a *mec*, and carries genes encoding a restriction-modification system (*hsdR, hsdS and hsdM*) in the J1 region. This system might play a role in the stabilisation of the element within the chromosome[8]. A few subtypes have been described for type V[274].

Type VI harbours type-4 *ccr*, class B *mec* and a specific J1 region[12].

Type VII (V_T) has type 5 (*ccrC2*), class C2b *mec* and *ccrC8*[6, 13, 236, 271]. Novel SCC*mec* type has been identified in a Swedish CA-MRSA strain. It carries type 5 *ccr* (*ccrC*) and class C1 *mec*[275, 276].

Type VIII (32,126 kb) is a novel SCC*mec* type found in a Canadian MRSA strain. It carries type 4 *ccr* and class A *mec*[14].

2.7.2.3 SCC types without the *mec* complex

SCC is a site specific mobile element inserted at the same site as SCC*mec* and may have the same function as in SCC*mec* or no function. Numerous SCCs without the *mec* gene complex have been found in *S. aureus* and CNS.

(i) The SCC *capI* (35kb) encodes the type 1 capsular polysaccharide and contains truncated *ccrC.* It is found in *S. aureus* (NCC8325)[277].

(ii) The SCC_{12263} (23kb) carries a functional type 1 *ccr* gene complex. It has been detected in a methicillin-susceptible *S. hominis* strain (GIFU12263)[278].

(iii) The SCC_{476} (22.8 kb) is found in MSSA476 strain. It carries type 1 *ccr* gene complex, *far* gene encoding resistance to fusidic acid, and type I restriction modification system genes[137, 279].

(iv) The SCC composite island (57kb) is found in *S. epidermidis* strain (ATCC 12228). It carries type2 *ccr*, type 4 *ccr* and SCC*pbp4*[273]. Recently identified GrMSSA isolate M06/0075 harbors a SCC*mec* IID remnant that lacks part of *mecI* and totally *mecR1*, *mecA* and IS*431*[280]. A similar SCC*mec* IID has been identified also previously[233].

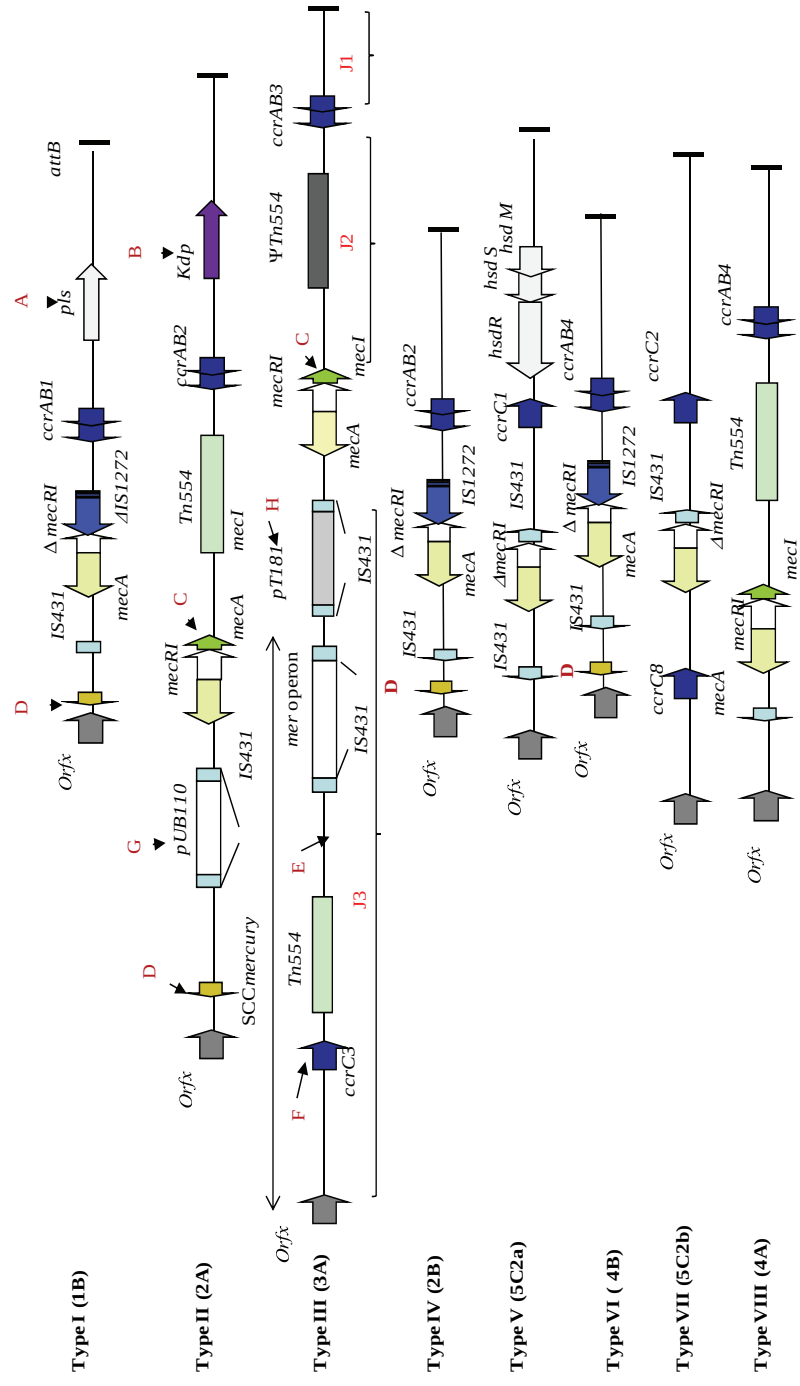


Figure 6. Schematic structures of MRSA SCC*mec* elements of types I to VIII based on the findings of [6, 8, 9, 11-14]. Horizontal arrows within the cassette indicate the direction of SCC*mec* elements. Appropriate letters (A–G) indicate the eight region loci that defined by multiplex PCR.

2.8 Tools for typing staphylococci

First, some terms closely related to the typing methods need to be clarified: a bacterial isolate can be defined as a single isolation in pure culture from a clinical specimen for which no information is available aside from its genus and species. A strain is an isolate that can be distinguished from other isolates of the same genus and species. Clones (genetically related isolates) are identical by a variety of typing methods or so similar that they are assumed to be derived from a same parent. Outbreak strains are defined as two or more isolates detected in the same place at the same time period, and they share the same or similar genotype patterns. They are presumed to be clonally related since they have common phenotypes. Epidemic strains are defined as two or more isolates in two or more places and they have the same genotype patterns, which suggest that the isolates may be derived from a common source. A sporadic strain is defined as a strain isolated from one person, and it has a unique genotype pattern[127, 281, 282]. These definitions are used in this study.

Typing methods are used as tools to help determine possible sources of microbial infections and to determine the spread of bacteria. Microbial typing contributes to the understanding of the epidemiology of infection. This information is helpful for prevention and control of infectious diseases in both hospitals and communities[281, 283, 284].

In practice, typing is most valuable in outbreak investigations when it is applied to small sets of isolates that are epidemiologically related. For instance, monitoring and limiting the intra- and inter hospital spread of staphylococcal strains requires the use of rapid and accurate epidemiologic typing systems. Currently, several typing techniques have been developed for typing staphylococci [284, 285]. The main task of any typing method is to determine whether epidemiologically related isolates are also genetically related. Traditionally this has depended on the comparison of phenotypic characteristics, such as biotypes, serotypes, bacteriophage types and antimicrobial susceptibility testing. During the past two decades these phenotyping methods have been largely replaced by genotyping methods based on analysis of DNA or other molecules. The genotyping methods include restriction-based methods like pulsed-field gel electrophoresis (PFGE), random amplified polymorphic DNA (RAPD) and plasmid profiles, Southern hybridisation based methods like ribotyping and binary typing, PCR-based typing methods like *mecA, nuc* typing and SCC*mec* typing, and DNA sequencing based methods like multilocus sequence typing (MLST),

spa sequencing typing and multiple-locus variable-number tandem-repeat typing (MLVA). The merging of molecular methods for typing MRSA has greatly helped to evaluate strain interrelationships[2, 286–291].

The criteria for evaluation of typing techniques can be divided into two categories: performance criteria (efficacy) and convenience criteria (efficiency)[281–284]. The performance criteria include (i) Typability, the ability of a technique to type all isolates tested by it. (ii) Reproducibility, the ability of a typing technique to give the same result on independent occasions, separated in time and/or place[292]. (iii) Discriminatory power, the ability of a technique to give a different result to two unrelated strains sampled randomly from the population of a given species. Discriminatory power should be high for outbreak investigation to be successful. The convenience criteria evaluate the practicality of the typing technique. They include flexibility (the technique can type any pathogen by modifying the protocol), rapidity (saving time), simplicity (ease of use), and the costs of the method (costs of the necessary apparatus and material)[284]. All typing systems can be characterised in terms of typability, reproducibility, discriminatory power, flexibility, rapidity, simplicity and the expenses[284, 290]. Some of the most widely used staphylococcal typing methods are described briefly in this review (Table 3).

2.8.1 Phage typing

Bacteriophages or phages are viruses that are able to infect and lyse bacterial cells. The differential ability of phages to infect certain cells is based upon the availability of corresponding receptors on the cell surface for the phage to bind[285]. Different strains have a different group of receptors, leading to variable lysis profiles. Phage typing classifies bacteria based on the pattern of resistance or susceptibility to an international set of 24 phages. These phages are used at a standard dilution known as routine test dilution (RTD) and at 100x RTD[293, 294]. Phage typing has been the traditional typing method and it has been very valuable in epidemiologic investigation over the past decades [284, 291, 295]. Limitations of the phage typing method include a lack of any systematic biological basis and technical difficulty[213, 291].

2.8.2 Antimicrobial susceptibility testing

Antimicrobial susceptibility testing is frequently used in the clinical microbiology laboratory. The ensuing antibiogram indicates the pattern of *in vitro* resistance or susceptibility of an organism to a repertoire of antimicrobial agents[296, 297]. It is inexpensive and rapid compared to other conventional methods[291]. There are globally used guidelines for antibiogram testing provided by CLSI (http://www.clsi.org). Antimicrobial susceptibility testing often provides the first indications of an emerging outbreak, despite the fact that its discriminatory power is poor. In addition, susceptibility pattern of some isolates are variable by the time, transfer and storage[285, 298]. The genetic relation between isolates cannot be identified by antimicrobial susceptibility testing[291].

2.8.3 Ribotyping

The most common Southern hybridisation method used is ribotyping[299]. Ribotyping consists of restriction endonuclease fingerprinting of ribosomal RNA (rRNA) genes visualised by Southern hybridisation with an rRNA probe. In this technique, genomic DNA is first isolated and digested with restriction enzymes and then the DNA fragments are separated in gel electrophoresis. The DNA fragments are transferred onto a nylon membrane and then hybridised with labeled DNA probes specific for 16S and 23S rRNA[248, 300]. It can be used for all isolates and its reproducibility is good, which makes it a suitable method for long-term studies of staphylococcal epidemiology. However, for short term studes its discriminatory power is too low[291].

2.8.4 Pulsed-field gel electrophoresis

PFGE is one of the earliest molecular methods[301] commonly used in the typing of human bacterial pathogens and investigation of disease outbreaks. It is an important method for short-term studies of staphylococcal epidemiology[291] but not suitable for long term studies. In this technique, the bacterial chromosome is first isolated and then digested with restriction enzymes to produce pieces small enough to be resolved by pulsed electrophoresis in agarose. This digestion yields several linear molecules of bacterial chromosome that usually is circular[281–283, 302-304]. Genomic restriction digests of various bacterial species can be analysed by PFGE with only minor modifications in the protocol[305]. The PFGE method for various bacteria has been harmonised to increase and improve the epidemiological

surveillance on a worldwide scale. An example of this is the harmony project for *S. aureus*[306] (http://www.harmony-microbe.net/).

PFGE types are determined with Bionumeric software (Applied Maths, Kortrijk, Belgium). However, PFGE is not flawless. For example, DNA degradation can occur in the gel preventing typing of some strains[307], and also some strains can not be typed by PFGE due to DNA methylation of the restriction enzyme (*Sma*I) recognition sites[308]. Additionally, it is time–consuming, the PFGE equipment is relatively expensive and the inter-laboratory exchange of results is difficult[282].

2.8.5 SCC***mec*** **typing**

Polymerase chain reaction (PCR) is one of the most effective molecular biology techniques developed in recent decades. It was described by Saiki and coworkers[309]. In this technique, multiple copies of a target DNA segment can be produced. It relies on previous knowledge of the nucleotide sequences of the target sites. Many PCR-based methods for SCC*mec* typing have been developed.

(i) The short- and long-range PCRs have been developed to determine the structure of the *mec* complex and identify the different types of *ccr* gene[7, 270].

(ii) A single-step multiplex PCR assay has been developed by Oliveira and de Lencastre. The method is based on the detection of *mecA* and eight loci in SCC*mec* region, seven loci in J regions (A, B, D, E, F, G and H) and one locus (C) in the *mec* complex (*mecI* gene). The SCC*mec* types can be classified into I, IA, III, IIIA, IV and IVA[232]. These two methods are most often used.

(iii) A multiplex PCR assay was developed by Zhang and coworkers. The J1 region of eight SCC*mec* elements (I, II, III, IVa–IVd andV) can be identified by this method [310].

(iv) A multiplex PCR assay was developed by Boye *et al.* It contains four primer sets for identifying the five main SCC*mec* types (I-V) [311].

(v) The PCR scheme developed by Kondo *et al*. consists of six multiplex PCRs. A multiplex PCR with primer set 1 (M-PCR 1) identifies five types of *ccr* genes, M-PCR 2 identifies class A to class C *mec*, M-PCRs 3 and 4 identify specific open reading frames in the J1 regions of type I and IV and of type II, III, and V SCC*mec* elements, respectively, M-PCR 5 identifies the

transposons Tn*554* and copy Tn*554* integrated into the J2 regions of type II and III SCC*mec* elements, and M-PCR 6 identifies plasmids pT*181* and pUB*110* integrated into J3 regions. Almost all the SCC*mec* types can be identified by this method [312].

(vi) Recently, Milheirico and coworkers updated the multiplex PCR assay described in 2002 by Oliveira *et al.* The updated method improves the detection of SCC*mec* types I to IV and includes the determination of the structure of SCC*mec* types V and VI [313].

(vii) Staphylococcal *ccrB* sequence typing was developed by Oliveira and co-workers. The method based on the sequence of an internal fragment of *ccrB* gene can classify the SCC*mec* types from I to IV[12]. A staphylococci *ccrB* sequence typing database Internet website has been developed (http://www.*ccr*btyping.net). Occasionally, these methods give different results in SCC*mec* typing of the same MRSA isolate[314].

2.8.6 Multilocus sequence typing

Multilocus sequence typing (MLST) is a typing technique based on nucleotide sequences of internal fragments of seven housekeeping genes. It can be used for all bacterial species[315–318]. A different number is given to each different sequence of the same allele. The alleles of the seven genes define an allelic profile designated sequence type (ST). The algorithm based upon related sequence types (BURST) is used to define clonal complexes (CCs) and to study the evolutionary events within an *S. aureus* population (www.eburst.mlst.net).

In 2000, MLST *S. aureus* scheme was developed by Enright *et al*[2] (www.mlst.net). In 2003, two MLST *S. epidermidis* schemes were developed[319, 320]. Wang scheme is based on the MLST *S. aureus* scheme[2] but Wisplinghoff's scheme is different and was set on MLST home page. In 2007, a new MLST *S. epidermidis* scheme was described by Thomas *et al*[321] and the Wisplinghoff scheme was replaced by the Thomas scheme on the MLST home page www.mlst.net. MLST typing is an excellent method for studying the molecular evolution of bacteria and is a highly discriminatory method. MLST typing data can be shared between laboratories via the Internet; other typing methods are very poor in this point. The drawback of the MLST method is that it is costly, lengthy (many steps), time consum-

ing and not useful for investigation of local outbreaks. Nor does it tolerate even a single nucleotide mistakes in sequencing[282, 283].

2.8.7 *spa* typing

This method was described by Frenay. It is based on the sequence of the polymorphic X region of the MRSA protein A (*spa*) locus, which contains a number of 24 bp repeat units[322]. The repeat units have a slow point mutation rate but faster changes occur in the number of repeats due to the gain or loss of repeats[323–325]. A *spa* type is composed of different repeats, each of which represents 24 nucleotides (eight codons). The repeats of the *spa* types are determined with software Ridom StaphType (Ridom GmbH, Würzburg, Germany). By using this software the results can be exchanged between laboratories worldwide. It also means that a common nomenclature (http://www.spaserver.ridom.de/) can be used. The discriminative power of *spa* typing is almost as good as that of PFGE, but it is lower than the power of MLST. *spa* typing can be used for both short- and long-term studies of MRSA epidemiology[324]. *spa* typing has rapidly become an important and most widely used method and it currently is the primary technique for typing MRSA in some laboratories. Because only a single locus is sequenced in *spa* typing, compared to seven loci in MLST, it is less expensive, less laborious and less time consuming. However, there are two nomenclature systems available[324, 326], which would make difficult to compare results.

2.8.8 Multiple-locus variable-number tandem-repeat typing

Multiple-locus variable-number tandem repeat analysis (MLVA) is a typing technique based on a variable numbers of tandem repeats (VNTR). Currently, the MLVA technique is used for typing many bacterial species[282, 327–336]. The first MLVA typing for *S. aureus* was developed by Sabat *et al.*[337]. It was based on five VNTR loci (*sdr, clfA, clfB, ssp* and *spa* gene). Later, many MLVA schemes have been developed[282, 338–341]. In 2009, a new MLVA scheme for *S. aureus* was described[342]. The target genes in this scheme are VNTR09-0 (*sspA*), VNTR61-01, VNTR61-02, VNTR67-01, VNTR21-01, VNTR24-01 (*spa*), VNTR63-01 and VNTR81-02(*coa*). The main advantage of this method is its portability. MLVA typing for *S. epidermidis* was developed by Johansson *et al.*[343]. It was based on five VNTR loci (Se1-Se5). The MLVA database Internet website was developed by Le Feche and Denoeud ([344, 345

(http://minisatellites.upsud.fr/MLVAnet/querypub1.php). In general, MLVA is a highly discriminatory typing method and it is easy to use. MLVA is as valuable as PFGE, but MLVA typing data can be easily shared between laboratories. However, MLVA techniques are still in the development stage.

2.8.9 Tools for typing of Finnish MRSA

The system for typing Finnish EMRSA isolates (MRSA isolates that share the same typing patterns and are isolated from two or more persons in several hospitals) has been constantly updated. From the beginning of 1992 to 2001 there were four typing methods: PFGE, phage typing, ribotyping and antibiogram[127], and PFGE was the primary method for the identification of Finnish MRSA strains. The EMRSA were named according to the geographic location where they were first identified. In the beginning of 2002, phage typing and ribotyping were replaced by MLST, *Spa* typing and SCC*mec* typing. The new FIN names with numeric codes have replaced the old names[346]. In 2008, the *spa* typing became the primary method. The different EMRSA strains were named according to the results gained from these methods.

Table 3. Comparison of typing methods of staphylococci (adapted from: [281, 283, 284, 336]). *MLVA method is still in the developmental stage

Methods	Simplicity (Ease of use)	Rapidity (no culture time)	Typability[a]	Discriminatory power[b]	Reproducibility[c]	Interpretation	Standard nomenclature	Comparability[d]	Cost
Phage typing	Moderate	≤ 3 days	Partial	Poor	Poor	Moderate	No	Poor	Low
Anti-biogram	Easy	≤ 2 days	All	Poor	Good	Easy	No	Poor	Low
PFGE	Difficult	≤ 4 days	All	Good	Good	Difficult	No	Difficult	Medium
Ribotype	Moderate	≤ 5 days	All	Moderate	Fair	Difficult	No	Moderate	Medium
SCC*mec* typing	Easy	≤ 2 days	All	Fair	Excellent	Easy	Yes	Excellent	Low
MLST	Difficult	≤ 7 days	All	Good	Excellent	Moderate	Yes	Excellent	High
spa sequencing	Moderate	≤ 3 days	All	Good	Excellent	Moderate	Yes	Excellent	High
MLVA*	Easy	≤ 3 days	All	Excellent	Excellent	Easy	Yes	Excellent	Low

[a] Typability: the ability of the method to provide a clear result for each isolate analysed.
[b] Reproducibility: Independency of the operator, time and place.
[c] Discriminatory power: The ability of the method to differentiate unrelated isolates.
[d] Comparability: Ease of exchanging results electronically between laboratories

3 AIMS OF THE STUDY

The purpose of this study was to study the genetic characteristics and putative horizontal transfer of the mobile genetic element SCC*mec* in Finnish *S. aureus* isolates and coagulase-negative staphylococci.

The specific aims were:

1. To study the diversity of the genomic backgrounds of epidemic MRSA isolates in comparison to methicillin-sensitive *S. aureus* (MSSA) isolates in Finland.

2. To study the molecular characteristics of bacteremic methicillin-resistant *S. epidermidis* (MRSE) strains, and to compare SCC*mec* of *S. epidermidis* with that of *S. aureus.*

3. To study the SCC*mec* elements in the carriers of methicillin-resistant staphylococci in a long-term care facility in Finland and especially the structure of the SCC*mec* elements of methicillin-resistant coagulase-negative staphylococci (MR-CNS) and MRSA isolated from the same person at the same time.

4. To study the SCC*mec* elements in clinical human isolates of *Staphylococcus sciuri*, the species suggested as the origin of *mecA* genes.

4 MATERIALS AND METHODS

4.1 Bacterial strains and the patients (I, II, III, IV)

The study material included a total of 528 bacterial isolates belonging to *Staphylococcus* species (Table 4). The isolates were stored at -70˚C in sterilised skim milk. In the studies I, II and IV, one isolate per patient was studied. In study III, one to five isolates per patient were studied. The control strains used are listed in Table 5.

In study I, the 299 clinical isolates of MSSA were collected by the clinical microbiology laboratory of the Helsinki University Central Hospital (HUCH) during the three last months of 2001. These isolates were from a variety of infection sites. HUCH serves several hospitals and health care centres in Southern Finland with a population of nearly 1.4 million. In addition, study I included 24 EMRSA isolates derived from the THL culture collection, which includes all clinical and carriage MRSA isolates collected since the early 1990s.

In study II, 60 clinical MRSE blood isolates were studied, including 22 MRSE isolates collected from a neonatal intensive care unit (NICU) of Children's Hospital of Helsinki between March 1997 and May 1998, and 38 MRSE isolates that previously had been characterised as true bactermic isolates and were considered to represent the most prevalent clones causing MRSE bacteraemia in four different units at HUCH in 1990–93[347].

In study III, a one day point–prevalence study of the nasal carriage of staphylococci was carried out six months after an outbreak of MRSA in a long-term care facility (LTF). 127 CNS and 24 *S. aureus* isolates were obtained from nasal swabs of 76 patients. One swab per patient was taken from both nostrils.

In study IV, the fifteen *S. sciuri* clinical human isolates of three subspecies isolated from various sites were provided by Dr Jérôme Etienne, Centre National de Référence Staphylocoques, Lyon, France[348].

4.2 Identification of staphylococcal isolates (I, III)

The swabs were cultivated on nonselective sheep blood agar (SBA, CM1008, Oxoid, United Kingdom) and incubated overnight at 37˚C.

All the isolates were identified by standard laboratory methods, including the catalase test, coagulase test, conventional biochemical tests and API ID 32 Staph (BioMe´rieux, Marcy l'Etoile, France). In some instances, the

GenoType® Staphylococcus (Hain Lifescience, Germany) test was performed.

4.3 Antibiotic susceptibility testing (I, II, III)

Antibiotic susceptibility testing was performed by disc diffusion method and the results were interpreted using CLSI guidelines[349]. The following antibiotics were tested: ampicillin, penicillin, cephalexin, cefuroxime, gentamicin, tobramycin, erythromycin, clindamycin, chloramphenicol, ciprofloxacin, rifampin, fusidic acid, trimethoprim- sulfamethoxazole, tetracycline, vancomycin, teicoplanin and mupirocin (AB Biodisk, Solna, Sweden). Minimal inhibitory concentration (MIC) to oxacillin was tested by using E-test (E-test; AB Biodisk, Solna, Sweden). The MRSA strains were verified by testing for the presence of the nuc and *mecA* genes as described previously[350].

Table 4. Bacterial isolates and methods used in the study

Study	*Staphylococcus* species	Properties	Site of isolation (No. of isolates)	Year of isolation	Methods used
I	*S. aureus*	Clinical isolates MSSA	Wound (118), urine (38), upper respiratory tract (27), skin (27), abscess (24), eye (21), ear (20), blood (10) and other sites (14)	2001	Conventional microbiological methods Antimicrobial susceptibility testing, PFGE Ribotyping MLST
		Epidemic MRSA	Various sites (24)	1992-2002	
II	*S. epidermidis*	Bacteremic MRSE isolates	Blood (38) Blood (22)	1990-1993 1997-1998	Antimicrobial susceptibility testing, PFGE, SCC*mec* typing and MLST
III	*S. aureus*	Nasal carriage isolates	Nose (18)	2004	Conventional microbiological methods Antimicrobial susceptibility testing, PFGE, SCC*mec* typing and MLST
	Coagulase negative staphylococci		Nose (127)		
IV	*S. sciuri* *S.rodentium* *S.carnaticus*	Clinical human isolates	Various sites (8) Various sites (5) Various sites (2)	1990-1997 (Marsou *et al.*1999)	PFGE Southern hybridisation Dot blot hybridisation

Table 5. The control strains used in this study

Species and strain	Procedure	Study	Reference/ Source
S. aureus MSSA (ATCC25923), *Escherichia coli* (ATCC25922), *Pseudomonas aeruginosa* (ATCC27853)	Antimicrobial susceptibility testing	I, II, III	American Type Culture Collection (ATCC, USA)
S. aureus (NCTC 8325) Citrobacter koseri	PFGE Ribotyping	I, II, III, IV I	Harmony collection (http://www.harmony-microbe.net/microtyping.htm) THL Culture Collection
MRSA Iberian clone (HPV107) UKEMRSA-16 (96/32010) Brazilian clone (HSJ216) Pediatric clone (HDE288) Finnish EMRSA (FIN-22, IH 101468)	SCC*mec* typing Southern hybridisation Dot blot hybridisation	II, III, IV	Harmony collection (http://www.harmony microbe.net/microtyping.htm THL Culture Collection
S. epidermidis (ATCC 12228)	MLST	II	American Type Culture Collection (ATCC, USA)
S. aureus, Finnish MRSA 1061 Finnish EMRSA FIN-2b (IH 99524)	Southern hybridisation Dot blot hybridisation	IV	 P. Kuusela *et al.,* 1994 THL Culture Collection

4.4 Molecular genotypes (I-IV)

4.4.1 DNA extraction (I, II, III, IV)

In studies I, II and IV, the bacterial genomic DNA was prepared by the rapid guanidium thiocynate (GES) method. The concentration of the eluted DNA was determined with UV/VIS Sectrophotometer Lambda 10 (Perkin Elmer, Berlin, Germany). In study III, the template DNA was prepared by dissolving a 1-µl loopful of bacteria in 150 µl of lysis buffer followed by incubation at 95oC for 10 min and centrifugation at 3,000 x g for 5 min. An aliquot of the supernatant was used as PCR-template. The oligonucleotide primers for all PCR reactions used in this study are listed in Table 6.

4.4.2 Pulsed field gel electrophoresis (I, II, III, IV)

For PFGE, one bacterial colony was grown overnight in 3 ml of brain heart infusion (BHI; Difco Laboratories, Detroit, MI) broth at 37°C with shaking. The cells were pelleted by centrifugation at 13000 xg for 1 min. Plugs were prepared by resuspending the pellets in 200 µl of EC buffer [6 mM Tris-HCI (pH 7.5), 1 M NaCl, 100 mM EDTA, 0.5% Brij 58, 0.2% Na-deoxycholate, 0.5% sodium lauroyl sarcosine], supplemented with 20 µl of lysostaphin (1mg/ml; Sigma, St Louis, Missouri, USA), and 200 µl of molten 1.5 % Seakem Gold agarose (Cambrex, Rockland, Maine, USA). Plugs were placed in EC buffer for one hour at 37°C, followed by one wash with TE buffer (10mM Tris-HCI, 1mM EDTA, pH8.0) for one hour at 55°C. A portion of the plug was digested with 35 U of SmaI enzyme (Roche Diagnostics GmbH, Mannheim, Germany). Separation was performed in 1.1% Seakem Gold agarose in 0.5xTBE (0.045 M Tris-borate, 0.001 M EDTA) in a CHEF-DRIII system (Bio-Rad Laboratories, Richmond, California, USA) or Gene Navigator TM system (Pharmacia, Uppsala, Sweden). Running conditions were according to the Harmony protocol[303]. The PFGE patterns were analysed by BioNumerics software (version 2.0, Applied Maths, Kortrijk, Belgium). After initial classification by computer assisted analysis, the patterns were further interpreted according to established guidelines[281]. The PFGE patterns with seven or more band differences were considered different types, and with one to six band differences as subtypes.

4.4.3 Ribotyping (I)

In study I, ribotyping was performed as described previously[127]. Ribotype patterns with one band difference were considered different types. The patterns were determined by visual analysis and were designated by letters.

4.4.4 Multilocus sequence typing (I, II)

MLST for *S. aureus* (Study I) was performed as previously described by Enright *et al*., and MLST for *S. epidermidis* (Study II) was carried out by using the MLST scheme described by Thomas et al.[321]. The Vector NTI program (Invitrogen Corporation, Carlsbad, California, USA) was used for sequence analysis.

Table 6. Oligonucleotides used in the PCR detection and sequencing in this study. *These PCR fragments were used as probes.[49] Locus **A**: upstream of the *pls* gene; locus **B**: *kdp* operon; locus C: *mecI* gene; Locus **D**: *dcs* region; Locus **E**: between integrated pI258 -Tn*554*; Locus **F**: between Tn*554* and *orfX*; Locus **G**: left junction between IS*431*- pUB*110*; Locus **H**: left junction between IS*431* - pT*181*

Method	Target element	Forward primer	Nucleotide sequence 5- 3`	Reverse primer	Nucleotide sequence 5- 3`	GenBank accession number	Reference
M-PCR typing	*mecA**	MECAP4	TCCAGATTACAACTTCACCAGG	MECAP7	CCACTTCATATCTTGTAACG)	Y00688	232
	Locus A*	CIF2F2	TTCGAGTTGCTGATGAAGAAGG	CIF2 R2	ATTTACCACAAGGACTACCAGC	AB033763	
	Locus B*	KDP F1	AATCATCTGCCATTGGTGATGC	KDP R1	CGAATGAAGTGAAAGAAAGTGG	D86934	
	Locus C*	MECI P2	ATCAAGACTTGCATTCAGGC	MECI P3	GCGGTTTCAATTCACTTGTC	D86934	
	Locus D*	DCS F2	CATCCTATGATAGCTTGGTC	DCS R1	CTAAATCATAGCCATGACCG	AB033763	
	Locus E*	RIF4 F3	GTGATTGTTCGAGATATGTGG	RIF4 R9	CGCTTTATCTGTATCTATCGC	AB037671	
	Locus F*	RIF5 F10	TTCTTAAGTACACGCTGAATCG	RIF5R13	GTCACAGTAATTCCATCAATGC	AB037671	
	Locus G*	IS431 P4	CAGGTCTCTTCAGATCTACG	pUB110R1	GAGCCATAAACACCAATAGCC	D86934	
	Locus H*	IS431 P4	CAGGTCTCTTCAGATCTACG	pT181 R1	GAAGAATGGGGAAAGCTTCAC	AB037671	
ccr and *mec* genes typing (M-PCR1)	*mecA*	mA1	TGCTATCCACCCTCAAACAGG	mA2	AACGTTGTAACCACCCCAAGA	D86934	312
	*ccrA1-ccrB**	α1	AACCTATATCATCAATCAGTACGT	βc	ATTGCCTTGATAATAGCCITCT	AB033763	312
	*ccrA2-ccrB**	α2	TAAAGGCATCAATGCACAAACACT	βc	ATTGCCTTGATAATAGCCIT	D86934	
	*ccrA3-ccrB**	α3	AGCTCAAAAGCAAGCAATAGAAT	βc	ATTGCCTTGATAATAGCCITCT	AB037671	
	ccrA4-ccrB4	α4.2	GTATCAATGCACCAGAACTT	βc4.2	TTGCGACTCTCTTGGCGTTT	AB063172	
	Class A	mA7	ATATACCAAACCCGACAACTAA	mI 6	CATAACTTCCCATTCTGCAGATG	D86934	
	Class B	mA7	ATATACCAAACCCGACAACTAA	IS7	ATGCTTAATGATAGCATCCGAATG	AB033763	
	Class C	mA7	ATATACCAAACCCGACAACTAA	IS2	TGAGGTTATTCAGATATTTCGAT	AB121219	
Single PCR (SCC *mec*, individual DNA	*ccrA1*	α1	AACCTATATCATCAATCAGTACGT	βc	ATCTATTTCAAAAATGAACCA	AB033763	7
	ccrA2	α2	TAAAGGCATCAATGCACAAACACT	βc	ATCTATTTCAAAAATGAACCA	D86934	249 278
	ccrA3	α3	AGCTCAAAAGCAAGCAATAGAAT	βc	ATCTATTTCAAAAATGAACCA	AB037671	7
	ccrB	α c	ATTGCCTTGATAATAGCCITCT	βc	ATCTATTTCAAAAATGAACCA	AB063172	270
	ccrC	γ F	CGTCTATTACAAGATGTTAAGGATAAT	γR	CCTTTATAGACTGGATTATTTCAAAATAT	AB121219	
	ccrA2- ccrA3	α2	TAAAGGCATCAATGCACAAACACT	*ccrA3*R	CTGACATGTAGCTAAGACTTGAACT	D86934/AB037671	
	*ccrA3-*ΨTn*544*	α3	AGCTCAAAAGCAAGCAATAGAAT	*cad2*	TAACTGCAACGGTCATCAGGGT	AB037671/AB037671	
	IS*431R –mecA*	IS2	TGAGGTTATTCAGATATTTCGATGT	mA1	TGCTATCCACCCTCAAACAGG	AB033763	

	IS*1272- mecA*	IS5	AACGCCACTCATAACATATGGAA	mA6	TATACCAAACCCGACAAC	AB037671	
	mec I- mecA	mcI4	CAAGTGAATTGAAACCGCCT	mA6	TATACCAAACCCGACAAC	AB033763	
	mecR- mecA	mcR2	CGCTCAGAAATTTGTTGTGC	mA6	TATACCAAACCCGACAAC	D86934	
	dcs region- mec	*DCS* R1	CTAAATCATAGCCATGACCG)	mA1	TGCTATCCACCCTCAAACAGG	AB033763/D86934	
	IS*1272- mecI*	IS5	AACGCCACTCATAACATATGGAA	*mcI3*	CAAAAGGACTGGACTGGAGTCCAAA	AB033763/D86934	
	IS*1272-mec (MS)*	IS5	AACGCCACTCATAACATATGGAA)	*mcR3*	GTCTCCACGTTAATTCCATT	AB033763/D86934	
	IS*1272-mec (PB)*	IS5	AACGCCACTCATAACATATGGAA)	*mcR4*	GTCGTTCATTAAGATATGACG	AB033763	
	mecR1 (MS)	*mcR3*	GTCTCCACGTTAATTCCATT	*mcR4*	GTCGTTCATTAAGATATGACG	D86934/AB037671	
	mecR1 (PB)	*mcR5*	CAGGGAATGAAAATTATTGGA	*mcR2*	CGCTCAGAAATTTGTTGTGC	D86934/AB037671	
	mecI	*mcI3*	CAAAAGGACTGGACTGGAGTCCAAA	*mcI4*	CAAGTGAATTGAAACCGCCT	D86934/AB037671	
	mer operon	*merA2*	TCTTCACAGCCTGTGCATGTCATGCCT	*merG*	TGATACCGCGAATGAATCAAAGGT	AB037671/D86934	
	Tnp*A* (Tn*544)*	Tnp*A1016*	TGTGATGTAAATTCTATTCCAGT	Tnp*A636*	TGAGATCAAAGGAAGTTAAGCAAAT-TATTGATG		
MLST (*S. aureus*)	Carbamate kinase	*arcC*-up	TTGATTCACCAGCGCGTATTGTC	*arcC* - dn	AGGTATCTGCTTCAATCAGCG	AJ271387	2
	Shikimate dehydro-genase	*aroE*–up	ATCGGAAATCCTATTTCACATTC	*aroE* -dn	GGTGTTGTATTAATAACGATATC	AJ271289	
	Glycerol kinase	*glpF*-up	CTAGGAACTGCAATCTTAATCC	*glpF*-dn	TGGTAAAATCGCATGTCCAATTC		
	Guanylatekinase	*gmk*- up	ATCGTTTTATCGGGACCATC	*gmk*- dn	TCATTAACTACAACGTAATCGTA	AJ271251	
	Phosphate acetyl-transferase	*pta*-up	GTTAAAATCGTATTACCTGAAGG	*pta*-dn	GACCCTTTTGTTGAAAAGCTTAA		
	Triosephosphate isomerase	*tpi*- up	TCGTTCATTCTGAACGTCGTGAA	*tpi*- dn	TTTGCACCTTCTAACAATTGTAC	AJ271482	
	Acetyl coenzymeA acetyltransferase	*yqiL*- up	CAGCATACAGGACACCTATTGGC	*yqiL*- dn	CGTTGAGGAATCGATACTGGAAC	AJ252295	
MLST (*S. epidermidis*)	Carbamate kinase	*arcC*-up	TGTGATGAGCACGCTACCGTTAG	*arc*- dn	TCCAAGTAAACCCATCGGTCTG	AF269525	321
	Shikimate dehydro-genase	*aroE*-up	CATTGGATTACCTCTTTGTTCAGC	*aroE*-dn	CAAGCGAAATCTGTTGGGG		
	ABC transporter	*gtr*-up	GATATAAGAATAAGGGTTGTGAA	*gtr*-dn	GTGATTAAAGGTATTGATTTGAAT		
	DNA mismatch repair protein	*mutS*-up	CAGCCAATTCTTTTATGACTTTT	*mutS*-dn	GTAATCGTCTCAGTTATCATGTT		
	Pyrimidine operon regulatory protein	*pyrR*-up	GTTACTAATACTTTTGCTGTGTTT	*pyrR*-dn	GTAGAATGTAAAGAGACTAAAATGAA		
	Triosephosphate isomerase	*tpiA*-up	ATCCAATTAGACGCTTTAGTAA	*tpiA* -dn	TTAATGATGCGCCACCTACA		
	Acetyl coenzyme A acetyltransferase	*yqiL*-up	C CACGCATAGTATTAGCTGAAG	*yqiL*-dn	CTAATGCCTTCATCTTGAGAAATA		

The alleles and the allelic profiles for each of the seven housekeeping loci and the STs were obtained from the MLST database (http://www.mlst.net, http://sepidermidis.mlst.net) for *S. aureus* and *S. epidermidis*, respectively.

4.4.5 SCC*mec* typing (II, III)

The initial classification of the SCC*mec* types was performed by multiplex PCR (M-PCR) as previously described[232] with slight modifications described in study II. The M-PCR detects eight loci (A through H) within SCC*mec* and uses *mecA* as an internal control.

SCC*mec* elements typing: In study II, for checking for the presence of individual genes and other DNA areas within the SCC*mec* element, we used the short and long-range PCRs as previously described[270]. The amplified products were run through 0.7% Sea Kem agarose (FMC BioProducts, Rockland Me, USA) gel in 0.5xTBE buffer at 100 V for one hour.

In study III, analysis of the *ccr* types (*ccrAB*1-4 and *ccrC*) and *mecA* (as an internal control) was performed by M-PCR1 and the *mec* classes A, B, and C was performed by M-PCR2 as previously described by Kondo and coworkers[312]. The amplified products were analysed by gel electrophoresis in 2 % Sea Kem agarose gel in 0.5xTBE buffer at 100 V for two hours.

4.4.6 Southern hybridisation and Dot blot hybridisation (IV)

In study IV, testing for the presence of seven loci (A-H) representing different types of *S. aureus* SCC*mec* regions, *mecA*, the recombinase genes in type I, II and III SCC*mec* (*ccrAB1*-3) and *pls* gene in *S. sciuri*, dot blot hybridisation was used for all 15 strains, and Southern hybridisation was used for only three *S. sciuri* strains. These methods were performed as described previously [49]. Thirteen digoxigenin-labeled probes (Table 6) were designed to differentiate between the eight *S. aureus* SCC*mec* loci in M-PCR[232]. Also probes for *pls*, *mecA* and *ccrAB1*-3 genes were used. For *pls* probe see reference[49]. More details are in the original article IV. PFGE was carried out as previously described for *S. aureus*[303].

4.5 Ethical aspects (studies I-IV)

Based on Finnish laws, the THL must conduct infectious disease surveillance and research in this area. Also, Finnish clinical microbiology laboratories must report certain infectious disease agents and cases to the NIDR. Thus, no ethics committee approval was required for studies I, II, and IV. My coworkers and I were at liberty to collect the samples from the residents in study III with the approval of the Ministry of Social Affairs and Health as well as of the data protection authority. In addition, permission for sampling was requested and obtained from each individual patient.

5 RESULTS

5.1 Genomic diversity of MSSA in Southern Finland (I)

This study was designed to examine the genetic backgrounds of both MSSA and MRSA PFGE was applied to determine the clonal relationships between 299 clinical MSSA isolates and 24 representative EMRSA strains in Finland.

5.1.1 Demographic data of the patients

A total of 299 patients were enrolled in the study, 57% of whom were females. The median age of all patients enrolled in the study was 56 years (age range, 1 to 95 years). A majority of paediatric patients (74%) were males (Figure 7).

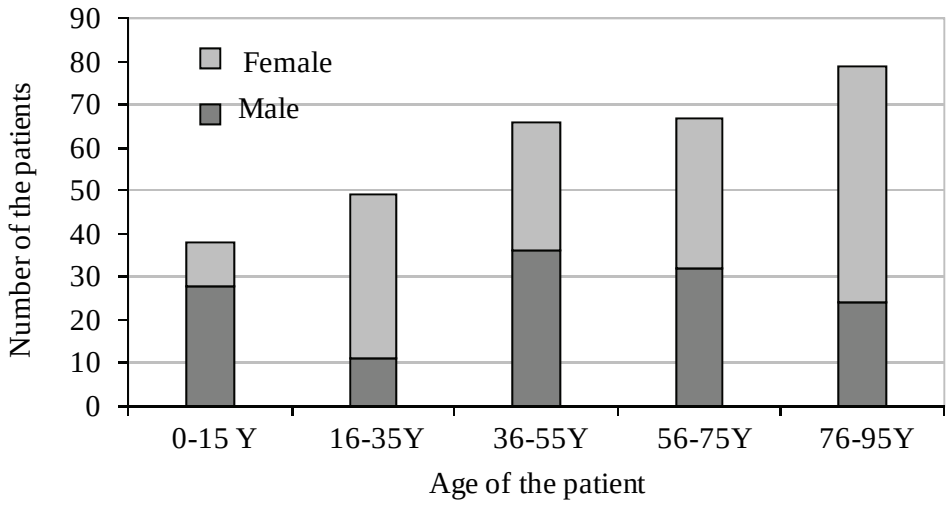


Figure 7. the numbers of the patients according to age and gender

5.1.2 Genotypic characteristics of MSSA

Among the 299 MSSA strains, 30 different PFGE types were identified. Sixty-eight percent (N=204) of MSSA strains had a genotype related to eight of the 24 epidemic MRSA strains. The remaining MSSA strains with unrelated genotypes to EMRSA (N=95) formed a heterogeneous

group showing 22 different genotypes (Figure 8). Of the twelve most prevalent MSSA strains (i.e. MSSA isolated from 5 or more persons during the study period), six were related to EMRSA (Figure 8).

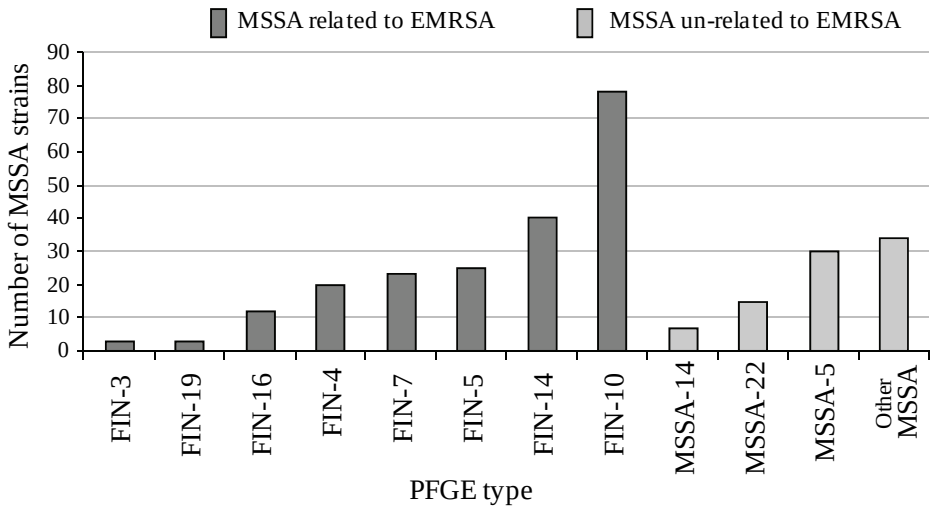


Figure 8. Distributions of PFGE types among Finnish MSSA strains

Ribotyping and MLST were carried out to confirm the clonal relationship between MSSA and EMRSA isolates. We selected 49 MSSA isolates for ribotyping. These isolates were grouped to twelve most prevalent MSSA strains (i.e., MSSA isolated from five or more persons during the study period): six MSSA strains (20 isolates) which were PFGE subtypes of EMRSA strains, and six MSSA strains (29 isolates) which were unrelated to EMRSA. Twenty different ribotype profiles were distinguished between these strains. Three of the six MSSA strains related to EMRSA strains had ribotype profiles similar to those of the corresponding EMRSA strains and two of the six MSSA strains unrelated to EMRSA shared a ribotype with MRSA.

For MLST, one selected isolate from each group of prevalent MSSA strains was chosen. Twelve different allelic MLST profiles (ST) were found. The MSSA strains related to EMRSA strains had the same ST profiles as the corresponding EMRSA strains. The variation of ribotype and MLST profiles within PFGE types is shown in Figure 9. MSSA-FIN-MSSA-FIN-7 and MSSA-FIN-16 subtypes showed the same ribotype and STs as that of the corresponding EMRSA (Figure 9; clusters 2, 4 and 5).

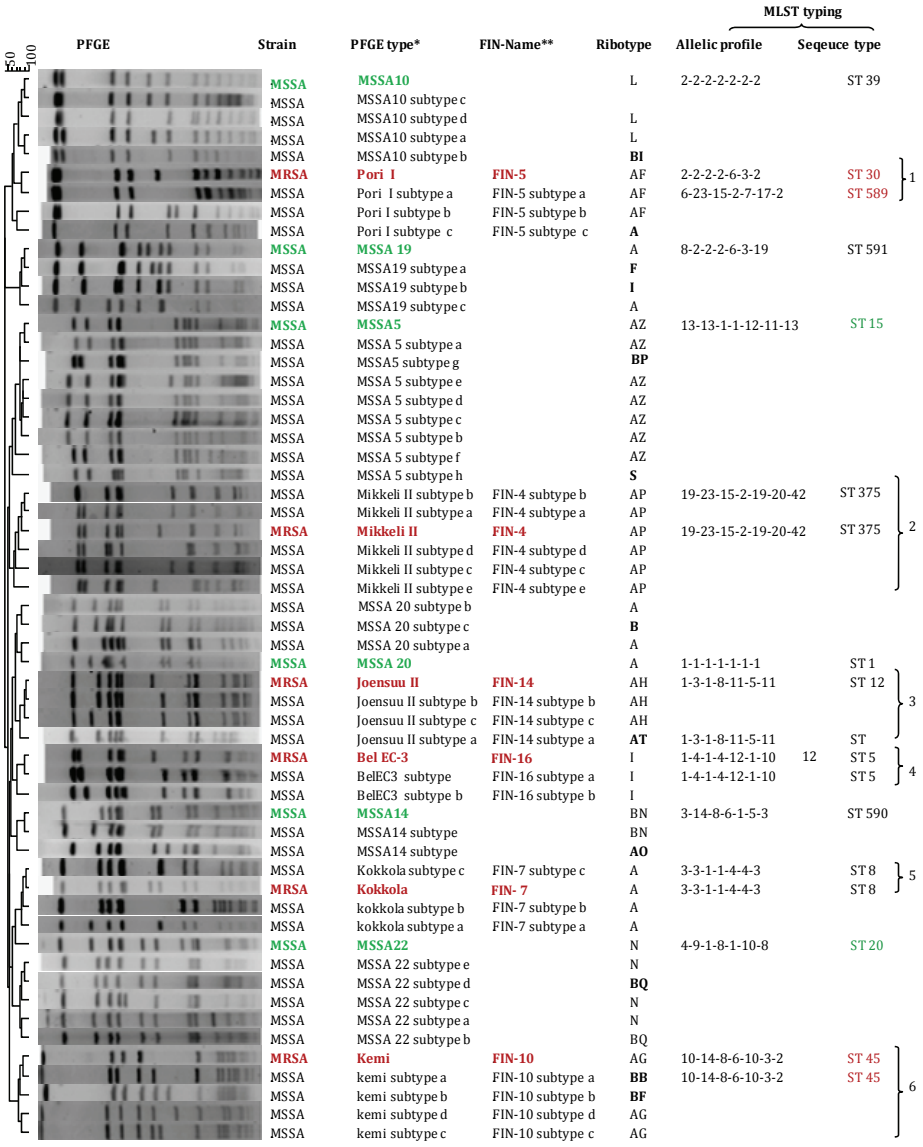


Figure 9. Molecular characteristics of the Finnish MSSA strains according to PFGE, Ribotype and MLST analysis. Cluster 1: sequence type different from that of the corresponding EMRSA with an identical ribotype. Clusters 2, 4 and 5: ribotype and sequence type the same as that of the EMRSA. Clusters 3 and 6: sequence type the same as that of the EMRSA with more than one ribotype.
*) Nomenclature based on old EMRSA naming system used before 2005
**) Nomenclature based on a new EMRSA naming system[346].

The MSSA-FIN-14 and MSSA-FIN-10 subtypes showed sequence types similar to those of the corresponding EMRSA also of more than one ribotype (Figure 9; clusters 3 and 6). On the other hand, MSSA-FIN-5 subtype showed sequence type (ST589) differing from that of the corresponding EMRSA (ST30) but still had the same ribotype as EMRSA (ST30) (Figure 9; cluster 1).

5.2 Molecular epidemiological characteristics of clinical MRSE strains (II)

In this study, 60 bacteremic MRSE strains were genotyped by PFGE, SCC*mec* typing and MLST methods for analysis of the clonality of MRSE strains and the SCC*mec* types in *S. epidermidis.*

5.2.1 SCC*mec* types in clinical MRSE strains

First, the SCC*mec* types of MRSE strains were examined using the M-PCR232. Sixty MRSE strains were positive for *mecA* and negative for loci H and B. The SCC*mec* type IA was identified in four of sixty strains. The remaining 56 strains had six different PCR patterns that did not correspond to any of the previously known patterns and are thus classified as new patterns (P1–P6) (Figure 10). All these patterns (Figure 1 in Study II) were studied using a simplex PCR. SCC*mec* type IA possessed the ccrA1 and class B *mec* complex as expected. The SCC*mec* patterns (P1–P6) included *ccrA2, ccrA3* and both class A and class B *mec* complexes. Three of them also had ccrC (P1, P4 and P6).

In addition, some genes were amplified to recognise the locations of these genes in the SCC*mec* region. Amplification of the DNA regions from *ccrA2* to *ccrA3* and from *ccrA3* to *ψTn554* produced amplicons. Also, the DNA regions from *IS1272* to *mecA*, from mecI to *mecA*, from mecRI (PB) to *mecA*, and from *IS1272* to *mecR1* (MS) were amplified. The DNA regions from *IS1272* to *mecI*, and from IS1272 to *mecR*1(PB), and the transposon *Tn544* (TnpA), could not be amplified from DNA yielding any of the SCC*mec* patterns (P1–P6). The DNA region from the *dcs* region to *mecA* was amplified in the cases of P1, P4, P5 and P6. The mer operon was present in DNA of all six patterns except P3 (Table 3 and Figure 2 in Study II).

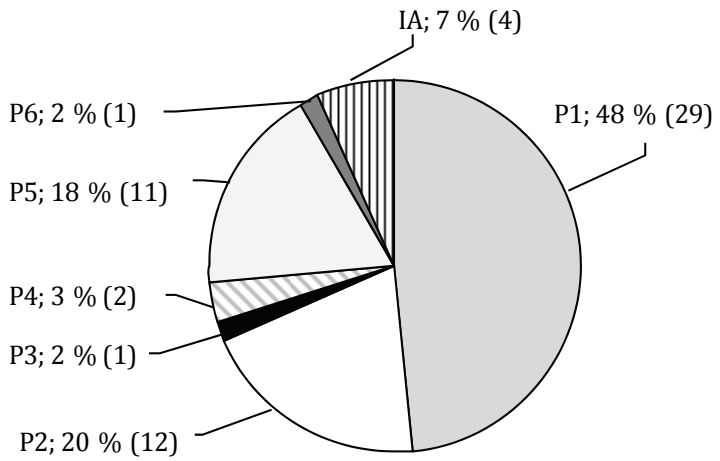
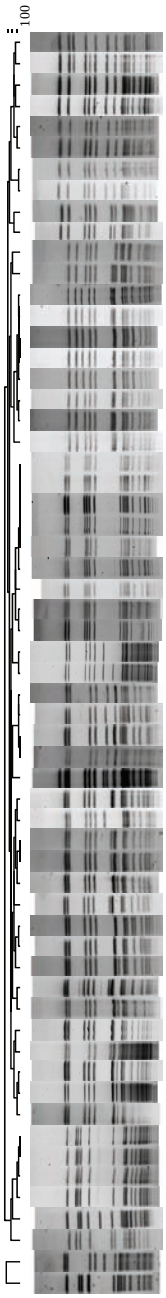


Figure 10. Distribution of SCC*mec* multiplex patterns of MRSE
P1, IIIA (loci F, E and C) and IVA (loci G and D)
P2, IIIB (locus C) and IV (locus D)
P3, IIIB (locus C) and IVA (locus G without locus D)
P4, IIIA (loci F, E and C) and IV (locus D)
P5, IIIB (locus C) and IVA (loci G and D)
P6, IIIA (loci F and C, without locus E) and IV (locus D)
IA, (loci A, D)

5.2.2 The PFGE types in clinical MRSE strains

The 60 MRSE isolates were analysed for their clonality by PFGE. Eleven PFGE types (FIN-SE-1 to FIN-SE-11) were found with a number of subtypes (Figure 11). The most prevalent PFGE type (FIN-SE-1, n = 18) was found dur-ing 1990–1992 in three units. FIN-SE-6 was found in both the PHU and AHU-A, but with an interval of 3 years. All 12 different representative PFGE types (including one subtype) belonged to ST 2 (study II, Table 2).



FIN-SE-2b	NICU	P2 (D,C,*mecA*)			IV+IIIB	
FIN-SE-2c	NICU	P2 (D,C,*mecA*)			IV+IIIB	
FIN-SE-2	NICU	P2 (D,C, *mecA*)			IV+IIIB	.
FIN-SE-2	**NICU**	**P2 (D,C, *mecA*)**	***ccrA2 ,ccrA3,*** **class.A, B**	***mer* Operon and *copy-Tn554***	**IV+IIIB**	**ST2**
FIN-SE-2c	NICU	P2 (D,C, *mecA*)			IV+IIIB	
FIN-SE-2	NICU	P2 (D,C, *mecA*)			IV+IIIB	
FIN-SE-2	NICU	P2 (D,C, *mecA*)			IV+IIIB	
FIN-SE-2	NICU	P2 (D,C, mecA)			IV+IIIB	
FIN-SE-5	AHU-A	P4 (F,D,E, *C,mecA*)			IV+IIIA	
FIN-SE-5	**AHU-A**	**P4 (F, D,E, C,mecA)**	***ccrA2 ,ccrA3,ccrC*** **Class A, B**	***mer* Operon and copy-*Tn554***	**IV+IIIA**	**ST2**
FIN-SE-7b	NICU	P1 (F,G, D,E, C, *mecA*)			IVA+IIIA	
FIN-SE-7a	NICU	P1 (F,G, D,E, C, *mecA*)			IVA+IIIA	
FIN-SE-7	NICU	P1 (F,G, D,E, C, *mecA*)			IVA+IIIA	
FIN-SE-7	NICU	P1 (F,G, D,E, C, *mecA*)			IVA+IIIA	
FIN-SE-7	NICU	P1 (F,G, D,E, C, *mecA*)			IVA+IIIA	
FIN-SE-7	NICU	P1 (F,G, D,E, C, *mecA*)			IVA+IIIA	
FIN-SE-7	**NICU**	**P1 (F,G, D,E, C, *mecA*)**	***ccrA2 ,ccrA3,ccrC*** **Class A, B**	***mer* Operon and copy-*Tn554***	**IVA+IIIA**	**ST2**
FIN-SE-7a	NICU	P1 (F,G, D,E, C, *mecA*)			IVA+IIIA	.
FIN-SE-7a	NICU	P1 (F,G, D,E, C, mecA)			IVA+IIIA	
FIN-SE-7a	NICU	P1 (F,G, D,E, C, *mecA*)			IVA+IIIA	
FIN-SE-8	NICU	P5 (G,D,C, *mecA*)			IVA+IIIB	
FIN-SE-8	NICU	P5 (G,D,C, *mecA*)			IVA+IIIB	
FIN-SE-8	NICU	P5 (G,D,C, *mecA*)			IVA+IIIB	
FIN-SE-8	NICU	P5 (G,D,C, *mecA*)			IVA+IIIB	
FIN-SE-8	**NICU**	**P5 (G,D,C, *mecA*)**	***ccrA2 ,ccrA3*** **Class A, B**	***mer* Operon and copy-*Tn554***	**IVA+IIIB**	**ST2**
FIN-SE-8	NICU	P5 (G,D,C,mecA)			IVA+IIIB	
FIN-SE-8	NICU	P5 (G,D,C, *mecA*)			IVA+IIIB	
FIN-SE-8	NICU	P5 (G,D,C, *mecA*)			IVA+IIIB	
FIN-SE-8a	NICU	P5 (G,D,C, *mecA*)			IVA+IIIB	
FIN-SE-1d	NICU	P2 (D,C, *mecA*)			IV+IIIB	**ST2**
FIN-SE-1f	NICU	P2 (D,C, *mecA*)			IV+IIIB	
FIN-SE-6	PHU & AHU-A	P1 (F,G, D,E, C, *mecA*)			IVA+IIIA	
FIN-SE-6a	PHU & AHU-A	P1 (F,G, D,E, C, *mecA*)			IVA+IIIA	
FIN-SE-6	PHU & AHU-A	P1 (F,G, D,E, C, *mecA*)			IVA+IIIA	
FIN-SE-6	**PHU & AHU-A**	**P1 (F,G, D,E, C, *mecA*)**	***ccrA2 ,ccrA3,ccrC*** **Class A, B**	***mer* Operon and copy-*Tn554***	**IVA+IIIA**	**ST2**
FIN-SE-2d	NICU	P2 (D,C,mecA)			IV+IIIB	
FIN-SE-1	**NICU, PHU, AHU-B**	**P1 (F,G, D,E, C, *mecA*)**	***ccrA2 ,ccrA3,ccrC*** **Class A, B**	***mer* Operon and copy-*Tn554***	**IVA+IIIA**	**ST2**
FIN-SE-1d	**NICU**	**P2 (D,C, mecA)**			**IV+IIIB**	**ST2**
FIN-SE-1c	NICU, PHU, AHU-B	P1 (F,G, D,E, C, *mecA*)			IVA+IIIA	
FIN-SE-1c	NICU, PHU, AHU-B	.			IV+IIIB	
FIN-SE-1a	NICU, PHU, AHU-B	P1 (F,G, D,E, C, *mecA*)			IVA+IIIA	
FIN-SE-1c	NICU, PHU ,AHU-B	P1 (F,G, D,E, C, mecA)			IVA+IIIA	
FIN-SE-1b	NICU, PHU, AHU-B	P1 (F,G, D,E, C, *mecA*)			IVA+IIIA	
FIN-SE-1	NICU, PHU, AHU-B	P1 (F,G, D,E, C, *mecA*)			IVA+IIIA	
FIN-SE-1a	NICU, PHU, AHU-B	P1 (F,G, D,E, C, *mecA*)			IVA+IIIA	
FIN-SE-1c	NICU, PHU, AHU-B	P1 (F,G, D,E, C, *mecA*)			IVA+IIIA	
FIN-SE-1a	NICU, PHU, AHU-B	P1 (F,G, D,E, C, *mecA*)			IVA+IIIA	
FIN-SE-1	NICU, PHU, AHU-B	P1 (F,G, D,E, C *mecA*)			IVA+IIIA	
FIN-SE-1e	NICU, PHU, AHU-B	P1 (F,G, D,E, C, *mecA*)			IVA+IIIA	
FIN-SE-1a	NICU, PHU, AHU-B	P1 (F,G, D,E, C, *mecA*)			IVA+IIIA	ST2
FIN-SE-1a	NICU, PHU, AHU-B	P1 (F,G, D,E, C, *mecA*)			IVA+IIIA	
FIN-SE-1a	NICU, PHU, AHU-B	P1 (F,G, D,E, C, *mecA*)			IVA+IIIA	
FIN-SE-4	AHU-B	A, G, D, mecA			IA	
FIN-SE-4	**AHU-B**	**IA (A, G, D, *mecA*)**	***ccrA1*, class B**		**IA**	**ST2**
FIN-SE-4	AHU-B	A, G, D, *mecA*			IA	
FIN-SE-4	AHU-B	A, G, D, *mecA*			IA	ST2
FIN-SE-10	**PHU**	**P5 (G,D,C, *mecA*)**			**IVA+IIIB**	**ST2**
FIN-SE-11	**AHU-A**	**P5 (G,D,C, mecA)**			IVA+IIIB	ST2
FIN-SE-9	**NICU**	P6 (F,D,C, mecA)			IV+IIIA	ST2
FIN-SE-3	**NICU**	P2 (D,C, *mecA*)			IVA+IIIB	ST2

Figure 11. Genetic background information of the Finnish MRSE strains analysed by PFGE, SCC*mec* typing and MLST. NICU, neonatal intensive care unit; PHU, paediatric haematology unit; AHU-A, adult haematology unit A; AHU-B, adult haematology unit B.

5.3 Carriage of MR-staphylococci and their SCC*mec* types in a long-term care facility (III)

One hundred and twenty-seven CNS and 24 *S. aureus* isolates were isolated from the nasal swabs of 76 patients during a clinical surveillance study in a long-term care facility in 2004. In this study, standard microbiological techniques were applied for species identification and antimicrobial susceptibility tests for search for methicillin-resistance. SCC*mec* typing methods were also used to examine the structure of the SCC*mec* elements of MR-CNS strains and for structural comparison of the SCC*mec* elements of MRSE and MRSA isolated from the same person at the same time.

5.3.1 The epidemiological background of the patients

The median age of 76 patients was 80 years (range, 35 to 99 years), 36% were male (n =27), 26% used antimicrobials (n =20), and 5% used foreign devices (n =4). The median length of nursing stay was 9 months (range, 1 to 90 months).

5.3.2 Nasal carriage of CNS in a long-term care facility

Seventy-three of 76 patients (96%) were colonised with a staphylococcal species; 67 (92%) were colonised by at least one CNS strain, 49 (73%) by only CNS and 18 (27%) by CNS in combination with S. aureus. Only S. aureus was found in six persons (8%). Forty-one (41) of 67 CNS (61%) carriers were colonised with at least one MR-CNS strain, and two of them carried MRSA as well. Methicillin-susceptible CNS strains were found in twenty-six (39%) patients, and one of them carried also MRSA. 127 isolates were obtained from the 67 patients with CNS, 61 (48%) were shown to be methicillin-resistant (Figure 12).

Figure 12. The rate of methicillin resistance within each CNS species: *S. epidermidis* isolates (n= 82), *S. capitis* isolates (n=15), *S. haemolyticus* isolates (n=7) and *S. hominis* isolates (n=2)

5.3.3 Distribution of the SCC*mec* types between carriages of MR-CNS strains

The 61 MR-CNS isolates tested by M-PCR[232], and three MRSE strains could be classified as SCC*mec* type I and two as SCC*mec* type IV. Among the other MR-CNS species, one MR-*S. capitis* isolate could be classified as SCC*mec* type IA. Only *mecA* was amplified in one MRSE isolate, two MR-*S. capitis* isolates and one MR-*S. hominis* isolate. The remaining 51 MR-CNS isolates, including 43 MRSE isolates, seven MR-*S. capitis* isolates and one MR-*S. haemolyticus* isolate, could not be interpreted as belonging to any of the currently described SCC*mec* types[232].

The SCC*mec* elements of strains representing all multiplex SCC*mec* PCR patterns were tested [312]**.** Among 49 MRSE isolates, three (6%) had SCC*mec* type I, two (4%) type II, 18 (37%) type IV, and nine (12%) type V. Three isolates were of a nontypeable SCC*mec* type, as neither the *ccr* genes nor the *mec* genes could be amplified. The remaining 14 isolates had *ccr* and *mec* complex gene combinations for which no names have been previously assigned. Among the ten MR-*S. capitis* isolates, three had SCC*mec* types IA, II, and IV, and two harboured type V. The remaining five isolates harboured a new SCC*mec* type. The one MR-*S. haemolyticus* isolate harboured SCC*mec* type IV, and the one MR-*S. hominis* isolate harboured a new SCC*mec* type (Figure 13).

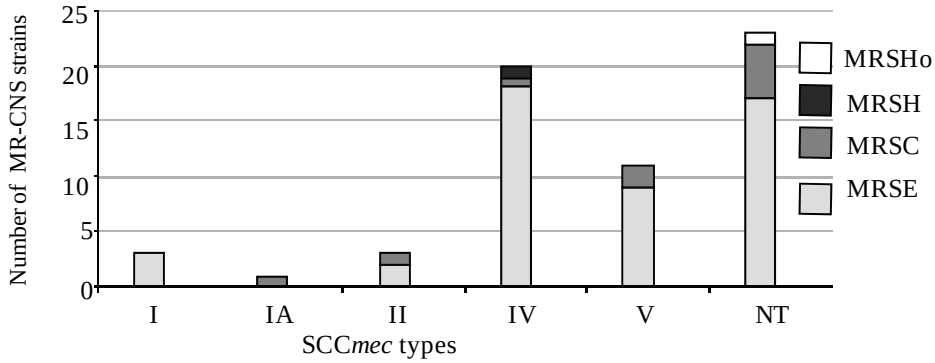


Figure 13. The distribution of SCC*mec* types amongst MR-CNS. NT = new SCC*mec* types (with different combinations of *ccr* and other *mec* genes or only *mecA*). MRSHo= MR-*S. hominis*, MRSH= MR-*S. haemolyticus*, MRSC= MR-*S. capitis*, MRSE= MR-*S. epidermidis*

5.3.4 The structure of the SCC*mec* elements of MRSE and MRSA isolated from the same person

Two patients were colonised by both MRSE and MRSA. The first one carried a MRSA strain and three MRSE strains. The MRSA strain and one of the MRSE strains harboured SCC*mec* type V (*ccrC* and class C), while the two MRSE strains were of different SCC*mec* types, type II (*ccrA2* and class A) and a new SCC*mec* type (*ccrC* and *mec*, class A). The second carrier had MRSA and MRSE, both of which harboured SCC*mec type* V. The MRSE multiplex SCC*mec* patterns varied among the isolates (Table 7). The two MRSA strains were representatives of a Finnish epidemic strain (FIN-7 and FIN-7 subtype), and the four MRSE strains had unique PFGE profiles (Study III, Table 2 and Figure 2).

Table 7. Distribution of the SCC*mec* types/elements within the MR staphylococcal isolates in two patients colonised with MRSA and MRSE.

	Staphylococcal species	SCC*mec*-type	Amplified loci by the Oliveira strategy	Amplified loci by the Kondo strategy	
				ccr type	*mec* class
First patient	MRSA	V	*mecA*, F	*ccrC*	C
	MRSE	NT II V	*mecA*, B, F *mecA*, C, D, A *mecA*, E (very faint), F, A	*ccrC* *ccrA2* *ccrC*	A A C
Second patient	MRSA	V	*mecA*, F	*ccrC*	C
	MRSE	V	*mecA*, E (very faint), F, A	*ccrC*	C

5.3.5 SCC*mec* regions of different *S. aureus* SCC*mec* types in *S. sciuri*

The presence of areas homologous to different types of *S. aureus* SCC*mec* regions in *S. sciuri* DNA was examined. Dot blot hybridisation was used for all 15 strains and, additionally, Southern hybridisation was used for three strains (see the article IV for information about N920212, N950120, and N960546). Thirteen digoxigenin-labeled probes designed to differentiate among type I to IV SCC*mec* regions were used in the PCR amplifition reactions. All the strains had unique PFGE profiles. Twelve of the 15 *S. sciuri* strains carried the *pls* gene. *mecA* hybridisation for the 15 *S. sciuri* strains showed that seven MS-*S. sciuri* strains had a single weak band by *mecA*-positive.. Of the remaining eight strains 4 had a single strong *mecA*-positive band and 4 others had (Table 1 and Figure 1 in Study IV). Locus A probe gave negative results in all 15 *S. sciuri* strains. Locus C probe gave negative results in the strains without $mecA_{S.\ aureus}$. Locus G, locus H and *ccr* gene probes gave positive result in dot bloting for 12 strains and also these probes bound to negative control DNA. Four *S. sciuri* strains did not hybridize with any of the *ccr* probes. Loci B, D, and F probes gave negative results in all the *S. sciuri* that contained only the *S. sciuri* copy of *mecA*.

6 DISCUSSION

One of the most important questions in the molecular evolution of *S. aureus* and MRSA development is the possible role of horizontal gene transfer. In this study, the possibility of horizontal transfer of SCC*mec* elements between the Finnish *S. aureus* and CNS was studied. Furthermore, the genetic characteristics of their SCC*mec* elements were analysed.

6.1 Genomic backgrounds of MSSA and EMRSA (I)

Do the MRSA strains develop from MSSA strains that get the SCC*mec*? Are there certain *S. aureus* strains that are more prone to accept and acquire methicillin-resistance? In study (I), these questions were addressed by studying the genomic diversity of 24 representative Finnish EMRSA strains in comparison to 299 clinical MSSA strains. A high genomic diversity consisting of 30 PFGE profiles was identified among the MSSA isolates. The majority of the MSSA strains (68%) were related to eight of the EMRSA strains and the MSSA unrelated to EMRSA showed heterogeneous group of 22 PFGE profiles. This is consistent with the findings of Goering and coworkers[351] who showed that globally MRSA and MSSA strains shared the same genetic backgrounds with higher diversity in MSSA.

It was previously elucidated that three of the EMRSA strains were more likely to be acquired from a community than from a hospital setting. These CA-MRSA strains were usually sensitive to antibiotics other than β-lactams[127]. In this study, the genotypes of five EMRSA are common (63%) among the MSSA strains. Two of them are CA-MRSA (FIN-4 and FIN-14) and three others are non-multi-resistant MRSA (FIN-10, FIN-7 and FIN-5) which have been found in hospitals and the community. The five EMRSA strains all carry the small size SCC*mec* type IV suggesting that this type of SCC*mec* is more readily transmissible between staphylococci than the other SCC*mec* types. Thus, SCC*mec* type IV might be the most successful SCC*mec* type turning MSSA strains into MRSA within the human normal microbiota. Five percent (5%) of the MSSA strains related to EMRSA showed one intra hospital EMRSA genotype (FIN-16). It is multi-resistant and carries SCC*mec* type IA. However, according to Lina and coworkers, SCC*mec* type IV might have developed from SCC*mec* type I[352]. The MSSA-FIN-5 subtypes possess identical ribotypes

but showed a sequence type different (ST589) from the corresponding EMRSA (ST30) (Figure 9; cluster 1). In the case of this particular strain, the different MLST profiles within the same PFGE-subtypes and one ribotype reflect the high discriminatory power of MLST in comparsion to PFGE and ribotyping (Figure 10)[291]. In general, a good correlation was observed between the MLST and PFGE results.

Within the eight clusters of MSSA related to EMRSA, most of the variability between the subtypes of any genotype occurred in the mid-range molecular weight area (674 kb-175kb) of PFGE profiles. This area is known to harbour the *mecA* complex (Figure 9). The results suggest that the acquisition of SCC*mec* from unknown donor (s) to several MSSA background genotypes has occurred. Previously, it has been reported that major MRSA clones have arisen frequently from epidemic MSSA clones[272]. The early MRSA and MSSA isolates, which were isolated in Denmark and the United Kingdom, suggest that the current MSSA isolates serve as early recipients of the *mecA* gene in Europe [353]. Moreover, the horizontal transfer of SCC*mec* has been shown to occur in a hospitalised patient during antibiotic treatment [354]. Also the loss of SCC*mec* from the chromosome has been observed *in vivo* and *in vitro*[355, 356].

Some of the MSSA genotypes unrelated to EMRSA were not encountered among all MRSA. For example, the two strains, MSSA5 and MSSA22, possess ST15 and ST20. In the MLST database, these two STs are only encountered among the MSSA strains. This result suggests that there may be some MSSA genotypes that can acquire SCC*mec* more easily than others. According to several earlier studies[13, 137, 273, 277–280], there are some MSSA strains that harbour the remnants of SCC*mec* without the *mec* complex. The existence of the SCC*mec* remnants might be native in the genome of some MSSA, or it could be that the MSSA is a former MRSA that has lost its *mecA*. Also, it may be that the SCC*mec* elements can transfer separately.

6.2 Genotypes of clinical MRSE strains (II)

In this study, the most notable finding is the limited heterogeneity in the genomes of bacteremic MRSE isolates which were collected from four different units at HUCH in two distinct time periods (Figure 11). There were 11 PFGE types among the 60 MRSE isolates, and all the PFGE

types belonged to ST2. Moreover, a single clone was present in four units at HUCH for nearly a decade, which may have resulted in PFGE type diversity over the years. The MRSE clone with ST 2 is known to have circulated also worldwide [321, 357, 358].

6.3 The prevalence of the nasal carriage of MR-CNS in a LCTF (III)

The results show that the nasal carriage of CNS was common among the 76 patients in a long- term care facility setting. The prevalence of MR-CNS was slightly higher (48%) than that in a similar study in the United States (40%)[359]. The present study also showed that the most prevalent CNS species was MR-*S. epidermidis*. The rate of methicillin-resistance varied between CNS species (Figure 12).

6.4 SCC*mec* types of CNS (II, III, IV)

6.4.1 SCC*mec* types in clinical MRSE strains

The study II shows that a single MRSE strain may possess two SCC*mec* types: SCC*mec* type IV (or IVA) and IIIA (or IIIB), a phenomenon known to occur also in MRSA. This is supported by several findings. First, the SCC*mec* patterns (P1, P2, P4 and P6) showed amplicons of the same molecular weight as expected for the loci that would represent two different SCC*mec* types (Figures 11 and 12), but SCC*mec* P3 and P6 do not have all the loci which represent the two different SCC*mec* types. Consistent to this, a SCC*mec* type IV lacking the *dsc* gene (locus D) and a SCC*mec* type III lacking the locus E have been previously reported[233, 360]. Second, the results show that the six SCC*mec* patterns carry two *ccr* complexes, *ccrAB*2 and *ccrAB3* and all had two *mec* complexes, class A and class B. None of the SCC*mec* patterns P1-P6 possessed the transposon Tn*554*. In accordance with the result, strains carrying multiple *ccr* complex genes have been encountered[13, 236, 237, 273, 361] and a SCC*mec* III lacking the Tn*554* has been reported in MRSA[233, 312] and MRSE[133]. Additionally, the IS*1272* and *mecI* and *mecR1* (PB) are not located in the same cassette. The DNA region between *ccrA*2 and *ccrA*3 produces an amplicon suggesting that the two probable SCC*mec* cassettes would be located closely side by side. The hypothesized arrangement of the P1–P6

SCC*mec* patterns is in agreement with all PCR results, based on the condition that there is an inversion of *ccrAB2* (Figure 2 in Study II,). It is also hypothesised that the *ccr* and *mec* gene complexes have gone through complex recombination and rearrangement processes, generating novel types of SCC*mec* elements[8]. When comparing the SCC*mec* types of MRSE with those that existed in the EMRSA strains at the same time (without paying attention to the epidemiological background), they all share the property of being carriers of SCC*mec* types; IIIA, IA and IVA in1991-1992 and III, IIIB and IV in 1997–1998 [346].

6.4.2 SCC*mec* types in nasal carriage of MR-CNS (III)

The majority of the MR-CNS isolates (62%) possessed a previously known SCC*mec* type I, IA, II, and IV, while 37% had a variety of new *ccr* and *mec* combinations or had only *mecA* without *ccr* and *mec* complexes. The SCC*mec* type IV was the most prevalent among the *S. epidermidis* strains (37%; 18/49). The prevalence of SCC*mec* type IV among the *S. epidermidis* strains was notable in the early 1970s[320]. Among MR-CNS that carried SCC*mec* type IV, there were MR-CNS strains that harboured SCC*mec* type IV (*mecA* and locus D) and other MR-CNS strains that harboured a modified SCC*mec* type IV which represent four different multiplex SCC*mec* patterns[232] (Table 2 and Figures 1 and 2 in Study III). Modified patterns were also found in MR-CNS strains, which were harbouring SCC*mec* type II that contained the additional locus A and lacked locus B (*kdp* operon). MR-CNS strains had SCC*mec* type V, which have four different multiplex SCC*mec* patterns.

Thirty-seven percent (37%) of the strains harboured a new SCC*mec* type, some of which are encountered in MRSA. Among these strains, there were ones which carried known SCC*mec* types with the additional *ccr* gene (type IV and *ccrC* or type V and *ccrA4*). Strains containing both type SCC*mec* IV and *ccrC* and strains carrying multiple *ccr* genes have been reported[232, 249, 326, 358, 362]. Some of our strains carried combinations of *ccr* and *mec* that could not be interpreted as belonging to any of the currently described SCC*mec* types. Only the *mecA* gene was amplified in other MR-CNS strains. Failure to amplify the *ccr* and *mec* class may indicate that the target sequence for primers may have changed. Previous studies have shown that some staphylococcal strains carry *ccr* complex genes without *mec* complex genes and vice versa, while some MRSA

strains carry neither *ccr* nor *mec* complexes, instead they carry only the *mecA* gene[312, 357, 358, 363].

In this study, the use of the PCR methods for detecting only the sequence in the junkyard regions for SCC*mec* typing in MR-staphylococci raises some concerns. The junkyard regions do not contain specific loci for a specific SCC*mec* type. Locus A is present in types II, IV, and V, but previously it was thought to be part of SCC*mec* type I and IA only. Locus B is present in type V and in three new types, but previously it has been defined to be specific for SCC*mec* type II. Also, the SCC*mec* types IV and V contained a variety of loci. Therefore, the detection of these intervening sequences provides valuable additional information on the discrimination of SCC*mec* types. The results demonstrate the high diversity of SCC*mec* types in MR-CNS. This finding is concordance with other studies[133, 357, 358, 360, 361, 363, 364].

6.4.3 Co-existence of similar SCC*mec* types in MRSE and MRSA of the same patient

The point prevalence study on nasal carriage of CNS was conducted six months after an outbreak of MRSA in a LTF in Northern Finland in 2004. The MRSA outbreak was caused by a strain that had not been previously encountered in Finland, namely FIN-22 with SCC*mec* type V. The MRSA nasal carriage of the patients was analysed [365] and it showed that each of the five different MRSA strains had SCC*mec* type V. Two patients carried both MRSE and MRSA strains concurrently and these strains also shared the SCC*mec* type V (*ccrC*, class C). However, differences in the J region sequences were identified between MRSA and MRSE strains (additional loci E and A in MRSE) (Table 7). Although we are not able to rule out the possibility that the similar SCC*mec* cassettes were acquired through different routes, this observation supports the possibility of SCC*mec* transfer. However, if such a transfer has happened, it was not complete. Further studies revealing the mechanisms of SCC*mec* transfer are needed. The hypothesis for the transfer of SCC*mec* between *S. epidermidis* and *S. aureus* has been presented previously[320, 326, 361, 366].

6.4.4 SCC*mec* region in *S. sciuri*

The results showed that the *S. sciuri* strains with only $mecA_{S.sciuri}$ did not carry the *mecI* gene (locus C). In accordance with previous studies, it showed that the presence of *mecI* was always connected to the presence of a copy of $mecA_{S.\ aureus}$ .This study also revealed *mecR1* and *mecI* adjacent to *mecA* genes of the *S. aureus* type but not adjacent to those of the *S. sciuri* type[110, 217, 367]. Possibly, native *S. sciuri* does not harbour the *mecI* gene. The IS*431* or integrated plasmids pUB*101* and pT*181* (Loci G and H) were sometimes part of the *S. sciuri* genome. The majority of strains (11/15) had recombinase genes (*ccr* –gene). Three regions of SCC*mec* (loci B, D and F) were not part of the *S. sciuri* genome.

7 CONCLUSION AND FUTURE CONTRIBUTIONS

According to the results, non-multi-resistant MRSA, including community-acquired MRSA strains, harbour genotypes found amongst clinical MSSA strains. These findings suggest that horizontal transfer of SCC*mec* elements from unknown donor(s) to several MSSA background genotypes has occurred in Finland. Also, some of the MSSA genotypes unrelated to EMRSA or any MRSA encountered in our laboratory data base were now found. It would be of interest to search for integration site (*orfX* and *attR*/*attL)* in the MSSA strains related and unrelated to MRSA, and to compare them to each other in order to understand why some strains acquire the SCC*mec* and some do not. Moreover, it would be interesting to study the horizontal gene transfer between staphylococcal strains *in vitro* and *in vivo*.

Among the clinical MRSE strains, SCC*mec* elements of two cassette types (III and IV), which seem similar to the SCC*mec* types of MRSA, were detected. To confirm these findings, the SCC*mec* regions of these strains should be sequenced.

Among the patients in the LTCF, nasal carriage of MR-CNS was common. A higher variety and frequency of SCC*mec* types was found in CNS than in *S.aureus*. Many unknown types were identified amongst the MR-CNS strains. These types have not yet been identified in MRSA. The SCC*mec* type IV, with a variety of it, was most prevalent among the *S. epidermidis* strains. Based on the sharing of SCC*mec* type V between MRSA and MR-CNS, horizontal transfer of SCC*mec* elements is speculated. Moreover, the MR-*S. capitis* was the second most common species after *S. epidermidis*, and it carried all SCC*mec* types known to date and some new types. Whole genome sequencing of the *S. capitis* would provide some answers regarding whether the horizontal transfer of genes is happening continuously between these staphylococcal species. The MR-CNS may represent the gene pool for the continuing creation of new SCC*mec* types from which MRSA might originate. However, it is already known that some parts of SCC*mec* regions are present in the genome of clinical *S. sciuri*.

8 THE MAIN FINDINGS

1. The majority of Finnish MSSA and EMRSA strains had the same genomic background.

2. Finnish MSSA strains acquired SCC*mec* from an unknown source by horizontal gene transfer mechanisms.

3. The SCC*mec* complexes of bactermic MRSE strains were unique combinations of elements SCC*mec*type III and IV.

4. MRSE was the most prevalent CNS species among the residents of the LTCF.

5. A diversity of SCC*mec* types, with many unknown types were recognized amongst nasal carriage MR-CNS strains.

6. Horizontal transfer of SCC*mec* elements is speculated to be based on the sharing of SCC*mec* type V by MRSA and MRSE.

7. *pls* gene was encountered less frequently in the *S. sciuri* than in *S. aureus* and some parts of the SCC*mec* were found in the *S. sciuri* genome.

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